BUTTERWORTH HEINEMANN

Methods of Nutritional Biochemistry

A method for the determination of betaine in tissues using high performance liquid chromatography

Mei-Heng H. Mar, Todd W. Ridky,* Sanford C. Garner,† and Steven H. Zeisel

*Department of Nutrition, Schools of Public Health and Medicine, University of North Carolina at Chapel Hill, Chapel Hill, NC USA; *Department of Chemistry, University of North Carolina at Chapel Hill, Chapel Hill, NC USA; and †Department of Surgery, Duke University School of Medicine, Durham, NC USA*

Betaine is a major metabolite of choline in liver and kidney and may be an important product of choline metabolism in other tissues. The available methods for assay of betaine, however, are time consuming and relatively insensitive. We describe a modification of published methods that provides a sensitive and specific assay for betaine by derivatization and HPLC separation with UV quantitation. Betaine and other water-soluble choline metabolites are extracted from biological samples and separated by HPLC based on mobility of ^{14}C-labeled internal standards. The betaine fraction is collected and derivatized with 4'-bromo-phenacyl triflate. The betaine-triflate derivative is quantitated by UV absorbance and the result is corrected for possible losses due to incomplete extraction recovery and incomplete derivatization by simultaneous measurement of radioactivity from the derivatized ^{14}C-betaine internal standard. Betaine concentrations determined with this procedure are reported for several adult and fetal rat tissues. (J. Nutr. Biochem. 6:392–398, 1995.)

Keywords: betaine; choline; HPLC; assay

Introduction

Betaine is the final oxidation product of choline metabolism.[1,2] In liver, betaine is an important donor of methyl groups for the regeneration of homocysteine.[3–5] In the kidney cells,[6,7] bacteria,[8–11] and in cells of many plants,[12,13] betaine serves as an osmolyte. We have recently found that betaine is a major metabolite of choline in isolated human trophoblasts[14] and in the placenta of rats (unpublished data). Betaine is present in milk[15]; however, its role in infant nutrition is not known.

Address reprint requests to Dr. Zeisel at the Department of Nutrition, Schools of Public Health and Medicine, CB# 7400, University of North Carolina at Chapel Hill, Chapel Hill, NC 27599-7400, USA.

Received January 27, 1995; revised February 15, 1995; accepted February 17, 1995.

Nutritional Biochemistry 6:392–398, 1995

655 Avenue of the Americas, New York, NY 10010

0955-2863/95/$10.00
SSDI 0955-2863(95)00046-3

Administration of betaine may protect the liver from ethanol-induced fatty infiltration.[5]

The previous methods for betaine assay have involved chemical conversion to betaine periodide,[16] which has a sensitivity of approximately 50 nmol of betaine. The concentration of betaine in most tissues is low, and a comparatively large amount of sample is required when the periodide method is used. Another method used betaine:homocysteine methyltransferase and radiolabeled betaine to assay betaine concentrations.[17] Unfortunately, the methyltransferase is not commercially available, and its purification from liver is time consuming. Recently, Lever et al.[18] and Minkler et al.[19] published methods for assay of betaine using nonenzymatic derivatization to an ultraviolet-absorbing compound. We have modified these published methods to use a single high performance liquid chromatography (HPLC) column and solvent system to separate betaine from other carboxylic acid–containing compounds and spectrophotometric detection of a 4′-bromophenacyl triflate derivative of betaine[19] with correction for extraction recovery and derivatization efficiency with a ^{14}C-betaine internal standard.

Methods and materials

Reagents

All reagents were obtained from Fisher Chemicals unless otherwise noted: 2-(2-ethoxyethoxy)ethanol (Aldrich E455-0), 4-bromobenzoyl chloride (Aldrich B 5,920-9), acetic acid (HPLC grade), acetonitrile (HPLC grade), activated carbon (Aldrich 16,155-1), ammonium acetate, anhydrous diethyl ether, choline chloride, [methyl-^{14}C] (NEC-141, New England Nuclear, Boston, MA), choline oxidase (Sigma Chemical Co., St. Louis, MO USA), diazald (Aldrich D 2,800-0), ethanol, magnesium oxide, methylene chloride, pentane, petroleum ether, potassium hydroxide, sodium phosphate (monobasic), sulfur dioxide (Aldrich 29,569-8), and trifluoromethanesulfonic acid (Aldrich 15,853-4).

Apparatus

The equipment used included a diazomethane generator (Aldrich AL-180), a separatory funnel (1 L), HPLC with a UV detector, and an HPLC column (Pecosphere-3x8C Cartridge, 0258-0167, Perkin Elmer, Norwalk, CT USA). The safety equipment used included a fume hood, and a blast shield (Aldrich Co., Milwaukee, WI).

Safety precautions

Because of the highly toxic and explosive nature of diazomethane, all reactions involving its preparation and use should be carried out in an efficient chemical fume hood and behind a safety shield. Rough surfaces are proven initiators of detonations of diazomethane, so all apparatus used for generating diazomethane should be Clear-Seal joint. The complete generating apparatus is available from Aldrich Chemical Company. Sulfur dioxide is a corrosive gas. A stainless steel body regulator is needed to dispense SO_2 from the storage cylinder. The gas cylinder must be secured to prevent accidental tipping and valve damage.

Procedures

The derivatizing agent, 4′-bromophenacyl triflate, is synthesized from an intermediate compound, 4′-bromo-2-diazoacetophenone, which is formed in a two-step procedure that must be completed as a continuous process in which diazomethane is generated and added immediately to a solution of 4-bromobenzoyl chloride in diethyl ether.

Synthesis of 4′-bromo-2-diazoacetophenone. We assembled the diazomethane generating equipment as described in the Aldrich technical information bulletin number AL-180. We used a 1 L round-bottom flask to collect the diazomethane solution and a 250 mL round-bottom flask for the reaction vessel. We then added 84 mL of 2-(2-ethoxyethoxy)ethanol, 48 mL of diethyl ether, and a solution of 15.06 g of KOH in 24 mL water to the reaction vessel. A separatory funnel was placed over the reaction vessel and the funnel was charged with a solution of Diazald (30.12 g in 271 mL of diethyl ether). The reaction vessel was warmed to 65°C with a water bath.

The Diazald solution was then added so that the rate of addition equaled the rate of distillation. When all the Diazald had been added, 60 mL of diethyl ether was slowly added and the distillation was continued until the distillate was colorless. The collected diazo-

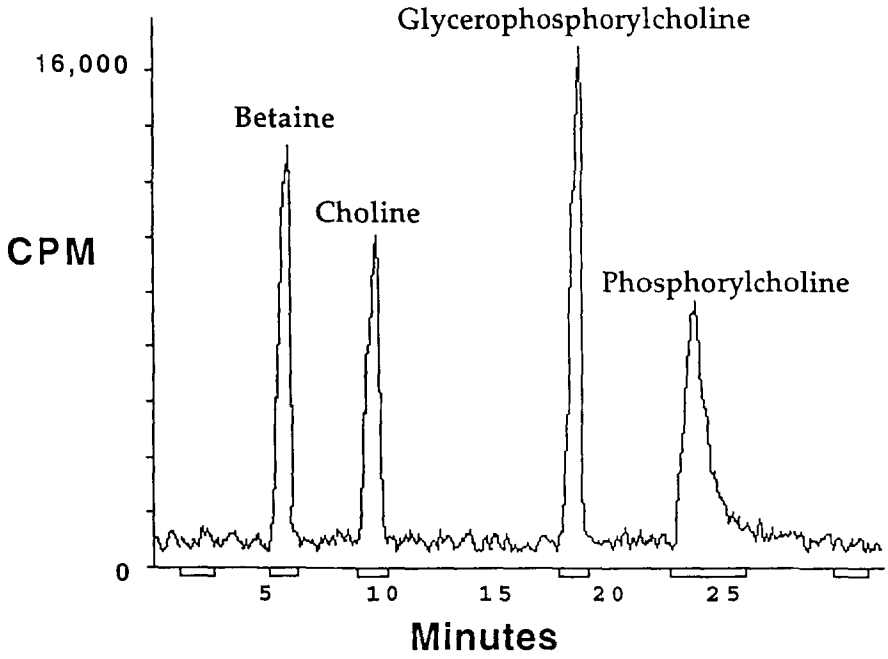

Figure 1 HPLC separation of betaine from other choline metabolites. Betaine, choline, glycerophosphocholine, and phosphocholine may be separated from tissue extracts by HPLC of the water-soluble material from a chloroform/methanol/water extraction. Radiolabeled choline metabolites are added before extraction to serve as markers for on-line radiometric detection and for the calculation of recovery efficiency. This procedure also separates betaine from carnitine, a potential interfering substance in the derivatization.

methane solution was kept cold in an ice bath. The total volume of the diazomethane/ether solution was approximately 400 mL. If necessary, ether was added to get a final volume of 400 mL.

We added 4-bromobenzoyl chloride solution (8.8 g of 4-bromobenzoyl chloride in 40 mL of diethyl ether), with stirring, to the freshly generated diazomethane/ether solution over a period of 20 to 30 min. The reaction mixture was incubated in an ice bath for 8 to 9 hr and left to stand at room temperature for 3 hr. The solvent was then evaporated in a vacuum. The residue was recrystallized with an ether/petroleum ether mixture (50/50, vol/vol). The product had the appearance of fine yellow needles with a melting point of 123.5 to 124°C.[11] The yield in our laboratory was 92% (8.3 g). The crystals of 4′-bromo-2-diazoacetophenone may be stored in a desiccator at room temperature until needed to complete the synthesis of triflate.

Synthesis of 4′-bromophenacyl triflate. In a dry ice–isopropanol bath, 50 mL of anhydrous sulfur dioxide was condensed in a 100 mL round-bottomed flask containing a stirring bar and fitted with a Claisen head, gas inlet tube, and calcium sulfate–filled drying tube. 4′-Bromo-2-diazoacetophenone, 2.25 g (0.1 mol), was added and stirred for 5 min. Then 1.5 g (0.9 mL, 0.1 mol) of anhydrous trifluoro-methanesulfonic acid was added. The flask was closed and we continued stirring for 15 min and then let the flask stand for 30 min. without the cooling bath.

At the end of the reaction, the solvent was evaporated under the hood by continuous stirring. We dissolved the brown residue in 100 mL of boiling methylene chloride. The solution was treated twice with 5 g of activated carbon and filtered through filter paper. The filtrate was evaporated to dryness. The residue was recrystallized from pentene-methylene chloride (80:20, vol/vol). The yield in our laboratory was 2.3 g (64%) of 4′-bromophenyacyl triflate as colorless flake, m.p. 137 to 138°C.

Preparation of ^{14}C-betaine. ^{14}C-betaine is required as a marker for radiometric detection of the betaine peak from the HPLC separation and as an internal standard for recovery of betaine from tissue. It is prepared from choline chloride, [methyl-^{14}C] in a reaction catalyzed by choline oxidase.[1] Sodium phosphate buffer (100 μL, 0.2 M, pH 7.8), containing 1 μmol of ^{14}C-choline chloride and 0.08 U of choline oxidase was incubated for 1 hr at 37°C. At the end of the incubation, 400 μL of methanol and 200 μL of chloroform were added to extract the product. One hundred microliters of $CHCl_3$ and 100 μL of water were

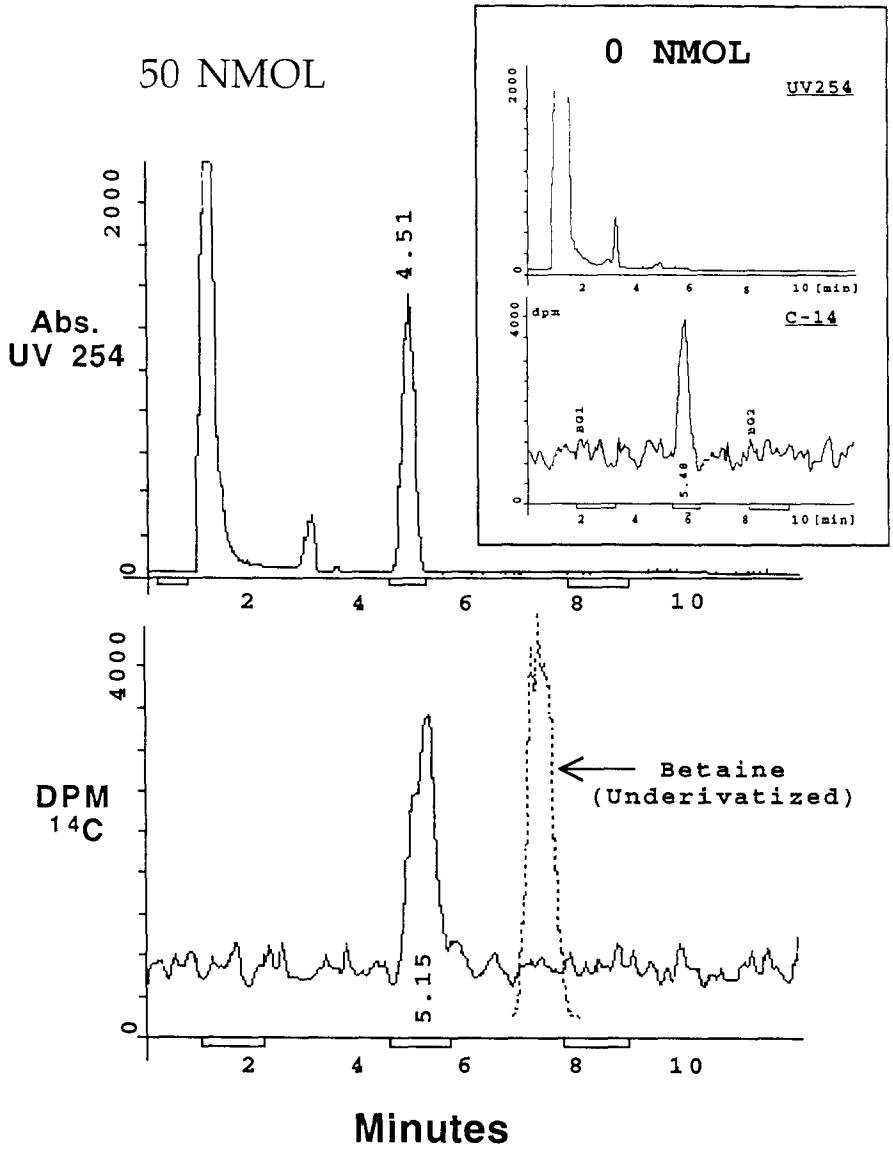

Figure 2 HPLC of betaine derivative. UV absorption and ^{14}C-betaine profiles obtained after derivatization of 50 nmol of betaine are illustrated. The insert in the upper half of the figure shows the lack of UV absorbance when 0 nmol of unlabeled and underivatized betaine were added to the radiolabeled betaine. The peak shown by the dashed line in the lower half of the figure was taken from another HPLC run and illustrates the shift in elution position that occurs with derivatization.

added to separate the aqueous and organic phases. The aqueous phase (top) containing ^{14}C-betaine was dried down and ^{14}C-betaine was purified by HPLC (see HPLC procedure below).

Betaine extraction

Sample preparation. Tissue samples were collected by freeze-clamping in liquid nitrogen and are stored at −80°C until assayed. Tissues were pulverized under liquid nitrogen, approximately 100 mg of pulverized tissue was placed in a 1.5 mL microfuge tube, and 400 μL of MeOH/$CHCl_3$ (2:1) mixture was added immediately. Approximately 10,000 dpm of ^{14}C-betaine was added to the extraction medium to permit detection of eluted peaks with an on-line radiometric detector and to serve as an internal standard in the derivatization procedure. The samples are extracted using the method of Bligh and Dyer.[20] The extraction medium was vortexed vigorously and left at −20°C for at least 1 hr. At the end of the extraction, samples were centrifuged at 5,000 rpm for 5 min. The supernatant was trans-

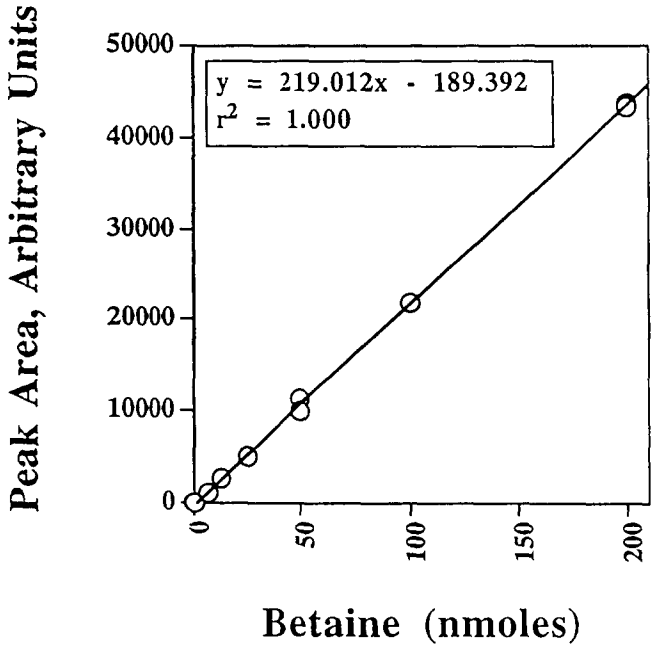

Figure 3 Betaine standard curve. Betaine standards ranging from 0 to 200 (0, 6.25, 12.5, 25, 50, 100, and 200) nmol were derivatized and the peak areas were determined by UV absorbance. Duplicate samples were included at each concentration.

ferred to a new tube. The residue was then re-extracted with 250 μL of MeOH/$CHCl_3$/water (2:1:0.8). The supernatants were combined and 100 μL of $CHCl_3$ and 100 μL of water were added to form two phases. The entire aqueous phase (top) was dried in a Speed-Vac (Savant instruments, Farmingdale, NY) and resuspended in 10 μL of water and 125 μL of methanol. After centrifugation, 100 μL of the supernatant was injected onto the HPLC. The betaine fraction (*Figure 1*) was collected and dried down for derivatization.

Betaine standard curve. Aqueous betaine standards ranging from 6.25 to 200 nmol, plus a blank (0 sample), were prepared to generate a calibration curve (*Figure 3*). The standards were also spiked with ^{14}C-betaine but are not carried through the extraction procedure. Otherwise, the standards were treated in exactly the same manner as the samples during the derivatization. The lowest standard was reliably distinguished from the blank.

HPLC separation of betaine. Betaine and other water-soluble choline metabolites were separated by the HPLC system of Pomfret et al.[21]

HPLC system. The HPLC system consisted of a microprocessor-controlled solvent delivery system (Series 4, Perkin-Elmer), a Gilson 231/401 autoinjector (Gilson, Inc., Middleton, WI USA), a Gilson model 118 UV-VIS detector (Gilson, Inc.), a radioactivity monitor (LB506C, Berthold, Inc., Nashua, NH USA), an Isco Foxy Fraction collector (Isco, Inc. Lincoln, NE USA), a Berthold data system (LB 506 C-1 HPLC system, Berthold Analytical Instrument Inc.), a silica guard column (Supelguard LC-Si: 2cm, Supelco, Inc., Bellefonte, PA USA), and a silica column (Pecosphere-3x8C Cartridge, Perkin-Elmer). The column was kept at room temperature and the flow rate was 1.5 mL/min. Buffer A contained acetonitrile/ethanol/acetic acid/1 M ammonium acetate/water/0.1 M sodium phosphate (800/68/2/3/127/10, vol/vol). Buffer B contained the same constituents but in different portions (400/68/44/88/400/10, vol/vol).

The column was equilibrated for 6 min at a flow rate of 1.5 mL/min in 100% Buffer A. After injection, Buffer A was delivered for 6 min, which elutes betaine from the column (*Figure 1*). Over a period of 15 min, solvents were switched to 100% Buffer B using a concave gradient (Perkin-Elmer series 4, curve 3), during which time choline and glycerophosphorylcholine (GPCho) were eluted. Buffer B was then delivered for 10 min, eluting phosphorylcholine (PCho). The initial conditions were restored (100% Buffer A) over 1 min using a linear gradient. Fractions were collected and dried in a vacuum centrifuge (Speed-Vac; Savant).

Table 1 Betaine concentrations in biological tissues

Tissue	Betaine concentration (nmol/g)
Adult liver (female)	589 ± 80
Placenta	2,615 ± 277
Fetal liver	338 ± 17
Fetal brain	193 ± 7
Fetal carcass remnant	115 ± 7

Tissues were collected from adult, nonmated female Sprague–Dawley rats. Placentas and fetal tissues were collected from pregnant rats on the 17th day of gestation. All animals were fed AIN-76A formulation pelleted diet (Dyets, PA). Results are mean ± standard error for at least five samples per point.

Derivatizing procedure. The dried betaine fraction was dissolved in 20 μL of methanol and 180 μL of acetonitrile, and 20 μL of a 10% suspension of magnesium oxide in water was added. After vortex mixing, 50 μL of derivatizing agent (100 mM 4′-bromo-phenacyl triflate in acetonitrile) was added. All the tubes were vortexed in a multitube vortexer for 5 min. After centrifugation for 5 min at 2,000 rpm, the supernatant was transferred to an autosampler and injected into the HPLC system.

The HPLC system was as described earlier. Only Buffer A was used to elute the betaine derivative. The run time was 12 min. For quantifying the betaine derivatives, a UV detector was added in front of the radiometric detector (dual channel detection) (*Figure 2*).

Interfering substances

Compounds containing a carboxylic acid group were very likely to react with 4-bromophenacyl triflate as described by Ingalls et al.[22] and Minkler et al.[19] Choline, betaine aldehyde, betaine, and carnitine were tested in the reaction. Choline and betaine aldehyde were not reactive with the derivatizing agent. Both betaine and carnitine, however, reacted to form the derivative. Although the retention time of derivatized betaine and carnitine are identical, betaine was effectively separated from carnitine during the initial HPLC separation procedure.

Results and discussion

Betaine is one of the major choline metabolites in the liver. Although sensitive methods for assay choline metabolites exist, assaying betaine has been more difficult. A complete description of the fate of ingested choline, however, must include determination of betaine formation as well as other choline metabolites. There are several advantages of using the procedures as described here. First, it allows us to collect all major water-soluble choline metabolites, including betaine. Using this extraction and HPLC separation procedure, betaine can be separated from other potential interfering components found in liver, primarily carnitine. Although choline and betaine aldehyde do not react with derivatizing agent, carnitine does, and the retention times of the carnitine derivative and the betaine derivative are identical. Second, only a single HPLC system is required for the assay. The HPLC system that is used for the separation of betaine from other choline metabolites and from carnitine can also be used for quantifying betaine derivatives by adding a UV detector in front of the radioactivity detector. Only one solvent system (Buffer A) is needed for elution of the betaine derivative, and the run time can be reduced to 10 to 12 min per sample.

Both the extraction of betaine and the derivatization with triflate may be less than complete for some samples. However, the assay method incorporates an internal standard (^{14}C-betaine) that provides information to correct the measured quantity of derivatized betaine for losses in either extraction or derivatization. The internal standard is added to the tissue sample at the first step of the extraction and the radioactivity in the betaine derivative peak is measured in series with the ultraviolet absorption profile.

We have used this procedure to assay betaine in several tissues, including

placenta, liver of both adult and fetal rats, and fetal brain. The results obtained with these tissues are shown in *Table 1*.

This assay procedure is approximately 10 times as sensitive as the periodide method of Barak and Tuma,[16] (6.25 nmol vs. 50 nmol of betaine). The assay is also quite reproducible, with an intraassay coefficient of variation (CV) of 4.9% and an interassay CV of 9.5% for samples assayed on 6 separate days. When liver samples were spiked with known amounts of pure betaine, the recovery was 93.4 ± 4.8% (mean ± SEM).

Acknowledgments

This work was funded by a grant from the National Institutes of Health (AG09525).

References

1 Zhang, J., Blusztajn, J.K., and Zeisel, S.H. (1992). Measurement of the formation of betaine aldehyde and betaine in rat liver mitochondria by a high pressure liquid chromatography-radioenzymatic assay. *Biochim. Biophys. Acta* **1117,** 333–339

2 Zeisel, S.H. and Blusztajn, J.K. (1994). Choline and human nutrition. *Ann. Rev. Nutr.* **14,** 269–296

3 Finkelstein, J.D., Martin, J.J., Harris, B.J., and Kyle, W.E. (1982). Regulation of the betaine content of rat liver. *Arch. Biochem. Biophys.* **218,** 169–173

4 Finkelstein, J.D., Martin, J.J., Harris, B.J., and Kyle, W.E. (1983). Regulation of hepatic betaine-homocysteine methyltransferase by dietary betaine. *J. Nutr.* **113,** 519–521

5 Barak, A.J., Beckenhauer, H.C., Junnila, M., and Tuma, D.J. (1993). Dietary betaine promotes generation of hepatic S-adenosylmethionine and protects the liver from ethanol-induced fatty infiltration. *Alcohol Clin. Exp. Res.* **17,** 552–555

6 Garcia-Perez, A., and Burg, M.B. (1991). Role of organic osmolytes in adaptation of renal cells to high osmolality. *J. Membr. Biol.* **119,** 1–13

7 Nakanishi, T. and Burg, M.B. (1989). Osmoregulatory fluxes of *myo*-inositol and betaine in renal cells. *Am. J. Physiol.* **257,** C964–C970

8 Landfald, B. and Strom, A.R. (1986). Choline-glycine betaine pathway confers a high level of osmotic tolerance in Escherichia coli. *J. Bacteriol.* **165,** 849–855

9 Smith, L.T., Pocard, J.A., Bernard, T., and Le, R.D. (1988). Osmotic control of glycine betaine biosynthesis and degradation in Rhizobium meliloti. *J. Bacteriol.* **170,** 3142–3149

10 Abee, T., Palmen, R., Hellingwerf, K.J., and Konings, W.N. (1990). Osmoregulation in Rhodobacter sphaeroides. *J. Bacteriol.* **172,** 149–154

11 Cayley, S., Lewis, B.A., and Record, M.T.J. (1992). Origins of the osmoprotective properties of betaine and proline in Escherichia coli K-12. *J. Bacteriol.* **174,** 1586–1595

12 McCue, K.F. and Hanson, A.D. (1992). Salt-inducible betaine aldehyde dehydrogenase from sugar beet: cDNA cloning and expression. *Plant Mol. Biol.* **18,** 1–11

13 Eichenberger, W., Araki, S., and Muller, D.G. (1993). Betaine lipids and phospholipids in brown algae. *Phytochemistry.* **34,** 1323–1333

14 Garner, S.C., Chou, S.-C., Mar, M.-H., Coleman, R.A., and Zeisel, S.H. (1993). Characterization of choline metabolism and secretion by human placenta trophoblasts in culture. *Biochem. Biophys. Acta.* **1168,** 358–364

15 Rohlfs, E.M., Garner, S.C., Mar, M.-H., and Zeisel, S.H. (1993). Glycerophosphocholine and phosphocholine are the major choline metabolites in rat milk. *J. Nutr.* **123,** 1762–1768

16 Barak, A.J. and Tuma, D.J. (1979). A simplified procedure for the determination of betaine in liver. *Lipids.* **14,** 860–863

17 Martin, J.J. and Finkelstein, J.D. (1981). Enzymatic determination of betaine in rat tissue. *Analyt. Biochem.* **111,** 72–76

18 Lever, M., Bason, L., Leaver, C., Hayman, C.M., and Chambers, S.T. (1992). Same-day batch measurement of glycine betaine, carnitine, and other betaines in biological material. *Anal. Biochem.* **205,** 14–21

19 Minkler, P.E., Ingalls, S.T., Kormos, L.S., Weir, D.E., and Hoppel, C.L. (1984). Determination of carnitine, butyrobetaine, and betaine as 4′-bromophenacyl ester derivatives by high-performance liquid chromatography. *J. Chromatog.* **336,** 271–283

20 Bligh, E.G. and Dyer, W.J. (1959). A rapid method of total lipid extraction and purification. *Can. J. Biochem. Physiol.* **37,** 911–917

21 Pomfret, E.A., daCosta, K., Schurman, L.L., and Zeisel, S.H. (1989). Measurement of choline and choline metabolite concentrations using high-pressure liquid chromatography and gas chromatography-mass spectrometry. *Anal. Biochem.* **180,** 85–90

22 Ingalls, S.T., Minkler, P.E., and Hoppel, C.L. (1984). Derivation of carboxylic acids by reaction with 4′-bromophenacyl trifluoromethanesculfonate prior to determination by high-performance liquid chromatography. *J. Chromatog.* **299,** 365–376