Induction and Synthesis of Metallothionein in Isolated Perfused Rat Liver

Myna Panemangalore and Frank O. Brady†‡

With the technical assistance of Penny Lindgren and Peter Kolbeck

From the Division of Biochemistry, Physiology and Pharmacology, The University of South Dakota School of Medicine, Vermillion, South Dakota 57069

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The induction and synthesis of metallothionein was studied in the isolated perfused rat liver, using Cd(II) as the inducing metal. Perfusing the liver with 25 to 100 µg of Cd(II) for 180 min revealed that the amount of Cd(II) in metallothionein increased with the dosage of Cd(II), saturation being achieved at 75 µg. Zn(II) in metallothionein decreased to about 60% of the level found in control perfused livers and was not altered thereafter. A time course study (75 µg of Cd(II)) indicated that Cd(II) incorporation into metallothionein increased with time for the entire 180 min of perfusion. After an initial decrease at 30 min, Zn(II) in metallothionein increased only slightly. To ascertain whether de novo synthesis of cadmium thionein was being observed, the effects of transcriptional and translational inhibitors on metallothionein synthesis was studied both in vivo and ex vivo. For in vivo experiments, donor rats were pretreated 4 h prior to removal of the liver with intraperitoneal injections of actinomycin D, cordycepin, or cycloheximide, and the livers were subsequently perfused with Cd(II). For ex vivo experiments, the inhibitors were added to the perfusion medium 60 min prior to the addition of Cd(II). Actinomycin D significantly decreased the incorporation of Cd(II) into metallothionein in vivo; cordycepin and cycloheximide were inhibitory in vivo and ex vivo. Zn(II) incorporation into metallothionein decreased in all cases. Up to 90% of the Cd(II) dose was retained by the liver throughout the duration of perfusion, and the rest was secreted into the bile. The latter was dose-dependent and increased when livers were pretreated with the inhibitors. Perfusing with Cd(II) did not significantly alter the Zn(II) content of the livers even in the animals receiving inhibitors in vivo. These data indicate that perfusion of the liver with Cd(II) results in the synthesis of cadmium thionein, and this de novo synthesis is regulated at transcriptional and translational levels.

Metallothioneins are inducible cytoplasmic proteins synthesized in certain mammalian tissues in response to transition metal ions. Originally, this protein was isolated from equine kidney cortex as a cadmium- and zinc-binding protein by Margoshes and Vallee (1). Subsequently, it was characterized and found to be a unique protein containing virtually no aromatic amino acids, but being rich in cysteine (32%) and binding cadmium and zinc (2, 3). This has been followed by the isolation and purification to homogeneity of metallothioneins from livers and kidneys of various species, including man (4–12). In addition to being inducible by cadmium and zinc, mercury and silver are also known to induce metallothionein synthesis (13, 14), while copper has been implicated in the synthesis of another low molecular weight protein, chelatin, in both liver and kidney (15), although there are reports of copper thionein induction by copper salts in pig (8) and rat liver (16), as well as the detection of it in human fetal liver (17).

Under normal conditions, zinc thionein has been detected in the testis of rat (18), in horse liver (6), and in human liver (4). There is ample evidence to show that the apoprotein, thionein, may have a special role in zinc homeostasis and a generalized role in heavy metal metabolism (19–24). Numerous studies have demonstrated the involvement of zinc thionein in the detoxification of heavy metals in general and cadmium in particular (25–29). That there is de novo synthesis of cadmium thionein after exposure to cadmium salts (either dietary or parenteral) is indicated not only by the incorporation of [14C]cystine (30) and [105]Cd(II) and [35S]cystine (31) into thionein but also by an increased synthesis of a specific mRNA for cadmium-binding protein (32). Various stresses like starvation and dietary restriction are known to increase liver zinc and cause hypozincemia (33, 34). A relationship between zinc accumulation in the liver and thionein synthesis has been clearly demonstrated (13) and a similar relationship between copper accumulation and chelatin synthesis implied (35). Increased levels of cadmium could disturb the endogenous zinc pool and result in an apparent increase in intracellular zinc (36). Not only administration of cadmium, but also of copper (37) and mercury (38), has been reported to result in the induction of zinc thionein which in turn is superinducible by actinomycin D, while cadmium thionein and copper chelatin do not respond to actinomycin D (37). Thus, it is possible that fluctuations in intracellular levels of zinc might be an important mechanism in the induction of, and subsequent control of the levels of, zinc thionein.

As a first step toward determining if such a fluctuation in intracellular zinc levels is an important mechanism in the induction process, we have chosen to study the induction and synthesis of metallothionein using cadmium as the inducing cation in the isolated perfused rat liver. In using this technique, we have attempted to exclude confusion that could arise from fluctuations in the endogenous metal pool which could lead to the migration of metal ions from peripheral...
tissues into the blood and thus to the liver. The response obtained in the ex vivo system, we hope, simulates the in vivo response and is thus preferable to an in vitro system.

MATERIALS AND METHODS

Male rats weighing 200 to 250 g were obtained from Sasco, Inc. (Omaha, Neb.). Upon arrival they were housed in wire-bottomed cages in a temperature- and light-controlled room. They were maintained on Purina rat chow and tap water ad libitum. The animals were not starved prior to being used as liver donors.

The technique of isolated liver perfusion has been described in detail and reviewed elsewhere (39, 40). The method we followed was that developed by Millar (41). A commercially manufactured perfusion apparatus was used in these studies (MRA Corp., Clearwater, Fla.). The surgical removal of the liver and the perfusion method is briefly outlined below.

A rat was anesthetized with ether, the abdomen and anterior thorax were shaved and cleaned with 70% ethanol, and the animal was attached to an operating board. The abdomen was opened with a long midline incision, and the intestines were retracted to expose the bile duct and the portal vein. The liver lobes were lifted forward, and the whole area was draped with gauze dipped in saline. Then, heparin (100 units) was injected into the abdominal vena cava, after which the bile duct and the portal vein were cannulated, and the vena cava and a major pancreatic branch of the portal vein were ligated. Thereafter, the thorax opening was opened and a plastic cannula was inserted into the thoracic vena cava with the tip pointing toward the liver. The liver was then excised along with the diaphragm without incising the gastrointestinal tract and was rinsed with saline and adjusted so that the thoracic cannula pointed vertically downward and the portal and bile cannulae were positioned horizontally, pointing outward. The liver was then mounted on a moistened gauze-lined platform in the perfusion apparatus. A defined perfusion medium was circulating through the apparatus. The medium was continually oxygenated with humidified 95% O2, 5% CO2. The portal cannula was connected to the circulating medium and after flowing through the liver the medium exited through the thoracic cannula which was then infused into the apparatus and collected the medium for recirculation. The bile secreted by the liver was collected in a tube. The bright red color of the perfusing medium in perfusions conducted beyond 120 min, a solution (1.5 ml) was added to the perfusion medium. Control livers were perfused for the desired length of time in the absence of Cd(II). 

Experiment 2—The time course of metallothionein induction was measured using a perfusion medium containing 75 μg of Cd(II) added at time zero with perfusion periods of 30, 90, and 150 min. Control livers were perfused only until time zero.

Translational and transcriptional inhibitor experiments were conducted with the inhibitors being administered both in vivo and ex vivo.

Experiment 3—In the in vivo inhibitor experiments, actinomycin D (1 mg/kg of body weight), cycloheximide (10 mg/kg of body weight), and cordycepin (10 mg/kg of body weight) (all purchased from Sigma Chemical Co.) were each injected intraperitoneally in rats 4 h prior to removal of the liver for perfusion. Metallothionein in the isolated perfused rat liver was determined with a dose of 75 μg of Cd(II) and a total perfusion time of 120 min. As controls, pretreated livers were perfused without Cd(II).

Experiment 4—For the ex vivo inhibitor experiments, livers were perfused for 10 min, then one of the inhibitors was added to the perfusion medium (10 mg/kg of body weight) and the perfusion was continued for 60 min at which time a dose of 75 μg of Cd(II) was added to the perfusion medium and the perfusion was continued for an additional 60 min. The total perfusion time was thus 130 min. Control livers were perfused similarly in the absence of Cd(II) or in the absence of inhibitors with or without Cd(II) for 60 min.

In all cases at the end of a perfusion the livers were quick-frozen on dry ice and stored at -20°C for further analysis. Samples of the perfusion medium were drawn at regular intervals during each perfusion and analyzed for Cd(II). Secreted bile was measured, frozen, and stored at -20°C for subsequent analysis. All livers were individually processed and analyzed. Each liver was weighed, homogenized in 0.01 M Tris-HCl, pH 8.6, containing 0.25% sucrose, and centrifuged at 105,000 × g. A suitable aliquot of the high speed supernatant was then chromatographed on a column of Sephadex G-10 (2.5 × 30 cm). This procedure is described in detail elsewhere (37). The fractions collected were monitored for Zn(II) and Cd(II) content, using a flame atomic absorption spectrophotometer (Perkin Elmer model 303). The metallothionein peak was located in the M, = 10,000 region, and the Zn(II) and Cd(II) contents were quantitated. Data was expressed as micrograms of Zn(II) or Cd(II) in metallothionein per g of liver, or as nanogram atoms of Zn(II) or Cd(II) per g of liver.

Aliquots of both the liver homogenate and the high speed supernatant were digested with concentrated H2SO4 and 30% H2O2, the residue was dissolved in 1 N HCl, and the Zn(II) and Cd(II) contents were determined. After suitable dilution, the Zn(II) and Cd(II) contents of bile were quantitated by flameless atomic absorption spectrophotometry using a carbon rod atomizer (Varian Techtron AA6A, Palo Alto, Calif.). The bile from each series of experiments was pooled and 10 ml of pooled bile was chromatographed on a column of Sephadex G-75 as described above. The fractions were monitored for protein in the 980 nm and for Cd(II). The column fractions were pooled together to obtain three major pools which were then analyzed by polyacrylamide gel electrophoresis, using the method of Davis (43). After staining with 0.5% Coomassie blue, the gels were scanned at 600 nm on a recording spectrophotometer (Gilford Instruments Inc., Oberlin, Ohio). Replicate unstained gels were sliced, eluted with saline, and the extracts were monitored for protein at 280 nm and for Cd(II).

Statistical analysis of all data was carried out using Fischer’s paired t test to determine significant differences between means.

RESULTS

Dose Response and Time Course Perfusions—To determine if metallothionein could be induced by Cd(II) in the isolated perfused rat liver, livers from normal stock diet-fed rats were perfused with exogenous Cd(II) for a period of 150 min following equilibration of the livers for 30 min. Even at the 100 μg level of Cd(II) no toxic effects were evident in the livers. Results of this dose response experiment are presented in Fig. 1. All of the exogenous Cd(II) was absorbed by the perfused livers within the first 15 min, as indicated by the absence of Cd(II) in the perfusate 15 min after addition, which is somewhat faster than reported by other workers (44). As can be seen in Fig. 1, the content of Cd(II) in metallothionein increased as the amount of Cd(II) added to the perfusion medium is increased.
medium was increased, saturation of Cd(II) in metallothionein being attained at a level of 75 μg of Cd(II). The Zn(II) in the metallothionein of Cd(II)-exposed livers decreased to about 60% of the level found in control livers perfused without Cd(II), and this level of Zn(II) was not altered as the amount of Cd(II) in the perfusate was increased.

The time course of metallothionein induction was studied in livers perfused for increasing lengths of time with a fixed amount of added Cd(II), 75 μg. The results are presented in Fig. 2. This data shows that the Cd(II) in metallothionein increased with the length of time of perfusion. The Cd(II) in metallothionein was still increasing at 150 min although somewhat more slowly than initially. After an initial decrease at 30 min, the Zn(II) in metallothionein of Cd(II)-exposed livers perfused for 90 and 150 min increased slightly, but this increase was not statistically significant. In this experiment we also observed that the amount of Cd(II) bound to high molecular weight proteins in the liver cytosol decreased as the duration of perfusion increased, and more Cd(II) was found associated with metallothionein, reflecting the transfer of Cd(II) from high molecular weight proteins to metallothionein as the time of perfusion increased.

Effect of Inhibitors on the Synthesis of Metallothionein—To ascertain if de novo synthesis of cadmium thionein was occurring in the isolated perfused livers in response to exposure to Cd(II), the effects of transcriptional and translational inhibitors on the synthesis of cadmium thionein in perfused livers were studied. The inhibitors used were actinomycin D, cordycepin, and cycloheximide, and these were administered in vivo to the rats prior to removal of the livers for perfusion or ex vivo in the perfusion medium. For the in vivo studies donor rats were treated with intraperitoneal injections of the inhibitor 4 h prior to removal of the liver, and in the ex vivo experiments, the inhibitors were added to the perfusion medium 60 min before the addition of Cd(II). Inhibitor-treated controls [no Cd(II)] were also included in these studies to determine whether the inhibitors themselves would influence metallothionein induction because of either their toxicity or the stress these drugs could exert on the animals.

Actinomycin D Effect on Metallothionein Synthesis—The results of experiments in which actinomycin D was used as an inhibitor of metallothionein synthesis in the perfused liver are shown in Fig. 3. The data are split into two classes: the results when the inhibitor was administered in vivo to the animal prior to removal of the liver and the results when the inhibitor was added to the perfusion medium at the beginning of perfusion (ex vivo). Four types of perfusions are compared: 1) controls, receiving neither actinomycin D nor cadmium; 2) cadmium-exposed; 3) cadmium- and actinomycin D-exposed; and 4) actinomycin D-exposed.
The effects of actinomycin D on the synthesis of metallothionein were varied depending on the route of administration. In vivo actinomycin D significantly decreased the amount of Cd(II) incorporated into metallothionein as compared with controls (p < 0.01), but it was without effect on Cd(II) incorporation when administered (ex vivo). Zn(II) incorporation into metallothionein was significantly decreased as compared with controls by exposure to Cd(II) in both sets of experiments (p < 0.001). Treatment with actinomycin D [±Cd(II)] significantly decreased the amount of Zn(II) incorporated into metallothionein only when administered ex vivo (p < 0.001).

Cordycepin Effect on Metallothionein Synthesis—Cordycepin, an inhibitor of the processing of nuclear mRNA which blocks the incorporation of poly(A), gave more clear cut results when used as an inhibitor of metallothionein synthesis in the isolated perfused rat liver. The results of these experiments with cordycepin are presented in Fig. 4, using the same format of presentation as for actinomycin D.

Cordycepin significantly inhibited the incorporation of cadmium into metallothionein as compared with controls when administered in vivo and ex vivo (p < 0.001). It also decreased significantly the amount of Zn(II) incorporated into metallothionein in vivo ± Cd(II) (p < 0.001) and ex vivo ± Cd(II) (p < 0.001). It, however, did not decrease significantly the amount of Zn(II) incorporated when administered ex vivo – Cd(II).

Cycloheximide Effect on Metallothionein Synthesis—Cycloheximide, an inhibitor of protein synthesis, also inhibited the synthesis of metallothionein in the isolated perfused rat liver. The results of experiments utilizing cycloheximide are presented in Fig. 5 in a manner identical with Figs. 3 and 4 for actinomycin D and cordycepin.

Cycloheximide decreased significantly the incorporation of Cd(II) into metallothionein as compared with controls when administered in vivo (p < 0.001) and ex vivo (p < 0.01). Zn(II) incorporation into metallothionein was inhibited by cycloheximide.

The results of experiments utilizing cycloheximide are presented in Fig. 5 in a manner identical with Figs. 3 and 4 for actinomycin D and cordycepin.
imide in both cases when Cd(II) was absent.

From the data of the inhibitor experiments presented so far, it is apparent that neither the in vivo nor the ex vivo treatment with transcriptional and translational inhibitors increased the amount of Zn(II) in metallothionein indicating thereby that no stress was involved in which the available amount of Zn(II) in the livers was increased.

**Metal Contents of Liver and Bile after Perfusion**—The total Cd(II) and Zn(II) contents of the perfused livers are shown in Table I. Since 98% of the Zn(II) and Cd(II) in the liver homogenate was found in the high speed supernatant, numbers in Table I are presented as averages of both values. Perfusion of the livers with varying amounts of Cd(II), in the absence of Cd(II), or after treatment in vivo or ex vivo with different inhibitors and then exposure to Cd(II) did not alter total liver Zn(II) levels. These ranged between 15 and 19 μg/g of liver, wet weight. No Cd(II) was detected in the control livers while in Cd(II)-exposed livers, the Cd(II) content varied from 68 to 72 μg/liver, indicating that up to 90% of the administered dose of Cd(II) (75 μg) was retained by the liver during the duration of the perfusions.

The bile produced by the perfused livers was also analyzed for total Cd(II) and Zn(II) content, and these results are presented in Table II. The amount of Cd(II) in bile increased as livers were perfused with greater amounts of Cd(II), and the zinc content increased only when 75 or 100 μg of Cd(II) were added during perfusion. However, there was no change in the amount of Cd(II) secreted in the bile from livers perfused for different durations. Pretreatment of the livers with any of the inhibitors either in vivo or ex vivo increased the Cd(II) content of the bile from about 10 to 19 μg/ml, with cycloheximide causing the greatest secretion of Cd(II) in the bile. But with ex vivo cordycepin or cycloheximide treatment in Cd(II) bile in the bile did not increase similarly. The Zn(II) content of the bile in all experiments ranged between 2 and 5 μg/ml indicating that a small amount of Zn(II) is consistently secreted into the bile under all conditions. Exposure to either 100 μg of Cd(II) or to any of the inhibitors tended to decrease the volume of bile secreted by the perfused liver.

In order to determine the distribution of Cd(II) in bile, all the bile collected was pooled into two distinct pools: Pool I, bile from all Cd(II)-perfused livers; and Pool II, bile from inhibitor-treated and Cd(II)-perfused livers (Table III). Sephadex G-75 column chromatography of the Pool I bile showed that 86% of the Cd(II) was bound to constituents which eluted below the Mr = 10,000 region compared to 14% in the region above Mr = 10,000. On the other hand, in the bile from Pool II, more than 30% of the Cd(II) was bound to constituents in the region above 10,000 daltons. Also, bile from inhibitor-treated livers contained 21% less protein than the bile of Pool I. Polycrylamide gel electrophoresis of the pooled fractions of the two regions indicated the presence of three separate proteins from the Mr ≥ 10,000 region pool and one protein from the Mr < 10,000 region pool. No attempts were made to quantitate either the protein or Cd(II) bound to them, although cadmium was detected in gel slice extracts.

Lastly, Sephadex G-75 fractionation of the perfusion medium (not containing erythrocytes) indicated that 98% of the Cd(II) added to the medium was bound to albumin (bovine serum albumin, Fraction V), while the rest was bound to free amino acids. This suggests albumin as a Cd(II)-transport protein.

**DISCUSSION**

Isolated liver perfusion offers distinct advantages over whole animal studies in that it preserves the control system associated with intact cells and provides a milieu similar to that in intact animals. At the same time, the advantage of an in vitro system is retained where strict control over the experimental conditions can be maintained, while eliminating interactions with other tissues (37). The results of our experiments using such an in vitro technique have indicated that perfusion of the liver with Cd(II) resulted in synthesis of cadmium thionein. Initially, the Zn(II) in the pre-existing metallothionein was partially displaced by exogenous Cd(II), but subsequently additional Cd(II) was incorporated into metallothionein. Furthermore, that there was de novo synthesis of cadmium thionein and that this synthesis was regulated at both transcriptional and translational levels was confirmed by the inhibitor studies. Also, of significance was the secretion of Cd(II) into the bile, illustrating the presence of enterohepatic circulation of Cd(II).

The presence of metallothionein in livers of normal animals has been reported by Chen et al. (46), by Kagi and Vallee (2), and in normal human livers by Bühler and Kagi (4). The finding of Zn(II) in metallothionein of normal, control perfused livers confirms these reports. The displacement of Zn(II) in metallothionein by Cd(II) has been observed in the livers of rats repeatedly dosed with subcutaneous injections of Cd(II) (47, 48). Webb (25), however, was unable to obtain displacement of Zn(II) by Cd(II) in the presynthesized metallothionein of the livers of rats treated with Cd(II). This was confirmed later by Webb and Virschoye (26) while investigating the role of metallothionein in protection against acute Cd(II) toxicity. Irons and Smith (49) have recently shown that in livers of rats treated for prolonged periods with CdCl₂, the saturation of metallothionein with Cd(II) approached 80% while the remaining metal sites bound Zn(II). Weser et al. (12) have shown that in Cd(II)-treated rats, liver metallothionein contained Cd(II) and Zn(II) in the ratio of 1:2.4. This indicates the presence of Zn(II) in cadmium thionein but not displacement. In our experiments, there is evidence for partial displacement of Zn(II) in metallothionein, but incorporation of Zn(II) into thionein is not entirely ruled out. Perhaps there is both displacement and incorporation occurring but more data is needed to prove this convincingly. However, the absence of displacement of Zn(II) by Cd(II) in metallothionein of in vivo actinomycin D-treated rats and the very small amount of pre-existing zinc thionein in the ex vivo treated rats in our experiments (Fig. 3) are more difficult to interpret, and there is no evidence for such changes in the literature. As the other two inhibitors, cordycepin and cycloheximide, did not cause such changes, the unique effect of actinomycin D on Zn(II) in metallothionein may be related to the toxic side effects of the inhibitor which could have caused transient alterations in the intracellular Zn(II) pool of the liver. Our data also indicate that ex vivo pretreatment of the liver with inhibitors was not satisfactory as the inhibitions obtained were not distinct, making interpretation difficult as compared to the in vivo results. Perhaps 80 min of ex vivo exposure to the inhibitor is not sufficient to produce changes similar to those obtained by 4 h of in vivo exposure.

There is both direct and indirect evidence demonstrating the de novo synthesis of metallothionein in the livers of Cd(II)-exposed rats. The presence of Cd(II) in the nuclei of liver cells and the correlation between the disappearance of free Cd(II) with the appearance of a Cd(II)-binding protein, using in vivo and in vitro experiments, was reported by Bryan and Hidalgo (50). Synthesis of metallothionein in a cell-free polysomal system has also been reported, supporting the contention that metallothionein synthesis is regulated by changes in the size of the pool of thionein mRNA (51). Squibb et al. (24) have also shown that zinc thionein synthesis is regulated at translational as well as transcriptional levels.
Metallothionein Synthesis in the Perfused Rat Liver

There is evidence to substantiate de novo synthesis of metallothionein after induction with Cd(II) under in vivo conditions in rat liver following oral or parenteral administration of Cd(II) (31, 52). The simultaneous incorporation of $^{109}$Cd(II) and $^{[35S]}$cysteine into hepatic metallothionein of Cd(II)-treated rats provides additional evidence of de novo metallothionein synthesis. Under ex vivo conditions, we have also obtained incorporation of $^{[35S]}$cysteine into rat hepatic cadmium thionein.

In rats given subcutaneous injections of Cd(II), Webb (53) found that metallothionein synthesis in the liver was inhibited by cycloheximide but not by actinomycin D, indicating regulation of synthesis at the translational level. This is similar to the report of Shaikh and Lucy (30), who also observed regulation at the translational level. In contrast, however, Shaikh and Smith (54) have reported differently in a recent paper. In rats treated with subcutaneous injections of 3 mg of Cd(II)/kg body weight, they found that both actinomycin D and cycloheximide inhibited the incorporation of $^{14}$C- and $^{[35S]}$cysteine labels into hepatic cadmium thionein. Our results with the isolated perfused liver system support these findings and indicate that synthesis of metallothionein following induction by Cd(II) is regulated at both levels of transcription and translation and is thus similar to the regulation of zinc thionein synthesis reported by Cousins’ group (24).

Although perfusion of the liver with Cd(II) did not alter the Zn(II) content of the liver in the present experiments, there is a report in the literature which shows that total liver Zn(II) increases following a 24-h exposure to Cd(II) and declines only after 18 days (55). It is known that hepatic zinc levels increase in response to hyperzincaemia, resulting in induction of zinc thionein synthesis (23).

Relatively small quantities of Cd(II) (30 pbb) were observed in bile collected from bile duct-catheterized rats injected with 25 µg of Cd(II)/kg body weight, although the amount of Cd(II) in bile did increase with increased dosage of Cd(II) (56). A significant dose-dependent excretion of Cd(II) in the bile has been reported by Cherian and Vostal (57). They were able to recover most of the Cd(II) in the bile bound to a low molecular weight molecule, identified as glutathione, and were able to recover most of the Cd(II) in the bile bound to a Cd(II) in bile did increase with increased dosage of Cd(II) 25 mg of Cd(II)/kg body weight, although the amount of in bile collected from bile duct-catheterized rats injected with thionein synthesis (23).

There is evidence to substantiate de novo synthesis of metallothionein in rat liver. The isolated liver perfusion technique can thus be used to study induction, synthesis, and metabolism of metallothionein under conditions more controlled than whole animal studies. Hopefully, we will be able to use this system to answer some of the questions which have been raised concerning the physiological role and function of metallothionein (59).

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M Panemangalore and F O Brady


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