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Ceruloplasmin is found in milk and amniotic fluid and may have a nutritional role

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Ceruloplasmin is a copper binding, α_2-globulin of blood plasma, made by the liver, but also expressed by some other secretory tissues, including the choroid plexus of brain and the mammary gland. During studies of neonatal copper transport in the rat, it was determined that amniotic fluid contains ceruloplasmin, based on the presence of azide-inhibitable p-phenylene diamine (pPD) oxidase activity eluting like serum ceruloplasmin in ion exchange chromatography. When ^{67}Cu-labeled (serum-derived) ceruloplasmin was injected into the amniotic sac of fetal rats near term, there was a rapid transfer of the ^{67}Cu to the liver and carcass. Uptake of injected ionic ^{67}Cu(II) was less rapid. Subsequently, the presence of ceruloplasmin in milk was sought and confirmed in several species, using enzymatic and immunologic methods. Based on pPD oxidase activity inhibitable with N_3^-, milks from humans, cows, and pigs all contained similar concentrations of ceruloplasmin and more than in mouse milk. There was no correlation between milk and serum ceruloplasmin oxidase activities among the species. As in amniotic fluid, ceruloplasmin was calculated to account for a substantial portion of the total copper present. A systematic study of non-colostrum human milk indicated by immunoassay that mean ceruloplasmin concentrations were 4.7 mg/L and copper concentrations 9.3 μmol/L during the first 5 days, post-partum. These values dropped about 50% (to 2.3 mg/L and 4.3 μmol/L, respectively) by the end of the first month of lactation. Ceruloplasmin concentrations of about 2 mg/L were maintained during long-term breast feeding, although total copper concentrations continued to decline. Newborn rats fed ^{67}Cu in milk preferentially absorbed ceruloplasmin-derived copper over ionic copper mixed with milk, whereas 4-week-old weanling rats absorbed both forms equally well. It is concluded that ceruloplasmin is a significant copper binding component of milk and amniotic fluid and more nutritionally available than other ingested copper in the perinatal period. © Elsevier Science Inc. 1996 (J. Nutr. Biochem. 7:632–639, 1996.)

Keywords: ceruloplasmin; perinatal nutrition; milk; amniotic fluid; copper

Introduction

Ceruloplasmin is the main copper binding component in mammalian blood plasma, accounting for 60 to 70% of the total copper in human and rat plasma,[1–4] An α_2-glycoprotein of about 132,000 Da, with 6 non-dialysable copper atoms per molecule,[5] plasma ceruloplasmin probably has at least three different functions. For one, it is a source of copper for cells in many tissues.[4,6–8] It is also able to scavenge a variety of oxygen radicals,[9,10] which would explain its role as an acute phase reactant and the increase in its rate of expression and secretion by the liver in response to inflammation.[3,11] A third function of ceruloplasmin, to help in the release of iron from cells in liver and some other tissues, is strongly suggested by effects of copper deficiency and ceruloplasmin infusions on iron metabolism,[12–14] by the liver and brain accumulation of iron in humans with genetic aceruloplasminemia,[15,16] and by analogy with a homologous protein of yeast (FET3) that sits on

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the outside of the cell membrane and is involved in iron transport.[17–19]

Although the liver is the primary source of ceruloplasmin circulating in the blood, it is not the only tissue where ceruloplasmin is expressed. Indeed, ceruloplasmin mRNA or its synthesis has now been detected in many tissues, including those involved in the production of proteins for other body fluids: the choroid plexus of the brain (which synthesizes proteins for cerebrospinal fluid),[20–22] Sertoli cells (and testes) which provide proteins for seminal fluid,[23,24] as well as uterus,[21,25,26] placenta, and/or yolk sac,[6,21,26] which have their own secretions. (Expression has also been reported for the lung,[26–28] as well as for the heart and kidney.*[,29]) In studies of perinatal copper transport in rats[6] in which we compared uptake of copper by the placenta and fetus after intravenous infusion of ^{67}Cu-labeled ceruloplasmin or ionic Cu(II) into maternal blood, we found that ceruloplasmin-copper was the preferred (and perhaps even the only) form taken up, short term. Moreover, we rediscovered that ceruloplasmin was present in amniotic fluid, an observation made earlier by Chan et al.[30] This lead us to investigate whether the protein in amniotic fluid might play a special role in the nutritional transport of copper during gestation, since the fetus ingests amniotic fluid. The results of that study were positive (reported in this article) and lead us to speculate that ceruloplasmin might also be in the milk. If so, it would be produced by the mammary gland and be expressed in that tissue. In concurrent studies on the expression of liver ceruloplasmin in rats bearing mammary tumors (Dunning DMBA-5A) and regulation of ceruloplasmin expression by estrogen,[31] we found that the mammary tumors themselves expressed ceruloplasmin mRNA (R. Middleton, N. Madani, and M.C. Linder), as suggested also by some earlier assays of Kunapuli et al.[32] At the same time, Jaeger et al.[33] reported expression of ceruloplasmin mRNA in rat mammary gland, supporting our conjecture. However, in the latter studies, expression of the ceruloplasmin message was not dependent on, or correlated with, lactation, suggesting its expression in these cells might not be related to milk production (so that ceruloplasmin might not even be in the milk). In contrast, a Russian report indicated that ceruloplasmin was indeed in human milk.[34]

The studies reported here provide conclusive evidence of the presence of ceruloplasmin in the milk of humans and other mammals and confirm its occurrence in amniotic fluid. They also indicate that ceruloplasmin may play a role in perinatal copper nutrition and be a specific vehicle for transfer of the metal from mother to offspring, perhaps in analogy with the role of lactoferrin in milk iron transfer.[35–37]

Methods and materials

Animals, treatments, and studies with radioactive copper

Pregnant and non-pregnant Sprague Dawley rats were obtained from Simonson Laboratories (Gilroy, CA USA). The fetal rats, studied 1 to 3 days before birth, had body weights of 3.5 ± 1.6 g and liver weights of 0.23 ± 0.10 g (mean ± SD, for 24 pups). Newborn pups had body weights of 9.3 ± 2.0 g (n = 8), and liver weights of 0.29 ± 0.11 (n = 8). Radioactive copper [^{67}Cu(II) in 0.1 N HCl] was obtained from Los Alamos National Laboratories or from the reactor at the University of Missouri (courtesy of Dr. Kurt Zinn). ^{67}Cu-labeled ceruloplasmin was produced in single donor rats injected 18 to 24 hr before sacrifice i.p. with 2 to 5 mCi ^{67}Cu(II) as the 1:1 molar complex with nitrilotriacetate (NTA) in 0.9% NaCl (pH between 5 and 7). One mL samples of plasma were immediately fractionated on 50 mL columns (0.8 × 60 cm) of Sephadex G150 (Pharmacia, Piscataway, NJ USA), equilibrated in PBS (phosphate-buffered saline; 0.9% NaCl; 10 mM Na phosphate, pH 7.0). Three mL comprising the peak of the ceruloplasmin fraction were taken as ceruloplasmin-copper for (a) injection into the amniotic sac of fetuses while under pentobarbital anesthesia (50 to 100 mg/kg administered i.p. to the dam); (b) for mixing with cow's milk to feed to newborn pups. ^{67}Cu-NTA in PBS was also injected directly into fetal amniotic sacs or mixed with cow's milk for the feeding studies. Doses of actual copper injected into amniotic fluid were 4 ng in PBS. In the feeding studies, pups received 50 μL portions of milk containing about 4 ng Cu labeled with ^{67}Cu and attached to ceruloplasmin or in the form of the NTA complex. Pups were fed by gavage, using very fine, flexible plastic tubing attached to a 1-mL syringe. Sacrifice of animals was by exsanguination while under pentobarbital anesthesia, as previously described.[6] Whole fetuses were rinsed thoroughly with 0.9% NaCl and blotted before opening of the peritoneal cavity to remove the GI tract and liver (which were separately counted for radioactivity). The GI tract was carefully removed from the fetuses and pups to avoid potential spillage of ^{67}Cu remaining in the tract. Livers were also rinsed before counting to minimize contamination. Samples of plasma, as well as whole organs, GI tracts, and carcasses were placed in vials and counted in a gamma counter (Tracor Analytic, IN USA). Data for radioactivity were corrected for background, decay, and for the efficiency of counting in relation to height in the vials.

Milk and serum samples

Human milk samples were initially obtained from volunteers involved in the LaLeche League and from personal friends and were from various stages of lactation ranging from 14 to 65 days post partum. Later, a systematic serial study was initiated, in which samples were collected by Lisa Wooten, RN, from volunteers interviewed in the maternity unit of St. Jude's Hospital (Fullerton, CA USA), after obtaining permission from the California State University Human Use Committee and the IRBs of the hospital. Of the 10 women recruited to give samples on days 1, 3, 5, and 30 post partum, 9 gave three serial samples during the first week and 4 gave additional samples at 30 days. (One only gave a sample on the first day, which was not included in the study.) The women ranged in age from 20 to 36 years (30 ± 7; Mean ± SD). All but two infants were full term (39 ± 0.9 weeks); one was 29 weeks and the other 32 weeks. All but three were vaginal deliveries, the rest by Caesarian section. Half of the infants were first born; three of the nine were males.

Sow's milk was obtained from the Swine Unit of the Department of Animal and Veterinary Science, California Polytechnic University, Pomona, CA USA, with the assistance of Steve Wickler, PhD, DVM, and Edward Fonda, PhD, Professors of Animal Science. Sows had been lactating from 2 to 10 weeks. One sample of colostrum was obtained 1 day after birth. Oxytocin was injected i.v. to release the milk. Cow's milk was obtained from the same institution and from Excelsior Farms (Corona, CA USA), but without oxytocin; lactational stage was not recorded. Two milk samples from one mouse were obtained courtesy of Dr. Jeffrey

*We have evidence for expression by rat kidney (P. Cerveza and M.C. Linder, unpublished)

Klein (Beckman Instruments, Fullerton, CA USA) whose family pet had given birth some days before. The mouse was milked with the help of suction applied with a 20 μL Pipetman (Rainin, Emeryville, CA USA). All milks were immediately placed on ice and either frozen for later processing or processed prior to freezing, to remove the fat. Nonfat milk was retrieved from underneath the fat layer after centrifugation at 4°. Samples not used immediately were frozen in aliquots (–20°). Work with serum ceruloplasmin has established that it keeps well when frozen in its original fluid and can withstand several rounds of freezing and thawing.[38]

A few samples of pig serum were obtained from some of the same lactating animals used to obtain milk. Bovine serum samples were obtained from non-pregnant cattle taken at slaughter, courtesy of Dr. Christine Goode (from our department). Mouse serum was from non-pregnant adult, female BALB-C mice in our university colony. Rat serum was from 3-month-old virgin female Sprague Dawley animals.

Ceruloplasmin enzyme and immunoassays

Ceruloplasmin oxidase activity was assayed with p-phenylene diamine,[39] $\pm N_3^-$ and with o-dianisidine,[40] as previously described.[41] Human ceruloplasmin was assayed by rocket immunoelectrophoresis, using rabbit antibody purchased from Dako (Glostrup, Denmark) (150 μL per 25 mL 1% agarose gel in 27 mM Tricine Buffer IV, pH 8.6) and a refrigerated Biophoresis unit (BioRad, Richmond, CA USA). Human serum ceruloplasmin standards used for quantitation were from Behring Diagnostics, Inc. (N Protein Standard SY; Westwood, MA USA). Antibody was added to the agarose (Standard Low m_T; Bio-Rad) at about 55°, just before pouring the plates. Samples (5 to 8 μL) were placed in wells on 10 × 10 or 10 × 15 cm, 1.5 mm-thick, glass plates immediately before application of current (100 V for a minimum of 4 hr, optimally overnight) at 9°, or 150 V for a minimum of 2 hr at 4°). Finished plates were soaked in deionized water for 15 to 30 min and then pressed under several layers of filter paper and a weighted glass plate for 30 min. The glass plates were then soaked in 0.9% NaCl for 15 to 30 min, and pressed as before. Pressed gels were dried completely with a hair dryer or in a gel drying apparatus, and were then immersed in Brilliant Blue R Staining solution (Sigma; St. Louis, MO USA) for a minimum of 15 min. Gels were destained with two to 3 changes of 10% acetic acid until a clear background was obtained, then dried as before. Areas under each rocket were calculated from height × width at half height. Quantities were determined from a curve made with area values for the three or more standards run on every plate. Each sample was assayed in duplicate or triplicate.

Copper analyses

The copper content of fluid samples was determined by furnace atomic absorption spectroscopy, using a Zeeman 800A spectrometer from Varian (Mulgrave, Victoria, Australia) with automatic sampling, duplication, resloping after every 10 samples, and restandardization after every 20. Copper standard stock solution was from Aldrich Chemical Co., Inc. (Milwaukee, WI USA) and diluted in 0.1% nitric acid (trace metal grade; Fisher Scientific, Pittsburgh, PA USA).

Results

Copper and ceruloplasmin in amniotic fluid, and its uptake by the fetus

As already indicated, in the course of studies on uptake of ceruloplasmin and non-ceruloplasmin copper by placenta and fetus of rats near term,[6] amniotic fluid samples were also taken and found to contain some ceruloplasmin, based on oxidase activity determined with pPD. Further analysis of comparable samples confirmed these results, giving values for oxidase activity that almost doubled in the last days before birth (*Table 1*), increasing from 3 to 7% those of serum. Most of the oxidase activity was inhibited by N_3^-, a characteristic of ceruloplasmin. Total copper content was significant and increased from 4 to 17% of serum. Total protein concentrations of amniotic fluid increased about 2 fold, in parallel with ceruloplasmin concentrations, in the last days before birth, whereas copper concentrations increased 4 fold. Using the specific activity of serum ceruloplasmin (an oxidase activity of 0.17 nmol/min/mL corresponding to 750 ng ceruloplasmin copper/mL), it was calculated that about 2/3 of the total copper in amniotic fluid (or 30 ng Cu/mL) 4 days before birth was accounted for by ceruloplasmin, and that this rose to 50 ng/mL just before birth.

Because amniotic fluid is a substance ingested by the fetus and contains proteins produced by the mother, we wondered whether the ceruloplasmin might be playing a nutritional role. To begin to address this issue, we injected ^{67}Cu-labeled ceruloplasmin (isolated from rat serum) into the amniotic sacs of fetuses, to observe whether the radiolabeled copper would appear in their tissues. Also, as we had observed that ceruloplasmin rather than ionic copper is the preferred source of maternal serum copper taken up by the fetus via the placenta,[6] we wondered whether the same might not be true for amniotic fluid copper (ingested by the fetus). Thus, half the fetuses in a litter were injected with ceruloplasmin-^{67}Cu the other half with ^{67}Cu-labeled ionic copper (as the 1:1 molar complex with NTA). The data in *Table 2* show that copper from both sources appeared in the livers of the fetuses already after one hour, and that uptake of ceruloplasmin-copper was much greater than uptake of ionic copper. (The same doses of actual copper were administered.) Much of the radiolabel was still in the amniotic fluid. (Exact calculations of the amounts remaining in the latter were not possible, as the volumes of fluid per fetus could not be accurately measured.) Some of the radioactivity also appeared in the uterus and placenta, although it is unclear whether part of this was contamination. (Tissues were washed in saline, but this may not have been enough to remove copper that became associated with these tissues

Table 1 Ceruloplasmin and copper in rat amniotic fluid and serum

	Amniotic Fluid		Normal Rat Serum
	–4 days	–1–2 days	
	(3)	(5)	(4)
Ceruloplasmin oxidase activity (10^5 IU/ml)[0]	0.7 ± 0.1	1.1 ± 0.0 (0.37)*	17.5 ± 3
Total copper (ng/ml)	45 ± 5	192 ± 22	1200 ± 150
Total protein (mg/ml)	1.5 ± 0.5	3.2 ± 0.7	710 ± 50

Values are Mean ± SD, for amniotic fluid samples taken 1–2 or 4 days before birth, and for serum samples of 3 mo old female virgin rats (numbers of samples in parentheses).
[0]with p-phenylenediamine.
*Activity in the presence of N_3^-.

Table 2 Fetal uptake of ^{67}Cu from ceruloplasmin and ^{67}Cu-NTA in amniotic fluid

Tissue	^{67}Cu in Tissue/Organ (% dose)	
	From Ceruloplasmin	From Cu-NTA
Fetal liver	0.32 ± 0.03*	0.05 ± 0.04
Placenta	1.7 ± 0.8	1.1 ± 0.4
Uterus (1/2)	20	3.7
Amniotic fluid (1.0 ml)	22	9.8

*Uptake significantly greater than from Cu-NTA ($p < 0.01$).
^{67}Cu-ceruloplasmin or ^{67}Cu(II)-NTA (nitrolotriacetate) was injected into the amniotic sacs of individual fetuses 1 h before sacrifice, in situ. [Fetuses in one half of the uterus were treated with ceruloplasmin and those in the other half with Cu-NTA, all in the same pregnant rat dam near term.] Doses of Cu per fetus were calculated to be 4 ng in 50 µl phosphate-buffered saline, pH 7.0, from either source. Mean values ± SD are for the individual fetuses and their placentas. There were 6 fetuses in each group.

during dissection of the fetuses.) In any event, the results imply that copper in amniotic fluid is ingested and absorbed by the fetus. They also suggest that there may be some special mechanism in the intestine of the fetus that allows preferential uptake of ceruloplasmin-copper.

Ceruloplasmin in milk, and its uptake by the newborn

By extension of our studies with amniotic fluid, we hypothesized we would find ceruloplasmin also in the milk and that this ceruloplasmin might be a means of transferring copper to the newborn. *Table 3* shows that we found oxidase activity, using pPD and the other commonly used substrate for ceruloplasmin, o-dianisidine, in the milk of pigs and humans. Levels of o-dianisidine oxidase activity were particularly high in the case of the pig. Almost all of the pPD oxidase activity was inhibited by azide, a characteristic of ceruloplasmin. The presence of ceruloplasmin in milk was also confirmed immunologically, using antibody against human serum ceruloplasmin in rocket immunoelectrophoresis (*Figure 1*).

Table 3 Ceruloplasmin in milk and colostrum detected by measuring azide-inhibitable p-phenylenediamine or o-dianisidine oxidase activity

	Ceruloplasmin oxidase activity		
	With p-Phenylenediamine (10^5 IU/ml)		
	($-N_3-$)	($+N_3-$)*	With o-Dianisidine (U/L)
Pig colostrum (1)	14.4	1.8 (12%)	190
Pig milk (5)	4.5 ± 1.7	0.0 ± 0.0 (0%)	60 ± 40
Human milk (6)	3.7 ± 0.8	0.5 ± 0.3 (14%)	1.2 ± 0.6

Colostrum was from day one of lactation. Pig and human milk samples were from 2–10 weeks of lactation. In the case of the human milk, one woman gave 3 of the samples. Values are Means – SD for the numbers of samples indicated.
*Significant inhibition ($p < 0.001$) of pPD oxidase activity by azide is characteristic of ceruloplasmin). Residual activity (as percent) is shown in parentheses.

Further assays of ceruloplasmin oxidase activity were performed on milk samples from four mammals, to establish whether ceruloplasmin is generally found in milk and to make a rough comparison of the levels present (*Table 4*). Milk samples were from a range of lactational stages except in the case of the mouse which was from a few days after birth. pPD oxidase activities were similar in milks from humans, cows and pigs, but lower in the case of the mouse (which is known to have a lower serum pPD oxidase activity).[41] Values for ceruloplasmin oxidase activities of serum are also shown. These were for samples from some of the same lactating sows but otherwise from non-lactating, non-pregnant adult females of the same species. The data suggest that there is less ceruloplasmin oxidase activity in milk than in serum in all species and that there is no correlation between serum and milk levels across the species.

A more systematic study of the ceruloplasmin and copper contents of human milk was then instituted. Serial samples of milk were collected from nine women on days 1, 3, and 5 after birth, and from four of the same women 1 month later. Two samples from mothers breast feeding more than a year were also analyzed. The data are summarized in *Figure 2*. Values for ceruloplasmin and total copper in milk were essentially unchanged during the first 5 days, and values for individual samples spanned a 2 fold range. Immunoassays indicated that there was an average of 4.7 ± 1.3 mg ceruloplasmin protein per L of milk (Mean ± SD; $n = 22$), and 602 ± 241 µg/L total copper (Mean ± SD; $n = 22$), or 9.3 ± 3.7 µmol total Cu/L. By immunoassay, ceruloplasmin concentrations dropped about 50% by the end of the first

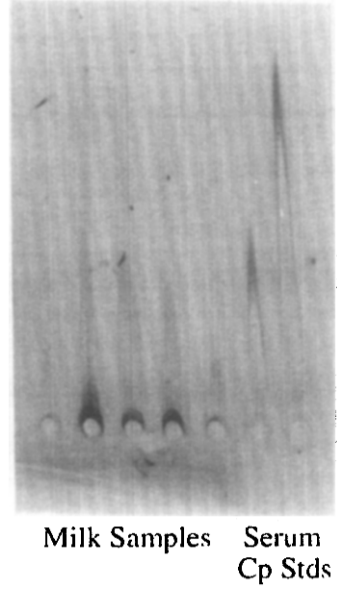

Figure 1 Rocket immunoelectrophoresis of ceruloplasmin in human serum and milk. This procedure was used to quantitate the ceruloplasmin protein concentrations in samples of human milk. The figure shows an example of a portion of a gel plate stained for protein after running several samples/dilutions of human milk (left wells), as well as human serum standards (right two wells) in agarose gel electrophoresis, as described in Methods.

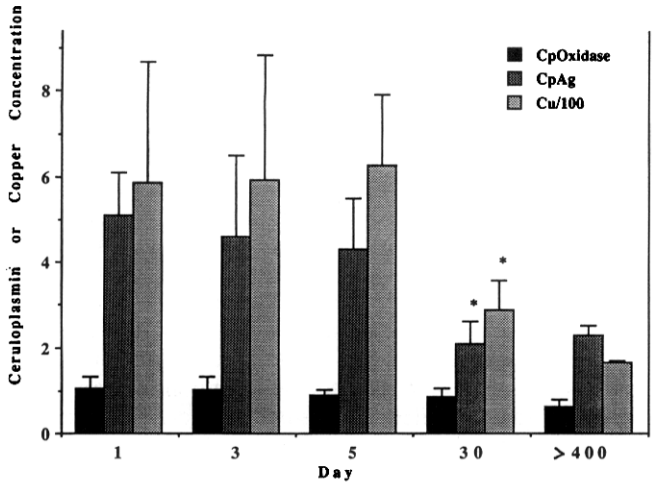

Figure 2 Ceruloplasmin and copper concentrations of human milk at various stages of lactation. Serial samples were collected from 9 women on specific days after birth (abscissa), as indicated. Values are Means ± SD for the samples from all the women on days 1 through 5 and for samples from four of the women on day 30. A few of the values in each group were not factored in because they were more than 3 SD from the Mean. Hence, Means are for 7–8 determinations on days 1–5, and 4 determinations on day 30. [The mean values for the four women who gave serial samples through day 30 did not significantly differ from those of the group as a whole, during the first week.] Values for samples of milk from two women still breast feeding after 1 year and 2 mo and 1 year and 9 mo were averaged and shown on the far right (>400). [In this case, the error bar indicates difference from the average.] From left to right, successive bars indicate: ceruloplasmin measured as pPD oxidase activity (10^5 IU/mL); ceruloplasmin measured by immunoassay (mg/L); and total copper in the milk (μg/L). [Values for the latter were divided by 100 to bring them into scale.] Values for the protein content of the milks were 15 ± 4, 16 ± 1, 14 ± 3, 8 ± 2, and 6 and 4 mg/mL, on days 1, 3, 5, 30 and >400 post partum, respectively. Starred (*) bars indicate a statistically significant difference from days 1–5 ($P < 0.001$) by student's t-test.

month post partum, and the same was the case for the total copper content. Oxidase activities were more variable and did not show the same mean fall. Analyses of the milk of the two women involved in long-term breast feeding suggested that there was a further 50% decline in total copper over time, but no further drop in ceruloplasmin concentrations.

Absorption of copper from ceruloplasmin or Cu-NTA (mixed with cow's milk) was also studied, using rat pups. Each received about 4 ng of copper from either ^{67}Cu-labeled ceruloplasmin or Cu(II)-NTA (by gavage). [This is less than 0.5% of the estimated daily intake of copper experienced by rat pups (calculated from Suttle[42]).] In the case of the newborn pups (1 to 2 days old), the percent dose absorbed and found in livers and carcasses from 3 to 25 hr later is shown in *Table 5*. Copper was absorbed from both sources, and the percent dose present in liver and carcass increased with time. Again, accumulation of radioactivity derived from ionic copper was significantly less rapid than that derived from ceruloplasmin-copper. However, when the same kind of experiment was performed with 4-week-old weanling rats (*Table 6*), there was no longer a difference between the availabilities of ceruloplasmin and ionic copper. This implies that the mechanism allowing preferential absorption of ceruloplasmin-copper is only operating directly before and after birth.

Table 4 Ceruloplasmin oxidase activity (measured with pPD) in milk and serum of several mammals (10^5 IU/mL)

	Milk	Serum
Cow	3.7 ± 0.7 (6)	5.8 ± 1.1 (3)
Pig	2.7 ± 0.9 (13)	52.9 ± 10.4 (6)
Human	2.6 ± 2.8 (10)	17.7 ± 4.3 (59)*
Mouse	0.4, 1.2 (2)	3.9 ± 0.3 (9)

Milk samples were from various stages of lactation (see Methods for details). Serum samples were were from virgin females except in the case of the pig, where they were from sows giving the milk samples (see Methods). Values are Mean ± SD(N).
*From Linder et al.[38]

Discussion

We have found that ceruloplasmin is present in milk and amniotic fluid and that it is more available for absorption than non-ceruloplasmin copper in the perinatal period. Much of the copper needed for growth within the womb and during the suckling period is transferred to the fetus from the mother during gestation.[3,43,44] As with iron,[45] stores of copper accumulate in the liver particularly just before birth. Probably most of this copper is transferred directly from the maternal circulation via the placenta,[6,46,47] where specific receptors for ceruloplasmin have been identified. However, our data suggest that some copper is also transferred via the amniotic fluid, and perhaps more importantly, that significant amounts of copper may be provided to the growing infant via the milk, and by the ceruloplasmin in the milk.

Our studies indicate that ceruloplasmin accounts for a small, but significant, portion of the total copper in milk and amniotic fluid. In the case of the rat, it would appear to account for about two-thirds and one-quarter of the total in amniotic fluid 4 and 1 to 2 days before birth, respectively. In the case of milk from the human, for which we have the best documentation, about 3% of the total copper could be accounted for by ceruloplasmin based upon the immunoas-

Table 5 Absorption of copper from ceruloplasmin (Cp) and Cu-NTA when fed to newborn rat pups in milk (Percent of dose)

	Liver		Carcass (minus liver, GI)	
Cu-Source	^{67}Cu-Cp	^{67}Cu-NTA	^{67}Cu-Cp	^{67}Cu-NTA
Time after feeding				
3–4 hours (4)	1.6 ± 1.4	0.5 ± 0.4	3.2 ± 2.2	1.4 ± 0.5
19 hours (4)	13 ± 4	1.1 ± 0.2*	9.8 ± 2.3	3.9 ± 2.0*
24–25 hours (8)	12 ± 5	5.4 ± 1.2*	8.0 ± 1.9	5.4 ± 1.3*

Day-old rat pups were fed cow's milk containing ^{67}Cu-labeled copper (about 4 ng) added in the form of purified rat serum ceruloplasmin or Cu-NTA at various times before sacrifice. Values are Mean ± SD for groups or newborns (N = 4 or 8, as indicated). Starred (*) values indicate a significant difference between giving copper as ceruloplasmin of ionic form ($p < 0.01$).

Table 6 Absorption of copper from ceruloplasmin (Cp) and Cu-NTA when fed to weanling rats in milk

	Absorption (percent of dose)			
	4 Hours		24 Hours	
Cu Source	^{67}Cu-Cp	^{67}Cu-NTA	^{67}Cu-Cp	^{67}Cu-NTA
Organ				
Liver	6.0 ± 3.4	5.6 ± 3.0	23 ± 2	23 ± 6
Kidney	0.9 ± 0.5	0.6 ± 0.3	5.2 ± 0.1	3.0 ± 0.1
Spleen	0.4 ± 0.1	0.1 ± 0.1*	2.1 ± 0.2	1.1 ± 0.3*
Stomach	86 ± 10	89 ± 9	33 ± 5	28 ± 10
Intestine	8 ± 6	4 ± 3	37 ± 5	46 ± 20

Mean values ± SD, for 3 animals per group. *$p < 0.01$ for difference from ceruloplasmin-Cu.

says (assuming 2.3 ng of Cu per μg of ceruloplasmin[3]) and about 7% based on ceruloplasmin oxidase activity. This was the case both for milk produced during the first 5 days post partum and for 1 month later. In long-term breast feeding, it accounted for about 5%. During the first week of lactation, human milk had an average of 4.7 mg ceruloplasmin per L (22 determinations). After 30 days or more, the value was 2.3 mg/L (six determinations). These are first reported quantitative data on concentrations of milk ceruloplasmin determined by immunoassay. The only other data available (Kiyosawa et al.[48]) are based on assays of its ferroxidase activity.[13,49] The authors translated ferroxidase activity into concentrations of ceruloplasmin protein, reporting values about 10 fold higher than our's: about 40 mg/L for samples less than 1 month after parturition, and 17 mg/L for those more than 1 month after birth. The reason for the discrepancy may have to do with the difference in assays, our's being more specific.

The total copper concentrations of our milk samples averaged 9.3 μmol/L over the first 5 days, and 4.4 μmol/L after 1 month, with values of 2.6 μmol/L in the case of the samples taken more than 1 year after birth (*Figure 2*). These various values are within the range of those previously reported by Salmenpera et al.,[49] although they are higher in the case of the samples of early milk. The Finnish group was examining samples collected during 12 months of lactation, and it is unclear just when and how many samples were analysed at the different time points. Nevertheless, their 1 month values match our's exactly, as do those for long-term lactation. Thus, it is possible that our values for the first 5 days (about 9.3 μmol/L) are more representative of what occurs at this time. Our findings also confirm that you will get different results depending upon lactational stage. Thus, for example, Suttle[42] reports an average value of 250 ng Cu/mL (3.8 μmol/L) for human milk, and other mean values in the literature range from 400 to 700 ng Cu/mL (or 6 to 11 μmol/L).[3]

The ceruloplasmin-copper content of the human milk based on pPD oxidase activity was about twice that calculated from the ceruloplasmin-protein content obtained by immunoassay. This discrepancy may reflect variations in the specific activity and/or copper content of ceruloplasmin. The specific activity of serum ceruloplasmin (oxidase activity) and its average copper content has been known to change, in relation to the physiological or nutritional state of the mammal[3,38,50] and depending on the proportion of a low copper (or apo) form of the ceruloplasmin present.*[,51–53] The maximum copper content of milk and serum ceruloplasmins may also not be identical. Even the amino acid sequence may not be identical. We, as yet, know next to nothing about the structure and/or isoforms of milk ceruloplasmin.

When we compared ceruloplasmin and non-ceruloplasmin (ionic) copper as sources of copper for intestinal absorption by fetal, newborn and weanling rats, we found that both forms were nutritionally available. However, in the case of the near-term fetus and the newborn pups, copper from ceruloplasmin appeared to be taken up and accumulated more rapidly than the exchangeable, ionic (non-ceruloplasmin) copper. This was no longer the case after weaning, suggesting that preferential uptake of ceruloplasmin-copper is confined to the perinatal period and ends after milk ingestion has ceased. However, an additional point from these studies worth noting is that most of the administered copper (of either form) was still in the digestive tract of the pups 24 hr after intake (*Tables 5* and *6*). So it is very possible that either a good portion of both forms of copper in milk is never absorbed, or that it rapidly returns to the GI tract after absorption (as for example via the bile). This remains to be explored.

Since the copper in ceruloplasmin is non-exchangeable and buried within its structure,[5] release of its copper (for absorption) would require either digestion or unfolding of the protein (or pinocytosis of the whole molecule). In the case of ceruloplasmin in blood, which is a major source of copper for cells,[3,4,54] unfolding and copper release are thought to be mediated by specific cell surface receptors.[6,47] Abundant evidence indicates that serum ceruloplasmin binds to surface receptors detected in the plasma membrane of the cells of many tissues where its copper is taken up from the blood.[3,55–57] We also know there are ceruloplasmin receptors in placental membranes.[6,47] So the possibility arises that intestinal absorption of ceruloplasmin-copper is also mediated by specific receptors located in the brush border, at least during the perinatal period.

In the case of the iron in milk, there is considerable evidence for such a mechanism involving the protein lactoferrin.[35,58] Lactoferrin is a homolog of plasma transferrin and the main milk iron binding protein of humans, monkeys and some other species.[37,59,60] It is relatively resistant to digestive enzymes, particularly in the immature digestive tract. Lactoferrin is found almost intact in the infant's stool[58] and has even been detected in the urine of human infants,[61,62] implying absorption of the whole protein. The intestinal brush border contains specific receptors that recognize holo (iron containing) lactoferrin and bind the apoprotein less firmly.[36,56,59,63] Uptake of iron from Fe-lactoferrin has also been demonstrated with enterocytes in vitro.[64] The tight binding of iron to lactoferrin in the milk

*Wang, T.P., Shoho, J.R., Vargas, E.J., Shulze, R.A., and Linder, M.C. (1996). Identification and characterization of ceruloplasmin species in rat plasma. Submitted

also appears to restrict the growth of bacteria that are beginning to populate the intestine of the newborn, thus reducing the likelihood of colic.[65]

Like lactoferrin, ceruloplasmin is somewhat resistant to degradation by digestive enzymes.[66] Similarly, its metal ions are unavailable to the environment by diffusion. By analogy with serum ceruloplasmin and lactoferrin, for which there are cell surface receptors on internal organs, there might be ceruloplasmin receptors in the intestinal brush border. These might allow a selective uptake of copper from milk ceruloplasmin ingested by the infant and reduce its availability to intestinal bacteria.

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