Threonine kinetics in preterm infants fed their mothers' milk or formula with various ratios of whey to casein[1-4]

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ABSTRACT

Background: Plasma threonine concentrations are elevated in infants fed formula containing a whey-to-casein protein ratio of 60:40 compared with concentrations in infants fed formula containing a ratio of 20:80 or human milk (60:40).

Objective: We studied whether degradation of excess threonine was lower in formula-fed infants than in infants fed their mothers' milk.

Design: Threonine kinetics were examined in 17 preterm infants (gestational age: 31 ± 2 wk; birth weight: 1720 ± 330 g) by using an 18-h oral infusion of $[1\text{-}^{13}C]$threonine at a postnatal age of 21 ± 11 d and weight of 1971 ± 270 g. Five infants received breast milk. Formula-fed infants ($n = 12$) were randomly assigned to receive 1 of 3 formulas (5.3 g protein/MJ) that differed only in the whey-to-casein ratio (20:80, 40:60, and 60:40).

Results: Threonine intake increased significantly in formula-fed infants with increasing whey content of the formula (48.5, 56.4, and 63.2 $\mu\text{mol} \cdot \text{kg}^{-1} \cdot \text{h}^{-1}$, respectively; pooled SD: 2.2; $P = 0.0001$), as did plasma threonine concentrations (228, 344, and 419 μmol/L, respectively; pooled SD: 75; $P = 0.03$). Despite a generous threonine intake by infants fed breast milk (58.0 $\pm$ 16.0 $\mu\text{mol} \cdot \text{kg}^{-1} \cdot \text{h}^{-1}$), plasma threonine concentrations remained low (208 $\pm$ 41 μmol/L). Fecal threonine excretion and net threonine tissue gain, estimated by nitrogen balance, did not differ significantly among groups. Threonine oxidation did not differ significantly among formula-fed infants but was significantly lower in formula-fed infants fed than in infants fed breast milk (17.1% compared with 24.3% of threonine intake, respectively).

Conclusion: Formula-fed infants have a lower capacity to oxidize threonine than do infants fed breast milk.   *Am J Clin Nutr* 1999;69:105–14.

KEY WORDS   Threonine oxidation, threonine flux, plasma threonine concentration, stable isotopes, breast milk, infant feeding, infant formula, milk protein, whey-casein ratio, preterm infants

INTRODUCTION

An important goal in infant feeding is to provide an optimal balance of amino acids that maximizes protein synthesis and tissue accretion without exceeding the capacity for degradation and elimination of surplus amino acids (1–3). Low-birth-weight infants are considered to be particularly vulnerable to amino acid deficiencies and excesses because of their immaturity (4). In recent years, much attention has been focused on refining the amino acid composition of formulas based on cow-milk protein by altering the whey-to-casein ratio of the milk protein. The current aim in the formula feeding of full-term infants is to produce metabolic responses similar to those in breast-fed infants (5), which are measured principally by plasma amino acid concentrations as an index of amino acid metabolism.

Studies conducted in both full-term (6–9) and preterm (10–12) infants showed no effect of altering the whey-to-casein ratio of formula on growth or nitrogen balance. However, these studies consistently showed that plasma threonine concentrations were increased in infants fed formulas with increased whey contents. The 2-fold increase in plasma threonine concentrations observed in preterm infants fed whey- compared with casein-dominant formulas was strikingly greater than the 1.3-fold increase in threonine intake (10–12). Additionally, compared with infants fed human milk, formula-fed full-term (9) and preterm (12, 13) infants had higher plasma threonine concentrations that could not be explained by differences in threonine intake.

Picone et al (9) suggested that a low availability of threonine within the immunologic proteins of human milk, such as secretory immunoglobulin A (sIgA), may explain the differences in plasma threonine concentrations in full-term infants. However, several authors (14–16) showed that fecal excretion of sIgA in low-birth-weight infants represented only ≈10% of sIgA intake, which would translate into a nonsignificant fecal loss of total dietary threonine from human milk.

FIGURE 1. Principal pathways of threonine catabolism (modified from reference 17). Threonine is catabolized in the cytosol by threonine dehydratase to yield 2-ketobutyric acid. Ketobutyric acid can be transaminated to 2-aminobutyric acid by alanine aminotransferase or further degraded by branched-chain α-keto acid dehydrogenase (BCKDH) or pyruvate dehydrogenase (PDH) to propionyl coenzyme A in mitochondria. Threonine is also catabolized in mitochondria by threonine dehydrogenase to 2-amino-3-ketobutyric acid, which can be further degraded mainly to glycine and acetyl CoA in vivo. Cofactors include pyridoxal (vitamin B-6, or nicotinamide adenine dinucleotide) and coenzyme A. *Denotes the fate of the labeled carbon of L-[1-^{13}C]threonine through the degradative pathways.

Determinants of the plasma concentration of indispensable amino acids, other than specific amino acid intake and absorption, include rates of amino acid oxidation and net utilization for protein synthesis. Amino acid flux and oxidation can be measured safely in infants by using amino acids labeled with stable isotopes. To our knowledge, there has been no report of in vivo measurements of threonine flux and oxidation in infants.

Two major pathways for threonine degradation in mammals are known (**Figure 1**). The pathway initiated by threonine dehydrogenase has been shown to account for >80% of total threonine degradation in pigs (18) and rats (17). In humans, however, the threonine dehydrogenase pathway has not been detected (19) and further study of threonine conversion to glycine, using more sensitive analytic methods, is necessary (18).

We hypothesized that rates of threonine oxidation relative to threonine intake would be lower in formula-fed infants than in infants fed human milk. The objective of this study was to measure plasma threonine concentrations and rates of threonine intake, flux, and oxidation to carbon dioxide in low-birth-weight infants fed formulas with various whey-to-casein ratios and in infants fed breast milk. Rates of fecal threonine excretion as well as rates of threonine utilized for net protein gain were estimated. We also examined the possibility of conversion of threonine to glycine.

SUBJECTS AND METHODS

Subjects

Eighteen low-birth-weight infants were recruited from the transitional care nursery at Women's College Hospital. Selection criteria included gestational age ≤34 wk (20), appropriate weight for gestational age (21), and body weight between 1600 and 2200 g at the time of the study. Infants could tolerate full oral feeds of 460–502 kJ·kg^{-1}·d^{-1} (110–120 kcal·kg^{-1}·d^{-1}), were without acute or chronic disease or congenital anomalies, and were not receiving antibiotic therapy. The clinical characteristics of these infants are shown in **Table 1**. Informed, written consent was obtained from one or both parents. The experimental protocol and study procedures were approved by the Human Subject Review Committee of The Hospital for Sick Children and the Research Ethics Board of Women's College Hospital.

Diet and experimental design

The decision to feed the babies either with formula or with their mothers' milk (preterm milk) was predetermined by the parents. Twelve formula-fed infants were randomly assigned to receive 1 of 3 experimental formulas. The 3 formulas, manufactured for this study by Wyeth-Ayerst Laboratories, Philadelphia,

TABLE 1
Characteristics of preterm infants fed formulas with various whey-to-casein ratios or their mothers' milk (preterm milk)[1]

| | Formula (whey-to-casein ratio) | | | Preterm milk |
	60:40 (*n* = 4)	40:60 (*n* = 4)	20:80 (*n* = 4)	(*n* = 6)
Birth weight (g)	1755 ± 248	1643 ± 186	1899 ± 528	1628 ± 335
Gestational age (wk)	32 ± 1	32 ± 2	33 ± 2	30 ± 3
Postnatal age at study (d)	20 ± 9	21 ± 10	16 ± 12	25 ± 14
Weight at study (g)	1983 ± 97	1868 ± 192	2058 ± 334	1973 ± 373
Weight gain during study (g·kg^{-1}·d^{-1})	16 ± 6	16 ± 6	16 ± 2	16 ± 5

[1] $\bar{x}$ ± SD. There were no significant differences among groups.

TABLE 2
Nitrogen and amino acid composition of the formulas and of preterm milk

| | Formula (whey-to-casein ratio) | | | |
	60:40	40:60	20:80	Preterm milk
Nitrogen				
Total (g/L)	2.30	2.30	2.30	2.1 ± 0.44[1]
Nonprotein nitrogen				
(g/L)	0.27	0.24	0.20	0.58 ± 0.07
(% of total N)	12	10	9	23 ± 5
Amino acids (μmol/L[2])				
Histidine	1767	1837	2120	2250 ± 730
Isoleucine	6605	5852	5518	5395 ± 1072
Leucine	11538	11621	11538	11238 ± 2336
Lysine	7802	7427	7277	6655 ± 1559
Phenylalanine	3717	3916	4115	3794 ± 1318
Threonine	6995	6074	5154	5813 ± 1129
Tyrosine	3268	3268	3389	4284 ± 1860
Glycine	3797	3651	3505	4407 ± 428
Valine	7676	7489	7395	6658 ± 1250
Arginine	3147	3399	3714	4064 ± 687
Alanine	7014	6153	5415	6199 ± 314
Aspartic acid[3]	9305	8729	7905	10211 ± 2192
Glutamic acid[4]	19730	20480	21160	17413 ± 4291
Proline	10000	11429	12857	9857 ± 1735
Serine	7927	7719	7510	7624 ± 1490

[1] $\bar{x}$ ± SD for 5 milk samples collected during the threonine infusion study.

[2] Tryptophan and sulfur amino acids were not measured.

[3] Reflects the sum of asparagine and aspartic acid.

[4] Reflects the sum of glutamine and glutamic acid.

contained equal amounts of protein (15 g/L) and energy (2803 kJ/L, or 670 kcal/L) and were similar except for their protein sources, which differed with respect to the whey-to-casein protein ratio. The formula with the 20:80 ratio was made from reconstituted skim milk powder. Whey protein, produced by electrodialysis, was added to the skim milk powder to attain the 40:60 and 60:40 whey-to-casein ratios. The 3 formulas were provided, ready to feed, in 125-mL glass bottles. Each study formula originated from a single lot. Samples of each formula were kept at −20°C until analyzed for nitrogen and amino acid contents (**Table 2**). The protein, fat, and carbohydrate concentrations of the formulas used were similar to those in SMA formula (Wyeth-Ayerst Laboratories) and the vitamin and mineral concentrations were similar to those in SMA Preemie formula (Wyeth-Ayerst Laboratories). Formula-fed infants were nourished via nasogastric tubes or by bottle every 3 h.

Six infants fed preterm milk were also recruited. Infants enrolled in the preterm milk group received their mothers' milk, mainly as expressed milk that was fed by nasogastric tube or by bottle every 3 h. Mothers were permitted to attempt to breast-feed their infants if they so desired. Mothers expressed their milk 4–6 times/d either manually or with the use of a breast pump. The milk was stored in sterile polypropylene containers as individual milk expressions and was kept either at 5°C and used within 24 h of expression or at −20°C and used within a few days.

To obtain a representative sample of preterm milk consumed daily by the infants for subsequent analysis and to ensure that these infants received a homogenous composition of milk throughout the 24-h tracer-infusion periods, milk from individual expressions was pooled, portioned, and sampled daily according to the following procedure. On each morning of the 4-d balance study, milk expressions were thawed and warmed to 37°C in a water bath. The milk from each container was then transferred to a sterile graduated polypropylene beaker and the total volume measured. The milk was warmed and gently mixed with a magnetic stirrer. A portion of the milk was transferred into polypropylene containers fitted with screw-cap lids and kept frozen at −20°C until analyzed for nitrogen and amino acid concentrations (Table 2). The milk was transferred, in equal volumes, into 8 graduated containers (Mead Johnson Canada Ltd, Ottawa), capped, and placed in the refrigerator before feeding. Two mothers attempted to breast-feed their babies during the study. The volume of milk ingested by the 2 infants during breast-feeding was measured by the test-weighing method (22) and was found to be small, 15% and 0.9% of total daily intake.

The infants were studied over 7 d. The studies were carried out while the infants were in cribs or standard incubators and did not interfere with routine care. Body weight (±1 g) was measured daily and body length was measured with a length board at entry into the study. After 3 d of feedings, a metabolic balance study was started at 1200 on day 3 and ended at 1200 on day 7. The 4-d balance study included two 24-h tracer-infusion periods that were separated by a 48-h washout period. Each tracer-infusion period included a 6-h baseline period and an actual tracer-infusion time of 18 h as outlined in **Figure 2**. The first tracer administered was always L-[1-^{13}C]phenylalanine and the second tracer used was always L-[1-^{13}C]threonine. The description and results of the L-[1-^{13}C]phenylalanine infusion study are reported elsewhere. Baseline breath $^{13}CO_2$ enrichment before the L-[1-^{13}C]threonine tracer infusion did not significantly differ from baseline breath $^{13}CO_2$ enrichment measured before the L-[1-^{13}C]phenylalanine tracer infusion [0.0087 ± 0.0003 compared with 0.0089 ± 0.0004 atoms percent excess (APE); $\bar{x}$ ± SEM, $n = 17$; $P = 0.3$ by paired Student's *t* test].

Isotopic tracer

L-[1-^{13}C]Threonine (99% 1-^{13}C, allo-free) was obtained from Cambridge Isotope Laboratories, Woburn, MA. The enrichment and enantiomeric purity of the L-[1-^{13}C]threonine were determined to be 99% and >98%, respectively, by gas chromatography–mass spectrometry analysis of the *N*-trifluoroacetyl methyl ester derivative (using a Chirasil Val-D fused-silica capillary column from Cambridge Isotope Laboratories).

Solutions of the tracer (10.7 g/L) were prepared in sterile water by the pharmacy department of Women's College Hospital. The solutions were sterilized by passage through a 0.22-μm filter (Millipore Corp, Bedford, MA) under laminar flow and were aliquoted into single-dose vials. Each batch was shown to be sterile.

Tracer administration and sample collection

The oral administration of the amino acid tracer doses simulated the primed, constant-isotope-infusion technique (23). On the day of the tracer-infusion study, the L-[1-^{13}C]threonine solution was drawn into 6 unit dose syringes (Oral-Topical Exacta-Med Dispenser; Baxa Corporation, Denver) to deliver a primed (15 μmol/kg), constant infusion (15 μmol·kg^{-1}·h^{-1}) to the infants for 18 h. The syringes were tested for accuracy (±0.01 mL) and precision (CV: 0.4%).

A schematic outline of the tracer-infusion studies is shown in Figure 2. The tracer doses were administered directly into the
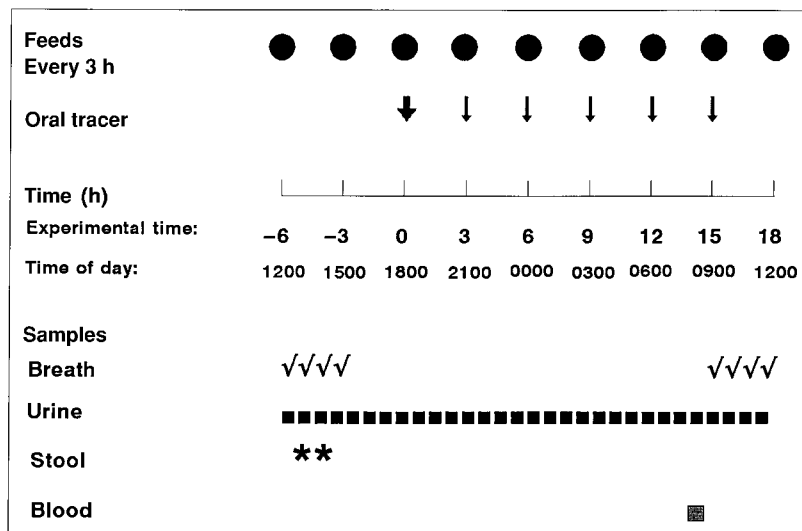
FIGURE 2. Outline of the 18-h oral tracer-infusion study conducted in low-birth-weight infants fed 1 of 3 study formulas or their mothers' milk. The L-[^{13}C]threonine infusion began at 1800 on the seventh day of the study. Stool was collected on the fourth day of the study.

feeding tube or bottle nipple immediately before each 3-h feeding starting at 1800 and ending the following day at 1200. The priming dose was given with the first tracer dose.

Breath samples were collected before and then during the last 2 h of the 18-h tracer infusion. A transparent thermoplastic hood was placed over the infant's head and upper body while the infant rested in his or her crib or isolette. Room air or incubator air was drawn through the hood at a rate of ≈ 1.5 L·kg^{-1}·min^{-1} and into an indirect calorimeter to measure carbon dioxide production ($\dot{V}CO_2$). An LB-2 Medical Gas Analyzer (SensorMedics; Beckman, Palo Alto, CA) was used to measure carbon dioxide concentration. The air flow rate was measured with a mass flow meter (model 5810, 0–10 L/min; Brooks Instrument Division, Emerson Electric Company, Stouffville, Canada) and was adjusted to achieve a carbon dioxide concentration in the hood of ≈ 0.5 %. Accuracy of the carbon dioxide analyzer and of the mass flow meter was within 1%. During breath collection, 10-min samples of air exiting the carbon dioxide analyzer at 500 mL/min were bubbled through 10 mL of a 1 mol NaOH/L solution by using a spiral reflux condenser to completely trap the carbon dioxide (24). The breath samples were transferred to evacuated glass tubes (Vacutainer brand 6441, 100 × 16 mm; Becton Dickinson Inc, Mississauga, Canada) and stored at −20°C until analyzed for $^{13}CO_2$ enrichment.

Urine was collected with a condom urine collector every 3 h throughout the 4-d balance study, starting on day 3 at 1200 and ending on day 7 at 1200. Two 3-h baseline samples of urine were collected before starting the tracer infusion and five 3-h samples of urine were collected during the tracer infusion and stored at −20°C until analyzed for [1-^{13}C]threonine and [1-^{13}C]glycine enrichment. Aliquots of each intact 3-h urine sample collected during the 4-d balance study, representing 10% of the volume, were pooled for analysis of nitrogen concentration and stored at −20°C. The infants continued to wear a diaper over the urine collector and were clothed in their usual apparel.

Stools were collected by inserting a plastic liner into the diaper. The plastic liner containing stool was then removed and stored at −20°C until analyzed for nitrogen and amino acid con-

centrations. Complete 96-h stool collections were not possible for all infants because one infant developed a diaper rash. For most infants, therefore, stools were collected during the first 24 h of the balance study.

Blood, ≈ 1 mL, was collected into a heparin-containing syringe by venipuncture or by heel prick immediately before either the 0900 or 1200 feeding, which was either 15 or 18 h after the start of the tracer infusion. Plasma was stored at −70°C until analyzed for amino acid concentration and [1-^{13}C]threonine and [1-^{13}C]glycine enrichment.

Analytic procedures

Amino acids in 250 μL urine and in 100 μL plasma were derivatized to their *N*-heptafluorobutyryl-*O*-isobutyl esters according to the method of Ford et al (25). The derivatized amino acids were separated on a gas chromatograph (model 5840A; Hewlett-Packard, Mississauga, ON) fitted with a capillary column (Hewlett-Packard Ultra 2) coupled directly to a quadrapole mass spectrometer (Hewlett-Packard model 5985) under conditions of negative chemical ionization and selected ion monitoring. Threonine and glycine were analyzed in separate injections. Selected ion chromatographs were obtained by monitoring mass-to-charge ratios of 351 and 352 for [1-^{13}C]threonine and 307 and 308 for [1-^{13}C]glycine, corresponding to the unenriched (*m*) and enriched (*m* + 1), respectively. Areas under the peaks were integrated by a Hewlett-Packard 1000E series computer.

Labeling of [1-^{13}C]glycine produced from the L-[1-^{13}C]threonine tracer was undetectable in both urine and plasma by gas chromatography–mass spectrometry (results not shown). Gas chromatography–combustion isotope ratio mass spectrometry (GC-CIRMS) was therefore used because of its potential for measuring [1-^{13}C]amino acid enrichments as low as 0.01 APE, thus providing a greater sensitivity than gas chromatography–mass spectrometry (0.2 APE). Isotopic enrichments of urinary free glycine and of glycine derived from urinary hippurate at baseline and at the end of the tracer infusion were determined by analysis of their *N*-propyl,*N*-acetyl derivative (26) by GC-CIRMS with an Orchid system consisting of a 20-20 mass

spectrometer with a gas chromatography combustion interface (Europa Scientific Ltd, Crewe, United Kingdom) and an HP5890 series II gas chromatograph (Hewlett-Packard). Free glycine and hippurate were first extracted from 2 mL urine according to the method described by Ballevre et al (18).

The isotopic enrichment of ^{13}C in breath carbon dioxide was measured with a dual-inlet isotope ratio mass spectrometer (model 602D; VG Micromass, Cheshire, United Kingdom). Mass spectrometry analysis was performed by using techniques described previously (24). Carbon dioxide enrichment from baseline samples and from those taken during the last 2 h of tracer infusion were expressed as APE $^{13}CO_2$ over a reference standard of compressed carbon dioxide gas.

Amino acid concentrations (not including tryptophan, methionine, and cysteine or cystine) in samples of the 3 formulas, human milk, and stool were determined by liquid chromatography of precolumn phenylisothiocyanate derivatives. Sample preparation and protein hydrolysis were performed as described by Sarwar et al (27). The amino acids were derivatized with phenylisothiocyanate and separated on a Pico-Tag Amino Acid Analysis Column (3.9 × 150 mm; Waters Chromatography Division, Millipore Corporation, Milford, MA) (27). Samples were hydrolyzed and run in duplicate. Plasma amino acid concentrations were measured by ion-exchange chromatography with postcolumn ninhydrin reaction and visible colorimetric detection with a system 7300 high-performance amino acid analyzer (Beckman Instruments, Mississauga, ON).

The total nitrogen content of the formulas, human milk samples, and urine samples was measured in duplicate with a micro-Kjeldahl, nonautomated procedure (28). The total nitrogen content of the stool samples was measured in duplicate by the micro-Kjeldahl procedure by using a Kjeltec 1030 Auto Analyzer (Tecator Inc, Herndon, VA).

Data analysis

Isotopic steady state in the metabolic pool was indicated by the attainment of plateaus in urinary [1-^{13}C]threonine and breath $^{13}CO_2$ enrichments. Attainment of a plateau was defined by considering the slope and SD of the points on the plotted enrichment curve. The plateau for urinary [1-^{13}C]threonine was defined by 2–4 points, representing a minimum of 6 h of urine collection, and corresponded to urine collected between 9 and 18 h after the start of tracer infusion. In the 17 studies combined, the CVs for enrichment measurements ($\bar{x} \pm$ SD) for baseline and plateau enrichments were, respectively, $0.4 \pm 0.4\%$ and $1.9 \pm 2.3\%$ for [1-^{13}C]threonine and $4.2 \pm 2.5\%$ and $4.4 \pm 2.8\%$ for breath $^{13}CO_2$. Enrichment due to the tracer at isotopic steady state (E_p), either in mol fraction above baseline × 100 (MF%) for urinary and plasma amino acids or in APE for breath carbon dioxide, was calculated according to standard equations (29).

Threonine flux was measured from the dilution of the infused tracer in the metabolic pool at isotopic steady state, as described previously (23, 29, 30).

$$Q = i\,[(E_i/E_p) - 1] \qquad (1)$$

where Q is the flux of amino acids through the free amino acid pool ($\mu mol \cdot kg^{-1} \cdot h^{-1}$), i is the infusion of the amino acid tracer, and E_i and E_p are the isotope abundance of the infusate (MF%) and of the urinary amino acid at isotopic steady state (MF%), respectively.

The rate of threonine oxidation to carbon dioxide was determined from the rate of excretion of $^{13}CO_2$ ($F^{13}CO_2$), expressed in $\mu mol \cdot kg^{-1} \cdot h^{-1}$ and from the enrichment of urinary threonine at the plateau. The rate of tracer oxidation was calculated as

$$F^{13}CO_2 = (FCO_2)(ECO_2)(44.6)(60)/(W)(0.80)(100) \qquad (2)$$

where FCO_2 is the carbon dioxide production rate (in cm^3/min), ECO_2 is the $^{13}CO_2$ enrichment above background at isotopic steady state (in APE), W is body weight in kg of the infant, and 0.80 takes into account that 80% of the $^{13}CO_2$ released by amino acid oxidation is expired and the remainder is retained in the body (31). The constants 44.6 $\mu mol/cm^3$ and 60 min/h serve to convert FCO_2 to $\mu mol/h$ and 100 changes APE into a fraction of 1. The rate of threonine oxidation to carbon dioxide (Ox) was calculated according to the following equation:

$$Ox = F^{13}CO_2(1/E_p - 1/E_i) \times 100 \qquad (3)$$

The possible contribution of threonine to glycine was monitored by measuring the enrichment of [^{13}C]glycine. Analysis of data obtained from GC-CIRMS involved the following. Because of a limitation in the capacity for analyzing a large number of samples by GC-CIRMS, baseline urine samples and the last 3-h urine sample collected between 15 and 18 h after tracer infusion began were analyzed for [^{13}C]glycine. The enrichment due to the tracer at isotopic steady state (in APE) was calculated by subtracting baseline enrichment from plateau enrichment and then multiplying by a factor of 7 to take into account the dilution caused by combustion of the N-propyl,N-acetyl derivatized glycine. The fractional contribution of plasma threonine to the glycine pool was estimated as E_{pgly}/E_{pthr}.

Apparent nitrogen retention ($mg \cdot kg^{-1} \cdot d^{-1}$) was calculated by subtracting nitrogen losses in urine and estimated losses in stool from nitrogen intake during the 4-d balance study. On the basis of a study conducted in a similar group of infants fed similar formulas and human milk (32), nitrogen loss in stool was estimated to represent 15% of nitrogen intake in formula-fed infants and 12% of nitrogen intake in infants fed preterm milk. Fecal excretion of threonine ($\mu mol \cdot kg^{-1} \cdot d^{-1}$) was determined by multiplying the fecal threonine concentration ($\mu mol/g$ nitrogen) by estimated fecal nitrogen excretion ($g \cdot kg^{-1} \cdot d^{-1}$).

Results are expressed as means ± SDs. Analysis of variance was used for comparison among formula groups. If the F value was significant at $P < 0.05$, then post hoc comparison of differences between groups was performed by using the Student-Neuman-Keuls multiple-range test with an α of 0.05, which was considered statistically significant. Whenever a nonsignificant difference was found ($P > 0.1$), results from formula groups were pooled and compared with results from the group fed preterm milk by unpaired Student's t tests for equal or unequal variances. When a significant difference was found ($P < 0.05$) among formula groups, analysis of variance was used for comparison among the 4 groups (preterm milk group and the 3 formula groups). Statistical analyses were performed with SAS software (SAS Institute Inc, Cary, NC).

RESULTS

Of the 18 infants enrolled, 17 infants completed the 7-d study. One infant fed preterm milk was removed from the study before

TABLE 3
Energy, total amino acid, threonine, and nitrogen intakes; apparent nitrogen balance; and fecal threonine excretion of preterm infants fed formulas with various whey-to-casein ratios or their mothers' milk (preterm milk)

| | Formula (whey-to-casein ratio) | | | | | | |
	60:40	40:60	20:80	SD[1]	P[2]	Preterm milk	P[3]
Energy intake[4]							
$(kJ\cdot kg^{-1}\cdot d^{-1})$	469	469	448	13	0.6	485 ± 38^5	0.2
$(kcal\cdot kg^{-1}\cdot d^{-1})$	112	112	107	3	0.6	116 ± 9	0.2
TAA intake $(\mu mol\cdot kg^{-1}\cdot d^{-1})^6$	18242	18021	17048	822	0.4	18749 ± 6472	0.6
Threonine intake $(\mu mol\cdot kg^{-1}\cdot d^{-1})$	1157[a]	1004[b]	813[c]	51	0.001	1042 ± 394	—
Nitrogen intake $(mg\cdot kg^{-1}\cdot d^{-1})$	380	380	363	17	0.3	455 ± 132	0.1
Nitrogen balance $(mg\cdot kg^{-1}\cdot d^{-1})^7$	249	240	234	11	0.2	280 ± 82	0.1
Fecal threonine excretion $(\mu mol\cdot kg^{-1}\cdot d^{-1})$	155	133	132	16	0.2	152 ± 48	0.6

[1] The root mean square error of the ANOVA model comparing the formula groups.

[2] Determined by the ANOVA model comparing the formula groups. Means with different superscript letters are significantly different, $P < 0.05$ (Student-Neuman-Keuls test).

[3] Determined by Student's *t* test comparing preterm milk and pooled formula groups, whenever the comparison between formula groups was not significant at $P < 0.05$.

[4] Based on an energy content of 2803 kJ/L (670 kcal/L) for formulas and preterm milk (34).

[5] $\bar{x} \pm$ SD.

[6] Total amino acids measured (*see* Table 2).

[7] Apparent nitrogen balance (*see* text).

completing the threonine tracer period because of severe diaper rash. There were no significant differences in characteristics of the infants fed the 3 formulas nor between the infants fed formula and those fed preterm milk (Table 1). The mean daily weight gain of the infants matched intrauterine rates for age (33).

The nitrogen and amino acid compositions of the 3 study formulas and the mean values for preterm milk sampled during the threonine tracer-infusion period are shown in Table 2. Daily energy, nitrogen, and total amino acid intakes by infants fed the 3 study formulas and by those fed preterm milk are shown in **Table 3.** These measurements were not significantly different among formula groups nor between the preterm milk group and the pooled formula group. Threonine intakes, however, differed significantly among formula groups as expected. There were no significant differences in apparent nitrogen balance among the 3 formula groups or between the pooled formula group and the preterm milk group. Stool threonine concentrations did not differ among infants fed the 3 formulas but was slightly ($P = 0.05$) higher in infants fed preterm milk than in the pooled formula group (2.87 ± 0.29 compared with 2.50 ± 0.27 mmol/g N, respectively). Fecal excretion of threonine, however, did not differ among formula groups nor between the preterm milk group and the pooled formula group.

Mean intakes of threonine from the diet and the tracer are presented in **Table 4**. Threonine intake during tracer infusion differed significantly by design among the formula groups and increased as the whey content of the formula increased from 20% to 40% and from 40% to 60%. Threonine intake of infants fed preterm milk was more variable, but the mean was within the range of values for the formula-fed infants.

Kinetic aspects of threonine metabolism are also summarized in Table 4; these were calculated from the plateau enrichment values for urinary threonine and breath carbon dioxide and rates of breath carbon dioxide production. Isotopic enrichment of threonine was similar in plasma and urine (plasma-to-urine enrichment ratio of 0.966 ± 0.010; $\bar{x} \pm$ SEM, $n = 14$), thus validating the measurement of $[^{13}C]$threonine enrichment in urine as opposed to plasma (23, 30). The mean ($\pm$SEM) CV of urinary

threonine enrichment above baseline at plateau was $5.2 \pm 1.6\%$ ($n = 14$). This degree of variation in urinary amino acid enrichment is similar to that reported with constant intragastric feeding and tracer infusion (30) as well as with 2-h feeding and tracer infusion (23). In breath carbon dioxide, the mean ($\pm$SEM) CV of enrichment above baseline at plateau was $14.0 \pm 1.7\%$ ($n = 17$), which is somewhat higher than that obtained during constant intragastric infusion of L-[1-^{13}C]leucine ($6.4 \pm 1.4\%$, $n = 7$) (30). This difference in results was presumably due to differences in feeding schedules between the 2 studies. In this respect, the precision of our method for determining amino acid oxidation may have been somewhat compromised because the tracer was administered every 3 h, in accordance with ethical regulations, as opposed to continuous intragastric infusion. We considered this variation to be acceptable and to permit the detection of important differences in rates of threonine oxidation between feeding groups.

As shown in Table 4, plasma threonine concentrations of the infants fed the study formulas increased significantly with increasing threonine intake. Despite a generous mean threonine intake in infants fed preterm milk (numerically similar to the intake of the infants fed the 40:60 formula), plasma threonine concentrations remained low in the preterm milk group. Plasma concentrations of other amino acids in both infants fed formulas and those fed preterm milk were consistent with previously published aminograms (10–12). Plasma threonine flux was significantly higher in infants fed the formula containing the 60:40 whey-to-casein ratio than in infants fed the 40:60 and 20:80 formulas. There was no significant difference in the amount of the tracer dose oxidized ($F^{13}CO_2$) among formula groups. Although mean threonine oxidation rates tended to increase with increasing threonine intake, differences among formula groups were not significant. Threonine oxidation in infants fed preterm milk was significantly higher than in the combined formula group, whether expressed as an absolute value ($\mu mol\cdot kg^{-1}\cdot h^{-1}$) or as a percentage of intake or percentage of flux. Mean rates of carbon dioxide production (in mL/min) were similar among feeding groups (results not shown).

TABLE 4
Threonine kinetics in preterm infants fed formulas with various whey-to-casein ratios or their mothers' milk (preterm milk)

	Formula (whey-to-casein ratio)					Preterm milk	
	60:40 ($n = 4$)	40:60 ($n = 4$)	20:80 ($n = 4$)	SD[1]	P[2]	($n = 5$)	P[3]
Threonine intake ($\mu mol \cdot kg^{-1} \cdot h^{-1}$)	63.2[a]	56.4[b]	48.5[c]	2.2	0.0001	58.0 ± 16.0[4]	—
Plasma threonine ($\mu mol/L$)	419[a]	344[a,b]	228[b]	75	0.03	208 ± 41	—
Plasma threonine flux ($\mu mol \cdot kg^{-1} \cdot h^{-1}$)	221.4[a]	183.0[b]	181.2[b]	20.0	0.03	170.0 ± 28.8	—
Tracer dose oxidized (%)	5.0	5.2	4.6	1.6	0.9	8.2 ± 2.2	0.0002
Threonine oxidation							
($\mu mol \cdot kg^{-1} \cdot h^{-1}$)	10.9	9.6	8.3	3.0	0.5	15.6 ± 6.2	0.02
(% of intake)	17.2	17.0	17.2	4.9	1.0	24.3 ± 4.9	0.01
(% of flux)	4.9	5.2	4.6	0.8	0.9	8.3 ± 2.2	0.002

[1] The root mean square error of the ANOVA model comparing the formula groups.

[2] Determined by the ANOVA model comparing the formula groups. Means with different superscript letters are significantly different, $P < 0.05$ (Student-Neuman-Keuls test).

[3] Determined by Student's t test comparing preterm milk and pooled formula groups, whenever the comparison between formula groups was not significant at $P < 0.05$.

[4] $\overline{x}$ ± SD.

The conversion of threonine to glycine in low-birth-weight infants fed formula and preterm milk is presented in **Table 5**. A low amount of [^{13}C]glycine enrichment derived from the threonine tracer was detected in the urine of the infants by GC-CIRMS. Enrichment in [^{13}C]glycine from the threonine tracer did not appear to be affected by feeding regimen. Enrichment in [^{13}C]glycine from hippurate could not be measured because of low hippurate concentrations in the urine samples. The mean fraction of glycine flux that was derived from threonine ranged from 1% to 1.5%. The mean fractional conversion rate of threonine to glycine ([^{13}C]glycine/[^{13}Cthreonine) was higher ($P < 0.01$) in infants fed the 3 formulas than in infants fed preterm milk. The actual rate of threonine conversion to glycine, expressed as $\mu mol \cdot kg^{-1} \cdot h^{-1}$, could not be calculated because glycine flux was not measured in this study.

DISCUSSION

The main purpose of this study was to measure threonine kinetics in low-birth-weight infants to gain insight into the significance of the plasma threonine concentration in response to feeding breast milk and formula. To our knowledge, this is the first report of threonine kinetics in infants and the first demonstration of the conversion of threonine to glycine in humans, presumably through the threonine dehydrogenase pathway.

We assumed that the infants were in a steady state rate of growth, given their stable condition, constant feeding schedules, and normal growth rates. The plasma threonine concentration was considered to be an indication of the size of the free threonine pool, which is determined by the balance between rates of threonine entering the pool from dietary intake and endogenous appearance (protein breakdown) and rates of threonine leaving the pool for oxidation and nonoxidative disposal (mainly protein synthesis). In formula-fed infants, plasma threonine concentrations rose sharply with increasing threonine intake and whey-to-casein ratios, as observed in other investigations (10–12). We found that rates of threonine oxidation to carbon dioxide did not change significantly despite the large and significant differences in plasma concentration observed in formula-fed infants. Threonine oxidation therefore appeared to be taking place at a maximal rate. This inability of low-birth-weight infants to dispose of excess dietary threonine is not necessarily attributable solely to an immature threonine degradative pathway because adults respond similarly to excess threonine intakes (19, 35).

This study represents the most comprehensive attempt to date to examine threonine balance in human neonates. Within the limitations of the measurements, some of which had large CVs, we could account for only a portion of the extra threonine ingested by infants fed the 60:40 formula compared with the 20:80 formula. The most striking differences between these 2 groups were the 1.8-fold expansion of the plasma threonine pool (191 $\mu mol/L$) and the 2.6-fold increase in urinary threonine excretion (from $0.8 ± 0.3$ to $2.1 ± 0.9$ $\mu mol \cdot kg^{-1} \cdot h^{-1}$) in the group fed the 60:40 formula. Of the extra 14.7 μmol threonine $\cdot kg^{-1} \cdot h^{-1}$ ingested by

TABLE 5
Urinary [^{13}C]glycine enrichment and the fraction of glycine flux derived from threonine ([^{13}C]glycine/[^{13}C]threonine) in preterm infants fed formulas with various whey-to-casein ratios or their mothers' milk (preterm milk)

	Formula (whey-to-casein ratio)					Preterm milk	
	60:40 ($n = 4$)	40:60 ($n = 4$)	20:80 ($n = 4$)	SD[1]	P[2]	($n = 5$)	P[3]
[^{13}C]Glycine (mol fraction %)[4]	0.0883	0.1118	0.0859	0.019	0.2	0.0769 ± 0.019[5]	0.2
[^{13}C]Glycine/[^{13}C]threonine (%)	1.38	1.51	1.16	0.24	0.2	0.96 ± 0.14	0.01

[1] The root mean square error of the ANOVA model comparing the formula groups.

[2] Determined by the ANOVA model comparing the formula groups.

[3] Determined by Student's t test comparing preterm milk and pooled formula groups.

[4] Mole fraction above baseline $\times$ 100.

[5] $\overline{x}$ ± SD.

the group fed the 60:40 formula compared with the 20:80 formula, up to 18% might be oxidized, 5% excreted in stool, 9% excreted in urine, and up to 40% accumulated in plasma and tissues (assuming a distribution of threonine within total body water at 85% of body weight). We speculate that there may be threonine pools or channels of threonine disposal other than glycine that were not measured in this study.

Infants fed preterm milk had lower plasma threonine concentrations relative to threonine intake compared with formula-fed infants, which is consistent with previous studies (9, 12, 13). Estimated fecal threonine excretion was not higher in infants fed preterm milk than in formula-fed infants. This result is consistent with other studies reporting low fecal excretion of immunologic proteins such as sIgA in low-birth-weight infants (14–16). Urinary threonine losses (results not shown) were not greater in infants fed preterm milk than in formula-fed infants. Based on weight gain and nitrogen balance results, there was no evidence for an increased utilization of threonine for protein gain by infants fed preterm milk. A major finding of this study is that low-birth-weight infants fed their mother's milk had significantly higher rates of threonine oxidation than did formula-fed infants (Table 4). Threonine intakes of infants fed preterm milk were more variable than those of formula-fed infants because of differences in the threonine content of breast milk from mother to mother. Nevertheless, threonine oxidation expressed as a percentage of threonine intake was significantly higher in infants fed preterm milk than in infants fed formula. The increased rate of threonine oxidation to carbon dioxide in infants fed preterm milk therefore appeared to be the major determinant of these infants' lower plasma threonine concentrations relative to threonine intake. A partial explanation for the lower plasma threonine concentrations in infants fed preterm milk may be that ingested threonine present in sIgA and other immunologic proteins is retained within the gastrointestinal tract (9). Nevertheless, this situation would only accentuate the differences in oxidation of absorbed threonine between infants fed human milk and those fed formula.

The reason for the higher threonine oxidation rate in low-birth-weight infants fed preterm milk is not known at this time. To explain this finding, one must consider the following determinants of threonine oxidation, ie, the activity and regulation of the threonine-degrading enzymes and the supply of substrate to the site of oxidation.

1) Threonine dehydratase activity may have been increased in infants fed preterm milk compared with formula. This enzyme is highly sensitive to the hormonal milieu, which has been shown to differ between infants fed formula and those fed human milk (36, 37). Human milk may contain a substance, such as a growth factor or a hormone, that enhances the activity of this enzyme and that is lacking in formula. In addition, the activity of this enzyme is influenced by the protein content of the diet (38) and can be induced not only by a high-protein diet (18) but also by the addition of certain glucogenic amino acids (39) and glutamic acid to the diet (40, 41). Rats have been shown to respond to low-protein diets with reduced threonine dehydratase activity (38). The formula-fed infants ingested a quantity of total amino acids similar to that ingested by infants fed preterm milk, but the type of protein and the amino acid composition differed. Of possible interest is the elevated free glutamic acid present in human milk and not in formula (42).

2) Threonine dehydrogenase activity may also have been enhanced with human milk feeding and the glycine produced may have been immediately oxidized. Threonine dehydrogenase activity, however, does not appear to respond to dietary changes nor to hormonal stimuli as does threonine dehydratase activity (18, 43). A high demand for glycine exists during the neonatal period and the glycine content of human milk and formulas does not provide enough glycine to meet this demand (44). A high rate of endogenous glycine synthesis, mainly from serine, is assumed to occur (44). Endogenous glycine (including that from threonine) may, presumably, be preferentially conserved for tissue accretion rather than be oxidized. We do not know whether glycine produced from threonine first equilibrates with the glycine pool before it is oxidized, in which case the label would be substantially diluted and not detectable in breath carbon dioxide, or whether a certain obligatory amount of glycine produced from threonine is oxidized through metabolic channeling.

3) Finally, the transport process of threonine uptake into tissues (mainly liver) for subsequent oxidation may be more efficient in low-birth-weight infants fed preterm milk than in infants fed formula. This process has not been studied in human infants. Hepatic threonine uptake into liver has been shown to be low compared with the uptake of other amino acids in pigs (45) and rats (46). Moundras et al (39) showed that the induction of threonine dehydratase was associated with an increase in the fractional hepatic extraction of threonine and a drop in circulating plasma threonine. Clearly, more research is needed to define the mechanism underlying the differences in threonine oxidation rates between formula-fed infants and those fed their mothers' milk.

Glycine flux was not measured in this study. However, we do have an estimate of glycine flux in a stable, growing group of formula-fed infants (23) of 560 μmol$\cdot$kg$^{-1}\cdot$h^{-1}. By using this value for glycine flux, the mean rate of conversion of threonine to glycine in formula-fed infants in the present study was 7.6 μmol$\cdot$kg$^{-1}\cdot$h^{-1}, which represents 44% of total threonine degradation. This is in contrast with studies conducted in pigs (18), in which glycine production accounted for ≈80% of total threonine degradation. The reason for the species difference is not known. The average amino acid composition of human and sow milk are comparable; however, piglets grow at ≈4 times the rate of human infants (47). Because of their higher growth rate, pigs may have a higher requirement for the endogenous synthesis of glycine. This issue needs further study.

In conclusion, this study showed that infants fed human milk have a greater capacity to oxidize threonine to carbon dioxide, presumably through the threonine dehydratase pathway, than do formula-fed infants. Infants fed human milk are thus better able to moderate their plasma threonine concentrations, in contrast with formula-fed infants, who appear unable to increase threonine degradation (by either the threonine dehydratase or the threonine dehydrogenase pathway) enough to handle an increased threonine load. This study suggests that the threonine content of whey-dominant formulas should be reduced to amounts approximating that in cow milk protein, ie, a whey-to-casein ratio of 20:80. Although this strategy for improving formulas can be appreciated on the basis of earlier studies (6–12, 48), the present study points the way to understanding the metabolic mechanisms that underlie the differences in plasma threonine concentrations between formula-fed and human milk–fed infants.

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