Intrinsic labeling of milk iron: effect of iron status on isotope transfer into goat milk

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Extrinsic labeling with radioiron is commonly used in studies on bioavailability of iron in milk. Because of differences in the distribution of added radioiron and native iron among ligands in milk, we found it important to explore the possibility of intrinsic labeling of milk iron. In this study, we have evaluated different conditions in the lactating goat to optimize transfer of ^{59}Fe into milk. Maximum ^{59}Fe concentration in milk was found at 10–15 hr post injection, indicating a carrier-mediated process. After iron depletion induced by bleeding, iron transfer into milk in the mammary gland was found to be low. After iron repletion, however, iron transfer increased. This study suggests that maternal iron status affects iron transfer into milk and that during iron depletion, hematopoietic organs may have higher affinity for transferrin-bound iron than the mammary gland. Iron transport into milk was lower in dairy goats than in a young goat which had not been used in milk production. This suggests that mammary development may enhance iron uptake in the gland, possibly via transferrin receptors.

Keywords: goat; milk; intrinsic labeling; iron transfer; iron status

Introduction

In milk, iron is bound to several ligands such as components of the casein micelle, ligands in the globular membrane of the fat droplets, and to some ligands in the whey compartment; however, this distribution varies among species.[1] Lactoferrin, an iron-binding protein, occurs in high concentration in the whey fraction of human milk. In milk from several other species, transferrin is more significant and in some species transferrin and lactoferrin occur in similar concentrations.[2] Like serum transferrin, lactoferrin is a glycoprotein consisting of a single peptide chain (≈78 kD)[3,4] possessing two domains each of which binds one ferric ion in the presence of carbonate or bicarbonate ions.[5] The lactoferrin-iron association constant is high, about 260 times higher than that of serum transferrin.[6] Furthermore, lactoferrin releases iron at much lower pH (pH 2) than transferrin (pH 4).[7] In spite of its high affinity to iron, lactoferrin occurs mostly as an apoprotein in human milk, being saturated to only 4% of its iron binding capacity.[8,9] Nevertheless, lactoferrin-bound iron comprises 25–30% of the total iron content in human milk.[8,10] This suggests that other ligands in human milk have affinity to iron and are therefore likely to affect iron absorption in the gastrointestinal tract.

Saarinen et al.[11] and McMillan et al.[12] concluded, by feeding extrinsically labeled milk, that human milk iron is highly available to the infant. This is supported by the data of Siimes et al.[13] who found that, in spite of low iron content in human milk, iron deficiency is rare in breast-fed infants during the first six months of life. In our laboratory, however, we have found that, when added to human milk in vitro, an iron isotope will mainly be bound to lactoferrin.[14] It is possible that iron is not completely exchangeable in milk when a ligand with an unusually high affinity to iron (lactoferrin) is present in unsaturated form.[15] In that case the observations reported by Saarinen et al.[11] and McMillan et al.,[12] made by feeding extrinsically labeled human milk, might only be relevant for the lactoferrin-bound iron, leaving the availability of iron from other fractions in human milk still unknown.[14]

This work was supported by the Swedish Council for Forestry and Agricultural Research, and the Swedish Nutrition Foundation.
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Received March 14, 1990; Accepted January 9, 1991.

The purpose of this study was to investigate the possibility of labeling all iron-binding fractions in milk intrinsically by intravenous administration of radioactive iron to lactating mothers, in order to assess total bioavailability of iron from milk. Since administration of radioactive isotopes to lactating women is prohibited ethically, an experimental animal had to be chosen. In this study we used the lactating goat. Recovery of administered dose, peak radiolabel appearance in milk, and influence of maternal iron status on isotope recovery in milk were determined. Isotope recovery in animals with different lactation history was also studied.

Materials and methods

Healthy goats in full lactation (Swedish "Lantras") (n = 5) were used in this study. One goat (No. 1) came from an experimental stable and had never been used for dairy milk production; she was in her second lactation (4 mo.). The other goats (Nos. 2–5) were bought from a dairy goat farm and were all at a similar stage of lactation (0.4–1.2 mo.). Goat No. 2 was primiparous and goats Nos. 3–5 were in their fourth lactation. All goats were in a phase of high milk production (1033 ml/d, range: 514–1725) during the experimental period (*Figure 1*). For the extrinsic labeling experiments, additional milk samples were obtained from four other goats that were in their third or fourth lactation (1.5–3 mo. of lactation).

Experimental design

The design of the experiment is presented schematically in *Figure 2*. Iron status of the goats was altered by bleeding or supplementing the animals with iron for periods of several weeks. At four time points, ^{59}Fe was administered intravenously and milk samples were collected for 5 days and counted (isotope transfer studies).

Bleeding period (iron deprivation)

All goats were first bled 35 ml/d for a period of 2–3 weeks; and for a second period, all goats were bled 35 ml/d for 1–3 additional weeks.

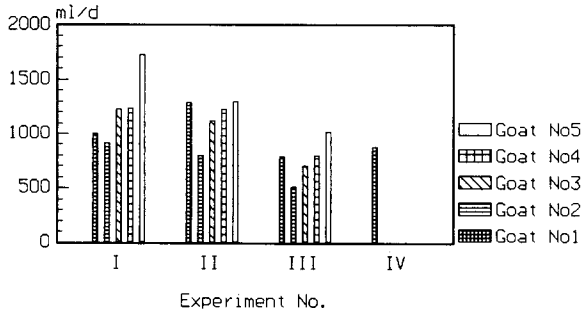

Figure 1 Milk production per day during the treatment periods (I: after bleeding; II: end of iron depletion period; III: after iron repletion; IV: after additional iron repletion).

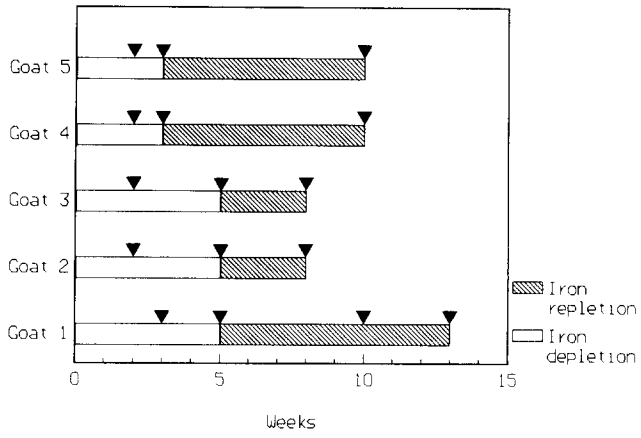

Figure 2 Experimental design (isotope transfer studies are indicated by triangles).

Supplementation period (iron repletion)

All goats (except goat No. 2, control) were supplemented orally with 50 mg iron/day and 74 mg/week intramuscularly for a period of 3–7 weeks. One goat (No. 1) was supplemented for a second period (3 weeks) in the same manner as the previous period.

Isotope administration

At the end of each treatment period (bleeding or supplementation), blood samples were collected for hematological evaluation. The mammary glands were emptied and background radioactivity in the milk was measured. Immediately after, a dose of 3.7 MBq ^{59}Fe-citrate was injected intravenously. Milk samples were collected by hand-milking at regular intervals for the following 5 days, at first hourly but then gradually at longer intervals. The mammary glands were emptied each time. In order to keep milk production as high as possible, the goats were milked twice a day during periods when milk samples were not collected.

Isotope concentration and recovery

Milk samples were thoroughly mixed and the radioactivity measured in samples of 5 ml in a gamma counter (Nuclear Chicago 1186 with a well-type NaI detector). Isotope concentration in milk or milk fractions was calculated as percent of administered dose per ml of milk or per g fraction. Cumulative isotope concentration was calculated as the cumulative percent of administered activity in milk collected during a period of 100 hr (total cpm of all milk samples collected), divided by the total milk volume (ml). Isotope recovery was calculated as percent of administered activity in milk produced during a period of 100 hr.

Milk fractionation

Goat milk samples were ultracentrifuged (140,000 × g, 1 hr, +4° C) in a Beckman ultracentrifuge (fixed angle rotor: Ti 70.1), and the fat, casein, and whey fractions were separated for gamma counting.

Table 1 Calculated iron losses during bleeding, and iron supplements during repletion (mg Fe).

Goat No	Period I iron losses	Period II iron losses	Total iron losses	Total iron supplem.
1	149	105	254	3000[a] 225[b]
2	103	176	279	
3	101	164	256	1150[a] 150[b]
4	101	50	151	2800[a] 150[b]
5	105	52	157	2800[a] 150[b]
Mean[c]	112 ± 9	109 ± 27	221 ± 27	2438 ± 432

[a] Oral.
[b] i.m.
[c] Mean ± SEM.

Iron status

Hematological parameters (Hb, and TIBC) were analyzed to evaluate iron status of the animals during each experiment. Estimation of iron losses by bleeding (during the two first experiments) was based on the fact that iron losses represent 0.35% (wt/wt) of the total hemoglobin loss. Calculated average iron losses were 112 mg (the first bleeding period) and 221 mg (total iron losses) (*Table 1*).

Extrinsic labeling of milk

Milk samples from four goats (20 ml) were labeled extrinsically by dropwise adding ^{59}Fe-citrate solution (400 Bq/ml) to fresh milk samples (8–10° C) while stirring. Samples were kept at room temperature for 30 min and then ultracentrifuged. Radioactivity in fat, whey, and casein fractions was determined in the gamma counter.

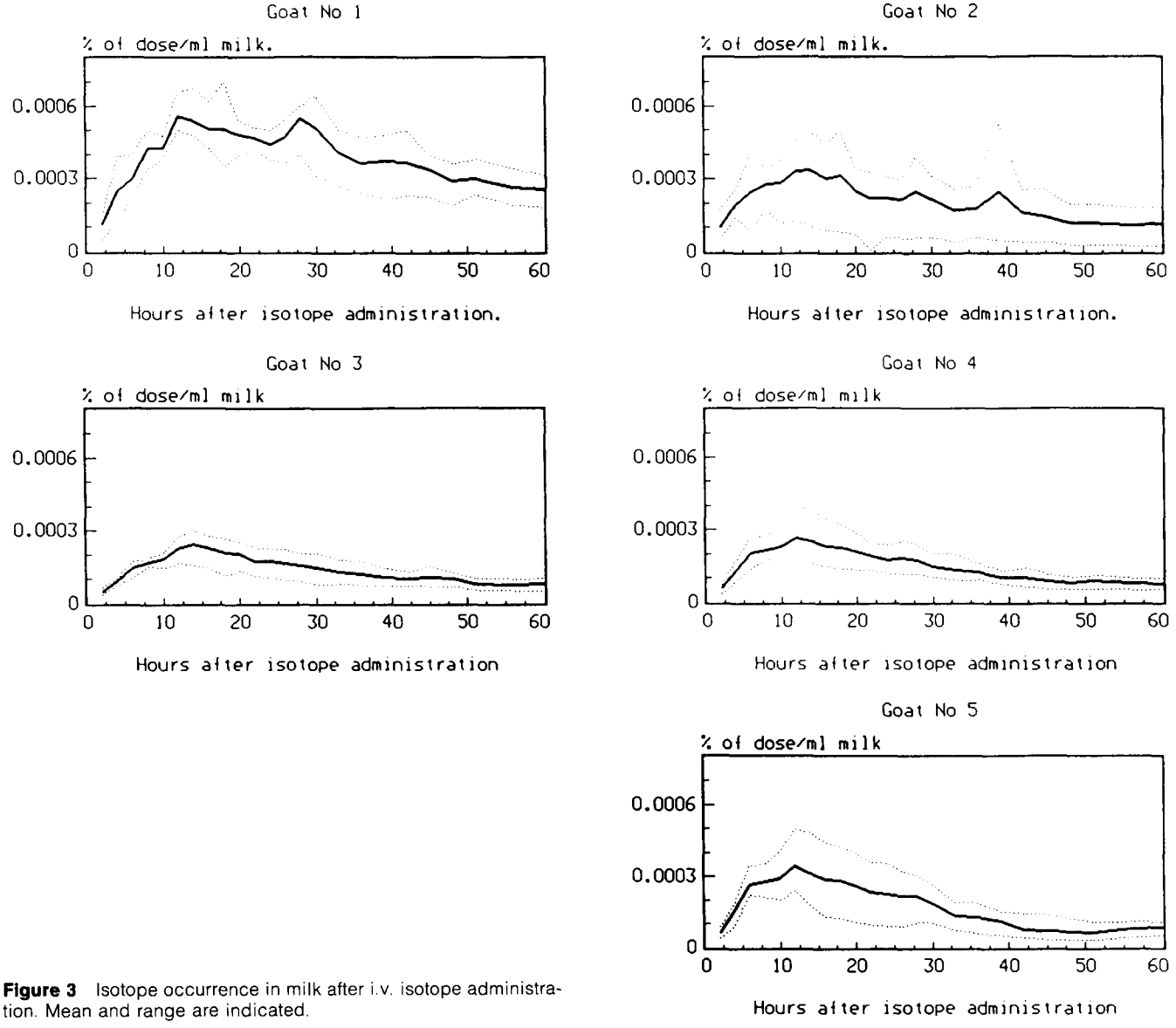

Figure 3 Isotope occurrence in milk after i.v. isotope administration. Mean and range are indicated.

Results

Maximum isotope concentration was found to be within the range of 21–141 cpm/ml which corresponds to between 0.0001–0.0007% of the injected dose. Total recovery in all milk produced within 100 hr was 0.3–1.2%. Peak concentration of ^{59}Fe was reached 10–15 hr after intravenous injection, which then declined slowly to reach a plateau 40–70 hr after injection (*Figure 3*).

Hemoglobin values confirmed that the goats were anemic (Hgb: 69.7 g/L; TIBC: 13.9 μM) after bleeding (reference value according to Jones[16]: 75–127 g/L) and that following iron supplementation, all goats had improved their iron status (Hgb: 83.6 g/L; TIBC: 12.3 μM). This improvement was significant for hemoglobin ($P < 0.05$) but not for TIBC. Neither Hgb, TIBC, nor total amount of iron supplemented showed any significant correlation to cumulative isotope concentration. However, quantity of blood loss (*Figure 4*) and duration of supplementation (*Figure 5*) were significantly correlated to cumulative isotope concentration. The partial correlation coefficients (r) were 0.935 ($t = -5.27$, $P = 0.0062$) and 0.778 ($t = 2.77$, $P = 0.039$), respectively. As iron depletion continued, the cumulative isotope concentration declined but increased with the progress of iron repletion.

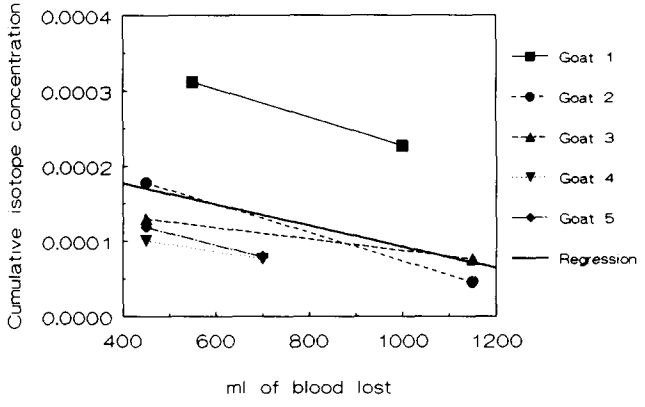

Figure 4 Correlation between cumulative isotope concentration and ml of blood lost during bleeding.

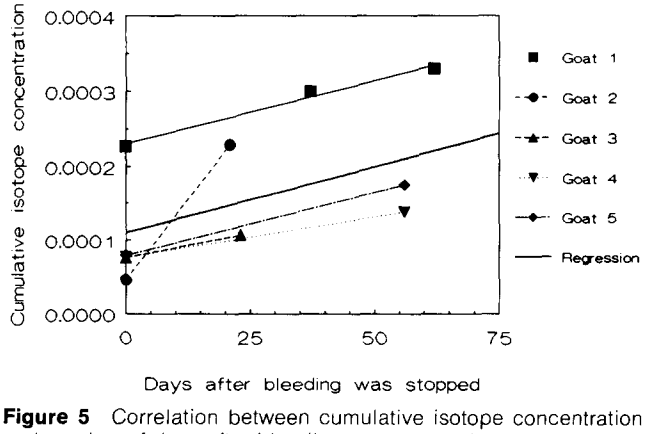

Figure 5 Correlation between cumulative isotope concentration and number of days after bleeding was stopped.

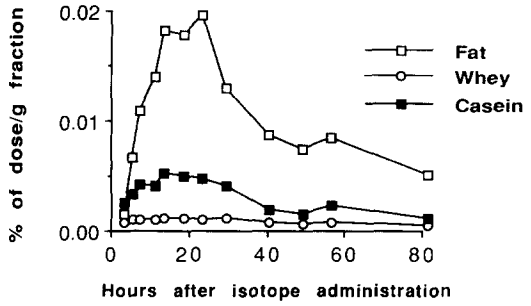

Figure 6 Percentage of ^{59}Fe in the three main fractions in goat milk.

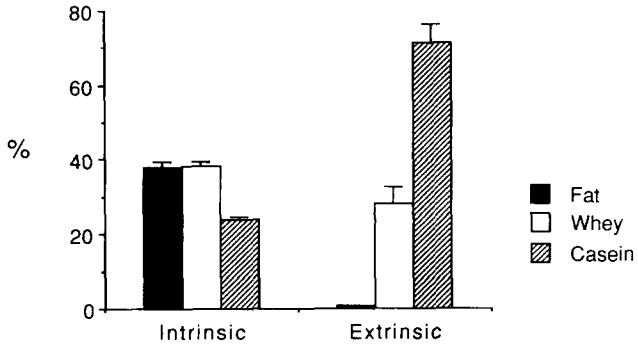

Figure 7 Distribution of extrinsically and intrinsically administered ^{59}Fe among the main milk fractions in goat milk.

The concentration of administered activity in the three main iron fractions in milk—casein, fat, and whey—is shown in *Figure 6*. The isotope was detected in all fractions, but appeared earlier in the casein fraction than in the fat and whey fractions. Highest isotope concentration was found for the fat fraction, with the whey fraction being lowest.

Isotope added extrinsically showed a different distribution in the milk compared to the intrinsic label. Of the extrinsic label, 60–80% was found in the casein fraction, 17–40% in the whey, and only 0.5–0.6% in the fat fraction (*Figure 7*).

Discussion

Iron is necessary for basic functions in most mammalian cells. Therefore, a mechanism by which iron uptake from the plasma pool is regulated seems to be essential. Serum transferrin is the principal iron carrier in blood and a specific receptor for transferrin appears to be the key to iron uptake in most cells.[17] Cellular iron uptake is regulated by the amount of transferrin receptors expressed on the cell surface and by the receptor-transferrin binding constant.[18] Iron, together with heme, has been suggested to regulate protein synthesis,[19] and transferrin has been shown to be one of several cellular growth factors.[20] Since rapidly proliferating cells have a high demand for iron, these cells express many transferrin receptors on their surfaces.[20] In the mammary gland, iron is needed for cell proliferation as well as for production of milk. The

transfer of iron into milk is probably mediated and regulated by transferrin receptors expressed on the surface of the milk-producing cells,[18,21-25] suggesting that milk iron concentration could depend on maturity of the mammary gland.

During anemia, the amount of available, circulating plasma iron will be reduced and most cells will respond with an increase in transferrin receptors. The hematopoietic cells, which have the highest iron demand of all mammalian cells, will express a higher number of receptors than most other cells, and possibly with higher affinity for transferrin than the mammary gland.[18] Our results indicate that the mammary gland might respond more slowly in increasing the number of exposed transferrin receptors during the iron depletion than do the hematopoietic organs, thus being less efficient when competing for available circulating iron. During iron repletion, however, mammary cells seem to compete more efficiently for iron, possibly by more slowly down-regulating the number of transferrin receptors, than do the hematopoietic cells. This is indicated by the rapid increase in cumulative isotope concentration in milk from goat No. 2 during the phase of iron repletion (exclusively from diet), even though she did not receive any additional iron supplements during this period. The amount of iron ingested does not seem to affect this supposed mechanism by which iron secretion to milk is related to maternal iron status.

We also considered the possibility that the physiological state of the animal, in particular its lactation history, could affect milk isotope concentration. For this reason, isotope concentration was investigated in milk from goats with different numbers of previous lactation periods. In the youngest goats (Nos. 1 and 2), isotope concentration was higher than in the older goats. This might indicate that the number of transferrin receptors is higher in mammary glands of younger goats, as a result of a more active cellular proliferation.[20]

The late appearance of the peak isotope concentration at 10–15 hr after intravenous isotope administration may indicate that iron uptake from plasma and subsequent secretion into milk is achieved by a receptor-mediated active transport mechanism. Calcium and iodine, which are known to be secreted by passive diffusion in the mammary gland, and tungsten and radium, which do not have any known physiological function in the organism, all appear in bovine and goat's milk at peak concentration within 4 hr after intravenous injection.[26,27] While little is known about receptor-mediated transport mechanisms from blood to milk, it seems reasonable to assume that such a process for iron would involve several steps including transferrin, transferrin receptors, and passage through membrane bilayers which could be slower than simple diffusion processes.

Our results show that when an iron isotope is administered to lactating animals, the isotope appears in all three main fractions of the milk and that the isotope appears faster in casein than in the fat fraction. The whey fraction is, in contrast to the casein and fat fractions, diluted by the water constituent of milk and shows the lowest isotope concentration. The high isotope concentration in the fat fraction may be explained by the presence of transferrin receptors in the apical plasma membrane that surrounds the fat globules. If the process of iron transfer into the cell is slow, this membrane would be expected to be high in ^{59}Fe activity.

The difference in distribution of the intrinsic and extrinsic radioiron indicates that iron is not easily exchanged between different ligands located in various fractions of milk. This difference in distribution is also likely to be due to specific receptor-mediated transfer of iron into milk fractions in the mammary gland in contrast to non-specific association of iron in an in vitro, virtually cell-free system (milk).

As the iron content in milk is generally low compared to other body fluids and tissues (blood, liver, and muscle), a low recovery of iron isotope in milk was expected. Our interest has been to use intrinsically labeled iron in our studies on milk iron bioavailability, but as these results of low recoveries of administered radioiron in milk indicate, this may be difficult. In this experiment, maximum radioactivity per ml of milk was about 140 cpm. We may consider that a goat kid (7–8 kg of weight) is given 500 ml of this intrinsically labeled milk and that about 20% of the iron is absorbed. The resulting absorbed activity (14,000 cpm) is then diluted by a total blood volume of 750 ml, which yields about 18 cpm/ml blood to be measured in a gamma counter (with a background activity of 25–28 cpm). By increasing the sample volume to 5 ml and the counting time, it might be possible to obtain 1,800 cpm in the sample. With 560 cpm from background, this would result in ≈1,300 net cpm counted. Thus, with a sensitive counter, the isotope concentration may be adequate for absorption studies. The possibility of feeding the offspring several doses of labeled milk should also be considered. Furthermore, if the lactating mother is properly selected, it is likely that the peak concentration of administered ^{59}Fe in the milk could be sufficiently high for bioavailability studies.

In conclusion, our results suggest that to optimize radioiron labeling of milk, the lactating goat should be primiparous, anemic, and fed a diet rich in highly available iron 2–3 weeks before the experiment is started. Then one can expect the transferrin receptors to be numerous in the mammary gland, partly because of the active cellular development and partly because of the lack of circulating iron in the state of anemia. Therefore, in the first phase of recovery from anemia, higher secretion of iron to the milk can be expected.

Acknowledgments

The authors' thanks are due to Mr. Sten-Åke Liedén for his valuable assistance during many late nights and early mornings.

References

1 Fransson, G-B. and Lönnerdal, B. (1983). Distribution of trace elements and minerals in human and cow's milk. *Pediatr. Res.* **17,** 912–915

2 Masson, P.L. and Heremans, J.F. (1971). Lactoferrin in milk from different species. *Comp. Biochem. Physiol.* **39B,** 119–129

3 Querinjean, P., Masson, P-L., and Heremans, J.F. (1971). Molecular weight, single-chain structure and amino acid composition of human lactoferrin. *Eur. J. Biochem.* **20,** 420–425.

4 Weiner, R.E. and Szuchet, S. (1975). The molecular weight of bovine lactoferrin. *Biochim. Biophys. Acta* **393,** 143–147

5 Masson, P.L. and Heremans, J.F. (1986). Metal-combining properties of human lactoferrin (red milk protein) 1. The involvement of bicarbonate in the reaction. *Eur. J. Biochem.* **6,** 579–584

6 Aisen, P.and Leibman, A. (1972). Lactoferrin and transferrin: A comparative study. *Biochim. Biophys. Acta* **257,** 314–323

7 Mazurier, J. and Spik, G. (1980). Comparative study of the iron-binding properties of human transferrins. I. Complete and sequential iron saturation and desaturation of the lactotransferrin. *Biochim. Biophys. Acta* **629,** 399–408

8 Fransson, G-B. and Lönnerdal, B. (1980). Iron in human milk. *J. Pediatr.* **3,** 380–384

9 Goldsmith, S.J., Eitenmiller, R.R., Barnhart, H.M., Toledo, R.T., and Rao, V.N.M. (1982). Unsaturated iron-binding capacity of human milk. *J. Food Sci.* **47,** 1298–1304

10 Lönnerdal, B. (1984). Iron in breast milk. In *Iron Nutrition in Infancy and Childhood* (A. Stekel, ed.), Nestle Nutrition Workshop series, Vol. 4, pp. 95–118, Nestle, Vevey/Raven Press, New York

11 Saarinen, U.M., Siimes, M.A., and Dallman, P.R. (1977). Iron absorption in infants: High bioavailability of breast milk iron as indicated by the extrinsic tag method of iron absorption and by the concentration of serum ferritin. *J. Pediatr.* **91,** 36–39

12 McMillan, J.A., Oski, F.A., Lourie, G., Tomarelli, R.M., and Landaw, S.A. (1977). Iron absorption from human milk, simulated human milk, and proprietary formulas. *Pediatrics* **60,** 896–900

13 Siimes, M., Salmenperä, L., and Perheentupa, J. (1984). Exclusive breast-feeding for 9 months: Risk of iron deficiency. *J. Pediatr.* **104,** 196–199

14 Fransson, G.-B. and Lönnerdal, B. (1983). Distribution of added ^{59}Fe among different fractions of human and cow's milk. *Nutr. Res.* **3,** 108–109

15 Lönnerdal, B. (1985). Dietary factors affecting trace element bioavailability from human milk, cow's milk and infant formulas. *Prog. Food Nutr. Sci.* **9,** 35–62

16 Jones, B. (1975). Simulated near-in fallout in goats and its effects. *Acta Vet. Scand. Supplement* **58,** 1–106

17 Irie, S. and Tavassoli, M. (1987). Review: Transferrin-mediated cellular iron uptake. *Am. J. Med. Sci.* **293,** 103–111.

18 Sigman, M. and Lönnerdal, B. (1988). Transferrin receptors and iron uptake of rat mammary gland membranes during lactation. In *Trace Elements in Man and Animals,* Vol. 6, (L.S. Hurley, C.L. Keen, B. Lönnerdal, and R.B. Rucker, eds.), pp. 433–434, Plenum Press, New York

19 Marcus, D.L. and Freedman, M.L. (1986). Role of heme and iron metabolism in controlling protein synthesis. *J. Am. Ger. Soc.* **34,** 593–600

20 Larric, J.W. and Cresswell, P. (1979). Modulation of cell surface iron transferrin receptors by cellular density and state of activation. *J. Supramol. Struct.* **11,** 579–586

21 Grigor, M.R., Wilde, C.J., and Flint, D. (1988). Transferrin receptor activity in rat mammary epithelial cells. *Biochem. Int.* **17,** 747–754

22 Shulman, H.M., Ponka, P., Wilczynska, A., Gauthier, Y., and Shyamala, G. (1989). Transferrin receptor and ferritin levels during murine mammary gland development. *Biochim. Biophys. Acta* **1010,** 1–6

23 Sigman, M. and Lönnerdal, B. (1990). Response of rat mammary gland transferrin receptors to maternal dietary iron during pregnancy and lactation. *Am. J. Clin. Nutr.* **52,** 446–450

24 Sigman, M. and Lönnerdal, B. (1990). Characterization of transferrin receptors on plasma membranes of lactating mammary tissue. *J. Nutr. Biochem.* **1,** 239–243

25 Sigman, M. and Lönnerdal, B. (1990). Relationship of milk, iron, and the changing concentration of mammary tissue transferrin receptors during the course of lactation. *J. Nutr. Biochem.* **1,** 572–576

26 Lengeman, F.W., Swanson, E.W., and Monroe, R.A. (1957). Effect of season on secretion of iodine in milk. *J. Dairy Sci.* **40,** 387–393

27 Sansom, B.F. and Garner, R.J. (1966). The metabolism of radium in dairy cows. *Biochem. J.* **99,** 677–681