THE COMPOSITION OF HUMAN MILK AS A MODEL FOR THE DESIGN OF INFANT FORMULAS: RECENT FINDINGS AND POSSIBLE APPLICATIONS

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INTRODUCTION

Human milk is assumed to be the ideal food for the infant at least up to the age of 5 or 6 months, ensuring optimal growth and development (ESPGAN, 1982). In many respects human milk, the most natural food available, is unique. The nutritional composition of

https://doi.org/10.1079/NRR19940004 Published online by Cambridge University Press

human milk varies from mother to mother, from day to day, during the day and even during a feed, and is generally suited to the individual needs of the infant. There is little doubt that human milk serves a role in infant physiology greater than being a supply of energy and nutrients. For instance, the immunological properties of human milk (immunoglobulins, bacteriostatic proteins, living cells, antiviral lipids) are well documented. In developing countries these established beneficial properties can be translated into demonstrable advantages to the breast fed over the bottle fed infant, in terms of reduced morbidity and mortality (Jason *et al.* 1984; Hanson *et al.* 1994). Controversy persists, however, about whether breast feeding protects infants in Western countries from infectious diseases and, if so, the magnitude of this effect. Four of the five epidemiological studies in industrialized countries published between 1970 and 1984 that met the methodological standards set by Bauchner *et al.* (1986) concluded that breast feeding was not protective against infections. Only one of the studies did show a protective effect of breast feeding against gastrointestinal infections among infants younger than four months. Newer studies continue to suggest only a minimal protective effect of breast feeding in industrialized countries, with the clearest impact on gastroenteritis (Wright *et al.* 1989; Howie *et al.* 1990; Rubin *et al.* 1990).

While human milk is superior for the newborn infant, milk substitutes play a necessary role in infant nutrition when breast feeding is not possible, desirable or sufficient. The search for human milk substitutes has been conducted since the Stone Age, but it was not until sanitation practices developed in the late nineteenth century that feeding these substitutes, mostly based on cows' milk, to infants could be safely accomplished. Since then, there have been progressive attempts to bring the composition of these formulations closer to human milk. Important modifications included reduction of the protein and electrolyte content, addition of vitamins, trace elements and lactose, alteration of the casein:whey protein ratio, and substitution of unsaturated vegetable fats for butterfat. With the addition of taurine some years ago a new phase in the development of infant formulas has commenced. Presently, research is concentrating on those substances in human milk which serve other than traditional nutritional roles. Attempts are in progress to supplement infant formulas with protective and trophic factors so far unique only to human milk. The final aim is not necessarily to mimic the composition of human milk in every respect, but to achieve physiological effects as in breast fed infants.

In this paper, the relevant aspects of the composition of human milk and of the current infant formulas will be reviewed, and an outline of some of the expected developments in the composition of infant formulas will be given.

ENERGY

The energy requirements of infants reflect levels of energy intake that will promote health, adequate growth, optimal body composition, and levels of physical activity appropriate for their developmental age. The average energy density of mature human milk is generally used as a basis for the assessment of the infant's energy requirement, and thus as a guideline for the energy content of infant formulas.

Traditionally, the energy density of human milk has been assessed by analysis of its macronutrient composition, obtained by manual or mechanical expression from the breast. To calculate the energy density of the milk from its macronutrient composition, the standard Atwater conversion factors are used. The mean energy value of mature milk obtained by this method is around 700 kcal/l (Department of Health and Social Security, 1977). This method of estimating energy density is subject to error, however, as milk that

has been obtained by whole breast expression may not be identical in composition to suckled milk. In particular, the fat content, a major determinant of energy content, may be higher in expressed milk, which may result in an overestimation of the energy density of suckled milk (Lucas *et al.* 1990). Another potential problem of this method of estimating energy density is that the Atwater factors used to estimate metabolizable energy from macronutrient composition may be inappropriate for young infants, as nutrient absorption may be less than that accounted for in these conversion factors. The recent development of the doubly labelled water method for determining energy expenditure, energy stored in new tissue, and water (and therefore milk) intake has made it possible to estimate the energy density of suckled breast milk. Using this method, the metabolizable energy content of breast milk has been found to be 530 kcal/l and 580 kcal/l at 6 weeks and 3 months of age respectively (Lucas *et al.* 1990). The metabolizable energy values obtained for infants fed formula with a calculated energy density of 680 kcal/l at these ages were 600 and 660 kcal/l respectively, suggesting that the use of the Atwater factors may indeed lead to an overestimation of the metabolizable energy intake in the first weeks of life.

These data have important potential consequences for infant nutrition. It may be that current guidelines for energy intake in infancy are too high and thus need revision (Prentice *et al.* 1988). This may explain why breast fed infants grow less rapidly than formula fed infants, despite their lower total daily energy expenditure (Butte *et al.* 1990; Heinig *et al.* 1993).

Further research is needed to confirm the energy values for suckled milk found by Lucas *et al.* (1990), and to determine whether the energy density of infant formulas should be lowered towards these values.

PROTEIN

PROTEIN QUANTITY

It is now generally accepted that the true protein content of mature milk is only about 8–10 g/l and that the earlier overestimation was due to a large proportion of nitrogen that is not part of human milk protein (Hambraeus *et al.* 1978; Räihä, 1985; Harzer *et al.* 1986). This so-called non-protein nitrogen fraction includes about 20–25% in human milk (Hambraeus *et al.* 1978; Harzer *et al.* 1986).

Since some of the protective whey proteins of human milk, particularly secretory IgA and lactoferrin, are quite resistant to low pH and the action of proteolytic enzymes and are to a significant extent excreted in the infant's stools, the nutritionally available protein of mature human milk may be as low as 6–8 g/l (Räihä, 1985; Davidson & Lönnerdal, 1987).

Through the last decades, there has been a trend towards lowering the protein levels of infant formulas, in order to make them more similar to human milk. A major reason for this decrease has been the finding that formula fed infants have elevated plasma concentrations of urea and of specific amino acids, which suggests that these infants may be exposed to unnecessary metabolic stress (Järvenpää *et al.* 1982*a*, *b*; Janas *et al.* 1985). Several studies evaluated the effects of lowering the protein content of formula to values of 11–13 g/l, while varying the casein:whey protein ratio (Räihä *et al.* 1986*a*, *b*; Picone *et al.* 1989; Lönnerdal & Chen, 1990). Infants receiving such a low protein formula had growth rates and indices of protein nutritional status similar to those of breast fed infants. During the first four weeks of life, however, Räihä *et al.* (1986*a*, *b*) observed that the infants fed the low protein formula (12·5 g/l) had blood urea nitrogen and urine nitrogen concentrations that were significantly lower than those of the breast fed group. The fact that this finding could not be confirmed by other investigators, even when a formula with a protein level of as low as 11 g/l was given, may at least be partly explained by differences

in non-protein nitrogen levels between the formulas used (Donovan & Lönnerdal, 1989). In none of the studies could plasma amino acid patterns identical to those of breast fed infants be produced, suggesting that factors other than the amino acid pattern of human milk influence plasma amino acid levels (Picone *et al.* 1989). Reducing the protein content of formula to values of around 13 g/l has been observed to result in low plasma tryptophan values relative to those of breast fed infants, presumably owing to inadequate intake and reduced bioavailability of tryptophan from bovine whey protein (Janas *et al.* 1987). Low plasma tryptophan levels may be of concern as tryptophan is an essential amino acid and a precursor of serotonin and niacin. Addition of free tryptophan to a low protein formula has been found to result in plasma tryptophan levels similar to those of breast fed infants, and to influence the infants' sleep latency (Hanning *et al.* 1992; Fazzolari-Nesci *et al.* 1992; Steinberg *et al.* 1992).

PROTEIN QUALITY

The concentration of whey proteins in human milk decreases from early lactation and continues to fall. These changes result in a casein:whey protein ratio of about 10:90 in the first days of lactation and of about 45:55 in mature milk (Harzer *et al.* 1986; Kunz & Lönnerdal, 1992). The optimal casein:whey protein ratio of infant formulas is still a point of controversy. Growth rates do not differ between infants fed whey predominant formulas and those receiving casein predominant formulas (Harrison *et al.* 1987; Janas *et al.* 1987; Lönnerdal & Chen, 1990). Theoretically, whey predominant formulas may offer some minor advantages for newborns because they form a finer, softer curd than casein predominant formulas leading to higher gastric emptying rates, which are more comparable to those of breast fed infants (Nakai & Li-Chan, 1987; Billeaud *et al.* 1990). Further, during the first two months a whey predominant formula was found to induce a faecal flora somewhat closer to that of breast fed babies than a casein predominant formula (Balmer *et al.* 1989). It should be noted, however, that the casein predominant formula used in this study had higher protein and phosphate contents than the whey predominant one, and therefore presumably a higher buffering capacity. The functional consequences of the observed differences in gastric emptying and intestinal flora still have to be demonstrated. The same holds true for the putative advantages of casein predominant formula being more satisfying or less allergenic.

Since there is no convincing evidence that whey predominant formulas are superior in composition or physiological effect, the Scientific Committee for Food, set up by the European Communities, recommended in their opinion expressed on 17 September 1993 not to adhere any longer to different minimal values for the protein content of infant formula dependent on the casein:whey protein ratio.

Human milk casein and its subunits represent the least understood and characterized class of proteins in human milk. Important differences exist between human and bovine caseins. β-Casein is the predominant casein in human milk, whereas cows' milk contains a large proportion of α-caseins (Eigel *et al.* 1984; Kunz & Lönnerdal, 1990). Additionally, physicochemical differences between human and bovine caseins affect curd formation, which in turn influences gastric emptying and intestinal transit time (Nakai & Li-Chan, 1987; Billeaud *et al.* 1990). Partial enzymic dephosphorylation and/or rennet modification may improve coagulation characteristics and digestibility of bovine caseins for infant feeding (Nakai & Li-Chan, 1987; Li-Chan & Nakai, 1989). The physiological roles of casein in mineral absorption and in providing fragments with immunomodulating and opioid-like activities need further study (Migliore-Samour & Jollès, 1988; Daniel *et al.* 1990).

LACTOFERRIN

Of the protective whey proteins in human milk, the iron binding glycoprotein lactoferrin is the second most abundant one, being present in colostrum and mature milk in concentrations of about 5–7 and 1–2 g/l respectively (Hambraeus *et al.* 1978; Goldman *et al.* 1982). Lactoferrin is remarkably resistant to degradation by proteinases (Davidson & Lönnerdal, 1987).

The possible physiological functions of lactoferrin have recently been reviewed by Iyer & Lönnerdal (1993). Lactoferrin has been suggested to have bacteriostatic activity, to enhance iron absorption, and to stimulate mucosal proliferation. The bacteriostatic and bactericidal effects of lactoferrin *in vitro* have been studied in a wide range of microorganisms. Lactoferrin withholds iron from invading organisms by its high iron affinity as well as its slow rate of change to a conformation in which the iron site is exposed (Chung & Raymond, 1993). Next to iron withholding, other mechanisms may be involved in the antibacterial action of lactoferrin, and a synergic mechanism with lysozyme and/or IgG has been proposed (Iyer & Lönnerdal, 1993). Convincing *in vivo* data which confirm the *in vitro* findings are still lacking. Limited hydrolysis of bovine lactoferrin resulted in potent antibacterial activity, suggesting that lactoferrin latently contains at least one antibacterial peptide region that is released when the molecule is hydrolysed. The bactericidal activity of the peptide fragments did not have any relation to iron chelation (Saito *et al.* 1991). The putative bactericidal domain, lactoferricin, has recently been isolated and described in bovine and human lactoferrin. The region is distinct from the iron binding region. The antimicrobial peptide of bovine lactoferrin was found to be more active than that of human lactoferrin (Bellamy *et al.* 1992). Further studies are required to determine whether digestion of either human or bovine lactoferrin *in vivo* generates potent bactericidal peptides in sufficient quantities to be of biological importance for neonates.

The precise role of lactoferrin in iron absorption has not yet been defined. Clinical trials in which infant formulas were supplemented with bovine lactoferrin have failed to demonstrate an improvement in iron absorption (Fairweather-Tait *et al.* 1987; Schulz-Lell *et al.* 1991). Possible explanations for these negative results are (1) the species specificity of the human lactoferrin receptor (if this receptor is involved in iron transport), and (2) inappropriateness of the composition of the formulas used (high in citrate and phosphate, low in bicarbonate). Chierici *et al.* (1992) reported, however, that infants fed a lactoferrin supplemented formula had higher ferritin levels than those fed the control formula, and not significantly different from the breast fed group. Both the experimental and the control formulas contained no added iron. One possible flaw of this study was the fact that the lactoferrin supplemented formula contained more endogenous iron than the non-supplemented control formula. Recently, Davidsson *et al.* (1994) surprisingly observed that iron absorption in infants was significantly lower from human milk with its native content of lactoferrin than from lactoferrin free human milk, which suggests that human milk lactoferrin may have no direct role in the enhancement of iron absorption. More studies are needed to define the role of lactoferrin in iron transport better. Lactoferrin could well be involved in iron metabolism, acting as a regulator of iron absorption when iron stores are adequate and as an enhancer of iron absorption in deficiency states (Iyer & Lönnerdal, 1993).

The hypothesis that lactoferrin acts as a growth factor for the intestine remains to be confirmed *in vivo* (Nichols *et al.* 1987; Iyer & Lönnerdal, 1993).

In the coming years, it will be possible to perform *in vivo* supplementation trials using human lactoferrin produced in the mammary glands of transgenic dairy animals. Transgenic dairy calves have already been generated carrying the human lactoferrin fusion

gene (Krimpenfort, 1993). It is hoped that these studies will lead to a better understanding of the biological functions of human milk lactoferrin.

IMMUNOGLOBULINS

Secretory IgA comprises over 90% of the immunoglobulins in human milk. The highest concentrations of sIgA (~ 9 g/l) are found in colostrum. Mature milk has sIgA levels averaging 1–2 g/l (Harzer & Bindels, 1985; Goldman & Goldblum, 1989). Human milk sIgA is directed against enteric and respiratory immunogens that have triggered the maternal enterobronchial mammary gland pathways (Goldman & Goldblum, 1989). Secretory IgA antibodies in human milk neutralize bacterial toxins and virulence factors and inhibit adherence and proliferation of bacteria on epithelial surfaces by binding to the bacterial adhesins (Goldman & Goldblum, 1989; Davin *et al.* 1991).

In order to improve the immunological composition of infant formulas, in theory IgA derived from human milk or produced through genetic engineering should be used. It is extremely difficult, however, to harvest large amounts of IgA from human milk for commercial purposes. Additionally, large scale production of sIgA by recombinant DNA technology may be a daunting task since the molecule has four different types of peptide chains, the formation of antigen combining sites requires the rearrangement of four different groups of genes, once the relevant peptides are produced it would be necessary to link them, and many different antibodies would be required to create a broad range of specificities (Goldman, 1989).

As a more feasible alternative to human IgA, bovine antibodies could be used. Whereas sIgA is the predominant class of immunoglobulins in human milk, IgG_1 is predominant in cows' milk. Even though it is structurally different, it appears to have the same function (Facon *et al.* 1993). Attempts have been made to increase the immunoglobulin concentration of cows' milk and to manipulate its specificity by immunization. In several studies infants were prophylactically supplemented with immunoglobulins isolated from the milk of cows immunized to specific pathogens. Davidson *et al.* (1989) demonstrated that bovine colostrum with high antibody titre against four human rotavirus serotypes, given as a supplement, was highly effective in protecting hospitalized infants against rotavirus infection. Additionally, administration of colostrum from rotavirus immunized cows prevented rotavirus infections in infants living in an orphanage (Ebina *et al.* 1985). Turner & Kelsey (1991) reported that administration of bovine milk antibodies to human rotavirus did reduce rotavirus associated illness but not rotavirus infection. Brunser *et al.* (1992) failed to prevent rotavirus and *Escherichia coli* infections in infants living in low socioeconomic conditions by providing a formula containing bovine milk antibodies against these pathogens. The investigators postulated that the dose of immunoglobulin given may have been too low and/or the age of the infants (majority 9–12 months) may have had an influence on the digestive enzymes and thereby on the unprotected passage of the antibodies through the intestine.

Larger scale controlled clinical trials are needed to prove the efficacy of passive oral immunization with milk antibodies from immunized cows, to ascertain whether the risks of such antibody supplementation are minimal, and to determine the minimal effective dose (Goldman, 1989; Boesman-Finkelstein & Finkelstein, 1991). It is not inconceivable that in the future immunoglobulin fortified infant formulas will be developed, which can be given to non-breast fed infants susceptible to infections by intestinal pathogens, such as those that are immunodeficient (Shield *et al.* 1993) and/or those in contaminated environments such as hospitals (Goldman, 1989; Facon *et al.* 1993).

NON-PROTEIN NITROGEN

Many non-protein nitrogen components have been identified, including urea, uric acid, ammonia, creatine and creatinine, free amino acids, nucleic acids and nucleotides, polyamines, carnitine, low molecular weight peptide hormones, growth factors, the amino sugars *N*-acetylglucosamine and *N*-acetylneuraminic acid (sialic acid), and the amino alcohols choline and ethanolamine (Carlson, 1985*a*; Atkinson *et al.* 1989). Some of this nitrogen contributes to the pool available for synthesis of non-essential amino acids. For instance urea nitrogen, which accounts for 30–50% of the non-protein nitrogen fraction, is partly hydrolysed to ammonia by intestinal microorganisms, with subsequent intestinal absorption of the released ammonia. From studies with isotopically labelled urea, it has been estimated that about 13–23% of dietary urea is retained and available for amino acid synthesis (Heine *et al.* 1986; Fomon *et al.* 1988).

Other non-protein nitrogen compounds may be involved in the development of the newborn infant. Taurine and nucleotides have been claimed to be conditionally essential substances and are dealt with later. Non-protein nitrogen components with clear trophic characteristics are polyamines (spermine, spermidine, putrescine) and epidermal growth factor. Polyamines are known to be involved in cell proliferation and differentiation in many tissues, including the gastrointestinal tract (Pegg, 1986; Pollack *et al.* 1992). Whereas human milk has been found to contain considerable amounts of putrescine, spermine, and spermidine, standard cows' milk based infant formulas contain no detectable polyamines or only very small amounts (Pollack *et al.* 1992; Romain *et al.* 1992). Additionally, epidermal growth factor may be involved in maturational processes of the newborn (Kidwell & Salomon, 1989). It can be expected that this rapidly emerging research area will give us significant information with regard to the importance of human milk for gut proliferation and maturation.

LIPIDS

LONG CHAIN POLYUNSATURATED FATTY ACIDS

Fats are vital for normal growth and development. In addition to providing energy, fats supply essential fatty acids and are the vehicle for fat-soluble vitamins and hormones in milk. Recently, the essential fatty acid requirements of newborns have received increasing attention. Particular interest has focused on the importance of long chain polyunsaturated fatty acids with 20 and 22 carbon atoms (LCP). These fatty acids are important structural components of cell membrane phospholipids, particularly those of the central nervous system and of retinal photoreceptors, and serve as precursors for the synthesis of eicosanoids (British Nutrition Foundation, 1992).

Docosahexaenoic acid (DHA) and arachidonic acid (AA) constitute a large proportion of the total lipids in brain and retina and their accretion primarily occurs during the last trimester of pregnancy and the first year of life (Clandinin *et al.* 1980*a*, *b*). Fetal accretion of LCP may result from placental transfer (Kuhn & Crawford, 1986). Postnatally, human milk provides the breast fed infant with preformed AA and DHA. Term human milk has an AA content of about 0·5% and a DHA content of about 0·3% of total fatty acids (Koletzko *et al.* 1992). The mean LCP levels of colostrum and preterm milk are higher (Rönneberg & Skåra, 1992; Foreman-van Drongelen *et al.* 1994).

The available evidence strongly suggests that a dietary supply of LCP is desirable for the preterm infant. Preterm infants fed formulas without LCP develop poor AA and DHA status, suggesting that the preterm infant is unable sufficiently to elongate and desaturate linoleic acid and α-linolenic acid to their long chain derivatives (Carlson *et al.* 1991). The

DHA status of preterm infants is positively related to their visual acuity, whereas their AA status positively correlates with their first-year growth and with scores on development tests (Carlson *et al.* 1992*a*, 1993*a*, *b*). Feeding formulas supplemented with fish oil to preterm infants improves their DHA status and visual function (Carlson *et al.* 1991, 1993*b*; Birch *et al.* 1992) but their AA status deteriorates and may result in poorer growth (Carlson *et al.* 1991, 1992*b*).

Both the ESPGAN Committee on Nutrition (1991) and the British Nutrition Foundation (1992) recommend enrichment of premature formulas with AA and DHA. There are already some premature formulas available which are supplemented with DHA and either AA or its precursor, the δ-6 desaturation product γ-linolenic acid. In term infants, however, supplementation of γ-linolenic acid was not able to prevent the fall in AA, which suggests that it may not be sufficient simply to bypass the first δ-6 desaturation step (Makrides *et al.* 1993*a*).

It is to be expected that within the next few years all premature formulas will contain both preformed DHA and AA. In the absence of a clear understanding of the actual LCP requirements of preterm infants, it seems prudent to aim at LCP levels at least approximating to those of preterm human milk, as the most important period for brain AA and DHA accumulation is the third trimester of gestation, and as the preterm infant is exposed to a significantly reduced intake of LCP compared to what it would have obtained by placental transfer had it been born at term.

The degree to which a dietary source of preformed LCP is also essential for term infants is an area of active investigation. As in preterm infants, the levels of AA and DHA in plasma and erythrocyte phospholipids of term, formula fed infants are lower than of breast fed infants (Clark *et al.* 1992; Makrides *et al.* 1993*a*, *b*). Decreasing the linoleic acid:α-linolenic acid ratio of a term formula to about 4:1 improves the DHA status of the infants, although not to values as in breast fed infants, but it worsens their AA status (Clark *et al.* 1992). Recently it has been demonstrated that breast fed infants have higher DHA concentrations in their brain cortical phospholipids, and higher AA and DHA concentrations in subcutaneous tissue, compared to infants fed formula (Farquharson *et al.* 1992, 1993).

The observed differences in LCP levels of brain cortex and subcutaneous tissue between breast fed and formula fed infants may affect physiological function. Term, breast fed infants have been found to have a better visual function (visual evoked potential acuity) at four to five months of age than infants fed a formula devoid of LCP (Birch *et al.* 1992; Makrides *et al.* 1993*b*). Additionally, at 3 years of age, breast fed infants have been found to have a significantly better visual function (stereo acuity and letter matching ability) than infants fed a corn oil formula during the first year of life (Uauy *et al.* 1992; Birch *et al.* 1993). The scores on the tests of visual function at 3 years were correlated with the DHA status at 4 months of life. It should be noted, however, that the formula used in this study was deficient in α-linolenic acid. Supplementing infant formula with fish oil and evening primrose oil (containing γ-linolenic acid) improved the DHA status and visual acuity of term infants. AA levels of these infants were reduced below levels of infants fed the non-supplemented formula, which did not, however, negatively affect growth (Makrides *et al.* 1993*a*).

The British Nutrition Foundation (1992) recommends that infant formulas should contain AA and DHA in amounts similar to those of human milk, although the Task Force acknowledges that addition of these LCP is of most importance for preterm formulas. Farquharson *et al.* (1992, 1993) concluded from their studies that a minimum daily requirement of 0·2 g DHA/100 g fatty acids (or 30 mg DHA/d) should be supplied in formulas designed for term infants to prevent the cerebrocortical deficiency of DHA. In

their most recent opinion, expressed on 17 September 1993, the Scientific Committee for Food stated not to object to the possibility of adding them in infant formulas provided that the resulting content in n-3 and n-6 LCP is similar to that present in human milk in Europe, and that the eicosapentaenoic acid content does not exceed that of DHA.

It is as yet unknown whether the effect of dietary DHA on neural maturity is long lasting. Longer term effects of lower concentrations of DHA in cerebrocortical phospholipids on neuronal integrity and function need urgent study (Cockburn, 1994). However, the fact that mature human milk contains both AA and DHA in considerable amounts may already serve as a rationale to add AA and DHA to term infant formulas, provided the amounts do not exceed those of human milk.

STRUCTURED LIPIDS

Another topical issue with respect to infant formula fat is the development of structured triacylglycerols. The fatty acids in human milk triacylglycerols have a highly specific positional distribution (Martin *et al.* 1993). Especially the positional distribution of palmitic acid in human milk has received increasing attention. Palmitic acid constitutes about 22% of mature human milk lipids and 70–75% of it is esterified at the sn-2 position (β-position) of the triacylglycerol (Freeman *et al.* 1965; Christie, 1986; Martin *et al.* 1993). The palmitic acid in vegetable oils commonly used in infant formulas and in chicken egg, on the other hand, is predominantly esterified at the sn-1 and sn-3 positions (Freeman *et al.* 1965; Tomarelli *et al.* 1968; Christie, 1986). Pancreatic colipase dependent lipase selectively hydrolyses the fatty acids at the sn-1 and sn-3 positions, yielding free fatty acids and a 2-monoacylglycerol (Bernbäck *et al.* 1990). The 2-monoacylglycerol is a well absorbed form of most fatty acids since it readily forms micelles with bile acids and cannot form insoluble soaps with cations like calcium and magnesium. Therefore, the absorption of palmitic acid is likely to be greater when it is esterified at the sn-2 position than when it is attached predominantly at the sn-1,3 positions (Small, 1991). Indeed, mixtures of coconut oil and palm olein are better absorbed by rats if the proportions of palmitic and stearic acids in the sn-2 position are increased by chemical randomization (Lien *et al.* 1993).

Several studies have focused on the effect of the triacylglycerol configuration on intestinal fat absorption in preterm infants (Brooke 1985; Verkade *et al.* 1989). Carnielli *et al.* (1994) recently evaluated the effects of two formulas for preterm infants, differing only in the isomeric position of palmitic acid, on the absorption of fat and fatty acids and on mineral balance in a crossover study in preterm infants. The palmitic acid content of both formulas was 25–26% of total fatty acids. Although total fat absorption was not significantly different between the two groups, infants who were given the formula with palmitic acid predominantly esterified at the sn-2 position showed a significantly higher palmitic acid absorption compared with the infants that obtained the control formula with palmitic acid mainly attached to the sn-1,3 positions. This effect on palmitic acid strongly correlated with faecal calcium excretion. Absolute calcium retention of infants given the sn-2 palmitate formula was increased by more than 20 mg/kg daily.

Only one study has reported on the influence of the triacylglycerol structure on the absorption of fatty acids in term infants (Filer *et al.* 1969). In this early study, a formula based on natural lard (containing palmitic acid mainly at the sn-2 position) was compared with one based on randomized lard. The absorption of all fatty acids was improved in the infants receiving the formula containing natural lard, the effect being most pronounced for palmitic and stearic acids.

Palmitic acid is not the only fatty acid to show a specific preference for a particular position in human milk triacylglycerols; oleic acid and stearic acid are mainly located at the

sn-1 position, whereas linoleic acid is located mainly in the sn-1 and sn-3 positions. AA and DHA are found primarily esterified in the sn-2 (~ 50%) and sn-3 (~ 45%) positions of human milk (Martin *et al.* 1993).

Because human milk is the natural source of fat for the newborn, the structure of its triacylglycerols may be used as a reference point for the design of lipid sources for infant formulas. Thus, adaptation of the triacylglycerol structure of infant fat to approximate that of human milk more closely seems a logical step in the further improvement of infant formula. The triacylglycerol structure of infant formula lipids could be modified by the process of 1,3-enzymic interesterification or by chemical randomization (Lien *et al.* 1993; Quinlan & Moore, 1993).

CHOLESTEROL

The widespread occurrence of atherosclerosis in developed countries has increased the emphasis on prevention of this disorder. One question that has been the subject of numerous studies is whether or not the infant's diet influences blood cholesterol or lipoprotein concentrations later in life. It is well documented that infants given human milk have higher plasma total cholesterol and plasma LDL cholesterol concentrations and a higher LDL:HDL cholesterol ratio than formula fed infants (Jooste *et al.* 1991; Kallio *et al.* 1992; Hayes *et al.* 1992). These differences, which gradually diminish at the age of one year, are primarily attributable to the relatively high cholesterol content, 100–150 mg/l, of human milk (Clark & Hundrieser, 1989; Lammi-Keefe *et al.* 1990), rather than to its relatively high level of saturated fatty acids (Carlson *et al.* 1982; Hayes *et al.* 1992). Wong *et al.* (1993) recently observed, using the ^2H_2O method, that the 6-fold greater cholesterol intake of breast fed infants resulted in a 3-fold suppression of cholesterol synthesis compared with formula fed infants. This down-regulation of cholesterol synthesis did not prevent increases in plasma total cholesterol and LDL-cholesterol concentrations in the breast fed group.

Data from animal studies suggest that there may be long term effects of infant diet on cholesterol metabolism. Adult baboons that were breast fed during infancy have lower HDL cholesterol concentrations, higher LDL+VLDL:HDL cholesterol ratios, lower cholesterol production and bile acid excretion rates, more extensive arterial lesions and an increased bile cholesterol saturation index (which may promote gallstone formation) compared with those fed formula (Mott *et al.* 1990, 1991). Additionally, short term exposure to high dietary cholesterol in early life has been found to increase arterial sensitivity to further cholesterol insult in adult rabbits in terms of enhanced atherogenesis, despite normalization of plasma cholesterol in these animals (Subbiah *et al.* 1989).

The question of whether there are also long term effects of breast versus formula feeding on serum cholesterol concentrations in humans has not been solved. Among children of 2·5 years (Ward *et al.* 1980) and of 7–12 years of age (Hodgson *et al.* 1976), those that were breast fed had higher serum cholesterol levels than those fed formula. Other investigators studying children 1–8 years of age observed no differences in serum cholesterol concentrations related to breast *v.* formula feeding (Huttunen *et al.* 1983; Fomon *et al.* 1984; Jooste *et al.* 1991). Measurement of plasma total cholesterol alone, however, may not be sufficient to identify the effects of early postnatal cholesterol ingestion on cholesterol homeostasis later in life (Hamosh, 1988). Fall *et al.* (1992) recently presented data which showed that adult men born during 1911–30 who were either exclusively breast fed during the first year of life or exclusively bottle fed from birth had higher mortality ratios from ischaemic heart disease and higher serum total cholesterol and LDL cholesterol concentrations than men who were either both breast and bottle fed or breast fed but

weaned before one year. No information was available on the fat composition of the bottle feeds, but it is to be expected that these formulas contained a high percentage of (cholesterol-rich) milk fat.

Cholesterol is not an essential nutrient; the human fetus is able to synthesize cholesterol endogenously from the 11th week of gestation and the cholesterol needed for brain myelinization is entirely synthesized within the brain (Carr & Simpson, 1981; Edmond *et al.* 1991). However, as breast feeding is the natural method of feeding an infant, it has been postulated that the cholesterol level of human milk must be considered physiological, and it has been questioned whether infant formulas in this respect are sufficient at the moment (Kallio *et al.* 1992). The hypothesis that exogenous cholesterol may influence the biochemical composition and function of the small intestinal microvillus membrane could not be confirmed in a study in neonatal pigs (Engelhardt *et al.* 1991). Van Biervliet *et al.* (1992) reported that addition of cholesterol to formula affected maturation of the HDL particles and postulated that exogenous cholesterol may promote adequate delivery of cholesterol and AA to the developing brain. Others hypothesize that cholesterol is present in human milk merely because it is needed for the secretion of milk fat, and are not in favour of adding cholesterol to infant formulas as an increased LDL:HDL cholesterol ratio is associated with increased atherogenic risk in adults, and as expansion of the LDL pool may result in a decrease of the LCP-rich HDL pool by down-regulation of hepatic LDL receptors (Hayes *et al.* 1992).

A clear understanding of the role of cholesterol in human milk will depend on future research. Additional well controlled human studies are warranted to answer the question whether or not addition of cholesterol to infant formulas is desirable.

CARBOHYDRATES

Until now, the carbohydrate contribution of human milk has been attributed for the most part to the disaccharide lactose, generally neglecting the fact that human milk is a rich source of oligosaccharides. The latter are complex sugars composed of D-glucose, D-galactose, L-fucose, *N*-acetylglucosamine, and *N*-acetylneuraminic acid (sialic acid). They may be classed either as acidic or neutral according to the presence or absence of sialic acid (Kunz & Rudloff, 1993). Oligosaccharides are synthesized in the mammary gland by the action of several enzymes, which add specific monosaccharides to the core structure. Some of the fucosyloligosaccharides in human milk have structural similarity to blood group determinants of the mother (ABH secretor and Lewis secretor type), as a result of the activity of fucosyltransferases common to the two systems (Viverge *et al.* 1990).

More than 100 oligosaccharide structures in human milk have been characterized so far (Kunz & Rudloff, 1993). Oligosaccharides represent about 27% of total carbohydrates in colostrum, decreasing to 19% by day 30 until a value of 15–16% is reached by day 60 (Coppa *et al.* 1991, 1993). The major oligosaccharide in human milk is lacto-*N*-tetraose (Galβ1-3Glc*N*Acβ1-3Galβ1-4Glc), followed by monofucosylated lacto-*N*-fucopentaose I and II. These three carbohydrates add up to approximately 50–70% of the complex carbohydrates (Kunz & Rudloff, 1993).

Sialyllactose is the only complex oligosaccharide present in both human milk and bovine milk (Parkkinen & Finne, 1987; Neeser *et al.* 1991). Human milk sialyllactose consists primarily of the (α2-6) isomer, whereas bovine sialyllactose is mainly in the (α2-3) form (Parkkinen & Finne, 1987; Kunz & Rudloff, 1993).

The pattern of urinary oligosaccharides in breast fed infants is strongly related to that of the milk they ingest, which might be explained by intestinal absorption of intact oligosaccharides from human milk (Coppa *et al.* 1990).

It is well recognized that oligosaccharides containing *N*-acetylglucosamine stimulate the growth of *Bifidobacterium* species. Already in 1954, it was found that such oligosaccharides from human milk stimulated the growth of *Bifidobacterium bifidum* subsp. *pennsylvanicus*, which was originally isolated from the faeces of breast fed infants (Gauhe *et al.* 1954). However, this strain is exceptional in that it is unable to utilize glucose and requires D-glucosamine derivatives for cell wall synthesis (Veerkamp, 1969). *N*-acetylglucosamine appears not to promote the *in vitro* growth of *B. infantis*, *B. breve* and *B. longum* (Petschow & Talbott, 1991).

As enteropathogens use the oligosaccharide portion of glycolipids and glycoproteins as targets for attachment of whole bacteria and toxins, human milk oligosaccharides might prevent intestinal attachment of microorganisms by acting as receptor analogues competing with intestinal ligands for binding. Neutral oligosaccharides from human colostrum caused inhibition of adhesion to uroepithelial cells of a strain of *E. coli* isolated from an infant with urinary tract infection (Coppa *et al.* 1990). Additionally, human milk oligosaccharides inhibited the adherence of *Streptococcus pneumoniae* to human pharyngeal or buccal epithelial cells, the inhibitory activity being in the same concentration range as that of synthetic lacto-*N*-tetraose and lacto-*N*-neotetraose (Andersson *et al.* 1986). In a similar way, fucose containing oligosaccharides from human milk could abolish the binding activity of *Vibrio cholerae* (Holmgren *et al.* 1983). Sialyl(α2-3)lactose was found to inhibit haemagglutination of *Campylobacter pylori* and of S-fimbriae carrying strains of *E. coli* which may cause meningitis and neonatal sepsis in newborns (Korhonen *et al.* 1985; Evans *et al.* 1988), but its concentration in human milk may be too low to exert a significant inhibiting effect (Schroten *et al.* 1993).

Another possible function of human milk oligosaccharides is to provide the infant with sialic acid. Human milk contains about 1 g/l of oligosaccharide derived sialic acid during the first week of lactation, a value which decreases to about 250 mg/l at 6–8 weeks (Carlson, 1985*b*). Whey and casein predominant formulas have been reported to contain only 50–70 and 10–30 mg/l of oligosaccharide derived sialic acid respectively (Carlson, 1985*b*; Neeser *et al.* 1991). It is known that mammalian species, including man, have the capacity to synthesize sialic acid from simple sugars and phosphoenolpyruvate. However, the relative capacity for synthesis by the neonate has not been studied (Carlson, 1985*b*). There is evidence from animal studies that exogenous administration of sialic acid can significantly increase its content in synaptosomal regions of the brain, and is associated with desirable early and long term modifications of behaviour (Morgan & Winick, 1980, 1981).

More basic and clinical research is warranted to clarify outstanding questions regarding the possible functions and gastrointestinal metabolism of the various oligosaccharides. Assuming that human milk oligosaccharides indeed contribute to the wellbeing of the baby, it is to be foreseen that eventually infant formulas will be supplemented with oligosaccharides. However, it is as yet too early to decide which of the more than 100 human milk oligosaccharides would be the best candidates for supplementation and in which amounts these complex sugars should be added to infant formulas.

MINERALS AND TRACE ELEMENTS

CALCIUM AND PHOSPHORUS

Compared with cows' milk, human milk is very low in calcium and phosphorus. Values for mature milk range from 200–350 and 110–160 mg/l for calcium and phosphorus respectively (Gross *et al.* 1980; Anderson, 1992). Although calcium and phosphorus levels of human milk are significantly lower than those of current infant formulas, bone

mineralization is similar in breast and formula fed infants (Hillman *et al.* 1988; Mimouni *et al.* 1993).

The low phosphorus content of human milk is held to be advantageous to the infant for several reasons (Manz, 1992). Firstly, owing to the low phosphorus and protein content of human milk, its buffering capacity is poor; this is suggested as one of the factors responsible for the low pH and the characteristic bacterial flora of the intestine of breast fed infants (Bullen & Willis, 1971; Balmer & Wharton, 1989). Secondly, owing to the immature renal handling of phosphates in the newborn, high phosphorus intakes substantially increase serum phosphorus levels (Manz, 1992). Increased serum phosphorus and parathyroid levels, and decreased serum ionized calcium levels have been observed during the first week of age in infants receiving a formula with a high phosphorus content, regardless of its Ca:P ratio (Specker *et al.* 1991). High serum phosphorus levels can have clinical consequences. Occasionally, hypocalcaemic tetany may occur in otherwise healthy term infants receiving a high phosphorus formula (Venkataraman *et al.* 1985). Finally, a high phosphorus intake is a risk factor for the development of metabolic acidosis in high risk term infants (Kalhoff *et al.* 1990).

It is not unlikely that in the future both calcium and phosphorus levels of infant formulas will be further reduced to levels closer to human milk, provided that mineral homeostasis and bone mineralization prove to be adequate. Calcium and phosphorus could be reduced to levels of around 400 and 200 mg/l respectively without negatively affecting bone mineralization of term infants (Vainsel, 1992).

IRON

Human milk has a very low iron content of about 0·5 mg/l, yet term breast fed infants rarely exhaust their iron stores until after four months of age (Saarinen *et al.* 1977; Siimes *et al.* 1979; Calvo *et al.* 1992). This is attributed to the high absorption of iron from breast milk (Saarinen *et al.* 1977; Fomon *et al.* 1993). Iron absorption from infant formulas is significantly lower (Saarinen & Siimes, 1977; Fomon *et al.* 1993), probably at least partly owing to their higher calcium content and the presence of bovine casein and whey proteins (Hurrell *et al.* 1989; Hallberg *et al.* 1992).

The optimal iron level in infant formulas is still a major controversy. The Committee on Nutrition of the American Academy of Pediatrics (1992) recommends that iron fortified formula be used for all formula fed infants during the first year of life. These formulas should contain between 1 and 2 mg of iron per 100 kcal, or between 7 and 13 mg/l (Committee on Nutrition, American Academy of Pediatrics, 1976). Most iron fortified formulas available in the United States contain iron levels at the upper limit of this recommendation. Iron fortified formulas available in Europe generally contain lower amounts of iron, 5–8 mg/l. These lower amounts have been found to be equally effective in supporting normal iron status (Bradley *et al.* 1993; Haschke *et al.* 1993). Even a formula with an iron content as low as 3 mg/l could prevent infants from developing iron deficiency during the first 6 months of life (Haschke *et al.* 1993).

Whether iron fortification of formula is preferable right from birth is uncertain. It can be argued that the body iron content at term birth of most infants is sufficient to support haematopoiesis until about 4 months of age (Dallman, 1986; Aggett *et al.* 1989). Another argument against the initial fortification of formula with iron is that omission of iron gives a faecal flora somewhat closer to breast milk (Mevissen-Verhage *et al.* 1985; Balmer & Wharton, 1991). The impression that low iron formulas are associated with fewer gastrointestinal side effects is not supported by controlled studies (Oski, 1980; Nelson *et al.* 1988).

SELENIUM, MOLYBDENUM AND CHROMIUM

Selenium functions as an integral cofactor for glutathione peroxidase (*EC* 1.11.1.9), which catalyses the destruction of peroxides, is part of the selenoprotein Type I iodothyronine deiodinase, and is a component of several other selenoproteins of which the metabolic functions are not yet understood (Rotruck *et al.* 1973; Arthur *et al.* 1993). At the present time, proper data on the selenium requirement of the infant are lacking; selenium intakes and status of the exclusively breast fed infant should therefore serve as the basis for recommendations on infant feeding. Selenium levels of mature human milk are in the range 12–20 μg/l, whereas unfortified milk based formulas generally contain between 3 and 9 μg/l of intrinsic selenium (Roekens *et al.* 1985; Kumpulainen *et al.* 1987; Dörner *et al.* 1990). The higher selenium intake of the breast fed infant as well as the higher availability of human milk selenium are reflected in higher serum selenium concentrations and a higher selenium retention compared to formula fed infants (Kumpulainen *et al.* 1987; Dörner *et al.* 1990; McGuire *et al.* 1993).

It is to be expected that in the near future all infant formulas will be fortified with selenium to levels found in mature human milk. Supplementation of infant formulas with sodium selenite has been found to be effective in maintaining selenium status comparable to that observed in human milk fed infants (Kumpulainen *et al.* 1987; Litov *et al.* 1989; McGuire *et al.* 1993). American formula manufacturers have already started supplementing their infant formulas with sodium selenite. In Europe, addition of selenium has to await the formalization of the latest 1993 amendment to the EC Directive. Sodium selenite, selenate, selenomethionine, and selenium-enriched yeast, provided that the selenomethionine concentration of these yeasts is well standardized, will then be permitted for use in European infant formulas. The optimal form of selenium supplementation still requires further research. The potentially most promising candidate for organic selenium supplementation could be selenocysteine, the bioactive form of selenium found in glutathione peroxidase and other selenoproteins. Selenomethionine has the drawback that it is metabolized like methionine and therefore is non-specifically incorporated into a large number of proteins (Behne *et al.* 1991). Selenocysteine, on the other hand, is specifically inserted into glutathione peroxidase and other selenoproteins by a selenocysteine specific tRNA which differs from that for cysteine. Selenocysteine can therefore be seen as the 21st amino acid in terms of ribosome mediated protein synthesis (Böck *et al.* 1991).

As the chromium content of infant formulas (5–25 μg/l) is much higher than that of mature human milk (0·2–0·5 μg/l), the addition of chromium to infant formulas is not necessary (Deelstra *et al.* 1988; Foucault *et al.* 1989; Kumpulainen, 1992). The same holds true for molybdenum: levels found in infant formula and mature human milk are 15–200 and 1–3 μg/l respectively (Casey & Neville, 1987; Bougle *et al.* 1988; Foucault *et al.* 1989).

VITAMINS AND CONDITIONALLY ESSENTIAL SUBSTANCES

The vitamin levels of infant formulas are based on the values found in mature human milk, corrected for losses during processing and storage.

β-CAROTENE

Human milk, particularly colostrum, contains considerable amounts of β-carotene. The concentration of β-carotene in human milk decreases from 2·13 mg/l at day 1 to 0·4 mg/l at day 5 of lactation. Owing to the high concentrations of β-carotene in colostrum and early breast milk, the serum level of β-carotene of breast fed infants increases rapidly during this

period to normal adult levels, whereas it does not rise in formula fed infants (Ostrea *et al.* 1986).

It has recently been demonstrated in infants that β-carotene can be converted to retinal by an intestinal mucosal enzyme (Lakshman *et al.* 1993). Thus, β-carotene may serve as a source of vitamin A in the neonatal period. Additionally, β-carotene may provide the infant's defence against oxygen toxicity by quenching singlet oxygen and free radicals (Ostrea *et al.* 1986; Krinsky, 1988). Future research should better define the role of dietary β-carotene in protecting the infant against oxygen toxicity, as well as in enhancing immune function (Bendich, 1991).

INOSITOL

Inositol, a component of membrane phospholipids and of compounds involved in signal transduction, is present in mature human milk in an amount of 250–300 mg/l (Bromberger & Hallman, 1986; Pereira *et al.* 1990). Serum inositol of preterm infants correlates significantly with inositol intake, the concentration being higher in infants receiving human milk than in those receiving unsupplemented formulas, which are low in inositol (Bromberger & Hallman, 1986; Pereira *et al.* 1990). Recently, it was reported that inositol supplementation to preterm infants with respiratory distress syndrome during the first week of life was associated with increased survival, a lower incidence of bronchopulmonary dysplasia, and a lower incidence of retinopathy of prematurity (Hallman *et al.* 1992). The authors suggested that inositol may increase surfactant availability through increased synthesis, release or recycling. Varying amounts of inositol have already been added to some formulas. Future research should further evaluate the importance of dietary inositol during the neonatal period, especially among high risk preterm infants.

TAURINE

Taurine is now added to nearly all infant formulas, although the effects of taurine supplementation on cholesterol synthesis, bile acid excretion, fat and vitamin D absorption, and auditory brainstem evoked responses have been shown only in infants born preterm (Tyson *et al.* 1989; Wasserhess *et al.* 1993; Zamboni *et al.* 1993).

NUCLEOTIDES

The importance of dietary nucleotides in infant nutrition has been the subject of active research for the last decade. Human milk is known to contain a significant amount of nucleotides (Gil & Sanchez-Medina, 1982; Janas & Picciano, 1982). Nucleotide supplemented formulas have been available in Spain and Japan for a number of years, and have more recently been introduced in the United States of America.

The clinical evidence for the claimed beneficial effects of dietary nucleotides is yet far from convincing. The results of Gil *et al.* (1986*a*) who found that nucleotides enhanced the growth of bifidobacteria in the faecal flora of infants could not be confirmed in a very recent study of Balmer *et al.* (1994). In this last study, at 2 weeks of age the reverse effect was noted with more nucleotide supplemented infants colonized with *E. coli* and a reduction in the counts of bifidobacteria. Dietary nucleotides only marginally influenced the essential fatty acid status of infants (Gil *et al.* 1986*b*; DeLucchi *et al.* 1987). Effects of dietary nucleotides on the gastrointestinal system and on hepatic growth and function have only been observed in animal studies (Uauy *et al.* 1990; Bustamante *et al.* 1994; Novak *et al.* 1994). Nucleotide supplementation appeared significantly to increase all plasma lipoprotein concentrations in preterm, but not in term infants (Sanchez-Pozo *et al.* 1994). A quite fascinating finding that

warrants further study has been that dietary nucleotides increased indices of cell mediated immunity in infants without, however, influencing incidence and severity of infections (Carver *et al.* 1991). Very recently, it was reported that infants living in a contaminated environment, fed a nucleotide supplemented formula, experienced less diarrhoea than controls receiving an unsupplemented formula (Brunser *et al.* 1994). The only significant difference between the two groups, however, was the number of first episodes of diarrhoea. The total number of episodes of diarrhoea, the total duration of episodes, and the pattern of enteropathogens isolated did not differ between the supplemented infants and the controls. More clinical research needs to be done to determine efficacy, safety, and optimal level of supplementation, and to elucidate what population of infants will derive clear benefit from dietary nucleotides, before routine supplementation of infant formulas with nucleotides can be recommended (Quan & Barness, 1990).

CONCLUDING REMARKS

Through the last decades, infant formulas have been developed that closely approach human milk in nutrient composition. As knowledge has accumulated about the effects and action of different substances in human milk which serve other than nutritional roles, some of them have already been incorporated into infant formulas. Whether all nutritional and metabolic components of human milk confer unequivocal benefit to the infant in terms of growth and development is difficult to determine. For many substances, such as hormones and hormone binding proteins, vitamin binding proteins, growth factors, enzymes, and various non-protein nitrogen components, it will be extremely difficult to demonstrate clear functional advantages of supplementation, while the costs of addition of these substances will generally be substantial. Therefore, attempts to improve the composition of infant formulas should be applauded, but the benefits of compositional modifications should be carefully weighed against the costs. Unlike the rigid USA guidelines, it is to be hoped that the European guidelines for clinical testing of infant formulas, which are currently being drawn up, will provide a reasonable balance between protecting public health against opportunistic supplementations and leaving room for scientific innovations.

Obviously, no formula can supplant mother's milk as the ideal food for healthy term infants. The biological properties of human milk make it uniquely suited to the human infant. In the years to come, researchers will continue to attempt to identify and explain the role of different substances in human milk in the hope of incorporating all the benefits provided by human milk into infant formulas. It will be a major challenge for the industry to concentrate efforts on those substances which have a clear physiological function and thus are worth their price.

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Printed in Great Britain