13 Betaine

Betaine: osmolyte and methyl donor

Michael Lever; Sandy Slow; Per M. Ueland

13.1 What is betaine?

In the 1860s, Scheibler isolated an organic base from sugar beet (*Beta vulgaris*) which he named 'betaine' (Scheibler, 1869, 1870), and he showed that it was *N,N,N*-trimethylglycine (►Fig. 13.1). Betaine is a highly polar but neutral zwitterionic compound that is very soluble in water. As its isolation shows, betaine is a natural product, and now commercially produced betaine is a by-product of the sugar beet industry. Subsequently other natural products have been isolated which are chemically related to betaine, and these are generically called 'betaines'; some examples are shown in ►Fig. 13.1. For this reason the compound Scheibler described is often called 'glycine betaine'. Although several betaines are found in the human diet (de Zwart et al., 2003), the only other betaine that has a known metabolic role in mammals is carnitine.

Retail outlets selling dietary supplements are likely to have betaine, usually called 'trimethylglycine' or 'TMG', on their shelves with claims that it gives health benefits ranging from preventing vascular disease to correcting autism. These claims are not justified by the available evidence, and one of the aims of this chapter is to present a realistic assessment of what role betaine might have in health, and the limitations on our current understanding.

13.2 Sources and availability of betaine

Betaine either comes from our food, or we convert dietary choline into betaine. The relative importance of these sources has not been well documented, but it could be expected to vary with dietary patterns. Both choline and betaine are essential for human well-being, but although dietary choline can, in principle, supply all the requirements for both choline and betaine, the converse is not true. Nevertheless when the dietary supply of choline is limiting, the choline-sparing effect of dietary betaine (Dilger et al., 2007) could become nutritionally important.

In western style diets, betaine is mostly obtained from cereals, particularly wheat (Sakamoto et al., 2002; Zeisel et al., 2003; de Zwart et al., 2003). Another important source is the beet family, which includes popular vegetables such as spinach, chard and beetroot. Several estimates of daily intake have given essentially concordant results (Slow et al., 2005; Cho et al., 2006; Chiuve et al., 2007; Bidulescu et al., 2007; Detopoulou et al., 2008), and most people in the various population groups that were studied had intakes between 100 and 300 mg/day. The actual value will not be the same in different

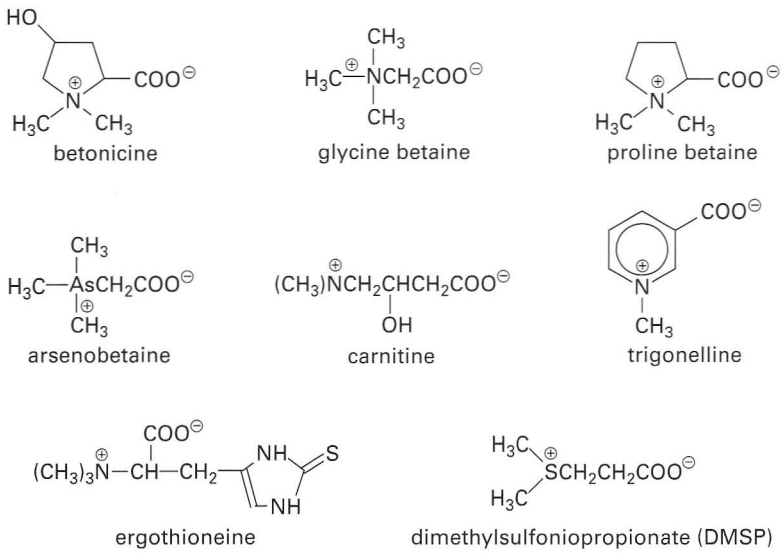


Fig. 13.1: Structures of betaine (glycine betaine) and analogues that are found in the diet.

populations, because it depends both on the diet and on the growing conditions of food plants; this latter is because many plants use betaine as an osmolyte, and accumulate more of it when grown on saline soils or when water deprived (Slow et al., 2008). In some of the earlier literature there were suggestions that the daily intake of betaine might be commonly over a gram a day (Craig, 2004; Olthof and Verhoef, 2005), but more recent estimates support a lower intake, and we have found (Elmslie, unpublished) that it is difficult to design an acceptable diet to supply more than approximately 800 mg/day long-term without supplementation. A factor which affects the actual betaine content of the diet, is food preparation. Betaine is highly water soluble, and boiled vegetables lose most of their betaine in the cooking water. By contrast, betaine is stable to oven cooking (de Zwart et al., 2003).

Because betaine occurs entirely as a simple solute, which is highly water soluble and is not protein bound, it would be expected that the bioavailability of dietary betaine and of betaine supplements would be similar, and this is the case (Atkinson et al., 2008). The details of betaine uptake are not well documented, but this uptake is efficient. There are at least three betaine transport systems in the mammalian gut (Thwaites and Anderson, 2007). In chickens (Kettunen et al., 2001c), as in rats (Slow et al., 2009), betaine is actively accumulated in the intestinal linings where this accumulation appears to be osmoregulated (Kettunen et al., 2001a) and stabilizes the mucosal structure (Kettunen et al., 2001c). Labeled betaine supplied orally to chickens is rapidly distributed through the body into various tissues and the methyl groups used to form tissue methionine (Kettunen et al., 2001b). Betaine absorption is obviously similarly rapid in humans (Schwahn et al., 2003; Atkinson et al., 2008) and there is no reason to believe that the mechanisms are not similar. Of the other betaines in the diet (de Zwart et al., 2003; Slow et al., 2005) only proline betaine (present in large amounts in citrus foods, including orange juice, and in legume sprouts) needs to be considered, because it inhibits the

renal resorption of glycine betaine (Lever et al., 2004; Atkinson et al., 2007) and can lead to an apparently abnormal excretion of betaine by healthy subjects. The effect is not sufficient to acutely affect plasma homocysteine concentrations (Atkinson et al., 2007), although it suggests that we need to be cautious about using orange juice in experimental protocols involving one-carbon metabolism. Trigonelline (found in coffee and legume sprouts) does not have a similar effect and trigonelline does not explain the homocysteine-raising effect of coffee (Slow et al., 2004b).

13.3 Betaine functions and metabolism

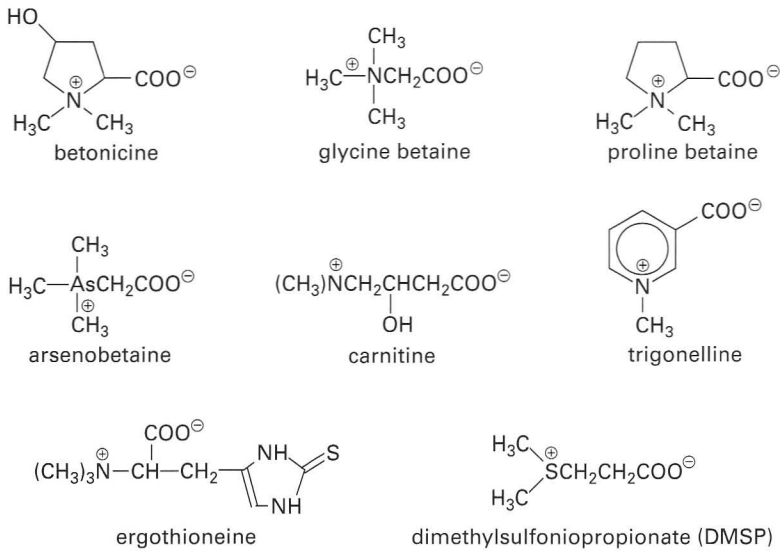
13.3.1 Physiological roles of betaine

Betaine has two important roles in mammalian physiology. One is as a major osmolyte, accumulated in most tissues to assist cell volume regulation (Feng et al., 2001; Schliess and Häussinger, 2002; Lang, 2007). The other role is as a methyl donor for the remethylation of homocysteine to methionine. Tissue betaine concentrations are higher than plasma concentrations in almost all rat organs (Slow et al., 2009), and often reach millimolar concentrations (exceptional concentrations, >100 mM, are possible in the renal medulla). The osmoregulated betaine transporter BGT-1 was originally identified in the kidney (Yamauchi et al., 1992; Kempson and Montrose, 2004) and it has since been shown that it is expressed in many tissues (Warskulat et al., 1995, 2004, 2009; Zhang et al., 1996; Denkert et al., 1998; Weik et al., 1998; Petronini et al., 2000; Olsen et al., 2005; Rainesalo et al., 2005). There are several other mammalian betaine transporters (Thwaites and Anderson, 2007), including the carnitine transporter OCTN2 (Pochini et al., 2004) and betaine transport by these other systems could be osmoregulated in some cells (Petronini et al., 2000; Alfieri et al., 2002, 2004; Anas et al., 2007): none of the transporters are specific for betaine. Osmolyte-mediated volume regulation is under tight control (Häussinger, 2004) and its disruption has serious consequences, including apoptosis (Häussinger, 1996; Dmitrieva and Burg, 2005; Reinehr and Häussinger, 2006). Thus an adequate supply of betaine is needed for tissue integrity. As with most osmolytes, betaine is also a 'compensatory' or 'counteracting' solute (Gilles, 1997) that enhances the stability of proteins, and it is (along with other chemically related osmolytes containing a trimethylamine functional group) particularly effective at countering the denaturing effect of urea (Yancey and Somero, 1979; Yancey and Burg, 1990; Burg et al., 1996; Venkatesu et al., 2009), a function that is particularly important in the renal medulla (Burg et al., 1996).

The second function for tissue betaine is a supply of methyl groups for the remethylation of homocysteine to methionine by the enzyme betaine-homocysteine methyltransferase (BHMT). The two functions of betaine interact, because BHMT is osmoregulated (Delgado-Reyes and Garrow, 2005; Schäfer et al., 2007), with high tonicity reducing its expression, so that betaine metabolism (and hence the mobilization of methyl groups) decreases when osmolyte concentrations need to be maintained.

13.3.2 Metabolic pathways involving betaine

A claim that humans cannot convert choline to the betaine (Barak et al., 2002; Sparks et al., 2006) is an error and dietary choline is undoubtedly an important source of



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betaine, and hence methyl groups. Betaine is an intermediate in the metabolism of choline, and dietary choline is an important source of betaine, and hence methyl groups, for humans. Choline is converted to betaine in two steps involving the enzymes choline dehydrogenase and betaine aldehyde dehydrogenase (►Fig. 13.2). Choline dehydrogenase and betaine aldehyde dehydrogenase are often grouped together as the 'choline oxidase' system, which can cause confusion (Olthof et al., 2005a; Li et al., 2007; Mato et al., 2008; Batra and Devasagayam, 2009). Choline oxidase (EC 1.1.3.17) is correctly a bacterial enzyme that catalyzes the conversion of choline to betaine by molecular oxygen (Rozwadowski et al., 1991; Fan and Gadda, 2005). In mammals choline dehydrogenase (EC 1.1.99.1) converts choline to betaine aldehyde (►Fig. 13.2), which is then oxidized to betaine by a separate enzyme (Zhang et al., 1992), betaine aldehyde dehydrogenase (EC 1.2.1.8), the two enzymes being co-localized in mitochondria (Chern and Pietruszko, 1999). Betaine synthesis from choline probably occurs only in the liver and kidney as the enzymes necessary for its oxidation have not been found outside these tissues, although there is an early report of choline oxidation to betaine in the hamster intestinal mucosa (Flower et al., 1972). The primary control point of this process is the transport of choline into the mitochondria (Porter et al., 1992; Kaplan et al., 1993; O'Donoghue et al., 2009). Choline can enter the mitochondria via a high capacity non-saturable diffusion process, dependent on a high membrane potential, or it can be transported via a low-capacity high affinity choline transporter, which has been identified in the inner membrane in isolated rat liver and kidney mitochondria (Porter et al., 1992; Kaplan et al., 1993; O'Donoghue et al., 2009). It has been estimated that at physiological choline concentrations the transport-mediated process is dominant in both liver and kidney (90% and 96%, respectively) (Kaplan et al., 1993; O'Donoghue et al., 2009). The choline transport rate in the kidney is estimated to be approximately five to six times that of the liver, possibly reflecting a higher synthesis of betaine from choline in the kidney where betaine is accumulated to high levels and functions as an osmolyte (O'Donoghue et al., 2009). The uptake of choline into both liver and kidney mitochondria is not coupled to betaine efflux, which is believed to be by passive diffusion as no active transport process has been identified.

In the reaction mediated by betaine homocysteine methyltransferase (BHMT) a methyl group is transferred from betaine to homocysteine, forming dimethylglycine (DMG) and methionine (►Fig. 13.2). The BHMT remethylation pathway contributes as much as 50% of the homocysteine methylation capacity of the liver (Finkelstein and Martin, 1984), implying an important role for betaine in the generation and maintenance of methionine and *S*-adenosylmethionine (SAM) concentrations. BHMT is a zinc metalloprotein (Garrow, 1996; Millian and Garrow, 1998), accounting for most of the bound zinc in the liver. It is cytosolic, makes up 0.5–2% of the soluble liver protein and is associated with the microtubules (Sandu et al., 2000). It has been highly conserved in chordate evolution and no metabolic abnormalities associated with low or absent BHMT are known, implying an essential role. In humans, BHMT is expressed in the liver, renal cortex and lens (Sunden et al., 1997), but although BHMT is thought to be primarily a liver and kidney enzyme, it is expressed in other cells and in early embryonic development (Anas et al., 2008). Disruption of BHMT should cause disturbed homocysteine metabolism, which in turn will lower SAM concentrations and reduce methylation capacity, and this is shown in mice when BHMT activity is inhibited by the potent and specific inhibitor *S*-(δ-carboxybutyl)-DL-homocysteine. This causes significant increases

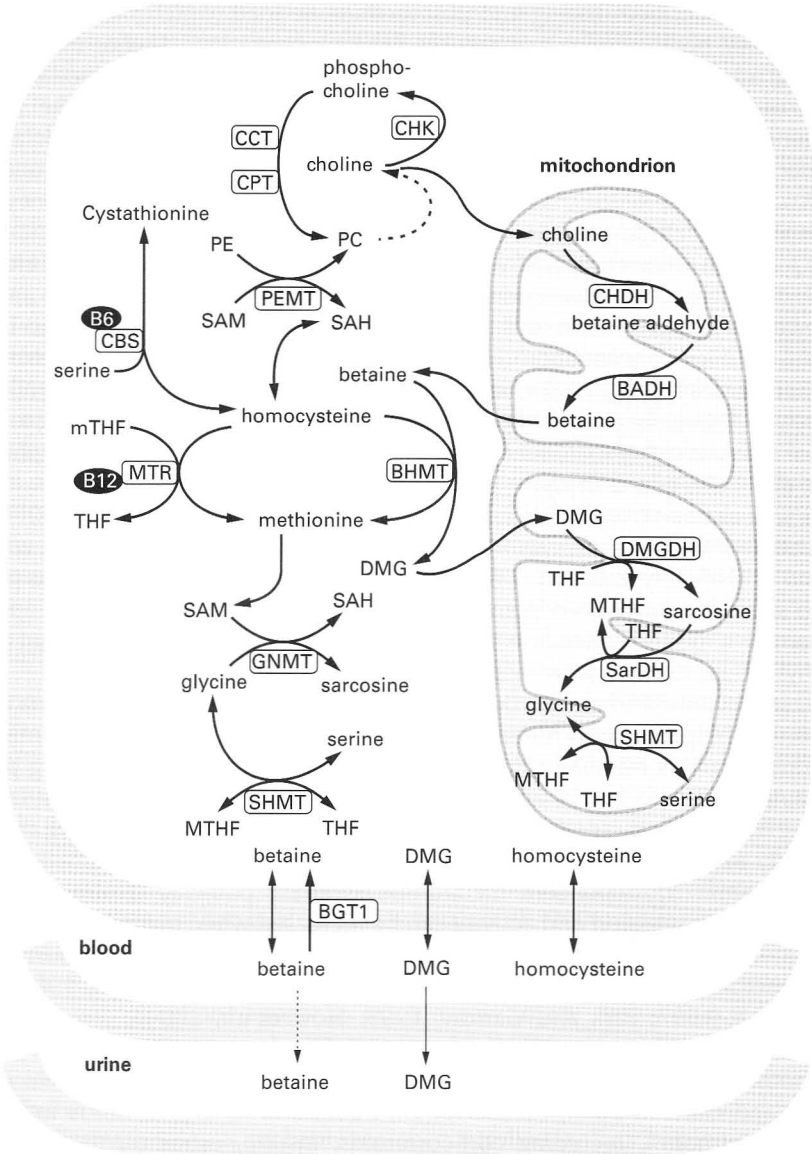

Fig. 13.2: Paths for betaine metabolism. *Metabolite abbreviations:* PE, phosphatidylethanolamine; PC, phosphatidylcholine; THF, tetrahydrofolate; mTHF, 5,10-methylenetetrahydrofolate; MTHF, 5-methyltetrahydrofolate; DMG, *N,N*-dimethylglycine; SAM, *S*-adenosylmethionine; SAH, *S*-adenosylhomocysteine. *Enzyme abbreviations:* CCT, CTP:phosphocholine cytidyltransferase; CPT, CDP-choline:diacylglycerol cholinephosphotransferase; CHK, choline kinase; CBS, cystathionine β-synthase; PEMT, phoshoethanolamine methyltransferase; CHDH, choline dehydrogenase; BADH, betaine aldehyde dehydrogenase; MTR, methionine synthase; BHMT, betaine-homocysteine methyltransferase; DMGDH, dimethylglycine dehydrogenase; GNMT, glycine *N*-methyltransferase; SarDH, sarcosine dehydrogenase; SHMT, serine hydroxymethyltransferase; BGT1, an osmoregulated betaine transporter.

(up to 7-fold) of both fasting and post-methionine load (which mimics the fed-state) plasma total homocysteine and reduces SAM concentrations (Collinsova et al., 2006). To date, 25 single nucleotide polymorphisms (SNPs) have been identified in the *BHMT* gene, of which only four encode non-synonymous changes that alter the amino acid sequence, none of the amino acid substitutions are near the active site or at intermolecular protein-protein contacts (Li et al., 2008). Of the *BHMT* SNPs, only one, the 742 G>A (c.716G>A), R239Q, is relatively common with a minor allele frequency of >10% (Li et al., 2008). There seems to be no difference in the stability or enzymatic activity of the Q isoform when compared with the R isoform (Weisburg et al., 2003). However, Li et al. (2008) have recently reported that *in vitro* the K_m for homocysteine and betaine is lower for the Q isoform. In humans, *BHMT* 742G>A was found not to be related to plasma tHcy concentration (Heil et al., 2000; Morin et al., 2003; Weisberg et al., 2003; Fredriksen et al., 2007), but a recent large epidemiological study demonstrated a decrease in dimethylglycine (the product of the BHMT reaction) according to the number of 742A alleles (Fredriksen et al., 2007), suggesting that this polymorphism could have metabolic effects *in vivo*.

As well as BHMT, mammals express another gene product called BHMT2. This name was coined when a gene was identified that encoded a putative protein with a 73% amino acid identity to BHMT (Chadwick et al., 2000). The gene product has proven not to be stable but might co-oligomerize with BHMT (Li et al., 2008), and it appears that its name is a misnomer because it does not use betaine as a substrate; instead it transfers methyl groups from *S*-methylmethionine (Szegedi et al., 2008).

Plasma betaine and urinary betaine excretion are determinants of plasma homocysteine, and this presumably reflects the importance of BHMT in one-carbon metabolism. Betaine becomes the major determinant of fasting homocysteine during pregnancy (Velzing-Arts et al., 2005), and is almost certainly the main determinant in the non-fasting state. It is particularly important when the supply of folate is limiting as the BHMT pathway appears to compensate for the lower homocysteine remethylation via the folate/vitamin B_{12}-dependent methionine synthase pathway (Holm et al., 2007). BHMT gene expression and activity can be increased when methyl donor availability (betaine, choline or various betaine analogues) is increased, particularly when dietary methionine is limiting (Emmert et al., 1996; Finkelstein et al., 1982a,b, 1983; Park et al., 1997; Park and Garrow, 1999; Slow et al., 2004a; Slow and Garrow, 2006). BHMT activity is subject to feedback inhibition, strongly by DMG and weakly by methionine (Allen et al., 1993). It is also regulated by osmotic stress (Delgado-Reyes and Garrow, 2005; Schäfer et al., 2007) and its expression is influenced by various hormones, including corticosteroids, insulin, estradiol, thyroid hormones and testosterone (Finkelstein et al., 1971; Park and Garrow, 1999; Shibata et al., 2003; Wijekoon et al., 2005; Ratnam et al., 2006). In an elderly cohort we found that the strongest predictors of plasma betaine were estradiol and cortisol (Storer et al., unpublished), which is consistent with the control of BHMT in rat liver (Finkelstein et al., 1971).

Aside from methionine, the other product of the BHMT-catalyzed reaction is *N,N*-dimethylglycine (DMG; ►Fig. 13.2). This is the only known route in mammals to this metabolite, so the presence of DMG is evidence that homocysteine has been methylated to methionine by BHMT. DMG is a potent feed-back inhibitor of BHMT (Finkelstein et al., 1972) and is further metabolized by the mitochondrial enzyme

dimethylglycine dehydrogenase; this is an oxidative demethylation, coupled to the electron-transport chain. Dimethylglycine dehydrogenase is a flavoprotein and converts the methyl group into a one-carbon unit attached to tetrahydrofolate (forming 5,10-methylene THF). The other product is monomethyl glycine, sarcosine, which, in turn, can be demethylated by sarcosine dehydrogenase, a very similar (indeed, highly homologous) enzyme to dimethylglycine dehydrogenase, with glycine as the final product. Sarcosine is not an unambiguous marker of this pathway because it is also the product of the methylation of glycine by SAM, catalyzed by glycine *N*-methyltransferase. Glycine methylation by glycine *N*-methyltransferase controls methionine and SAM concentrations, and hence the distribution of methyl groups. Elevated methionine leads to increased expression of glycine *N*-methyltransferase (Rowling et al., 2002) and thus causes an increased consumption of *S*-adenosylmethionine, and consequently homocysteine. Because much of the homocysteine so produced is remethylated to methionine the carbon skeleton of homocysteine is not lost. As a result of this cyclic process, a large dose of betaine makes only a small difference to the plasma homocysteine concentration; plasma homocysteine is not an equilibrium concentration but a steady-state concentration (Hoffer, 2004; Mudd et al., 2007), and increasing the supply of methyl groups only results in a small shift in the steady state concentrations of methionine and homocysteine.

13.3.3 Plasma and urine betaine

Plasma betaine concentrations are usually much lower than tissue concentrations (Slow et al., 2009). Despite the importance of betaine as an osmolyte, plasma concentrations are little affected by osmotic stress, and appear to remain stable for years (Lever et al., 2004). Concentrations are higher in men than in women (Lever et al., 1994a; Konstantinova et al., 2008a), and this sex difference is also seen in rats (Slow et al., 2009). Thus it would seem that plasma betaine is an inherent and presumably genetically controlled individual characteristic, however, plasma betaine concentrations are affected by the dietary intake of betaine (Alfan et al., 2004; Schwab et al., 2006; Atkinson et al., 2008, 2009). Plasma betaine increases in a dose-related manner with increased betaine intake (Schwab et al., 2006), but when subjects change their daily betaine intake the fasting plasma concentrations appear to reach a new steady state within a few days (Alfan et al., 2004; Atkinson et al., 2009) (►Fig. 13.3). This observation explains why there appears to be long-term stability despite the dietary effects on plasma concentrations, and individual subjects maintain similar plasma betaine concentrations for years (►Fig. 13.4). Plasma betaine is probably mainly controlled by liver betaine concentrations (Slow et al., 2009), which, in turn, are controlled by the activity of BHMT. Given the control of BHMT, the overall effect is that plasma betaine concentrations are highly individual (Lever et al., 2004, 2009a), but plasma concentrations of the betaine metabolite, dimethylglycine, do not show similar control (Lever et al., 2009a); plasma dimethylglycine does increase slightly after a betaine load, but the effect is small and transitory. The supply of folate (necessary for the *de novo* generation of methyl groups) also affects plasma betaine concentrations (Melse-Boonstra et al., 2005) and they are lowered by folate deficiency in some patients (Allen et al., 1993). Folate-deficient patients had elevated serum dimethylglycine, which is consistent with an increased use of betaine as a source of methyl groups (Allen et al., 1993).

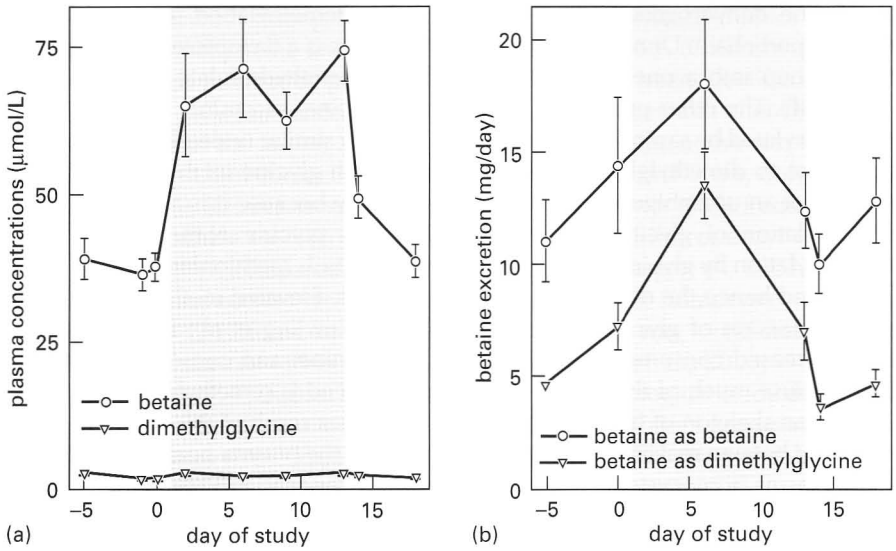

Fig. 13.3: Effect of a betaine supplement (1 g/day), taken during the shaded period, on 8 healthy young male subjects. Data are given as means and standard errors of the means. (a) Plasma betaine and *N,N*-dimethylglycine concentrations. (b) Urinary excretion of betaine and *N,N*-dimethylglycine (expressed as mg betaine lost). Data from Atkinson et al. (2009).

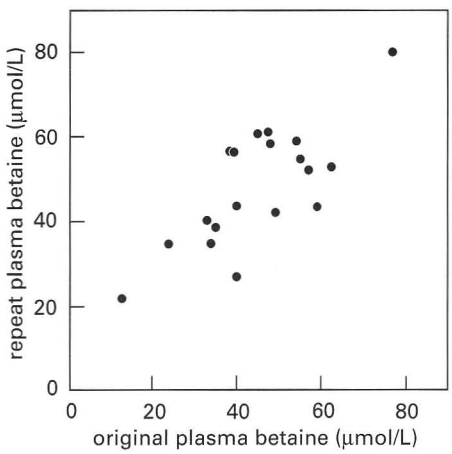

Fig. 13.4: Reproducibility of plasma betaine concentrations in normal adult subjects, samples taken 3 years apart; $r = 0.80$. Data from Lever et al. (2004).

Minimal betaine is excreted in the urine. Normally, the fractional clearance is less than 1.5% (Lever et al., 1994a) and the urinary excretion does not correlate with plasma concentrations. When normal subjects receive a betaine supplement there is at most a small and transient increase in the urine excretion (Schwab et al., 2006; Atkinson et al., 2008, 2009), and after two weeks on a supplement normal subjects are not excreting significantly more betaine than before the supplementation period

(►Fig. 13.3) even though plasma betaine concentrations remain higher. Similarly, urinary betaine excretion is not significantly affected by osmotic stress (Lever et al., 2007c), and instead, individual subjects appear to excrete an approximately constant amount of betaine for weeks and possibly years. The urinary betaine excretion, as measured by the ratio to creatinine, is stable through a day, and collecting 24-hour urine samples does not seem to add to the information given by the creatinine ratio (Lever et al., 2007c, 2009a). Therefore, random urine samples are sufficient for assessing betaine excretion, because the betaine:creatinine ratio is not significantly affected by either food intake or hydration status. Urinary dimethylglycine increases after a betaine load, although this only accounts for a small proportion of the betaine supplied and appears to be transitory (Atkinson et al., 2009). Although the fractional clearance of dimethylglycine is not as low as that of betaine (Lever et al., 2005) the predominant route for its clearance is probably mitochondrial by dimethylglycine dehydrogenase rather than urinary clearance.

Some ingested betaine could be stored in tissues, and plasma concentrations do not change significantly under conditions that would be expected to affect tissue concentrations. However, in the longer supplementation studies (Alfthan et al., 2004; Atkinson et al., 2009) most of the betaine in the supplements appears to have been metabolized, and this must have involved the conversion of homocysteine to methionine. The small (sometimes undetectable) changes in plasma homocysteine show the limitation of this approach for lowering homocysteine, because the methionine produced will be metabolized to produce homocysteine. Studies of the fate of methyl-labeled betaine fed to chickens supports the concept of rapid distribution to tissues and the transfer of the methyl groups (Kettunen et al., 2001b).

13.3.4 Effect of a methionine load

When a load of methionine is supplied to humans or other mammals, plasma homocysteine increases. This is expected because excess methionine is metabolized by glycine *N*-methyltransferase, an enzyme controlled by the methionine supply, thus generating homocysteine. The catabolism of the carbon skeleton of methionine almost entirely proceeds through cystathionine β-synthase (►Fig. 13.2), but this step is limiting and controlled by methionine and *S*-adenosylmethionine; most of the homocysteine is remethylated, completing a cycle. Thus supplying a methionine load predictably increases the steady-state concentration of homocysteine, and the steady-state ratio of methionine to homocysteine will depend on the supply of methyl groups for remethylation (Hoffer, 2004; Mudd et al., 2007). Tissue betaine is the major reserve of preformed methyl groups: this means that the supply of betaine is the major determinant of the post-methionine load increase in homocysteine (Holm et al., 2004). The methionine load test could be regarded as a test for betaine sufficiency.

13.4 Betaine in development

Maternal nutrition is important for normal human development, and in particular the supply of methyl groups is vital at all stages from conception to early infancy (Zeisel, 2009a,b). The role of different sources of methyl groups changes during development.

13.4.1 Early embryogenesis

In the mouse early pre-implantation embryo betaine is transported via a single saturable transporter, SIT1 (encoded by *Slc6a20a*) that is different from the osmoregulated betaine transporter BGT1 (Hammer and Baltz, 2002; Anas et al., 2007, 2008). SIT1 betaine transport is Na^+ and Cl^- dependent, and it is active a few hours after fertilization. It is present only in the fertilized egg and at lower levels at the two-cell stage, and is completely absent by the four-cell stage (Anas et al., 2007, 2008). SIT1 also transports proline and it is inhibited by several methylamino acids and proline derivatives *in vitro* (Anas et al., 2007), however, it has been suggested that its primary function *in vivo* is to mediate betaine accumulation and retention (Anas et al., 2008). One- and two-cell mouse pre-implantation embryos cultured in the presence of betaine have ≈4-fold higher betaine levels than those cultured without. Similarly, betaine appears to accumulate to relatively high levels *in vivo*, with intracellular concentrations corresponding to ≈6–7 mM (Anas et al., 2008) and is comparable to the average concentration of ≈4 mM in rat liver (Slow et al., 2009). The accumulated betaine remains present until the blastocyst stage (Lee et al., 2009).

Developing embryos depend on obtaining betaine from the mother, and the earliest stages of embryogenesis takes place in the oviduct. Mouse oviducts contain significant amounts of betaine (≈0.5 millimolar) averaged over the whole organ (Anas et al., 2007), and it is possible that this tissue store is an important supply of betaine that can be transferred via SIT1 to the pre-implantation embryo. The activation of betaine transport via SIT1 after fertilization and its transient presence during a short period of development implies that betaine has an important function during pre-implantation embryo development, however, the mechanism by which betaine exerts a beneficial effect is not known. It has been shown *in vitro* that betaine protects pre-implantation embryo development against increased osmolarity (Biggers et al., 1993; Dawson and Baltz, 1997; Hammer and Baltz, 2002) but it is yet to be established that it performs this role *in vivo*. The accumulated betaine could be important for methylation reactions including the maintenance of the methylation profile of DNA, which is an important control mechanism that regulates gene expression. The methylation profile of DNA (whether the gene is 'imprinted' or 'non-imprinted') depends on the stage of pregnancy (Delaval and Feil, 2004; Swales and Spears, 2005; Trasler, 2006; Zeisel, 2009a). However, for betaine to be utilized as a methyl donor, BHMT must be expressed and active in the pre-implantation embryo. BHMT mRNA transcript has been found to be expressed from the four-cell stage to the blastocyst. High levels of transcript, comparable to that found within the maternal liver, is found at the morula stage, which then decreases sharply in the blastocyst (Lee et al., 2009). Active BHMT protein is found in the inner cell mass of the blastocyst, but not the morula and it is only detectable for 48 h after blastocyst hatching, indicating that BHMT activity is transient and restricted to the blastocyst (Lee et al., 2009). This suggests that the betaine accumulated by the two-cell stage might be stored in the pre-implantation embryo and subsequently utilized as a supply of methyl groups via BHMT in the blastocyst, and thus betaine could have an important role as a source of methyl groups in the pre-implantation embryo. However, the consequences for pre-implantation embryo development should the betaine supply or BHMT activity be disrupted is as yet unknown. Notably, folate does not appear to be taken up by the mouse pre-implantation embryo at any stage (Baltz, personal communication),

suggesting that the BHMT pathway might be the only means of maintaining methylation capacity, thus having a supply of betaine might be crucial at these early developmental stages.

Choline is also actively transported in the pre-implantation embryo via a Na^+-independent transporter, whose activity can be detected from the one-cell stage and increases 100-fold in the conceptus between the two-cell and blastocyst stages of development (Van Winkle et al., 1993). The primary role of choline in the pre-implantation embryo is thought to be for phospholipid synthesis (Van Winkle et al., 1993), which is required by blastocysts for morphological changes and growth. It has been shown that choline is incorporated into phosphatidylcholine and lysolecithin in mouse pre-implantation embryos, whereas the omission of choline from culture media inhibits the hatching of hamster blastocysts (Van Winkle et al., 1993). It is not known whether any of the transported choline is oxidized to betaine or if the oxidation pathway is active in the pre-implantation embryo.

13.4.2 Pregnancy and fetal development

The near-term rat fetus has been reported to have a betaine content of <2 mM whereas the placenta has >4 mM (Zeisel et al., 1995). In humans, maternal plasma betaine and dimethylglycine concentrations decline until gestational week 20 and thereafter remain constant, whereas plasma choline concentrations increase continuously during pregnancy (Molloy et al., 2005; Ueland et al., 2005; Velzing-Aarts et al., 2005). Maternal plasma homocysteine concentrations are reduced during gestation (Molloy et al., 2005; Wallace et al., 2008) with the lowest concentrations occurring in the second trimester (Velzing-Aarts et al., 2005). Plasma choline is a positive predictor of homocysteine, whereas from gestational week 20 onward maternal plasma betaine is a strong negative predictor of homocysteine (Molloy et al., 2005; Velzing-Aarts, 2005). It is believed that these changes during pregnancy are important to ensure choline availability for placental transfer, with subsequent use by the growing fetus. Choline is actively transported across the placenta and is $\approx$3-fold higher in the fetal circulation compared with those of the mother (Molloy et al., 2005; Velzing-Aarts et al., 2005; Friesen et al., 2007). Similarly, betaine and dimethylglycine are elevated 1- to 2-fold higher in the fetal circulation as assessed from umbilical cord blood at birth (Molloy et al., 2005; Friesen et al., 2007).

The inverse relation between maternal plasma betaine and homocysteine reflects the role of betaine in one-carbon metabolism during normal pregnancy. This suggests that a low betaine status during pregnancy might predispose to pregnancy complications associated with high homocysteine (Velzing-Aarts et al., 2005). In contrast to the pre-implantation embryo, BHMT expression has not been detected in the mouse fetus until gestational day 10 (Fisher et al., 2002), suggesting that expression is switched off in the post-implantation embryo only to be switched on again in the early fetus: thus the BHMT expression profile appears to be biphasic and dependent on the stage of pregnancy. It is also probable that fetal betaine is important for osmoregulation: however, the importance and partitioning of betaine between these two important competing functions (methyl donor and osmolyte) during human pregnancy has not been elucidated.

13.4.3 The relevance of choline versus betaine during pregnancy

Choline is an essential nutrient and is particularly crucial during fetal and neonatal life to ensure optimal neurodevelopment in rodents (Zeisel, 2000, 2009a). Normally, most of the betaine needs of the body can be met by choline oxidation. During pregnancy, the majority of choline is utilized for the production of phospholipids, including phosphatidylcholine and sphingomyelin, which are involved in membrane synthesis, and are crucial for normal spinal cord and brain development. The embryo/fetus develops in a high choline environment where the concentration in amniotic fluid is up to 10-fold higher than in maternal blood (Zeisel, 2006a, 2009a,b). In rats fed a diet considered to be adequate in choline, demands are so high during pregnancy that maternal liver choline concentrations are significantly depleted (Zeisel et al., 1995). The *de novo* synthesis of phosphatidylcholine, catalyzed by phosphatidylethanolamine methyltransferase (PEMT), is also up-regulated. This reaction requires the sequential addition of three methyl groups, provided by SAM, to phosphatidylethanolamine to form one molecule of phosphatidylcholine, thus utilizing a significant portion of SAM, and a supply of methyl groups other than choline is required (Zeisel, 2006a). As stated above, in humans, there is a negative association between betaine and homocysteine whereas maternal plasma choline concentrations positively correlate with homocysteine concentrations, suggesting there is also substantial phosphatidylcholine synthesis via PEMT during pregnancy (Molloy et al., 2005).

Choline oxidation is irreversible and diminishes the availability of choline for its other vital functions. Dietary betaine therefore spares choline and might be essential during pregnancy to ensure adequate choline for phospholipid and neurotransmitter synthesis. In addition, adequate dietary betaine can reduce maternal plasma homocysteine, which can have a direct deleterious effect on the developing embryo. Maternal flux through the choline oxidation pathway is thought to be increased during pregnancy providing betaine for homocysteine remethylation, thus supplying adequate methionine and methyl donors to the fetus for protein synthesis and methylation reactions (Molloy et al., 2005; Wallace et al., 2008) which could also explain the strong inverse association observed in human pregnancy between maternal plasma betaine and homocysteine concentrations. Similarly, maternal choline oxidation and dietary betaine intake are thought to be even more important for normal fetal development when the mother's supply of methionine or folate is limited or deficient, because it appears that the BHMT remethylation pathway compensates for the inadequate flux through the methionine synthase pathway (Molloy et al., 2005; Wallace et al., 2008).

13.4.4 Clinical significance of methylation in pregnancy

Failure to re-establish the methylation profile of non-imprinted genes or to maintain the methylation of imprinted genes during embryogenesis is associated with embryo loss, fetal abnormalities (Wolff et al., 1998; Paulsen and Ferguson-Smith, 2001; Wu et al., 2004; Niculescu et al., 2006; Waterland et al., 2006), perturbations in growth and placental formation/function *in utero* (Mann et al., 2004; Coan et al., 2005) as well as cancer (Feinburg and Vogelstein, 1983; Jones and Laird, 1999; Davis and Uthus, 2004), disturbed neurobehavioral processes (Robertson, 2005), obesity (Garfinkel and Ruden, 2004; Waterland et al., 2008), diabetes (Brownlee, 1995) and vascular disease

(Roher et al., 1993; Brownlee, 1995; Stadtman and Levine, 2000) in the newborn and adult progeny. Thus it is essential that the maternal dietary intake of methyl groups, both periconceptually and throughout gestation, is adequate to meet the requirements of the developing progeny. This is clearly illustrated by dietary manipulation in the *agouti* and *axin fused* mice, where maternal supplementation of folate, vitamin B12, methionine, choline and betaine before and during pregnancy permanently increased DNA methylation at the viable *yellow agouti* (A^{vy}) (Wolff et al., 1998) and *AxinFU* (Waterland et al., 2006) metastable alleles affecting gene expression in the offspring as well as their health and longevity (Wolff et al., 1998). Similarly in humans, folate supplementation before and in early pregnancy reduces the recurrence and occurrence of neural tube defects (spina bifida, anencephaly and encephalocele).

The periconceptual maternal dietary intake of choline and to a lesser extent betaine has been associated with neural tube defects, where the risk was lowest in women whose diets were rich in choline, betaine and methionine (Shaw et al., 2004). However, the association for betaine was weaker and disappeared when folate status was high, suggesting that the stronger association with choline might be more related to its other functions rather than as a source of methyl groups via its oxidation to betaine. This is not surprising given that the BHMT pathway does not appear to be active in the post-implantation embryo and is only found in the early fetus at a time when neural tube closure is nearly complete (Fisher et al., 2002). However, because betaine is a requirement, the observed association could illustrate the choline-sparing effect of dietary betaine; women with high dietary betaine intakes can supply more choline to the developing embryo at time when it might be a more crucial nutrient for neural tube development. Similarly, a low periconceptual maternal betaine status or dietary intake might cause epigenetic disturbances during pre-implantation embryo development, a time when the betaine/BHMT pathway appears to be active, which could subsequently cause aberrant gene expression in the post-implantation embryo and, in turn, lead to an increased risk of neural tube defect.

It has been suggested that homocysteine metabolism via the BHMT pathway might be more important than metabolism via the folate-dependent methionine synthase pathway in late pregnancy (Ueland et al., 2005; Velzing-Aarts et al., 2005). In pre-eclampsia, homocysteine concentrations are elevated in both the maternal and fetal circulation and one hypothesis was that the high homocysteine concentrations might have been caused by a choline and betaine insufficiency. However, compared with uncomplicated pregnancies, maternal and fetal choline and betaine concentrations were higher in pre-eclampsia and the association between maternal plasma choline and homocysteine previously documented (Molloy et al., 2005) was not found (Braekke et al., 2007). These findings do not necessarily mean that the BHMT pathway is any less important in late pregnancy, or that too much betaine or choline increases the risk of pre-eclampsia. It could be that the elevations in both choline and betaine indicate that the BHMT pathway might be down-regulated in pre-eclampsia and the increase in choline and betaine is a consequence of the condition rather than a cause.

BHMT genotype could also contribute to the risk of adverse pregnancy outcomes and birth defects, but the number of studies that have investigated this are limited and the findings have been inconsistent. The BHMT A allele (742G>A; R239Q) has been associated with a decreased risk of neural tube defect, where the AA genotype in either the mother or the child was associated with decreased risk, albeit non-significantly

(Morin et al., 2003), whereas another study found no association with spina bifida and the A genotype (Zhu et al., 2005).The risk of orofacial clefts (cleft lip with or without cleft palate) has been found to be significantly lower in children with the AA genotype (Mostowska et al., in press), and by contrast, mothers homozygous for the A allele have an increased risk of placental abruption (Ananth et al., 2007). The sometimes seemingly contradictory findings have been attributed to possible differences in BHMT expression at different life stages. In adults BHMT functions predominantly in the liver, but little is known regarding the expression pattern in the developing embryo and it could be markedly different compared with the adult (Boyles et al., 2006). The mechanism by which the BHMT A allele affects risk is unknown although a recent *in vitro* study has shown that the apparent K_m values for homocysteine and betaine are considerably lower for the Q enzyme when compared to the wild-type R enzyme (Li et al., 2008) and it has been suggested that the A genotype could create a highly efficient enzyme variant *in vivo* (Mostowska et al., in press).

13.4.5 Neonatal development

Plasma, liver and kidney betaine concentrations change substantially between neonatal and adult life in rats (Wijekoon et al., 2005), all transiently increasing several-fold post-weaning, and remaining high, peaking at 28 days of age (1 week post-weaning) until day 42, before declining by day 56 (Rafter et al., 1991; Clow et al., 2008). The increases in plasma and tissue betaine appear to be a response to dietary intake, with the accumulation occurring because liver clearance is lower than intake (Clow et al., 2008). Choline oxidation to betaine is still occurring because rat pups fed a betaine-free diet still show a substantial transient increase in total liver betaine content, but animals fed a diet containing betaine have larger increases in plasma and tissue betaine, although the osmotic stresses on the animals, a major determinant of tissue betaine content, does not appear to have been controlled. Liver BHMT activity is high pre-weaning (day 14–21) before declining to remain constant from day 21 to 27. Choline dehydrogenase activity gradually increases to a plateau between 35 and 42 days of age. Pup dietary betaine intake post-weaning has little effect on either BHMT or choline dehydrogenase activity, neither does maternal dietary intake pre-weaning (Clow et al., 2008).

Neonatal rats and humans both excrete remarkable amounts of betaine (Davies et al., 1988), and this high excretion does not appear to occur *in utero* (Davies et al., 1992) but commences at birth (Trump et al., 2006). Excretion might increase for a short time after birth (Davies et al., 1992), but this is probably an artifact of expressing excretion as a ratio to creatinine, which is not reliable in the neonatal period (Trump et al., 2006). Certainly betaine excretion declines during human infancy and childhood (▶Fig. 13.5) although it does not reach the lower adult levels until the early teenage years (Lever et al., 2009a), and the neonatal betaine excretion might reflect the immaturity of the neonatal kidney. Because most of this betaine is presumably derived from choline it might compromise the supply of choline and consequently methyl groups (Davies et al., 1992; Holmes et al., 2000), and it is probable that the loss is of the same order as the supply, although the increase in the choline content of breast milk in the first few days should give a margin of safety (Holmes et al., 2000). However, this is probably not true if milk formulas are used (Holmes et al., 2000), and including additional betaine or choline in these formulas might be advisable.

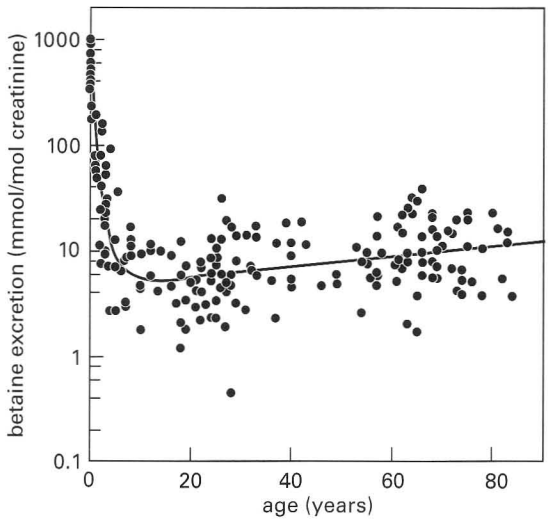

Fig. 13.5: Changes in betaine excretion with age (Lever et al. 2009a). Minimum at approximately 13 years.

13.5 Clinical importance

The possibility that disturbances in betaine intake, metabolism or excretion have clinical consequences has been canvassed for some years. Because betaine has important roles in normal physiology we would predict that a betaine insufficiency would have adverse consequences.

13.5.1 Betaine, obesity and fat metabolism

A major part of the world's betaine production is used by the animal industries. It has been known for many years that including betaine in animal feeds leads to better meat, more lean muscle mass and less fat (Eklund et al., 2005). Although this is best documented in pigs, similar effects are well known in poultry (Wang et al., 2004; Zhan et al., 2006), and can also be shown in lambs before rumen development (Fernández et al., 2000). How betaine affects body fat is not clear, and many mechanisms have been proposed (Eklund et al., 2005). Betaine is an osmolyte in intestinal cells and probably affects nutrient digestibility and partitioning, and increasing the availability of methionine has been invoked to explain increased protein production. Small animal studies also support a role for betaine, e.g., in the agouti mouse model the transgenerational amplification of obesity is prevented when animals are fed a diet high in betaine and its precursor choline (Waterland et al., 2008), and these results suggest that epigenetic methylation is involved. There is evidence in finishing pigs that betaine decreases lipogenesis by decreasing the mRNA expression of the relevant enzymes (Huang et al., 2008), and although it is suggested that this effect is mediated by growth hormone (which is elevated by betaine treatment), an epigenetic effect is possible. Despite the effects of betaine supplements on other mammals and on poultry, they have not been

observed in humans. When otherwise healthy obese adult subjects were supplied a betaine supplement for 12 weeks no effect was seen on body fat as assessed by bioelectrical impedance or body mass index (BMI) (Schwab et al., 2002). By contrast, cross-sectional data (Konstantinova et al., 2008a) show a highly significant negative association between markers of obesity (BMI, percent body fat and waist circumference) and plasma betaine concentrations.

The effect on obesity implies an interaction between betaine and lipid metabolism. In several human cross-sectional studies (Lever et al., 2005, 2007b; Konstantinova et al., 2008a) plasma betaine is inversely related to lipid markers (triglycerides, apolipoprotein B, LDL-cholesterol), suggesting that low plasma betaine is associated with a greater vascular risk; however, betaine supplementation of healthy subjects, which raises plasma betaine concentrations, increases plasma cholesterol (Schwab et al., 2002). Indeed, Olthof et al. (2005b) questioned the safety of betaine supplementation on the basis of a small elevation in plasma triglycerides and LDL cholesterol in subjects receiving betaine supplements, although the significance of these results has been questioned (Zeisel, 2006b). Animal studies suggest a possible resolution of this apparent contradiction by suggesting that betaine affects the partitioning of lipid in the body, and in particular increases the export of tissue triglycerides. In normal rats, betaine supplementation (particularly when methionine is restricted) leads to increased hepatic production of apolipoprotein B (Sparks et al., 2006) and increased plasma LDL and triglycerides, and these changes are associated with a large decrease in tissue lipid, which has been confirmed in other studies (Hayes et al., 2003). When the water-soluble components of lean and obese Zucker rat livers were compared (Serkova et al., 2006), the largest differences were in betaine (approx. 4-fold lower in the obese animals) and methionine (more than 10-fold higher in the obese animals), which is consistent with what is seen in human cross-sectional studies because liver concentrations determine plasma concentrations. Other mechanisms, however, might be involved; betaine has also been shown to decrease fatty acid synthesis, in pigs (Huang et al., 2008), by decreasing the expression of the enzymes involved in lipogenesis. In an apolipoprotein E-deficient mouse model, which develops atherosclerotic lesions (Lv et al., 2009), betaine supplementation increased plasma cholesterol (but not triglycerides) but at the same time decreased atherosclerotic lesion areas. The difference was attributed to lowered TNF-α expression in the supplemented animals and hence less inflammation. Another mouse model, with a disruption of the methylenetetrahydrofolate reductase gene (with the result that the animals tend to have elevated plasma homocysteine) was given a diet supplemented with betaine, and in this model the plasma triglycerides decreased on long-term (1 year) supplementation whereas HDL-cholesterol increased; other improvements in the atherogenic risk factor profile were also observed (Schwahn et al., 2007). Another relevant observation on the connection between betaine and obesity is that making rats obese by supplying them with a high-fat diet more than doubles their betaine excretion (Kim et al., 2009).

The diverse effects of betaine supplementation on plasma lipids implies that the outcome also depends on other clinical conditions, and additionally in rats there is some evidence that diet also affects the results (Hayes et al., 2003). In the human cross-sectional studies low plasma betaine concentrations are associated with elevated plasma lipids and other components of the metabolic syndrome (Konstantinova et al., 2008a). Similarly, in a population that was being treated for dyslipidemia (Lever et al., 2005,

2007b) the plasma betaine concentrations were particularly low and consistent with the results reported by Konstantinova et al. (2008a). It is possible that in these studies many of the subjects, with elevated plasma lipids, had a betaine deficiency and that there is an association between betaine deficiency and the lipid abnormalities. A possible connecting theme is an association with mitochondrial function. The protective effect of betaine on the electron transport system has been attested in plant root mitochondria (Hamilton and Heckathorn, 2001), and suggested in rats (Ganesan et al., 2007). Mitochondrial dysfunction could be involved in many of the diseases where abnormalities of betaine homeostasis occur, and such an indirect connection would also explain the correlations between betaine and carnitine (Lever et al., 2005, 2007b) and a correlation between plasma betaine and coenzyme Q in acute coronary syndrome patients (unpublished data). Causal connections are not necessary because they could all be consequences of a common underlying pathology.

It is interesting that the reported studies of betaine supplementation in rats, mice and domestic animals are often longer-term than any reported in a human population. The overall impression is that long-term betaine supplementation is unlikely to be harmful and could be beneficial. The small increases in plasma lipids reported in some studies are not clinically significant, they might only apply to betaine-replete subjects and might not persist. By contrast, the loss of tissue lipid could be a long-term gain.

13.5.2 Betaine, diabetes and the metabolic syndrome

It has been known for years that more than 20% of patients with diabetes mellitus excrete abnormal amounts of betaine in their urine (Lever et al., 1994b; Dellow et al., 1999) while maintaining near-normal plasma concentrations, and the fractional clearance in some of these patients exceeds 100% (normal <1.5%), which suggests that there is an active process exporting the betaine. Abnormal betaine excretion is also frequent in other patient groups, e.g., in chronic renal failure patients (Lever et al., 1994b), in patients attending a lipid disorders clinic (Lever et al., 2005, 2007b) and in patients being treated with fibrates (Lever et al., 2009b). In patients with diabetes there is a positive correlation between the urinary excretion of betaine and of another renal osmolyte, sorbitol, and this has not been seen in other groups, which suggests that in at least some of the patients with diabetes a different mechanism is involved, possibly involving an overproduction of sorbitol.

As already noted, in the metabolic syndrome there is a tendency for plasma betaine to be lower than in a healthy population (Konstantinova et al., 2008a). The divergent associations of the substrate (choline) and product (betaine) of mitochondrial choline dehydrogenase could reflect disruption of this pathway as part of the mitochondrial dysfunction that prevails in metabolic syndrome. Elevated urinary betaine loss is common in metabolic syndrome patients (Lever et al., 2009a), so abnormal loss of betaine might be a factor in causing betaine insufficiency, which could be a common feature of the metabolic syndrome. Similarly, because some of the lipid disorders patients were persistently excreting abnormal amounts of betaine for years (Lever et al., 2007a) there must be some stress on the tissue betaine supply in these patients (tissue betaine is variable and much higher than the controlled circulating betaine concentrations). These are the population groups that are most likely to benefit from betaine supplementation.

13.5.3 Betaine, homocysteine and vascular disease

High doses (6 g/day or more) of betaine, alone or in combination with B-vitamins, have been used for years to treat patients with genetic disorders that cause homocystinuria (Wilcken et al., 1983, 1985; Ogier de Baulny et al., 1998; Yap, 2003). Because the betaine supply is the main determinant of plasma homocysteine under non-fasting conditions, several investigators have studied the consequences of more modest supplementation on plasma homocysteine, and it has been confirmed that there is a dose-related reduction of fasting homocysteine (by 20%) and post methionine load homocysteine (by 29–40%) sustained as long as betaine is administered (Schwab et al., 2002; Olthof et al., 2003; Steenge et al., 2003; Olthof and Verhoef, 2005). The methionine produced by methylating homocysteine can only be metabolized by conversion to homocysteine and in this cyclic process the steady-state concentrations of plasma homocysteine only change by a small amount compared with the large amount of betaine being processed (Hoffer, 2004; Mudd et al., 2007). This is a different effect from that of folate supplementation because folate provides a recyclable cofactor for the *de novo* synthesis of methyl groups, and reduces only fasting homocysteine, whereas betaine directly supplies the methyl groups and is consumed in the process of forming methionine (►Fig. 13.2).

That total plasma total homocysteine is a vascular risk factor is well-attested and recently reaffirmed (Loscalzo, 2006; Herrmann et al., 2007; Ueland and Clarke, 2007), but the results of prospective, secondary trials have thrown doubt on whether homocysteine is causal (Bønaa et al., 2006; HOPE, 2006). A significant reduction (by B-vitamin supplementation) in mean plasma total homocysteine concentrations did not significantly reduce the incidence of subsequent events. If the supply of methyl groups is crucial then betaine should be immediately and directly effective because the methyl groups of dietary betaine rapidly appear in tissue methionine, and similarly if the betaine supply is restricted then its function as an osmolyte is compromised. The groups where betaine deficiency is most likely to be found are patients with diabetes or metabolic syndrome, and it is possible that elevated homocysteine in some of these patients is an indicator of betaine status. There are few studies that directly test a connection between betaine supply and vascular disease. Short or medium-term betaine supplementation does not improve flow-mediated vasodilation, a marker of endothelial function, despite reduced homocysteine (Olthof and Verhoef, 2005; Olthof et al., 2006). One study demonstrated impaired vasodilation in subjects given betaine, no effect from low doses of folic acid and enhanced vasodilation in subject given folic acid at doses exceeding those required to obtain maximal homocysteine reduction, suggesting improved endothelial function by mechanisms independent of homocysteine (Moat et al., 2006).

A recent study on 3000 healthy Greek men and women demonstrated low plasma levels of inflammatory markers, such as C-reactive protein, interleukin-6 and TNF-α, in subjects with high intake of choline and betaine (Detopoulou et al., 2008). Because inflammation plays a role in atherogenesis, high intake of choline and betaine could protect against cardiovascular disease. However, two recent large prospective studies, based on the participants in the Dutch PROSPECT-EPIC cohort (Dalmeijer et al., 2007) and in the Atherosclerosis Risk in Communities (ARIC) study (Bidulescu et al., 2007), respectively, found no association between intake

of choline and betaine and cardiovascular disease. These negative or contradictory findings could be related to the large measurement error of intake estimates for micronutrients such as choline and betaine (Bidulescu et al., 2009), or they might result from the selection of the study populations, and for clarification we must wait for the results of future studies.

13.5.4 Betaine and liver disease

Betaine is known to ameliorate the adverse effects of alcohol on the liver, in particular, fatty liver (Barak and Tuma, 1983; Barak et al., 1996, 1997), and this has been attributed to maintaining SAM levels and minimizing the accumulation of *S*-adenosylhomocysteine, SAH (Barak et al., 2003; Kharbanda et al., 2005). Alcohol is reported to inhibit methionine synthase, increasing the requirement for betaine to sustain methylation (Kharbanda, 2009). Methylation is particularly important in the liver for the *de novo* synthesis of phosphatidylcholine, which is essential for normal VLDL assembly and secretion, and thereby minimizes fatty accumulations (Kharbanda et al., 2007; 2009). Betaine can also improve liver health in non-alcoholic fatty liver diseases, and in liver disease induced by xenobiotics (Oliva et al., 2009) or bile salts (Graf et al., 2002). The proposed mechanism, involving the supply of methyl groups for phosphatidylcholine synthesis, is plausible, but other mechanisms have been suggested, including reduced endoplasmic reticulum stress (Ji and Kaplowitz, 2003; Ji, 2008) and epigenetic effects (Graf et al., 2002), and also betaine is a major liver osmolyte. The use of betaine in treating these conditions was reviewed by Craig (2004).

13.5.5 Betaine and other diseases

The first clinical role attributed to betaine was a negative one, as an osmoprotectant that enables bacteria (particularly *Escherichia coli*) to grow in urine that has enough salt and urea to normally preclude growth (Chambers and Kunin, 1985, 1987). This is important in urinary tract infections. The common invasive bacteria do not synthesize betaine (except from choline) but have betaine transport systems that are highly efficient, and to enable growth they accumulate betaine against concentration gradients greater than 10^7, so the actual betaine concentration in urine is not a clinically significant factor affecting growth, and patients with high betaine excretion might not be at a higher risk of developing infections.

Claims that betaine (TMG) alleviates autism do not appear to be supported by any controlled clinical studies, only by the suggestion that there is an impaired methylation capacity in autism (James et al., 2004). There might be an association between betaine and cognitive function, based on a study in which an elderly group (>70 years) was supplemented with folate and cobalamin (Eussen et al., 2007), which raised the plasma betaine concentrations, interestingly, there appeared to be an association between this increase in betaine and memory performance. Betaine supplementation has been suggested for other disorders involving disturbed methyl metabolism but few have been investigated (Craig, 2004).

13.6 Clinical laboratory aspects of betaine

13.6.1 Laboratory measurement

Measuring the concentration of betaine poses a challenge for the analyst. As a consequence of its high water solubility, and low solubility in organic solvents, it is not easily extracted from an aqueous solution. Its absorbance spectrum has no peaks in the visible or near-UV regions, and it has only a weak absorbance close to 200 nm where most metabolites absorb light, further, the quaternary amine group is unreactive, and the carboxyl group is highly deactivated and thus harder to derivatize than most carboxylates. An early approach exploited the low solubility of quaternary ammonium tri-iodides (Barak and Tuma, 1979, 1981), which enabled tissue concentrations to be measured; another approach was to use betaine homocysteine methyltransferase in enzymatic assays (Martin and Finkelstein, 1981), and when combined with derivatization of the product and mass spectroscopic detection this led to a method with sufficient sensitivity to measure blood concentrations (Allen et al., 1993). These methods were insensitive and time-consuming.

Nuclear magnetic resonance (NMR) has been extensively used to measure betaine, and has the advantages of being non-destructive, needing minimal sample preparation and rapidly providing a large amount of data. Almost all have used proton NMR, although ^{13}C-NMR has been used to measure betaine in bacteria (Ko et al., 1994), and the possibility of using ^{14}N-NMR has been explored (Balaban and Knepper, 1983; Bell et al., 1991). Proton NMR is attractive because of the strong singlet resonance from the nine identical protons on the three methyl groups, making for a relatively sensitive assay (Davies et al., 1988; Bell et al., 1991; Lundberg et al., 1995). It is possible to measure betaine directly in water after suppressing the water signal and techniques are available to suppress the protein resonances. The main limitation on the use of NMR is sensitivity. It has been shown to be a rapid and convenient technique for measuring betaine in urine (Lee et al., 2006) and tissues (Bedford et al., 1998), and recently NMR has been widely adopted in metabolomic studies (Beckonert et al., 2007; Martin et al., 2009; Bollard et al., 2010). A problem with measuring betaine in biological samples by proton NMR is the near co-resonance, at neutral pH, of the methyl protons of betaine and of trimethylamine *N*-oxide (TMAO); the choline signal is also close enough to interfere. The signals can be separated by changing the pH of the solution so that TMAO, and possibly betaine also, are protonated (Bell et al., 1991; Bedford et al., 1998; Lee et al., 2006). Overlooking this has led to mis-identifications in the past (Bell et al., 1991). It is probable that this confusion continues, e.g., the signals reported by Martin et al. (2009) as 'TMAO' are probably betaine, and the protocol recommended by Beckonert et al. (2007) will confuse these metabolites. These problems can be avoided, and because of its advantages NMR is likely to become increasingly popular for measuring betaine as more sensitive instruments become widely available, and probe technology improves.

High-performance liquid chromatography (HPLC) is widely used to measure betaine, although detection is a problem. Underivatized betaine has been chromatographed and detected by refractive index changes (Wolff et al., 1989) and this had sufficient sensitivity for measuring the high concentrations of tissue betaine, but lacks the sensitivity needed to measure the betaine in plasma or serum, and for these samples the betaine needs to be derivatized first. Most derivatization methods depend on alkylating

the carboxyl group to add a UV-absorbing or fluorescent functional group, and the commonest derivatizing agents are 2′-bromophenacyl bromide (Laryea et al., 1998; Clow et al., 2008), 2′-bromophenacyl triflate (Lever et al., 1992; Mar et al., 1995) and 2-naphthacyl triflate (Storer and Lever, 2006); others can be used (Sakamoto et al., 2002; Happer et al., 2004; Storer and Lever, 2006). Preparing derivatives that have a higher UV absorbance, or are fluorescent, makes possible a simple method using a 10 µl or smaller plasma sample (Storer and Lever, 2006). The betaine derivatives are quaternary ammonium cations and can be separated by ion-exchange based chromatography systems (Sakamoto, 2002; Storer et al., 2006). Derivatization often leads to the derivatization of the betaine metabolite, *N,N*-dimethylglycine (Laryea et al., 1998; Storer et al., 2006), but this derivative is a double derivative, alkylated both on the nitrogen and the carboxyl to give a quaternary amine with two chromophores (Storer et al., 2006) which elutes too early on silica columns for reliable quantification. The alumina columns that made the separation possible (Storer et al., 2006) are no longer available, but similar separations can be made on titania or zirconia columns (unpublished data). Capillary electrophoresis (Zhang et al., 2002) is not sufficiently sensitive for measuring blood concentrations (Storer et al., 2006).

Betaine determination using mass spectrometry has a long history, being implemented directly on ester derivatives (Rhodes et al., 1987) or in combination with gas chromatography after conversion to volatile derivatives (Allen et al., 1993). However, mass spectrometry is now used in conjunction with liquid chromatography, which avoids the need for extraction and derivatization steps. Liquid chromatography-tandem mass spectrometry (LC-MS/MS) has become the method by which most betaine assays are conducted (Koc et al., 2002; Holm et al., 2003; Ueland et al., 2007). The detection is made highly selective by using multiple-reaction monitoring (MRM), and because high resolution is not necessary in the chromatography this can be made more rapid than with UV or fluorescence detection; e.g., betaine and related compounds can be separated and simultaneously quantified in under 5 min using less than 5 µl of sample (Holm et al., 2003). By these means, plasma betaine, choline and dimethylglycine can be measured simultaneously on over 300 samples in a day (Ueland et al., 2007) using one analytical system. This is the way that recent large epidemiological studies of betaine metabolism have become possible (Ueland et al., 2005; Fredriksen et al., 2007; Ueland et al., 2007; Konstantinova et al., 2008a,b; Johansson et al., 2009).

13.6.2 Clinical biochemistry of betaine

Formal reference ranges for plasma and urine concentrations of betaine and its metabolite, *N,N*-dimethylglycine are not available, and reports from different countries either include a small number of subjects or they focus on subsets of the populations. Fortunately these reports are consistent (▶Tab. 13.1) despite the variety of analytical methods used and the differences in the population groups that have been selected. Serum or plasma betaine tends to be higher in adult males than in females, and is usually in the 20–70 µmol/l range; less is known about serum or plasma dimethylglycine concentrations, but they are usually below 10 µmol/l. Urine betaine excretion is less well documented, but adults normally excrete between 2 and 35 mmol betaine per mole creatinine (▶Tab. 13.1).

Tab. 13.1: Reference range data for betaine and dimethylglycine.

Literature reference	*n*	Blood (μmol/l)		Urine (mmol/mole creatinine)	
		Betaine	DMG	Betaine	DMG
Allen et al. (1993)					
serum and urine	60	18–73[a]	1.4–5.3[a]	2–56[a]	1.2–12.2[a]
Lever et al. (1994)					
Plasma ♀	35	12–56[a]			
Plasma ♂	37	11–83[a]			
Urine	76			1.5–32.5[a]	
Holm et al. (2003)					
Serum fasting	59	28–42[b]	1.4–2.2[b]		
Plasma fasting	60	27–41[b]	1.3–2.0[b]		
Serum non-fasting	46	37–48[b]	1.6–2.5[b]		
Plasma non-fasting	60	36–47[b]	1.6–2.5[b]		
Schwab et al. (2006)					
Serum	10	27–67[a]			
Holm et al. (2007)					
Plasma 50–64 years ♀	5381	14.9–58.6[a]			
Plasma 50–64 years ♂	5221	23.2–71.1[a]			

(*Continued*)

Tab. 13.1: (*Continued*)

Literature reference	*n*	Blood (μmol/l)		Urine (mmol/mole creatinine)	
		Betaine	DMG	Betaine	DMG
Konstantinova et al. (2008)					
Plasma 47–49 years ♀	2062	16.9–56.2[a]			
Plasma 47–49 years ♂	1657	26.3–72.1[a]			
Plasma 71–74 years ♀	1860	20.4–62.3[a]			
Plasma 71–74 years ♂	1466	26.5–73.8[a]			
Lever et al. (2009a)					
Plasma ♀	74	17–60[a]			
Plasma ♂	81	21–78[a]			
Plasma ♀	37		0.4–12.3[a]		
Plasma ♂	43		0.8–9.6[a]		
Urine	160			1.8–35.9[a]	
Urine	52				0.4–30.5[a]

DMG, *N,N*-dimethylglycine. [a]95% range; [b]interquartile range. The two Lever et al. studies are not independent, and the later estimates differ slightly because more elderly subjects were recruited after the earlier report.

What is the clinical value of measuring betaine or dimethylglycine? Because there is no correlation between betaine excretion and plasma betaine concentration in either cross-sectional studies or within individual subjects (Lever et al., 2009a), these are potentially separate tests. Despite its high individuality, plasma betaine concentrations do not appear to have significant diagnostic value, being remarkably stable despite significant changes in tissue betaine (Lever et al., 2004). Although plasma betaine concentrations tend to be lower in the metabolic syndrome and in response to stress, the difference is not likely to have a diagnostic application. It is affected by dietary betaine intake, but the large changes seen in supplementation studies are not typical, and the small changes on a normal diet would not confound interpretation (Holm et al., 2003; Lever et al., 2004, 2005). Urine betaine excretion, measured as the ratio to creatinine, is potentially more useful; as well as the abnormally high betaine excretion in diabetes and the metabolic syndrome, high excretion is common in chronic renal failure patients without diabetes, and interestingly, an abnormally low betaine excretion is also found in some patients with diabetes or with chronic renal failure, although no clinical significance has yet been attached to this. Elevated excretion is also associated with fibrate therapy (Lever et al., 2009b), with particularly high excretions in patients with features of the metabolic syndrome who are being treated with a fibrate. Elevated betaine excretions also show individual persistence (Lever et al., 2007a). Random urine samples are adequate to identify abnormal urine excretion, because the betaine to creatinine ratio is at least as reliable as the 24-h excretion (Lever et al., 2009a), and is not significantly affected by food intake. This test could identify potential betaine deficient patients. As previously noted, urine betaine excretion might be elevated as a result of consuming foods containing proline betaine (Atkinson et al., 2007), and supplying betaine supplements in orange juice probably accounts for the different (although both low) estimates of urinary loss made by Schwab et al. (2006) and Atkinson et al. (2009), making it advisable to avoid using orange juice in investigations of one-carbon metabolism.

Although it has low individuality, plasma dimethylglycine concentrations could have diagnostic value (Lever et al., 2009a) as a marker of abnormal one-carbon metabolism because it is only produced via the betaine-homocysteine methyltransferase catalyzed reaction, and the plasma dimethylglycine to betaine ratio might be the preferred test (McGregor et al., 2001), but more work is needed to establish that these measures would add to the information available from more accessible tests. No clinical information has so far been identified in measures of urine dimethylglycine excretion, which correlate with both plasma dimethylglycine and with urine betaine excretion.

13.7 Summary

Betaine is an essential metabolite with important roles as a tissue osmolyte and as a reserve of metabolically available methyl groups. It is not an essential component of the diet because the requirements for betaine can be met by the metabolism of dietary choline, assuming that the supply of choline is sufficient. However, dietary betaine is efficiently absorbed and readily used, and this reduces the demand for choline, which is an essential nutrient with different roles in mammalian physiology. Betaine cannot be readily converted into choline and can only partly replace dietary choline.

The betaine supply could be inadequate in some patients with diabetes mellitus or with features of metabolic syndrome, one reason being that many of these patients lose excessive amounts of betaine in their urine. These patients typically present with dyslipidemia and elevated plasma homocysteine. It is possible that the betaine deficiency contributes to the health problems of this increasing section of the population, and that a modest level of betaine supplementation will be beneficial, however, long-term prospective studies on appropriate human populations have not been conducted to establish this. Betaine also has important roles in human development, in the period immediately before and after conception as well as later in pregnancy, and in the first few weeks after birth. The recent improvements in analytical methods and the availability of food composition data for choline and betaine have motivated clinical and epidemiological studies on the relation between choline and betaine status and disease risk, mainly for conditions previously investigated in relation to folate status. Human data are sparse, the number of studies is limited and no large placebo-controlled intervention trials on betaine or choline supplementation have been published; the claims of those marketing 'TMG' as a panacea are not based on reliable evidence. Betaine and human health is a research area in its infancy, but with the potential to generate data leading to rational strategies for disease prevention.

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This book contains num. figs. and tabs.

ISBN 978-3-11-021448-2 • e-ISBN 978-3-11-021449-9

Library of Congress Cataloging-in-Publication Data

Vitamins in the prevention of human diseases / edited by Wolfgang Herrmann, Rima Obeid.
p. ; cm.
ISBN 978-3-11-021448-2 (alk. paper)
1. Vitamins. 2. Vitamin therapy. I. Herrmann, Wolfgang, Prof. II. Obeid, Rima.
[DNLM: 1. Vitamins–pharmacology. 2. Vitamins–therapeutic use. QU 160]

QP801.V5V58 2010
612.3'99–dc22 2010028998

Bibliographic information published by the Deutsche Nationalbibliothek
The Deutsche Nationalbibliothek lists this publication in the Deutsche Nationalbibliografie; detailed bibliographic data are available in the Internet at http://dnb.d-nb.de.

Project management: Dr. Petra Kowalski.
Production editor: Simone Pfitzner.
Production manager: Sabina Dabrowski.
Typesetting: Compuscript, Shanon – Ireland.
Cover image: Dr. Arthur Siegelman/Visuals Unlimited/gettyimages
Printing and binding: Druckhaus „Thomas Müntzer", Bad Langensalza.