Role and Function of Nucleotides in Infant Nutrition

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Nucleotides are low-molecular-weight biologic compounds that play a major part in almost all biologic processes. Their main roles include the following (1–3):

• Nucleic acid precursors: nucleotides make up the monomeric units of DNA and RNA.
• Energy-transfer molecules: adenosine triphosphate (ATP) is the major source of cellular chemical energy.
• Physiologic mediators: cyclic adenosine monophosphate (cAMP) acts as second messenger; cyclic guanosine monophosphate (cGMP) regulates many cellular events; adenosine is known to be a potent vasodilator; guanosine triphosphate (GTP) is involved in signal transduction, etc.
• Activated intermediates: for example, uridine diphosphoglucose (UDP glucose) is an intermediate in glycogen and glycoprotein synthesis, and other nucleotides are intermediates in the synthesis of phospholipids and serve as methyl sulfate donors.
• Structural components of coenzymes: such as nicotinamide adenine dinucleotide (NAD), flavin adenine dinucleotide (FAD), and coenzyme A (CoA), involved in many metabolic pathways.
• Allosteric effectors: controlling the regulatory steps of major metabolic pathways.
• Cellular agonists: extracellular nucleotides trigger intracellular signal-transduction cascades including the cAMP and inositol–calcium pathways.

METABOLISM OF NUCLEOTIDES

Cell nucleic acids and nucleotides are continuously synthesized, degraded, and salvaged, particularly in tissues with a rapid cellular turnover such as the gut and the immune system. Nucleotide pools are derived from three potential sources: de novo synthesis, salvage (recycling of preformed bases and interconversion into the desired compound), and the diet. The relative contributions of the salvage pathway and the diet to this pool in vivo are poorly understood.
De Novo Synthesis and Catabolism of Nucleotides

There is a close relation between amino acid metabolism and nucleotide base synthesis. Nucleotides contain either a purine or a pyrimidine base (nitrogen-containing bases). Almost all the atoms in both kinds of bases are derived directly or indirectly from amino acids. Thus the purine core of adenosine and guanine is synthesized de novo in mammalian cells from glycine, aspartate, glutamine, tetrahydrofolate derivatives, and CO$_2$. The first step is the formation of phosphoribosylpyrophosphate (PRPP) from ribose-5-phosphate and ATP. PRPP and glutamine are then involved in a condensation reaction catalyzed by PRPP aminotransferase, an enzyme that is inhibited by feedback from AMP and GMP, so that the synthesis is governed by the needs of the cell. After 10 steps, monophosphates are converted into di- and triphosphates.

The pyrimidine ring is synthesized de novo in mammalian cells from aspartate, glutamine, and CO$_2$. In this pathway, the ring is formed first, and then the sugar phosphate is added. The first reaction in pyrimidine synthesis is the formation of carbamoyl phosphate from glutamine and CO$_2$. Subsequent steps yield orotate, which reacts with PRPP (the ribose-5-phosphate donor) to render ornithine monophosphate (OMP). Uridine monophosphate (UMP) is then synthesized by OMP decarboxylation. Uridine triphosphate (UTP), cytidine triphosphate (CTP), and deoxyderivatives are obtained from UMP.

Uric acid is the end product of purine base catabolism in primates and humans, whereas other species can convert it to more soluble catabolites. The end products of pyrimidine base catabolism are β-alanine (from cytidine and uracil) and β-aminoisobutyrate (from thymine). Both are soluble, easily excreted products.

The Salvage Pathway

The endogenous supply of nucleotides is maintained through both the de novo synthesis described earlier and through a salvage pathway, a mechanism to recover the bases resulting from endogenous nucleotide and nucleic acid breakdown present in many cell types. This pathway might be enhanced when nucleotides are provided by the diet and is much less energy consuming for the cell than is the de novo process (4). Thus an exogenous source of nucleotides, such as dietary supplements, that spares the cost of the de novo synthesis may be especially important during the period of rapid growth experienced by young infants.

There are not enough data regarding the relative contribution of each of the three potential sources (de novo, salvage, and the diet) to the body’s pools, or how this contribution changes depending either on the tissue studied or on the special metabolic conditions. Two important questions have to be answered: first, whether there is a need for dietary nucleotide supplementation; and second, the extent to which the different sources of nucleotides are used under given metabolic conditions.
Contribution of Dietary Nucleotides

Dietary nucleotides contribute to the salvage pathway by providing preformed nucleosides and nitrogenous bases. Nucleotides are ingested in the form of nucleoproteins, from which nucleic acids are liberated in the intestinal tract by the action of proteases. After this, pancreatic nucleases degrade nucleic acids to a mixture of mono-, di-, tri-, and polynucleotides. Phosphoesterases supplement the action of these nucleases, producing mononucleotides that are converted into nucleosides by the action of intestinal alkaline phosphatase. Finally, nucleosidases release the sugar moiety, yielding free nitrogenous bases. The evidence suggests that a mixture of nucleosides and nitrogenous bases is offered to the enterocyte for absorption (5). Figure 1 summarizes the digestion of nucleoproteins and the absorption of nucleosides.

Absorption and Metabolism of Dietary Nucleotides

Dietary nucleotides are absorbed mainly as nucleosides by a combination of a highly efficient Na\(^+\)-dependent active transport and facilitated diffusion. It appears that nucleosides are predominately absorbed in the upper part of the small intestine. Two facts support this observation: first, the cells of this part of the small intestine have,
in general, the greatest absorptive capacity; second, several studies have suggested that these cells have a low capacity for de novo synthesis (6–8).

Experiments carried out in healthy mature animals have shown that 90% of the ingested nucleotides are absorbed. Nevertheless, there is growing evidence that in the process of absorption, dietary nucleotides are extensively metabolized in the gastrointestinal and liver tissues before their entry into the systemic circulation. In fact, experiments carried out with rats have shown that only 5% of the absorbed purines are incorporated into intestinal nucleic acids, and a relatively small amount appears in hepatic cells (7–9). Similar results were obtained by Simmonds et al. (10,11) when free guanine was added to the diet of pigs, and only minor incorporation into pig tissues was found (<1%).

Stable isotope approaches in mice, using $^{13}$C nucleotides, also have shown that purine nucleotides undergo almost complete degradation during their passage through the enterocytes (12). In this study, the contributions of dietary nucleotides to RNA synthesis in vivo were estimated by feeding pregnant mice a diet containing $[^{13}\text{C}]$-labeled nucleotides for 5 days. It was found that only 0–0.2% of tissue RNA purine nucleotides came from the diet, whereas this incorporation percentage reached 4% in the case of uridine incorporation into hepatic and mucosal RNA. These results are in agreement with in vitro studies carried out by He et al. (13) in which the uptake and transport of nucleosides were studied in intestinal cell lines (Caco-2 and IEC-6). After a 2-hour incubation with radiolabeled cytidine or uridine, a small amount of transported pyrimidine nucleoside (10–15%) was detected from both directions in the cell monolayer (apical to basolateral and basolateral to apical), whereas the purine nucleoside could not be detected. It was therefore concluded that the metabolism of radiolabeled cytidine was complete during transport. These studies show that, although dietary purines are metabolized during absorption, a modest but perhaps critically important fraction of dietary uridine does become incorporated into nucleic acid. It is tempting to speculate that this might represent a specific and functionally important fraction or subfraction of RNA.

In general, all the experiments summarized earlier are in agreement with the observation (14) that in well-nourished animals, the de novo synthesis of both purines and pyrimidines from amino acid precursors is capable of supporting the cellular needs for nucleic acid synthesis.

It has been suggested that when protein intake is reduced, salvage of nucleotides is increased. Boza et al. (15) showed that the incorporation of purine nucleotides into RNA of Caco-2 cells is rather limited, but it becomes important when cells are nutritionally stressed by glutamine deprivation. Animal studies also support this hypothesis. In a recent study, mice infected with Staphylococcus aureus and fed a protein-free diet showed a higher RNA content in small intestinal cells when a mixture of nucleosides was administered intraperitoneally (16). Based on these observations as well as many others discussed later, it has been proposed that nucleotides behave as "semiessential" or "conditionally" essential nutrients (17,18). These nutrients may became essential when the endogenous supply is insufficient for normal function, even though their absence from the diet does not lead to a classic clinical deficiency.
syndrome. Therefore, not only when protein intake is decreased but also in situations in which there is an increased demand for nucleotide synthesis (after gut injury, sepsis, or surgical trauma, or during rapid growth such as in pregnancy and the neonatal period), some tissues with a rapid turnover, such as the gut and the immune system, may increase the salvage of exogenous nucleotides by reducing their catabolism. This hypothesis is supported by several studies suggesting that nucleotide supplementation of diets may be of clinical significance.

BIOLOGIC EFFECTS OF DIETARY NUCLEOTIDES

Gastrointestinal Effects

Intestinal Growth and Development

The involvement of dietary nucleotides in intestinal growth and maturation has been extensively documented (19-21). Thus Uauy et al. (19) found that the administration of nucleosides (0.8% wt/wt of diet) to weanling rats over a 2-week period increased the DNA content, villous height, and maltase activity when compared those in with rats fed a nucleotide-free diet. Carver (20) also reported an increase in small bowel weight (as a percentage of body weight) as well as of weight/length ratio in weanling mice fed a 0.21% (wt/wt) nucleotide-supplemented diet; however, disaccharidase activities were not affected. Other studies carried out in adult rats have shown that deprivation of dietary nucleotides diminishes the activity of enzymatic markers of villous tip cell differentiation, whereas nucleotide supplementation increases brush-border enzymatic activities in the same cells. No significant changes were observed in the crypts (21).

Recovery after Injury

The effects described are much more pronounced in the case of animals recovering after gut injury induced by exposure to sublethal radiation doses (22) or after lactose-induced chronic diarrhea (23). In the former study, nucleotide supplementation of the rat diet (0.8% wt/wt) over a 10-day period was correlated with a decrease in animal mortality and intestinal inflammation, and with higher maltase- and sucrase-specific activities in the jejunum and ileum. However, no differences were shown for mucosal protein or DNA content, villous height, and so on. In the latter study, rats fed a nucleotide-supplemented diet (0.25% wt/wt) over a 4-week period showed higher levels of intestinal maltase and sucrase activity compared with rats fed a nonsupplemented diet. A greater villous height-to-crypt depth ratio and reduced numbers of intraepithelial lymphocytes also were observed in the rats fed the nucleotide-supplemented diet.

Starvation and refeeding with nucleotide-free diets also have been shown to alter the intestinal mucosa. Thus adult rats subjected to starvation for 5 days and later given a nucleotide-free diet showed slower normalization in the jejunum and incomplete normalization of differentiation markers in the ileum (24). Nucleotide
supplementation of the diet caused increased mucosal weight in the jejunum and ileum, with an increase in protein and DNA content and brush-border enzyme activity. However, this nucleotide supplementation did not lead to any striking changes in nonstarved control rats. It is well known that removing intraluminal nutrient supply and giving parenteral nutrition retards cell turnover and induces atrophy and dysfunction, but Tsujinaka et al. (25) recently showed that supplementation of total parenteral nutrition (TPN) solutions with nucleotides improved mucosal permeability in rats. All these findings imply that dietary nucleotides may be important for rapid cell replication at a time of increased need.

There is evidence that dietary nucleotides also may have an effect on healing in the human intestinal mucosa. In a clinical trial on small-for-gestational age neonates whose intestinal mucosa was shown to be functionally impaired by intrauterine malnutrition, babies fed a formula supplemented with nucleotides gained more weight and length and had a greater head circumference than did those fed a nucleotide-free formula (26). Dietary nucleotide supplementation also promoted healing of small bowel ulcers in experimental ileitis in rats after the intraperitoneal administration of indomethacin (27). However, these same investigators have recently shown that dietary nucleotides aggravate dextran sulfate sodium-induced distal colitis in rats by enhancing the concentration of proinflammatory cytokines and myeloperoxidase-specific activity in the colon (28).

It has been shown that nucleotides are involved in small intestinal maturation during TPN and prevent TPN-associated intestinal atrophy (29,30). The addition of a source of purines and pyrimidines to a standard TPN regimen in rats stimulates the proliferative activity of crypt cells, as assessed by mucosal wet weight, DNA and RNA content, villous height, and maltase- and sucrase-specific activities.

Gut Microflora

It is well known that the intestinal microflora of breast-fed infants is different from that of infants fed cow’s milk formula. Bifidobacteria usually predominate in the gut of breast-fed infants, whereas gram-negative bacteria are the predominant microorganisms in infants fed cow’s milk formula.

Gil et al. (31) carried out a study to determine the effects of nucleotide supplementation of an adapted formula on the microbial pattern of the feces in newborn term infants. No differences in bacterial counts could be demonstrated after 1 or 4 weeks. Only when the values were expressed as percentages of total fecal bacterial counts could slight differences be observed between supplemented and unsupplemented formulas, with a higher percentage of bifidobacteria and a lower percentage of enterobacteria in the feces of infants fed the supplemented formula. However, the percentages remained very different in both cases from those in breast-fed infants. This lack of influence of nucleotide supplementation on the fecal flora was confirmed in a similar but larger study with a higher level of nucleotide supplementation (32). No advantage of nucleotide supplementation could be found at ages 2, 4, or 7 weeks.
Hepatic Effects

The liver has a large capacity for nucleotide synthesis; it is believed to be the supplier of nucleosides for other tissues. Nevertheless, dietary nucleotide deprivation impairs liver structure and function. Thus Novak et al. (33) reported that weanling mice fed a nucleotide-free diet had decreased liver weight and glycogen content, and increased hepatic cholesterol, liver phosphorus, and serum bilirubin, when compared with animals fed a nucleotide-supplemented diet (0.21% wt/wt). López-Navarro et al. (34) showed that deprivation of dietary nucleotides changes the morphology of the hepatocyte, and demonstrated both nuclear and cytosolic alterations. This same group found a decrease in protein synthesis in the liver as well as in the small intestine in adult rats fed a nucleotide-free diet for 7 days (35).

The role of nucleotides on liver regeneration after injury has been assessed by using hepatectomy or different models of chemically induced cirrhosis. Ogoshi et al. (36,37) reported that a parenterally administered nucleotide/nucleoside mixture (10% of amino acid nitrogen) improved hepatic function and promoted an earlier restoration of nitrogen balance after liver injury or 70% hepatectomy in rats. Gil’s group in Granada (38,39) carried out two studies in rats made cirrhotic by thioacetamide administration. These investigators concluded that dietary nucleotide supplementation is decisive in ensuring hepatocyte recovery after liver damage, by attenuating the inflammatory reaction, interfering with collagen metabolism (antifibrotic properties), and correcting plasma and liver microsomal fatty acid profile. Therefore it would appear that the repair and growth of the liver, a major organ of purine and pyrimidine biosynthesis, can be improved by providing an external supply of preformed nucleotides and nucleosides.

Effects on Immunity

Like the intestine, the immune system is a rapidly dividing tissue. The role of nucleotides in immune tissue has recently become evident. Although the mechanism is not known, dietary nucleotides may contribute to the pool of nucleotides available to proliferating lymphocytes, which turn over rapidly and thus have increased nucleotide requirements. The levels of various enzymes involved in nucleotide metabolism vary with different stages of lymphocyte activation or function, and salvage of exogenous purines and pyrimidines is increased in activated lymphocytes, in which de novo purine and pyrimidine biosynthesis also is dramatically increased (40,41). Increased intracellular nucleotide pools and the expression of large numbers of transmembrane nucleoside transporters are consequences of inducing lymphocyte proliferation (1).

Cellular Immunity

Many effects of dietary nucleotides on cellular immunity have been described. The main ones are as follows:

• Lymphocytes from animals fed a nucleotide-free formula have a significantly decreased proliferative response to mitogens (42).
• Nucleotide-free diets lead to a decrease in interleukin-2 (IL-2) production in response to mitogens in mice. The addition of either uracil or RNA to the diet corrects this. The effect is much more marked in animals recovering from protein-energy malnutrition (43,44).
• Dietary nucleotides influence the delayed-type cutaneous hypersensitivity response by maintaining the T-helper cell population in the murine spleen (45,46).
• Nucleotide-free diets produce a decrease in the phagocytic capacity of murine macrophages (47).

In most of these studies, the addition of RNA or uracil restores immune function, and this may be related to the suggested limited capacity of rapidly proliferating lymphocytes to salvage pyrimidines (48).

• Mice fed diets containing nucleotides have a greater resistance to challenge with S. aureus and Candida albicans (49). In contrast, oral RNA or intraperitoneally administered individual nucleosides have no effect against methicillin-resistant S. aureus infection in mice (50).
• The addition of nucleoside–nucleotide mixtures improves gut morphology and reduces the incidence of bacterial translocation in protein-deficient mice (51).
• Dietary nucleotides partially counteract the immunosuppressive effects of dexamethasone in Cryptosporidium parvum–challenged mice (52).
• Dietary nucleotides and nucleosides aggravate colonic damage and inflammation in chemically induced experimental colitis in rats (53).

Humoral Immunity
The effects of nucleotides on humoral immunity are as follows:
• Mice fed a nucleotide-free diet show a depressed humoral response to T cell–dependent antigens, an effect that is reversed by the addition of a nucleotide–nucleoside mixture to the diet (54).
• RNA supplementation of cell-culture media leads to (a) increased antibody production to T cell–dependent antigens in murine spleen cells (55), and (b) increased immunoglobulin G (IgG) and IgM levels in response to T cell–dependent stimuli in mononuclear cells from human peripheral blood (56).

Human Infant Studies
As discussed earlier, there is evidence from both animal studies and in vitro studies that the inclusion of either nucleic acids or nucleotides in the diet can be beneficial for immune and gastrointestinal function under conditions in which there is an increased demand for nucleotide synthesis. In light of this, several studies have been carried out on the impact of dietary nucleotides in both preterm and term newborn infants and in children with malnutrition or diarrhea.

The capacity of mononuclear cells to exert natural killer (NK) cell activity (cytotoxic lymphocytes) and to produce IL-2 was investigated by Carver et al. (57). Term
infants were either exclusively breast-fed \((n = 9)\), fed a nucleotide enriched formula (SMA\(^+\), \(n = 13\)), or fed the same formula without added nucleotides (SMA\(^-\), \(n = 15\)), for 4 months. The nucleotide supplementation levels per liter in SMA\(^+\) were 12 \(\mu\)mol AMP, 6 \(\mu\)mol GMP, 6 \(\mu\)mol inosine monophosphate (IMP), 62 \(\mu\)mol cytidine monophosphate (CMP), and 15 \(\mu\)mol UMP. During the study, mothers recorded the duration of infections as well as the highest temperature reached during any illness. Both the incidence and the severity of infections were very low, with no differences between dietary groups. Red blood cell and hematocrit values did not differ between the groups. NK cell activity was greater in breast-fed infants and infants fed the nucleotide-supplemented formula than in infants fed the unsupplemented formula at age 2 months, whereas at 4 months, no difference was found. IL-2 production at age 2 months was greater with the nucleotide-supplemented formula, the values being similar to those of the breast-fed group. At 4 months, no differences in IL-2 production were found between the formula groups.

Another way to study the response of the immune system to dietary nucleotides is to assess the immune response to vaccination. Pickering et al. (58) carried out a study on newborn infants receiving either a conventional formula \((n = 107)\) or the same formula supplemented with nucleotides (Similac Advance, \(n = 101\)) for 12 months. A third group of infants \((n = 103)\) was exclusively breast-fed for 2 months, and then fed with human milk or the conventional formula until age 12 months. The immunization schedule recommended by the American Academy of Pediatrics was followed: *Haemophilus influenzae* type B (HIB), diphtheria–pertussis–tetanus (DPT), and oral poliovirus vaccine (OPV) were given at age 2 and 4 months, followed by DPT and HIB at 6 months. The vaccine response, assessed by HIB and diphtheria antibody titers, was greater at age 7 months in children receiving the nucleotide-supplemented formula than in those receiving the unsupplemented formula. The higher HIB antibody titer persisted in the nucleotide-supplemented group at 12 months. The nucleotide-supplemented group had higher HIB antibody concentrations throughout the study than did children fed human milk for <6 months, but the values were not different from those fed human milk for >6 months. The enhanced responses to diphtheria and HIB are consistent with an effect on T cell–dependent responses. However, the mechanisms whereby dietary nucleotides enhance the antibody responses to HIB or diphtheria remain unknown.

Martinez-Augustin et al. (59) evaluated the influence of dietary nucleotide supplementation on the intestinal permeability to lactulose/mannitol and to \(\beta\)-lactoglobulin and \(\alpha\)-casein in preterm infants during the first month of life. Of the 27 preterm infants enrolled in the study, 11 were fed a standard low-birthweight milk formula, and 16 were fed the same formula supplemented with nucleotides at levels similar to those found in human milk. Blood and urine samples were obtained at ages 1, 7, and 30 days. Neither the intestinal permeability to saccharides nor the intestinal absorption of \(\beta\)-lactoglobulin or \(\alpha\)-casein was affected by the addition of nucleotides to the formula. However, serum concentrations of IgG to \(\beta\)-lactoglobulin at age 30 days were higher in preterm neonates fed the supplemented formula than in those fed the control formula. The authors concluded that nucleotide
supplementation might be beneficial in achieving optimal maturation of the immune system.

Another study (60) evaluated the influence of dietary nucleotide supplementation on total immunoglobulin levels in preterm infants, following a similar protocol to that described earlier. Twelve infants received a standard low-birthweight milk formula, and 12 received the same formula supplemented with nucleotides at levels similar to those found in human milk. Serum samples were obtained at ages 10 and 20–30 days and 3 months. Cord blood also was obtained. Cord blood levels of IgG were lower than described in normal term neonates, corresponding to the low transfer time of maternal IgG across the placenta. During the first 3 months of postnatal life, a significant decrease in IgG was observed in both groups, but no differences were detected at any time between the two formula groups. Serum IgM concentrations showed a completely different pattern: low levels of this immunoglobulin were detected in cord blood, whereas a progressive increase in IgM concentration with age was observed. At ages 20–30 days, as well as at 3 months, higher IgM concentrations were detected in the serum of infants fed the nucleotide-supplemented formula than in those fed on the conventional formula. IgA was reported in the serum only after 20–30 days because of the low concentrations before this time. The levels were increased at 3 months, especially in infants fed the supplemented formula.

Recently Martínez-Augustin et al. (60) evaluated the influence of dietary supplementation on the recovery of infected and malnourished children with diarrhea. Twenty-two children younger than 3 years (mean age, 17.1 months) admitted to the Hospital Albina R. de Patino (Cochabamba, Bolivia) because of persistent diarrhea (for >15 days) were included in the study. Children were randomly assigned to a control group (n = 11), which received a formula without nucleotides added (Nieda), or to an experimental group (n = 11), which received the same formula supplemented with nucleotides. Blood samples were obtained at the beginning of the study and 3 weeks after recovery. Total IgG, IgA, and IgM and specific IgG against β-lactoglobulin and α-casein were determined. Refeeding after malnutrition did not produce any significant changes in either specific or total IgG concentrations. Serum IgA decreased significantly during the refeeding period in infants fed the nucleotide-supplemented formula. In contrast, serum IgM increased during this period, and significant differences were found in the group fed the standard formula. However, no differences were related to the nucleotide supplementation.

These results are consistent with a previous study that showed that healthy infants consuming a nucleotide-fortified formula (n = 194) for 3 months experienced fewer first episodes of diarrhea than did those fed a control formula (n = 190) (61). However, the investigators found that neither the characteristics of the episodes (hospital admission rate, total number of episodes, total number of days with diarrhea, duration of episodes) nor the pattern of enteropathogens isolated was affected.

NUCLEOTIDES IN HUMAN MILK

Nucleic acids in human milk are likely to originate from intact or lysed cells contained in the milk. Indeed Colostrum, which contains many more cells than does
mature milk, has a much higher nucleic acid concentration. Human milk contains significant amounts of preformed nucleotides. At least 13 different acid-soluble nucleotides have been found, of which pyrimidine nucleotides represent the most abundant fraction (62,63). The nucleotide composition of human milk differs appreciably from that of other species. For example, cow's milk–based infant formulas contain significant quantities of orotic acid, whereas this nucleotide cannot be found in human milk. It has been shown that high levels of dietary orotic acid cause fatty liver; however, this effect is unique to the rat (64,65).

As much as 25% of the total nitrogen in human milk is nonprotein nitrogen (66). This fraction of human milk has been partially characterized and includes compounds such as urea, free amino acids, nucleic acids, nucleotides, peptides, amino sugars, creatinine, creatine, amino alcohols, uric acid, ammonia, carnitine, and polyamines. Several of these nitrogenous compounds have been recognized as modulators of important neonatal physiologic functions and play specific roles in neonatal development (67,68).

Nucleotides are reported to account for 0.5–5% of human milk nonprotein nitrogen, ranging from 4 to >70 mg/l. Most of the nucleotides contained in human milk are monophosphates (AMP, GMP, IMP, CMP, and UMP) and diphosphates [adenosine diphosphate (ADP), guanosine diphosphate (GDP), inosine diphosphate (IDP), cytidine diphosphate (CDP), and uridine diphosphate (UDP)]. However, the nucleotide composition of human milk is very complex. In addition to mono- and dinucleotides, combinations of nucleotides with other components such as hexoses, N-acetyl-exosamines, and cyclic nucleotides are encountered.

Table 1 shows the mean nucleotide content of human milk at 2, 4, 8, and 12 weeks of lactation, as reported by Janas and Picciano (63). Five lactating women were followed up longitudinally. The cytidine and uridine nucleotides measured in this study comprised the first and second largest fractions, respectively, of the total nucleotide content. Progression of lactation is accompanied by a decrease in the levels of CMP and AMP, whereas levels of IMP increase (Table 1).

**TABLE 1. Nucleotide content of human milk**

<table>
<thead>
<tr>
<th>Nucleotide</th>
<th>2 wk</th>
<th>4 wk</th>
<th>8 wk</th>
<th>12 wk</th>
<th>Mean</th>
</tr>
</thead>
<tbody>
<tr>
<td>CMP</td>
<td>18.5</td>
<td>16.5</td>
<td>13.0</td>
<td>10.0</td>
<td>14.4</td>
</tr>
<tr>
<td>UMP</td>
<td>5.5</td>
<td>8.0</td>
<td>4.3</td>
<td>4.4</td>
<td>5.5</td>
</tr>
<tr>
<td>AMP</td>
<td>7.1</td>
<td>5.0</td>
<td>4.5</td>
<td>4.2</td>
<td>5.1</td>
</tr>
<tr>
<td>IMP</td>
<td>4.6</td>
<td>6.4</td>
<td>6.6</td>
<td>8.4</td>
<td>6.6</td>
</tr>
<tr>
<td>GMP</td>
<td>3.2</td>
<td>4.2</td>
<td>3.4</td>
<td>4.6</td>
<td>3.9</td>
</tr>
<tr>
<td>UDP</td>
<td>3.9</td>
<td>4.5</td>
<td>3.8</td>
<td>5.0</td>
<td>4.3</td>
</tr>
<tr>
<td>CDP</td>
<td>9.7</td>
<td>10.6</td>
<td>15.1</td>
<td>11.4</td>
<td>11.8</td>
</tr>
<tr>
<td>ADP</td>
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<td>1.9</td>
<td>1.0</td>
<td>1.0</td>
<td>1.6</td>
</tr>
<tr>
<td>GDP</td>
<td>2.2</td>
<td>2.1</td>
<td>2.2</td>
<td>2.2</td>
<td>2.2</td>
</tr>
</tbody>
</table>

Values expressed as µmol/l.
ADP, adenosine diphosphate; AMP, adenosine monophosphate; CDP, cytidine diphosphate; CMP, cytidine monophosphate; GDP, guanosine diphosphate; GMP, guanosine monophosphate; IMP, inosine monophosphate; UDP, uridine diphosphate; UMP, uridine monophosphate.
From Janas and Picciano (63).
The nucleotide content of human milk was compared with that of colostrum in a study by Gil et al. (62). Pooled colostrum and mature milk from four women was analyzed. Human milk at 3 months of lactation contained ~63 µmol/l of nucleotides—that is, ~50% of the amount of nucleotides present in human colostrum. The results presented in Table 2 show a decrease of nucleotide concentration with advancing lactation (Table 2).

Leach et al. (69) recently introduced the concept of total potentially available nucleotides (TPAN). In their opinion, nucleotide quantification of human milk should include measurement of polymeric nucleotides (nucleic acids), nucleosides, and nucleotides containing adducts. They used three different types of enzymatic hydrolysis, involving nuclease P1 (to convert polymeric nucleotides into mononucleotides), nucleotide pyrophosphatase (to release nucleotides from adducts), and alkaline phosphatase (to release the nucleosides). This complete enzymatic digestion is a reasonably accurate reflection of the \textit{in vivo} process and allows the measurement of the entire nucleotide fraction of human milk. The authors analyzed >100 individual samples from four different European sites at different stages of lactation. Table 3

### TABLE 2. Nucleotide contents of human colostrum and mature milk

<table>
<thead>
<tr>
<th>Nucleotide</th>
<th>Stage of lactation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2 d</td>
</tr>
<tr>
<td>CMP</td>
<td>55.1</td>
</tr>
<tr>
<td>UMP</td>
<td>17.7</td>
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<td>AMP</td>
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<tr>
<td>GMP</td>
<td>3.3</td>
</tr>
<tr>
<td>GDP-mannose</td>
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</tr>
<tr>
<td>UDP-Achexosamines</td>
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</tr>
<tr>
<td>UDP-hexoses</td>
<td>2.8</td>
</tr>
<tr>
<td>UDP</td>
<td>2.2</td>
</tr>
</tbody>
</table>

Values expressed as µmol/l.

AMP, adenosine monophosphate; CMP, cytidine monophosphate; GDP, guanosine diphosphate; GMP, guanosine monophosphate; UDP, uridine diphosphate; UMP, uridine monophosphate.

From Gil and Sanchez de Medina (62).

### TABLE 3. Potentially available nucleosides and total potential available nucleotides in pooled human milk by stage of lactation

<table>
<thead>
<tr>
<th>Nucleoside</th>
<th>2 d</th>
<th>3–10 d</th>
<th>1 mo</th>
<th>3 mo</th>
<th>Mean (range)</th>
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<tr>
<td>Cytidine</td>
<td>71</td>
<td>86</td>
<td>102</td>
<td>96</td>
<td>88 (33–146)</td>
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<tr>
<td>Uridine</td>
<td>26</td>
<td>32</td>
<td>48</td>
<td>47</td>
<td>38 (21–67)</td>
</tr>
<tr>
<td>Guanosine</td>
<td>21</td>
<td>30</td>
<td>45</td>
<td>28</td>
<td>31 (19–92)</td>
</tr>
<tr>
<td>Adenosine</td>
<td>21</td>
<td>29</td>
<td>46</td>
<td>31</td>
<td>32 (13–97)</td>
</tr>
<tr>
<td>TPANs</td>
<td>137</td>
<td>177</td>
<td>240</td>
<td>202</td>
<td>189 (82–402)</td>
</tr>
</tbody>
</table>

Values expressed as µmol/l.

TPANs, total potential available nucleotides.

From Leach et al. (69).
shows the mean values for each nitrogenous base at different stages of lactation, expressed as nucleoside equivalents as well as TPAN. The mean TPAN of pooled samples was lowest in colostrum, but showed no consistent upward or downward trend in transitional milk, early mature milk, or late mature milk. Using the process described earlier, Leach et al. (69) were able to determine the percentage of nucleotides found in human milk as poly- or monomeric nucleotides, nucleosides, or adducts. The amount of each nucleoside found in each form and the amounts of each form contributing to the TPAN are shown in Tables 3 and 4.

The nucleotides in these samples were present in predominantly polymeric and monomeric forms. Consistently, only low concentrations of nucleoside and adducts were present, uridine being the predominant nitrogenous base in this nucleotide fraction. Using the percentages and values of TPAN in human milk (Table 3), it is possible to calculate the free nucleotide concentrations in this study. These are (per liter) 33 xmol cytidine nucleotide, 14 xmol uridine nucleotide, 10 xmol guanosine nucleotide, and 11 xmol adenosine nucleotide, for an average total of 68 xmol nucleotides/l. This calculation includes the contribution of mono-, di-, and triphosphonucleotides. Janas and Picciano (63) measured mono- and diphosphonucleotide concentrations and reported mean values (per liter) of 26.2 xmol cytidine nucleotide, 9.8 xmol uridine nucleotide, 6.1 xmol guanosine nucleotide, and 6.7 xmol adenosine nucleotide, for an average total of 55.2 xmol nucleotides/l (including inosine monophosphate). The study by Janas and Picciano is the only one reporting consistent data on IMP concentrations. Leach et al. (69) suggested that the reason for this may be the action of adenosine deaminase (present in human milk), which converts adenosine to inosine. If this enzyme acts in vitro, the absence of inosine could constitute a sample preparation artifact.

The most recent characterization of the nucleotide content of human milk is that carried out by Thorell et al. (70), who analyzed the concentration of nucleic acids and ribonucleotide metabolites in the milk of 14 mothers at 3–24 weeks of lactation. Expressed as nucleotide equivalents, 68 ± 55 xmol/l was present as nucleic acid, 84 ± 25 xmol/l as nucleotides, and 10 ± 2 xmol/l as nucleosides. As previously described, the nucleotide/nucleoside profile showed a substantial predominance of pyrimidines, and uric acid also was found in high concentrations. Enzymes capable of degrading nucleotides in the milk were found in this study, suggesting that the

<table>
<thead>
<tr>
<th>Nucleoside</th>
<th>Cytidine</th>
<th>Uridine</th>
<th>Guanosine</th>
<th>Adenosine</th>
<th>TPANs</th>
</tr>
</thead>
<tbody>
<tr>
<td>Polymeric NTs</td>
<td>57 ± 12</td>
<td>19 ± 7</td>
<td>59 ± 21</td>
<td>47 ± 11</td>
<td>48 ± 8</td>
</tr>
<tr>
<td>Monomeric NTs</td>
<td>37 ± 13</td>
<td>36 ± 12</td>
<td>34 ± 14</td>
<td>35 ± 10</td>
<td>36 ± 10</td>
</tr>
<tr>
<td>Nucleosides</td>
<td>5 ± 5</td>
<td>18 ± 14</td>
<td>1 ± 2</td>
<td>5 ± 4</td>
<td>8 ± 6</td>
</tr>
<tr>
<td>Adducts</td>
<td>1 ± 1</td>
<td>27 ± 12</td>
<td>7 ± 15</td>
<td>13 ± 9</td>
<td>9 ± 4</td>
</tr>
</tbody>
</table>

NTs, nucleotides; TPANs, total potential available nucleotides.
From Leach et al. (69).
TABLE 5. Nucleotide content of human milk (μmol/l) (mean ± SEM) changes in nucleotide levels after incubation of human milk

<table>
<thead>
<tr>
<th>Compound</th>
<th>Fresh milk (n = 14)</th>
<th>24 h/23°C (n = 3)</th>
<th>4 h/37°C (n = 1)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nucleic acid*</td>
<td>68 ± 55</td>
<td>NM</td>
<td>NM</td>
</tr>
<tr>
<td>CMP</td>
<td>66 ± 19</td>
<td>-9.7</td>
<td>-4.8</td>
</tr>
<tr>
<td>UMP</td>
<td>11 ± 5.3</td>
<td>-7.0</td>
<td>-5.1</td>
</tr>
<tr>
<td>GMP</td>
<td>1.5 ± 1.6</td>
<td>-3.3</td>
<td>—</td>
</tr>
<tr>
<td>AMP</td>
<td>5.7 ± 4.9</td>
<td>-8.3</td>
<td>-1.7</td>
</tr>
<tr>
<td>Cytidine</td>
<td>5.4 ± 1.6</td>
<td>+6.0</td>
<td>+11</td>
</tr>
<tr>
<td>Uridine</td>
<td>4.9 ± 1.3</td>
<td>+12.5</td>
<td>+15</td>
</tr>
<tr>
<td>Inosine</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Guanosine</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Adenosine</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Guanine</td>
<td>0.8 ± 1.3</td>
<td>+2.6</td>
<td>—</td>
</tr>
<tr>
<td>Hypoxanthine</td>
<td>—</td>
<td>—</td>
<td>-0.8</td>
</tr>
<tr>
<td>Xanthine</td>
<td>—</td>
<td>—</td>
<td>+3.8</td>
</tr>
<tr>
<td>Uric acid</td>
<td>69 ± 12</td>
<td>+11.4</td>
<td>+6.5</td>
</tr>
</tbody>
</table>

Values expressed as μmol/l, mean ± SEM.

* Nucleic acids expressed as nucleotide equivalents.

AMP, adenosine monophosphate; CMP, cytidine monophosphate; GMP, guanosine monophosphate; NM, not measured; UMP, uridine monophosphate.

nucleotide profile could have been affected by catalysis during storage of the milk in the breast. Incubation of fresh human milk for 24 hours at 23°C caused partial transformation of CMP and UMP to cytidine and uridine, respectively. GMP and AMP were partly transformed to guanine and uric acid. This indicates that human milk contains the complete set of enzymes necessary to convert purine nucleotides to uric acid. However, pyrimidine nucleotides remained as nucleotides or nucleosides (Table 5).

Thorell et al. (70) also incubated fresh human milk (for 4 hours at 37°C) with a fetal small intestine homogenate to simulate the physiologic digestion of nucleotides. Again the metabolic products of this digestion were mainly cytidine, uridine, and uric acid (Table 5). In conclusion, pyrimidine nucleotides are partially degraded during storage in the breast and digestion in the small intestine. The products of these processes are cytidine and uridine (pyrimidine nucleosides), which are readily absorbed in the small intestine. Conversely, purine nucleotides also are partially degraded during storage in the breast and digestion in the small intestine, so they are found mainly as uric acid, the final metabolic end product of purine catabolism, which is excreted and cannot be used further. Thus the bioavailability of pyrimidine nucleotides seems to be substantially greater than that of purine nucleotides. These results are in agreement with those obtained in vivo by Boza et al. (12,71), who quantified the incorporation of exogenous nucleotides into murine tissue RNA when nucleotides were included in the diet. As we pointed out earlier, they observed that 4% of tissue RNA pyrimidine nucleotides came from dietary sources, whereas exogenous purine nucleotides could account for only 0–0.2% of the total pool of purine nucleotide tissue RNA.
CONCLUSIONS

In summary, pyrimidine nucleotides are (a) present in disproportionately high quantity in human milk, (b) better preserved during storage in the breast and digestion in the small intestine than are purine nucleotides, and (c) better absorbed and incorporated into tissue RNA than are purine nucleotides. These facts are consistent with the work of Pizzini et al. (44), who showed that various beneficial effects of dietary nucleic acids may be reproduced by uracil alone.

A dietary source of nucleotides may be particularly important for infants whose tissue needs are increased (preterm neonates, newborn infants with malnutrition or chronic diarrhea, and so on), but what should be added and how much? Two facts must be considered: first, the nucleotide content of infant formulas derived from bovine milk is considerably lower than and different in composition from that of human milk (72); and second, infant formulas are generally developed and manufactured to be as similar to human milk as possible. Therefore, and particularly in the United States, some companies have started to add nucleotides to their formulas to reach the levels present in human milk, a practice that also was initiated in Japan in 1965 and in some European countries (for example, Spain, in 1983) (72). So far, no deleterious effects have been reported. The European Commission’s Scientific Committee for Food published guidelines in 1991 and 1996 on nucleotide supplementation of infant formulas (73,74). These allow the use of the following nucleotides and their sodium salts: CMP, UMP, AMP, GMP, and IMP, at maximal concentrations of 2.5, 1.75, 1.5, 0.5, and 1.0 mg/100 kcal, respectively, for a total maximum of 5 mg/100 kcal. The committee also stated that the total nucleotide concentration should be of the same order of magnitude as the free nucleotides in human milk.

REFERENCES


DISCUSSION

Dr. Moro: Do you have an idea of the effects of temperature on the nucleotides present in human milk? I mean pasteurization or freezing of the milk.

Dr. Boza: I do not know about human milk, but I can tell you what happens to nucleotides added to cow's milk formula submitted to UHT treatment. They are partially converted into nucleosides, but they are still absorbable, and this is actually the main form in which nucleotides are absorbed. They do not appear to be converted into uric acid or other nonabsorbable forms.

Dr. Hernell: You showed that there is an enhanced antibody response to β-lactoglobulin. Do you interpret this as being an indication of a more mature immune system? If so, could that not actually be a risk factor for intolerance?

Dr. Boza: I agree entirely with you. There could be a risk to oral tolerance of this kind of protein. Unfortunately there are no further studies on this subject, and the investigators did not follow up the infants, so we do not know whether or not they developed increased allergy to cow's milk proteins.

Dr. Hernell: You also mentioned that the formula had a nucleotide composition similar to that of human milk. One of the points we made in our article (1) is the uncertainty of human milk composition—it depends on how you handle the milk after it has been expressed from the breast up to the time of analysis, because of inevitable degradation.

Dr. Boza: Again I agree entirely with you. The method of collecting the samples and their subsequent storage is very important in determining the actual mononucleotide content of human milk. You have shown that degradation occurs because of the presence of enzymes in human milk that can degrade all the way up to uric acid, especially purine nucleotides, so the most important thing is to freeze the milk rapidly after collection.

Dr. Räihä: Many of the effects you showed in the articles you referred to may be especially beneficial in preterm infants. Do you know whether there are more nucleotides in preterm milk than in mature milk?
**Dr. Boza:** I do not know. I have no data on the content of nucleotides in preterm milk. I agree with you that the benefits of dietary nucleotides are more likely to affect preterm infants than, say, 1-year-old children.

**Dr. Read:** Could you comment a little more on the studies relating to the gut. Do you see a proliferation of normal gut as opposed to damaged gut, and if so, is the growth balanced or is crypt proliferation increased selectively, which might indicate some danger of cancer?

**Dr. Boza:** The results we obtained were much more pronounced when there was gut injury—a situation in which there is an increased rate of cell proliferation. We found in healthy growing rats that there was an increase in cell proliferation at the level of the crypts.

**Dr. Endres:** A while ago at an ESPGAN meeting, the Pickering group presented data similar to yours, showing increased vaccination titers after ingesting formula that had been supplemented with nucleotides. In the discussion it was stated that the soy formula they used had a natural content of nucleotides. Is it true that soy proteins contain nucleotides?

**Dr. Boza:** The formula that Pickering was using in that study contained added nucleotides. I do not know the nucleotide content of soy protein in the raw state.

**Dr. Bachmann:** We usually associate immunologic effects more with purines than with pyrimidines. We think of adenosine deaminase deficiency or purine nucleoside phosphorylase deficiency, but these are to do with the purine side, whereas you seem to advocate the pyrimidine side more. Maybe purines are low because the enterocytes that need to regenerate themselves reuse purines better via the salvage pathway. I am somewhat confused about your article. On the one hand, you raise concerns about purines and pyrimidines, whereas on the other hand, you emphasize that purines may be important with respect to T and B lymphocytes. We know from work in the 1980s by Baker and others that the oxyglucose moiety in nucleosides is more important in influencing the immune response than are the normal hexoses. So my question is, do you have any idea if we have to deal with deoxynucleotides?

**Dr. Boza:** If one is considering de novo synthesis, it does not matter whether deoxyglucose is used because the purine ring and the sugar ring are resynthesized at the same time. From the studies I have looked at, it seems that there is almost no salvage of purine nucleotides. Not only are they almost entirely catabolized to uric acid, but they also are tightly controlled and regulated because they can exert important effects on the systemic circulation and because they regulate many metabolic pathways. A large increase in the concentration of this nucleotide could have major consequences in some pathways.

**Dr. Vigi:** It puzzles me that these small quantities of substances that are produced normally in the body and that represent only a fraction of the total metabolism of nucleotides can exert such important effects. You cannot explain all these on the supply theory because all the effects appear to be obtainable with different added nucleotides—for example, the effect on diarrhea in poor populations, the effect on the response to immunization, and so on. We even hear talk about nucleotides having an effect on lipid metabolism, so everything could be included! Might it not be better to concentrate on more proven effects? I would like a general comment. This is not a question but a request for an opinion.

**Dr. Boza:** My opinion about nucleotide supplementation is that in healthy newborn infants, with the quantities we are adding to formulas, we will hardly see any effects at all. But in infants receiving a limited protein intake, or in those prone to developing infections because of their environment, or with gut injury or malabsorption syndromes, then the small quantities we have been discussing may make a real difference. I am not speaking about the quantity that we are giving, only about the quantity the enterocytes are not catabolizing. We need to focus on the role of the enterocyte in the salvage of purines and pyrimidines in the diet. That is the most important point.
Dr. Vigi: So you are not in favor of additional nucleotides in starting formulas for normal infants?

Dr. Boza: I am not saying that. I do not think there are good enough studies as yet showing the beneficial effects. We need specific controlled trials to show whether added nucleotides may spare amino acids normally used in nucleotide synthesis, so they can be used for other purposes.

Dr. Bohles: There is a lot of orotate in cow's milk. Is there any orotate in human milk?

Dr. Boza: There is no orotate in human milk. Orotate is one of the precursors of pyrimidine synthesis and is present in cow's milk. It has been shown in rats that it can cause hepatic fatty acid accumulation, which is unique to the rat.

REFERENCES