Proton nmr studies of betaine excretion in the human neonate: consequences for choline and methyl group supply

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Proton NMR spectroscopy was used to determine the magnitude and temporal variation of betaine excretion in the human neonate and infant with respect to choline supply and demand. The results show that betaine excretion, which is present 1 day after birth, increases to reach a maximum of nearly 1.5 mol/mol creatinine at 2–3 months of age. Excretion then declines and is less than 0.2 mol/mol creatinine after 1 year. Comparison of estimated total excretion from these results with the calculated dietary choline (the precursor of betaine) intake from breast milk or proprietary feeds was made. The results indicated that during part, if not most, of the neonatal period betaine excretion might exceed the choline intake. The apparent insufficiency of dietary choline to satisfy both metabolic needs and the high betaine excretion suggests that of the two routes for remethylation of homocysteine to methionine, the folate-dependent pathway may be more important in the neonate. Consideration of the demand on methyl group supply for biosynthesis and for choline incorporation into phospholipids emphasizes the vital role played by remethylation in development and the possible need for alternative (endogenous) sources of choline.

Keywords: betaine; proton NMR spectroscopy; choline; neonate; methylation; nutrition

Introduction

In the course of investigating a number of inherited metabolic disorders in young children using ^{1}H-nmr spectroscopy, we detected large amounts of betaine in the urine of both affected and healthy neonates.[1,2] No betaine could be detected in urine from adult subjects using ^{1}H-nmr spectroscopy. However, betaine could be detected (≈0.2 mol/mol creatinine) on the first day after birth and excretion (expressed as creatinine equivalents) had increased after the first 7 days of life in human neonates;[2] in rats a peak of excretion was reached after 35–40 days (Rafter et al., unpublished).[2] We present here a study of betaine excretion in human subjects from the neonatal period to 20 months of age. The results are discussed with respect to the likely origin of betaine, its possible role in the body, and the demands on both betaine and choline in the developing infant. We also consider the related problem of methyl group supply, particularly in the perinatal period. A preliminary report of part of this work has been presented.[3]

Methods and materials

Patients

The patients studied were either from Northwick Park Hospital or The London Hospital. Ethical permission for the

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study was obtained. Urine samples were collected from healthy babies (aged 1–7 days) and older children. Samples were also obtained from children suffering from non-metabolic disorders including asthma, nocturnal enuresis, and psychosocial problems, as well as from children admitted for minor surgical procedures such as circumcision and tonsillectomy. Children who were acutely ill and those with evidence of hypoxia or ischaemia were excluded from the study. Urine samples were frozen and stored at −20° C until nmr analysis. Information on current nutritional intake and medications was recorded.

Estimation of betaine in neonatal feeds

To determine the betaine content of the major feeds that neonates receive, samples of breast milk, bovine milk, and various proprietary feeds were analyzed using ^{1}H-nmr spectroscopy. All feeds contained very high contents of lactose, e.g., the concentration in breast milk exceeds 250 mmol/L. The proton spectrum of lactose gives rise to an intense triplet from the C4 proton at 3.30 ppm, which partly overlaps the methyl resonance of betaine. Therefore fractionation of the samples was carried out to eliminate the lactose signals. The solid feeds were dissolved in water according to the manufacturer's instructions. A sample of each feed (1 mL) was acidified to a pH of 1 then applied to a Dowex (AG 50W-X8, 100–200 mesh H^+) cation exchange column. The column was then washed with 10 mL of H_2O to remove lactose and acidic components, and the basic components (including betaine) were eluted using 2 M ammonium hydroxide. Both fractions were then adjusted to pH 6–7, lyophilized, then redissolved in 0.5 mL of ^2H_2O before spectroscopy. A standard betaine solution (2 mmol/L) was treated similarly.

Estimate of choline demand (incorporation) in the human neonate

These data *(Table 2)* were derived from published values. The weights of tissues and organs are based on the measurements of Widdowson and Dickerson[4] (liver and brain) and Dickerson and Widdowson[5] (skeletal muscle) for newborn humans. The phospholipid content for liver (20 g/kg) was interpolated from the value for the human fetus at 32 weeks gestation (18.7 g/kg),[6] and the adult level (25 g/kg).[7] The phospholipid content for human skeletal muscle (psoas) was from Fletcher[8] (adult value) and for newborn brain from Svennerholm and Vanier.[9] Phosphatidylcholine (+ lysophosphatidylcholine) content and sphingomyelin (7%) content for liver, skeletal muscle (adult value), and brain (newborn) were from Kwitervich et al.,[7] Fletcher,[8] and Svennerholm and Vanier,[9] respectively. An approximate value for the molecular weight of phosphatidylcholine was taken as 600.

Nmr spectroscopy

All urine samples were analyzed at room temperature on either a Bruker AM250 or WH400 spectrometer (Queen Mary College, London, UK) operating at 250MHz and 400MHz for protons, respectively. A 500 μL sample of urine was placed in a 5mm nmr tube (Goss Scientific Instruments, Ingatestone, UK) to which 50 μL ^2H_2O (Goss Scientific Instruments, Ingatestone, UK) and 20 μL of 500 mmol/L sodium-3-(trimethylsilyl)-2,2,3,3-tetradeuteropropionate, ($TSPd_4$), (BDH Chemicals Ltd, Poole, UK) were added to act as a field frequency lock (to maintain the correct spectrometer field frequency) and a chemical shift reference

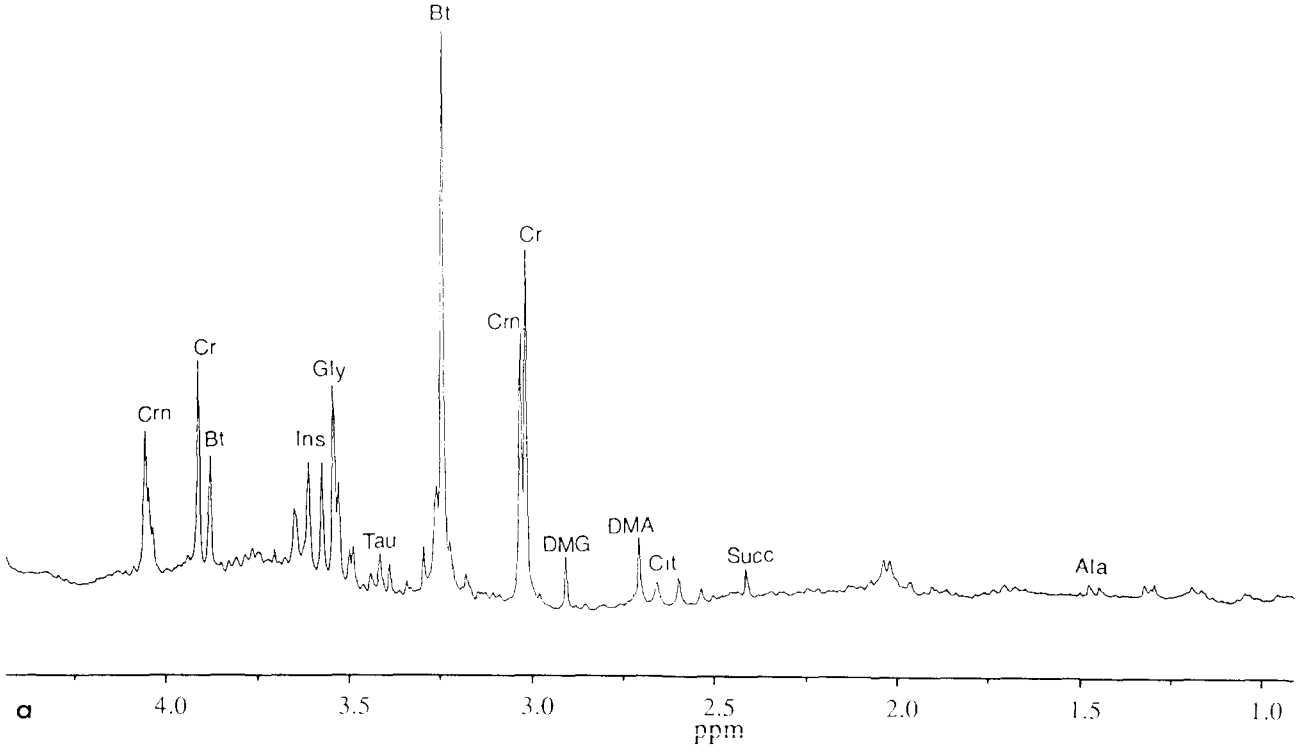

Figure 1 ^{1}H-nmr spectra of urine from human infants. The spectra were recorded at room temperature at 250MHz: (a) a 2-day-old neonate, the sum of 224 data accumulations; (b) a 3-month-old infant, the sum of 209 data accumulations; (c) a 3¼-year-old infant, the sum of 240 data accumulations. Chemical shifts are given with respect to $TSPd_4$. Ac, acetate; Ala, alanine; Bt, betaine; Cit, citrate; Cr, creatine; Crn, creatinine; DMA, dimethylamine; DMG, dimethylglycine; Gly, glycine; Ins, inositol; Lac, lactate; Succ, succinate; Tau, taurine; TMNO, trimethylamine-N-oxide.

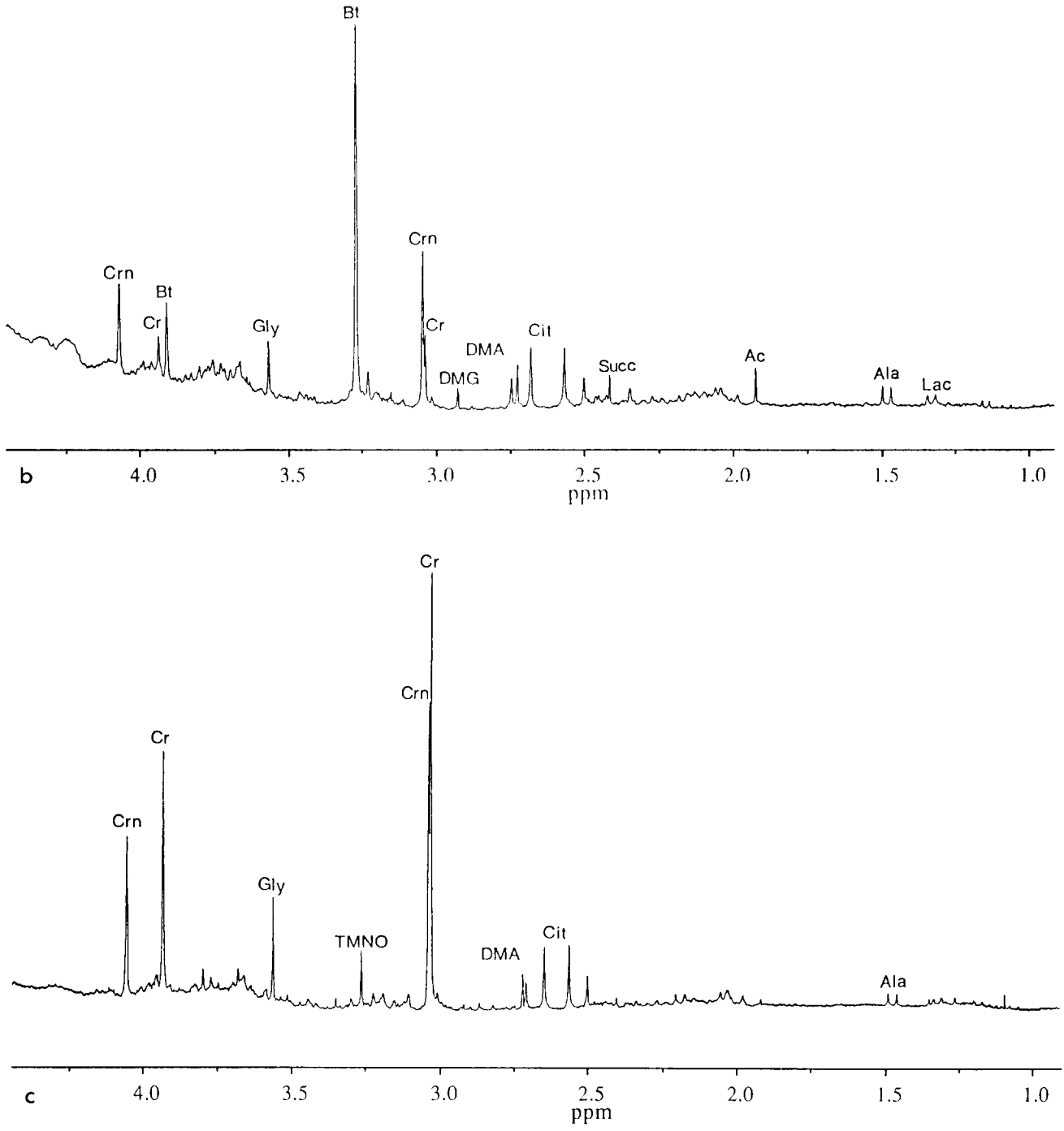

Figure 1 *continued*

signal, respectively. No further modification of the sample was necessary. A single pulse experiment was carried out using a 45° pulse angle and a 2-second delay between pulses. The water signal was suppressed by presaturation during the relaxation delay.

Results and discussion

Spectra from (a) a 2-day-old neonate, (b) a 3-month-old infant, and (c) a 3¼-year-old child are shown in *Figure 1*. All spectra show prominent signals from the methyl and methylene resonances of creatinine at 3.05 and 4.08 ppm, respectively. Spectra (a) and (b) also show resonances from the same groups in creatine at 3.04 and 3.94 ppm. In addition, in spectra (a) and (b) signals from the trimethyl-protons (3.26 ppm) and methylene protons of betaine (3.90 ppm) are clearly visible. In spectrum (c) the resonance at 3.26 ppm is not from betaine, as there is no corresponding signal at 3.90 ppm, but rather from trimethylamine-N-oxide (TMNO), which can be identified by the titration behavior of its chemical shift (pK_a = 4.5).

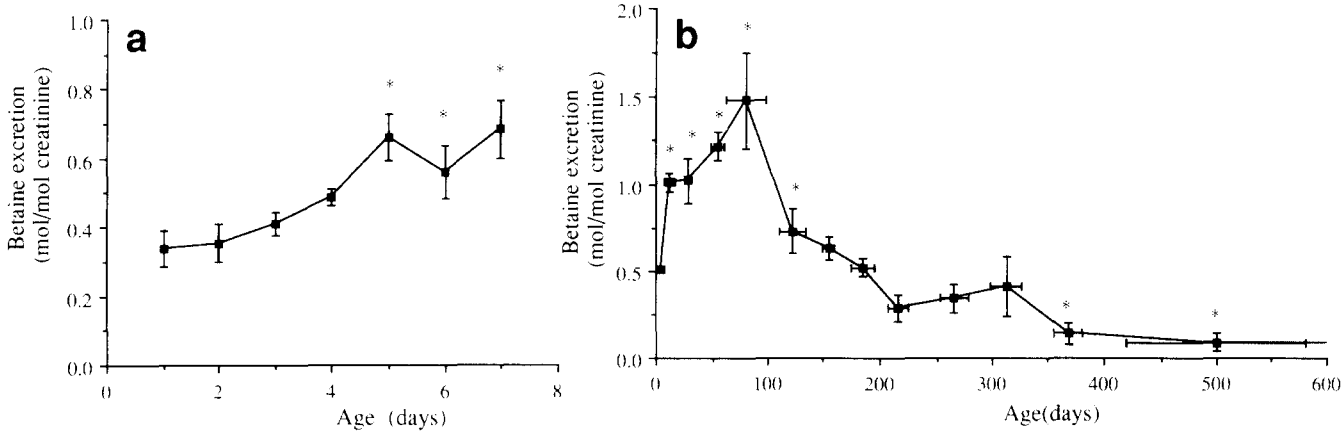

Figure 2 Betaine excretion in human neonates and infants. Betaine excretion is expressed as a molar fraction of creatinine excretion. Results are given as the mean ± 1 SEM calculated from the ^{1}H-nmr spectra. (a) Results for the first 7 days of life. The numbers of observations are as follows: day 1: 9; day 2: 10; day 3: 8; day 4: 7; day 5: 8; day 6: 5; day 7: 5. Asterisks (*) indicate significant differences ($P < 0.05$) from the mean for day 1 by analysis of variance (ANOVA). (b) Results for the first 20 months. The horizontal bars indicate the age ranges. The numbers of observations for each age range are between 6 and 13 except for the first week where $n = 52$. Asterisks (*) indicate significant differences ($P < 0.05$) from the mean for week 1 by ANOVA.

Figure 2 shows the betaine excretion expressed in terms of the ratio of the betaine:creatinine concentration ratio plotted against age. It can be seen that betaine excretion continues to rise after the early neonatal period, reaching a maximum somewhere between 2 and 3 months of age. The excretion then declines rapidly to <0.05 mol/mol creatinine at 3 years of age. Small amounts of a related metabolite dimethylglycine *(Figures 1a and 1b)* were detected but excretion was never more than 20% that of betaine.

At least 80% of the standard betaine solution was recovered after extraction. However, there were no detectable resonances at 3.26 or 3.90 ppm that corresponded to the position of the betaine signals in the proton spectra of any of the neonatal feed extracts. We estimate that a betaine concentration as low as 0.05 mmol/L would be detected because the trimethyl-resonance consists of nine equivalent protons. Similar spectra were obtained for all the feeds analyzed and we therefore conclude that the betaine content in the original feeds is less than 0.02 mmol/L.

Betaine excretion

These results show, therefore, that healthy infants excrete betaine in substantial amounts from birth, and that excretion increases until 2–3 months of age before declining. The possibility that the origin of excreted betaine is from dietary betaine would seem to be unlikely based on our measurements in a variety of neonatal feeds.

Two questions need to be addressed: first, why is the betaine excretion so much higher in neonates compared with adults, and second, what are the consequences of betaine loss for choline metabolism?

We have previously suggested that the excretion of betaine may be due either to leakage from the immature kidney or to an imbalance of betaine supply (from choline in the diet) versus utilization.[2] Bagnasco et al.[10] found that in the adult rabbit kidney, inner medulla betaine was present at a concentration of 17 μmol/g in diuresis, which rose to 35 μmol/g in antidiuresis. They suggested that betaine functioned in this organ as an osmolyte. It may be that the betaine content increases with age as the concentrating ability of the kidney improves.

The only known endogenous source of betaine is from choline via the choline oxidase system, which catalyses choline conversion to betaine via betaine aldehyde.[11,12] The greatest amount of activity of this enzyme occurs in the liver and kidney.[13] There is no information that we are aware of on the activity of the kidney enzyme in the neonatal period. However, Zeisel and Wurtman[14] found that the activity in the neonatal rat liver was very low during the first 10 days of life and then increased in an exponential manner to reach adult levels by 40 days. This increasing activity parallels the increase in betaine excretion until days 35–40, when excretion declines[2] (Rafter et al., unpublished). We are not aware of any information on the variation of choline oxidase activity in human organs during development.

If betaine accumulation occurred in the fetus, release of betaine postpartum might account for its excretion. However, this explanation could not account for the continuous increase in betaine excretion over 2–3 months, and we have not detected significant betaine concentrations (<0.2 mmol/L) in amniotic fluid.

Betaine could play an important role in the transfer of methyl groups to homocysteine to form methionine by the enzyme betaine-homocysteine methyltransferase (BHMT) *(Figure 3)*. The same transfer can also be catalysed by the enzyme 5-methyltetrahydrofolate-homocysteine methyltransferase (FHMT) using 5-methyltetrahydrofolate as a methyl group donor. Both

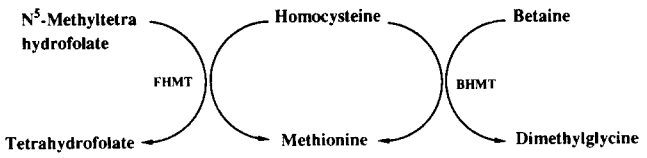

Figure 3 The formation of methionine by the action of 5-methyltetrahydrofolate-homocysteine (FHMT) and betaine-homocysteine methyltransferases (BHMT).

enzymes are present in the newborn rat and human liver in activities comparable with the adult liver, and their relative contribution to methyl transfer is unknown. There is evidence that although both systems are important for methyl group provision, the FHMT system is by far the most significant, at least in adults.[15] However, Finkelstein and Martin[16] found that if rats were injected with homocysteine, hepatic betaine was depleted. Human liver choline oxidase activity is much lower than that in the rat liver,[17] however, and we have found that the betaine content of adult human liver biopsies is much lower than in adult rats (Bell et al., unpublished). The conversion of betaine to dimethylglycine does occur in infants, as we have found the latter in the urine in variable amounts in both healthy children and patients *(Figure 1)*.[18] In addition, betaine has been used for treating infants with homocystinuria caused either by impairment of the FHMT reaction[19] or cystathionine synthase deficiency,[20] and we have detected increased dimethylglycine excretion after betaine therapy (Burns et al., unpublished). There is evidence therefore that betaine can supply methyl groups when FHMT activity is reduced, but its contribution in the healthy child, particularly in the neonatal period, is uncertain.

Choline supply and demand

In our previous report[2] we suggested that the choline intake was sufficient to account for the betaine excreted without allowing for incorporation of choline in the body. However, in the calculations below we show that this may not be true for all stages of development, especially when the body's choline demand is included.

Zeisel et al.[21] analysed the choline content of human milk, bovine milk, and several infant formulas. Choline was found in the free form and as phosphatidylcholine and sphingomyelin. Using their data for human milk we calculated the total choline intake for babies aged 1–30 days assuming an average daily milk intake of 150 mL/kg. This will be an overestimate for most children in the first 3 days after birth, when the milk intake is usually much lower (40–90 mL/kg). The intake for babies on proprietary feeds (based on bovine milk) over the same age range is shown assuming a similar intake. The results are shown in *Table 1* together with the estimated mean 24-hour betaine outputs for the corresponding days. These were calculated using our measured betaine:creatinine ratios *(Figure 2)* and a value for the mean creatinine output in the neonatal period of 105 μmol d^{-1} kg^{-1}.[22] The choline intake is greater than the betaine excretion on days 1 and 3 but the difference narrows considerably over the next 4 days. From days 7–9 the difference is marginal and by day 15 betaine excretion may exceed choline intake. Formula feeds based on bovine milk (without supplementation) would seem to supply less choline than betaine excreted at all times. As the choline content of breast milk remains at a similar level from days 15–90,[21] the disparity between choline intake and betaine excretion may be even greater in the 2–3 month period, when betaine excretion is at its peak *(Figure 2)*. Thus, during much of the neonatal period,

Table 1 Choline supply from human breast milk, bovine milk, and a typical proprietary feed compared with betaine excretion in the human neonate

Choline source	Age (days) 1	3	5	7	9	15	30
	Choline intake (choline + phosphatidylcholine + sphingomyelin) (μmol/kg body wt^{-1}/day^{-1})						
Human breast milk (hindmilk)	71	130	107	89	81	80	75
Bovine milk	53	53	53	53	53	53	53
Formula feed	59	59	59	59	59	59	59
	Betaine output (μmol/kg body $weight^{-1}$/day^{-1})						
	35	42	68	71	85	107	111
	Choline intake (human milk) − Betaine output (μmol/kg body $weight^{-1}$/day^{-1})						
	36	88	39	18	−4	−27	−36

Total choline was calculated using the data of Zeisel et al.[21] from the sum (choline + phosphatidylcholine + sphingomyelin). The intake of both human and formula feeds by the 1–30-day-old neonate was assumed to be approximately 150 mL/kg body wt^{-1}/day^{-1}. Daily betaine output was calculated from the betaine/creatinine ratio (Figure 2) and using a value for creatinine excretion of 105 μmol/day^{-1}/kg body wt^{-1}.[22] If the calculation is made by multiplying the concentration of betaine in urine by a mean urinary volume of 60 mL/kg body wt^{-1},[40] similar results are obtained.

when the choline requirement is particularly high, it is possible that dietary choline intake is insufficient for the body's requirements.

The greatest quantitative need for choline is presumably for phosphatidylcholine and to a lesser extent sphingomyelin, both of which are incorporated into membranes. An estimate for the body's requirement for choline for phosphatidylcholine synthesis in the neonatal period (1–30 days) can be made, making a number of assumptions. It is assumed that most of the new choline phospholipid is incorporated into liver, muscle, and brain, which contain the greatest bulk of cell membranes and the calculation is therefore an underestimate. Second, the mean rate of neonatal growth is taken as 25 g per day based on a mean growth rate for males and females of 750 g over the first month.[23,24] Third, it is assumed that the relative weights of liver,[4] muscle,[5] and brain[25] are constant over the same period. This would tend to underestimate the choline demand in the early neonatal period, when brain growth is relatively greater, and overestimate it in the later period when brain grows more slowly. Another factor leading to an underestimate of choline incorporation is the increase in phospholipid content of tissues that occurs during growth. The results are shown in *Table 2*. It can be seen that the addition of choline utilization as phosphatidylcholine to that lost as betaine excretion *(Table 1)* gives a net negative choline balance throughout most of the neonatal period. The discrepancy may be even greater because our calculations do not include choline demand in other tissues, and other fates. We have also assumed that dietary choline absorption is 100%, i.e., there is no loss in the intestine. It is known, however, that newborns have a reduced ability to digest phospholipids compared with adults[26] because of a lack of bile salts and pancreatic phospholipases.[27] There is also evidence that a variable amount of ingested choline is degraded in the gut, and excreted as trimethylamine-N-oxide *(Figure 1c)*.[28,29]

Alternative sources of choline

If the equivalent of most (if not all) of the choline intake is excreted as betaine alternative, choline sources become very important. The only known pathway for net choline synthesis in the body is by conversion of phosphatidylethanolamine to phosphatidylcholine by three successive methylations via the enzyme complex phosphatidylethanolamine-N-methylase.[30] The source of the methyl groups is initially from S-adenosyl methionine and ultimately from methionine. This enzyme has been characterized in the rat liver[30] but in the adult brain the activity was thought to be very low.[31] However, Blusztajn et al.[32] have detected N-methylase activity in both the neonatal and adult rat brain. Ethanolamine entering the brain could therefore provide an alternative source of choline provided that the supply of methyl groups was sufficient.

Methionine from the diet is thought to provide the greatest source of methyl groups in the adult. Krebs et al.[33] have calculated that the methionine content of the adult diet is equivalent to the daily requirement of methyl groups for creatine synthesis. Any requirements for methyl groups in excess of this need would therefore have to come from remethylation of homocysteine. In *Table 3* we calculated the methionine intake from breast milk using the data of Macy[34] and Rassin et al.,[35] for free methionine plus methionine as protein (mainly in lactalbumin and casein). The minimum creatine requirement based on the increase in skeletal muscle creatine, plus creatine and creatinine excretion is also given in *Table 3*. It can be seen that the methionine intake is well in excess of the creatine

Table 2 Estimate of choline demand (for phospholipid synthesis) in the human neonate

Tissue	Mass (% total body wt.)	Phospholipid content (g/kg organ^{-1})	Phosphatidylcholine content (g/kg organ^{-1})	Phosphatidylcholine (g/kg body wt^{-1}.)	Phosphatidylcholine (mmol/kg body wt^{-1}.)
Liver	5	20	8.8	0.44	0.73
Skeletal muscle	20	7	4.0	0.80	1.33
Brain	13	24	10.8	1.40	2.33

Whole body phosphatidylcholine (sum)	4.40	(mmol/kg body wt.$^{-1}$)
Whole body phosphatidylcholine + sphingomyelin (7%)	4.71	(mmol/kg body wt.$^{-1}$)
Daily choline demand for new tissue synthesis	118	μmol/25 g new body wt.$^{-1}$
Daily choline demand (day 1) = 118/3.5 =	34	μmol/kg body wt.$^{-1}$
Daily choline demand (day 30) = 118/4.2 =	28	μmol/kg body wt.$^{-1}$

It is assumed that the major demands for choline incorporation are for phosphatidylcholine and sphingomyelin in membranes and that skeletal muscle, liver, and brain are the main sites of phosphatidylcholine deposition. The calculations of choline demand also assume that a mean gain in body weight of 25 g per day occurs based on a mean body weight on day 1 of 3.5 kg and on day 30 of 4.2 kg.[23,24] The data are based on newborn values with the exception of the skeletal muscle (adult psoas) phospholipid and phosphatidylcholine contents. For further details see the Materials and methods section.

Table 3 Methionine content of human breast milk and bovine milk compared with creatine demand in the growing neonate

Age		Days 1–5	Days 6–10	Day 15+
Methionine intake	Breast milk	250	240	120
	Bovine milk	800	800	800
(μmol/kg body wt^{-1}/day^{-1})				
Creatine demand [skeletal muscle: (creatine + phosphocreatine) + excretion: (creatine + creatinine)]		150	175	200
(μmol/kg body wt^{-1}/day^{-1})				

The methionine data is from Macy.[34] The amount of methionine as the free amino-acid is very low compared with that in milk proteins, principally casein and lactalbumin.[35] The total methionine content of human milk declines over days 1–30.

requirement in the first 10 days of life in contrast to adults. This is perhaps to be expected as methionine is also needed for new protein synthesis. However, from day 15 forward the methionine intake is much lower than the creatine requirement. It is of interest that the methionine content of bovine milk is much higher than human milk, whereas the choline content is less.

These calculations therefore emphasize the vital role of remethylation of homocysteine for regeneration of methionine *(Figure 3)*. They also imply that net methylation of homocysteine by betaine would seem to be very unlikely in neonates more than 5 days old in view of the near equivalence of choline intake to betaine excretion. This would, in effect, require three methyl groups (from S-adenosyl methionine) to form choline to gain one methyl from the betaine-homocysteine methyltransferase system. (Two more methyl groups could eventually be recovered as "active" formaldehyde via the formyl-tetrahydrofolate system if betaine was further degraded through dimethylglycine and sarcosine to glycine).[36] Thus, the alternative methylating system based on methyltetrahydrofolate and 5-methyltetrahydrofolate-homocysteine methyltransferase would seem to be the major net supplier of methyl groups in the healthy neonate. Nevertheless, we recently found evidence for substantial activity of BHMT in the neonatal period by measuring a high rate of dimethylglycine production (equivalent to creatinine generation on a molar basis) in a patient with multiple acyl CoA dehydrogenase deficiency.[37]

The choline concentration in neonatal plasma measured immediately after birth is some 5–6 times that in adult plasma, similar to the rat.[15,38] It then falls rapidly over the next 2 weeks, approaching adult levels after only 7 days in contrast to the rat (after 18 days). In the human, and perhaps also in the rat, the fall may be due to a combination of falling choline content in the milk, increasing betaine excretion, and increasing demand on choline for phospholipid biosynthesis.

Whatever the explanation, the loss of such a large amount of betaine could be significant in situations of marginal choline intake, particularly if accompanied by a protein-deficient diet. Such situations exist in the malnourished child and also may occur in children receiving special formula feeds used in the treatment of inborn errors of metabolism. These diets, in addition to being low in protein, contain much less phosphatidylcholine than human milk.[39]

Preferential conversion of choline to betaine may result in insufficient choline for the developing brain and loss of betaine in the urine, and if sustained under these conditions, might cause ensuing methyl-group depletion and consequent severe metabolic repercussions. However, further studies of betaine output where choline intake is known to be marginal are required to determine this point.

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