THE PARTICULATE HALF-CYSTINE-RICH COPPER PROTEIN OF NEWBORN LIVER. RELATIONSHIP TO METALLOTHIONEIN AND SUBCELLULAR LOCALIZATION IN NON-MITOCHONDRIAL PARTICLES POSSIBLY REPRESENTING HEAVY LYSOSOMES

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SUMMARY: The particulate half-cystine-rich copper protein of newborn liver can be partially purified by centrifugation of the heavy mitochondrial fraction through glycogen-sucrose or sucrose density gradients. The resulting sediments contained about 4% copper, about 20% half-cystine and a 2 to 3-fold increase in β-glucuronidase specific activity. The copper protein is not a true mitochondrial constituent and the data are consistent with its localization in a distinct population of heavy lysosomes. The amino acid composition of the polypeptide isolated from the crude insoluble copper protein is strikingly similar to that of metallothionein, suggesting that the neonatal protein represents a copper-rich form of metallothionein.

The liver of newborn animals contains much larger concentrations of copper than other tissues (1-3). An insoluble crude copper protein containing more than 4% copper and more than 25% half-cystine has been isolated from newborn bovine and newborn human liver under copper-free conditions (4-6). Homologous control material from adult livers of either species contained less than one-thirtieth the copper yield and less than one-tenth the copper concentration found in the newborn. The extraordinarily high copper content of this protein suggests that it has a storage or detoxifying function for copper in the newborn animal.

Previous work showed that the particulate neonatal copper protein was localized predominantly in the mitochondrial fraction (4) and it was designated neonatal hepatic mitochondrocuprein. The subcellular soluble fraction, which contains copper bound to metallothionein (7-9) as well as to superoxide dismutase, accounts for only a small proportion of the total tissue copper of newborn liver. This paper presents evidence that the particulate neonatal copper protein is localized in a distinct population of heavy lysosomes and

that it represents a copper-rich form of metallothionein.

MATERIAL AND METHODS: Bovine liver was removed within 5 minutes after death from animals one day old and transported from slaughter-house to laboratory in plastic bags with ice external to the bag. Liver pulp was scraped from the interior of the organ with glass knives and homogenization was begun about one hour after death of the animal. Precautions were taken to prevent contamination with extraneous copper as previously described (4,6). All fractionation procedures were carried out at 0-4°. The liver pulp was suspended in 0.25M sucrose and homogenization and preparation of subcellular fractions were carried out as described by DeDuve et al (10).

The heavy mitochondrial fraction was further fractionated on glycogen-sucrose (11) or sucrose density gradients of 26 ml total volume. The glycogen-sucrose gradient was linear from 0 to 33.7 g glycogen in 43.5 g sucrose per 100 g water (gradient Gh(22) employed by Beaufay et al (11) for sedimentation of lysosomes). The sucrose gradient contained a cushion of 4 ml of 60% sucrose in the bottom of the tube above which was layered 22 ml of solution linear from 35% to 50% (w/w) sucrose. The sample, containing about 45 mg of protein suspended in 3 ml of 0.25M sucrose, was layered on top of the gradient which was centrifuged in a Spinco SW 25 rotor at 50,000 g for 2 h. After centrifugation, the gradient layers above the sediment were removed with a Pasteur pipette and all subfractions were washed by suspension in 0.25M sucrose and centrifugation at 20,000 g for 20 min. The entire material on the gradient was included in the sum of the copper analyses.

Copper, protein and amino acids were determined as previously described (4,6). β-glucuronidase activity was determined by the method of Fishman (12). Succinic dehydrogenase was assayed spectrophotometrically with phenazine methosulfate and dichlorophenolindophenol (13) after pre-incubation of the samples in the presence of phosphate and KCN at 37° (14).

RESULTS: The present study confirms the selective localization of the bovine neonatal copper protein in the heavy mitochondrial fraction which contained

TABLE 1

DISTRIBUTION OF COPPER AND ENZYMATIC ACTIVITIES AMONG SUBCELLULAR FRACTIONS FROM NEWBORN BOVINE LIVER

Fraction	Protein* (mg)	Copper		β-glucuronidase		Succinic dehydrogenase	
		Total* (μg)	Ratio# (μg/mg)	Total units*	Specific activity+	Total units*	Specific activity‡
Cytoplasmic extract plus nuclear fraction	153.2	109.7**	0.7	19740	129	3026	19.8
Nuclear fraction	23.2	33.3	1.4	1840	79.4	346	14.9
Heavy mitochondrial fraction	18.6	49.0	2.6	1080	57.9	1190	63.9
Light mitochondrial fraction	15.5	10.0	0.6	4460	288	696	44.9
Microsomal fraction	24.0	1.7	0.1	3540	148	196	8.2
Supernatant fraction	60.1	14.8	0.2	7220	121	83	1.4

*Protein, total copper and total enzymatic activities expressed as units per g fresh tissue.

#μg copper per mg protein.

+nmoles of phenolphthalein released from phenolphthalein glucosiduronic acid per h per mg protein.

‡nmoles indophenol reduced per min per mg protein.

**Total copper in the unhomogenized liver was 121.0 μg per g fresh tissue.

TABLE 2

DISTRIBUTION OF COPPER AND β-GLUCURONIDASE ACTIVITY AMONG SUBFRACTIONS OBTAINED BY CENTRIFUGATION OF THE HEAVY MITOCHONDRIAL FRACTION FROM NEWBORN BOVINE LIVER THROUGH GLYCOGEN-SUCROSE OR SUCROSE DENSITY GRADIENTS

Particulate Subfraction	Protein* (mg)	Copper Total* (μg)	Copper Ratio# (μg/mg)	β-glucuronidase Total units*	β-glucuronidase Specific activity+
A. GLYCOGEN-SUCROSE DENSITY GRADIENT					
Total heavy mitochondrial fraction	18.6	49.0	2.6	1080	57.9
Top subfraction	2.9	1.9	0.7	49	17.0
Main yellow band	8.7	9.5	1.1	331	38.0
Bottom subfraction	0.63	3.1	4.9	260	413
Sediment	0.89	34.2	38.4	121	136
B. SUCROSE DENSITY GRADIENT					
Total heavy mitochondrial fraction	16.0	43.2	2.7	1445	90.4
Top subfraction	1.8	1.0	0.6	43	24.1
Main yellow band, upper 2/5	6.2	2.3	0.4	125	20.2
Main yellow band, lower 3/5	3.7	8.2	2.2	433	117
Sediment	0.76	32.9	43.2	198	260
(Soluble Material)	(2.0)	(2.0)	(1.0)	(560)	(280)

* Protein, total copper and total β-glucuronidase activity expressed as units per g fresh tissue.

\# μg copper per mg protein.

\+ nmoles of phenolphthalein released from phenolphthalein glucosiduronic acid per h per mg protein.

more than 50% of the particulate (non-supernatant-fraction) tissue copper with almost a 4-fold increase in copper concentration compared to that of the total homogenized preparation (Table 1). This fraction showed a 3-fold increase in succinic dehydrogenase specific activity, used as a marker for mito-

chondria, and a decrease in β-glucuronidase activity, used as a marker for lysosomes.

Further fractionation of the heavy mitochondrial fraction on either glycogen-sucrose or sucrose density gradients yielded 3 visible subfractions: a narrow pale band at the interface of sample and gradient, a dense yellow band somewhat below the midpoint of the gradient, and a small sediment. Table 2 shows that the sediments contained 70% of the heavy mitochondrial fraction copper with a 15-fold increase in copper concentration and a 2 to 3-fold increase in β-glucuronidase specific activity. Succinic dehydrogenase activity in the sediments was, in contrast, reduced to less than 2% of the total activity and less than 25% of the specific activity present in the total heavy mitochondrial fraction. The bulk of the succinic dehydrogenase total activity was recovered in the main yellow band which contained mitochondria.

Amino acid analyses showed that the sucrose gradient sediments contained the half-cystine-rich protein moiety of the copper protein as well as its copper. Table 3 shows that the gradient sediment contained more than 22% half-cystine with an amino acid composition very broadly similar to that of the detergent-insoluble crude copper protein (4,6) previously isolated from the whole mitochondrial fraction. The detergent-insoluble residue from the gradient sediment showed a slight increase in purity in the direction of the more highly purified half-cystine-rich polypeptide (6) isolated from the crude copper protein after sulfitolysis.

DISCUSSION: The particulate copper protein of newborn liver is selectively concentrated in density-gradient sediments which contain less than 5% of the heavy mitochondrial fraction protein and show decreased succinic dehydrogenase activity but which show increased β-glucuronidase specific activity. These results indicate that the half-cystine-rich copper protein is not a true mitochondrial constituent and are consistent with its localization in lysosomes. The selective concentration of this non-mitochondrial copper protein in the heavy mitochondrial fraction in the preceding subcellular

TABLE 3

AMINO ACID COMPOSITION OF THE SUCROSE DENSITY-GRADIENT SEDIMENT COMPARED WITH THAT OF THE DETERGENT-INSOLUBLE CRUDE COPPER PROTEIN AND THAT OF THE HALF-CYSTINE-RICH POLYPEPTIDE ISOLATED FROM THE CRUDE COPPER PROTEIN

Amino acids as moles/100 moles total amino acids recovered.

	A. From heavy mitochondrial fraction		B. From whole mitochondrial fraction	
Amino Acid	Total Gradient Sediment without detergents	Detergent-insoluble* residue from gradient sediment	Detergent-insoluble* crude copper protein	Cys-rich polypeptide+ isolated from crude copper protein
Cys‡	22.2	26.8	25.9	39.3
Lys	7.5	9.1	6.2	10.9
His	1.3	1.4	1.3	
Arg	3.5	3.5	3.6	1.9
Asp	6.0	4.9	5.1	1.5
Thr	4.0	3.8	3.6	2.6
Ser	8.2	9.4	7.6	11.6
Glu	7.1	5.5	6.8	2.0
Pro	5.1	5.0	5.5	8.2
Gly	8.9	9.5	11.5	9.3
Ala	7.6	7.3	7.4	8.8
Val	5.2	4.6	4.8	4.0
Met	1.2	0.5	0.9	
Ile	3.2	2.2	2.6	
Leu	5.4	3.6	4.2	
Tyr	1.6	1.2	1.2	
Phe	2.5	1.7	1.8	

*Material insoluble after successive extractions with deoxycholate, Tween 80 and dodecylsulfate (4).

+Half-cystine-rich polypeptide obtained from the crude copper protein after sulfitolysis, gel filtration and ion-exchange chromatography as previously described (6) followed by oxidation (15) and preparative paper electrophoresis.

‡Determined as cysteic acid after performic acid oxidation (16).

fractionation step, as opposed to the occurrence of the bulk of the lysosomes in the light mitochondrial fraction (10), suggests that the copper protein may be localized in newborn liver in a distinct population of heavy

lysosomes. Such a distinct population of heavy lysosomes has been found in spleen (17,18). The localization of a major portion of the copper of newborn liver in lysosomes is in agreement with the hisotological data of Goldfischer and Bernstein (19).

The amino acid composition of the half-cystine-rich polypeptide isolated from the crude insoluble copper protein after sulfitolysis (Table 3, fourth column) shows striking similarities to that of equine metallothionein (7). The polypeptide from newborn liver has been found to have a molecular weight of the order of 7000 (6). Assuming 7 residues of lysine, the tentative residues per molecule in the polypeptide are as follows (comparable values for metallothionein (7) shown in parentheses): Cys, 24 (20); Lys, 7 (7); Arg, 1 (1); Asp, 1 (3); Thr, 2 (2); Ser, 7 (7); Glu, 1 (3); Pro, 5 (3); Gly, 6 (6); Ala, 6 (6); Val, 2 (2); Met, 0 (1). The bovine neonatal polypeptide contains no methionine and contains only one-third as much aspartic and glutamic acids as found in adult equine metallothionein. The neonatal copper protein also differs from adult equine metallothionein in its much higher copper content. Its insolubility and the presence of predominantly cystine rather than cysteine may be related to the method of preparation. It seems probable that particulate neonatal copper protein, as isolated from the mitochondrial fraction, represents a copper-rich polymerized form of metallothionein which sequesters copper in the newborn animal.

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REFERENCES

1. Lorenzen, E.J., and Smith, S.E. (1947) J. Nutr. 33, 143-154.
2. Underwood, E.J. (1971) Trace Elements in Human and Animal Nutrition (3rd ed.), pp 57-115, Academic Press, New York.
3. Evans, G.W. (1973) Physiol. Rev. 53, 535-570.
4. Porter, H., Johnston, J., and Porter, E.M. (1962) Biochim. Biophys. Acta. 65, 66-73.
5. Porter, H., Sweeney, M., and Porter, E.M. (1964) Arch. Biochem. Biophys. 104, 97-101.
6. Porter, H. (1971) Biochim. Biophys. Acta 229, 143-154.

7. Kagi, J.H.R., Whanger, P.D. and Bethune, J.L. (1973) Fed. Proc. 32, 942.
8. Evans, G.W., Majors, P.F. and Cornatzer, W.E. (1970) Biochem. Biophys. Res. Commun. 40, 1142-1148.
9. Bremner, I. in Trace Element Metabolism in Animals, University Park Press, Baltimore, in press.
10. DeDuve, C., Pressman, B.C., Gianetto, R., Wattiaux, R., and Appelmans, F. (1955) Biochem. J. 60, 604-617.
11. Beaufay, H., Jacques, P., Baudhuin, P., Sellinger, O.Z., Berthet, J., and DeDuve, C. (1964) Biochem. J. 92, 184-205.
12. Fishmann, W.H. (1967) Meth. Biochem. Anal. 15, 77-145.
13. King, T.E. (1967) Meth. Enzym. 10, 322-331.
14. Caplan, A.I., and Greenawalt, J.W. (1968) J. Cell Biol. 36, 15-31.
15. Hirs, C.H.W. (1956) J. Biol. Chem. 219, 611-621.
16. Schram, E., Moore, S., and Bigwood, E.J. (1954) Biochem. J. 57, 33-37.
17. Bowers, W.E., and DeDuve, C. (1967) J. Cell. Biol. 32, 339-348.
18. Houghton, P.B., Jones-Williams, W. and Davies, M. (1971) Biochem. J. 123, 28p-29p.
19. Goldfischer, S. and Bernstein, J. (1969) J. Cell Biol. 42, 253-261.