Hexoneogenesis in the Human Breast during Lactation
Agneta L. Sunehag, Kathryn Louie, Jessica L. Bier, Stelios Tigas and Morey W. Haymond


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Lactose is the major osmotic agent in milk. Therefore, lactose synthesis indirectly regulates milk volume. The aim of this study was to determine the source of glucose and galactose in lactose. Six healthy lactating women were studied twice, during a 24-h fast and during ingestion of a mixed macronutrient drink (Sustacal) using [U-13C]glucose and [2-13C]glycerol. Six additional lactating women were studied on one single occasion during ingestion of glucose labeled with [1,13C]glucose.

Using the ratios of [13C6] enrichments of glucose in lactose and plasma glucose and that of galactose in lactose and plasma lactose, we determined that 98 ± 3% of glucose and 68 ± 7% of galactose in lactose were derived from plasma glucose in the fed state, and 72 ± 4 and 51 ± 3%, respectively, after a 24-h fast. Virtually identical results (97 ± 6 and 64 ± 4%, respectively) were obtained during the glucose feeding study. On the basis of the [13C1] enrichment of glucose and galactose in lactose (derived from [2-13C]glycerol), glycerol contributes to the production of galactose but not glucose within the breast.

Thus, plasma glucose is an important source of lactose, but significant amounts of glucose and galactose in lactose are generated within the breast, a process denoted hexoneogenesis. In this process, glycerol is a precursor for milk galactose but not glucose. (J Clin Endocrinol Metab 87: 297–301, 2002)
written consent was obtained from each of them. They are the same lactating subjects reported Tigas et al. (14). The subjects were studied in two protocols (see below). Before being accepted in the study, they underwent a general physical examination and had normal hemoglobin and screening tests for liver and renal function and a negative pregnancy test. The ages of the subjects averaged 27 ± 3 yr (protocol 1) and 30 ± 2 yr (protocol 2); the weights were 69 ± 5 and 67 ± 3 kg, and body mass index was 26 ± 3 and 25 ± 3 kg/m², respectively. The women were between 6 wk and 3 months postpartum. All infants were healthy, born at term, and exclusively breast-fed at the time of the study. Each lactating woman was provided iron supplementation over the course of these studies, if not already taking iron as part of her postpartum management prescribed by her obstetricians.

Protocol 1: Sustacal feeding and short-term fasting. In protocol 1, one woman was studied on two occasions, in random order, once in the fed state and once during a 24-h fast. The studies were separated by 2–4 wk. Each woman and her infant were admitted to the Metabolic Research Unit of the Children’s Nutrition Research Center (CNRC) or the General Clinical Research Center on the evening before study. At 1800 h on the evening of admission, two iv catheters were introduced in the antecubital fossae or forearm veins under EMLA (Astra Pharmaceuticals, Wayne, PA) cream analgesia, one for infusion and the other (in the contralateral arm) for blood sampling. On both study occasions, subjects were fed a supper meal of 10 kcal/kg at 1800 h and were subsequently fasted (except for water) overnight. At 0600 h, after the overnight fast, baseline milk and blood samples (5 ml) were obtained, and the subjects received the following: 1) an oral dose of deuterium oxide (0.1 g/kg) to measure the rapidity of equilibration of maternal body water with milk water to estimate the existence, if any, of dead volume of milk in the breast; 2) a primed constant rate infusion of [U-13C]glucose to measure the fraction of galactose and glucose in lactose derived from the plasma glucose space; and 3) a primed constant rate infusion of [2-13C]glycerol to measure the fraction of glucose and galactose in milk lactose derived from plasma glycerol (14). In the fed protocol, the women drank 90 kcal (90 ml) of Sustacal (Mead Johnson Nutritional, Evansville, IN) every 30 min between 0600 h and 1800 h, providing a total of approximately 2160 kcal and approximately 33 μmol/kg-2 min-1 of carbohydrate. During the fasting study, the women drank an equal volume of water but received no calories from 0600 h until 1800 h on the day of study.

In addition to the baseline samples, blood and milk samples (6–10 ml) were collected at 3-h intervals. The infants were breast-fed approximately every 3 h (from both breasts at each nursing), and milk samples were collected before and immediately after each feeding.

In protocol 1, we attempted to maintain energy neutrality and used a commercially available preparation containing unlabeled complex carbohydrates. As a result, we were unable to estimate the contribution of dietary glucose to milk production. Thus, protocol 2 was designed to address this issue.

Protocol 2: Oral glucose load. Each woman was studied on one occasion using a protocol identical to that outlined above except: 1) she received oral glucose enriched with [1-13C]glucose every 15 min at a rate of 33 μmol/kg-2 min-1 instead of Sustacal but no 3H2O, [U-13C]glucose, or [2-13C]glycerol was administered; and 2) the duration of study was 9 h.

Plasma analysis

Enrichments of [1-13C]glucose, [U-13C]glucose, and the glucose isomers, were measured by gas chromatography mass spectrometry (GCMS) using the penta-acetate derivative and of [2-13C]glycerol using the triacetate derivative as previously described (15). Deuterium enrichments in both plasma and milk (defatted) water were measured using isotope ratio mass spectrometry (16). All measurements were made in the Stable Isotope Core Laboratory of the CNRC.

Milk analyses

Defatting milk. A homogeneous sample of milk (1 ml) is pipetted into microcentrifuge tubes (1.5-ml conical, screw-capped tubes, CEL Associates Inc., Houston, TX). The tube is capped and placed upside down in a 20-ml scintillation vial and centrifuged for 10 min at 3,000 rpm (900× g) at 4 C in a refrigerated centrifuge (Beckman Coulter model J-68, Beckman Coulter Instruments, Inc., Falo Alto, CA). Without disturbing the layer of milk fat, the supernatant is carefully decanted into a new microcentrifuge tube.

Deproteinizing milk. To a 1.5-ml microcentrifuge tube, 50 μl defatted milk, 450 μl deionized water, 500 μl BaOH, and 500 μl ZnSO4 are added. The tubes are capped tightly, vortexed, and centrifuged for 10 min at 3,000 rpm (900× g) at 4 C. The defatted, deproteinized supernatant is used for lactose hydrolysis.

Lactose hydrolysis. Defatted, deproteinized milk (150 μl) and 50 μl (0.35 U) β-galactosidase in a final buffer concentration of 93 mm sodium phosphate (pH 7.3), 1 mm MgCl2, and 112 mm 2-mercaptoethanol are added to 1.5-ml microcentrifuge tubes. The reaction mix is incubated at 37 C for 1 h.

Derivatization of hydrolyzed products. Fifty microliters of each hydrolyzed sample are aliquoted into 4-ml vials and taken to dryness under nitrogen at room temperature. Fifty microliters of acetic anhydride/pyridine (2:1) are added to each sample, and the samples are heated to 60 C for 10 min or allowed to sit overnight at room temperature. Then, the samples are dried under nitrogen at room temperature, reconstituted in 100 μl ethyl acetate, and transferred to autosampler vials.

GCMS analysis. The derivatized samples are analyzed using a Hewlett-Packard Co. GCMS (GC 6890, MS 5973) with a SP-17 column (30 m × 0.25 mm × 0.25 μm; Supelco, Inc., Bellefonte, PA). The conditions for the GC are the following: injector, 250 degree (splitless injection of samples); oven, 70 C for 1.0 min; ramp, 30 C/min to 280 C; hold at 280 C for 5 min. The positive ionization mode is employed with methane as the reagent gas and selected monitoring of m/z 331–337 for glucose and galactose. The SP-17 column separates the a- and β-anomers of glucose and galactose. We have used the a-anomers of both sugars for the analyses. These peaks are very well separated, with a difference in retention time of approximately 0.25 min.

Calculations

Standard product precursor relationships were calculated. The percentage of product, i.e. glucose and galactose in milk lactose, that was derived from the precursor pool (plasma glucose), i.e. percentage milk sugar ← plasma glucose, was calculated using the following equation:

% milk sugar ← plasma glucose = \left(\frac{[13C]_{\text{milk sugar}}}{[13C]_{\text{plasma glucose}}}\right) \times 100 \quad (\text{protocol 1})

or

% milk sugar ← plasma glucose = \left(\frac{[13C]_{\text{milk sugar}}}{[13C]_{\text{plasma glucose}}}\right) \times 100 \quad (\text{protocol 2}),

where milk sugar is either the glucose or the galactose moiety in lactose.

[13C] enrichment is derived from the [U-13C]glucose tracer (protocol 1) and [13C] enrichment is derived from the [1-13C]glucose tracer (protocol 2).

The contribution from glycerol to milk glucose and galactose produced from other sources than plasma glucose via processes within the breast (percentage milk sugar ← glycerol) was calculated using the following equation:

% milk sugar ← glycerol = \left(1 - \left(\frac{[13C]_{\text{milk sugar}}}{[13C]_{\text{plasma glucose}}}\right)\right) \times \left(\frac{\left([13C]_{\text{milk sugar}}/13C_{\text{plasma glucose}}\right) - \left([13C]_{\text{milk sugar}}/13C_{\text{plasma glucose}}\right)}{[13C]_{\text{plasma glucose}}}\right) \times 100.


Statistical analysis

All data are expressed as mean ± se. The product/precursor relationships were compared using a paired t-test.
Results

The results of the maternal adaptation to fasting and feeding are provided in the accompanying manuscript (14). The results of this manuscript focus exclusively on the method of analysis of the milk carbohydrates and on the impact of feeding and fasting on the product precursor relationships between the milk and the maternal plasma space.

Reproducibility of the lactose analysis

A variety of methodologies for milk hydrolysis were attempted, including commercial over-the-counter lactase preparations. Initial results were always promising, but over time the enrichments in glucose would decrease, indicating dilution by unlabeled glucose. Eventually, we identified that these products were stabilized with starch (and reacted to iodine). The procedure described above (using β-galactosidase from E. coli) was found to be stable and reproducible over time. The coefficient of variation for preparation and GCMS analyses of 10 aliquots of a sample was 5% for glucose and 6% for galactose. The SP-17 GC column separates the α- and β-anomers of glucose and galactose, respectively, in the following order: α-galactose, α-glucose, β-glucose, and β-galactose (Fig. 1). Figure 1 depicts the GCMS total ion chromatogram of a sample of glucose and galactose standards and a sample of hydrolyzed milk, demonstrating a complete separation of the glucose and galactose moieties.

Equilibration of 2H2O in milk

Figure 2 depicts the results of the time course of deuterium enrichment in plasma and breast milk water during the fed and fasted conditions. Within 3 h of the oral administration of the 2H2O, the plasma and milk water enrichments of 2H were essentially identical and remained so throughout the study period, thus eliminating the possibility of any significant pools of preformed milk.

Effects of Sustacal and fasting (protocol 1)

Analysis of milk samples obtained before and after each feeding gave essentially identical values (data not shown). The values obtained after the feedings were used because, theoretically, they would contain the most recently synthesized lactose. Table 1 provides the enrichments of [13C1] (derived from [2,13C]glycerol) and [13C4] (derived from [U-13C]glucose) enrichments (%) in plasma glucose, milk glucose, and milk galactose represent the mean of the data obtained after the 9- and 12-h feedings in the fed and fasted limb of protocol 1.

![Graph showing enrichment of 2H2O in plasma and milk water in the fed and short-term fasted states.](image)

**TABLE 1.** The [13C1] (derived from [2,13C]glycerol) and [13C4] (derived from [U-13C]glucose) enrichments (%) in plasma glucose, milk glucose, and milk galactose represent the mean of the data obtained after the 9- and 12-h feedings in the fed and fasted limb of protocol 1.

<table>
<thead>
<tr>
<th>Isotopomer</th>
<th>Fed</th>
<th>Fasted</th>
</tr>
</thead>
<tbody>
<tr>
<td>[U-13C]glucose</td>
<td>[13C1] 0.57 ± 0.05</td>
<td>[13C1] 1.33 ± 0.09</td>
</tr>
<tr>
<td></td>
<td>[13C4] 0.51 ± 0.04</td>
<td>[13C4] 0.98 ± 0.10</td>
</tr>
<tr>
<td>Milk glucose</td>
<td>[13C1] 0.36 ± 0.04</td>
<td>[13C1] 0.68 ± 0.07</td>
</tr>
<tr>
<td>Milk galactose</td>
<td>[13C4] 2.04 ± 0.37</td>
<td>[13C4] 3.38 ± 0.58</td>
</tr>
<tr>
<td>Plasma glucose</td>
<td>[13C6] 0.66 ± 0.45</td>
<td>[13C6] 0.65 ± 0.55</td>
</tr>
<tr>
<td>Plasma galactose</td>
<td>[13C6] 0.70 ± 0.45</td>
<td>[13C6] 0.68 ± 0.55</td>
</tr>
</tbody>
</table>

If glycerol is not a substrate for production of glucose and galactose in lactose within the breast, we would predict that the ratios between the enrichment of [13C1] (derived from the [2-13C]glycerol tracer) in milk glucose/plasma glucose and milk galactose/plasma glucose would be equal to the corresponding [13C1] ratios. During both feeding and fasting, the ratios between the [13C1] milk glucose and [13C1] plasma glucose (76 ± 5 and 64 ± 5%, respectively) and the corresponding [13C4] ratios (see above) were not different, dem-
onstrating that essentially no plasma glycerol contributed to the glucose formed within the breast and incorporated into lactose. In contrast, during both feeding and fasting, the ratios between $[^{13}C_1]$ milk galactose and $[^{13}C_1]$ plasma glucose (130 ± 16 and 80 ± 6%, respectively) were significantly higher ($P < 0.01$) than the corresponding $[^{13}C_6]$ ratios (see above), demonstrating that glycerol contributed significantly to the synthesis of galactose within the human breast (Fig. 4). The means of the individual ratios differ slightly from those that can be calculated using the mean enrichments provided in Tables 1 and 2.

**Effects of oral glucose (protocol 2)**

During ingestion of labeled glucose alone, the ratios between the $[^{13}C_1]$ enrichment (from the $[1-^{13}C]$glucose derived from the meal) in milk glucose/plasma glucose and in galactose/plasma glucose were virtually identical (97 ± 6 and 64 ± 4%, respectively) to those observed with $[U-^{13}C]$glucose described above. We have previously demonstrated that in these women 94% of glucose appearing in plasma was derived from the meal glucose (based on the $[1-^{13}C]$ enrichments in plasma glucose and that of the glucose drinks) (14). The above milk data demonstrate that 83% of lactose is derived from plasma glucose. Thus, during the continuously fed condition, we would estimate that dietary carbohydrate contributes 78% (0.94 × 0.83 × 100) of the substrate necessary to produce lactose.

**Discussion**

The present studies demonstrate that in humans the vast majority of lactose (83%) is derived from the plasma glucose pool during feeding of a mixed macronutrient drink or with glucose alone. However, we have also demonstrated that the human breast is capable of synthesizing both the glucose and galactose moieties of lactose from substrates other than plasma glucose. In the fed condition, 20% of lactose is derived from substrates other than plasma glucose, and this fraction is increased to 40% during short-term fasting. Surprisingly, we observed unequal labeling of the glucose and galactose moieties in lactose suggesting different pools and/or processes for de novo synthesis of glucose and galactose. Under conditions of nutrient ingestion, nearly all of the glucose in lactose is derived from the plasma glucose space, but only 68% of galactose. These results are similar to those observed in ruminants (6, 7). Neville et al. (5) observed, under nonsteady state conditions during an insulin clamp, that milk galactose enrichment was only approximately 70% of that of plasma glucose. Again, these results are consistent with those of the present study. During short-term fasting, the fraction of glucose and galactose in lactose derived from plasma glucose decreased to 72 and 51%, respectively. Conversely, 28% of the glucose and 49% of the galactose in lactose were synthesized from substrates other than plasma glucose. We have denoted this de novo synthesis of glucose and galactose in the breast hexoneogenesis, implying that this process is similar to hepatic gluconeogenesis, except that we doubt that any free glucose is released back into the maternal circulation due to the low activity of glucose-6-phosphatase in lactating mammary tissue (6, 12, 17), and that another hexose, galactose, is produced in this process. Whether this is the maximum fraction of lactose that can be synthesized by hexoneogenesis is unknown and will require additional studies employing a longer duration of fasting. However, significant use of other hexoneogenic substrates by the breast during fasting would potentially spare maternal glucose for her use.

Several explanations might be considered for the dilution of the plasma glucose label measured in the milk glucose and galactose. The first is the possibility of hidden pools of preformed milk within the breast. This seems unlikely because of the turnover of milk with each feeding. In addition, using a small oral dose of deuterated water, we demonstrated that plasma and milk water were in complete equilibrium after 3 h. The dilution in galactose, in both the fed and short-term

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### TABLE 2. The $[^{13}C_1]$ enrichments (%) (derived from $[1-^{13}C]$glucose) in plasma glucose, milk glucose, and milk galactose represent the data obtained after the 9-h feeding in protocol 2

<table>
<thead>
<tr>
<th>Tracer/isotopomer</th>
<th>$[^{13}C_1]$glucose</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasma glucose</td>
<td>2.37 ± 0.07</td>
</tr>
<tr>
<td>Milk glucose</td>
<td>2.29 ± 0.16</td>
</tr>
<tr>
<td>Milk galactose</td>
<td>1.52 ± 0.11</td>
</tr>
</tbody>
</table>

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**Fig. 3.** The fraction of milk galactose and glucose derived from plasma glucose in the fed and fasted conditions in protocol 1 (based on the $[1-^{13}C]$glucose tracer); during glucose feeding in protocol 2 (based on the $[1-^{13}C]$glucose tracer in the glucose drink).

**Fig. 4.** The contribution from glycerol based on the $[^{13}C_1]$ enrichment from the $[2-^{13}C]$glycerol tracer (protocol 1).
fasted states, suggests that under both conditions a substantial amount of the carbon is derived from nonglucose substrates, presumably entering the breast from the plasma. Although we cannot delineate the contributions of amino acids, lactate, and pyruvate to this processes, we have demonstrated that glycerol contributes substantially to the galactose formed by hexoneogenesis and that this contribution is most likely from direct uptake of the glycerol into the breast rather than the uptake of maternal glucose derived from glycerol via gluconeogenesis in the mother’s liver and/or kidney. Comparing the ratios of the enrichments of \([^{13}C_1}\) (derived from \([2-^{13}C]\)glycerol) and \([^{13}C_6}\) (derived from \([U-^{13}C]\)glucose) between milk galactose and plasma glucose (in protocol 1), we demonstrated that under fed and fasted conditions, glycerol accounted for about 24 and 14%, respectively, of the galactose formed within the mammary gland from other sources than plasma glucose. This would suggest that about 76 and 86%, respectively, of the galactose generated via hexoneogenesis must be derived from other substrates than glycerol.

In contrast to galactose, it is of great interest that the glycerol contributed little or nothing to the glucose found in lactose. This again is consistent with studies in bovine mammary gland incubation studies and perfused goat mammary gland (9, 12). However, it is important to note that during fasting the glucose in lactose was also derived from substrates other than maternal plasma glucose. We presumed that the substrates available for the formation of glucose-1-phosphate for the subsequent conversion to galactose would have been the same pool of glucose-1-phosphate available as a precursor of glucose. The glycerol labeling of galactose, but not glucose in milk lactose, demonstrates that these hexoses are being derived from at least two separate pools, presumably within the breast. This is both reassuring and disturbing at the same time; it is reassuring in that little or no glucose-6-phosphatase is present in at least bovine (8) and rat (17) mammary tissue and disturbing in the sense that we have no clear idea as to the precursor pool for glucose produced within the breast for lactose synthesis. One consideration is that of intramammary gland glycogen. However, to our knowledge no such pool exists in sufficient quantity to dilute the glucose in lactose over the time of these studies. Elucidation of the source(s) of this unlabeled glucose in lactose formed within the mammary gland will require further study.

In the fed state (under the conditions of these studies), we conclude that oral glucose is an important source of substrate for both the glucose and galactose moieties in human milk. If one can extrapolate from the ingestion of oral glucose (labeled with \([1-^{13}C]\)glucose) to that of a normal mixed meal, our data would suggest that up to 75% of the milk lactose is derived directly from the meal carbohydrate. In conclusion, the results from these studies demonstrate that although adequate carbohydrate supply is important for lactogenesis, processes independent of glucose availability are required to maintain lactose production in both the fed and short-term fasted conditions.

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Address all correspondence and requests for reprints to: Morey W. Haymond, M.D., CNRC, 1100 Bates Street, Houston, Texas 77030. E-mail: mhaymond@bcm.tmc.edu.

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