Manipulating the sulfur amino acid content of the early diet and its implications for long-term health

William D. Rees
The Rowett Research Institute, Greenburn Road, Bucksburn, Aberdeen AB21 9SB, UK

Epidemiological studies of human populations show that poor growth in utero predisposes an individual to the later development of type 2 (non-insulin-dependent) diabetes mellitus and hypertension in adulthood. This phenomenon is not confined to man; feeding pregnant rats diets moderately deficient in protein has a similar effect, programming the adult blood pressure and glucose metabolism of the offspring. A restriction in the amino acid supply was thought to cause poor fetal growth. However, recent experiments have shown that this is not the case and instead have implicated the metabolism of the S-containing amino acids. Many semi-synthetic experimental diets contain an imbalance in S-containing amino acids, forcing the animal to synthesise a sizeable part of its cysteine requirement from methionine. Unfortunately, when the diet is low in protein, the oxidation of amino acids is reduced, perturbing methionine metabolism and increasing levels of homocysteine. It is this interaction between protein content and composition of the diet which influences neonatal viability and may also determine the long-term health of the offspring. An excess of homocysteine is known to affect levels of two of the main mediators of cellular methylation reactions, S-adenosyl methionine and methylene tetrahydrofolate. S-adenosyl methionine is the methyl donor for the methylation of newly-synthesised DNA, regulating chromatin assembly and gene expression. The balance between S-adenosyl methionine and the methylated derivatives of folic acid may be critical for the development of differentiating cells and the long-term regulation of gene expression.

Fetal programming: Metabolic syndrome: Hypertension: Homocysteine: DNA methylation

There is a growing body of evidence suggesting that the early diet influences the subsequent susceptibility to disease. All adult cells develop from precursor stem cells, so it is not surprising that the fetal and neonatal periods of life are particularly sensitive to inadequate nutrition. Small defects introduced at an early stage are likely to propagate through the subsequent lineages and affect whole tissues. As well as affecting the mature body size there is evidence in both man and experimental animals which links impaired fetal growth with lifelong effects on metabolism, blood pressure, bone function, the immune system and brain development (Godfrey & Barker, 2000). The maternal diet is an important determinant of fetal growth and, therefore, a major factor influencing disease susceptibility in the offspring.

The fetus draws its nutrient supply from the maternal circulation, the composition of which depends on both the intake and metabolism of the mother. During the course of gestation maternal metabolism changes in order to meet the differing demands of the fetus and to support mammary development. The mother also provides a buffer against day-to-day variations in the diet, and in the event of a shortage can mobilise nutrients from her own tissues in an attempt to make up the deficit. These nutrients support the fertilised oocyte as this one precursor cell undergoes a complex process of growth, while at the same time an elaborate signalling system controls the development of the three-dimensional body structure (for example, see Morris-Kay et al. 2001). Chemical gradients (Patten & Placzek, 2000) and signals generated by cell-to-cell contacts (McNeil, 2000) commit individual undifferentiated stem cells into particular lineages. Initially, trophectoderm cells produce the placenta, while the inner cell mass forms the rest of the body. Once the neural tube has formed the major axes are defined, enabling the process of limb and visceral development to begin.

In vitro studies have been essential in elucidating the mechanisms that control the process of differentiation. Embryonal carcinoma cells were used initially (Strickland

Abbreviations: CpG, cytosine residue 5’ to guanosine residue; IGF2, insulin-like growth factor 2; SDH, cystathionine γ-lyase.
Corresponding author: Dr William D. Rees, fax +44 1224 715349, email wdr@rrri.sari.ac.uk
models for the fetal origins of disease. Fetal growth restriction programmes a number of different functions, including growth, glucose tolerance and blood pressure, in the adults. In the simplest model the experimental animals are fed a predetermined proportion of the total amount of stock diet consumed by controls with *ad libitum* access (Bertin *et al.* 1999). With this global deprivation it is difficult to ascribe the effects to any single nutrient. However, a number of studies of single nutrient deficiencies have shown that reducing the protein content of the diet is all that is required to programme the offspring (for review, see Gardner *et al.* 1998; Ozanne & Hales, 1999). The rat normally requires a diet containing approximately 180 g crude protein (N × 6.25)/kg during gestation. Feeding an isoenergetic diet containing 90 g crude protein/kg is sufficient to set the animals on a different growth trajectory, resulting in adult body weights that are 10–20% lower than those of the controls. More importantly, the offspring of the protein-restricted dams go on to show elevated adult blood pressure (Langley & Jackson, 1994) and changes in glucose tolerance. These latter effects result from functional differences in the liver, islet cells and adipose tissue (Snoeck *et al.* 1990; Desai *et al.* 1997; Shepherd *et al.* 1997). As a result the animals exhibit a type of insulin resistance that has many of the characteristics of human maturity-onset (type 2) diabetes.

Unfortunately, the results of intervention trials in human subjects suggest that protein supplementation does not improve maternal or fetal growth (Kramer, 2000). Thus, there has been some controversy as to whether the protein-deficient pregnant rat is an appropriate rodent model of the epidemiological observations in man. Further confusion arises because of a number of apparently conflicting results. Subtle differences in the amino acid composition of the diets impact on the development of hypertension (Langley-Evans, 2000). Also, the critical window seems to be somewhat unclear and unconnected with growth retardation. The low-protein diet needs to be fed for just the first 4 d after conception to increase the blood pressure of the offspring (Kwong *et al.* 2000). It has been suggested that a perturbation of pre-implantation growth is sufficient to alter the allocation of cells in the blastocyst. By changing the number of cells in the inner cell mass relative to those in the trophectoderm the entire growth trajectory of the fetus may be disturbed. However, other studies suggest the protein-deficient diet causes little or no effect on the overall growth of the fetus up to day 19 of gestation; indeed, the pups of the dams fed the low-protein diet are somewhat larger than the controls (Rees *et al.* 1999). It is only in the very last stages of gestation, between days 19 and 22, that there is a 15–20% reduction in fetal growth. Other experiments suggest that growth restriction caused by global feed restriction from day 15 onwards can also have a long-term effect on glucose tolerance, affecting critical organs such as the endocrine pancreas (Garofano *et al.* 1997). All these observations suggest that the programming of fetal growth and development is not the result of a simple growth restriction, but is the product of more fundamental metabolic changes in mother or fetus.

Diet composition and fetal growth

Useful as they are for the determination of mechanisms of differentiation, *in vitro* studies are unable to replicate the complex nutrient interactions that occur in the whole animal. The pregnant rat fed deficient rations is one of the most widely used and extensively characterised animal
Homocysteine is well recognised as a highly-toxic non-protein amino acid. With sizeable amounts of homocysteine being produced as a result of the conversion of methionine to cysteine, there is the danger of homocysteine accumulating. The removal of homocysteine depends on cystathionine synthase and SDH (Fig. 3). Homocysteine is first combined with serine to form cystathionine, which is then cleaved to form cysteine and 2-oxobutyrate. Although the activity of cystathionine synthase remains constant

**Table 1. Sulfur amino acid content of semi-synthetic diets**

<table>
<thead>
<tr>
<th></th>
<th>Cysteine</th>
<th>Methionine</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mg/g</td>
<td>% estimated requirement</td>
</tr>
<tr>
<td>Estimated minimum requirement (AIN-93G)*</td>
<td>3.7</td>
<td>1.2</td>
</tr>
<tr>
<td>Casein diet (g casein/kg)</td>
<td>180</td>
<td>0.8</td>
</tr>
<tr>
<td></td>
<td>90</td>
<td>21</td>
</tr>
</tbody>
</table>

during the course of pregnancy, the activity of SDH changes. In animals fed a normal diet the activity of SDH remains high during the first 4 d of pregnancy, but by day 11 has fallen to about 25% of the starting activity (Rees & Hay 2001). This decline probably corresponds to a general decrease in amino acid oxidation in the middle of gestation that spares amino acids to support the increasing demands for fetal growth. In the animals fed the protein-deficient diet the need to conserve amino acids occurs earlier, so the enzyme activity, which is already lower than that in the non-pregnant animals, falls rapidly to 25% of the starting activity by day 4 of gestation (Rees & Hay, 2001). Unfortunately, the animals fed the casein-based experimental diets still need to oxidise similar amounts of homocysteine to meet their cysteine requirement. Thus, the fall in SDH activity may explain why the circulating levels of homocysteine in dams fed low-protein diets are double those of the dams fed the high-protein diet on day 4 of gestation (WD Rees, SM Hay, C Antipatis, J McConnell, L Pietric and DS Brown, unpublished results). As homocysteine is not normally released from the cells, the intracellular levels may be even higher.

The decrease in SDH activity that occurs in protein deficiency is probably caused by endocrine changes. One group of hormones known to change in response to protein intake is the glucocorticoids. These hormones also regulate the expression of the SDH gene, since the promoter contains a glucocorticoid response element (Su & Pitot, 1992). Indeed, even relatively mild stresses such as laparotomy are able to induce the expression of the enzyme (Ogawa et al. 1999; Leonhardt & Cardoso, 2000; Rountree et al. 2001). Analysis of the DNA methylation of homocysteine is one of the critical molecules that accept methyl groups from S-adenosyl homocysteine (SAHcy) as cofactor. These reactions include the methylation of DNA. SAHcy, S-adenosyl homocysteine.

**Homocysteine and the derivatives of folic acid**

The other pathway for homocysteine metabolism involves methionine synthase, which utilises methylene tetrahydrofolate to re-synthesise methionine (Fig. 4). Folic acid is well recognised as an essential nutrient during pregnancy, and a deficiency leads to an increase in the incidence of neural tube defects (van der Put et al. 2001). If substantial quantities of methylene tetrahydrofolate are used for the remethylation of homocysteine, then the pool of this active derivative of folic acid becomes depleted (James et al. 1997). An excess of methionine or homocysteine is therefore in some ways similar to a dietary folate deficiency. A number of different amino acids, particularly glycine, provide the methyl groups required for the synthesis of methylene tetrahydrofolate and determine the availability of methylated folates. This replenishment of the methylated folate pool may explain the reversal of the hypertensive effect observed when the casein-based diet is supplemented with glycine (Dunn et al. 2001). The re-methylation of homocysteine to reform methionine potentially sets up a cycle that transfers methyl groups from methylene tetrahydrofolate into S-adenosyl methionine (Fig. 4). Thus, the balance of these two essential intermediates in methyl transfer reactions is determined by the amount of homocysteine present in the system. The functional methylene tetrahydrofolate deficiency generated by excess homocysteine may affect all the essential metabolic pathways that require this cofactor. These pathways include nucleotide and choline synthesis. The consequences of a deficiency in the products of these pathways remain to be seen. For example, the semi-synthetic experimental diets are almost devoid of preformed nucleotides, so the entire supply must be synthesised de novo. In adults depletion of the folate pool leads to a reduction in the deoxy nucleotide triphosphate pools and the rate of DNA synthesis (Jackson et al. 1997). The effect of stalling DNA synthesis during the differentiation of cells is unknown, but may, for example, disrupt chromatin formation.

**Methionine homocysteine and DNA methylation**

DNA is one of the critical molecules that accept methyl groups from S-adenosyl methionine. Changes in methylation are an essential step in cell differentiation and part of normal fetal development (Martin et al. 1999; Leonhardt & Cardoso, 2000; Rountree et al. 2001). Analysis of the DNA from the fetuses of dams fed the methionine-imbalanced
low-protein diets showed that in some tissues, particularly the fetal liver, the methylation of DNA was increased (Rees et al. 2000). This process may be an important mechanism linking the metabolic changes to the development of fetal cells.

The functional consequences of DNA methylation are wide ranging. About 5% of the cytosine residues in mammalian DNA are methylated at the 5 position of the cytosine ring. However, this modification is not randomly distributed, but occurs in a highly organised manner. First, it depends on the neighbouring base and normally occurs at dinucleotides with the sequence of cytosine residue 5′ to guanosine residue (CpG). If the structure of DNA were random, then there is an equal probability that a cytosine residue would be followed by any of the four bases. However, the sequence CpG is found at only about one-third of expected frequency in mammalian genomes. In most parts of the genome about 70% of cytosine residues followed by a guanosine residue are methylated. These simple repetitive DNA sequences, which are regularly dispersed throughout the genome, are the target for specific methyl CpG-binding proteins that anchor the DNA to the nuclear matrix. Since these regions map to the boundaries of active chromatin and intronic regions of genes, they have the capability to control access for transcription and regulate gene expression (Epplen et al. 1996).

Second, there are groups of CpG residues where only about 30% of the cytosine residues are methylated (Gardiner-Garden & Fromer, 1987). These regions, known as CpG islands, are found either within or adjacent to about half the sequences that encode genes. Methylation of cytosine residues in the CpG islands has both positive and negative effects on the expression of the neighbouring genes. Numerous studies suggest that hypermethylation of CpG islands within the promoter correlates with decreased expression of the gene. On the other hand, hypermethylation of CpG sequences downstream of the promoter correlates with increased expression. Once established, the CpG methylation status of a particular gene is normally preserved during cell division. Changes in DNA methylation are particularly important during neoplastic progression and correlate with the activity of the promoter (Wong et al. 1999). As with the matrix attachment sites, the CpG islands are also believed to be important in regulating chromatin structure and therefore the accessibility of DNA for transcription.

DNA methylation can have very widespread effects on gene expression and the most dramatic of these effects is the silencing of an entire X chromosome (Avner & Heard, 2001). Methylation also silences large parts of other chromosomes, so that only the paternally- or maternally-inherited gene is expressed. One of the best characterised of these systems is the insulin-like growth factor 2 (IGF2) and IGF2/mannose 6-phosphate receptor genes which are expressed from the maternally and paternally-inherited alleles respectively (for a recent review, see Wolff, 2000). Other imprinted genes include a number of essential growth factors and regulators. The importance of this system in regulating fetal growth is illustrated by studies of mice with targeted mutations of the IGF2 gene. Normal mice have only one active copy of the IGF2 gene. Deleting the methylation-sensitive region activates the silenced copy of the IGF2 gene, doubling IGF2 production and causing fetal overgrowth. Deleting the maternally-inherited IGF2/mannose-6-phosphate receptor, which controls IGF2 removal, also causes fetal overgrowth (Wang et al. 1994). However, it is not necessary to delete whole sections of DNA to cause changes in growth, much milder insults can also alter methylation and growth. Exceptionally-large offspring are a common result of embryo transfer in ruminant species. There is now evidence that this large-offspring syndrome is coupled to changes in the expression of IGF2 receptor (Khosla et al. 2001; Young et al. 2001). Manipulating the diet can also regulate epigenetic gene expression. Methyl-supplemented diets containing methionine, betaine, folic acid and vitamin B12 have been shown to alter patterns of epigenetic gene expression in the agouti mouse (Wolff et al. 1998). It is not yet clear whether the imprinted genes are major targets with regard to the development of hypertension and metabolic disease following growth retardation in utero. The perturbation of S-adenosyl methionine levels may also interfere with the changes in methylation that affect both alleles during normal differentiation.

The methylation reactions involve two separate DNA methyltransferase systems. Interestingly, as well as S-adenosyl methionine, the methyltransferases also require Zn as a cofactor (Bestor, 1992). Zn is essential during pregnancy, and deficiencies are teratogenic. The first system involves a maintenance methylase (coded by the DNA methyltransferase 1 gene) which copies the pattern of methylation from the template strand onto the newly-synthesised DNA and preserves the existing pattern during each round of cell replication. There is, therefore, a clear mechanism for a heritable programming of gene function through changes in DNA methylation. This pattern is preserved during the differentiation of cells as the maintenance methylases copy the pattern to the newly-synthesised strand.

In the second class of reaction de novo methylases (coded for by the DNA methyltransferase 2 and 3 genes) introduce new patterns during differentiation of tissues. These enzymes are essential for normal fetal development, and mutations are lethal for the developing fetus (Okano et al. 1999). It is possible that high levels of intracellular S-adenosyl methionine may cause a substrate-driven increase in methylation affecting the establishment of new methylation patterns. The product of the transmethylation reaction, S-adenosyl homocysteine, can also affect the outcome, since it acts as a product inhibitor of the enzyme (O’Gara et al. 1996). If it is not removed quickly enough there is the possibility that its accumulation may inhibit the DNA methyltransferase activity during key methylation reactions.

The methyl transferases are only part of a complex system that regulates chromatin formation. A complex of proteins, which includes several methyl CpG-binding proteins and histone acetyl transferase, binds to newly-synthesised DNA and regulates its packaging into chromatin (Fig. 5). This complex is important in deciding whether the DNA is packaged into open or condensed chromatin and determines subsequent gene expression. It is important not
Fig. 5. Interactions between metabolism and chromatin formation. The DNA molecule is wound around a histone former. As the cell replicates it is uncoiled and replicated by the DNA polymerase complex (DNA pol). A complex of DNA methyltransferase (DNMT), transcriptional activators or CpG-binding proteins (TA) and histone acetyl transferase (HAT) bind to the newly-synthesised strand. This complex utilises S-adenosyl methionine (SAM) and acetyl-CoA (Ac-CoA) to modify the DNA and histones whilst forming the new chromatin structure. Methyl THF, methylene tetrahydrofolate; SAHcy, S-adenosyl homocysteine; dNTP, deoxy nucleotide triphosphates.

Methyl
THF
SAHcy
dNTP
DNA pol
TA
DNMT
HAT
AcCoA
SAM

to oversimplify the complex interactions that occur when chromatin is remodelled. The process of DNA methylation may depend on or generate an altered chromatin state that suppresses gene expression (Fuks et al. 2000). Histone acetylation is another essential part of the remodelling process and depends on acetyl-CoA as its substrate. This factor raises interesting questions on the role of acetate metabolism in determining chromatin structure. The metabolic interactions which control gene expression through long-range chromatin interactions are an exciting new type of gene–nutrient interaction, and one which is just beginning to be explored.

Future prospects

There is clear evidence that protein deficiency changes the flux through the S amino acid cycle. It becomes of increasing importance to know whether this mechanism is the only one that programmes adult disease or whether other factors such as restricted cellular growth also play a role. One of the principal criticisms of the protein-deficiency model in the rat is that protein deficiency itself is not a recognised problem in man. The recent animal data from the rodent models is providing increasing evidence for complex interactions between dietary protein content, quality and micronutrient composition. All these factors can be involved in the nutritional regulation of chromatin structure and the long-term programming of gene expression. These data suggest that the methylation reactions and the micronutrients involved in them may be far more important than the bulk amino acid supply. Indeed, it may be possible to produce a small but symmetrical growth retardation without any impact on long-term health. It is only when the metabolism of the S amino acids is disturbed that long-term changes are programmed in cell function.

Whilst all these studies have concentrated on the role of the S amino acids during pregnancy, it is important to realise that similar processes continue in a number of cell types during adult life. For example, the immune system, the gut and endothelial cells are continually renewing themselves from stem cells. Homocysteine is a recognised risk factor for diseases associated with these cells, suggesting that these metabolic perturbations may have much more widespread implications for dietary manipulation.

Acknowledgements

This work was supported by the Scottish Office Rural Affairs Department.

References


Manipulating early diet


