

# Choline and choline esters in human and rat milk and in infant formulas<sup>1-3</sup>

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**ABSTRACT** Large amounts of choline are required in neonates for rapid organ growth and membrane biosynthesis. Human infants derive much of their choline from milk. In our study, mature human milk contained more phosphocholine and glycerophosphocholine than choline, phosphatidylcholine, or sphingomyelin ( $P < 0.01$ ). Previous studies have not recognized that phosphocholine and glycerophosphocholine exist in human milk. Concentrations of choline compounds in mature milk of mothers giving birth to preterm or full-term infants were not significantly different. Infant formulas also contained choline and choline-containing compounds. In infant formulas derived from soy or bovine milk, unesterified choline, phosphocholine, glycerophosphocholine, phosphatidylcholine, and sphingomyelin concentrations varied greatly. All infant formulas contained significantly less phosphocholine than did human milk. Soy-derived formulas contained significantly less glycerophosphocholine ( $P < 0.01$ ) and phosphocholine ( $P < 0.01$ ) and more phosphatidylcholine ( $P < 0.01$ ) than did human or bovine milk or bovine milk-derived infant formulas. Rat milk contained greater amounts of glycerophosphocholine (almost 75% of the total choline moiety in milk) and phosphocholine than did human milk. When dams were provided with either a control, choline-deficient, or choline-supplemented diet, milk composition reflected the choline content of the diet. Because there are competing demands for choline in neonates, it is important to ensure adequate availability through proper infant nutrition. Although the free choline moiety is adequately provided by infant formulas and bovine milk, reevaluation of the concentrations of other choline esters, in particular glycerophosphocholine and phosphocholine, may be warranted. *Am J Clin Nutr* 1996;64:572-6.

**KEY WORDS** Choline, phosphocholine, glycerophosphocholine, human milk, bovine milk, infant formulas, human, rat

## INTRODUCTION

Choline is a dietary nutrient that is crucial for normal function of cells and essential for many mammalian species (1). It is a precursor for the biosynthesis of the phospholipids phosphatidylcholine and sphingomyelin and of choline plasmalogens, which are essential constituents of membranes (2) (Figure 1). Choline is important for the structural integrity of cell membranes, methyl metabolism, transmembrane signaling, lipid-cholesterol transport and metabolism, and normal brain development (3, 4). Choline is also needed to make acetylcholine, an important neurotransmitter (4, 5), and is the major source of

methyl groups in the diet: its metabolite, betaine, participates in the methylation of homocysteine to form methionine (4).

Neonates require especially large amounts of choline for sustaining growth as well as for normal maintenance of tissue mass (6). Fetuses derive all nutrition from their mother's blood across the placenta, but neonates consume only a single food—milk—that contains a high concentration of choline (7, 8). Previously, we showed that pregnant rats have depleted concentrations of choline-containing metabolites in liver and that lactation exacerbates this depletion (9). Supplementation with choline (to the pregnant rat or newborn pup) results in changes in hippocampal function with life-long enhancement of the spatial memory capacity of rats exposed to extra choline during critical periods perinatally (10-12). This suggests that choline nutrition during the newborn period may be marginal, and is critical for normal brain development. Therefore, the choline compounds in milk may be very important. In an earlier report, we were not aware that milk might contain phosphocholine or glycerophosphocholine (8). In the present study, we examined the differences in choline and choline ester composition of mature human milk from human mothers who delivered either pre- or full-term infants, of bovine milk, and of commercial infant formulas. In addition, we examined how maternal diet may influence choline and choline ester composition and concentration in lactating rats.

## SUBJECTS AND METHODS

### Human subjects

Healthy women who delivered preterm or full-term infants and were breast-feeding were recruited. The mothers' ages ranged from 18 to 40 y; 17 women gave birth to preterm infants and 16 women gave birth to full-term infants. The study methods and collection procedures were explained to each mother by the staff at the milk bank (Triangle Mother's Milk

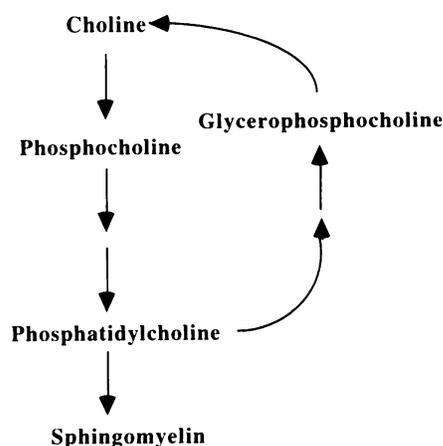
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**FIGURE 1.** Milk contains choline, phosphocholine, glycerophosphocholine, phosphatidylcholine, and sphingomyelin. The metabolic pathways illustrated are present in many tissues, including mammary tissue.

Bank, Wake Medical Center, Raleigh, NC) and signed, written consent was obtained from each mother before the study. The study was approved by the Human Subjects Committee of the University of North Carolina at Chapel Hill.

### Animals

Timed-pregnant Sprague-Dawley rats (Charles River Breeding Laboratories, Raleigh, NC) were received on day 8 of gestation. All rats were housed in individual opaque polyethylene cages with stainless steel tops. Animals were maintained in an air-conditioned environment at a temperature of 25 °C and were exposed to a 12-h light cycle (0600 to 1800). AIN-76A semipurified diet (13, 14) containing 1.1 g choline chloride/kg diet (Dyets, Inc, Bethlehem, PA) and water ad libitum were provided. On day 12 of gestation, dams were divided into three experimental groups ( $n = 6/\text{group}$ ) and provided with either the AIN-76A diet, the AIN-76A choline-deficient diet, or the AIN-76A diet along with drinking water (ad libitum) supplemented with 3.5 g choline chloride/L and 50 mmol sodium saccharin/L (10). Water containing 50 mmol sodium saccharin/L was provided ad libitum to rats receiving the control and choline-deficient diets. These experimental treatments were continued through postnatal day 15. This study was approved by the Animal Subjects Committee of the University of North Carolina at Chapel Hill.

### Infant formulas and human, bovine, and rat milk

Infant formulas were purchased locally and prepared according to package instructions. Commercial powdered bovine-derived formulas were as follows: Enfamil with iron (Mead Johnson, Evansville, IN), Similac with iron (Ross Laboratories, Columbus, OH), S.M.A. with iron (Wyeth-Ayerst Laboratories, Philadelphia), Gerber with iron (Gerber Products Co, Fremont, MI), Goodstart (Carnation Co, Glendale, CA), and Follow-up (Carnation Co). Commercial powdered soy-derived formulas were as follows: ProSobee Soy (Mead Johnson), Isomil (Ross Laboratories), Nursoy (Wyeth-Ayerst Laboratories), and Gerber Soy (Gerber Products Co). Distilled water was used to dilute the formulas. Aliquots were immediately frozen and then stored at  $-80\text{ }^{\circ}\text{C}$  until analyzed for choline and choline esters.

Mature human milk from lactating women was obtained from the hospital milk bank during postnatal days 27–32 (Triangle Mother's Milk Bank). At their homes, or in the milk bank, mothers emptied their breasts of milk by using a pump and mixed the milk; the milk was then immediately frozen and stored at  $-20\text{ }^{\circ}\text{C}$ . Frozen milk samples were taken to the milk bank and immediately stored at  $-20\text{ }^{\circ}\text{C}$  overnight. Thereafter, milk was stored at  $-80\text{ }^{\circ}\text{C}$  until assayed. Pasteurized bovine milk (homogenized; Maola Milk and Ice Cream Co, New Bern, NC) was purchased locally. Aliquots were immediately frozen at  $-80\text{ }^{\circ}\text{C}$  until assayed. Human and bovine milk samples were stored for 3 mo at  $-80\text{ }^{\circ}\text{C}$  until analyzed for choline and choline esters.

On postnatal day 15, lactating rat dams were removed from their pups 2 h before milk was collected. To stimulate milk let-down, rats were injected with oxytocin (1.0 IU/100 g body wt, subcutaneously; LyphoMed, Inc, Rosemont, IL) 30 min before being anesthetized with sodium pentobarbital (0.1 mL/100 g body wt, intraperitoneally; University of North Carolina Hospitals Pharmacy, Chapel Hill, NC). Milk was expressed manually and collected in duplicate into labeled tubes ( $\approx 0.5\text{--}1.0\text{ mL}$ ) and immediately frozen in liquid nitrogen and stored at  $-80\text{ }^{\circ}\text{C}$  until assayed.

### Analysis of milk and infant formulas

Choline-containing compounds in infant formulas and in human and rat milks were measured with HPLC and gas chromatography–mass spectrometry (15, 16). Samples of milk were extracted by the method of Bligh and Dyer (17) and a [ $^{14}\text{C}$ ]methyl and a [ $^2\text{H}$ ]methyl internal standard were added for each metabolite to permit peak collection and correction for recovery during the analysis. The aqueous phase was suspended in water-methanol and injected onto a normal phase silica HPLC column (Pecosphere-3CSi, 3  $\mu\text{mol/L}$  cartridge; Perkin-Elmer, Norwalk, CT). Choline, glycerophosphocholine, and phosphocholine were eluted with a binary, nonlinear gradient of acetonitrile:ethanol:acetic acid:1 mol ammonium acetate/L:water:0.1 mol sodium phosphate/L (800:68:2:3:127:10, by vol, changing to 400:68:44:88:400:10, by vol) at room temperature with a flow rate of 1.5 mL/min. Peaks were detected with an on-line radiometric detector (Berthold, Pittsburgh).

Fractions that cochromatographed with the [ $^{14}\text{C}$ ]methyl standards were collected and dried under a vacuum. Glycerophosphocholine and phosphocholine were hydrolyzed in 6 mol HCl/L at 95 °C for 1 and 24 h, respectively, to liberate choline (15). Phosphatidylcholine and sphingomyelin were isolated from the organic fraction by applying the reconstituted organic residues to a thin-layer chromatography plate (Si250-PA; JT Baker, Inc, Phillipsburg, NJ) and were developed in chloroform:methanol:water (65:30:4, by vol). The bands that cochromatographed with the phosphatidylcholine external standard (99% dipalmitoyl; Sigma Chemical Co, St Louis) were identified by iodine vapor, scraped, and hydrolyzed in 6 mol methanolic HCl/L at 95 °C for 1 h to liberate choline. Choline was converted to the propionyl ester and demethylated with sodium benzenethiolate. This was then isolated with gas chromatography–mass spectrometry (Hewlett Packard, Andover, MA) (15). Deuterated internal standards were used to correct for variations in recovery. Sphingomyelin was measured by phosphorous content after thin-layer chromatography. Samples

were digested in concentrated perchloric acid at 200 °C, and phosphorus was quantified after reaction with molybdate by measuring absorbance at 825 nm (18).

### Statistical analysis

Data were analyzed by using a one-way analysis of variance (ANOVA) to compare the differences of means among three or more groups (Macintosh STATVIEW 512<sup>+</sup>; Abacus Concepts, Inc, Berkeley, CA).

## RESULTS

Human milk is the gold standard from which we calculate the nutrient requirements of infants aged between 0 and 6 mo (19–21). Mature breast milk choline concentrations were significantly different ( $P < 0.05$ ) in mothers who delivered preterm compared with full-term infants but choline ester concentrations were not (Table 1). Because total choline and choline ester concentrations were not significantly different, data presented in Figure 2 are from the combined groups ( $n = 33$ ). Mature human milk had significantly higher phosphocholine concentrations (718  $\mu\text{mol/L}$ ) than did either bovine milk or infant formulas ( $P < 0.01$ ) (Figure 2). Bovine milk and bovine-derived infant formulas had the same or higher glycerophosphocholine concentration (400–800  $\mu\text{mol/L}$ ) than did human milk (415  $\mu\text{mol/L}$ ). Soy-derived infant formulas had lower glycerophosphocholine concentrations ( $\leq 115 \mu\text{mol/L}$ ) ( $P < 0.01$ ). Human milk phosphatidylcholine and sphingomyelin concentrations were not significantly different from those in bovine milk or bovine-derived infant formulas. Soy-derived infant formulas had more phosphatidylcholine than did human milk or bovine-derived formulas ( $P < 0.01$ ). Soy-derived formulas had less sphingomyelin than did human milk ( $P < 0.01$ ). Unesterified choline concentrations in mature human milk were 30–80% lower than in either bovine milk or infant formulas [mature human milk has significantly lower free choline than does colostrum-transitional human milk (8)].

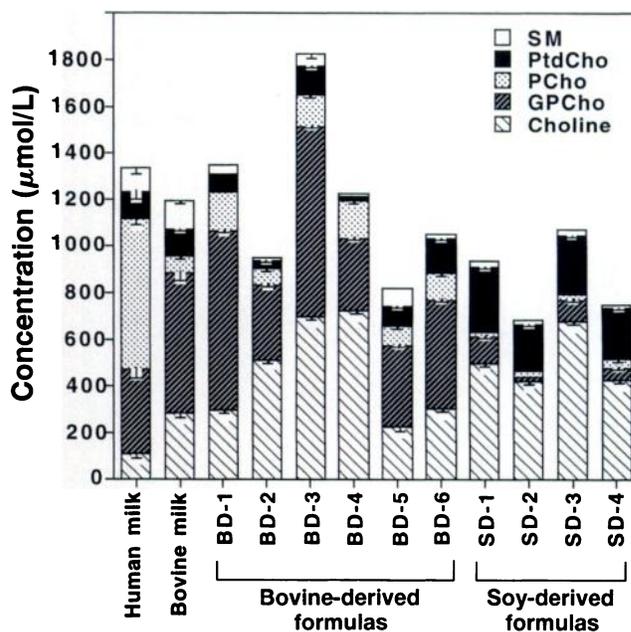
These results update our earlier report on the choline content of milks, and our report that rat milk contains glycerophosphocholine and phosphocholine (8, 22). They show, for the first time, that phosphocholine and glycerophosphocholine are important constituents of human milk and infant formulas. The large amount of phosphocholine in the milk of humans and rats may either be formed by phosphatidylcholine-specific phospholipase C activity (23) or by choline kinase activity (24).

**TABLE 1**  
Choline-containing compounds in human milk<sup>1</sup>

Metabolite	Preterm ( $n = 17$ )	Full-term ( $n = 16$ )
	$\mu\text{mol/L}$	
Choline	98 $\pm$ 45	116 $\pm$ 22 <sup>2</sup>
Glycerophosphocholine	379 $\pm$ 42	362 $\pm$ 70
Phosphocholine	639 $\pm$ 118	570 $\pm$ 136
Phosphatidylcholine	90 $\pm$ 13	82 $\pm$ 6
Sphingomyelin	104 $\pm$ 9	124 $\pm$ 9

<sup>1</sup>  $\bar{x} \pm \text{SE}$ . Human milks were prepared and analyzed as described in the text. Preterm and full-term refer to whether the women gave birth to preterm or full-term infants.

<sup>2</sup> Significantly different from preterm,  $P < 0.05$ .



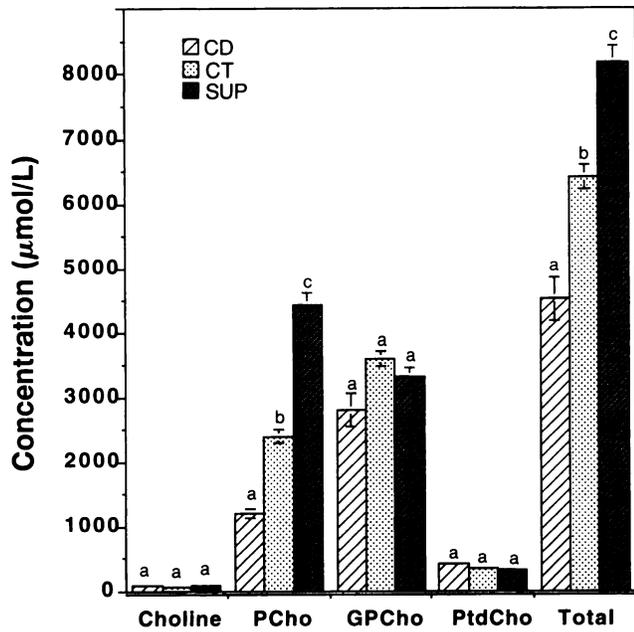
**FIGURE 2.** The choline and choline ester content of milk is different in human milk than in bovine milk and infant formulas. Human and bovine milk and infant formulas were prepared and analyzed as described in the text. Data are expressed as mean concentrations in human milk ( $n = 33/\text{point}$ ), bovine milk ( $n = 3/\text{point}$ ), and infant formulas ( $n = 3/\text{point}$ ). Commercial powdered formulas, which were either bovine-derived (BD) or soy-derived (SD), were as follows: BD-1, Enfamil with iron (Mead Johnson); BD-2, Similac with iron (Ross Laboratories); BD-3, S.M.A. with iron (Wyeth-Ayerst Laboratories); BD-4, Gerber with iron (Gerber Products Co); BD-5, Goodstart (Carnation Co); BD-6, Follow-up (Carnation Co); SD-1, ProSobee Soy (Mead Johnson); SD-2, Isomil (Ross Laboratories); SD-3, Nursoy (Wyeth-Ayerst Laboratories); and SD-4, Gerber Soy (Gerber Products Co). Variability of data is indicated as the SEM within the stacked bar for the data; when error bars are not shown the error was smaller than could be indicated by an error bar. SM, sphingomyelin; PtdCho, phosphatidylcholine; PCho, phosphocholine; GPCho, glycerophosphocholine.

Glycerophosphocholine is thought to be generated when phosphatidylcholine is hydrolyzed by phospholipase A activity (25). The bioavailability of these two choline esters in milk is different from that of choline or phosphatidylcholine (26).

Consumption of either a choline-deficient or choline-supplemented diet by lactating rat dams resulted in significant changes in the phosphocholine concentration of their milk (Figure 3). Milk phosphocholine concentration was reduced by 50% with the choline-deficient diet ( $P < 0.05$ ), whereas other choline compounds were unchanged. Milk phosphocholine concentrations were increased when rats consumed the supplemented diet ( $P < 0.05$ ), whereas other choline compounds were unchanged. Thus, dietary variation caused a fourfold change (comparing the choline-deficient and supplemented diets) in milk phosphocholine content.

## DISCUSSION

Studies have consistently shown that milk quantity and nutrient composition are affected by maternal nutritional status (27–29). Choline moieties in milk can be derived by active uptake from maternal circulation as well as by de novo syn-



**FIGURE 3.** The total choline content in rat milk varies directly with the choline content of the dam's diet. Rat dams were fed either a control diet (CT), a choline-deficient diet (CD), or a choline-supplemented diet (SUP) for 25 d. Milk samples were collected and analyzed as described in the text.  $\bar{x} \pm \text{SEM}$ ;  $n = 6$  per group. PCho, phosphocholine; GPCho, glycerophosphocholine; PtdCho, phosphatidylcholine. Bars with different superscript letters are significantly different,  $P < 0.05$ .

thesis within the mammary gland (30, 31). Human milk contains choline in the form of choline, phosphocholine, glycerophosphocholine, sphingomyelin, and phosphatidylcholine (22). Although choline concentrations were significantly lower in mature milk from mothers who delivered preterm infants, it is unlikely that developmental problems and liver disease that affect newborn preterm infants are related solely to a lower supply of choline (free choline) via preterm breast milk. We have suggested that formulas and milks with different compositions might deliver different amounts and forms of choline to target tissues. This may have consequences for the relative balance between use of choline as a methyl donor (via betaine), acetylcholine precursor (via choline), or phospholipid precursor (via phosphocholine and phosphatidylcholine) (26). Our study indicates that dietary intake of choline can influence the composition of milk.

These findings are particularly interesting because other investigators have previously described a significant, lifelong enhancement of the spatial memory capacity of rats exposed in utero or postnatally to choline supplementation (10, 11). At 60 d of age, these rats performed more accurately in a radial-arm maze task than did rats from control dams (11, 12). Choline-induced spatial memory enhancement correlated with altered distribution and morphology of septal neurons (32). The two critical periods when choline availability affects rat brain function are gestation days 12–17 and postnatal days 15–30 (10, 11, 32). The prenatal period is associated with neurogenesis of cholinergic cells involved in memory (33–35). The postnatal period is associated with synaptogenesis (36). It is possible that the ingestion of milks with different choline composition and content could affect brain development.

Therefore, until we fully understand perinatal choline requirements, it seems best to emulate the composition of human breast milk when milk substitutes are designed. ✉

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