Ceruloplasmin and Copper Transport During the Latter Part of Gestation in the Rat (43619)

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Abstract. **We have examined the tissue uptake of ^{67}Cu from ceruloplasmin versus albumin and transcuprein, after its intravenous administration to pregnant rats, in the last 4 days of gestation. ^{67}Cu infused as *in vivo*-labeled ceruloplasmin remained on ceruloplasmin in the maternal circulation over the 4- to 6-hr time period examined, as determined by gel chromatography and immunoreactivity. That infused as *in vitro*-labeled serum was initially on transcuprein and albumin but soon also with new ceruloplasmin. On the basis of percent dose as well as total actual Cu transferred (taking into account the sizes of the two plasma Cu pools), ceruloplasmin was the preferred source of Cu for most tissues. Total uptake of Cu from ceruloplasmin was seven times greater than that from albumin and transcuprein for the placenta, whole fetus, and fetal liver. It was 2- to 6-fold greater for other tissues (except liver and kidney). When synthesis of maternal ^{67}Cu-ceruloplasmin (from ^{67}Cu administered on albumin and transcuprein) was inhibited with cycloheximide, uptake by nonhepatic tissues was reduced markedly. In the *fetal* circulation, entering ^{67}Cu was initially associated with transcuprein and α-fetoprotein (or albumin), but then also appeared with ceruloplasmin. Specific receptors for ceruloplasmin were detected on membranes from the placenta as well as fetal liver; mRNA for ceruloplasmin was detected on the endoplasmic reticulum-bound polyribosomes of placenta/yolk sac, and of fetal and maternal liver. We conclude that Cu destined for the fetus is delivered mainly or exclusively by ceruloplasmin. It may enter via placental receptors, arriving in fetal plasma in ionic form, for later incorporation into fetal ceruloplasmin. The importance of ceruloplasmin as a source of plasma Cu for nonhepatic organs is also confirmed.** [P.S.E.B.M. 1993, Vol 203]

In most mammals, copper, iron, and zinc accumulate in the liver during the latter part of gestation (1–4). The accumulation of copper is primarily in metallothionein, which deposits both in the cytosol and nuclei of hepatocytes (5–6). The prenatal accumulation of these metals assures their availability immediately after birth and during the suckling period when milk is the primary food. Milk is low in these trace elements (1, 4, 7), although they are needed for rapid growth. Neonatal liver concentrations of Cu(II) and iron fall dramatically during suckling, and in humans the concentrations of these elements rarely (if ever) rise again to those present at birth (1, 4). Mice and rats differ from the human in continuing to deposit some liver copper in the first week of life (1, 4) because their milk has a much higher copper content (7), but even in these species, liver copper concentrations then fall rapidly until weaning (1, 4).

Little is known about the way copper is transferred to the fetus during gestation, although it is thought that the placenta is the main mediator of fetal uptake. McArdle and Erlich (8) have documented the transfer of radiocopper to fetal mice after intravenous injection of ^{64}Cu(II) to their dams. Uptake of copper by the fetus was detected by 4 hr after injection. Rates of uptake appeared to peak not at the end of gestation but 5 days before birth. Mas and Sarkar (9) reported the transfer of radioactive copper across the placenta when given as the dihistidine complex, and found radioactivity with a component the size of ceruloplasmin in placental extracts. Just recently, they reported that ceruloplasmin (as well as other forms of Cu) can be sources of this element for cultured human trophoblasts (10).

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In adult, nonpregnant mammals, plasma copper is transported by at least three different proteins. Albumin has long been recognized as a major carrier, having (in most species) a single high affinity copper binding site at the N terminus (11, 12). Numerous studies have implicated albumin in the transfer of newly absorbed copper from the intestine to the liver (and kidney) (13–17). These are the tissues in which most of the incoming dietary copper is first retained. Another plasma protein named transcuprein, with an even higher affinity for copper (15, 18, 19), also plays a role in this process. This 270-kDa protein is labeled in parallel with albumin when radiocopper is administered intraduodenally, intravenously, intraperitoneally, or *in vitro* (15), and it loses copper in parallel with albumin as copper accumulates in the liver. Transcuprein is not a polymer of either albumin or ceruloplasmin, as it fails to react with polyclonal antibodies to either protein in double immunodiffusion (15) or in Western blotting. It rapidly exchanges copper with albumin by direct molecular interaction (15, 20). Rat transcuprein has been isolated and partially characterized (18, 20, 21). Together, albumin and transcuprein appear to account for about 25% of the copper in human (and rat) plasma (22–25) and are the main components of the exchangeable plasma copper pool.

The third plasma protein involved in copper transport is ceruloplasmin, the well-known α_2-globulin that accounts for about 60% of plasma copper (4, 22, 23). It contains six to seven copper atoms per molecule in nondialyzable form and is thus not part of the exchangeable copper pool. Copper is incorporated during synthesis by the liver, after entry of dietary copper into hepatocytes. Ceruloplasmin secreted by the liver then becomes the major source of copper for cells in peripheral tissues (26–30), and is thought to deliver this copper via receptors on the cell surface (31–33). Ceruloplasmin mRNA has been cloned in several species (34–36), and cDNAs have been used to show that the gene is expressed not just in liver but also in the brain (especially the choroid plexus; 33, 37), and by Sertoli cells (38). Cells in these sites produce proteins for the cerebrospinal and seminal fluids, respectively. Messenger RNA for ceruloplasmin has also been detected in the uterus and placenta/yolk sac of rats during gestation (34, 39, 40), which suggests that ceruloplasmin might be secreted from these organs into the cord blood and/or amniotic fluid. It has also been detected in cells of the mammary gland (41; N. Madani and M. C. Linder, unpublished observations), and fetal and newborn lungs appear to express the message (40). Pregnancy (42, 43) and estrogen treatment (44–47) increase concentrations of circulating ceruloplasmin, which suggests that it plays a role in gestation.

The studies described here were designed to determine whether ceruloplasmin or ionic copper (binding to albumin + transcuprein) or both are the best maternal blood sources for transfer of copper to the fetus, by what mechanism transfer occurs, and the initial fate of the copper in the fetus after transfer.[2]

Materials and Methods

Animals and Treatments. All rats used were females of the Sprague-Dawley strain, from Simonson Laboratories (Gilroy, CA) and Lab Pets (Riverside, CA). First-time pregnant rats, and in some cases their siblings, were received midway through pregnancy. For 12 of the dams, the stage of pregnancy was timed. In the case of nontimed dams, pup gestational age was determined by crown-to-rump length and weight, comparing values with those from the timed pups, as described previously (2). Rats were infused intravenously into the tail vein with ^{67}Cu-labeled samples under light pentobarbital anesthesia (15 mg/kg ip) and were sacrificed by exsanguination under heavy pentobarbital anesthesia (50 mg/kg), with heparin treatment (48). In one study, rats were treated with cycloheximide (5 mg/100 g body wt; 49) 30 min before and 1.75 hr after intravenous injection of ^{67}Cu(II)-treated serum (where Cu(II) is bound to albumin and trancuprein) to inhibit formation of ^{67}Cu-ceruloplasmin by maternal tissues.

Whole blood was collected from the vena cava in the thoracic cavity and centrifuged to obtain plasma that was either used directly or frozen until use. Other organs removed were the liver, kidney, spleen, heart, brain, as well as placentae, and usually the uterus. Half of the fetuses were taken whole for determination of their radioactivity. The other fetuses were used as sources of fetal blood (from the carotid arteries) and liver. Blood from each half litter was pooled and centrifuged. The plasma was used directly or frozen until use. In most cases, some amniotic fluid was also collected by syringe before removal of the fetuses within the uterus. To obtain samples for determination of ceruloplasmin mRNA or membrane receptors, tissues were immediately chilled in ice-cold, sterile, phosphate-buffered saline solution (0.9% NaCl, with 10 m*M* potassium phosphate) before homogenization in appropriate buffers. A membrane fraction for binding studies was obtained as described previously (33). Samples for RNA extraction were prepared by standard procedures, also as described previously (50, 51).

Radioisotopes and Isotopic Samples. $^{67}CuCl_2$ was obtained in millicurie quantities from Los Alamos National Laboratory (Los Alamos, NM) with specific activities in the range of 4000 μCi/μg, upon receipt. Smaller quantities (usually 1 mCi) of non-carrier-added product, made from ^{67}Zn, were obtained from the reactor facility of the University of Missouri, courtesy

[2] A preliminary report of these findings was presented at the Seventh International Symposium on Trace Elements in Man and Animals in 1991 (3).

of Dr. Kurt Zinn. For preparation of ^{67}Cu-labeled ceruloplasmin, a donor rat was injected with 3–5 mCi of ^{67}Cu, in the form of the neutralized, nitrilotriacetate (NTA) complex (Cu:NTA; 1 mole:1 mole) in 0.9% NaCl. Twelve to 19 hr later, the donor rat was sacrificed, and 1.0-ml portions of plasma were fractionated on 50-ml columns of Sephadex G-150, equilibrated with 0.9% NaCl (buffered with 10 m*M* potassium phosphate, pH 7.0). A single radioactive peak was obtained that gave a single radioactive band in polyacrylamide gel electrophoresis characteristic of ceruloplasmin. The three most radioactive fractions were pooled for tail vein injection into animals (1.0 ml/rat). Albumin + transcuprein-labeled samples were prepared by adding 2–3 ng of Cu (labeled with ^{67}Cu(II)-NTA) to 1.0-ml portions of donor rat plasma (from untreated rats) 2–50 hr before injection. (Samples were kept frozen at −20°C if not used the same day.) Binding of radioisotope to serum components fractionated on Sephadex G-150 was identical whether or not serum samples were first brought to pH 5.5 and back to 7.0 before injection (52). Lowering the pH to facilitate albumin binding thus appeared to make no difference and was not usually applied.

Radioactive samples were counted in a multisample gamma counter (Gammatrac 1191; Tracor Analytic, Mountain View, IL). Counts per minute were corrected for time and isotopic decay, as well as for efficiency, which varied with the volume of the sample. Individual fetuses, fetal livers, placentae, and maternal organs were counted directly, as were samples of serum, column fractions, etc.

Chromatography. Sephadex G-150 chromatography was on 50-ml or 25-ml columns equilibrated with either phosphate-buffered saline (see above), or 20 m*M* potassium phosphate buffer (pH 7.0). Samples of 1.0 ml and 0.50 ml were applied to the larger and smaller columns, respectively. Fractions of 1.0 ml or 0.5 ml were collected for counting and other analyses.

Ceruloplasmin Assays. Ceruloplasmin oxidase activity was measured as described previously, using *p*-phenylene diamine as a substrate (48). In some cases, ceruloplasmin was identified by immunoprecipitation with specific rabbit anti-rat ceruloplasmin polyclonal antibody raised in our laboratory. Ceruloplasmin samples were also identified by their characteristic migration (versus bromphenol blue) in disk gel electrophoresis on polyacrylamide, as described previously (53). Gels were either stained with amido black or sliced (25–30 slices) and counted for ^{67}Cu.

Ceruloplasmin Receptor Assays. Receptor assays were carried out in the presence and absence of an excess of nonradioactive (300 μM) Cu(II) (the 1:1 molar NTA complex) as described previously for microsomal membranes (33). This concentration provides maximum competition in the binding assay. For this, portions (1–3 mg) of membrane protein were incubated with 50 pmol of ^{67}Cu-labeled ceruloplasmin in a total volume of 900 μl for 1 hr at room temperature before separation of bound and free ceruloplasmin by Airfuge (Beckman Instruments, Palo Alto, CA). Total binding and binding in the presence of excess Cu-NTA were recorded as described previously (33). To demonstrate binding kinetics, larger (25–50 mg) portions of membranes from placenta were incubated with varying amounts of ^{67}Cu-ceruloplasmin (in the picomolar to nanomolar range) in the absence and presence of an 100-fold excess of nonradioactive ceruloplasmin, or 300 μM "cold" Cu-NTA. Data were treated as described by Scatchard (54) to determine the apparent binding constant.

Ceruloplasmin mRNA. Total ceruloplasmin mRNA was determined using extracts of total RNA obtained from portions of guanidine thiocyanate-treated tissue, as described previously (50, 51). The distribution of ceruloplasmin-mRNA to free and endoplasmic reticulum-bound polyribosomes was also examined. For this, postnuclear supernatants were fractionated on discontinuous sucrose gradients, and total RNA was extracted from the appropriate fractions, as described previously (50, 51). For mRNA determinations, 30-g portions of RNA were slot-blotted and hybridized with [^{32}P]cDNA for rat ceruloplasmin, using a plasmid obtained from Julian Mercer and David Danks (Royal Children's Hospital and Murdoch Institute, Melbourne, Australia). Densitometry of autoradiographs developed from the slot blots was performed with a Beckman model 24 spectrophotometer equipped with a scanner. Control blots were made with [^{32}P] cDNA for ferritin and tubulin.

Results

Transport of copper from mother to fetus was studied in Sprague-Dawley rats 1–4 days before the end of gestation. The characteristics of these rats are summarized in Table I. Data are grouped according to treatment and to the principal times (after injection) in which the tissue distribution of radiocopper was examined. There were no significant differences in body weight or other parameters among the groups. In the case of the virgin (nonpregnant) rats examined, the weights of their organs were about the same as those of their pregnant siblings.

Uptake of Copper from Ceruloplasmin versus Albumin and Transcuprein. Radioactive copper in the form of *in vivo*-labeled ^{67}Cu-ceruloplasmin or ^{67}Cu(II)-treated plasma was injected into the rats, by tail vein, while under light anesthesia, 1 and 4 hr before death. Figure 1 shows how the samples used for injection chromatographed on Sephadex G-150, and how radioactivity was distributed among components of maternal serum at the two time points examined. In the case of the *in vivo*-labeled ceruloplasmin, plasma samples were

Table I. Body and Organ Weights (g) of Experimental Animals (mean ± SD)

	Body wt	Liver	Kidney	Spleen	Heart	Brain	Placenta	Uterus	Fetus	Fetal liver	No. in litter	Length (mm)
Pregnant rats												
After ^{67}Cu-ceruloplasmin												
1 hr (n = 6)	344 ± 49	12.9 ± 0.6	1.7 ± 0.1	0.9 ± 0.2	1.0 ± 0.1	1.7 ± 0.2	0.47 ± 0.10	1.4, 0.9 (2)	3.3 ± 1.9	0.22 ± 0.13	12 ± 2	37 ± 8
4 h (n = 5)	361 ± 43	12.7 ± 1.4	2.0 ± 0.3	0.9 ± 0.3	0.9 ± 0.1	1.6 ± 0.2	0.45 ± 0.07	1.1 ± 0.3	2.6 ± 1.0	0.18 ± 0.04	12 ± 2	36 ± 6
All times[a]	325 ± 91	13.1 ± 1.7	1.7 ± 0.2	0.9 ± 0.2	1.0 ± 0.2	1.6 ± 0.2	0.48 ± 0.08	1.1 ± 0.3 (8)	3.3 ± 1.6	0.24 ± 0.10	12 ± 2	36 ± 6
After ^{67}Cu-NTA												
1 hr (n = 8)	342 ± 21	12.0 ± 0.8	1.8 ± 0.1	0.7 ± 0.1	0.9 ± 0.1	1.7 ± 0.1	0.47 ± 0.06	1.5 ± 0.8 (5)	3.5 ± 1.3	0.22 ± 0.08	13 ± 2	37 ± 5
4 hr (n = 5)	362 ± 21	11.1 ± 1.8	1.8 ± 0.2	0.7 ± 0.1	0.9 ± 0.1	1.5 ± 0.1	0.52 ± 0.08	1.0 ± 0.5	3.9 ± 1.6	0.22 ± 0.05	13 ± 2	37 ± 5
All times[a]	347 ± 26	12.0 ± 1.2	1.8 ± 0.2	0.7 ± 0.1	0.9 ± 0.1	1.6 ± 0.1	0.59 ± 0.26	1.2 ± 0.6	3.7 ± 1.5	0.23 ± 0.10	13 ± 2	36 ± 7
Nonpregnant rats												
All times (n = 4)	291 ± 37	12.4 ± 4.0	2.5 ± 0.6	0.8 ± 0.3	1.1 ± 0.3	1.8 ± 0.2	—	—	—	—	—	—

[a] Includes some rats sacrificed at 2 and 6 hr.

taken from donor rats 12–19 hr after an intraperitoneal injection of a large dose of ^{67}Cu(II) (see Materials and Methods). Fractionation of 1.0 ml on a 50-ml column of Sephadex G-150 gave a single peak eluting in the position of ceruloplasmin oxidase activity (Fig. 1A), and a single band migrating in the position of ceruloplasmin (Rf 0.6) in polyacrylamide gel electrophoresis (data not shown). Radioactivity in maternal serum taken 1 or 4 hr after intravenous injection of peak fractions of this ^{67}Cu-ceruloplasmin also eluted as a single symmetrical peak in the position of ceruloplasmin (Ve about 23 ml) (Fig. 1B) and could be precipitated with rabbit, anti-rat ceruloplasmin antiserum (data not shown).

Fresh plasma treated *in vitro* with nanogram quantities of ^{67}Cu-NTA, to label albumin and transcuprein (see Materials and Methods), gave a different chromatographic distribution of the radiolabel (Fig. 1C). About one third of the radioactivity was on a peak in the void volume (Ve about 17 ml) and the rest was on a peak eluting in the position of albumin standard (Ve about 30 ml). (By definition, the former peak is what we call transcuprein, and the radioactivity in this peak is precipitable with antibody against an M_r 270,000 protein we have isolated and begun to characterize [18, 20, 21]. The other label is on albumin [15, 21].) One hour after intravenous injection of this solution (Fig. 1D), the ^{67}Cu in maternal serum was still mainly with transcuprein and albumin, but more was with the transcuprein fraction. By 4 hr, however, most was with a peak eluting in between, namely with ceruloplasmin, representing that synthesized by the pregnant rat from the injected ^{67}Cu(II). Production of this ^{67}Cu-ceruloplasmin could be severely inhibited with cycloheximide (see later).

Distribution of radioactivity to maternal and fetal tissues from the two different injected sources was followed and compared. The data on uptake as percentage of dose are summarized in Tables II and III. Values are based on total radioactivity per organ, 1 and 4 hr after injection of ^{67}Cu-ceruloplasmin or ^{67}Cu-(transcuprein + albumin). They represent accumulation of *radioactivity*, rather than actual copper. Values for actual copper accumulation are presented later on.

Four hours after giving ^{67}Cu-ceruloplasmin, three quarters of the ^{67}Cu was still circulating in the maternal blood with the ceruloplasmin that had been injected (Table II). (Also see Fig. 1A.) Four hours after injection of ^{67}Cu(II) bound to transcuprein and albumin (Fig. 1C), more than 80% of the radioactivity had left the maternal blood (Table II). Already at 1 hr, and increasingly with time, detectible amounts of radioactivity were transferred to the placenta and fetus from either copper source (Tables II and III), but more was transferred in the case of injections of ^{67}Cu-ceruloplasmin. On the basis of percentage of dose per organ (shown), or percentage of dose per gram (not shown), the placenta seemed to be as avid for copper as the liver when copper was given as ceruloplasmin and less avid when it was given as ^{67}Cu(II) bound to albumin + transcuprein (Table II). There was a significant uptake of copper by the uterus from both copper sources (Table III), and accumulation of ^{67}Cu from ceruloplasmin was greater than from albumin + transcuprein for maternal heart, brain, and spleen, and less for liver and kidney (Table III), as described previously for nonpregnant, adult rats (26).

Fetal blood and tissues also accumulated more radioactivity over time when ^{67}Cu was given as ceruloplasmin (Tables II and III). On the basis of percentage of dose of radioactivity, the whole fetus accumulated three times more ^{67}Cu from ceruloplasmin over 4 hr than from ionic copper attached to albumin and transcuprein (Table III). There was a 7-fold difference in the case of fetal plasma and a 2-fold difference in the case of fetal liver (Table II).

As mentioned earlier, the data in Tables II and III show the accumulation of radioactivity by maternal and fetal tissues rather than the accumulation (or uptake) of *actual copper*. The latter can be calculated from the specific activity of the injected ^{67}Cu once it has entered and been mixed with the copper in the endog-

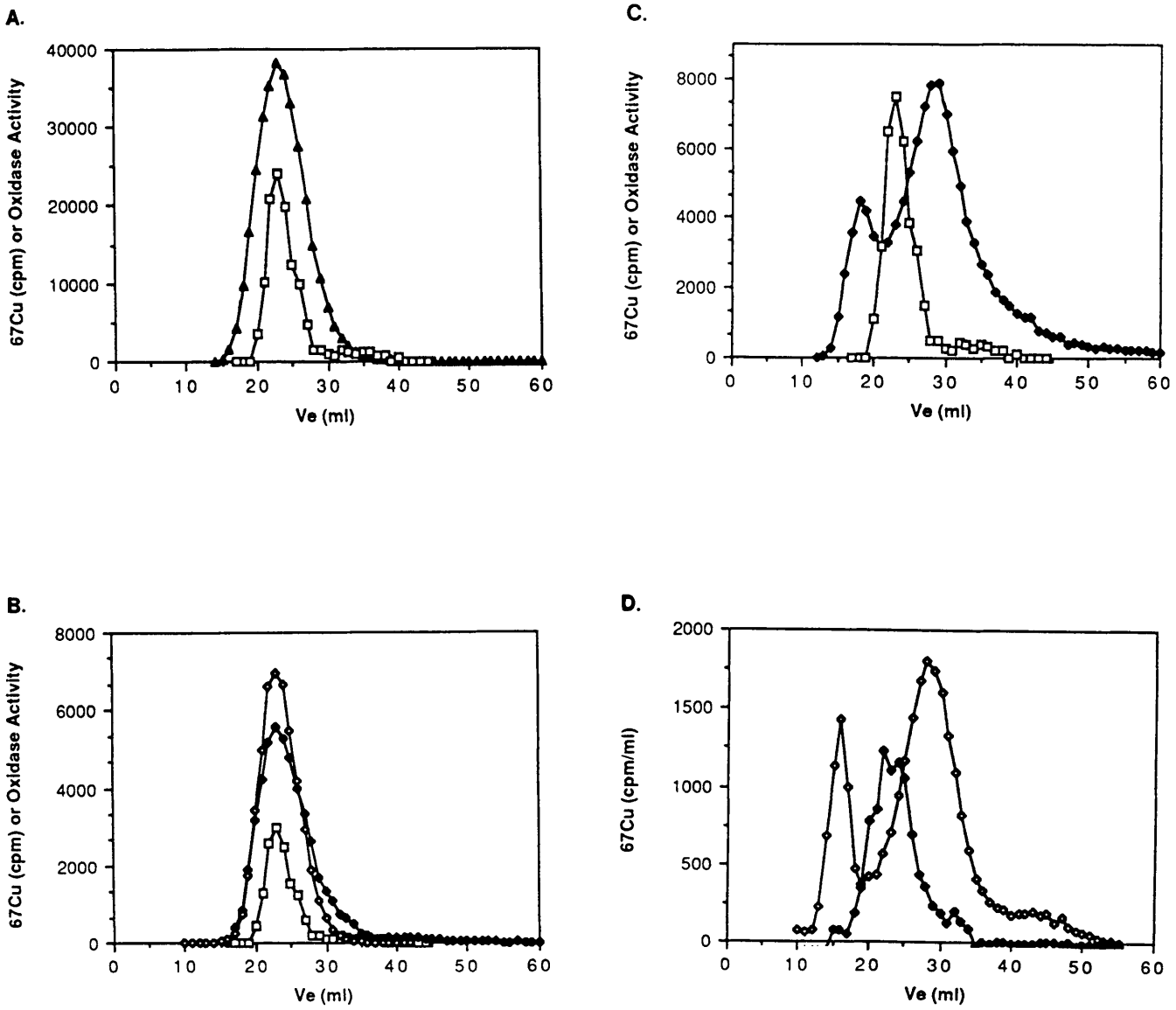

Figure 1. Gel chromatography of ^{67}Cu-labeled samples used for intravenous infusion, and serum samples from the maternal circulation taken 1 and 4 hr after infusion. One-milliliter samples were chromatographed on 50-ml columns of Sephadex G-150. Ceruloplasmin and albumin standards eluted at 23 ml and 28 ml in these columns, respectively. (A) An example of the partially purified ^{67}Cu-ceruloplasmin used in the intravenous infusions, showing elution of radioactivity (▲) in relation to ceruloplasmin oxidase activity (□). (B) Maternal serum at 1 hr (◇) and 4 hr (◆) after tail vein infusion of the ^{67}Cu-ceruloplasmin in (A). Elution of ceruloplasmin oxidase activity is also shown (□). (C) Nonradioactive rat serum to which 2 ng of Cu (as ^{67}Cu(II)-NTA) had been added *in vitro* (◆), which was infused as ^{67}Cu attached to albumin + transcuprein. (The comparative elution of ceruloplasmin oxidase activity is shown again.) (D) Maternal serum at 1 hr (◇) and 4 hr (◆) after infusion of albumin + transcuprein-bound ^{67}Cu (mixture shown in [C]). For the 4-hr sample, cpm values have been multiplied by two to make them more visible.

enous ceruloplasmin (or transcuprein + albumin) plasma pool. Such calculations were carried out for some of the tissues (Fig. 2), using mean values for total ^{67}Cu per organ (Tables II and III), estimates of the amounts of copper injected, and reasonable assumptions about plasma volumes and sizes of plasma copper pools. (Details are described in the figure legend.) The results are given as total ng Cu transferred or accumulated over 4 hr (Fig. 2). Since the plasma pool of ceruloplasmin-copper into which ^{67}Cu was injected is roughly twice as large as that of albumin + transcuprein-copper, the data show that ceruloplasmin was actually seven or eight times more effective than transcuprein and albumin at delivering copper to the placenta and fetus. It was 2.5 times as effective in the case of the uterus, in comparison with the data as percentage of dose (where differences were half as great; Table III). The same approach can be applied to the data for the other tissues (not shown). Thus, ceruloplasmin was clearly the most important source of copper for the placenta and fetus, as well as for most maternal tissues.

Indeed, it seemed possible that even in the case of rats given ^{67}Cu(II) attached to albumin + transcuprein,

Table II. Distribution of ^{67}Cu to Maternal and Fetal Tissues at Various Times after Intravenous ^{67}Cu-Ceruloplasmin or ^{67}Cu(II) Attached to Albumin and Transcuprein[a]

Condition	^{67}Cu (% total dose/tissue or organ)				
	Maternal plasma	Maternal liver	Placenta	Fetal plasma	Fetal liver
After ^{67}Cu-ceruloplasmin					
1 hr	74 ± 6 (8)	4.3 ± 0.3 (6)[b]	5.1 ± 1.4 (6)[b]	0.07 ± 0.38 (8)[b]	0.09 ± 0.14 (6)
2 hr	74 ± 3 (3)[b]	7.2 ± 0.4 (3)	4.5, 12.2 (2)	0.05 ± 0.01 (3)	0.50, 0.47 (2)
4 hr	73 ± 5 (8)[b]	6.1 ± 2.1 (5)	5.6 ± 1.8 (5)[b]	0.15 ± 0.08 (8)[b]	1.03 ± 0.43 (5)[b]
After ^{67}Cu(II)-labeled plasma					
1 hr	43 ± 30 (9)	10.4 ± 0.9 (8)	2.7 ± 0.7 (7)	0.04 ± 0.02 (9)	0.06 ± 0.04 (8)
2 hr	12 ± 5 (3)	9.1 ± 4.8 (3)	1.5, 1.3 (2)	0.02 ± 0.01 (3)	0.15, 0.13 (2)
4 hr	18 ± 8 (8)	10.4 ± 3.7 (5)	2.2 ± 0.9 (5)	0.02 ± 0.01 (8)	0.52 ± 0.15 (5)

[a] Total radioactivity recovered in each tissue or organ, assuming plasma is 5% of body weight. Data for placentae, fetal plasma, and liver were totals/litter. Values are mean ± SD for the numbers of litters or dams indicated in parentheses.
[b] $P < 0.01$ for difference from rats infused with nonceruloplasmin ^{67}Cu, determined by Student's t test.

Table III. Uptake of ^{67}Cu from Ceruloplasmin or Other Plasma Proteins by Maternal Tissues and the Fetus, 1–4 Days before Term[a]

Condition	^{67}Cu uptake (% total dose)					
	Maternal kidney	Maternal spleen	Maternal heart	Maternal brain	Uterus	Whole fetuses
After ^{67}Cu-ceruloplasmin						
1 hr (6)	1.2 ± 0.4	0.24 ± 0.07	0.53 ± 0.11[b]	0.14 ± 0.04[b]	1.0 ± 0.8 (3)	0.15 ± 0.05
2 hr (3)	2.0 ± 0.2	0.31 ± 0.05[b]	0.58 ± 0.21	0.12 ± 0.11	0.43, 0.45 (2)	0.71, 0.52 (2)
4 hr (5)	2.2 ± 1.3	0.24 ± 0.05	0.54 ± 0.16[b]	0.10 ± 0.08	0.69 ± 0.24	1.57 ± 0.40[b]
After ^{67}Cu(II)-labeled plasma						
1 hr (6)	2.5 ± 0.8	0.19 ± 0.04	0.17 ± 0.07	0.05 ± 0.03	1.0 ± 0.7 (5)	0.18 ± 0.14
2 hr (3)	5.9 ± 3.7	0.12 ± 0.04	0.14 ± 0.06	0.03 ± 0.01	0.57, 0.47 (2)	0.12, 0.18 (2)
4 hr (5)	2.4 ± 0.7 (4)	0.22 ± 0.07	0.24 ± 0.13	0.04 ± 0.02	0.68 ± 0.46	0.52 ± 0.15

[a] Values are mean ± SD for the numbers indicated. Data were obtained as in Table II and are for the same animals.
[b] $P < 0.01$ for difference from rats infused with nonceruloplasmin-^{67}Cu, determined by Student's t test.

uptake of ^{67}Cu by the placenta and fetus might still be mainly (or even exclusively) from ceruloplasmin. From previous data (Fig. 1D), it was apparent that maternally synthesized ^{67}Cu-ceruloplasmin appeared in the plasma within an hour or two of injecting ^{67}Cu(II) on albumin + transcuprein. It would thus be available to tissues before the 4-hr time point.

To test this theory, we examined the effect of inhibiting synthesis of maternal ^{67}Cu-ceruloplasmin with cycloheximide. Two pairs of pregnant rats were intravenously injected with ^{67}Cu(II) (on albumin + transcuprein). One pair was treated with cycloheximide 30 min before and 1.75 hr after the ^{67}Cu injection, and both pairs were sacrificed 4 hr after the ^{67}Cu. The results (Table IV) were clear-cut. The cycloheximide-treated animals had much higher levels of ^{67}Cu in their livers and much less in the plasma, consistent with an inhibition of the synthesis of ^{67}Cu-ceruloplasmin by the maternal liver and its reduced release into the plasma. The latter was confirmed by chromatography (Fig. 3). Inhibition of ^{67}Cu-ceruloplasmin synthesis clearly reduced uptake of ^{67}Cu by placenta and fetal tissues, and a reduction in ^{67}Cu uptake was also observed for most other maternal tissues. Uptake was half as great as otherwise in the case of placenta, and about one third as great in the case of the fetus and fetal liver. Uptake was 25–50% reduced for maternal kidney, heart, spleen, and brain. This confirms that ceruloplasmin is the most important source of copper for these tissues, and suggests it might, at least for some, be the only source.

Copper in the Fetal Circulation. The form of the copper appearing in the fetal circulation after administration of ^{67}Cu to the dam was also examined by gel chromatography and immunoprecipitation (Fig. 4). Independent of source, ^{67}Cu in the fetal plasma was initially attached mainly to a component eluting in the void volume of Sephadex G-150 columns (Fig. 4A) and to a component eluting in the position of albumin. In fetal plasma, α-fetoprotein substitutes mainly for albumin. It is of similar size and also has a similar high affinity copper binding site (55, 56). A similar distribution of radioisotope was obtained when traces of

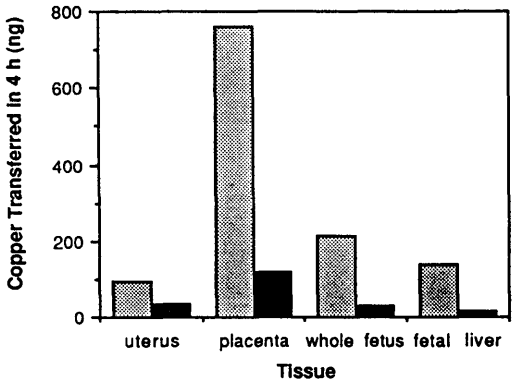

Figure 2. Estimates of actual nanograms of Cu transferred from the maternal circulation to pregnancy and fetal tissues over 4 hr, from ceruloplasmin (shaded bars) and from albumin + transcuprein (closed bars). Mean values for percentage of dose of radioactivity per organ (in Table II) and sizes of plasma copper pools (in ng Cu/ml) were used to calculate the specific activity of ^{67}Cu in ceruloplasmin as well as in albumin + transcuprein (as percentage of dose per ng Cu) after injection of the radioactive samples into the plasma. Assumptions used were plasma volume = 5% of body wt; body wt = 360 g; ceruloplasmin Cu = 750 ng/ml of plasma, totaling 13.5 μg/whole animal; albumin + transcuprein Cu = 300 ng/ml, totaling 5.4 μg/animal. Less than 50-ng portions of Cu were injected as ceruloplasmin or transcuprein + albumin, a negligible amount in relation to the sizes of the inherent copper pools labeled by the radioisotope. The values shown are mean percentage of dose × pool size, in nanograms. Values for placenta and uterus represent amounts retained at the 4-hr time point (i.e., they do not take into account any copper transferred to the fetus).

^{67}Cu(II)-NTA were added to fetal serum *in vitro* before gel chromatography (Fig. 4B). On the same columns, ceruloplasmin oxidase activity from adult rat plasma eluted between the transcuprein and albumin/α-fetoprotein peaks (Fig. 4B).

Evidence that the void volume peak was indeed transcuprein was obtained by immunoprecipitation. Antibody raised against the ^{67}Cu-binding protein in the void volume peak from adult rat plasma, purified by methods described previously (18), was capable of precipitating ^{67}Cu from ^{67}Cu-transcuprein in adult rat plasma (as for example the void volume radioactivity in Fig. 1B). This antibody also precipitated ^{67}Cu from the void volume peak of fetal plasma, but it failed to precipitate ^{67}Cu-albumin. (Specifically, addition of 400 and 800 μl of antiserum to 400 μl of ^{67}Cu-labeled sample precipitated 70 and 173 cpm [or 5 and 11% of the total radioactivity], respectively, while precipitating −7 to +8 cpm in the case of ^{67}Cu albumin [<0.01% of the total cpm]. Background was −7 and +8 cpm [obtained with no addition of antibody].) Additional antiserum was not available for further studies, leaving open the possibility that some of the unprecipitated ^{67}Cu was also with another protein (or proteins) eluting in the same position (although it is unclear what this or these might be).

Four hours after injection of copper radioisotope into the dams, ^{67}Cu in fetal plasma was still associated with the peak ascribable to transcuprein (Fig. 4C), but an additional peak of radioactivity had emerged, eluting between that of transcuprein and albumin/α-fetoprotein, i.e., in the position of ceruloplasmin. The beginnings of such a peak were already present in some fetal plasma samples taken at earlier times (even in one case at 1 hr; Fig. 4D). Treatment of the fractions of this peak (Ve about 23 ml) with antiserum against rat ceruloplasmin confirmed that the tracer copper had been incorporated into that protein (data not shown).

Assays of ceruloplasmin oxidase activity confirmed that the fetus had circulating ceruloplasmin. The level was only about one fifth that in the adult rats (mean ± SD, 3.2 ± 1.5 [n = 9] vs 17.5 ± 2.4 [n = 4] units with 1 unit being 10^{-5} IU/ml plasma). It rose to one third of adult values by Day 2 after birth (5.2 ± 1.5 [n = 3]

Table IV. Effect of Inhibiting Ceruloplasmin Formation with Cycloheximide on Tissue Deposition of Intravenous Injected ^{67}Cu(II) Given as Albumin Plus Transcuprein to Pregnant Rats[a]

Tissue	Total ^{67}Cu (% dose)		Organ wt (g)	
	−Cycloheximide	+Cycloheximide	−Cycloheximide	+Cycloheximide
Plasma	30, 35	10, 20	16.5, 14.8	17.3, 15.7
Liver	31, 25	63, 50	10.6, 9.9	11.0, 9.8
Kidney	6.4, 5.1	3.5, 4.1	1.7, 1.5	1.9, 1.6
Spleen	0.30, 0.33	0.18, 0.15	0.6, 0.5	0.5, 0.3
Heart	0.42, 0.51	0.35, 0.25	0.9, 0.8	1.0, 0.9
Brain	0.15, 0.12	0.09, 0.08	1.7, 1.6	1.6, 1.7
Muscle	11.4, 13.4	9.6, 11.4	107, 100	110, 103
Placentas	8.6, 9.7	4.0, 4.4	5.4, 5.2	6.2, 5.1
Fetuses	2.7, 2.3	0.6, 1.0	77, 61	68, 66
Fetal livers	1.0, 0.9	0.3, 0.3	5.3, 4.6	4.8, 5.7
Fetal plasma	0.12, 0.12	0.03, 0.05	3.8, 3.0	3.4, 3.2

[a] Pairs of pregnant rats were given intravenous ^{67}Cu(II) attached to albumin + transcuprein (as in Fig. 1C) by tail vein, 4 hr before sacrifice. Two rats were injected with cycloheximide intraperitoneally 30 min before and 1.75 hr after the intravenous ^{67}Cu to suppress formation of ^{67}Cu-ceruloplasmin. Data are values for radioactivity, as percentage of dose/organ for individual rats. Assumptions were plasma volume = 5% of body wt and muscle = 35% of body wt.

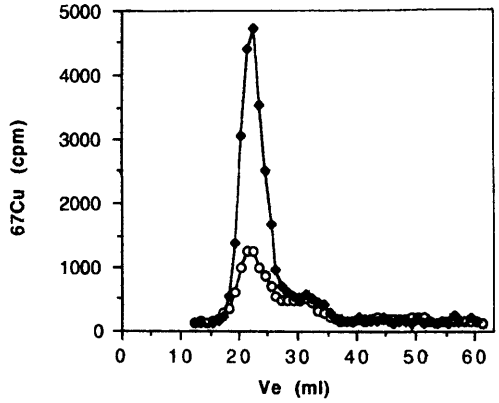

Figure 3. Effect of cycloheximide treatment on production of ^{67}Cu-ceruloplasmin by maternal tissues. Samples of plasma from pregnant rats injected intravenously with equal doses of ^{67}Cu(II) on albumin + transcuprein 4 hr before, were fractionated on Sephadex G-150, as in Figure 1. One rat received cycloheximide before and during the ^{67}Cu uptake period (○), the other did not (♦). (Similar results were obtained for another pair of rats.) Samples were run on the same column and decay-corrected to the same time.

units). Maternal (pregnant) rats had the same level of ceruloplasmin oxidase activity as their nonpregnant siblings (16.3 ± 3.1 [n = 10] vs 17.5 [n = 4] units, respectively), and there appeared to be traces of ceruloplasmin in the amniotic fluid also (1.3 ± 1.0 [n = 8] units of oxidase activity/ml).

Expression and Translation of Ceruloplasmin by Maternal and Fetal Tissues. The capacities of fetal and maternal tissue to produce ceruloplasmin were also examined by assaying for expression of ceruloplasmin mRNA. Relative concentrations of the message were determined on three subfractions of tissue RNA (see Materials and Methods): that associated with the free polyribosomes; that with the polyribosomes bound to the endoplasmic reticulum (ER) (used to produce proteins destined for cell membranes or for secretion); and that associated with the mRNP fraction (which is not being translated). Equal portions of RNA from each fraction were slot-blotted onto nylon membranes, and hybridized with [^{32}P]cDNA for rat ceruloplasmin. Figure 5A shows the distribution of ceruloplasmin and tubulin mRNA among the RNA fractions obtained from livers of nonpregnant, control rats. The bulk of the ceruloplasmin mRNA was with the ER-bound polyribosomes, and that for tubulin was with the free polyribosomes, as we would expect. Figure 5B shows a representative autoradiographic result for tissues from a pregnant rat and her litter. (This was replicated in five additional rats.) It shows that, also in maternal liver, most of the ceruloplasmin mRNA was associated with the ER-bound polyribosomes. (That on free polyribosomes represents mRNA being initiated for ceruloplasmin synthesis at a stage before completion of signal peptide translation [57].) Some ceruloplasmin mRNA was also detected in the mRNP fraction (representing mRNA not being translated, and tRNA). The same results were obtained for fetal liver. Indeed, the apparent degree of hybridization of the radioactive probe with equal amounts of RNA from maternal and fetal liver fractions was very similar, suggesting that both tissues were equally active in terms of ceruloplasmin protein synthesis and secretion. However in placenta/yolk sac, mRNA for ceruloplasmin was even more abundant (or concentrated) than in fetal and maternal liver.

Receptors for Ceruloplasmin on Placental Membranes and Membranes of Other Tissues. Finally, placentae and other maternal and fetal tissues were probed for their content of membrane-associated ceruloplasmin receptors. Aliquots of placental microsomal membranes were incubated with various amounts of ^{67}Cu-ceruloplasmin, and the amount of radioactivity bound was determined, as described previously (33). Figure 6 shows that binding appeared to obey saturation kinetics at low concentrations and that most of the binding could be diluted by the addition of a 100-fold excess of nonradioactive ceruloplasmin, indicative of specific binding. Binding was half-maximal at about 0.5 nM, indicating an affinity of the ceruloplasmin receptor about 10 times higher than that obtained previously for other tissues (31, 32). Total binding of ^{67}Cu-ceruloplasmin was also reduced in the presence of 300 μM Cu(II)-NTA, as shown previously for other rat tissue membranes (33). Total binding to placental membranes was about the same as that for maternal and virgin adult liver, and was reduced 50 ± 12% (n = 6) in the presence of excess Cu-NTA. (Reduction was 53 ± 15% [n = 5] in the case of maternal liver, and 72 ± 73% [n = 2] in the case of virgin liver, in these studies, done with 2 mg of membrane protein and 50 pmol of ^{67}Cu-ceruloplasmin.)

Discussion

We have compared the uptake of copper from the two major sources of this element found in the blood circulation in pregnancy, in the rat. Our findings indicate that ceruloplasmin is clearly more effective than ionic copper (attached to albumin and transcuprein) in delivering this element to the fetus. This was apparent from the data for radioactivity expressed as percentage of dose (per organ/tissue or per gram), in which ^{67}Cu from ceruloplasmin achieved a significantly higher concentration in the placenta and fetus, and accumulated much more rapidly with time. It was even more apparent when actual copper transfer was calculated, taking into account the sizes of copper pools ascribable to ceruloplasmin and albumin + transcuprein in the maternal circulation. For such calculations (Fig. 2), it was assumed that ceruloplasmin and albumin + transcuprein comprise 60% and 25% of plasma copper, respectively (4, 22, 23, 55). On this basis, it was calculated

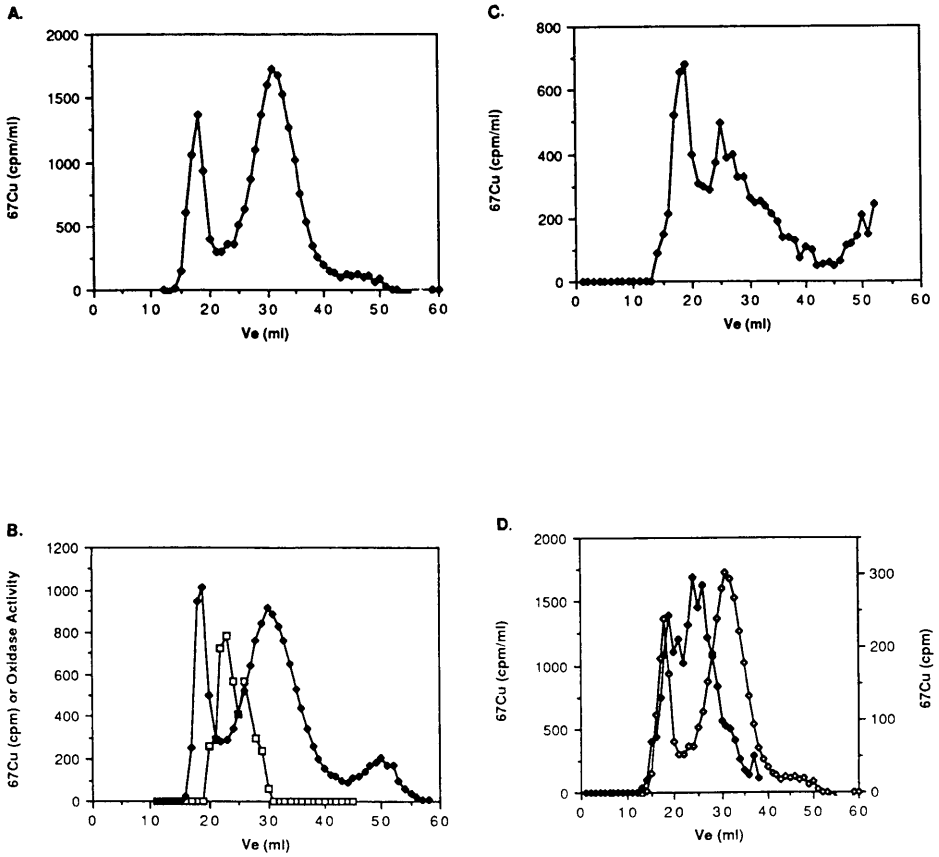

Figure 4. (A) Chromatography of fetal plasma samples taken at different times after infusion of ^{67}Cu components into the maternal circulation (as in Fig. 1). Fetal plasma from rats sacrificed 1 hr after infusion of ^{67}Cu-ceruloplasmin. (The same kind of chromatograph was obtained when ^{67}Cu-labeled transcuprein + albumin was infused.) (B) Fractionation of 1.0 ml of fetal plasma labeled *in vitro* with 2 ng of ^{67}Cu(II)-NTA. The elution position of ceruloplasmin oxidase activity (□) is also indicated. (C) ^{67}Cu-labeled components in fetal plasma 4 hr after maternal infusion of ^{67}Cu-ceruloplasmin. (The same kind of chromatograph was obtained after infusion of ^{67}Cu-labeled transcuprein + albumin.) (D) Another profile of ^{67}Cu-binding components in fetal plasma (♦) less commonly obtained 1 hr after infusion of ^{67}Cu (on albumin + transcuprein) into the mother, in comparison with the more usual profile (◇) also shown in (A).

that more than 200 ng of Cu were transferred to the fetuses (as a litter) from maternal plasma ceruloplasmin in 4 hr, as compared with less than 30 ng when Cu was given attached to albumin + transcuprein. This is a very large difference. Indeed, since copper given as albumin and transcuprein rapidly disappeared from the circulation and was replaced by ceruloplasmin (Fig. 1B), even the ^{67}Cu injected as transcuprein + albumin may have entered placental tissue from ^{67}Cu-ceruloplasmin newly synthesized by the dam. The likelihood of this was confirmed by our finding that inhibition of endogenous ^{67}Cu-ceruloplasmin synthesis (from ^{67}Cu on albumin + transcuprein) reduced uptake of ^{67}Cu by almost all organs. The reduction was particularly strong in the case of the placenta and fetus, which emphasized the importance of ceruloplasmin for fetal uptake. The finding that maternal organs (other than liver) also depended largely on ceruloplasmin for copper confirms the results of our earlier studies in nonpregnant rats (26).

In the mouse studies of McArdle and Erlich (8), ^{64}Cu(II) was administered intravenously as the histidine complex, which would have labeled the exchangeable plasma copper pool. Uptake by placenta and fetus was observed, but the data do not exclude the possibility that, in this species as well, ceruloplasmin made from the injected copper initiated most of the uptake. There was almost immediate radioactivity in the placenta, but the proportion due to the radiolabeled blood in the organ is unknown. Mas and Sarkar (9) showed in preliminary work that perfusion of placentae with Cu(II)-histidine can result in an uptake of Cu and its release to the fetus. Thus, the placenta may well take up ionic or chelated copper when ceruloplasmin is unavailable. However, in recently published work (10), they reported that ceruloplasmin (labeled with ^{67}Cu by *in vitro* manipulation) can indeed be a source of copper for cultured human trophoblasts, which further supports our findings. Using comparable amounts of Cu, they found that individually, Cu-histidine or chloride and ceruloplasmin gave the same initial rates of uptake (and greater rates than Cu-albumin) in serum-free medium. Since the concentration of ceruloplasmin-Cu is several orders of magnitude greater than that of free Cu

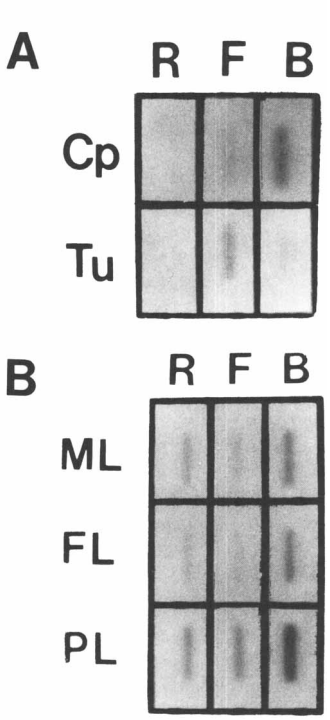

Figure 5. Messenger RNA for ceruloplasmin on free and ER-membrane-bound polyribosomes of fetal and maternal tissues. Equal portions (30 μg) of RNA extracted from free (F) and ER-bound polyribosomes (B) and from the post-ribosomal/mRNP fraction (R) of discontinuous sucrose gradients (see Materials and Methods) were slot-blot-hybridized with [^{32}P]cDNA for rat ceruloplasmin or tubulin. (A) Data are for liver RNA fractions of a virgin rat probed with [^{32}P] cDNA for ceruloplasmin (Cp) or tubulin (Tu). (B) Shown are Representative autoradiographic data for a pregnant rat and her litter, showing hybridization of the ceruloplasmin probe with fractions of RNA from maternal liver (ML), fetal liver (FL), and placenta/yolk sac (PL).

or copper histidine (4), this implies that ceruloplasmin is a much better source under physiologic conditions. All of these findings support the concept that ceruloplasmin is normally the main or only source of copper for the placenta and fetus, in humans as well as rats.

It seems likely that most of the copper destined for the fetus has gone there via the placenta, although Thomas and Schreiber (39) have proposed that some may go via the uterus, at least in the rat. In our studies, the placenta achieved an isotope concentration substantially greater than that of the uterus when ceruloplasmin was the source. Total copper accumulated from ceruloplasmin by the placenta (not including any transferred to the fetus) was more than seven times that accumulated by the uterus. This in itself suggests the uterus is not as important as the placenta for fetal transport.

Placental cells appeared to have membrane receptors for ceruloplasmin. As a result, it seems likely that the receptors are on the pathway taken by maternal ceruloplasmin copper to the fetus. Studies by Percival and Harris (30) and our own laboratory (15, 21, 33) indicate that transfer of copper from ceruloplasmin is mainly a cell surface event not involving endocytosis. The protein moiety of ceruloplasmin does not enter as fast as the copper, *in vivo* (26) or with cells in culture (30), and ceruloplasmin-copper uptake is not inhibited by monensin (33) (an inhibitor of endocytosis/exocytosis). Placental receptors had an apparent affinity for ceruloplasmin somewhat grater than that reported previously for other cells and tissues (31, 32, 45). However, binding by placental (and fetal liver) membranes displayed the characteristic, seen previously, that binding of the protein was inhibited or reversed by an excess of Cu(II) (21, 33). (Uptake of ceruloplasmin-copper by cells is also inhibited by an excess of Cu(II), at least in cell culture [33].)

Our data suggest that, in rats, copper first enters the fetal circulation in ionic form, bound principally to nonceruloplasmin proteins, especially transcuprein. Evidence for the presence of transcuprein in fetal rat plasma is (i) that tracer ^{67}Cu is associated with a peak in the void volume of Sephadex G-150 columns; (ii) that this same peak appears when tracer ^{67}Cu(II) is added to fetal plasma *in vitro*; and (iii) that antibody raised against purified adult rat transcuprein precipitates ^{67}Cu associated with the fetal transcuprein peak. Albumin also appears to bind some of the copper in the fetal circulation (as well as ^{67}Cu(II) added to fetal plasma *in vitro*), and it seems likely that α-fetoprotein is also involved, as it contains the same high affinity copper binding site (56, 58).

Whether some copper enters the fetal circulation also on ceruloplasmin is still unclear. Certainly early on, most of it appears to be on transcuprein and albumin, but a small portion could be on ceruloplasmin, and this is clearly the case at later times, after ^{67}Cu has reached the fetal liver. We have confirmed the findings of Aldred *et al.* (34) and Thomas and Schreiber (39) that the rat placenta (fused to a remnant of the yolk sac) has mRNA for ceruloplasmin. Data from Gitlin's laboratory indicate that the message is actually with the yolk sac portion (40). We have shown that this ceruloplasmin message is being translated and that the product is most likely being secreted, as the message is associated with ER-bound polyribosomes. This would also explain the report of Mas and Sarkar (9) that ^{67}Cu from dihistidine becomes associated with ceruloplasmin in placental/yolk sac extracts. Perhaps some of the ceruloplasmin from the yolk sac is entering the fetal circulation.

It certainly is still possible that the uterus is also in some way involved in ceruloplasmin secretion and copper transport to the fetus (as suggested by Thomas and Schreiber; 39). Fleming and Gitlin's (40) results indicate that the concentration of ceruloplasmin mRNA in uterus near term is much greater than that in the yolk sac/placenta, a point also noted by Thomas and Schreiber (39).

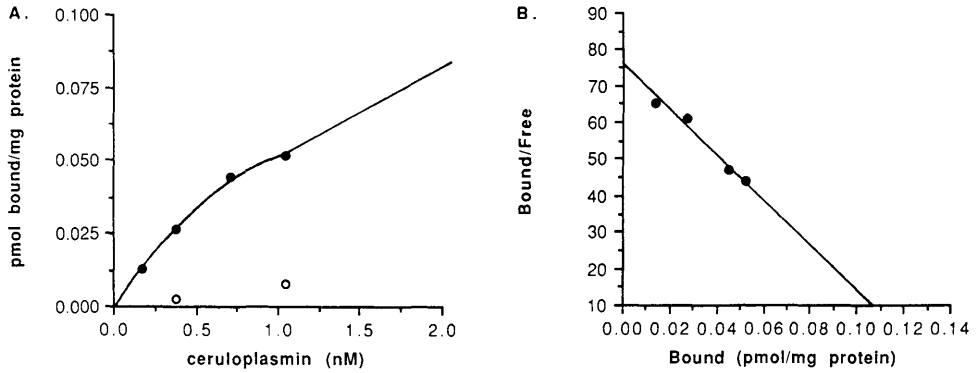

Figure 6. Binding of ^{67}Cu-ceruloplasmin to placental membranes. Aliquots of microsomal membranes from placentae of rats 1 day before term were incubated with various concentrations of ^{67}Cu-ceruloplasmin, partially purified from the plasma of a donor rat, for 2 hr at 25°C, before separation of free and bound radioactivity by ultracentrifugation. (A) Total binding of radioactivity (●); binding in the presence of a 100-fold excess of nonradioactive ceruloplasmin was tested at some concentrations (○). Not shown is binding that was measured at much higher concentrations (up to 200 n*M*), which was only partially (30–50%) inhibited by excess "cold" ceruloplasmin (due to nonspecific binding). (B) Data from (A) plotted according to Scatchard (54).

Our data agree with those of both of the latter laboratories (39, 40) that fetal rat liver is capable of producing ceruloplasmin, and our data strongly suggest, in addition, that it is indeed producing and secreting ceruloplasmin: The mRNA is associated with the ER-bound polyribosomes. Thus, in the rat, the fetal liver is likely to be a significant source of ceruloplasmin in the fetal circulation. (In contrast, the guinea pig may not express any liver ceruloplasmin mRNA until after birth [59], and the same may be true of the pig [60].) The data of Fleming and Gitlin (40) suggest that the fetal (and newborn) *lung* may also contribute ceruloplasmin to the blood plasma of the rat. In humans, the fetal circulation contains ceruloplasmin already well before birth (42, 43), and there are traces of it in amniotic fluid (61, 62), as we have now also shown for the rat. These various findings confirm the importance of ceruloplasmin in copper transport, not just for the normal mammalian adult, but also for the pregnant adult and the developing fetus, as it prepares for birth.

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