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Report of the Scientific Committee on Food on the Revision of Essential Requirements of Infant Formulae and Follow-on Formulae

(adopted on 4 April 2003)

Contains a corrigendum made on 17/09/2007 on page 60, chapter 4.7.2 Carnitine

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LIST OF ABBREVIATIONS

AA	arachidonic acid
ADI	acceptable daily intake
ALA	α-linolenic acid
AMP	adenosine 5'-monophosphate
ATP	adenosine triphosphate
BSSL	Bile salt stimulated lipase
CFM	cereal-fruit meal
CFR	code of federal regulations (USA)
CFU	colony forming units
CLA	conjugated linoleic acid
CMP	cytidine 5'-monophosphate
DBP	vitamin D binding protein
DHA	docosahexaenoic acid
DHGLA	dihomo-γ-linolenic acid
DNA	deoxyribonucleic acid
ECF	extra-cellular fluid
EGRAC	erythrocyte glutathione reductase activity
EPA	eicosapentaenoic acid
EU	European Union
FA	fatty acids
FAD	flavin adenine dinucleotide
FMN	flavin mononucleotide
FNB	Food and Nutrition Board
GMP	guanosine 5'-monophosphate
GRAS	generally recognized as safe
HDL	high-density lipoprotein
HiB	*Haemophilus influenzae* type B
ID	iron deficiency
IDA	deficiency anaemia
IMP	inosine 5'-monophosphate
LA	linoleic acid
LCPUFA	long-chain polyunsaturated fatty acids
LCT	long-chain triglycerides
LDL	low-density lipoprotein
LSRO	Life Sciences Research Office
MCM	milk-cereal meal
MCT	medium-chain triglycerides
MCV	medium corpuscular volume
MM	molecular mass
MRI	magnetic resonance imaging
MUFA	monounsaturated fatty acids
NAD	nicotine adenine dinucleotide
NE	niacin equivalents
NPN	non-protein nitrogen
NPR	net protein ratio
NPU	net protein utilisation
PC	phosphatidyl choline
PE	phosphatidyl ethanolamine
PER	protein efficiency ratio

PGA	pteroyl glutamic acid
PI	phosphatidyl inositol
PRI	population reference intake
PS	phosphatidyl serine
PTH	parathyroid hormone
PUFA	polyunsaturated fatty acids
RAAR	relative amino acid rating
RAST	radio allergen sorbent test
RDA	recommended dietary allowances
RE	retinol equivalents
RNA	ribonucleic acid
RNIs	reference nutrient intakes
SAFA	saturated fatty acids
SAM	S-adenosyl methionine
SD	standard deviation
TE	tocopherol equivalents
TN	total nitrogen
TPD	true protein digestibility
UHT	ultra-high temperature
UL	tolerable upper intake level
UMP	uridine 5'-monophosphate
USA	United States of America
VLDL	very low-density lipoprotein
VMM	vegetable-meat meal
$1,25(OH)_2D$	1,25-dihydroxy-vitamin D
25(OH)D	25-hydroxy-vitamin D

TERMS OF REFERENCE AND BACKGROUND

Terms of Reference

The Committee was asked to revise the essential requirements of infant formulae and follow-on formulae intended for the feeding of infants and young children.

Background

The Community had adopted harmonised rules on the composition of infant formulae and follow-on formulae (Directive 91/321 EEC, amended by Directive 96/4/EC, hereinafter referred to as the Infant Formulae Directive) in 1991 and 1996. Such rules include essential requirements for these products. These essential requirements were based on a number of opinions of the Committee (SCF, 1983, 1989, 1991, 1993a, and 1995). It was considered necessary to review and update the scientific basis of the above Community legislation taking into account scientific and technical developments. In addition, an updated scientific basis was also expected to contribute to the ongoing process of the Codex Alimentarius in revising the existing Codex standard on infant formulae (CAC, 2002). In the context of its review, the Committee was also requested to address specifically the issue of nucleotide content and of the content of fructo-oligosaccharides (FOS) and galacto-oligosaccharides (GOS) in these products.

Acknowledgements

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I. INTRODUCTION AND GENERAL ASPECTS

1. GENERAL ASPECTS OF INFANT FEEDING

The adequate supply of nutrients through the provision of safe foodstuffs with a balanced composition is even more important for infants than for healthy children and adults during any other period of the life. The very rapid rate of growth of healthy infants born at full term, who double their weight in only 4-5 months after birth, results in relatively high requirements of energy and nutrients per kilogram body weight. In addition to meeting maintenance requirements, infants must cover the energy and substrate needs for the synthesis and deposition of newly formed tissues. The resulting large metabolic requirements contrast with the limited capacity of young infants to compensate for an unbalanced nutrient supply due to small body stores of nutrients and immature homeostatic mechanisms. For example, the activity of some metabolic pathways and the renal capacity to concentrate urine is still low in infants during the first 3-4 months of life (Koletzko, 2003; Wharton and Scott, 1996). Young infants may be unable to synthesize sufficient amounts of certain substrates otherwise considered non-essential or dispensable, because the limited capacity of specific metabolic synthesis pathways would not always suffice to match high requirements. Some substrates considered dispensable in adults or older children may become conditionally indispensable substrates for infants that should be supplied with the diet.

In addition to immediate consequences of infant feeding on growth, body composition, health and well-being, a number of recent studies have also provided indications that the quantity and quality of nutrient supply during infancy has important long-term consequences on organ development and function, health and disease risks as well as cognitive ability in later life. This phenomenon has been referred to as early metabolic programming or metabolic imprinting of long-term health and development (Barker, 1994; Lucas, 1998).

In consideration of the particular risk of infants to experience untoward effects by diets with providing either too low or too high supplies of specific substrates (Fomon, 2001), and the fact that during the first months after birth usually one sole milk source must meet all the infant's dietary requirements, special efforts are required to secure an adequate dietary composition for infants.

2. BREAST-FEEDING

The Committee strongly supports breast-feeding as the ideal way of feeding infants born at term, and it recommends that efforts are made to continue to promote and support breast-feeding in the European Union. In most cases breast-feeding provides an adequate supply of nutrients to support healthy growth and development of infants, it provides anti-infective protection, and it forms a unique biological and emotional basis for the health and well-being of both mother and child (WHO, 1986; Kunz *et al.*, 1999; Koletzko *et al.*, 2000). Comparative studies in affluent countries have indicated important advantages of breast-feeding over formula-feeding for the recipient infants, such as lower incidence rates of gastrointestinal and respiratory infections (Forsyth, 1995), reduced rates of later juvenile type diabetes (Hypponen *et al.*, 1999) and obesity (von Kries *et al.*, 1999; Toschke *et al.*, 2002), as well as better long-term cognitive achievements (Anderson *et al.*, 1999; Mortensen *et al.*, 2002).

Considering the many benefits of breast-feeding, the Committee fully supports the recommendations of the Fifty-Fourth World Health Assembly (2001) to promote and support exclusive breast-feeding for six months as a public health recommendation for populations. The Committee is also in agreement with the conclusions of the WHO expert consultation on the optimal duration of exclusive breast-feeding that some infants, for example infants with high requirements of specific nutrients such as iron, may benefit from the introduction of nutrient sources other than breast milk prior to the age of 6 months (WHO, 2001b; ESPGHAN, 2002).

3. INFANT FORMULAE AND FOLLOW-ON FORMULAE

Infants who cannot be fed at the breast, or should not receive breast milk, or for whom breast milk is not available, require breast milk substitutes of high quality (Thirty-Ninth World Health Assembly, 1986). Standards for such breast milk substitutes, namely infant formulae manufactured from cows' milk or soya, have been established in the European Union by the Infant Formulae Directive. This Directive was based upon the following documents: 1) First report on the essential requirements of infant formulae and follow-up milks based on cows' milk proteins (SCF, 1983); 2) The minimum requirements for soya-based infant formulae and follow-up milks (SCF, 1989); 3) First addendum to the aforementioned reports (SCF, 1991); 4) Report on infant formulae claimed to be "hypoallergenic" or "hypoantigenic", and Second addendum concerning the essential requirements of infant formulae and follow-up milks based on cows' milk proteins and the minimal requirements for soya-based infant formulae and follow-up milks (SCF, 1993a); and 5) Report on essential requirements for infant formulae and follow-on formulae (SCF, 1995).

According to the Infant Formulae Directive, "infant formulae" means foodstuffs intended for particular nutritional use by infants during the first four to six months of life and satisfying by themselves the nutritional requirements of this category of persons, whereas "follow-on formulae" means foodstuffs intended for particular nutritional use by infants aged over four months and young children and constituting the principal liquid element in a progressively diversified diet of this category of persons.

4. GENERAL PRINCIPLES ADOPTED IN THE COMMITTEE'S SCIENTIFIC REVIEW ON REQUIREMENTS OF INFANT FORMULAE AND FOLLOW-ON FORMULAE

The Committee adopted the following principles:

1. All infant formulae must be safe and meet the normal nutritional requirements of infants born at term when used as the sole source of nutrition during the first months of life. Infant formulae may also continue to be used during the later part of infancy as part of a progressively diversified diet.

2. All follow-on formulae must be safe and meet the normal nutritional requirements of generally healthy infants and young children, when used as the principal liquid element in a progressively diversified diet after the timely introduction of complementary foods.

3. The formulation of infant formulae and of follow-on formulae must be based on sound

medical and nutritional principles. Their use should have been demonstrated, by scientific evidence, to be safe and beneficial in meeting the particular nutritional requirements and to promote normal growth and development of the infants for whom they are intended (ESPGHAN, 2000; Koletzko *et al.*, 2002).

4. Although the composition of human milk of healthy, well-nourished women can provide some guidance for the composition of infant formulae and follow-on formulae, gross compositional similarity is not an adequate determinant or indicator of the safety and nutritional adequacy of dietary substitutes in infants (ESPGHAN, 2000; COMA, 1996). The composition of human milk is remarkable for its variability, as the content of many nutrients changes during lactation, or throughout the day, or differs among women (Kunz *et al.*, 1999; Rodriguez *et al.*, 1999). Moreover, there are considerable differences in the bioavailability and metabolic effects of similar contents of many specific nutrients in human milk and formulae, respectively. Thus, conclusions on the suitability and safety of nutrient contents in infant formula cannot be simply based on its similarity to human milk composition.

5. A more useful approach to evaluate the adequacy of infant formula composition is the comparison of physiological (e.g. growth patterns), biochemical (e.g. plasma markers) and functional (e.g. immune response) outcomes in infants fed formulae with those in populations of healthy infants who have been exclusively breast-fed for four to six months (ESPGHAN, 2001; COMA, 1996; Koletzko *et al.*, 2002).

6. Although the feeding of infant formulae and follow-on formulae has a history of apparently safe use, the outcomes of formula-fed infants are not equal to those of breast-fed populations (Koletzko *et al.*, 2000; Forsyth, 1995; Hypponen *et al.*, 1999; von Kries *et al.*, 1999; Toschke *et al.*, 2002; Anderson *et al.*, 1999; Mortensen *et al.*, 2002). Therefore, it is unreasonable to assume that the range of infant and follow-on formulae that have been in use so far would represent the optimal composition that might be achieved.

7. Infant formulae and follow-on formulae should only contain components in such amounts that serve a nutritional purpose or other benefit. A documented safety of components in particular amounts in adults or older children does not establish their safety in infants. The inclusion of unnecessary components may put a burden on metabolic and other physiologic functions of the infant. Those components taken in the diet which are not utilised or stored by the body have to be excreted, often as solutes in the urine. Since water available to form urine is limited and the infant's ability to concentrate urine is immature during the first months of life, the need to excrete any additional solutes will reduce the margin of safety, especially under conditions of stress, such as fever, diarrhoea or during weight loss.

8. The goal of establishing minimum and maximum values of nutrients is to provide safe and nutritionally adequate infant formula products that meet the normal nutritional requirements of healthy babies. Minimum and maximum values should be based on adequate scientific data that establish the needs for practically all infants in the target populations, and the absence of adverse effects (ESPGHAN, 1997). In the absence of an adequate scientific evaluation, minimum and maximum values should at least be based on an established history of apparently safe use. The establishment of minimum and maximum values also should take into account where possible other factors such as bioavailability and losses during processing and shelf-life. Minimum and maximum

values refer to total nutrient contents of infant formulae and follow-on formulae as prepared ready for consumption according to the instructions of the manufacturer. The Committee recognises the importance of the quality of the water that is used for reconstituting powdered formulae.

When establishing minimum and maximum values of nutrients per 100 kcal, with consideration of reference nutrient intakes established by the Committee (SCF, 1993b) and other scientific bodies, calculations are based on a reference infant with a weight of 5 kg consuming per day an amount of infant or follow-on formula with an energy content of 500 kcal (100 kcal/kg body weight per day).

9. The general considerations and the suggested values for minimum and maximum levels of nutrients described here for infant formulae and follow-on formulae may also serve as the basis for defining revised compositional criteria for foods for special medical purposes for infants, unless the disease conditions for which such foods for special medical purposes are to be used necessitate other compositional aspects.

10. As part of the review on the requirements of infant formulae and follow-on formulae, the Committee reviewed the scientific basis for the current European standards for the presentation of such products (Directive 91/321/EEC, Article 9) and recommends that some of these claims should be reconsidered (cf. chapter X). The Committee also recommends that mechanisms and criteria should be developed for the communication not only of relevant compositional properties, but possibly also of a limited number of selected other effects of infant formulae or follow-on formulae if they have been demonstrated beyond doubt in rigorous studies with adequate scientific standards, and the evidence has been accepted by an independent scientific body reviewing such data.

11. The Committee recommends that the addition of new ingredients to infant formulae or follow-on formulae, or of established ingredients in newly determined amounts, beyond the established standards on formula composition, should be made possible if their benefit, suitability and safety for particular use by infants have been established by generally accepted scientific data. The Committee notes the absence of clear guidance on the nature and extent of the information that should be submitted in support of the suitability and safety of modifications to be introduced. This makes it difficult for interested manufacturers to design and undertake studies for this purpose. Thereby, a real need to establish general guidelines is identified. As part of this report, the Committee proposes general principals for such evaluations (see chaper XI) and recommends that the scientific evidence to support modifications of infant formulae and follow-on formula beyond the established standards is overseen and evaluated by an independent scientific body prior to the introduction of such products to the market.

II. ENERGY CONTENT

1. CURRENT REQUIREMENTS CONCERNING MINIMUM AND MAXIMUM ENERGY CONTENT IN INFANT FORMULAE AND FOLLOW-ON FORMULAE MARKETED IN THE EU

The minimum and maximum energy contents of both infant and follow-on formulae, as described in the Infant Formulae Directive are given in Table 1. The minimum energy content is similar in infant and follow-on formulae, whereas the maximum content is slightly higher (by 6.7%) in follow-on formulae than in infant formulae.

Table 1. Energy content of infant formulae and follow-on formulae when reconstituted as instructed by the manufacturer

Energy	Infant Formulae		Follow-on Formulae	
	Minimum	Maximum	Minimum	Maximum
kJ/100 mL	250	315	250	335
kcal/100 mL	60	75	60	80

The values refer to the product ready for use

2. COMMENTS ON THE EXISTING REQUIREMENTS CONCERNING ENERGY IN INFANT FORMULAE AND FOLLOW-ON FORMULAE IN THE INFANT FORMULAE DIRECTIVE

It is nowadays recognised that the comparison of outcomes in infants fed dietary products with those observed in healthy infants who have been exclusively breast-fed for four to six months constitutes a much more appropriate method of investigation. For the reasons mentioned in chapter I, the breast-fed baby rather than the composition of breast milk is considered as the reference for infants artificially fed.

2.1 Energy density of human milk

It has been very recently shown that the energy density of human milk was lower than previously thought (Butte *et al.*, 2001). Butte *et al.* (2001) demonstrated that mean milk energy outputs measured during full lactation in 24 well-nourished women were similar (502-526 kcal/day). These values are 5-10% less than estimates used in the FAO/WHO/UNU (1985) recommendations and US Recommended Daily Allowances (RDA) (FNB, 1989), which assumed a milk energy concentration of 701 kcal/L and milk production rates of 819-848 mL/day and 750 mL/day, respectively. The values reported by Butte *et al.* (2001) are in accordance with an energy intake of 502 kcal/day for infants during the 4-6 months of life, based on an average volume of milk intake of 0.78 L/day (Heinig *et al.*, 1993a) and an average caloric density of human milk of 651 kcal/L (Nommsen *et al.*, 1991), as reported in the recently published US recommendations (FNB, 2002a).

2.2 Growth of breast-fed and formula-fed infants

It is well documented that the pattern of growth of formula-fed infants differs from that of breast-fed infants (Powers, 2001). The Euro-Growth study collected anthropometric data from 319 infants who were exclusively breast-fed according to the 1995 WHO recommendations for at least 4 to 5 months, and compared them to anthropometric data of infants who were fed by other modes (Haschke *et al.*, 2000). The pattern of growth of the former children showed higher weight during the first 2 to 3 months of life and lower weight and length from 6 to 12 months. Breast-fed infants tend to gain less weight and usually are leaner than are formula-fed infants in the second half of the first year of life (Dewey, 2001). The difference in average attained weight at 12 months is approximately 600 to 650 grams. Butte *et al.* (2000b) also clearly showed that intakes of energy, protein, fat, and carbohydrate were lower in breast-fed than in formula-fed infants at 3 and 6 months and were positively correlated with weight gain and Fat Free Mass (FFM) gain, but not with Fat Mass (FM) gain. No differences in nutrient intakes were observed at 12 and 24 months.

This difference of growth pattern between breast- and formula-fed infants seems to be the result of differences of composition between the two diets, but may be also due to differences in infant self-regulation of energy intake. There is evidence that breast-fed infants self-regulate their energy intake at a lower level than do formula-fed infants (Heinig *et al.*, 1993a). When solid foods are introduced, the breast milk intake declines spontaneously (Heinig *et al.*, 1993b). The mechanisms of self-regulation of energy intake in formula-fed infants are not known and the risk of overfeeding seems to be higher in this population. An important question is why breast-fed infants achieve a greater metabolic efficiency for the energy consumed than formula-fed infants. Body temperature and minimal observable metabolic rate are lower in breast-fed than in formula-fed infants, which may be part of the explanation (Dewey, 1998a).

Evidence to date suggests that there are no apparent adverse consequences associated with the lower intake and slower weight gain of breast-fed infants: compared to formula-fed infants, they do not differ in activity level, and they experience less illness and appear to have enhanced cognitive development (Anderson *et al.*, 1999; Angelsen *et al.*, 2001; Dewey, 1998a; Mortensen *et al.*, 2002; Rao *et al.*, 2002). It might be more appropriate to raise the question why formula-fed infants consume more energy than breast-fed infants. Butte *et al.* (1990) have suggested that the higher protein content of infant formula may alter the infant's hormonal status and drive higher energy intake. More information is needed on the balance between water and energy requirements. The Committee suggests that research in this area should be encouraged.

The comparison between breast-fed and formula-fed infants is much less consistent with regard to linear growth, with more than half of the published studies showing no difference by feeding mode (Dewey, 1998a). When differences in length gain were detected, the magnitude of the percentage gap between breast-fed and formula-fed infants was considerably less than the percentage gap in weight gain. Growth in head circumference is not related to feeding mode. New growth charts based on infants breast-fed throughout the first year of life are needed and are being developed by WHO (Dewey *et al.*, 1995; Dewey, 1998b; Garza and De Onis, 1999).

The Euro-Growth study also showed that between 12 and 36 months of age, anthropometric differences between infants breast-fed for at least 4 to 5 months and infants fed by other modes were small and clinically non relevant (Haschke *et al.*, 2000). At 24 months of age, the

219 children who had been fed according to WHO recommendations during infancy had lower length and weight (each by 0.12 z-score). This difference is not significant. Such a difference corresponds to a difference in length of approximately 3 millimetres and a difference of weight of 150 grams. These differences were smaller than the usual measurement errors for length (6 millimetres) and weight (239 grams) at that age. The US National Health and Nutrition Examination Survey (1988-1994) also showed that the differences in growth status observed at 8-11 months of age between breast- and formula-fed babies had dissipated by 12-23 months of age; there were no significant differences up to 5 years of age (Hediger *et al.*, 2000).

2.3 Energy requirements of infants

The energy requirements of infants and young children are the energy intakes that will balance Energy Expenditure (EE) at a Physical Activity Level (PAL) consistent with normal growth and development and allow for deposition of tissues at a rate consistent with health. In older children, adolescents and adults, energy requirements are based on Basal Metabolic Rates (BMR) and an allowance for PAL. Because it was not possible at that time to specify with any confidence the allowance for a desirable PAL in infants and young children, the 1985 FAO/WHO/UNU recommendations for the energy intake from birth to 10 years of age were derived from the observed intakes of healthy, thriving children (FAO/WHO, 1985). Energy requirements of infants were based on energy intakes compiled by Whitehead *et al.* (1981) from the literature predating 1940 and up to 1980; 5% was added to compensate for underestimation of intake. Implicit in this approach is the assumption that *ad libitum* intakes reflect desirable intakes. However, energy intakes are not inherently constant but are influenced by external factors. For instance, downward secular trends in the energy intakes of infants have been attributed to changes in breast-feeding rates, the formulation of infant formula, and the timing of food supplementation (Whitehead *et al.*, 1981).

Estimated average requirements of energy for European children aged 0-36 months were described by the Committee in 1993 (SCF, 1993b). They were derived from the 1985 FAO/WHO/UNU report, without the addition of the above-mentioned 5% increase for a possible underestimate of intakes from breast milk in the first year of life (Table 2).

Table 2. Estimated average requirements of energy for children aged 0-12 months (SCF, 1993b)

Age (months)	Average weight (kg)		Intake (kcal/kg body weight)	Estimated average energy requirements (kcal/day)	
	Boys	*Girls*		*Boys*	*Girls*
1	4.0	4.0	115	455	455
3	6.0	5.5	100	598	550
6	8.0	7.5	96	766	718
9	9.0	8.5	96	861	813
12	10.0	9.5	96	957	909

Recommendations for Estimated Energy Requirements (EER) for male and female infants from birth to one year of age have been published in 2002 by the US Food and Nutrition Board (FNB, 2002a) (Tables 3 and 4). These recommendations were based on the data of Butte *et al.* showing very clearly that the total daily energy expenditure in infants and the energy content of breast milk were lower than previously assumed (Butte, 1996; Butte, 2001;

Butte *et al.*, 2000a). These data are detailed above in section 2.1 (Energy density of human milk) and below in section 2.4 (Energy expenditure: comparison between breast-fed and formula-fed infants). Taking into account these important data, the Committee supports the use of the 2002 Food and Nutrition Board recommendations, which are more appropriate for determining the energy intake of formula-fed infants and the minimum and maximum energy content of infant and follow-on formulae than the 1993 SCF recommendations. With the exception of male infants at the age of 1 month, the EER for male and female infants published by the Food and Nutrition Board in 2002 are lower than the estimated average requirements published by the SCF in 1993. At 1, 3, 6, 9 and 12 months of age, the differences between 1993 SCF and 2002 FNB recommendations for male infants are as follows: - 3.6%; + 4.4%; + 15.6%; + 13.4%; +11.8%. At 1, 3, 6, 9 and 12 months of age, the differences between 1993 SCF and 2002 FNB recommendations for female infants are as follows: + 3.6%; + 5.3%; + 17.4%; +16.6%; +15.5%.

2.4 Energy expenditure: comparison between breast-fed and formula-fed infants

Deriving energy requirements on the basis of energy expenditure and deposition has become possible with available data on Total Daily Energy Expenditure (TDEE), growth and body composition (Prentice *et al.*, 1988) and has a better scientific basis than the observational approach. The opportunity now exists to compare current recommendations with measurements of TDEE obtained via the Doubly Labelled Water (DLW) technique. The DLW technique is now accepted as the best method available in infants up to 12 months of age since physical activity does not vary as markedly as in older children, adolescents and adults. The use of the DLW technique is strengthened given the limitations of available predictive equations for BMR, which are problematic since many of the underlying measurements were made under conditions such as sedation which influence BMR. Provided that the diet is appropriate to meet the infants' nutritional requirements, energy deposition, which is the synthetic energy cost of growth, can be derived from Total Body Fat (TBF) and FFM gains as measured by body composition assessment techniques.

Table 3. Estimated Energy Requirement (EER) for boys from birth to one year of age (FNB, 2002a)

Age (months)	Reference weight (kg)	TDEE (kcal/day)	Energy Deposition (kcal/day)	EER (kcal/day)
1	4.4	292	180	472
2	5.3	372	195	567
3	6.0	434	138	572
4	6.7	496	52	548
5	7.3	550	46	596
6	7.9	603	42	645
7	8.4	648	20	668
8	8.9	692	18	710
9	9.3	728	18	746
10	9.7	763	30	793
11	10.0	790	27	817
12	10.3	817	27	844

*TDEE = Total Daily Energy Expenditure

Several studies aimed at measuring TDEE and energy deposition have been recently published. They showed that TDEE of breast-fed infants is lower than TDEE of formula-fed infants. The mean percentage difference from formula-fed infants is as follows: 12% at 3 months, 7% at 6 months, 6% at 9 months, 3% at 12 months, and 1% at 18 months, respectively.

Table 4. Estimated Energy Requirement (EER) for girls from birth to one year of age (FNB, 2002a)

Age (months)	Reference weight (kg)	TDEE (kcal/day)	Energy Deposition (kcal/day)	EER (kcal/day)
1	4.2	274	164	438
2	4.9	336	164	500
3	5.5	389	132	521
4	6.1	443	65	508
5	6.7	496	57	553
6	7.2	541	52	593
7	7.7	585	23	608
8	8.1	621	22	643
9	8.5	656	22	678
10	8.9	692	25	717
11	9.2	719	23	742
12	9.5	745	23	768

*TDEE = Total Daily Energy Expenditure

De Bruin *et al.* (1998) investigated prospectively in 46 healthy, full-term infants the effect of at least 4 months of formula-feeding compared with exclusive breast-feeding on macronutrient and energy intake, TDEE, energy deposition, and growth. Metabolizable Energy Intake (MEI) was assessed from macronutrient intake by Test Weighing (MEI-TW) and from the sum of TDEE and energy deposition (MEI-Pred). At 1-2, 2-4, 4-8, and 8-12 months of age, MEI-Pred averaged 103±9, 94±8, 89±8, and 85±5 kcal/kg/day for boys, and 96±14, 90±6, 80±8, and 78±4 kcal/kg/day for girls, respectively. No significant difference between formula-fed and breast-fed infants was found with respect to weight, length, head circumference, TBF, FFM, and TDEE at all ages, or for gain in length, weight, TBF, and FFM. MEI-TW was significantly different between feeding groups at 1-4 months of age (formula-fed being greater than breast-fed, $p < 0.005$). This feeding effect, however, was not significant for MEI-Pred (kcal/day). MEI-TW differed from MEI-Pred only in breast-fed infants aged 1-4 months ($p < 0.05$ at 2-4 months). De Bruin *et al.* concluded from their study that energy requirements in infants are lower than the recommendations in guidelines currently in use.

Butte had also suggested in 1996 the need to revise energy requirements in infants and young children (Butte, 1996; Torun *et al.*, 1996). Current recommendations were considered too high for children aged less than 2 years. However, an unresolved issue pertinent to the revision of energy recommendations was whether differences in energy utilization observed between breast-fed (BF) and formula-fed (FF) infants in early infancy persist into the second year of life. TDEE, sleeping metabolic rate, anthropometry, and body composition were measured in 76 infants (Butte *et al.*, 2000a). Average total energy requirements were 533, 620, 711, 809, 890, and 993 kcal/day at 3, 6, 9, 12, 18 and 24 months, respectively. Energy

deposition (in kcal/day) decreased significantly over time (p=0.001) and was lower in BF than in FF infants (p=0.01). Energy requirements were ~80% of current recommendations (Davies, 1998). Energy requirements differed by age (p=0.001), feeding group (p=0.03), and sex (p=0.03). Adjusted for weight or fat-free mass and fat mass, energy requirements still differed by feeding group but not by age or sex. Mean National Center for Health Statistics weight-for-age and weight-for-length z-scores did not differ by feeding group (Hamill *et al.*, 1979).

2.5 Feeding in early childhood and later growth, and nutritional status

Recent data strongly suggest that the type of feeding and rate of growth during infancy and early childhood may play a role for an increased risk of overweight later in life. A pattern of rapid weight gain during the first 4 months of life seems to be associated with an increased risk of overweight status at age 7 years, independent of birth weight and weight attained at age 1 year (Stettler *et al.*, 2002). In a population survey of 848 full term singletons from a 10% random sample of the Avon longitudinal study of pregnancy and childhood, children who showed catch-up growth between zero and two years were fatter and had more central fat distribution at age five years than other children (Ong *et al.*, 2000). Moreover, infants who were fed breast milk more often than infant formula, or who were breast-fed for longer periods, had a lower risk of being overweight during older childhood and adolescence (Armstrong and Reilly, 2002; Butte, 2001; Gillman, 2001; Martorell *et al.*, 2001; Toschke *et al.*, 2002; von Kries, 1999). Early introduction of dairy products in young infants before the age of 2 months has also been shown to be associated with an increased risk of insulin dependent diabetes mellitus in later life (Virtanen *et al.*, 1993).

3. MINIMUM AND MAXIMUM ENERGY CONTENT IN INFANT FORMULAE AND FOLLOW-ON FORMULAE

The Committee proposes the following levels of energy contents of infant formulae and follow-on formulae:

- For infant formula a minimum of 60 kcal/100 mL (no change from the Infant Formulae Directive) and a maximum of 70 kcal/100 mL (instead of 75 kcal/100mL).

- For follow-on formula a minimum of 60 kcal/100 mL (no change from the Infant Formulae Directive) and a maximum of 70 kcal/100 mL (instead of 80 kcal/100 mL).

The Committee is not aware of data describing the actual energy intake from infant or follow-on formulae from birth to 7 months of age, in the presence or absence of complementary feeding from the 5^{th} to 7^{th} months of age. Therefore, a model calculation was performed to demonstrate the effect of changes in energy content of both infant and follow-on formulae on the energy intake in infants between 4 and 7 months of age. For this calculation the recommended feeding regimen for the first year of life was used, which has been developed by the German Research Institute for Child Nutrition (Kersting *et al.*, 1994). This has the advantage that the recipes of the complementary meals in addition to formula are fixed and their energy content has been estimated.

It has to be kept in mind that the model described by Kersting *et al.* (1994) is not the result of observational studies but has been designed to check whether the progressive introduction of complementary feeding recommended by the Research Institute of Child Nutrition at Dortmund was in accordance with the recommendations of the German Society of Nutrition.

The energy content of the complementary meals has been added to the energy content of the formula [minimum and maximum energy limits of the formula expressed in kcal/100 mL - as defined in the Infant Formulae Directive (present infant formula and present follow-on formula) and by the new proposal (proposed infant formula and follow-on formula) - multiplied by the amount of formula known to be consumed by the infant].

According to this regimen infants are fed exclusively on breast milk or formula for the first four to six months of life. A Vegetable Potato-Meat Meal (VPMM) is introduced at the earliest at the beginning of the 5th month, about four weeks later a Cow's Milk-Cereal Meal (CMCM) is added and again about one month later a Cereal-Fruit Meal (CFM) while breast milk (or formula) is continued in decreasing amounts. Infant formulae at present provide 60 to 75 kcal/100 mL whereas follow-on formulae provide 60 to 80 kcal/100 mL. Infant and follow-on formulae modified according to the new proposal would provide 60 to 70 kcal/100 mL.

In the absence of complementary food

During the 5th to 7th months of life an infant is assumed to consume 870 mL of formula per day. The recommended energy intake at that age is 670 to 770 kcal per day (SCF, 1993b), whereas the estimated energy requirements for boys ranges from 596 to 668 kcal/day and the estimated energy requirements for girls ranges from 553 to 608 kcal/day for girls, according to FNB (FNB, 2002a), respectively. The calculated energy intakes are shown in Table 5.

In the presence of complementary food

- During the 5th month of life at the earliest an infant is assumed to consume 600 mL of formula and a VPMM, which provides 200 kcal. The recommended energy intake at that age is 650 to 690 kcal/day for the SCF while the EER is 598 kcal/day for formula-fed boys and 553 kcal/day for formula-fed girls according to FNB. The calculated energy intakes are shown in Table 5.

- During the 6th month of life an infant is assumed to consume 400 mL of formula, a VPMM providing 194 kcal and a CMCM providing 216 kcal. The recommended energy intake is 720-760 kcal/day for the SCF while the EER is 645 kcal/day for formula-fed boys and 593 kcal/day for formula girls according to FNB. The calculated energy intakes are shown in Table 5.

- During the 7th month of life an infant is assumed to consume 240 mL of formula per day and a VPMM providing 225 kcal, a CMCM providing 216 kcal and a CFM providing 183 kcal. The recommended energy intake is between 770 and 810 kcal/day for the SCF while the EER is 668 kcal/day for formula-fed boys and 608 kcal/day for formula-fed girls according to FNB. The calculated energy intakes are shown in Table 5.

The minimum and maximum energy intake from the different types of formula (present infant formula, present follow-on formula and proposed infant formula and follow-on formula), with or without complementary feeding, have been compared to the estimated average requirements (SCF, 1993b) and to the EER (FNB, 2002a) (Table 6).

In the absence of complementary feeding, the new proposal of an upper energy limit of 70 kcal/100 mL leads to a decrease of the energy intake, representing 7% of the recommended

energy intake of the Committee as compared to the present infant formula.

Table 5. Energy intake (minimum-maximum) in kcal/day from the 5th to the 7th months of age, arising from formula alone [present infant formula (IF), present follow-on formula (FOF), proposed IF and FOF] or formula + complementary feeding

Age	Presence of complementary feeding	Type of formula	Energy intake from formula alone (minimum-maximum)	Energy intake from formula + complementary feeding (minimum-maximum)
5th-7th months	-	Present IF Proposed IF	522-653 522-609	- -
5th month	+	Present IF Present FOF Proposed IF and FOF	360-450 360-480 360-420	560-650 560-680 560-620
6th month	+	Present IF Present FOF Proposed IF and FOF	240-300 240-320 240-280	650-710 650-730 650-690
7th month	+	Present FOF Proposed IF and FOF	144-192 144-168	768-816 768-792

Table 6. Percentage of the estimated average energy requirements (SCF, 1993b) and the EER (FNB, 2002a) represented by the minimum and maximum energy intake from present infant formula (IF), present follow-on formula (FOF) and proposed IF and FOF, with or without complementary feeding.

Age	Presence of complementary feeding	Type of formula	Percentage of the estimated average energy requirements (SCF, 1993b)		Percentage of the EER (FNB, 2002a)			
			Minimum energy intake	Maximum energy intake	Minimum energy intake		Maximum energy intake	
					Boys	*Girls*	*Boys*	*Girls*
5th-7th months	-	Present IF Proposed IF	68-78	85-97 79-95	78-88	86-94	98-110 91-102	107-118 100-110
5th month	+	Present IF Present FOF Proposed IF and FOF	81-86	94-100 99-105 90-95	94	101	109 114 104	118 123 112
6th month	+	Present IF Present FOF Proposed IF and FOF	86-90	93-99 96-101 91-96	101	110	110 113 107	120 123 116
7th month	+	Present FOF Proposed IF and FOF	95-100	101-106 98-103	115	126	122 119	134 130

The decrease of energy intake represents 7 to 8% of the EER (FNB, 2002a) for both boys and girls as compared to the present infant formula.

It should be emphasised that an energy intake below the recommended intake does not mean that individually this intake is deficient and can be harmful for the subject. Moreover, while no change is proposed for the lower energy limit as compared to the Infant Formulae Directive, the model calculation leads to a low energy intake, representing a surprising 68-78% of the recommended energy intake of the SCF in the absence of complementary feeding,

whatever the formula (present and proposed infant formula). This could suggest an insufficient lower limit of energy content in formulae but is completely contradictory with the fact that infant formulae have been satisfactorily used for the growth and development of infants within the European Union from birth to one year of age since 1994, when the Infant Formulae Directive was implemented. The figure is higher when the lower energy intake is compared to the EER (FNB, 2002a): 78-88% for boys and 86-94% for girls, respectively.

The model calculation gives no matter of debate for the 7th month of life. While no change has been proposed for the lower energy limit, the model calculation leads again to a surprisingly low energy intake in infants receiving complementary feeding, representing 81-86% of the recommended energy intake of the SCF for the 5th month and 86-90% for the 6th month, whatever the formula (present infant formula, present follow-on formula and proposed infant and follow-on formulae). In comparison, the lower energy intake represents 94-101% of the EER (FNB, 2002a) for the 5th month and 101-110% for the 6th month, respectively.

This strongly suggests, as already discussed in section 3.4 (Energy expenditure), that the 1993 recommendations of the SCF for energy intake in infants from birth to one year of age are higher than the actual energy requirements of infants.

4. CONCLUSION AND RECOMMENDATION

Based on the reviewed data showing that the total daily energy expenditure in infants and the energy content of breast milk are lower than previously assumed, the Committee proposes to reduce the maximum energy content of both infant formulae and follow-on formulae as compared to the limits defined by the Infant Formulae Directive. There is no evidence to justify a differentiation in minimum and maximum energy levels between infant formulae and follow-on formulae. Therefore, the Committee proposes the following energy contents of the product as prepared ready for use for both infant formulae and follow-on formulae:

Minimum	**60 kcal/100 mL** **250 kJ/100 mL**
Maximum	**70 kcal/100 mL** **295 kJ/100 mL**

III. PROTEIN CONTENT

1. INTRODUCTION

The protein content and quality of infant formula and follow-on formula is regulated in the Infant Formulae Directive.

In 1993, the Committee accepted the use of partial hydrolysates of protein as the protein source of infant formula (SCF, 1993a). In 1995, an additional report abolished the differentiation between modified and unmodified cows' milk protein (SCF, 1995).

For both kinds of formulae only cows' milk protein and soy bean protein isolate and, for infant formula, partially hydrolysed protein are identified as sources of protein. Accordingly, infant formulae on the market can be divided into three categories, which differ by their protein source and minimum protein content. The minimum content required is higher for partially hydrolysed and soy protein-based infant formula to account for a potentially lower biological value.

Follow-on formulae with all kinds of protein sources have the same requirements for the protein content.

The presently available infant and follow-on formulae and their permitted protein contents are given in Table 1.

The protein contents in g/100 mL are calculated from the permitted energy values (60 to 75 kcal/100 mL and 60 to 80 kcal/100 mL for infant formulae and follow-on formulae, respectively).

Table 1. Types of formulae and their protein contents in the EU

Protein source		**Infant formula**		**Follow-on formula**	
		minimum	maximum	minimum	maximum
Cow's milk	g/100 kcal	1.8	3.0		
	g/100 mL	1.08	2.25		
Partially hydrolysed protein	g/100 kcal	2.25	3.0	2.25 g/100 kcal	4.5 g/100 kcal
	g/100 mL	1.35	2.25	1.35 g/100 mL	3.6 g/100 mL
Soy protein isolate	g/100 kcal	2.25	3.0		
	g/100 mL	1.35	2.25		

2. GENERAL CONSIDERATIONS ON PROTEIN IN FORMULAE

2.1 Protein composition of infant formula and human milk

The protein content of an infant formula must provide both for the need of nitrogen and of indispensable amino acids for maintenance of the body and for growth i.e. protein deposition.

The percentage of indispensable amino acids of total amino acids required for maintenance is lower (20-30%) than that required for growth (40%). Snyderman *et al.* (1962) have shown, that while providing sufficient indispensable amino acids in the diet of infants, the intake of "unessential nitrogen" could become limiting for growth and that growth could be restored by adding such nitrogen either as glycine or urea. They could show that N^{15} from labelled ammonium chloride and urea was incorporated into plasma proteins and haemoglobin under conditions of a low protein diet.

The definition of indispensable amino acid as "one which cannot be synthesised by the animal organism out of materials ordinarily available to the cells at a speed commensurate with the demand for normal growth" (Borman *et al.*, 1946) implies that for the synthesis of dispensable amino acids precursors must be available and that in certain circumstances the need for these amino acids may become greater than the synthesising capacity. Of the dispensable amino acids only glutamate and serine are synthesised *de novo*, the other conditionally indispensable amino acids all need precursors. Glycine which is low in human milk can become limiting in premature infants when serine synthesis is inadequate (Jackson *et al.*, 1981).

In addition, consumption of any amino acid in functional pathways other than for protein synthesis will detract from the available amino acid pool and either has to be replaced by the diet or by endogenous synthesis (Reeds, 2000).

The quantity of nitrogen in human milk changes dramatically, especially during the first days of life, from about 400 mg/100 mL in colostrum to about 180 mg/100 mL in mature milk. Expressed as a percentage of the energy content protein accounts for 17% in colostrum and for 7% in mature human milk (Räihä, 1994b). More important, the concentration of different proteins changes with duration of lactation: the whey-casein ratio is 90:10 in colostrum and becomes 55:45 in mature milk and 50:50 in late lactation. The decrease in total protein content is due mainly to a decrease in secretory immunglobulin A (sIgA) and in lactoferrin. These two bioactive proteins, which, together with lysozyme, comprise about 30% of the total protein in mature human milk, are resistant to low pH-values and to digestive proteolytic enzymes. Between 3 and 10% of the milk proteins are considered to be nutritionally unavailable to infants as sources of amino acids (Picone *et al.*, 1989b).

In addition, human milk contains between 20 to 25% of its total nitrogen as non-protein nitrogen (NPN). Up to 50% of the NPN-fraction is urea-nitrogen. Both breast-fed and formula-fed infants retained about 13% of ^{15}N labelled urea (Lönnerdal, 1994a). The ability to salvage both endogenous and dietary urea hydrolysed in the gut decreases after the age of six weeks in breast-fed infants and is probably dependent on the actual nitrogen requirement (Steinbrecher *et al.*, 1996). If and how much of the urea-nitrogen is available for net *de novo* synthesis of amino acids and body proteins, mainly through bacterial metabolism in the colon, is not precisely known (Fuller and Reeds, 1998). The composition of NPN of human milk and infant formula is given in Table 2. The amount and composition of NPN in infant formula depends on the processing of the protein sources. It is higher in ion-exchange and electrodialysed whey (26% and 14 to 18%, respectively) than in ultrafiltrated whey (6 to 8%) and in skim milk powder (6 to 7%). In formula based on soy protein isolates it can vary between 1 and 25% of the total nitrogen, dependent on whether or not the protein source has been partly hydrolysed (Donovan and Lönnerdal, 1989b)

Nitrogen from peptides in infant formula can be higher than in human milk. Nitrogen from free amino acids is more variable in infant formula than in human milk and depends in part on

additions of amino acids. Both peptide and amino acid nitrogen which contribute 5 to 7% of the total nitrogen in human milk and between 2 to 8% of the total nitrogen in infant formula will be available for protein synthesis.

Table 2. Nitrogen in mature human milk and cows' milk-based infant formula (Lönnerdal, 1994a; Miera, 1998)

	Mature human milk		**Infant formula**	
	mg/100 mL	%	mg/100 mL	%
Total nitrogen	148-195	100	230-300	100
Non-protein nitrogen	35-50	20-25=100	14-43	8-17=100
Nitrogen in peptides	9	20	5-17	20-30
Nitrogen in free-amino acids	4	10	0-1	4-15
Urea nitrogen	9-23	25-50	4-19	20-40
Creatine N	0.4		0.8-2	4-8
Creatinine N	0.2-0.8		0.1-0.5	1-2
Ammonia N	0.2-0.3		-	1-10
Orotic acid N	-		0.2-2	1-3
Uric acid N	0.4-0.5		0-0.2	0-1
Carnitine N	0.05-0.1		0.1-0.2	< 0.5
Sialic acid N	1-8	1.5	0.3-1	
Nucleotides N	0.3		-	
Polyamines N	0.02		-	
Choline N	0.3		-	

Taurine is the predominant free amino acid in human milk (4 to 5 mg/100 mL or 0.3 to 0.4 mmol/L) (Agostoni *et al.*, 2000). In infant formula it is only present if added.

Subtracting non-nutritional proteins and the NPN-fraction from the total crude protein content (nitrogen x 6.38), one arrives at a minimal protein content of 0.85 g/100 mL in human milk (about 1.27 g/100 kcal) (Räihä, 1985).

Available quantities of amino acids from human milk do not correspond to the quantities and patterns of amino acids determined after hydrolysis, because of the partial unavailability of some digestion-resistant proteins. It has been shown, that the pattern of nutritionally available amino acids remains essentially the same throughout lactation, while the total concentrations in human milk change considerably (Harzer and Bindels, 1987).

A simple imitation of the amino acid pattern of human milk is therefore not sufficient in devising infant formula. Feeding ten healthy term infants each an isocaloric cows' milk-based formula with protein contents (total N x 6.38; 9% non-protein N) of 1.12, 1.33 and 1.48 g/100 mL (corresponding to 1.63, 1.95 and 2.18 g/100 kcal) for 12 weeks and comparing growth development, serum total protein, prealbumin, urea nitrogen, albumin and plasma amino acids and urinary sulphate excretion with the values from 10 infants breast-fed exclusively, showed that despite an amino acid pattern of the formula close to that of human milk (with the exception of lower contents of tryptophan, tyrosine and arginine per g of protein) and comparable protein intakes per kg body weight and day between infants fed formula 1.33 and 1.48 and human milk at 4 weeks, between formula 1.12 and human milk fed infants at weeks 8 and 12, there were significant differences in the plasma amino acids of formula and breast-

fed infants at all times. Infants fed the highest protein containing formula had higher urea nitrogen serum levels and sulphate excretion than breast-fed infants at all times. There were no significant differences in other blood parameters and there was normal growth development in all four feeding groups (Picone *et al.*, 1989b). The aim to be achieved is optimal growth and development of body composition, body functions and body metabolism by supplying sufficient nitrogen and (non-dispensable) amino acids in available form. However, "optimal" growth and development is not a well-defined criterion. Dewey *et al.* (1996) have described and discussed the five principal possibilities to determine the protein requirements of infants (and children):

A. The model of the fully breast-fed infant. This was applied in the FAO/WHO/UNU report (1985) under the assumption that fully breast-fed infants thrive and therefore, that the protein and amino acid intake per kilogram body weight calculated from concentration multiplied by volume intake corresponded to the requirement. New data on breast-fed infants from 1 to 6 months of age and taking into account an only partial metabolic availability of NPN (46-61%) resulted in revised (10-26%) lower protein intakes of breast-fed infants than in 1985 (see Table 3).

Table 3. Revised estimates (Dewey *et al.*, 1996) for nitrogen and protein intakes of breast-fed infants (taken from Table 29 of the 1985 FAO/WHO/UNU report on energy and protein requirement)

Age	**n**	**Breast milk intake**	**Weight**	**Total nitrogen intake**		**Crude protein (N x 6.25/1000)**		**Adjusted* protein intake**	
months		g/day	kg	mg/day	mg/kg/day	g/day	g/kg/day	g/day	g/kg/day
1	37	794	4.76	1723	362	10.8	2.26	9.3-9.7	1.95-2.04
2	40	766	5.62	1486	264	9.3	1.65	7.9-8.3	1.41-1.48
3**	37	764	6.3	1406	233	8.8	1.46	7.5-7.9	1.19-1.25
3***	61	812	6.24	1472	236	9.2	1.48	7.9-8.3	1.27-1.33
4	41	782	6.78	1408	208	8.8	1.3	7.5-7.8	1.11-1.16
6	12	881	7.54	1486	197	9.3	1.23	8.0-8.4	1.05-1.11

* Based on milk protein concentration plus 46-61% of the NPN (protein = 6.25 x nitrogen)
** From Butte *et al.*, 1984
*** From Heinig *et al.*, 1993a

To achieve the same crude protein intake an infant of one month of age would have to consume 210 mL/kg/day of an infant formula with the presently lowest permitted protein content of 1.08 g/100 mL, whereas a six months old infant would have to consume 116 mL/kg/day.

B. The factorial approach, taking into account estimates of the nitrogen requirements for maintenance and for growth, efficiency of conversion from dietary protein into body protein, and epidemiological data. Again the nitrogen needs for maintenance which had been defined as 120 mg/kg/day can be revised according to new data to 90 mg/kg/day and availability of growth data for breast-fed infants allow to calculate protein gain more precisely. Additions to the calculated required nitrogen increment for growth to account for individual variation are no longer proposed. The interindividual coefficient of variation for nitrogen needs also revision, whereas the 70% efficiency of conversion of dietary protein to body protein is retained in the new estimate (Dewey *et al.*, 1996) (see Table 4). By definition the level of protein intake

adequate for formula-fed infants is higher than the observed protein intake of breast-fed infants.

Table 4. Revised estimates (Dewey *et al.*, 1996) for adequate level of protein intake for infants according to revised factorial approach (CV = Coefficient of Variation)

Age	CV (%) growth	CV (%) total	Adequate level	
			Nitrogen	Protein (N x 6.25)
months			mg/kg/day	g/kg/day
0-1	24	17.6	431	2.69
1-2	24	15.9	326	2.04
2-3	25	14.4	245	1.53
3-4	26	13.9	219	1.37
4-5	29	14.2	200	1.25
5-6	32	14.6	190	1.19
6-9	34	14.2	175	1.09
9-12	46	15.6	163	1.02

To achieve the adequate level of crude protein intake, a one month old infant would have to consume 250 mL/kg/day, a six months old infant 112 mL/kg/day of an infant formula with the lowest permitted protein content in the EU.

C. A direct approach through controlled clinical studies. Because formula-feeding cannot rely on the empirical safety and adequacy of breast-feeding in which the composition and intake cannot voluntarily be influenced, prospective study designs for formulae under controlled conditions and with defined end points are to be preferred. It is important to test the nutritional value of proteins in formulae under strictly controlled energy intakes and intakes of other nutrients, including micronutrients.

D. The operational approach by using protein-energy ratios. This approach requires both the knowledge of the safe level of protein intake and the mean requirement for energy at all ages. Both are difficult to estimate and the results are based on assumptions. For exclusively breast-fed infants protein-energy ratios at age 3 to 4 months are 8 to 8.5%, when the protein concentration is 9.6 to 10 g/L (true protein including 46% utilisation of the NPN fraction), energy density is 670 kcal/L and a conversion factor of 5.65 kcal per g of protein is applied (Dewey *et al.*, 1996). For older infants extrapolation would require several more assumptions. The present European regulation prescribes protein energy ratios from 7.2 to 12% for infant formula and from 10 to 18% for follow-on formula.

E. An approach via the determination of the metabolic needs for individual amino acids. This requires the knowledge of the need for amino acids both for growth and maintenance. Whereas the need for growth can be assumed from the amino acid pattern of body proteins the needs for maintenance are essentially not known and can be calculated in different ways. Dewey *et al.* (1996) recommend retaining the amino acid pattern of human milk. Table 5 shows amino acid requirements for infants 3 to 4 months old based on observed intakes that supported satisfactory growth (FAO/WHO, 1985), data from infants between 8 and 112 days of age studied by Fomon *et al.* (1973) and the minimum required values calculated for infants 0 to 6 months of age by Dewey *et al.* (1996).

Table 5. Requirement of indispensable and some conditionally indispensable amino acids by infants from three different published sources (Dewey *et al.*, 1996; FAO/WHO, 1985; Fomon *et al.*, 1973) in mg/kg body weight/day

			Dewey *et al.*, 1996		
	Fomon *et al.*, 1973 [8-112 days]	FAO/WHO, 1985 [3-4 months]	0-1 months [bw 3.6 kg]	1-3 months [bw 4.7 kg]	3-6 months [bw 6.3 kg]
Histidine	26	28	-	-	-
Isoleucine	66	70	59	43	32
Leucine	132	161	109	75	54
Lysine	101	103	116	85	63
Cystine	23	-	-	-	-
Methionine	24	-	-	-	-
Cystine + methionine	-	58	64	37	27
Phenylalanine	57	-	-	-	-
Tyrosine	-	-	-	-	-
Phenylalanine + tyrosine	-	125	114	88	60
Threonine	59	87	63	45	34
Tryptophan	16	17	22	16	11
Valine	83	93	72	51	38

2.2 Protein composition of follow-on formula

The higher protein content that is presently required for follow-on formula corresponds to the mixture of two parts cows' milk plus one part water (3.7 g protein/100 kcal) that has traditionally been used for older infants in Europe. The Committee considered a higher protein content acceptable because of a matured renal function and metabolic interconversion system after the age of 4 months without stressing a need for it (SCF, 1983).

A high protein content of follow-on formula is justified when available complementary food is low or deficient in protein (Wharton, 1994). This is not the case in Europe. With introduction of complementary food, which is regulated to have a high protein content in the European Union (at least 3 g/100 kcal or 12% of energy in all kinds of baby foods) the total dietary protein intake increases significantly. There are indications that an excessive protein intake in infancy and young childhood not only constitutes an unnecessary stress on metabolism and kidney function but also has undesirable effects on long-term health by increasing the risk of overweight. Nielsen *et al.* (1998) described in a retrospective study of 339 Danish infants that the median protein intake at about 10 months of age was 3.9 g/kg/day (10^{th} percentile 2.7; 90^{th} percentile 5.4), the highest protein intake corresponding to more than 16% of the energy intake. Infants breast-fed for more than 7 months gained 200 g less in weight and 7 mm less in length from age 5 to 10 months. Infants with the highest protein intake (>16% of energy) gained 260 g more weight in 10 months, than infants with lower intakes.

Healthy Swedish and Italian infants studied between three and 12 months of age received either of three infant formulae (1.3; 1.5; 1.8/2.0 g protein/100 mL) from age 3 or 5 months onwards and in addition complementary (weaning) food after termination of breast-feeding, ready-to feed in Sweden and home-prepared in Italy. Total protein intake was higher in Italian

than in Swedish infants both at age 6 and 12 months: 6 months Italy 3.5 to 3.8 g/kg/day, Sweden 1.9 to 2.2 g/kg/day; 12 months Italy 3.7 g/kg/day (18% of energy), Sweden 2.6 g/kg/day (11% of energy). Growth for weight and length was normal in both groups and comparable. Serum urea was higher in Italian infants (4.9 mmol/L) than in Swedish infants (3.8 mmol/L) at 12 months of age (Åkeson *et al.*, 2000).

In a longitudinal study on 354 German infants and children protein intake was 1.6 and 1.4 g/kg/day in breast-fed boys versus 2.5 g/kg/day in formula-fed boys at ages 3 and 6 months (corresponding to 7 and more than 10% of energy intake). Protein intake increased thereafter to a maximum at 12 months of age (3 g/kg/day, corresponding to more than 13% of the energy intake). Similar findings were reported for girls. The median protein intake as percentage of energy intake remained between 13 and 14% until the age of 3 years (Alexy *et al.*, 1999).

One detailed prospective cohort study of 40 infants breast-fed for 4 months and 36 infants formula-fed for 4 months demonstrated that early feeding mode does influence the body composition in early infancy but that these differences do not persist into the second year of life. Energy and protein intakes were lower in breast-fed infants at 3 and 6 months of age, thereafter no differences were observed until 24 months. Weight and length of formula-fed infants was higher at 3 and 6 months, thereafter up to the age of 24 months no differences between feeding groups were observed. Daily intakes of energy, protein, fat, carbohydrate were positively correlated with weight and fat free mass, but not with fat mass or percent fat mass at 3, 6 and 12 months of age. The percentage of energy intake as protein was positively correlated to weight gain and fat free mass gain at 3 and 6 months, but not to fat mass (Butte *et al.*, 2000b). This study seems to imply that a comparable intake of both energy and protein after the period of exclusive breastmilk or formula-feeding does not result in group differences for weight gain and body composition.

Very high protein intakes at 2 years of age were observed in 112 French children followed from 10 months to 8 years of age and measured for weight and length and skinfold thickness (two sites) at 10 months, 2, 4, 6 and 8 years of age. Protein intake as percentage of energy intake was <14.8 in the lowest quartile, 14.8 to 18% in the next two quartiles and more than 18% in the highest quartile. There was a statistically significant correlation between the percentage of protein at two years of age with the body mass index and subscapular skinfold thickness at 8 years of age after adjustment for energy intake at 2 years of age and parental body mass index. Moreover, the percentage of protein at 2 years of age was negatively associated with age at adiposity rebound. This has been interpreted as a risk for obesity in later life by high protein intake in early life (Rolland-Cachera *et al.*, 1995). Similar results were reported in 150 Italian children, followed from birth to 5 years of age. Children with a BMI above the 90th percentile at 5 years had a higher protein intake at one year than those who were not overweight (<90th percentile). The protein intake both in the overweight and in the non-overweight group was very high, 22 and 20% of energy, respectively (Scaglioni *et al.*, 2000). In the latter study there was also a tendency of breast-feeding to protect against obesity later in life.

Dorosty *et al.* (2000) have attempted to reproduce the results of Rolland-Cachera by following a cohort of 889 British children born in 1991 and 1992 from birth to 5 years of age. Ten anthropometric measurements were performed in 5 years and two 3-day dietary records were taken at 8 and 18 months of age. Protein intakes as percentage of energy intake were 14.6±2.2% at 18 months. They were not correlated with age of adiposity rebound. Only parental body mass index or obesity were significantly correlated to an early adiposity

rebound, which is considered as predictive of future adiposity (Rolland-Cachera *et al.*, 1984).

The lower protein content of human milk, as compared to infant formula, has been considered to possibly reduce the risk of later obesity (Koletzko and von Kries 2001; FAO/WHO, 2002a). A review of 18 published studies comparing breast fed and formula fed populations (six retrospective studies, ten prospective, one cohort, one case control) between 1945 and 1999 and involving in total nearly 20,000 subjects found a positive relationship between breast-feeding and later obesity in two studies, a negative (protective) relationship in four studies and no effect in ten studies (Butte *et al.*, 2001). A protective effect of breast-feeding was seen in a large retrospective cross-sectional study on almost 10,000 children at the age of 5 years in Bavaria (von Kries *et al.*, 1999), in a survey of 15,000 adolescents whose mothers participated in the Nurses' Health Study II (Gillman *et al.*, 2001), and in a large cohort of 32,000 Scottish children, 27% of whom were breast-fed when seen at the age of 6 to 8 weeks, and who were reinvestigated at the age of 39 to 42 months (Armstrong and Reilly, 2002). 2500 children examined at the age of 3 to 5 years in the course of the third National Health and Nutrition Examination Survey (NHANES III) were found to be at a decreased risk of being overweight at the age of 3 to 5 years if ever breast-fed (Hediger *et al.*, 2001). A school based study in the Czech Republic in some 33,000 children found breast feeding associated with a 20% risk reduction of both overweight and obesity until the age of 14 years (Toschke *et al.*, 2002). From these studies the question remains open if it is breast-feeding *per se* or the lower protein intake connected with breast-feeding, which protect against overweight in later life, or if simply any excessive protein intake during infancy (three times the recommended intake and more or more than 14% of the energy intake) predisposes to later adiposity and related diseases and, if so, by what means (Martorell *et al.*, 2001; Metges, 2001). High protein intakes can have impact on glomerular filtration rate and will increase the renal solute load, they can lead to elevations of certain plasma amino acid levels with metabolic effects on the hormone system (stimulation of insulin and insulin-like growth factor type 1 secretion) and the production of neurotransmitters, which are not sufficiently investigated (Michaelsen *et al.*, 2002). As excessive protein intakes do not appear to have beneficial effects on the health of the child they should be avoided.

2.3 Effects of processing on nutritional value

The nutritional value of protein is influenced by its amino acid composition, by protein hydrolysis but also by heat-treatment, especially in the presence of iron, vitamin C and lactose in these products. Sarwar *et al.* (1989) have demonstrated in rats that heat treatment (122-132°C for 5 to 8 minutes) applied in the manufacture of ready-to-feed liquid infant formulae reduces the apparent and true digestibility of protein (74 to 76%; 88 to 90%) compared to powder forms of infant formula (79 to 83%; 93 to 97%) and that the true digestibility of lysine, methionine and cystine in liquid products was 5 to 13% lower than in powder products. Rats fed liquid products during two weeks showed decreased plasma lysine levels. Bioavailability of tryptophan in rats was reduced from two commercial heat-treated liquid products (83 to 84%) compared to powdered products (90 to 95%) but not impaired from two others (92 to 95%). The concentration of bioavailable tryptophan was generally lower in liquid products (Sarwar and Botting, 1999).

Infant formulae both in powdered and liquid forms contain furosine and hydroxymethyl-furfural as indicators of early Maillard reactions and liquid products contain double the amount of advanced Maillard reaction products. This results in a reduction of available lysine and is accompanied by a 30% reduction of tryptophan in soluble proteins (Birlouez-Aragon, 1999). In powdered infant formula (n=44) 5% of total lysine was destroyed, 8% inactivated,

in liquid sterile infant formula (n=23) the respective values were 9 and 10%, measured as furosine, the acid hydrolysis product of fructoselysine containing proteins (Erbersdobler and Hupe, 1991). Prolonged heat treatment under alkaline conditions promotes lysinoalanine formation in soy protein and reduces its digestibility (Liener, 1994). A recent analysis of nine commercial powdered formulae (one infant formula and eight follow-on formulae, three of the latter were based on soy protein isolate) and 12 commercial liquid formulae (six infant formulae and six follow-on formulae) demonstrated that lysinoalanine as indicator of thermal damage to the protein was below the detection limit (0.5 µg/g protein) in powdered products. Liquid infant formulae had lower contents of lysinoalanine than liquid follow-on formulae. Among these the highest concentrations were found in in-can sterilised products (>300 µg/g protein), whereas ultra-high temperature (UHT) treated liquid follow-on formulae had intermediary values (D'Agostina *et al.*, 2003).

Ultra-high temperature treated (142° for 2 to 3 seconds) liquid formulae based on electrodialysed whey-casein mixtures (1.3 and 1.5 g protein/100 mL) fed to healthy full-term infants until the age of 6 months resulted in comparable growth, haemoglobin, ferritin, zinc and copper levels as in breast-fed infants or infants fed powdered whey-casein formula (1.3 g protein/100 mL). Plasma amino acid levels of the infants fed the lower-protein UHT-formula were most similar to breast-fed infants. Blood urea nitrogen was lowest in breast-fed infants and highest in the infants fed the UHT-formula with the higher protein content and similar to the values of infants fed the powder formula (Lönnerdal and Hernell, 1998). In 12 premature infants fed for five days each UHT-treated and in-can sterilised formulae with known lactulosyllysine and lysinoalanine contents in a cross-over design, no effects of the in-can sterilised product compared to the UHT treated product with respect to creatinine clearance, electrolyte excretion or signs of renal tubular damage were found (Langhendries *et al.*, 1992), Even if these data seem to indicate that human infants tolerate these compounds better than rats, their contents in infant formula and follow-on formula should be kept as low as technically possible.

2.4 Recommendation

The Committee proposes there should be a requirement for adequate clinical testing before the commercialisation of all formulae which contain protein sources or protein hydrolysates which have no established use in infant formulae and/or to which processing technologies have been applied that can affect the bioavailability of nitrogen compounds (cf. chapter XI).

3. CURRENT REQUIREMENTS CONCERNING PROTEIN IN INFANT FORMULAE AND FOLLOW-ON FORMULAE IN THE INFANT FORMULAE DIRECTIVE

3.1 Calculation of protein

The protein content is calculated from the total nitrogen content by multiplying with a factor of 6.38 in the case of cows' milk protein and with a factor of 6.25 in the case of soy protein isolate and partial hydrolysates of protein for both infant formula and follow-on formula.

3.2 Sources of protein

For infant formula only cows' milk protein - unmodified or increased in whey protein - soy protein isolates and protein partial hydrolysates are permitted. Neither the protein source of

the partial hydrolysates nor the degree of hydrolysation are explicitly specified. Infant formula bearing a claim for "adapted protein" must have a whey protein to casein ratio of at least one.

For follow-on formula only cows' milk protein and soy protein isolates are permitted. The use of partially hydrolysed protein is not explicitly excluded. L-Amino acids may only be added to both infant and follow-on formulae for the purpose of improving the nutritional value of the protein. Taurine must be added to infant formula based on protein hydrolysates in amounts to achieve at least 5.25 mg/100 kcal (42 μmol/100 kcal) and L-carnitine must be added to infant formulae based on protein hydrolysates and soy protein isolates to achieve a content of at least 1.2 mg/100 kcal (7.5 μmol/100 kcal).

Nucleotides (CMP, UMP, AMP, GMP and IMP) may be added to infant and follow-on formulae in total amounts of 5 mg/100 kcal.

Choline and inositol may be added to infant and follow-on formulae in unspecified amounts.

3.3 Protein content

The minimum and maximum protein density values are given in Table 1.

The maximum protein content of an infant formula bearing a claim for "adapted protein" is 2.5 g/100 kcal.

3.4 Protein quality

The Infant Formulae Directive defines the chemical index of a protein as the lowest of the ratios between the quantity of each indispensable amino acid of the test protein and the quantity of each corresponding amino acid of the reference protein. Reference protein for infant formula is human milk protein as defined in section 3.5. Reference proteins for follow-on formula are casein (from cows' milk) and human milk protein.

The chemical index is not used to determine the quality of protein in infant formula based on cows' milk protein and protein partial hydrolysates. Soy protein isolate in infant formula has to have a minimal chemical index of 80% in comparison to human milk protein.

Protein in follow-on formula must have a chemical index of at least 80%, with reference to casein or human milk protein.

Infant formula based on cows' milk protein and based on partially hydrolysed protein has to contain per energy value at least the same available quantity of each indispensable and some conditionally indispensable amino acid as human milk protein. However, methionine and cystine may be added in that calculation. Infant formula based on soy protein isolate must contain the same amount of available methionine per energy value as human milk. For infant formula made with partially hydrolysed protein a PER (protein efficiency ratio) and NPU (net protein utilisation) value at least identical to casein is also requested.

Follow-on formula manufactured from cows' milk protein or from or with soy protein isolate has to contain the same amount of available methionine per energy value as human milk.

3.5 Amino acid pattern of the reference proteins human milk and cows' milk casein

The amino acid pattern is given in Annex VI of the Infant Formulae Directive. These data were taken from FAO Nutritional Studies No 24 (1970). They are based on 8 casein samples with a mean nitrogen content of 2.08 g/100 g from which a crude protein content of 13.3 g/100 g by multiplication with the factor 6.38 was calculated and on 35 samples of human milk with a mean nitrogen content of 0.19 g/100 g from which a crude protein content of 1.2 g/100 g (factor 6.38) was calculated.

Annex VI of 91/321/EEC: The amino acid composition of casein and human milk protein (g/100 g protein)

	Casein	Human milk
Arginine	3.7	3.8
Cystine	0.3	1.3
Histidine	2.9	2.5
Isoleucine	5.4	4.0
Leucine	9.5	8.5
Lysine	8.1	6.7
Methionine	2.8	1.6
Phenylalanine	5.2	3.4
Threonine	4.7	4.4
Tryptophan	1.6	1.7
Tyrosine	5.8	3.2
Valine	6.7	4.5

The values of Annex V were obtained by multiplying the amino acid concentrations in mg/100 mL from the FAO report by the coefficients 0.36 or 1.5. These coefficients were obtained by dividing the true protein content (α-amino N x 6.38) by the energy density.

Annex V of 91/321/EEC: Indispensable and conditionally indispensable amino acids in human milk (mg/100 kJ or mg/100 kcal)

	per 100 kJ	per 100 kcal
Arginine	16	69
Cystine	6	24
Histidine	11	45
Isoleucine	17	72
Leucine	37	156
Lysine	29	122
Methionine	7	29
Phenylalanine	15	62
Threonine	19	80
Tryptophan	7	30
Tyrosine	14	59
Valine	19	80

4. COMMENTS ON THE EXISTING REQUIREMENTS FOR PROTEIN

4.1 Calculation of protein content

When different authors refer to "protein" contents in food, e.g. infant formula they do not mean the same. In general, there are three possible methods to determine the protein content. For labelling purposes one method has to be chosen.

4.1.1 Calculation from nitrogen content

Crude protein is calculated from the total nitrogen content of a food multiplied by a conversion factor, as a rule 6.25. Because different food proteins contain differing amounts of nitrogen, different nitrogen conversion factors have been proposed. FAO/WHO use a factor of 6.25 for all their reports on protein requirement and quality.

For milk and milk products of all kinds a conversion factor of 6.38 has been determined by Hammarsten (1883). The Infant Formulae Directive applies this factor to products based on intact cows' milk proteins. The conversion factor of 6.38 for milk protein is a compromise for the different protein fractions of cows' milk. From the amino acid sequence of different protein fractions the nitrogen content can be calculated per g of that protein. For caseins conversion factors varying between 6.08 and 6.71 are thus obtained (>7.3 for casein macropeptides). For β-lactoglobulins and α-lactalbumins factors between 6.14 and 6.40 derive. For the NPN fraction, which on average is 5% (4-8%) of total nitrogen, in whey however 25-30%, a composite factor of 3.60 is calculated. Part of the NPN, especially amino acids and peptides are of nutritional value. Taking into account the different amounts of different protein fractions and their genetic variation a conversion factor for proteins in raw milk of 6.34 has been calculated. If the NPN is taken into account, a conversion factor of 6.20 to estimate the crude protein content will result for a NPN content of 5%, and of 6.07 for a NPN content of 10% (Karmann and van Boekel, 1986).

Crude protein includes all NPN-containing substances. In cows' milk this NPN amounts to 25-30 mg/100 mL or 5 to 6% of total nitrogen. 50% of this NPN is urea-nitrogen, up to 10% are free amino acids.

True protein has been defined most often as total nitrogen minus NPN multiplied by the appropriate conversion factor. However, this calculation excludes nitrogen that is partially metabolically available to the body (amino acids, small peptides, urea, aminosugars, nucleotides, carnitine, and choline). In cows' milk-based infant formula approximately 8 to 18% of total nitrogen is NPN (Donovan and Lönnerdal, 1989b; Miera, 1998). Table 6 illustrates the variability in the nitrogen composition of 15 infant formulae based on intact cows' milk protein and of one soy protein isolate-based formula.

Products with low whey protein content and those with added ultrafiltrated whey (e.g. product 2) have lower NPN percentages. Remarkable is the high NPN content of the soy formula. Free amino acids and acid-soluble peptides together are 28 to 44% of NPN, whereas urea-nitrogen is missing in soy based formula and is between 19 to 41% of NPN in formulae based on cows' milk protein.

Assuming that all of the peptides and amino acids and 17% of the urea content, therefore 95.4% of total nitrogen in formula was bioavailable. Fomon *et al.* (1995a) applied a corrected conversion factor (i.e. 6.38 x 0.954) of 6.09 to convert total nitrogen to protein.

Table 6. Nitrogen composition of 15 infant formulae based on cows' milk protein and of one infant formula based on soy protein isolate (Miera, 1998)

Product	Amino acids added	Casein/Whey Ratio	Total N mg/100 mL	NPN mg/100 mL	NPN in% of total N	Urea-N in% of NPN	Peptide N in% of NPN	Free AA-N in% of NPN
1	taurine + arginine	40:60	234	38	16	41	n.a.	14
2		40:60	262	20	8	19	n.a.	2
3		40:60	256	39	15	26	30	4
4		40:60	248	44	18	22	32	2
5	taurine/ arginine	40:60	219	31	14	38	n.a.	7
6	taurine/ arginine	40:60	228	34	15	41	29	9
7	taurine	40:60	264	24	9	26	37	4
8	taurine	40:60	277	36	13	38	n.a.	4
9	taurine	40:60	258	27	10	33	n.a.	4
10	taurine	40:60	226	24	11	28	39	5
11	arginine	50:50	289	38	13	39	n.a.	8
12	taurine	70:30	293	22	8	41	23	5
13	taurine	70:30	299	24	8	29	18	5
14	taurine	77:23	302	26	9	37	28	4
15	taurine	80:20	247	19	8	34	18	15
16	soy; taurine methionine		269	68	25	0	n.a.	4

n.a. = not analysed

For human milk protein content calculations Dewey *et al.* (1996) propose the term "adjusted protein" which is true protein (total nitrogen minus NPN) plus 46-61% of NPN multiplied by 6.25.

In its 1998 report the Life Sciences Research Office (LSRO) accepted this method of calculation for the setting of the minimum protein content of infant formula and named it confusingly "true protein" (calculated from α-amino nitrogen x 6.25). α-Amino nitrogen determinations are unreliable, if not based on the analysis of the total amino acid content or, alternatively, on the determination of the total nitrogen and the NPN content and assuming that 2 to 8% of total nitrogen is nitrogen from amino acids and small peptides. Under the assumption that 15% of total nitrogen is NPN and amino acid and peptide-nitrogen together make up on average 5% of total nitrogen, α-amino nitrogen would be 90% of the total nitrogen content, which multiplied by 6.25 would give the "true" protein content as defined in the LSRO report.

4.1.2 Protein determination

Some authors have determined protein by colorimetric methods (e.g. Lowry) and applied correction factors (e.g. Heinig *et al.*, 1993a). All colorimetric methods tend to overestimate the protein content compared to the Kjeldahl method, especially if non-human milk standards are used in the determination (Donovan and Lönnerdal, 1989a). Infrared analysis is another possible method, which determines true protein (Fleischer Michaelsen *et al.*, 1994).

4.1.3 Protein as sum of amino acids

Another possibility, which again excludes urea, is to calculate protein as the sum of anhydrous amino acids quantitatively determined after hydrolysis of the sample. This is, however, very demanding on analytical procedures (Donovan and Lönnerdal, 1989a; Sarwar *et al.*, 1996).

The following Table 7 compares the contents of total nitrogen to the sum of amino acid nitrogen after hydrolysis and to the calculated α-amino nitrogen (total nitrogen minus NPN + peptide N + free amino acid N) in 16 infant formulae (Miera, 1998).

Table 7. Total nitrogen content, nitrogen content from total amino acids and calculated α-amino nitrogen

Formula	Total nitrogen	Total amino acid nitrogen		Calculated α-amino nitrogen mg/100 mL	% of TN
	mg/100 mL	mg/100 mL	% of total nitrogen		
1	234	191	82	-	-
2	262	242	92	-	-
3	256	208	81	231	90.1
4	248	197	79	223	90.0
5	219	182	83	-	-
6	228	192	84	211	92.6
7	264	222	84	234	-
8	277	223	80	-	-
9	258	225	87	-	-
10	226	197	87	209	92.5
11	289	222	77	-	-
12	293	256	87	271	92.4
13	299	275	92	293	98.0
14	302	267	88	283	93.8
15	247	219	89	231	93.4
16	269	245	91	-	-

The sum of the anhydrous amino acids (g/100 mL) (corrected for analytical losses) is lower than the crude protein content (total nitrogen x 6.38) by 6 to 13%. Using the conversion factor 6.25 it is still lower by 4 to 11%. By dividing the sum of anhydrous amino acids (g/100 mL) by total nitrogen (g/100 mL) a median conversion factor of 5.9 (range 5.55-5.99) results. A calculation of the amino acid-true protein content by multiplying the total nitrogen content by 5.90 resulted in protein content values which differed maximal by 6% from the sum of anhydrous amino acids and determined it within 1.5% in six of nine formulae.

The resulting "protein" contents (g/100 mL) from different calculation models are given in the following Table 8 for comparison. The crude protein content calculated with the conversion factor of 6.25 is between 2 and 3% lower than when it is calculated with the factor 6.38. The true protein content (TN minus NPN multiplied by factor) is between 7 and 17% lower than the crude protein content.

Table 8. "Protein" content of infant formulae calculated from total nitrogen, non-protein nitrogen (NPN) and amino acid analysis by different methods (g/100 mL)

	Label claim	Crude protein	Crude protein	Sum anhydrous amino acids	True protein	True protein	α-amino N x 6.38	α-amino N x 6.25	(TN-NPN) + 46% NPN x 6.38	(TN-NPN) + 46% NPN x 6.25	"True protein" (LSRO)
		N x 6.38	N x 6.25		TN-NPN x 6.38	TN-NPN x 6.25					
1	1.5	1.49	1.46	-	1.25	1.23	-	-	1.38	1.35	1.32
2	1.7	1.67	1.64	-	1.55	1.51	-	-	1.61	1.58	1.47
3	1.6	1.63	1.60	1.44	1.38	1.36	1.47	1.44	1.52	1.49	1.44
4	1.5	1.58	1.55	1.37	1.30	1.28	1.42	1.39	1.45	1.42	1.40
5	1.4	1.40	1.37	-	1.20	1.17	-	-	1.31	1.28	1.23
6	1.5	1.45	1.43	1.30	1.24	1.21	1.35	1.32	1.36	1.33	1.28
7	1.8	1.68	1.65	1.56	1.53	1.49	1.49	1.46	1.61	1.58	1.49
8	1.8	1.77	1.73	-	1.54	1.51	-	-	1.66	1.63	1.56
9	1.8	1.65	1.61	-	1.48	1.44	-	-	1.56	1.53	1.45
10	1.5	1.44	1.41	1.33	1.29	1.26	1.33	1.31	1.37	1.34	1.27
11	1.8	1.84	1.81	-	1.60	1.57	-	-	1.74	1.70	1.63
12	1.9	1.87	1.83	1.73	1.73	1.69	1.73	1.69	1.81	1.77	1.65
13	1.9	1.91	1.87	1.78	1.75	1.72	1.87	1.83	1.84	1.80	1.68
14	1.9	1.93	1.89	1.81	1.76	1.72	1.81	1.77	1.85	1.81	1.70
15	1.7	1.58	1.54	1.58	1.46	1.43	1.47	1.44	1.52	1.49	1.39
16	1.8	1.68	1.67	-	1.26	1.26	-	-	1.52	1.49	1.51

The sum of anhydrous amino acids measured after hydrolysis is the best indicator for the content of protein, peptide and free amino acids in infant formula. It corresponds well with the estimation of the protein content from the sum of α-amino nitrogen multiplied by 6.25. However, both methods are demanding with respect to analysis. Comparable results are obtained by calculating the "adjusted true" protein content from total nitrogen and NPN under the assumption that 46% of the latter is available for protein metabolism and using a conversion factor of 6.25. Only the total nitrogen and NPN needs to be analysed. Alternatively, the method proposed in the LSRO report, simplified as outlined in section 4.1.1 (90% of TN multiplied by factor), could be applied. This method, which requires the determination of the total nitrogen content only, underestimates the protein content in products in which the measured α-amino nitrogen is more than 90% of TN.

4.1.4 Conclusion and recommendation

The present requirements for protein in the Infant Formulae Directive rely on the crude protein content calculated from total nitrogen multiplied by 6.38 or 6.25 for cows' milk-based and soy protein isolate based formula, respectively. This calculation includes the NPN which is around 15% of total nitrogen in cows' milk-based and significantly higher in some soy protein based formula. The NPN consists of approximately 35% of probably 100% available amino acids and peptides, and on average 33% urea-nitrogen, part of which (depending on age and nitrogen need) can be assumed to be metabolically available.

Changing the conversion factor to 5.9 would calculate a protein content corresponding better with the sum of anhydrous amino acids and with the "adjusted" protein calculation proposed by Dewey *et al.* (1996) for human milk: "adjusted" protein is 6.25 x [(total nitrogen minus NPN) plus 46 to 61% of NPN]. In this calculation it is assumed that amino-nitrogen accounts

for about 35% of NPN and is 100% available and that 17 to 40% of the remaining NPN can be utilised, overall 46 to (61%) of NPN. In Table 8 the more conservative percentage of 46% has been chosen in the calculation of the "adjusted" protein content. This calculation method is technically easy and relies on two determinations, total nitrogen and NPN. When the modified LSRO method, which requires the determination of the total nitrogen content only, is applied (see section 4.1.1), the minimum permitted crude protein content of infant formula in the EC of 1.8g/100 kcal converts to a "true" protein content of 1.59 g/100 kcal. In order to achieve the same "adjusted" protein intake as a breast-fed child (see Table 3), a one-month old infant would have to consume 585 to 610 kcal/day or 123 to 128 kcal/kg body weight/day of an infant formula with the minimum permitted protein content in the EU. This would require a volume between 164 to 213 mL/kg/day, depending on the energy content of the formula (at present 75 or 60 kcal/100 mL).

Although the Committee is not aware of data that indicate problems in the supply of adequate levels of intake of protein from infant formula under the present regulation which relies on the crude protein content of such formulae, the Committee cannot exclude that this is mainly due to the fact that manufacturers of infant formula generally aim at intermediary levels of the permitted protein contents (2 to 2.25 g of protein/100 kcal). The Committee considers it therefore advisable to request that the NPN content be taken into account and be not higher than 15% of the total nitrogen content, in order to guarantee a minimum amount of amino nitrogen available for protein synthesis.

The Committee proposes to determine the crude protein content of all types of infant formula and follow-on formula (total nitrogen x 6.25). In addition, the NPN content must not be higher than 15% of the total nitrogen content in formula based on intact proteins.

The label information on crude protein allows calculating the total nitrogen content and thereby provides useful information on the potential renal solute load of a formula which is predominantly determined by the nitrogen content.

4.2 Protein sources

4.2.1 Proteins permitted

At present, only cows' milk protein and soy bean protein isolate are identified as protein sources in formulae in the EU (in addition hydrolysates of protein of unspecified degree of hydrolysis and of unspecified origin). The restriction to these sources was made because only these kinds of products were manufactured and marketed at the time of the opinions of the Committee.

There have been requests to permit, like in the Codex standard for infant formula, other protein sources both from milk from other animals and from plants.

4.2.2 Other milks

4.2.2.1 Protein and amino acids

Milk from goats, sheep and horses have a higher protein content than human milk. Total concentration of amino acids (g/L) (without tryptophan) was determined to be 33.6 in the milk of cows, 25.7 in goats, 54.1 in sheep and 15.8 in horses (Davis *et al.*, 1994). All milks were most abundant in glutamate plus glutamine, leucine and proline, which were

approximately 20%, 10% and 10%, respectively of the total amino acids. The percentage of indispensable amino acids (except tryptophan) on a weight base to total amino acids was about 43% in cows', goat's and sheep's milk and 38% in horse's milk (human milk 40%), the percentage of branched chain amino acids around 20% in the first three and 18% in the latter. The percentage of total sulphur amino acids was the same as in human milk (around 3.5%), however, the cystine percentage was lower (around 0.8% in cow, goat and sheep, 1.1% in horse versus 2.0% in human milk) while the methionine percentages were correspondingly higher. The methionine to cystine ratio was higher in all four animal milks (2 to 3.8) compared to human milk (1.1) (Davis *et al.*, 1994). Whereas there are significant differences in the amino acid pattern of animal milk compared to human milk, cows' milk does not appear to be more advantageous with regard to the amino acid pattern than other milk (Urbiené *et al.*, 1997).

4.2.2.2 Digestibility

No systematic studies on the digestibility of these other milks were available to the Committee apart from the data by FAO (1970), neither does it has reason to suppose it to be different from that of cows' milk.

4.2.2.3 Allergenicity

An increased or decreased allergenicity of milk other than cows' milk has not been demonstrated. The high incidence of cows' milk protein allergy is most probably the consequence of a predominance of cows' milk in infant feeding. Cross reactivity to goat and sheep milk has been shown in children with cows' milk allergy never having received goat or sheep milk (Gjesing *et al.*, 1986; Dean *et al.*, 1993). These authors consider goats' and sheep's milk as the least suitable alternatives in children with cows' milk protein intolerance.

4.2.2.4 Bioavailability of nutrients

Bioavailability of zinc was found to be lowest in sheep's milk compared to cows' and goats' milk (which were both lower than human milk). Calcium availability was similar in different milks (Shen *et al.*, 1995).

4.2.3 Plant proteins (vegetables and legumes)

4.2.3.1 Soy protein-based infant formulae

Infant formula based on soy protein isolate may be used as as a breast-milk substitute for non-breast-fed vegetarian infants, as an alternative for cows' milk protein intolerant infants and, because of the absence of lactose, it is suitable for lactose-intolerant and galactosaemic infants. [It is normally produced from soy protein isolate (minimum protein content 65% of the dry extract), practically free of galactooligosaccharides (carbohydrate content less than 0.5%). Soy protein isolate still contains phytate (between 1 and 2%), which can inhibit the absorption of iron and zinc. Phytate can be decreased by treatment with phytases (Lönnerdal *et al.*, 1999), however, this technique appears not to be applied by European manufacturers. The Committee requested in its 23rd report (SCF, 1989) that every effort should be made to reduce the concentrations of trypsin inhibitors, lectins, goitrogenic substances and phytooestrogens by controlled heat treatment and/or extraction (Liener, 1994).

Early reports on negative effects of soy-based infant formulae on growth, enhanced morbidity

and decreased immunologic reactivity were done with formulae made from soy flour (Zoppi *et al.*, 1982 and 1979), whereas recent studies with soy protein isolate-based formulae (2.45 g protein/100 kcal) with and without the addition of nucleotides indicate that the recipient infants have normal immune development (Ostrom *et al.*, 2002).

Fomon *et al.* (1973) fed 13 infants *ad libitum* a formula based on methionine supplemented soy protein isolate containing 1.1 g of protein (N x 6.25) and 67 kcal/100 mL (1.64 g/100 kcal or 6.6% energy) for 28 days exclusively and thereafter combined with some complementary food (7.7 and 6.15% of total energy and protein intake, respectively) up to 112 days. They showed that these infants did not differ from normal breast-fed infants in gains in length and weight and albumin levels in serum and had similar nitrogen balance data. However, intake of energy was slightly higher than in infants fed a cows' milk-based formula with 1.77 g of protein/100 kcal. In a study designed to estimate the requirement of sulphur amino acids of infants up to the age of 112 days a beneficial effect of L-methionine supplementation (7.5 mg/100 kcal) with respect to nitrogen balance was only seen at a protein content of the soy-based formula of 1.8 g/100 kcal. However, with respect to weight gain and/or serum concentrations of urea nitrogen and albumin, a beneficial effect of methionine supplementation could be demonstrated at protein concentrations of the formula of 2.2 and 2.6 g/100 kcal in 19 and 15 infants, respectively (Fomon *et al.*, 1986).

The Committee decided in 1989 to base the minimum protein requirement for infant formula based on soy protein isolate fortified with L-methionine (32 mg/100 kcal) on growth studies in infants fed a formula providing 9% of energy as protein (Fomon *et al.*, 1979). Although it is suggested, that the protein content of soy infant formula need not be higher than that of cows' milk-based infant formula when providing the same amounts of available amino acids no studies of sufficient power with such formulae have been performed (Churella *et al.*, 1994).

The nucleotide content of soy protein based formulae is much higher (about 310 mg/L) than in either human milk (68 to 72 mg/L) or cows' milk-based infant formula (8 to 72 mg/L) (Kuchan *et al.*, 2000).

Soy protein is rich in isoflavones which can for example bind to oestrogen receptors and interact with enzyme systems influencing oestrogenic activity (Setchell, 2001). The total content in ready-for-use products in the USA was determined to be 20-47 µg/mL (Murphy *et al.*, 1997; Setchell *et al.*, 1998; Johns *et al.*, 2003), mainly the glycosides of genistein (65%) and daidzein. A four-month old infant fed such soy formula will receive 22 to 45 mg per day or 6 to 11 mg/kg body weight per day. Accordingly, plasma levels of daidzein and genistein in infants fed soy formula were significantly higher (654-1775 ng/mL), than in infants fed cows' milk formula (9.4±1.2 ng/mL) after 4 months or human milk (4.7±1.3 ng/mL) (Setchell *et al.*, 1998). It is noted that adverse effects of soy-based formulae on reproduction, development, carcinogenesis and immunology have been observed in animals (Badger *et al.*, 2002; Essex, 1996; Newbold *et al.*, 2001; Setchell *et al.*, 1998; Yellayi *et al.*, 2002). To date, despite the wide-spread use of soy-based formulae for example in the USA, there are only limited data addressing the safety of soy-based infant formulae and follow-on formulae, other than noting the absence of case reports of adverse effects in those fed soy-based infant formulae. The limited epidemiological data available are described below.

Strom *et al.* (2001a) performed telephone interviews in 811 adults aged between 20 and 34 years who had participated as infants during the years 1965 to 1978 in feeding trials with soy-based formula (n=248; 120 males) or cows' milk formula (n=563; 295 males). Data were

collected in adulthood for self-reported height, weight, body mass index, pubertal maturation, menstruation, reproduction and education levels. Female subjects of the original soy group had a higher rate of regular use of antiasthmatic and antiallergic drugs (18.8% *vs.* 10.1%, p=0.047), while males showed a similar but non-significant trend (15.8% *vs.* 10.2%, p=0.08). Females previously fed on soy formulae had a lower prevalence of sedentary activities (8.9±3.4 hours/week *vs* 9.6±3.5 hours/week, p=0.05) while there was no group difference for males. There were no differences in height, weight, incidence of thyroid disease (Strom *et al.*, 2001b) or pubertal development between the groups previously fed the two types of formuale. Duration of menstruation was slightly longer (by 0.37 days) and more painful in the soy-fed group. Pregnancies were reported by 42% of women fed soy-formula and by 48% of women in the cows' milk formula group. Outcomes of pregnancies were not different, neither were there differences in the occurrence of cancer, hormonal disorders, sexual orientation or birth defects in the offspring between the groups. No conclusions can be drawn on possible effects on fertility in men previously exposed to soy-based formula, considering their relatively young age at the time of the follow-up study. The Committee notes, however, that the potential effects of exposure to oestrogenic substances during infancy on subsequent male fertility need to be evaluated.

A retrospective epidemiological study by Fort *et al.* (1990) found that children with autoimmune thyroid disease were significantly more likely to have been fed soy formulae in infancy: the frequency of previous feedings with soy based formulae in infancy was 31% in 59 patients with autoimmune thyroid disease, but only 12% in their 76 healthy siblings ($p<0.01$) and 13% in healthy nonrelated controls ($p<0.02$). There was no group difference in the frequency and duration of breast feeding. The aglucons of genistein and daidzein were demonstrated to inhibit the activity of thyroid peroxidase purified from porcine thyroid glands when present at concentrations of 1 to 10 μM, resulting in iodinated isoflavone compounds. Four months old infants fed soy protein formulae were shown to have plasma levels of isoflavones in the range of 1 to 4 μM/L (Setchell *et al.*, 1998). The presence of at least 150 μM of iodine per litre in the incubation mixture completely protected against the isoflavone mediated thyroid peroxidase inactivation (Divi *et al.*, 1997).

A preliminary report in abstract form did not indicate any oestrogenic hormonal effects in children fed soy formula (Businco *et al.*, 2000).

In infants at risk for atopic diseases there was no difference in the cumulative incidence of atopic diseases after 5 years between children fed on soy or cows' milk-based formula (Chandra, 1997). In fourty-nine children with a history of peanut allergy identified in a cohort study of 13,971 preschool children there was a significant independent association with the intake of soy infant formula or soy milk (odds ratio 2.6; 95 percent confidence interval, 1.3 to 5.2) (Lack *et al.*, 2003).

Both cows' milk protein and soy protein isolate may be regarded as nutritionally adequate in infant formula. However, in view of some remaining uncertainties on the short- and the long-term effects of a high isoflavone intake in infancy and on the potential to influence allergic and autoimmune disease, the Committee is of the opinion that soy-based formula should be reserved for specific situations only and that cows' milk-based formula should be the standard choice.

4.2.3.2 Protein and amino acids, digestibility, bioavailability of nutrients, allergenicity of other plant proteins

The same considerations as for alternative milks and for soy protein isolate have to be made. It is well known that some indispensable amino acids are deficient in plant proteins and that the digestibility of these proteins is significantly less than that of milk proteins.

The increased phytic acid content of plant proteins, that can inhibit the availability of minerals and trace elements, has to be considered and adequately tested for. If necessary, the contents of minerals whose absorption is impaired have to be adapted (Lönnerdal *et al.*, 1984). Reduction of phytate content increased the absorption and availability of zinc and copper in rhesus monkeys and rats and of iron in infants (Davidsson *et al.*, 1994a; Lönnerdal *et al.*, 1999). Moreover, phytochemicals like flavonoids occur in plant proteins and their effects on health have to be assessed before the use of the plant protein in the manufacture of formula.

4.2.4 Recommendation

The Committee concludes that at present there is no documented benefit in the choice of other animal milk proteins or of plant proteins over cows' milk protein in the manufacture of infant formula. If other protein sources are to be used, their suitability and safety must be assessed before commercialisation. Data required are amino acid contents and availability, allergenicity, digestibility and technical processing, and constitutuents other than protein and nitrogenous compounds. Controlled clinical studies are needed to assess the nutritional safety and nutritional value.

4.3 Protein hydrolysates

Hydrolysed protein is permitted in the manufacturing of infant formula intended for healthy non-breast-fed infants at risk for atopic diseases. The method and extent of hydrolysis and processing must be documented but are not regulated. The minimum protein level is 2.25 g/100 kcal. The protein content is calculated with a conversion factor of 6.25 and both taurine (42 μmoles/100 kcal) and L-carnitine must be added (7.5 μmoles/100 kcal).

The amount of immunoreactive protein must be less than 1% of nitrogen containing substances in the formulae to substantiate a claim on reduced antigen content. Objective and scientifically verified data as proof to the claimed properties must be available. The methods for verification are mentioned in the report of the Committee from 1993: molecular properties of the hydrolysate, assessment of the actual reduction of antigenicity compared with the original protein by *in vitro* as well as by *in vivo* (animal) tests. However, from recent studies on the clinical effectiveness in preventing or delaying the manifestation of atopic diseases in infants at risk, it appears that data on the degree of hydrolysation and the residual antigenicity of a hydrolysate do not predict clinical effectiveness.

Moreover, the Committee proposed in 1993 that the nutritional efficacy of all products should be demonstrated by a longitudinal study on weight and height development over at least 3 months, involving at least 20 babies born at full term and aged less than one month at the beginning of the study, which should include, if necessary, data on plasma levels of albumin and short half-life proteins, and on the plasma amino acid profile.

The results of clinical studies depend on the type of protein hydrolysed (milk protein, soy protein, serum albumin, collagen), and the extent and method of hydrolysation, and of

processing (Rudloff and Kunz, 1997). Rigo *et al.* (1989) demonstrated that feeding a whey hydrolysate formula (1.6 g protein/100 mL; N x 6.38) to 11 term infants during the first six days of life resulted in higher concentrations of the sum of indispensable amino acids and of the ratio of indispensable amino acids to total amino acids in plasma than in 10 breast-fed infants and nine infants fed a whey-predominant conventional formula (1.5 g protein/100 mL). The threonine level in plasma was more than double the level in breast-fed infants. The same changes were observed in 13 term infants receiving the whey hydrolysate formula for 33 days. Whereas gain in weight, length, head circumference and the serum levels of retinol binding protein, prealbumin, transferrin and IgG were comparable to those of breast-fed infants, there was less gain in skinfold thickness and a lower total protein level in serum in the infants fed the hydrolysate formula (Rigo *et al.*, 1994a). The plasma levels of tyrosine, proline and phenylalanine were significantly decreased, the threonine level increased in the formula group.

In a further study the same authors evaluated the effects on growth, serum proteins, and plasma amino acids of five different protein hydrolysate formulae (protein content N x 6.38) fed to 71 infants during the first month of live in comparison to 23 breast-fed infants. Three formulae were based on whey-hydrolysate and produced roughly comparable disturbances of the plasma amino acid pattern: elevation of threonine, isoleucine, lysine and citrulline and decrease of tyrosine. However, whereas the gain in weight, length and head circumference was comparable to breast-fed infants with two of the formulae (1.6 g protein/100 mL), the feeding of one whey-hydrolysate formula (1.7 g protein/100 mL) resulted in a reduction of weight, length and head circumference gain by 50%, 15% and 30%, respectively. Two other hydrolysate formulae (1.5 g protein/100 mL) based on soy/collagen (50:50) and a whey/casein (50:50) mixture also resulted in impaired growth, reduced serum transferrin levels and plasma amino patterns reflecting their amino acid composition (Rigo *et al.*, 1994b). Metabolic balance studies were performed in ten infants receiving the suspect whey-hydrolysate formula and 5 infants on the whey-casein-hydrolysate formula and compared to twelve infants on human milk and 86 infants on conventional infant formulae. Nitrogen absorption in infants receiving the whey hydrolysate was significantly lower than in those on conventional formula, but was similar to that observed in the group of breast-fed infants. However, nitrogen utilisation (nitrogen retained/nitrogen absorbed) was significantly lower (62%) than both in conventional formula (66%) and human milk (77%) fed infants. Fat absorption, calcium, phosphorus and magnesium absorption and retention were similar to those infants fed conventional formulae. The infants fed the whey-casein-hydrolysate formula absorbed only 72% of nitrogen, 38% of fat, 27% of calcium, 75% of phosphorus and 30% of magnesium, which is significantly lower than the values observed in infants fed human milk or conventional formula. Nitrogen utilisation was reduced to 55% (Rigo *et al.*, 1995).

Vandenplas *et al.* (1993) compared the nutritional value of a whey hydrolysate formula (1.5 g protein/100 mL; 2.2 g protein/100 kcal) and a whey-predominant formula (1.4 g protein/100 mL; 2.1 g protein/100 kcal) based on whole protein in 20 and 25 healthy infants from birth to 3 months of age. (For both formulae the protein content was calculated as N x 6.25.) In spite of a significantly lower volume intake in the hydrolysate group, there were no differences in weight gain and length. Blood urea levels and urine urea excretion were significantly higher in the group fed the hydrolysate. Serum zinc and iron binding capacity were significantly higher in the hydrolysate group. All other haematologic and blood chemistry values were similar.

A recent study evaluated the effects of feeding three different extensively hydrolysed protein formulae to healthy term infants from 6 weeks to 6 months of age on growth, plasma amino

acids, haematology and trace elements in comparison to breast-fed infants and to infants receiving a whey-predominant standard formula for the same length of time. The two casein-hydrolysate formulae (true protein (α-amino nitrogen x 6.25) content 1.9 g/100 mL corresponding to 2.8 and 2.7 g/100 kcal) differed from the whey-hydrolysate formula (1.6 g protein/100 mL, 2.3 g/100 kcal) in fat, calcium and iron content. The conventional formula contained less protein (1.3 g/100 mL; 2 g/100 kcal), less calcium, iron and copper. There were no differences in weight and length and weight/length gain in the five feeding groups. The infants receiving the casein-hydrolysates demonstrated the highest blood urea nitrogen levels. All hydrolysate formulae increased the plasma levels of threonine, valine, phenylalanine, methionine, tryptophan and branched-chain amino acids above values seen in breast-fed infants, with tryptophan and threonine level highest in infants fed the whey-hydrolysate. Tyrosine levels in infants fed the casein-hydrolysate were lowest. Haemoglobin and transferrin receptor concentrations were similar in all feeding groups, ferritin levels were lowest in infants fed the casein-hydrolysates. Serum zinc and copper concentrations did not show differences, confirming earlier studies on zinc absorption (Hernell and Lönnerdal, 2003). Extensively hydrolysed protein formulae, however, are at present not covered by the Infant Formulae Directive.

In an unselected population of more than a thousand infants the feeding of a partially hydrolysed-whey formula (1.64 g protein/100 mL or 2.25 g protein/100 kcal; protein N x 6.38) instead of or supplementing breast-feeding did not show a difference in growth parameters at three and six months compared either to exclusively breast-fed or partially breast-fed infants supplemented with cows' milk protein formula or that formula alone (Exl *et al.*, 2000).

Infant formula with a reduced antigen content due to partial hydrolysis of their protein source are intended as (prophylactic) formulae for non-breast-fed healthy infants at risk of atopic diseases. More antigen-reduced formulae due to extensive hydrolysis of their protein source and suitable for the treatment of infants with demonstrated protein allergy are not infant formula but foods for special medical purposes and shall not be discussed further. However, compositional and nutritional differences between partial-hydrolysates exist. This concerns the molecular weight pattern of the hydrolysed protein and the eventual presence of potentially active peptides, due to different enzymes employed in hydrolysis and subsequent ultrafiltration, different heat treatment, etc. These differences will have effects on the nutritional value and on the intended action of preventing sensitisation and inducing oral tolerance. Pre-clinical testing of formulae based on protein hydrolysates with respect to their physicochemical properties (extent of hydrolysis, size exclusion chromatography, peptide weights) and to their immunological properties (residual antigenicity; *in vitro* and in animal models, immunogenicity in animal models) by standardised and validated methods should precede clinical tests (Leary, 1992). Whereas the effects of some hydrolysate formulae have been evaluated in numerous published studies, others are put on the market without competent clinical testing of their nutritional safety. This is unacceptable and is illustrated by the examples cited above.

Ingestion of hydrolysed protein accelerates gastric emptying compared to intact proteins, it induces faster and higher total amino acid, indispensable amino acid and branched-chain amino acid increases in plasma in combination with higher insulin levels and higher insulin/glucagon ratios. This effect could be stimulatory for protein synthesis but could also result in increased amino acid oxidation (Calbet and MacLean, 2002).

4.3.1 Conclusion and recommendation

Infant formula based on hydrolysed proteins are insufficiently characterised by declaring the protein source and amount and by designating them as low-degree (partial) or high-degree (extensive) hydrolysates with respect to their nutritional value.

Hydrolysis of proteins and protein mixtures is done with different enzymes, the hydrolysate can be processed in different ways and the resultant "protein" is insufficiently characterised by the declared protein value alone, even if it fulfils the regulatory criteria concerning amino acid patterns and contents. However, the dossiers on the nature of the hydrolysate and the data on eventually performed clinical studies are not readily available. It appears difficult to identify products already on the market from published literature. Moreover, additional compositional variations in infant formulae containing hydrolysates that have already been studied can modify the nutritional value of the formula and clinical outcome parameters. This has been clearly demonstrated by the examples cited. Whereas it has been demonstrated that hydrolysates of whey-casein mixtures with low protein contents similar to formula with intact proteins can lead to normal growth and plasma amino acid patterns comparable to breast-fed infants, it has been shown for some products that they were nutritionally inadequate. It is unknown if such products were removed from the market. The inherent claim that hydrolysates result in less allergic diseases cannot be deduced from technical data alone and needs substantiation in clinical trials. Surprising is the total lack of clinical studies published on follow-on formulae based on partially hydrolysed proteins.

The Committee proposes there should be a requirement for adequate clinical testing before commercialisation of protein hydrolysate-based formulae.

4.4 Protein content

4.4.1 The model of human milk

It is customary to state that infant formula - to a lesser degree follow-on formula - because it has to substitute for human milk in non-breast-fed infants, should be as similar as possible to human milk. Although it can be assumed that human milk provides adequate amounts of energy and nutrients for growth and development of infants breast-fed exclusively by well-nourished mothers during the first months of life (except for vitamins D and K), this does not mean that copying the composition of human milk as far as possible will guarantee the same effect on growth and development. Instead breast-milk substitutes have to be designed with a view to create similar nutritional and physiological effects as breast-milk feeding.

Moreover, human milk is a food of changing composition during the lactational period, during 24 hours and during one meal, whereas an infant formula is a product of constant composition and, therefore, must be a compromise on the safe side, both as to the amount of protein as to the quality of the protein. Protein requirements of formula-fed infants may be greater than requirements of infants receiving human milk due to differences in utilisation of the proteins.

4.4.2 Protein requirements from studies with infant formulae compared to the human milk model

The protein intake of breast-fed infants was recalculated by Dewey *et al.* (1996) on the basis of "true or adjusted protein" (α-amino nitrogen x 6.25) (see Table 3).

Assuming an energy density of human milk of 67 kcal/100 mL and the measured volume intakes one arrives at protein energy ratios of 1.7 to 1.84 g/100 kcal at one month of age and of 1.28 to 1.34 g/100 kcal at age two months. It further declines thereafter.

Fomon *et al.* (1995a) tested in 15 male infants a formula with 1.56 g/100 kcal at ages 8 to 27 days thereafter gradually decreasing in protein density to 1.25 g/100 kcal at 84 days of age. This formula was fed from day 84 to 111. A control group of 13 infants received formula with 2.14 g protein/100 kcal. A group of 261 infants fed formulae with protein densities between 1.98 to 2.67 g/100 kcal served as reference. Gains in length and levels of serum urea nitrogen were significantly less in the experimental group, whereas gains in weight and serum albumin levels did not differ between the three groups. The authors concluded that the protein energy ratio of the experimental formula was below the safe level of intake, and that the protein energy ratio in the control formula was higher than the safe dietary intake.

Protein in this study was calculated from nitrogen content multiplied by 6.09. Applying the factor 6.38 as requested in the Infant Formulae Directive the protein energy ratios would have been 1.63 to 1.31 g/100 kcal for the experimental formulae, 2.24 g/100 kcal for the control formula and 2.07 to 2.8 g/100 kcal for the reference formulae.

Räihä *et al.* (1986) compared the effects on weight, length, head circumference development, on serum albumin, blood urea nitrogen and urinary nitrogen excretion of breastmilk, low-protein infant formula (1.8 g/100 kcal) and "normal" protein infant formula (2.2 g/100 kcal) fed ad libitum in 10 infants each for three months. There were no significant differences in weight, length, head circumference and serum albumin. However, during the first four weeks of life blood urea nitrogen and nitrogen excretion were lower in the low-protein formula group. Thereafter they were similar to the breast-fed group. The "true protein" calculated from amino acids in the formulae was 1.6 and 2.14 g/100 kcal, respectively. The protein intake in the low-protein formula group was lower than in breast-fed infants during the first month of life. Räihä (1994a) recommended formulae with a crude protein content of 2.2 g protein/100 kcal during the first month of life, 2.0 g/100 kcal in the second month, 1.8 g/100 kcal in the third month and 1.6 g/100 kcal in the fourth month.

Fomon *et al.* (1999) tested a cows' milk-based formula from day 8 to 112 of life in 16 male infants that contained 1.7 g protein/100 kcal or 1.15 g protein/100 mL. Protein was calculated as total nitrogen x 6.11(according to the crude protein calculation in the EU this corresponds to 1.2 g protein/100 mL or 1.8 g protein/100 kcal) and compared it to earlier data from infants fed cows' milk formula (1.8 to 2.7 g/100 kcal) and breast-fed infants. Infants receiving the experimental formula gained more weight and had higher body mass indices. Serum urea was similar to the breast-fed group. However, because energy intakes were significantly higher in the *ad libitum* fed group on the experimental formula, the authors concluded that the protein energy ratio of the experimental formula was not "safe" although the protein intake was judged to be adequate between one and 8 weeks of age.

Räihä *et al.* (2002) compared three whey modified cows' milk-based formulae in 29 infants each, two with 1.9 g protein/100 kcal (protein = total nitrogen x 6.38) and the other with 2.3 g protein/100 kcal, which were fed from birth to the age of 4 months. The NPN content was 10% of TN in all three formulae. The "adjusted" protein [(TN - NPN + 40% NPN) x 6.38] content was given as 1.8 and 2.2 g/100 kcal, respectively. Nitrogen retention (117 mg/kg/day) did not differ in the infants receiving the low- *versus* the higher-protein formula. The amino acid pattern of the formulae with a protein content of 1.8 g/100 kcal was remarkably similar to

the pattern of human milk after correction of the human milk pattern given in the publication for nitrogen. There was no difference in gains of weight and length, or in serum albumin levels. Serum urea concentrations were higher in the higher-protein formula-fed group. The lower-protein formulae were judged to meet needs of normal infants. In another study two whey-predominant formulae with protein contents of 1.83 g/100 kcal and 2.24 g/100 kcal were compared in 8 normal infants between 28 and 128 days of age in a cross-over design. Despite the lower protein intake with the low-protein formula nitrogen retention was similar in both groups (Ziegler, 2002).

These studies indicate that, depending on protein quality, whey adaptation and processing, a formula with a crude protein content of 1.9 g/100 kcal is safe in infants especially over the age of two months.

4.4.3 The minimum protein content in infant formulae

At present the Infant Formulae Directive requests at least 1.8 g of crude protein/100 kcal for cows' milk-based formula and 2.25 g/100 kcal for soy protein and partially hydrolysed protein formula. Protein is calculated as crude protein with a factor of 6.38 for the first and with a factor of 6.25 for the two latter.

A content of 1.8 g crude protein according to the Directive corresponds to 1.76 g crude protein calculated with a factor of 6.25, to 1.72 g "true" protein according to Fomon *et al.* (1995a), to 1.62 g "adjusted" protein according to Dewey *et al.* (1996), and 1.66 g according to Miera (1998) and 1.59 g according to the the simplified LSRO method (see section 4.1.1). The Expert Panel of the LSRO (1998) stresses the necessity to calculate the "true" minimum protein content for all kinds of formula from α-amino nitrogen to avoid unusual amounts of non-available nitrogen to be included into the protein content calculation. This report suggests 1.7 g "true protein"/100 kcal (α-amino nitrogen x 6.25) as the minimum protein level for all kinds of protein sources. Expressed as crude protein (N x factor 6.25) this corresponds to 1.87 g/100 kcal.

From the data in section 4.4.2 it appears that a crude protein content of 1.8 g/100 kcal in an infant formula is marginal for normal growth in young infants. The Committee does, therefore, not recommend feeding a formula with a crude protein content of 1.8 g/100 kcal to young infants. However, the Committee proposes to retain the present minimum level for the range of crude protein levels to be provided by infant formula, because it does not recognise an urgent necessity to increase the crude protein content of infant formula presently on the market and because an increase in the minimum level would be likely to lead to an unnecessary increase in the average protein content of infant formula.

The higher minimum levels for crude protein set in the Infant Formulae Directive for infant formula with intact proteins other than intact cows' milk protein, i.e. soy protein isolate, aims to correct for potentially less digestibility and therefore availability of the "protein" compared to intact cows' milk protein. There are insufficient data to justify a change in the minimum protein level (2.25 g/100 kcal) in soy formulae.

Any protein source which has not yet been evaluated should be clinically tested before being used in the manufacture of infant formula for its capability to support normal growth in comparison to infants either breast-fed or fed a reference formula.

To our knowledge there are no systematic studies to assess growth and biological parameters

of infant formulae with partially hydrolysed protein to determine the minimal safe protein content. Therefore, the Committee does not propose a change in the minimum protein content (2.25 g/100 kcal).

4.4.4 The minimum protein content in follow-on formulae

The minimum protein content required for follow-on formulae in the EU is no longer justified (see section 2.2). It is even discussed that the resultant protein intake of 13 to 20% of the energy intake in infants drinking follow-on formula and eating (protein-rich) complementary food (Schöch and Kersting, 1996; Nielsen *et al.*, 1998; Alexy *et al.*, 1999) could program these children for disease such as obesity in adult life, apart from inherent negative effects of a high protein intake on renal function and water balance. The weakness of most human studies in that field is that they are retrospective and do not provide data on absolute protein intake apart from protein-energy-ratios. Prospective longitudinal studies are needed to provide more conclusive information (Metges, 2001).

The Committee proposes to reduce the minimum crude protein content for follow-on formulae to 1.8 g/100 kcal. If the energy content of follow-on formula remained at 60 to 80 kcal/100 mL this would result in crude protein contents between 1.08 g and 1.44 g/100 mL. Changing the energy content of follow-on formula to 60 to 70 kcal/100 mL will result in minimum protein concentrations of 1.08 to 1.26 g/100 mL.

In analogy to infant formula, the Committee proposes to retain the minimum crude protein content (TN x 6.25) of follow-on formula based on soy protein isolate and protein hydrolysate (2.25 g/100 kcal).

A model calculation was performed to demonstrate the effect of changes in energy and protein contents of both infant and follow-on formulae based on intact cows' milk protein on the intake of protein in infants between 4 and 7 months of age. For this calculation the recommended feeding regimen for the first year of life was used, which has been developed by the German Research Institute for Child Nutrition (Kersting *et al.*, 1994). This has the advantage that the recipes of the complementary meals in addition to formula are fixed and their nutrient and energy content has been estimated.

According to this regimen infants are fed exclusively on breast-milk or infant formula for the first four to six months of life. A vegetable-potato-meat meal is introduced at the earliest at the beginning of the 5th month, about four weeks later a milk-cereal meal is added and again about one month later a cereal-fruit meal, while formula (or breast-milk) is continued in decreasing amounts.

Infant formula at present provides 60 to 75 kcal and 1.1 to 2.25 g crude protein/100 mL. Follow-on formula provides at present 60 to 80 kcal and 1.4 to 3.6 g crude protein/100 mL. Infant and follow-on formulae modified according to the new proposals would provide 1.08 to 2.1 g crude protein/100 mL.

A. During the 5th to 7th months of life an infant is assumed to consume 870 mL of infant formula per day. The recommended protein intake at that age is 14 g per day (SCF, 1993a).

   The calculated intakes are given in the following Table in g per day.

	Protein intake
Present infant formula	9 - 20
Proposed infant formula	9 - 18

B. During the 5th month of life at the earliest an infant is assumed to consume 600 mL of formula, either infant or follow-on formula and a vegetable-meat meal (VMM), which provides 200 kcal and 6.1 g protein.

The recommended protein intake is 14 g/day.

The calculated intakes are given in the following Table in g per day.

	Intake from formula	Total intake from formula + VMM
	protein	protein
Present infant formula	6 - 13.5	12 - 20
Proposed infant and follow-on formulae	6 - 12	12 - 18
Present follow-on formula	8 - 22	14 - 28

C. During the 6th month of life an infant is assumed to consume 400 mL of formula, a VMM which provides 194 kcal and 7.8 g of protein and a milk-cereal meal (MCM) which provides 215 kcal and 9.4 g protein. The recommended protein intake is 14 g protein per day.

The calculated intakes are given in the following Table in g per day.

	Intake from formula	Total intake from formula VMM plus MCM
	protein	protein
Present infant formula	4 - 9	22 - 26
Proposed infant and follow-on formulae	4 - 8	22 - 25.5
Present follow-on formula	5 - 14	22 - 31

D. During the 7th month an infant is assumed to consume 240 mL formula per day, and a VMM providing 225 kcal and 9.3 g protein, a MCM providing 216 kcal and 9.4 protein and a cereal- fruit meal (CFM), providing 180 kcal and 3.2 g protein per portion. The recommended protein intake is 15 g/day.

The calculated intakes are given in the following Table in g per day.

	Intake from formula	Total intake from formula, VMM, MCM and CFM
	protein	protein
Present infant formula	3 - 5	25 - 27
Proposed infant and follow-on formulae	3 - 5	25 - 27
Present follow-on formula	3 - 9	25 - 31

From this model calculation it appears that infants between 4 and 6 months who do not receive complementary food can have a protein intake at the safe level of protein intake as given in Table 4 if they are fed formula with the minimum permitted protein content. A

protein intake below the safe level of intake, by definition, does not mean that in individuals this intake would be deficient.

The use of infant or follow-on formulae with the minimum protein amount can result in total protein intakes in infants fed formula exclusively or in infants with little complementary food to critically low levels.

From this model calculation the lowering of the maximum protein content of follow-on formula appears to be desirable.

These model calculations also indicate that an increasing number of complementary meals tend to reduce the contribution of formula to the protein intake.

4.4.5 The maximum protein content in infant formulae

The Infant Formulae Directive sets a maximum value of 3.0 g crude protein/100 kcal for all types of formulae. For formulae based on intact cows' milk this is calculated with a factor of 6.38, for all others with 6.25.

This is lower than the maximum level proposed in the LSRO report (1998): 3.4 g crude protein/100 kcal (calculated from total nitrogen x 6.25).

There is no justification on nutritional grounds for a higher value than the current one. Formulae providing more than 12% of energy as protein are not needed by healthy infants. They increase the potential renal solute load of a formula which is predominantly determined by the protein content. Infants with extra water losses in the course of illnesses could become endangered by hypertonic dehydration.

4.4.6 The maximum protein content in follow-on formulae

The considerations and model calculations under section 4.4.4 lead the Committee to propose a reduction of the crude protein content of follow-on formula based on intact proteins and on hydrolysed protein to 3.0 g/100 kcal.

4.5 Protein quality

All calculations of protein requirements have to consider the protein quality. Determinations of protein requirements from measured protein intakes of breast-fed infants, by the factorial method or by nitrogen balances are valid only for the protein consumed. This is especially true during infancy with its rapid growth and the ressulting high needs for protein synthesis.

The Infant Formulae Directive requires protein quality to be assessed by the "chemical index" or amino acid score with human milk as the reference protein for both cows' milk and soy protein based formula and formula with hydrolysed protein and for follow-on formula. For follow-on formula alternatively casein can be used as the reference protein.

In addition, the protein efficiency ratio (PER) and net protein utilisation (NPU) for partial hydrolysates of protein must be similar to casein.

4.5.1 Amino acid score

The Infant Formulae Directive has converted the amino acid pattern of human milk protein (mg/1 g protein) (Annex VI) into minimum amino acid amounts that will have to be provided by an infant formula per 100 kcal, by multiplication of the pattern of Annex VI by the minimum protein content of 1.8 g/100 kcal in infant formula. A formula with a higher protein content can, therefore, compensate for possible deficits in the amino acid pattern of the protein used by providing more of that protein.

Not the quality of the protein *per se* as to its amino acid pattern in comparison to human milk (or casein) is assessed but the amounts of single amino acids per energy value in comparison to human milk.

This is named amino acid rating. This is, of course, only valid if the protein content of both formula and human milk is calculated in the same way and corrected for by digestibility (true protein digestibility - relative amino acid rating, TPD - corrected RAAR).

It has been shown in pig models, that the digestibility corrected amino acid pattern of human milk differs from the amino acid pattern of the protein (Darragh and Moughan, 1998). The same can be expected for proteins in infant formula. However, as long as there are no reliable data, the present requirements as to protein quality will have to suffice as the best compromise.

For formulae based on soy protein isolates the requirements for protein quality as determined by amino acid score rating should be the same as for other infant formulae.

The pattern given in Annexes V and VI of the Infant Formulae Directive will be discussed in section 4.6.

4.5.2 Protein Efficiency Ratio (PER) and Net Protein Utilisation (NPU)

PER and NPU are routinely determined in rats. There are some doubts as to the usefulness of these tests to assess protein quality for humans: rats require higher amounts of sulphur amino acids than humans, weanling rats cannot tolerate lactose, the standardised application of 10% protein in the feed for PER is not achieved with infant formulae, there is poor reproducibility and the costs are high. For protein hydrolysates a PER and NPU are required which corresponds to that of casein as a minimum.

While the determination of PER or NPU or NPR (net protein ratio) can give valuable information in preclinical testing of newly introduced protein sources (Sarwar *et al.*, 1989), these methods are not sufficient to characterise the nutritional value and to substitute for clinical testing. The Committee proposes that the obligatory request for PER and NPU in the Infant Formulae Directive is not necessary, and that it should be replaced by a requirement for clinical testing of any proteins that have not been evaluated before for use in infant formulae.

4.5.3 Other qualifying factors for protein

Methionine and cyst(e)ine cannot substitute completely for each other. Infants have a basic need for both. It should be considered not to allow the sum of both to be used in amino acid rating (this is the current rule). Whereas the methionine to cystine ratio on a weight basis is 1.1 in human milk, it is almost 3 in cows' and goat's milk. Ratios of 2 and more should not be

accepted in infant formula without clinical testing. Also the ratio of phenylalanine to tyrosine, which is about 1 in human milk should not be lower than 0.5 in infant formula.

For soy protein-based infant formula there is already a requirement in the Directive to provide the same amount of methionine per energy value as human milk. The Committee proposes to request complete amino acid rating as for the other types of infant formula.

If a specified taurine content is considered to be relevant logically this should not be restricted to formula with hydrolysed protein, but the addition should be permitted irrespective of the protein source.

4.6 Amino acids in reference proteins

4.6.1 Comparison to the FAO/WHO/UNU scoring pattern

The Infant Formulae Directive applies the amino acid pattern for human milk protein developed from a FAO report (1970) not the pattern suggested by FAOWHO/UNU in 1985 and not replaced since. In addition to the nine amino acids in the 1985 pattern (histidine, isoleucine, leucine, lysine, methionine plus cystine, phenylalanine plus tyrosine, threonine, tryptophan, and valine), the Infant Formulae Directive contains values for arginine and lists cystine and methionine, and phenylalanine and tyrosine separately.

The 1970 pattern makes reference to crude protein calculated with the factor 6.38, the FAO/WHO/UNU pattern from 1985 to crude protein calculated with the factor 6.25. If one corrects for this difference the following values result:

Table 9. Amino acid content of human milk (g/100 g crude protein)

	FAO, 1970	**FAO/WHO, 1985**	
	protein = N x 6.38	protein = N x 6.25	protein = N x 6.38
Arginine	3.9	-	-
Cystine	1.3	-	-
Histidine	2.5	2.6	2.55
Isoleucine	4.1	4.6	4.51
Leucine	8.8	9.3	9.11
lysine	6.8	6.6	6.47
Methionine	1.6	-	-
Methionine plus cystine	-	4.2	4.11
Phenylalanine	3.5	-	-
Threonine	4.5	4.3	4.21
Tryptophan	1.7	1.7	1.67
Tyrosine	3.3	-	-
Phenylalanine plus tyrosine	-	7.2	7.05
Valine	4.5	5.5	5.39

The differences are small. It must be considered if the reliability of values determined before 1970 is still acceptable. The same criticism could be offered for the 1985 values.

The Committee proposes to delete arginine from the scoring pattern in Annex V and Annex VI to of the Directive. There is no scientific evidence to prove that healthy full-term infants

need a specified amount of dietary arginine. The presently valid request for a minimum arginine content of 69 mg/100 kcal in infant formula has led to the addition of free arginine to 4 of 15 infant formulae analysed, whereas 5 of these 15 products did not comply with the requirement for arginine (Miera, 1998).

4.6.2 Other scoring patterns

The report of the LSRO from 1998 has combined and calculated the means of amino acid contents in human milk of more recent date (Davis *et al.*, 1994; Janas *et al.*, 1987; Sarwar *et al.*, 1996; Picone *et al.*, 1989a). However, one of these publications is on transitional milk (Sarwar *et al.*, 1996), and only one has analysed complete 24-hour collections of human milk (Davis *et al.*, 1994). These values are referenced to "true protein" (α-amino nitrogen x 6.25) and would therefore have to be corrected to become comparable to the FAO values. In converting these values to mg/100 kcal methionine and cystine and phenylalanine and tyrosine have been added, however, under the precautionary statement that their ratios should not exceed 2:1 or 1:2, respectively.

The LSRO report recommends the setting of maximum values per 100 kcal for each of the listed amino acids (two times the minimum values) to avoid the use of proteins with imbalanced amino acid patterns.

The Food and Nutrition Board of the Institute of Medicine (FNB, 2002a) has proposed a modified amino acid pattern of human milk, based on 4 references (Darragh and Moughan, 1998; Davis *et al.*, 1994; Heine *et al.*, 1991; Villalpando *et al.*, 1998). However, again the reference unit in the different sources is not the same although expressed in mg amino acid per g protein. For example "protein" is the sum of total anhydrous amino acids (Davis *et al.*, 1994, Heine *et al.*, 1991) or total nitrogen multiplied by 6.38 (Darragh and Moughan, 1998) or total nitrogen minus NPN multiplied by an unknown factor (Villalpando *et al.*, 1998). To avoid this problem it seems to be advisable to refer the individual amino acid content to the nitrogen content to avoid confusion about the nature of the protein calculation. This has been done for published studies on the amino acid content of human milk which report measurements of the total nitrogen content and/or the calculation method for the protein content (Table 10).

The studies listed in this Table cover milk samples from about 120 women from 4 weeks of lactation to late lactation and from pooled human milk bank samples. The Committee decided not to consider the data of FAO (1970) and of FAO/WHO/UNU (1985) and the studies by Donovan and Lönnerdal (1989b) and by Sarwar *et al.* (1996) because of a small sample size and because of early or unstated sampling periods.

Table 11 compares the amino acid pattern according to Annex VI of the Infant Formulae Directive (recalculated for one gram total nitrogen content by dividing protein by the factor 6.38), of the FNB (2002a) and the mean values from the studies listed in Table 10.

The main differences between the FNB values and the mean values in Table 11 compared to Annex VI concern significantly higher values for cysteine, isoleucine, leucine, phenylalanine, tyrosine and valine. The Committee proposes to revise Annex VI. Starting from the mean values in Table 11 and recalculating them with reference to crude protein (total nitrogen x 6.25) and taking into account the Committee's proposal to delete arginine from the scoring pattern the following pattern results (Table 12).

The values for indispensable and conditionally indispensable amino acids in human milk expressed as mg/100 kJ or 100 kcal given in Annex V of the Infant Formulae Directive will have to be revised based on the proposed amino acid pattern in Table 12 and a minimum crude protein content of 1.8 g/100 kcal.

Table 10. Amino acid content of human milk, expressed as mg per gram of nitrogen

	Lönnerdal and Forsum (1985)	**Darragh and Moughan (1998)**	**Bindels and Harzer (1985)**	**Janas *et al.* (1987)**	**Villalpando *et al.* (1998)**		**Räihä *et al.* (2002) mod. Nayman *et al.* (1979)**
N	milk bank	20	10	10	Mexico 40	Houston 40	milk bank
Stage of lactation	4-16 weeks	10-14 weeks	5 weeks	8 weeks	4-6 m		>1 m
Sampling	pooled bank milk	over 20 days, pooled	24 h, pooled	24 h, pooled	24 h, pooled		pooled bank milk
Arginine	157	200	281	184	168	184	172
Cystine (half)	111	173	108	101	167	134	133
Histidine	111	156	255	112	112	108	122
Isoleucine	242	333	376	306	292	331	300
Leucine	457	598	713	611	528	541	572
Lysine	314	406	522	365	366	408	361
Methionine	78	90	89	73	99	76	83
Phenylalanine	153	243	344	183	440	439	217
Threonine	217	316	344	251	248	242	256
Tryptophan	n.a.	n.a.	172	79	112	89	111
Tyrosine	201	241	369	191	292	299	233
Valine	253	327	376	267	286	331	317
Methionine + cystine	289	-	-	-	-	-	-
Phenylalanine + tyrosine	354	-	-	-	-		-
Total N mg/L	**2070**	**1800**	**1760**	**1800**	**1610/1570**		**1800**

n.a. = not analysed

Table 11. Amino acid content of human milk expressed as mg per gram of nitrogen from different sources

	Annex VI 91/321/EEC	**FNB 2002a***	**Mean value of data in Table 10**
Arginine	242	n.a.	192
Cysteine	83	140	132
Histidine	159	146	139
Isoleucine	255	363	311
Leucine	542	643	574
Lysine	427	439	392
Methionine	102	102	84
Phenylalanine	217	255	288
Threonine	281	299	268
Tryptophan	108	115	113
Tyrosine	204	299	261
Valine	287	357	308

* Protein converted to nitrogen by dividing by 6.38

Table 12. New proposed amino acid pattern for human milk protein expressed as g/100 g protein to replace Annex VI of the Infant Formulae Directive

Amino acid	g/100 g protein
Cystine*	2.1
Histidine	2.2
Isoleucine	5.0
Leucine	9.2
Lysine	6.3
Methionine*	1.3
Phenylalanine**	4.6
Threonine	4.3
Tryptophan	1.8
Tyrosine**	4.2
Valine	4.9

* Sum of cystine and methionine may be used as basis of calculation if the methionine:cystine ratio ≤2

** Sum of phenylalanine and tyrosine may be used as basis of calculation if the tyrosine:phenylalanine ratio ≤2

The new proposed values for indispensable and conditionally indispensable amino acids in human milk expressed as mg/100 kJ or 100 kcal are given in Table 13.

Table 13. New proposed values for indispensable and conditionally indispensable amino acids in human milk expressed as mg/100 kJ or 100 kcal

	mg/100 kJ	mg/100 kcal
Cystine*	9	38
Histidine	10	40
Isoleucine	21	90
Leucine	40	166
Lysine	27	113
Methionine*	6	23
Phenylalanine**	20	83
Threonine	18	77
Tryptophan	8	32
Tyrosine**	18	76
Valine	21	88

* Sum of cystine and methionine may be used as basis of calculation if the methionine:cystine ratio ≤2

** Sum of phenylalanine and tyrosine may be used as basis of calculation if the tyrosine:phenylalanine ratio ≤2

If infants of the age of one and three months, weighing 4.2 and 5.6 kg, consumed 650 and 800 mL/day of an infant formula with the minimum crude protein content of 1.8 g/100 kcal and an energy density of 67 kcal/100 mL, provided the protein corresponded to the amino acid pattern of Table 13, amino acid intakes as given in Table 14 would result.

Comparison with the recent data on amino acid requirements of infants in Table 5 shows that

these requirements are met.

Table 14. Amino acid intake in one- and three-month old infants (body weight 4.2 and 5.6 kg, respectively) consuming 650 and 800 mL of an infant formula with a crude protein content of 1.8/100 kcal with an energy density of 67 kcal/100 mL and the amino acid pattern proposed in Table 13

	One month old infant		Three months old infant	
	mg/day	mg/kg/day	mg/day	mg/kg/day
Cysteine	166	39	204	36
Histidine	174	42	214	38
Isoleucine	392	93	482	86
Leucine	724	172	890	159
Lysine	493	117	606	108
Methionine	100	24	123	22
Phenylalanine	362	86	444	79
Threonine	336	80	413	74
Tryptophan	140	33	172	31
Tyrosine	331	79	407	73
Valine	384	91	472	84

4.6.3 Recommendation

The Committee proposes new values for the amino acid pattern of the reference protein human milk as given in Tables 12 and 13.

The Committee proposes that for all types of infant formulae and follow-on formulae the per energy value the amount of indispensable and conditionally indispensable amino acids should at least correspond to the values in human milk as shown in Table 13. The Committee is of the opinion that the sum of methionine and cystine contents may be used as the basis for calculation if the methionine:cystine ratio does not exceed 2, and the sum of phenylalanine and tyrosine may be used if the tyrosine:phenylalanine ratio does not exceed 2.

4.7 Taurine, carnitine, nucleotides, choline and other nitrogen-containing compounds

4.7.1 Taurine

Taurine is a non-protein amino acid that is found in most tissues and in human milk at all lactational stages (3.4 to 8.0 mg/100 mL or 5.1 to 11.9 mg/100 kcal). It is practically absent in mature cows' milk (Rassin *et al.*, 1978) and formula based on cows' milk protein and soy protein isolates. It is added to many infant formulae without adverse effects and little evidence of benefit and mostly because it is found in human milk.

It has recognised functions in bile acid conjugation. Other roles of taurine in the scavenging of hypochlorous acid produced by activated neutrophils and macrophages during the respiratory burst (Cunningham *et al.*, 1998), in the detoxification of retinol, iron and xenobiotics and in calcium transport, myocardial contractility, osmotic regulation and in the central nervous system have been shown mostly in *in vitro* or animal experiments (Gaull, 1989). Taurine is found in high concentrations in foetal and neonatal human brain (Sturman, 1988). Infants fed parenterally developed low levels of taurine in plasma and urine and

changes in electroretinography which could be corrected by taurine supplementation (Sturman and Chesney, 1995).

Infants fed a taurine-supplemented (6 mg/100 mL) infant formula with a protein content of 2 g/100 mL (2.9 g/100 kcal) showed the same growth development from 2 to 12 weeks of age as infants breast-fed or receiving the same formula without taurine. However, blood urea nitrogen levels at 12 weeks were significantly lower than in infants fed the taurine-free formula and similar to breast-fed infants, as were the concentrations of indispensable amino acids in plasma and urine (Räihä *et al.*, 1996). The mechanism of this effect is unclear.

As previously noted (in section 4.5.3) if a specified taurine content is considered to be relevant logically this should not be restricted to formula with hydrolysed protein. The Committee considers that the requirement for a minimum content of taurine in formulae manufacturered from hydrolysed protein is not necessary.

The Committee proposes that, when added, taurine addition to any type of infant formula should be not exceeding 12 mg/100 kcal.

4.7.2 Carnitine

The addition of L-carnitine to infant formula based on soy protein isolate and hydrolysed protein to give a content of at least 7.5 μmoles/100 kcal (1.2 mg/100 kcal) is required in the EU and the Committee does not propose a change. This value is similar or somewhat higher than in human milk (0.9 to 1.2 mg/100 kcal) because of a presumed reduced bioavailability from formula (Warshaw and Curry,1980). Cow's milk is rich in carnitine (around 5 mg/100 kcal*) compared to human milk, therefore carnitine addition to cows' milk-based formula is not necessary. Carnitine is synthesised in the human body at a rate of approximately 0.3 mg/kg/day from lysine and using methionine as methyl donor (Rebouche and Seim, 1998). It is considered an indispensable nutrient for newborn infants (Rebouche, 1992) because of a temporarily compromised synthesising capacity. Its function is the transport across membranes of carboxylic acids that have been activated to the co-enzyme A level, thereby delivering substrates for oxidation and removing toxic compounds.

Infants receiving unsupplemented soy formula for 112 days showed lower serum levels of carnitine, higher levels of free fatty acids and an increased excretion of medium-chain dicarboxylic acids (Olson *et al.*, 1989). The minimal dietary carnitine requirement of a newborn infant has been estimated to be 1.7 mg/kg/day due to a practically absent endogenous synthesis (Scholte and de Jonge, 1987).

The Committee considers the addition of carnitine to follow-on formula is not necessary. Supply from appropriate complementary food and from endogeneous synthesis should be sufficient in older infants. Only the liver butyrobetaine hydroxylase, the last enzyme in carnitine biosynthesis, shows age-dependent low activity in young infants. The activity of the kidney enzyme and the other three biosynthetic enzymes in the liver and other tissues are not age-dependent (Vaz and Wanders, 2002)

4.7.3 Nucleotides and nucleosides

Nucleosides contain a nitrogenous base and a pentose, but no phosphate group. Nucleotides contain a nitrogenous base, a pentose and one or more phosphate groups. Nucleotides are found primarily intracellularly. They are the structural components of DNA and RNA.

*The figure has been changed from "50 mg/100kcal" to "5 mg/100kcal" following a corrigendum sent by EFSA notifying the Commission of the typing mistake (letter of 22/08/2007).

Nucleotides such as adenosine triphosphate (ATP) transfer chemical energy. Other nucleotides are involved in the synthesis of proteins, lipids and carbohydrates (e.g. nicotine adenine dinucleotide, NAD; flavin adenine dinucleotide, FAD).

Human milk contains free ribonucleosides and ribonucleotides (Gil and Sanchez-Medina, 1982; Topp *et al.*, 1993; Leach *et al.*, 1995; Schlimme and Schneehagen, 1995) (Table 15). In addition, human milk contains RNA and DNA, of which the major part is located within cells. Leach *et al.* (1995) reported data for "total potentially available nucleosides" released from previously frozen human milk samples after sequential treatment with sodium hydroxide, nuclease, pyrophosphatase, phosphatase and phosphoric acid. This method should detect also nucleotides hydrolysed from polymeric ribonucleotides (RNA and DNA) and adducts, and includes intracellular nucleosides. Leach and coworkers reported markedly higher concentrations of "total potentially available nucleosides", as compared to the reported amounts of free ribonucleosides and ribonucleotides (Table 15). Of the "total potentially available nucleosides", 48±8% were reported to be polymeric nucleotides, 36±10% monomeric nucleotides, 8±6% nucleosides and 9±4% adducts (nucleoside-phosphate-phosphate-X, e.g. uridine diphosphate galactose or NAD) (Leach *et al.*, 1995). However, it is not known which proportion of the "total potentially available nucleosides" in human milk is utilised by the breast fed infant in vivo. While human milk nucleotides might exert beneficial effects in the breast-fed infant, it is also possible that nucleotides and nucleosides, RNA and DNA might occur in human milk only as a by-product of milk formation that reflect metabolic activity of the mammary gland tissue, shedding of somatic cells and occurrence of microorganisms, without having a specific function for the infant. Accordingly, higher concentrations of ribonucleosides in colostrum than in mature milk have been interpreted as a consequence of the high metabolic activity of the mammary gland during the first days postpartum, along with higher contents of cellular components (Schlimme *et al.*, 2000).

Table 15. Reported mean contents of ribonucleosides, ribonucleotides, and so-called "total potentially available ribonucleosides" in mature human milk (mg/L)

	Adenine	**Cytosine**	**Guanine**	**Uracil**	**Total**
Ribonucleotides[1]	7.8	7.0	1.2	4.8	
Ribonucleosides[2]	0.4-1.8	1.0-1.2	0.1-0.3	0.1-1.7	
"Total potentially available nucleotides"[3]	10.6	29.3	6	12.6	67.2-67.5

[1] Gil and Sanchez-Medina, 1982
[2] Topp *et al.*, 1993; Leach *et al.*, 1995; Schlimme and Schneehagen, 1995
[3] Leach *et al.*, 1995

Nucleotides and nucleosides are synthesised *de novo* in human metabolism, and nucleotides liberated during nucleic acid catabolism can be reutilized via a salvage pathway. Hence nucleotides and nucleosides are considered to be dispensable nutrients. In breast-fed infants the dietary intake of preformed nucleotides accounts for only a minor fraction of the requirement. Uauy *et al.* (1994) estimated that only about 2% of nucleotide accretion in growing tissues of the infant is covered by the ingestion of nucleotides with breast milk. Even if the sum of nucleotides and nucleic acids in breast milk could be utilized by the infant they would account for only about 15% of nucleotide accretion (Uauy *et al.*, 1994). Thus, the infant must be capable of effectively synthesing nucleotides. Moreover, the assumption that the sum of nucleotides and nucleic acids in breast milk could be utilized may not be correct.

In human adults, the effect of ingested free nucleotides on urinary uric acid excretion indicates practically complete absorption of purines from such sources, whereas dietary RNA increases urinary uric acid excretion to a lesser extent, hence dietary RNA is not fully absorbed and utilised (PAG *Ad hoc* Working Group, 1975). The effect of DNA on uric acid levels is only about half of that of RNA (PAG *Ad hoc* Working Group, 1975). Human infants fed a formula based on soybean protein isolate, which contains relatively large amounts of soy RNA, were reported to have serum uric acid concentrations near the upper end of the reference range, suggesting that infants utilise soy RNA at least in part (Kuchan *et al.*, 2000). However, a systematic evaluation on the relative utilisation of free nucleotides, RNA and DNA in infants, for example with a comparison of their effects on uric acid excretion, is not available. In any case based on the quantitative considerations cited above it is unlikely that the nucleotide supply provided with human milk has marked effects on tissue accretion.

Even if dietary nucleotides, nucleosides, RNA and DNA would have little systemic effects, they may be utilised by some tissues such as intestinal mucosa cells. It should also be noted that the addition of nucleotides to the food provides an added source of NPN, and of phosphorus, which under certain circumstances might have relevant metabolic effects.

Several clinical trials have been presented that aim at evaluation of effects of nucleotide addition to formula in infants, but only two of the trials have studied formulae with nucleotide levels of 72 mg/L (Pickering *et al.*, 1998, Lasekan *et al.*, 1999).

Carver *et al.* (1991) studied 15 healthy term infants randomised to a formula without and 13 infants to a formula with 33 mg/L free nucleotides, as well as a reference group of 9 breast fed babies. There were no differences in clinical outcomes such as growth or infection rates. Natural killer cell activity and interleukin 2 production of peripheral blood mononuclear cells in vitro was significantly higher in infants fed formula with nucleotides than the control formula at the age of 2 months, but not at 4 months. The clinical relevance of this difference is not known.

Brunser *et al.* (1994) studied infants from a lower socioeconomic group in Chile fed formulae without (n=148) and with (n=141) 14.2 mg free nucleotides/100 g powder, which would yield a nucleotide concentration of about 2 mg/100 mL product as ready to feed. During the study period of 3 months, the group fed formula with nucleotides experienced a significantly lower number of first episodes of diarrhoea (74 *vs.* 102). No difference in the spectrum of enteropathogens found in stools and in growth parameters was found.

Martínez-Augustín *et al.* (1997 a and b) evaluated formula supplementation with about 11.6 mg nucleotides/L relative to a control diet in preterm infants. Lactose/mannitol ratios indicating intestinal permeability as well as serum concentrations of β-lactoglobulin were not different. Serum IgG antibodies to β-lactoglobulin on day 30 were higher in the nucleotide supplemented group, whereas antibodies to alpha-casein did not differ. The clinical relevance of this difference is not known.

Cosgrove (1998) studied infants born small for gestational age fed a formula with about 33 mg nucleotides/L (n=39) or a control formula without added nucleotides (n=35). Supplemented infants showed a greater gain in weight, length and head circumference between birth and 2 months, as well as between birth and 6 months (e.g. weight gain 0-6 months 80.1 *vs.* 71.8 g/week and kg weight at baseline, p=0.05).

Pickering *et al.* (1998) performed a multi-center trial with one year duration in term infants. The study was completed by 101 infants fed formula with 72 mg/L nucleotide, 107 infants fed a control formula, and 124 infants fed human milk. There were no differences between the formula groups with respect to indicators of growth and tolerance. Infants fed nucleotide supplemented formula had higher antibody titres to HiB and diphtheria at 7 months of age. Data on the incidence of diarrhoea were monitored only at 2 of the 13 study sites. The evaluation of the data from these two sites indicated a lower number of infants that suffered from at least one episode of diarrhoea (15 *vs.* 41%, $p<0.05$).

Lasekan *et al.* (1999) followed infants fed formula with 72 mg/L nucleotides (n=138) or unsupplemented control (n=147), of which about 80% completed the one year study. There were no group differences in growth, tolerance, adverse effects, the incidence of diarrhea, illness visits to physicians, antibiotic prescription, and antibody responses after vaccination to HiB and diphtheria, but anti polio virus type 1 titres were higher in the nucleotide group. Immune phenotype analysis showed an increased proportion of memory lymphocytes and a reduced proportion of naive lymphocytes in the supplemented group. The clinical relevance of these differences is not known.

The available studies show some indication for a possible modulation of immune phenotypes and antibody responses to vaccination by the addition of free nucleotides to infant formulae in healthy term babies. There are some indications for a potential protective effect against diarrhoea in compromised, and possibly also in healthy infants. An enhancing effect on growth has only been reported in infants born small for gestational age, in comparison to a control formula apparently not matched in nitrogen and phosphorus contents. No dose effect between nucleotide concentrations used and outcomes has been shown. There are no indications for relevant adverse effects of nucleotide addition in the concentrations tested.

The Infant Formulae Directive has approved the addition of nucleotides to infant formulae and follow-on formulae in concentrations up to 1.5 mg/100 kcal 5'-AMP, 2.5 mg 5'-CMP/100 kcal, 0.5 mg 5'-GMP/100 kcal, 1.75 mg 5'-UMP/100 kcal, 1.00 mg 5'-IMP/100 kcal, and a total concentration of up to 5 mg/100 kcal, which is similar to reported data for free ribonucleotides in human milk (about 4-6 mg/100 kcal).

The Protein-Calorie Advisory Group of FAO/WHO has recommended an upper limit of 2 g/day for the addition of nucleic acids to the diet of human adults (PAG *Ad hoc* Working Group 1975). Based on 70 kg body weight, this amount would be equivalent to an intake of about 28.6 mg/kg. In infants consuming a formula intake providing 100 kcal per kg and day with the current maximum level of 5 mg/100 kcal would thus be equivalent to a maximal daily nucleotide intake of 5 mg/kg. If the maximum level would be increased to 16 mg/100 kcal, the estimated daily nucleotide intake of an infant fed such formula would be 16 mg/kg and thus rather close to the recommended adequate level of intake set for adults. However, there are no data to indicate that this level of intake would have adverse effects in infants. No studies are available that evaluate a dose-response relationship between the concentrations of nucleotides in infant formula and relevant outcomes in infants. Thus, there is no adequate scientific basis at present to conclude that the addition of nucleotides in higher concentrations than presently permitted for infant formula would provide additional benefits.

In the absence of evidence of benefit of increasing the levels of added nucleotides permitted at present in infant formulae, the Committee recommends that the content of nucleotides, if added to infant formulae and in follow-on formulae, should not exceed 5 mg/100 kcal. If added the maximum nucleotide contents should be: cytidine 5'-monophosphate (CMP) 2.5

mg/100 kcal, uridine 5'-monophosphate (UMP) 1.75 mg/100 kcal, adenosine 5'-monophosphate (AMP) 1.50 mg/100 kcal, guanosine 5'-monophosphate (GMP) 0.50 mg/100 kcal, inosine 5'-monophosphate (IMP) 1.00 mg/100 kcal. Formula based on soy protein isolates should be excluded from the option of further addition of nucleotides because of their high natural contents.

4.7.4 Choline

Choline is a quaternary amine which is ubiquitously distributed in tissues. It can be produced in the body and is widely available in the diet. The extent to which it is a required dietary constituent under normal circumstances is not clear, although when the needs are increased, or the ability to form adequate choline impaired, then it is considered to be conditionally indispensable. Choline status may in part be determined by the availability of those compounds with which it shows close metabolic interaction: serine, methionine, folate, vitamin B_{12} and betaine.

Choline serves as the precursor for the synthesis of phosphatidyl choline (PC), the main phospholipid in brain, liver and other tissues. PC plays a role in normal membrane composition and signalling processes, the transport of cholesterol and lipid in blood, and normal brain development. Choline is oxidised to betaine, a major source of methyl groups for the conversion of homocysteine to methionine, and the subsequent formation of S-adenosyl methionine (SAM), and hence all methylation reactions (Zeisel, 1994). Choline is a substrate for the synthesis of the neurotransmitter, acetylcholine (Wecker, 1990).

In the newborn the plasma concentration of free choline is significantly greater than in adults falling to adult levels by one year. Newborn levels of phospholipid bound choline in plasma are substantially lower than for adults (Buchman *et al.*, 2001). In adults given parenteral feeding devoid of choline or in people fed choline deficient diets, there are decreased plasma concentrations of choline, evidence of liver dysfunction, including a propensity towards fatty infiltration (Zeisel, 1981; Buchman *et al.*, 1993; Zeisel 1994). These changes can be reversed with supplemental choline (Buchman *et al.*, 1992; Buchman *et al.*, 1995). Patients on parenteral nutrition developed hepatic steatosis which was reversed following 1 to 4 g/day of choline chloride for 6 weeks (Buchman *et al.*, 1995).

Choline is present in a number of different forms in milk, and the relative proportions of these differ amongst human- and cows' milk and soya-derived formula. Mature human milk contains 200 μmol/L (3.1 mg/100 kcal), but additional choline in PC and sphingomyelin might increase the total potentially available to 12.6 mg/100 kcal (Zeisel, 1994; LSRO, 1998). The choline content of human milk doubles during the first week after birth. The choline content of most formulae was comparable with that in colostrum, but below that of mature milk (Holmes *et al.*, 2000).

There is no requirement for choline identified within the Infant Formulae Directive. Requirements for infant formulae in the USA indicate a minimum of 7 mg/100 kcal for non-milk-based formulae (FDA, 1985). Codex Alimentarius (1994) has specified a minimum level of 7 mg/100 kcal for infant formulae in general. The Department of National Health and Welfare Canada (1995) established that choline content of formula be no less than 12 mg/100 kcal. The Food and Nutrition Board (FNB, 2000b) has set an Adequate Intake for choline for infants from 0 to 6 months of age as 125 mg/day (about 18 mg/kg/day). Doses of choline in the region of 8 to 20 g/day in adults have been associated with abnormal odour, hypotension, hepatic toxicity and gastrointestinal upsets (LSRO, 1998).

LSRO Expert Panel (1998) has recommended a minimum content of choline in infant formula of 7 mg/100 kcal, based upon limited information of lower content in human milk. A maximum content of choline in infant formula has been set at 30 mg/100 kcal, based upon extrapolation from adult data on the safe level of intake and allowing for potential age differences in metabolism.

The Committee proposes a minimum content of choline in infant formula of 7 mg/100 kcal and a maximum content of choline in infant formula of 30 mg/100 kcal.

4.7.5 Other nitrogen-containing compounds

In the absence of adequate scientific data no recommendations can be made on sialic acid, which is lower in infant formula than in human milk (Wang *et al.*, 2001), on amino-sugars, urea, orotic acid, creatine, creatinine, polyamines at present.

5. SUMMARY OF ALL CONCLUSIONS AND RECOMMENDATIONS

On the basis of additional information which has become available since the last report of the SCF in 1995 on protein in infant formula and follow-on formula, the Committee proposes the following changes:

I. INFANT FORMULAE AND FOLLOW-ON FORMULAE

1. Calculation of protein content

The Committee proposes to determine the crude protein content of all types of infant formula and follow-on formula (total nitrogen x 6.25). In addition, the NPN content must not be higher than 15% of the total nitrogen content in formula based on intact proteins.

2. Sources of protein

In view of some remaining uncertainties on the short- and the long-term effects of a high isoflavone intake in infancy and on the potential to influence allergic and autoimmune diseases, the Committee is of the opinion that soy-based formula should be reserved for specific situations only and that cows' milk-based formula should be the standard choice.

The Committee concludes that at present there is no documented benefit of milk proteins from animals other than cows, or of plant proteins, over cows' milk protein in the manufacture of infant formula. If other protein sources are to be used, their suitability and safety must be assessed before commercialisation. Data required are amino acid contents and availability, allergenicity, digestibility and technical processing, and constituents other than protein and nitrogenous compounds. Controlled clinical studies are needed to assess the nutritional safety and nutritional value.

3. Protein quality

The Committee proposes to require for all types of infant formula and follow-on formula that per energy value the amount of indispensable and conditionally indispensable amino acids

should at least correspond to the values in human milk as shown in Table 13. The Committee proposes to replace the amino acid values of Tables 13 and 12 for the values used in Annex V and VI, respectively, of the Infant Formulae Directive. Arginine should not be included in these tables. The Committee is of the opinion that the sum of methionine and cystine contents may be used as the basis for calculation if the methionine:cystine ratios does not exceed 2, and the sum of phenylalanine and tyrosine may be used if the tyrosine:phenylalanine ratio does not exceed 2.

The Committee proposes that the obligatory request for determining of Protein Efficient Ratio (PER) and Net Protein Utilisation (NPU) of protein hydrolysates in the Directive is not necessary, and that it should be replaced by a requirement for clinical testing of any proteins that have not been evaluated before in the manufacturing of formula. The Committee however emphasizes the importance of pre-clinical testing of new protein sources, hydrolysates and applied new technologies, which may include the evaluation of PER and NPU.

With respect to protein hydrolysates used in infant formulae and follow-on formulae, the Committee proposes that the source of protein or proteins on which hydrolysates are based should be declared. The Committee proposes to all formulae which contain new protein sources or protein hydrolysates which have no established use in infant formulae and/or to which processing technologies have been applied that can affect the bioavailability of nitrogen compounds should be clinically tested before their commercialisation. The Committee concludes that claims on a reduction of allergic disease risk by protein hydrolysates cannot be deduced from technical data alone but need substantiation in clinical trials.

4. Other requirements

The Committee supports the optional addition of taurine up to 12 mg/100 kcal to all types of formula without setting a minimum value.

The optional addition of nucleotides to infant formulae and follow-on formulae based on cows' milk protein or cows' milk protein hydrolysates should be maintained, while formulae based on soy protein isolates should be excluded from the option of further addition of nucleotides. If added, the maximum content of nucleotides should not exceed 5 mg/100 kcal, and that of individual nucleotides should not exceed: cytidine 5'-monophosphate (CMP) 2.5 mg/100 kcal, uridine 5'-monophosphate (UMP) 1.75 mg/100 kcal, adenosine 5'-monophosphate (AMP) 1.50 mg/100 kcal, guanosine 5'-monophosphate (GMP) 0.50 mg/100 kcal, inosine 5'-monophosphate (IMP) 1.00 mg/100 kcal.

II. INFANT FORMULAE

1. Calculation of protein content

The percentage of non-protein nitrogen (NPN) from total nitrogen in infant formulae based on intact proteins should not be higher than 15. The Committee proposes to determine the crude protein content of all types of infant formula and follow-on formula (total nitrogen x 6.25). In addition, the NPN content must not be higher than 15% of the total nitrogen content in formula based on intact proteins.

2. Protein content

The Committee proposes no change in the minimum crude protein content of 1.8 g/100 kcal for infant formula based on intact cows' milk protein. However, the Committee recommends that infant formula with this minimum protein content should be subjected to adequate clinical testing of its nutritional adequacy.

The Committee proposes no change in the minimum crude protein content of 2.25 g/100 kcal for infant formula based on soy protein isolates and hydrolysed protein, and no change in the maximum crude protein content (3.0 g/100 kcal) of infant formula based on all types of protein sources is proposed.

3. Protein quality

Infant formulae based on intact cows' milk protein, on soy protein isolate and on protein hydrolysates should provide per energy value the same amounts of each indispensable and conditionally indispensable amino acid as the reference human milk (Table 13). The sums of methionine and cystine contents and of phenylalanine and tyrosine contents may be used under the conditions described above.

4. Other requirements

The obligatory requirement for a minimum carnitine content of 1.2 mg/100 kcal in infant formulae based on soy protein isolates and protein hydrolysates should be maintained.

The Committee proposes to require a minimum choline content of 7 mg/100 kcal for all types of infant formula. The maximum choline content should not exceed 30 mg/100 kcal.

III. FOLLOW-ON FORMULAE

The Committee proposes to determine the crude protein content of all types of infant formula and follow-on formula (total nitrogen x 6.25). In addition, the NPN content must not be higher than 15% of the total nitrogen content in formula based on intact proteins.

1. Protein content

The Committee proposes for follow-on formulae the same minimum and maximum crude protein contents as for infant formula.

2. Protein quality

The Committee proposes no changes to the existing requirements for amino acid scoring (chemical index of at least 80% of the reference protein human milk as modified in table 13).

6. RECOMMENDATIONS FOR FUTURE WORK

The Committee underlines the necessity to generate reliable analytical data on the amino acid pattern of human milk protein.

The Committee acknowledges gaps in the knowledge of protein requirements of infants in the second half of the first year of life and recommends appropriate studies to fill these gaps.

IV. FAT CONTENT

1. INTRODUCTION

Dietary lipids are indispensable for normal growth and development. The dietary fat (i.e. predominantly triglyceride or triacylglycerol) is the predominant source of fuel energy for breast-fed and formula-fed infants. Oxidation of 1 g fat yields about 38 kJ (9 kcal) of energy, or twice as much as protein and carbohydrate. Besides energy, dietary lipids provide indispensable fatty acids and fat-soluble vitamins to the organism and are also necessary for efficient absorption of the fat-soluble vitamins, carotenoids and cholesterol. Furthermore, fats are carriers of flavours in the diet and contribute to its satiety value (Carey and Hernell, 1992).

Lipids in the form of triglycerides are stored in the adipose tissue as an energy reserve, while other lipids, i.e. phospholipids and cholesterol, are integral structural components of biological membranes, and thereby modulate important physiological functions such as fluidity, permeability, activity of membrane bound enzymes and receptors and electrical response to excitation (ESPGHAN, 1991). Fatty acids also play a direct role in gene regulation (Clarke and Jump, 1996).

Most of the naturally existing fats are mixtures of triglycerides. A triglyceride is composed of one molecule of glycerol to which three fatty acid molecules are esterified. The fatty acids (FA) account for more than 90% of the molecular weight. The effects of FA depend on the length of the carbon chain, degree of saturation, the number, structure and position of the double bonds, and to some extent also on their position in the triglyceride molecule (Small, 1991; Hunter, 2001). The unsaturated FA are characterised by the number of double bonds. Monounsaturated fatty acids (MUFA) have only one double bond, typically located between carbons 9 and 10 (calculated from the methyl end) of the acyl chain, e.g. oleic acid (C18:1 n-9) has 18 carbons and one double bond in that position. Polyunsaturated FA (PUFA) have 2 to 6 double bonds. The human organism is capable of synthesising saturated FA (SAFA) and MUFA from acetate, which is in contrast to the n-6 (ω-6) and n-3 (ω-3) PUFA, which must be provided by the diet. Linoleic acid (LA, 18:2 n-6) and α-linolenic acid (ALA, 18:3 n-3), which are the major dietary constituents of the n-6 and n-3 PUFA, respectively compete for the same enzyme systems (desaturases and elongases) as they are metabolised further in the organism to long-chain polyunsaturated fatty acids (LCPUFA) with a chain length of 20 or more carbons (Sprecher *et al.*, 1995).

Naturally occurring unsaturated fatty acids in plants and free-living fish are mainly *cis*-fatty acids. They can however be transformed into *trans*-isomers chemically by partial hydrogenation of vegetable and fish oils, or through the action of bacteria in the fore-stomach of ruminants. Small amounts of *trans* fatty acids, mainly 11-*trans* vaccenic acid, are found in the milk and meat of cow, sheep and goat. Industrially hydrogenated oils contain varying amounts of various *trans* isomers of fatty acids. The main sources of dietary *trans* fatty acids are deep-fried and industrially produced fatty foods and dairy products (ESPGHAN, 1994; FNB, 2002b).

A particular group of *trans* fatty acids are conjugated linoleic acids (CLA) that are formed by bacteria in the rumen and by desaturation of *trans* MUFA in the organism. The *cis*-9, *trans* -11 CLA, which is the predominant isomer in milk fat, has anti-carcinogenic properties in

experimental animal studies (Ip *et al.*, 1999) and the less abundant *trans*-10, *cis*-12 isomer has been found to reduce abdominal fat mass in adult humans (Risérus *et al.*, 2001), although it also seems to increase insulin resistance (Risérus *et al.*, 2002).

In addition to triglycerides, phospholipids and cholesterol are also part of a normal mixed diet and of human milk (Jensen, 1999). The most common dietary phospholipid is lecithin, which is often used as emulsifier of dietary lipids but also as a source of LCPUFA in infant formulae. Cholesterol is found in foods of animal origin including milk fat. Phospholipids and cholesterol are also synthesised in the human organism and the capacity for synthesis seems to be well developed at birth (Wong *et al.*, 1993; Bayley *et al.*, 1998). Plants contain small amounts of plant sterols, mainly sitosterol and campesterol, which are poorly absorbed (5-15%) from the intestine, but interfere with the absorption of cholesterol. The absorption of the corresponding saturated sterols sitostanol and campestanol is only 1-3% (Gylling and Miettinen, 1999; Igel *et al.*, 2002).

In the gastrointestinal tract the dietary lipids are hydrolysed by lipases to less water-insoluble constituents (i.e. mainly monoglycerides, free fatty acids, lysophospholipids, unesterified cholesterol and fat soluble vitamins), which together with bile salts form mixed micelles or unilamellar vesicles from which the products of fat digestion are absorbed into the enterocytes of the small intestinal mucosa (Staggers *et al.*, 1990; Hernell and Bläckberg, 1997). After reassembly to triglycerides, phospholipids and cholesterol- and fat-soluble vitamin esters in the enterocytes, the absorbed lipids form lipoprotein particles, allowing their transportin the blood for distribution to the various tissues. Medium-chain saturated fatty acids (C8:0 and C10:0), and to a lesser extent lauric acid (C12:0) are less dependent on a mixed micellar phase for absorption and may be transported as unesterified fatty acids bound to albumin directly to the liver via the portal vein (Carey and Hernell, 1992). The core of the lipoproteins is formed by triglycerides and esterified cholesterol while the surface coat is composed of free cholesterol, phospholipids, and proteins. The lipoproteins are commonly divided into four classes according to density: triglyceride-rich chylomicrons and VLDL (very low-density lipoprotein), originating in the intestine and liver, respectively and LDL (low-density lipoprotein) and HDL (high-density lipoprotein). LDL contains two-thirds of circulating cholesterol and is an important risk factor of arteriosclerosis. A high HDL-cholesterol concentration and a high HDL/LDL cholesterol ratio reduce the risk of arteriosclerosis (ESPGHAN, 1994).

2. TOTAL FAT

The maximum and minimum fat intakes that are physiologically tolerable by healthy, term infants are not well defined. It seems that the maximum tolerable intake is limited by the minimum requirements of protein, carbohydrate and micronutrients. In fact, it is not clear if there is a minimal metabolic requirement of dietary fat in early life besides that required to cover the needs for indispensable fatty acids and fat-soluble vitamins, including the provision of a vehicle for their absorption from the diet (Koletzko, 1999). A practical lower limit is set by the provision of an adequate energy density of infant formulae, simultaneously keeping the osmotic and metabolic burdens of the infant low (ESPGHAN, 1991). Breast-fed infants receive about half of their total energy from the energy-rich triglycerides, which constitute about 98% of the lipids in human milk. With the high fat content of human milk as the model, the Infant Formulae Directive recommends that the total fat content in infant formulae should not provide less than 40% and not more than 55% of the total energy in infant formulae, or 1.05-1.5 g/100 kJ (4.4-6.5 g/100 kcal). These figures are the same as the ESPGHAN

recommendation from 1991 and almost identical to the LSRO report from 1998. There are no new data to support a change of this recommendation.

The Infant Formulae Directive specifies for follow-on formulae a fat content of 30-59% of the energy or 0.8-1.5 g/100 kJ (3.3-6.5 g/100 kcal). In view of the fact that complementary feeding may be low in fat it seems prudent to increase the lower level to 35% of the energy or 0.9 g/100 kJ (4.0 g/100 kcal). On the other hand there is no reason to allow a higher maximum level than for infant formulae and 55% of total energy or 1.4 g/100 kJ (6.0 g/100 kcal) is therefore recommended. These levels are the same as the ESPGHAN recommendation of 1991.

Sesame oil and cottonseed oil have not been used in infant formulae and follow-on formulae. Sesame oil contains sesamolin and sesamin, which both have been identified as causing agents in contact dermatitis (Hayakawa *et al.*, 1987). Cottonseed may contain cyclopentenic fatty acids, which may have negative effects on fatty acid metabolism, e.g. desaturation of fatty acids (Phelps *et al.*, 1965). It seems prudent that these oils remain excluded from use in infant formulae and follow-on formulae.

2.1 Structured triglycerides

Besides overall fatty acid composition, the stereospecific distribution of fatty acids in a particular fat should be considered when effects of fatty acids are evaluated. Fatty acids can occupy any of the three positions on the glycerol backbone, designed as sn-1, sn-2 and sn-3, where "sn" denotes "stereospecific numbering". Different types of animal or vegetable fats often have saturated or unsaturated fatty acids in certain sn positions. For example bovine milk fat and pork fat (lard) contain mainly a saturated fatty acid in the sn-2 position, whereas tallow contains saturated fatty acids mainly in the sn-1 and sn-3 positions. Soybean oil and cocoa butter contain unsaturated fatty acids primarily in the sn-2 position and saturated fatty acids primarily in the sn-1 and sn-3 positions. The stereospecific position of fatty acids on triglycerides does affect the functionality of fats in food products.

It is technically possible by use of lipases or chemicals to synthesize triglycerides with a given fatty acid in a given sn-position of the triglyceride molecule, i.e. structured triglycerides. With a similar fatty acid composition the coefficient of fat absorption is higher from human milk than from infant formulae. Likely explanations for this are the unique structure of human milk triglycerides (Tomarelli *et al.*, 1968; Filer *et al.*, 1969) and the presence of the bile salt-stimulated lipase in human milk (Hernell and Bläckberg, 1997; Bernback *et al.*, 1990), but its absence from cows' milk used as the protein source in milk-based formulae. In human milk triglycerides 70% of the saturated fatty acid palmitic acid (16:0), which accounts for about 20-25% of total fatty acids in the milk, is esterified in the sn-2 position (Jensen, 1999). This position is relatively resistant to hydrolysis by colipase-dependent pancreatic lipase (Mattson and Volpenhein, 1964). Hence a major fraction of the palmitic acid in the milk triglycerides will not be released as free fatty acid but absorbed as sn-2 monoglyceride. Because free palmitic acid is prone to form insoluble calcium soaps (Tomarelli *et al.*, 1968), which are not readily absorbed but lost in the stool (Quinlan *et al.*, 1995) a large proportion of palmitic acid in the sn-2 position would, according to theory, improve fat absorption. There is some evidence to support this both in experimental animals (Tomarelli *et al.*, 1968; Mattson and Volpenhein, 1964) and in preterm (Carnielli *et al.*, 1995) as well as term infants (Lucas *et al.*, 1997; Carnielli *et al.*, 1996a). Absorption of both palmitic acid and calcium were more efficient from a formula with a structured, synthetic triglyceride yielding 66-76% of the palmitic acid in the sn-2 position as compared to a

formula based on vegetable oils with about 13% of the palmitic acid in that position (Carnielli *et al.*, 1996a). However, the effect on overall fat absorption was less (Lucas *et al.*, 1997; Carnielli *et al.*, 1996a). During reassembly of the sn-2 monoglycerides, which occurs mainly via the sn-2 monoacylglycerol pathway, a major fraction of the palmitic is retained in the sn-2 position of the newly formed triglyceride (Innis *et al.*, 1995). Hence, there are some data to support a beneficial physiological effect of one specific structured triglyceride on fat and calcium absorption. However, effects on other metabolic functions need to be further studied. There is virtually no information on physiological effects in infants of other structured triglycerides or their long-term safety (Hunter, 2001).

2.2 Medium-chain triglycerides (MCT)

Medium-chain triglyceride (MCT) oil is usually prepared from coconut oil and contains a mixture of fatty acids with varying chain length, usually C6:0 (1-2%), C8:0 (65-75%), C10:0 (25-35%) and C12:0 (1-2%) (Bach and Babayan, 1982). Compared to long-chain triglycerides (LCT), MCT are more rapidly hydrolysed by the gastrointestinal lipases and the hydrolysis products are less dependent on a mixed micellar phase for their absorption. Hence, under conditions where fat absorption is a limiting factor MCT fat improves fat absorption. However, there is no clear evidence of an improved energy balance of dietary MCT as compared to LCT typical of human milk, in preterm and healthy term infants (Brooke, 1980; Whyte *et al.*, 1986). MCT has provided as much as 50% of total fat in some formulae intended for preterm infants, which is considerably higher concentration than the 8-10% medium-chain fatty acids present in average human milk, of which less than 2% of total fatty acids is C8:0 and C10:0 (Jensen, 1989). Although, MCT may have a positive effect in preterm infants on the calcium balance and may reduce the occurrence of hypoglycaemia (Chapell *et al.*, 1986; Sulkers *et al.*, 1990; Sann *et al.*, 1988) higher intakes may have adverse effects (Whyte *et al.*, 1986). The predominant metabolic pathway for MCT is oxidation and there is a linear association between the intake of MCT and ketone bodies in serum and excretion of dicarboxylic acids (Wu *et al.*, 1993). Furthermore, the energy density of MCT is lower than for LCT. There is no evidence of a benefit of the addition of MCT oils to infant formulae intended for healthy, term infants or to follow-on formulae.

2.3 Phospholipids

Phospholipids constitute less than 1% of the dietary lipids in a normal diversified diet. In human milk the milk fat globule membrane stabilising the milk fat globule is rich in sphingomyelin, which is the major phospholipid accounting for almost 40% of total phospholipids, while phosphatidyl choline (PC), ethanolamine (PE), serine (PS), and inositol (PI) account for 28, 20, 9 and 6%, respectively. After the first few days the concentration of phospholipid, on average 0.5% (0.2%-1%) of total lipids, or 0.15-0.20 g/L) as well as the class distribution is remarkably constant irrespective of length of gestation and duration of lactation (Jensen, 1989; Bitman *et al.*, 1984).

Phospholipids may be used as a food additive, mainly because of its emulsifying properties. More recently, phospholipids, e.g. PC, which typically has a polyunsaturated fatty acid in the sn-2 position, has been used as a source of LCPUFA added to infant formulae. However, although the relative concentration of LCPUFA may be high in the phospholipid fraction of human milk, the major part of these fatty acids are supplied by the milk triglycerides. Bearing in mind that besides being important to membrane structure and function metabolites of sphingomyelin and glycerophospholipids, e.g. PC, have key functions in signal transduction affecting important cell functions (Cockcroft, 2001), it is proposed that formulae should not

contain phospholipids at substantially higher levels than present in human milk until the safety of such formulae for infants and young children have been properly documented. Directive 95/2/EC on food additives permits the addition of phospholipids to a maximum level of 1 g/L. Based on current knowledge the Committee proposes that the maximum level of phospholipid in infant formulae and follow-on formulae remains as 1 g/L, whether the addition is for technological or nutritional purposes, e.g. to be a source of LCPUFA.

2.4 Inositol

The most common form of inositol in mammalian tissues and cells is myoinositol, a six carbon cyclic sugar related alcohol. It is found in the free form as phosphorylated lipid derivatives, phosphoinositides, and in membranes as glycosyl-phosphatidyl inositol where it plays an important structural and functional role in signal transduction and metabolic regulation (Aukema and Holub, 1994; LSRO, 1998). Physiologically, inositol is an essential growth factor, which is readily synthesised in the body, but may need to be provided in the diet under certain conditions. The form in which inositol occurs most commonly in plants is as phytate, and when phytate is taken in the diet it may limit the bioavailability of minerals with which it forms complexes, such as iron, copper and zinc.

Inositol is present in high concentration in human milk, and decreases over the course of lactation. The reported concentrations range from 22 to 48 mg/100 kcal (Bromberger and Hallman, 1986; Ogasa *et al.*, 1975; Pereira *et al.*, 1990). Inositol levels in blood are high in neonates, leading to the suggestion that it plays an important role in early development. Over a three week period the serum concentration of myoinositol increased in infants receiving human milk, but not in those receiving formulae (Pereira *et al.*, 1990). A possible role has been suggested for the formation of surfactant and lung development (Anceschi *et al.*, 1988; Hallman *et al.*, 1992), the prevention of the development of retinopathy of prematurity (Friedman *et al.*, 2000), and necrotising enterocolitis (Carlson *et al.*, 1998). There are no studies on the effect of inositol on growth and development, nor on its safety.

In the USA, inositol needs to be added to non-milk-based formula at 4 mg/100 kcal (FDA, 1985).

The LSRO Expert Panel (1998) recommended that the minimum content of myoinositol should be 4 mg/100 kcal, and the upper level should be 40 mg/100 kcal, which is around the upper level reported for human milk.

The Committee proposes a minimum content of myoinositol of 4 mg/100 kcal and a maximum content of myoinositol of 40 mg/100 kcal for infant formulae, while no limits are suggested for follow-on formulae.

3. FATTY ACIDS

3.1 Saturated fatty acids

The Infant Formulae Directive set an upper limit of 15% each of total fatty acids for lauric acid (C12:0) and myristic acid (C14:0) because of their marked hypercholesterolaemic and atherogenic effects, which is more pronounced than for other saturated fatty acids. Although, there is no proof that the total fat intake and fat composition in infancy confers increased risk for coronary heart disease later in life, there are no studies that have properly evaluated the

possible hypercholesterolaemic effect of lauric and myristic acids in infant formulae and follow-on formulae, or the long term effects of unnecessarily high consumption of these fatty acids during infancy. It therefore seems appropriate to apply a cautious approach until convincing evidence on the long-term safety of higher levels are presented. It should be noted however that the harmful effect of saturated fatty acids on serum cholesterol has recently been questioned (Samuelson *et al.*, 2001).

Until this issue has been clarified it is proposed that the sum of myristic acid and lauric acid do not exceed 20%. The same maximum level is set for infant formulae and follow-on formulae. Although, the concentration of these fatty acids in the milk varies between mothers and is influenced by maternal diet the now proposed maximum level is still above that found in milk of most European mothers (Koletzko *et al.*, 1992).

3.2 *Trans* fatty acids

Trans fatty acids are unsaturated fatty acids that contain at least one double bond in the *trans* configuration. The *trans* configuration results in a greater bond angle than that of the *cis* configuration, which makes the carbon chain of a *trans* fatty acid more extended than that of the corresponding *cis* fatty acid, and with properties more comparable to a saturated fatty acid. Hence, in adults there is a positive linear trend between *trans* fatty acids intake and total and LDL cholesterol, and therefore increased risk of coronary heart disease (FNB, 2002b). The *trans* isomers of oleic acid (18:1n-9) and linoleic acid (18:2n-6), that are formed during partial hydrogenation of unsaturated vegetable oils have been suggested to have potential adverse effects on foetal and infant growth and development, possibly through inhibition of desaturation of the parent n-6 and n-3 fatty acids, linoleic acid (18:2n-6) and α-linolenic acid (18:3n-3), respectively to their respective LCPUFA metabolites (Koletzko, 1992; Sugano and Ikeda, 1996; Decsi *et al.*, 2001; Elias and Innis, 2001). In preterm infants inverse correlations between the exposure to *trans* fatty acids and body weight as well as indispensable fatty acids conversion were observed (Koletzko, 1992). In contrast, studies in term infants found no relation between *trans* fatty acids and length of gestation, birth weight or birth length (Elias and Innis, 2001). An inverse association between plasma phospholipid *trans* fatty acids and arachidonic acid was reported in children aged 1 to 15 years (Decsi and Koletzko, 1995). For these reasons the *trans* fatty acid content of formulae should be as low as practically feasible. This led in the Infant Formulae Directive to a maximum level of 4% of total fatty acids.

Considering that the concentration of *trans* fatty acids in bovine milk varies, that formulae with as much as 40% of the fat as milk fat are not unusual, and also taking the view that the use of hydrogenated oils in infant and follow-on formulae should be discouraged the committee sees a lower maximum level of *trans* fatty acids justified. The maximum level is now proposed to be 3% of total fatty acids. The maximum level should be the same in infant formulae and follow-on formulae.

3.2.1 Conjugated linoleic acid (CLA)

CLA is a collective term for a group of geometric and positional isomers of linoleic acid in which the *trans*/*cis* double bonds are conjugated, i.e. they are located without an intervening carbon not part of a double bond. Out of several identified isomers in food only two, *cis*-9, *trans*-11 (rumenic acid) and *trans*-10, *cis*-12, possess biological activity (Pariza *et al.*, 2001). The former has anticarcinogenic effects in animals while the latter seem to reduce fat deposition both in animals and humans (Risérus *et al.*, 2001), although it also seems to increase resistance to insulin in human adults (Risérus *et al.*, 2002), reduces milk fat content

(Masters *et al.*, 2002) and suppress the desaturation of linoleic and α-linolenic acids in cultured cells (Eder *et al.*, 2002). CLA is a natural constituent of dairy products and ruminant meats as a consequence of biohydrogenation in the rumen. While the *cis*-9, *trans*-11 isomer may be synthesized also from vaccenic acid (*trans*-11, octadecenoic acid) by mammalian cells by delta-9 desaturase endogenous production of *trans*-10, *cis*-12 does not occur because mammalian cells do not possess the delta-10 desaturase enzyme (Pariza *et al.*, 2001; Adlof *et al.*, 2000). These two isomers are present in both bovine and human milk at similar concentrations (about 0.5% of the fatty acids), with the *cis*-9, *trans*-11 isomer accounting for the major part (Pariza *et al.*, 2001; Jensen and Lammi-Keefe, 2001). It was recently shown that the concentration in the milk increases if the mother consumes supplements with CLA, while the fat content of the milk decreases (Pariza *et al.*, 2001). There are virtually no studies on the effects of CLA in infants (Elias and Innis, 2001).

Until more is known of the effect of CLA on the growing infant, there should be no voluntary addition of CLA to infant formulae and follow-on formulae, except for the natural contents contributed by the natural fat ingredients of the formulae.

3.3 Polyunsaturated fatty acids (PUFA)

3.3.1 Linoleic acid (LA) and α-linolenic acid (ALA)

Indispensable fatty acids are comprised of two separate families of fatty acids, the n-6 and the n-3 family, respectively. Mammals, including man, do not possess enzyme systems to insert a double bond at either the n-3 or n-6 positions of a fatty acid and therefore depend on dietary supply. Linoleic acid (LA, 18:2 n-6) and α-linolenic acid (ALA, 18:3n-3) are the true indispensable fatty acids from which the respective metabolites of the n-6 and n-3 series of fatty acids are synthesized by shared endogenous enzyme systems. The most important metabolites of LA are dihomo-γ-linolenic acid (DHGLA, 20:3n-6) and arachidonic acid (AA, 20:4 n-6) and of ALA are eicosapentaenoic acid (EPA, 20:5 n-3) and docosahexaenoic acid (DHA, 22:6 n-3). DHGLA, AA and EPA are precursors of the biologically active eicosanoids. AA and the eicosanoids are important factors in second-messenger systems and are indispensable for normal cell function. AA and DHA are major components of specific membrane phospholipids (Lauritzen *et al.*, 2001).

For LA (as glycerides) the Infant Formulae Directive allows a range in infant formulae from 70 mg/100 kJ (300 mg/100 kcal) to 285 mg/100 kJ (1200 mg/100 kcal), or 7-20% of total fatty acids, which is in the same order as found in average human milk, although the concentration varies considerably with the mother's diet. The minimum level is well above what is required to prevent deficiency (around 1% of energy) and the maximum level took into account that too high intakes of LA may have adverse effects on several functions, e.g. lipoprotein metabolism, immune functions, eicosanoid balance, and lipid peroxidation (ESPGHAN, 1991; LSRO, 1998; Lauritzen *et al.*, 2001).

The minimum level of LA is the same as the Codex standard of 1981, but lower than the ESPGHAN recommendation of 1991 [(120 mg/100 kJ (500 mg/100 kcal)], the LSRO report of 1998 (350 mg/100 kcal) and the British Nutrition Foundation (BNF) recommendation of 1992 (4% of energy, or 280-1130 mg/kg/day). While the Codex standard gives no maximum level for LA, the Infant Formulae Directive is in accord with the ESPGHAN recommendation [290 mg/100 kJ (1200 mg/100 kcal)], but lower than the LRSO recommendation (2240 mg/100 kcal).

Because it seems prudent to achieve a balance between saturated, monounsaturated and polyunsaturated fatty acids in infant formulae and follow-on formulae similar to what is considered healthy in children and adults it is now proposed that the minimum level of LA is increased to 120 mg/100 kJ (500 mg/100 kcal) while keeping the maximum level unchanged. Thus LA will account for 4.5 to 10.8% of the total energy in infant formulae and follow-on formulae.

For ALA a minimum level of 12 mg/100 kJ (50 mg/100 kcal) is allowed, but the Infant Formulae Directive does not state a maximum level. Rather the maximum level is regulated by the balance between n-6 and n-3 PUFA. The Directive allows a ratio range of linoleic acid to α-linolenic acid of 5 to 15. ALA typically contributes 0.5-1% of total fatty acids in human milk (Jensen, 1999).

The minimum level of ALA is now proposed to be set at 12 (24) mg/100 kJ or 50 (100) mg/100 kcal corresponding to 1 (2)% of total fatty acids. The concentration of ALA is further regulated by a proposed ratio between LA to ALA of 5-15 (20) to ascertain a proper balance between the precursors of the respective n-6 and n-3 fatty acid series, which is justified by their use of common enzymes for their metabolism to LCPUFA of the respective n-6 and n-3 series (Lauritzen *et al.*, 2001). The higher minimum level should be used in infant formulae to which no LCPUFA has been added (see below), while the lower level is appropriate in formulae containing at least 0.2% of the fatty acid as DHA. In such formulae the more generous ratio of 5-20 between linoleic and α-linolenic acid should suffice, while it is proposed that a ratio of 5-15 be applied for formulae containing less than 0.2% of the fatty acids as DHA.

While the Infant Formulae Directive set the same minimum level of LA for follow-on formulae as for infant formulae, no maximum level was set, and no limitation of the ratio between LA and ALA was set. It is now proposed that the minimum and maximum levels should be the same in follow-on formulae and infant formulae, i.e. 120 mg/100 kJ and (0.5 g/100 kcal) and 285 mg/100 kJ (1.2 g/100 kcal), respectively. It is further proposed that the same minimum and maximum levels of ALA as in infant formulae apply also for follow-on formula, i.e. 12 mg/100 kJ (50 mg/100 kcal) and 24 mg/100 kJ (100 mg/100 kcal), respectively. The ratio of LA to ALA should be 5-15 (or up to 20 if the formula contains at least 0.2% of fatty acids as DHA) applying the same principles as for infant formulae.

3.3.2 Arachidonic acid (AA) and docosahexaenoic acid (DHA)

Although the capacity to synthesize LCPUFA from LA and ALA, respectively may be higher in preterm infants than in term infants there is considerable interindividual variation in both preterm and term infants (Carnielli *et al.*, 1996b; Uauy *et al.*, 2000) and evidence to suggest that the capacity is still insufficient to meet the needs of all preterm infants (ESPGHAN, 1991; LSRO, 1998). The concentration of DHA is higher in plasma, erythrocyte membranes and even in the brain in infants that are fed human milk or infant formulae with added DHA as compared to infants fed formulae containing only the precursors LA and ALA but no LCPUFA (Martínez, 1992; Jørgensen *et al.*, 1996; Farquharson *et al.*, 1992; Makrides *et al.*, 1994; Jamieson *et al.*, 1994). This difference is considered a major reason for differences in neurodevelopmental and visual function (Lauritzen *et al.*, 2001; Jensen and Heird, 2002; Clandinin *et al.*, 1980b). Indeed, AA and DHA are the major n-6 and n-3 fatty acids of neural tissues (Martínez, 1992; Clandinin *et al.*, 1980 a and b) and DHA is a dominating fatty acid in phospholipids of the photoreceptor cells of the retina (Martínez, 1992; Rodríguez de Turco *et al.*, 1999). The evidence of causality is greater for preterm infants than for term infants

(Willatts and Forsyth, 2000), although the lack of consensus for term infants may be explained by the power of the studies (SanGiovanni *et al.*, 2000a and b), the concentration of ALA in the control formula (LSRO, 1998; Lauritzen *et al.*, 2001; Arbuckle *et al.*, 1994; Suerwald *et al.*, 1996), the level of DHA in human milk (Jørgensen *et al.*, 2001), or the formula under study (Lauritzen *et al.*, 2001) and the limitation of the tests used to assess functional outcome at these ages (Willatts and Forsyth, 2000; Carlson and Neuringer, 1999). Indeed, the supply of LA and ALA in the diet modulates the synthesis of AA and DHA. However, it seems as if the contribution of endogenous synthesis of DHA from ALA to tissue pools of infants does not match that of diets containing DHA (Farquharson *et al.*, 1992; Clandinin *et al.*, 1989). Similarly, neither dietary LA, nor its metabolite ALA, contributes to plasma AA to the extent that AA itself does (Gibson *et al.*, 2000; Jørgensen *et al.,* 1998).

From the current literature one can conclude that DHA has a potential benefit on visual acuity but there is yet no consensus that DHA or AA, or both, are indispensable nutrients for term infants, nor that an exogenous supply is truly beneficial, at least not after the first few months of life (LSRO, 1998; Lauritzen *et al.*, 2001; Jensen and Heird, 2002; Lucas *et al.*, 1999). However, there is no evidence to suggest that concentrations within the range found in human milk are harmful. A recent expert panel reviewed the literature and recommended that infant formulae for term infants should contain at least 0.2% of the fatty acids as DHA and 0.35% of the fatty acids as AA and concluded that these levels are at the lower end of the range of human milk DHA content world wide (Koletzko *et al.*, 2001). In contrast, the recent LSRO report gave no recommendation on addition of LCPUFA to formulae for term infants, nor were minimum or maximum levels set (LSRO, 1998). The FNB report of 1992 recommended a lowest daily intake of 20 mg/kg/day for each of AA and DHA and the FAO/WHO 40 mg/kg/day for AA and 20 mg/kg/day for DHA (FAO/WHO, 1994). Clearly, there is a need of larger, properly designed and controlled studies with longer follow-up with respect to functional outcomes in relation to intake levels of LCPUFA in term infants.

According to the Infant Formulae Directive, infant formulae for term infants do not need to contain fatty acids of longer chain length than 18 carbons. However, if such fatty acids are added to the formulae this is regulated by maximum levels because too high concentrations are not beneficial and may have harmful effects. The maximum level was set at 2% of total fatty acids for the n-6 and at 1% for the n-3 long-chain polyunsaturated fatty acids. These maximum levels were set using the concentration in breast milk of European women as reference (Koletzko *et al.*, 1992), and were the same as recommended by ESPGHAN in 1991 for preterm infants. Furthermore, if added there should be a proper balance between n-6 and n-3 PUFA and the concentration of EPA should not exceed that of DHA.

Having reviewed the available literature the Committee sees the evidence insufficient to set a minimum level of LCPUFA. The Committee considers that a statement relating to the presence of DHA in a formula should only be made if the content of DHA is not less than 0.2% of the total fatty acids. The proposed level is based on the available studies on effects of DHA in infants and the fact that the level is at the lower end of the range of human milk DHA content worldwide. To avoid relative deficiency of AA, which may have negative effects on growth (Innis *et al.*, 2002), and also to keep a proper balance between n-6 and n-3 LCPUFA for other reasons (Lauritzen *et al.*, 2001) the concentration should not be lower than that of DHA, and should not exceed 1% of total fatty acids. It is proposed that the concentration of n-6 LCPUFA should not exceed 2% of total fatty acids and that of n-3 LCPUFA 1% of total fatty acids.

3.3.3 Erucic acid

The Infant Formulae Directive allows a maximum level of erucic acid of 1% total fatty acids. It is proposed that this maximum is unchanged.

4. SUMMARY OF ALL RECOMMENDATIONS

4.1 Total fat content

For infant formulae the Committee proposes to maintain the current recommendation that the minimum fat content is 4.4 g/100 kcal and the maximum content 6.0 g/100 kcal, which correspond to 40-55% of the total energy content.

For follow-on formulae the Infant Formulae Directive requires a total fat content of 3.3-6.5 g/100 kcal or 30-59% of the energy. Recognising that complementary feeding may be low in fat the Committee sees it prudent to increase the lower level to 4.0 g/100 kcal (35% of the energy). On the other hand there is no reason to recommend a higher maximum level than in infant formulae, and 6.0 g/100 kcal (55% of the energy) is therefore proposed as the maximum level of fat also in follow-on formulae.

Sesame oil and cottonseed oil should remain excluded from use in infant formula and follow-on formula unless their suitability and safety are adequately demonstrated.

4.1.1 Phospholipids

Being aware of that phospholipids have key functions in signal transduction affecting important cell functions the Committee takes the view that until the safety for infants and young children have been properly documented formulae should not contain phospholipids at substantially higher levels than present in human milk. Directive 95/2/EC on food additives permits the addition of phospholipids to a maximum level of 1 g/L. Based on current knowledge the Committee proposes that the maximum level of phospholipid in infant formulae and follow-on formulae remain as 1 g/L, whether the addition is for technological or nutritional purposes e.g. to be a source of LCPUFA.

4.1.2 Inositol

The Committee proposes a minimum content of myoinositol of 4 mg/100 kcal and a maximum content 40 mg/100 kcal for infant formulae, while no limits are suggested for follow-on formulae.

4.2 Individual fatty acids

4.2.1 Saturated fatty acids

Noting that the potential negative effect of lauric acid and myristic acid on serum cholesterol concentration early in life is not known, it is proposed that the sum of myristic acid and lauric acid do not exceed 20% of total fatty acids. The same maximum level is set for infant formulae and follow-on formulae. Although, the concentration of these fatty acids in the milk varies between mothers and is influenced by maternal diet the now proposed maximum level is above that found in milk of most European mothers on a normal diet

4.2.2 Monounsaturated fatty acids (MUFA)

No minimum or maximum level for total monounsaturated fatty acids is proposed. The recommendation that erucic acid (C22:1 n-9) should account for at most 1% of the total fatty acids is maintained.

4.2.3 Polyunsaturated fatty acids (PUFA)

Linoleic acid (18:2 n-6) and α-linolenic acid (18:3n-3) are the true essential fatty acids from which the respective metabolites of the n-6 and n-3 series of fatty acids the long-chain polyunsaturated fatty acids (LCPUFA) are synthesized by shared endogenous enzyme systems. The most important metabolites of linoleic acid are dihomo-γ-linolenic acid and arachidonic acid and of α-linolenic acid eicosapentaenoic acid (20:5 n-3) and docosahexaenoic acid (22:6 n-3).

4.2.3.1 Linoleic acid

For both infant formulae and follow-on formulae a minimum level of 500 mg/100 kcal and a maximum level of 1200 mg/100 kcal (4.5-10.8% of the total energy content) is proposed. The minimum level is well above that required to prevent deficiency and the proposed range will allow a reasonable balance between saturated, monounsaturated and polyunsaturated fatty acids in formulae.

4.2.3.2 α- Linolenic acid

For both infant formula and follow-on formula it is proposed to set a minimum level of 50 mg/100 kcal (1% of total fatty acids) for formulae which are supplemented with long-chain polyunsaturated fatty acids (LCPUFA), i.e. arachidonic acid and docosahexaenoic acid, provided that the concentration of docosahexaenoic acid is at least 0.2% of total fatty acids (see below). For infant formulae and follow-on formulae, which are not supplemented with LCPUFA a minimum concentration of 100 mg/100 kcal (2% of total fatty acids) is proposed.

To ascertain a balance between the metabolites of linoleic acid and α-linolenic acid in the recipient infant it is recommended that the ratio of linoleic acid to α-linolenic acid should be 5-15 in infant formula and follow-on formula, which are not supplemented with long-chain polyunsaturated fatty acids. For formulae supplemented with long-chain polyunsaturated fatty acids (docosahexaenoic acid content ≥0.2% of all fatty acids) a more generous ratio of 5-20 is recommended.

4.2.4 Long-chain polyunsaturated fatty acids (LCPUFA, 20-22 carbons)

Having reviewed the available literature the Committee sees the evidence insufficient to set an obligatory minimum level of LCPUFA. The Committee considers that a statement relating to the presence of DHA in a formula should only be made if the content of DHA is not less than 0.2% of the total fatty acids. The proposed level is based on the available studies on effects of DHA in infants and the fact that the level is at the lower end of the range of human milk DHA content worldwide. To avoid relative deficiency of arachidonic acid, which may have negative effects on growth, and also to keep a proper balance between n-6 and n-3 LCPUFA for other reasons the concentration of n-6 LCPUFA should not be lower than that of DHA in all types of formulae with added LCPUFA.

It is proposed for both infant formulae and follow-on formulae that n-6 LCPUFA should not exceed 2% of total fatty acids and that of n-3 LCPUFA 1% of total fatty acids. Arachidonic acid should not contribute more than 1% of total fatty acids. The ratio between eicosapentaenoic acid and docosahexaenoic acid should be <1.

4.2.5 Trans fatty acids

Considering that *trans* fatty acids may have adverse effects on plasma lipoproteins, on metabolism of polyunsaturated fatty acids and possibly on growth, that the concentration of *trans* fatty acids in bovine milk varies, that formulae with as much as 40% of the fat as milk fat are not unusual, and also taking the view that the use of hydrogenated oils in infant and follow-on formulae should be discouraged the Committee proposes to lower the maximum level of *trans* fatty acids to 3% of total fatty acids. The maximum level should be the same in infant formulae and follow-on formulae.

4.2.6 Conjugated linoleic acid (CLA)

Until more is known about the effects of CLA on the growing infant, except for the CLA that is naturally present in the fat ingredients of the formula, there should be no voluntary addition of CLA to infant formula and follow-on formula.

V. CARBOHYDRATES

A. MOSTLY DIGESTIBLE CARBOHYDRATES

1. INTRODUCTION

Human breast-milk contains both digestible and indigestible carbohydrates. For infant formulae and follow-on formulae, on the contrary, only digestible carbohydrates are required and regulated in the Infant Formulae Directive, based on the Reports of the SCF (SCF, 1983; 1989; 1993a and 1995).

2. CURRENT REQUIREMENTS CONCERNING MINIMUM AND MAXIMUM CARBOHYDRATE CONTENT AND TYPE OF DIGESTIBLE CARBOHYDRATE IN INFANT FORMULAE AND FOLLOW-ON FORMULAE MARKETED IN THE EU

2.1 Amounts of carbohydrate

The minimum and maximum amounts of digestible carbohydrates are given in table 1.

Table 1. Minimum and maximum amounts of digestible carbohydrates

		Minimum	Maximum
Infant formula	g/100 kcal	7	14
	g/100 mL	4.2	10.5
Follow-on formula	g/100 kcal	7	14
	g/100 mL	4.2	11.2

Table 2 lists the minimum and maximum requirements for some individual carbohydrates.

Table 2. Regulation of the minimum and maximum content of specific carbohydrates

		Infant formula	Follow-on formula
Lactose*	minimum	3.5	1.8
[g/100 kcal]	maximum	-	-
Saccharose**	minimum	-	-
[in % of total carbohydrates]	maximum	20%	20%
Precooked/gelatinatinised starch	minimum	-	-
[in % of total carbohydrates]		30%	-
[in g/100 mL]	maximum	2	-

* Not applicable to formulae with >50% of protein as soy protein isolate

** In follow-on formula in addition to saccharose also fructose and honey

2.2 Types of carbohydrates

For infant formulae only lactose, maltose, saccharose, maltodextrins, corn-syrup solids, pre-cooked and gelatinised starch are permitted. Starches must be free of gluten by nature.

For follow-on formulae certain conditions apply to the use of lactose, saccharose, fructose and honey. Other digestible carbohydrates can be used provided they are free of gluten.

3. COMMENTS ON THE EXISTING REQUIREMENTS OF THE INFANT FORMULAE DIRECTIVE

3.1 General comments on the carbohydrate content of formulae and human milk

Digestible carbohydrates serve as essential sources of energy in the diet and moreover provide structural elements for the synthesis of glycolipids and glycoproteins. Disaccharides and polysaccharides from the diet are hydrolysed to monosaccharides which after absorption in the upper small intestine are converted to glucose in the liver. The human brain has a high need of energy, especially in infants, and there is an obligatory need for glucose, although ketones can partly substitute for glucose (Kraus *et al.*, 1974). On the basis of estimated glucose utilisation rates of the newborn brain of 27 μmol (4.9 mg)/100 g brain/min, which converts to 8 to 12 g glucose/kg body weight/day, the experts of the Life Science Research Office (1998) recommended a minimum total carbohydrate content of infant formula of 9 g/100 kcal.

Human milk contains predominantly lactose (galactose-β(1→4)-glucose), 55 to 70 g/L or 8.2 to 10.4 g/100 kcal, and in addition oligosaccharides, about 20 g/L in colostrum and 10 to 13 g/L in mature milk (Coppa *et al.*, 1994); according to other authors the concentration is between 5 to 8 g/L (Kunz *et al.*, 2000), whereas the content of monosaccharides is only about 1% of total carbohydrates (Coppa *et al.*, 1994). Human milk does not contain saccharose or fructose.

The more than 130 different oligosaccharides in human milk identified so far consist of glucose, galactose, N-acetylglucosamine fucose and sialic acid (N-acetylneuraminic acid) and in most cases lactose at their reducing end (Kunz *et al.*, 2000). They are for the most part not broken down by digestive enzymes of the human intestinal tract. *In vitro* studies with isolated acidic and neutral oligosaccharides from human milk showed that they were not hydrolysed (less than 5%) by human salivary amylase, human and porcine pancreatic amylase and brush border-membranes prepared from human and porcine duodenum (Engfer *et al.*, 2000; Gnoth *et al.*, 2000). These oligosaccharides are for a small part absorbed intact by the breast-fed child and excreted in the urine, minimally hydrolysed in the small intestine, and the majority reaches the colon and are substrates for bacterial hydrolysis and fermentation (Kunz *et al.*, 2000). Cows' milk contains only traces of oligosaccharides (0.03 to 0.06 g/L), mostly sialylated derivatives. Infant and follow-on formulae based on cows' milk protein can therefore contain sialylated oligosaccharides. From the sialic acid content in different formula fractions it was calculated that formula-fed infants consumed 28% of the amount of sialic acid containing oligosaccharides consumed by breast-fed infants (Sánchez-Díaz *et al.*, 1997).

Lactose, the predominant carbohydrate of human and cows' milk, is not completely hydrolysed in the small intestine either, although the enzyme lactase (a β-galactosidase) of the brush border of small-intestinal epithelial cells is fully active at birth and should enable the

hydrolysis of up to 62 g lactose/day (Auricchio *et al.*, 1965). Unhydrolysed lactose reaching the colon is salvaged by bacteria, resulting in the preferential growth of bifidobacteria and lactobacilli. While colonic bacteria from formula-fed infants preferentially produce propionate and butyrate, bifidobacteria and lactobacilli preferentially produce lactate and acetate that increase colonic water absorption, and lower the pH of the chymus (Parrett and Edwards, 1997).

It has been speculated that the β-lactose added to infant formula, under the influence of anions of acids such as phosphate and citrate, would be converted by mutarotation to its anomer α-lactose for which the K_M of β-galactosidase (lactase) is lower, and that therefore lactose in infant formula would be hydrolysed and absorbed to a greater extent than lactose in human milk. This would appear not to be of practical relevance in modern formulae with a low protein and phosphorus content (Zunft and Schulze, 1991). Lactose escaping hydrolysis in the small intestine is not the only factor responsible for the bacterial population typical for the breast-fed infant.

A specific need for lactose of the young infant has not been proven. The effects of monosaccharide absorption in the small intestine on absorption of water and sodium and of calcium by passive non-saturable diffusion are not restricted to lactose. Fractional calcium absorption was measured by a multitracer stable-isotope technique in 18 full-term healthy newborns who were fed from the age of 8 to 12 weeks in a cross-over design an isocaloric formula based on hydrolysed whey which contained 34 g carbohydrates/L, consisting of 70% lactose and 30% corn maltodextin (percent of calcium absorbed 67±12%) or 70% corn maltodextrins and 30% corn syrup solids (percent of calcium absorbed 56±15%). The absolute amount of calcium absorbed per day was 60 mg higher from the lactose-containing formula. The amount of absorbed calcium from both formulae was higher than from human milk because of an almost double calcium concentration in the formulae (Abrams et al., 2002). There was no effect on zinc absorption in this study.

The disaccharidases that hydrolyse maltose, saccharose, maltodextrins (sucrose-isomaltase, maltase-glucoamylase) in the brush-border membrane of the small intestinum are all fully active at birth (Auricchio *et al.*, 1965). In contrast, α-amylase activity is practically nil in the duodenal fluid of premature infants and term infants at birth (Lebenthal and Lee, 1980). Therefore, starch (amylopectin) hydrolysis is incomplete in infants below 6 months of age if dextrin units contain more than 30 glucose units. Nonetheless, one month-old infants fed 45 g/m^2/day (corresponding to 2.6 g/kg body weight/day) of corn starch utilised 99.8% of the ingested starch based on the analysis of glucose and starch in faeces (De Vizia *et al.*, 1975). Low-birth-weight infants (birth weight 1880 to 2700 g) utilised 88% of 3.5 g/kg body weight/day of corn starch (Senterre, 1980). However, these studies did not consider the possibility of colonic bacterial degradation of non-absorbed carbohydrate. Shulman et al., (1983) compared the effects of feeding glucose, glucose polymers and precooked corn starch which were substituted for saccharose in the basal diet for one meal at a dose of 1 g/kg bodyweight in 16 healthy infants aged between 3 and 4 weeks on breath $^{13}CO_2$, breath hydrogen and stool ^{13}C abundance taking into account the natural ^{13}C abundance in the different formulae. The calculated oxidation rate was comparable for the different carbohydrates. Mean peak hydrogen production was highest for starch and significantly higher with starch and polycose than with glucose. Starch derived carbon (3.7 and 13.1% of the ingested load) was detected in stools of two of four infants in whom it was measured. The authors concluded that 4-week old infants can utilise part of ingested cereal as source of energy which they do not absorb completely, that bacterial fermentation plays an important

role in utilisation, and that the capacity for bacterial fermentation can be exceeded by high intakes (Shulman *et al.*, 1983).

Also the chain length of glucose polymers influences their absorption and oxidation by young infants. Twelve infants aged 3 to 4 weeks fed either glucose, short-chain (3 to 8 glucose units) or long-chain (average length 43 units) glucose polymers naturally rich in ^{13}C at a dose of 2 g/kg in one meal of their usual formula oxidised comparable percentages as measured by increases in breath $^{13}CO_2$. They absorbed less long-chain glucose polymers, and with greater individual variation, than glucose or short-chain glucose polymers (Shulman *et al.*, 1986).

Salivary amylase and low pancreatic amylase activity may be sufficient to partly digest small amounts of starch and long-chain glucose polymers, and the mucosal glucoamylase may play an important role in spite of its optimal suitability for the hydrolysis of oligomers smaller than 10 glucose residues (Kelly and Alpers, 1981).

3.2 Hereditary disorders of metabolism or transport of sugars

Congenital deficiency of lactase (McKusick 223000) is a rare disease with an almost total lack of lactase in jejunal biopsy material at birth, lactose intolerance and severe diarrhoea when breast-fed. It has predominantly been reported in Finland.

Even less frequent is congenital lactose intolerance (McKusick 150220), a disorder of unknown aetiology, more serious than congenital lactase deficiency and with additional symptoms of lactosuria, renal tubular acidosis, hyperaminoaciduria, liver damage and cataracts.

Adult lactase deficiency or non-persistence of lactase activity (McKusick 223100) is not manifest in infancy.

Disaccharide intolerance I or sucrase-isomaltase deficiency (McKusick 222900) is a congenital disorder that manifests in non-breast-fed infants fed formulae which contain glucose polymers and/or saccharose. Homozygote frequency in Inuits is 4 to 10%. Glucose-galactose malabsorption (McKusick 606824) is a rare disease and clinically similar to congenital lactase deficiency.

Hereditary fructose intolerance (aldolase B or fructose-1-phosphate aldolase deficiency) (McKusick 229600) is a potentially fatal disease in infants fed fructose or saccharose containing formulae leading to hypoglycaemia, vomiting, malnutrition and liver cirrhosis. Its incidence in Switzerland may be as high as 1:20,000.

Incomplete fructose absorption of amounts higher than 0.5 to 1 g/kg bodyweight occurs in two of three children, leading to bloating, pain and watery diarrhoea. However, symptoms do not occur when glucose and galactose are simultaneously given.

Classical galactosaemia (galactose-1-phosphate uridyltansferase deficiency) (McKusick 230400) is a fatal disorder of infancy if unrecognised, with liver failure, failure to thrive, hyperbilirubinaemia, cataract and renal Fanconi syndrome. Its incidence is between 1:35,000 to 60,000.

Galactokinase deficiency (McKusick 230200) leads to cataracts if untreated. Its incidence is unknown.

The manifestation of these disorders depends on the carbohydrate present in their diet and their treatment consists in the elimination of the respective harmful sugar compound.

3.3 Total carbohydrates in infant formulae

In human milk lactose provides about 40% of the energy value. Under the present regulation, carbohydrates in infant formula may theoretically contribute between 28% and 56% of the energy, or 26% to 53% if one applies the energy conversion factor of 3.75 kcal/g carbohydrate proposed in the 14th report of the Scientific Committee on Food. However, taking into account both the minimum required contents of protein (1.8 and 2.25 g/100 kcal for cows' milk based formula and formula based on soy protein isolate and protein hydrolysate, respectively) and of fat (4.4 g/100 kcal) the carbohydrate content will provide for up to 51 to 53% of the total energy content (12.8 to 13.3 g/100 kcal). Assuming that a formula contains the maximum permitted amounts of protein (3 g/100 kcal) and fat (6.5 g/100 kcal) carbohydrates could provide 29.5% of the total energy content (7.3 g/100 kcal).

If, however, the permitted energy content for infant formula is limited to 60 to 70 kcal/100 mL, the permitted crude protein content remains at 1.8 to 3.0 g/100 kcal for formula based on cows' milk protein, and at 2.25 to 3.0 g/100 kcal for formula based on soy protein isolate and for formulae based on protein hydrolysates and the fat content is limited to 4.4 to 6 g/100 kcal, the carbohydrate contents given in table 3 would be possible. According to this table the minimum carbohydrate content would be 8.5 g/100 kcal providing 34% of energy, and the maximum carbohydrate content would be 13.5 g/100 kcal, providing 54% of energy.

Table 3. Potential carbohydrate content of infant formula composed according to the new proposals for energy, protein and fat content

Energy content		**60 kcal/100 mL**				**70 kcal/100 mL**			
		Minimum		Maximum		Minimum		Maximum	
Fat	g/100 kcal	4.4		6.0		4.4		6.0	
Protein intact cows' milk protein	g/100 kcal	1.8	3.0	1.8	3.0	1.8	3.0	1.8	3.0
Carbohydrate	g/100 kcal	**13.3**	**12.1**	**9.95**	**8.5**	**13.3**	**12.1**	**9.95**	**8.5**
	g/100 mL	8.0	7.3	5.7	5.1	9.3	8.5	7.0	6.0
	% of energy	***53***	***48***	***40***	***34***	***53***	***48***	***40***	***34***
Soy protein + protein isolate	g/100 kcal	2.25	3.0	2.25	3.0	2.25	3.0	2.25	3.0
Carbohydrate	g/100 kcal	**12.9**	**12.1**	**9.3**	**8.5**	**12.9**	**12.1**	**9.3**	**8.5**
	g/100 mL	7.7	7.3	5.6	5.1	9.0	8.5	6.5	6.0
	% of energy	***52***	***48***	***37***	***34***	***52***	***48***	***37***	***34***

The Committee proposes the total digestible carbohydrate content to be between 9.0 and 14.0 g/100 kcal.

3.4 Total carbohydrates in follow-on formulae

The carbohydrate content required for follow-on formula is derived from the proposed energy, protein and fat contents as given in table 4.

Table 4. Potential carbohydrate content of follow-on formula composed according to the new proposals for energy, protein and fat content

Energy content		**60 kcal/100 mL**				**70 kcal/100 mL**			
		Minimum		Maximum		Minimum		Maximum	
Fat	g/100 kcal	4.0		6.0		4.0		6.0	
Protein									
intact cows' milk protein	g/100 kcal	1.8	3.0	1.8	3.0	1.8	3.0	1.8	3.0
Carbohydrate	g/100 kcal	**14.2**	**13.0**	**9.95**	**8.5**	**14.2**	**13.0**	**9.95**	**8.5**
	g/100 mL	8.5	7.8	6.0	5.1	9.9	9.1	7.0	6.0
	% of energy	***57***	***52***	***40***	***34***	***57***	***52***	***40***	***34***
Soy protein + protein isolate	g/100 kcal	2.25	3.0	2.25	3.0	2.25	3.0	2.25	3.0
Carbohydrate	g/100 kcal	**13.75**	**13.0**	**9.25**	**8.5**	**13.75**	**13.0**	**9.25**	**8.5**
	g/100 mL	8.3	7.8	5.6	5.1	9.6	9.1	6.5	6.0
	% of energy	***55***	***52***	***37***	***34***	***55***	***52***	***37***	***34***

The present upper limit of 14 g carbohydrates/100 kcal is sufficient, whereas the minimum amount of 7 g/100 kcal is below the content that is required according to the new proposals for composition.

The Committee proposes a minimum carbohydrate content of 9.0 g/100 kcal and a maximum content of 14 g/100 kcal for follow-on formula**.**

3.5 Type of carbohydrate in infant formulae and follow-on formulae

3.5.1 Lactose in infant formulae

There is no firm evidence for an absolute need of lactose for infants. UDP-galactose needed for the formation of glycolipids, glycoproteins and oligosaccharides can be formed from glucose via glucose-1-phosphate and UDP-glucose by galactose-4-epimerase. However, other carbohydrates offer no advantage over the use of lactose in infant formula. The lactose from human milk can be sufficiently hydrolysed. Higher lactose contents than 10 g/100 kcal, however, could exceed the capacity for absorption but would be salvaged by colonic flora (MacLean et al., 1983).

The Committee is of the opinion that lactose should be the preferred carbohydrate in infant formula, except for formula based on soy protein isolate. The Committee proposes that for all infant formulae, except for those based on based on soy protein isolate, one should maintain the present requirement that at least 50% of the total minimum carbohydrate content (4.5 g/100 kcal) should be lactose. A partial substitution of other carbohydrates for lactose may be advisable for reasons of osmolality of selected products.

The Committee is of the opinion that formulae based only on soy protein isolate may be free of lactose, which is naturally not present in soy bean. Lactose-free formula based on soy protein isolate is the rule at present. Minimal lactose amounts resulting from contamination of ingredients in such formulae cannot be excluded.

The Committee proposes qualifying the permitted claim "free of lactose" for products which contain less than 10 mg of lactose per 100 kcal. This proposal is based on the empirical

guidance values for a galactose (both free and β-glycosidic) intake of 50 (to 200) mg/day for infants with classical galactosaemia (Arbeitsgemeinschaft Pädiatrische Stoffwechselstörungen, 1997).

3.5.2 Lactose in follow-on formulae

Considering that lactose confers some benefits, e.g. on calcium absorption (Abrams et al., 2002) also to the older infant, the Committee proposes the same requirements for lactose in follow-on formula as in infant formula.

3.5.3 Saccharose (and fructose) in infant formulae

Both saccharose and fructose are sweeter than lactose, glucose, maltose and glucosepolymers. Because of sweetness infants fed ad libitum tend to consume more volume of a formula containing saccharose compared to lactose. Fourteen infants fed from the 8th day until the 112th day of life isocaloric formulae based on soy protein isolate which contained either 6.8 g saccharose or 6.8 g corn starch hydrolysate per 100 mL for alternating periods of 4 weeks each had a higher volume and energy intake during the saccharose periods. However, there were no significant differences in weight gain and in weight gain per 100 kcal consumed between the feeding regimes. Because of the shortness of alternating feeding periods it could not be proven that the consumption of a sweeter formula would lead to greater weight gain (Fomon *et al.*, 1983). There is no proof that consumption of sweeter formulae by infants would promote a preference for sugar in later life. There is some evidence to show that dietary fructose (20% in the diet) impairs copper absorption in human adults (Reiser *et al.*, 1985).

Saccharose and fructose from infant formula can lead to life-threatening illness in infants with fructose intolerance, while offering no benefit for normal infants. The Committee considers the risk for infants with this metabolic disorder as serious and avoidable. More than 20 different mutations of the aldolase B gene have been described. The severity of the disease does not depend on the type of the mutation. The most common mutation A149P in Western Europe and Northern America has a heterozygote frequency of 1.3% in the UK, from which it can be calculated that 0.02% of all newborns would be homozygous and at risk of developing the symptoms of fructose intolerance if exposed to fructose (Kullbergh-Lindh *et al.*, 2002). Symptoms are more severe in young infants and occur at smaller doses of fructose. Fructose intake restriction to 10 mg/kg body weight/day has been necessary in patients of about 4 to 5 years of age to normalise growth velocity and serum urate levels (Mock *et al.*, 1983).

The Committee acknowledges, however, that saccharose may be helpful in camouflaging the bitter taste of protein hydrolysates.

In conclusion the Committee proposes to delete saccharose (and fructose) from the permitted carbohydrates for use in infant formula based on intact proteins.

The Committee suggests accepting the use of saccharose in formula based on protein hydrolysates in amounts of up to 20% of the total carbohydrate content. The label should then clearly state the presence of saccharose.

3.5.4 Saccharose (and fructose) in follow-on formulae

Although saccharose (and fructose) offers no advantage over other carbohydrates in follow-on formula, the Committee considers the use acceptable, because at the earliest age of

introduction of follow-on formula (4 months) many infants are introduced to complementary food and presumably to saccharose. The sum of fructose, saccharose (and honey) should be not more than 20% of the total carbohydrate content.

3.5.5 Maltose in infant formulae and follow-on formulae

In both types of formula maltose can be accepted without stating minimum or maximum levels.

3.5.6 Maltodextrins and corn-starch syrup solids in infant formulae

Maltodextrins have the advantage of causing a lower osmolality in products than mono- or disaccharides. Because of the chain-length specificity of intestinal glucoamylase glucosepolymers with 5 to 9 glucose units should be preferred. At present the type of glucosepolymers is not regulated. This is reflected in the variable pattern of distribution of the degree of polymerisation in 7 commercial formulae containing maltodextrin which ranged from 1 to 30 (Coppa *et al.*, 1994).

3.5.7 Maltodextrins and corn starch syrup solids in follow-on formula

No recommendations on the chain length of maltodextrins are considered necessary.

3.5.8 Glucose in infant formulae and follow-on formulae

Glucose should not voluntarily be added to infant or follow-on formula based on intact proteins, because its osmotic activity is higher than that of di-, oligo- und polysaccharides (1 g of glucose per 100 mL adds 58 mOsmol/kg).

In nine commercially available infant formulae labelled to contain lactose only small amounts of glucose (0.2 to 0.3 g/l) were found. In all infant formulae that contained maltodextrins there were also small amounts of glucose (0.15 to 1.2 g/l) (Coppa *et al.*, 1994).

Glucose additions to formulae based on hydrolysed protein for reasons of taste should be restricted to not more than 2.0 g/100 kcal in order to keep the osmolality of the product ready for consumption within reasonable limits.

3.5.9 Starches in infant formulae

Unmodified starches are made of long chains of glucose polymers forming amylose or amylopectin (Filer, 1988). Unmodified starches are considered unsatisfactory for some food uses because they lose desirable thickening properties when cooled and stored. Two methods used to modify starches are cross-linking and stabilization. Cross-linking prevents swollen starch granules from rupturing by bridging one starch molecule to another with either phosphate or adipic acid. Through the process of stabilization, acetyl groups are used to modify and stabilize the starch (Christian *et al*., 1999). Modified food starches are predominantly used in baby foods. The Joint FAO/WHO Codex Alimentarius Commission (CAC, 1994) stipulated that soy-based infant formulae and soy-based follow-on formulae should contain no more than 0.5 g of distarch phosphate, acetylated distarch phosphate, and phosphated distarch phosphate, used singly or in combination, per [100 mL] of a ready-to-drink product. The Infant Formulae Directive stated that only pre-cooked starch and gelatinized starch (naturally free of gluten) can be used as modified food starches in infant

formulae. No more than 2 g of pre-cooked starch and gelatinized starch should be added in [100 mL] of formula and these starches should provide no more than 30% of the total carbohydrate content. As described in Part 4 of Annex VI of the Directive 98/72/EC amending Directive 95/2/EC, starch sodium octenyl succinate (E 1450) is permitted at a maximum level of 20 g/L in infant formulae and follow-on formulae.

There are no biological and nutritional data to define a minimum level of modified food starches in infant and follow-on formulae. The total amount of modified food starches, precooked starch and gelatinized starch (naturally free of gluten) should not be above 2 g/100 mL and should not represent more than 30% of the total carbohydrate content of the formulae.

3.5.10 Starches in follow-on formulae

The Committee proposes to retain the present rules for starches in follow-on formula, that is, only precooked and gelatinised starches naturally free of gluten are permitted and no restriction is made for the starch content besides the one resulting from the requirement for lactose.

B. MOSTLY NON-DIGESTIBLE CARBOHYDRATES

1. INTRODUCTION

Non digestible carbohydrates (NDC) are a heterogeneous group of dietary substances that are derived principally from plants. The principle groups of dietary non digestible carbohydrates comprise fibre and non-starch polysaccharides, a certain proportion of dietary starches that are not digested and absorbed in the small intestine of healthy individuals, a certain proportion of disaccharides such as lactose that may be incompletely digested in the small intestine especially in young infants, oligo- and polysaccharides, and synthetic and modified complex carbohydrates such as thickening agents (ESPGHAN, 2003b).

The physiological effects of dietary NDC are at least partly related to their mechanical and physicochemical properties as they pass down the gut, including the retention of water, minerals and organic compounds. Moreover, NDC serve as a substrate for bacterial fermentation in the colon. These effects and the interactions of NDC with other dietary components can have nutritional and other physiological consequences which have been investigated largely in adults, while only few studies have been carried out in infants and children (ESPGHAN, 2003b). While diets rich in NDC may have marked benefits for gastrointestinal physiology and health, they may potentially also reduce energy intake or reduce the availability, digestion and absorption of fat, carbohydrates, minerals and other nutrients and increase faecal water losses. Faecal loss of energy may increase with increasing NDC intake by infants and young children (Hamaker *et al.*, 1991), and adverse effects through malabsorption and fermentative diarrhoea have been reported by high NDC intakes of infants during the weaning period (Rowland, 1986). Diarrhoea can also be caused by a high intake of fructose exceeding the capacity for absorption, and other NDC (Cole *et al.*, 1999). Therefore, the possible addition of NDC to infant formulae or follow-on formulae needs to be assessed with regard to potential nutrional and other physiological effects.

Non digestible carbohydrates may be added to formulae, follow-on formulae or to foods for special medical purposes for infants primarily for technological reasons, e.g. as stabilizers,

texturizers, or thickening agents, or as a source of fermentable substrates for the gut microflora.

Here the Committee attempts to review the current knowledge on non digestible carbohydrates that might be added to infant and follow-on formulae, to comment on the available knowledge on their suitability and safety for use in infant and follow-on formulae, and to assess whether limitations on the contents of non digestible carbohydrates, if added to infant and follow-on formulae, should be recommended. Included in this review are locust (carob) bean gum, guar gum, pectins, oligosaccharides (oligogalactosyl-lactose [GOS], oligofructosyl-saccharose [FOS]), other fructans, and carrageenan.

2. EUROPEAN LEGISLATION AND PREVIOUS COMMITTEE´S STATEMENTS ON THE CONTENT OF NON-DIGESTIBE CARBOHYDRATES IN INFANT FORMULAE AND FOLLOW-ON FORMULAE

The use of guar gum (E 412) is permitted for inclusion in infant formulae for infants in good health for technological reasons, at a maximum level of 1 g/L, where the liquid product contains hydrolysed proteins (part 1 of Annex VI of Directive 95/2/EC of 20 February 1995) and is in conformity with the conditions set in Annex IV of Directive 91/321/EEC, as amended by Directive 96/4/EC.

Follow-on formulae may contain, for technological reasons, carrageenan (E 407) at a maximum level of 0.3 g/L, locust bean gum (E 410) at a maximum level of 1 g/L, and guar gum (E 412) at a maximum level of 1g/L (Part 2 of Annex VI of Directive 95/2/EC). If more than one of these three substances is added to a follow-on formula, the maximum level established for each of those substances is lowered with that relative part as is present of the other substances together.

The Committee has previously commented on the use of oligofructosyl-saccharose and oligogalactosyl-lactose in infant formulae and in follow-on formulae (SCF, 2001d). The Committee had no major concerns of adding up to 0.8 g/100 mL of a combination of 90% oligogalactosyl-lactose and 10% high molecular weight oligofructosyl-saccharose in infant formulae and follow-on formulae, but recommended that further information is collected on the suitability and safety of the inclusion of digestion-resistant short chain carbohydrates in infant formulae and follow-on formulae. The Committee did not draw any conclusions on potential beneficial effects of resistant short chain carbohydrates in infant formulae and follow-on formulae.

The Committee has recently commented on the use of carrageenan in dietetic products for infants (SCF, 2003d). In the absence of any further information on possible absorption of carrageenan by the immature gut in the very young infant, the Committee reaffirmed its earlier view (SCF, 1999a) that it remains inadvisable to use carrageenan in infant formulae that are fed from birth, including those in the category of foods for special medical purposes. The Committee had no objection to the use of carrageenan, for technological reasons, in foods for older infants, such as follow-on formulae (SCF, 1983) and weaning foods.

3. NON-DIGESTIBLE CARBOHYDRATES

3.1 Gums

3.1.1 Locust bean gum

Locust (carob) bean gum (E 410) is refined from the endosperm of the carob tree, *Ceratonia siliqua*. It contains tannins and the carbohydrate component is a galactomannan polymer consisting of linked D-mannose units with side chains of D-galactose. Locust bean gum is widely used as a thickening agent.

The Committee has previously stated that no conclusive information was available on the potential deleterious effets of thickening agents on the bioavailability of dietary nutrients and growth in infants (SCF, 1999a). Using an *in vitro* model, Bosscher *et al.* showed in two studies that bioavailability of calcium, iron and zinc was decreased by thickening formulae with locust bean gum and guar gum whereas no detrimental effect on mineral absorption was observed by thickening formulae with added starch (Bosscher *et al.,* 2000; Bosscher *et al.,* 2001). In some, but not all animal studies, the addition of locust bean gum to the diet decreased growth (Vohra *et al.*, 2000). A recent review did not reveal any deleterious effect on mineral absorption of soluble non digestible carbohydrates (specifically pectins, gums, resistant starches, lactulose, oligofructose and inulin) (Greger, 1999).

Sievers and Schaub reported an increased frequency of defecation and the occurrence of metabolic acidosis in premature infants after the introduction of formula thickened with locust bean gum (Sievers and Schaub, 2003).

Allergy to locust bean gum has been documented in a 5-month-old girl fed a formula thickened with locust bean gum to improve regurgitation, who developed explosive vomiting, urticaria and a facial rash (Savino *et al.,* 1999). Rhinitis and asthma have been reported in adults exposed to locust bean flour at their workplaces (Scoditti *et al.*, 1996; van der Brempt *et al.*, 1992).

Data assessing the efficacy of thickening formulae for infants with gastroesophageal reflux disease (GERD) are scarce. In a recent review, Carroll *et al.* concluded that no study demonstrated a significant reflux-reducing benefit of thickened infant foods compared with placebo (Carroll *et al.*, 2002). One study reported a significant benefit of a formula thickened with locust bean gum, as compared with formula with rice flour (Borrelli *et al.*, 1997) on the lowering of intraesophageal acid exposure and a decrease of both symptom score and number of emesis episodes. In an open evaluation, formula thickened with locust bean gum seemed to induce clinical improvement of regurgitation and emesis in infants with GERD (Vandenplas and Sacré, 1986). However, although the number of acid reflux episodes was reduced during pH-monitoring, there was also an increase of the duration of the longest episode and no change in the total duration of acid exposure of the oesophageal mucosa, presumably because of slower clearance of the thickened refluxate from the esophagus. Orenstein et al. reported an increased incidence of coughing in infants receiving a thickened formula which has been attributed to decreased acid reflux clearance (Orenstein *et al.*, 1992).

The ESPGHAN Committee on Nutrition concluded that the thickening of formulae with locust bean gum, starch or other thickening agents may be beneficial in a limited number of selected infants, such as infants in who repeated possetting leads to a marked loss of energy

and induces failure to thrive. However, the ESPGHAN Committee recommended that the widespread use of thickened formula for infants with repeated possetting without any risk for health or well being should be discontinued (ESPGHAN, 2002b).

In conclusion, the Committee reaffirms its earlier view that it "is not persuaded that it is necessary to give thickened infant formulae to infants in good health", and that the information available on the potential effects on the bioavailability of dietary nutrients and growth in young infants is not conclusive (SCF, 1999a). It is therefore recommended that the use of locust bean gums should not be acceptable for use in infant formulae.

The Committee recommends maintaining the current maximum level of the use of locust bean gums in follow-on formulae of 1 g/L. The Committee further recommends maintaining the concept that if more than one of the three substances locust bean gum, guar gum or carrageenan are added to a follow-on formula, the maximum level established for each of those substances is lowered with that relative part as is present of the other substances together.

The Committee accepts that there is a case of need for use of locust bean gums in dietary foods for special medical purposes for therapeutic use in a small number of infants with gastro-oesophageal reflux disease under medical supervision, and the Committee considers its use in these products up to a maximum level of 10g/L acceptable.

3.1.2 Guar gum

Guar gum (E 412) is used as a stabiliser, thickener and texturiser. Although viscous gums may enhance the viscosity of luminal contents in the upper gastrointestinal tract, guar gum seems not to impair the bioavailability or utilisation of dietary nitrogen (Mariotti *et al.*, 2001). A double-blind controlled study in volunteers found that partially hydrolyzed guar gum did not affect intestinal absorption of carbohydrates, protein and fat, and that insulin release and exocrine pancreatic function were not affected (Alam *et al.*, 1998). Guar gum has been widely used in adults with the intention to induce body weight-reduction, although a recent meta-analysis of randomized trials suggested that guar gum is not effective in this regard (Pittler and Ernst, 2001). The most frequently reported adverse effects of guar gum were abdominal pain, flatulence, diarrhoea, and cramps. Gastric emptying and intestinal transit were delayed by the addition of guar gum to the diet of human volunteers, and duration of postprandial motor activity in the small bowel was markedly prolonged (Schonfeld *et al.*, 1997). A recent study reported that addition of guar gum to a semi-solid, low energetic meal had no effect on either the gastric emptying rate or rate of intestinal transit (van Nieuwenhoven *et al.*, 2001).

The Committee recommends guar gum should not be used in infant formulae. Considering that guar gums have been used for quite some time in follow-on formulae without the appearance of reports on adverse events, the Committee finds it acceptable to maintain the current maximum level of the use of guar gums in follow-on formulae of 1 g/L. The Committee further recommends maintaining the concept that if more than one of the three substances locust bean gum, guar gum or carrageenan are added to a follow-on formula, the maximum level established for each of those substances is lowered with that relative part as is present of the other substances together.

3.1.3 Pectins

Pectins (E 440) are soluble polysaccharides, the main sugar moieties of which are D-galacturonic acid and D-galacturonic acid methylester. Pectins are used as stabilisers and thickeners, and as texturisers. They are present in cell walls of all plant tissues. Foods for special medical purposes for infants to be used under medical supervision may contain pectins up to a maximum level of 10 g/L (Part 4 of Annex VI of the Directive 98/72/EC).

The Committee recommends that pectins should not be used in infant formulae and follow-on formulae in view of limited information on their potential effects in infants.

The Committee has no objections against the continued use of pectins up to a maximum level of 10 g/L in dietary foods for special medical purposes for infants to be used under medical supervision.

3.2 Oligosaccharides

Oligosaccharides are defined as molecules containing a small number (3 to about 10) of monosaccharide residues connected by glycosidic linkages (IUPAC-IUBMB Joint Commission on Biochemical Nomenclature, 1982). The number of monosaccharide residues defines the degree of polymerisation (DP). Some oligosacharides that are resistant to digestion in the human intestinal tract may promote the growth of bifidobacteriae and lactobacilli in the colon and thus induce prebiotic effects (Salminen *et al*, 1998; Cummings *et al*, 2001). A Bifidus-dominated flora is also typically found in breast fed infants, but in addition to human milk oligosaccharides also other human milk factors such as the relatively lower protein and phosphorus concentrations, the lactose content and the effects on the composition of colonic mucins appear to be relevant for the bifidogenic effects of human milk (Yoshioka *et al.*, 1983; Walker, 2000). The promotion of a Bifidus-dominated flora might have beneficial effects in infants, such as some protection against enteric infections. However, at this time there is little conclusive evidence on the relationship between a bifidobacteriae dominated flora and relevant outcomes on health and well-being in infants.

Human milk contains a complex mixture of more than 130 different oligosaccharides comprising a total concentration of 15-23 g/L in colostrum and 8-12 g/L in transitional and mature milk (Kunz *et al*, 1999; Kunz *et al.*, 2000). The carbohydrate chains of almost all oligosaccharides in human milk isolated so far contain lactose at the reducing terminal. Other monosaccharides are glucose, galactose, fucose, N-acetylglucosamine and sialic acid. Synthesis of oligosaccharides starts mainly from lactose moieties by means of transglycosyltransferases. Recently, human milk oligosaccharides were shown to be resistant to enzymatic digestion in the upper gastrointestinal tract (Engfer *et al.*, 2000). Among other functions human milk oligosaccharides may serve as substrates for colonic fermentation. It has been shown that human milk oligosaccharides induce an increase in the number of *Bifidobacteria* of colonic flora in breast-fed infants, accompanied with a significant reduction in the number of potentially pathogenic bacteria (Kunz *et al.*, 2000). Complex oligosaccharides have the ability of inhibiting the binding of pathogens to cell surfaces because they act as competitive receptors on the host cell surface, thereby preventing adhesion of a number of bacterial and viral pathogens.

Because of the variety, variability, complexity and polymorphism of their structure, it is currently not feasible to add a similar oligosaccharide composition as contained in human

milk to infant and follow-on formulae (Erney *et al.*, 2000). Alternatively, the addition of other oligosaccharides to infant formulae and to follow-on formulae has been proposed.

Oligofructosyl-saccharose (oligofructose; fructooligosaccharides, FOS) and oligogalactosyl-lactose (oligogalactose; galactooligosaccharides, GOS) have been used in dietetic products for infants. Oligofructose is produced from chicory roots and contains one molecule of saccharose to which between 1 and >60 fructose molecules are added. Oligofructose is not found in human milk. Oligofructosyl-saccharose naturally occurs in food (chicory, onion, artichoke, asparagus, and banana) but can be incorporated into beverages, confectionary, dairy products and other foods. Oligogalactose is produced from lactose with the help of a bacterial ß-galactosidase, contains one molecule of glucose and typically between 1 and 7 molecules of galactose. Oligogalactose is found only in trace amounts in human milk. Many fermented milk products contain oligogalactosyl-lactose obtained from lactose by β-galactosidases of lactic bacteria. Lactose-free formulae may also contain trace amounts of oligogalactosyl-lactose. In addition to oligogalactose, the preparation used in dietetic products for infants contains some 40% (wt/wt) of mono- and disaccharides. Oligofructose has a sweet taste considered to be about 0.3 times as sweet as saccharose (Wiedmann and Jager, 1997). The preparations of oligosaccharides under discussion here are partly digestable and hence cannot be considered as dietary fibre. It has been proposed that the utilisable caloric value of these oligosaccharides is in the order of 1.5 kcal/g (Carabin and Flamm, 1999).

Although it has been said that oligogalactosyl-lactose has been used as an ingredient in infant and follow-on formulae in Japan for some 15 years, studies of the safety and the effects on faecal flora in infants receiving these oligogalactosyl-lactose enriched formulae published in peer-reviewed journals are lacking.

The occurence of repeated anaphylactic reactions to inulin and oligofructose has been reported in one human adult (Gay-Crosier *et al.*, 2000).

No conclusive data are available on direct or indirect measures of water balance. Some studies in non-pregnant and in pregnant rats fed diets with added oligofructose showed a dose-dependent induction of loose and watery stools, and a reduced body weight (Carabin and Flamm, 1999). The effects on body weight might be related either to an interference with substrate absorption, or water homeostasis, or a combination of both. In a further feeding study, 50 male and 50 female rats received diets providing 0, 0.34-0.42, 0.85-1.0, or 2.2-2.7 g/kg per day of oligofructose. Male rats fed diets with oligofructose showed significant elevations of serum concentrations of sodium and chloride, and in some cases showed pathological changes of the kidneys, including degeneration of proximal tubular epithelial cells. Only the male rats receiving 0.85 g/kg also showed a significant increase of serum creatinine as well as increased mortality (Carabin and Flamm, 1999). In two studies in rats exposed to dietary oligofructose, also swelling of the appendices was noted (Carabin and Flamm, 1999). Although conclusions cannot be directly extrapolated from these observations to infants, they illustrate possible adverse effects of dietary oligofructose and oligogalactose on water balance.

In infants, oligosaccharides increased stool frequency and reduced stool consistency in a dose dependant manner. For measures of both these parameters, significant differences were found between the two concentrations of 0.4 g/100 mL or 0.8 g/100 mL of the mixture of 90% oligogalactose and 10% oligofructose. In one open study in term infants, infants with a mean age of 7 weeks fed a formula diet with 0.8 g/100 mL oligosaccharides, as well as modified protein, fat and lactose contents, showed watery or fluid stools in 27%, compared to 12% in a

control group fed regular infant formula (Veitl *et al.*, 2000). Although stools with low consistency are common in breast fed infants, human milk has a far lower renal solute load than infant formula. In a controlled trial in term infants, lower serum concentrations of prealbumin were reported in infants fed a formula diet with 0.8 g/100 mL oligosaccharides compared to the control group, which may or may not be related to a modulation of nitrogen retention. Some data on effects of dietary products with added oligosaccharides on growth are available, but the data are limited and include only short term observations.

The Committee considered whether the addition of oligosaccharides in a dosage that increases stool frequency and reduces stool consistency might induce a risk of inadequate water balance particularly in infants during the first months of life with renal immaturity and a poor ability to concentrate urine, especially if an additional stress on water balance is induced, for example by fever, hyperventilation resulting from pulmonary disorders, infectious diarrhoea, high dietary renal solute loads, or refusal of the infant to accept appropriate quantities of fluids (SCF, 2001a). Young infants have a very high water turnover, in the order of 200 mL/kg and day during the first two months of life (Goellner *et al.*, 1981). Interference with water balance may put young infants at risk of hypernatriaemic dehydration, as is exemplified by the relationship between dietary renal solute load and incidence rates of hypernatriaemic dehydration observed in the United Kingdom in the 1970s (Arneil and Chin, 1979; Sunderland and Emery, 1979; Manuel and Walker-Smith, 1980; Davies *et al*, 1979). However, the information available to the Committee, in particular with respect to effects on growth and markers of water balance, does not demonstrate adverse effects from the use of a formulae with up to 0.8 g/100 mL of a combination of 90% oligogalactosyl-lactose and 10% high molecular weight oligofructosyl-saccharose (SCF, 2001b).

3.2.1 Effects of oligofructosyl-saccharose (FOS) and oligogalactosyl-lactose (GOS) on the faecal flora

The ability of many bacterial strains of the colonic flora to metabolize oligofructosyl-saccharose and oligogalactosyl-lactose has been extensively studied in *in vitro* and *in vivo* animal studies. These results have been confirmed in healthy adult humans. Fermentation by the colonic microflora has been shown, both *in vitro* and *in vivo*, by the production of metabolites, gas, short chain fatty acids and microbial enzymes (Bouhnik *et al.*, 1999; Cummings *et al.*, 2001; Salminen *et al.*, 1998).

The ingestion of oligofructosyl-saccharose and oligogalactosyl-lactose can lead to an increase of the faecal population of *Bifidobacteria* in adult humans. The intensity of this bifidogenic effect is inversely correlated to the initial amount of *Bifidobacteria* in the faeces (Van Loo *et al.*, 1999). It has also been shown in adults that the ingestion of oligogalactosyl-lactose could give rise to an increase of *Lactobacilli* in faeces and to a decrease of potentially detrimental bacteria such as *Bacteroides*, *Clostridium* and *Enterobacteriacae* (Williams *et al.*, 1994). The studies reported in adults have been performed for short-term duration (usually 10-30 days) and realised with different prebiotic substances and a very wide range of daily supplementation.

The only randomised controlled study related to the addition of oligofructosyl-saccharose in an infant formula failed to find significant effects on bifidobacteriae counts in stools (studied with classical bacteriological methods) nor any stool pH lowering with daily doses of 1, 2 and up to 3 g of oligofructosyl-saccharose (Guesry *et al.* 2000). These results are contradictory with the well-known bifidogenic effect of FOS in adults (Van Loo *et al.*, 1999).

The use of a mixture of 10% oligofructosyl-saccharose (high molecular weight fraction obtained by hydrolysis of inulin extracted from chicory roots) and 90% oligogalactosyl-lactose (derived from lactose by the action of a bacterial β-galactosidase) in infant formula was recently studied in randomised clinical trials performed during the first weeks of life, with a follow-up of 4 to 12 weeks. Preterm and healthy term infants were given an infant formula supplemented with 0.4, 0.8 or 1 g/100 mL of the oligofructosyl-saccharose / oligogalactosyl-lactose mixture (Boehm *et al.*, 2002; Moro *et al.*, 2002; Rigo *et al.*, 2001; Schmelzle *et al.*, 2003). Bacteriological analysis of the stools was either performed using classical microbiological methods or the fluorescent *in situ* hybridisation (FISH) technique. A significant increase of total faecal bifidobacteria and/or a significant increase of the proportion of faecal bifidobacteria as a percentage of total faecal micro-organisms were observed in these studies. This effect was dose dependent, with a concentration of the oligofructosyl-saccharose/oligogalactosyl-lactose mixture of 0.8 g/100 mL of formula associated with a higher increase of the bifidogenic flora than the concentration of 0.4 g/100 mL (Moro *et al.*, 2002). In one of the 2 studies where this parameter was assessed, an increase of *Lactobacilli* in faeces was also demonstrated (Boehm *et al.*, 2002; Moro *et al.*, 2002). In the study performed in preterm infants, no significant change was observed as regards potentially harmful components of the faecal flora, particularly *Bacteroides*, *Clostridium* species, *Escherichia coli*, *Enterobacter*, *Citrobacter*, *Proteus*, *Klebsiella*, and *Candida* (Boehm *et al.*, 2002). It is difficult to draw firm conclusions since there were differences in the composition of the formula used in the different studies as regards the amount of oligofructosyl-saccharose and oligogalactosyl-lactose (0.4, 0.8 and 1.0 g/100mL) as well as the nature of lipid and protein contents of the enriched formula in 2 studies [presence of β-palmitate (total palmitic acid: 0.6 g/100 mL; 41% in sn-2 position) and partially hydrolysed whey protein] (Rigo *et al.,* 2001; Schmelzle *et al.*, 2003).

3.2.2 Health benefits of FOS and GOS in children

Modifications of the faecal microflora *per se* do not demonstrate the prebiotic nature of an ingredient, which by definition includes the demonstration of a beneficial effect on host health. Data on potential health benefits of oligofructosyl-saccharose and oligogalactosyl-lactose in infants are rare.

In a dose dependent manner, the use of oligofructosyl-saccharose and oligogalactosyl-lactose in infant formulae was associated with an increase of stool frequency and a reduction of both stool pH and stool consistency in infants, with the induction of more watery stools. Both stool frequency and stool consistency differed significantly with the use of two concentrations of 0.4 g/100 mL or 0.8 g/100 mL, respectively, of the mixture of 10% oligofructosyl-saccharose and 90% oligogalactosyl-lactose (Moro *et al.*, 2002). Similar observations were made in preterm infants supplemented with 1g/L of the same oligosaccharide mixture (Boehm *et al.*, 2002). The increase of osmolarity of the formula due to the presence of the short chain carbohydrate mixture was very low and estimated to be less than 5 mOsm/L (Boehm *et al.*, 2002). Increased stool frequency and softer stool consistency may provide a relevant benefit in those subgroups of infants that suffer from hard stools and constipation. No information is available as to whether or not the supplementation of infant and follow-on formulae with oligofructosyl-saccharose and/or oligogalactosyl-lactose may have a preventive effect on the incidence of infectious and allergic disorders. Potential clinical benefits of oligofructosyl-saccharose and oligogalactosyl-lactose in young infants need to be further assessed.

In conclusion, the Committee reaffirms its previous statement that is has no major concerns on the inclusion of up to 0.8 g/100 mL of a combination of 90% oligogalactosyl-lactose and

10% high molecular weight oligofructosyl-saccharose to infant formulae and follow-on formulae. It also reaffirms its previous comment that further information should be gathered on safety and benefits of this combination as well as other forms of oligosaccharides in infant formulae and follow-on formulae.

3.3 Fructans other than FOS

Fructans encompass both inulin and oligofructosyl-saccharose. Fructans are important storage carbohydrates in many plant families, each having its own fructan profile, but are also produced by algae, fungi and bacteria. Fructan molecules consist of monosaccharide units, usually one glucosyl unit and a varying number of fructosyl units. The most important commercially available types of fructans are inulin (mainly 2-60 units of fructose) and oligofructosyl-saccharose (Nordic Committee of Ministers, 2000). The absence of digestibility of these oligosaccharides has been demonstrated by *in vitro* and animal studies, as well as human studies.

Inulin-type fructans have been reported to stimulate calcium absorption in both experimental animals and in humans (Scholz-Ahrens *et al.*, 2002). There are preliminary indications of a hypotriglyceridemic effect of inulin-type fructans in humans with mild hypertriglyceridemia (Jackson *et al.,* 1999), which might point to a possible interference with lipid absoption. No study on fructans other than oligofructosyl-saccharose as an ingredient of infant formulae or follow-on formulae has been reported.

Young infants have an immature gut barrier and are considered at increased risk for allergic sensitisation. In view of a report on anaphylactic reactions to inulin and oligofructosyl-saccharose in a single 39-year old man after ingestion of vegetables (salsify, artichoke) and processed food containing inulin, the potential of sensitisation should be monitored in future evaluations (Gay-Crosier *et al.*, 2000).

The Committee concludes that based on the data available at this time, fructans other than oligofructosyl-saccharose should not be included in infant formulae and follow-on formulae.

3.4 Carrageenan

Carrageenan (E 407), a seaweed extract, is a high-molecular weight hydrocolloid (molecular weight 1.5-20 * 10^6). It is widely used as a thickener, stabiliser, and texturiser in a variety of processed foods in the Western diet. In 1959, carrageenan was granted GRAS (Generally Recognized as Safe) status in the United States. The Committee has previously established an acceptable daily intake (ADI) of 0-75 mg/kg body weight (SCF, 2003).

Carrageenan as a component of a barium enema solution produced positive skin prick test and RAST results in a patient with an anaphylactic reaction during a barium enema (Tarlo *et al.*, 1995). The patient had suffered from gastrointestinal symptoms which disappeared when a diet free of carrageenan was followed. Since carrageenan is widely distributed in common foods, it might account for the occurrence of allergic reactions to foods containing carrageenan, including milk products and formulae for infants (Tarlo *et al.*, 1995). In its recent comment on carrageenan, this Committee concluded that the data available do not support the hypothesis that breast cancer may be causally related to intakes of caraagenan (SCF, 2003).

The Committee reaffirms its view that infant formulae should not contain carrageenan, but

that it has no objection to the use of carrageenan in follow-on formulae up to a maximum level of 0.3 g/L. The Committee further recommends maintaining the concept that if more than one of the three substances locust bean gum, guar gum or carrageenan are added to a follow-on formula, the maximum level established for each of those substances is lowered with that relative part as is present of the other substances together.

VI. LIPID-SOLUBLE VITAMINS

1. INTRODUCTION

The vitamins A, E, D and K are lipid soluble; hence their absorption from infant formulae is related to the efficacy formula fat absorption. The lipid-soluble vitamins are stored in body fat depots, such as adipose tissue, and high intakes over longer periods of time may lead to their tissue accumulation. In addition to dietary intake, also their transplacental supply during pregnancy determines an infant's stores of lipid soluble vitamins at birth, which may vary considerably within populations and may modulate the dietary requirements during the first months of life.

2. VITAMIN A

A group of natural and synthetic substances with the vitamin activity of all *trans* retinol is called vitamin A. Retinol and retinyl esters are found in animal products, such as liver, butter, egg yolk and ocean fish, as well as other foods enriched with vitamin A. Retinol sources used in infant formulae and follow-on formulae are retinol, retinyl palmitate and retinyl acetate (Infant Formulae Directive). The biological value of substances with vitamin A activity is expressed as retinol equivalent (RE) with 1 RE equal to 1 µg all *trans* retinol.

The yellow pigment β-carotene found in plants is considered a provitamin of vitamin A since humans and other mammals are able to convert ß-carotene into retinol. The amount of 6 µg β-carotene is generally regarded to be equivalent to 1 µg RE (SCF, 1993b). However, recent stable isotopes studies in healthy human adults question this assumption (Tang *et al.*, 2000; Hickenbottom *et al.*, 2002). Hickenbottom *et al.* (2002) reported that the mean absorption of orally supplied β-carotene is only about 2%, and that only a mean 0.03 mol retinol are formed from one mol of β-carotene. The data available indicate a very large inter-individual variation. The rate of absorption of β-carotene and its bioequivalence to retinol in infants is unknown, thus a reliable equivalence factor for the vitamin A activity of β-carotene cannot be derived for infants.

Vitamin A modulates the growth and differentiation of epithelial and bone cells, serves in the form of 11-*cis*-retinal as an essential chromophor in retinal photoreceptors, and is required for reproduction, testosterone synthesis and the integrity of various immune functions (SCF, 1993b; Dawson, 2000; Stephensen, 2001; Clagett-Dame and De Luca, 2002). After cellular uptake and binding to specific cytosolic proteins, retinol acts as the ligand and activator of several nuclear receptors (retinoid A receptors alpha, beta and gamma, retinoid X receptors alpha, beta and gamma). Thereby, retinol modulates gene expression and regulates the synthesis of enzymes and other proteins.

2.1 Vitamin A deficiency

The first clinical sign of vitamin A deficiency is prolonged visual dark adaptation, which may progress to night blindness. Further clinical findings in severe vitamin A deficiency include xerosis of the conjunctiva, Bitot spots, keratomalacia and blindness, mucosal damage, dry skin and hyperkeratosis, microcytic anemia, growth failure, and increased intracranial pressure. A series of epidemiological and intervention studies in children living under poor

conditions have clearly documented a relationship between poor vitamin A supply and increased rates and severity of infections as well as mortality related to infectious diseases such as measles (D'Souza and D'Souza, 2002).

2.2 Vitamin A excess

Signs of chronic hypervitaminosis A in infants are reported as loss of appetite, dermal dryness, loss of hair, fissuring of the corners of the mouth, bone pain, hepatomegaly, increased intracranial pressure, and failure to thrive (Fomon, 1993). Toxicity has been reported in infants receiving daily intakes in the order of 7,200-36,000 µg per day over weeks and months (Woodard *et al.*, 1961; Perrsson *et al.*, 1965; Tunell *et al.*, 1965; Mahoney *et al.*, 1980). One death of an infant given approximately 27,000 µg retinol equivalents per day over 11 days was reported (Bush and Dahms, 1984).

The tolerable upper intake level recommended by the Committee for preformed vitamin A (retinol and retinyl esters) for children 1 to 3 year old is 800 µg RE/day. The tolerable upper level for children is based on the value of 3000 µg RE/day for adults (the LOAEL for teratogenicity), with correction for differences in basal metabolic rate compared to adults using scaling according to body surface area (body weight$^{0.75}$) (SCF, 2002b).

2.3 Intake with human milk

Human milk contains retinol and retinyl esters, which are effectively hydrolysed in the infant gut by the action of human milk bile salt stimulated lipase (BSSL) and pancreatic enzymes (Hernell and Blackberg, 1994). The total concentrations of preformed vitamin A in mature milk have been reported as about 150-1100 µg/L (Gebre-Medhin *et al.*, 1976; Jensen, 1995). Based on an assumed mean energy content of 680 kcal/L, a human milk content of preformed vitamin A of 150-1100 µg/L is equivalent to about 22-160 µg/100 kcal.

2.4 Desirable intakes

A Population Reference Intake (PRI) of 350 µg RE/day for infants 6-11 months was recommended by the SCF (SCF, 1993b) and some recent national recommendations (FSAI, 1999; CSH, 2000; AFSSA, 2001). Recently, also higher reference intakes of 500 µg RE/day for infants aged 0-4 months and of 600 µg RE/day for infants aged 4-12 months have been put forward (D-A-CH Referenzwerte, 2000). There are no indications that the use of formulae containing vitamin A levels according to the Infant Formulae Directive would provide an inadequate vitamin A supply.

In view of the existing uncertainties as to the relative equivalence of β-carotene and retinol in infants, the vitamin A activity in infant formulae and in follow-on formulae should be provided by retinol or retinyl esters, while any content of carotenes should not be included in the calculation and declaration of vitamin A activity. In this regard, the Committee notes that the respective regulation in the Commission Directive 96/5/EC on processed cereal-based foods and baby foods for infants and young children should be reconsidered.

2.5 Recommendation

The following ranges of preformed retinol or retinyl esters in infant formulae and follow-on formulae are recommended:

60-180 µg RE*/100 kcal
14-42 µg RE*/100 kJ

* RE = retinol equivalent, 1 µg RE = 1 µg all *trans* retinol.
It must be provided in the form of retinol or retinyl esters.

3. VITAMIN D

3.1 Description of vitamin D

The term vitamin D refers to a group of substances with anti-rachitic activity. Vitamin D_3 (cholecalciferol) is of animal origin and can both be supplied with the diet and be synthesised endogenously from 7-dehydrocholesterol (provitamin D_3) in the skin under exposure to ultraviolet light (SCF, 1993b; Bässler *et al.*, 2002). Vitamin D_2 (ergocalciferol) is of plant origin. Vitamin D_3 and vitamin D_2 have generally been considered to have equal biological potency, but recent data indicate that vitamin D_2 has less biological activity than vitamin D_3 (Trang *et al.*, 1998; Finch *et al.*, 1999). No data are available that would allow to calculate the bioequivalence of vitamins D_3 and D_2 in infants.

Vitamin D is hydroxylated primarily in the liver to 25-hydroxy-vitamin D_3 (25[OH]D) which is released into the circulation. Most of the circulating vitamin D is bound to an alpha-2 globulin with a molecular weight of 60,000, vitamin D binding protein (DBP). In the kidney, 25(OH)D is further hydroxylated to 1,25-dihydroxy-vitamin D ($1,25[OH]_2D$), the biologically most active vitamin D metabolite which is responsible for most the effects of vitamin D, particularly with respect to calcium metabolism. The concentration of $1,25(OH)_2D$ is subject to various regulatory mechanisms. The synthesis and concentration in plasma are enhanced by poor availability of calcium and phosphorus and by high concentrations of parathyroid hormone (PTH), while they are reduced by calcium and phosphorus sufficiency and by $1,25(OH)_2D$ itself. In addition to the concentration of unbound $1,25(OH)_2D$, the biological effects are influenced by the expression of a nuclear vitamin D receptor which is modulated by various factors, including genetic polymorphisms in the population (Jurutka *et al.*, 2001; Rizzoli *et al.*, 2001; Csaszar and Abel, 2001).

The major function of free $1,25(OH)_2D$ is to increase the intestinal absorption of calcium and phosphorus, which to some extent is also achieved by 25(OH)D. In addition, $1,25(OH)_2D$ stimulates tubular reabsorption of calcium and phosphorus in the kidney. Thus, vitamin D sufficiency is of particular importance during phases of rapid growth and bone mineralization, as is the case in infancy.

Vitamin D intakes are reflected by plasma or serum concentrations of 25(OH)D. Low plasma concentrations of 25(OH)D indicate a poor vitamin D availability, and high 25(OH)D plasma concentrations a high vitamin D availability or an availability that is exceeding requirements. Therefore, it is generally accepted to use 25(OH)D plasma concentrations as a marker for defining a range of adequate vitamin D supply (endogenous and exogenous).

3.2 Hypovitaminosis D

Vitamin D deficiency causes markedly reduced intestinal absorption and renal reabsorption of calcium and phosphate and induces the clinical syndrome of rickets. Recently, an increasing rate of rickets in infants and young children has been reported in Europe (Pal and Shaw, 2001;

Brunvand and Brunvatne, 2001) and in the USA (Welch *et al.*, 2000; Tomashek *et al.*, 2001; Rowe, 2001; McCaffree, 2001).

3.3 Hypervitaminosis D

Signs and symptoms of hypervitaminosis D include hypercalcaemia and its consequences, such as hypercalciuria, nausea, vomiting, irritability, muscle weakness, polydipsia and polyuria, and failure to thrive. Vitamin D is deposited in body fat stores, and overdoses can lead to prolonged liberation of high amounts from adipose tissue into the circulation (Bässler *et al.*, 2002). Hypervitaminosis D has been observed with the bolus administration of very high doses of vitamin D (Evliyaoglu *et al.*, 2001) that had been previously used for rickets prevention in some parts of Europe, for example until about 1990 in East Germany. However, at the current level of intakes with milk and complementary foods and with daily vitamin D supplements, no reports on hypervitamonosis D or toxic effects in European infants have been found in a literature search from 1995 to 2002.

The occurrence of hypercalcemia and hypercalciuria has been used as markers of excessive vitamin D intakes in populations. For example, surveys of the British Paediatric Association (BPA, 1956 and 1964) showed a high incidence rate of hypercalcaemia that were reported on a voluntary basis by British paediatricians, at a time when infants received vitamin D intakes in the order of 100 µg/day (4000 IU/day) (Fomon, 1993). From 1960 onwards a decline in the reported number of cases with infantile hypercalcaemia was noted, in some lose association with a reduction of vitamin D supply, while at the same time the reported number of cases with rickets increased particularly in the immigrant population. The report of the British Paediatric Association, however, concludes: "Unfortunately the results of these surveys do not provide a positive answer to the question of the part played by excessive intake of vitamin D in the pathogenesis of infantile hypercalcaemia nor do they indicate precisely the optimal daily requirement of vitamin D during the first years of life" and further "It remains speculative whether the decrease in hypercalcaemia noted from 1960 is a consequence of reduced vitamin D intake".

An important consideration here is that the occurrence of hypercalcaemia and in part also of hypercalciuria are not specific markers of vitamin D toxicity, because hypercalcaemia is not entirely due to vitamin D intake and metabolism. Hypercalcaemia may result from a variety of other conditions, such as abnormalities of genetic (e.g. idiopathic infantile hypercalcaemia, hypocalciuric hypercalcaemia, Williams' syndrome, metaphyseal dysplasia, adiponecrosis subcutanea, Morbus Jansen), endocrine (e.g. hyperparathyroidism, multiple endocrine neoplasia types I and II, thyroid disorders, adrenal insufficiency), pathophysiological (e.g. physical inactivity, malignant tumour) and nutritional nature (e.g. inadequate phosphorus intake). Therefore, individual case reports of hypercalcaemia, or epidemiological studies on the occurrence of hypercalcaemia, do not allow any firm conclusions on the causal effects of given vitamin D intakes and do not suffice as a basis for establishing an adequate upper level.
Some systematic clinical observations of the presence or absence of adverse effects of high vitamin D intakes in infants are available that give indications on the dimension of the vitamin D dosage that may induce adverse effects in infants. Jeans and Stearns (1938) reported retarded growth in infants supplemented with 45-112 µg vitamin D/day, in addition to dietary intake. In contrast, Fomon *et al.* (1966) observed no adverse effects in infants on growth nor on serum calcium concentrations provided with 34.5-54.3 µg vitamin D/day, suggesting that the threshold for adverse clinical effects in infants might be somewhere above an intake of 50 µg/day. In a controlled trial, Hövels *et al.* (1983) measured similar serum 25(OH)D concentrations with supplements of either 500 (12,5 µg) or 1000 IU (25 µg) vitamin

D_3 per day, in addition to dietary vitamin D intakes. Similarly, Houhala *et al.* (1985) reported no signs of hypercalcemia or other adverse effects in infants supplemented with either 10 or 25 µg vitamin D/day, in addition to dietary intakes. Vervel *et al.* (1997) did not find an increase of hypercalcemia in infants receiving 25 µg vitamin D/day in addition to vitamin D-fortified formulae, relative to infants receiving 12.5 µg vitamin D/day.

Thus, no adverse effects were found in controlled studies found in infants at vitamin D intakes >25 µg/day to 54.3 µg/day (Hövels *et al.*, 1983; Ala-Houhala *et al.*, 1985; Vervel *et al.*, 1997; Fomon *et al.*, 1966), while adverse effects on growth occurred in some infants at 45-112 µg vitamin D/day in addition to dietary intake (Jeans and Stearns, 1938).

3.4 Desirable intakes

Reported levels of vitamin D in human milk are reported to be in the order of 4-110 IU/L (0,015-0,4 µg/100 kcal), with up to 10-fold higher values in the summer than in the winter (Fomon, 1993; Jensen, 1995). However, numerous case reports indicate that the human milk content of vitamin D does not provide full protection against the occurrence of rickets (Biser-Rohrbaugh and Hadley-Miller, 2001; Tomashek *et al.*, 2001; Mughal *et al.*, 1999).

The Committee determined the PRI for vitamin D for infants aged 6-11 months as 10-25 µg/day (SCF, 1993b). Recent national reference intakes for infants are in the range of 7 µg/day (FSAI, 1999), 5-10 µg/day (Gezondheidsraad, 2000), 10 µg/day (D-A-CH Referenzwerte, 2000), 10-15 µg/day (CSH, 2000) to 20-25 µg/day (AFSSA, 2001). An infant's vitamin D supply with infant formulae and follow-on formulae in Europe currently is in the order of 7-20 µg vitamin D_3/day or 280-800 IU/day. In addition to the supply with human milk, infants in Europe are often also supplemented with vitamin D_3 as an additional measure for rickets prevention, frequently with a daily dose of the supplement of 10-12.5 µg vitamin D_3/day or 400-500 IU/day.

The tolerable upper intake level recommended by the Committee for infants 0-24 months of age is 25 µg vitamin D/day. Two endpoints are used in the derivation of this value, namely hypercalcaemia, which can be considered an adverse effect, and the serum concentration of 25(OH)D greater than the upper reference level (SCF, 2002c).

The Committee is aware that vitamin D supply may become particularly critical in some populations of infants during the second part of the first year of life, when the volume of formula consumed as part of a progressively diversified diet is considerably lower than during the first months after birth. Therefore, the Committee considered it appropriate to set a higher maximum value for vitamin D contents in follow-on formulae than in infant formulae.

No conclusive evidence has come to the attention of this Committee that would allow a comparative assessment of the biological activity of dietary vitamin D_3 and vitamin D_2 in infants. Therefore, it is recommended to continue to use vitamin D_3 in infant formulae and follow-on formulae, rather than vitamin D_2, until such comparative data might become available.

3.5 Recommendation

The following ranges of vitamin D in infant formulae and follow-on formulae are recommended:

Infant Formulae	Follow-on Formulae
1-2.5 µg vitamin D*/100 kcal	1-3 µg vitamin D*/100 kcal
40-100 IU vitamin D*/100 kcal	40-120 IU vitamin D*/100 kcal
0.25-0.6 µg vitamin D*/100kJ	0.25-0.6 µg vitamin D*/100kJ

* Vitamin D should be supplied the form of vitamin D_3 (cholecalciferol) of which 10 µg is equal to 400 IU of vitamin D.

4. VITAMIN E

Vitamin E denotes all natural and synthetic derivates of tocol and tocotrienol that have the biological activity of RRR-α-tocopherol, which differ in the position of the methyl groups in their side chains. Vitamin E acts as a chain-breaking antioxidant in tissues and is considered essential for the protection of unsaturated lipids in biological membranes against oxidative damage (Traber and Sies, 1996).

The d-stereoisomer of α-tocopherol, RRR-α-tocopherol, has a high biological activity and is the principal isomer occurring in animal tissues. Since the free alcohol form of α-tocopherol is relatively unstable, more stable tocopherol esters are commonly used in the production of infant food products. Compared to 1 mg of RRR-α-tocopherol (= 1 mg α-tocopherol-equivalent, α-TE; 100% activity), the relative activity of 1 mg RRR-α-tocopherylacetate is considered as 91%, RRR-α-tocopheryl-hydrogensuccinate 81%, all-rac-α-tocopherol 74%, all-rac-α-tocopheryl-acetate 67%, all-rac-α-tocopheryl-hydrogensuccinate 60%, RRR-β-tocopherol 57%, RRR-γ-tocopherol 10%, and RRR-δ-tocopherol 1% (Bässler *et al.*, 2002).

Dietary vitamin E is absorbed in the small intestine after uptake into mixed micelles, which requires the presence of bile acids and pancreatic enzymes to form monoglycerides that are incorporated into micelles. Prior to absorption, vitamin E-esters need to be hydrolysed by the action of human milk bile salt stimulated lipase or pancreatic lipolytic activity (Hernell and Blackberg, 1994). Vitamin E absorption appears to be a passive, non-saturable process without a specific carrier. The efficacy of absorption is reported to be in the range of 25-60% and is related to the efficacy of fat absorption (Fomon, 1993; Bässler *et al.*, 2002). After absorption, tocopherols are incorporated into triglyceride rich lipoproteins, chylomicrons and VLDL. Following triglyceride lipolysis, most circulating vitamin E is found in the cholesterol rich lipoproteins, LDL and HDL, which allow the uptake into tissues. Requirements are related to the number of double bonds in unsaturated lipids that are to be protected against peroxidation (ESPGHAN, 1998). Vitamin sufficiency is considered at plasma α-tocopherol concentrations >0.6 mg α-tocopherol/100 mL plasma or >0.7 mmol α-tocopherol/mol plasma lipids (Tsang *et al.*, 1997).

4.1 Vitamin E deficiency

Vitamin E deficiency induces increased lipid peroxidation and elevated peroxide levels in blood and tissues. A functional test that indicates vitamin E deficiency is an increased red cell haemolysis after exposure to hydrogen peroxide *in vitro*. Infants with reduced vitamin E levels show enhanced haemolysis of red blood cells exposed to hydrogen peroxide, anaemia and increased exhalation of ethane and pentane (Fomon, 1993; Socha *et al.*, 1997; van Zoeren-Grobben *et al.*, 1998). Chronic vitamin E deficiency in children with fat malabsorption or with a genetic defect in a transfer protein induces severe and irreversible neurological damage with spinocerebellar ataxia and muscle dysfunction (Sokol 1994; Socha

et al., 1997; Ouahchi *et al.*, 1995).

4.2 Vitamin E excess

The toxicity of very high vitamin E intakes is very low (SCF, 1993b). At daily intakes of about 800-1200 mg vitamin E in adults, an antagonistic action against vitamin K and anticoagulative effects has been observed (Corrigan and Marcus, 1974; Elmadfa and Bosse 1985; Meydani *et al.*, 1998). At high concentrations vitamin E may act as a pro- rather than an antioxidant, which has been observed at daily intakes of 1050 mg D-α-tocopherol in human adults (Brown *et al.*, 1997; Kontush *et al.*, 1996; Alessi *et al.*, 2002). No toxicity studies are available in healthy term infants, although hepatic and renal lesions have been reported in preterm infants after administration of a parenteral vitamin E preparation with additional components (Bell, 1989). On the basis of the dosages provided to preterm infants and the assumption that term infants are not more susceptible to adverse effects than preterm babies, Bell suggested that a vitamin E content of 10 mg/100 kcal in infant formula might be considered to induce no adverse effects (Bell, 1989).

4.3 Desirable intakes

Mature human milk contents of vitamin E are approximately 2-5 mg/L (about 0.5-1.6 mg α-TE/g PUFA) (Fomon, 1993; Jensen, 1995). The SCF set the Population Reference Intake (PRI) for infants aged 6-11 months at 0.4 mg α-TE/g PUFA (SCF, 1993b). Recent national reference intakes for infants are 4 mg/day (D-A-CH Referenzwerte, 2000; AFSSA, 2001) or 0.6-0.8 mg/g PUFA (CSH, 2000).

4.4 Recommendation

The Committee recommends that the minimum vitamin E content should be 0.5 mg α-TE/g polyunsaturated fatty acid expressed as linoleic acid, but in no case less than 0.1 mg per 100 kJ (0.5 mg/100 kcal).

Since vitamin E requirements are considered to increase with the number of double bonds contained in the dietary fatty acid supply (SCF, 1997; ESPGHAN, 1998), the following factors of equivalence should be used to adapt the minimal vitamin E content to the formula fatty acid composition:

0.5 mg α-TE/1 g linoleic acid (18:2n-6)
0.75 mg α-TE/1 γ-linolenic acid (18:3n-3)
1.0 mg α-TE/1 g arachidonic acid (20:4n-6)
1.25 mg α-TE/1 g eicosapentaenoic acid (20:5n-3)
1.5 mg α-TE/1 g docosahexaenoic acid (22:6n-3)

A maximum content of 5 mg α-TE/100 kcal is recommended for infant formulae and follow-on formulae.

5. VITAMIN K

Vitamin K_1 (phylloquinone) is formed in the chloroplasts of green plants and found in green plants and their fruits. Various forms of vitamin K_2 (menaquinones with different side chains) are synthesised by some microorganisms, such as the gut bacteria *Escherichia coli* and

Bacteroides fragilis (SCF, 1993b). However, it is not clear to which extent vitamin K synthesised by the colonic microflora are absorbed and utilised by the human host organism (Suttie, 1996).

Phylloquinones and menaquinones are incorporated into mixed micelles, a process that depends on the availability of bile salts and lipases. They are then absorbed in the jejunum by a saturable, energy dependant mechanism. Absorption increases at lower pH-values. The absorption rate in newborn infants is estimated as only about 30%, in contrast to about 60-80% in adults (Bässler *et al.*, 2002). After absorption, vitamin K is incorporated into chylomicrons and VLDL and distributed to tissues.

Vitamin K is an essential cofactor for the microsomal carboxylation of glutamyl residues to γ-carboxyglutamic acid. Gamma-carboxyglutamic acid is needed for the synthesis of prothrombin, the plasma proteins C, S and Z as well as proteins in bone, such as osteocalcin, and proteins in various other tissues (Lipkin and Kowdley, 2002).

5.1 Intake with human milk

Human milk contains low concentrations of vitamin K provided mostly by phylloquinone. These low concentrations are difficult to analyze precisely, and only limited data are available. Vitamin K concentrations between about 0.6 and 10 µg/L have been reported (von Kries *et al.*, 1987; Fomon, 2001). There is general agreement that the supply with human milk does not suffice to meet the requirements of all young infants. Therefore, vitamin K supplementation in addition to the supply with breast milk is generally recommended in early infancy.

5.2 Vitamin K deficiency

In newborn and young infants, vitamin K deficiency may induce a severe coagulopathy with neonatal haemorrhagic disease or a late onset bleeding, which can lead to intracranial bleeding, permanent damage, and death (Sutor *et al.*, 1999). Infants who have an increased risk for vitamin K deficiency bleeding are breast fed infants, who get a far lower dietary vitamin K supply than infants fed current formulae, and infants with lipid malassimilation even in mild and subclinical forms (Greer, 2001).

5.3 Vitamin K excess

There is little toxicity of very high intakes of vitamin K_1 and vitamin K_2 (Fomon, 1993). Very high vitamin K concentrations may induce DNA damage in mammalian cells in experimental settings (Webster *et al.*, 2000; Morgan, 1995). Parenteral administration of pharmacological doses (1-2 mg vitamin K) has been associated with a possible increase of malignancies in later childhood, but the evidence for an association between administration of phylloquinone to neonates and childhood cancer is not convincing (von Kries, 1998; SCF, 2003a). No adverse effects of enteral vitamin K supply in term infants have been observed. Given that there is no evidence for risk associated with high intakes of phylloquinone in adults, no numerical upper safe level of intake for phylloquinone has been set by the Committee (SCF, 2003a).

5.4 Desirable intakes

The Committee concluded that the human vitamin K requirement is about 5 µg/kg per day but no specific population reference intake for infants was defined (SCF, 1993b). National

reference intakes for infant have recently been set at 5-10 µg/day (CSH, 2000; AFSSA 2001) and at 4 µg/day for young infants 0-4 months and of 10 µg for older infants (D-A-CH Referenzwerte, 2000).

In view of the serious and life-threatening consequences of vitamin K deficiency, throughout Europe the provision of vitamin K supplements to young infants is recommended, for infants fed breast milk and partly also for infants fed formulae. Different forms and dosages of administration are used. The daily supplementation of 25 µg vitamin K_2 to breast fed infants has been found to be effective in preventing vitamin K deficiency bleeding without indications of any adverse effects (Cornelissen *et al.*, 1997). The amount of vitamin K required to normalise prothrombin levels in newborns has been estimated as 3-5 µg/day (Olson, 1984). The levels of vitamin K supplied with current infant formulae (≥4 µg/100 kcal) suffice to provide an effective protection against vitamin K deficiency, while there are no indications of adverse effects (Suzuki *et al.*, 2001).

5.5 Recommendation

The following ranges of vitamin K in infant formulae and follow-on formulae are recommended:

4-20 µg vitamin K_1/100 kcal
1-5 µg vitamin K_1/100 kJ

VII. WATER-SOLUBLE VITAMINS

1. INTRODUCTION

1.1 Objective

To provide a minimum level of each vitamin in the formula, which when consumed in normal amounts will ensure that the infant is able to grow and develop normally and not be at risk of developing an inadequate nutritional status.

To ensure that the maximum level of each vitamin in the formula, which when consumed in normal amounts will ensure that the infant is not exposed to the risk of an excess.

1.2 Background

In the First Report on the Essential Requirements on Infant Formulae and Follow-up Milks based on cows' milk protein (SCF, 1983) minimal composition requirements were defined for water-soluble vitamins, but no maximal levels were set. However, it was determined that the food for neonates and young infants should not only supply them with all the materials and energy needed for growth and for the development of various tissues, but also be capable of being metabolized and anything given in excess to be eliminated. A reasonable safety margin should be selected to allow for further possible reduction in their tolerance in the event of illness and possible errors made by the parents in the preparation of bottle feeds. The average composition of human milk was taken as determining the composition of breast-milk substitutes, allowing for the variability in composition, but no allowance was made for the variability in the needs amongst infants. Where maximal levels were defined they were chosen to be 2 to 3 times the minimum values. A Table of minimum values was drawn up, and this has remained unchanged since.

Values for Population Reference Intakes (PRI) for nutrient and energy intakes for the European Community were published in 1993, as part of a more general determination of the energy and nutrient needs of the population (SCF, 1993b). This document contained estimates of the amounts of vitamins which would need to be consumed by the general population in order to cover the needs of the majority of the population. This document included PRI for infants. Thus the PRI are greater than the average needs of the population by a margin (usually in the region of plus 2 SD) to allow for those with especially high needs.

The current document has drawn upon the data available in the literature, together with reviews and interpretations (Dietary Reference Values for food energy and nutrients for the United Kingdom, 1991; Nutrient and Energy intakes of the European Community, 1993b; Bates and Prentice, 1994; LSRO, 1998). To a considerable extent all of these reports draw on the same base of information which is generally inadequate. They adopt varying approaches to their interpretation of these data.

The amount of a nutrient available from a formula should be adequate to prevent a deficiency and at least equivalent to that normally present in human milk. In the absence of alternative approaches, most usually the content of water-soluble vitamins in human milk has been used in making a judgement on the needs of the infant, based upon the intake of infants being breast-fed by normal mothers of adequate nutritional status. Although it may be desirable to

be able to identify other functional outcomes of direct relevance to health, the data are seldom available to enable this assessment. The composition of human milk is variable and due account has to be made of intrinsic biological factors which contribute to this variability, and also analytical factors which vary amongst studies. The biological availability may vary, depending upon whether the nutrient is bound or free, and the particular form in which the nutrient is normally present in human milk.

Given the intrinsic difference amongst women and their infants, it remains unclear whether the minimum content of infant formula should represent the mean for a population (Beaton, 1985) or the approach adopted in the derivation of PRI, for example, in which some notional value is selected which is adequate to cover the presumed needs of the entire population (mean +2 SD). If the value selected for the minimum content of infant formula was equivalent to the mean consumption observed within a population of infants being fed human milk, then those infants which normally consume milk from their own mother with a higher nutrient content would, while consuming an infant formula, be receiving less than they would have from human milk. It is possible that their needs were not being met. Therefore, in order to ensure that no infant received less of a nutrient than they would if they were consuming the milk from their own mother it would be necessary to provide an amount greater than the mean. This is the approach adopted in setting the PRI. Therefore, the minimum content of water-soluble vitamins present in a formula should be, at least, the PRI for that nutrient (not the average content in human milk).

The LSRO (1998) identified an upper desirable limit for the vitamin content of infant formula. This upper desirable limit was selected as the maximum content of any particular vitamin from those formulations which were currently available on the market (often up to 4 times the minimum). One important factor which will determine the upper level of a vitamin within any infant formula is the level of that vitamin in the cows' milk from which the formula has been derived (Fomon and Ziegler, 1989). This approach to setting an upper desirable limit does not have any sound biological basis, but is based upon a history of apparently safe use. There may be considerable difference in the water-soluble vitamin content of human milk compared with cows' milk, by a factor as high as 10 fold, for example for riboflavin (Wharton, 1989). Thus, basing the recommendation of a maximum level on what is currently observed in marketed products is not of itself a secure basis for setting an upper limit. As human milk is superior to infant formula as a source of nourishment for young infants (Howie *et al.*, 1990; Forsyth *et al.*, 1993), it should not be presumed that improvements cannot be made to the current composition of formulae. Therefore, it is suggested that if an arbitrary approach is to be adopted it should err on the side of caution.

Those components taken in the diet which cannot be utilised or stored by the body have to be excreted, often as solutes in the urine. In the infant, the water available to form urine is limited, and therefore the need to excrete any additional solutes will reduce the margin of safety, especially under conditions of stress, such as during fever or diarrhoea or especially during weight loss (Fomon and Ziegler, 1989). As there is no particular physiological requirement for excessive amounts of water-soluble vitamins for infants and young children, any excess would have to be excreted. For infant formula, a differentiation should be drawn between the natural presence of a nutrient and that which is added either for technological reasons or to allow for losses incurred during shelf life. For most nutrients the evidence is not strong with which to enable a judgement on the upper limit which would carry an increased risk. Tolerance will vary amongst individuals, with age and other circumstances. However, once adequate allowance has been made to ensure that the normal requirements have been met, a reasonable margin of safety based upon the amount normally present, would not be

expected to require an intake in excess of two to five times the requirement, unless there is clear evidence to justify an alternative. Nutrients added for technological reasons would not be expected to be present in amounts greater than five times the requirement, without clear evidence to justify an alternative.

The effect of the previous considerations would be to increase the minimum level from that which is currently set. For some water-soluble vitamins there is no strong evidence to indicate a special risk in providing greater amounts in the diet than those required for normal physiological function. However, the risk of any adverse effects will increase if intakes in excess of the physiological needs are continued over time, although the intake at which a given individual might develop adverse effects can not be known with certainty. As there is no known health benefit in providing a substantial excess and there are no scientific evaluations in infants, an upper limit between three and five times the requirement has been proposed for those vitamins where there is reasonable evidence of potential adverse effects, via niacin, pyridoxal, folic acid and ascorbic acid. For these four vitamins an estimate was made of the intake for an infant with a weight of 5 kg consuming a formula with an energy content of 500 kcal (100 kcal/kg body weight per day). This was compared with a judgement of the daily upper limit for that vitamin, based upon the daily upper limit which has been defined for 1-3 year olds (for niacin, pyridoxal and folic acid: SCF, 2002a, 2000a and 2000b; and for ascorbic acid: FNB, 2000a), on the assumption that for an infant up to 6 months of age the upper limit would be one third that for a 1 to 3 year old. Thus, although an upper limit of five times the requirement falls below the estimated safe upper limit for niacin and pyridoxal, this is not so for folic acid and ascorbic acid. Using these criteria for folic acid and ascorbic acid, an upper limit of two to three times the requirement would be more appropriate. For the others it would be undesirable for the product to contain more than five times the requirement as a matter of course.

There are a number of approaches which have been used for assessing the adequacy of a vitamin (Fidanza, 1991):

- Under normal circumstances the absorption of water-soluble vitamins through the gastrointestinal tract is effective. For most, there are only modest, or no reserves within the body. Thus levels of consumption in excess of requirements are usually absorbed with any excess appearing in the urine. Therefore, the level of excretion of an oral load in urine is often used to assess body status. If a substantial proportion of an administered dose is retained then it is presumed that the body was previously deficient in that vitamin. Conversely, if a substantial proportion is excreted then it is presumed that the body is replete. These conclusions assume a normal capability to absorb, metabolise and excrete the vitamin.

- Measurement of the concentration of vitamin in a transport pool in the blood compartment, usually in plasma or serum, but sometimes in red cells or white cells.

- If there is a storage pool which can be sampled, an assessment of the reserves.

- Many, if not all, water-soluble vitamins have metabolic functions which include acting as cofactors for enzyme catalysed reactions. An assessment of the activity of the holo enzyme, and its stimulation by the addition of the exogenous cofactor, can be used as a functional measure of vitamin status. For example, the activity of erythrocyte glutathione reductase activity (EGRAC) with and without added exogenous riboflavin has been used to assess functional riboflavin status.

- For any of these measures the response to a loading test with the vitamin, gives a measure of the extent to which the concentration might be increased or function enhanced in the presence of additional vitamin.

There is evidence that a number of the water-soluble vitamins can be synthesised by the normal metabolic activity of the colonic microflora. The extent to which any vitamin synthesised in this way might be available to the host, thereby contributing to overall status, is not clear and generally it has been assumed that this potential route is unlikely to be of functional significance.

In this chapter the following approach has been used:

- There is a brief overview of each vitamin;

- There is a comment on Assessment of Nutrient Requirements for Infant Formulae (ANRIF) (LSRO, 1998); and

- There is a brief review of recent literature in relation to the PRI.

A recommendation is made on the minimum level in infant formula and a maximum level expressed as the prudent guidance level in g/100 kcal (g/100 kJ). The figures proposed are based upon an infant of 5 kg, with an energy intake of 500 kcal/day, that is a volume of intake of 750 mL/day of a formula containing 67 kcal/mL.

2. THIAMINE, B_1

Thiamine is a water-soluble compound made up of pyrimidine and thiazole nucleus linked by a methylene bridge (relative molecular mass, MM, 300.84). It is heat stable, but is destroyed rapidly at pH <5.5. It is available as the mononitrate and hydrochloride.

As the body stores are low a regular intake is required. Large doses are poorly absorbed, and beri-beri develops when habitual intake is less than 0.2 mg/1000 kcal.

Status has been assessed by:

- activation coefficient of erythrocyte transketolase (EGRAC);

- urinary excretion before and after loading dose; and

- blood levels.

Thiamine is well absorbed in the jejunum through a saturable, carrier mediated system, and intracellular phosphorylation leads to metabolic trapping. At high concentrations absorption may take place at a slower rate through passive diffusion with a maximum rate of absorption of 8-15 mg/day.

At high intakes any excess is rapidly cleared by the kidney and excreted in urine.

In its active co-enzyme form thiamine pyrophosphate plays an integral part in carbohydrate

metabolism, specifically, the oxidative decarboxylation of α-keto acids and pyruvate and the activity of transketolase in the pentose pathway. It is also involved in decarboxylation of branched chain amino acids, and possibly has a direct effect in nerve conduction.

Deficiency may present as infantile beri-beri. Thiamine deficiency has also associated with sudden infant death syndrome and megaloblastic anaemia.

2.1 Milk content

Classical beri-beri has not been reported for infants of healthy well nourished mothers and therefore it can be assumed that the thiamine content of human milk from healthy women is adequate for an otherwise healthy term infant.

In human milk, Fomon and McCormick (1993) report that for mature milk thiamine content is 154 to 328 µg/L (23 to 35 µg/100 kcal). In their review of 8 human studies Bates and Prentice (1994) report 170 µg/L (25 µg/100 kcal) for milk from healthy women. The thiamine content during early lactation has been reported as 20-133 µg/L (3 to 20 µg/100 kcal) (Nail *et al.*, 1980; Dostalova *et al.*, 1988), and for mature milk as 200 µg/L (30 µg/100 kcal) (Picciano, 1995). Fomon and McCormick estimate mean ±1SD was 112-217 µg/L (for many infants intake less than 160 µg/day [32 µg/100 kcal]).

The thiamine content of pasteurized whole cows' milk has been reported to be 370-460 µg/L (55 to 68 µg/100 kcal) (LSRO, 1998).

2.2 Existing regulations and recommendations

There have been few studies to establish the requirements for thiamine during infancy. Balance studies were carried out in 7 infants aged 1 to 10 months at ranges of intake from 140 to 200 µg/day, and no evidence of deficiency was identified (Holt *et al.*, 1949).

In 1985 the Center for Food Safety specified that infant formulae should not contain less than 40 µg/100 kcal of thiamine (FDA, 1985). For an infant up to six months of age, the RDA for thiamine is 300 µg/day (60 µg/100 kcal for a 5 kg infant consuming 100 kcal/kg/day) (FNB, 1989).

The LSRO Expert Panel recommended a minimum thiamine content in infant formulae of 30 µg/100 kcal. The LSRO Expert Panel considered the adequacy of thiamine intakes by exclusively breast-fed term infants as the primary evidence to support its recommendations. The Expert Panel based its recommendations on the knowledge that thiamine concentrations increase from the early stages of lactation to a mean of 200 µg/L (30 µg/L, range 23 to 35 µg/L) in mature milk and on the absence of evidence to indicate that thiamine deficiency occurs in exclusively breast fed infants from well nourished mothers. An intake of 0.75 L/day of human milk containing 200 µg/L of thiamine would provide a thiamine intake of about 150 µg/day which is equivalent to 30 µg/100 kcal for the 5 kg infant consuming 500 kcal/day. The LSRO Expert Panel found no reason to suspect that the formula-fed infant has a greater thiamine requirement than the infant fed human milk.

There is no clearly defined toxicity from excess consumption of thiamine. The LSRO Expert Panel recommended a maximum content of thiamine in infant formula of 200 µg/100 mL based on the 90th centile of FDA analysis of currently marketed infant formulae.

Within the EU the Infant Formula Directive specifies that the minimum thiamine content of infant formulae should be 40 µg/100 kcal. No maximum has been set.

For infants aged 6-11 months the PRI set by the Committee is 0.3 mg/day (SCF, 1993b). Due to the lack of systematic oral dose-response studies and the low toxicity, the Committee did not set a numerical tolerable upper intake level (SCF, 2001b).

2.3 Recommendation

The Committee makes the following recommendation on the thiamine content of infant formulae and follow-on formulae: minimum 60 µg/100 kcal, maximum 300 µg/100 kcal.

3. RIBOFLAVIN, B_2

Riboflavin is a water-soluble vitamin that functions primarily as a component of two flavin co-enzymes flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD) (MM 376.36). It is widely distributed in nature, and is a permitted food colour. Riboflavin is stable when dry but deteriorates rapidly in alkaline solution, with deterioration being accelerated by light. It leaches into cooking water, and with pasteurization the losses are about 20%.

Riboflavin is readily absorbed from small intestine, through a special transport mechanism involving phosphorylation to flavin mononucleotide. Little riboflavin is stored in the body, and urinary excretion reflects recent dietary intake and saturation of tissue stores.

FMN and FAD have widespread role in range of biochemical reactions, as components of several enzymes that catalyse many oxidation/reduction reactions in numerous metabolic pathways. They are required for lipid degradation, synthesis of steroids and glycogen, metabolism. They help maintain integrity of mucous membranes, skin, eyes and nervous system. They have important interactions with iron metabolism.

A deficiency of riboflavin leads to symptoms which are widespread, but non-specific. There is degradation of riboflavin when newborns are exposed to phototherapy, and in premature infants the absorptive capacity is reduced and stores are especially low at birth.

Virtually non-toxic when given orally, excess excreted in urine.

Assess status: EGRAC, blood or urine concentration.

3.1 Milk content

The reported riboflavin content of mature human milk varies widely, from 274 to 580 µg/L (41 to 87 µg/100 kcal) (Thomas *et al.*, 1980; Nail *et al.*, 1980; Dostalova *et al.*, 1988; Roughead and McCormick, 1990). Bates and Prentice (1994), calculate a mean of 380 µg/L (57 µg/100 kcal), from 10 studies in Western countries. Methodological problems with analyses may lead to under-estimate, and using state of the art methodologies for the total riboflavin content of human milk Roughead and McCormick (1990), estimate mean 570 µg/L (85 µg/100 kcal). Picciano (1995) suggest typical values for human milk are likely to be around 400 to 600 µg/L (60 to 90 µg/100 kcal).

The riboflavin content of cows' milk is variable with season, breed etc. For pasteurised milk,

a value of 914 µg/L (136 µg/100 kcal) is considered typical, but values as high as 1,900 µg/L have been reported (LSRO, 1998).

Greene *et al.* (1990) compared biochemical indices of term (68) and pre-term (14) infants fed human milk or infant formula. Serum riboflavin concentrations of human milk fed infants were lower at all time periods (up to 112 days postpartum) compared with those fed formula. It cannot be determined whether there were any functional implications associated with this difference. Bamji *et al.* (1991) provided biochemical evidence of impaired riboflavin status of exclusively breast fed infants. Of 55 infants fed only human milk, 19 (35%) had elevated EGRAC index. The riboflavin concentrations in the milk were at the lower end of the range reported in the literature (221 µg/L, 33 µg/100 kcal: methodologically may have been an underestimate in women of low socio-economic group.)

3.2 Existing regulations and recommendations

In 1985, in the USA the Code of Federal Regulations specified a lower limit for riboflavin of 60 µg/100 kcal (FDA, 1985). In 1989 the RDA for riboflavin was set at 400 µg for infants up to 6 months of age (80 µg/100 kcal for a 5 month old infant consuming 100 kcal/kg/day). Based upon this the recommended minimum riboflavin content in infant formulae is 80 µg/100 kcal.

The LSRO Expert Panel concluded a maximum riboflavin content in infant formulae of 300 µg/100 kcal based on the 90th centile of the FDA analyses of infant formula.

Within the EU the Infant Formulae Directive specifies that the minimum riboflavin content of infant formulae should be 60 µg/100 kcal. No maximum has been set.

The PRI for infants aged 6-11 months set by the Committee for riboflavin is 0.4 mg/day (SCF, 1993b). The Committee did not set a numerical tolerable upper intake level (SCF, 2000c).

3.3 Recommendation

The Committee makes the following recommendation on the riboflavin content of infant formulae and follow-on formulae: minimum 80 µg/100 kcal, maximum 400 µg/100 kcal.

4. NIACIN, B_3

Niacin is the generic form for nicotinic acid (pyridine 3-carboxylic acid), nicotinamide (nicotinic acid amide) and the co-enzyme forms of the vitamin (NAD and NADP). Pyridine nucleotides activate many dehydrogenases and are essential for electron transport and other cellular respiratory reactions. Niacin is stable in foods and is not destroyed by heat or prolonged storage.

Humans are able to synthesise niacin *de novo* from tryptophan, therefore the niacin content of foods is expressed as niacin equivalents (preformed niacin plus 1/60th of amount of tryptophan).

Niacin is rapidly absorbed by carrier mediated mechanism at low concentrations and by passive diffusion at higher concentrations. It circulates in plasma in a bound form and enters

tissues by passive diffusion, where it is fixed as pyridine nucleotides. It is taken into red cells by an anion-transport system and accumulates at high concentration.

A deficiency of niacin leads to characteristic skin changes, known as pellagra; and may include gastrointestinal symptoms and central nervous symptoms. In adults nicotinic acid has been used in the treatment of hypercholesterolaemia and dose related adverse effects have been reported. Flushing is the most common side effect and can occur at relatively low doses (Rader *et al.*, 1992). Chronic toxicity, in the form of hepatotoxicity, hyperglycaemia, hyperuricaemia and adverse ophthalmological effects, has been reported frequently, following therapy with nicotinic acid over extended periods of time. There are no reports of acute toxicity with nicotinamide, but high doses over longer periods of time have been associated with liver dysfunction.

4.1 Milk content

The concentration of niacin in human milk is much higher than in cows' milk. The FNB give the typical daily secretion of preformed niacin as being 1.0 to 1.3 mg in 750 mL (FNB, 1989). Picciano (1995) cited a range of 1100 to 2300 µg/L of niacin (164 to 343 µg/100 kcal). About 70% of NEs in human milk are derived from tryptophan (Schanler, 1989). The total niacin in cows' milk (preformed plus NE from tryptophan), is in the range 710 to 1300 µg/L (106 to 194 µg/100 kcal) (Jensen, 1995). There are no studies which provide specific data from which niacin requirements might be derived.

4.2 Existing regulations and recommendations

As recommended by the Committee on Nutrition of the American Academy on Pediatrics in its 1983 report to the FDA (1985), the USA Code of Federal Regulations specifies that infant formula contain a minimum level of 250 µg preformed niacin/100 kcal: no maximum value is specified. The 1989 RDA for niacin is expressed in niacin equivalents: 5 mg NE/day for the infant from birth to 6 months of age (1 mg/100 kcal for a 5 kg infant consuming 100 kcal/kg/day) (FNB, 1989).

The LSRO Expert Panel recommended a minimum niacin content in infant formulae of 550 µg/100 kcal. The Expert Panel recommended a maximum niacin content in infant formulae of 2000 µg/100 kcal based on the 90th centile of the FDA analyses of infant formulae.

Within the EU the Infant Formulae Directive specifies that the minimum niacin content of infant formulae should be 800 µg/100 kcal. No maximum has been set.

The PRI for niacin for infants aged 6-11 months set by the Committee is 5 mg/day (SCF, 1993b). The Committee choose a tolerable upper intake levels for children 1-3 years of 2 mg/day for nicotinic acid and of 150 mg/day for nicotinamide (SCF, 2002a).

4.3 Recommendation

The Committee makes the following recommendation on the naicin content of infant formulae and follow-on formulae: preformed niacin as nicotinamide, minimum 300 µg/100 kcal, maximum 1200 µg/100 kcal.

5. PANTOTHENIC ACID, B_5

Pantothenic acid is formed from pantoic acid and β-alanine. It is a biological component of Co-enzyme A, and widely found in virtually all living cells. Pantothenic acid is freely soluble in water, stable in neutral solution, but unstable to acids, bases and heat. Supplements of pantothenic acid are available as the calcium salt or the alcohol, which are more stable, highly soluble in water and rapidly converted to the free acid in the body.

Pantothenic acid is absorbed through a saturable sodium dependent mechanism, and by passive diffusion at higher concentrations. Its biological availability from food might be only 50% that of pure vitamin based upon patterns of urinary excretion (Tarr *et al.*, 1981). It is transported around body to tissues primarily as bound forms in erythrocytes.

Pantothenic acid is essential for the function of Co-enzyme-A and the majority of tissues transport the compound into cells through an active sodium co-transport mechanism. It performs multiple roles in cellular metabolism and in the synthesis of many essential metabolites. Urinary excretion is highly correlated with dietary intake.

A deficiency of pantothenate is said to be extremely rare, but deficiency has been associated with burning feet syndrome.

Pantothenate appears to have important interactions with other nutrients.

Supplementation with thiamine (5 mg) or riboflavin (5 mg) might increase mean serum and urinary excretion of pure pantothenic acid in adults (Koyangi *et al.*, 1969).

Animal studies suggest a possible sparing action of B_{12} on the basis that they may share a common carrier mediated transport system in a number of tissues.

In guinea pigs there may be a beneficial interaction with ascorbic acid.

5.1 Milk content

The mean concentration of pantothenic acid in human milk has been reported as 6.7 mg/L (1 mg/100 kcal) (Johnston *et al.*, 1981); the mean minus 1 SD as 1.9 mg/L (284 ug/100 kcal) (Song *et al.*, 1984); and the range as 2 to 2.5 mg/L (269 to 552 µg/100 kcal) (Picciano, 1995); and 0.48 to 2.45 mg/L (269 to 552 µg/100 kcal) (Plesofsky-Vig, 1996). The differences have been attributed to maternal dietary variation, or analytical approaches.

For pasteurized whole cows' milk the range has been reported as 3100 to 3600 µg/L (465 to 530 µg/100 kcal (Jensen, 1995).

Other than the content in milk, there is no other basis for deriving an estimate of the dietary requirement for pantothenic acid as there are no satisfactory indices of pantothenate status. Urinary excretion reflects dietary intake, and for adults an excretion below 1 mg/day has been considered inadequate. Therefore best estimates have been based on consumption of breast fed infants, because there are no reports of deficiency in infants who are breast-fed by adequately nourished mothers.

5.2 Existing regulations and recommendations

Infant formulae in the USA should contain a minimum level of 300 μg/100 kcal of pantothenic acid (FDA, 1985). The estimated safe and adequate daily dietary intake as established by the FNB is 2000 μg/day (400 μg/kg or 400 μg/100 kcal for the 5 kg infant consuming 500 kcal/day).

The LSRO Expert Panel recommended maintaining the minimum content of pantothenic acid in infant formulae at 300 μg/100 kcal. Based on mean minus 1SD for levels in human milk (1.9 mg/L, 284 μg/100 kcal), which was rounded off to 300 μg/100 kcal.

The LSRO Expert Panel recommended a maximum content of 1200 μg/100 kcal based on the 90^{th} centile of the FDA analyses of infant formulae.

Within the EU the Infant Formulae Directive specifies that the minimum pantothenate content of infant formulae should be 300 μg/100 kcal. No maximum has been set.

The Committee did not set a PRI for pantothenate (SCF, 1993b). Owing to the lack of systematic oral dose response studies and the very low toxicity of pantothenic acid, the Committee did not derive a numerical tolerable upper intake level (SCF, 2002d).

5.3 Recommendation

The Committee makes the following recommendation on the pantothenic acid content of infant formulae and follow-on formulae: minimum 400 μg/100 kcal, maximum 2,000 μg/100 kcal.

6. PYRIDOXINE, B_6

Pyridoxine embraces a group of chemically related compounds including pyridoxamine and pyridoxal which are found in animal products and pyridoxine which is found in plants. All forms are taken up from small intestine and converted to pyridoxal phosphate in tissues. A proportion of vitamin B_6 in plant-based foods is biologically unavailable because it is present as pyridoxine glycosides that are not hydrolysed by intestinal enzymes. All three forms are readily soluble in water, light sensitive and relatively heat stable in acid medium, but heat labile in alkaline medium. Pyridoxine hydrochloride, the most heat stable form, is used to fortify infant formulae.

Pyridoxine requires riboflavin, zinc and magnesium for its normal function. Pyridoxine deficiency may result in low blood levels of vitamin C, increased excretion of calcium, zinc and magnesium, and reduced copper absorption.

Vitamin B_6 stored in liver with about 50% being present in muscle bound to glycogen phosphorylase. Pyridoxal phosphate acts as a co-enzyme in the metabolic transformation of amino acids, decarboxylation, transamination and racemization, the metabolism of lipids and nucleic acids and in glycogen metabolism. The metabolism of vitamin B_6 is strongly altered in premature infants (Zempleni, 1995).

Pyridoxine is primarily absorbed in the jejunum by a non-saturable, passive process and is transported in the plasma bound to albumin.

The dietary requirement for pyridoxine varies in relation to the dietary consumption of protein (Hansen *et al.*, 1996).

There was an outbreak of pyridoxine related convulsive seizures in early 1950's, which was associated with an hypochromic microcytic anaemia, vomiting, diarrhoea, failure to thrive, listlessness, hyper-irritability, seizures. The concentration of pyridoxine in the formula associated with the symptoms was on average 62 µg/L (9.2 µg/100 kcal; 4.1 µg/g protein), compared with 92 µg/L (13.7 µg/100 kcal; 5.6 µg/g protein) in the formula that was not associated with symptoms (Borschel, 1995).

6.1 Milk content

The pyridoxine content of milk increases as the maternal intake of the vitamin increases and correlates with measures of pyridoxine status, which in turn is affected by stage of lactation, length of gestation and oral contraceptive use. The pyridoxine content of human milk increases over the course of lactation (LSRO, 1998). In mature milk the concentration may vary from 70 to 310 µg/L (10.4 to 46.3 µg/100 kcal), and in their summary Bates and Prentice (1994) estimated that for 17 studies in unsupplemented women the pyridoxine content was 150 µg/L (22 µg/100 kcal). The primary form of pyridoxine is pyridoxal, 81%, and pyridoxal phosphate, 7%.

For cows' milk the range is range 400 to 650 µg/L (60 to 97 µg/100 kcal), of which pyridoxal is 80% and pyridoxamine 20% (LSRO, 1998).

The possibility that breast fed infants might have marginal, poor of deficient pyridine status is reviewed in LSRO (1998). Indices of pyridoxine status tend to be lower in breast-fed than formula-fed infants. Biochemical evidence of marginal pyridoxine status has been reported in infants breast-fed by mothers having intakes less than 2 mg/day, and whose milk provided less than 100 µg/day. The pyridoxine content of mother's milk, and the intakes of infants, parallelled the level of maternal supplementation from 2 to 27 mg/day. Infants of mothers who take daily supplements containing 10 to 27 mg pyridoxine hydrochloride, have intakes of 160 to 280 µg/day (24 to 42 g/100 kcal), and appear to have adequate pyridoxine status, with increased levels of pyridoxal phosphate in plasma and a decrease in the stimulation index for erythrocyte alanine amino transferase (Kang-Yoon *et al.*, 1992; Kang-Yoon *et al.*, 1995). The protein content of mature human milk is about 10.6 g/L. A pyridoxine intake of 50 µg/day should be adequate in preventing deficiency symptoms in most exclusively breast fed infants below 6 months of age, based upon minimum pyridoxine intake of 5.56 µg/g protein and an average milk consumption of 0.75 L/day (Borschel, 1995).

A formula providing 5.56 µg pyridoxine/g protein was sufficient in preventing convulsions in most young infants, whereas 4.13 µg/g protein was not sufficient in preventing deficiency symptoms in all infants (Borschel, 1995). Most standard formulae provide 14.2 to 18.0 g/L protein, with some formulae providing up to 22 g/L. Based on the highest level of protein and an average formula intake of 0.75 L/day, a pyridoxine intake of 104 µg/day should be sufficient in maintaining optimal pyridoxine status in these infants (Borschel, 1995). The pyridoxine content of most infant formulae is around 400 µg/L, or higher (~60 µg/100 kcal). Therefore, formula-fed infants consuming 0.75 L/day would consume greater than 300 µg/day pyridoxine (Borschel, 1995).

It has been concluded that a pyridoxine intake of 50 µg/day by breast-fed infants or 104

µg/day by formula-fed infants appears to prevent biochemical indicators of reduced status, according to Borschel (1995). However, an intake of 160 µg/day may be needed to reduce biochemical evidence of marginal pyridoxine status for most infants aged four to six months, based upon the evidence of Kang-Yoon *et al.* (1992). On this basis the mother would have to have an intake greater than 10 mg/day, to ensure that the infant fed human milk achieved a pyridoxine intake of 100 µg/day.

Although pyridoxine toxicity has not been reported for infants, there is some evidence for adverse effects in adults taking higher doses in the region of 150 to 200 mg/day.

6.2 Existing regulations and recommendations

Based on the Committee on Nutrition of the American Academy on Pediatrics recommendations (1985), the USA Code of Federal Regulations specifies that infant formula contain no less than 35 µg/100 kcal of pyridoxine, but does not specify a maximum level (FDA, 1985). Further, because pyridoxine is involved in protein metabolism, and the requirement is related directly to the level of protein in the diet, pyridoxine should be present at a level of at least 15 µg for each g protein in excess of 1.8 g protein per 100 kcal of infant formula. Thus, the minimum content recommended for pyridoxine in infant formulae is 30 µg/100 kcal.

Although deficiency manifestation have not been reported in infants fed formulae providing 100 µg pyridoxine per L (15 µg/100 kcal for a 5 kg infant consuming 100 kcal/kg/day), an intake of 130 µg/day (26 µg/100 kcal for a 5 kg infant consuming 100 cal/kg/day), may be required to achieve adequate pyridoxine status. The value of 26 µg/100 kcal was rounded to 30 µg/100 kcal. At the recommended maximum protein content in infant formulae (3.4 g/100 kcal) a pyridoxine content of 30 µg/100 kcal results in an intake level of 8.8 µg pyridoxine/g protein, which is well above 5.6 µg/g estimated as necessary to prevent deficiency.

The LSRO Expert Panel recommended a maximum content of pyridoxine in infant formulae of 130 µg/100 kcal based on the 90th centile of the FDA analyses of infant formulae.

Within the EC the Infant Formulae Directive specifies that the minimum pyridoxine content of infant formulae should be 35 µg/100 kcal. No maximum has been set.

The PRI for infants aged 6-11 months set by the Committee for pyridoxine is 0.4 mg/day (SCF, 1993b). However, as noted above there are data which indicate that a lower intake is acceptable for normal function. For children aged 1-3 years, the Committeed has set a tolerable upper intake level of 5 mg/day (SCF, 2000a).

6.3 Recommendation

The Committee makes the following recommendation on the pyridoxine content of infant formulae and follow-on formulae: minimum 35 µg/100 kcal, maximum 165 µg/100 kcal.

7. FOLATES

Folate is a general term for compounds that have a common vitamin activity and includes the synthetic form of the vitamin folic acid (pteroyl glutamic acid, PGA) and a wide variety of derivatives. Folic acid, the synthetic form, is not present in nature and has about twice the

activity of natural folates.

Pteroyl glutamic acid consists of three subunits, pteridine, *p*-aminobenzoic acid and glutamic acid, and has a molecular mass of 441. It is odourless and tasteless, and is virtually insoluble in water, alcohol, acetone, chloroform, and ether; but is soluble in hydrochloric acid and sulphuric acids. The disodium salt is freely soluble. Aqueous solutions are heat sensitive and decompose rapidly in the presence of light. The derivatives of PGA are predominantly present in the body and foods as reduced folates, with variable numbers of glutamate residues.

Folate-deficiency has not been identified in breast-fed infants, even in those with folate deficient mothers.

The majority of folate is absorbed within the proximal small intestine, following hydrolysis of polyglutamates to monoglutamate. Folate monoglutamates are transported by energy dependent carrier mediated processes. Non-saturable, passive diffusion occurs at high intraluminal concentrations. Folate monoglutamates are the main circulating and transport forms, with polyglutamates being the main intracellular forms.

Folate is functionally important for one carbon transfer reactions, including those involved in amino acid metabolism (serine/glycine and homocysteine/methionine interconversion), purine and pyrimidine synthesis, and the formation of the primary methylating agent, S-adenosyl methionine (SAM).

Folate deficiency is associated with a reduction in *de novo* DNA biosynthesis and thus impairment of cellular replication: recognised haematologically as macrocytic anaemia. Poor folate status in the periconceptual period has been associated with increased risk of neural tube defects in the offspring. Poor folate status may lead to hyperhomocysteinaemia, which has been associated with and increased risk of vascular disease.

Excess dietary folate tends to be excreted in the urine rather than stored in the body. Consumption of high levels of folic acid has been associated with increased risk of neurological damage in older adults caused by masking B_{12} deficiency.

7.1 Milk content

A wide range of folate concentrations have been reported in human milk, from 24-141 µg/L (3.8 to 20.9 µg/100 kcal) (LSRO, 1998), in part due to the difficulties in reliably determining the folate content of milk, uncertain storage methods, in part due to sampling differences and possibly to inherent differences amongst women (Bates and Prentice, 1994). For pasteurized cows' milk, the folate concentration has been reported to range from 50 to 60 µg/L (8.0 to 9.5 µg/100 kcal) (LSRO, 1998).

Salmenpera *et al.* (1986) followed 200 infants of healthy well-nourished mothers, all of whom were given a folate supplement (0.1 mg/day). In infants fed a formula containing 35 µg/L (5 µg/100 kcal, FAO/WHO recommended level), 69 to 94% had plasma folate levels below the lowest levels for the breast-fed infants. Those infants weaned earliest had the lowest haemoglobin concentrations and highest MCV at 9 months of age. The authors concluded that infants fed the recommended level were at risk of developing folate deficiency.

7.2 Existing regulations and recommendations

The folate requirement established by the Joint FAO/WHO Expert Group (1970) was 5 µg/kg body weight, but was based on very limited data.

The regulations in the USA specified the minimum folic acid in infant formulae as 4 µg/100 kcal (FDA 1985), based upon the Committee on Nutrition of the American Academy on Pediatrics (1985). There was no maximum identified.

The recommendation that the minimum content of folic acid in infant formula be 11 µg/100 kcal, was based on the most reliable and valid data on total folic acid levels in human milk for which the mean minus 1SD value was taken as 71 µg/L or 10.6 µg/100 kcal by LSRO. The recommendation that the maximum content of folic acid in infant formulae be 40 µg/100 kcal was based on the 90th centile of the FDA analyses of infant formulae.

Within the EU the Infant Formulae Directive specifies that the minimum folate content of infant formulae should be 4 µg/100 kcal. No maximum has been set.

The PRI for infants aged 6-11 months set by the Committee for folate is 50 µg/day (SCF, 1993b). For children aged 1-3 years, the Committee set a tolerable upper intake level for folic acid of 200 µg/day (SCF, 2000b).

7.3 Recommendation

The Committee makes the following recommendation on the folate content of infant formulae and follow-on formulae: as folic acid, minimum 10 µg/100 kcal, maximum 30 µg/100 kcal.

8. COBALAMIN, B_{12}

Cobalamin is a water-soluble vitamin and a member of a family of related molecules known as corrinoids which contain a corrin nucleus made from a tetrapyrrolic ring structure. The centre of the tetrapyrrolic ring nucleus contains a cobalt ion that can be attached to a methyl, deoxyadenosyl, hydroxy, or cyano groups.

Cobalamin is present in all or most animal tissues, but ultimately derived from bacteria, fungi or algae. Yeasts fruit and vegetables do not contain cobalamin. Populations on strict vegetarian diets have the lowest dietary intakes of cobalamin. The major form which is used in food fortification is cyanocobalamin.

Heating food can result in losses of cobalamin activity from 10 to 90%.

Cobalamin absorption in the lower ileum is mediated by intrinsic factor secreted in the stomach (1.5-2.0 µg/day), but a low level of absorption can also take place by diffusion which is not mediated by intrinsic factor (about 1.2% exposure). Cobalamin enters cells mainly through receptor-mediated endocytosis.

Cobalamin is stored in the liver and at birth the liver of a full-term infant contains 25-30 µg cobalamin with the total body content being 30-40 µg. There is no, or only a limited, increase in the concentration of cobalamin in breast milk following supplementation of replete women.

Cobalamin is excreted in urine and faeces, with entero-hepatic recycling through bile (up to 0.2% pool/day, up to 10 μg, with all but ~1 μg being reabsorbed, and <1 μg excreted in urine).

Cobalamin is involved in the synthesis of DNA, the synthesis and transfer of single-carbon units such as methyl groups and in the synthesis of methionine and choline. It is a cofactor for methionine synthase (pivotal in one carbon metabolism), and methylmalonyl CoA mutase (even chained fatty acid synthesis).

Those at risk of cobalamin deficiency are strict vegans and those with gastrointestinal pathology. Deficiency may give rise to hyperhomocysteinaemia, and leads to macrocytic anaemia, and a demyelinating neuropathy.

8.1 Milk content

Bates and Prentice (1994) report a mean of 10 studies from developed countries as 0.51 μg/L. The range of values for milk from unsupplemented mothers is 0.16 to 0.64 μg/L (0.02 to 0.09 μg/100 kcal). The wide range of values may reflect analytical difficulties rather than intrinsic biological variability (Picciano, 1995). The concentration is relatively stable up to 12 weeks, with a progressive decrease to about 50% by 27 to 35 weeks post-partum.

The cobalamin content of pasteurized whole cows' milk is 3 to 5 μg/L (0.4 to 0.7 μg/100 kcal (Jensen, 1995).

Deficiency of cobalamin is rare in infants, although it can occur with a congenital deficiency of intrinsic factor, or as the result of an inadequate intake from human milk of mothers consuming a strict vegetarian or vegan diet, or rarely infants given goat's milk. When a deficiency has been reported in infants fed human milk, the cobalamin content of the milk has been 0.03 to 0.09 μg/L (0.004 to 0.013 μg/100 kcal). Formula-fed infants had higher cobalamin concentrations and lower urinary methyl malonic acid excretion than breast-fed infants (Specker *et al.*, 1990a and b). At intakes of 0.37 μg/day (RDA 0.3 μg/day) urinary methyl malonic acid excretion became inversely related to intake. Herbert (1996) suggested that an oral dose of cobalamin 0.1 μg/day corrects deficiency.

Oral doses of cobalamin up to 100 g/day are said to be non-toxic, with excessive amounts being excreted in the urine.

8.2 Existing regulations and recommendations

Based on the Committee on Nutrition of the American Academy on Pediatrics (1985), regulations in the USA require that infant formula contain no less than 0.15 μg/100 kcal of cobalamin (FDA, 1985). A maximum is not specified.

The LSRO Expert Panel recommended a minimum content of cobalamin in infant formulae of 0.08 μg/100 kcal (0.54 μg/L; 0.4 μg/day). This is based on the mid range of mean cobalamin levels in human milk and represents an intake above that reported to be associated with biochemical evidence of deficiency (i.e. <0.37 μg/day). This amount of cobalamin will provide a sufficient intake to meet the RDA from birth to 6 months (0.3 μg/day; 0.06 μg/100 kcal) for a 5 kg infant consuming 100 kcal/kg/day and provides an additional margin of safety.

The LSRO Expert Panel recommended a maximum content of cobalamin in infant formulae of 0.7 µg/100 kcal based on the 90th centile of the FDA analyses of infant formulae.

Within the EU the Infant Formulae Directive specifies that the minimum cobalamin content of infant formulae should be 0.1 µg/100 kcal. No maximum has been set.

The Committee set the PRI for cobalamin for infants aged 6-11 months as 0.5 µg/day (SCF, 1993b). No numerical tolerable upper intake level has been set, since there are no clearly defined adverse effects produced by vitamin B_{12} that can be used to define a LOAEL or NOAEL (SCF, 2000d).

8.3 Recommendation

The Committee makes the following recommendation on the cobalamin content of infant formulae and follow-on formulae: minimum 0.1 µg/100 kcal, maximum 0.5 µg/100 kcal.

9. ASCORBIC ACID, VITAMIN C

L-ascorbic acid is a six carbon compound structurally related to glucose. L-ascorbic acid has a molecular mass of 176 and is a strong reducing agent. Its oxidised derivative is L-dehydroascorbic acid and both forms have biological activity and are interconvertible by an oxidation/reduction reaction. Most tissue ascorbic acid exists in the reduced form (90%).

Most plants and animals synthesise ascorbic acid, but humans and guinea pigs lack hepatic L-glulono-gamma-lactone oxidase, which is necessary for the conversion of 2-keto-L-gulonolactone to L-ascorbate.

L-ascorbic acid decomposes rapidly in water, due to rapid oxidation with atmospheric oxygen, but there is no evidence of heat degradation.

L-ascorbic acid is absorbed by a sodium dependent, active transport process, with efficiency being up to 98% at low doses. L-ascorbic acid is transported in plasma as the free anion and is primarily lost to the body through urinary excretion.

L-ascorbic acid acts as a strong reducing agent, and has diverse biochemical functions, being involved in the synthesis of collagen, neurotransmitter formation, carnitine synthesis, and enhancing iron absorption.

A clinical deficiency of L-ascorbic acid leads to a clinical condition identified as scurvy.

9.1 Milk content

Bates and Prentice (1994) estimated the mean L-ascorbic acid content of human milk from 11 studies of unsupplemented women from western countries, as 55 mg/L. However, there is wide variation, from 30 to 100 mg/L (4.5 to 15 mg/100 kcal), with a progressive decrease during the course of lactation (LSRO, 1998).

The L-ascorbic acid concentration in cows' milk is lower, about 8 to 20 mg/L (1.3 to 3.3 mg/100 kcal) (LSRO, 1998).

9.2 Existing regulations and recommendations

Based on recommendation of the Committee on Nutrition of the American Academy on Pediatrics (1985), the regulations in the USA set a minimum level for L-ascorbic acid in infant formulae as 8 mg/100 kcal, while no maximum level is specified (FDA 1985).

The LSRO Expert Panel recommended a minimum content of L-ascorbic acid in infant formulae of 6 mg/100 kcal, which is consistent with RDA of 30 mg (equivalent to mean concentration in human milk of 40 mg/L assuming consumption of 0.75 L/day) and is equivalent to 6 mg/100 kcal for a 5 kg infant consuming 100 kcal/kg/day.

The Expert Panel recommended a maximum content of L-ascorbic acid in infant formulae of 15 mg/100 kcal. This would results in an intake of 15 mg/kg/day for an infant consuming 100 kcal/kg/day (on a body weight basis equivalent to 1 g/kg/day in 70 kg adult man (NOAEL). The Expert Panel recommended that research be undertaken to establish NOAEL for vitamin C in infancy.

Within the EU the Infant Formula Directive specifies that the minimum L-ascorbic acid content of infant formulae should be 8 mg/100 kcal. No maximum has been set.

The PRI for L-ascorbic acid set by the Committee is 20 mg/day (SCF, 1993b). However, this is now considered to be low, and a number of countries have set higher levels based upon newer evidence.

9.3 Recommendation

The Committee makes the following recommendation on the ascorbic acid content of infant formulae and follow-on formulae: minimum 10 mg/100 kcal, maximum 30 mg/100 kcal.

10. BIOTIN

D-Biotin is a water-soluble and has a bicyclic ring structure, one of which contains sulfur.

All biotin is formed from micro-organisms and it is widely distributed in foods at low levels. It is usually present as biocytin, covalently bound to enzymes, and is relatively rich in liver, yeast, egg yolk, soy flour and cereals.

The preferred methods for assessing status are the urinary excretion of 3-hydroxyisovaleric acid, or biotin itself (Zempleni, 1995).

The biotin present in a bound form in food is by biotinidase in pancreatic secretions. Biotin is absorbed directly by a highly specific transporter and by non-specific peptide transporters. It is synthesised by the colonic microflora and probably absorbed, although the extent is not known. Some anticonvulsant drugs and alcohol may inhibit absorption.

Unabsorbed biotin is excreted in faeces and absorbed excreted in urine. Steroid hormones and some anticonvulsant drugs may accelerate catabolism and excretion (Mock, 1996).

Its function is as co-enzyme for several carboxylases, where it acts as a carrier for active bicarbonate: pyruvate carboxylase, methyl crotonyl carboxylase, propionyl Co-enzyme-A

carboxylase, acetyl Co-enzyme-A carboxylase. Therefore plays important role in metabolism of carbohydrates, fats and amino acids. Biotin may have a role in the regulation of gene expression.

A clearly characterised deficiency syndrome has been demonstrated following prolonged consumption of large amounts of egg whites (biotin bound by avidin and not available for absorption), during parenteral nutrition for intestinal failure, and in some infants on special formulae. Deficiency has generally been considered to be rare, but there is recent evidence of biochemical deficiency during pregnancy. Signs of deficiency include erythematous seborrhoeic dermatitis, alopecia, conjunctivitis and abnormalities of the central nervous system (hypotonia, lethargy and developmental delay). Toxicity is not thought to be a problem because of biotin is relatively insoluble in water, which limits its availability for absorption. Up to 60 mg/day for a period of 6 months was not associated with any adverse effects (Dakshinamurti, 1994), and 5-10 mg/day was given to infants as part of their parenteral nutrition without adverse effect (Fomon McCormick, 1993).

10.1 Milk content

Biotin in human milk is nearly all free and water-soluble. The average content is 6 µg (24 nmol/L) and the range 5-9 µg/L (0.75-1.3 µg/100 kcal) (Salmenpera *et al.*, 1985; Hirano *et al.*, 1992; Bates and Prentice, 1994; Picciano, 1995). Estimate consumption between 0-6 months is about 5 µg/day.

In pasteurized cows' milk there is 20-47 µg/L (3-7 µg/100 kcal) (LSRO, 1998).

There is no evidence of dietary deficiency in breast-fed or formula-fed infants. Infants on 0.75 L/day, with 5-9 µg/L (0.75-1.34 µg/100 kcal) would obtain 3.8-6.8 µg/day. Mean minus 1SD breast-fed infant, 5 µg/L; for model infant 0.75 µg/100 kcal, suggest at or above requirements.

There is no RDA. The FNB considered 10 µg/day safe and adequate dietary intake for 0-6 months of age (large safety factor built in).

10.2 Existing regulations and recommendations

The LSRO report recommended a minimum content of 1 µg/100 kcal, based on mean minus 1 SD contents of human milk, 0.5-0.75 µg/100 kcal, rounded to 1. A maximum content of 15 µg/100 kcal was recommended, based on the 90th centile of FDA analyses of infant formulae.

Within the EU the Infant Formulae Directive specifies that the minimum biotin content of infant formulae should be 1.5 µg/100 kcal (0.4 µg/100kJ) while no maximum level has been set.

The Committee set no PRI for biotin (SCF, 1993b). The Committee also concluded that it could not derive a numerical tolerable upper intake level due to the lack of systematic oral intake dose-response studies (SCF, 2001c).

10.3 Recommendation

The Committee makes the following recommendation on the biotin content of infant formulae and follow-on formulae: minimum 1.5 µg/100 kcal, maximum 7.5 µg/100 kcal.

Table 1. Requirements of water-soluble vitamins of infants (in mg or µg per day)

Vitamin	EU PRI	USA DRIs, 2001 Adequate Intake (AI)	FAO/WHO, 2001a Recommended Nutrient Intake (RNI)
	(6-11 months)	(0-6 months)	(0-6 months)
B_1, thiamine	0.3 mg	0.2 mg	0.2 mg
B_2, riboflavin	0.4 mg	0.3 mg	0.3 mg
B_3, niacin	5 mg NE	2 mg preformed	2 mg preformed
B_5, pantothenate	-	1.7 mg	1.7 mg
B_6, pyridoxal	0.4 mg	0.1 mg	0.1 mg
Folate, as folic acid	50 µg	65 µg	80 µg
B_{12}, cobalamin	0.5 µg	0.4 µg	0.4 µg
Biotin	-	5 µg	-
C, L-ascorbate	20 mg	40 mg	25 mg

Table 2. Current requirements for water-soluble vitamins in infant formulae and follow-on formulae and recommended levels (in µg or mg per 100 kcal)

Vitamin	Infant Formulae Directive	USA (LSRO)		SCF recommendation	
	Minimum	Minimum	Maximum	Minimum	Maximum*
B_1, thiamine	40 µg	30 µg	200 µg	60 µg	300 µg
B_2, riboflavin	60 µg	80 µg	300 µg	80 µg	400 µg
B_3, niacin	800 µg NE	550 µg	2000 µg	300 µg preformed**	1,200 µg preformed**
B_5, pantothenate	300 µg	300 µg	1200 µg	400 µg	2,000 µg
B_6, pyridoxal	35 µg	30 µg	130 µg	35 µg	165 µg
Folate, as folic acid	4 µg	11 µg	40 µg	10 µg	30 µg
B_{12}, cobalamin	0.1 µg	0.08 µg	0.7 µg	0.1 µg	0.5 µg
Biotin	1.5 µg	1 µg	15 µg	1.5 µg	7.5 µg
C, L-ascorbate	8 mg	6 mg	15 mg	10 mg	30 mg

* The recommended maximum level has been set at five times the minimum where there is no evidence that this level might be associated with any adverse effect. For niacin and pyridoxal the maximum recommended is below the estimate of the safe upper level estimated from an extrapolation of the daily upper limit determined for 1-3 years old children (SCF, 2002a and 2000a). For folic acid and ascorbic acid the recommended maximum has been set at three times the minimum, and for each this value is close to an estimate of the safe upper level estimated from an extrapolation of the daily upper limit determined for 1-3 year old children (for folic acid: SCF, 2000b; for ascorbic acid FNB, 2000a): see text.

** As nicotinamide

VIII. MINERALS AND TRACE ELEMENTS

A. IRON

1. INTRODUCTION

Iron is essential for virtually every living organism. The dominating function of iron in the human body is as the oxygen-binding core of haemoglobin, the red pigment of blood transporting oxygen from the lungs to all tissues. During the progress of iron deficiency (ID), haemoglobin synthesis in the bone marrow is restricted resulting in anaemia. Anaemia caused by ID is called iron deficiency anaemia (IDA), distinguishing this condition from other causes of anaemia, such as infection, inflammation, haematological disorders and other nutritional deficiencies.

IDA is the most common micronutrient deficiency in the world with about 600 million individuals affected (DeMaeyer and Adiels-Tegman, 1985). Rapid growth makes infants and young children a particular risk group for IDA. In 1980, the WHO estimated the worldwide prevalence of anaemia in children below 4 years of age to be 43%, with a higher prevalence in developing regions (51%) than in industrialized countries (12%) (Fairweather-Tait, 1996). A recent WHO communication reports for the same age group 20% for children in industrialized countries and 39% in non-industrialized countries (WHO, 2002).

Human milk has unique properties, making exclusive breast-feeding a superior form of nourishment for infants during the first half of infancy. In affluent populations, exclusive breast-feeding prevents IDA during the first half of infancy in healthy term infants (Pisacane *et al.*, 1995; Siimes *et al.*, 1984). However, in socio-economically disadvantaged populations infants may be at risk of developing ID or even IDA if exclusively breast-fed beyond 4 months (Calvo *et al.*, 1992; Domellöf *et al.*, 2001). In affluent societies, healthy term infants fed infant formulae unfortified with iron seldom develop IDA before 4 months of age, but are at higher risk than are breast-fed infants after that age (Moffatt *et al.*, 1994; Pizarro *et al.*, 1991). For this reason, most infant formulae are fortified with iron, but the optimal level of added iron is still an open question.

2. IRON REQUIREMENTS IN INFANCY

2.1 Determinants of iron stores at birth

The term newborn infant has a total body iron content averaging about 75 mg/kg body weight (Widdowson and Spray, 1951), which can be compared with 55 mg/kg for an adult. Although, placental iron transport is negligible during the first two trimesters, it rises progressively to about 4 mg daily towards the end of the third trimester (Lukens, 1995). Consequently, both birth weight and gestational age are major determinants of total body iron content at birth. The amount of circulating haemoglobin at birth is a function of the haemoglobin concentration and the blood volume. The average haemoglobin concentration of cord blood from normal term infants is 170 g/L (range 135 to 210 g/L) (Lukens, 1995). The average blood volume at birth is 85 mL/kg, depending mostly on birth weight but to some extent also on timing of umbilical cord clamping (Brugnara and Platt, 1998). Thus, for infants with similar birth weights, individual variations in haemoglobin concentration and blood volume at

birth are major causes of differences in body iron content.

2.2 Requirements for growth

In adult humans, there is little exchange of iron between the body and the environment, since body iron is recycled and physiological iron losses are small. In the infant, however, the recycling yields a deficit since a substantial part of iron turnover is diverted to growing tissues. In a 6-month old infant ~0.5 mg iron/day is needed for expansion of the blood mass and 0.1 mg/day for growth of muscle and other tissues. Since iron requirements excluding growth would equal iron losses (0.15 mg/day), infant growth increases iron requirements 5-fold to approximately 0.75 mg/day. As a consequence of reduced haemoglobin concentration and redistribution of iron to storage sites, the infant is able to double its birth weight without exhausting its reserves, independent of external iron (Lukens, 1995). For the term, normal infant this occurs at about 5 months of age and, soon thereafter, iron utilization from the diet becomes critical for maintenance of iron balance (Oski, 1993). In proportion to body weight the need for dietary iron is greater during late infancy than during any other period of life (Dallman, 1992). Based on certain assumptions, such as total body iron at birth and basal iron losses Oski estimated the average requirement for absorbed iron during the first year of life to be 280 mg, averaging 0.8 mg/day (Oski, 1993). Such intake is virtually impossible to achieve with unmodified complementary foods, suggesting that additional iron is needed, either as iron addition to foodstuffs or as separate iron supplements to cover the estimated needs (WHO, 1998).

2.3 Iron stores at birth affect requirements after birth

A randomised trial of iron supplementation of Swedish and Honduran infants of normal birth weight, in which the infants were all exclusively breast-fed until 6 months of age, showed significant differences in iron status between the Swedish and Honduran infants already at 4 months of age. At 9 months, when the infants were partially breast-fed, the proportion of IDA was <3% in the unsupplemented Swedish infants, suggesting that the iron requirements of these infants are lower than previously assumed. In contrast, 28% of the unsupplemented Honduran infants were classified as IDA at 9 months, suggesting a higher iron requirement in socioeconomically disadvantaged populations (Domellöf *et al.*, 2001).

3. ADDITION OF IRON TO INFANT FORMULAE

Despite the documented preventive effect on ID of adding iron to infant formulae, IDA in infancy is still a major public health problem, not only in developing countries (Haschke and Male, 1996; ESPGHAN, 2002a), but also in certain populations in industrialized countries, among whom non-fortified formulae and unmodified cows' milk are still widely used (Daly *et al.*, 1996; Freeman *et al.*, 1998). In the United States 13% of 1-year olds fulfil the criteria of ID (Looker *et al.*, 1997). A recent prospective, multi-centre cohort study from 11 European areas (the Euro-Growth study) showed in the same age group an overall prevalence of 7.2% ID and 2.3% IDA, using multiple criteria to define ID (Male *et al.*, 2001). The prevalence of anaemia was 9.4% of which more than 40% was associated with normal iron status, and an increased frequency of recent infections. Data from the Swedish cohort of the Euro-Growth study further illustrates the difficulty to interpret such prevalence data. Of the healthy, well-nourished Swedish infants (61% were breast-fed at 6 months and 91% received iron-fortified cereal and milk-based follow-on formulae), 26% had serum ferritin values <12 μg/L at 12 months, indicating ID, and 13% haemoglobin concentrations <110 g/L, indicating anaemia

(Persson *et al.*, 1998). This could suggest that the phytate-rich complementary food caused impaired iron status due to poor bioavailability despite sufficient iron intake. However, the proportion of infants with low haemoglobin and low serum ferritin values did not differ between Euro-Growth countries (Haschke and Male, 1996). Furthermore, also in this cohort only a few infants had both low haemoglobin and low serum ferritin, which casts doubt on the validity of the reference values used for haemoglobin and ferritin in infancy (ESPGHAN, 2002a; Emond *et al.*, 1996). Breast feeding, addition of iron to infant foods including formulae, and the avoidance of unmodified cows' milk in the diet of young children are key measures in combating IDA (ESPGHAN, 2002a; AAP, 1999).

4. IRON IN BREAST MILK AND INFANT FORMULAE

The concentration of iron in human milk is ~0.3 mg/L, which is approximately the same as in cows' milk, but the difference in bioavailability is reported to be at least five-fold in favour of human milk. The iron content of commercially available infant formulae varies widely, ranging from about 1 mg/L in unfortified formula to as much as 15 mg/L in some iron-fortified formulae. Table 1 shows the various iron levels recommended by different authorities for use in infant formulae. It is obvious that there is no consensus with respect to the fortification level (Table 1).

Table 1. Recommended iron contents of infant formulae

Year	Authority	Minimum (mg/L)	Maximum (mg/L)
1977	ESPGHAN	7	-
1981	Codex Alimentarius Commission	1 mg/100 kcal	-
1991	European Commission	3	11
1998	Life Science Research Office	1.3	11
1999	American Academy of Pediatrics	4	12

In 1977, the European Society for Pediatric Gastroenterology and Nutrition recommended 7 mg/L as the minimal level, but no upper level for iron was set, nor was the use of unfortified formulae (0.7-1.4 mg/L) explicitly discouraged (ESPGHAN, 1977). The current Codex Alimentarius Commission standard is a lowest level of 1 mg iron/100 kcal and no upper level set (CAC, 2002). The European Commission currently recommends an iron content of 3-11 mg/L (Directive 91/321/EEC), and the Life Science Research Office Report 1998, prepared for the USA Food and Drug Administration, recommends a minimal iron content of 0.2 mg/100 kcal (corresponding to ~1.3 mg/L) and a maximal level of 11 mg/L. The most recent recommendation from the American Academy of Pediatrics is to avoid all use of unfortified formulae, and to provide iron-fortified formulae containing 4-12 mg/L to all infants who are not breast-fed or are partially breast-fed throughout the first year of life (AAP, 1999).

In the United States, most formulae on the market are fortified at the upper level of the current recommendations, i.e. 10-12 mg elemental iron/L. However, low-iron and unfortified formulae still account for 9 -30% of the market share (AAP, 1999), whereas in Europe, infant formulae are generally fortified at lower levels (4-7 mg/L), although, as in the United States, unfortified formulae are still available and used (AAP, 1999; Ruiz *et al.*, 1996).

5. ABSORPTION OF IRON FROM BREAST MILK AND INFANT FORMULA

The iron concentration in human milk is low (0.2-0.4 mg/L). This is thought partly compensated by its high bioavailability, often cited as 50% (Saarinen *et al.*, 1977). However, the fractional absorption of iron from human milk is highly variable and it was shown recently that it changes with infant age and total dietary iron intake (Domellöf *et al.*, 2002). Conversely, reported iron absorption from infant formulae and cows' milk ranges from 2 to 19%, with an average ~10% (ESPGHAN, 1977). The reason for this difference is not fully understood. Cows' milk contains almost four times more calcium and 6 times more phosphorus than mature human milk. Hallberg *et al.* (1992) studying iron bioavailability from human and cows' milk in healthy adults, suggested that >70% of the variation in iron bioavailability could be explained by differences in calcium content. The clinical relevance of this is uncertain, as calcium and phosphorus fortification of formula seem to have no effect on the iron status of infants (Dalton *et al.*, 1997). In mature human milk, the relation between whey protein and casein is about 60:40, whereas in cows' milk, it is 20:80. The effects of this difference in protein composition on iron absorption were studied by Hurrell *et al.* (1989), who found that both bovine casein and whey protein hampered iron absorption, an effect most probably reflecting the iron-binding properties of these proteins.

Ascorbic acid is a known enhancer of iron absorption. Cows' milk is much lower in ascorbic acid than is human milk, which may be another reason for more efficient absorption of iron from the latter (Lönnerdal, 1997). However, this difference is met by routinely generously fortifying modern formulae with ascorbic acid.

Finally, human breast milk, in contrast to cows' milk, contains a high concentration of the iron-binding whey protein lactoferrin, which may facilitate absorption of iron from human milk. Lactoferrin has a reasonably high affinity for iron, is fairly stable against intestinal proteolytic digestion, and binds to species-specific receptors in human intestinal mucosa (Hernell and Lönnerdal, 2002). The concentration of these receptors changes with age, and is highest during infancy. Several studies have failed to demonstrate that addition of bovine lactoferrin to infant formulae improve iron absorption, or iron status (Lönnerdal and Hernell, 1994; Hernell and Lönnerdal, 2002; Fairweather-Tait *et al.*, 1987; Schulz-Lell *et al.*, 1991; Chierici *et al.*, 1992). Using cross-over design, Davidsson *et al.* (1994b) studied the effect of human lactoferrin. Eight infants were randomised to receive breast milk from their own mothers. Infants were fed the milk untreated or after treatment to remove the lactoferrin. The milks also were extrinsically labelled with a stable iron isotope, allowing quantification of the amount of iron absorbed. Unexpectedly, iron absorption was higher from the lactoferrin-reduced milk. Thus a promoting effect of lactoferrin on iron absorption remains to be documented.

Assuming a daily intake of 800 mL breast milk and an iron concentration of 0.3 mg/L, of which 50% is absorbed, an exclusively breast-fed 4-month old infant would absorb 0.12 mg/kg/day of iron daily. This is approximately sufficient for covering the estimated basal iron losses (20 μg/kg/day) (Oski, 1993) but does not allow for net gain of iron. To achieve the same iron absorption from formula (assuming that 10% is absorbed), the iron content of formulae would need to be fivefold higher than that in human milk, or 1.5 mg/L.

Atkinson *et al*. (1996) compared iron status in infants fed formulae fortified with iron to 1.5, 7, or 12 mg/L, respectively, and found lower serum ferritin at 6 months in the 1.5 mg group. In a study of healthy term Swedish infants, there was no significant difference in haemoglobin and iron status at 6 months between infants fed a formula containing 2 mg iron/L from age 1

month as compared with those fed the same formula containing 4 mg iron/L, or exclusively breast-fed infants (Hernell and Lönnerdal, 2002). This suggests that during the first half of infancy, a fortification level of 2 mg/L is sufficient for healthy term infants in an affluent society such as Sweden.

Using different assumptions (that is, 7% absorption and an iron requirement of 0.06 mg/kg/day [Hokama, 1994]), the American Academy of Pediatrics Committee on Nutrition concluded that a formula containing 12 mg iron/L was necessary, although their recommended iron content is 4-12 mg in formulae intended for use throughout infancy and they also conclude that there is little or no advantage of iron fortification at a level greater than 1.2 mg/100 kcal, or 8 mg/L (AAP, 1999).

Obviously, it is difficult to make recommendations on iron contents based only on theoretical calculations from absorption figures and estimates of iron requirements. Carefully designed and controlled clinical studies with sufficient statistical power carried out in different strata of the infant population are essential to verify the adequacy of the iron level suggested from the available absorption and requirement studies.

6. FROM WHAT AGE IS IRON ADDITION TO FORMULA NEEDED?

Directly after birth, there is a sudden increase in tissue oxygen tension, which is reflected by a marked decrease in plasma erythropoietin, in turn leading to virtual cessation of erythropoiesis and haemoglobin synthesis during the first 6-8 weeks of life. (Lukens, 1995; Brugnara and Platt, 1998). Coincident with the ensuing decline in haemoglobin is a shift of iron from the circulating haemoglobin mass to storage sites. It is not until the haemoglobin level falls from the 170 g/L to about 120 g/L that erythropoiesis resumes. After the rapid decrease in haemoglobin during the first months of life, there is a slow decrease until a nadir is reached at about 8-18 months of age, when mean haemoglobin is usually 110- 20 g/L. After 2 years of age mean haemoglobin slowly increases to 135-140 g/L at 12 years of age (Guest *et al.*, 1938; Dallman and Siimes, 1979). Isotope studies have shown that iron from dietary sources does not appear in the circulating red cells in appreciable amounts until the age of 4-6 months (Smith *et al.*, 1955), which supports that the infant is not in need of exogenous iron until after that age. Thus the addition of iron to infant formulae intended for term, normal infants would not be necessary before age 4 months. This is supported by a recent study. Comparing breastfed infants with infants fed unfortified infant formula (<1 mg iron/L) or a fortified formula (5 mg iron/L) from birth to 3 months of age. Tuthill *et al*. (2002) found no difference in haemoglobin or iron status (MCV, ferritin) at either 3 or 12 months of age.

Contrasting with this however, it has been shown that infants do absorb iron tracer already at age 2 months, or even before, which has been interpreted as if also breast-fed infants need iron supplementation from early age (Fomon *et al.*, 1995b; Garby and Sjölin, 1959). Furthermore, although earlier studies showed that infants fed high-level iron-fortified formulae from the first few weeks of life increased their haemoglobin from ages 3-4 months (Marsh *et al.*, 1959; Andelman and Sered, 1966), more recent studies with lower but different iron levels have not confirmed this (Lönnerdal and Hernell, 1994; Hernell and Lönnerdal, 2002). There are, however, indications that infants have immature regulation of iron absorption and haemoglobin synthesis during the first 6 months (Domellöf *et al.*, 2001 and 2002). If so, absorption of tracer iron and the haemoglobin response to iron supplementation may not necessarily reflect a true need for exogenous iron.

As mentioned above and (Sturgeon, 1956) the amount of total body iron at birth may vary considerably, depending on birth weight and other factors. To allow for such variation in initial iron stores between individuals within the same population and also between populations of different socio-economic situations (Domellöf *et al.*, 2001), taking into consideration that IDA is the latest stage of iron deficiency, it seems clearly justified to recommend the addition of iron to infant formulae intended for use also before 4 months of age.

7. CLINICAL STUDIES OF DIFFERENT IRON CONTENTS IN FORMULAE

Even severe IDA in infancy may pass unnoticed, as symptoms such as paleness, fatigue, and developmental or behavioural disturbances are quite subtle. Impaired neurodevelopment and growth have been suggested as markers of iron deficiency or mild iron-deficiency anaemia, which can be assessed and quantified in addition to the common haematological and biochemical determinants of iron status in clinical studies of infants and children.

7.1 Haematologic outcome

In the early American studies on the effect of iron contents in infant formulae, an iron concentration of 12.7 mg/L was generally used (Marsh *et al.*, 1959; Andelman and Sered, 1966). Such formulae effectively prevented development of ID and IDA throughout infancy (Moffatt *et al.*, 1994; Walter *et al.*, 1993). Saarinen and Siimes (1978) showed that feeding a formula with 11 mg iron/L from early infancy resulted in significantly higher levels of serum ferritin at 6-12 months compared with feeding an unfortified, home-prepared cows' milk formula. Concentrations of 1.1 and 12.8 mg iron/L were compared in a study on Canadian term infants from very low-income families. The lower iron level gave significantly lower haemoglobin and iron status at 6 months (Moffatt *et al.*, 1994).

As formulae have become increasingly adapted - modified to resemble human milk more closely - several investigators have challenged the need for such high iron levels. Bradley *et al.* (1993) compared the effect of formulae fortified with 12.7 or 7.4 mg of elemental iron/L fed from early infancy to age 12 months, and showed no significant difference in growth or in iron status, except for a small but significant difference in ferritin in favour of the 12.7 mg group at 12 months. The authors concluded that the lower iron level could safely be used without risking development of iron deficiency. Haschke *et al.* (1993) compared lower iron levels (i.e. 3 *vs.* 6 mg iron/L) from age 3 months in healthy term infants with those in a breast-fed control group. There was no difference in iron status at 9 months between the two formula groups. Low serum ferritin (<10 μg/L) was slightly more common in the breast-fed group at ages 6 and 9 months.

Two Swedish studies compared haemoglobin values and iron status in healthy term infants receiving exclusively infant formula, with varying iron levels, with that of exclusively breast-fed infants during the first 6 months of life. In the first study, a formula containing 4 mg iron/L was compared with one containing 7 mg/L (Lönnerdal and Hernell, 1994), and in the second study, a formula with 2 mg iron/L was compared with one containing 4 mg/L (Hernell and Lönnerdal, 2002). There was no significant difference in haemoglobin levels or iron status between groups at 6 months in either of these studies.

7.2 Neurodevelopmental outcome

Several studies have shown impaired development and behavioural disturbances in infants with iron-deficiency anaemia (Roncagliolo *et al.*, 1998; Lozoff *et al.*, 1998), and in infants as well as in school children, iron deficiency can have adverse effects on cognition that are reversible with iron treatment (Idjradinata and Pollitt, 1993; Bruner *et al.*, 1996).

Moffatt *et al.* (1994), in their study of infants from very low income families, also compared the effect of feeding iron-fortified (12.8 mg/L) or unfortified formula (1.1 mg/L) on developmental status. The results indicated that, apart from a difference in anaemia and ID from age 6 months, psychomotor development assessed by the Bayley scales of infant development differed at ages 9 and 12 months in favour of the group fed iron-fortified formula. This difference had, however, disappeared at age 15 months, although by that time, 46% of the original cohort had been lost to follow-up. With a similar design, Williams *et al.* (1999) randomised 100 infants at age 6 to 9 months, whose mothers had already chosen to feed them with unmodified cows' milk, to either iron-fortified cows' milk formula (12 mg/L) or unmodified cows' milk up to the age of 18 months, after which both groups returned to unmodified cows' milk until age 24 months. Both groups experienced a decrease in age-specific developmental scores as measured by the Griffith scales, but the decline was greater in the group receiving unmodified cows' milk; the difference reached statistical significance at age 24 months but not at age 18 months. Daly *et al*. (1996) reported the haematological outcome of the same subjects, showing that the group receiving iron-fortified milk had significantly better iron status at all time points (i.e. ages 12, 18, and 24 months) despite switching back to the original milk at age 18 months.

Morley *et al.* (1999) randomised 493 infants at age 9 months to continuing cows' milk or receiving a formula containing 0.9 or 12 mg iron/L. The infants were followed up until age 18 months, when iron status and development were assessed. Although the infants fed the iron-fortified formula also had the highest serum ferritin, the authors could not show any difference in developmental score measured with the Bayley test.

Various studies have thus been performed using neurodevelopment outcome to assess iron requirement in infancy, but so far, the results have been inconclusive. The fact that different populations have been studied at different ages with different developmental tests makes it difficult to compare the studies. In socio-economic deprived populations in particular, IDA may merely be a marker of other nutritional deficiencies, which confuse the picture (Lozoff *et al.*, 1998). However, it also is possible that an early neurodevelopment insult caused by iron-deficiency anaemia may not result in detectable psychomotor delay during infancy, but symptoms such as deficits in attention and school performance may appear later.

7.3 Growth

Several studies have addressed the issue of whether iron supplementation affects growth, but the results have been contradictory. Iron supplementation to anaemic (Aukett *et al.*, 1986) or malnourished children (Angeles *et al.*, 1993; Morais *et al.*, 1993) has resulted in improved growth; in the latter case, possibly because of reduced morbidity. Some studies have shown no effect of iron intake on growth, although a higher intake affected iron status (Moffatt *et al.*, 1994; Daly *et al.*, 1996; Morley *et al.*, 1999), indicating that iron deficiency was not a growth-limiting factor in the infants studied. Two studies have in fact shown a negative effect on body growth when iron supplements were give to iron sufficient infants (Idjradinata *et al.*, 1994; Dewey *et al.*, 2002). It seems as if iron replete infants may be more susceptible to this

complication, although the mechanism is unknown.

8. POTENTIAL NEGATIVE EFFECTS OF ADDED IRON

While there are no indications that large iron stores confer a benefit to the individual, a high daily intake of iron may in fact have negative consequences. Besides infections and the possible adverse effect on growth mentioned above these include competition with respect to absorption of other minerals (Solomons, 1986), prooxidant effects (Schneider and Leibolod, 2000) and aggravating symptoms in diseases in which iron absorption is increased (Lynch, 1995).

8.1 Mineral absorption

There has been concern that the iron used to fortify formulae could compete with other divalent cations during absorption. A high iron/zinc molar ratio (i.e. >2:1) in the diet, especially where the total amount of ionic species is >25 mg, has been implicated as a cause of impaired zinc absorption (for review, see Solomons, 1986). The recent discovery of the divalent metal transporter 1 (DMT1) (Einstein and Blemings, 1998; Andrews, 1999) explains why there is a competition between absorption of iron and divalent cations. Those of most relevance for infants and young children are copper and zinc. There are indications from clinical studies in infants that infant formulae with high iron content (7 mg/L or higher) may have negative effect of copper absorption (Lönnerdal and Hernell, 1994; Haschke *et al.*, 1993), while the evidence for zinc from such studies are lacking (Bradley *et al.*, 1993; Haschke *et al.*, 1986).

8.2 Infections

Iron is a known trophic factor for several pathogenic bacteria, and there has also been concern that iron supplementation in breast-fed infants might saturate human milk lactoferrin, thus diminishing its anti-infective properties (Andersson *et al.*, 2000). Parenteral iron treatment has been associated with the exacerbation of malaria and with neonatal sepsis (Oppenheimer, 1989). For these reasons, it has been suggested that iron supplementation, or the addition of iron to infant formulae, might increase the incidence of gastroenteritis and other infections in infants. However, several studies have failed to confirm this theory for iron-fortified formulae. One study even showed that added iron provided a small but significant protection against diarrhoea (Scariati *et al.*, 1997).

8.3 Other adverse effects

Because of its pro-oxidant effects, excess iron has been implicated as a potential risk factor for cancer (Stevens *et al.*, 1994), as well as for coronary heart disease (Ascherio *et al.*, 1994). While several recent studies suggest an association between dietary iron intake and increased risk of colorectal cancer in the adult population (Deneo-Pellegrini *et al.*, 1999; Kato *et al.*, 1999; Wurzelmann *et al.*, 1996) a recent meta-analysis did not find support for the theory that iron intake is associated with coronary heart disease (Danesh and Appleby, 1999). These studies all were performed in adults, and data are scarce on the effects of iron supplementation or fortification of formulae on oxidative stress in infants.

Hereditary haemochromatosis is a common genetic disorder, especially in individuals of European origin, affecting as many as 1 in 300 people (Merryweather-Clarke *et al.*, 1997).

Infants and children with this disorder most often have not yet been diagnosed, and unnecessary iron supplementation or iron containing formulae might theoretically aggravate their iron overload.

We have found no conclusive proof so far that iron-containing infant formulae confer important adverse effects. However, to minimize this risk, it seems prudent to keep the recommendded level of iron content as low as considered adequate to secure an appropriate iron supply.

9. CONCLUSION

The risk for an infant to develop ID depends on socio-economic conditions, maternal nutritional status during pregnancy, birth weight, postnatal growth, duration of breast feeding, quality of complementary feeding, and the age at which complementary feeds are introduced. For the healthy, term infant the risk of developing IDA is low before the age of 4-6 months, but increases thereafter and probably reaches its peak between 12 and 18 months of age. Promotion of breast-feeding and use of iron-fortified complementary foods seems an efficient strategy to prevent ID during infancy.

When breast-feeding is not possible, iron-fortified infant formulae should be used, and when breast-feeding is discontinued iron-fortified infant formulae or follow-on formulae should be used rather than cows' milk or formulae not fortified with iron. However, based on present knowledge it seems prudent to keep the fortification level of infant formulae and follow-on formulae as low as possible, as long as iron deficiency is prevented, also taking differences in bioavailability of the fortification iron into account.

The lower level of added iron to an infant formula is not critical during the first few months, but becomes important after 4-6 months when iron stores begin to be depleted. This is particularly true when the formula (or follow-on formula) constitutes a major part of the infant's diet, or when the infant consumes only small amounts of iron from other dietary sources. In populations at low risk of developing IDA, a fortification level of 0.3 mg/100 kcal (2 mg iron/L) for cows' milk-based infant formulae seems to suffice, at least during the first 6 months of life. In populations at high risk of developing IDA, it seems prudent to provide a higher level of iron fortification during the first half of infancy, not only to prevent IDA during this early period but also to prevent non-anaemic ID - that is, depleted iron stores. A maximum level of 1.3 mg/100 kcal (8 mg/L) is proposed.

The current recommendation of iron content in follow-on formulae, intended for use from the fifth month of life as the liquid part of a diversified diet, is 1-2 mg/100 kcal, or 6-16 mg/L (Infant Formulae Directive). These levels appear unnecessary high with respect to current knowledge (reviewed above), and therefore a minimum level of 0.6 mg/100 kcal (4 mg/L) and a maximum level of 1.7 mg/100 kcal (10 mg/L) is now proposed. Although there is no reason that the upper level should exceed that of infant formulae intended for use after the first half of infancy.

Soy protein isolates used in infant- and follow on formulae contain phytic acid (myoinositol hexaphosphate [IP_6] and other inositol phosphates), which is an inhibitor of iron and zinc absorption (Hurrell *et al.*, 1992). This is the reason for the current proposal of 50 to 100% higher iron levels in infant- and follow-on formulae based on soy protein than in those based on cows' milk protein (Infant Formulae Directive). The bioavailability of iron from a soy

protein-based formula can be increased to the level of cows' milk protein-based formulae. This can be achieved by considerably reducing the phytate content, or by increasing the ratio of ascorbic acid to iron molar ratio in the formula (Davidsson *et al.*, 1994a). Hertrampf *et al.* (1986) concluded from a study on bioavailability of iron in soy-based formulae that it was higher than expected. However, since the majority of soy-protein based formulae on the European market is not considerably reduced in phytic acid, and in the absence of sufficient data on the long term (several months) effect on iron status of feeding exclusively soy protein-based formulae with different iron levels it is proposed that the minimum and maximum fortification level in soy-protein based infant formulae and follow-on formulae is 1.5 times higher than in the cow milk protein-based formulae.

The iron contents of infant formulae or follow-on formulae intended for use after age 6 months must take into account the iron intake from other dietary sources, which varies between countries and populations. A higher iron content is needed in formulae intended after the first 6 months of infancy, particularly if the amount of iron provided by other dietary sources is low.

10. RECOMMENDATION

The Committee makes the following recommendations for the iron content of infant formulae and follow-on formulae:

Type of Formulae	Iron content	
	mg/100 kcal	mg/100 kJ
Cows' milk-based infant formula	0.3 - 1.3	0.07 - 0.32
Soy protein-based infant formula	0.45 - 1.9	0.11 - 0.48
Cows' milk-based follow-on formula	0.6 - 1.7	0.13 - 0.40
Soy protein-based follow-on formula	0.9 - 2.5	0.20 - 0.60

B. CALCIUM

At birth about 99% of the calcium in the body is part of the structural matrix of bone, with the remainder being physiologically active as a free calcium pool within cells and the extracellular fluid. Within cells, calcium acts as a second messenger, modulating the transmission of hormonal signals, and regulating enzyme function. It is involved in blood coagulation, nerve conduction, muscle contraction and reproductive functions.

Although net calcium absorption and calcium retention may be related to the calcium intake, there are a range of other factors either in the diet (lactose and phytate), or related to metabolic regulation which influence the absorption, around 30 to 50% of dietary calcium, and retention of calcium (Arnaud and Sánchez, 1996). Thus calcium status represents the integration of the absorption of calcium within the gastrointestinal tract, the deposition and resorption of calcium in bone and the excretion or retention of calcium in the kidney. Hormonal factors, such as calcitonin, parathyroid hormone and vitamin D, play a clear role in this integrated function, but the metabolic handling of other nutrients such as sodium, phosphorus, iron, zinc and magnesium are also known to exert an influence. In this regard the intake and metabolism of phosphorus is of particular importance, as effective calcium

retention requires that adequate phosphorus is available. For this reason the ratio of calcium to phosphorus within formula should be specified.

The calcium content of human milk decreases over the course of lactation, and a summary of the data from 24 studies gave a range between 194 and 268 mg/L (29 to 40 mg/100 kcal), (Atkinson *et al.*, 1995; LSRO, 1998). The calcium content of cows' milk is much higher with about 1246 mg/L (186 mg/100 kcal) (LSRO, 1998). The calcium content of cows' milk protein based infant formula is between 63 and 82 mg/100 mL, and of soy-protein based formula is around 105 mg/100 kcal, although for each absorption may be lower than for human milk (LSRO, 1998). The ratio of calcium to phosphorus in human milk is about 2:1 and in most infant formulae is between 1.3:1 and 1.5:1 (Steichen and Koo, 1992).

Although previously, a lower limit had been specified for the calcium content of formula, and a ratio of calcium to phosphorus, no upper limit for calcium content had been specified. The LSRO (1998) recommended a minimum calcium content of 50 mg/100 kcal. The mean minus 1 SD concentration of the mineral in human milk, which is often used as a first guidance for defining adequate concentrations in formulae, is estimated at 220 mg/L or about 33 mg/100 kcal. Mean absorption from human milk is about 58%, and from cows' milk about 38% of intake. Therefore to achieve a calcium absorption equivalent to that from human milk (based on mean minus 1 SD contents), formula content must be at least 50 mg/100 kcal. LSRO recommended a maximum calcium content of 140 mg/100 kcal which is less than the 90th centile of currently available formula.

The PRI for calcium is 50 mg/100 kcal for infants 6-11 months (SCF, 1993b). The Infant Formulae Directive sets a minimum for calcium in infant formula of 50 mg/100 kcal while no maximum is set, and the calcium/phosphorus ratio shall be not less than 1.2 nor greater than 2.0.

Recommendation

The minimum calcium content in infant formulae should be maintained at 50 mg/100 kcal, and the maximum level should be 140 mg/100 kcal. The ratio of calcium to available phosphorus (based on measured bioavailability, or calculated as 80% of total phosphorus in cows' milk protein based formulae and as 70% of total phosphorus in soy protein based formulae) should be not less than 1.0 nor greater than 2.0. This recommendation also applies to follow-on formulae.

C. PHOSPHORUS

Phosphorus is an integral part of the inorganic matrix of bone, has an important homeostatic function as a metabolic buffer, and is an integral part of a range of compounds which play central roles as structural cellular components and in intermediary metabolism (phospholipids and phosphoproteins, nucleic acids and adenosine triphosphate). Therefore during growth the retention of phosphorus is determined by the rate of bone growth and the rate of lean tissue accumulation. The ratio of calcium to phosphorus retention will vary with the relative proportions of bone and lean tissue growth.

Phosphorus is absorbed in the small intestine by diffusion and by active transport, and the overall body content is determined by varying the tubular reabsorption of phosphorus in the

kidney. At low intakes phosphorus retention is mediated by parathyroid hormone and vitamin D.

The phosphorus content of human milk have been reported to range from 107 to 164 mg/L (16 to 24 mg/100 kcal) (Fomon and Nelson, 1993), peaking in early lactation and decreasing as lactation progresses (Atkinson *et al.*, 1995; Fomon and Nelson, 1993). Motil *et al.* (1997) reported average concentration falling from 184±16 (SD) mg/L (27±2.4 mg/100 kcal) at 6 weeks of lactation to 155±17 mg/L (23±2.5 mg/100 kcal) at 24 weeks. About 85% of the phosphorus in human milk is absorbed.

Conventional cows' milk protein-based infant formulae currently marketed in the USA contain 310-420 mg phosphorus/L (about 46 to 63 mg/100 kcal) (LSRO, 1998). The phosphorus content of soy-based formula may be much higher, because the phytic acid contained in the formula binds phosphorus, about 28% of the mass of phytic acid. Phytic acid may represent 1.5% of soy-protein by weight. Therefore a significant proportion of the phosphorus contained in soy formulae may be unavailable.

In the past a minimum content but no maximum content for phosphorus had been specified. Phosphorus metabolism is closely linked with calcium metabolism. During early life an excessive intake of phosphorus may be associated with hypocalcaemia, because of the relative sensitivity of the maturing kidney and increased renal losses. The hypocalcaemia may be symptomatic and may be associated with bone changes of osteopaenia, which if severe can manifest as rickets. The currently available formulae might result in higher blood levels of phosphorus or increased renal excretion, but differences in bone mineral content have not been attributed to this finding (LSRO, 1998). Given the potential for these interactions consideration has been given to defining an upper limit for phosphorus.

The LSRO (1998) Expert Panel recommended a minimum for available phosphorus of 20 mg/100 kcal and a maximum for available phosphorus of 70 mg/100 kcal. These values are given as available and apply to all formulae, including those which contain appreciable amounts of phytate. FDA analysis of 90th centile of infant formulae is 97 mg/100 kcal: on the assumption that phytate phosphorus accounts for 30% of the total in isolated soy-protein based formulae, the 90th centile for available phosphorus would be 70 mg/100 kcal.

Concerns expressed by the LSRO Expert Panel that hyocalcaemia in the neonatal period might occur as a result of consuming a formula which contained the minimum calcium (50 mg/100 kcal), with the maximum phosphorus to give a calcium to phosphorus ratio of 0.71 to 1. Therefore, the Expert Panel added the recommendation that the ratio of calcium to phosphorus should not be less than 1.1 to 1.

The Infant Formulae Directive sets a minimum for phosphorus in infant formulae of 25 mg/100 kcal, and a maximum of 90 mg/100 kcal. The PRI for phosphorus for 6-11 months is 300 mg/d (SCF, 1993b). If it is assumed that a 6 month old weighs 8 kg, then the PRI for phosphorus for a 5 kg infant consuming 100 kcal/kg/d, would be 37.5 mg/100 kcal.

Recommendation

The minimum for bioavailable phosphorus should be 20 mg/100 kcal, and the maximum 70 mg/100 kcal. The proportion of bioavailable phosphorus content may either be based on measured data in the respective formula, or be based on a mean phosphorus absorption of

about 80% from cows' milk protein-based formulae and about 70% from soy protein isolate-based formulae, which results in the following recommended minimum and maximum levels:

Cows' milk protein-based and protein hydrolysates only infant formulae and follow-on formulae: minimum 25 mg/100 kcal, maximum 90 mg/100 kcal.

Soy protein based infant formulae and follow-on formulae: minimum 30 mg/100 kcal, maximum 100 mg/100 kcal.

D. MAGNESIUM

The metabolism of magnesium is tightly regulated. It is an integral constituent of bone mineral and as the second most abundant intracellular cation plays a fundamental role in many aspects of intermediary metabolism, as a cofactor for many different enzymes and as a modulator of physiological processes. Status is hard to define as plasma concentrations are maintained within narrow limits. It is absorbed in the distal ileum and colon, through a regulated process which may involve vitamin D and is influenced by the presence and body status of sodium, potassium and calcium. The plasma concentration and its availability to metabolism are regulated through a combination of gastrointestinal absorption, bone deposition and resorption and renal excretion. Gastro-intestinal absorption may be enhanced by lactose. Renal handling is influenced by parathyroid hormone, calcitonin, glucagon, insulin and probably vitamin D.

The bioavailability of dietary magnesium is high. The concentration in human milk appears to be regulated within narrow limits, 31.4 to 35.7 mg/L (Atkinson *et al.*, 1995) or 17-28 mg/d (Lönnerdal, 1997). Cows' milk may contain 120 to 130 mg/L (18-19 mg/100 kcal) (Atkinson *et al.*, 1995; Greer, 1989; Lönnerdal, 1995). Formulae have been reported to contain 40-70 mg/L (6-12 mg/100 kcal) (LSRO, 1998).

High doses of magnesium are toxic, and untoward effects have been reported in newborn infants treated with antacids or whose mothers were treated with magnesium sulphate for hypertensive disorders of pregnancy (Brand and Greer, 1990; Tsang, 1972). Magnesium toxicity from formulae has not been reported.

The LSRO Expert Panel recommended a minimum magnesium content of 4 mg/100 kcal, based on mean minus 1 SD contents in human milk of 26 mg/mL or 4 mg/100 kcal, and a maximum of 17 mg/100 kcal.

For children 1-3 years, no numerical tolerable upper intake level has been set for magnesium by the Committee (SCF, 2001d). The Infant Formulae Directive sets a minimum for magnesium of 5 mg/100 kcal, and a maximum of 15 mg/100 kcal. A PRI for magnesium has not been set, but for adults the acceptable range of intakes is 150-500 mg/day (SCF, 1993b). Guidance for children is given, and for infants 6-11 months this is 7 mg/kg/day (7 mg/100 kcal), slightly higher than the intake from breast milk at 6 months.

Recommendation

The minimum level for magnesium in infant formulae and follow-on formulae should be 5 mg/100kcal, and the maximum 15 mg/100 kcal.

E. SODIUM, POTASSIUM AND CHLORIDE

Sodium is the principal cation in extracellular fluid and is present in the body mainly in the ionised form, which represents around 96%. The amount and concentration of sodium determines the volume of extra-cellular fluid (ECF). Potassium is the major intracellular cation, contributes to the intracellular osmotic activity and in part determines the intracellular fluid volume. Sodium and potassium are lost from the body by extra renal routes, but the body content of both cations is regulated predominantly by renal excretion. The exchange of sodium for potassium across the cell membrane provides an energy gradient which enables nutrient uptake through membrane transport processes and is fundamental for the propagation of the action potential in nerves and muscles. Chloride is the principal anion in the ECF and together with sodium contributes more than 80% of the osmotic activity. It is an essential component of the membrane transport systems.

Dietary requirements are based upon the need to match inevitable losses through renal and extra-renal routes, and to meet the needs for net tissue deposition. Intakes which exceed the capacity of the kidney for excretion are associated with adverse effects in the short and longer term. Minimum and maximum criteria were defined in 1983, and have not been changed since.

Wack *et al.* (1997) have recently suggested that intermittently the electrolyte content of breast milk might be less than optimal. Thus although on average the concentration of electrolytes may remain constant, there is variation within and between women, which they speculate might impact upon infant nutrition, and lead to deficiencies on occasion (Asnes *et al.*, 1982; Hill and Bowie, 1983; Shaffer and Feretti, 1990). Wack *et al.* (1997), in breast milk from thirty mothers, reported the average sodium concentration to be 0.87±0.45 (SD) mEq/100 kcal between 60 and 240 days, potassium concentration to be 1.65±0.27 mEq/100 kcal, and chloride concentration to be 1.68±0.69 mEq/100 kcal. Motil *et al.* (1997) reported the composition of breast milk from eleven adults and eleven adolescents between 6 and 24 weeks postpartum. The concentration of potassium was 1.92±0.24 mEq/100 kcal, and sodium concentrations were 0.50±0.14 mEq/100 kcal in adults or 0.80±0.23 mEq/100 kcal in adolescent mothers.

It should be noted that the mineral content of water may vary widely depending upon its source (Rottoli *et al.*, 1997; Willershausen *et al.*, 2000; Pomeranz *et al.*, 2002), and if used to re-constitute a powdered formula can influence the characteristics and composition of the resulting preparation. The balance of water and solutes such as sodium consumed by the infant is important if adverse effects are to be avioded in the short and longer term (Campfield *et al.*, 1994; Eke and Nte, 1996; Bruce and Kliegman 1997; Pomeranz *et al.*, 2002).

The LSRO (1998) recommended a minimum sodium content of 25 mg/100 kcal. Because of the high variation of sodium contents reported for human milk, there is reluctance to follow the concept of using mean levels minus 1 SD in human milk as a guidance for infant formula contents. Moreover, it is noted that recently there are increasingly reports on hypernatraemic dehydration in infants if lacatation and breast-feeding are not well established. Therefore the minimum sodium content should be based upon the RDA of 120 mg/day for infant less than six months. The maximum level for sodium recommended by LSRO was 50 mg/100 kcal, based on the 90th centile of FDA analyses of infant formula.

For potassium, LSRO proposed a minimum content of 60 mg/100 kcal, based on mean minus 1 SD contents in mature human milk of 400 mg/L or 60 mg/100 kcal. The proposed

maximum level was 160 mg/100 kcal based upon 90th centile of FDA analysis of infant formula (LSRO, 1998).

For chloride, the minimum recommended level was 50 mg/100 kcal, based on mean minus 1 SD contents in human milk of 320 mg/L or 48 mg/100 kcal, rounded to 50 mg/100 kcal. The proposed maximum content was 160 mg/100 kcal based upon the 90th centile of FDA analysis of infant formula (LSRO, 1998).

The Infant Formulae Directive sets for infant formulae a minimum for sodium 20 mg/100 kcal, and a maximum of 60 mg/100kcal, a minimum for potassium of 60 mg/100 kcal, and a maximum of 145 mg/100 kcal: a minimum for chloride of 50 mg /100 kcal, and a maximum of 125 mg/100 kcal.

There is no PRI set for sodium or for chloride (SCF, 1993b). The PRI for potassium was estimated factorially and for infants aged 6-11 months was set at 800 mg/d or 20 mmol/d, or around 4 mEq/100 kcal. The PRI for potassium is similar to the maximum recommended for infant formula by LSRO (1998) of 4.1 mEq/100 kcal, and by the Committee (SCF, 1993b), 3.8 mEq/100 kcal. For breast milk the mean potassium content plus 2 SD would be 2.2 to 2.4 mEq/100 kcal (Motil *et al.*, 1997; Wack *et al.*, 1997). The mean ± 2SD for the chloride content of breast milk would be 3.05 mEq/100 kcal (Wack *et al.*, 1997)

Recommendation

It is proposed that for sodium the minimum content of infant formulae should be 20 mg/100 kcal and the maximum 60 mg/100 kcal, unchanged from the present. This recommendation applies also to follow-on formulae.

It is proposed that for potassium the minimum content of infant formulae and follow-on formulae should be 60 mg/100 kcal, and the maximum 160 mg/100 kcal.

It is proposed that for chloride the minimum content of infant formulae and follow-on formulae should be 50 mg/100 kcal and the maximum 160 mg/100 kcal.

F. COPPER

Copper is required as an essential dietary trace element. It is required for cellular metabolism in enzymatic and non-enzymatic systems. It is absorbed from the diet in the upper jejunum by active and passive processes, stored in the liver and kidney and excreted in the bile to be lost in faeces. Copper status is not easy to determine, and the homeostatic mechanisms which control copper distribution and metabolism are incompletely understood. However, severe deficiency may manifest with abnormal collagen and bone development, sideroblastic anaemia and neutropaenia. Newborns have a high liver content of copper which is drawn upon for growth during the first six months of life.

The copper content of human milk is not directly influenced by maternal dietary intake of copper, and is generally higher than cows' milk. In the 1980s the concentration of copper in infant formulae was reported to vary very widely, with some milks having almost undetectable amounts (Lönnerdal *et al.*, 1983). Copper interacts with other divalent cations, such as iron and zinc, for gastrointestinal absorption. Absorption and availability may be influenced by the carbohydrate content of the diet, with reduced availability from phytate

containing diets, or diets containing fructose. Diets which contain excess copper have been reported to lead to toxicity and liver damage during childhood (O'Neill and Tanner, 1989; Lönnerdal, 1998), and the levels of copper in the tap water delivered through copper pipes has been reported to lead to toxicity, associated with levels in infant formula between 9 to 26.4 mg/L (Eife *et al*, 1999; Dieter *et al.* 1999).

The LSRO Expert Panel recommended a minimum content of 60 μg/100 kcal (0.4 mg/L), based on published report as mean - 1SD of human milk during first three months (220 μg/L, 33 μg/100 kcal). Based on 90th centile of infant formula, the recommended maximum is 160 μg/100 kcal.

The Infant Formulae Directive sets for infant formulae a minimum for copper of 20 μg/100 kcal, and a maximum of 80 μg/100 kcal. The PRI for copper is set at 37.5 μg/100 kcal (SCF, 1993b). For children 1-3 years, this Committee set a tolerable upper intake level for copper of 1 mg/day (SCF, 2003b).

Recommendation

The minimum copper content of infant formulae and follow-on formulae should be 35 μg/100 kcal, and the maximum 100 μg/100 kcal.

G. ZINC

Zinc is essential for growth and development. Zinc is a constituent of more than 200 metalloenzymes and plays a key role in the synthesis of genetic material and the regulation of gene expression as well as in cell division, epithelial integrity, cellular immunity and sexual maturation.

The young infant has a high zinc requirement to support the very rapid growth of early infancy. Symptoms of zinc deficiency include impaired growth and altered cognition in children, loss of appetite and taste sensitivity, eye and skin lesions, alopecia, diarrhoea, susceptibility to infections, delayed healing of wounds and reproductive failure (FAO/WHO, 2002b).

Prolonged use of high doses of zinc results in a reduction of copper absorption, as it has been shown in patients with Wilson's disease (Yuzbasiyan-Gurkan *et al.*, 1992). Iron supplementation could decrease zinc absorption (O'Brien *et al.*, 2000). However, at levels present in food and at realistic supplementation levels, zinc absorption appears not be significantly affected by iron and copper (FAO/WHO, 2002b). Phytate, which is present in significant amounts in plant-based foods, particularly grains and legumes, reduces zinc absorption from the gastrointestinal tract through complexation and precipitation (Oberleas *et al.*, 1966).

A large variability of zinc concentration in human milk (from 0.5 to 4.7 mg/L) has been shown during the course of lactation and among individuals. Zinc supplementation does not influence zinc concentrations of human milk in well-nourished lactating women (Krebs *et al.*, 1995). Milk zinc concentration declines sharply over the early weeks of lactation. Indeed, concentrations of zinc in human milk decrease from ~4 mg/L at 2 weeks to ~3 mg/L at 1 month, ~2 mg/L at 2 months, ~1.5 mg/L at 3 months and ~1.2 mg/L at 6 months (Krebs *et al.*,

1995). With an estimation of milk intake of 0.78 L/day, calculated daily zinc intake is ~2.2 mg at 1 month, ~1.6 mg at 2 months, ~1.2 mg at 3 months, and ~0.9 mg at 6 months (FNB, 2001). Mean measured daily zinc intake of exclusively breast-fed infants was 2.3 mg at 2 weeks, 1 mg at 3 months, 0.8 mg at 5 months and 0.5 mg at 7 months (Krebs *et al.*, 1994). Zinc deficiency is very rare in breast-fed term infants during the first 6 months of life in industrialised countries (Krebs and Westcott, 2002). However, concerns have been raised on the consequences of marginal intakes of zinc on growth of older exclusively breast-fed infants (Walravens *et al.*, 1992). The zinc concentration of cows' milk ranges from 3 to 5 mg/L (Lönnerdal *et al.*, 1981). A stable isotope study of zinc absorption from different milks and formulae performed in healthy adults showed the following results: human milk: 41±9%; cows' milk: 28±15%; cows' milk formula: 31±7%; soy protein formula: 14±4% (Sandström *et al.*, 1983). The low availability of zinc from soy protein formula has been attributed to its high phytate content (Lönnerdal, 1994b). Using infant Rhesus monkeys and suckling rat pups as animal models, Lönnerdal *et al.* showed that the bioavailability of human milk in monkeys was 65% compared to 54% for monkey milk, 46% for casein-based infant formulae, 60% for whey-predominant formula, and only 27% for soy formula (Lönnerdal *et al.*, 1988). In contrast, zinc absorption from dephytinized soy formula was 45%. In suckling rats, zinc absorption from conventional soy formula was only 16% versus 47% for dephytinized soy formula. These results show that the low bioavailability of zinc from soy formula can be overcome by the removal of phytate. In a recent study, Abrams *et al.* (2002) showed that the absorption of zinc in a partially hydrolysed whey infant formula containing 6.7 mg/L of zinc was 32±11%. In a Japanese study, the zinc concentration of erythrocytes was not different in infants fed a formula containing 1.0-1.5 mg/L of zinc and in breast-fed infants (Hatano *et al.*, 1985). Longitudinal zinc balances have been performed in breast-fed and formula-fed infants (Sievers *et al.*, 1992). In view of the urinary and faecal zinc losses measured, a daily intake of 0.3-0.5 mg zinc/kg body weight is considered to be sufficient to ensure a zinc retention equivalent to breast-fed infants. This requires a zinc concentration of 2-3 mg/L dpending on milk volume intake. For breast-fed infants, zinc absorption increases with the decrease in milk zinc content over the course of lactation (Krebs and Hambidge, 1986).

The zinc content of infant and follow-on formulae has been defined in the Infant Formulae Directive (Tables 4 and 5).

Table 4. Zinc content of infant formulae and follow-on formulae containing cows' milk protein only as defined in the Infant Formulae Directive

Zinc	Infant Formulae		Follow-on Formulae	
	Minimum	Maximum	Minimum	Maximum
mg/100 kcal	0.5	1.5	0.5	-

Table 5. Zinc content of infant formulae and follow-on formulae containing soy protein as defined in the Infant Formulae Directive

Zinc	Infant Formulae		Follow-on Formulae	
	Minimum	Maximum	Minimum	Maximum
mg/100 kcal	0.75	2.4	0.75	-

The Expert Panel of LSRO recommended a minimum zinc content of 0.4 mg/100 kcal and a maximum zinc content of 1 mg/100 kcal in infant formulae containing either cows' milk protein only or soy protein (LSRO, 1998).

Daily Reference Nutrient Intakes (RNIs) of zinc proposed by different bodies are shown in Table 6.

Table 6. Daily Reference Nutrient Intakes (RNIs) of zinc proposed by different bodies

Reference	RNI (mg/day)			Age group
SCF, 1993b	4.0			6-12 months
AFSSA, 2001	-			0-6 months
	-			7-12 months
Spanish Dietetic and Food Science Association	3.0			0-1 year
COMA, 1991	4.0			0-6 months
	5.0			7-12 months
CSH, 2000	4.0			0-3 months
	4-5			4-5 months
	5.0			6-11 months
D-A-CH, 2000	1.0*			0-4 months
	2.0			5-12 months
FNB, 2001	2.0			0-6 months
	2.5			7-12 months
FAO/WHO, 2002b	HB[1]	MB[2]	LB[3]	
	1.1[a]	2.8[b]	6.6[c]	0-6 months
	0.8[a]	-	-	7-12 months
	2.5[d]	4.1	8.4	7-12 months

* Estimated value.
[1]HB: high bioavailability diet.
[2]MB: moderate bioavailability diet.
[3]LB: low bioavailability diet.
[a] Human milk infants receiving maternal milk only.
[b] Formula-fed infants.
[c] Formula fed-infants receiving a phytate-rich vegetable protein-based formula.
[d] Not applicable to infants consuming human milk only.

The UL of zinc intake has been set by the FNB to 40 mg/day for an adult man (FNB, 2001) and to 45 mg/day by the FAO/WHO Joint Expert Consultation (FAO/WHO, 2002b). The FAO/WHO Joint Expert Consultation also suggested an extrapolation in relation to basal metabolic rate for children and other age groups, meaning an UL of zinc intake of 23-28 mg/day for children, which is close to what has been used in some of the zinc supplementation studies. The UL of zinc intake has been set from a single study by the FNB to 4 mg/day for infants 0-6 months and 5 mg/day for infants 7-12 months (Walravens and Hambidge, 1976; FNB, 2001). The Committee recently set a tolerable upper intake level for zinc of 25 mg/day for adults and of 7 mg/day for children from 1 to 3 years of age (SCF, 2003c).

The following proposals can be made for formulae containing cows' milk protein only. Since zinc absorption from human milk, whey-predominant and casein-predominant infant formulae

has been shown to be similar, a minimum zinc content of 0.5 mg/100 kcal would allow to a 5 kg cows' milk formula-fed infant consuming 100 kcal/kg to achieve the RNI recently established by the FNB for zinc (2.0 mg/day) (FNB, 2001). The same minimum zinc content can be set for follow-on formulae since complementary feeding brings an additional amount of zinc sufficient to cover the RNI established from 7 to 12 months by the FNB (2.5 mg/day) (Krebs, 2000; FNB, 2001). A maximum zinc content of 1.5 mg/100 kcal would allow the zinc intake of a 5 kg-formula-fed infant not to markedly exceed the upper tolerable level of zinc intake established by the FAO/WHO Joint Expert Consultation (FAO/WHO, 2002b) and the upper level of tolerable zinc intake of 7 mg/day established by this Committee for children aged 1-3 years (SCF, 2003c).

The following proposals can be made for formulae containing soy protein. Since zinc absorption from soy formula is low (~25%), a minimum zinc content of 0.75 mg/100 kcal would allow a 5 kg-soy formula-fed infant consuming 100 kcal/kg to fulfil his requirements. A maximum zinc content of 2.4 mg/100 kcal would allow the zinc intake of a 5 kg-soy formula-fed infant to be under the UL of zinc intake established by the FAO/WHO Joint Expert Consultation (FAO/WHO, 2002b).

Recommendation

The minimum zinc content should be 0.5 mg/100 kcal for infant and follow-on formulae containing cows' milk protein and protein hydrolysates only. The maximum zinc content should be 1.5 mg/100 in both infant formulae and follow-on formulae containing cows' milk protein and protein hydrolysates only.

The minimum zinc content should be 0.75 mg/100 kcal for infant and follow-on formulae containing soy protein. The maximum zinc content should be 2.4 mg/100 kcal in both infant formulae and follow-on formulae containing soy protein.

H. CHROMIUM

Chromium potentiates the action of insulin both *in vitro* and *in vivo*, and thereby influences carbohydrate, lipid and protein metabolism. Chromium deficiency has only been described in patients on long-term parenteral nutrition deficient in chromium, presenting with hyperglycemia, weight loss, ataxia and peripheral neuropathy, as well as in infants with severe malnutrition (Brown *et al.*, 1986; Hopkins *et al.*, 1968).

Human milk has an average concentration of trivalent chromium of 0.25 µg/L (FNB, 2001). Thus, mean intake of trivalent chromium in a breast-fed infant is estimated to be 0.15-0.2 µg/day. The mean concentration of trivalent chromium in cows' milk and infant formulae was reported to be 0.83 and 4.84 µg/L, respectively (Cocho et *al.*, 1992). There are no data on the bioavailability of chromium in infant formulae.

No specification is made regarding a minimum and maximum content of chromium in infant and follow-on formulae in the Infant Formulae Directive. The Expert Panel of LSRO did not recommend minimum or maximum chromium contents for infant formulae (LSRO, 1998).

The Committee on Medical Aspects of Food Policy (COMA) has set no Reference Nutrient Intakes (RNIs) and suggested that the adequate level of intake (AI) was 0.1-1 µg/kg/day for children and adolescents (COMA, 1991). AI reflects the observed mean chromium intake of

infants fed mainly or exclusively breast milk. Since data on the essentiality and metabolism of chromium were so sparse, the Committee and the French Food Safety Agency (AFSSA) were unable to specify any requirements (SCF, 1993b; AFSSA, 2001). The FNB did not define as well a recommended dietary intake (RDA) for chromium and proposed an AI of 0.2 µg/day in infants from birth to 6 months based on consumption of chromium from human milk. Since the chromium content of a well balanced diet has been estimated to be 13.4 µg/1,000 kcal, an AI of 5.5 µg/day has been proposed in infants from 7 to 12 months based on consumption of chromium from human milk and complementary feeding (FNB, 2001). Germany, Austria and Switzerland proposed an AI of 1-10 µg/day and 20-40 µg/day in infants 0-4 months and in infants 4-12 months, respectively (D-A-CH Referenzwerte, 2000). Because of the very few adverse effects associated with a high chromium intake originating from food, a tolerable upper intake level (UL) could not be determined (FNB, 2001).

Recommendation

There are no biological or nutritional data to define a minimum and maximum content of chromium in infant formulae and follow-on formulae.

I. MANGANESE

Manganese is involved in the formation of bone and in amino acid, cholesterol, and carbohydrate metabolism. Manganese is a component of many enzymes. Glycosyl transferases are specifically activated by manganese. Manganese deficiency is associated in animals with growth retardation, impaired glucose tolerance and skeletal malformations (Keen *et al.*, 1994). Data on manganese deficiency in humans are very scarce and limited to experimental conditions. Main symptoms were scaly dermatitis, hair depigmentation associated with hypocholesterolemia and reduced vitamin K-dependent clotting proteins (FNB, 2001). Manganese toxicity has been demonstrated for people who inhale manganese dust. The most prominent effect is central nervous system pathology, especially in the extra pyramidal motor system. Neurotoxicity can also be observed after ingestion of manganese. The effects could be more severe in the developing brain. Manganese toxicity has been shown in children receiving long-term parenteral nutrition (Fell *et al.*, 1996), and plays a role in cholestasis as well as in nervous system abnormalities in these children, as assessed by MRI studies (Fok *et al.*, 2001; Quaghebeur *et al.*, 1996). It has been recently shown that dietary exposure to high levels of manganese during infancy can be neurotoxic to rat pups and result in developmental deficits (Tran *et al.*, 2002).

Average manganese content of human milk has been estimated as 3.5 µg/L, with a slight decrease over the course of lactation (Casey *et al.*, 1985). Thus, manganese intake of a breast fed infant is estimated to be 2.5-3 µg/day. The concentration of manganese in soy formula (200-300 µg/L) and in cows' milk is much higher (25-100 µg/L) than in human milk (Lönnerdal *et al.*, 1981; Lönnerdal *et al.*, 1983; Lönnerdal, 1994b). A large range of values (10-200 µg/100 kcal) has been reported for manganese content of various formulae (Stastny *et al.*, 1984). At 3 months, human milk fed infants received significantly less manganese (0.42 µg/kg/day) than formula fed infants (183 µg/kg/day). However, mean serum manganese concentrations of infants receiving human milk or formula were similar with mean values of 4.4 and 4.7 µg/L, respectively. The absorption of manganese from human milk (8.2 ± 2.9%) is significantly higher than from soy formula (0.7 ± 0.2%), cows' milk (2.4 ± 1.7%) and whey predominant cows' milk formula (3.1 ± 2.8%) (Davidsson *et al.*, 1989). In a Japanese study, the manganese concentration of erythrocytes was significantly higher in formula-fed than in

breast-fed infants at 1 to 5 weeks of age, but this difference was no longer present between 6 and 52 weeks of age (Hatano *et al.*, 1985).

No specification is made regarding a minimum and maximum content of manganese in infant and follow-on formulae in the Infant Formulae Directive. In the Commission Directive 1999/21/EC of 25 March 1999 on dietary foods for special medical purposes (FSMPs), the minimum and maximum content of manganese in nutritionally complete foods intended for use by infants have been set to 50 and 200 µg/100 kcal, respectively. The Expert Panel of LSRO recommended a minimum manganese content of 1 µg/100 kcal and a maximum manganese of 100 µg/100 kcal for infant formulae (LSRO, 1998).

According to the population intake, the Committee considered it preferable to give a safe and acceptable range of 1-10 mg/day (SCF, 1993b). The COMA was unable to set a RNI for manganese (COMA, 1991). However, a safe intake was defined above 16 µg/kg/day in infants and children. The safe intake was judged to be the level or range of intake at which there is no risk of deficiency, and below a level where there is a risk of undesirable effects (COMA, 1991). The Belgian High Council of Hygiene set an AI of 0.3-0.6 mg/day in infants <6 months and of 0.6-1.0 mg/day in infants 7-12 months (CSH, 2000). In the D-A-CH reference values, no AI was set for infants <4 months and an AI of 0.6-1.0 mg/day was set for infants 4-12 months (D-A-CH Referenzwerte, 2000). No RDA was set for manganese by the FNB (FNB, 2001). AI of manganese reflects the observed mean manganese intake of infants fed principally breast milk. AI was set at 0.003 mg (3 µg)/day in infants from birth to 6 months. In view of the much higher concentration of manganese in foods other than human milk, the AI was set at 0.6 mg/day in infants 7-12 months by the FNB.

Due to the limitation of human data and the lack of information from animal studies, the SCF could not recommend an UL neither for children over 1 year nor for adolescents or adults (SCF, 2000e). Although neurotoxicity has been described in animals and in humans chronically exposed to a high dose of manganese, the FNB estimated that it was not possible to establish an UL for infants aged 0-12 months. However, the source of intake should be from food and formula only (FNB, 2001). The UL was set at 2 mg/day in children 1-3 years and at 11 mg/day in adults. The SCF recommended in 1999 an upper level of manganese concentration of 0.5 mg/L in natural mineral waters (SCF, 1999b).

Based on the mean content of manganese in human milk of 3-4 µg/L (~0.5 µg/100 kcal) and on the lower absorption of manganese in cows' milk and in soy-formula, the minimum manganese content should be 1 µg/100 kcal. In spite of the absence of a well identified UL for manganese in infants, there is increasing evidence of the neurotoxicity of high exposure to manganese. Therefore, a maximum manganese content of 100 µg/100 kcal is proposed, which is below the estimated LOAEL in adults for manganese contents in water (4.2 mg/L).

Recommendation

The minimum manganese content should be 1 µg/100 kcal and the maximum manganese content should be 100 µg/100 kcal for both infant formulae and follow-on formulae.

J. MOLYBDENUM

Molybdenum acts as a cofactor of 3 enzymes in humans: xanthine oxidase, aldehyde oxidase and sulfite oxidase. While molydenum deficiency has never been observed in healthy humans,

it has been described in a single patient on long-term parenteral nutrition deficient in molybdenum, presenting with night blindness, tachycardia and amino acid intolerance (Abumrad *et al.*, 1981).

Molybdenum has an average concentration of 2 μg/L in human milk (FNB, 2001). Thus, molybdenum intake of a breast fed infant is estimated to be 1.5-2 μg/day. Mean concentration of molybdenum in cows' milk and soy milk is much higher (50 μg/L) than that of human milk (Tsongas *et al.*, 1980). The concentration of molybdenum in infant formulae ranges from 2 to 30 μg/L (Picciano, 1985). There are no data available on the bioavailability of molybdenum in cows' milk and infant formulae.

No specification is made regarding a minimum and maximum content of molybdenum in infant and follow-on formulae in the Infant Formulae Directive. The Expert Panel of LSRO did not recommend a minimum or maximum molybdenum content for infant formulae (LSRO, 1998).

There are no reliable estimates of human requirements for molybdenum and no recommended intake has been established by this Committee or by AFSSA (SCF, 1993b; AFSSA, 2001). The COMA has not set a RNI value for molybdenum (COMA, 1991), nor did the FNB set a RDA for infants aged 0-1 year (FNB, 2001). The COMA set an AI of 0.5-1.5 μg/kg/day in infants, children and adolescents, whereas the FNB set an AI of 2 μg/day in infants from birth to 6 months based on consumption of molybdenum from human milk, and an AI of 3 μg/day in infants from 7 to 12 months by extrapolation from the AI of 0-6 months infants. In the D-A-CH reference values, an AI of 7 μg/day and 20-40 μg/day is set for infants <4 months and infants 4-12 months, respectively (D-A-CH Referenzwerte, 2000). The Belgian High Council of Hygiene proposed an AI of 15-30 μg/day and 20-40 μg/day in infants 0-6 months and in infants 7-12 months, respectively (CSH, 2000). Because of the lack of data on adverse effects of a high molybdenum intake in infants aged 0-1 year, the UL could not be established in this age group. However, the only source of intake for young infants should be from food and formula, in order to prevent high levels of intake (FNB, 2001).

Recommendation

There are no biological or nutritional data to define a minimum and maximum content of molybdenum in infant formulae and follow-on formulae.

K. FLUORIDE

The primary role of fluoride is improving caries resistance. It has also been suggested that fluoride may play a role in bone mineralisation and maintenance of peak bone mass, as well as for normal growth in humans (Bergmann and Bergmann, 1991). Fluoride deficiency is associated with an increase of the prevalence of dental caries. The main adverse effects of a chronic excessive fluoride intake are enamel (dental) and skeletal fluorosis.

Mean fluoride content of human milk ranges from 0.007 to 0.011 mg/L (FNB, 1997). Thus, mean fluoride intake of a breast-fed infant is estimated to be 0.005-0.01 mg/day. Mean fluoride concentration in cows' milk is 0.022±0.007 mg/L (Koparal *et al.*, 2000). Most of the fluoride contained in the diet of a formula-fed infant comes from the water used in diluting concentrated liquid or powdered formulae. Fluoride intake may rise if the water source is

fluoridated. Fluoridation of water is widely practised in the USA and Canada, as well as in some European countries. The fluoride concentration of ready-to-feed formulae ranges from 0.1 to 0.2 mg/L in the USA and from 0.15 to 0.3 mg/L in Canada. A recent survey showed that the mean fluoride content of soy-based ready-to-feed formulae was higher (0.26 mg/L) than that of cows' milk protein-based ready-to-feed formulae (0.17 mg/L) (Van Winkle *et al.*, 1995). In the USA, the average dietary fluoride intake by children living in optimally fluoridated communities (i.e. associated with a high degree of protection against caries and a low prevalence of the milder forms of enamel fluorosis) is around 0.05 mg/kg/day, ranging from 0.02 to 0.10 mg/kg/day (FNB, 1997). In many industrialized countries, a supplementation of fluoride of 0.25 mg/day is provided to infants lower than 2 years living in communities with drinking water concentration under 0.3 mg/L of fluoride. In two studies performed in Germany and Turkey, mean fluoride concentration in infant formulae was estimated to be 0.029 ± 0.014 mg/L and 0.022 ± 0.007 mg/L, respectively (Bergmann and Bergmann, 1991; Koparal *et al.*, 2000). Latifah and Razak (1989) reported that fluoride in several brands of infant formulae approximated the mg/L level in the water used for the preparation of the formulae. An infant consuming 600-700 mL/day of formula would be fed 0.23 to 0.27 mg/day of fluoride. Most foods have fluoride concentrations well below 0.05 mg/100 g (Taves, 1983). The SCF recommended in 1999 an upper limit of fluoride concentration of 1.5 mg/L in natural mineral waters (SCF, 1999b).

No minimum and maximum contents of fluoride in infant and follow-on formulae are specified in the Infant Formulae Directive. The Directive on Foods for Special Medical Purposes did not set a minimum content of fluoride and recommended a maximum content of fluoride of 200 μg/100 kcal (Directive 1999/21/EC). The Expert Panel of LSRO recommended a minimum level of zero and a maximum level of 60 μg/100 kcal for the fluoride content of infant formulae (LSRO, 1998).

No specific recommendations have been made by the Committee since there appeared to be no specific physiological requirements of fluoride (SCF, 1993b). No RNIs or UL were determined by the COMA (COMA, 1991). However, the COMA defined a safe intake of fluoride of 0.22 mg/kg/day in infants under 6 months and 0.12 mg/kg/day in children from 6 months to 6 years. No RDA was set by the FNB for fluoride. According to the mean content of fluoride in human milk, AI was established at 0.01 mg/day for infants from birth to 6 months. Based on the relationship described above between prevalence of caries, prevalence of enamel fluorosis, and fluoride intake, AI was set at 0.5 mg/day for infants from 7 to 12 months. Since a chronic fluoride intake of less than 0.10 mg/kg/day has been shown to be associated with a low prevalence of enamel fluorosis, the FNB stated an UL of 0.7 mg/day in infants from birth to 6 months and an UL of 0.9 mg/day from 7 to 12 months (FNB, 1997).

Recommendation

There is no need for defining a minimum level for the fluoride content of both infant formulae and follow-on formulae. Based on the aforementioned data and considering that infants may be exposed to an additional intake from fluoride containing water or fluoride supplements, the maximum fluoride content in both infant formulae and follow-on formulae should be 100 μg/100 kcal.

L. IODINE

Iodine is an essential component of the thyroid hormones thyroxine (T_4), containing 65% by weight of iodine, and its active form triiodothyronine (T_3), containing 59% by weight of iodine. Therefore, the dietary requirement of iodine is determined by normal T_4 production by the thyroid gland without stressing the thyroid iodide trapping mechanism or raising TSH levels. Thyroid hormones are involved in the maintenance of metabolic rate and cellular metabolism. Thyroid hormones are also necessary for the development of the nervous system in the fœtus and infant. Iodine deficiency disorders include mental retardation, cretinism, hypothyroidism, goiter and growth impairment (Delange, 1994). Severe iodine deficiency is a major cause of mental retardation worldwide, with more than 10 million cases of cretinism. Mild deficiency is associated with goitre in 5-20% of schoolchildren. A meta-analysis of 18 studies has shown that iodine deficiency alone may reduce the mean IQ scores by 13.5 points (Bleichrodt and Born, 1994). Excess iodine may disrupt thyroid function and secondarily lead to the induction of hypothyroidism with or without goitre, hyperthyroidism (thyrotoxicosis) and changes in the incidence and types of thyroid malignancies (Braverman, 1994).

Iodine content of human milk varies markedly as a function of the iodine intake of the population. It ranges from 10-20 μg/L to more than 300 μg/L in Europe and from 30 to 490 μg/L in the USA (FNB, 2001). Thus, mean iodine intake of a breast fed infant can be estimated to 60 μg/day in Europe and 120 μg/day in the USA. Iodine is also present in cows' milk (average level: 150 μg/kg) probably as a result of the use of supplemented cattle feeds and iodophores as teat sterilants. Iodine content of cows' milk varies from whole cows' milk (27-47 μg/kg) to summermilk (90 μg/kg) and wintermilk (210 μg/kg) (SCF, 2002d).

The iodine content of infant and follow-on formulae has been defined in the Infant Formulae Directive (Table 1).

Table 1. Iodine content of infant formulae and follow-on formulae as defined in the Infant Formulae Directive

Iodine	Infant Formulae		Follow-on Formulae	
	Minimum	Maximum	Minimum	Maximum
μg/100 kcal	5	-	5	-

The Expert Panel of LSRO recommended a minimum iodine content of 8 μg/100 kcal and a maximum iodine content of 35 μg/100 kcal in infant formulae (LSRO, 1998). The Commission Directive on FSMPs set a minimum and maximum iodine content in nutritionally complete foods intended for use by infants of 5 and 35 μg/100 kcal, respectively.

Daily RNIs of iodine proposed by different bodies are shown in Table 2.

The FNB could not establish an UL for infants 0-12 months (FNB, 2001). The UL was 200 μg/day of iodine in children 1-3 years for the FNB as well as for the SCF (SCF, 2002d). FAO/WHO Joint Expert Consultation set probable safe upper limits of 150 and 140 μg/kg/day in infants 0-6 months and 7-12 months, respectively (FAO/WHO, 2002b). However, the report also states that the upper limit probably should be one that provides a daily iodine intake of no more than 100 μg/kg.

Table 2. Daily RNIs of iodine proposed by different bodies

Reference	RNI (μg/day)	Age group
SCF, 1993b	50	6-12 months
AFSSA, 2001	40 50	0-6 months 7-12 months
Spanish Dietetic and Food Science Association	35	0-1 year
COMA, 1991	50 60	0-3 months 4-12 months
CSH, 2000	90	0-1 year
D-A-CH, 2000	50 (Switzerland)	0-1 year
	40 (Austria and Germany) 80 (Austria and Germany)	0-4 months 5-12 months
FNB, 2001	110 130	0-6 months 7-12 months
FAO/WHO, 2002b	15 μg/kg	0-1 year

Measurement of urinary iodine excretion is recommended for assessing iodine status. Iodine nutritional status is considered to be normal when urinary iodine is above 100 μg/L. In a study of 160 healthy French infants aged 10 days to 6 years (mean: 17.7±2.5 months), urinary iodine concentration ranged from 4 to 1042 μg/L (median ± SD: 195.5±21.6 μg/L). Iodine deficiency (defined by urinary iodine <100 μg/L) was found in 24% of cases (Pouessel *et al.*, 2003). In another French study, the prevalence of iodine deficiency was 18% among 10 month-old infants (Valeix *et al.*, 1992). In a study performed in 241, 1 month-old Italian infants, the prevalence of iodine deficient infants as defined above was 63% (Rapa *et al.*, 1999). In a recent study performed in 111 healthy Belgian children aged 6 months to 3 years, 21% of the subjects had a urinary iodine concentration < 50 μg/L (Delange *et al.*, 2001). Even if few infants presented in these 4 studies with severe iodine deficiency (urinary iodine <20 μg/L), iodine status was not optimal in these populations. Because of the large discrepancies in the UL for iodine between the FNB and the FAO/WHO Expert consultation, the Committee encourages further studies on the iodine status of infants and young children living in the European Union.

A minimum content of iodine of 5 μg/100 kcal in infant and in follow-on formulae does not allow fulfilling the requirements set by most European national authorities. To achieve the previously mentioned RNI for iodine (40-60 μg/day), a 5-kg formula-fed infant consuming 100 kcal/kg would need to receive 10 μg/100 kcal. Because excess iodine can inhibit thyroxine synthesis, it seems appropriate to set an upper limit of iodine in infant and follow-on formulae. However, it is difficult to propose a maximum limit since the iodine content of cows' milk is not constant and depends on seasons and hygienic or agricultural techniques. For a limit of iodine of 100 μg/kg/day as set by the Joint expert consultation of FAO/WHO (FAO/WHO, 2002b) and with the assumption that the total daily intake is from infant formula, with a daily intake of 100 kcal/kg (150 mL/kg), the upper limit of the iodine content of infant formulae would be 100 μg/100 kcal. An upper limit of iodine of 75 μg/100 kcal in infant formulae was proposed by the US Food and Drug Administration in 1985 (FDA, 1985).

This upper limit is above the current upper range of iodine concentration in cows' milk as it has been described above.

Recommendation

The minimum iodine content should be 10 μg/100 kcal and the maximum iodine content should be 50 μg/100 kcal for both infant formulae and follow-on formulae.

M. SELENIUM

Selenium is necessary for the activity of glutathion peroxydase, which protects against oxydative damage in intracellular structures. At least 15 other selenoproteins have been characterised, as iodothyronine deiodinases, which are essential for the conversion of tetraiodothyronine (T_4) to its physiologically active form, triiodothyronine (T_3). Between 60 and 80% of selenium in human plasma is accounted for by selenoprotein P, which has antioxidant activity and may be involved in selenium transport (FAO/WHO, 2002b). Biochemical evidence of selenium depletion, i.e. a decline in blood glutathion peroxydase activity, has been observed in patients on long-term parenteral or enteral feeding. In humans, selenium deficiency is mainly associated with Keshan disease, an endemic cardiomyopathy occurring in young children and women of child-bearing age in different regions of China, and possibly with Kashin-Beck disease, a musculoskeletal disorder (Yang and Xia, 1995). Both Keshan disease and Kashin-Beck disease occur in areas where the availability of soil selenium is low. The available studies show that the development of chronic selenium poisoning, or selenosis, is associated with selenium intakes over 0.85 mg/day (14 μg/kg body weight for a 60 kg adult). Selenosis is characterised by skin lesions and neurological abnormalities such as hypoaesthesia and hyperreflexia (FNB, 2000a).

The wide range of breast milk selenium concentrations depends on selenium consumed in natural foods, which reflects the content of the soils where they are grown. Selenium prophylaxis, either through soil selenium fertilisation or maternal supplements, is effective in raising breast milk selenium concentration (Dorea, 2002). In general, the selenium content of human milk is highest in colostrum (Ellis *et al.*, 1990). In spite of wide variation, the median selenium concentration from studies worldwide are 26, 18, 15, and 17 μg/L in colostrum (0-5 days), transitional milk (6-21 days), mature milk (1-3 months) and late lactation (>5 months), respectively (Dorea, 2002). In a recent Australian study, mean concentration of selenium in cows' milk varied from 20.6 ± 4.8 to 23.8 ± 4.7 μg/L and was close to that of human milk (Tinggi *et al.*, 2001). The mean selenium concentration in 24 brands of commercial infant formulae was 49.0 ± 11.6 μg/L, with a range from 26 to 68 μg/L (al-Saleh and al-Doush, 1997). Infants fed a soy formula supplemented with selenium (22-28 μg/L) had plasma and erythrocyte selenium values lower than those of infants fed human milk (Johnson *et al.*, 1993). However, plasma and erythrocyte glutathione peroxydase activities were normal, indicating that the physiologic requirement for selenium was being met. The same biological pattern was observed in German infants fed cows' milk formulae as compared to breast-fed infants (Jochum *et al.*, 1995)

No minimum contents of selenium in infant and follow-on formulae are specified in the Infant Formulae Directive, while a maximum selenium content of 3 μg/100 kcal was set for infant formulae with added selenium. The Expert Panel of LSRO recommended a minimum level of 1.5 μg/100 kcal and a maximum level of 5 μg/100 kcal for the selenium content of infant

formulae (LSRO, 1998). The Commission Directive on FSMPs set a minimum and maximum selenium content in nutritionally complete foods intended for use by infants of 1 and 3 µg/100 kcal, respectively.

The RNIs for selenium in adults are based on the amount of selenium needed to maximise synthesis of the selenoprotein glutathion peroxydase as assessed by the plateau in the activity of the plasma isoform of this enzyme. However, no functional criteria of selenium status have been demonstrated that reflect response to dietary intake in infants. The AI for infants aged 0-6 months (15 mg/day) is based on an average volume of human milk intake of 0.78 L/day and an average concentration in human milk of 18 µg/L (FNB, 2000a). The AI for infants aged 7-12 months has been estimated to 20 µg/day, by adding selenium intake from human milk (average volume of human milk intake of 0.6 L/day) and selenium intake from complementary feeding (Lambeck *et al.*, 1984). Daily RNIs of selenium proposed by different bodies are shown in Table 3.

Table 3. Daily RNIs of selenium proposed by different bodies

Reference	RNI (µg/day)	Age group
SCF, 1993b	8	6-12 months
AFSSA, 2001	- -	0-6 months 7-12 months
COMA, 1991	10 13 10	0-3 months 4-6 months 7-12 months
CSH, 2000	10 13 15	0-3 months 4-5 months 6-11 months
D-A-CH, 2000	5-15 7-30	0-4 months 5-12 months
FNB, 2000a	15 20	0-6 months 7-12 months
FAO/WHO, 2002b	6 10	0-6 months 7-12 months

The UL of selenium intake has been set by the FNB to 45 µg/day in 0-6 month infants and 60 µg/day in 7-12 month infants (FNB, 2000a). The Committee set an UL for children 1-3 years of 60 µg/day (SCF, 2000f).

A minimum selenium content of 3 µg/100 kcal would allow a 5 kg cows' milk protein based formula-fed infant consuming 100 kcal/kg to achieve the RNI recently established by the FNB for selenium (15 µg/day) (FNB, 2000a). A maximum selenium content of 9 µg/100 kcal would allow a 5 kg-formula-fed infant to receive a selenium intake close to the UL of selenium intake established by the FNB (FNB, 2000a).

Recommendation

The minimum selenium content should be 3 µg/100 kcal and the maximum selenium content should be 9 µg/100 kcal for both infant formulae and follow-on formulae.

IX. PROBIOTICS

1. INTRODUCTION

Human breast-milk promotes the development of a microbial flora in the colon which is largely predominated by lactobacilli and bifidobacteria (up to 90% of the total flora) (Harmsen *et al.*, 2000). Due to the microbial production of lactic and acetic acids the faecal pH decreases which inhibits the colonisation by potentially pathogenic bacteria.

Modern infant formulae with low contents of protein, high contents of lactose and high calcium-phosphate ratios do not, as a rule, have the same quantitative and qualitative effects on the gut microflora of the infant. The detectable effects on the microflora depend very much on the method applied, because of difficulties in cultivation of obligate anaerobic species. Molecular methods based on identification of microbal DNA are now available for quantitative studies (Tannock, 2000) and may be combined with classical bacteriological methods (AFSSA, 2003). In recent years two approaches have been proposed to achieve an intestinal flora more similar to that of breast-fed infants: the addition of non-digestible carbohydrates that may act as prebiotics to formulae, such as oligofructosyl-saccharose and oligogalactosyl-lactose, and the addition of bacterial cultures such as specific strains of lactobacilli and bifidobacteriacae considered as probiotics to formulae. Prebiotics have been defined as "non-digestible food ingredients that beneficially affect the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon, and thus improve host health" (Gibson and Roberfroid, 1995). Probiotics have been considered to be "microbial food supplements that beneficially affect the host by improving his intestinal microbiological balance" (Fuller, 1989) or "microbial cell preparations or components of microbial cells that have a beneficial effect on the health and well being of the host" (Salminen *et al.*, 1999).

Although the Infant Formulae Directive does not specify that the addition of live bacteria to infant and/or follow-on formulae is permitted, except for the purpose of acidification of formula (Directive 95/2/EC, Annex VI, parts 1 and 2), several formulae with added live bacteria considered as "probiotics" have been introduced into the European market in recent years.

2. LEGISLATION

2.1 Legislation on bacteria in infant formulae

The addition of microbes to infant or follow-on formula is not mentioned in the Infant Formulae Directive. However, article 3 of the Directive permits the use of food ingredients whose suitability for the particular nutritional use of infants has been proven by generally accepted scientific evidence.

For the production of acidified infant and follow-on formulae non-pathogenic L(+)-lactic acid producing bacterial cultures are permitted.

Whereas in fermented dairy products bacteria, yeasts and moulds are added for fermentation purposes, to metabolise lactose to lactic acid with a reduction of the pH to ≤4.6 and

consecutive changes in the physico-chemical properties of the milk constituents (Tamime, 2002), probiotic bacteria are added e.g. freeze-dried to the finished formula product and not incubated for any length of time under temperature control. Therefore, the bacteria do not have to be those permitted in fermented dairy products.

Microbiological criteria for infant formula and follow-on formula have not yet been harmonised in the EU.

There is no special regulation in the EU neither on criteria for bacteria claimed to be "probiotic" nor on their use in the manufacturing of food or on claims of foods containing them.

The US Food and Drug Administration (FDA) has accepted as of 19 March 2002 the notice on Substances Generally Recognised as Safe (GRAS) submitted by Nestlé USA for the use of *Bifidobacterium lactis* strain Bb12 and *Streptococcus thermophilus* strain Th4 as ingredients in milk-based formula that is intended for consumption by infants four months and older and who are not immunocompromised (FDA, 2002). The FDA has, however, not made its own determination regarding the GRAS status of the two notified stains and has not made any decisions in relation to label claims.

3. SCIENTIFIC REPORTS ON THE USE OF PROBIOTIC BACTERIA IN FOODS

3.1 General reports

A group of Experts for Food Hygiene and Veterinary Medicine convened in 1997 at the Federal Institute for Health Protection of Consumers and Veterinary Medicine in Berlin (BgVV Working Group, 1999) to define probiotics, probiotic food, identify microorganisms with probiotic properties (*Lactobacillus acidophilus* group, *Lactobacillus casei* group, *Bifidobacterium* spp), effective doses (10^8 to 10^9 microorganisms), set up safety testing requirements (production of biogenic amines, activation of pro-carcinogens, induction and/or destruction of thrombi with the aid of specific hydrolases, activation of platelet aggregation, binding to fibrinogen and fibronection, degradation of mucin, haemolytic activity, transmission of resistance to antibiotics), set up criteria for genus and strain identification and stability, consider influences of food processing and handling (survival of microorganisms, food matrix, packaging, shelf-life) and proof of claimed effects with the specific food product under specified conditions in randomised double-blind placebo-controlled trials. Considered effects were improved lactose hydrolysis in lactase deficient subjects, prevention or mitigation of gastrointestinal infections, modulation of immune responses, inactivation of enzymes and decreased production of harmful metabolic substances. Claims on health-promoting effects would have to be based on scientific evidence. Legislative actions including the definition of criteria for the use of the term "probiotic" for a bacterial strain, of conditions of use of a probiotic strain in the manufacturing of probiotic foodstuffs and the extent of permissible claims were proposed.

A committee of Swedish scientists convened by the Swedish Nutrition Foundation reviewed and evaluated human studies on health effects of probiotics and prebiotics (Andersson *et al.*, 2001). The group concluded that certain probiotic bacteria may improve symptoms of lactose intolerance, acute rotavirus-induced diarrhoea and possibly also other forms of infectious

diarrhoea while reports on possible effects on serum cholesterol and on irritable bowel syndrome are still inconclusive.

A Joint FAO/WHO Expert Consultation on Evaluation of Health and Nutritional Properties of Probiotics in Food Including Powder Milk with Live Acid Bacteria (2001b) focussed mainly on the evaluation of the available scientific evidence for the properties, functionality, benefits, safety and nutritional features of probiotic foods:

- probiotic strains must survive the passage through the digestive tract and proliferate in the gut;

- they belong primarily to two genera: *Lactobacillus* and *Bifidobacterium*;

- there are no *in vitro* tests to predict the probiotic activity of a strain;

- strains should be named according to the International Code of Nomenclature;

- probiotic strains should be deposited in an internationally recognised culture collection;

- strain identification should be performed by phenotypic tests followed by genetic identification with methods as DNA/DNA hybridisation, 16SRNA sequencing (Blaut *et al.*, 2002) or similar methods;

- beneficial effects must be related to dosage regimens and duration of use of each individual product or strain;

- effects considered and evaluated comprised prevention and therapy of infectious diarrheal diseases and of sequelae of antibiotic treatment, *Helicobacter pylori* infection, inflammatory bowel diseases, prevention or delaying the onset of cancer, alleviation of constipation, modulation of mucosal or systemic immune responses including allergic diseases, benefits on cardiovascular diseases and urogenital disorders and infections, possible beneficial or adverse effects in healthy subjects;

- the necessity for well designed in vivo trials was underlined;

- safety considerations should include transmission of antibiotic or drug resistance inherent in some probiotic microorganisms; the exclusion of *Enterococcus* strains as probiotic microorganisms was recommended;

- labelling should include the microbial species or strain and its viable concentration, claims would have to be substantiated;

- stock cultures should be maintained under appropriate conditions and be checked periodically for strain identity and probiotic properties;

- dried milk powders with live lactic acid bacteria should preserve adequate numbers of viable probiotic bacteria with stable probiotic properties throughout shelf-life;

- the regulatory status of probiotics as a component in food should be established on an international level.

In the Guidelines for the Evaluation of Probiotics developed subsequently (FAO/WHO, 2002c) the need to know the strain identity, established by the most valid methodology is repeated. A flow-sheet for evaluation of probiotic bacteria and of foods that contain probiotic bacteria and are labelled as such is provided. This report also emphasised the need to fully evaluate the safety of probiotics, in particular with respect to potential infectious risks in subjects with a compromised immune defense and subjects at increased risk for endocarditis.

3.2 AFSSA report on the use of probiotic foods in infants

The specific aspects of the modification of the intestinal flora by nutritional means, including the use of probiotics, in infants were evaluated by a French working group (AFSSA, 2003). The tasks were to clarify the aims and risks connected with such a modification, to describe the desirable microbial profile and the means and consequences of its achievement also on other body functions, and to deliberate on how to communicate possible effects.

This working group excluded genetically modified and non-viable microorganisms from its definition of probiotics.

The speciation of probiotic strains both by classical bacteriological and modern molecular methods and the deposition of identified strains in an internationally recognised culture collection were considered necessary.

Two distinct periods of modification of intestinal colonisation were defined: the first week of life, when breast-fed infants develop a flora dominated by bifidobacteria and formula-fed infants possibly a more complex flora, and the period when complementary feeding is started.

The fermentative capacity, profile of short-chain fatty acids and pH in faeces varies among infants: acetic acid and lactic acid dominate until one month of age in breast-fed infants and higher concentrations of propionic acid and butyric acid appear later, whereas formula-feeding favours propionate and butyrate production and a near neutral pH of the faeces. The possible health consequences of the enzymatic activities of the more diversified flora of formula-fed infants compared to that of breast-fed infants are not known. For infants without lactose-intolerance the lactose-fermenting capacity of probiotic bacteria confers no appreciable benefit.

It is not clear whether a diminished bacterial translocation that has been observed in animal experiments with the supply of certain probiotic strains would also occur in infants. There is some evidence that certain probiotic bacteria exert strain-specific effects on the intestinal mucosal and systemic immune system. The application of *Lactobacillus rhamnosus* GG (5.10^8 colony forming units (CFU)/day over one month) to infants and young children (age 2 month to 16 month) allergic to cow's milk protein and of *Lactobacillus GG* or *Bifidobacterium lactis* Bb12 added to a hydrolysed formula to previously breast-fed infants suffering from atopic dermatitis ameliorated the clinical symptoms (Majamaa and Isolauri, 1997; Isolauri *et al.*, 2000). *Lactobacillus GG* (10^{10} CFU) given as a supplement two to four weeks before delivery to pregnant women at risk of atopic disease and to their children during six months decreased the incidence of atopic eczema during the first two years of life (Kalliomäki *et al.*, 2001). Confirmation by further investigations appears necessary.

In young children and partly also in infants, several strains of *Lactobacilli* have shortened and decreased the symptoms of infectious enteritis, especially Rotavirus diarrhoea (Rosenfeldt *et*

al., 2002; Rautanen *et al.*, 1998; Shornikova *et al.*, 1997), and both *Lactobacillus rhamnosus* GG and *Bifidobacterium lactis* Bb12 have been shown to prevent diarrheal diseases (Saavedra *et al.*, 1994; Guandalini *et al.*, 2000; Szajewska *et al.*, 2001). There are some preliminary indications that the risk of other infectious diseases such as *Helicobacter pylori* infections and of necrotising enterocolitis might by reduced by some probiotics. However, it remains to be determined if the modification of the intestinal flora in young infants of good health is of any appreciable benefit to their health.

For safety reasons probiotics should not be given to immunocompromised or premature infants, based on current knowledge. In addition to the requirements on strain identity, viability, strain stability, and number of probiotic bacteria in a food until the end of its shelf life, it is recommended that instructions for preparation, storage and heating of the formula ready for consumption be specific to guarantee the survival of the desired amount of microorganism until the time of feeding.

The working group recommends that nutritional, physiological and therapeutic effects be demonstrated by appropriate clinical studies described in detail. Formulae with added probiotic microorganisms should be labelled with the exact name of the strain and its concentration (number of microorganisms per weight unit of formula as ready for consumption). The label should include recommendations as to the amount and duration of consumption, and on storage and preparation.

The French working group has not made proposals as to the communication of proven beneficial effects of probiotics to the consumer via claims.

4. COMMENTS AND CONCLUSIONS

4.1 Use of probiotics in infant formulae and follow-on formulae

The Committee recognises the necessity to come to a decision at Community level on the use of bacteria generally considered as probiotics in infant formulae and/or follow-on formulae. The Committee consulted previous expert reports, but in view of time restrictions was unable to perform a full review of the available evidence on the inclusion of probiotic bacteria into infant formulae and follow-on formulae, and it recommends that a full review should be performed in the future. The Committee notes that the available information is still limited, and many studies in young infants have been done in non-European countries and in selected subpopulations of infants that are at increased risk of infectious or atopic diseases. The Committee recommends that infant formulae with microorganisms regarded as probiotics should only be introduced into the market if their benefit and safety have been evaluated according to the principles outlined in chapter XI of this report.

Follow-on formulae with added bacteria regarded as probiotics have been for since about three years. The Committee has no reason to object to the addition of bacteria regarded as probiotics to follow-on formulae, provided the requirements described below are fulfilled.

4.2 Requirements for the use of probiotic bacteria in follow-on formulae

Only bacterial strains with identity and genetic stability demonstrated by cultural and molecular methods should be used, if they can be considered as generally safe when added to the individual food and have been shown to survive the gastrointestinal passage, have the

capacity to proliferate in the gut for the duration of consumption and can modify the intestinal milieu (for example pH, short chain fatty acids). The identity of the probiotic strain should be described by molecular methods in a dossier and be available to the food control authorities. The content of viable bacteria should be such throughout shelf-life as to achieve 10^6 to 10^8 colony forming units per gram of formula prepared as ready for consumption. Processing, packaging and storage should not impair the viability of the bacteria.

4.3 Labelling requirements for follow-on formulae with added probiotic bacteria

In case of the addition of microorganisms, it is proposed to allow a label statement on the name of the bacterial strain, the number of microorganisms per g of powder or per 100 mL of the formula as prepared ready for consumption, and the duration of guaranteed microorganism content. The instructions for storage, preparation, heating and handling should take into account the possible impaired viability of probiotic strains when exposed to heat or oxygen.

The term “probiotic(s)” should only appear on formula labels if beneficial health effects in recipient infants have been established by adequate clinical trials and the results have been evaluated by an independent scientific body. The Committee considers claims on effects of probiotic bacteria on modification of the risk for specific health disorders as inappropriate unless such effects have been demonstrated by adequate scientific evidence following the guidance outlined in chapter XI of this report.

X. COMMENTS ON THE PRESENTATION OF INFANT FORMULAE AND FOLLOW-ON FORMULAE

As part of the review on the requirements of infant formulae and follow-on formulae, the Committee reviewed the scientific basis for the current European requirements for the presentation of such products (Article 9 of the Infant Formulae Directive).

1. In consideration of the numerous health and other benefits of breast-feeding, the marketing of breast milk substitutes should not interfere with breast-feeding, and with the promotion of and the support for breast-feeding.

2. The labelling and presentation of infant formulae and follow-on formulae should clearly identify their respective roles as a breast milk substitute and as the liquid part of a diversified diet and should not in any way discourage breast-feeding. The products should be labelled in such a way as to avoid any confusion between infant formula, follow-on formula and foods for special medical purposes.

3. The Committee has reviewed the scientific basis for the compositional criteria of descriptions of infant formulae as laid down in the Infant Formulae Directive (Annex IV). The Directive defined the conditions for claims on aspects of formula composition ("adapted protein", "low sodium", "sucrose free", "lactose only", "lactose free", "iron enriched") as well as one functional property ("reduction of risk of allergy to milk proteins").

4. With respect to these hitherto accepted claims, the Committee notes that the criteria used for the term "adapted protein" does not necessarily describe a protein of more superior quality for the feeding of infants. Therefore, the Committee suggests removing this claim.

5. The Committee suggests removing the claim "low sodium". All infant formulae and follow-on formulae have a limitation of sodium contents within well-defined ranges, and no products with high sodium content are recommended or permitted for healthy infants.

6. The Committee considers the claim "lactose free" to be of considerable relevance for the choice of products intended to be fed to infants who do not tolerate lactose. The acceptable maximum amount of residual lactose, if any, in formulae considered "lactose free" should not exceed 10 mg per 100 kcal. This proposal is based on the empirical guidance values for a safe intake of galactose (both free and β-glycosidic) for infants with classical galactosaemia).

7. Similarly, the Committee suggests removing the claim "sucrose free" or "saccharose free" for infant formulae, as the Committee has recommended that sucrose (saccharose) should not be added to infant formulae except for infant formulae based on protein hydrolysates where it may be used in limited amounts to achieve an acceptable taste.

8. The Committee further notes that the criteria used for the term "iron enriched" will become superfluous if the recommendation that all infant formulae should contain iron within the range specified in this report is adopted. Thus the Committee suggests

deleting this claim.

9. The Committee suggests permitting nutrition labelling of docosahexaenoic acid (DHA) content in infant formulae and in follow-on formulae if DHA contents equal at least 0.2% of total fatty acid contents.

10. The Committee concludes there is no scientific foundation to base a claim that a formula induces "reduction of risk of allergy to milk proteins" or is "hypoallergenic" on a content of immunoreactive protein of less than 1% of nitrogen-containing substances, as is presently the case. The Committee suggests that such a limited content of immunoreactive protein could indicate the compositional characteristics of a formula as being based on a "protein hydrolysate". However, the demonstration of a content of immunoreactive protein of less than 1% of nitrogen-containing substances cannot predict any potential preventive effect on the risk of developing an allergy to milk proteins (ESPGHAN, 1993; von Berg *et al.*, 2003). Such functional effects can only be based on controlled clinical trials following generally accepted scientific standards. Therefore, the Committee recommends that any such claims that a formula induces "reduction of risk of allergy to milk proteins" or is "hypoallergenic" should only be allowed if adequate scientific evidence has been accepted by an independent scientific body reviewing such data.

11. The Committee recommends that the source of the protein or proteins used for the production of the hydrolysate should be declared. Moreover, the Committee recommends not using the term "partial hydrolysate" since there is no agreed definition of "partial" or "extensive" hydrolysates and the effects of protein hydrolysates on prevention of allergic manifestations appear not to be strictly related to the degree of hydrolysis, but may depend also on other properties of the hydrolysates and the formulae produced thereof (ESPGHAN, 1993; von Berg *et al.*, 2003).

12. The Committee recommends that the scientific basis for claims for infant formulae, as laid down in Annex IV of the Infant Formulae Directive, should be reconsidered, based on current scientific knowledge. The Committee recommends that mechanisms and criteria should be developed for the communication not only of relevant compositional properties, but possibly also of selected other effects of infant formulae or follow-on formulae if they have been demonstrated beyond doubt in rigorous studies with adequate scientific standards, and the evidence has been accepted by an independent scientific body reviewing such data.

13. The Committee notes that some dietetic products intended for infants with minor and mostly transient health complaints, such as repeated possetting or intestinal discomfort, are currently marketed as Dietary Foods for Special Medical Purposes. Neither the nature of the complaints concerned nor the recently adopted definition of Dietary Foods for Special Medical Purposes (Directive 1999/21/EC) justifies such presentation for the vast majority of these products. The Committee also notes that such presentation has implications for the labelling and marketing practices of these products. The Committee recommends that the scientific basis for the use, potential benefits and compositional aspects of such products should be reviewed.

XI. MODIFICATION OF THE COMPOSITION OF INFANT FORMULAE OR FOLLOW-ON FORMULAE BEYOND THE ESTABLISHED STANDARDS

The Committee is aware of continuing improvements in the understanding of the complex composition of human milk, in dietary effects on physiological outcomes in the infant, and in food technology, which have led and will continue to lead to innovative modifications of infant formulae and follow-on formulae. The addition of new ingredients or of established ingredients in newly determined amounts that deviate from the established guidance on formula composition, the reduction or elimination of current constituents, or any other modification of formula composition should be made possible if the benefit, suitability and safety for particular use by infants have been established by generally accepted scientific data and this is overseen and evaluated by an independent scientific body prior to the introduction of such modified products into the market. Here we propose some general principles that should be followed in the evaluation of modifications of infant formulae or follow-on formulae with regard to their suitability and safety for particular nutritional use by infants. The Committee is aware, however, that details of the appropiate evaluation process of specific modifications will have to be decided on a case by case basis, depending on the nature of those modifications.

1. The introduction of modifications to an infant formula or a follow-on formula beyond the established standards should be based on and justified by defining an expected benefit (nutritional, functional, technological, or other). Any such modification should be based on a systematic review of the relevant existing literature and other information available in the area of the proposed innovation to develop a clear hypothesis or hypotheses on the expected benefits, and on safety considerations which might need to be addressed in an ensuing further evaluation. The hypotheses outlined in the systematic review of the relevant existing information should form the basis for the choice of potential further pre-clinical or clinical studies that may need to be performed to evaluate a modification. In addition, general nutritional assessments may be needed. Based on this systematic review and the description of the specific innovation, its suitability and safety for the intended use, and the extent of potential additional scientific data that may be necessary, should be determined by an independent scientific body prior to the commercialization of the modified product.

2. A detailed description of the modification in the composition and/or process should be provided. Complete compositional data of the modified or new product, in particular information on those compounds that may be altered or newly formed during the process, including possible contaminants, should be provided. In case of added ingredients a full description of the new ingredient(s) should be provided, such as their chemical name according to IUPAC nomenclature rules, CAS number (if any), synonyms, trade names and abbreviations, molecular and structural formulae, molecular weight, purity and impurities. If a mixture of compounds is used, the constitutents of the mixture and proportion of each component should be reported. In case of natural extracts, particular attention should be given to other components of the extract and possible allergenic residues from the original source. Specifications, manufacturing processes, methods of analysis in formulae, and reaction and fate in formulae to which the ingredient is added should be reported as outlined in the SCF guidance document on safety evaluation of sources of nutrients (SCF, 2001a).

3. Further required data are the way of addition of new ingredients, and a description of the technological processes applied to the formula, with particular emphasis on the dry mixing *vs.* wet mixing steps. When the innovation includes addition of a new ingredient submitted to further processing, potential effects on the ingredient and other components of the formula should be characterized. When innovation is limited to the use of a new source of a nutrient or ingredient authorized by the EU legislation or evaluated by the Committee, which is added by dry mixing and for which no interference with other formula ingredients is to be expected, pre-clinical assessment may be limited to the ingredient itself following the guidance previously provided by the Committee (SCF, 2001a).

4. All available biological and toxicological information should be reported, such as bioavailability of added or modified compounds from the formula, subsequent metabolic fate and biological distribution, biological effects and underlying known mechanisms, any known interactions with other components of the formula, impact of added or modified compounds on gastrointestinal physiology and the absorption of other nutrients, and any toxicological information. Safety data should be applicable to the population for which the product is intended as far as possible.

5. Information on *in vitro* or *in vivo* studies in animal models or other experimental settings should be provided if they may help to establish information on nutritional adequacy, potential benefits, and safety of the proposed innovation. When innovation involves a change in protein source, or any technological process that may affect protein quality, pre-clinical assessment should include not only amino-acid profile, but also a relevant measurement of amino-acid availability (for instance lysine blockage and protein efficiency).

6. If the innovation falls under the definition of a novel food, the Novel Food procedure as described in Directive 258/97/EEC should apply, and the respective SCF guidelines (SCF, 1997) should be followed.

7. In case sources of nutrients or other ingredients are used that contain or are derived from genetically modified organisms, the SCF guidance on safety evaluation of GMOs (SSC, 2003) should be followed.

8. In addition to preclinical assessment, clinical trials may often be needed to further characterise the suitability and safety of modifications that extend beyond the established standards of infant formulae and follow-on formulae. Clinical studies should only be undertaken if there is reasonable assurance on the probable suitability and safety in infants from the preclinical assessment. The gold standard for the evaluation of outcomes in infants fed a modified infant formula should be the comparison with outcomes seen in healthy infants who have been exclusively breast-fed for four to six months, but comparative studies with established formulae may also be useful. If clinical studies are to be performed, they should follow the general principles of Good Clinical and Good Laboratory Practices. Ethical approval should be acquired and informed parental consent obtained, and this should be declared in the report of results. The study design should consider from the outset the statistical power of the study, and the confidence limits of any differences found should be included in the reports on the studies. All infants participating in clinical trials should be characterised with regard to factors which might affect the planned outcomes. A core data set that should be reported for all nutritional studies in infants has been proposed

(ESPGHAN, 2003a). Blind randomisation with respect to the allocation of test and reference formulae should be used whenever the innovation under evaluation allows this approach. The possibility of unexpected adverse outcomes should be anticipated and addressed by adequate clinical monitoring of participants, and by incorporating into the study design arrangements for the independent scrutiny of the accumulating data. Preliminary pilot studies of the proposed study design may be useful to identify and anticipate outcomes and issues which would inform definitive studies and enable protocols to be adapted, and they also would enable the views of the infants' carers to be taken into account.

9. A growth study in infants should be performed if the modification may reasonably be expected to have an effect on growth. Examples where a growth study would appear necessary are changes in energy density beyond established limits, significant changes in macronutrient composition, new or markedly modified nitrogen sources, or possible alterations of bioavailability of macronutrients. Results should be compared either to those achieved with a reference formula, or to breast fed populations, considering that maximal weight gain is not always optimal weight gain. Studies to evaluate infant growth should have a duration of at least 3 months, should preferably start from birth, and should include, as a minimum, monthly measures of the growth parameters weight, length and head circumference. Growth studies should be designed to have a power to detect a difference in weight gain equal to 0.5 standard deviations. It is desirable to also include some measure of body composition, such as assessments of skinfold thickness and arm circumference, as well as a measure of infant formula intake, but this is considered optional.

10. In specific cases, the monitoring of adverse responses by consumers, medical doctors and other health care professionals after the introduction of a new infant formula or a significantly changed infant food into the market may be useful. The effective execution of such an "post market monitoring" would require the definition of further details such as the the specific questions to be addressed and information to be collected, conditions of data collection (e.g. spontaneous reports by consumers or health professional, or active surveillance methods), and the conditions of data evaluation by an independent scientific body.

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ANNEX to the Report of the Scientific Committee on Food on the Essential Requirements for Infant Formulae and Follow-on Formulae

SUMMARY TABLE OF RECOMMENDATIONS ON THE COMPOSITION OF INFANT FORMULAE AND FOLLOW-ON FORMULAE

	Infant Formulae	**Follow-on Formulae**
Energy density (kcal/100 mL)	60-70	60-70
Nutrients (per 100 kcal, unless otherwise stated)		
Protein[1]		
Cow's milk protein	1.8-3 g[2]	1.8-3 g
Soy protein Protein hydrolysates	2.25-3 g	
L-carnitine addition to soy protein and protein hydrolysates formulae	≥1.2 mg	no requirement
Addition of taurine	≤12 mg	
Nucleotides, if added[3]	≤ 5 mg	
Choline	7-30 mg	no requirement
Fat		
Total fat	4.4-6 g	4.0-6.0 g
Phospholipids	≤1 g/L	
Inositol	4-40 mg	no requirement
Lauric and myristic acids	Together ≤20% of total fatty acids	
Linoleic	0.5-1.2 g	
Formulae without added LCPUFA		
α-linolenic	≥100 mg	
Linoleic/α-linolenic ratio	5-15	
Formulae with added LCPUFA		
α-linolenic[4]	≥50 mg	
Linoleic/α-linolenic ratio[4]	5-20	
n-6 LCPUFA	≤2% of total fatty acids	
Arachidonic acid	≤1% of total fatty acids	
n-3 LCPUFA	≤1% of total fatty acids	
Ratio EPA/DHA (wt/wt)	<1	
Cottonseed/sesame oils	No use of these type of oils	
Conjugated linoleic acid (CLA)	No intentional addition	
Trans fatty acids	≤3% of total fatty acids	
Erucic acid	≤1% of total fatty acids	

[1] Calculation of protein content: N x 6.25, non-protein nitrogen (formulae made from intact protein) ≤15% of total nitrogen.
[2] Infant formulae containing 1.8 g/100 kcal should be clinically evaluated.
[3] Maximum content per nucleotide as specified in the text.
[4] If DHA content ≥0.2% of total fatty acids.

	Infant Formulae	Follow-on Formulae
Carbohydrates		
Total carbohydrates	9-14 g	
Lactose in cows' milk protein- and protein hydrolysates formulae	≥4.5 g	
Lactose in soy protein formulae	No requirement	
Saccharose	None in cows' milk protein and soy protein formulae ≤20% of total carbohydrates in protein hydrolysates formulae	Sum of saccharose, fructose, honey ≤20% of total carbohydrates
Fructose	None	
Glucose	No intentional addition to formulae based on intact proteins, ≤2 g in formulae based on protein hydrolysates	
Maltose, maltodextrins	Unrestricted	
Starches	≤30% of total carbohydrates (≤2 g/100 mL) as precooked or gelatinised naturally gluten-free starches No starches modified by enzymatic cross-linking or stabilisation	Gluten-free carbohydrates only
Vitamins		
Vitamin A	60-180 µg RE	
Vitamin D	1-2.5 µg	1-3 µg
Vitamin E	≥0.5 mg αTE/g PUFA (corrected for double bond, see footnote[5]) - 5 mg	
Vitamin K	4-20 µg	
Vitamin B_1 (thiamine)	60-300 µg	
Vitamin B_2 (riboflavin)	80-400 µg	
Vitamin B_3 (niacin)	300-1200 µg NE	
Vitamin B_6 (pyridoxine)	35-165 µg	
Vitamin B_{12} (cobalamin)	0.1-0.5 µg	
Pantothenic acid	400-2000 µg	
Folic acid	10-30 µg	
Vitamin C (ascorbic acid)	10-30 mg	
Biotin	1.5-7.5 µg	

[5] 0.5 mg α-TE/1 g linoleic acid (18:2n-6); 0.75 mg α-TE/1 γ-linolenic acid (18:3n-3); 1.0 mg α-TE/1 g arachidonic acid (20:4n-6); 1.25 mg α-TE/1 g eicosapentaenoic acid (20:5n-3); 1.5 mg α-TE/1 g docosahexaenoic acid (22:6n-3).

	Infant Formulae	Follow-on Formulae
Trace Elements		
Iron		
Cow's milk protein and protein hydrolysate formulae	0.3-1.3 mg	0.6-1.7 mg
Soy protein formulae	0.45-1.9 mg	0.9-2.5 mg
Calcium	50-140 mg	
Calcium/Phosphorus-Ratio	1.0-2.0	
Phosphorus	Cows' milk protein- and protein hydrolysate formulae: 25-90 mg Soy protein formulae: 30-100 (Bioavailable phosphorus, if measured: 20-70 mg)	
Magnesium	5-15 mg	
Sodium	20-60 mg	
Chloride	50-160 mg	
Potassium	60-160 mg	
Chromium	No recommended minimum and maximum levels	
Manganese	1-100 µg	
Molybdenum	No recommended minimum and maximum levels	
Fluoride	≤100 µg	
Iodine	10-50 µg	
Selenium	3-9 µg	
Copper	35-100 µg	
Zinc		
Cow's milk protein and protein hydrolysate formulae	0.5-1.5 mg	
Soy protein formulae	0.75-2.40 mg	

European Food Safety Authority

EXECUTIVE DIRECTOR

SANCO A- J40 J3
04. 09. 2007
Deadline: 25/09/2007
DG | DDG | 03 | A | B | C | D | E | F | G

Parma, 22 August 2007
CGL/HK/PRI/jj (2007)/2326289

Ms Paola Testori Coggi
Deputy Director General SANCO
European Commission
BRU-B232 04/095
B – 1049 Brussels

Subject: **Request to the European Commission for a Corrigendum to the SCF Report on the revision of essential requirements of infant formulae and follow-on formulae (adopted on 4 April 2003)**

Dear Ms. Testori Coggi, Dear Paola,

With reference to the Report on the revision of essential requirements of infant formulae and follow-on formulae which was adopted by the Scientific Committee on Food (SCF) on 4 April 2003 (Ref. SCF/CS/NUT/IF/65 Final dated 18 May 2003), I have been notified of a typo-mistake concerning a figure given for the content of carnitine in cow's milk (page 60 attached, section 4.7.2 Carnitine). After checking with the expert involved in the drafting of the above-mentioned report, it has been confirmed that the figure of 50 mg/100 kcal is wrong and should be read as follows:

4.7.2 Carnitine

"...Cow's milk is rich in carnitine (around 5 mg/100 kcal) compared to human milk,..."

Considering that the above-mentioned Report of the SCF is managed by the European Commission, I am therefore requesting you to proceed with a corrigendum to amend the figure. Ms. Helen Lee from your services has been informed of the above.

Yours sincerely,

Catherine Geslain-Lanéelle

Enclosure: The SCF Report – page 60

Copy.: B. Mathioudakis, L. Helen (SANCO)

changes in electroretinography which could be corrected by taurine supplementation (Sturman and Chesney, 1995).

Infants fed a taurine-supplemented (6 mg/100 mL) infant formula with a protein content of 2 g/100 mL (2.9 g/100 kcal) showed the same growth development from 2 to 12 weeks of age as infants breast-fed or receiving the same formula without taurine. However, blood urea nitrogen levels at 12 weeks were significantly lower than in infants fed the taurine-free formula and similar to breast-fed infants, as were the concentrations of indispensable amino acids in plasma and urine (Räihä *et al.*, 1996). The mechanism of this effect is unclear.

As previously noted (in section 4.5.3) if a specified taurine content is considered to be relevant logically this should not be restricted to formula with hydrolysed protein. The Committee considers that the requirement for a minimum content of taurine in formulae manufacturered from hydrolysed protein is not necessary.

The Committee proposes that, when added, taurine addition to any type of infant formula should be not exceeding 12 mg/100 kcal.

4.7.2 Carnitine

The addition of L-carnitine to infant formula based on soy protein isolate and hydrolysed protein to give a content of at least 7.5 μmoles/100 kcal (1.2 mg/100 kcal) is required in the EU and the Committee does not propose a change. This value is similar or somewhat higher than in human milk (0.9 to 1.2 mg/100 kcal) because of a presumed reduced bioavailability from formula (Warshaw and Curry, 1980). Cow's milk is rich in carnitine (50 mg/100 kcal) compared to human milk, therefore carnitine addition to cows' milk-based formula is not necessary. Carnitine is synthesised in the human body at a rate of approximately 0.3 mg/kg/day from lysine and using methionine as methyl donor (Rebouche and Seim, 1998). It is considered an indispensable nutrient for newborn infants (Rebouche, 1992) because of a temporarily compromised synthesising capacity. Its function is the transport across membranes of carboxylic acids that have been activated to the co-enzyme A level, thereby delivering substrates for oxidation and removing toxic compounds.

Infants receiving unsupplemented soy formula for 112 days showed lower serum levels of carnitine, higher levels of free fatty acids and an increased excretion of medium-chain dicarboxylic acids (Olson *et al.*, 1989). The minimal dietary carnitine requirement of a newborn infant has been estimated to be 1.7 mg/kg/day due to a practically absent endogenous synthesis (Scholte and de Jonge, 1987).

The Committee considers the addition of carnitine to follow-on formula is not necessary. Supply from appropriate complementary food and from endogeneous synthesis should be sufficient in older infants. Only the liver butyrobetaine hydroxylase, the last enzyme in carnitine biosynthesis, shows age-dependent low activity in young infants. The activity of the kidney enzyme and the other three biosynthetic enzymes in the liver and other tissues are not age-dependent (Vaz and Wanders, 2002)

4.7.3 Nucleotides and nucleosides

Nucleosides contain a nitrogenous base and a pentose, but no phosphate group. Nucleotides contain a nitrogenous base, a pentose and one or more phosphate groups. Nucleotides are found primarily intracellularly. They are the structural components of DNA and RNA.