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The Ontogeny and Induction by Zinc of Hepatic Chick Embryo Metallothionein[1] (42706)

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Abstract. To avoid the maternal influences inherent in mammalian models, the chick embryo was examined for the effects of development on the ontogeny of hepatic metallothionein (MT) induction. Livers from embryos were examined for total and cytosolic zinc (Zn) and copper (Cu), and cytosolic MT on 12, 14, 16, and 18–21 days of incubation (DI) as well as 1, 7, and 14 days posthatch (ph). Cytosolic Zn levels fell, from a high on 12 DI (45.04 ± 1.05 ng/μg DNA), to 25.79 ± 1.05 ng/μg DNA on 19 DI and then increased to 44.73 ± 3.47 ng/μg DNA by 1 day ph. Cytosolic Cu followed a reverse pattern, with a high of 24.10 ± 0.74 ng/μg DNA on 16 DI. Both ^{109}Cd radioassay analysis and gel filtration chromatography of hepatic cytosols showed a developmental pattern for MT similar to that of cytosolic Zn. These results are in contrast to the developmental pattern of the rat. To establish if yolk Zn levels limit hepatic MT in the 1-day neonate, the yolks of unincubated fertile eggs were supplemented with 26, 52, or 78% of the endogenous Zn (768 μg/yolk). As a result, hepatic MT of 1-day neonates increased by 3.9-, 4.7-, and 7.1-fold, respectively. Thus the initial level of Zn in the yolk has a significant impact on the final concentration of MT in neonatal liver. A study of MT induction in the 18-DI embryo revealed that yolk sac administration of Zn (550, 2750, 4950 μg/yolk) increased hepatic MT by 5.8-, 23-, and 39-fold, respectively. This demonstrates the inducibility of MT even at the point of minimum endogenous MT (18 DI). In summary, our results show that (1) a marked difference exists between the developmental patterns of MT in avian and mammalian species, and (2) chick embryo hepatic MT is highly responsive to exogenous Zn introduced into the yolk.

Metallothionein (MT) is a small, cysteine-rich, metal binding protein which is thought to have a role in normal zinc (Zn) homeostasis. This has been proposed based partly on the observation that hepatic MT levels are elevated during several physiologically relevant states: fasting (1), various stresses (2), and development (3, 4). The fact that MT levels are increased during the late fetal or early neonatal period in a number of species (4), coupled with the ability of MT to activate apo-enzymes *in vitro* (5), has led researchers to suggest MT may be used to store Zn for use in periods of high demand during development (6, 7, 8). Most studies investigating the role of MT in development have used mammalian models and have treated the dam by various means to affect fetal MT (9, 10, 11). Observations from a mammalian model, however, are confounded by maternal influences. This has been particularly well demonstrated by Quaife *et al.* (12). They showed that the large increases in maternal glucocorticoids, which are seen as parturition approaches, account for a large part of the elevation in fetal mouse MT mRNA seen during that same period. This finding accentuates the need to remove maternal influences when examining the role of MT in Zn homeostasis during fetal development.

To eliminate maternal influences, we used the self-contained system of the developing chick egg to examine changes in hepatic MT which occur during the later half of incubation (Days 12–21) as well as to determine the effects of supplemental Zn on those levels. We report a biphasic developmental pattern of hepatic MT which is markedly different than that previously reported for either the rat (13) or the chick (14). We also demonstrate the responsiveness of hepatic MT levels in the developing embryo liver to supplemental yolk Zn as a result of either acute

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or extended exposure. These data support the contention of Richards and Steele (15) that the avian embryo has great utility for studying trace element metabolism during development.

Materials and Methods. *General.* Fertile eggs from Cornell K-strain white leghorns were incubated at 37.5°C and 85% humidity, with rotation occurring once an hour. Candling for fertility was routinely conducted at 10 days of incubation (DI). Zinc solutions were made by mixing Zn acetate with sterile distilled deionized water in sterile glassware.

For the determination of total liver Cu and Zn, tissues were dried at 100°C for 24 hr and digested by boiling in nitric acid followed by perchloric acid. For this procedure, 1 g of dried tissue and 10 ml of concentrated nitric acid were combined in a round bottom digestion flask. The sample was boiled until 5 ml remained and was then cooled. An additional 7–8 ml of nitric acid was then added and the sequence was repeated a second time (boil to 5 ml, add 8 ml nitric, boil to 5 ml). When the volume reached 5 ml for the third and last time, 5 ml of concentrated perchloric acid was added to the cooled flask. The mix was boiled until all of the nitric acid was dissipated. The remaining perchloric acid was carefully boiled off until only 2–3 ml remained. This solution was then transferred to a 10-ml volumetric flask and brought up to volume with distilled deionized water. Following further dilution, samples were analyzed for Cu and Zn concentration by atomic absorption spectrophotometry (AAS, Varian Instrument Co.). Metal concentrations per gram dry weight were reajusted to a wet tissue basis, after which they were divided by the DNA value per gram of fresh tissue and expressed as nanograms per microgram DNA.

Pooled liver samples were homogenized in 3 vol of ice-cold buffer (0.25 *M* sucrose, 0.01 *M* Tris, 1% Na azide, 5 m*M* dithiothreitol, pH 7.4) and centrifuged at 100,000*g* for 60 min, and the supernatant (cytosol) was removed. Cytosols were stored under nitrogen gas as a precaution against oxidative degradation of MT. Diluted cytosols were analyzed for Cu and Zn concentration by AAS. MT levels were determined on heat-treated cytosols (2 min, 100°C) by the ^{109}Cd radioassay of Eaton and Toal (16). These results were confirmed by gel filtration of pooled cytosolic samples (Sephadex G75 superfine, 2.5 × 60-cm column, Pharmacia Inc., Piscataway, NJ). DNA analysis was performed on fresh tissue samples at each time point in the developmental study to account for variations in cell number per gram liver. Sample preparation was by the perchloric acid method of Shibko *et al.* (17). Total DNA was determined by a modified diphenylamine procedure (18). Sample means for total and cytosolic metals or cytosolic MT were then adjusted to account for differences in DNA content at each time point.

Data were analyzed using the analysis of variance procedure available in PROC GLM of the SAS statistical package (19). Differences in treatments were determined by sets of relevant orthogonal contrasts whenever possible. Differences between all treatment means were determined using the Waller–Duncan Bayesian *K*-ratio *t* (LSD) rule (20).

Developmental profile for MT, Cu, and Zn. At 12, 14, 16, and 18–21 DI embryos were removed from the egg and killed by decapitation. After chicks hatched (Days 1, 7, and 14), they were killed via cervical dislocation. Livers, minus gall bladders, were quickly removed and placed on ice. Five grams of liver were pooled to make a single observation, four observations per time point. This resulted in varying numbers of individual embryos per time point: from 45 livers per observation at 12 DI to 5 livers at 1 day posthatch (ph). The pooled tissue samples were frozen at −20°C until later analysis for cytosolic Zn, Cu, and MT, as well as total hepatic Zn and Cu. In addition, at each time point five yolk sacs (plus contents) were removed, stripped of surrounding membranes, dried, and later analyzed for the determination of yolk Zn and Cu.

Supplementation of yolks with Zn (chronic exposure). Prior to incubation, eggs were weighed and assigned to one of five treatment groups: no treatment (control), 0.9% NaCl, or 200, 400, or 600 μg Zn per egg. Test solutions, at a constant volume of 0.1 ml/egg, were administered directly into the yolk. To accomplish this, eggs were candled to locate the yolk and a small hole was made in the shell directly over the yolk shadow using

a sterile 1.5-in., 18-gauge needle. Solutions were then introduced into the yolk using a sterile 1-ml syringe and a 1-in., 23-gauge needle. The hole was sealed using a small drop of a quick drying glue and the egg was set for incubation. One day following hatch, chicks were killed by cervical dislocation and livers removed and frozen for later analysis of Cu, Zn, and MT.

Acute Zn exposure and embryonic MT. Eggs were incubated under normal conditions until 17 days at which time fertile eggs were assigned to one of four treatment groups: 0.9% Na acetate, 10 mg Zn/kg egg weight (approximately 550 μg per egg), 50 mg/kg (2750 μg/egg), or 90 mg/kg (4950 μg/egg). At this time in chick development, the yolk sac rests between the legs of the embryo, at the bottom of the egg. A high-intensity candling lamp was used to ascertain the positioning of the embryo and solutions were administered as described above. Following introduction of treatments into the yolk sac, eggs were allowed to incubate for 24 hr, after which embryos were sacrificed by decapitation. Livers were removed and frozen for later analysis.

Results. Total hepatic Zn and Cu, as well as cytosolic Zn and Cu, followed an inverse relationship during the second half of incubation (Fig. 1). Cytosolic Zn levels started high (45.04 ± 1.05 ng/μg DNA at 12 DI) and fell to a low of 25.79 ± 1.05 ng/μg DNA by 19 DI. The level of Zn then increased by 1 day ph to a level of 44.73 ± 3.47 ng/μg DNA. Cytosolic Cu, on the other hand, was initially low (4.95 ± 0.21 ng/μg DNA 12 DI), increased to a peak of 24.10 ± 0.74 ng/μg DNA at 16 DI, and then fell to a low of 3.79 ± 0.42 ng/μg DNA by 1 day ph. The elevation in total and cytosolic Cu corresponds to a greater decline of yolk Cu seen during that same period (Fig. 2). G75 superfine chromatography of hepatic cytosols demonstrated that almost all of the change in cytosolic Zn occurred in the MT region, whereas changes in cytosolic Cu were reflected in both the high MW and MT peaks (Fig. 3).

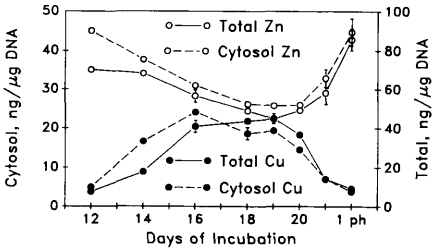

FIG. 1. Total hepatic and cytosolic zinc (Zn) and copper (Cu) concentration during development. For total hepatic metals, pooled hepatic tissue from embryos during various stages of development were dried and subjected to nitric:perchloric digestion. Cytosolic samples were obtained from fresh portions of these samples. Digested samples and cytosolic fractions were analyzed for Zn and Cu by AAS. Metal concentrations were corrected for DNA level to account for changes in cell number per gram of tissue. Refer to Materials and Methods for specific details of the analytical procedure. Each point represents the mean ± SEM of four observations.

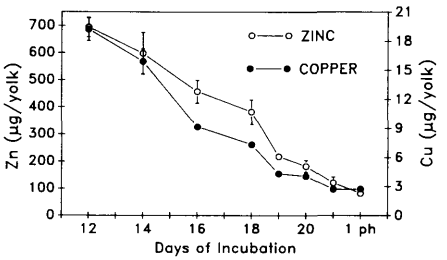

FIG. 2. Changes in total yolk metal levels during the later half of development. At several time points during development embryos were killed and yolk sacs were removed. Dried yolks were nitric:perchloric digested and analyzed for Zn and Cu by AAS. Each point represents the mean ± SEM of five observations.

During development, overall changes in MT content followed the same pattern as seen in cytosolic Zn. This was true whether MT was determined by G75 superfine chromatography (which is a measure of metal associated with MT) or ^{109}Cd radioassay analysis (Fig. 4). Regardless of the method used, cytosolic MT levels range from 2.3-fold higher than 14-day ph levels (at 12 DI and 1 day ph) to levels of only 53% of 14-day ph levels on 18 and 19 DI. After chicks hatched, cytosolic MT fell so that by 14 days ph values had returned to levels commonly found in adult (>28 days ph) male chicks.

Analysis of unincubated, fertilized eggs demonstrated that 94.9% of total egg Zn (809.5 ± 21.9 μg/58.8 g egg) and 58.1% of total egg Cu (38.4 ± 3.5 μg/egg) was found in the yolk. Because Zn is found primarily in

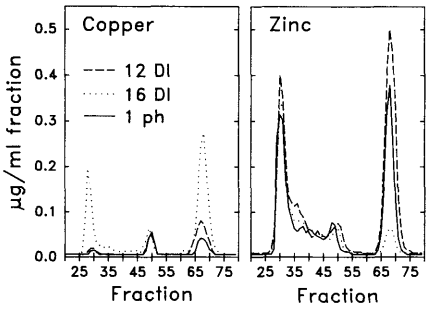

FIG. 3. Sephadex G75 superfine chromatography of hepatic cytosols from various time points during chick embryo development. Liver cytosols from each time point were pooled and 3 ml of the pool was loaded on a 2.5 × 60-cm column of Sephadex G75 superfine and eluted with 0.002 *M* Tris-acetate (pH 7.4). Eluent was collected in 3.07 ml fractions and subsequently analyzed for Cu and Zn levels by AAS. The elution volume for Zn MT (V_e MT = 205.7 ml) was determined by using DEAE-purified chick liver MT.

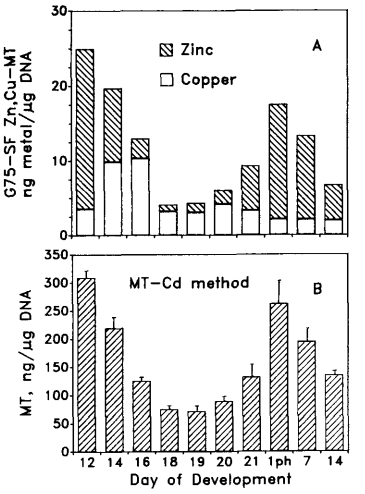

FIG. 4. (A) Developmental changes in the level of Zn and Cu bound to the MT region of G75 superfine chromatograms. Hepatic cytosols from each time point examined were pooled and 3 ml of this pool was loaded to a calibrated column of Sephadex G75 superfine (2.5 × 60 cm). Three-milliliter fractions were collected and analyzed for Zn and Cu by AAS. Results are expressed as ng of Zn and Cu as MT/µg DNA. (B) ^{109}Cd radioassay analysis of hepatic cytosols from embryos during development. Cytosolic fractions of pooled hepatic tissue were analyzed for MT concentrations using the ^{109}Cd radioassay. Results were corrected for variations in DNA levels per gram of tissue. Each time point represents the mean ± SEM of four observations.

the yolk, we chose to supplement Zn via this route rather than through the air sac. In our first study, we supplemented the yolks of unincubated, fertilized eggs with Zn and examined the effects on MT following the full term of incubation (1 day ph). As shown in Fig. 5, our data demonstrate that the introduction of 200, 400, or 600 µg Zn resulted in the elevation in hepatic MT by 3.9-, 4.7-, and 7.1-fold, respectively (neonatal hepatic MT = 0.246 (yolk Zn dose) + 31.79; r^2 = 0.979).

Figure 4 reveals that the level of existing MT was at a minimum (significantly below the level found in 14-days ph liver) at 18–19 DI. This led us to question whether the 18-day-old embryo exhibits a diminished capacity for the accumulation of MT. To test this, we injected from 550 µg (75% of the initial level of yolk Zn) to 4950 µg Zn (675%) into the yolk sac of 17-day-old embryos *in ovo*. Figure 6 shows that the exogenous Zn was readily incorporated into hepatic MT within 24 hr. Levels of MT were 5.9-, 23.2-, and 39.4-fold higher for the 550-, 2750-, and 4950-µg Zn groups, respectively (18-day hepatic MT = 0.06 (yolk Zn dose) + 10.78, r^2 = 0.999).

Discussion. The developmental pattern of MT reported herein for the chick embryo is considerably different from profiles (both MT protein and MT mRNA) which have

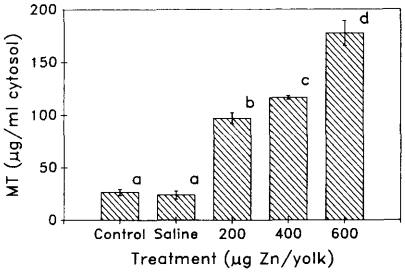

FIG. 5. The effect of supplemental Zn, injected into yolks prior to incubation, on hepatic MT concentrations of 1-day-old chicks. Prior to incubation, fertile eggs were administered one of four levels of supplemental Zn: none or 200, 400, or 600 µg. The eggs were then incubated without further intervention. Day-old chicks from each group were killed and their livers were saved as 5-g pools to give four statistical observations per treatment. Cytosols from these pools were analyzed for MT using the ^{109}Cd radioassay. Bars represent a mean ± SEM of four observations. Different superscripts denote statistical significance ($P < 0.05$).

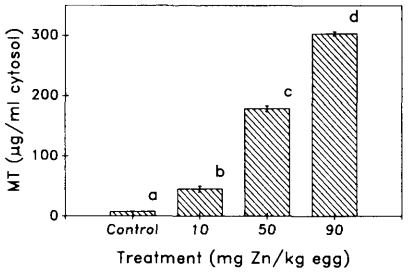

FIG. 6. The effect of exogenous Zn, given in the yolk sac at 17 DI, on hepatic MT levels of 18-day embryos. On Day 17 of incubation, the yolk sacs of developing chick embryos were supplemented with one of four levels of Zn: none or 550, 2750, or 4950 μg. After 24 hr embryos were killed and livers were removed and pooled (5 g/pool, four observations/treatment). Cytosols from these pools were analyzed for MT using the ^{109}Cd radioassay of Eaton and Toal (16). Bars represent means ± SEM of four observations. Different superscripts denote statistical significance ($P < 0.05$).

been previously reported either for mammals (3, 4, 7, 13) or for the chick (14). Using the turkey embryo, *in ovo,* Richards *et al.* (21) have presented a partial profile which corresponds well with the early portion of our profile. Our profile, as seen in Fig. 4, demonstrates that there are two times during development when MT levels are elevated (2- to 2.3-fold greater than those of adults): at 12 DI and at 1 day ph. Equally interesting is our observation that hepatic MT is only 53% of adult levels at 18 DI, indicating that MT bound Zn is variable during development. We also noted that elevated posthatch levels of MT were transient; they declined rapidly so that by 14 days ph, adult levels of 135.8 ± 6.5 ng MT/μg DNA were reached (Fig. 4). The fact that the level of MT changes during development suggests two possible mechanisms: (1) the level of MT is responding only to hepatic changes in metal concentrations, and (2) modest changes in trace mineral requirements occur during hepatic development and MT levels change to sequester metals to meet these needs. Our data do not allow us to differentiate between the two possibilities.

In another study on the chick embryo, Sandrock *et al.* (14) found that the percentage ^{115m}Cd bound per milliliter of supernatant from heat-treated homogenates increased progressively from Day 15 of incubation until 1 day posthatch. These levels were reported to remain high during the first week of life. However, there appeared to be little or no correlation between total Zn levels and ^{115m}Cd binding in these supernatants suggesting their method may not be measuring only MT.

Sandrock *et al.* (14) suggested that an elevation in hepatic Cu at 16 DI stimulated the accumulation of hepatic MT. While we also observed an increase in hepatic Cu at this time point, our findings suggest the opposite effect; i.e., as cytosolic Cu increases, total hepatic MT and cytosolic Zn fall. Some possible reasons for the discrepancies include differences in the preparation of the cytosolic fraction used for the analysis of MT (Sandrock *et al.* used the low-speed supernatant from a heat-treated homogenate while we use a 100,000*g* supernatant of nonheat-treated homogenates) and the poor separation of Zn and ^{115m}Cd binding proteins these researchers obtained following G-50 chromatography (which may include additional, non-MT Cd/Zn binding proteins). A recent paper by Chakraborty and Biswas (22) failed to detect significant amounts of MT in the 14-DI chick embryo using SDS–PAGE on carboxymethylated cytosols. However, it is possible that their method for analyzing MT is not applicable to samples with a low concentration of MT which contain large amounts of Cu in them (as we found in 14-DI embryos).

Our data support a report by Richards and Weinland on the changes in hepatic Zn, Cu, and Fe which occur during turkey embryo development (23). These data (on a per gram of dry weight basis) show a pattern remarkably similar to that of our chick embryo data (DNA corrected data is given in Fig. 1). In this study, no attempt was made to analyze the distribution of Cu and Zn within the liver. However, in an earlier report (21) pertaining to the metal levels of turkey embryos maintained in long-term shell-less culture, this group reported chromatographic data from turkey embryos incubated *in ovo* for 15, 18, 21, and 24 days (of the 28-day total incubation period for turkey eggs). These data also suggest that the hepatic level of MT is high early in development and declines to

a low several days prior to hatch. A report on basal and induced aryl hydrocarbon hydroxylase activity (AHHase) in the chick embryo is pertinent to our findings (24). Hamilton *et al.* found a basal pattern of AHHase activity which is nearly identical to the pattern we are now reporting for MT. This would suggest that basal levels of several inducible proteins follow a similar pattern during chick embryo development. This possibility deserves further consideration.

In addition to the profile we present, our G75 superfine chromatography data demonstrate that the Cu and Zn composition of MT is considerably variable (Fig. 4A). Richards *et al.* (21) observed this previously by studying the turkey embryo liver at 15, 18, 21, and 24 DI. In our study, the ratio of Cu to Zn in the G75 MT peak is 0.16 at 12 DI. This increased to 3.9 at 16 days and fell to 0.14 by 1 day ph. The concept of varying metal composition in MT during development is not unique to avians, however. Bakka and Webb (4) previously observed this phenomenon in a study which compared the MT developmental profiles from several mammalian species. They felt that the ratio varied simply as a reflection of changes in hepatic metal levels. Our data do not contradict this possibility.

Although it is tempting to compare our findings in the chick to those from mammals, there are at least two factors which must be considered before doing so. First, it must be recognized that although the gestation period of rats and mice is identical to the incubation period of the chick egg (21 days), the stages of development do not coincide. The most obvious example is that at the end of their respective incubation/gestation periods, chicks are fully mobile, while rats and mice are maternally dependent and thus relatively immature. The second factor is that the primary energy source for the chick embryo is yolk lipids (particularly in the later half of incubation) which is in contrast to the mammalian fetus which utilizes maternal glucose. These factors are likely to have a profound influence on both the time course and the magnitude of MT levels during chick development.

Quaife *et al.* (12) showed that the level of MT during murine development is highly dependent upon maternal glucocorticoid levels. Using metyrapone as a specific inhibitor of croticosterone synthesis, these researchers found that both fetal and maternal MT mRNA levels could be depressed. It is important to realize, however, that the developmental change in MT was not completely eliminated, suggesting an underlying level of MT synthesis which is not glucocorticoid dependent. Similarly, Andrews *et al.* (3) found that the primary elevation in MT mRNA in the developing mouse occurred before glucocorticoid levels increased (Day 12 of gestation). A recent paper by Andrews *et al.* (25) has sought to elucidate the role of maternal Zn status on the levels of MT and MT mRNA in the developing rat liver. Using deficiencies of various minerals (Cu, Zn, Fe) from 12 days of gestation on, this group was able to show that only Zn deficiency significantly depresses the levels of MT and MT mRNA. While these data demonstrate the importance of maternal Zn status on the ontogeny of fetal MT mRNA (and hence MT) levels, alterations in chromatin structure which occur following Zn deficiency (26) coupled with the importance of Zn in various enzyme systems, particularly DNA and RNA polymerases (27), makes one question whether or not the effects are specific to MT transcription.

It is possible that the late gestational elevation in hepatic MT seen in mammals is a compound effect of two stimuli (one being glucocorticoids, the other undetermined). The pattern of MT accumulation we observe in the chick embryo may reflect a separation of these two stimuli. Kern *et al.* (7) proposed that the partitioning of Zn within MT could signal the shift from cell proliferation to cell differentiation during hepatic development in the rat. The first peak in MT concentration that we see in the chick may be correlated to this function and therefore reflect a need for MT unique to development. In contrast, the second peak may be related to the stress of hatching. Starting at Day 19 of incubation, the yolk sac begins to be internalized and the embryo begins to position itself for hatching (28). The last day in the shell is characterized by movement in an attempt to hatch. As strenuous activity is known to induce MT (2), this effort could be

the stimuli for the second peak. This was also suggested to be the reason for the increases in hepatic Zn seen toward the end of turkey embryo development (23).

Several studies have focused on the induction of fetal MT by exogenous metals using mammalian models (9, 29–31). Mercer and Grimes (11) used these observations, and particularly the possibility that Cd is a specific inhibitor of placental Zn transport, to suggest that fetal Zn is not involved in the stimulation of fetal MT mRNA levels just prior to parturition. Another possibility is that maternal Cd exposure adversely affects placental transfer of all nutrients. For example, Samarawickrama and Webb (32) demonstrated that maternal Cd exposure as low as 1.25 mg/kg adversely affects placental circulation and inhibits embryonic DNA synthesis. This exemplifies the problems inherent in interpreting data derived from mammalian models.

The use of an avian embryo/egg provides an opportunity to examine the influence of exogenous metals on MT levels during development without the concern of maternal and placental influences (15). Prasad and Datta (33) found that 10 μmole Cd/kg egg weight could induce the synthesis of hepatic MT in 14-day-old embryos. The method by which metals were introduced into the egg was not described. Using an *ex ovo* system of maintaining turkey embryos in long-term shell-less culture Richards (34) demonstrated that in 17-day cultured embryos (4 days *in ovo*, 13 days *ex ovo*) synthesis of hepatic and yolk sac MT was increased by exogenous Zn injected into the chorioallantoic membrane. However, these results are confounded by the well-characterized alterations in trace mineral metabolism (i.e., reduced hepatic Cu and elevated hepatic Zn and Fe) which occur in the shell-less culture (21).

In our studies, we added Zn directly into the yolks of fertilized eggs or into the yolk sacs of developing embryos. As 95% of total egg Zn is found in the yolk, we felt that this created a more physiologically relevant method of exposure. In our first experiment, yolks of fertile unincubated eggs were supplemented with Zn to levels which were 26–78% greater than those of nontreated controls (768 μg Zn). Our goal was to determine whether neonatal hepatic MT levels were related to the initial level of Zn in the yolk. We found that this was indeed the case; hepatic MT, examined in 1-day neonates which received supplemental Zn, responded progressively to 200, 400, and 600 μg added Zn (3.9-, 4.7-, and 7.1-fold, respectively; Fig. 5). This indicates that at least one of the factors affecting neonatal hepatic Zn, and particularly MT-bound Zn, is the initial concentration of Zn in the yolk.

Our second experiment was conducted to determine the potential for MT induction in 18-day-old chick embryo liver. Normally the levels of Zn and MT at this point in development are low (Fig. 4). This suggested that the ability to produce MT may be compromised at this point during development. Our results illustrate that even at that point when its levels are lowest, the concentration of hepatic MT is responsive to exogenous Zn (Fig. 6). Although Experiments 1 and 2 are very different (the first being chronic, the second acute exposure), it is interesting to compare the accumulation of MT following the addition of similar amounts of metal to the yolk. In Experiment one, the addition of 600 μg Zn to the yolk resulted in hepatic MT levels of 175 μg/ml cytosol (1.837 μg/μg DNA) at 1 day ph. In contrast, a similar dose of Zn given at Day 17 of incubation resulted in hepatic MT levels less than 30% as great (45.05 ± 2.8 μg/ml or 0.423 μg/μg DNA). This may be related to a decline in Zn transport from the yolk at this point in development. A reduced inducibility from 15 and 19 DI was also observed by Hamilton *et al.* (24) in their studies on basal and induced aryl hydrocarbon hydroxylase activity in the chick embryo, suggesting that this trend reflect developmental changes not unique to MT.

Overall, our data show a developmental pattern of hepatic MT levels which has been demonstrated previously only in part (21). The appearance of two periods of elevated hepatic MT suggests that this protein may possess a function which is unique to development. Unfortunately, our results do not rule out the possibility that hepatic MT levels are responding simply to variations in total hepatic metal concentrations. An answer to this question may come from a study of fac-

tors influencing the movement of Zn and Cu from the yolk. By introducing exogenous Zn into the yolk, either prior to incubation or during late development, we demonstrated that MT was highly responsive to changes in yolk Zn. While avian embryo models have been previously utilized by others (21–23, 33), our data reaffirm the previously recognized potential (15) of the avian embryo, *in ovo,* for the study of MT's role during development.

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