Growth, development and differentiation: a functional food science approach

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Abbreviations: APRT, adenosine phosphoribosyltransferase; cDNA, complementary DNA; DEXA dual-energy X-ray absorptiometry; DHA, docosahexaenoic acid; EFA, essential fatty acids; EGF, epidermal growth factor; GLUT, glucose transporter; hGH, human growth hormone; HGPRT, hypoxanthine phosphoribosyltransferase; HPA, hyperphenylalaninaemia; IDDM, insulin-dependent diabetes mellitus; Ig, immunoglobulin; IGF, insulin-like growth factor; LPH, lactase–phlorizin-hydrolase; MUC1, high-molecular mass glycoprotein; NPN, non-protein nitrogen; PAH, phenylalanine hydroxylase; PCD, programmed cell death; PKU, phenylketonuria; PPAR, peroxisome proliferator activated receptors; PUFA, polyunsaturated fatty acids; SGLT1, sodium-dependent glucose transporter 1; SI, sucrase–isomaltase; SPA, single-photon absorptiometry; SREBP, sterol regulatory element binding protein; XME, xenobiotic-metabolizing enzymes.

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Abstract

Few other aspects of food supply and metabolism are of greater biological importance than the feeding of mothers during pregnancy and lactation, and of their infants and young children. Nutritional factors during early development not only have short-term effects on growth, body composition and body functions but also exert long-term effects on health, disease and mortality risks in adulthood, as well as development of neural functions and behaviour, a phenomenon called 'metabolic programming'. The interaction of nutrients and gene expression may form the basis of many of these programming effects and needs to be investigated in more detail. The relation between availability of food ingredients and cell and tissue differentiation and its possible uses for promoting health and development requires further exploration. The course of pregnancy, childbirth and lactation as well as human milk composition and the short- and long-term outcome of the child are influenced by the intake of foods and particularly micronutrients, e.g. polyunsaturated fatty acids, Fe, Zn and I. Folic acid supplementation from before conception through the first weeks of pregnancy can markedly reduce the occurrence of severe embryonic malformations; other potential benefits of modulating nutrient supply on maternal and child health should be further evaluated. The evaluation of dietary effects on child growth requires epidemiological and field studies as well as evaluation of specific cell and tissue growth. Novel substrates, growth factors and conditionally essential nutrients (e.g. growth factors, amino acids, polyunsaturated fatty acids) may be potentially useful as ingredients in functional foods and need to be assessed carefully. Intestinal growth, maturation, and adaptation as well as long-term function may be influenced by food ingredients such as oligosaccharides, gangliosides, high-molecular-mass glycoproteins, bile salt-activated lipase, pre- and probiotics. There are indications for some beneficial effects of functional foods on the developing immune response, for example induced by antioxidant vitamins, trace elements, fatty acids, arginine, nucleotides, and altered antigen contents in infant foods. Peak bone mass at the end of adolescence can be increased by dietary means, which is expected to be of long-term importance for the prevention of osteoporosis at older ages. Future studies should be directed to the combined effects of Ca and other constituents of growing bone, such as P, Mg and Zn, as well as vitamins D and K, and the trace elements F and B. Pregnancy and the first postnatal months are critical time periods for the growth and development of the human nervous system, processes for which adequate substrate supplies are essential. Early diet seems to have long-term effects on sensory and cognitive abilities as well as behaviour. The potential beneficial effects of a balanced supply of nutrients such as I, Fe, Zn and polyunsaturated fatty acids should be further evaluated. Possible long-term effects of early exposure to tastes and flavours on later food choice preferences may have a major impact on public health and need to be further elucidated. The use of biotechnology and recombinant techniques may offer the opportunity to include various bioactive substances in special dietary products, such as human milk proteins, peptides, growth factors, which may have beneficial physiological effects, particularly in infancy and early childhood.

Growth: Development: Differentiation

1. Introduction

There are few other aspects of food supply and the metabolism of food ingredients that are of greater biological importance than the feeding of mothers during pregnancy and lactation, and of their children. The rapid growth of fetuses, infants and children, which double their body weights within only 6 weeks *in utero* and 4–5 months after birth respectively, depends on the supply of very large amounts of nutrients per kg body weight through the placenta, human milk and children's diets. Marginal nutrient supplies are usually more critical in a developing and growing organism than in steady-state situations during adulthood. The ability to effectively utilize and compensate for unbalanced supplies is severely limited in the fetus and in young children due to small endogenous stores of a number of relevant substrates, and in many cases also due to immature metabolic pathways (e.g. amino acid metabolism) and physiological functions (e.g. renal conservation of substrates). Nutritional factors during early development have important immediate and short-term effects on growth, body composition and body functions. In addition, accumulating data indicate long-term effects of nutritional and metabolic factors during critical time periods of development on later physiological and metabolic processes, a phenomenon referred to as 'metabolic programming' (Barker, 1994). For example, epidemiological studies have suggested long-term effects of intrauterine and postnatal nutrient supply on the prevalence of obesity in adulthood (Ravelli *et al.* 1976) and on the risk of developing diabetes, hypertension, hypercholesterolaemia, CHD, and other disorders during adult life (Barker, 1994). The rate of death from all cardiovascular disease and from CHD (Fig. 1) in adulthood was found to be significantly related to body weight at birth (Osmond *et al.* 1993; Barker, 1994). Standardized mortality from CHD was also closely related to weight at 1 year in males but not in females (Fig. 2). These findings indicate the potential of influencing long-term health and life expectancy by modulations of maternal diet in pregnancy and of postnatal infant feeding. Moreover, the type of postnatal infant feeding has been related to long-term outcomes such as the later incidence of insulin-dependent diabetes mellitus (IDDM) (Virtanen *et al.* 1991; Gerstein, 1994) and cognitive development (Lucas *et al.* 1992; Lanting *et al.* 1994), and a lasting effect of Ca intake during childhood and adolescence on bone mineral density and the risk of fractures in old age has been proposed (Ribot *et al.* 1995). This programming of permanent effects of the physiology and function of the organism during critical time periods of early development appears to be of major importance in preventive health strategies. Improved knowledge on the cellular and molecular mechanisms of programming and of the complex physiological and nutritional factors relevant to the health and well-being during the periods of reproduction and childhood is required. A better definition of the optimal supply of relevant nutrients and other food ingredients is expected to help optimize dietary intakes during these critical periods of life which should improve the chances that infants and children have to utilize fully their genetic potential, as well as maintain maternal health.

2. Nutrient–gene interaction, genetic regulation

2.1. Introduction

The perinatal period is attended by important modifications in energy metabolism (Girard *et al.* 1992). *In utero*, the fetus receives a continuous intravenous supply of substrates for growth and oxidative metabolism. Immediately after birth, the maternal supply of substrates ceases abruptly and the newborn has to withstand a brief period of starvation before being fed at intervals with milk, a high-fat and low-carbohydrate diet. The sucking–weaning transition is also characterized by profound changes of nutrition (Girard *et al.* 1992). Towards the end of the sucking period, the milk is progressively replaced by the solid food diet of the adult, the composition of which is usually lower in fat and higher in carbohydrates. The successful adaptation of neonates to these changes in nutrition requires important modifications

Fig. 1. Standardized mortality ratios for CHD below age 65 years, showing a statistically significant relationship with birth weight in 5585 women and 10 141 men born between 1911 and 1930. (Data from Barker, 1994.)

Fig. 2. Standardized mortality ratios for CHD below age 65 years, showing a statistically significant relationship with weight at age 1 year in 10 141 men, but not in 5585 women born between 1911 and 1930. (Data from Barker, 1994.)

of energy metabolism in the vast majority of organs, intestine, liver, muscle, adipose tissue and brain. Postnatal development is also associated with differentiation processes and a high growth rate, involving specific requirements of nutrients such as amino acids (protein synthesis) or fatty acids (e.g. brain growth). Thus, nutrients can be considered to cause energy metabolism modifications but also to play a major role in organ growth and/or functional differentiation.

2.2. Modulation of gene expression participates in the adaptations of energy metabolism

The adaptations of energy metabolism to the nutritional environment imply the modulation and/or emergence of metabolic pathways. This can be achieved through changes in the efficiency of a given step (specific transporters, enzymes) by allosteric or phosphorylation–dephosphorylation mechanisms, or by translocation of a protein into a different cellular compartment. However, many of these adaptations also imply a change in the amount of a given protein. This phenomenon is usually related to a change of the transcription rate of the corresponding gene.

A good example of such a mechanism is the appearance at birth in mammals of phosphoenolpyruvate carboxykinase (*EC* 4.1.1.49), an enzyme of the gluconeogenic pathway allowing the *de novo* production of glucose by the liver (Girard *et al.* 1992). This pathway is essential for the survival of the newborn mammal, which undergoes a brief period of starvation followed by the ingestion of a diet low in carbohydrates, the milk. The transcription rate of this gene is extremely low during the fetal period and increases abruptly in the first hours after birth, allowing the newborn to maintain glucose homeostasis.

Another example is the lipogenic pathway in the rat species. This pathway allows synthesis of fatty acids from glucose when this substrate is ingested in excess of energy requirements. During the sucking period, the capacity of this pathway is kept very low because the small quantity of glucose ingested is essentially directed towards oxidative processes. When the rat is weaned onto the high-carbohydrate diet, this pathway is switched on and excess glucose will be converted into fatty acids, ultimately stored as triacylglycerols in the adipose tissue. The expression of one of the key enzymes of this pathway, fatty acid synthase (*EC* 2.3.1.85) is extremely low during the sucking period but increases when the rat is weaned onto the adult high-carbohydrate diet, but not if weaning occurs onto a high-fat diet, clearly underlining the importance of the nutritional environment in this process. This phenomenon is due to the activation of the gene transcription process at weaning (Foufelle *et al.* 1996). Thus, modulation of gene expression must be considered as an integral part of the adaptations occurring during development.

Although it has been known for a long time that nutrients can regulate the expression of specific genes in prokaryotes (the lactose operon in *Escherichia coli* for instance) or in primitive eukaryotes such as yeasts, the demonstration that a similar phenomenon occurs in higher eukaryotes is recent. The regulation of specific gene expression in mammals in response to changes in nutrition has become a major aspect of modern nutrition, due to the emergence of molecular biology that has allowed the cloning of most of the genes involved in the regulation of energy metabolism. Recently, it has been demonstrated that major (glucose, fatty acids, amino acids) or minor (Fe, vitamins) dietary constituents participate, in concert with hormones, in the regulation of gene expression in response to nutritional changes (see for instance Clarke & Abraham, 1992).

2.3. Examples of gene regulation by nutrients

2.3.1. Carbohydrates. In the liver and adipose tissue, excess glucose, after its metabolism into pyruvate through glycolysis is converted into fatty acids by the lipogenic pathway. The expression of three enzymes of the combined glycolytic–lipogenic pathway has been shown to respond to an increased glucose concentration: L-pyruvate kinase (*EC* 2.7.1.40; liver), fatty acid synthase and acetyl-coA carboxylase (*EC* 6.4.1.2; liver and adipose tissue) (Foufelle *et al.* 1992; Vaulont & Kahn, 1994). *In vivo*, it has been shown in rats that high-carbohydrate diets induce the transcription

of these genes, whereas transcription is inhibited by starvation or a high-fat diet. During the sucking period in rats, the expression of these enzymes is kept low and dramatically increases at weaning on to a high-carbohydrate diet. *In vitro* studies have shown that glucose is the primary inducer of the gene transcription. This effect requires that glucose is metabolized at least into glucose-6-phosphate which might be the signal metabolite. The response of transcription to high glucose is a very rapid phenomenon (less than 1 h). Glucose response elements, which bind specific transcription factors of the USF/MLTF family have been characterized on these genes although the mechanism linking glucose-6-phosphate to transcription factors is presently unknown (Foufelle *et al.* 1996). In the β-cells of the islets of Langerhans, glucose induces the transcription of the insulin gene on which glucose response elements have also been characterized (Docherty & Clark, 1994). This feature is obviously relevant to the diabetic syndrome.

2.3.2. Fatty acids. *In vitro* studies have shown that the transcription of a number of genes of adipocytes is increased in the presence of fatty acids. This is the case, for instance, for phosphoenolpyruvate carboxykinase, an enzyme involved in this tissue in the provision of α-glycerophosphate necessary for the esterification of fatty acids (Antras-Ferry *et al.* 1995). Similarly, the expression of the fatty acid binding protein ap2, which binds fatty acids into the cell, is strongly stimulated by fatty acids (Grimaldi *et al.* 1992). Fatty acid response elements have been characterized in the promoter of these genes. They bind a transcription factor called peroxisome proliferator activated receptors (PPAR) which can be activated by the binding of fatty acids or a metabolite of fatty acids (prostaglandin for instance) (Schoonjans *et al.* 1996).

Fatty acids have also been shown to inhibit gene expression in rats. The addition of a small amount (20–30 g/kg) of polyunsaturated fatty acids (PUFA) of the *n*-3 or *n*-6 families to a high-carbohydrate fat-free diet decreases markedly the lipogenic capacity and the activity of lipogenic enzymes (Clarke, 1994). In contrast, monounsaturated and saturated fatty acids have no effects. Interestingly enough, this effect seems to be specific to the liver since lipogenesis is not affected in the adipose tissue. The decrease induced by PUFA of the activity of lipogenic enzymes such as fatty acid synthase, acetyl-CoA carboxylase or glucose-6-phosphate dehydrogenase (*EC* 1.1.1.49), is clearly linked to an inhibition of gene transcription as shown in studies using primary cultures of rat hepatocytes (Clarke, 1994). At the present time the cellular and molecular mechanisms involved in the inhibitory effect of PUFA on gene transcription have not been elucidated.

2.2.3. Cholesterol. A very interesting series of studies has been performed by the group of Brown and Goldstein on the effect of cholesterol on gene expression (Wang *et al.* 1994). Cholesterol represses the expression of genes involved either in the synthesis of cholesterol (cytoplasmic hydroxymethylglutaryl-CoA synthase, *EC* 4.1.3.5) or in its uptake from external sources, the LDL receptor (LDL are lipoproteins rich in cholesterol). In the absence of cholesterol, the transcription of these genes is activated by a transcription factor called sterol regulatory element binding protein (SREBP). SREBP is usually hooked onto the endoplasmic reticulum where it can be cleaved by a protease. SREBP can then be transferred into the nucleus and activates the transcription of relevant genes. In the presence of cholesterol, the protease is inhibited and SREBP can no longer enter into the nucleus and stimulate gene transcription (see Fig. 3).

2.3.4. Amino acids. In yeast, amino acid starvation results in the activation of several genes involved in N metabolism and the mechanisms involved are now known (Kilberg *et al.* 1994). In mammalian cells, amino acid availability also modulates the expression of some genes as shown, for instance, for asparagine synthetase (*EC* 6.3.5.4) which is responsible for the biosynthesis of asparagine from aspartate and glutamine. When cultured cells are transferred to a medium lacking asparagine, the concentration of asparagine synthetase mRNA increases. It must be underlined, however, that the signalling mechanism exhibits a broad substrate specificity since availability of other amino acids controls the asparagine synthetase mRNA

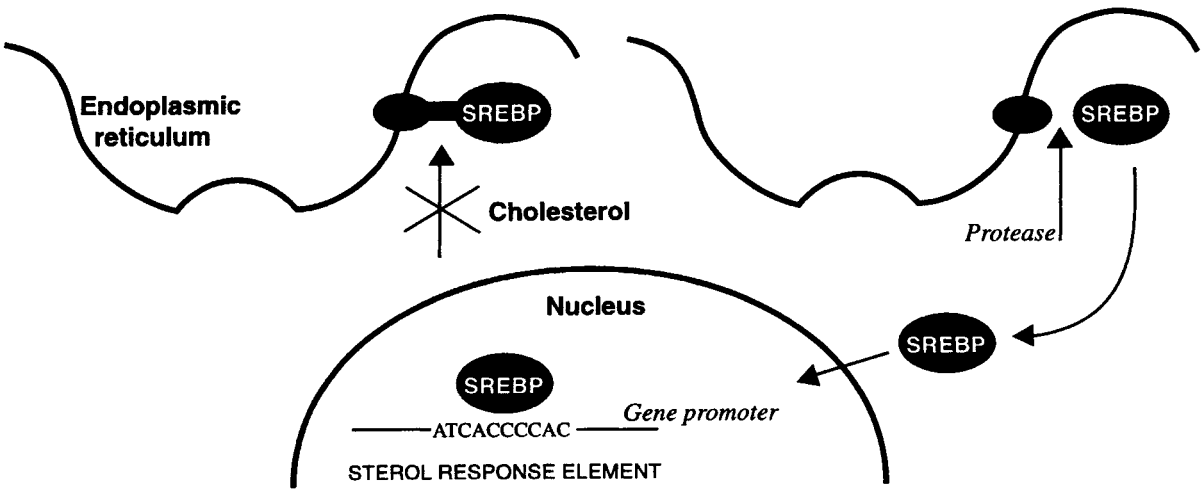

Fig. 3. Model for the cholesterol-dependent control of gene transcription. SREBP, sterol regulatory element binding protein.

concentration as well. The mechanisms for the increase in asparagine synthetase mRNA concentration could involve both *cis*-acting elements contained in the mRNA itself and affecting its stability, as well as in the genomic promoter sequence (Kilberg *et al.* 1994). The signal metabolite could be, in fact, the degree of occupancy of the transfer RNA as in yeast, although evidence is clearly lacking.

2.4. Nutrients and cell differentiation

The fetal–neonatal period is also characterized by the differentiation of a number of organs. One important question, which is presently totally unaddressed, is whether nutrients could affect differentiation *per se* and thus modulate physiological functions on a long-term basis. Two examples are listed here.

2.4.1. Fatty acids. One example of such a mechanism stems from *in vitro* studies on pre-adipocyte cell lines. It has been shown that fatty acids are not only able to modulate the transcription rate of specific genes in differentiated adipocytes, but are also able to induce preadipocyte differentiation into adipocytes through their action on a specific isoform of a PPAR transcription factor (Schoonjans *et al.* 1996). Obviously, if this happens also *in vivo*, it would imply that perinatal nutrition could modulate the number of adipocytes and, thus, be a crucial determinant of a possible expansion of this tissue, in relation to the obesity syndrome.

2.4.2. Retinoic acid. Vitamin A or retinol can be oxidized to retinoic acid in cells. In addition to retinol, β-carotene may also be a source of retinoic acid. Retinoic acid and retinoid X receptors which belong to the family of steroid–thyroid hormone receptors have been cloned (De Luca, 1991). They are, in fact, ligand-activated transcription factors (of the same family as the PPAR, see p.S9). It has been shown in numerous studies that retinoic acid is a potent morphogen and that it can affect fetal development (De Luca, 1991). Thus, vitamin A or β-carotene deficiency or overload might have major consequences on tissue differentiation and fetal development.

2.5. Concluding remarks

These examples underline the idea that gene regulation by nutrients, a process we induce each time we eat a food of specific composition, includes a very wide range of mechanisms involved in the regulation of crucial pathways as well as in cell differentiation. Since the fetal–neonatal period is concomitant with marked changes in the nutritional environment, it represents a period in which these mechanisms are particularly important.

As for other components of cell functions, it is very likely that gene regulation by nutrients is subject to variations linked to genetic polymorphisms among individuals, of which some could ultimately lead to pathologies. This obviously opens new fields for genetic studies on nutrient-related pathologies (obesity, for instance).

3. An overview of programmed cell death (apoptosis)

A type of cell death which does not involve primary cell membrane disintegration and tissue inflammatory responses was identified some 70 years ago, and the term apoptosis was applied by analogy with the deciduous loss of leaves by trees. The process was identified by characteristic histomorphological changes in the cell nuclei (Kroemer *et al.* 1995). Although apoptosis is sometimes used as a synonym for programmed cell death (PCD), it has become apparent that apoptosis can be caused by noxious or toxic events which might affect several cell components and not just primarily the nucleus. PCD applies to the entire process and phenomenology of this form of cell death and thus embraces the intrinsic mechanisms and regulatory processes involved. PCD might not necessarily be induced by external toxic stimuli, but rather by signals which are a fundamental component of cellular and tissue physiology. The distinction between the two terms PCD and apoptosis is subtle, and not invariably respected. Clearly it is misleading to characterize PCD on the basis of nuclear morphological changes since these are not always the primary events in PCD (Kroemer *et al.* 1995; Hale *et al.* 1996; Vaux & Strasser, 1996; Nagata, 1997) but one should not be distracted overmuch by the distinction: rather interest should focus on the processes involved in the induction, mediation, regulation and process of the cellular events, and the ways in which dietary components might affect these mechanisms which are fundamental to tissue differentiation, development and function.

PCD is a more appropriate description of a variety of intrinsic cellular events, involving cytoplasmic as well as nuclear processes which precede loss of intracellular and cellular membrane integrity, should these occur at all. However, non-physiological stimuli can effect mechanisms involved with PCD, and the process or its dysfunction can be an integral part of oncogenesis and autoimmune disorders (Hale *et al.* 1996).

All multicellular organisms use PCD to remove superfluous and damaged cells in tissues and organs, particularly, but not exclusively, in proliferating tissues. There is a strong evolutionary conservation of the mechanisms of PCD and the nematode *Caenorhabditis elegans* is a valuable model for the system in higher species, including man, in whom several genes and effectors of PCD are homologous to cell death genes and products in *C. elegans* (e.g. proto-oncogene bcl-2 is homologous to the nematode 'cell-suicide gene' ced3) (Kroemer *et al.* 1995; Vaux & Strasser, 1996).

The induction mechanisms of PCD are more complex in higher animals in whom co-operativity between the different tissues of organs plays an important part in determining PCD. For example, the extracellular matrix exerts some control over differentiation and morphogenesis of organs by specific effects on constituent cells, tissue-specific gene expression, and cell death (Roskelley *et al.* 1995). This is important for the differentiation of tissues and organs (e.g. the gastrointestinal tract) throughout life as well as during embryogenesis.

PCD can be envisaged to comprise three stages: induction, the effector stage, and degradation (Fig. 4) (Kroemer *et al.* 1995). There are at least two principal routes of inducing PCD. One involves genotoxic events damaging DNA; the other involves receptor-mediated stimuli such as specific death signals, the absence of rescue signals such as growth factors, and contradictory or conflicting signals (Kroemer *et al.* 1995; Nagata, 1997).

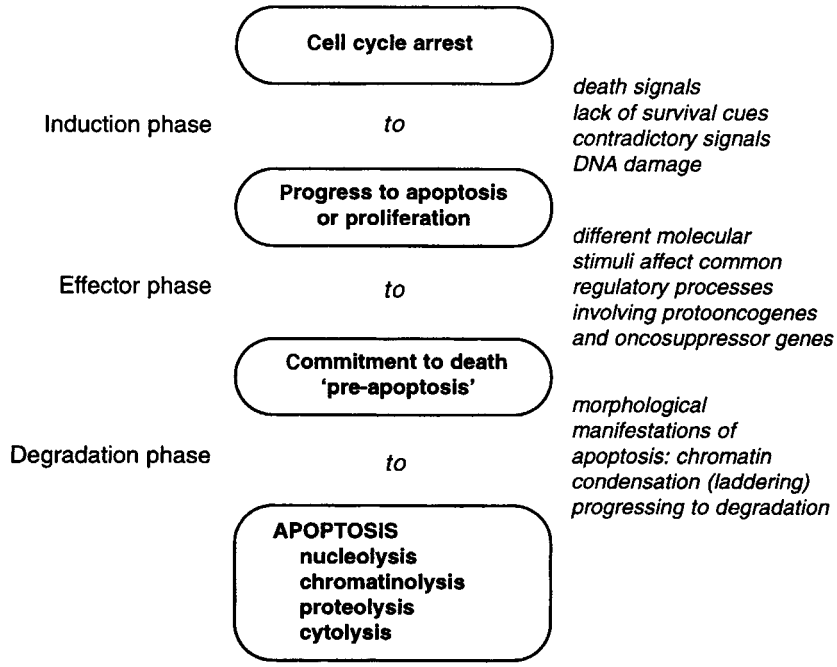

Fig. 4. The stages of programmed cell death and apoptosis. (From Kroemer *et al.* 1995.)

The origin of signals can be systemic or local (e.g. extracellular matrix) (Hakomori & Igarashi, 1995) humoral cytokines (Nagata, 1997), or cellular such as cytotoxic T cells. The signal and transduction systems stimulating PCD include steroids (Evans-Storms & Cidlowski, 1995), cytokines such as tumour necrosis factor and nerve growth factor and interleukins, all of which operate through their cognate receptors (Cosman, 1994; Nagata, 1997).

The responsiveness to such stimuli may also be determined by the nature of the cell surface receptors which might change during ontogenesis (and, for that matter, oncogenesis). The surface receptors include glycosphingolipids, lectins and sphingosines and their respective roles have not been totally clarified. It is noteworthy that inherited defects of cell-surface CD40 cause a human X-linked immunodeficiency syndrome associated with defective PCD, and analogous syndromes in mice are associated with congenital abnormalities of Fas receptors and ligands (Cosman, 1994; Nagata, 1997). Changes in structural components of cellular membranes (e.g. ceramide formation) can induce signal transduction cascades (Ballou *et al.* 1996) leading to cell death.

The heterogeneity of signals is matched by the variety of effector mechanisms which are stimulated. These involve tyrosine kinase, protein kinase C, and other kinases, Ca permeability routes and intracellular proteases. These initiate an amplification cascade affecting a number of genes, several of which are known proto-oncogenes (e.g. c-myc) or tumour suppressors (e.g. p53) and which are common both to PCD and to the regulation of normal cell differentiation, function and intermediate metabolism (Kroemer *et al.* 1995; Hale *et al.* 1996; Nagata, 1997). This highlights the central conundrum of PCD, namely what is the ultimate determinant of whether a cell undergoes PCD or not? Is there a threshold event? It has been suggested that one basic determinant is the stage of the cell cycle when the cell receives the relevant signals (Meikrantz & Schlegel, 1995) or the availability of an appropriate nutrient supply (that is, do some stressors such as a specific nutrient excess or deficiency induce cell death by more direct means than, say, oxidant damage or simple starvation?).

Intrinsic effector systems for cell death programmes involve cytoplasmic and nuclear metabolic and functional disintegration with ultimate morphological damage. Within the mitochondria there is a decrease in transmembrane potential, energy uncoupling of the respiratory chain, and increased production of reactive O species. These increase oxidative damage and increase leakage of mitochondrial Ca. In the cytoplasm there is a loss of anabolic activities with a corresponding activation of proteases, disruption of the cytoskeleton and of the endoplasmic reticulum: similarly in the nucleus the endonucleases are activated and there is nucleolysis with some activation of enzymes usually associated with repair activity.

The mechanisms and regulation of PCD and apoptosis in abnormal cell proliferation have focused on their role in cancer and the possibility that external events or compounds might induce, suppress, regress, or protect against cancer by affecting these processes. The potential role of dietary components influencing these processes is being investigated actively in relation to functional food science. In other physiological areas there might exist other opportunities to explore and exploit a better understanding of these processes in cellular proliferation and differentiation. These are outlined in the following paragraphs.

PCD of uterine cells enables blastocyst implantation and placentation (Welsh, 1993). Prostaglandins, leukotrienes, platelet-activating factor, and transforming growth factor are thought to induce this process. In embryogenesis there is extensive mesodermal PCD to eliminate undifferentiated

cells (Sanders & Wride, 1995; Wride & Sanders, 1995). The extracellular matrix and the regulation of cell adhesion molecules and integrins, perhaps involving tumour necrosis factor-α as a cellular growth and differentiation factor, have been shown in the regulatory processes in the morphogenesis of the limb bud (Hurle *et al.* 1995).

Again extracellular matrix and specified cell adhesion molecules control endothelial cell position and behaviour and enable the definition of mechanisms directing endothelial cell differentiation, commitment, migration and organization into a tube as the fundamental components of vasculogenesis (Baldwin, 1996). PCD has also been shown to be integral to the development of the conduction processes and to be involved in the pathogenesis of congenital cardiac structural and electrical conduction abnormalities as well as acquired cardiovascular disease (James, 1993). The role of PCD in intestinal mucosal development and differentiation is discussed elsewhere: a similar dependence on PCD has been demonstrated in renal organogenesis (Igarashi, 1994).

It is in haematopoiesis and in the development of the immune system that there might be some particular aspects of relevance to functional food science. Haematopoiesis and PCD involve an extensive multigene cytokine network which has positive regulators such as colony-stimulating factors and interleukins and negative regulators such as transforming growth factor-β and tumour necrosis factor (Krammer *et al.* 1994; Cidlowski *et al.* 1996; Sachs, 1996). Apoptosis in T and B lymphocytes is involved in all fundamental processes in the immune system and PCD appears to be vital in selecting or favouring development of specific lymphocytes and eliminating cells which are sensitized to autoantigens. Factors which would interfere with this process would be undesirable and there exists a possibility that similar selective processes might underlie the acquisition of immunotolerance to ingested antigens in foods or the development of adverse immune reactions to food constituents.

In the central nervous system neurones and glia synthesize and secrete cytokines which affect the differentiation and function of nerve cells (Dragunow & Preston, 1995; Kawata, 1995; Sei *et al.* 1995). Disturbance of these cytokine-mediated interactions may lead to neuronal dysfunction and/or cell death and contribute to the pathogenesis of central nervous system diseases. The functional and cellular differentiation underlying sexual dimorphism in the central nervous system might depend on the effects of steroids on PCD in the relevant regions of the brain.

Many of the phenomena regarded as inevitable consequences of ageing, including nerve cell death, involve processes of PCD and apoptosis, and a better understanding of these events might in due course lead to a more sophisticated and rational scientific basis on which to base functional food science and its application to avoid the depredations of ageing (Zakeri & Lockshin, 1994).

4. Supply of food ingredients before and during pregnancy

The nutritional status of the mother before and during pregnancy may influence fertility, the course of pregnancy and the incidence and severity of complications during gestation and birth, lactation (Rasmussen 1992), and the short- and long-term health and development of the baby. During recent years scientific understanding of the physiology of energy metabolism, weight gain and particularly the effects of micronutrient status and their clinical consequences have considerably improved. The dietary supply of certain nutrients may have beneficial or preventive aspects both for the course of gestation and for the offspring.

4.1. Physiological aspects of nutritional requirements in pregnancy

The range of weight changes that occurs in pregnant women is wide and ranges from a loss of weight to a gain of 25 kg or more. A normal weight gain during pregnancy for most women with normal prepregnancy weight is in the order of 11–15 kg (Institute of Medicine, 1990). In addition to food intake, weight gain is also influenced by medical disorders such as development of oedema associated with pre-eclampsia. The weight increase and substrate deposition in maternal and fetal tissues is not only due to increased dietary intake during pregnancy, but also supported by a variety of complex adaptations of gastrointestinal, endocrine and metabolic functions. The effects of food ingredients on these adaptations as well as on placental function and the efficacy of materno-fetal substrate transfer are not well understood and require further exploration. Substrate needs during pregnancy are not only met by the food consumed during this period of time, but food intake before or between pregnancies may be of major importance for providing adequate availability of a number of nutrients, e.g. Ca and Fe. Also, it is increasingly appreciated that substrate needs during pregnancy may show marked inter-individual variability, partly due to genetic heterogeneity in a population as is the case for metabolic pathways involving folic acid.

4.1.1. Energy. Based on a factorial approach calculating the additional energy requirements for the deposition of protein and fat as well as the energetic cost of tissue synthesis and enhanced maintenance metabolism, the total energy cost of pregnancy is assumed to be in the order of 334·72 MJ (80 000 kcal) (Food and Agriculture Organization/World Health Organization/United Nations University (FAO/WHO/UNU), 1985). In women who maintain their previous physical activity, this results in an additional energy allowance of about 837 kJ/d (200 kcal/d) equivalent to an increase of 9 % over the energy needs of 9·2 MJ (2200 kcal) of a non-pregnant, moderately active woman, or if adapted to an estimated increase of energy needs during the course of pregnancy, about 628 kJ/d (150 kcal/d) during the first and about 1464 kJ/d (350 kcal/d) during the second and third trimesters of pregnancy (FAO/WHO/UNU, 1985). However, energy needs may be lower with reduced physical activity, particularly towards the end of pregnancy.

While moderately reduced energy intakes have little effect on pregnancy outcomes, severe energy deprivation during pregnancy results in reduced infantile birth weight. During the 6-month period of the Dutch famine with an available energy supply below 4184 kJ/d (1000 kcal/d) and a protein intake of no more than 30–40 g/d, average birth

weight fell by 200 g (Ravelli *et al.* 1976). The effect was most marked if the hunger period coincided with the latter part of pregnancy. Later studies have confirmed these observations. In underweight women, a close relationship between maternal and neonatal body weights has been observed, while the correlation is much less close in pregnant women with a normal body weight (Luke & Petrie, 1980). Not only total body weight, but also the organ weights of liver, spleen, heart, adrenal gland, kidneys, skeleton and thymus are reduced in newborn infants of mothers who are underweight (Naeye *et al.* 1969), and placental size and number of cells by 15–20 % in pregnancy with intrauterine infantile growth failure or even up to 50 % in severe maternal malnutrition (Winnick, 1970). In malnourished South American women, average placental weights were reduced by about 15 %, and there was a marked reduction of the placental peripheral villi surface, the site of materno-fetal nutrient transfer, which may aggravate the restricted nutrient supply to the fetus in malnourished mothers. These findings are of concern, since neonatal birth weight is the strongest predictor of infant morbidity and mortality, and epidemiological studies have found strong associations of low birth weights with increased risks for cardiovascular disease and total mortality rates in adulthood (Barker, 1994).

Several studies on energy supplementation during pregnancy in malnourished women found an increase in average birth weight and a reduction of the rate of low-birth-weight infants (Lechtig *et al.* 1975; Prentice *et al.* 1987), while intervention studies with an increased energy intake in apparently healthy women in Europe did not have significant effects on neonatal weight. However, dietary supplementation in Canadian women with twin pregnancies resulted in an increase of average birth weight by 80 g, and reductions of preterm deliveries and the rate of very-low-birth-weight infants by 30 and 50 % respectively (Dubois *et al.* 1991). In conclusion, energy supplements during pregnancy appear to be beneficial in selected populations at risk of very low intakes or increased demands.

4.1.2. Protein. With a factorial approach again based on calculated materno–fetal accretion, the additional protein requirements for the four successive 10-week periods of a normal pregnancy have been estimated as 0·6, 1·8, 4·8 and 6·1 g/d, respectively (Hytten & Leitch, 1971), and an increase of recommended protein intakes by about 6 g/d (expressed as milk or egg protein) over non-pregnancy values has been recommended (FAO/WHO/UNU, 1985). Even though this represents an increase of about 30 % over recommended intakes in non-pregnant women, and hence the recommended relative increment of protein intake is clearly higher than that of energy, the protein intakes of most pregnant women in Europe tend to exceed minimal requirements by far, with the possible exception of small subgroups consuming low-protein diets. It has been proposed that protein-enriched diets during pregnancy may be beneficial for the prevention of pregnancy-induced hypertension and pre-eclampsia, but conclusive evidence to support these hypotheses is missing (Roberts *et al.* 1974; Williams *et al.* 1981).

4.1.3. Carbohydrates. Glucose serves as the major energy source for the fetus, comprising about 90 % of the energy supply. Hence, maternal carbohydrate metabolism during gestation is of potential relevance to the optimal supply for the fetus. Little is known about the effects of dietary habits, particularly the total amount and relative composition of sugars and starches on gestation, and potential implications for clinically relevant outcome variables such as macrosomia, postpartal hypoglycaemia, or increased risk of developing glucose intolerance later in life.

4.1.4. Lipids. Although there is a tendency in some pregnant women to aim at a low consumption of dietary fat (Hachey, 1994), there are no conclusive data to demonstrate the safety of maternal low-fat diets or immediate benefits for pregnant women and the fetus and infant. On the other hand, pregnant women appear to have high requirements for lipid-soluble vitamins and PUFA. During pregnancy, the concentrations of blood lipids and their constituent fatty acids increase considerably. Amounts (mg/l) of plasma phospholipid-associated essential fatty acids (EFA) were reported to increase during the course of pregnancy by about 40 % and those of the long-chain PUFA arachidonic acid (20:4*n*-6) and docosahexaenoic acid (DHA, 22:6*n*-3) by about 23 and 52 % respectively (Al *et al.* 1995). It has been proposed that a high supply of long-chain *n*-3 fatty acids may be beneficial for fetal development because of the importance of these compounds for neural tissue development (Koletzko, 1992), and that they may improve some obstetric complications, particularly lessen the severity of pregnancy-induced hypertension (Secher & Olsen, 1990). Moreover, observations in the population of the Faroe islands which consumes a diet rich in fish suggested that this high intake of *n*-3 fatty acids may increase average birth weight by prolonging gestation (Olsen *et al.* 1986). However, in this population there were also apparent adverse effects of *n*-3 fatty acids, including higher rates of blood loss on delivery, which may be explained by fish-oil-induced suppression of platelet aggregation, and a higher perinatal mortality. An intervention with supplementation of *n*-3 long-chain PUFA was reported to prolong pregnancy without any detrimental effects on growth of the fetus or the course of delivery (Olsen *et al.* 1992). Supplementation of fish oil with vitamins and minerals has been considered to reduce the frequency of pre-eclampsia, but a double-blind placebo-controlled trial did not find any effect of fish-oil supplementation on the occurrence of pregnancy-induced hypertension (Onwude *et al.* 1995).

Pregnancy may be associated with DHA mobilization from maternal stores. Under the present dietary conditions, pregnancy is associated with a reduction of the EFA status and particularly of the DHA status. After delivery normalization takes place, but recovery of the DHA status appears to be still incomplete after 6 months (Al *et al.* 1995). Throughout pregnancy, the DHA content of plasma phospholipids of primigravida is significantly higher than that of multigravida, and a negative relationship was observed between DHA content and gravida number, which is reflected in neonatal DHA status and may have functional consequences for infant growth and development. Since increased supplies of selected PUFA during pregnancy may well have some benefits, the possible benefits as well as the potential risks of such strategies should be carefully evaluated.

4.1.5. Vitamins, minerals and trace elements. Relative to the increased energy needs (about 10%), the recommended relative increase of some other nutrients is markedly higher, for example, for folate the reference intake increases by 100% in pregnancy (Scientific Committee for Food, 1993). Based on these considerations, the potential for an inadequate intake of one or more of these specific nutrients during pregnancy is greater than for total energy.

Calcium. During the latter part of pregnancy, the fetus has a high rate of Ca accretion in the order of 250–300 mg/d. This appears to be partly accounted for by extensive adjustments in Ca metabolism during pregnancy, particularly the inhibitory effects of placental oestrogen on maternal bone resorption that result in an enhanced release of parathyroid hormone and, hence, increased maternal Ca absorption, decreased urinary excretion and enhanced Ca retention (Cole *et al.* 1987). Although low dietary Ca intakes of malnourished pregnant women have been associated with reduced bone mineral densities of the newborn infants (Ramam *et al.* 1978), in well-nourished mothers no enhancement of neonatal Ca accretion by increased maternal intakes has been demonstrated. However, at relatively low intakes, maternal body stores of Ca may be utilized and depleted to meet fetal needs (Duggin *et al.* 1974). In women with multiple pregnancies, in particular, low Ca intakes may increase the risk of osteomalacia at a later age.

Although dental caries is relatively common during pregnancy, there is no evidence for a causal link of its rate of occurrence with dietary Ca intakes.

Low Ca intakes during pregnancy have also been associated with the occurrence of EPH-gestosis (oedema, proteinuria and hypertension) as well as eclampsia (Burke *et al.* 1943). In a controlled study, supplementation of the diets of pregnant women with Ca was associated with a reduced systolic blood pressure at term and a lower incidence of pregnancy-induced hypertension (Villar *et al.* 1987). In contrast, another study found no effect of a Ca supply on the incidence of pregnancy-induced hypertension, although mean blood pressure was lowered.

Magnesium. Mg status is related to neuromuscular excitability, and it has been proposed that an additional Mg supply may contribute to prevention of eclampsia and other complications of pregnancy. However, controlled trials have not provided conclusive evidence for an improved course of pregnancy or delivery (Spatling & Spatling, 1988; Sibai *et al.* 1989).

Iron. The total Fe needs for pregnancy have been estimated to be in the order of 300–850 mg (Bothwell *et al.* 1979; Hallberg, 1988). Although the efficacy of Fe absorption is markedly enhanced in pregnancy, the incidence of Fe deficiency and Fe-deficient anaemia during pregnancy remains a sizeable problem that may increase maternal and fetal morbidity and mortality (Llewellyn-Jones, 1965), cardiovascular stress associated with increased complication rates before and at birth (Banks & Beutler, 1988) and an elevated risk of delivering low-birth-weight and premature infants (Scholl & Hediger, 1994). At particular risk of poor Fe status in pregnancy are women with vegetarian or predominantly vegetarian diets, because of the relatively low absorption of non-haem Fe. Thus, foods that promote net Fe absorption may be beneficial for some pregnant women.

Zinc. Maternal Zn deficiency is highly teratogenic in rodents, and in monkeys it induces abnormal fetal brain development (Worthington-Roberts 1985). Studies in human pregnancies found that maternal leucocyte Zn levels during pregnancy were correlated with later infantile birth weight (Wells *et al.* 1987) and inversely related to the rate of low-birth-weight infants (Neggers *et al.* 1991). Moreover, a better maternal Zn status was associated with lower rates of pregnancy-induced hypertension, protracted delivery and maternal complications at birth and the rate of premature rupture of membranes (Sikorski *et al.* 1990). In contrast, another controlled study evaluating Zn supplementation in the second and third trimesters of pregnancy in Europe did not find any effect. To what extent these differing results were influenced by variations in Zn status of the respective populations studied, and may be reproduced in other populations, remains to be clarified.

Iodine. In parts of Europe, the I status of many pregnant women is inadequate with a sizeable prevalence of increased thyroid size in mothers and newborn infants (World Health Organization, 1993). Poor I status is associated with an increased risk of miscarriage in early gestation and preterm delivery as well as compromised mental development of the infant (Xue-Yi *et al.* 1994).

Fluoride. Since postnatal F^- supply has a strong preventive effect on the incidence of dental caries, the question was raised whether an increased F^- supply to pregnant women may contribute to protecting pre-eruptive teeth of the unborn child, but conclusive evidence to answer this question is not available.

Folic acid. Folic acid is essential for the synthesis of pyrimidines, purines and hence of DNA and RNA, as well as amino acids and neurotransmitters; thus, an adequate folate status is of importance to allow undisturbed cell multiplication and growth during pregnancy. Following observations of a relation between folic acid status and the occurrence of neural tube defects in the offspring, several controlled clinical trials have demonstrated that folic acid supplied before conception and during the first weeks of pregnancy can markedly reduce the incidence of neural tube defects, such as spina bifida and anencephaly, by 40–70% (Butterworth & Bendich, 1996). This important preventive effect is apparently associated with a folic acid-responsive derangement in homocysteine metabolism in a genetically determined subgroup of the population (Steegers-Theunissen *et al.* 1991). Poor folic acid status of pregnant women has also been associated with miscarriages, repeated abortions, pregnancy length and neonatal outcome as well as unexplained sterility (Pietrzik *et al.* 1992; Bung *et al.* 1993, 1995). It has been recommended that all pregnant women should be supplemented with a daily dose of 0·4 mg folic acid from before conception to at least four completed weeks after conception (Scientific Committee for Food, 1993). In view of the postulated key role of folic acid status in the aetiology of atherosclerosis by means of modulating homocysteine metabolism, the question of a potential programming effect of intrauterine folic acid supply on the later risk of cardiovascular diseases has been raised (Pietrzik *et al.* 1992).

5. Modulation of growth

5.1. Introduction

The term 'growth' expresses the increase in number and size of cells of a particular species and it refers to changes in body dimensions. Growth is a phenomenon usually associated with increase in length and weight whereas the term 'development' is a physiological concept indicating the progressive differentiation of tissues and organs with acquisition of their specific functions. All mammals start life as a single cell; during the early part of the gestation the fertilized ovum divides many times and different kinds of cells develop during the process of differentiation and arrange themselves to form part of the various organs of the body. The general principles of growth apply to all species but the rate of cell division is genetically determined and depends on nutrient supply and utilization. Regardless of the exact time that differentiation occurs it always results in the transformation of the parental cell into a large number of morphologically different progeny cell types. The rapidity of physical growth is regulated during the life cycle and is modulated by genetics, a variety of growth factors that interact with target cells, as well as environment and diet (Hernández & Argente, 1992; Philips, 1995).

Human growth hormone (hGH) or somatotropin is essential for normal postnatal growth. It is released from the anterior pituitary gland on stimulation by growth hormone-releasing hormone or somatocrinin, a factor produced by the hypothalamic region of the brain. Like other pituitary hormones, hGH acts on target tissues, primarily the liver, to cause synthesis and release of a second hormone mediator, insulin-like growth factor (IGF)-I, also called somatomedin C, into the systemic circulation. IGF-I is a growth-accelerating peptide that acts directly on cartilage to promote bone growth. Deficiency of hGH production causes metabolic alteration and growth failure (Philips, 1995).

Different requirements for growth of different cell types have been established (Sato *et al.* 1982) but despite considerable progress in tissue culture systems we are still a long way from identifying all the stimulatory and inhibitory macromolecules that regulate the growth of all the human cell types *in vivo*. Most growth factors are polypeptides or small proteins with molecular masses that vary from 1 to 40 kDa and bind to specific cell surface receptors, with pleiotropic effects on cells including changes in gene expression (Watson *et al.* 1987). By binding to their receptors, growth factors modify the activity of the membrane-bound enzyme adenylate cyclase (*EC* 4.6.1.1), using the GTP-binding protein as an intermediate so that the level of cAMP in the cell is altered. This, in turn affects the activity of cAMP-dependent protein kinases which phosphorylate specific target proteins, regulating their activity. Other possible second messengers to carry signals from growth receptors to the cell include Ca, inositol triphosphate and diacylglycerol (Lodish *et al.* 1995).

In addition to genetic factors, neurohormonal and tissue-specific growth factors, growth is also affected by a number of metabolic and environmental factors which include the availability of nutrients. Recommended daily nutrient intakes have been established for all periods of life and both sexes to support an adequate growth of the human being (National Research Council, 1989). However, there is a lack of information about how semi-essential nutrients can affect growth in specific periods of life and in particular situations including disease states.

The rapidity of physical growth in the normal infant during the first months of life is remarkable and unmatched during later times of life. Moreover, physiological and developmental changes during infancy are as notable as the speed of physical growth. Changes in the rates of physical growth and in the allocation of dietary intake of energy and protein for growth and maintenance occur as a continuum rather than in discrete stages, but the progression of changes during the early months of life is very rapid.

Human milk provides all nutrients necessary to support adequate growth of the term infant during the first 4–6 months of life. Furthermore, in addition to universally recognized nutrients, human milk contains a number of semi-essential nutrients, enzymes, hormones and growth factors which appear to have a role in supporting infantile growth (Koldovsky & Strbak, 1995). However, there is a lack of information about how those nutrients and factors interact with the growth process and how they affect specific tissue growth.

5.2. Methods for the determination of growth

Anthropometric measurements such as weight, length, head circumference, skinfold thickness, limb circumference, mid-arm cross-sectional area, BMI etc. at various ages during infancy have been published in many developed countries and are useful in evaluating the size of an infant in relation to the size of his or her peers (Fomon & Nelson, 1994). In the infant the Quetelet index is difficult to interpret since the body mass increases rapidly from birth to 4 months of age and the percentage of body weight contributed by body fat also increases rapidly during that period. Reference data for increments in size and other indices of nutritional status are more sensitive in children beyond infancy, particularly in alerting to the possibility of illness or nutritional inadequacy (Fomon, 1991).

Data on body composition at various ages are one of the bases for the factorial approach in estimating the requirements for various nutrients for growth. Chemical composition of animals is determined by direct whole-body analysis. The most extensive data on the composition of the term infant are those published by Widdowson (1982), but data beyond infancy are scanty. The most useful indirect methods of estimating various aspects of body composition in the infant are determination of total body water from the concentration of a suitable tracer in body fluid, i.e. heavy water, extracellular water, and determination of natural abundance of ^{40}K in the whole body (Forbes, 1987). Measurements of both bioelectric impedance and of total body electrical conductivity can estimate non-invasively the fat-free body mass of infants and children (Mayfield *et al.* 1991; Houtkooper *et al.* 1992). Many new techniques are available for measurement of total body fat, although only a few can be used in general practice or in epidemiological research (Deurenberg, 1992).

The urinary excretion rates of endogenous creatinine and 3-methyl-histidine have been proposed as indices of muscle

mass, and the urinary excretion rates of endogenous hydroxyproline and type I and type III procollagen propeptides as indices of growth rates (Trivedi *et al.* 1991). Measuring cell kinetics represents a new approach in evaluating cell and tissue growth. Cell kinetics can be used to measure the proliferation rate of cells, the phases of the cell cycle and the percentage of cells in cycle, to plot the position of dividing cells and determine the size of the proliferation compartment and to follow the movements of labelled cells. Cell kinetic studies received a boost when [^{3}H]thymidine was introduced in the 1950s. Recently, several new techniques have been used in cell kinetics, i.e. incorporation of bromodesoxyuridine and labelling of cell antibodies (Kember, 1993). There is a need for new methods to evaluate the specific organ and tissue growth applicable in a wide range of conditions.

5.3. Growth factors in human milk and their influence on infant growth

A large number of hormones and growth factors are present in human and bovine milks (Koldovsky & Thornburg, 1987; Strbak, 1991). Non-peptide hormones (thyroid hormones, cortisol, progesterone, pregnanediol, oestrogens and artificial contraceptives) and peptide hormones and growth factors (erythropoietin, hGH, growth hormone releasing factor, gonadotropin-releasing hormone, epidermal growth factor (EGF), insulin, IGF-I, nerve growth factor, gastrointestinal regulatory peptides and thyroid–parathyroid hormones) have been isolated and quantitated in human milk (Koldovsky & Strbak, 1995). The orogastric effects of hormones and growth factors on infant growth require further elucidation. However, there is some evidence that a number of hormones in human milk may contribute to the intestinal maturation of sucking infants. For example, oral administration of EGF to 10-d-old sucking rats resulted in changes in protein and DNA content of colonic mucosa (Koldovsky & Thornburg, 1987).

5.4. Potential roles of non-protein nitrogen compounds as growth modulators

Non-protein N (NPN) compounds are present in most foods mainly as nucleic acids, free amino acids, small peptides and other minor compounds. The concentration of nucleic acids in foods depends on the number of cells of the original biological tissue. Thus, meat, fish and vegetal seeds are rich in nucleic acids whereas fruits have a low content (Gil & Uauy, 1995*b*). NPN accounts for 18–30 % of the total N in human milk. Some of this N, namely urea, contributes to the pool available for synthesis of non-essential amino acids in infants. Other NPN components may have particular roles in tissue growth. Among the NPN known to have specialized roles in the ontogeny of the human newborn are the growth factors, namely EGF, amino sugar oligosaccharides, free amino acids like taurine, arginine and glutamine, amino alcohols of phospholipids i.e. choline, nucleotides and nucleic acids and polyamines.

5.5. Human milk oligosaccharides and growth

In addition to lactose, the carbohydrates of human milk include nucleotide sugars, glycolipids, glycoproteins, and oligosaccharides. Viverge *et al.* (1990) have isolated three oligosaccharide fractions representing 13–18 g/l; the concentration varied with the mother's genetic ability to synthesize specific fucosyl linkages. Approximately eighty neutral and sialic acidic oligosaccharides have been isolated and identified (Newburg & Neubauer, 1995).

Human milk oligosaccharides appear to be synthesized by some of the same glycosyltransferases that participate in the synthesis of glycoprotein and glycolipid cell surface components. Thus, it is reasonable to postulate that some of those compounds can act as analogues to host cell surface receptors for pathogens. Anderson *et al.* (1986) reported that specific oligosaccharides can inhibit binding of *Streptococcus pneumoniae* and *Hemophilus influenzae* to their receptors and Cravioto *et al.* (1991) described an oligosaccharide that inhibits adherence of enteropathogenic *E. coli* to their receptors. Other authors have reported that specific fucosylated oligosaccharides inhibit binding of invasive strains of *Campylobacter jejuni* (Ruiz-Palacios *et al.* 1992) and the toxicity of *E.coli in vivo* (Newburg *et al.* 1990).

Gangliosides are glycosphingolipids that contain sialic acid (*N*-acetylneuraminic acid) as part of their carbohydrate moiety. GM_1, a milk ganglioside present in human milk, binds to *E. coli* and *Vibrio cholerae* toxins and may contribute to infant protection against infection by those enteropathogens (Laegrid *et al.* 1986).

Since lactating mothers differ genetically in their ability to produce various oligosaccharides, this variability might influence the susceptibility of breast-fed infants to enteric disease. The influence of supplementing infant milk formulas with oligosaccharides on the susceptibility of infants to gastrointestinal diseases, namely acute diarrhoea, is one of the current fields of intense investigation.

5.6. Free amino acids and tissue growth

The free amino acid pool of human milk is small compared with the total amount of milk amino acids in protein. Free amino acids contribute only 10 % of the total NPN in human milk. Glutamine and taurine are the free amino acids found in higher concentrations in human milk. While clinical effects of low dietary intakes of taurine have not been demonstrated, there is a concern about the possibility of subclinical deficiency, particularly in the premature infant since its ability to synthestize taurine may be limited (Gaull *et al.* 1977).

Free glutamine accounts for about 20 % of the total glutamine pool in human milk. Glutamine is currently extensively investigated because of its importance in cell and tissue culture and because it serves as a preferred respiratory fuel for rapidly proliferating and growing cells, such as enterocytes and lymphocytes. Moreover, it is a regulator of acid–base balance through the production of urinary NH_3, a carrier of N between tissues and an important precursor of nucleotides, amino sugars and proteins (Lacey & Wilmore, 1990). There is increasing evidence that glutamine may become a conditionally essential amino acid in critically ill patients, and glutamine appears to be important for the maintenance of small-intestinal structure and functionality (Newsholme & Carrié, 1994).

Arginine and ornithine are also present in human milk as free amino acids, although the first is found in higher

concentrations in milk proteins. Arginine has multiple biological properties, including the ability to stimulate anabolic hormone secretion: intravenous and enteral administration of arginine increases both insulin and hGH secretion (Cynober, 1994). Several studies show that arginine given to patients, as well as in various experimental stress models, acts by improving N balance, accelerating wound healing, and restoring depressed immunity. Dietary supplements of arginine have been shown to inhibit tumour growth in animals, probably by activating the immune system. However, in cancer patients arginine stimulates tumour protein synthesis, suggesting that arginine might have separate stimulatory effects on the tumour and on the immune system, the outcome depending on which effect predominates (Garlick & McNurlan, 1994). Arginine has also been shown to enhance the growth-hormone-releasing-hormone-induced hGH rise in patients with anorexia nervosa (Ghigo *et al.* 1994). Moreover, oral administration of arginine enhances the hGH response to growth hormone releasing hormone in short children (Loche *et al.* 1993).

Ornithine shares with arginine the ability to stimulate hGH secretion. In addition, ornithine as its α-ketoglutarate salt generates various molecules, i.e. glutamine. Ornithine ketoglutarate has been shown to improve N balance in various acute and chronic malnutrition states. It increases muscle protein anabolism in moderate catabolic states and reduces protein catabolism in hypercatabolic states (Cynober, 1994).

Arginine and ornithine are precursors of NO and polyamines respectively. These metabolites participate intimately in permeability and adaptive responses of the gut. Recent animal studies showed improved morphology after ornithine ketoglutarate administration, acting perhaps through increased polyamine synthesis (Cynober, 1994). It is controversial whether exogenous arginine can be a relevant precursor of polyamines.

5.7. Polyamines and tissue growth

Polyamines are detectable in relatively high quantities in human and rat milk. Artificial infant formulas do not contain appreciable amounts of polyamines, specifically putrescine and spermidine, and spermine is undetectable. Thus, formula-fed infants are not exposed to polyamines nor to any potential effects that these compounds may have on the developing intestine. It is noteworthy that food contains polyamines and that polyamines are produced by the gastrointestinal microflora. Thus, the direct uptake by enterocytes of preformed polyamines could contribute to the polyamine cellular pool. Indeed, putrescine and spermidine uptake has been shown in isolated rat enterocytes (Cynober, 1994).

5.8. Dietary nucleotides and tissue growth

Human milk is the exclusive source of dietary nucleotides for infants during the first months of life, and its nucleotide profile (Gil & Sánchez-Medina, 1982; Gil & Uauy, 1995*a*) differs markedly from that of cow's milk and most infant formulas (Gil & Sánchez-Medina, 1981; Gil & Uauy, 1989, 1995*a*). Preformed nucleotides may be of importance for the growth of tissues with a rapid turnover (Van Buren *et al.* 1985; Nuñez *et al.* 1990; Uauy *et al.* 1990; Gil & Uauy, 1995*b*), particularly bone marrow, leucocytes and the intestinal mucosa which preferentially use the nucleotide salvage pathway to fulfil their purine and pyrimidine nucleotide requirements (Mackinnon & Deller, 1973; Savaiano & Clifford, 1981; LeLeiko *et al.* 1983; Cohen *et al.* 1984).

Dietary nucleotides may modulate lipoprotein and fatty acid metabolism in human early life (Gil *et al.* 1986*b*, 1988; De-Lucchi *et al.* 1987; Pita *et al.* 1988; Morillas *et al.* 1994; Sánchez-Pozo *et al.* 1994), and they may enhance the growth of bifidobacteria and limit that of enterobacteria in the gut of newborn infants (Gil *et al.* 1986*a*; Gil & Uauy, 1995). Dietary nucleotides may affect small-intestinal growth in experimental animals (Gil & Uauy, 1995) and may have a role in the maintenance of the immune response both in animals (Van Buren *et al.* 1983, 1985; Pizzini *et al.* 1990; Kulkarni *et al.* 1992) and in human subjects (Carver *et al.* 1991; Gil & Uauy, 1995).

5.8.1. Nucleotides and small intestine growth. A number of factors are involved in the regulation of the renewal of the absorptive epithelium and in the repair of the epithelium under pathological conditions (Shiner *et al.* 1990). N-containing nutrients appear to be important for gut growth. At weaning, protein modulates the ontogenic changes in tissue DNA synthesis and plays a role in completing the growth of the rat's gastrointestinal tract (Buts & Nyakabasa, 1985). Dietary nucleotides have been shown to influence gut development and repair after injury. Uauy *et al.* (1990) reported that intestinal disaccharidase activities are increased in rats during development by dietary nucleotides, and DNA, lactase (*EC* 3.2.1.23), sucrase (*EC* 3.2.1.48) and maltase (*EC* 3.2.1.20) activities increase with a nucleotide-supplemented diet in animals after chronic diarrhoea (Nuñez *et al.* 1990; Bueno *et al.* 1994). Dietary nucleotides promote the enterocyte growth in tissue culture (He *et al.* 1993; Sanderson & He, 1994) and improve the intestinal repair in an animal model of radiation injury (Uauy *et al.* 1994). Recent studies on the potential roles of exogenous nucleotides on proliferation, differentiation and apoptosis of human small-intestinal epithelium have shown that AMP may have an important role in controlling the dynamic balance of cellular turnover in the developing human small intestine (Tanaka *et al.* 1996). Moreover, dietary nucleotides influence the gene transcription in the intestine (LeLeiko *et al.* 1995). Animals receiving a purine and/or pyrimidine-free diet have a decreased protein synthesis and RNA throughout the intestine and specific mRNA for the enzymes hypoxanthine phosphoribosyltransferase (HGPRT) and adenosine phosphoribosyltransferase (APRT) (LeLeiko *et al.* 1987). A 35-base pair region identified in the HGPRT promoter is necessary to confer sensitivity to exogenous purines as a site for binding to trans-acting regulatory proteins (Walsh *et al.* 1990, 1992).

5.8.2. Nucleotides and liver growth. Extracellular nucleotides and nucleosides have been reported to modulate hepatocyte growth (Ohyanagi, 1989; Gil & Uauy, 1995*b*) and regeneration and to play an important role in the synthesis of glycogen (Buxton *et al.* 1986). Ogoshi *et al.* (1985, 1988) reported that parenterally administered

nucleotides improved the hepatic function and promoted earlier restoration of the N balance after liver injury or partial hepatectomy. Moreover, it has been observed that adenosine administration partially prevents cirrhosis induced by CCl_4 in rats due to a stimulation of total hepatic collagenase activity and is able to counteract the drastic decrease in adenine nucleotides (Hernández-Muñoz *et al.* 1990).

Deprivation of dietary nucleotides results in a transient decrease in acid-soluble nucleotides and RNA content in rat liver as well as in a decreased protein synthesis rate (López-Navarro *et al.* 1995). Dietary nucleotides have improved liver structural recovery and binuclearity in experimental cirrhosis induced by thioacetamide (Torres *et al.* 1996). In that model dietary nucleotides led to a lower number of stellate cells and to a lower collagen deposition.

5.9. Long-chain polyunsaturated fatty acids and cell growth

Despite recent advances in neonatal care, low-birth-weight infants do not achieve first year growth equivalent to that of infants born at term. It has not been clarified how the administration of long-chain fatty acids to infants may affect growth and specific tissue growth and differentiation. Direct evidence that normalized growth might relate to arachidonic acid status came from the observation that formula supplemented with marine oil but no arachidonic acid decreased the concentrations of plasma phosphatidylcholine arachidonic acid (Carlson *et al.* 1991) and reduced weights compared with standard formula without marine oil (Carlson *et al.* 1992). Phosphatidylcholine arachidonic acid declined in preterm infants fed on non-supplemented formulas, and weight fell progressively beginning at 2 months of age. The nadir of plasma phosphatidylcholine arachidonic acid and growth was further reduced by formula containing marine oil compared with the non-supplemented formulas (Carlson *et al.* 1993).

Koletzko & Braun (1991) have investigated whether birth weight correlates with the postnatal EFA status in premature infants. A significant and positive correlation between body weight and plasma triacylglycerol content of arachidonic acid and total *n*-6 long-chain-PUFA was found as well as a negative correlation with α-linolenic acid.

There is only limited information about how intakes of *n*-6 and *n*-3 long-chain-PUFA may affect specific tissue growth. Animal studies using diets supplemented with both types of fatty acids have shown that in addition to plasma and erythrocyte cell membranes, small intestine, liver, kidney, lung and heart are affected in their fatty acid composition. Depending on the composition of the diet, susceptibility to oxidation may be affected, which might influence tissue growth and function (Suarez *et al.* 1996*a*,*b*).

5.10. Early growth and later obesity

In animal studies, early overfeeding may have lasting effects on nutrient utilization and body composition (Davis *et al.* 1973; Lewis *et al.* 1986). In male infants of pregnant women who suffered from starvation, obesity in young adulthood was more prevalent if the mothers were exposed to the famine during the first half of gestation, while the incidence of later obesity was reduced if starvation occurred during late gestation and the early postnatal period (Ravelli *et al.* 1976). Also, food composition, and particularly protein intake, in early childhood has been suggested as a predictor of later risk of obesity. In view of the high prevalence of obesity in Europe and its major importance for public health and health-care costs, the potential modulation of later obesity by early food choice needs to be further explored.

6. Maturation of the gastrointestinal tract

6.1. Introduction

Digestion and hydrolysis of macro- and micronutrients by the gastrointestinal tract are essential prerequisites for long-term survival of mammals including man. Proteins, fats and carbohydrates are digested and hydrolysed by a variety of potent excretory glands and by the brush-border enzymes of the small intestine as well as by bacterial breakdown within the large intestine. As for carbohydrates, a cascade of hydrolytic events finally leads to the presence of monosaccharides within the lumen of the gastrointestinal tract which are transported across the microvillus membrane by highly specialized transporters. Carbohydrates with high molecular mass in the form of amylose and amylopectin are hydrolysed by α-amylase (*EC* 3.2.1.1) of the saliva and the pancreas. α-Amylase can hydrolyse 1-4-α-glycosidic bonds which are present in both amylose and amylopectins. The branches of 1-6-α-glycosidic side chains in amylopectins remain after the action of α-amylase as α-limit-dextrins, and are further hydrolysed by sucrase–isomaltase (SI) (Gray, 1967). Hydrolysis of starches is dependent on the age of the infant. In the first 6 months of life, activity of α-amylase is low and reaches full activity at the end of the first year of life (Lentze, 1986). Defects in sugar digestion occur because of disturbances within the combined action of pancreatic α-amylase and that of intestinal brush-border enzymes. Decreased digestion and hydrolysis of carbohydrates will induce either osmotic diarrhoea and/or bacterial overgrowth within the small intestine as well as bacterial breakdown of carbohydrates within the colon.

Malnutrition is very often combined with chronic diarrhoea and damage of the gastrointestinal mucosa as a consequence of lack of protein and energy. Key factors in this devastating cascade are the brush–border membrane of the small intestine and its hydrolysing and absorptive capacity. Important observations with regard to the hydrolytic capacity of intestinal disaccharidases, which are responsible for sugar hydrolysis, have come through the study of their intracellular pathways and processing of enzyme molecules in normal and altered human as well as animal mucosa using various techniques of molecular biology. The knowledge of these investigations has considerably increased our understanding as to how carbohydrates are hydrolysed and absorbed from the intestinal epithelial cell.

6.2. Development of sugar hydrolases and transporters

The morphological development of the small intestine starts in the 9th week of gestation from the proximal to the distal part of the gut (Hauri, 1986). Small villi develop over a

stratified epithelium of several layers. The first crypts are seen at the age of 10–11 weeks gestation within the duodenum and jejunum, and at 11–12 weeks in the ileum and colon. The changes into a columnar epithelium occur together with the appearance of secondary lumina visible by electronmicroscopy and parallel invagination of mesenchymal cells and extrusion of surface cells (Naim *et al.* 1988). The development of a brush-border membrane is seen together with crypt development in the 10–12th week of gestation. At the same time brush-border membrane hydrolases start to appear. Lactase–phlorizin-hydrolase (LPH), SI and maltase–glucoamylase are first detectable at the 10th week of gestation (Dahlqvist & Lindberg, 1965). Their enzymic activities increase during gestation. As SI and maltase–glucoamylase reach their full activities by the 25th week of gestation (Jirsova *et al.* 1965–6), the activity of LPH remains low until the 28th week of gestation and increases slowly between the 32nd and 34th weeks of gestation (Dahlqvist & Lindberg, 1966). For the nutrition of very immature premature babies with very low birth weight between the 26th and 28th weeks of gestation this could play a role in the digestion and hydrolysis of lactose given in breast milk or infant formula. After introduction of lactose-containing milk the activity of LPH matures quickly to normal enzymic activities. The glucose transporters in the small intestine develop during gestation at about the same time as the sugar hydrolases. Sodium-dependent glucose transporter 1 (SGLT 1), glucose transporter (GLUT) 5 and GLUT 2 appear at the 11th week of gestation as seen by the expression of specific mRNA in human fetal intestine (Davidson *et al.* 1992).

6.3. Biosynthesis of intestinal brush-border membrane hydrolases

Mature intestinal epithelial cells are highly polarized and are composed of two main membranous regions: the apical cell membrane, with its unique feature of a brush border, and the basolateral membrane. The microvillar membrane is characterized by a network of microvilli which contain the important glycoproteins responsible for the hydrolysis and absorption of micronutrients and minerals. For the degradation of various sugar and peptide molecules of different composition and chain length, the intestinal disaccharidases SI, maltase–glucoamylase, LPH, trehalase and a variety of peptide hydrolases are present within the microvillar region of the columnar epithelia in order to digest carbohydrate molecules and oligopeptides from nutritional intakes. The disaccharidases are the best studied brush-border hydrolases. Their enzymic activities and their distribution throughout the gastrointestinal tract as well as their age dependency have been investigated by many groups of researchers. The biogenesis of the disaccharidases produced and processed by the mature enterocyte has been elucidated in mammals as well as in man, demonstrating common pathways within the translational and post-translational routes.

SI and LPH are the best studied brush-border membrane hydrolases in all species including man. The data accumulated from these studies have led to a general understanding as to how these hydrolases are synthesized and processed within the small-intestinal enterocyte. After transcription a single-chain precursor (pro-SIh) rich in mannose (high mannose precursor) is produced in the rough endoplasmic reticulum. This contains carbohydrate residues which are *N*-glycosylated and has an apparent molecular mass of 210 kDa in human subjects (Hauri *et al.* 1980; Ghersa *et al.* 1986). From the rough endoplasmic reticulum the pro-SIh is transported to the Golgi apparatus where trimming of the mannose residues and addition of complex carbohydrates occur to yield pro-SIc (molecular mass 245 kDa) (Hauri *et al.* 1982). The complete primary structure of the pro-SI from rabbit is composed of 1827 amino acid residues containing the two active catalytic subunits isomaltase (140 kDa) and sucrase (120 kDa) which are associated by oncovalent, ionic interactions (Sjöström *et al.* 1980). After complex glycosylation in the Golgi the pro-SIc is translocated and inserted into the microvillus membrane by vesicular transport directly into the apical microvillus membrane. The exact route for the transportation of glycoproteins from the Golgi to the microvillar membrane remains to be established. The time course of transport of the pro-SIc from the Golgi into the brush-border membrane in a human colon carcinoma cell line (CaCo-2 cells) is rather slow (Herskovics *et al.* 1981). Similar transport kinetics were also obtained when the biosynthesis of SI was investigated in the organ culture of human intestinal explants (Naim *et al.* 1988). Insertion of pro-SIc into the microvillus membranes is obtained by anchoring a single hydrophobic segment of the molecule which is located at the N-terminus of isomaltase (Hauri *et al.* 1986). After insertion into the microvillar membrane pro-SIc is cleaved into sucrase and isomaltase by pancreatic proteases (Naim *et al.* 1988). The mature catalytic enzymes sucrase and isomaltase cleave various substrates including sucrose, isomaltose, maltose, maltotriose and amylose as well as α-limit dextrins which are derived from the hydrolysis of amylopectins. SI, together with maltase–glucoamylase, plays a major role in starch digestion during the first month of life as α-amylase in human infants is not developed during the first 6 months of life (Danielsen *et al.* 1981). Striking structural and functional similarities suggest that intestinal SI, human lysosomal α-glucosidase and *Schwanniomyces occidentalis* glucoamylase are derived from a common ancestral gene (Naim *et al.* 1991).

LPH, as the only β-glycosidase of the brush-border membrane, has been reported in earlier work to be synthesized also as a single-chain precursor with a molecular mass of 150 kDa (Sjöström *et al.* 1983). However, conflicting results were obtained on the structure and identification of the precursor molecules. In the pig a precursor protein of 200 kDa was observed (Danielsen *et al.* 1984). The same group reported in a more recent publication that the precursor molecule of LPH in the pig small intestine is a membrane-bound polypeptide of 225 kDa which is intracellularly cleaved after complex glycosylation (Mantei *et al.* 1988). Similar data were obtained in CaCo-2 cells (Herskovics *et al.* 1981). In human intestinal epithelial cells a high-mannose precursor of 215 kDa was demonstrated in intestinal explants maintained in organ culture (Hauri, 1986). Here the intracellular cleavage of the high-mannose precursor occurs during the translocation of the

molecule across the Golgi before complex glycosylation takes place. The mature form of LPH is then inserted into the membrane with a molecular mass of 160 kDa. The primary structure of the human lactase molecule is known and comprises 1927 amino acids in man and 1926 amino acids in the rabbit (Messer & Kerry 1967). The place at which the mature form of lactase is cleaved from its precursor is position 866 of the whole molecule. In contrast to most other brush-border membrane hydrolases the mature lactase is anchored within the lipid bilayer from its carboxyl end of the protein chain (Messer & Kerry, 1967).

Maltase–glucoamylase hydrolyses 1-4-α-glycosidic-linked glucose polymers including maltose and maltotriose (Naim *et al.* 1989). The enzyme is developed early in gestation and contributes to the digestion of starch after birth. The biosynthesis of maltase–glucoamylase is similar to that of SI as a precursor molecule of 225 and 245 kDa. The former represents the high mannose and the latter the complex glycosylated precursor of maltase–glucoamylase in the pig small intestine (Danielsen *et al.* 1984). The biosynthesis and processing of maltase–glucoamylase in human intestinal biopsy specimens does not involve intracellular or extracellular proteolytic modifications, in contrast to SI and LPH (Pfeffer & Rothman, 1987).

6.4. Intestinal absorption of glucose and fructose

Our current understanding of glucose (galactose) and fructose is that the monosaccharides are transported by different methods into the intestinal absorptive epithelial cells. Whereas SGLT 1 is responsible for the active transport of glucose or galactose with equimolar amounts of Na against a concentration gradient into the cytoplasm of the enterocyte (Crane 1975), fructose undergoes facilitated transport by the GLUT 5 transporter which is also located on the brush-border membrane (Davidson *et al.* 1992). Once taken up into the enterocyte, Na^+ is exchanged with K^+ by the Na^+, K^+-ATPase (*EC* 3.6.1.3) which is located in the basolateral membrane and glucose is pumped into the intracellular space by another glucose transporter protein, GLUT 2. GLUT 2 has also been shown to be localized within the basolateral membrane. The function of SGLT 1 is essential for survival of a given species such as man. When absent or deficient, as in congenital glucose–galactose malabsorption, the malfunction of SGLT 1 is a lethal factor.

The absorption of these simple monosaccharides when present in the intestinal lumen is dependent on a variety of factors contributing to the rate of absorption. It is dependent on age, composition of food and species. In mice a relationship between the type of diet and sugar uptake was demonstrated. When fed on a high-carbohydrate, low-protein chow, the uptake of glucose remained high, and dropped considerably when the mice were put on a low-carbohydrate, high-protein diet (Karasov *et al.* 1983). Moreover, it was shown that a strong correlation exists between glucose uptake and the type of natural diet within various vertebrate species. The more herbivore the species is, the more glucose is absorbed; the more carnivore the species is, the less glucose is taken up (Riby *et al.* 1993). Fructose absorption depends strongly on the presence of other carbohydrates within the intestinal lumen. Fructose given together with glucose, galactose, sucrose or starch is better absorbed than fructose alone (Fujisawa *et al.* 1991) whereas the presence of sorbitol or dextrin leads to fructose absorption as with fructose alone. Species differences have a considerable influence on fructose absorption which depends entirely on the composition of the natural diet. Whereas the carnivores (cats) have a low fructose absorption after weaning, the rat and the rabbit increase their fructose absorption considerably after weaning (Buddington & Diamond, 1989).

The complete mechanism for fructose absorption from the human intestine remains to be elucidated. A model of fructose absorption can be deduced from animal studies as well as from human studies. The rate of fructose absorption is influenced by glucose, but also by the amino acid glycine. Two mechanisms have been proposed to explain this effect. Fujisawa *et al.* (1991) speculate that a mechanism in the brush-border membrane exists which they call the disaccharidase-related transport system. This speculation is based on their findings that the specific inhibitor of SI, acarbazone, decreases fructose absorption when given with sucrose, whereas absorption increases without the inhibitor. Another explanation could well account for this effect: glucose absorption as well as glycine absorption increases the water flow from the lumen into the intercellular space by osmosis. Therefore, a solvent drag occurs leading to an enhanced uptake of fructose. The same mechanism applies when starches or sucrose are present at the same time as fructose because of the rapid hydrolysis of these carbohydrates to glucose and/or fructose by the action of maltase–glucoamylase and SI. In order to elucidate this effect fructose absorption should be studied in individuals with SI deficiency which would be the equivalent model to the rat intestinal fructose uptake studies with acarbazone. Except for LPH, the activities of other sugar hydrolases as well as SGLT 1 can be influenced by substrates (Buddington *et al.* 1991; Quan & Gray, 1993). The close presence of sugar hydrolases and sugar transporters within the microvillar membrane of the small-intestinal enterocyte as well as the transporters for amino acids is the guarantee of a steady uptake of sugars derived from various sources.

6.5. Oligosaccharides and mucins

Besides the most abundant sugar lactose, human milk contains more than 130 different oligosaccharides and is unique among all mammalian species for its content of higher oligosaccharides, i.e. larger than lactose. For a long time the oligosaccharide fraction in human milk has been overlooked although it is the third largest solute (up to 18·5 g/l) and present in higher amounts than protein (Egge *et al.* 1983). As oligosaccharides escape the hydrolysis in the small intestine, two possible functions are discussed. One function would be the intact absorption of these components serving as substrates for organ maturation such as the brain, where rapid synthesis of sialoglycoproteins and gangliosides occurs (Sabharwal *et al.* 1991). Their role in the large bowel as 'dietary fibre' and their fermentation would be of significant value in nutrition. The oligosaccharides in human milk are based on five monosaccharide residues: sialic acid, *N*-acetylglucosamine, fucose, glucose

and galactose. All oligosaccharides possess a lactose moiety at their reducing end, with sialic acid (when present) and fucose at the non-reducing end. The chain length varies between three and eleven units. The oligosaccharide composition of human milk shows temporal and individual variations (Miller *et al.* 1994). At the same stage of lactation the variation in oligosaccharide content was shown to be fourfold.

Recent findings on the chemical structure of oligosaccharides in milk have demonstrated structural homologies to carbohydrates carried by glycoproteins and glycolipids on cell surfaces. Such oligosaccharides are very antigenic and were targets of monoclonal antibodies in the search for specific binding to human cancer cells. Similar novel oligosaccharides which can inhibit antigen–antibody reactions have been detected in human milk (Fievre *et al.* 1991; Kitagawa *et al.* 1991). They are useful hapten inhibitors to study the binding specificities of anti-carbohydrate antibodies produced as mucins on cancer cells. This observation has great clinical implications with respect to breast cancer. High-molecular-mass glycoproteins (MUC1) in milk and lactating tissue have been found to contain up to 80% of carbohydrates. The low level of expression of MUC1 in healthy, undifferentiated (non-lactating) breast tissue, and its presence in many, particularly metastasizing, breast tumours has established a very useful marker in breast cancer screening. The exact nature of MUC1, its biological role and expression, has been studied in milk and mammary tissue (Patton *et al.* 1995). MUC1 as expressed in tumours activates B- and T-lymphocytes. These epitopes represent underglycosylated forms of MUC1 characteristic of breast and pancreatic cancer. It is also responsible for keeping ducts and lumens, such as the mammary ducts, open. Here it binds also to L-selectin which is expressed on the surface of leucocytes. By this action leucocytes are bound to the lumen and excreted into the milk (Welply *et al.* 1994). It also escapes digestion and is excreted in the stools of breast-fed infants. In the colon it binds to micro-organisms, particularly to the fimbrins of *E. coli*, contributing to the host defence of the breast-fed infant (Cravioto *et al.* 1991; Schroten *et al.* 1993).

6.6. Probiotic substances in milk or milk substitutes

There are indications that certain ingested micro-organisms added to milk or milk products may exert some physiological effects and promote health in human infants. Such an example is the use of Lactobacillus GG added to the milk formula for premature infants. After administration of these bacteria to premature infants it was noted that the bowel was colonized by Lactobacillus GG, and no clinical side-effects were seen. However, no clinical benefit was noted either (Millar *et al.* 1993). As far as potential effects on fermentation are concerned, Lactobacillus GG given to premature infants had no effect on production of short-chain fatty acids in stools. The observed small increase in ethanol excretion is unlikely to have any clinical significance (Stansbridge *et al.* 1993). Whether or not pre- and probiotics modulate gut maturation and have relevant health benefits in infancy remains to be elucidated.

6.7. Dietary regulation of xenobiotic metabolism

The nutritional status or specific nutrients may influence the metabolic capacity of the liver, but also of other organs (intestinal mucosa). The exact mechanism of drug–nutrient interaction remains unknown. Moreover, the health effects of such interactions have to be explored.

Diet could modulate the metabolism either through an action on substrate availability or by modifying key enzymes of metabolism. The influence of starvation on conjugation and glucuronidation illustrates this point (Mandl *et al.* 1995).

One important discovery of recent years is that some components of food (antioxidants like butylated hydroxytoluene) are able to modulate the activity of key enzymes of phase 1 or 2 metabolism, by modifying gene expression (Kashfi *et al.* 1994). This is a promising area that requires further work. In fact the modulation of gene expression by nutrients is well described in the context of the influence of food on carbohydrate or lipid metabolism but the effect of macro- or micronutrients on xenobiotic-metabolizing enzymes (XME) remains unelucidated.

Diet may also influence XME by inducing specific pathology e.g. steatosis. The part played by nutrients and morphological alteration in the modification of XME has to be established (Leclercq *et al.* 1996).

Specific components of food (*n*-3 PUFA), even at very low concentrations (piperine from black pepper, naringenine from grapefruit) may modify the activity of specific isoforms of XME (e.g. glucuronosyltransferases) (Speck *et al.* 1991). This could lead to interesting developments in several fields. (1) Fundamental research: it will help in studying this metabolic reaction in detail by using those nutrients as activators or specific inhibitors. (2) Those nutrients which can be used at low dose could, thus, be considered as 'toxico-modulators'. (3) Such compounds have been proposed as therapeutic adjuvants, allowing reduction of the dose of expensive drugs, or drugs with a low safety-therapeutic index. The discovery of new nutrients with new targets could constitute a very promising area. Finally, the validation of experimental models (and particularly *in vitro* models) allowing the study of drug–nutrient interactions would give a new input in this area.

7. Development of the immune system

7.1. Introduction

Positive effects of particular foods or food ingredients on the human immune system (e.g. inhibition of CHD and cancer development etc.) could conceivably be related to early nutritional events or may only be seen after decades of intake or lifestyle changes. An important unresolved issue is whether there are critical time periods during which a provision may be especially beneficial to the immune system. In view of these difficulties, *in vitro* surrogate markers for study end-points are frequently used. A surrogate end-point can be defined as a laboratory measurement or a physical sign used as a substitute for a meaningful end-point that measures directly how a patient feels, functions or survives. Changes to a surrogate end-point induced by a dietary intervention are expected to reflect changes in a

biologically meaningful end-point. However, surrogate end-points do not always reflect the true clinical outcome and can be misleading or even meaningless (Fleming & DeMets, 1996). Other important limitations of studies reporting effects on the development of immunity are:

short duration;
lack of standardized tests;
lack of correlation of *in vitro* and/or *in vivo* findings with immune protection or immune suppression;
lack of demonstration of health-enhancing effects in a developing normal, presumably non-deficient population of infants, but with changing nutritional requirements.

7.1.1. Which constituents of the immune system to investigate? The immune system is a highly complex regulatory cellular and humoral system of protection and stimulation directed to avoid danger to the host. On this basis a possible role of diet in cancer prevention could be taken as summary evidence for beneficial effects of the diet on the immune system. This extrapolation, however, seems highly conjectural since it would argue that a number of cancers are due to deficient immune surveillance mechanisms. This may or may not be the case and a number of other protective mechanisms could be equally plausible.

The present report focuses separately on published evidence mostly in infants and children and, where appropriate, in normal individuals and animals. Parenteral micronutrient supplementation in disease states or after surgery, low level toxicity and multiple chemical sensitivities will not be addressed.

7.1.2. Special considerations for the immune system of the developing child. There is a dearth of reliable information of the effects of vitamins, saturated and unsaturated fatty acids, trace minerals and other normal food constituents on the infant's developing immune system. The majority of reports examine the effects of corrections of severe or moderate deficiencies on the immune responses in infants and children (or animals). Very little is known of the effects of supplementations, either in line with recommendations or above, in a non-deficient population. As indicated earlier, most measurements have been related to surrogate end-points and the relationships between administration of test substances and their effects on the immune system are not strongly causal.

7.2. Antioxidants and vitamins

7.2.1. In general. Antioxidant vitamins generally enhance different aspects of cellular and non-cellular immunity. The antioxidant function of these micronutrients could, at least in part, enhance immunity by maintaining the functional and structural integrity of important immune cells. Multiple effects attributed to antioxidants include risk reduction of a variety of chronic diseases (Messina & Messina, 1996), anti-(retro)-viral activity (Formica & Regelson, 1995), immune enhancement in animals (Forni *et al.* 1986; Gebhard *et al.* 1990,), in man (Chavance *et al.* 1989; Penn *et al.* 1991; Rall & Meydani, 1993), and evidence that certain vitamins alone or in combination and other micronutrients given in levels above the current recommendations have critical, beneficial effects on human immune responses (Bendich, 1995).

7.2.2. Vitamin A. The effects of vitamin A supplementation on measles in deficient and non-deficient children have been the subject of several recent reports (Coutsoudis *et al.* 1992, 1995; Rosales & Kjolhede, 1994; Semba *et al.* 1995; Stabell *et al.* 1995; Keusch, 1996). In general, in the last decade epidemiological, immunological, and molecular studies have yielded substantial evidence for a central role. The recent discovery of retinoic acid and retinoid X receptors has provided a molecular basis for the action of vitamin A and its metabolites at the level of gene activation. β-Carotene supplementation enhances the expression of functionally associated molecules on human monocytes (Hughes *et al.* 1996) and also enhances immune responses to poor immunogens, which may be relevant to infants receiving vaccines which are characterized by low seroconversion rates (Semba, 1996), however under certain conditions seroconversion rates may be negatively affected too (Semba *et al.* 1995). No difference was found (using whole-blood culture techniques) in the *in vitro* proliferative responsiveness of T-cells to concanavalin-A and tetanus toxoid of children with normal or low–normal concentrations of vitamin A or Zn (Kramer, 1996).

7.2.3. Vitamin C. Vitamin C has gained great scientific and media attention through the promotions of its effects on the common cold. Early reports of systemic conditioning of infants following an increased intake of vitamin C during development seem to have been unfounded (Gerster & Moser, 1988). Studies which failed to identify a positive effect on the symptoms of the common cold have recently been critically reviewed and it now seems likely that there is a reduction in clinical symptoms associated with intake of 2–3 g ascorbic acid/d at the onset of the cold (Hemila, 1996).

7.2.4. Vitamin B complex. Few studies have addressed the effects of supplementation with individual B vitamins and/or the coenzyme CQ10. When studied, positive enhancing effects on cell-mediated immunity, CD4 : CD8 ratios, delayed type hypersensitivity and antibody production have been reported (Gebhard *et al.* 1990; Miller & Kerkvliet, 1990; Folkers *et al.* 1993). Tumour inhibition by high dietary pyridoxine may be mediated by immunological mechanisms that are lacking in the genetically immunodeficient (athymic) mice in which these studies have been carried out (Gebhard *et al.* 1990).

7.2.5. Vitamin E. Vitamin E, in its role as a potent antioxidant and immunostimulant, has received a great deal of attention (Tengerdy, 1990; Shor Posner *et al.* 1995; Liang *et al.* 1995; Finch & Turner, 1996; Liang & Watson, 1996). Vitamin E supplementation enhances humoral and cell-mediated immunity, and augments the efficiency of phagocytosis in laboratory animals and human subjects. Vitamin E deficiency has been suggested to contribute to alterations of the neonatal neutrophil function, and supplementation with 120 mg/kg over the first 14 d of life of healthy premature infants has been shown to increase phagocytosis (Chirico *et al.* 1983).

7.2.6. Vitamin D. There is now increasing evidence that the hormonal form of vitamin D, 1,25-dihydroxycholecalciferol ($1,25(OH)_2D_3$), is involved in the regulation of the

immune system. $1,25(OH)_2D_3$ exerts most of its actions after it has bound to its specific receptors which are present in monocytes and activated lymphocytes. The hormone inhibits lymphocyte proliferation and immunoglobulin production in a dose-dependent fashion. It interferes with T-helper cell function, reducing T-helper cell-induction of immunoglobulin production by B-cells and inhibits the passive transfer of cellular immunity by T-helper cells *in vivo*. Expression of Class II antigen by lymphocytes and monocytes is also affected. In experimental *in vivo* studies $1,25(OH)_2D_3$ is particularly effective in preventing autoimmune diseases (Schwartz, 1992; Mathieu *et al.* 1994; Thomasset, 1994). There is no information on the effects of vitamin D or its metabolites on the developing human immune system.

7.3. Multiple micronutrient supplementation studies

Trace elements perform important functions in growth and development. However, little information exists about dietary requirements of them during the demanding period of infancy. Although several factors influence the dietary needs of these essential elements, the basis for establishing dietary needs in infants is hindered by the dearth of studies that have assessed their bioavailability and effects on the immunity in this age group. Thus, until it has been conclusively shown otherwise, the physiological response to human milk is used as the standard for infant feeding practices (Milner, 1990).

Key questions such as the risks to human health of altered environmental distribution of Zn, assessment of Zn status in man, effects of Zn status in relation to other essential metals on immune function, reproduction, neurological function and others remain (Sherman, 1992; Walsh *et al.* 1994; Prasad, 1995; Ripa & Ripa, 1995; Sazawal *et al.* 1996). Zn supplementation has recently been shown to reduce persistent diarrhoea in children (Sazawal *et al.* 1996).

In vitro and *in vivo* studies show that antioxidants generally enhance different aspects of cellular and non-cellular immunity. The antioxidant function of these micronutrients could, at least in part, enhance immunity by maintaining the functional and structural integrity of important immune cells (Chew, 1995).

Effects of trace minerals on the outcome of pregnancy have been reviewed. About 30 % of pregnant women suffer from Fe deficiency worldwide, and while its effects on neonatal Fe status are not severe, adverse sequelae include impaired neonatal immune status (Allen, 1986; for review, see Bryan & Stone, 1993). Low maternal intakes of Cu, Mn, and Se have not been associated with adverse outcomes of pregnancy. Se deficiency, however, appears to result in immunosuppression affecting neutrophil function, antibody production, cytotoxicity and lymphocyte proliferation (Kiremidjian Schumacher & Stotzky, 1987).

7.4. Fatty acids

Several lines of evidence support the role of dietary lipids as regulators of the immune system. This is demonstrated by studies examining lipid alteration of the immune response to allergens, malignancy, autoimmune disease, sepsis, trauma, and transplantation. Both the quantity and quality of lipid are important in immunoregulation. Both cell-mediated and humoral immunity are affected by dietary lipids. Multiple mechanisms probably contribute to the overall effects of lipids, including alteration of arachidonic acid metabolism, changes in cell membranes, production of inflammatory cytokines, and impairment of the reticuloendothelial system (Perez & Alexander, 1988; Yetiv, 1988; Fernandes *et al.* 1990; Melnik *et al.* 1991; Watanabe *et al.* 1994; Endres, 1996; Hellerstein *et al.* 1996).

7.5. Arginine

Many of the known roles of arginine (e.g. in immune function, wound healing, and protection against NH_3 intoxication) are mediated by a metabolic pathway synthesizing NO in the liver. Both stimulatory and suppressive functions have been identified with a prominent role of the macrophage (Barbul, 1990; Rodeberg *et al.* 1995; Suzuki *et al.* 1995; Krenger *et al.* 1996; Marcinkiewicz *et al.* 1996). Particular effects on the developing human immune system are uncertain. Oral administration may be less effective than parenteral administration (Torre *et al.* 1993). A number of reports investigate the potential benefits of arginine supplementation during surgical and other periods of stress in human subjects and in experimental models. Beneficial effects on wound healing, reduction of postoperative septicaemias etc. cannot always be attributed entirely to arginine, since it was often given with other dietary modulations (Daly *et al.* 1990; Cerra *et al.* 1991; Seidman *et al.* 1991; Kemen *et al.* 1995; Senkal *et al.* 1995; Braga *et al.* 1996; Kudsk *et al.* 1996; Marcinkiewicz *et al.* 1996).

7.6. Nucleotides

Dietary sources of preformed purines and pyrimidines seem to be important for optimal function of the cellular immune response. It was previously assumed that nucleotides were not needed for normal growth and development, but the results described in the present review demonstrate a need for nucleotides in the response to immunological challenges. The need for sources of preformed nucleotides in defined formulas such as parenteral and enteral formulas and infant formulas is suggested in some studies (Carver, 1994; Kulkarni *et al.* 1994; Rudolph, 1994). An exogenous source of nucleotides from the diet may optimize the function of rapidly dividing tissues when growth is rapid and the diet is low in nucleotides. Studies performed in human infants are, at most, inconclusive (Carver, 1994; Kulkarni *et al.* 1994) and further studies are required to assess in infants the interesting findings about dietary nucleotides reported in experimental models (Gil & Uauy, 1989; Sanchez Pozo *et al.* 1994, 1995; Ortega *et al.* 1995; Lopez-Navarro *et al.* 1996; Navarro *et al.* 1996).

7.7. Maturation of the immune system in formula-fed v. *breast-fed infants*

In a small study of systemic and secretory immunity of breast-milk-fed *v.* formula-fed infants it was shown that

there is a general stimulation of responsiveness by cytokines in milk and a reduction of specific responses by antigen exclusion. Bottle-fed infants reached a similar level of immunological maturity by 3 months of age, exhibiting raised levels of serum antibodies against gut organisms and milk proteins and demonstrating increased non-specific activation of lymphoid cells (Stephens, 1986; Stephens *et al.* 1986*a*,*b*)

7.7.1. Effects of antigen transfer via breast milk on the infant's immunity. Dietary antigen excretion into breast milk seems to be a general phenomenon and has been reported for milk, egg, wheat proteins and parasite antigens (Kilshaw & Cant, 1984; Troncone *et al.* 1987; Petralanda *et al.* 1988). Excreted amounts are in the range of μg/l. The immunological significance of transfer of dietary antigens during breast-feeding is still unclear. It is generally accepted that breast-feeding reduces the risk of food allergic reactions and also of atopy in a population at risk (uni- or biparental history of atopy) but sensitizing effects in infants have also been described (Warner, 1980; Gerrard & Shenassa, 1983; Savilahti *et al.* 1987; Lindfors & Enocksson, 1988). Studies by Chandra *et al.* (1986, 1989*a*), Zeiger *et al.* (1992) and others (Halken *et al.* 1993*a*; Vandenplas *et al.* 1995) suggest that elimination of (significant) dietary antigen transfer via breast milk for 6 months (amongst other preventive measures) in a population at risk reduces the probability of a food-specific sensitization (and possibly atopic symptoms) for up to 48 months, an effect which persists even after the diet of the infant has been liberalized. The following points merit special consideration.

7.7.2. Maternal diet during pregnancy and effects on the infant's immunity. There are no reports which demonstrate any preventive (or sensitization) effects in infants with a parental history of atopy (Fälth-Magnusson *et al.* 1987; Lilja *et al.* 1989; Fälth-Magnusson & Kjellman, 1992).

7.7.3. Maternal diet during pregnancy and lactation. In view of the complexity of the studies and the number of confounding variables, it is not entirely surprising that a number of studies have come to different conclusions. Maternal diet, while continuing breast-feeding, has been shown to be of moderate benefit in the reduction of atopic manifestations (mainly eczema) and milk allergy in infants or children (Chandra *et al.* 1989*a*,*b*; Sigurs *et al.* 1992; Zeiger *et al.* 1992; Zeiger & Heller, 1995). Other studies have failed to show this effect (Lilja *et al.* 1989).

7.8. Role of the gut flora and probiotic bacteria in the infant's immunity and gut defence

Little is known about the immunomodulating capacity of the first bacteria colonizing the gut. Breast-fed infants develop a typical intestinal flora and this has been linked to a certain resistance to enteric infections. However, breast milk contains a host of other immunomodulating factors and it is difficult to claim any causality in these studies. Infants given breast-milk substitutes with various strains of lactic acid-producing bacteria may also exhibit some resistance to infections. In a small clinical study of thirty-nine children, protective effects of the supplementation of an infant formula with oligosaccharides, fermented milk or lactic acid-producing bacteria on the reduction of the incidence of acute diarrhoea were reported. A randomized controlled feeding trial with *Bifidobacterium breve* in ninety-one very-low-birth-weight infants demonstrated effective colonization, fewer abdominal signs and better weight gain (Kitajima *et al.* 1997). An enhancement of the circulating antibody-secreting cell response was observed in infants with rotavirus diarrhoea supplemented with a strain of *Lactobacillus casei,* compared with a placebo group (Kaila *et al.* 1992). The duration of this response and other protective or longer-term effects are unknown. Other small studies reported an enhancement in the phagocytic activity of granulocyte populations in the blood of human volunteers after consumption of fermented milk with *Lactobacillus acidophilus* and *B. bifidum* (Schiffrin *et al.* 1995). It is unresolved whether studies in a larger number of unselected infants under different conditions in different countries would yield similar encouraging results.

The gut microflora is an important constituent in the intestine's defence barrier. Probiotic bacteria have been suggested to affect different aspects of gut defence: immune exclusion, immune elimination and immune regulation.

7.8.1. Immune exclusion and elimination. Although many clinical benefits have been ascribed to consumption of candidate probiotic strains in gastrointestinal disease (Isolauri *et al.* 1991; Saavedra *et al.* 1994), only a few human studies have assessed the effects of these bacteria on gut defence mechanisms. Early reports associated clinical observations with the effects on the intestinal microflora (Niv *et al.* 1963). Oral bacteriotherapy affected microbial imbalances shown during rotavirus infections in infants (Isolauri *et al.* 1994). Oral introduction of probiotic micro-organisms has been associated specifically with reduction of intestinal inflammation (Majamaa & Isolauri, 1997) and an increase in circulating antibody-secreting cells in the serum as an indicator of the intestine's immunological barrier function (Kaila *et al.* 1992). In children with rotavirus diarrhoea, probiotic bacteria administered during the diarrhoeal phase of the infection promoted clinical recovery and enhanced intestinal immunoglobulin A (IgA) responses (Kaila *et al.* 1992).

7.9. Effects of formulas with protein hydrolysates on the infant's immune responses

Extensively hydrolysed casein formula has been used in the treatment of children with cow's milk protein allergy and/or intolerance. Recently, ultrafiltrated whey hydrolysates have been also been used therapeutically (Halken *et al.* 1993*b*). In an attempt to prevent and/or modulate the risk of developing food-allergic and atopic manifestations in infants and children, less extensive (partial whey hydrolysates) (Chandra, 1991; Vandenplas *et al.* 1992, 1995) and extensively hydrolysed formulas (Chandra *et al.* 1989*a*; Zeiger *et al.* 1989; Halken *et al.* 1993*a*; Zeiger & Heller, 1995; Oldaeus *et al.* 1997) have been used. Although these studies have been performed in infants of different (atopic) family and ethnic background, with different nutritional habits and different measures of control for confounding variables and a lack of standardized diagnostic protocols, they could be summarized as follows. (1) Infants with a high-risk family

background of atopic disease are likely to benefit from exclusive breast-feeding for 4–6 months with some added benefit if the mother avoids certain foods such as milk, eggs, fish and possibly nuts (including peanuts) during lactation. The benefits include a reduction in the incidence of cow's milk and food allergies and atopic eczema for up to 4 years. (2) If exclusive breast-feeding for 4–6 months cannot be sustained, the use of a hydrolysed infant formula may help reduce the overall incidence of atopic manifestations in the child at risk. (3) Preventive effects of hydrolysed formulas in infants with a normal risk of developing atopic manifestations and the respective benefits of extensively *v.* less extensively hydrolysed infant formulas need to be further evaluated.

7.10. Insulin-dependent type 1 diabetes mellitus and cow's milk exposure in infancy

Insulin-dependent diabetes mellitus (IDDM) is considered to be a chronic autoimmune disease characterized by gradual β-cell destruction mediated by autoreactive T-lymphocytes during an asymptomatic prediabetic phase of varying duration (Knip, 1992). In a Finnish study, associations of infant feeding patterns and milk consumption with cow's milk protein antibody titres were studied in newly-diagnosed diabetic children, sibling-control children and birth-date- and sex-matched population-based control children. Inverse correlations were observed between the duration of breast-feeding, or age at introduction of dairy products, and antibody titres. High IgA antibody titres to cow's-milk formula were associated with a greater risk of IDDM both among diabetic-population-control and diabetic-sibling-control pairs. The results suggested that young age at introduction of dairy products and high milk consumption during childhood increase the levels of cow's milk antibodies and that high IgA antibodies to cow's milk formula are independently associated with increased risk of IDDM (Vaarala *et al.* 1996). Similar associations between bovine serum albumin antibodies and onset of IDDM have also been found in a low-incidence French population (Levy Marchal *et al.* 1995). Human T-lymphocyte cultures of cells taken from affected children allowed the detection of bovine serum albumin-specific T-cells which were mapped to the ABBOS peptide (pre-bovine serum albumin position 152–169) previously identified as a possible immunological mimicry epitope which could explain the cross-reactivity with pancreatic islet cell antigens (Cheung *et al.* 1994). A currently ongoing prospective dietary intervention trial in children genetically at risk will be able to address the causality of this highly intriguing association and, it is hoped, open the way for a primary nutritional prevention strategy.

8. Bone growth and mineralization

8.1. Cell biology of bone growth

Bone growth and mineralization is an ongoing process during human fetal and postnatal development, stabilizing at about 21 years of age. Skeletal Ca content increases from 30 g in the neonate to 1200 g in the adult, and skeletal P from 17 to 700 g. Bone tissue possesses a series of enzymic mechanisms that permit mineralization of its extracellular matrix. This matrix is composed of collagen, proteoglycans and other non-collagen proteins in which insoluble mineral salts of hydroxyapatite and small amounts of other salts of Mg, sodium carbonate and citrate are deposited, converting it into a structure capable of supporting the organism. The two most important bone cell types are osteoblasts and osteoclasts. Osteoblasts are responsible for the formation and organization of the extracellular matrix and its subsequent mineralization. Osteoclasts are large motile multinucleated cells, located on bone surfaces, responsible for the resorption of bone matrix. Bone growth or bone modelling is the result of two processes: first formation of new bone and then resorption to maintain the same structural form with, as a net result, acquisition of bone mass. At the age of about 18 years, both male and female adolescents have reached 95–99 % of their individual peak bone mass. After adolescence the processes of bone resorption and formation become quantitatively in balance and this situation is referred to as bone remodelling. After 35–40 years of age the processes of bone resorption and formation become uncoupled and net bone loss will occur, eventually leading to osteoporosis (Price *et al.* 1994; Anderson, 1996*a*,*b*). The obvious strategies to prevent, or at least delay, the onset of osteoporosis include: (a) optimizing the attainment of peak bone mass in adolescents, and (b) preventing bone loss in later life.

8.2. Methodological aspects in bone-mass-related studies

In the interpretation of the bone-mass-related results of the various studies one needs to be aware of the actual technique used. The first non-invasive methods for measuring bone mass were based on quantitative evaluation of standard radiographs. During the last decade gamma- or X-ray techniques were developed based on the variable effect of matter on the passage of radiation. Most studies were performed using either single photon absorptiometry (SPA) or dual-energy X-ray absorptiometry (DEXA). SPA is a relatively simple technique using ^{125}I as the radioactive source. Its application is limited to the peripheral skeleton, particularly the radius. DEXA is becoming more and more the preferred technique to assess bone density at various sites of the skeleton with only minimal radiation exposure. Although calibration of DEXA instruments seems to be a rather trivial exercise, it relies strongly on highly specific software, with the results that measurements on one patient made with instruments of different brands do not necessarily result in similar bone mineral density data (Slosman *et al.* 1995). An additional feature of DEXA is its ability to measure whole-body mineral content as well as body composition. Appropriate DEXA software for infants has been developed recently and reference values from the first studies are now beginning to become available in the literature (Rigo *et al.* 1996). The fact that SPA and DEXA data cannot be compared with each other is illustrated by the fact that SPA produces a measurement of bone mineral content (g/cm) and assumes that the site of measurement is a small cylinder of constant width, whereas DEXA produces a measurement of bone mineral density (g/cm^2) by correcting

Table 1. Additional increment in bone mineral density (BMD) (as a percentage) following supplementation of the diet with calcium or dairy products, in children and adolescents from five different studies (From Kerstetter, 1995)

	Johnston *et al.* (1992)*					
	Prepubertal	Pubertal	Lloyd *et al.* (1993)	Lee *et al.* (1994)	Andon *et al.* 1994)†	Chan *et al.* (1995)
Subject no., total	22 twin pairs	23 twin pairs	94	162	248	48
Sex	Boys and girls	Boys and girls	Girls	Boys and girls	Girls	Girls
Entry age (years)	6·9 (SD 1·4)	10·6 (SD 2·0)	11·9 (SD 0·5)	7·2 (SD 0·2)	11·4 (SD 0·8)	11·1 (SD 1·0)
Intervention duration (months)	36		18	18	6	12
Baseline Ca intake (mg/d) (placebo)	908		935	280	888	728
Total Ca intake (mg/d) (diet + supplement)	1612		1370	580	1315 1618	1437
Supplemental Ca source	Ca citrate malate		Ca citrate malate	$CaCO_3$	Ca citrate malate	Dairy foods
BMD determination	DEXA		DEXA	SPA	DEXA	DEXA
Change in BMD‡						
Midshaft radius	+5·1	−0·1				
Distal radius	+3·8	+2·9		+3·1		NS
Lumbar spine	+2·8	−1·0	+2·9			+9·9
Femoral neck	+1·2	−0·4				NS
Ward's triangle	+2·9	−0·4				
Greater trochanter	+3·5	+0·2				
Total body			+1·3		+1·0 +2·2	+6·6

DEXA, dual-energy X-ray absorptiometry; SPA, single photon absorptiometry.
* Two age groups were studied: prepubescent (6·9 (SD1·4) years) and pubescent (10·6 (SD2·0) years).
† Two levels of dietary Ca (1315 and 1618 mg/d) were studied.
‡ Change in BMD = percentage increase in supplemented group minus increase in unsupplemented group.

the bone mineral content for the projected area of bone (Slosman *et al.* 1995). A shortcoming of the usual expression of bone mineral density obtained by DEXA (g/cm^2) is that this areal bone mineral density does not take the age-related increase in bone thickness into account. Therefore Cowell *et al.* (1995) have developed a measure of true bone mineral density, volumetric bone mineral density (g/cm^3), and demonstrated its usefulness in assesssing patients with phenylketonurea (PKU), chronic renal failure and chronic asthma.

8.3. Peak bone mass and relative risk of osteoporosis

The relative importance of peak bone mass on the subsequent risk of osteoporosis has recently been reviewed by Ribot *et al.* (1995). They started with the available *in vitro* evidence on the relationship between low bone mass and the risk of osteoporosis relating to the mechanical properties of bone. The relevant *in vivo* studies include both cross-sectional surveys and at least eleven prospective studies. From these studies it can be deduced that the relative risk of osteoporosis for each 1 SD decrease in bone mineral density is increased by a factor of between 1·7 and 2·7. On comparing this value with the relative risk of CHD for a 1 SD rise in serum cholesterol or that of stroke for 1 SD increase in blood pressure being 2·1 and 1·3 respectively, it is clear that low peak bone mass is a very strong risk factor for later osteoporosis. Another appealing value relating to the relevance of optimizing or increasing peak bone mass can be deduced from the study by Gilsanz *et al.* (1991) on comparing the development in peak bone mass in white and black girls. They found a 10–20 % higher bone density in black girls relative to white girls which is likely to correspond to an additional 10–20 years of protection against the decline in skeletal mass, and might explain the relatively low prevalence of osteoporosis in black women. About 80 % of the variance in bone mineral density is accounted for by genetic factors (Pocock *et al.* 1987), thus leaving only 20 % of the variance to be influenced by environmental factors such as diet. For this reason the investigation of twin pairs is very attractive because the genetic bias may thus be minimized (Johnston *et al.* 1992). From a study with postmenopausal twins in Britain, Spencer *et al.* (1995) reported a genetic linkage between the vitamin D receptor genotypes and bone mineral density. The degree to which this might explain the genetic factors is as yet unclear and a recent study from Denmark failed to find any significant association between common allelic variations at the vitamin D receptor locus and bone mineral density (Jørgensen *et al.* 1996).

8.4. Bone growth and mineralization in infants and young children

The literature on the effects of different diets during infancy on bone mineral content at 2 or 5 years of age is not yet consistent. Exclusive feeding of breast milk during the first 6 months of life supports a bone growth considered adequate even though breast milk contents of Ca and vitamin D are relatively low. Infant formulas in general, and formulas for premature infants in particular, contain higher levels of these nutrients to meet the dietary requirements and to provide a safety margin to correct for a likely lower bioavailability. Surprisingly, Bishop *et al.* (1995) reported a strong positive association between the amount of human milk consumed and bone mineral content at the age of 5 years in their multi-centre cohort of prematurely born infants. The authors raised two possible hypotheses to interpret their finding. Bone mineral depletion in preterm infants fed on unsupplemented human milk might 'programme' these infants to be conservative with bone mineral and to reduce the overall rate of growth so that

'over-mineralization' occurs at a later stage when the intake of bone mineral substrates is normal. A second possibility is that one or more of the human milk growth factors might survive breast milk pasteurization and the immature digestion system and end up via the circulation at the target organ. Until one of these hypothetical mechanisms is further substantiated, the general goal in the nutrition of premature infants remains to provide enough mineral supplementation to allow attainment of bone mineral content comparable to that accrued *in utero* and to support catch-up growth in the first year of life. Several studies comparing bone mineralization in term-born breast-fed infants with that in infants fed on formulas containing moderate or high Ca content conclude that the bone mineral content of formula-fed infants is higher at the ages of 2 and 5 years (Demirini & Tsang, 1995). Whether this effect of cumulative Ca intake during the first 2 years of life will be retained until adolescence is still unclear. A negative impact on bone growth and mineralization has been reported for a number of chronic conditions during infancy and early childhood like cystic fibrosis, IDDM, cerebral palsy, leukaemia, renal disease, growth hormone deficiency and anorexia nervosa (Shaw & Bishop, 1995). In a group of fifty-five children with milk allergy showing a broad distribution of daily Ca intake (quartiles: 409, 663, 950 and 1437 mg Ca/d), a clear correlation was found between Ca intake and bone mineral density, thus underlining the vulnerability of this group and illustrating the efficacy of dietary measures or supplementation of Ca to achieve normal bone growth (Henderson & Hayes, 1994).

8.5. Calcium supplementation in children and adolescents and bone health

Despite the relatively poor contribution diet is supposed to make to the variance of peak bone density, a substantial number of studies have been published in recent years to address the effects of nutrition, particularly of Ca, on bone density in children and adolescents. These studies have been reviewed by Kerstetter (1995) who concluded that the cross-sectional and correlation studies have yielded rather mixed results. Surprisingly, more consistent findings were obtained from the five recent prospective Ca or dairy supplementation studies in children and adolescents published between 1992 and 1995. In Table 1 the basic data from these studies (Johnston *et al.* 1992; Lloyd *et al.* 1993; Andon *et al.* 1994; Lee *et al.* 1994; Chan *et al.* 1995) are compared. In all studies the baseline Ca intake was less than 1000 mg/d and the amount of supplemented Ca ranged from 300 to 700 mg/d. The percentage increase in bone mineral density in all the supplemented groups amounted to 1–10 % and was significant in all studies. Of those intervention studies, the one by Chan *et al.* (1995) is most appealing because in this study a near doubling of the Ca intake (from 728 to 1437 mg/d) was reached only by supplementation with dairy products and the increase in total body bone mineral density amounted to 7 %. However, the conclusion which one might draw on superficial reading of the review by Kerstetter (1995) that dairy products outperform other Ca supplements cannot be substantiated yet. Nevertheless, it is remarkable that in the study by Chan *et al.* (1995) the supplemented dairy products also contained some vitamin D and extra P, which can at least be interpreted as showing that P has no negative effect on peak bone mass accretion in adolescents under these conditions. In conclusion, the results from the Ca and dairy-product supplementation studies summarized have clearly demonstrate that it is possible to increase peak bone mass at the end of adolescence simply by dietary means.

8.6. Nutrients other than calcium and environmental factors involved in bone growth

The crystal salt of bone resembles hydroxyapatite ($Ca_{10}(PO_4)_6(OH)_2$) which contains Ca and P in the proportion 2.15:1 (w/w); in addition approximately 60 % of the body Mg and 30 % of the body Zn are present in the skeleton. It is, therefore, obvious that P, Mg and Zn are also important nutrients in the process of bone mineralization. Although there is little information about conditions in developed countries where Mg or Zn would represent single limiting factors causing impaired bone growth, it seems reasonable and prudent to include these elements proportionally in supplements, possibly also taking their different rates of absorption into account. The case for P is more delicate and, except for premature infants where P intake can be a limiting factor for bone growth, much more concern has been shown about excessive dietary intakes of P (Calvo & Park, 1996). High P intake results in an increased serum P concentration which initiates several hormonal respones, of which an increase in parathyroid hormone level effects the balance between bone mineralization and bone resorption (Anderson, 1996*a*,*b*). It is for this reason that in several supplementation studies no phosphate was given in order to shift the Ca : P ratio as much as possible in the direction of Ca. As already mentioned, the results from the study by Chan *et al.* (1995) give support to a concept where the absolute amount of Ca is more important than the Ca : P ratio.

A nutrient which is most important in bone metabolism is vitamin D. The major role of dietary vitamin D is to function as precursor for 25-hydroxy- and 1,25-dihydroxycholecalciferol which maintain the plasma Ca concentration within very narrow limits. This is accomplished by varying the proportion of dietary Ca absorbed and excreted. As the body becomes vitamin D depleted, the efficiency of Ca absorption decreases from 30–50 % to no more than 15 %. In addition, 1,25-dihydroxycholecalciferol has a direct effect on osteoblast production and thus on bone formation and mineralization (Anderson, 1996*a*,*b*). The major source for vitamin D in human subjects is exposure to sunlight which enables cutaneous synthesis of vitamin D. However, several groups ranging from premature infants to institutionalized elderly people may not be able to receive sufficient exposure to sunlight and thus require dietary vitamin D.

The involvement of vitamin K in bone metabolism is through its action on maturation of osteoblastic bone proteins by carboxylation of their glutamate residues (Vermeer *et al.* 1996). Vitamin K may be generated by the intestinal microflora or obtained from dietary sources such as green vegetables and meat. Except for the fact that it depends on the quantity of fluoride ingested and the time of exposure,

the role of F in bone health is still poorly understood. It has been used as a therapeutic agent in bone pathology including osteoporosis, but also cases of skeletal fluorosis are reported with radiologically demonstrable abnormal bone densification (Boivin *et al.* 1993). Of the minerals for which a role and/or essentiality in man is still unclear, B has been connected with the mechanical properties of bone (Mastrmatteo & Sullivan, 1994). Because the effects of B supplementation are not striking, and plausible mechanistic explanations still need to be presented, it is uncertain whether B will become an important issue in bone health research. In a cross-sectional study in about 500 children and adolescents (aged 8–17 years) bone mineral density was found to be related to diet, weight-bearing exercise and daylight hours spent outdoors (Gunnes & Lehman, 1995). Next to an effect in accordance with the literature with respect to dietary Ca it is surprising that the authors found a positive correlation between bone mineral density and saturated fat, fibre and vitamin C. Any interpretation of these correlations can only be speculative. The positive association between fat intake and bone mineral density might be attributed to an increased intake of vitamin D with the fat or might possibly be mediated by a higher cholesterol level. The positive association between bone mineral density and fibre intake is even more puzzling because one might expect exactly the opposite based on impairment of Ca absorption by dietary fibre. As a possible explanation for the association between vitamin C and bone mineral density, the authors indicate the fact that vitamin C is a cofactor in collagen synthesis. Moreover, they point to a possible link between high intakes of Ca, fibre and vitamin C as constituents of a more wholesome diet. Considering the importance of a high Ca intake in achieving maximal bone growth and peak bone mass, it seems logical to prevent any negative dietary interactions which might interfere with maximal Ca absorption. Of the dietary interactions affecting Ca absorption reviewed by Licata (1993), the possible negative effect of caffeine is mentioned because it increases urinary Ca excretion. However, this effect is considered to be fairly unimportant relative to other factors and is also not consistently found in all the studies. A clear negative effect on Ca absorption is represented by dietary oxalate-exemplified by the poor Ca absorption (only 5 %) from oxalate-rich spinach. Although some authors report a stimulating effect of lactose on Ca absorption in humans, others fail to find any significant effect. Essentially the same holds true for the effect of protein on Ca absorption: if there is any stimulating effect, it will be rather small. In view of the marginal gain on fractional Ca absorption which can possibly be obtained by optimizing the nutritional matrix, selecting Ca salts with a high bioavailability may be of particular relevance. In this respect an increase in fractional Ca absorption of 0·26 to 0·36 which can be achieved by replacing $CaCO_3$ with calcium citrate–malate is illustrative (Peacock, 1991).

9. Nutrient effects on development of neural functions and behaviour

9.1. Introduction

Pregnancy and the first postnatal months are critical time periods for the growth, development and differentiation of the human nervous system. There is good evidence that availability of nutrients during these critical time periods affects brain growth and development and can have long-term programming effects on an individual's central nervous functions. In contrast to some other mammalian species, in man the peak growth rate of the brain ('brain growth spurt') occurs both pre- and postnatally, with continued relatively rapid growth well into the second year of life. Between the 24th week of gestation and the time of term birth, brain weight increases more than fivefold. The disproportionate speed of brain growth, compared with total body growth, is apparent from the fact that at age 2 years the weight of the human brain has already reached 80 % of adult weight, while whole body weight at this age is less than 18 % of adult weight. An adequate substrate supply is essential for a physiological brain composition and differentiation during this rapid perinatal growth.

9.2. Physiology of neural development

Development of neuronal tissues is characterized by the sequential occurrence of mitosis, cell migration, differentiation, synaptogenesis, apoptosis and synaptic reorganization. These consecutive steps begin during pregnancy at different gestational ages and occur over a certain time period in different brain areas; therefore, there is an overlap in time of the various brain development steps in different areas of the brain (Reisbick, 1996).

Mitosis of germinal cells located along the neural tube and the brain ventricles, which form neuronal and glia cells, begins in the sixth embryonic week, peaks in the second trimester and is almost complete by the end of the second trimester, even though a small number of cells may form during the last trimester of gestation (Jacobson, 1991; Rakic, 1995). Under the influence of hormones, cell adhesion molecules and other local factors, the created cells migrate to the brain nuclei and cortex and the ganglia of the peripheral nervous system (Jacobson, 1991; Kandel *et al.* 1991). Although largely occurring during pregnancy, nervous cell migration particularly in the superficial layers of the cerebral and cerebellar cortices continues to occur after term birth (Rakic, 1995). Cell differentiation into particular neurons may begin during migration but generally is only completed at its final destination (Kandel *et al.* 1995). The differentiation of neuronal cells is modulated by induction through surrounding cells, the kind of cell innervated, growth factors and hormones such as glucocorticoids, and nutrient intakes. For example, pre- and postnatal protein–energy malnutrition has marked effects on brain cell differentiation (Cravioto & Cravioto, 1996), and long-term depletion of the *n*-3 PUFA DHA induced altered brain cell levels of monoamines and monoamine receptors in rats. When neurons have migrated to their destination and reached their differentiation, they grow and form synaptic connections (Kandel *et al.* 1995). Most cell growth in the human cerebellar cortex occurs between 32 weeks of gestation and 11 months after term birth (Rakic, 1995). Synaptogenesis in primates peaks in the first 2–3 months of life (Rakic, 1995), and the number of synapses in the human visual cortex increases twofold between the ages of 2 and 8 months after birth, which is paralleled by a marked increase

in the number of neurotransmitter receptors (Huttenlocher *et al.* 1982). The number of synapses formed is far greater than the number of synapses later found in adult brains. The early rapid synaptogenesis depends on an extensive formation of highly fluid cell membranes and, hence, on sufficient availability of substrates required for this membrane formation. Brain structure and function is also greatly influenced by apoptosis, since cell death affects between 20 and 80 % of the neuronal cells formed during gestation before the time of birth (Rosenzweig *et al.* 1996), which appears to be regulated by genetically programmed apoptosis, protecting neurotrophic factors, synaptogenesis, hormones and eicosanoids. In the surviving cells, the synaptic connections are reduced and rearranged at a rapid rate initially, but continuing throughout the life of the organism in relation to stimulation and learning. This synaptic rearrangement appears to be regulated by many different modulators, including Ca channels, second messengers such as phosphatidyl inositol, protein kinases, membrane lipids and eicosanoid hormones (Reisbick, 1996; Wainwright, 1997).

9.3. Nutrition and neural development

In Europe and other developed countries, an associated secular increase both of adult height and adult intelligence quotients has been observed over the last few decades, and the hypothesis has been raised that both these effects are related to the early nutrient supply.

Several studies have documented that severe general malnutrition in infancy or early childhood, which is characterized by a combined deficiency of energy, protein and many other substrates, is associated with a marked reduction of cognitive ability (Cravioto & Cravioto, 1996; Kretchmer *et al.* 1996). However, the adverse effects on neural development cannot be attributed entirely to nutrient depletion, but may also be modulated by other factors typically associated with severe childhood malnutrition, including infections, poverty, psychological depression and lack of adequate stimulation. Evidence of organic effects of malnutrition on brain growth and maturation was provided by the demonstration of cerebral atrophy and lasting organic brain damage (Ambrosius, 1966; Stoch *et al.* 1982; Houscham & Devilliers, 1987) and by electrophysiological evidence of impaired information processing, such as altered auditory evoked brain stem potentials (Barnett *et al.* 1978; Bartel *et al.* 1986).

9.3.1. Protein. Randomized studies in premature infants demonstrated that a low protein intake during the early postnatal period resulted in poorer results of orientation, habituation and stability clusters when tested with the neonatal behaviour assessment scale (Bhatia *et al.* 1991), and in a markedly reduced mental and psychomotor development when assessed at an age of 18 months postterm with the Bayley scales of infant development (Morley & Lucas, 1993).

9.3.2. Iodine. Today I deficiency is considered the most common cause of nongenetic inborn neurological damage on a worldwide basis, and causes cretinism with severe mental retardation (Stanbury, 1994). In I-deficient populations, an early I supply beginning before or at the time of conception can prevent neural damage of the infant, whereas a later start of I supply in the second or third trimester of pregnancy or after birth is associated with a smaller preventive effect (Xue-Yi *et al.* 1994).

9.3.3. Iron. Fe uptake into the brain is mediated by transferrin receptors on the endothelial surface of brain microvasculature, reaches its maximum during the period of rapid brain growth and peak myelinogenesis (Taylor & Morgan, 1990) and continues throughout life. Analysis of Fe distribution in the human brain during childhood with magnetic resonance imaging showed the highest concentrations in the globus pallidus, caudate nucleus, putamen and substancia nigra, while the cortex and cerebellum had substantially lower contents. Fe serves as an essential cofactor in a variety of cellular and metabolic functions, including the synthesis of dopamine, serotonin, catecholamines and possibly γ-aminobutyric acid as well as myelin formation (Kretchmer *et al.* 1996), while Fe overload has toxic effects. In young rats deprived of Fe in early postnatal life, total brain Fe content was severely depleted to 27 % of that of controls and was resistant to later restoration despite aggressive treatment (Dallman & Spirito, 1977). Sustained early Fe deficiency in rats causes persistent behavioural and learning deficits, leading to the hypothesis that Fe sufficiency throughout the critical phases of early brain development is crucial to the achievement of normal brain Fe content, function and behaviour. Several studies in children have clearly documented that severe Fe depletion resulting in Fe-deficiency anaemia results in a poor attention span, poor performance in the Bayley mental development index, low intelligence scores, some degree of perceptual disturbance and altered affective behaviour (Lozoff & Brittenham, 1986; Beard *et al.* 1993; Pollitt, 1993; Sheard, 1994; Kretchmer *et al.* 1996). Although children with Fe depletion without anaemia also showed behavioural abnormalities in some studies, these abnormalities appear to have been influenced by poor environmental conditions that were associated with the occurrence of Fe depletion (Lozoff *et al.* 1996). At this time there is no conclusive evidence that poor Fe status without anaemia has adverse effects on neural development in children.

9.3.4. Zinc. In animal experiments, Zn deprivation was shown to adversely affect brain growth, learning ability, memory and activity (Smart, 1974; Halas & Sanstead, 1975, 1980; Peters, 1979). Low-birth-weight infants showed an improvement of motor development when supplemented with Zn (Friel *et al.* 1993). In a recent study in Indian children aged 6–35 months who were not malnourished, daily supplementation with 10 mg elemental Zn (as Zn gluconate) for a period of 6 months resulted in a significant increase of observed activity (Sazawal *et al.* 1996).

9.3.5. Polyunsaturated fatty acids. Some 50–60 % of the structural matter in the central nervous system is composed of lipids, which almost entirely serve structural functions in cell membranes and myelin. Much of the rapid lipid accretion during brain growth comprises lipids that can be synthesized *de novo* in the fetus and infant, e.g. cholesterol. In addition, the rapid brain growth occurring perinatally and the extensive synaptogenesis occurring during the first months of life require the incorporation of relatively large amounts of essential PUFA, primarily the highly unsaturated long-chain PUFA DHA and arachidonic

acid (Koletzko, 1992). In experimental studies, the addition of DHA and arachidonic acid to fetal mouse brain cultures increased the number, diversity and complexity of synaptic contacts (Tixier-Vidal *et al.* 1986). In addition to cell growth and synaptogenesis, long-chain PUFA as well as eicosanoids formed from long-chain PUFA may influence neural cell apoptosis (Finstad *et al.* 1994; Wainwright, 1997). Experimental studies in rodents and in non-human primates indicated that the degree of long-chain PUFA incorporation into the developing brain influenced reflex development, memory, discrimination learning, retinal function and visual acuity (Neuringer, 1993; Wainwright, 1993), i.e. functions related to the efficacy of information processing.

The human fetal supply with preformed long-chain PUFA by a materno-fetal placental transfer (Koletzko & Müller, 1990) may be influenced by the maternal dietary long-chain PUFA intake. Also, the postnatal long-chain PUFA supply in human milk is affected by maternal diet and can be altered by dietary supplements provided to lactating women (Harris *et al.* 1984; Koletzko *et al.* 1992). In contrast to human milk, most infant formulas do not contain preformed DHA and arachidonic acid. Although newborn infants have the ability to synthesize long-chain PUFA from EFA precursors, the rate of synthesis appears to be low (Demmelmair *et al.* 1995; Sauerwald *et al.* 1997) and long-chain PUFA levels in blood lipids (Decsi & Koletzko, 1995) and brain (Farquharson *et al.* 1992; Makrides *et al.* 1994) of formula-fed infants are significantly lower than those found in breast-fed infants.

Some randomized intervention studies comparing diets without and with a supply of preformed long-chain PUFA both in premature infants (Uauy *et al.* 1990; Carlson & Werkman, 1996) and in healthy term infants (Makrides *et al.* 1993, 1995; Agostoni *et al.* 1995) found indications of improved retinal and visual function and of cognitive development in infants receiving long-chain PUFA, while other studies found no appreciable advantage (Innis *et al.* 1996). Effects of supplementing additional long-chain PUFA during pregnancy or lactation on functional development of the infant have not been reported.

9.4. Early nutrition and development of taste preferences

Preferences of taste and smell are critical factors in selecting foods and drinks throughout life. There are some indications that sensory perception of tastes and flavours may be modulated by early exposure.

Observations of fetal swallowing following the ingestion of sweet- and bitter-tasting substances into the amniotic fluid suggest that the fetus is sensitive to sweet- and bitter-tasting substances (de Snoo, 1937; Liley, 1972). It has been concluded that both the olfactory apparatus and taste perception are fully developed *in utero* (Beauchamp *et al.* 1991). Molecules carrying flavours and aromas may cross the placenta, and it has been proposed that early sensory exposure may modulate the acquisition of later flavour preferences. In animal studies, such effects of intrauterine exposure to flavour-rich foods on postnatal food choices have been documented.

Premature infants tested between 33 and 40 weeks post-conception and newborns during the first hours after birth show a clear and reproducible preference for sweet tastes (Beauchamp & Mennella, 1980). In blinded controlled studies, the sucking behaviour of breast-fed infants is altered by supplementing their mothers with encapsulated garlic compared with placebo, and repeated consumption of garlic modifies the infantile response to this taste (Menella & Beauchamp, 1993). Also the volatile flavours of vanilla and alcohol modulate infantile sucking behaviour (Beauchamp & Mennella, 1980; Mennella & Beauchamp, 1991). The question of to what extent food choices during childhood and adult life are modulated by pre- and postnatal experience is of major importance and needs to be further explored.

9.5. Methodological aspects

An optimization of the quality of nutrient intakes during pregnancy, lactation and infancy may well have the potential of improving developmental outcomes in the recipient infants. Since even small improvements of the population means by such potential effects are of major public health significance, they need to be carefully examined. If plausible hypotheses can be raised and supported by data from experimental models, epidemiological studies or pilot intervention trials, they should be subjected to rigorous testing with adequate scientific methodology in double-blind placebo-controlled randomized trials. While some electrophysiological measures of human neural function can be assessed with sufficient precision to justify interventions in small groups, behavioural methods tend to have a greater degree of variation and, therefore, effects on such outcome variables require large studies, particularly if one considers that dietary factors must be expected to have relatively small effects compared with genetic and other environmental influences. The results of such studies can be greatly influenced by the study design and factors such as the time points chosen for testing effects as well as the adequacy of the method chosen for testing the targeted effect. Large sample sizes may be required, for example, to show an effect of a dietary supplement, resulting in a comparably small mean difference of developmental scores due to the many other variables that influence such end-points. Therefore, the realization of adequate trials to test for long-term developmental effects of early food choices will usually require a large budget. In view of the major public health importance of the questions addressed by such trials, it appears justified that well-designed trials addressing relevant and pertinent questions are supported by public funds.

10. Production of bioactive factors for inclusion into food products

Interest in the production of human milk proteins, peptides, growth factors and other bioactive substances with the use of biotechnology and recombinant techniques is growing. The inclusion of such substances into dietary products may have beneficial physiological effects particularly in infancy and early childhood, for example defence against infectious agents, the optimization of nutrient uptake from the diet as well as the differentiation and growth of cells and tissues. Micro-organisms and transgenic animals can now be used

for the production of bioactive proteins (Lönnerdal, 1996). However, the benefits and safety of each substance must be evaluated in adequate studies in cells, animal models and clinical studies before routinely adding them to products for infants to improve their nutrition, health or development. Proper manufacturing conditions must be developed for introducing such substances into foods. The importance of post-translational modifications must also be taken into account. Some proteins may require proper glycosylation or phosphorylation for physiological activity.

Several human milk proteins have been cloned and sequenced. The majority were cloned at the level of complementary DNA (cDNA). In a few cases the entire gene has been characterized. One of the first milk proteins which was cloned from a mammary gland library was α-lactalbumin (Hall *et al.* 1987). Other human milk proteins that have been cloned include lysozyme, lactoferrin, human β-casein and κ-casein. For the production of human milk proteins and bioactive factors, several expression systems can be used, such as bacterial expression of recombinant proteins. The expression vector can be constructed so that the protein is made available in the supernatant fraction or in the bacteria. Bacterial expression will lead to the production of proteins which are not phosphorylated or glycosylated. If this property is needed for biological functions, yeast or fungi can be used as expression systems. *Saccharomyces cerevisiae* was used to produce human lactoferrin (Liang & Richardson, 1993) and human β-caseins. *Aspergillus nidulans* and *Aspergillus oryzae* were also used to produce human lactoferrin (Ward *et al.* 1992*a*,*b*). Alternatively, living cells such as baby hamster kidney cell can be used to produce human lactoferrin (Ward *et al.* 1992*a*). As such expression systems will still provide proteins with different phosphorylation and glycosylation, physiological function may be altered. Tissue-specific expression of human milk proteins in transgenic animals should result in recombinant protein glycosylations and phosphorylation similar to the native human milk proteins.

The architecture of the transgene DNA that is introduced into the germline of animals by microinjection plays an important role in the level of expression of the transgene. DNA that is introduced by microinjection into the pronucleus is usually inserted randomly into the genome as head-to-tail concatemers. Because the eukaryotic genome is organized into topologically constrained domains, random integration can lead to position effects in which the transgene expression is influenced by the surrounding chromosomal sequences. Thus in many cases the level of transgene expression will vary over several logarithms, depending on the site of integration, and expression may be observed in <50% of the positive transgenic mice. Because of the expense and time required to generate transgenic livestock, this presents a major problem (Stowell *et al.* 1991). To date, human lactoferrin (Rosen *et al.* 1996) and human lysozyme (Kim *et al.* 1994) have been expressed in transgenic mice with reasonable expression in the milk. Human lactoferrin was recently produced in transgenic cows (Maga *et al.* 1994). Whether the glycosylation pattern of human lactoferrin within the cow's mammary cells will alter the biological function remains to be elucidated.

Similar to the gene farming of human milk proteins and other important functional proteins in transgenic animals, such bioreactors are used to produce human hormones and growth factors. In transgenic rabbits, hGH was produced in milk in suitable amounts without being affected by the transgene expression (Limonta *et al.* 1995). Bovine growth hormone was also placed by a recombinant technique into the milk of transgenic mice (Thépot *et al.* 1995). In addition, human IGF1 was expressed in the milk of transgenic mice (Hadsell *et al.* 1996) and rabbits (Brem *et al.* 1994). Some oligosaccharides which may play an important role in promoting growth and differentiation of intestinal epithelial cells have successfully been expressed in the milk of transgenic mice (Prieto *et al.* 1995). The enormous potential of these methodologies for improving food products needs to be further explored.

11. Commentary on biomarkers

Drawing on experience from child health and development it is possible to use experience of inborn errors of metabolism to demonstrate the evolution and use of biomarkers to detect and predict a disease arising from an imbalance between systemic homeostasis and essential and non-essential dietary components. A good example is hyperphenylalaninaemia (HPA) which arises from an inborn error of metabolism of the essential amino acid phenylalanine (Scriver & Clow, 1980; Güttler, 1984). First of all, however, it is helpful to consider some general aspects of the strategy of biomarkers.

Biomarkers can be defined as indicators of actual or possible changes of systemic, organ, tissue, cellular and sub-cellular structural and functional integrity which can be used, either singly or in batteries, to monitor health and exposure to compounds in populations and individuals: as such they are the essence of chemical pathology or clinical biochemistry. Thus although the term 'biomarker' might itself be relatively novel, the concept is far from being so: the use of biochemical biomarkers dates at least from the discriminatory use of the sweet taste of glycosuria to diagnose and name diabetes mellitus. This intolerance of glucose illustrates a disturbance of the usually tightly controlled internal milieu as conceived by Claude Bernard, and of the processes involved in maintaining this state, i.e. homeostasis. These mechanisms lie at the core of the regulatory adaptations which maintain a steady state in the face of changes in environment, including our interaction with desirable and non-desirable components of the diet, which, of course, is one of our most intimate interactions with the environment. Homeostasis is regulated by cellular and systemic mechanisms which, in turn, are dependent on gene-mediated responses. Genetic heterogeneity amongst people underlies the intrapopulation variability of the interaction between nurture and nature. These generalizations apply equally to exposure to abnormal compounds and to excessive or inadequate exposure to normal components in the diet or environment.

Homeostasis comprises several processes, some of which act synchronously, but many of which act in a specific sequence, each being triggered by the relative efficiency of the preceding process. Some of these mechanisms are

specific for individual compounds. This applies to essential nutrients or generic groups of nutrients, but for non-nutrients and xenobiotics there appears to be a limited number of common protective mechanisms. In the chain arising from threatened or actual toxic exposure there would first be compensatory adaptations such as metabolic biotransformations (phase I: oxidation, hydrolysis, reduction; phase II: conjugations), excretion, and sequestration of compounds (for example in adipose tissue, bone, hair, skin); when these are inadequate biochemical and histopathological features follow with structural cellular damage and functional derangement. These lead to tissue damage with clinical manifestations (e.g. neurological and muscular toxicity, retinal toxicity, immunotoxicity, hepatotoxicity, nephrotoxicity, bone marrow damage, teratogenicity, cytotoxicity, cancer, methaemoglobinaemia, haemolysis) and overt disease states many of which are major health issues (e.g. cancers, cardiovascular disease, obesity, osteoporosis, adverse reactions to foods and food additives and contaminants, atopic disease, behavioural abnormalities, fetotoxic effects). This chain shows the series of genotypic (genetic), and phenotypic (biochemical and clinical) biomarkers.

The underlying assumption here is that all diet-related disease arises from an inappropriate interaction between diet and systemic homeostasis and that the fundamental basis of this imbalance lies in the relative imbalance of dietary exposure and the genetic controlled response. At this level the genome and its product would serve as an ideal biomarker: unfortunately the processes involved might not have been identified or might not be accessible to practical and ethical sampling techniques. Alternative and less immediate biomarkers have to be used. However, the more remote the biomarker is from the primary event the more it is attenuated and subject to confounding factors. It becomes less specific. On the other hand the more immediate it is to the basic interaction the more quantitatively related and predictive it becomes: biomarkers represent Garrod's concept that genetic factors determine the nature of chemical metabolism and human biochemical heterogeneity.

More immediate biomarkers are not only potentially more predictive, they might also provide more immediate outcomes which could be used to assess interventions in a reasonable timescale and, in turn, replace the temporally and aetiologically remote outcomes which are so often the foci of epidemiological studies of diet and health: for example the diseases mentioned earlier are probably both metabolically and temporally remote from their aetiology.

Insight into the metabolic processes involved in the particular issue being investigated can inform the choice of tissue or fluid to be sampled and the phenotypic marker to be measured. This applies also to genotypic biomarkers designed to assess adaptive phenomena but it is possible to measure some predictive genotypic markers in tissues other than those in which the gene product is expressed. Whatever the situation the selection of biomarker(s) should be dictated by the problem being considered rather than by the accessibility of tissue or fluid to be measured or the ease of an assay.

These general points are represented by the disease PKU which epitomizes the interaction between nature and nurture and the benefits which can be attained by an appropriate manipulation of the diet. PKU is a manifestation of HPA which results from the altered activity of the principally hepatic enzyme phenylalanine hydroxylase (*EC* 1.14.16.1; PAH), functional defects of which arise from intrinsic defects in the apoenzyme or from defective synthesis of its cofactor (tetrahydrobiopterin) (Scriver & Clow, 1980; Güttler, 1984; Scriver, 1991; Scriver *et al.* 1996; Güttler & Guldberg, 1996). The condition was recognized in 1933 when a mother with a mentally retarded child sought help on the peculiar smell of her child's urine. She saw several doctors, one of whom even referred her to a psychiatrist for help with her delusion. In the end one physician, Dr Asbjorn Folling confirmed the peculiar smell. He attributed it to the presence of phenylpyruvic acid in the urine and he called the condition 'imbecillitas phenylpyruvica' thereby incorporating both the initial clinical and biochemical biomarkers into the condition's nomenclature (Güttler, 1984; Scriver, 1991). He developed the '$FeCl_3$' colorimetric test of urine to detect the excess metabolite and used it to screen mentally retarded children and adults for the condition (Scriver & Clow, 1980). In 1950 Horst Bickel appreciated the implication of raised blood phenylalanine concentrations in PKU patients and explored the use of a phenylalanine-free mixture of amino acids to feed affected infants. It was found that blood phenylalanine levels fell and that the urine abnormality decreased. There was also some clinical and behavioural improvement, but the intervention was too late to affect the established developmental delay and mental damage. Nonetheless this progress in the phenotypic biomarkers demonstrated the potential clinical and economic benefits of screening and early diagnosis in the hope that early dietary intervention could prevent the neurological damage.

Early dietary management was effective, but many cases went undetected because the $FeCl_3$ biomarker was relatively insensitive and non-specific (Scriver & Clow, 1980). Phenylpyruvic acid is one of a number of normal metabolites of phenylalanine which are found in excessive amounts in the urine in PKU as a consequence of the increased activity of alternative pathways for the metabolism of phenylalanine. Other variables affected the production of these metabolites and it was appreciated that blood phenylalanine concentrations would be a better biomarker, but the analytical techniques (paper chromatography) available at that time were costly and time consuming. In the late 1950s the abnormal gene product, i.e. defective PAH, was identified, but since this activity was in the liver this did not provide an effective biomarker. The finding of this functional defect reinforced the opinion that screening should be targeted at the HPA.

Guthrie solved the problem in 1961 by establishing a semiquantitative bioassay based on the inhibition of bacterial growth by high concentrations of phenylalanine. The test was done on blood drops collected on filter card from infants at 4–7 d of age by which time the babies would have fed and, in contrast to *in utero*, would have had the opportunity to challenge the activity of their endogenous PAH with a phenylalanine load. The biomarker for definitive diagnosis and monitoring of management was direct quantitative measurement of blood phenylalanine concentrations. This enabled an appreciation that these concentrations could correlate with the efficiency of management and with behavioural and other clinical outcomes. However, not all

patients responded similarly or predictably. Amongst this heterogeneity was an atypical group with HPA which was, in the mid 1970s, found to be secondary to a defect in the synthesis of the tetrahydrobiopterin cofactor. Thus, in the midst of the phenotypic and molecular heterogeneity which was becoming increasingly obvious, it was realized that PKU or HPA was not the result of a single gene defect. The allele for PAH has since been found on chromosome 12q, and that for the affected stage in tetrahydrobiopterin synthesis is on chromosome 4 (Güttler & Guldberg, 1996; Scriver *et al.* 1996).

HPA is, thus, evident as a complex entity with many clinical and metabolic phenotypes. Consistent with this heterogeneity and with the variation in dietary tolerance for phenylalanine, almost 300 mutations in the PAH gene have been identified. Some of these genetic mutations have been specifically correlated with the activity of their enzyme products and with the severity of the clinical disease (Scriver, 1991; Güttler & Guldberg, 1996; Scriver *et al.* 1996).

In the context of biomarkers, HPA represents a continuum of genotypic, biochemical (PAH, tetrahydrobiopterin defect), metabolic (HPA), and clinical (the PKU syndrome) phenotypic biomarkers of an abnormal interaction between a dietary component and an individual's ability to achieve effective homeostasis at customary dietary exposure.

The genotypic biomarker may be used for definitive diagnosis and family screening and possibly for prognostication, but the metabolic biomarker is most useful for monitoring and tailoring the dietary reduction of phenylalanine. The urinary biomarker (PKU) is no longer of much use, and the clinical phenotypic biomarker is one of a medical tragedy.

PKU and HPA demonstrate the movement of biomarkers from remote and non-specific outcomes (many other conditions including other inborn errors of metabolism cause epilepsy and developmental delay) to more specific and informative outcomes, and ultimately to the basic genomic mutation offering the opportunity of appropriately designed diets. It is feasible that in due course a similar heterogeneity will be found in the metabolism of other nutrients, and that this will explain many of the conflicting phenomena found in epidemiological studies of the interaction between diet and health and in apparent heterogeneity of nutritional requirements. Such biomarkers should also provide more definitive indicators of response to interventions, thereby enabling shorter and more definitive epidemiological studies. Whether or not people would accept the corollary of this, namely specific diets tailored to their genotypic or surrogate metabolic characteristics, depends probably on the nature of the diets involved and on the people's motivation in the context of the much less dramatic and immediate effects of an inappropriate diet compared with that experienced with PKU. The integration of markers of susceptibility with appropriate follow up of long-term outcomes by epidemiological studies of characterized populations will demonstrate the relevance or otherwise of formal interventions.

12. Conclusions

Food supply and the metabolism of food ingredients in women during pregnancy and lactation and in their children have implications for long-term health and child development. Epidemiological evidence and studies performed in infants have highlighted the fact that maternal and intrauterine influences are of special importance during the development of the infant and child. Early nutrition modulates growth and functional development of the organism and appears to exert life-long programming effects that modulate health, disease and mortality risks in adulthood, neural function and behaviour, and quality of life.

The field of nutrient–gene interaction is in a phase of rapid expansion and development. There are several areas where dietary modulation of gene expression could exert beneficial effects, for example with respect to lipid metabolism and risk of cardiovascular disease. Applied research should further elucidate the interaction of nutrients and gene expression. The genes affected by specific nutrients, e.g. amino acids, must be characterized in animal models as well as the underlying cellular and molecular mechanisms. Since the vast majority of studies have been performed on animal models or animal cell lines, strategies must be defined to approach these questions in human subjects, for example by using human cell lines responding to nutrients. Once the mechanisms and relevance to man have been clarified, then development and testing of existing or new 'functional foods' could be performed both in animals and human subjects.

The relation between nutrients and differentiation needs to be further explored by *in vivo* models, studying food effects on cell differentiation and later performance. Concomitantly, the effects of nutrients on cell differentiation in *in vitro* studies should be strongly encouraged.

The course of pregnancy, childbirth and lactation as well as human milk composition and the short- and long-term outcome of the child may be influenced by the intake of foods and particularly micronutrients, e.g. PUFA, Fe, Zn and I. Folic acid supplementation from before conception through the first weeks of pregnancy was reported to markedly reduce the occurrence rates of severe embryonic malformations, including anencephaly and spina bifida. The potential of exerting beneficial effects for mother and child by modulating maternal nutrient supply should be further explored.

The evaluation of dietary effects on child growth requires epidemiological and field studies as well as evaluation of specific cell and tissue growth. Novel substrates, growth factors and conditionally essential nutrients (e.g. growth factors, amino acids, unsaturated fatty acids) may be potentially useful as ingredients in functional foods and need to be assessed carefully with respect to their potential effects on growth, maturation and development of specific cell types, tissues and organs under different physiological conditions. In particular, the potential modulation of later obesity by early food choices needs to be further explored.

Intestinal growth, maturation, intestinal adaptation and regulation and long-term function may be influenced by food ingredients. The roles of compounds such as dietary oligosaccharides, gangliosides, high-molecular-mass glycoproteins, bile salt-activated lipase, pre- and probiotics as to their physiological functions in the developing organism need to be further explored. The interaction of the appropriate genes of intestinal substrate transporters and their

substrates in early childhood is not well understood and needs to be clarified.

There are indications for some beneficial effects of functional foods on the developing immune response *in vitro* and *in vivo*, for example induced by antioxidant vitamins, trace elements, fatty acids, arginine, nucleotides, and altered antigen content in infant foods. A general unresolved issue is related to the lack of reliable surrogate outcome markers when investigating the effects of nutrition on the developing immune response and possible long-term benefits.

Peak bone mass at the end of adolescence can be increased by dietary means, which is expected to be of long-term importance for the prevention of osteoporosis at older ages. Future studies should be directed to the combined effects of Ca and other constituents of growing bone, such as P, Mg and Zn, as well as vitamins D and K, and trace elements F and B. In addition to observational and intervention studies, *in vitro* and animal model studies might provide a basis for new concepts on the interaction and optimal relative proportions of the several macro- and micronutrients involved in bone growth and mineralization.

Pregnancy and the first postnatal months are critical time periods for the growth and development of the human nervous system, processes for which adequate substrate supplies are essential. Early diet may have long-term effects on the structure and function of the nervous system, sensory and cognitive abilities as well as behaviour. The potential beneficial effects of a balanced supply of nutrients such as I, Fe, Zn and PUFA need to be explored in further detail.

The question of a possible relationship between early exposure to tastes and flavours and later food choice preferences may have a major impact on public health and needs to be further elucidated.

Bioactive factors such as human milk proteins, peptides, growth factors and other substances may be produced for use in food products with the use of biotechnology and recombinant techniques. The inclusion of such substances into dietary products may have beneficial physiological effects particularly in infancy and early childhood, for example defence against infectious agents, the optimization of nutrient uptake from the diet as well as the differentiation and growth of cells and tissues. The enormous potential of these methodologies for improving food products needs to be further explored.

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