The hepatic concentration of a low molecular weight, metal-binding protein in 1- to 4-day-old rats is 20 times that in 70-day-old adults. Biochemical purifications were performed to characterize this metal-binding protein in the liver of 1- to 4-day-old rats. Gel filtration of hepatic cytosol on Sephadex G-75 demonstrated that the major Zn-containing fraction of newborns had a relative elution volume and low absorbance at 280 nm, similar to the metallothionein fraction of Zn-treated adult rats. These fractions were further purified by DEAE-Sephadex A-25 chromatography and two major subfractions (MT-A and MT-B) were obtained from both the newborns and Zn-treated adults, with the corresponding subfractions eluted in buffer of similar conductivity. MT-A and MT-B of newborns also exhibited mobilities on polyacrylamide gel electrophoresis similar to the corresponding subfractions from Zn-treated adults. Both subfractions from the newborns had an abundance of half-cystine and a deficiency in most aromatic amino acids which are characteristics of metallothionein. Therefore, according to all of the criteria commonly used for the identification of metallothionein, these data demonstrate that the metal-binding protein which is highly concentrated in newborn liver is metallothionein. The variation of the concentration of hepatic and renal metallothionein with age was also determined. The hepatic concentration peaks at 1 to 4 days of age and falls rapidly after that, reaching the adult level at 28 days of age. However, the renal concentration showed no variation with age.

Since the first isolation of a cadmium-binding protein from equine renal cortex by Margoshes and Vallee (1), similar proteins have been isolated from the liver and/or kidney of humans (2, 3), rats (4, 5), mice (6), rabbits (7), sheep (8), pigs (9), California sea lions (10), and chickens (11). These proteins have a low molecular weight (6,000 to 10,000) and are referred to as MT due to their association with metals and their high cysteine content (12, 13). Evidence indicates that the binding of cadmium and zinc to MT may involve the sulfhydryl groups of cysteine residues (2, 11, 12).

The concentration of MT in tissues can be increased by administration of metals such as cadmium, zinc, mercury, silver, copper, and bismuth (9, 14-16). In addition, this protein can also be induced by food restriction (17), various stresses (18), and a number of alkylating agents (19). The physiological role of this protein is not known. However, it has been speculated that the binding of metals to MT provides a detoxification mechanism against certain heavy metals (20-22). It has also been suggested that MT may function in the homeostasis of essential trace elements, such as copper and zinc (23-25).

It has been shown that, as compared to adults, hepatic concentrations of zinc and copper are higher in bovine, sheep, and human fetuses (26-28) and also in newborn rats (29-31). Since both zinc and copper bind to MT (2, 12), the high level of zinc and copper in newborn rat liver could be due to the presence of higher levels of MT in newborns relative to adults. This is especially possible for zinc, since hepatic zinc is located primarily in the cytoplasm (32, 33) and metallothionein is a cytoplasmic protein (4, 5). Metal-binding proteins have been noted in the cytoplasm of the liver of newborn rats (34-36) and human and rat fetuses (37, 38). In these studies, however, the proteins were neither isolated and identified nor quantitated. Thus, the objectives of this investigation were 3-fold. The first was to compare the concentration of metal-binding proteins in newborn rat liver with that in adult liver. Second, isolation and identification of the metal-binding protein from newborn rat liver were performed. Finally, it was of interest to estimate the hepatic and renal concentrations of metal-binding protein during rat development.

MATERIALS AND METHODS

Animals—Sprague-Dawley rats with free access to Purina Lab Chow and tap water were used throughout. Rats 5 weeks of age and younger were born in our laboratory, while 10-week-old male rats were purchased from Bio-Lab (White Bear, MN). The mother and offspring were kept in plastic cages with Bed-O-Cobbing bedding (Andersens Cob Division, Delphi, IN).

Estimation of the Concentration of Metal-binding Protein in Newborn Rat Liver—To estimate the concentration of metal-binding protein in newborn rat liver as compared to that in adults, the method of Potrowski et al. (39) as described by Kotsonis and Klaassen (40) was used. Livers were removed from rats 1 to 4 days old and 70 days old under ether anesthesia. The livers were homogenized in 1.15% KCl (7 ml/g) with a motor-driven Potter-Elvehjem homogenizer with a Tellon pestle. Mercuric chloride (550 μg of Hg) labeled with 203Hg (2 mCi/mmol, New England Nuclear) was added to 3.5 ml of liver homogenate. One milliliter of 1% trichloroacetic acid was added and then the amount of 203Hg in the trichloroacetic acid supernatant was determined with a Packard model 5130 Auto.Gamma scintillation spectrometer. The amount of 203Hg in the supernatant reflects the amount of low molecular weight proteins that bind Hg. In the entire study, we were not concerned with any high molecular weight metal-binding proteins that may be present in newborn rat liver.

Isolation of Hepatic Metal-binding Protein—Since it was suspected that the low molecular weight mercury binding protein in the liver of newborn rats might be MT, MT was also isolated for comparative purposes from adult rats that had been pretreated with zinc, which is known to increase the amount of MT in the liver (15). Approximately 6 g of liver from 1) untreated 10-week-old male rats; 2) 10-week-old male rats treated intraperitoneally with ZnCl2 (10 mg of Zn/kg) at 48 and 24 h before death; and 3) rats between 1 and 4...
days of age was homogenized in 1.15% KCl (3 ml/g). Each homogenate was centrifuged at 9,000 \( \times \) g for 20 min and the supernatant from this was further centrifuged at 100,000 \( \times \) g for 1 h. The cytosol fraction was chromatographed on a Sephadex G-75 column (70 x 2.6 cm) equilibrated with 10 mM Tris-acetate buffer, pH 7.4, and eluted with the same buffer at 30 ml/h at 4°C. Effluent fractions were collected at 10-min intervals. The absorbance at 280 nm and the amount of Zn in the effluent were determined with a Beckman model 25 spectrophotometer and a Beckman model 444 atomic absorption spectrophotometer, respectively. The amount of the low molecular weight, mercury-binding protein in the effluent was estimated by the method of Piotrowski et al. (39) as described above with a slight modification. A mixture of 0.5 ml of effluent and 3.0 ml of liver homogenate (prepared by homogenization of untreated adult rat liver in 1.15% KCl at 7 ml/g) was used instead of 3.5 ml of liver homogenate.

The fractions with a high concentration of metal-binding protein were pooled and mixed with approximately 15 to 20 \( \mu \)Ci of \( ^{199m} \text{ZnCl}_2 \) (carrier free, Amersham/Searle). These fractions were further purified on a DEAE-Sephadex A-25 column (40 x 2.6 cm) equilibrated with 10 mM Tris-acetate buffer, pH 7.4, at 4°C, as described by Bremner and Young (41). After the sample was applied to the column, 10 mM Tris-acetate buffer was used as the eluant for 20 fractions (10 min each) at a rate of 30 ml/h to remove any free \( ^{199} \text{Zn} \). Then a linear gradient of Tris-acetate buffer (10 mM to 200 mM, pH 7.4) was used as the eluant. The amount of \( ^{199} \text{Zn} \) and the conductivity of the effluent were determined with a Packard model 5130 Auto-Gamma scintillation spectrometer and a YSI model 31 conductivity bridge (Yellow Springs Instrument), respectively.

**Disc Gel Electrophoresis of Hepatic Metal-binding Protein**—The purities of the two major protein subfractions isolated by ion exchange chromatography were determined by disc polyacrylamide-gel electrophoresis at 2 mA/gel with a running pH of 9.5 and bromphenol blue as the tracking dye (42, 43). A concentration of 2.5% acrylamide was used in the concentrating gel and 7.5% in the separating gel. The gels were stained with 0.05% Coomassie brilliant blue and excess stain was removed by a charcoal-type diffusion destainer (Bio-Rad). The gels were scanned at 650 nm using a Gilford 240 spectrophotometer.

**Amino Acid Analysis**—The subfractions from ion exchange chromatography were dialyzed against deionized water, concentrated by lyophilization, oxidized with performic acid as described by Hirs (44), and hydrolyzed with 6 \( \text{N} \) HCl at 110°C for 24 h, and analyzed with a Beckman-Spinco model MS amino acid analyzer (Beckman-Spinco, Palo Alto, CA).

**RESULTS**

The concentration of low molecular weight mercury-binding protein in the liver of 1- to 4-day-old rats was 1.41 \( \pm \) 0.040 pmol of Hg bound/g of liver (mean \( \pm \) S.E. of nine rats). The amounts of mercury bound to the hepatic high molecular weight proteins (trichlo-
Metallothionein in Newborn Rats

NEWBORN

FIG. 3. Sephadex G-75 elution profiles of the hepatic cytosol of 1- to 4-day-old rats. The column was eluted with 10 mM Tris-acetate buffer, pH 7.4, at a rate of 30 ml/h. After the first 30 ml of effluent, 10-min fractions were collected. The concentrations of mercury-binding proteins and Zn and the absorbance at 280 nm were determined as described under "Materials and Methods."

Isolation of Metal-binding Protein from Neonatal Rat Liver—To determine whether or not this low molecular weight Hg-binding protein from newborn rat liver is MT, the protein was isolated. The cytosol fractions of livers from untreated adults, Zn-treated adults, and 1- to 4-day-old rats were chromatographed on Sephadex G-75 columns (Figs. 1 to 3). In the untreated adult (Fig. 1), no major Zn-binding peaks were evident after the initial high molecular weight proteins were eluted. In the liver of the Zn-treated adult which contained low molecular weight mercury-binding protein at a concentration of 0.849 μmol of Hg bound/g of liver, a major peak of Zn radioactivity in counts per min; ---, conductivity of the effluent in nmho/cm.

FIG. 4. Purification of the metal-binding protein from newborn and Zn-treated rat liver by DEAE-Sephadex A-25 chromatography. The proteins were eluted with a linear gradient of buffer created by mixing 300 ml of 10 mM Tris-acetate and 300 ml of 200 mM Tris-acetate gradually as described under "Materials and Methods." - - , Zn radioactivity in counts per min; ---, conductivity of the effluent in mmho/cm.

roacetic acid-precipitable) in newborns and adults were 2.08 ± 0.040 and 3.41 ± 0.009 μmol/g of liver, respectively.

Amino acid composition of hepatic metallothionein of 1- to 4-day-old newborn and Zn-treated adult rats

Expressed as residues per molecule. Results of one analysis of proteins oxidized with performic acid and hydrolyzed with 6 M HCl at 110°C for 24 h (44). Values are based on minimum molecular weight as shown. His was not found. Tyr was absent, since no appreciable peaks of Tyr and halogenated tyrosine appeared on the ion exchange chromatogram. The absence of Tyr and Trp is shown by the lack of absorbance of the unoxidized protein at 280 nm.

TABLE I

Amino acid composition of hepatic metallothionein of 1- to 4-day-old newborn and Zn-treated adult rats

Expressed as residues per molecule. Results of one analysis of proteins oxidized with performic acid and hydrolyzed with 6 M HCl at 110°C for 24 h (44). Values are based on minimum molecular weight as shown. His was not found. Tyr was absent, since no appreciable peaks of Tyr and halogenated tyrosine appeared on the ion exchange chromatogram. The absence of Tyr and Trp is shown by the lack of absorbance of the unoxidized protein at 280 nm.

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>Newborn</th>
<th>Adult</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lysine</td>
<td>7.06 (7)</td>
<td>11.13 (11)</td>
</tr>
<tr>
<td>Arginine</td>
<td>0.38 (0)</td>
<td>0.12 (0)</td>
</tr>
<tr>
<td>Aspartate</td>
<td>6.15 (6)</td>
<td>4.47 (4)</td>
</tr>
<tr>
<td>Threonine</td>
<td>2.75 (3)</td>
<td>3.89 (4)</td>
</tr>
<tr>
<td>Serine</td>
<td>8.94 (9)</td>
<td>10.93 (11)</td>
</tr>
<tr>
<td>Glutamate</td>
<td>1.63 (2)</td>
<td>1.58 (2)</td>
</tr>
<tr>
<td>Proline</td>
<td>5.18 (5)</td>
<td>1.71 (2)</td>
</tr>
<tr>
<td>Glycine</td>
<td>6.01 (6)</td>
<td>6.95 (7)</td>
</tr>
<tr>
<td>Alanine</td>
<td>3.93 (4)</td>
<td>3.45 (3)</td>
</tr>
<tr>
<td>Half-cystine</td>
<td>15.68 (16)</td>
<td>17.31 (17)</td>
</tr>
<tr>
<td>Valine</td>
<td>2.63 (3)</td>
<td>2.13 (2)</td>
</tr>
<tr>
<td>Methionine</td>
<td>0.89 (1)</td>
<td>0.91 (1)</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>1.32 (1)</td>
<td>0.08 (0)</td>
</tr>
<tr>
<td>Leucine</td>
<td>1.72 (2)</td>
<td>0.39 (0)</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>1.13 (1)</td>
<td>0.09 (0)</td>
</tr>
<tr>
<td>Total residues</td>
<td>66</td>
<td>64</td>
</tr>
</tbody>
</table>

Minimum molecular weight

a Corrected for standard losses.

b Determined as cysteic acid.

c Determined as methionine sulfoxide.

d Calculated from amino acid analysis data as described by Delaage (46).
A and MT-B–Disc polyacrylamide gel electrophoresis of the studies on this protein were not performed. The Zn-binding middle Zn-binding subfraction was so minor that further, each showing three Zn-binding subfractions (Fig. 4). The profiles from a DEAE-Sephadex A-25 column were very sim-
ilar, with a conductivity of 1.8 to 1.9 mmho/cm and MT-
A eluted at a conductivity of 3.25 mmho/cm.

The Zn-containing peak was evident at V, / V, of approximately 2 (Fig. 2), which is similar to that reported for MT from a number of species (2, 10, 45). This major Zn-containing peak had a low absorbance at 280 nm and was the only prominent mercury-binding component that could be detected by the method of Piotrowski et al. (39). The elution profile of hepatic cytosol from newborn rats (Fig. 3) strikingly resembled that of the Zn-treated adults in that there was a major Zn-containing (and Hg binding) peak at V, / V, approximately 2 with a low absorbance at 280 nm. The amount of zinc in the high molecular weight region of the elution profile was minimal compared with that in the peak at V, / V, of approximately 2. This indicated that our preoccupation with the low molecular weight protein as the major metal-binding protein in newborn rat liver was justified.

This major Zn-containing protein from both the newborn and Zn-treated adult rats was labeled with 65Zn and further purified by cation-exchange chromatography. The elution profiles from a DEAE-Sephadex A-25 column were very sim-
ilar, each showing three Zn-binding subfractions (Fig. 4). The middle Zn-binding subfraction was so minor that further studies on this protein were not performed. The Zn-binding subfractions of the newborn were eluted at the same conductiv-
ty as those from the Zn-treated adult; MT-A eluted in buffer having a conductivity of 1.8 to 1.9 mmho/cm and MT-
B eluted at a conductivity of 3.25 mmho/cm.

Electrophoretic Characteristics of Neonatal Hepatic MT-
A and MT-B—Disc polyacrylamide gel electrophoresis of the MT-A and MT-B from both newborn and Zn-treated adult livers showed one visible band per gel (Fig. 5). The electrophoretic mobility of MT-A was 0.41 for both the newborn and Zn-treated adult rats relative to bromphenol blue, while that of MT-B was 0.57 and 0.58 for newborns and adults, respectively.

Amino Acid Composition of Neonatal Hepatic MT-A and
MT-B—The amino acid compositions of the neonatal MT-A and MT-B were very similar to that of the Zn-treated adult (Table I). Each contained a high amount of half-cystine: 16 to 17 residues/molecule in the newborn and 17 to 21 in the adult. There also existed substantial amounts of lysine and serine (7 to 11 residues/molecule) and an absence of tyrosine, tryptophan, and histidine in these subfractions. However, the newborn MT contained 1 residue of phenylalanine per mole-
cule which was essentially absent in the adult MT.

Recovery of Hepatic Metal-binding Protein—In terms of the capacity to bind mercury, approximately 90% of the low molecular weight protein in both the newborn and Zn-treated adult rats was recovered after G-75 chromatography, while further purification by ion exchange chromatography gave a final yield of approximately 61% from both sources.

Relative Levels of Hepatic and Renal MT in Rats of
Different Ages—The maximum concentration of hepatic MT occurred in the 1- to 4-day-old age group (Fig. 6). These neonates had levels 20 times that found in adults, whereas fetal liver MT levels at the 19th day of gestation were 14 times adult levels. The concentration of MT decreased rapidly after 4 days of age and approximated adult levels at 28 days of age. The concentration of MT in kidneys did not change signi-
ificantly with age.

Fig. 6. The relative concentrations of MT in rats at various ages. Rat liver and kidneys were homogenized and the concentrations of MT were estimated by the method of Piotrowski et al. (39) as described under “Materials and Methods.” An age of -2 days repre-
"
extremely low absorbance at 280 nm of the newborn Zn-containing protein and the MT of Zn-treated adults suggests that tryptophan and tyrosine are absent. The newborn subfractions contained 1 residue of phenylalanine per molecule which is absent in MT from adult rats. All subfractions contained appreciable amounts of lysine, serine, and glycine as shown previously in MT of various species (7, 11, 13). Therefore, because of the fulfillment of the aforementioned criteria, it is concluded that the two subfractions (MT-A and MT-B) of the newborn liver are metallothioneins.

The separation of MT from newborn rat liver into two subfractions conforms to findings that two forms of MT are present in adult equine kidneys (12) and rabbit (7) and rat subfractions conforms to findings that two forms of MT are present in adult equine kidneys (12) and rabbit (7) and rat liver (15, 17). The electrophoretic mobilities of the newborn MT-A and MT-B found in this study (0.41 and 0.57) are similar to those of the two forms of MT isolated from adult rat liver (15, 17).

A change in the concentration of MT in the liver of rats with age was determined using the method of Piotrowski et al. (39). However, this assay is based on the assumption that trichloroacetic acid precipitates all Hg-binding substances except MT. Thus, it could overestimate the amount of MT if other nonprecipitable mercury-binding substances exist. Gel filtration of the hepatic cytosol of both the newborn and Zn-treated adult showed that the major Zn-containing protein at V₀/Vₛ of approximately 2 coincided with the major Hg-binding nonprecipitable protein detected by the assay of Piotrowski et al. (39). Moreover, in the G-75 elution profiles, there were no Hg-binding peaks eluted after the MT peak. This suggests that there apparently were no low molecular weight substances in the newborn liver cytoplasm to interfere with this method. In addition, gel filtration on Sephadex G-75 of the trichloroacetic acid supernatant obtained from the liver of 1- to 4-day-old rats showed only one peak at V₀/Vₛ of approximately 2 when the amount of ²⁰⁹Hg in the effluent was monitored. Thus, this method gave a valid estimation of MT in our system.

The function of the high concentration of MT in neonatal rat liver is not known. The relatively high concentration of MT in newborn liver might play a role in the homeostasis of copper and zinc, elements necessary for growth, as suggested by the findings of previous studies with adult animals (23-29). If this hypothesis is true, it is not surprising that the liver of the newborn rat has a high concentration of MT since it is rapidly growing. The evidence that the hepatic concentrations of copper and zinc are higher in newborn rats and human fetuses also tends to support this idea (28-30).

Acknowledgment—We are greatly indebted to Dr. Joe Kimmel of the Department of Biochemistry for the amino acid analysis.

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K L Wong and C D Klaassen


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