Copper transport to mammary gland and milk during lactation in rats

STEPHANIE A. DONLEY, BERNARD J. ILAGAN, HISUN RIM, AND MARIA C. LINDER
Department of Chemistry and Biochemistry, and Institute for Molecular Biology and Nutrition, California State University, Fullerton, California 92834-6866

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Donley, Stephanie A., Bernard J. Ilagan, Hisun Rim, and Maria C. Linder. Copper transport to mammary gland and milk during lactation in rats. Am J Physiol Endocrinol Metab 283: E667–E675, 2002. First published June 4, 2002; 10.1152/ajpendo.00115.2002.—The delivery of copper to mammary gland and milk and the effects of lactation were examined in rats. Traces of $^{67}$Cu/$^{64}$Cu were injected intraperitoneally or intravenously into virgin rats or lactating rats (2–5 days postpartum), and incorporation into blood, milk, and tissues was monitored. In virgin rats, most of the isotope first entered the liver and kidney. In lactating rats, almost 60% went directly to the mammary gland. Uptake rates and copper contents of the mammary gland were 10-fold higher in lactation. $^{67}$Cu/$^{64}$Cu appeared in milk and milk ceruloplasmin as rapidly as in mammary tissue and when there was no $^{67}$Cu/$^{64}$Cu-ceruloplasmin in the maternal plasma. Milk $^{125}$I-labeled albumin entered milk much more slowly. Milk ceruloplasmin (10 mg/l) had 25% of the plasma. Plasma $^{125}$I-labeled albumin entered mammary tissue and when there was no $^{67}$Cu/$^{64}$Cu-ceruloplasmin in the maternal plasma. Plasma $^{125}$I-labeled albumin entered milk much more slowly. Milk ceruloplasmin (10 mg/l) had 25% of the plasma. Thus lactation markedly enhances the avidity of the mammary gland for copper, diverting most of it from liver and kidney to that tissue. Also, the primary source of milk ceruloplasmin is the mammary gland and not the maternal plasma.

ceruloplasmin; copper-67; copper-64; iodine-125 albumin; pregnancy; virgin rats

IT IS WELL DOCUMENTED that, during gestation in mammals, the fetus accumulates stores of certain metal ions in the liver. These are used up during the rapid growth of the infant after birth (22, 26). Copper is one of the metal ions to which this observation pertains and where such changes in liver stores have been documented (21, 22, 26). Thus, in most mammalian offspring at birth, the concentration of copper in the liver is higher than at any other time in the normal life of that species (21). In the human, concentrations at birth are $\sim 75 \mu g/l$; during suckling, they fall to $\sim 20 \mu g/g$; in the normal adult, they are $\sim 5 \mu g/g$ (18, 21). In rats, concentrations at birth are $\sim 30 \mu g/g$, compared with $\sim 5 \mu g/g$ in the adult (20, 21). The importance of this fetal copper store has been emphasized since the time of Widdowson et al. (57) because, as they pointed out, the copper content of milk (the initial food of the infant) is relatively low: $\sim 0.25 \mu g/l$ in the case of the human, but higher in the case of pigs and rats (50). Nevertheless, studies of the total copper content of rat pups during suckling indicate that they are rapidly accumulating whole body copper (16, 21, 50, 53, 58), which means they must be getting it from the milk. If so, the mammary gland must be actively taking up this trace element and transferring it to the milk, although this has not been specifically examined.

Recent studies on milk from our laboratory have indicated that ceruloplasmin, a copper-binding protein known for its abundance in the blood plasma, is present in the milk of the several mammals so far examined (59). Moreover, we have obtained evidence in rats that the copper in ceruloplasmin, when fed to pups (59), is more readily available for absorption than is nonceruloplasmin copper. Initial studies with piglets indicate that there is a specific ceruloplasmin receptor in the intestinal brush border that disappears after weaning (27). In analogy with the intestinal receptor for lactoferrin, which is thought to provide for specific iron uptake from the milk (10), a ceruloplasmin receptor may allow preferential intestinal uptake of ceruloplasmin copper. A copper transport role for blood plasma ceruloplasmin has been documented by studies in several laboratories, which have shown that ceruloplasmin is a good source (or preferential source) of copper for many tissues (see Ref. 23). This is particularly the case for the placenta and fetus during gestation (19). At the same time, ceruloplasmin is not essential for copper metabolism, in that absorption, tissue distribution, and excretion of copper are not abnormal in the knockout mouse (33). Copper in ceruloplasmin is not dialyzable or exchangeable, and it is thought that delivery from ceruloplasmin must involve specific receptors for this protein that have been detected in the plasma membranes of most cells (21, 49, 51). Other sources of copper in the plasma are albumin and transcuprein, the latter a form of macroglobulin (Liu N, Lo LSL, Goforth J, Vivas E, Sebastian S, Ackary SH, Tsai M, and Linder MC, unpublished observations). These proteins bind Cu(II) when it first enters the blood, as from the diet, and seem to target it to the liver and kidney, where most of the copper is initially de-
posed (22, 23, 25) or where it is brought for biliary excretion (21).

mRNA for ceruloplasmin has been detected in mammary tissue (15, 28) and in the cells known to produce the milk (15). Its role in providing ceruloplasmin to the milk has been questioned, because ceruloplasmin mRNA is also detected in the mammary gland in the absence of lactation and in male as well as female rats (15). However, we have shown in pigs that mammary gland expression increases markedly at the end of pregnancy and especially in conjunction with lactation (7), and that changes in the levels of mammary gland mRNA expression parallel those of ceruloplasmin production for the milk. Both of these findings imply that milk ceruloplasmin is produced by the mammary gland.

Potential roles for ceruloplasmin have multiplied in recent years. Expression of the protein or its mRNA has been detected in a variety of tissues, including certain cells in the central nervous system and elsewhere, where it also occurs in glycosphatidylinositol-anchored form (39, 45). Ceruloplasmin has long been recognized as capable of scavenging oxygen radicals (12, 13), which would explain its increased synthesis by the liver during inflammation. An additional role in the exit and entry of iron from/to hepatocytes and other cells is the object of intense current scrutiny on the basis of involvement of another blue copper protein (Fet3) in yeast iron uptake (4, 5, 8); the identification of another potential homolog, hephaestin, in connection with intestinal (and perhaps also placental) iron transport (9, 55); and observations that ceruloplasmin deficiency, due to copper deficiency or mutations in the ceruloplasmin gene, causes iron accumulation (14, 36, 37, 43, 44, 61).

The studies reported here were designed to increase our understanding of copper transport during lactation and the involvement of the mammary gland, by use of the rat as a model. This had not been previously studied. Indeed, we were unable to find reports of even the copper content of mammary tissue. The specific questions addressed were the following. How rapidly does the mammary gland take up copper from the maternal circulation, and how does lactation influence this process? Is plasma ceruloplasmin the preferred source of copper for the mammary gland? How rapidly does copper enter the milk after being absorbed by mammary tissue? Does incorporation of copper into milk ceruloplasmin lag behind the appearance of nonceruloplasmin copper in the milk (i.e., does plasma ceruloplasmin enter the milk in significant amounts)? Our results show that the mammary gland is very active in copper nutrition and metabolism during lactation and is the primary source of ceruloplasmin entering rat milk.

MATERIALS AND METHODS

Rats and treatments. All studies were preapproved by the Institutional Animal Care and Use Committee of California State University, Fullerton (protocol nos. 98-R-05 and 01-R-02). Sprague-Dawley rats were obtained from Simonsen Laboratories (Gilroy, CA) and maintained on normal rat chow. Virgin and lactating rats were used at 3–5 mo of age. Lactating rats, received −10 days before the end of gestation, were used on days 2–5 postpartum. Milk was collected into a clean sterile syringe upon manual massage of the mammary gland while animals were under anesthesia with xylazine and ketamine (13 and 87 mg/kg sc, respectively). Milk release was promoted by injection of oxytocin (−240 IU/kg ip). Rats were injected intraperitoneally or intravenously (in the tail vein) with 67Cu or 64Cu(CuII) as the 1:4 copper-nitritolactate complex (Cu-NTA), as previously described (19). The 67Cu radioisotope was obtained as a mixture of 64Cu and 67Cu chloride, in HCl, from the Nuclear Reactor Facility of the University of Missouri, at Columbia; the 64Cu was 64CuCl2 from the Mallinckrodt Institute of Radiology Facility of Washington University, St. Louis, MO (courtesy of Dr. Debra McCarthy). Doses of radioactive copper injected in 0.6 ml of 0.9% NaCl were 50–300 μCi, containing 20–100 ng of copper. In some cases, rats were injected intraperitoneally with 125I-labeled rat albumin (5 or 25 μg albumin in 0.9% NaCl, buffered with 10 mM K phosphate) to monitor its appearance in maternal blood, mammary gland, and milk. 125I-albumin was prepared by treating freshly isolated rat albumin with 125I-NaI, obtained from Amersham (Amersham Pharmacia Biotech, Buckinghamshire, UK) by use of Iodogen (Pierce, Milwaukee, WI) and the protocol from Amersham. Albumin was isolated from frozen rat plasma by binding to Cibacron Blue (Affigel Blue; Bio-Rad, Hercules, CA), applied in 20 mM K phosphate buffer, pH 7.1, and eluted with 1.4 M NaCl in the same buffer. Rats were euthanized by exsanguination, after treatment with heparin and while under anesthesia with pentobarbital sodium, as previously described (19, 33). Blood plasma was obtained by centrifugation. Whole organs and tissues were excised. These, as well as portions of milk and plasma, were directly counted for radioactivity using a Cobra II gamma counter (Packard Instruments, Downer’s Grove, IL). The content of 67Cu or 125I in blood and organs was calculated as a percentage of the dose. Rat milk and serum samples were stored frozen at −20°C.

Assays of copper and ceruloplasmin. The total copper content of rat milk was determined by furnace atomic absorption spectroscopy, using a Zeeman 800 AA instrument from Varian (Sugarland, TX). Samples of milk were diluted 10-fold with distilled, deionized water before analysis. Standards, made from a 1,005 μg/ml stock solution ("copper atomic absorption standard"; Aldrich Chemical, Milwaukee, WI), were diluted in 1% ultrapure HNO3 (trace metal grade; Fisher Scientific, Pittsburgh, PA). Tissue copper was determined by atomic absorption after wet ashing of 100-μg samples with nitric acid and peroxide, as previously described (59). Ceruloplasmin was assayed by a sandwich EIA/ASA developed with antisera against the 0.6 Rf form of rat plasma ceruloplasmin (33), as previously described (30). The content of 67Cu in milk ceruloplasmin was determined by immunoprecipitation, after removal of the casein, as previously indicated (7), by use of the antibody against the 0.6 RF form of rat ceruloplasmin raised in a goat (30). Immunoprecipitation of ceruloplasmin from rat plasma was accomplished by the same procedure with and without the addition of protein A beads (Sigma, St. Louis, MO). For immunoblotting, milk and serum ceruloplasmin samples were partially purified by one round of ion exchange chromatography, with DEAE-Sepharose CL-B in 100 mM K phosphate, pH 6.8, and elution with 300 mM of the same buffer, as previously described (33).
Size-exclusion chromatography. Samples (0.5) of plasma were applied to 25-ml columns of Sephadex G150 or Sephacryl S200, and 60 fractions (0.5 ml) were collected for analysis of radioactivity, total protein (absorbance at 280 nm), and, in some cases, ceruloplasmin (by immunoprecipitation).

**SDS-PAGE and immunoblotting.** Samples of proteins, including immunoprecipitates, were subjected to SDS-PAGE by standard procedures, as previously described (7). Stacking and resolving gels were of 5 and 7.5% acrylamide, respectively. Prestained and unstained high molecular weight standards were from Bio-Rad. Staining was with Coomassie Blue R250. For immunoblotting, samples were transferred onto polyvinylidene difluoride membranes (Millipore, Bedford, MA) by use of Tobin buffer (25 mM Tris, 192 mM glycine, 1.3 mM SDS, and 15% methanol, pH 8.3) and the Hoefer semidry apparatus. The primary antibody was the same as that used for immunoprecipitation; the secondary antibody was rabbit anti-goat IgG conjugated to alkaline phosphatase (Bio-Rad).

**RESULTS**

**Uptake of copper by mammary gland and other tissues during lactation.** To examine the avidity of mammary tissue for circulating copper, lactating rats at 2–5 days postpartum were injected intraperitoneally with 20- to 100-ng doses of $^{67}$Cu(II) as the Cu-NTA complex (see MATERIALS AND METHODS) and killed at various times thereafter. Figure 1, A and B, shows the total radioactivity (percent dose) absorbed by mammary gland, liver, and kidney over time. The copper tracer was rapidly absorbed by the tissues, and maximum uptake was achieved by 1 h. Unexpectedly, most of the copper administered was absorbed by the mammary gland. More than twice as much tracer entered this tissue than entered the liver, and uptake by the kidney was seven times less than into mammary tissue. This contrasts sharply with what normally occurs in virgin (38, 56) and even pregnant rats (19), where almost all of the incoming copper initially enters the liver and kidney. As expected, absorption of the tracer by other tissues of the lactating rats was much lower (Fig. 1C).

Our finding that uptake of the administered copper into mammary tissue was so large and immediate implied that plasma ceruloplasmin (Cp) was not the source of the copper entering the mammary gland. Copper is incorporated into Cp during its synthesis by the liver, and in virgin rats and male rats, $^{67}$Cu only appears in Cp some hours after tracer injection (19, 54, 56). To confirm this for lactating rats, samples of maternal plasma were fractionated by size-exclusion chromatography to identify which plasma proteins bound

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**Fig. 1.** Time course of uptake of $^{67}$Cu into mammary gland and other tissues of lactating rats after ip injection as copper-nitrilotriacetate (Cu-NTA). Data are mean values for percent dose in individual organs. Error bars represent SD for 3 or 4 rats at each time point examined, with the exception of hours 2 and 78, where single rats were involved. Mammary gland (MG; ●), liver, (L; ○), and kidney (K; ▲) are shown in A and B, which are 1–8 and 6–126 h after isotope injection, respectively. In C, %dose in kidney (K; ○) is compared with that in spleen (Sp; dashed line, ■), heart (H; ●), and brain (Br; dotted line).
$^{67}$Cu during the 1st h after isotope injection. An example of the results is given in Fig. 2A, in this case taken 30 min after injection. As seen previously, radioactive copper was associated with two components, one eluting in the void volume of Sephacryl S200 (and Sephadex G150; data not shown), the other eluting with albumin. Neither peak coincided with the elution of Cp. Samples taken 60 min after isotope injection gave the same results (data not shown).

To further confirm that ceruloplasmin in the maternal blood plasma was not labeled with radioactive copper during the 1st h after injection, the ceruloplasmin was precipitated from plasma with specific antibody, as verified by SDSPAGE and Western blotting. No radioactivity could be immunoprecipitated: for 15 samples of serum (50 μl), 0 ± 0 (means ± SD) counts/min (cpm) were recovered in the washed precipitates compared with from 3,600 to 10,300 cpm in the supernatants and washes of the different samples.

After high concentrations of tracer were attained in the first few hours (Fig. 1A), radioactivity in the mammary gland declined rapidly, indicating that the element was being transferred elsewhere, presumably into the milk. Figure 2, B and C, shows the time course of $^{67}$Cu appearance in milk in relation to blood plasma, mammary gland, and liver. The data are given in terms of percent dose per milliliter of milk or blood plasma, or as percent dose per gram of tissue in the case of mammary gland and liver.

The time course of appearance of tracer copper in the milk virtually paralleled that of its entry into mammary gland (Fig. 2, B and C). Milk and tissue radioactivities both reached peak concentrations 1–2 h after administration, after which they fell off dramatically. This implies that the copper that had entered the mammary gland was rapidly transferred to the milk. It is noteworthy that the concentration of $^{67}$Cu achieved by the milk (per ml) was considerably higher than that achieved by the mammary tissue itself, or the liver (per g) (Fig. 2, B and C), implying a targeting and compartmentalization by mammary gland of the copper going to the milk. Also, concentrations of isotope in the blood plasma were much lower than in the milk and mammary gland. This was so during the entire time period examined, further emphasizing the rapidity of transfer of copper from the blood to the mammary gland and milk.

Copper uptake by the mammary gland of virgin rats. Because the mammary gland was so avid for copper during lactation, we wondered whether even in the absence of lactation uptake might be high. Therefore, parallel uptake studies were carried out in virgin rats of the same age. Also, to ensure that the route of isotope administration by injection through the abdom-
portion of the radioisotope entered mammary tissue: rats compared with lactating rats, only a very small dose in a given tissue 1 h after injection. In the virgin the uptake of\textsuperscript{67}Cu(II) by their tissues after intraperitoneal injection of copper: 80 vs. 20
did not a function of contamination or route of delivery. Because the lactating mammary gland appeared to be so active in copper uptake and transport, we deduced it would also have a higher copper content than in the virgin state. This was the case. Three samples of lactating mammary tissue had copper contents of 12.9 ± 0.1 (SD) \(\mu\)g Cu/g wet wt, whereas samples from two virgin rats had 2.9 and 3.0 \(\mu\)g Cu/g. Liver copper concentrations were 5.4 ± 1.4 and 4.0 or 4.4 \(\mu\)g Cu/g (\(n = 3\) and 2, respectively) in the lactating and virgin rats.

Ceruloplasmin in the milk. The ceruloplasmin and copper contents of rat milk were also assayed, as was the time course of appearance of tracer copper in milk ceruloplasmin. The copper concentration of the rat milk at 2–5 days postpartum averaged 3.3 ± 0.6 (SD) mg/l (\(n = 8\)), and the ceruloplasmin concentration (measured by ELISA) was 10 ± 3 mg/l (\(n = 7\)). The proportion of radioactive copper in the milk associated with ceruloplasmin at various times after administration of the isotope was also measured after precipitation with specific antibody. The proportion of total tracer with ceruloplasmin was identical at all time points examined (1–122 h) and averaged 27 ± 8% SD; \(n = 24\). This indicates that copper entering the mammary gland went as rapidly into ceruloplasmin as it did into other forms of copper entering the milk. It also proves that the ceruloplasmin in the maternal blood circulation could not have been the primary source of the ceruloplasmin in the milk: by the time maximal labeling of milk ceruloplasmin had occurred, little or no ceruloplasmin labeled with \textsuperscript{67}Cu had appeared in the maternal blood circulation (see \textit{Uptake of copper by mammary gland and other tissues during lactation}).

That the copper released into the milk and milk ceruloplasmin was absorbed by the suckling rat pups was verified, as indicated in Table 2. Groups of six pups were suckled for 8 or 24 h on dams that had earlier been injected with \textsuperscript{67}Cu. (The time of injection vs. start of suckling varied widely.) Clearly, intestinal absorption of copper had occurred, although compared with what was in the gastrointestinal tract, only a small

Table 1. \textit{Whole body and organ weights of virgin and lactating rats}

<table>
<thead>
<tr>
<th></th>
<th>Virgin Rats</th>
<th>Lactating Rats</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight, g</td>
<td>219 ± 26(12)</td>
<td>245 ± 19(33)*</td>
</tr>
<tr>
<td>Liver weight, g</td>
<td>8.1 ± 1.1</td>
<td>9.9 ± 1.4(22)</td>
</tr>
<tr>
<td>Mammary gland, g</td>
<td>2.6 ± 1.2</td>
<td>12.1 ± 2.9*</td>
</tr>
<tr>
<td>Kidney, g</td>
<td>1.6 ± 0.1</td>
<td>1.7 ± 0.2</td>
</tr>
<tr>
<td>Spleen, g</td>
<td>0.6 ± 0.1</td>
<td>0.6 ± 0.1</td>
</tr>
<tr>
<td>Heart, g</td>
<td>0.8 ± 0.1</td>
<td>1.0 ± 0.3</td>
</tr>
</tbody>
</table>

Values are means ± SD for the no. of determinations (rats) indicated in parentheses. (If there are no parentheses, \(n\) is the same as the nearest value higher in the column.) *\(P < 0.005\) for difference from virgin rats by Student’s \(t\)-test.

Figure 3 shows the results of studies in which virgin and lactating rats were compared directly in terms of the uptake of\textsuperscript{67}Cu by tail vein vs. intraperitoneal injection.

Table 2. \textit{Body and organ weights of rat pups and evidence for absorption of \textsuperscript{67}Cu during suckling of dams injected with tracer}

<table>
<thead>
<tr>
<th></th>
<th>Body (with Contents)</th>
<th>GI Tract</th>
<th>Liver</th>
<th>Spleen</th>
</tr>
</thead>
<tbody>
<tr>
<td>Weight, g</td>
<td>14.6 ± 1.6</td>
<td>1.3 ± 0.2</td>
<td>0.7 ± 0.2</td>
<td>0.4 ± 0.3</td>
</tr>
<tr>
<td>(\textsuperscript{67}Cu, %)</td>
<td></td>
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</tr>
<tr>
<td>8 h</td>
<td>92 ± 2(6)</td>
<td>7.6 ± 2.3(6)</td>
<td>0.19, 0.13(2)</td>
<td></td>
</tr>
<tr>
<td>24 h</td>
<td>89 ± 3(3)</td>
<td>10.8 ± 2.3(3)</td>
<td>0.09, 0.27(2)</td>
<td></td>
</tr>
</tbody>
</table>

Values are means ± SD for the no. of litters indicated in parentheses. For each litter, pooled data for 6 pups were collected. \(\textsuperscript{67}Cu\) uptake is given as a percentage of the total recovered in the gastrointestinal (GI) tract, liver, and spleen. Actual uptake could not be calculated because rats began suckling at various times after their dams had been given the radiotracer.
proportion was in liver and spleen of the pups. Radioactivity in other parts of the carcasses was not measured.

The nature of rat milk ceruloplasmin was also examined. Ceruloplasmin in pooled samples of milk was partially purified by ion exchange chromatography and then analyzed by Western blotting after separation by SDS-PAGE with a specific antibody raised in goats against the 0.6 Rf form of rat serum ceruloplasmin (33). Figure 4 shows an SDS-PAGE gel and Western blot for rat milk and plasma ceruloplasmin, obtained after partial purification by ion exchange chromatography (see MATERIALS AND METHODS). In several studies and as shown here, immunoreactive bands in plasma and milk were of identical size, with an apparent average molecular mass of 138 kDa, as indicated.

Appearance of albumin in the milk. To further confirm that most of the ceruloplasmin in the milk was not coming from the maternal blood plasma, traces of $^{125}$I-albumin were administered to lactating rats to label the maternal plasma pool, and amounts of radioactive albumin in the milk and plasma 1 h later were recorded. One hour after injection, there was a large amount of $^{125}$I-albumin in the maternal blood plasma and very little in the milk: $1,226 \pm 400$ (SD) cpm/ml ($\times 10^{-3}$) in the plasma (3 rats); $36 \pm 30$ cpm/ml ($\times 10^{-3}$) in the milk from the same animals (thus $2.9 \pm 2.2\%$ of that in plasma). Size-exclusion chromatography of the plasma samples indicated that 64% of the $^{125}$I was with the albumin at this time (Fig. 5A). The rest (35%) was associated with a larger protein. In the case of the milk (Fig. 5B), 26% of the radioactivity was with albumin, and the rest was with the larger component (1 h after $^{125}$I-albumin injection). On the basis of radioactivity actually with albumin, the ratio of $^{125}$I in milk to plasma albumin was 0.012. Thus, 1 h after injection, very little $^{125}$I-albumin had made its way from the plasma into the milk.

**DISCUSSION**

We have demonstrated in the rat that lactation has a marked effect on how copper is distributed to tissues once it has entered the blood. As confirmed here for the normal state, most of the copper entering the blood plasma first travels directly to the liver and kidney (38, 47, 54, 56). In lactation, this changes markedly, 60% of the administered tracer now going directly to the mam-
mary gland. Our studies, the first to follow this process, showed that lactation caused a 20-fold increase in the rate of copper uptake by the mammary gland. This was accompanied by a 6-fold increase in mammary tissue mass, as well as a rise in tissue copper concentrations from 3 to 13 μg/g wet wt. Total copper in the mammary gland thus went from ~8 to ~160 μg, exactly paralleling the 20-fold increase in rate of copper uptake.

Despite the diversion of so much incoming copper to the mammary gland, the concentration of copper in the liver was not diminished by lactation. This is consistent with earlier reports that intestinal copper absorption increases in pregnancy (11, 46) and that biliary copper excretion diminishes (52), only returning to normal in the 3rd wk of lactation, when pups begin to ingest solid food.

Copper that entered the mammary gland went almost immediately into the milk, and ~25% went into milk ceruloplasmin. The time course of radiocopper appearance in this protein exactly paralleled that for the nonceruloplasmin fraction, suggesting a common pathway for entry of the metal into these two milk compartments. The mammary cells concentrated the copper before releasing it to the milk. This was evident from the finding that, 1 h after injection, the concentration of tracer in the milk was 20-fold higher than that of the blood plasma or mammary tissue as a whole, and that of mammary tissue was 4.5 higher than in plasma. The exact mechanisms by which copper entered mammary epithelial cells and found its way into the milk and milk ceruloplasmin remain to be established. However, it is possible that copper entered the cells via a transporter, CTR1, first identified in yeast (62). Export of copper from cells and its insertion into ceruloplasmin within hepatocytes have been linked to the function of two P-type ATPases, ATP7A and ATP7B, defective in Menkes and Wilson diseases, respectively, and found within the trans-Golgi network (TGN) or at the cell surface (33, 40, 60). Copper is delivered to these transporters by the copper “chaperone” ATOX1/HAH1 (4, 17, 42). In contrast to other cells, the mammary epithelium expresses both of these ATPases (1, 2, 6, 53), so it is unclear whether only one or both are involved in conveying the incoming copper to milk. Ackland et al. (1) found that lactation enhanced expression of ATP7A in the TGN of breast epithelial cells, as well as in the TGN and endosomes of PMC42 breast carcinoma cells treated with lactational hormones (3). However, mutation of the other ATPase, ATP7B, in the “toxic milk” mouse (6, 53) results in a lower copper content of the milk, implying that this ATPase also has a role in milk production.

The ceruloplasmin content of rat milk, determined by the ELISA method here employed, was in the range of what we had previously reported for milk from humans and pigs (7, 59); similarly, our total milk copper values were in the range of those previously reported for rats and mice (6, 7, 50, 52, 59). Our values for the copper content of the nonlactating mammary gland (3 mg/g) appear to be the first ones published for any species. Lower values of 9.7 and 4.0 mg Cu/g (dry wt), respectively, for lactating mammary gland of mice with and without the “toxic milk” mutation (modeling Wilson disease), have just been reported (53), but the day of lactation was not given and might make a big difference (7, 59).

The findings presented here prove conclusively what we had surmised from previous studies, namely, that much of the ceruloplasmin in milk comes from mammary cells and not from the maternal blood plasma. Most of the tracer 67Cu entered the mammary gland, milk, milk ceruloplasmin, and other maternal tissues already within the 1st h after injection. During that period, no 67Cu/64Cu-labeled ceruloplasmin could be detected in the maternal plasma, confirming what had been documented previously for nonlactating rats (19, 56). This is because ceruloplasmin is not part of the exchangeable plasma copper pool. Newly absorbed incoming copper does not bind directly to plasma ceruloplasmin but must first enter the liver and be pumped by ATP7B into the TGN, where it binds to ceruloplasmin newly synthesized on endoplasmic reticulum-bound polyribosomes (34, 60). This must happen before 67Cu/64Cu-ceruloplasmin appears in the blood. Because no tracer-labeled ceruloplasmin was present in the maternal plasma in the 1st h, that in the milk must have come from cells of the mammary gland producing the milk. mRNA for ceruloplasmin is expressed by the milk-producing epithelial cells of the mammary gland (15). Indeed, we have documented in pigs that total amounts of ceruloplasmin mRNA in the mammary gland increase markedly with lactation (7). Consistent with these findings are those of Puchkova et al. (41), who found in rats that radiolabeled ceruloplasmin appeared in the milk much more rapidly than in blood plasma after injection of a 14C-labeled amino acid.

This does not mean that some milk ceruloplasmin did not come from the maternal blood plasma. It is well known that there is paracellular leakage of plasma proteins into the milk (32). We confirmed this by use of 125I-labeled rat plasma albumin, showing that small amounts appeared in the milk within the 1st h after injection into lactating dams. This is consistent with recent findings (48) on the gradual appearance in milk of 125I-labeled recombinant human serum albumin after its injection into rats. At 1 h, we found that the concentration of rat 125I-albumin in milk was about one-eightieth of that in maternal plasma. One would expect Cp to be transferred at a similar or slower rate, because it is larger. If so, and assuming a Cp concentration of 275 μg/ml, one can calculate that ~3.5 μg/ml of the ceruloplasmin in rat milk might have come paracellularly from the plasma, which is one-third of the total detected.

Evidence from the studies reported here is consistent with previous findings, obtained by exon-specific PCR and Northern analysis, indicating that the liver and mammary gland produce the same kind of ceruloplasmin (7). The apparent sizes of the ceruloplasmins detected by immunoblotting in plasma and milk were also identical, as previously reported by us for pigs (7, 24) and humans (31).
In contrast to what we have reported for certain tissues of nonlactating and pregnant rats (19, 54, 56), ceruloplasmin did not appear to be the blood plasma carrier delivering copper to the secretory epithelium of the mammary gland. Instead, as with delivery of copper to liver and kidney, the exchangeable plasma copper pool appeared to be involved. In normal rats, this pool is comprised principally of copper bound to the macroglobulin/transcuprein, and that bound to albumin (23, 25, 28). Both of these proteins bind copper very tightly and rapidly exchange it with each other (23, 25, 28). The same two components (transcuprein and albumin) appeared to be the only plasma substitu-
ents to carry radiocopper during the 1st h after its administration, as shown by the gel filtration profiles obtained in Sephadex G150 and Sephacryl S200. Moreover, it is worth noting that the proportion of radioac-
tivity binding to the macroglobulin/transcuprein component (eluting in or near the void volume) was larger in the case of the plasma from lactating rats than is observed in rats not in lactation (23, 25, 28, 54, 56). This suggests that transcuprein expression may be upregulated in lactation.

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dation, as shown by the gel

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