PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 176, 392–402 (1984)

The Tissue-Specific Accumulation of Hepatic Zinc Metallothionein following Parenteral Iron Loading[1] (41888)

CHARLES C. McCORMICK

Department of Poultry and Avian Sciences, Cornell University, Ithaca, New York 14853

Abstract. The synthesis in various tissues of the unique metal-binding protein, metallothionein, can be influenced by the administration of certain trace elements. Zinc and cadmium, both of which bind to metallothionein, are most widely recognized as potent inducers. Preliminary results in our laboratory suggested that iron loading causes a marked accumulation of hepatic zinc metallothionein. We present in this report the effects of parenteral iron administration on metallothionein concentration in various tissues. Male chicks (300–350 g) received (ip) either a single injection (+1 Fe) of iron (10 mg Fe/kg, as $FeCl_3$), two injections (+2 Fe) given 24-hr apart, three injections (+3 Fe) each given 24-hr apart, or an equivalent volume of 0.9% saline (control). Twenty-four hours following the final injection, chicks were killed and tissues analyzed for cytoplasmic zinc and metallothionein (Zn–MT). The parenteral administration of ferric iron, $FeCl_3$, resulted in a marked tissue-specific accumulation of zinc as metallothionein. In chicks given +2 Fe, hepatic Zn–MT increased more than 10-fold with a third injection (+3 Fe) causing no further change. The concentration of Zn–MT in renal and pancreatic tissue was unaffected by iron loading. An increase in hepatic Zn–MT was evident prior to detectable changes in total hepatic iron. The administration of other ferrous iron compounds at a similar rate produced comparable changes in hepatic Zn–MT. Feeding excess dietary iron, however, had no effect on liver Zn–MT levels even though similar hepatic iron concentrations were attained. Our results indicated that parenteral administration, but not feeding, of various iron compounds causes a marked increase in zinc metallothionein, specifically in liver tissue.

Presently, there is a considerable body of literature concerning the unique metal-binding protein, metallothionein. Although isolated more than 20 years ago (1), a consensus regarding the biological role of this inducible protein does not exist. It is well recognized that a nutritionally required trace element, zinc, as well as an extremely toxic metal, cadmium, both induce the synthesis of metallothionein in various tissues. Accordingly, a function in the metabolism of these elements has been suggested (2–6). In addition to zinc and cadmium, the synthesis of metallothionein can be influenced by other elements, copper and mercury (7–9), as well as steroid hormones (10–12), stressors (13–15), and certain chemicals, e.g., carbon tetrachloride (13) and isopropanol (16). Although a unifying mechanism has not been established, there is little doubt that transcriptional events are involved in the process of metallothionein induction.

The influence of elements, other than those cited above, on the induction of tissue metallotionein has received less attention. The several studies conducted have reported equivocal results. Piotrowski and Szymanska (17) examined the ability of 19 different metals to induce the synthesis of hepatic and renal metallothionein-like proteins. They concluded that cadmium, zinc, copper, and cobalt were capable of inducing metallothionein synthesis in liver while cadmium, zinc, copper, and mercury induced metallothionein in kidney. Winge *et al.* (18) reported that silver salts were potent inducers of both hepatic and renal metallothionein. Others have indicated that bismuth (19) is capable of inducing renal metallothionein. A recent study by Eaton *et al.* (20) showed that copper and cobalt did not induce hepatic metallothionein but that nickel produced a two- and threefold increase in hepatic and renal metallothionein. In addition, and in contrast to results of Winge *et al.* (18), silver did not significantly increase renal or

[1] Supported by NIH Grant AM 33058 and Hatch Funds provided through the College of Agriculture and Life Sciences, Cornell University.

0037-9727/84 $1.50

hepatic metallothionein. Differences in the route of administration, dosing schedules, animal strains, and method of metallothionein quantitation were cited as possible causes for discrepancies.

Recent preliminary work (21) in our laboratory directed toward an investigation of an antagonism between zinc and iron indicated that iron may be capable of causing a pronounced increase in zinc metallothionein, specifically in hepatic tissue. Other tissues which are sensitive to changes in zinc status, such as kidney and pancreas, were unaffected. We demonstrate in this report that parenteral administration, but not feeding, of various iron compounds causes a more than 10-fold increase in hepatic zinc metallothionein.

Materials and Methods. Five-week-old male chicks (300–350 g), Leghorn strain, received (ip) either a single injection of iron (+1 Fe) as ferric chloride ($FeCl_3$), two injections (+2 Fe) given 24 hr apart, three injections (+3 Fe) each given 24 hr apart, or an equivalent volume of 0.9% saline (control). Each injection of iron was given at a rate of 10 mg Fe/kg body wt from a solution containing 20 mg Fe/ml. Feed and water were provided *ad libitum* throughout the experiment. Twenty-four hours following the final injection, chicks were killed by cervical dislocation and tissues excised, immediately placed in ice, and subsequently frozen (−20°C). Pooled cytosol were prepared from separate samples of three tissues, liver, kidney, and pancreas. Each sample of hepatic cytosol was prepared from hepatic tissue from two chicks. Each pancreatic and renal cytosol sample was prepared from tissues obtained from three chicks. An equal portion of each tissue sample was combined (1:2 wt/vol) in ice-cold buffer (0.25 *M* sucrose/0.01 *M* Tris–HCl/0.01% NaN_3, pH 8.6) and homogenized using a Potter–Elvejhem glass homogenizer. The homogenate was centrifuged at 100,000*g* for 1 hr at 4°C to yield a final supernatant (cytosol) which was collected and immediately stored at −20°C.

Tissue metallothionein was isolated and quantitated by gel-filtration chromatography. Three milliliters of cytosol were applied to a 2.5 × 50-cm column of Sephadex G-75 (medium) equilibrated in 0.01 *M* Tris–HCl/0.01% NaN_3, pH 8.6, at 4°C. Cytosol was eluted in the same buffer (flow rate of 0.3 ml/min) and collected in 3-ml fractions. The zinc content of each fraction was determined by atomic absorption spectrophotometry, AAS (AA575, Varian Associates, Inc., Palo Alto, Calif.). Fractions containing zinc metallothionein (1.8 × Vo) were summed and expressed as μg Zn/ml cytosol. Columns were calibrated with DEAE-purified rat hepatic metallothionein to verify the elution volume of metallothionein. Total cytosol zinc was also determined by AAS following an appropriate dilution (1:20) with deionized water.

To confirm the identity of iron-induced hepatic metallothionein eluting from G75 chromatography, nondenaturing polyacrylamide gel electrophoresis (PAGE) was performed. Fractions containing the third peak from G75 chromatography (see Fig. 2, fractions 52–65) were pooled and applied directly to a column (0.9 × 10 cm) containing anion-exchange media (DEAE–Sephadex A25, Pharmacia Fine Chemicals Co., Piscataway, N.J.) equilibrated in 0.002 *M* Tris–acetate buffer, pH 7.4. The procedure has been previously described (22). The single DEAE chromatographic peak was concentrated by ultrafiltration using YM2 membrane filters (Amicon Corp., Danvers, Mass.). An approximately equal amount of zinc from the DEAE concentrate of iron-injected and zinc-injected (5 mg/kg) chicks, as well as zinc MT_A from rat liver, was subjected to vertical slab PAGE. The discontinuous buffer system described by Davis (23) was employed using 7.5% total acrylamide and 2.5% Bis. Gels were cast in a vertical apparatus (Hoeffer Scientific Instruments, San Francisco, Calif.) with dimensions of 14 cm W × 12 cm H × 1.5 mm thick and subjected to constant current (20 mA) with cooling. Electrophoresis was terminated when the tracking dye reached the bottom of the gel. Gels were stained overnight in 0.25% Coomassie R250/7% acetic acid/25% methanol and destained by diffusion.

In addition to electrophoretic analysis of hepatic DEAE-purified material from iron- and zinc-induced chicks, amino acid composition analysis was conducted on the same samples. Samples were lyophilized in ampules and hydrolyzed *in vacuo* for 24 hr at 110°C in constant-boiling HCl containing ethanethiol as a reducing agent. Cysteic acid was determined following performic acid oxidation (for

16 hr) by the method of Hirs (24) and hydrolyzed as above in constant-boiling HCl. All determinations were performed on a Beckman Model 119 Cl amino acid analyzer. Three replicate analyses were performed.

Whole-tissue zinc and iron determinations were also conducted on liver samples. Equal portions of liver from two chicks were pooled and treated as a single sample. A total of four samples from each treatment were subjected to acid digestion using sulfuric and nitric acids. The zinc and iron concentration of the tissue digest was determined by AAS and results expressed as micrograms per gram wet weight tissue.

A second experiment was conducted to investigate the effect of ferrous (Fe^{2+}) iron salts on hepatic zinc metallothionein concentrations. Five-week-old male chicks received (ip) either two injections of saline (0.9% NaCl), or two equivolume injections of iron (1 mg Fe/100 g body wt) as ferric chloride ($FeCl_3$), ferrous sulfate ($FeSO_4$), or ferrous ammonium sulfate ($Fe(NH_4)_2(SO_4)_2$). A fourth group received two injections of a similar volume of equimolar ammonium sulfate ($(NH_4)_2SO_4$).

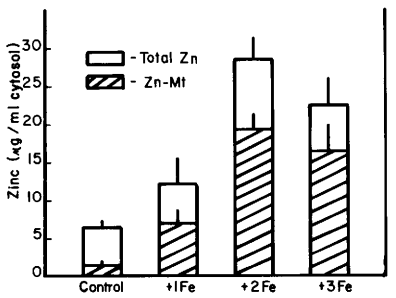

FIG. 1. Total zinc and zinc metallothionein content of liver cytosol from iron-loaded chicks. Five-week-old chicks were given (ip) either one injection (+1 Fe) of iron as ferric chloride (10 mg Fe/kg body wt), two injections (+2 Fe) given 24-hr apart, three injections each at 24-hr intervals, or an equivalent volume of saline (control). Twenty-four hours following the final injection, chicks were killed and liver cytosol was prepared. Zinc metallothionein (Zn–MT) was determined by atomic absorption spectroscopy following gel filtration chromatography (G-75). Total cytosol zinc was also determined by AAS. Total Zn and Zn–MT increased significantly following one injection (+1 Fe) of iron. An additional significant increase was observed after two injections (+2 Fe) with no further change in +3 Fe chicks. Bar height represents a mean ± SEM of three samples each composed of pooled tissue.

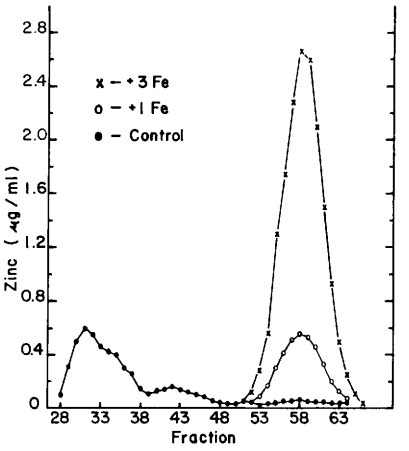

FIG. 2. A typical chromatogram of liver cytosol derived from iron-loaded or control chicks. See legend to Fig. 1 for description of experimental treatments. Three milliliters of liver cytosol (1:2 w/v) was applied to a column (2.5 × 50 cm) containing G-75 and eluted in 3-ml fractions. Zinc metallothionein eluted in a symmetrical peak centered around fraction 57. Profiles from cytosol of chicks given three iron injections (+3 Fe) were similar to that from chicks given two injections (+2 Fe).

The second injection in all groups was given 24 hr following the initial injection. Twenty-four hours following the final injection, chicks were killed and livers excised and frozen. Hepatic cytosol zinc and metallothionein were determined as previously described.

A third experiment was conducted to determine if elevated liver iron, which is effected by feeding, would influence hepatic metallothionein levels. Male chicks (1 week of age) were fed a soy-based purified diet (25) containing either no additional iron supplement (basal), or 1200 or 2000 ppm added iron as ferrous sulfate. Chicks were fed the experimental diets for a period of 2 weeks at which time chicks were killed and tissues excised and frozen. Total liver zinc and iron analyses were performed by AAS following acid digestion of tissue. Metallothionein determinations were also conducted on hepatic cytosol as previously described.

Data were analyzed statistically by one-way analysis of variance and mean differences determined by Duncan's Multiple-Range Test (26).

Results. Iron loading caused a marked increase in hepatic zinc metallothionein (Zn–MT) as illustrated in Fig. 1. When compared

to control values, the concentration of liver cytosolic Zn–MT increased approximately 5-fold in chicks given a single injection of iron (+1 Fe). An elevation of more than 10-fold was observed in liver cytosol from chicks given two injections (+2 Fe) with no further change occurring in those given three injections (+3 Fe). A typical chromatogram of liver cytosol from control chicks and those given +1 Fe or +3 Fe is shown in Fig. 2. The change in the profiles of +1 or +3 Fe was exclusively in the last eluting peak. There was no detectable change in zinc concentration of fractions other than those containing Zn–MT. We determined that 80–90% of the increase in cytosol total zinc was accounted for in Zn–MT. These results suggest that a maximum in the iron-induced accumulation of zinc metallothionein occurs after two administrations (ip) of iron in the form of $FeCl_3$ and that zinc bound to metallothionein accounts for the majority of increase in total cytosolic zinc. In a following study, two injections of various other iron compounds were used since this dose appeared to result in a maximum response.

Confirmation of metallothionein as the third chromatographic peak from G-75 (Fig. 2) was accomplished by anion exchange chromatography, gel electrophoresis, and amino

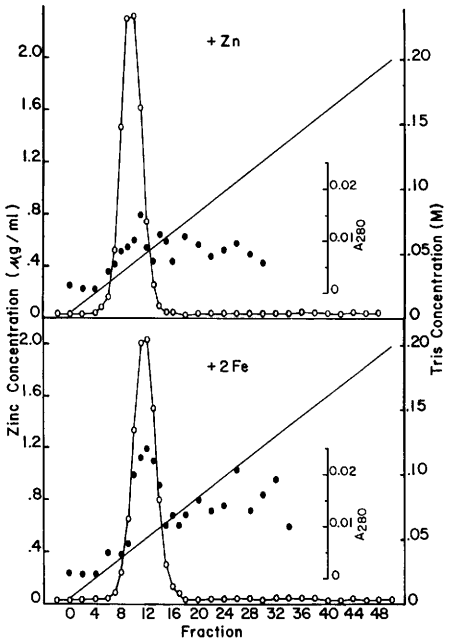

FIG. 3. Anion exchange chromatography of zinc-containing fractions from G75. The chromatograms show a comparison of zinc-binding material obtained from G75 fractionation of hepatic cytosol from both zinc-injected (+Zn) and iron-injected (+2 Fe) chicks. Five-week-old chicks were given ip zinc as zinc sulfate (5 mg/kg) and killed 24 hr later. Iron was administered as described in Fig. 2. Fractions (52–65, Fig. 2) were pooled, and an approximately equal amount of zinc was applied directly to an anion exchange (DEAE-Sephadex A25) column (0.9 × 10 cm) equilibrated in 0.002 *M* Tris–Acetate, pH 7.4. Following a wash, the columns were eluted with a gradient (0.002 *M* to 0.2 *M* Tris–Acetate, pH 7.4, total volume of 100 ml) in 2-ml fractions. The zinc concentration (open circles) and absorbance at 280 nm (solid circles) of each fraction is shown.

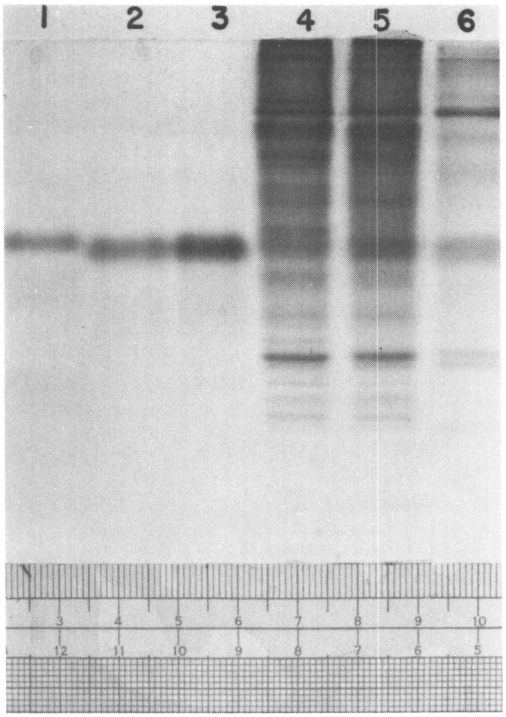

FIG. 4. Polyacrylamide gel electrophoresis of DEAE-purified hepatic zinc-binding material from zinc- and iron-injected chicks. Fractions containing anion exchange peaks shown in Fig. 3 were concentrated by membrane filtration and an aliquot applied to vertical slab gels (7.5% T, 2.5% Bis) as described under Methods. Lane 1, 0.22 μg zinc as rat liver metallothionein isoform A; Lane 2, 0.42 μg zinc DEAE concentrate from +2 Fe chicks; Lane 3, 0.60 μg zinc DEAE concentrate from +Zn chicks; Lane 4, 13.6 μl cytosol from "control" chicks; Lane 5, 13.6 μl cytosol from +2 Fe chicks; Lane 6, 13.6 μl supernate of heat-treated (80°C for 5 min) +2 Fe cytosol. Dye front is represented by upper edge of rule.

acid compositional analysis. Approximately 90% of the zinc in the third peak from G75 chromatography of hepatic cytosol from both zinc- and iron-injected chicks adhered to and later eluted (simultaneously and in a single peak) from DEAE columns as shown in Fig. 3. The electrophoretic analysis of concentrate from these peaks is shown in Fig. 4. A single comigrating band was obtained with both samples. In addition, both exhibited mobility which was similar to that of rat DEAE-purified hepatic metallothionein isoform A. These resuts are consistent with the nature of chick and rat metallothioneins (22), i.e., that the single form of chick tissue metallothionein is similar to the A isoform of rat tissue. Amino acid compositional results of both iron- and zinc-induced DEAE-purified material are shown in Table I. The amino acid composition of both samples was virtually identical. Our analysis, however, did vary somewhat from two other analyses of chick metallothionein (27, 28).

The effect of iron loading on Zn–MT concentration was not observed in other tissues. Shown in Fig. 5 are data of renal and pancreatic cytosol zinc and Zn–MT from iron-loaded chicks. Neither the kidney nor the pancreas appeared to respond to iron loading. There was no significant difference in the concentration of either cytosol zinc or Zn–MT from kidney and pancreas of chicks given up to three injections of iron (+3 Fe). As these tissues are sensitive to changes in zinc status as well as capable of synthesizing Zn–MT, these results indicate that iron loading effects an increase in tissue Zn–MT via a mechanism which is specific to hepatic tissue.

Since liver is a known storage site for iron, we examined changes in total liver iron in an attempt to relate an elevation in liver iron to the increase in Zn–MT. Figure 6 illustrates changes in total hepatic zinc and iron of iron-loaded chicks. The changes observed in total hepatic zinc were similar qualitatively to changes that occurred in cytosolic zinc and Zn–MT. Chicks given +1 Fe exhibited a slight increase in total hepatic zinc. Those given +2 Fe or +3 Fe showed a much greater increase (200%) and were not significantly different be-

TABLE I. AMINO ACID COMPOSITIONAL ANALYSES OF DEAE-PURIFIED ZINC-BINDING MATERIAL FROM CHICK LIVER INDUCED BY EITHER IRON OR ZINC INJECTIONS

	Percentage of total residues			
Amino acid	+Zn[a] (this study)	+2 Fe (this study)	Zn–MT (Ref. 27)	Cd, Zn–MT (Ref. 28)
Aspartic	10.0	9.6	9.5	9.8
Threonine	3.8	3.5	1.3	1.7
Serine	7.8	7.7	9.0	12.6
Glutamine	7.1	8.4	3.8	3.8
Proline	5.0	5.0	8.5	5.0
Glycine	7.6	7.1	6.7	6.5
Alanine	8.4	8.2	9.3	9.7
Cysteine[b]	21.1	21.5	31.6	31.8
Valine	2.8	2.9	1.7	1.8
Methionine	2.4	2.6	1.6	1.9
Isoleucine	2.1	2.1	0.3	0
Leucine	2.7	2.8	0.4	0
Tyrosine	0.6	0.8	0	0
Phenylalanine	1.3	1.3	0	0
Histidine	1.4	1.4	1.7	0.9
Lysine	11.8	11.2	10.0	9.3
Arginine	4.1	4.0	4.5	5.0
Tryptophan	ND[c]	ND	ND	ND

[a] Triplicate analysis of DEAE-purified material obtained from hepatic cytosol of zinc-injected chicks (+Zn) or iron-injected chicks (+2 Fe). See Fig. 3 for description of treatments. Amino acid compositional analysis of zinc (Zn–MT) and cadmium, zinc–metallothionein (Cd, Zn–MT) of chick tissue from two additional studies is also shown.

[b] Determined as cysteic acid.

[c] Not determined.

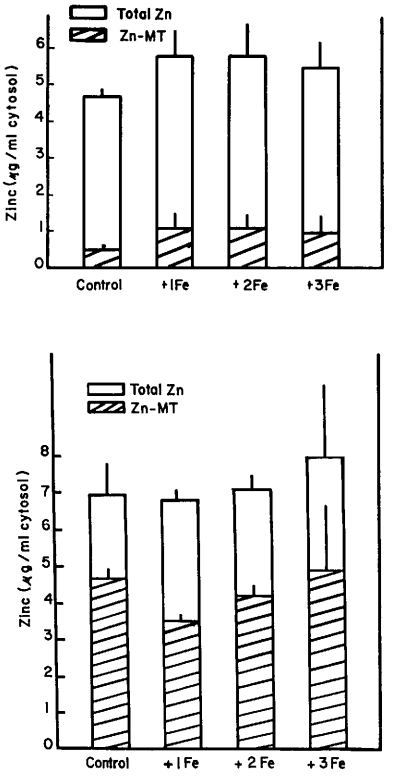

FIG. 5. Renal (top) and pancreatic (bottom) cytosolic zinc (total Zn) and zinc metallothionein (Zn-MT) iron-loaded and control chicks. Chicks were given (ip) either saline (control) or one injection (+1 Fe), two injections (+2 Fe), or three injections (+3 Fe) of iron each at 24-hr intervals. For either tissue, there were no significant differences between treatments with either variable. Bar height represents a mean ± SEM of three samples each composed of pooled tissue.

tween treatments. Liver total iron was not affected by two iron injections (+2 Fe), a treatment which resulted in a maximum increase in Zn-MT (Fig. 1), but was significantly elevated by +3 Fe. The results indicate that in chicks given +2 Fe the concentration of hepatic Zn-MT is maximum prior to detectable changes in the concentration of total hepatic iron. The latter was noted only in +3 Fe chicks, those which exhibited no further change in hepatic Zn-MT.

A second experiment was conducted to determine whether the administration of other iron salts would alter hepatic Zn-MT levels. The initial experiment utilized the more oxidized form of iron, the ferric (Fe^{3+}) form. In the following study, a direct comparison of a reduced iron salt was performed. The two compounds tested were ferrous sulfate and ferrous ammonium sulfate. The latter was chosen so that a comparison could be made of a similar salt, ammonium sulfate, in the absence of iron. Chicks were given two injections (ip) of either (i) one of the various iron solutions, (ii) equimolar ammonium sulfate or (iii) saline. Injections were given 24-hr apart and chicks killed 24 hr after the final injection. The results of this study are shown in Fig. 7. As previously observed, the administration (+2 Fe) of iron as ferric chloride resulted in a 10-fold increase in hepatic Zn-MT. A similar administration of other iron compounds given at an equal rate (10 mg Fe/kg body wt) caused a comparable increase in hepatic Zn-MT. Equimolar injections of ammonium sulfate did not change the concentration of liver Zn-MT suggesting an effect which is unique to the chemical nature of iron. Since there was no significant difference in the concentration of hepatic Zn-MT from chicks given either of the three iron compounds, the data suggest that the form of iron which is administered parenterally is of little importance in terms of effecting changes in hepatic Zn-MT.

A final experiment was conducted to determine if the route of administration of excess iron was an important factor in iron-induced Zn-MT accumulation and to establish definitively that elevated hepatic iron is not a causal factor. One-week-old chicks were fed a purified diet containing excess dietary iron. Chicks

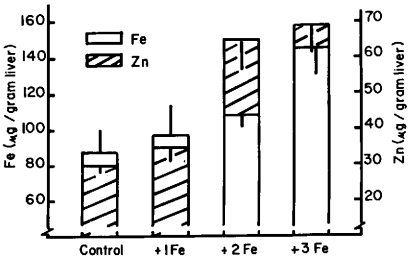

FIG. 6. The effect of iron loading on the concentration of total liver zinc and iron. Description of experimental treatments is given in the legend to Fig. 1. Total liver zinc and iron were determined after acid digestion by AAS. Total liver zinc of chicks increased significantly after one or two injections of iron whereas total liver iron did not. The latter variable increased only after three injections, a treatment which had no further effect on liver zinc. Bar height represents a mean ± SEM of three samples.

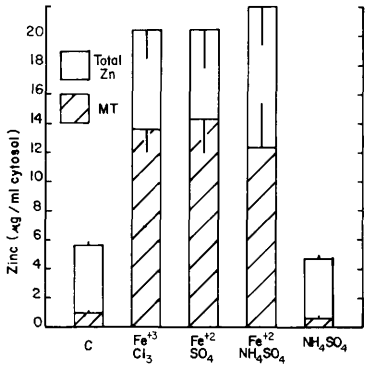

FIG. 7. Hepatic zinc metallothionein and the influence of various iron compounds administered parenterally. Chicks were given (ip) two injections administered 24-hr apart of either saline (C) or one of three iron compounds, ferric chloride ($FeCl_3$), ferrous sulfate ($FeSO_4$), ferrous ammonium sulfate ($FeNH_4SO_4$). Each iron injection was given at a rate of 10 mg Fe/kg body wt. A fifth group was given a similar volume of equimolar ammonium sulfate ((NH_4SO_4)). Twenty-four hours following the final injection, chicks were killed and hepatic cytosolic zinc (total Zn) and zinc metallothionein (MT) were determined. Bar height represents a mean ± SEM of three observations each a pooled sample of hepatic tissue from two chicks. The various iron compounds were equally effective in causing increased hepatic cytosolic MT.

were fed these diets for a period which was considered to be of sufficient duration to cause elevated hepatic iron, i.e., 2 weeks. The results of this study are shown in Table II. Feeding 2000 ppm added iron caused a marked elevation in total hepatic iron to a concentration which was similar to that observed previously in chicks given three injections of iron. This dietary treatment resulted, however, in no significant change in total hepatic zinc. Moreover, there was no significant change in the concentration of hepatic cytosol zinc metallothionein. These results confirm the suggestion that elevated hepatic iron is not in itself responsible for iron-induced hepatic zinc metallothionein accumulation. In addition, they indicate that the route of administration of iron compounds is an important factor.

Discussion. The results of the present study show that parenterally administered iron causes a marked tissue-specific accumulation of a zinc-binding protein which we suggest is metallothionein. We base this suggestion on anion exchange chromatography, gel electrophoresis, and amino acid composition from which the similarity of zinc- and iron-induced hepatic material is apparent. The amino acid composition (Table I) of both preparations, although virtually identical, varied somewhat from the two other reports concerning chick tissue metallothioneins (27, 28). While we have no firm explanation for these differences, the results may indicate either copurification of nonmetallothionein-like proteins or microheterogeneity between variant forms of metallothionein (29). Microheterogeneity has been noted even in apparently pure preparations (30) since the number of moles of individual amino acids were frequently noninteger. Bremner and Davies (14) have also indicated that although all of their preparations typically had high cysteine and usually no aromatic amino acid residues, there were sometimes marked variations in the proportions of many other amino acids.

To the author's knowledge, this report is

TABLE II. THE EFFECT OF FEEDING EXCESS DIETARY IRON ON HEPATIC ZINC, IRON, AND ZINC METALLOTHIONEIN[a]

Diet	Iron (μg/g liver)	Zinc (μg/g liver)	Zinc metallothionein (μg zinc/ml cytosol)
Basal	84.9 ± 11.5 (4)	30.2 ± 0.7 (4)	0.41 ± 0.08 (3)
1200 ppm Fe	125.6 ± 11.1 (4)	31.4 ± 1.3 (4)	0.56 ± 0.18 (3)
2000 ppm Fe	145.4 ± 11.3 (4)	32.4 ± 0.6 (4)	0.41 ± 0.07 (3)

[a] Chicks at 1 week of age were fed an iron-adequate purified diet containing no additional iron supplement (Basal), 1200 ppm or 2000 ppm added iron as ferrous sulfate. After 2 weeks, chicks were killed and liver analyses performed as described in text. Each value represents a mean ± SEM of 3–4 observations. Number in parenthesis indicates number of observations.

the first to demonstrate an extensive iron-induced increase in zinc-bound hepatic metallothionein. The liver was the most responsive tissue and, therefore, presumably the target organ since neither kidney nor pancreas responded. Yoshikawa and Ohta (31) have indicated that iron (Fe^{2+}) is among some 15 elements which have the ability to induce metallothionein, but reference to the original work (17) reveals that there are equivocal data. In a study of the influence of iron and 18 other metals on the level of metallothionein-like proteins (MTP) in liver and kidney of rats, Piotrowski and Szymanska (17) cited preliminary findings which suggested that iron may influence the concentration of hepatic MTP. More extensive experiments within the same study, however, showed that massive injections of ferric chloride did not affect hepatic MTP levels. This was true even in rats which received six to eight doses (subcutaneous) of iron as ferric chloride as a rate of 200 mg Fe/kg (cf. 10 mg Fe/kg used in the present study). While we are uncertain as to why our results are not consistent with those of the above authors', there are some critical differences between the two studies. First was the difference in the route of iron administration. In the present study, intraperitoneal injections were given in contrast to the subcutaneous injections administered by the above workers. Second, there was a difference in the animal model utilized; chicks in the present versus rats in the above study. And finally, the method of analysis of metallothionein differed substantially in the two studies. In the present study, the more conventional method of gel filtration chromatography was employed to isolate and quantitate zinc metallothionein. Piotrowski and Szymanska (17) employed a radiochemical method based on tracing metallothionein in a TCA supernatant of tissue homogenate.

Which of the above three possibilities most likely explains the disparity in experimental results is difficult to determine. The radiochemical metallothionein assay procedure has been shown (32) to give "reliable" results but has not been directly compared to the chromatographic procedure. It is likely that on a relative basis, there may be little real difference between the two procedures. Regarding the second possibility, species differences, it again is difficult to quantitate since a direct comparison of species regarding capacity to synthesize metallothionein has not been reported. We have conducted similar studies with weanling rats (data not shown) and have observed that the rat, when treated similarly with ferric iron, is much less responsive. Rats (200 g) were given either one or two injections (ip) of iron as $FeCl_3$ (10 mg Fe/kg body wt) in a protocol similar to that used with chicks. Saline-injected controls showed very little zinc metallothionein present (trace amount). Values of 2.7 ± 0.3 and 4.4 ± 0.2 μg Zn as MT/ml of liver cytosol were observed in rats given either one or two iron injections. When one compares these values to average values of 7 μg Zn and 18 μg Zn–MT for similarly treated young chicks, it is apparent that the chick is more responsive. While this observation may account for quantitative differences in results of the present study and those reported above, it would not seem to explain the lack of significant effect of iron. This leads finally to the initial suggestion of differences in the route of administration. Recently, Eaton *et al.* (20) examined the dose–response effects of various metal ions on rat liver metallothionein. Iron as ferrous sulfate was among the metals studied. Adult rats were given two intraperitoneal injections of iron at a rate of 16.8 mg Fe/kg. Analysis of metallothionein by the radiochemical method revealed an increase of 150–200% in hepatic metallothionein of iron-treated rats when compared to controls. While the response to intraperitoneally administered iron was small in comparison to that observed with either zinc or cadmium, the rat did respond to injected iron. This was in contrast to results from the previously cited report (17) but qualitatively similar to our results. A possible explanation for these differences may lie in the route of administration of iron. Eaton *et al.* (20). and our protocol employed intraperitoneal injections of iron while Piotrowski and Syzmanska (17) used subcutaneous injections. In the former two studies, a response to iron was observed whereas in the latter there was no consistent change in hepatic metallothionein concentration. While we can offer no explanation regarding physiological differences between the two routes of administration, the evidence suggests that the method administering iron warrants consideration.

Eaton *et al.* (20) suggested the small effect of some metals on metallothionein synthesis may be the result of the effects of stress rather than the metal ion per se. Perhaps there is a greater stress associated with intraperitoneal administration.

Additional data obtained in the present study indicate further that effect of iron on hepatic metallothionein is dependent on the method of effecting an elevation in iron status. Results of a feeding study in which the concentration of hepatic iron was increased to a value similar to that ultimately observed by injection showed no change in hepatic zinc metallothionein as a result of increased hepatic iron (Table II). These data indicate strongly that elevated hepatic iron alone is not causal in the process of iron-induced accumulation of hepatic zinc metallothionein and that the parenteral route of iron administration, as an important factor in the process, must be considered.

An inhibition by zinc and other divalent cations of iron binding and hence formation of the iron storage protein, ferritin, has been documented (33, 34). Recently, a zinc "storage and transferring" function of ferritin has been implicated (35). This arose primarily as a result of the observation that the association of zinc with ferritin increased with the administration of zinc. Our initial concern in the present study focused on the possibility that the zinc-binding material induced by iron administration may have been a subunit of ferritin (19,000–22,000 mol wt). The feeding study (Table II) provided evidence that an elevation of hepatic iron, a level which undoubtedly increased ferritin concentration, did not effect of change in the zinc-binding material of the third peak on G75 as seen in Fig. 2. Since tissues from both the injection and feeding studies were prepared in a similar manner and our additional qualitative analyses (Figs. 3 and 4 and Table I) indicated metallothionein characteristics, we are confident that the zinc-binding protein induced by iron injection is not a subunit of ferritin but in fact similar to metallothionein.

The mechanism by which iron loading effects an elevation in zinc metallothionein specifically in hepatic tissue is not known. The suggestion made previously (20) that the induction of metallothionein by metals other than those recognized as potent inducers is the result of stress rather than the metal ion per se may be relevant in the present study. Stress hormones such as adrenal glucocorticoids are well recognized (10–12) as possessing the ability to induce the synthesis of metallothionein. Although both liver and kidney are responsive in terms of glucocorticoid induction, the liver appears to be much much more sensitive. Hager and Palmiter (36) quantitated the extent of accumulation of metallothionein mRNA following the administration of a synthetic glucocorticoid, dexamethasone. Liver exhibited a 100-fold increase in the number of molecules of metallothionein mRNA whereas the kidney showed only a 3-fold increase. They suggested that the meager response of the kidney may be due to the relatively few glucocorticoid receptors present in that organ. Somewhat similar results relative to the differences in stress-induced metallothionein accumulation in liver and kidney were reported by Oh *et al.* (13). These workers found that various stresses which increase hepatic metallothionein have essentially no effect on renal metallothionein concentration. In view of the apparent difference between liver and kidney in terms of glucocorticoid sensitivity, it is then plausible that parenteral iron administration, specifically intraperitoneal injection, represents a potent stress causing the secretion of adrenal glucocorticoids which, in turn, stimulates the synthesis of metallothionein primarily in hepatic tissue. The net effect is the accumulation of metallothionein-bound zinc in the liver. Due to the lower sensitivity of kidney, one might anticipate little or no change in the concentration of metallothionein in this organ, which was in fact the result of the present study. The lack of change of zinc metallothionein in the pancreas may or may not be indicative of a mechanism involving glucocorticoids.

In addition to the possibility of stress as a cause of iron-induced metallothionein accumulation, an alternative mechanism involving elevated blood zinc as effected by parenteral iron is worthy of comment. Since zinc is recognized as a potent inducer of metallothionein in hepatic tissue, any treatment which results in an elevation of blood zinc may then indirectly effect an increase in tissue metallothionein. The administration of iron, a highly reactive element, may result in the liberation

of tissue zinc. We recognized this possibility due to a casual observation in the present study that a small percentage of the chicks treated with iron developed local tissue necrosis. Damaged tissue may release zinc, which may be the direct cause of increased hepatic metallothionein. Our data on the tissue specificity of iron effects, however, do not support such a conclusion. Elevated blood zinc would undoubtedly result in a general increase of metallothionein in each of the three tissues examined. Since this was not the result of our study, it is unlikely that elevated blood zinc is a causal factor.

In conclusion, the present work has demonstrated that intraperitoneal injections of various iron compounds results in a dramatic increase in hepatic zinc metallothionein. Other tissues such as kidney and pancreas, which are sensitive to metal-induced metallothionein synthesis, did not respond. Elevated hepatic iron, either from injection or feeding, was not correlated with iron-induced metallothionein accumulation. We suggested that stress-induced changes in adrenal cortical hormone secretion may account for the observed changes in hepatic metallothionein concentration.

The author is truly indebted to Dr. Curtis S. Fullmer for conducting the amino acid compositional analysis. It was performed at the Cornell University Amino Acid Analysis and Protein Sequencing Facility. This aspect was supported by D.O.E. Contract EV-02792 awarded to R. H. Wasserman.

1. Margoshes M, Vallee BL. A cadmium protein from equine kidney cortex. J Amer Chem Soc **79**:4813–4814, 1957.
2. Richards MP, Cousins RJ. Mammalian zinc homeostasis: Requirement for RNA and metallothionein synthesis. Biochem Biophys Res Commun **64**:1215–1223, 1975.
3. Kojima Y, Kagi JHR. Metallothionein, Trends Biochem Sci 3:90–93, 1978.
4. Chen RW, Whanger PD, Weswig PH. Biological function of metallothionein. I. Synthesis and degradation of rat liver metallothionein. Biochem Med **12**:95–105, 1975.
5. Nordberg GF, Piscator M, Lind B. Distribution of cadmium among protein fractions of mouse liver. Acta Pharmacol Toxicol **29**:456–470, 1971.
6. Webb M. Binding of cadmium ions by rat liver and kidney. Biochem Pharmacol **21**:2751–2765, 1972.
7. Bremner I, Young BW. Isolation of (copper, zinc)—thioneins from the livers of copper injected rats. Biochem J **157**:517–520, 1976.
8. Durnam DM, Palmiter RD. Transcriptional regulation of the mouse metallothionein-I gene by heavy metals. J Biol Chem **256**:5712–5716, 1981.
9. Piotrowski JK, Trojanowska B, Wisniewska-Knypl JM, Bolanowska W. Mercury binding in the kidney and liver of rats repeatedly exposed to mercuric chloride; induction of metallothionein by mercury and cadmium. Toxicol Appl Pharmacol **27**:11–19, 1974.
10. Etzel KR, Shapiro SG, Cousins RJ. Regulation of liver metallothionein and plasma zinc by the glucocorticoid dexamethasone. Biochem Biophys Res Commun **89**:1120–1126, 1979.
11. Failla ML, Cousins RJ. Zinc accumulation and metabolism in primary cultures of adult rat liver cells. Regulation by glucocorticoids. Biochim Biophys Acta **543**:293–304, 1978.
12. Klaassen CD. Induction of metallothionein by adrenocortical steroids. Toxicology **20**:275–279, 1981.
13. Oh SH, Deagen JT, Whanger PD, Weswig PH. Biological function of metallothionein. V. Its induction in rats by various stresses. Amer J Physiol **234**:E282–E285, 1978.
14. Bremner I, Davies NT. The induction of metallothionein in rat liver by zinc injection and restriction of food intake. Biochem J **149**:733–738, 1975.
15. Sobocinski PZ, Canterbury WJ, Jr, Mapes CA, Dinterman RE. Involvement of hepatic metallothionein in hypozincemia associated with bacterial infection. Amer J Physiol **234**:E399–E406, 1978.
16. Swerdel M, Cousins RJ. Characterization of the induction of metallothionein in liver by isopropanol. Fed Proc **42**:821, 1983.
17. Piotrowski JK, Szymanska JA, Influence of certain metals on the level of metallothionein-like proteins in the liver and kidneys of rats. J Toxicol Environ Health **1**:991–1002, 1976.
18. Winge DR, Premakumar R, Rajagopalan KV. Metal-induced formation of metallothionein in rat liver. Arch Biochem Biophys **170**:242–252, 1975.
19. Szymanska JA, Mogilnicka EM, Kaszper BW. Binding of bismuth in the kidneys of the rat: The role of metallothionein-like proteins. Biochem Pharmacol **26**:257–258, 1977.
20. Eaton DL, Stacy NH, Wong K-L, Klaassen CD. Dose-response effects of various metal ions on rat liver metallothionein, glutathione, heme oxygenase, and cytochrome P-450. Toxicol App Pharmacol **55**:393–402, 1980.
21. McCormick CC. The influence of iron loading on hepatic and pancreatic zinc metabolism in the chick. Fed Proc **41**:782, 1982.
22. McCormick CC. Induction and accumulation of metallothionein in liver and pancreas of chicks given oral zinc: A tissue comparison. J Nutr **114**:191–203, 1984.

23. Davis BJ. Disc electrophoresis—Method and application to human serum proteins. NY Acad Sci **121**:404–427, 1964.
24. Hirs CHW. Determination of cystine as cysteic acid. In: Hirs CHW, ed. Methods of Enzymology. New York, Academic Press, Vol 11:pp59–62, 1967.
25. Fox MRS, Briggs GM. Salt mixtures for purified-type diets. III. An improved salt mixture for chicks. J Nutr **72**:243–250, 1960.
26. Snedecor GW, Cochran WG. Statistical Methods. Ames, Iowa Univ Press, pp258–296, 1967.
27. Oh SH, Nakaue H, Deagen JT, Whanger PD, Arscott GH. Accumulation and depletion of zinc in chick tissue metallothioneins. J Nutr **109**:1720–1729, 1979.
28. Weser U, Rupp H, Donay F, Linnemann F, Voelter W, Voetsch W, Jung G. Characterization of Cd, Zn-thionein (metallothionein) isolated from rat and chicken liver. Eur J Biochem **39**:127–140, 1973.
29. Kojima Y, Berger C, Vallee BL, Kagi JH. Amino acid sequence of equine renal metallothionein-1 B. Proc Natl Acad Sci USA **73**:3413–3417, 1976.
30. Buhler HF, Kagi JH. Human hepatic metallothioneins. Fed Eur Biochem Soc Lett **39**:229–234, 1974.
31. Yoshikawa H, Ohta H. Interaction of metals and metallothionein. In: Foulkes EC, ed. Biological Roles of Metallothionein. New York, Elsevier/North-Holland, pp11–24, 1982.
32. Kotsonis FN, Klaassen CD. Comparison of methods for estimating hepatic metallothionein in rats. Toxicol Appl Pharmacol **42**:583–588, 1977.
33. Macara IG, Hoy TG, Harrison PM. The formation of ferritin from apoferritin—Inhibition and metal ion-binding studies. Biochem J **135**:785–789, 1973.
34. Macara IG, Hoy TG, Harrison PM. Inhibition by zinc ions of ferritin formation in vitro. Biochem Soc Trans **1**:102–104, 1973.
35. Price D, Joshi JG. Ferritin: A zinc detoxicant and a zinc ion donor. Proc Natl Acad Sci USA **79**:3116–3119, 1982.
36. Hager LJ, Palmiter RD. Transcriptional regulation of mouse liver metallothionein-I gene by glucocorticoids. Nature (London) **291**:340–342, 1981.

Received June 10, 1983. P.S.E.B.M. 1984, Vol. 176.
Accepted April 19, 1984.