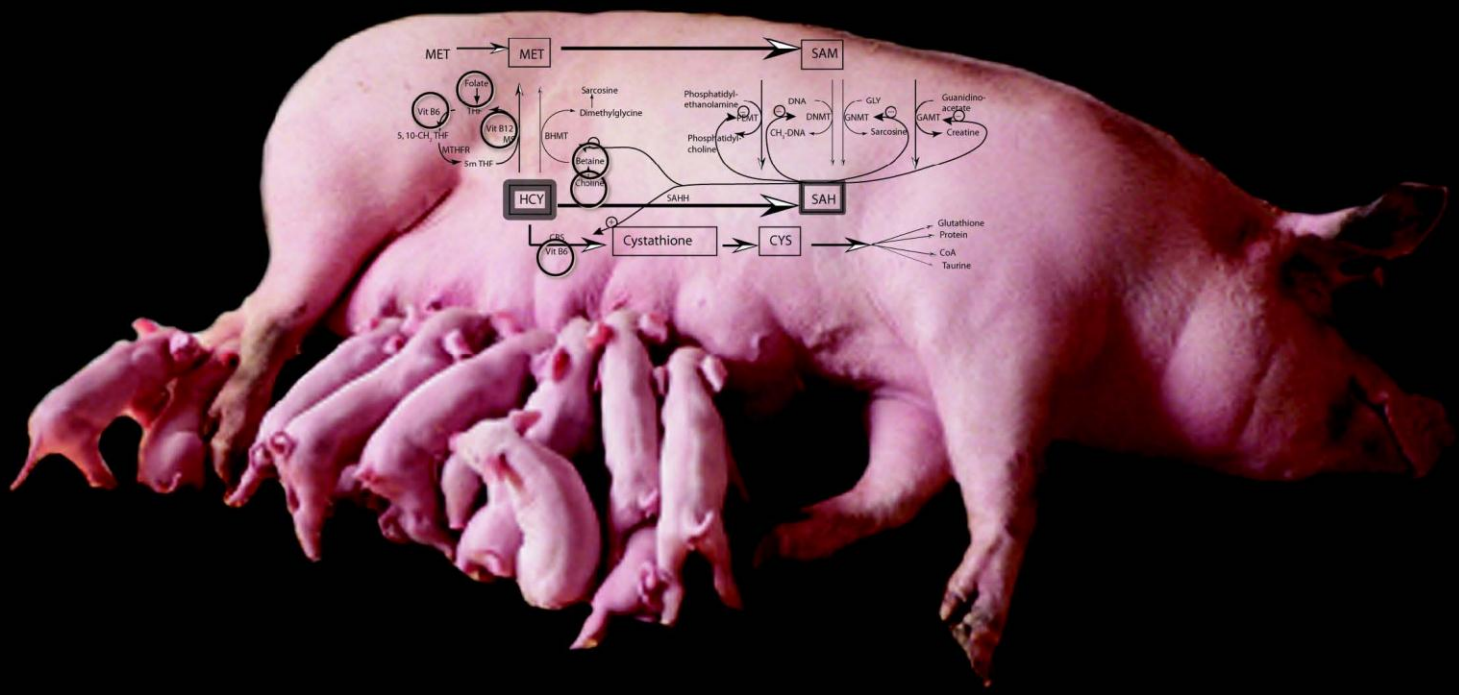
The Requirement of Pigs for Methyl Groups

Methionine B12 B6 Betaine Choline Folate



Glutathione Nitric Oxide Arginine DNA

Ulcers Disease resistance Placental insufficiency Embryonic death Intra-uterine growth retardation Litter size Post-natal survival Growth Body composition Reproduction

The Requirement of Pigs for Methyl Groups

A review compiled for Pork CRC Ltd

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Introduction

The addition of a methyl group to a metabolite increases the ease with which it is absorbed into cell membranes and increases its rate of metabolism; the addition of a methyl group to DNA alters gene expression. Methylation is essential for a variety of metabolic processes, including hormonal signalling, neurotransmission, protein synthesis, muscle contraction, cell growth and membrane integrity. In functional terms, methylation affects embryonic differentiation, fetal growth, immunity and metabolism. Abnormal methylation is associated with embryonic death, impaired fetal development, cancer and diseases of the brain, liver, kidneys and heart.

Methylation is accomplished within the methionine cycle and involves sources of methyl groups such as methionine, betaine, choline and folate, and co-factors such as vitamin B6 and vitamin B12. Although considerable research has been devoted to assessing the optimum dietary levels of these compounds for pigs in terms of growth, body composition and prevention of the symptoms of vitamin deficiencies, few studies have considered the full role of methylation in pig metabolism. As a result, the requirements of pigs for methyl donors and the effects of an imbalance in the supply of methyl donors are unknown. In contrast, progress in understanding the roles of intermediates involved in methylation reactions in humans has progressed greatly since it was discovered that accumulation of homocysteine, an intermediate in the methionine cycle, is associated with cardiovascular disease, Alzheimer’s disease, diabetes, osteoporosis, kidney disease and neural tube defects. This review was prompted by the observation that the level of homocysteine in pigs is typically several times greater than that considered harmful for humans and discusses probable causes and potential solutions.

The methionine cycle

Transmethylation involves the transfer of a methyl group from S-adenosylmethionine (SAM) to a compound, resulting in the production of S-adenosylhomocysteine (SAH) (Fig. 1). SAH is then converted to homocysteine (HCY), which may be remethylated to complete the cycle by forming methionine (MET), the precursor of SAM.

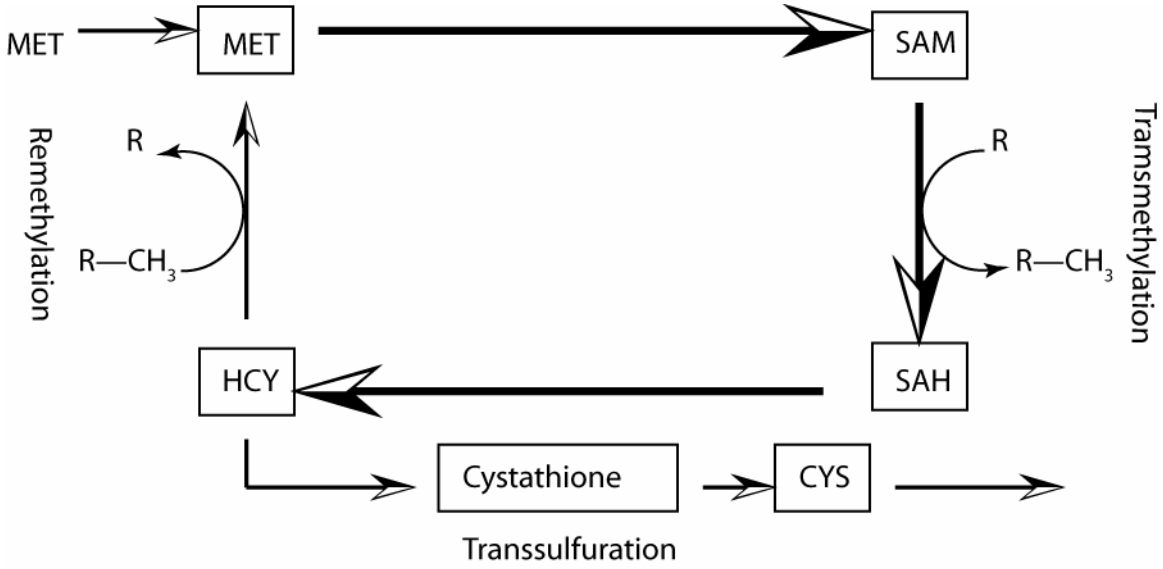

Figure 1. The elements of the methionine cycle (MET, methionine; SAM, S-adenosylmethionine; SAH, S-adenosylhomocysteine; HCY, homocysteine; CYS, cysteine; CH_3, methyl group).

If the entry of MET to the cycle is low, the deficit may be supplied by increased remethylation of HCY (Fig 2. a). If the amount of MET entering the cycle is in excess of requirements (Fig. 2b), methyl groups may be dissipated by converting HCY to cysteine (CYS). Because HCY represents a key control point in the cycle, high HCY levels are considered indicative of an imbalanced methionine cycle (Rees et al., 2006). In the following discussion, each of the reactions in the methionine cycle is described in detail.

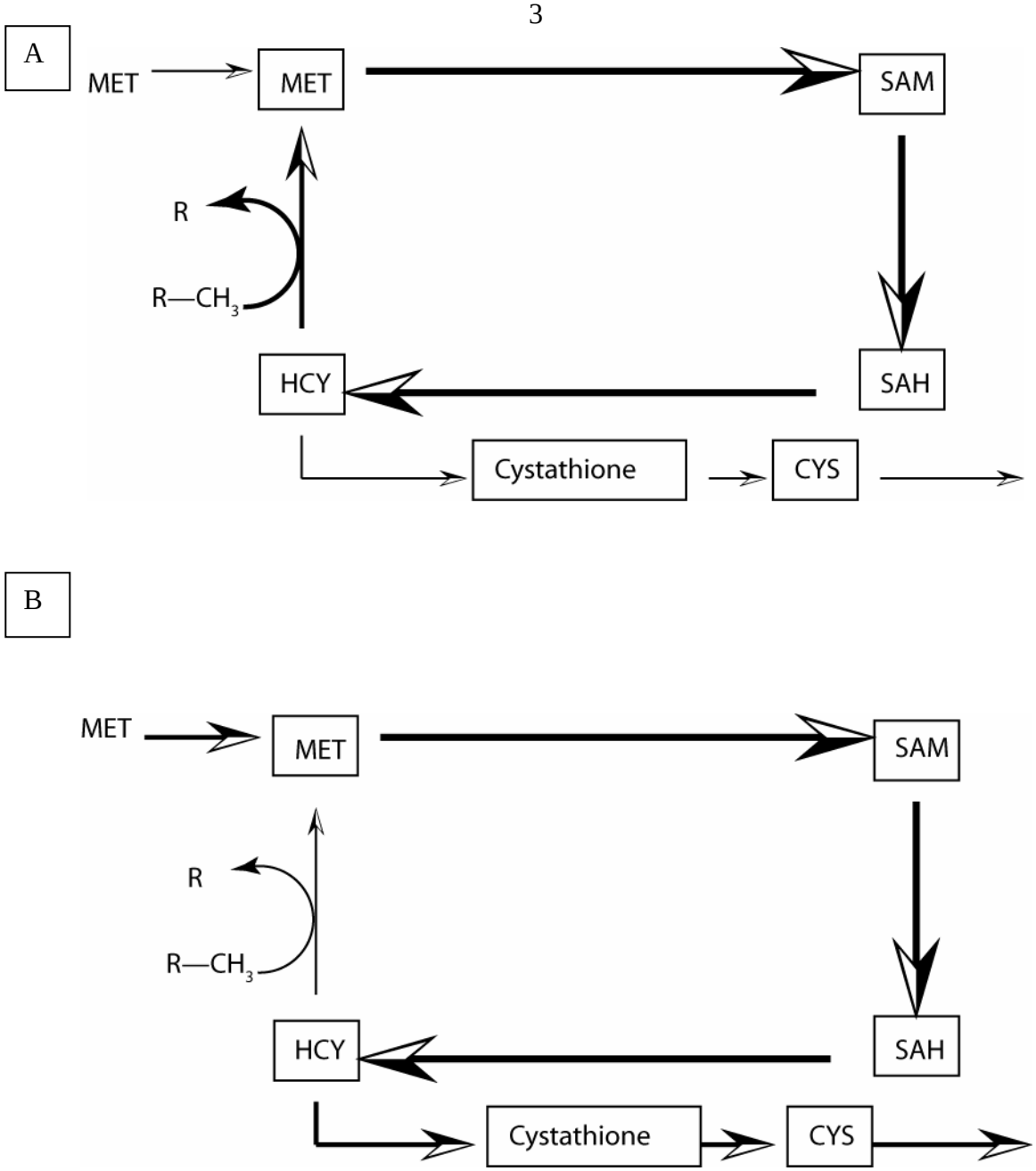

Figure 2. Flux through the methionine cycle when the input of methionine is low (A) or high (B). The thickness of arrow lines is scaled according to the magnitude of the flow along the indicated paths (MET, methionine; SAM, S-adenosylmethionine; SAH, S-adenosylhomocysteine; HCY, homocysteine; CYS, cysteine; CH_3, methyl group).

MET → SAM

The conversion of MET to SAM is catalysed by two isomers of the enzyme methionine adenosyl transferase, viz., MAT I and MAT III. These enzymes have different kinetic properties and play a role in stabilizing the conversion of MET to SAM (see later). In addition to its role as a methyl donor, SAM is a precursor for polyamines, which are essential for cell growth (Fukagawa, 2006). In rats, the pulsatile secretion of growth hormone in males is responsible for lower MAT activity than

in females, in which growth hormone secretion is more irregular and frequent, resulting in the continuous presence of this hormone (Oscarsson et al., 2001).

SAM → SAH

SAM is the most important donor of methyl groups in the body and is associated with more than 300 methylation reactions (Brosnan and Brosnan, 2006). These reactions encompass a range of functions such as the biosynthesis of small molecules (creatine, phosphatidylcholine, epinephrine), modification of macromolecules (DNA, RNA, proteins), detoxification of xenobiotics (thiols, arsenite) and activation of neurotransmitters (epinephrine, norepinephrine, dopamine) (Brosnan et al., 2007a). Of these reactions, those involving phosphatidylcholine and creatine synthesis (Fig. 3) account for the majority of methyl groups consumed in the methionine cycle. Although there is debate about the relative contribution of these two reactions to SAM metabolism (Stead et al., 2006; Mudd et al., 2007), there is agreement that together they account for more than two-thirds of the total demand for methyl groups.

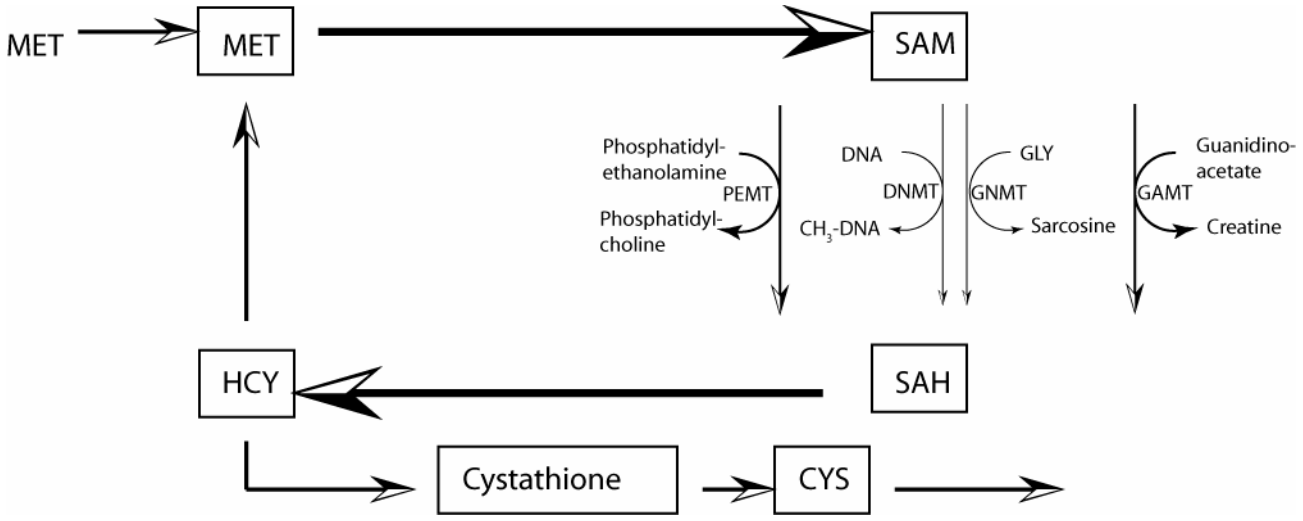

Figure 3. The main methyl acceptors and associated enzymes of the methionine cycle. The thickness of arrow lines is scaled according to the magnitude of the flow along the indicated paths (PEMT, phosphatidylethanolamine methyltransferase; DNMT, DNA methyltransferase; GNMT, glycine *N*-methyltransferase; GAMT, guanidinoacetate methyltransferase; MET, methionine; SAM, S-adenosylmethionine; SAH, S-adenosylhomocysteine; HCY, homocysteine; CYS, cysteine).

Creatine is formed in the liver by the transfer of a methyl group to guanidinoacetate and is then exported to muscle and brain tissue. Creatine is stored in muscle tissue and used as a source of energy during short, intense bursts of activity. Creatine also plays an important role in transporting high-energy phosphate compounds from the mitochondria, where they are produced, to the cytosol, where they are utilized (Brosnan et al., 2007b). In muscle, creatinine is formed from creatine by

irreversible non-enzymatic dehydration and loss of phosphate. This constant loss of creatine is proportional to muscle mass and must be replaced by resynthesis of creatine. It has been estimated that creatine synthesis is responsible for 33% of the demand for methyl groups in piglets (Brosnan et al., 2007a).

Phosphatidylcholine is synthesised in the liver from phosphatidyl ethanolamine and requires the transfer of three methyl groups from SAM. Phosphatidylcholine is the most abundant phospholipid in cell membranes and plays a key role in keeping membranes fluid and membrane receptors mobile. It is also an important component of bile chylomicrons, which assist in the absorption of lipids from the gut, and of lipoproteins, which transport lipids in the blood circulation.

Although quantitatively less significant than the contribution of SAM to the synthesis of creatine or phosphatidylcholine, SAM-mediated methylation of DNA is critical for the control of gene expression, without which life cannot be sustained.

SAH ↔ HCY

The conversion of SAH to HCY is catalysed by SAH hydrolase (SAHH) (Fig. 4).

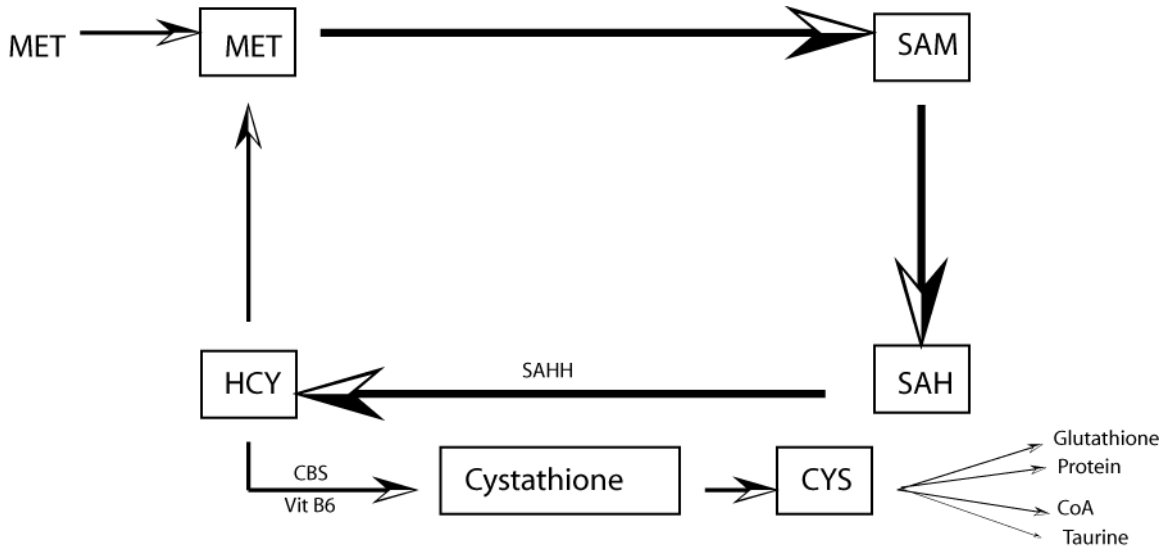

Figure 4. Transsulfuration reaction of the methionine cycle. The thickness of arrow lines is scaled according to the magnitude of the flow along the indicated paths (CBS, cystathionine β-synthase; MET, methionine; SAM, S-adenosylmethionine; SAH, S-adenosylhomocysteine; HCY, homocysteine; CYS, cysteine).

Although this is a reversible reaction with an equilibrium that favours the formation of SAH from HCY, under normal conditions removal of HCY ensures that the reaction proceeds from SAH to HCY (Sharma et al., 2006). Excess HCY is usually removed rapidly by transsulfuration to cysteine (CYS) via cystathione (Fig. 4).

HCY→ CYS

Transsulfuration is an irreversible process and reduces the amount of methyl groups in the methionine cycle. The transsulfuration pathway is highly active in the liver, kidneys, small intestine and pancreas (Brosnan et al., 2007a). The conversion of HCY to cystathione is catalysed by cystathionine β-synthase (CBS), which requires vitamin B6 as a co-factor. Cystathione is then converted to cysteine (CYS).

When excess protein is fed to rats, 89% of homocysteine produced is diverted to CYS synthesis. When adequate quantities of protein are fed, 46% of homocysteine is partitioned to CYS production. However, even when dietary protein intake is low, 34% of homocysteine is partitioned to CYS synthesis (Finkelstein and Martin, 1984). These findings indicate that although remethylation receives priority over transmethylation when methyl precursors are scarce, the amount of methyl groups lost to the transsulfuration pathway is still considerable. CBS activity is stimulated by glucagon and glucocorticoids and inhibited by insulin (Ulrey et al., 2005).

CYS is used for synthesis of glutathione (the most abundant anti-oxidant in the body), proteins, CoA, taurine (an osmolyte, membrane stabilizer, neurotransmitter and a component of bile acid; Brosnan and Brosnan, 2006) and as a source of S. In humans, the amount of CYS partitioned to glutathione production is usually slightly greater than that partitioned to protein synthesis, but protein synthesis has a higher priority for CYS than glutathione when protein intakes are low (Stipanuk et al., 2006).

HCY→ MET

HCY may be remethylated to form MET by one of two reactions in which methyl groups are derived either from 5-methyl tetrahydrofolate (5m-THF) or from betaine (Fig. 5).

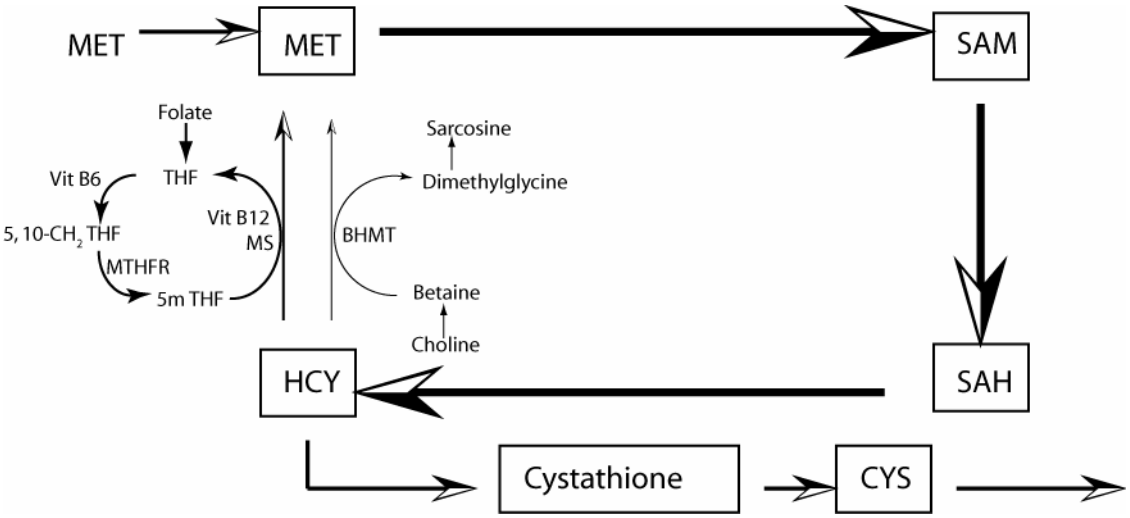

Figure 5. Remethylation reactions of the methionine cycle. The thickness of arrow lines is scaled according to the magnitude of the flow along the indicated paths (BHMT, betaine-homocysteine methyltransferase; MS, methionine synthase; MTHFR, 5,10-methylenetetrahydrofolate reductase; THF, tetrahydrofolate; MET, methionine; SAM, S-adenosylmethionine; SAH, S-adenosylhomocysteine; HCY, homocysteine; CYS, cysteine).

Methionine synthase (MS) catalyses the conversion of 5m-THF and HCY to MET and tetrahydrofolate (THF). In the folate cycle, THF is converted to 5,10-CH_2 THF, which is in turn converted to 5m-THF by methylenetetrahyrofolate reductase (MTHFR). The supply of THF is replenished by dietary folate. Vitamins B6 and B12 are required as co-factors for this cycle. Betaine-homocysteine methyltransferase (BHMT) catalyses the conversion of HCY and betaine to MET and dimethylglycine, a precursor of sarcosine. Betaine may be derived from the diet or from choline. Although the relative importance of these two pathways is not well understood, there is evidence from rodents showing that the BHMT reaction is quantitatively more important than the MS reaction (Reed et al., 2006). Published studies and model simulations indicate that HCY concentration is decreased by betaine supplementation when folate supply is low but HCY concentration is relatively insensitive to betaine when folate status is normal to high (Reed et al., 2006).

Regulation of the methionine cycle

Precise regulation of the methionine cycle is essential for two reasons. Firstly, a constant rate of methylation must be maintained to ensure that DNA is neither over- nor under-methylated (both of which have adverse effects) despite daily variation in the input of MET and co-factors. Secondly, although remethylation of HCY is essential to maintain the cycle when the entry of MET to the cycle is low, excess HCY must be removed from the cycle when MET input is high because accumulation of HCY results in inhibition of methylation.

Two isoforms of MAT are present in the liver; the activity of one (MAT I) is inhibited by its product, SAM, and that of the other (MAT III) is stimulated by SAM. As a result, the concentration of MET in the cycle is very constant when the entry of MET varies. In their model, Reed et al. (2004) showed that the concentration of MET varied by only 7% when the entry of MET to the system was varied by 600%. Although MET concentrations remain constant because of the actions of MAT I and MAT III, the concentration of their product, SAM, varies in proportion to the methionine input to the cycle (Reed et al., 2004). The sensitivity of SAM to MET input to the cycle and its ability to regulate the activities of several key enzymes in the cycle play a major role in regulating flow through the cycle. Enzymes that are regulated by SAM are shown in Fig. 6.

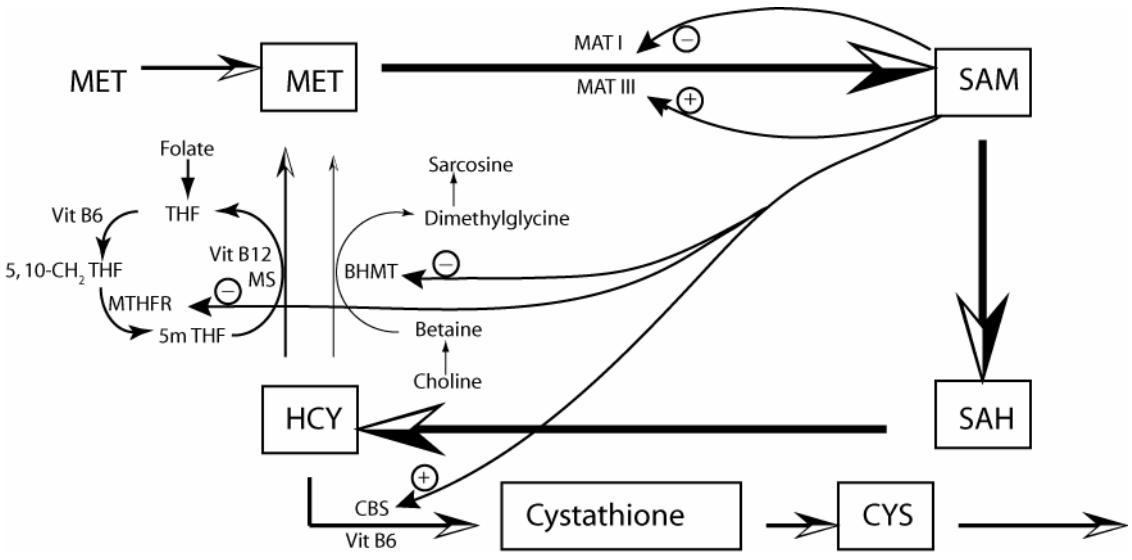

Figure 6. Allosteric regulation of enzymes of the methionine cycle by S-adenosyl methionine (SAM). A plus symbol indicates stimulation of enzyme activity and a minus symbol indicates inhibition of enzyme activity. The thickness of arrow lines is scaled according to the magnitude of the flow along the indicated paths (BHMT, betaine-homocysteine methyltransferase; MS, methionine synthase; MTHFR, 5,10-methylenetetrahydrofolate reductase; THF, tetrahydrofolate; MET, methionine; SAH, S-adenosylhomocysteine; HCY, homocysteine; CBS, CBS, cystathionine β-synthase; CYS, cysteine)

When the input of MET to the cycle increases, an elevated SAM concentration reduces the amount of methyl groups in the cycle by inhibiting the activities of enzymes involved in remethylation of HCY (MTHFR and BHMT) and stimulating the activity of the enzyme responsible for the irreversible loss of HCY from the cycle (CBS) (Fig. 6). Conversely, when the input of MET is low, SAM concentrations decrease and CBS activity returns to basal non-stimulated levels, conserving HCY for remethylation.

The methionine cycle is also affected by hormones (Ulrey et al., 2005). Glucocorticoids increase the conversion of MET to SAM, the conversion of SAM to SAH and the conversion of HCY to CYS. Glucagon increases conversion of SAM to SAH and conversion of HCY to CYS, whereas insulin has the opposite effect.

Several models have been developed to simulate the methionine cycle in humans (Reed et al., 2004, 2006; Nijhout et al., 2006; Reed et al., 2008). Nijhout et al. (2006) examined the efficacy of SAM- mediated regulation of the enzymes of the methionine pathway on variation in DNA methylation rate and concluded that methylation was remarkably stable at low input levels of MET. In their simulations, Reed et al. (2004) showed that methylation was only slightly decreased by folate deficiency. However, betaine deficiency resulted in a substantial decrease in methylation. It has been proposed that the primary role of the BHMT reaction is not to stabilize methylation but to regulate HCY clearance (Nijhout et al., 2006). Consistent with this role, excessive dietary MET administration in rats results in depletion of betaine (Finkelstein and Martin, 1986).

If SAM-mediated stimulation of transsulfuration is sufficient to prevent HCY levels from increasing, SAH levels remain constant. However, in the event of failure to maintain HCY levels within acceptable limits, the level of SAH will increase because the equilibrium of the reversible SAH–HCY reaction strongly favours SAH. High levels of SAH strongly inhibit methylation (Fig. 7) because most methyltransferases bind SAH with a higher affinity than SAM (Yi, et al., 2000). GNMT is less sensitive to inhibition by SAH than other methyltransferases, which ensures that methyl groups are disposed of when the supply is excessive (Williams and Schalinske, 2007).

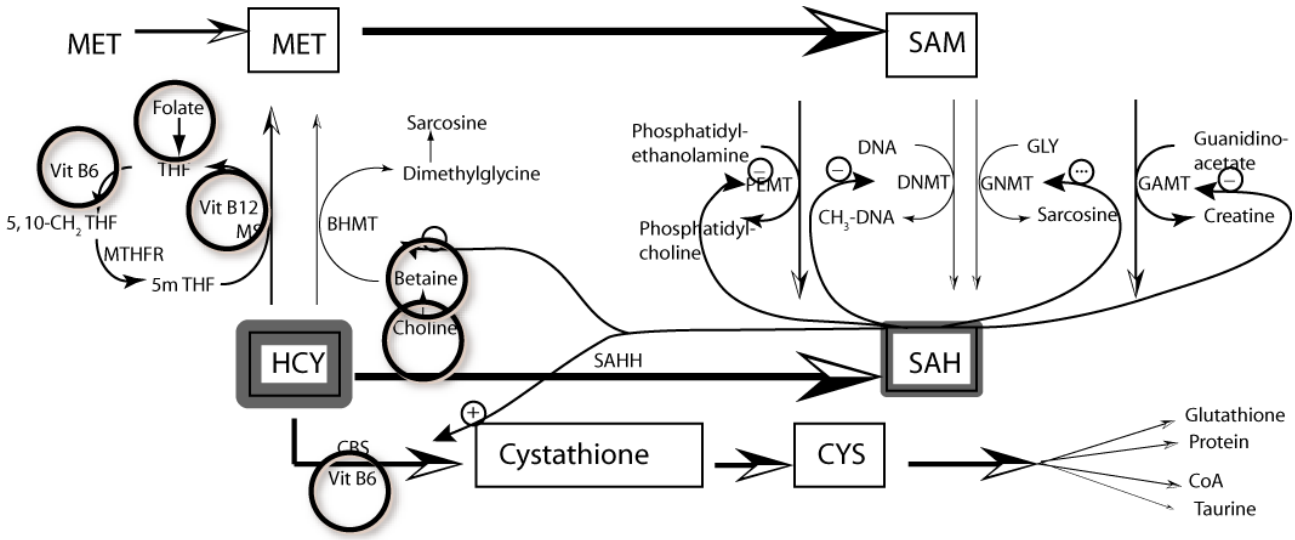

Figure 7. Allosteric regulation of enzymes of the methionine cycle by S-adenosylhomocysteine (SAH). Accumulation of HCY reverses the direction of the SAH–HCY reaction and increases the level of SAH. Deficiencies of compounds that may result in accumulation of HCY are encircled. A plus symbol indicates stimulation of enzyme activity and a minus symbol indicates inhibition of enzyme activity. The thickness of arrow lines is scaled according to the magnitude of the flow along the indicated paths (BHMT, betaine-homocysteine methyltransferase; MS, methionine synthase; MTHFR, 5,10-methylenetetrahydrofolate reductase; THF, tetrahydrofolate; PEMT, phosphatidylethanolamine methyltransferase; DNMT, DNA methyltransferase; GNMT, glycine *N*-methyltransferase; GAMT, guanidinoacetate methyltransferase; MET, methionine; SAM S-adenosyl methionine; HCY, homocysteine; CBS, cystathionine β-synthase; HCY, homocysteine; CYS, cysteine).

As SAH is a more potent regulator of DNA methyltransferases than SAM (Yi. et al., 2000; James et al., 2002; Ulrey et al., 2005), the level of HCY is considered to reflect the status of the methionine cycle (Rees et al., 2006). In humans, high HCY levels are associated with a wide range of diseases affecting the heart (atherosclerosis), nervous system (Alzheimer's disease, schizophrenia), kidneys (end-stage renal disease), bones (osteoporosis), metabolism (non-insulin-dependent diabetes) and fetal development (neural-tube defect). There are three routes by which accumulation of HCY may be prevented: conversion to MET by BHMT or MS or conversion to CYS via CBS. Thus, deficiencies of choline, betaine, folate or of the co-factors involved in these pathways, vitamins B6 and B12, may induce high levels of HCY (Fig. 7).

Hyperhomocysteinemia and the adequacy of methyl cycle intermediates in growing pigs

In a study involving 700 children aged between 4 days and 19 years, Monsen et al. (2003) determined that plasma HCY concentrations increase from a median of 6 μmol/L at 4 days of age to 8 μmol/L at 4 years of age, after which they decrease to 5 μmol/L at one year of age and remain stable until 7 years of age. Between 7 and 19 years of age, the level of HCY gradually increases to 8 μmol/L at 19 years of age. Further analysis of data for children older than six months showed that HCY concentrations above the 75th percentile (>7.85 μmol/L) were associated with folate and vitamin B12 levels. This indicates that growing children with HCY concentrations in excess of 7.85 μmol/L should be classed as hyperhomocysteinemic. Several authors have reported HCY concentrations in growing-finishing pigs that exceed 7.85 μmol/L: 170 μmol/L, Skomial et al. (2004); 40 μmol/L, Smolin et al. (1983); 26 μmol/L, Giguere et al. (2005); 20 μmol/L, Stangle et al., (2000); 14 μmol/L, Zhang and House, (2006); and 11 μmol/L, Ambrosi et al. (1999). Although the reason for this species difference is unclear, hyperhomocysteinemia in pigs may be indicative of an imbalance in the methionine cycle.

HCY and the demand for creatine synthesis

In week-old piglets, it has been estimated that one third of methyl groups consumed as methionine are partitioned to creatine synthesis (Brosnan et al., 2007a). This implies that one third of the HCY produced by piglets is a consequence of creatine synthesis. Stead et al. (2001) examined the effect of reducing methylation demand by supplementing the diets of hyperhomocysteinemic rodents with creatine. They found that creatine supplementation reduced HCY concentration by 27%. Even though sow milk only provides one third of the creatine accreted by week-old piglets (Brosnan et al., 2007a), creatine supplementation of piglets before or after weaning has no effect on growth or the creatine content of muscle (Miller at al., 1962; Guzik et al., 2000).

HCY and vitamin B12

House and Fletcher (2003) studied the vitamin B12 requirements of early-weaned (5–10 kg) pigs using the plasma concentration of HCY as a response indicator. They concluded that the pattern of responses in plasma vitamin B12 and HCY concentrations indicated that the vitamin B12 requirement of the early-weaned piglet was double the level recommended by the NRC (1998). Although supplementation with vitamin B12 for 15 days reduced plasma HCY concentrations, this did not have a significant effect on growth rate, feed conversion efficiency or feed intake.

HCY, betaine and choline

Emmert et al. (1998) examined the responses of growing pigs to betaine and choline supplementation of diets deficient in methionine and concluded that BHMT activity was not substantially affected by MET deficiency or by surfeit levels of betaine or choline. However, subsequent research has shown that BHMT activity has little effect on the rate of methylation at moderate or low MET input levels but has a substantial effect on methylation rate (Nijhout et al., 2006) and HCY level (Holm et al., 2005) when methionine input is high. In support of this, one of the trials conducted by Emmert et al. (1998) showed that betaine supplementation of a diet adequate in methionine increased the activity of renal BHMT by 70%. Siljander-Rasi et al. (2003) reported that neither betaine nor choline supplementation had a statistically significant effect on plasma HCY concentrations of growing-finishing pigs fed a low-energy diet when analysed separately. However, when the data for betaine and choline supplementation were pooled, plasma HCY concentrations were significantly decreased.

Hyperhomocysteinemia and the adequacy of methyl cycle intermediates in gestating sows

In a study involving 235 men and women, Rasmussen et al. (1996) proposed the following upper reference limits for adult HCY concentrations: 8.1 μmol/L for subjects less than 30 years old, 7.9 μmol/L for women 30–59 years old, 11.2 μmol/L for men 30–50 years old and 11.9 μmol/L for subjects older than 60 years. HCY concentrations are lower in pregnant women than in non-pregnant women (Holmes et al., 2005). Median plasma HCY concentrations of pregnant women increase from about 5 μmol/L in early pregnancy to about 8 μmol/L during the last trimester of pregnancy (Holmes et al., 2005; Milman et al., 2006). Although Milman et al. (2007) defined the upper reference value for plasma HCY concentration during pregnancy as 11 μmol/L, Holmes et al. (2005) showed that a plasma HCY concentration of 8 μmol/L or more was associated with a history of miscarriage. In pregnant women, elevated HCY concentrations are associated with fetal neural tube defects, fetal death in early pregnancy, placental dysfunction and preeclampsia (see Holmes et al., 2005). Mean plasma HCY concentrations in gestating pigs are threefold greater than in pregnant women, increasing from 17 μmol/L between day 30 and 110 of gestation to 24 μmol/L at parturition (Simard et al., 2007). The high HCY levels in gestating pigs may be indicative of an imbalance in the methionine cycle.

HCY and folic acid

In a study involving 400 healthy pregnant women, Milman et al. (2006) showed that plasma folate concentrations decrease during pregnancy and are negatively correlated with plasma HCY

concentrations, suggesting that progressive depletion of folate reserves during pregnancy is responsible for the increase in the level of HCY. Consistent with this, Holmes et al. (2005) reported that HCY concentrations of women in the third trimester of pregnancy are decreased by folate supplementation. Serum folate concentrations of sows also decrease during pregnancy (Harper et al., 1994). Supplementation of a basal diet containing 1 mg/kg folate, which is close to the level recommended by the NRC (1998) for gestating and lactating sows (1.3 mg/kg), with an additional 15 mg/kg folate reduced the HCY concentration of plasma in gestating sows by 10% and reduced that of the uterus by 30% (Guay et al., 2002a). In a review of folic acid supplementation of gestating sows, Lindemann (1993) concluded that folate supplementation during early gestation improves embryo and fetal survival, resulting in increases in litter size.

HCY and vitamin B12

Plasma vitamin B12 concentrations are twofold lower in gilts than in multiparous sows (Guay et al., 2002b). During gestation, plasma vitamin B12 concentrations of gilts decrease to a nadir at 30 d of gestation and subsequently increase to pre-gestational levels by the end of gestation (Simard et al., 2007). Vitamin B12 supplementation of a diet containing an adequate level of folate decreased plasma HCY concentrations of gestating gilts (Simard et al., 2007). Furthermore, the effect of vitamin B12 supplementation on plasma HCY levels persisted during lactation. Maximum residual responses in the gilts and their progeny were obtained when gestating gilts were given 100 and 200 μg/kg of supplementary vitamin B12, respectively. Plasma concentrations of cysteine in the gilts and their piglets were not affected by supplementary vitamin B12, indicating that the reduction in HCY level was not caused by increased activity of the transulfuration pathway but by increased vitamin B12-mediated remethylation of HCY catalysed by methionine synthase. The level at which the optimum effects of vitamin B12 on HCY were observed suggests that diets formulated using the NRC (1998) recommendation of 15 μg/kg vitamin B12 may result in an imbalance in the methylation cycle in gestating sows. Simard et al. (2007) reported that vitamin B12 supplementation of gestating sows increased litter size and weight at parturition ($P = 0.06$) and had a significant residual effect on litter size at weaning.

In their review of the literature, Matte et al. (2006) showed that beneficial embryo and uterine responses to folate were more prevalent among multiparous sows than gilts because vitamin B12 was more limiting than folate in gilts. They concluded that the NRC (1998) vitamin B12 recommendations may be 10 times less than those required to minimize HCY accumulation in first parity sows and suggested that effective realization of the beneficial effects of folate on sow prolificacy is dependent on definition of the optimum folate:vitamin B12 ratio.

HCY, choline and betaine

The preceding evidence indicates that methylation reactions in gestating sows fed diets formulated according to NRC (1998) recommendations may be constrained by the supply of folate or vitamin B12. If this were the case, a response to betaine or choline supplementation would be expected, as remethylation of HCY via this pathway is not reliant on the vitamin B12 or folate status of the animal. A study commissioned by the NRC involving 552 sows in 22 trials at nine research stations concluded that dietary supplementation of gestating sows with choline increases the number of live pigs born per litter (NRC-42 Committee on Swine Nutrition, 1976). In a subsequent review of the literature, NRC (1998) concluded that supplementation of gestating gilts fed grain–soybean diets with 434–880 mg/kg choline increases the number of piglets born alive and the number of piglets weaned. There do not appear to be any published trials in which gestating pigs have been supplemented with betaine.

HCY: the folate/vitamin B12 pathway vs the choline/betaine pathway

In humans, plasma concentrations of betaine, folate and vitamin B12 are correlated with HCY concentrations. In their study of pregnant women, Velzing-Aarts et al. (2005) observed that plasma folate concentration was the strongest predictor of plasma HCY concentration in early gestation but betaine was the strongest predictor of plasma HCY concentration in mid-to-late gestation. The strong association between betaine and HCY during pregnancy is at variance with that in non-pregnant humans, in whom betaine is less strongly associated with HCY than folate except when folate status is low (Holm et al., 2005). In non-pregnant humans, folate supplementation increases plasma betaine concentrations by up to 15%, indicating that increased flux through the folate pathway inhibits flux through the betaine pathway, sparing betaine and choline (Melse-Boonstra et al., 2005). In the study of Velzing-Aarts et al. (2005), both folate and choline plasma levels increased during pregnancy whereas those of betaine and vitamin B12 decreased. They suggested that the increase in plasma choline level represents a means of ensuring that sufficient choline is available for placental transfer to meet fetal requirements and that the change in the activities of the two remethylation pathways during gestation represents a mechanism for conserving methionine via a folate-independent route. Subsequently, Friesen et al. (2007) proposed that the increase in maternal choline levels and decrease in betaine levels during pregnancy is consistent with an increase in the conversion of choline to betaine to supply glycine to the fetus for glutathione (GSH) synthesis.

The NRC (1998) nutrient recommendations for swine list only one level of methionine for the duration of gestation, which may result in consumption of excess methionine during the first 70 days of gestation and a deficit of methionine during the last 44 days of gestation (Baker, 2005). When MET input to the methionine cycle is excessive, surplus methyl groups are dissipated from

the cycle by transsulfuration. Because GNMT is less inhibited by high SAH levels than other methyltransferases (Williams and Schalinske, 2007), the initial recipient of methyl groups from SAM is glycine. As the developing fetus has high demand for glycine, excess methionine may result in glycine deficiency (Rees et al., 2006). Glycine is important for the synthesis of collagen, elastin, bile acids, purines, porphyrins, creatine and GSH (Friesen et al., 2007). Most studies of the effects of methyl donors and their co-factors in gestating pigs have involved the application of treatments for the duration of gestation. As available evidence indicates that the optimum level and type of methyl donor differs according to the stage of gestation, further research to refine the requirements of gestating pigs not only for methyl donors and their cofactors but also for methionine is warranted.

The adequacy of methyl cycle intermediates in lactating sows

In a trial involving 393 sows and five experimental stations, Harper et al. (1994) demonstrated that serum folate concentrations increase during lactation. In a subsequent trial, Harper et al. (2003) reported that dietary folate supplementation of gilts had no effect on sow or litter performance even though folate supplementation almost doubled sow plasma folate concentrations at weaning. Furthermore, folate supplementation had no effect on reproductive performance in the subsequent parity.

Choline is present in milk in the form of membrane phospholipids in milk-fat globules. Levels of choline secretion into milk are generally maintained even at the cost of depleting liver reserves of choline (Pinotti et al., 2002). However, choline supplementation during lactation does not appear to affect sow feed intake, piglet survival or growth to weaning (Boyd et al., 1982; NRC 1998).

Hyperhomocysteinemia and polymorphisms of genes involved in the methionine pathway

Elevated levels of HCY may also be caused by gene polymorphisms. In humans, elevated HCY levels have been associated with gene polymorphisms that reduce the activities of CBS, MS and MTHFR (Friso and Choi, 2002; Brosnan et al., 2004; Reed et al., 2006). It is noteworthy that the MS and MTHFR variants exhibit nutrient–gene interactions: individuals with the MTHFRC677T polymorphism only display elevated HCY levels in the presence of low folate status and those with the MS polymorphism only display elevated levels of HCY in the presence of low vitamin B6 status (Friso and Choi, 2002). In a literature mining study Sharma et al. (2006) identified 17 other genes that modulate HCY levels. As pigs have been subjected to intense selection pressure, it is possible that a high prevalence of one or more polymorphisms associated with elevated HCY levels exists in this species.

Implications of an inadequate supply of methionine cycle intermediates in pigs

HCY concentrations in pigs are typically several-fold higher than in other species, which indicates that the supply of methionine cycle intermediates may be imbalanced. Although no studies have been conducted to determine the effects of elevated HCY levels on pig productivity and health, evidence from other species suggests that reduction of HCY levels in pigs may have beneficial effects.

Sharma et al. (2006) reviewed the literature for reports of genes affected by elevated levels of HCY and mapped these genes to their respective biochemical pathways. They found that 135 genes were affected by HCY. The biochemical pathways involved included those associated with the metabolism of glucose, insulin, lipids, lactate, calcium, chloride, the immune system (cytokines and anti-oxidant activity), the circulatory system (blood coagulation, blood pressure, vasodilation, nitric oxide and atherosclerosis), the nervous system, the cell cycle and cell death.

Homocysteine-mediated ulcer formation and disease susceptibility

Reactive oxygen species are a normal product of aerobic metabolism but if allowed to accumulate will damage cell membranes, DNA and proteins. Glutathione (GSH) constitutes the most important defence of the body against reactive oxygen species. GSH is particularly important for maintaining gut integrity and preventing gut disorders (Aw, 2005). In a reaction catalysed by glutathione peroxidase (GPx), GSH converts reactive oxygen species such as hydrogen peroxide to H_2O and is itself oxidised to its disulfite form, GSSG. Transsulfuration of methionine to cysteine for GSH synthesis is necessary for the maintenance of intestinal epithelial cells; exposure of the gut to reactive oxygen species increases the conversion of methionine to cysteine (Shoveller et al., 2005). However, when HCY levels are elevated, HCY undergoes auto-oxidation, forming reactive oxygen species such as hydrogen peroxide, lipid peroxides, peroxynitrite and superoxide (Hayden and Tyagi, 2004). Although the presence of these reactive oxygen species increases the production of GSH, hydrogen peroxide attenuates the activity of GSH by inhibiting the transcription of GPx (Upchurch et al., 1997) (Figure 8). Consequently, cell membranes are damaged and gut ulcers result.

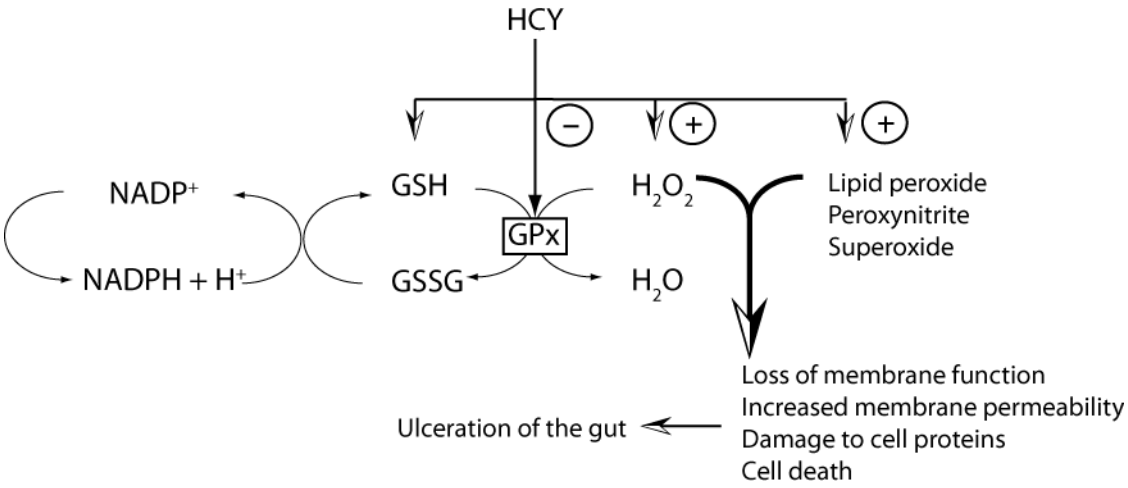

Figure 8. Mechanism by which elevated levels of homocysteine (HCY) in pigs may result in gut ulcers (see text for details). H_2O_2, hydrogen peroxide; GPx, glutathione peroxidase; GSH, glutathione; GSSG, oxidised glutathione; NAD, nicotinamide adenine dinucleotide phosphate. The thickness of arrow lines is scaled according to the magnitude of the flow along the indicated paths.

The incidence of gut ulcers in pigs of slaughter weight, which has been reported to be as high as 90% in the USA (Friendship, 2005), indicates that that the gut health of grower–finisher pigs is compromised under commercial production conditions. Gut lesions in growing pigs fed finely ground, pelleted diets are caused by high levels of reactive oxygen species, which appear to overwhelm the ability of the gut to produce sufficient GSH to prevent tissue damage (Eisemann and Argenzio, 1999). GSH synthesis is often limited by CYS, 50% of which is derived from the transsulfuration pathway (Brosnan and Brosnan, 2006). The liver has traditionally been thought of as the primary site of metabolism of dietary methionine and cysteine. However, recent evidence indicates that the gut of the young piglet extracts up to 52% of dietary methionine (Shoveller et al., 2005), two-thirds of which is used for the synthesis of cysteine (Riedijk et al., 2007). This indicates that the gut of pigs is exposed to a high load of reactive oxygen species under normal conditions. An elevated HCY level would exacerbate this situation by increasing the production of reactive oxygen species and impairing the ability of GSH to destroy them.

Several studies have shown that betaine supplementation improves gut health and improves the digestibility of dietary protein and fat in pigs (reviewed by Eklund et al., 2005). The relationship between high levels of HCY and depression of GSH synthesis represents a possible causative link between diet and the incidence of gut lesions in pigs, and deserves further investigation.

Homocysteine-mediated embryonic death and fetal intra-uterine growth retardation

Elevated levels of HCY result in cardiovascular disease in humans because HCY inhibits the production of endothelial nitric oxide (NO), a vasodilator that regulates blood flow (Stuhlinger et al., 2001). Impaired synthesis of NO in the placental vasculature will decrease the transfer of nutrients and oxygen to the fetus, which could result in intra-uterine growth retardation. As discussed previously, HCY induces oxidative stress by forming reactive oxygen species and inhibiting antioxidant activity. HCY decreases the availability of NO by promoting its degradation and impairing its synthesis (Fig. 9).

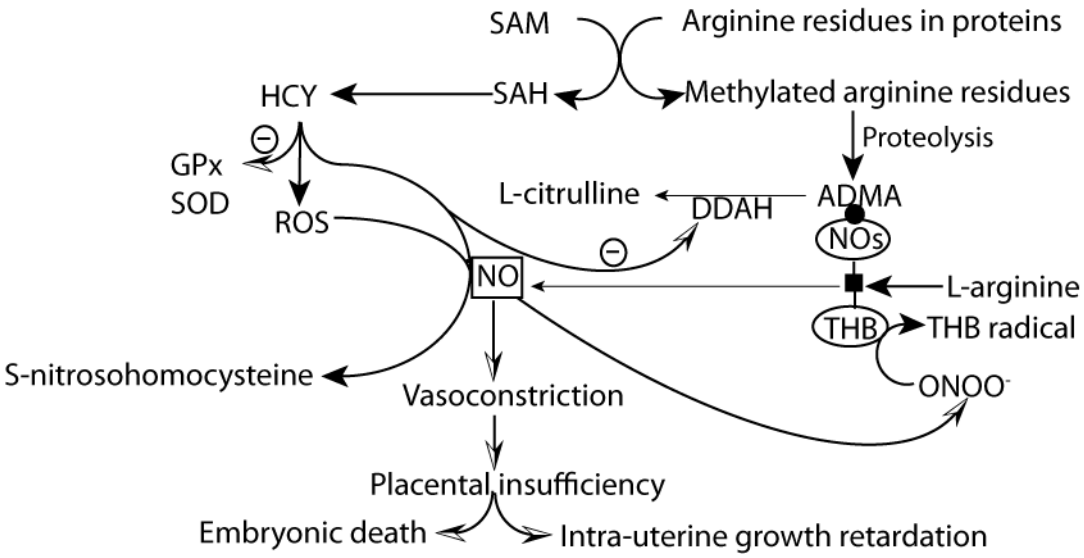

Fig. 9. Mechanism by which elevated levels of homocysteine in pigs may result in embryonic death or intrauterine growth retardation of surviving embryos (see text for details). SAM, S-adenosyl methionine; SAH, S-adenosyl homocysteine; HCY, homocysteine; GPx, glutathione peroxidase; SOD, superoxide dismutase; ROS, reactive oxygen species; NO, nitric oxide; ADMA, asymmetric dimethylarginine; NOs, nitric oxide synthase; THB, tetrahydrobiopterin; $ONOO^{-}$, peroxynitrite.

HCY promotes the degradation of NO by reacting directly with NO to form S-nitrosohomocysteine and indirectly by producing hydrogen peroxide and superoxide, which degrade NO (Stuhlinger et al., 2001). HCY decreases the synthesis of NO by impairing the activity of nitric oxide synthase (NOs) via two mechanisms. Asymmetric dimethylarginine (ADMA) is a competitive inhibitor of NOs and is released when proteins containing methylated arginine residues are degraded during protein turnover (Boger, 2004). Under normal conditions, ADMA-mediated inhibition of NOs activity is prevented by its conversion to L-citrulline by the enzyme, dimethyl-arginine dimethylaminohydrolase (DDAH). HCY directly inhibits the activity of DDAH by attacking a critical sulfhydryl group in DDAH (Stuhlinger et al., 2001). Consequently, ADMA accumulates and

decreases the synthesis of NO by competing for NOs. HCY also inhibits NOs activity indirectly by forming reactive oxygen species that react with NO to form peroxynitrites ($ONOO^-$), which convert tetrahydrobiopterin (THB), a co-factor required by NOs, to the trihydrobiopterin radical (Sharma et al., 2006). Thus, elevated levels of HCY decrease the synthesis of NO and increase its degradation. When NO levels are insufficient to maintain vasodilation, the consequent vasoconstriction may cause placental insufficiency, embryonic death and intra-uterine growth retardation.

A recent proteomic study revealed that piglets that have been exposed to intra-uterine growth retardation have decreased levels of proteins involved in immune function, defence against oxidative stress, protein synthesis, tissue growth, iron metabolism, carbohydrate metabolism and fatty acid metabolism, and increased levels of proteins involved in protein breakdown, ATP breakdown and response to oxidative stress (Wang et al., 2008). It is likely that many of these effects are mediated by insufficient transfer of methionine cycle intermediates to the fetus because induction of intra-uterine growth retardation in rats by uterine artery ligation increases levels of SAH and HCY (MacLennan et al., 2004). The postnatal consequences of intra-uterine growth retardation in pigs include reduced growth rate, feed efficiency, skeletal muscle fibre number and meat quality and increased muscle collagen and fat content, incidence of stillbirths, neonatal morbidity and mortality and susceptibility to necrotizing enterocolitis (Wu et al., 2006). Although hormonal therapy and dietary supplementation with energy, protein, antioxidants and arginine have been used to improve placental insufficiency in livestock (Wu et al., 2006), reduction of HCY levels may represent a more cost-effective approach to reduce the incidence of intra-uterine growth retardation in pigs.

Homocysteine-mediated epigenetic impairment of genetic potential

Epigenetic regulation of gene expression is accomplished by methylation of cytosine residues in regions near the promoter regions of genes and by methylation of histones, both of which inhibit gene expression (Van den Veyver, 2002; Frisco and Choi, 2002). After fertilization, the maternal and paternal genomes undergo extensive demethylation, followed by *de novo* remethylation at the time of implantation. Subsequently, DNA methylation plays a key role by silencing the expression of specific genes during the process of embryonic and fetal cell differentiation and the development of various tissues (Burdge et al., 2007).

High levels of HCY reverse the direction of the SAH–HCY reaction and the resulting accumulation of SAH inhibits methylation of DNA and of histones. Hypomethylation results in aberrant gene expression, which may exert a variety of effects in pigs, ranging from embryonic death to impaired productivity of surviving progeny (Fig. 10).

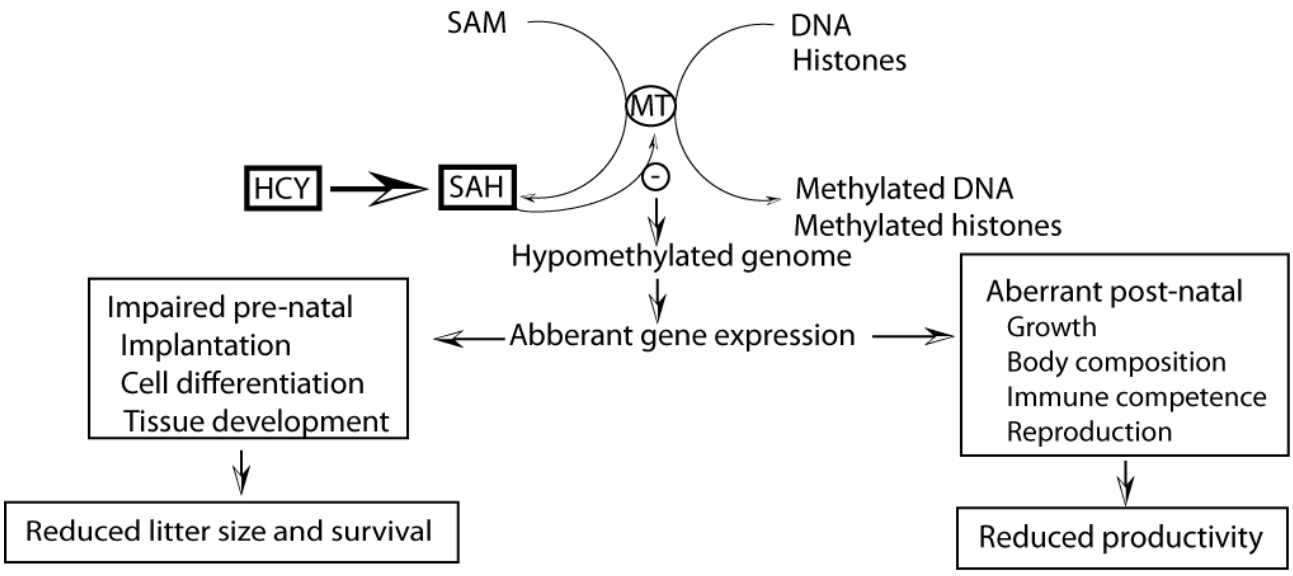

Fig. 10. The epigenetic mechanism by which an elevated level of homocysteine may induce embryonic death and impair the genetic potential of surviving progeny. SAM, S-adenosyl methionine; SAH, S-adenosyl homocysteine; HCY, homocysteine; MT, methyl transferase.

Aberrant methylation of a single key transcription factor during fetal development could alter many metabolic and developmental pathways (Burdge et al., 2007). Transcription factors such as the peroxisomal proliferator-activated receptor (PPAR) family function as lipid sensors and modulate the activities of a broad spectrum of genes involved in energy and fat metabolism, inflammatory processes, wound healing, reproduction, and embryonic development (Nunn et al., 2007). Altered expression of PPARs in the offspring of rats fed low-protein diets during gestation

appear to be a consequence of HCY-mediated depression of DNA methylation because folic acid supplementation of the pregnant dams prevented gene hypomethylation in the offspring (Lillycrop et al., 2005, 2008). However, deficiencies of vitamin B12, betaine or choline also induce epigenetic changes in gene expression (Wolff et al., 1998; Cooney et al., 2002). In addition to the mechanism illustrated in Fig. 10, HCY also inhibits the production of retinoic acid, which is also involved in fetal gene expression (Guay et al., 2002a).

A plethora of human and rodent studies (Barker, 1998, 2000; Godfrey, 1998) have established that maternal nutrient deficits during gestation, particularly of methionine (Rees, 2002), result in permanent changes in gene expression of the progeny in later life. In livestock species, maternal nutrient deficits during gestation may affect the growth rate, body composition, reproduction, immune competence, stress susceptibility and health of the progeny (Robinson et al., 1999; Harding, 2001; Cronjé, 2003a, 2003b; Bell, 2006). In sows, feed restriction during the last week of lactation is sufficient to induce epigenetic effects during the subsequent pregnancy that result in embryonic loss (Vinsky et al., 2007).

Given that there is evidence that supplementation with methionine cycle intermediates decreases HCY levels in gestating sows and has beneficial effects on litter size and weight (see previous discussion), further studies on the long-term effects of elevated maternal HCY concentrations on the health and productivity of the progeny are warranted.

Elevated HCY levels also affect methylation in adults. Yi et al. (2000) studied 58 healthy adult women and found that a moderate elevation in HCY concentration (from 7.3 ± 1.1 μM to 12.3 ± 1.8 μM) was sufficient to induce lymphocyte DNA hypomethylation. In humans and rodent models, DNA hypomethylation has been associated with central nervous system demyelation, reduced neurotransmitter synthesis, chemotaxis and macrophage phagocytosis, and altered membrane phospholipid composition, membrane fluidity, gene expression, and cell differentiation (Yi et al., 2000). Elevated HCY levels result in increased levels of reactive oxygen species, which have adverse effects on many aspects of reproduction such as oocyte maturation, fertilization, maintenance of pregnancy and parturition (Agarwal et al., 2005). Long-term studies on the effects of HCY levels on the health and productivity of sows across multiple parities are warranted.

Conclusions

No systematic studies have been undertaken to determine the requirements of pigs for methyl groups. With the exception of one study on piglets fed milk-replacer (Riedijk et al., 2007), no quantitative studies have been conducted on the dynamics of the methionine cycle in pigs. However, several studies have shown that HCY levels in pigs are high, which indicates that the supply of methyl donors or methionine cycle co-factors is inadequate. Studies showing that supplementation of diets considered nutritionally adequate with extra vitamin B12 and folate reduces HCY levels in pigs and increases sow productivity, which is consistent with the notion that current nutrient recommendations for pigs are insufficient for optimum methionine cycle activity. Although elevated HCY levels are associated with a wide range of adverse effects in humans, no large-scale epidemiological studies have been conducted to determine the consequences of elevated HCY levels in pigs. Although reduction of HCY levels in humans by supplementation with betaine, folic acid and vitamin B12 has beneficial effects on health (reviewed by Craig, 2004), few studies on supplementation of pigs with these compounds have considered effects other than growth rate and carcass composition. The potential benefits of research aimed at restoring the balance in the methionine cycle and reducing HCY levels include increased disease resistance, reduced incidence of gut lesions, reduced incidence of intra-uterine growth retardation and reduction of epigenetic impairment of genetic potential.

Recommendations

Development of a methionine cycle simulation model

Because of the complexity of the methionine cycle, a simulation model of the methionine cycle in pigs would be of great value in identifying areas in which investment in research into the adequacy of methyl donors in pigs would be most likely to yield useful outcomes. Reed et al. (2004) developed a model of the human methionine cycle because non-linearities among the components of the cycle render the response to [experimental] perturbation context-dependent and therefore non-intuitive and unpredictable. Several models involving various aspects of the human methionine cycle have been developed and validated by Reed's group and would be a useful starting point for the development of a porcine methionine cycle model and its incorporation into the AusPig model. It is suggested that the best initial use of such a model would be to identify the most likely reason for high HCY levels in pigs.

Establishing the benefits of reducing HCY levels in pigs

Although there is a surfeit of evidence on the adverse effects of elevated HCY levels in humans and rodent models, no comparable studies have been conducted with pigs. Therefore, it is important for the credibility of a research effort in this area to establish a link between HCY levels in pigs and various aspects of productivity. An epidemiological approach in which blood samples for HCY analysis are taken from pigs involved in CRC and other research projects for which reliable production data is available would be a most cost effective way of establishing that research aimed at reducing HCY levels in pigs would bear dividends for the industry. For example,

1. Data and samples from project 2C-105 0607 (Probiosis - a novel strategy for improved gut health and feed conversion efficiency..) could assist research into the association between HCY and ulcers.
2. Data and samples from projects currently investigating aspects of seasonal infertility and sow longevity could assist research into the association between HCY and epigenetic impairment of genetic potential, intrauterine growth retardation and long-term sow productivity.

Evaluation of the adequacy of methionine cycle intermediates

Several existing pork CRC projects could be expanded to include aspects of methyl donor adequacy and minimise the costs involved in setting up new projects. Examples of these are:

1. Projects 2E-102 0607 (The effects of dietary arginine during gestation on the subsequent litter size of gilts and sows) and 2E-103 0506 (Development of a selection marker for placental insufficiency) could be expanded to determine interrelationships between methyl donor and co-factor supply and HCY, NO and arginine.

2. Project 2D-112 0708 on nutritional manipulation during early pregnancy to increase embryo survival and litter size could be expanded to determine interrelationships between methyl donor and co-factor supply and embryo survival and litter size.
3. Project 2F-103 (The effects of exogenous PST ...on birth weight and growth performance of gilt and sow progeny) could be expanded to determine the effect of PST on the synthesis of the key methionine cycle regulatory intermediate, SAM.
4. Project 2B-104 0506 (Protein restriction and subsequent growth) could be expanded to determine the effect of low methionine and cystine supply on the activity of the methionine cycle.

Evaluation of the association between HCY levels and polymorphisms of genes involved in the methionine cycle

Because of the existence of gene polymorphisms associated with high HCY levels in other species and their interaction with folate and vitamin status, it is essential to quantify the prevalence of such polymorphisms among pigs. If such polymorphisms do exist among pig populations, it would be useful to determine the genetic profiles of all animals used for research into the adequacy of methionine cycle substrates and co-factors.

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