

A single form of metallothionein is present in both heavy metal induced and neonatal chicken liver

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Abstract. Multiplicity of metallothionein and their genes in higher animals are documented extensively in recent literature. In contrast, chicken liver produced apparently a single form of metallothionein upon heavy metal exposure. This protein was purified by gel filtration and ion exchange chromatography and another technique based on heat treatment and acetone fractionation, followed by ion exchange chromatography. In adult uninduced chicken liver the presence of metallothionein was below the detection limit. But, like mammalian system, chicken liver was found to contain high amount of metallothionein at neonatal stage. This naturally occurring neonatal chicken hepatic metallothionein was purified and compared with the heavy metal induced adult hepatic metallothionein. The biochemical and immunobiological comparative analysis of adult and neonatal hepatic metallothionein showed identical characteristics. The neonatal metallothionein expressed naturally was a zinc metallothionein and unlike few other mammalian neonatal metallothionein did not contain any copper. Metallothionein was undetectable in unfertilized eggs, in early embryos, and in postnatal chicken, from 4 weeks after birth. The highest level of this naturally occurring neonatal metallothionein was found in 1–4 day old neonatal liver, which was about 1.5% of the total cytosolic protein. This is the first reported evidence for the presence of ontogenically modulated expression of metallothionein in avian system. Possible biological role of neonatal metallothionein and their cellular interactions has been discussed.

Keywords. Metallothionein; isometallothionein; neonatal; chicken liver; ontogenic-regulation; zinc; biological role.

Introduction

Metallothionein (MT), a low molecular weight (*Mr*), cystein-rich, inducible heavy metal binding protein is present in most of the eukaryotic organisms—from yeast to man (Kagi and Nordberg, 1979; Karin, 1985). A complete picture of its physiological role is not yet established but it appears to protect organisms against toxic heavy metals and may play certain role in the cellular zinc, and copper homeostasis (Foulkes, 1982; Hamer *et al.*, 1985). MT may be involved in defense against stress, infection or radiation damage (Oh *et al.*, 1978; Sobocinski and Canterbury, 1982; Thornalley and Vasak, 1985). The biosynthesis of MTs are increased, when organism or cultured cell lines are exposed to heavy metals such as zinc, copper, cadmium and mercury (Durnam and Palmiter, 1981). In addition to this, MT genes are induced by a plethora of chemical, physical or environmental stimuli which includes glucocorticoid hormones (Failla and Cousin, 1978; Karin and Herschman, 1980), interferon (Friedman and Stark, 1985), interleukin-1 (Karin *et al.*, 1985), bacterial LPS

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Abbreviations used: MT, Metallothionein; *M_r*, molecular weight; AP, acetone purified; PAGE, polyacrylamide gel electrophoresis; DTT, dithiothreitol; HPLC, high performance liquid chromatography; Tris, Tris-(hydroxymethyl) amino methane.

(Durnam *et al.*, 1984) hepatotoxic solvents like isopropanol or carbontetrachloride (Oh *et al.*, 1978; Swerdel and Cousin, 1984), UV, X-ray or γ -ray irradiation (Shiraishi *et al.*, 1983; Lieberman *et al.*, 1983) and starvation, strenuous exercise and other stresses (Oh *et al.*, 1978; Webb and Cain, 1982). The induction is regulated mainly at the level of transcription (Hager and Palmiter, 1981; Richards *et al.*, 1984; Searle *et al.*, 1985; Karin, 1984). A higher level of MT expression during early developmental stages of mammalian species has also been noted (Wong and Klassen, 1979; Piletz *et al.*, 1983; Anderson *et al.*, 1983; Andrews *et al.*, 1984). Thus, MT is a protein of potentially great biological importance; biochemical and genetic studies would contribute significantly in our understanding of its function and regulation.

In our laboratory, the biochemical and immunological comparative analysis of MTs isolated from different species and different tissues showed species specific difference in the ratio of isometallothioneins, difference in the electrophoretic migration as well as some tissue specific immunological variations (Chakraborty and Maiti, 1985). Attention is currently being paid on the multiplicity and microheterogeneity of isometallothioneins and their plausible physiological significance (Wilson *et al.*, 1982; Klausner *et al.*, 1983; Hunziker and Kagi, 1985). The chicken system was found to be unique among higher animals, as they produce apparently only a single form of MT under heavy metal induction. Under similar condition primates produce 6–7 different isometallothionein (Koizumi *et al.*, 1985). The ontogenic regulation of MT has been studied in different mammalian species. Since the early development of avian and mammalian species have certain marked differences, our interest was primarily focussed on the nature of MT in developing chicken liver. The present paper describes the purification, biochemical and immunological characterization of MT in developing chicken liver.

Methods

White leghorn one day old chickens (average body weight 25 g), incubated fertilized eggs and developing neonatal chicks were obtained from State Poultry Farm, Tollygunge, (Govt. of West Bengal).

Purification

For MT induction animals were injected with 0.5–2.0 mg CdCl₂/kg body weight and sacrificed after 18–24 h; livers were collected. Two different methods were used for purification of hepatic MTs. One was the usual and established methodology of gel filtration combined with ion-exchange chromatography (Chakraborty and Maiti, 1985). Another simple procedure for MT purification, based on its unusual heat stability and solvent fractionation behaviour was developed (Winge *et al.*, 1975). Tissue homogenate (1:5w/v) in 10 mM Tris-HCl pH 8.6, 10 mM 2-mercaptoethanol was subjected to ultracentrifugation at 1,000,00 g for 50 min. The clear supernatant avoiding lipid layer was taken and heat-treated at 85°C for 10 min, chilled on ice and centrifuged at 20,000 g for 10 min. The clear supernatant (HTS) was incubated with 1–5 μ Ci ¹⁰⁹ CdCl₂ for 30 min at 25°C followed by selective step-wise acetone fractionation. Ice cold acetone was added dropwise to make the

supernatant 40% (v/v) in acetone, kept on ice for 30–60 min centrifuged at 20,000 g for 30 min. In the supernatant fresh acetone was added to make it 60% (v/v), stand on ice for 1 h, and centrifuged at 20,000 g for 30 min. This 60% acetone supernatant was made upto 80% (v/v) in acetone and kept at 0°C for 3 h and centrifuged. The 60–80% (v/v) acetone pellet was dissolved in 10 mM Tris-HCl pH 8.6 containing 5 mM 2-mercaptoethanol (or for spectrophotometric analysis, in H₂O). This acetone purified fraction (AP) was found to be rich in MT. DEAE-sephadex chromatography of acetone purified fraction in 10 mM Tris-HCl pH 8.6 with a linear gradient of 10–300 mM of Tris-HCl pH 8.6 yielded a single homogeneous form of MT.

Gel electrophoresis

Non-denaturing 7.5% Polyacrylamide gel electrophoresis (PAGE) was done according to Davis (1964) in a slab gel. The gels were stained with 0.25% Coomassie Brilliant blue R-250 and destained as described earlier (Chakraborty and Maiti, 1985). For autoradiography, the gels were covered with Saran Wrap and dried in a LKB-Slab gel drier immediately after electrophoresis and dried gels were exposed to Kodak-X-ray films. Proteins were measured by the Bradford's dye binding assay (Bradford, 1976) using bovine serum albumin as Standard.

Immunization and immunodouble diffusion assay

Rabbits were immunized with purified chicken apometallothionein by method essentially same as that of Granger and Lazarides (1979) the details of which will be published elsewhere. The chicken MT antisera was tested by Ouchterloney immunodouble diffusion. The gels were stained and destained as for Polyacrylamide gels. The results were confirmed with mouse MT antisera which also cross-reacts with chicken MT (Chakraborty and Maiti, 1985).

UV-Spectra

UV absorption spectra of neonatal and Cd induced adult chicken hepatic MT were measured, using 15–30 μg of purified MT in water, in a Shimadzu spectrophotometer. The change in pH was brought about by adding 1(N)HCl dropwise in the cuvette.

In vitro ¹⁰⁹Cd-labelling

Naturally occurring neonatal MT was incubated with 10 μCi carrier free ¹⁰⁹CdCl₂ in 10 mM Tris-HCl pH 8.6 and 0.5 mM dithiothreitol (DTT) at 4°C for 12–16 h in a total volume of 100 μl. The labelled proteins were separated from unbound free ¹⁰⁹CdCl₂ by passing it through a Sephadex G-25 column (0.8×10 cm) equilibrated and eluted with 10 mM Tris-HCl pH 8.6, 0.5 mM DTT. The specificity of the labelling was judged by the autoradiography of 7.5% nondenaturing PAGE of the labelled products.

Sulphydryl group and metal estimation

Sulphydryl groups were determined in purified MT fractions with 5,5'-dithiobis-2-nitrobenzoic acid (DTNB) in a buffered solution of 6 M guanidine hydrochloride and 50 mM EDTA at pH 8.0 (Elmann, 1959), absorbance changes were monitored at 412 nm spectrophotometrically. The metals were analyzed using Varian Atomic Absorbance Spectrophotometer, using standard samples of zinc, cadmium and copper as references. Absorbance was monitored at 213.9 nm for zinc and 324.7 nm for copper in acetylene flame.

High performance liquid chromatography (HPLC) and isoelectric focussing

HPLC separation of MT was carried out in LKB apparatus using ultropac TSK G, 2000 SW column (7.5×300 mm) at 27°C. The elute was monitored at 220 or 206 nm. The flow rate was 0.1 ml/min, and the column was previously calibrated with known M_r protein markers (Suzuki, 1980).

The isoelectric focussing of purified MTs were carried out in 7.5% Polyacrylamide gel rod, containing 2% carrier ampholine (Pharmalyte pI 3–10) in absence of any denaturing agent, and stained with Coomassie Brilliant blue G-250. The pIs were determined from parallelly run control gel rods.

Results

Isolation and purification of MTs

For large scale isolation from sources rich in MT the method based mainly on sephadex G-75 gel filtration followed by DEAE-ion exchanging chromatography gives good yield (Garvey *et al.*, 1982; Chakraborty and Maiti, 1985). For rapid isolation of MT from low amount of starting tissue material, we have adopted a methodology which gives good recovery in short time. This procedure is based upon the known heat stability and acetone precipitation behaviour of MT as described in methods section. We are using this method extensively in our study of the ontogenic regulation of MT synthesis in chicken. Figure 1 shows the typical DEAE-sephadex A-25 elution profile of MTs isolated from mouse, rat, golden hamster and chicken liver, after following identical induction and isolation procedure. The chickens, unlike most other higher animals produces apparently a single form of MT. Weser *et al.* (1973) have earlier isolated and characterized a MT from heavy metal induced chicken liver. They did not focussed on the isometallothionein pattern. The previous studies in our laboratory have clearly demonstrated that the ratio of isometallothioneins differs considerably from species to species and in some species like mouse and golden hamster one form is the major and another minor (Chakraborty and Maiti, 1985). Weser *et al.* (1973) isolated the MT after 14 days of the last heavy metal exposure, which might drastically affect the isometallothionein ratio (if they are really present). In our study, we followed the Standard procedure for induction and isolation, which could separate even 6 different isometallothioneins in human system (Koizumi *et al.*, 1985). The single form of chicken MT was further confirmed by

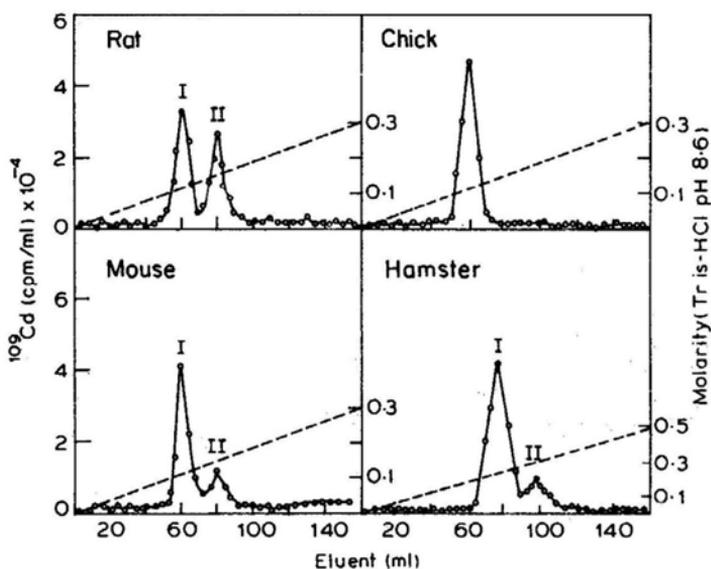


Figure 1. DEAE-sephadex A-25 column (6×1 cm) chromatography profiles of rat, mouse, golden hamster and chicken hepatic heavy metal induced MTs (sephadex G-75 pooled fraction, 3 mg). The proteins were eluted with a linear gradient 10–300 or 500 mM Tris-HCl pH 8.6 at a flow rate of 25 ml/h. The Cd-binding peaks denotes separated isoforms of MTs.

HPLC hooked with gel permeation* and isoelectric focussing. A single band of pI 4.1 was identified in isoelectric focussing gel.

Purification of MT from newborn uninduced chicken liver

The Cd-induced hepatic MT purified by Sephadex G-75 and DEAE-sephadex A-25 chromatography served as the standard for detection and identification of any similar protein in new born chick liver. Figure 2 (A and B) shows the elution pattern for cytosolic MT of Cd-administered chicken liver. Uninduced adult chicken liver subjected to identical isolation procedure did not show the presence of any MT like protein (data not shown). Three day old white leghorn chicken livers, when processed in identical manner, show the presence of a MT like protein. This protein can be easily *in vitro* labelled with ^{109}Cd . The *in vitro* ^{109}Cd labelled protein from neonatal livers on further fractionation in Sephadex G-75 and DEAE-sephadex A-25 chromatography behaved exactly like the hepatic induced MT (figure 2C and D). Neonatal hepatic MT was found to aggregate during lyophilization or prolonged storage. So, acetone fractionation procedure was adopted avoiding sephadex G-75 gel filtration step for neonatal MT purification.

The 60–80% acetone fraction was found to be rich in MT, comparable with Sephadex G-75 pooled protein. This scheme was found to be suitable for the quick isolation of MTs even from very low amount of starting material.

*Column (LKB 2135 Ultropack TSKG-3000 SW 7.5 \times 300 mm).

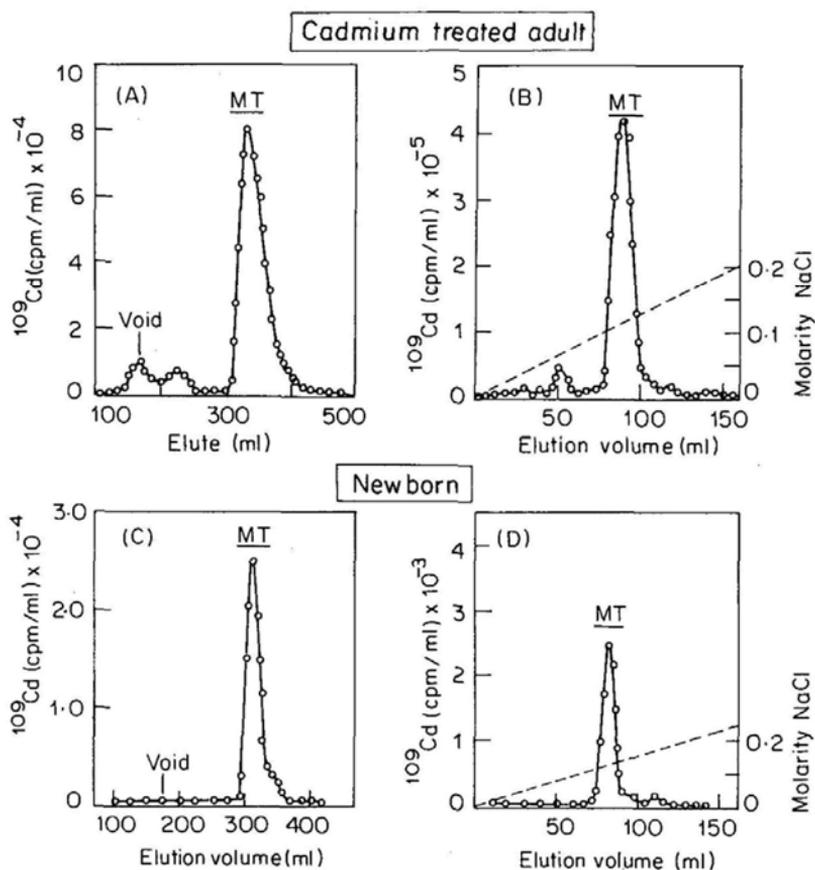


Figure 2. Purification of chicken adult Cd-treated and naturally occurring neonatal hepatic MTs. A and B show the sephadex G-75 column (90×2.6 cm) and DEAE-sephadex A-25 column (6×1cm) elution profile of Cd-treated adult hepatic MTs. C and D show the neonatal *in vitro* ^{109}Cd -labelled chicken hepatic proteins subjected to sephadex G-75 and DEAE-sephadex A-25 chromatography exactly like the adult Cd-induced samples.

Comparison of heavy metal induced and normal neonatal chicken liver MT

An extensive comparative study of the heavy metal induced chicken liver MT with the normal neonatal hepatic MT was carried out. Result of this study is presented in table 1. In neonatal stage and also under heavy metal induction, the chicken was found to be capable of producing apparently a single form of MT. The UV-absorbance spectrum of the neonatal MT was very similar to all other animal MTs and the heavy metal induced MT of its own system (figure 3). The total absence of the aromatic amino acid residues is reflected in the very low absorbancy at 280 nm. The higher absorbancy at 250 nm is due to the metal-thiolate linkages. This 250 nm absorbance is abolished upon acidification due to the acid lability of the metal-thiolate bonds (Furey *et al.*, 1986; Low and Stillman, 1980). The electrophoretic migration (R_f value) of the neonatal and heavy metal induced MTs were identical on polyacrylamide gels in the presence (figure 4B) or absence of sodium dodecyl sulphate (SDS) (table 1). The *in vitro* ^{109}Cd -binding property of the neonatal MT and

Table 1. Comparison of neonatal and heavy metal induced chicken hepatic MT.

Property	Neonatal MT	Induced MT
M_r determined by SDS-PAGE	7-8 K	7-8 K
R_f value in 7.5% non denaturing PAGE	0.42	0.42
M_r as determined by HPLC elution*	10 K	10 K
Metal content per mol of purified protein	6.8 g atom/mol \pm 0.1	6.7 g atom/mol \pm 0.1
No. of SH group/g atom of metal (ratio) per mol of protein	2.8	2.8
Absorbance 250 nm/280 nm ratio	High	High
Ability to <i>in vitro</i> bind ^{109}Cd .	+	+
Ability to cross-react with mouse MT-I antisera	+	+
Isoelectric point (pI)	4.1	4.1

*Due to non-globular shape of MT the M_r determined on size exclusion or gel filtration principle gives higher than actual value. Moreover, at alkaline pH used, isometallothionein separate in this column (data not shown).

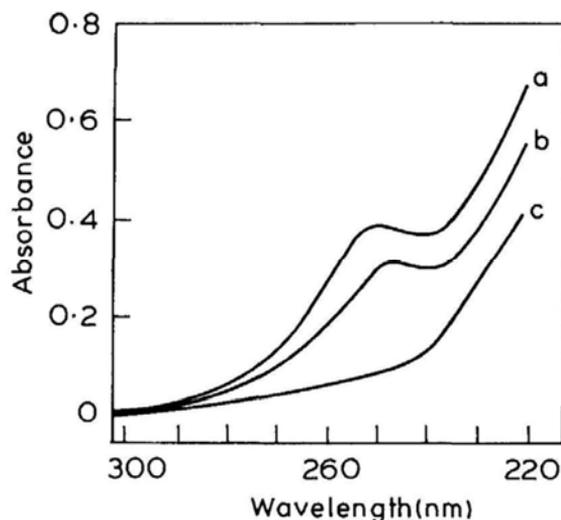


Figure 3. UV-absorption spectrum of neonatal chicken hepatic MT. The curve 'a' shows the spectrum at pH 7.0 of Cd-incubated neonatal MT, the curve 'b' shows the absorption spectra at pH 5.0 and the 'c' shows the UV-spectrum of the same sample at pH 2.0.

its co-migration with the *in vivo* ^{109}Cd labelled Cd-induced chicken MT further confirm this view (figure 4C). Another critical parameter, which can distinguish the MT isoforms are the isoelectric-points, which was also found to be identical for both of the chicken MTs (table 1). The immunological identity of the proteins were evident, as they formed precipitating lines of complete identity in Ouchterlony's immunodiffusion test (figure 5). The cross-reactivity of both the mouse MT-1 and chicken MT antisera with the chicken MT confirm our earlier observation that the mouse and chicken MT share immunological characteristics (Chakraborty and Maiti, 1985). Moreover, the HPLC elution patterns of both the MTs of chicken were same, and when a mixture of them were loaded on the column they are eluted as a single

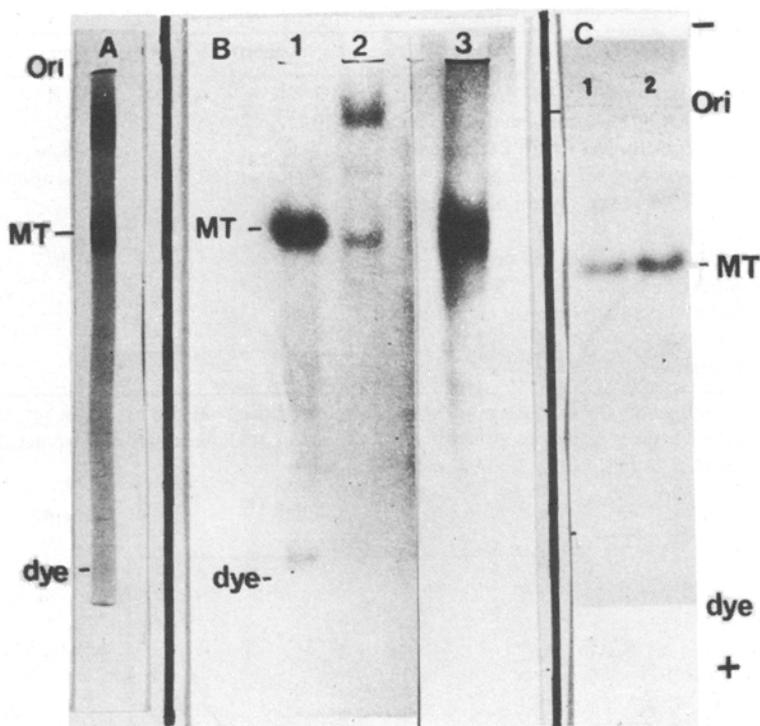


Figure 4. PAGE of chicken hepatic MT. A. Non-denaturing 7.5% PAGE of Cd-treated adult chicken hepatic MT of Sephadex G-75 peak (30 μ g). The MT-band is associated with the ^{109}Cd -radioactivity. B. Non-denaturing 7.5% PAGE of chicken hepatic proteins purified by the acetone fractionation scheme. Lane 1 and Lane 3 show the MTs purified from 1 day neonatal liver (20 μ g) and 20 day old embryonic liver (30 μ g) respectively. Lane 2 shows the Sephadex G-75 MT peak of the neonatal chicken liver (25 μ g). C. The autoradiogram of the 7.5% PAGE separated *in vivo* ^{109}Cd -induced adult chicken liver MT (Lane 1) and the *in vitro* ^{109}Cd -labelled chicken neonatal MT (Lane 2). The panel A and B are the Coomassie-blue stained polyacrylamide gels.

symmetric peak (table 1). The SH group and metal-ion ratio of both the MTs were found to be very comparable (table 1).

Neonatal chicken MT is a Zn-MT

Remarkable differences exist in the nature of the endogenous heavy metals associated with the naturally occurring neonatal hepatic MTs isolated from different animals. Fetal and neonatal mouse, Chinese hamster and rat MT contain both zinc and copper in appreciable amount (Bakka and Webb, 1981). While in the Syrian golden hamster and human fetal liver MT copper is the principal bound metal (Brady *et al.*, 1982). In contrast, the rabbit liver neonatal MT always contains zinc (Klaassen and Wong, 1982). In some species such as human fetas and infants, the zinc: copper ratio in the MT fraction varies with age. The MT isolated from late embryonic and the neonatal

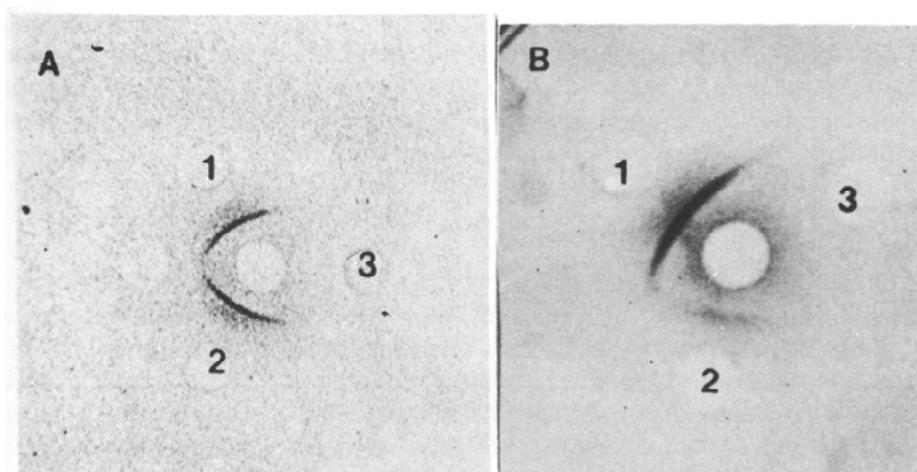


Figure 5. Immunodouble diffusion assay of chicken MTs against mouse or chicken antimetallothionein. **A.** The central well contained rabbit antiserum prepared against chicken hepatic MT. The well 1 and 2 contained purified hepatic MT of Cd-treated adult and uninduced chicken neonatal MT, well 3 contained preimmune serum. **B.** The central well contained rabbit antiserum (immunoglobulin-*G fraction) prepared against mouse hepatic MT-1. The well 1 and 3 contained chicken adult Cd-treated hepatic MT, chicken neonatal MT and uninduced adult chicken AP fraction of proteins.

chicken liver was found to be associated solely with zinc (table 1). There was no measurable copper in these samples. The metal and sulphhydryl content estimated from the same sample of MT showed that approximately all the metal binding sites of chick neonatal MT is saturated with zinc. The role of the neonatal MT in the metabolism and storage of zinc cannot be overlooked in these circumstances. The elevated MT in the neonatal liver has been postulated to serve as a transient store of essential heavy metals like zinc (Bell, 1979; Webb, 1979) and copper (Terao and Owen, 1977). In chicken the physiological involvement and interaction of neonatal MT must be sought in association with zinc and not with copper.

Developmental regulation of MT

In adult uninduced chicken liver MT is below our detection limit. This is reflected in the very low amount of protein in the 60–80% acetone purified fraction and the MT could not be detected even after *in vitro* ^{109}Cd labelling. This is also reflected in immunological assay (figure 5B, well 3). This has earlier been observed in other adult animals not exposed to inducing agents (Winge and Rajagopalan, 1972). After establishing the presence of a naturally occurring MT in 3 day old neonatal chicken liver, detection of hepatic MT during chicken development was studied. The MT was not detectable in both unfertilized eggs and embryos of upto 2 weeks of development. Egg white, egg yolk and the embryo were tested by heat-treatment, acetone fractionation scheme followed by *in vitro* ^{109}Cd -binding. Immunological assay also confirmed this result. This is consistent with the one earlier report, where only Cd-induced MT like protein was detected from 14 day old chick embryo (Prasad and

Datta, 1983). MT seems to appear during late maturation of the embryo and was abundant in 20 day embryo and 1–5 day old neonatal chicken liver. The concentration of protein (MT) declines steadily after the first week of birth and gradually disappears (below the detection limit) from one month old chicken liver but retains its capacity to be induced by heavy metals.

Discussion

We have isolated MT from chicken liver after heavy metal exposure. A naturally occurring MT was also isolated from the neonatal chicken liver. The MT isolated under these two experimental conditions has been found to have one distinct property from those of the mammalian MTs. While all the mammalian MTs are known to be a mixture of at least two isoforms (isometallothioneins) which are separable by DEAE-anion exchange chromatography and in PAGE under non-denaturing condition (Koizumi *et al.*, 1985; Karin, 1985), this avian MT consists of a single isoform. Both the DEAE-chromatography and PAGE pattern of chicken MT conclusively establish this fact. As previous studies in this laboratory have shown that the ratio of isometallothioneins can vary widely, and in some species like mouse and golden hamster, the amount of one isometallothionein is predominant over the other, we have taken special care to see if any minor isometallothioneins are present together with the main chicken hepatic MT or not. The results obtained from ¹⁰⁹Cd-labelling, HPLC, and isoelectric focussing ruled out this possibility. Many mammalian species have been shown to contain large amount of MT in their liver during prenatal and neonatal period (Ryden and Deutsch, 1978; Wong and Klassen, 1979; Bell, 1979, Charles-Shannon *et al.*, 1981). The mammalian hepatic MT at neonatal stage appears to be the mixture of two isometallothioneins like the adult induced ones (Webb and Cain, 1982). However, the validity of this phenomenon outside the mammalian domain has not been checked. We report here, the first evidence of such type of neonatal MT in avian species, containing only one form.

In the case of mammalian MT, whether the isoforms at neonatal stage are identical or different from the heavy metal induced MT isoforms have not been studied so far. We have done few comparative studies between the neonatal and heavy metal induced chicken MT. The biochemical and immunological properties like R_f value in denaturing and nondenaturing polyacrylamide gels, chromatographic elution pattern, HPLC analysis, isoelectric points and metal-sulphydryl ratios and immunological cross-reactivity suggest that these two MTs are identical. Whether there is any microheterogeneity within these isoforms (different species within a single form) can only be ascertained after amino acid sequence analysis which is now in progress.

Among all the vertebrates examined so far the multiple forms of MT have been detected under the experimental condition. However in the case of chicken it seems to be different. But, the number of MT isoforms and their genes are found to be variable among different vertebrates (Hunziker and Kagi, 1985; Klauser *et al.*, 1983; Karin, 1985). The complexity seems to be highest among the primates—where at least 6 different isoforms of MT and 12 genes including few pseudogenes, have been detected (Karin, 1985). Some of these mammalian isometallothioneins might have tissue specific expression and function (Chakraborty and Maiti, 1985; Schmidt and Hamer, 1986).

The developmental regulation of MT biosynthesis in chicken so far studied by us seems to be similar to what has been found in mammalian system (Bakka and Webb, 1981; Piletz *et al.*, 1983; Andrews *et al.*, 1984). The full significance of this phenomenon is yet to be ascertained. Detailed study might reveal some biological involvement of MT. As the neonatal MT is found to be associated with zinc, the essential heavy metal closely associated with protein and nucleic acid biosynthesis, it is tempting to speculate that the function of this neonatal protein is to serve as a transient store of zinc which is toxic at high concentration in free form. The partitioning of zinc between MT and the protein and nucleic acid biosynthesizing machineries during development is one area to be investigated properly. The chicken MT antisera will be used to assay the detailed time course of the change in MT level from embryo to adult chickens. We are also engaged in studying this phenomenon at genetic level through MT-messenger RNA quantitation which will answer whether this elevation of MT is due to MT-gene switching during development. The importance of zinc as a principal regulatory element in gene expression as well as in the cellular adaptation to stress is increasingly being felt (Bruckman and Zondek, 1939; Vallee, 1976; Brady, 1981; Fraker *et al.*, 1977; Good and Fernandes, 1979; Brady, 1982) and so association of neonatal chicken MT with zinc raises several interesting possibilities.

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