Metabolism of U^{13}C-labeled linoleic acid in lactating women

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Abstract Long chain polyunsaturated fatty acids are nutritionally important constituents of breast milk. The origin of these fatty acids in milk has not been clearly identified. We studied the contribution of maternal endogenous conversion of linoleic acid to milk dihomo-γ-linolenic and arachidonic acids, using stable isotope techniques. Six lactating women ingested 1 mg of [U-^{13}C]linoleic acid/kg body weight in the 2nd, 6th, and 12th week of lactation. Before and at several times during a 5-day period after tracer intake, samples of breath and milk were collected and the volume of daily milk production was recorded. Nutrient intakes were assessed with dietary protocols. The estimated oxidized proportion of the ingested labeled linoleic acid did not differ significantly with duration of lactation (2nd week: 18.9 ± 4.5%, 6th week: 24.0 ± 3.8%, 12th week: 17.7 ± 3.5%, mean ± SE), nor did transfer into milk as linoleic acid (12.7 ± 1.4%, 13.1 ± 2.5%, 11.7 ± 2.7%, respectively). About 0.2% of the tracer appeared in milk as dihomo-γ-linolenic acid and 0.02% as arachidonic acid. There were no statistically significant changes with time. We estimate that about 30% of milk linoleic acid is directly transferred from the diet, whereas about 11% of milk dihomo-γ-linolenic acid and 1.2% of milk arachidonic acid originate from direct endogenous conversion of dietary linoleic acid.—Demmelmair, H., M. Baumheuer, B. Koletzko, K. Dokoupil, and G. Kratl. **Metabolism of U-^{13}C-labeled linoleic acid in lactating women. *J. Lipid Res.* 1998.** 39: **1389–1396.**

Supplementary key words lactation • long chain polyunsaturated fatty acids • stable isotopes • endogenous synthesis

Breast milk is the most suitable diet for healthy infants and provides the necessary nutrients for the newborn and for infants up to an age of 6 months (1, 2). The nutritive value of milk is not only determined by the amount of protein and energy supplied, but it is recognized that vitamins, minerals, essential amino and fatty acids have to be available in sufficient quantities and in ratios that meet infantile requirements (3). In recent years there has been an ongoing debate about the importance of certain polyunsaturated fatty acids in the diet of newborns for their development. A deficiency of linoleic (18:2n–6) and α-linolenic (18:3n–3) acids causes obvious clinical symptoms (4). In addition, their C-20 and C-22 long-chain polyunsaturated derivatives (LCP), primarily arachidonic (20:4n–6) and docosahexaenoic acids, may be conditionally essential as some studies show functional disadvantages when babies are fed formulas without LCP (4–6).

The essential amino acids delivered in milk are constitutional components of proteins with genetically determined composition, hence the amino acid intake of the recipient infant with a given milk volume will only depend on the relative contribution of individual proteins and their absolute concentration in milk. In contrast, the fatty acid composition of milk fat is variable and subject to dietary influences (7). Fatty acids available from the circulation can be assembled to milk fat globules in the mammary gland and are secreted with breast milk. Thus, any fatty acid supplied with the diet or which is endogenously synthesized might occur to a certain extent in milk. On the other hand, it seems desirable that the infants have a sufficient supply of those fatty acids needed to meet their nutritional requirements, and some form of metabolic control of the influx of individual fatty acids into milk or at least some metabolic buffering of fluctuations in the fat composition of the dietary intake of the mother could be advantageous. Fatty acids that are mainly used as sources of energy by the baby are interchangeable, but the essential fatty acids and possibly also their desaturation and elongation products are mandatory for the growth and development of the infants (4, 8).

Some mammalian species like seals derive all their milk fat from breakdown of body stores (9). While this effect is much less pronounced in other mammalian species and in humans, there is a turnover of body fat which attenuates fluctuations of the dietary composition (10). Additionally, short term fluctuations might be partly compen-

Abbreviations: DOB, delta over baseline, i.e., increase of the δ^{13}C-value above prestudy value; GC–C–IRMS, gas chromatography–combustion–isotope ratio mass spectrometry; FID, flame ionization detector; HDL, high density lipoproteins; LCP, long chain polyunsaturated fatty acids with 20 or 22 carbon atoms; LDL, low density lipoprotein; VLDL, very low density lipoproteins.

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sated for by the distribution of fatty acids among different lipoprotein fractions, because the mammary gland obtains its supply of essential fatty acids mainly from chylomicrons and VLDL, and to a lesser extent from other lipoprotein fractions (11). Thus, not only the last meal but also the remainder of former meals contribute to the composition of the available lipid substrate, as LDL and HDL are catabolized in the liver and their fatty acids are incorporated into newly formed VLDL (12).

Three possible sources of milk LCP have to be considered: they may be directly transferred to milk from dietary intake of preformed LCP, be mobilized from body stores, or be synthesized from linoleic (18:2n–6) or α-linolenic acid, respectively, in the liver or in the mammary gland prior to their incorporation into milk. Mobilization and conversion are suggested as potential sources by the finding that there is a relatively constant ratio between n–6 and n–3 LCP (13, 14) and that the secretion of n–6 LCP with milk is not directly dependent on the diet. Mobilization from body fat or a large endogenous pool of LCP, which serves as precursor for milk production, would guarantee a more constant supply of LCP for the infant.

Quantitative determination of the dietary intake of 18:2n–6 and its output in milk does not enable a distinction between different sources of milk 18:2n–6. A possibility to elucidate the transfer of 18:2n–6 into milk either as 18:2n–6 or as one of its conversion products is offered by the use of a stable isotope-labeled analogue of natural dietary 18:2n–6. Using deuterated fatty acids, Hachey et al. (16) have demonstrated the direct transfer of dietary palmitic, oleic and linoleic acids into breast milk in a group of three mothers. In our study we supplied uniformly ^{13}C-labeled 18:2n–6 orally to lactating women in order to quantify the transfer of dietary 18:2n–6 to milk, its conversion to milk LCP, and its oxidation. Using gas chromatography–combustion–isotope ratio mass spectrometry as a more sensitive method of tracer detection, we could perform repeated measurements in the same subjects with reasonable tracer costs to look for alterations in the metabolism with progressing lactation. Furthermore, this method is suitable to detect even low conversion of 18:2n–6 into its LCP derivatives; thus we could investigate the contribution of endogenous elongation and desaturation to milk fat synthesis.

MATERIAL AND METHODS

Mothers delivering without complications in the Dept. of Obstetrics, Neuburg, Bavaria, Germany, and planning to fully breast feed their infants for more than 3 months were asked to participate in the study. The six participating women were aged 31 ± 3 years (mean ± SD) and had given birth to healthy, singleton full-term babies. The infants were exclusively breast fed for the duration of the study, with the exception of one mother during the third study period, who supplemented breast feeding with minor amounts of infant formula. The mothers had an average weight of 65.2 ± 8.7 kg (at 14 days after delivery) and a height of 167 ± 3 cm. They were in their first or second lactation. All mothers consumed omnivorous diets and none reported any health problems. They were studied in the second, sixth, and 12th week of lactation. During each observation period they were allowed to continue their usual dietary habits, but were instructed to avoid corn-based products (elevated natural abundance of ^{13}C) and to avoid eggs, because of their high 20:4n–6 content. During the study periods their food consumption was documented in dietary diaries, which were later evaluated by a dietitian, and they were asked to continue with their usual daily routines.

At the start of each study period, subjects received 1 mg per kg body weight of uniformly (98%) ^{13}C-labeled 18:2n–6 (Martek Bioscience, Columbia, MD) as free acid, dissolved in 2 ml of pure ethanol. The solution was dropped on a slice of buttered bread. The tracer was ingested together with a freely chosen breakfast.

The study protocol was approved by the ethical committee of the Medical Faculty, Ludwig-Maximilians-Universität, München.

Milk

Samples were obtained by manual expression of about 1 ml of milk both before and after feeding the infant from the same breast. Equal amounts of both samples were pooled to take into account the differences in fat content of fore and hind milk. Samples were taken during 5 days, for the first time in the morning of the first study day before tracer application, as well as 6 and 12 h later. The sampling was repeated during the following 4 days; thus a total of 15 samples were collected at time points 0, 6, 12, 24, 30, 36, 48, 54, 60, 72, 78, 84, 96, 102, and 108 h after tracer intake. Samples were stored at −20°C until analysis. The total volume of milk secreted per day was determined by weighing the infant before and after each feeding during the 5 day period.

Breath

Breath was collected by exhalation into 1-l breath bags, and 11-ml aliquots were transferred into evacuated glass tubes (Labco Ltd. Manchester, UK) for storage until measurement of the ^{13}C-content of the CO_2. Before breakfast on the first study day, a baseline sample was taken in duplicate and further samples were collected in hourly intervals until 12 h after start. During the following 4 days, samples were taken in the morning, around midday, and in the evening. The exact time points of sampling were recorded. Analyses were performed by isotope ratio mass spectrometry (delta S, Finnigan MAT, Bremen, Germany) after chromatographic purification of the CO_2 in a continuous flow inlet system. Total CO_2 production was assumed to be 300 mmol CO_2 per m^2 of bodysurface per hour. The estimation of Haycock, Schwartz, and Wisotsky (17) was used to calculate body surface from weight and height.

Dietary records

During each 5-day study period the subjects kept a dietary record where they noted all food ingested quantitatively. Using the dietary evaluation program Diät 2000 (Hard & Soft, Rimbach, Germany) the daily intakes of total calories, 18:2n–6 and 20:4n–6 were calculated from these records.

Analysis of milk samples

After thawing, the samples were mixed and total fat was extracted from 1 ml milk. After the addition of 4 ml chloroform–methanol 2:1, the samples were shaken and warmed to 37°C for 15 min. A further 1 ml of chloroform and 1 ml of water were added and the mixture was cooled down to 0°C in melting ice for 15 min, before phase separation was achieved by centrifugation at 1200 *g*. The chloroform phase was transferred into a preweighed 4 ml vial and dried under N_2 at 37°C. Residual water was

eliminated by drying over silica gel overnight and total fat was determined gravimetrically. The fat was reacted with 1.5 ml methanolic hydrochloric acid (3 m) for 1 h at 85°C to transfer fatty acids into their methyl esters. After neutralization of the reaction mixture with bicarbonate buffer, fatty acid methyl esters were extracted into 3 ml hexane which was taken to dryness under a gentle stream of nitrogen at room temperature, and taken up for storage into 1 ml hexane with 2 g/l butylhydroxytoluene added as an antioxidant (13). Aliquots of this solution were used undiluted for the isotopic analyses, while it was 5-fold diluted for the quantification of individual fatty acids by gas chromatography with flame ionization detection (FID).

Quantification was performed with a HP5890 series II GC (Hewlett-Packard, Waldbronn, Germany) equipped with a cool on-column injector (110°C for 1 min, then heated to 250°C with a rate of 50° per min) and a FID (250°C). Carrier gas (He) pressure was set to 1.3 bar at start and programmed to rise continuously with a rate of 0.01 bar/min during the analysis. Separation of the fatty acid methyl esters was achieved on a BPX70 column (SGE, Weiterstadt, Germany) with 50 m length and an inner diameter of 0.32 mm. The oven temperature program started at 130°C and after a 1-min delay was set to rise with 3°/min to 180°C, from there on with 4°/min to 200°C and then 1°/min to the final temperature of 210°C, which was kept for 8 min. Identification of individual fatty acids and determination of response factors was performed with fatty acid methyl ester standards (NuChek Prep, Elysian, MN; Sigma, Deisenhofen, Germany). Results are given as weight percentages of fatty acids with carbon chain lengths from 14 to 24 C-atoms.

Similar chromatographic conditions were used for the separation of fatty acid methyl esters for ^{13}C-analysis by gas chromatography–combustion–isotope ratio mass spectrometry (Hewlett-Packard GC interfaced to Finnigan MAT delta S mass spectrometer). For injection, a split/splitless injector in split mode (1:10) was used which was held at 250°C, and the head pressure was kept constantly at 1.5 bar for the whole duration of the analysis. The separated fatty acid methyl esters were combusted on line after they eluted from the separation column, and the $^{13}C/^{12}C$ ratio was determined in the resulting CO_2 (18). By relating the $^{13}C/^{12}C$ ratio of the sample components to the international PDB standard, their $\delta^{13}C$-values were obtained (19). The ^{13}C-contents are presented as differences between the $\delta^{13}C$-values measured at individual sampling times and the prestudy $\delta^{13}C$-value of the corresponding compound, commonly referred to as delta over baseline (DOB) values. From the $^{13}C/^{12}C$ ratio the percentage of ^{13}C in the compound was also calculated. By subtraction of the basal ^{13}C-percentage, the contribution of tracer ^{13}C to total ^{13}C content was determined.

Calculations

The amounts of individual fatty acids secreted per day were calculated from the volume of milk secreted per day, mean fat content, and weight % of individual fatty acids. Each of the three sampling points per day was assumed to represent one-third of the daily milk production, as samples were not taken at all feedings during the day. Multiplication of the amounts of fatty acids secreted with the percentage of ^{13}C contributed by the tracer yielded μmoles of tracer ^{13}C secreted with a milk sample. Amounts of ^{13}C were expressed as percentage cumulative recovery of the applied tracer (20).

For the modeling of the metabolism of [^{13}C]linoleic acid, the SAAM II software was used (SAAM Institute, Washington, DC). All statistical calculations were performed with the Software package for Social Sciences (SPSS Ver. 6.1.3, Chicago, IL). The comparisons between the time points were done using the Friedman-test for paired samples ($P \leq 0.05$ taken as significant).

TABLE 1. Dietary intake, daily milk production, and milk fat content of the participating breast-feeding women in the 2nd, 6th, and 12th week of lactation (n = 6, mean ± SE)

Variable	Week 2	Week 6	Week 12
Total energy intake (kcal/d)	2621 ± 115	2518 ± 90	2501 ± 186
Total fat intake (g/d)	110.7 ± 5.3	102.7 ± 5.7	123.2 ± 16.4
18:2n–6 intake (g/d)	9.5 ± 1.7	9.9 ± 0.5	9.9 ± 1.4
20:4n–6 intake (g/d)	0.55 ± 0.22	0.59 ± 0.21	0.76 ± 0.21
Milk production (ml/d)	670 ± 34	757 ± 50	735 ± 64
Fat content (g/100 g)	4.1 ± 0.9	3.9 ± 1.0	4.0 ± 1.0
18:2n–6 in milk (g/d)	3.0 ± 0.5	3.0 ± 0.4	3.0 ± 0.5
20:3n–6 in milk (g/d)	0.10 ± 0.04	0.10 ± 0.03	0.09 ± 0.04
20:4n–6 in milk (g/d)	0.12 ± 0.02	0.11 ± 0.01	0.10 ± 0.01

RESULTS

Dietary intakes, milk, and milk fat secretion as well as milk fatty acid composition are shown in **Table 1** and **Table 2**. Maximal ^{13}C-enrichments in breath-CO_2 were ob-

TABLE 2. Milk fatty acid composition of the 6 participating subjects in the 2nd, 6th and 12th week of lactation (% wt/wt of all fatty acids with ≥14 carbon atoms)

	Week 2		Week 6		Week 12	
Fatty Acid	Mean	SE	Mean	SE	Mean	SE
14:0	7.54	0.69	7.34	0.43	7.72	0.92
14:1t	—	—	—	—	0.02	0.01
14:1n–5[a]	0.47	0.04	0.55	0.03	0.47	0.04
15:1n–5	0.15	0.02	0.15	0.01	0.14	0.01
16:0	25.86	0.47	26.84	0.40	26.29	0.36
16:1t	0.30	0.03	0.3	0.02	0.26	0.02
16:1n–7[a]	2.11	0.16	2.41	0.17	2.03	0.16
17:0	0.44	0.02	0.45	0.02	0.41	0.01
17:1n–7	0.33	0.02	0.35	0.02	0.3	0.02
18:0	8.40	0.33	8.46	0.16	8.99	0.26
018:1t	0.55	0.21	0.82	0.22	1.08	0.42
18:1n–9	34.42	0.78	35.16	0.44	34.12	1.84
18:1n–7	2.04	0.18	2.03	0.17	2.75	1.04
18:2tt	0.25	0.02	0.31	0.02	0.30	0.05
18:2n–6	12.33	1.21	10.78	0.53	10.87	0.52
18:3n–6	0.17	0.01	0.18	0.02	0.17	0.02
18:3n–3	1.00	0.10	0.89	0.07	0.99	0.06
18:4n–3	0.56	0.07	0.47	0.01	0.49	0.13
20:0	0.48	0.04	0.5	0.04	0.47	0.04
20:1n–9	0.14	0.02	0.14	0.01	0.2	0.05
20:2n–6[a]	0.30	0.02	0.22	0.01	0.21	0.01
20:3n–6[a]	0.41	0.03	0.34	0.02	0.31	0.01
20:3n–9	0.02	0.01	0.02	0.01	0.02	0.01
20:3n–3	0.05	0.02	0.03	0.01	0.04	0.02
20:4n–6[a]	0.46	0.04	0.41	0.03	0.33	0.02
20:5n–3	0.13	0.04	0.08	0.02	0.13	0.05
22:0	0.09	0.02	0.08	0.02	0.07	0.02
22:1t	0.11	0.01	0.08	0.01	0.1	0.01
22:1n–9	0.15	0.07	0.08	0.02	0.1	0.03
22:2n–6	0.08	0.03	0.06	0.02	0.13	0.05
22:4n–6	0.08	0.01	0.07	0.01	0.05	0.02
22:5n–3	0.15	0.02	0.14	0.02	0.14	0.01
22:6n–3	0.29	0.08	0.16	0.06	0.25	0.07
24:0	0.10	0.04	0.07	0.04	0.02	0.01
24:1n–9	0.03	0.02	0.01	0.01	0.02	0.01
Total n–3	2.18	0.24	1.78	0.11	2.05	0.29
Total n–6	13.85	1.22	12.06	0.51	12.08	0.48
Total n–3 LCP	0.63	0.14	0.42	0.08	0.57	0.13
Total n–6 LCP[a]	1.35	0.07	1.1	0.05	1.03	0.06
n–6LCP/n–3LCP	2.81	0.65	3.24	0.63	2.26	0.42

[a] $P \leq 0.05$ in the Friedman test.

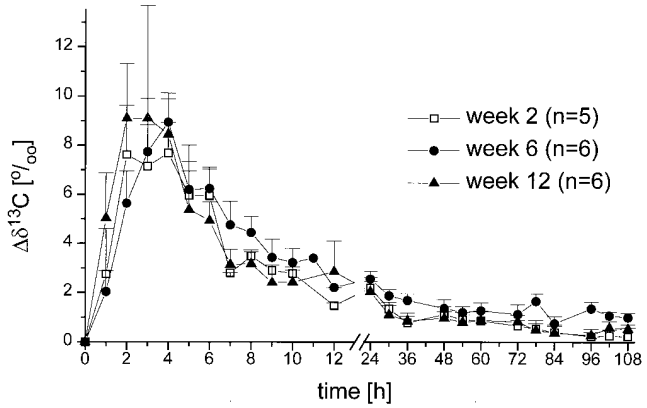

Fig. 1. Change of ^{13}C-enrichment of exhaled breath CO_2 over time in 6 lactating women after oral application of 1 mg/kg bodyweight of U-^{13}C-labeled linoleic acid ($\Delta\delta^{13}C$ over baseline, DOB, $^{0}/_{00}$, mean + SE).

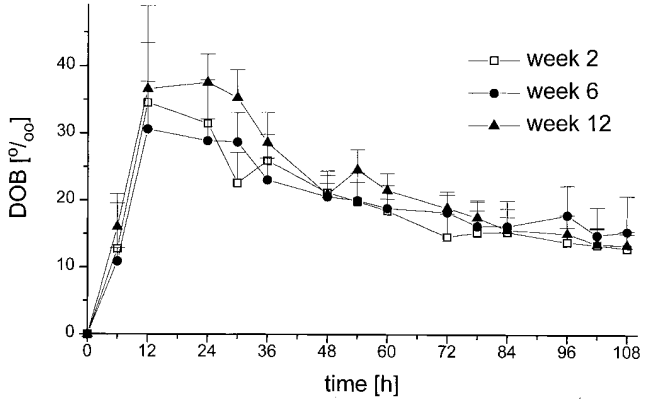

Fig. 3. Change of ^{13}C-enrichment in milk dihomo-γ-linolenic acid (20:3n–6) over time in 6 lactating women after oral application of 1 mg/kg bodyweight of U-^{13}C-labeled linoleic acid ($\Delta\delta^{13}C$ over baseline, DOB, $^{0}/_{00}$, mean + SE).

tained between 3 and 5 h after tracer intake; after 36 h enrichment values had almost returned to baseline (**Fig. 1**). There were no significant differences between different weeks of lactation at any time point of the study.

The ^{13}C-enrichments in milk 18:2n–6 peaked after 12 h, followed by a fast decrease of the ^{13}C content until 72 h (**Fig. 2**). For the remainder of the sampling period, the slope of the enrichment curve was considerably smaller, therefore ^{13}C excess was detectable even in the last sample taken 108 h after tracer ingestion. In milk dihomo-γ-linolenic acid (20:3n–6) enrichments were maximal between 12 and 24 h followed by a constant decrease thereafter (**Fig. 3**). Increases of the $\delta^{13}C$-values in 20:4n–6 were close to the limits of detectability (**Fig. 4**). A clear increase was observed already after 6 h, but a maximum could not be identified as enrichments varied between 1.5 and 3.5% until 108 h.

We used a compartmental model to calculate the recoveries at 108 h after tracer application. The principle of the model (**Fig. 5**) is not to describe the metabolic pathways, but it aims at describing the cumulative tracer recoveries and to elucidate possible product–precursor relationships. We can only determine cumulative recoveries in milk, because the components present at any time point reflect an average value of the enrichment during the last hours while the milk sampled was synthesized. As complete emptying of the fatty acid pools in the mammary gland by nursing cannot be expected, one needs to assume some mixing with earlier produced lipids. We defined tracer recovery as the percentage of the applied tracer 18:2n–6 that was excreted in milk. As we used a heterogeneous model, describing not only the transfer of 18:2n–6 between different compartments but also its conversion into other molecules, all tracer amounts were taken as moles of ^{13}C and expressed as percentage of the applied moles of tracer carbon.

It was assumed that after resorption the tracer enters pool 1 with fast turnover (e.g., chylomicrons), from which it could either directly enter the oxidative pathway, be transferred to milk, or move into the central plasma pool 2 (Fig. 5). For all these pathways it is assumed that no recy-

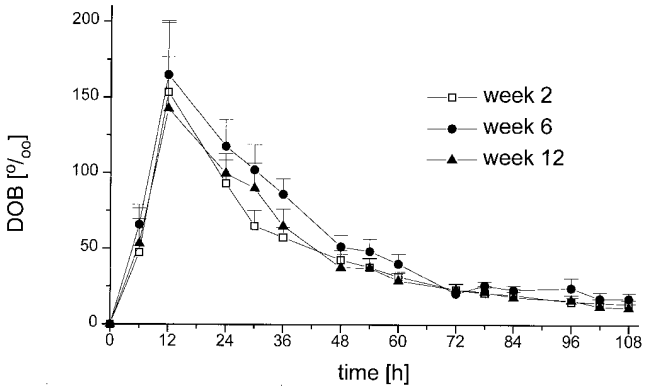

Fig. 2. Change of ^{13}C-enrichment in milk linoleic acid (18:2n–6) over time in 6 lactating women after oral application of 1 mg/kg bodyweight of U-^{13}C-labeled linoleic acid ($\Delta\delta^{13}C$ over baseline, DOB, $^{0}/_{00}$, mean + SE).

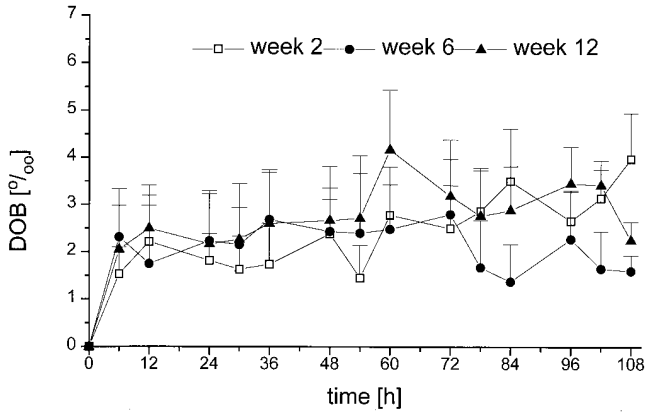

Fig. 4. Change of ^{13}C-enrichment in milk arachidonic acid (20:4n–6) over time in 6 lactating women after oral application of 1 mg/kg bodyweight of U-^{13}C-labeled linoleic acid ($\Delta\delta^{13}C$ over baseline, DOB, $^{0}/_{00}$, mean + SE).

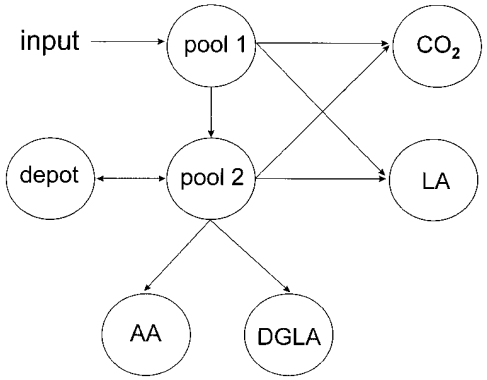

Fig. 5. Metabolic model used to describe tracer recovery after oral application of [^{13}C]linoleic acid to lactating women. Samples were obtained from the milk pools of 18:2n–6, 20:3n–6, and 20:4n–6 and from breath CO_2, while data for the other pools were adjusted by the modeling software. Arrows indicate the assumed metabolic flows.

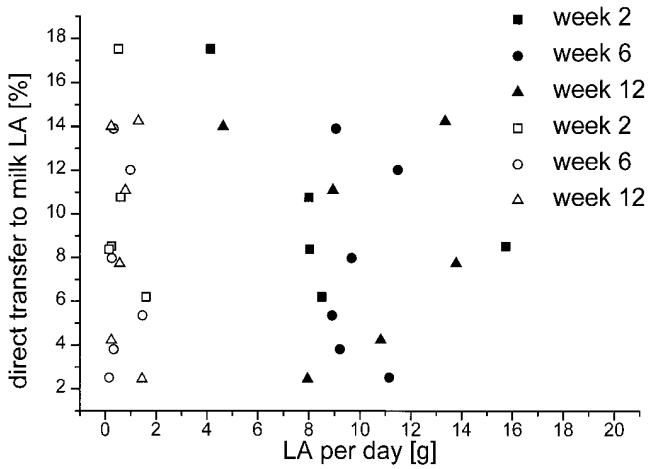

Fig. 6. The percentage of labeled dietary linoleic acid directly transfered into breast milk, as estimated by the tracer model, is not related to the daily dietary linoleic acid intake (solid symbols) or to the daily milk output of li-

cling into pool 1 is possible. From the central pool 2, the metabolites can be oxidized or transferred into milk either unchanged as 18:2n–6 or after conversion into LCP derivatives. There is no distinction whether the conversion takes place in the mammary gland or in the slow pool 2 (e.g., liver). The construction of the model demands the complete elimination of all tracer from the body, if time is infinite. It does not take into account the possibility that [^{13}C]linoleic acid is stored in tissues with very slow turnover, resulting in an unmeasurable return of the tracer into one of the pools sampled. Furthermore, losses due to conversion into other products not analyzed are neglected, e.g., incorporation of C_2 units from breakdown of 18:2n–6 into products not analyzed or loss of CO_2 during transfer through the bicarbonate pool. The cumulative recoveries estimated by this model (**Table 3**) correlated very closely with the data directly measured at or near 108 h after tracer application. There were no statistically significant changes over time, but a large variation between the individuals.

The mathematical model is not only used for direct description of the data. In addition, it is possible to estimate the percentage of tracer transferred into milk 18:2n–6 without passing the "depot" pool with slow turnover by calculating the percentages of the corresponding pathways and summing them up. **Figure 6** shows that neither dietary 18:2n–6 intake nor milk secretion of 18:2n–6 was related to the tracer proportion directly transferred into milk.

If one assumes that the orally applied 18:2n–6 tracer is representative for the total dietary 18:2n–6, the amount of 18:2n–6 transferred directly into milk fat can be calculated by multiplication of the total daily intake of 18:2n–6 times the percentage of direct transfer. In the second week 32.7 ± 10.3%, in the 6th week 24.0 ± 4.5%, and in the 12th week 28.0 ± 5.2% (mean ± SE) of milk 18:2n–6 were derived from the diet without passing the slow pool (no significant difference between different weeks of lactation).

Based on the same principles, the data of the recovery of 18:2n–6 converted to LCP fatty acids can be interpreted. Although only about 0.2% of the 18:2n–6 tracer was found in milk 20:3n–6, between 3 and 25% of milk 20:3n–6 were directly converted from dietary 18:2n–6 (**Fig. 7**). For milk 20:4n–6, the portion derived from 18:2n–6 does not exceed 3.2%, but there is a significant correlation between the results of the two fatty acids (Fig.

TABLE 3. Calculated cumulative recovery of orally applied [^{13}C] linoleic acid in different compartments in 6 lactating women in the 2nd, 6th and 12th week of lactation (% of dose, mean ± SE)

Compartment	Week 2	Week 6	Week 12
Breath CO_2	18.9 ± 2.2	24.0 ± 3.8	17.7 ± 3.5
Milk 18:2n–6	12.7 ± 1.4	13.1 ± 2.5	11.7 ± 2.7
Milk 20:3n–6	0.19 ± 0.04	0.20 ± 0.04	0.17 ± 0.04
Milk 20:4n–6	0.02 ± 0.01	0.02 ± 0.01	0.02 ± 0.01

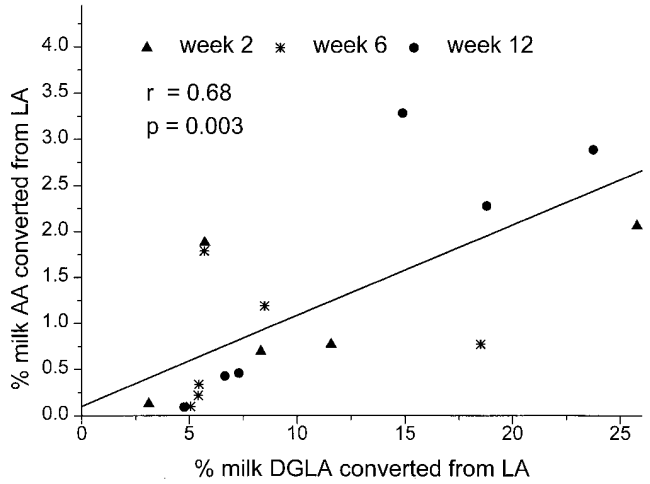

Fig. 7. Correlation of the percentage contributions of milk arachidonic acid and dihomo-γ-linolenic acid, respectively, formed directly from dietary linoleic acid.

7). As there were no significant differences between time points, data were pooled for regression analysis.

DISCUSSION

Dietary intakes, milk, and milk fat secretion of the mothers at the three study time points (Table 1) were within the range reported in other studies (21–23). There were no statistically significant changes over time but considerable variation among the individuals. Average fatty acid composition calculated from the total of 15 milk samples per subject and study periods at the various time points (Table 2) was similar to published values of human milk of European women (13, 24–27) and support the previous observations that average dietary intakes and human milk contents of linoleic acid are lower in central Europe than in the USA (24). Milk fatty acid composition showed only little intra-individual variation, but there was considerable variation among subjects. The percentage of 20:4n–6 and total n–6 LCP decreased with longer duration of lactation, as previously observed (25, 28). Although this change is statistically significant, the total amounts of these fatty acids secreted with milk per day did not change, due to an attenuation of the percentage change by the increase of total milk fat output. Also, the dietary records showed no unusual food habits and seemed not to differ from groups of central European lactating women studied by other investigators (29).

Hachey and co-workers have previously investigated the transfer of dietary palmitic, oleic, and linoleic acids into breast milk. They report that transfers during a 72-h period for the three fatty acids studied were between 10 and 12%. The data are similar to our finding that 12.5% of the labeled 18:2n–6 was recovered in milk until 108 h after application, although the subjects studied by Hachey et al. (16) were in a later stage of lactation. Although the authors supplied a standardized diet, coefficients of variation among the three studied subjects were up to 25%, whereas coefficients of variation for 18:2n–6 recovery in milk ranged from 27% (2nd week) up to 57% (12th week) in our study, with an additional contribution of free selection of individual diets to the variation.

A major difference between Hachey's (16) and our studies was the type and amount of tracers used. We used uniformly ^{13}C-labeled 18:2n–6 and detection of enrichments by gas chromatography–combustion–isotope ratio mass spectrometry, while Hachey et al. (16) applied positionally deuterated tracer molecules and detected them by organic mass spectrometry. As our tracer was more highly enriched and the detection method was more sensitive, we could reduce the amount of tracer from several grams to only about 70 mg per subject and test. Furthermore the use of ^{13}C instead of ^{2}H enabled us to measure also the conversion of the labeled substrate to CO_2. With the exception of one subject in the second week and another one in the 12th week, there was more tracer carbon exhaled than transferred into milk, but a significant relationship between both these parameters was not detected. Nevertheless, there was a trend that oxidation tended to increase with higher transfer of dietary 18:2n–6 into milk. As absorption of 18:2n–6 should be near 100% in healthy adults (30), it cannot be argued that differences in absorption were the reason for the different recoveries. Rather, it appears that under the given dietary conditions, lactation and oxidation were not competitive processes of 18:2n–6 metabolism, or 18:2n–6 was available in excess. In every subject at all time points a considerable portion of the 18:2n–6 tracer was not recovered during the 5-day periods, presumably because it was stored in body tissues, which could be a common factor governing the rates of oxidation and transfer into milk.

The oxidation rates we have found here cannot easily be compared to data reported for other fatty acids. We assumed that total CO_2 production in lactating women is equal to the rates found in healthy resting adults, but this assumption might not be completely valid because of the additional energy requirements for milk synthesis. Furthermore, our tracer was labeled in all positions of the carbon chain, while in most other studies fatty acids positionally labeled in the carboxyl group have been used. It has been demonstrated that there is an influence of the position of the labeled carbon atom on its rate of conversion into CO_2, because more atoms at even numbered positions escape this pathway during passage of the citric acid cycle (31). However, the cumulative recoveries we found here fall well into the range reported for palmitic or oleic acid (32, 33). We are not aware of other published data on the oxidation of ^{13}C labeled 18:2n–6 in humans.

A further focus of this study was to investigate potential changes in the 18:2n–6 metabolism with the duration of lactation, because it has been demonstrated that concentrations of essential fatty acids and their derivatives are low in plasma phospholipids after pregnancy and body stores cannot be repleted during lactation (34). While the proportion of milk n–6 LCP decreased, there were no significant changes in the other metabolic parameters studied. If a depletion of n–6 LCP in the body would have caused the decrease of milk fat LCP, this would have to be associated with markedly smaller body pools of these fatty acids. In the presence of smaller pools, an applied tracer would have been less diluted and hence would have led to higher ^{13}C-enrichment, if all other factors such as dietary intake of 18:2n–6 as well as dietary intake of and conversion to 20:3n–6 and 20:4n–6 are similar. We did not observe an increased enrichment with longer duration of lactation in our subjects, and tracer recoveries in milk LCP did not change; therefore, we assume that the activity of conversion and body pools of n–6 LCP did not undergo major changes during the study period. Our data do not yield any quantitative information on the contribution of dietary 20:4n–6 to the milk output of 20:4n–6, but at any time point the dietary 20:4n–6 supply exceeded milk output in all subjects: 20:4n–6 intakes calculated from the dietary diaries were 4.0 $\pm$ 1.2 (mean $\pm$ SE) times higher than milk contents were in the 2nd week, 4.6 $\pm$ 1.2 times in the 6th week and 9.2 $\pm$ 3.3 times in the 12th week. Thus, it may well be possible that under our study conditions the

major portion of 20:4n–6 secreted with milk was of exogenous origin, and results on endogenous turnover might differ if 20:4n–6 intakes are considerably lower.

The model we used to interpret the measured values describes the cumulative recovery data very well, but it contains several simplifications that might cause errors in the metabolic parameters determined. The basic assumption that all tracer will leave the body, if time is infinite, must not be true for our system, because we could not measure all possible routes for carbon atoms to leave the body. There might be exchange of C_2 units in intermediary metabolism with other substrates used for milk synthesis, although we did not find significant increases of the ^{13}C-content in other than n–6 milk fatty acids. However, we performed isotopic analyses only on milk fatty acids with 14 or more carbon atoms, thus we may have missed enrichment in short chain fatty acids.

The pool 1 (Fig. 5) should largely represent chylomicrons, because after absorption the tracer is transported via this fraction, and oxidized or incorporated into milk after uptake into corresponding tissues. In the liver the remaining lipids of this fraction are incorporated into VLDL, which we assume to be represented by pool 2. From this central pool, 18:2n–6 may be oxidized or be secreted into milk, and to simplify the model we also assume milk 20:3n–6 and 20:4n–6 to be derived from this compartment. Obviously this metabolic conversion does not take place in plasma, and it will take some time. This would require additional pools to represent the process or at least the introduction of delay times, but such complex models did not result in identifiable fitting solutions. Our data represent only the output of the substances in milk, so information about the pathways is limited. The failure to consider further pools of 20:3n–6 or 20:4n–6 might result in underestimation of the endogenous conversion, because we do not take into account the dilution of the tracer in these pools.

It was not possible to obtain an adequate fit of the data to a model without a pool with slow turnover, because in the later part of the study period there was still enrichment in 18:2n–6, which decreased only slowly (Fig. 2). We cannot assign a defined physiological equivalent to this pool, but it could largely represent fatty acids in LDL and HDL, in liver and in adipose tissue. Our way of expressing the data does not require knowledge of the size of the proposed pools, because we only describe the behavior of the labeled 18:2n–6. To obtain quantitative information we have to multiply the rates offered by the model calculation with known quantities, e.g. dietary intake, but as there is no information about the size of the "depot" pool, we could not deduce information about this flow.

The fractional transfer rates of a pool into a certain direction can be interpreted as the probability of a molecule in the pool to go along the pathway. By following the pathway of 18:2n–6 from inflow into pool 1 either directly or via pool 2 into milk, the probability to pass directly from the diet into milk can be estimated. There were no statistical differences with duration of lactation, therefore, data for all time points were pooled for identification of potential relationships to diet or milk production. Based on our model there is a wide range of direct transfer of dietary linoleic acid into milk, ranging from about 2 to 18% of the tracer dose, but there is no relationship either to dietary intake or to milk output. Further studies with more detailed investigation of lipoprotein metabolism as well as lipase and receptor activities might help to elucidate the underlying metabolic mechanisms. However, we can conclude from our results that about 30% of the milk output of 18:2n–6 originates from direct transfer from the diet, which is in accordance with the documented immediate response of milk fat composition to dietary changes as well as results obtained with deuterated fatty acids (12, 35). However, the influence of dietary intake on milk composition is moderated by the quantitatively larger contribution of fatty acids from body compartments with slow turnover, thus resulting in a relatively constant milk fatty acid supply to the recipient infant.

The times of maximal ^{13}C-enrichments of milk 20:3n–6 and 18:2n–6 almost coincide, which reflects an active endogenous synthesis for milk 20:3n–6, with an apparently sufficient Δ^6 desaturation activity to ensure an efficient and rapid conversion of 18:2n–6 to 20:3n–6. Based on our mathematical model, direct conversion of exogenous 18:2n–6 to 20:3n–6 contributed some 3–25% to milk 20:3n–6. In view of the expected dilution of newly synthesized 20:3n–6 in a pool of pre-existing 20:3n–6 in liver, plasma and the mammary gland, the true contribution of conversion to milk 20:3n–6 may be even higher than the calculated 3–25 %. There was a considerable inter- as well as intra-individual variation largely related to the variation of 18:2n–6 intake at the specific time points. Although there seemed to be subjects with high and low rates of endogenous linoleic acid conversion, final conclusions on possible inter-individual variation of synthesis rates are not possible due to the variable dietary supply of 18:2n–6 and 20:4n–6.

The calculated contribution of endogenous conversion to milk output of 20:4n–6 is less than 3%. Endogenous production of 20:4n–6 appears not to be necessary for milk synthesis under the conditions of our study because of the considerable dietary intake of 20:4n–6, which conceivably might act as an inhibitor of arachidonic acid synthesis. However, the data need to be interpreted with some caution. If we assume that the pool of 20:4n–6 is much larger than that of 20:3n–6, due both to a higher exogenous supply or larger body stores of 20:4n–6 than 20:3n–6, one needs to consider that milk 20:4n–6 may have originated from a large pool derived from sources we could not identify, and hence there is no proof that endogenous conversion is an unimportant source of milk 20:4n–6. The hypothesis of a large 20:4n–6 pool is supported by the time course of the increase of ^{13}C content in milk 20:4n–6, beginning already hours after tracer intake and continuing further for the whole study duration without any tendency to decrease. The observed large difference in calculated proportions of endogenous synthesis between milk 20:3n–6 and 20:4n–6 may have been influenced by a passage of the recovered 20:4n–6 through a

pool of 20:3n–6, which also serves as the precursor pool for milk 20:3n–6, and a dilution of tracer in this intermediate pool resulting in a decreased enrichment of newly formed 20:4n–6. Nonetheless, the observed correlation of the percentage contributions of endogenous synthesis of 20:4n–6 and 20:3n–6 to their output in milk indicates that LCP synthesis in lactating women is limited by Δ^6 desaturase activity, and it also emphasizes the significance of maternal 18:2n–6 conversion for provision of milk n–6 LCP, because otherwise one would expect dietary variation to have obscured this relationship.

In conclusion, our results demonstrate that the tracer approach is suitable to gain further understanding of the physiology of milk fat formation. While both body stores and diet are both important sources for milk fat, the data presented here indicate an endogenous synthesis of human milk n–6 LCP.

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