Elevated Tissue Betaine Contents in Developing Rats Are Due to Dietary Betaine, Not to Synthesis¹,²

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Abstract
The time course of betaine accumulation and activities of enzymes involved in betaine metabolism were studied in developing rats. In study 1, pups weaned on a nonpurified diet had a transient increase in liver and kidney betaine content followed by a decline after ~42–56 d. In study 2, dams and, following weaning, pups were fed an AIN-93G (betaine-free) or an AIN-93G betaine-supplemented diet (0.3%) to determine the source of the transient increase in betaine levels previously observed. In study 2, only rats fed betaine had an increase in plasma betaine concentration. Similarly, liver and kidney betaine contents increased postweaning; however, betaine levels returned to that found in rats fed a betaine-free diet by 49 d of age. The dietary content of betaine fed to dams did not affect pup betaine. The activities of choline dehydrogenase, an enzyme of betaine synthesis, and betaine:homocysteine methyltransferase (BHMT), which is the only known betaine-consuming enzyme in mammals, were also measured in study 2. Liver BHMT activity decreased after weaning, whereas liver and kidney choline dehydrogenase activity increased with age, possibly reaching a plateau by 42 d of age. We conclude that the transient increase in betaine reflects high dietary betaine and not a change in endogenous betaine synthesis. J. Nutr. 138: 1641–1646, 2008.

Introduction
There has been a recent resurgence in research on betaine (also known as trimethylglycine or glycine-betaine) largely due to a number of potential therapeutic uses, including the treatment of mild hyperhomocysteinemia (1), nonalcoholic steatohepatitis (2), and alcohol-induced liver damage (3). Betaine is also important to renal function, because it is an intracellular osmolyte in the mammalian kidney (4). Betaine’s potential use as a therapeutic agent relates either directly to betaine metabolism (Fig. 1) or is facilitated by indirect effects thereof. Betaine can be obtained through the diet and enter cells, including hepatocytes, either via the γ-aminobutyric acid/betaine transporter or the amino acid transport system A (5). Endogenous synthesis of betaine occurs following entry of choline into the mitochondrion (6,7) by the 2-step dehydrogenation of choline by choline dehydrogenase (CHDH³; EC 1.1.99.1) and betaine aldehyde dehydrogenase (EC 1.2.1.8) (8,9). The only known route of betaine catabolism in animals is by conversion to dimethylglycine via the cytosolic enzyme betaine:homocysteine methyltransferase (BHMT; EC 2.1.1.5), with the dimethylglycine being further catabolized to methylglycine (sarcosine) and glycine (8). The BHMT reaction allows betaine to act as a labile methyl donor remethylating homocysteine (Hcy) to methionine. The liver is the primary site of betaine synthesis and catabolism (8); however, kidney also has betaine synthetic capacity and very low levels of BHMT (10).

Betaine is normally low in liver, but recent work by this laboratory on Hcy metabolism in Zucker diabetic fatty (ZDF) rats unexpectedly found very high levels of hepatic betaine in 35-d-old rats [17.6 and 9.9 μmol/g in ZDF fa/? (heterozygous) and ZDF fa/fa (homozygous), respectively], with liver betaine content displaying a marked decrease (5.3 and 2.5 μmol/g in ZDF fa/? and ZDF fa/fa, respectively) by 77 d (11). The plasma betaine concentration was much lower than that found in liver (<0.5 μmol/L). In the case of the 35-d ZDF fa/? rats, correcting these values to intracellular water [liver tissue is 45% hepatocellular water by mass (12)], betaine would account for ~10% of the total 305 mOsm/kg intracellular osmotically active substances. The lower liver betaine content in the ZDF fa/? rats was implicated with increased removal of Hcy (11); however, the source of the accumulated betaine, synthesis or diet, was not assessed. Developmental changes in betaine metabolism have received little detailed study, but the findings of Wijekoon et al. (11) suggest substantial alterations from the neonate to adult stage. The liver betaine content has been shown to increase several-fold with weaning (13) and remains high for

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³ Abbreviations used: BHMT, betaine-homocysteine methyltransferase; CHDH, choline dehydrogenase; DMG, dimethylglycine; DSS, 2,2-dimethyl-2-silapentane-5-sulfonate; Hcy, homocysteine; ZDF fa/fa, Zucker diabetic fatty rats (homozygous); ZDF fa/?, Zucker diabetic fatty rats (heterozygous).
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origin. We examined the time course of betaine accumulation in tissues in developing rats whose dams were maintained on either a betaine-containing or betaine-free diet and, following weaning, pups raised on betaine-containing or betaine-free diets to determine the source of the accumulated betaine. Until now, there have been no long-term studies, to our knowledge, comparing betaine-free and supplemented diets over the duration of early development. The activities of betaine-metabolizing enzymes were also determined to test the hypothesis that betaine synthetic capacity correlates with betaine concentration in the developing rat. In addition, the developmental pattern of enzymes of betaine metabolism and the effects of betaine supplementation on their pattern may illustrate if these enzymes play a role in the transitory nature of betaine accumulation.

**Materials and Methods**

**Animals and sampling procedures**

All animals used were Sprague-Dawley rats obtained from the Memorial University of Newfoundland Animal Care Unit. All animal care and animal husbandry was approved by and conducted in accordance to protocols established by Memorial University of Newfoundland's Institutional Animal Care Committee.

**Study 1 (nonpurified diet)**

Rats that were 7, 14, 21, 28, 35, and 42 d of age were obtained from the Memorial University of Newfoundland Animal Care Unit. Rats consumed nonpurified diet (Purina 5008) ad libitum except for 7-, 14-, and 21-d-old pups, which were still nursing from dams fed that diet. On the day of organ collection, rats were anesthetized with an intraperitoneal injection of sodium pentobarbital (65 mg/kg). Kidneys, liver, heart, brain, and muscle were removed, in that order, freeze-clamped, ground into a powder, and stored at −80°C until assays were performed. Muscle and heart from more than 1 rat (ages 7 and 14 d only) were combined to obtain enough tissue to perform enzyme assays and betaine analysis. Betaine was analyzed by NMR.

**Study 2 (AIN 93G with or without 0.3% added betaine)**

Animals and tissue sampling. Ten pregnant (5-d gestation) rats were obtained from the Memorial University of Newfoundland Animal Care Unit and started on AIN 93G diets (17) with (n = 5) or without (n = 5) added 0.3% betaine (20 μmol/g of diet). Dams were fed the same diet after the pups were born. Pups were weaned on d 21 or 22 and fed either the same diet as the dam or the alternative diet (dam + betaine, pup − betaine; dam + betaine, pup + betaine; dam − betaine, pup + betaine; dam − betaine, pup − betaine). Liver, whole kidney, and arterial blood was taken from pups aged 13–15 d, 20–22 d (prior to weaning), 27–30 d, 34–36 d, 41–43 d, and 48–50 d. For simplicity, these sample periods will be referred to by the median sample day, for example 13–15, 20–22, and 27–30 are referred to as d 14, 21, and 28, respectively. Liver and kidneys were freeze-clamped and assayed for CHDH and BHMT activity and betaine. Plasma was obtained by centrifuging blood at 3000 × g; 15 min at room temperature and frozen −80°C until analyzed. Betaine in plasma was measured by HPLC and by 1H NMR for liver and kidney extracts as described below.

**Enzyme assays.** A piece of previously frozen liver or kidney was ground into a fine powder with liquid nitrogen. Samples were diluted 1:5 with 50 mmol/L potassium phosphate buffer (pH 7.0) and homogenized with a Polytron (Brinkman Instruments). An aliquot of the crude homogenate was reserved for the choline dehydrogenase assay. The rest of the homogenate was centrifuged at 16,000 × g; 30 min at 4°C and the supernatant was removed and used to measure BHMT activities. BHMT (0.3 mg protein) was assayed using previously described methods (18). CHDH was assayed following Haubrick and Gerber (9) with modified assay conditions, which included: 0.15 mg protein, 2 mmol/L [methyl-14C] choline chloride (0.2 μCi), and 1 mmol/L phenazine methosulfate. Assay
volume was 50 μL and samples were incubated for 7.5 min. Protein concentrations were determined by the Biuret method using bovine serum albumin as a standard.

**Betaine determinations.** Betaine levels in the liver and kidneys were measured by 1H NMR (Bruker AVANCE 500 MHz). Briefly, previously frozen tissues were ground into a fine powder and homogenized in 6% perchloric acid (1:5). Homogenates were centrifuged at 16,000 × g; 30 min at 4°C and the supernatant collected. Sample pH was adjusted to 9 by adding 20% KOH. The sample was centrifuged at 16,000 × g; 5 min to pellet the precipitate. The supernatant was diluted 1:1 with 100 mmol/L sodium phosphate buffer (pH 9); 65 μL D2O (Cambridge Isotope Laboratories) and 6.5 μL 109 mmol/L sodium 2,2-dimethyl-2-silapentane-5-sulfonate (DSS; Cambridge Isotope Laboratories) was added to 585 μL sample in phosphate buffer (pH 9). Samples were transferred to a 5-mm NMR tube and 1-dimensional NMR spectra were acquired on each sample at ambient probe temperature as described by Lundberg et al. (19). The resonance of DSS was set to a chemical shift of 0.00 ppm. We calculated betaine concentrations from a standard curve using the ratio of peak height to the peak height of DSS (9 protons at 0.00 ppm).

Betaine levels in the plasma were measured using HPLC, because we thought that NMR might not be sensitive enough to detect betaine levels in plasma. The HPLC method is based on Ganzera et al. (20) with minor modifications. First, 50 μL plasma was added to 50 μL of 100 mmol/L KH2PO4. This sample was added to 900 μL derivatizing solution containing 66 mg 18-Crown-6 and 1390 mg 2,4-dibromoacetophenone in 100 mL acetonitrile. Samples were heated at 80°C for 60 min, allowed to cool for 5 min, and centrifuged at 2000 × g; 5 min at room temperature. Samples were analyzed on a Waters HPLC system equipped with a LC spectrophotometer, a 717 Wisp autosampler, and a 600E system controller and were quantitated using Millennium software. Separation was achieved using a Supelcosil LC-SCX column (250 × 4.6 mm, 5 μm) and a mobile phase consisting of a 70:30 mix of methanol and 25 mmol/L choline chloride (pH 2) with a flow rate of 0.8 mL/min and a detection wavelength of 454 nm. Results obtained by the 2 analytical techniques for betaine quantification yielded comparable results for samples from the same animal.

**Plasma Hcy.** Samples were prepared and analyzed for plasma Hcy by HPLC as described by Vester and Rasmussen (21).

**Statistical analysis**

For the initial exploratory experiment (study 1), means were compared with 1-way ANOVA followed by Tukey’s post hoc test. Comparisons between groups in Study 2 were assessed by general linear model with the independent factors being the maternal and pups’ diet and the pups’ age as well as the interaction between these factors. When significant interaction was found between treatments, pairwise comparisons were made using Tukey’s post hoc test. The relationship between plasma betaine concentration and tissue betaine concentrations was analyzed by linear regression. Growth rates in study 2 were determined by fitting an exponential growth curve to the mass data for individual rats. For all statistical analyses, P < 0.05 was considered significant with the exception of growth rates that were compared by overlapping 95% CI. When necessary, prior to conducting ANOVA, data were log transformed to normalize residuals. Values presented are means ± SD.

**Results**

**Study 1 (nonpurified diet)**

The nonpurified diet contained ~20 μmol/g betaine (mean of 3 separate determinations measured by NMR). Tissue betaine was low until pups were weaned, at which point liver and kidney betaine contents increased markedly (Fig. 2). Betaine levels in liver and kidney peaked at d 28 (1 wk postweaning) to d 42 and began declining by d 56. Heart betaine had a small but sustained increase, whereas brain betaine content decreased continually during the experiment (Fig. 2).

**Study 2 (AIN 93G diet supplemented with 0.3% betaine)**

Having shown that Sprague-Dawley rats had a temporally transient increase in betaine when fed a nonpurified diet, which is a substantial source of betaine, we conducted all further experiments on rats fed a purified diet.

**Growth rates.** The diets did not affect the growth rate of rats as determined by fitting data to an exponential curve (P < 0.001; r² ≥ 0.93 for all; data not shown). The growth rates of pups in the 4 groups ranged from 4.8 to 5.5%/d.

**Betaine contents.** From d 14 to 21, prior to weaning, there was no effect of treatment on betaine in plasma, liver, or kidney (Fig. 3). Following weaning, plasma, liver, and kidney betaine increased in the pups fed a betaine-supplemented diet irrespective of the mothers’ diet. Throughout the experiment, only the pups’ diet affected betaine accumulation, whereas the mothers’ diet did not affect betaine content in the pups. From d 42 to 49, liver betaine content was not affected by the pups’ diets (Fig. 3B). Similarly, following a plateau, plasma (Fig. 3A) and kidney (Fig. 3B) betaine content decreased and the groups did not differ on d 49.
Enzyme activities. Neither maternal nor pup diets affected liver enzyme activities. Hepatic BHMT activity was high from d 14 to 21 (Fig. 4A) followed by a decline from d 21 to 27. Hepatic BHMT activity remained relatively constant after d 27 and CHDH gradually increased over time, reaching a plateau at 35–42 d (Fig. 4B). Kidney BHMT activity was low and difficult to detect (data not shown) and so these data were not analyzed. As in the liver, kidney CHDH increased in all groups over time (Fig. 4C).

Plasma betaine concentration and tissue accumulation. When liver or kidney betaine content was plotted in relation to plasma betaine concentration across the wide range of values found in this study, there were positive correlations with slopes of 15.2 and 13.9 μmol betaine·g tissue⁻¹/μmol betaine·L plasma⁻¹ for liver ($r^2 = 0.48; P < 0.001$) and kidney ($r^2 = 0.63; P < 0.001$), respectively (Fig. 5). These data indicate a substantial concentration gradient between the extracellular fluid (micromolar) and intracellular fluid (millimolar) of these tissues.

Plasma Hcy. Circulating levels of Hcy in rat pups were not affected by betaine feeding in dams or by betaine supplementation in the pups’ diet (Fig. 6). Because experimental treatment did not affect plasma Hcy concentration, data from all treatments combined illustrate that plasma Hcy was constant throughout the study from 14 to 42 d of age for all treatments but increased at d 48.

Discussion

The current study has confirmed the transient increase in betaine levels in young developing rats fed nonpurified and purified diets and has identified exogenous betaine supply as the source of this accumulated betaine. Thus, the accumulated betaine is not a...
result of endogenous synthesis, which would be suggestive of some physiological function in development. Instead, this phenomenon is a response to high dietary intake, with the accumulation likely representing hepatic betaine clearance being less than dietary intake in the younger rats in this study. However, betaine is synthesized by the rats fed the betaine-free diet, as illustrated by the substantial increase in total liver betaine content (Fig. 7), spanning well over an order of magnitude. This increase in the betaine-free groups is likely a result of choline metabolism; choline was present as 0.25% choline bitartrate in both diets. The similarity between betaine-free and -supplemented diets at d 42 and 49 suggests that in older rats, even if betaine must be synthesized, hepatic betaine is maintained at a regulated level.

Maternal betaine dietary content had a negligible effect on pup betaine content or enzymes of betaine metabolism. This indicates that either the maternal synthesis of betaine for the pups was sufficient or the pups could synthesize adequate betaine. Given the high total choline (including all choline-containing compounds) and very low betaine concentration of rat milk (22), it seems unlikely that maternal betaine supply to pups is an important factor in development. This also suggests that the elevated liver BHMT activity (Fig. 4A) prior to weaning may be involved in removal of betaine produced by the metabolism of any excess choline-containing compounds supplied in the milk. The high BHMT activity in preweaned rats, along with the normally high choline utilization associated with this period of high growth, may also partly explain why there was no accumulation of betaine until the postweaning stage when liver BHMT activity decreased.

Betaine feeding and subsequent elevation of betaine levels had little effect on enzymes of betaine metabolism (Fig. 4). A number of studies have shown activation of liver BHMT with increased dietary betaine or the betaine precursor choline (10,23–25); however, many of these studies required a methionine-deficient diet to display large increases in BHMT activity. The consistent pattern between liver and kidney CHDH, and among treatments within each tissue, indicates a strong developmental trend of increasing choline oxidative capacity, consistent with previous studies (14,15).

A substantial concentration gradient has been demonstrated between the extracellular fluid and the intracellular fluid of the liver and kidney (Fig. 5) and the tissue content rise in parallel with increasing plasma betaine; however, it is unclear if increased plasma betaine is due to insufficient betaine removal via BHMT or if the transporters responsible for betaine uptake into the liver and kidney are below saturation. In the later case, the increasing plasma concentration may result in an increased rate of inward transport and establish a higher intracellular concentration.

Betaine levels decreased in the supplemented rats by the end of the study to the same level as that found in rats fed the betaine-free diet (Fig. 3). This was not coincident with an increase in BHMT activity, which remained constant from d 35 to 49 (Fig. 4A). An increase in transport capacity into the hepatocyte would also fail to explain the decline in betaine, because even in the betaine-free diet where intracellular betaine concentrations would be in the mmol/L range, the intracellular betaine concentration in the liver would be expected to greatly exceed the Michaelis constant for BHMT, ~48 μmol/L in rat liver (26). Although daily food consumption was not determined in the current study, it is well established that food intake decreases with age. For example, Chen and Nyomba (27)
reported an ~30% decrease in daily food intake (as percent body mass) from d 21–28 to d 42–49. The liver is the primary tissue of betaine metabolism and removal and it may be expected that liver betaine levels would decline in advance of plasma if flux through hepatic BHMT is the limiting site of betaine metabolism. Consistent with a decrease in dietary intake and a subsequent reduced requirement to metabolize betaine, the liver betaine levels decline before plasma or kidney levels. However, the present study indicates that there is still need for further study of hepatic betaine metabolism and handling, particularly in developing animals where marked changes occur.

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