Nucleotides in milk

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Introduction

Human milk contains significantly higher levels of nucleotides (NT) and NT derivatives compared with conventional infant formulas. NT can be synthesized endogenously, and thus are not essential nutrients. Dietary NT, however, are reported to affect the immune system, small intestinal growth and development, lipid metabolism and hepatic function (Table I). The terms “semi-” or “conditionally” essential have therefore been used to describe the role of dietary NT in human nutrition. Indeed, these nutrients may become 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. Conditions under which these nutrients may become essential include certain disease states, periods of limited nutrient intake or rapid growth, and the presence of regulatory or developmental factors which interfere with full expression of the endogenous synthetic capacity [1]. Under these conditions, dietary intake of the nutrient spares the organism the cost of de novo synthesis or NT salvage, and may optimize tissue function.

Nucleotide metabolism

NT and their related metabolic products play key roles in many biological processes. They serve as nucleic acid precursors, physiological mediators, components of coenzymes, and sources of cellular energy. NT consist of a nitrogenous base, a pentose sugar and one or more phosphate groups. The nitrogenous base is either a purine or a pyrimidine, whose atoms are derived primarily from amino acids (Fig. 1). A purine or pyrimidine base linked to a pentose molecule constitutes a nucleoside (NS). A NT is a phosphate ester of a NS, and may occur in the mono-, di- or triphosphate forms. The pentose is either ribose or deoxyribose; the riboNT and deoxyriboNT serve as the monomeric units of RNA and DNA, respectively. RNA and DNA are linear polymers consisting of four different NT linked together by 5’-3’ phosphodiester bonds [2]. Purines and pyrimidines can be synthesized de novo. However, de novo NT synthesis is a metabolically costly process which requires a great deal of energy in the form of ATP, in addition to amino acid precursors. An alternative mechanism for maintenance of cellular NT pools is the NT salvage pathway, in which pre-formed purine and pyrimidine bases and NS are converted to NT. The salvage pathway conserves energy, and permits cells incapable of de novo synthesis

Table I: Reported effects of dietary nucleotides in humans and in animals.

<table>
<thead>
<tr>
<th>Human</th>
<th>Animal</th>
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<tbody>
<tr>
<td>Promotion of small intestinal growth</td>
<td>+</td>
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<tr>
<td>Increased small intestinal disaccharidase activity</td>
<td>+/-</td>
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<tr>
<td>Intestinal hyperemia</td>
<td>+</td>
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<tr>
<td>Protection against diarrheal disease</td>
<td>+</td>
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<tr>
<td>Effects upon stool flora</td>
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<td>Enhanced cellular immunity</td>
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<td>Enhanced humoral immunity</td>
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<td>Effects upon hepatic composition</td>
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<td>Increased blood levels of LCP</td>
<td>+/-</td>
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<td>Effects upon serum lipoproteins</td>
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1 long chain polyunsaturated fatty acids
to maintain NT pools. NT synthesis and salvage are regulated at several points in order to maintain an appropriate balance of NT [2].

Rapidly dividing tissues require a constant supply of NT to manufacture nucleic acids. During cell replication, the enzymes of purine and pyrimidine synthesis are elevated and the DNA pool is increased. RNA and protein synthesis occurs continuously, although at varying rates during the cell cycle. Tissues such as regenerating liver, embryonic tissue, intestinal mucosal cells and erythropoietic cells are geared towards DNA replication and RNA synthesis.

**Nucleotide absorption**

Oral intake of NT, NS and nucleic acids increases serum and urinary degradation products in animals [3], and adult humans [4, 5]. Dietary nucleic acids have the greatest influence upon serum uric acid levels [4], and a maximum safe limit of RNA in the diet of 2 g/day has been suggested for adults. These observations provide presumptive evidence of NT absorption.

Orally ingested nucleic acids are degraded by pancreatic nucleases to a mixture of mono-, di-, tri-, and polyNT. Intestinal polynucleotidases or phosphoesterases supplement the action of pancreatic nucleases in producing mononucleotides from nucleic acids. The liberated NT are hydrolyzed to nucleosides by alkaline phosphatase and nucleotidases, and may be broken down further by nucleosidases to produce purine and pyrimidine bases [2] (Fig. 2). Investigations in animals suggest that NS are the primary form absorbed, and that over ninety percent of NS and bases are absorbed into the enterocyte [1, 3, 6].

Transport of nucleosides into the enterocyte occurs via both facilitated diffusion and specific Na+-dependent carrier mediated mechanisms. The upper region of the small intestine has the greatest absorptive capacity [7]. Once absorbed, most of the nucleosides and bases are degraded rapidly within the enterocyte, and catabolic products are excreted in the urine and intestine [3, 8]. Purine NT, NS and bases are degraded in humans to uric acid, while pyrimidines are degraded to beta-alanine and beta-aminoisobutyric acid [2].

**Fig. 1: Biosynthetic origin of the atoms in purine and pyrimidine bases.** Pyrimidine bases are six-membered rings, and include uracil (U), cytosine (C) and thymine (T). Purine bases have a second five-membered ring, and include adenine (A), guanine (G), hypoxanthine and xanthine.
Catabolic enzymes for purines and pyrimidines predominate over anabolic enzymes in the small intestine, which also may serve as an extrarenal route for the elimination of uric acid in man. The highest levels of purine catabolic enzymes are found in the upper alimentary tract [6]. Chinsky et al. [9] found that adenosine deaminase was one of the most abundant proteins of the epithelial lining of the alimentary mucosa in mice. Levels were low at birth, and achieved very high levels within the first few weeks of life. Witte et al. [10] reported that from the tongue to the ileum, diverse epithelial cell types lining the lumen of the mouse gastrointestinal tract strongly coexpress each of the five key purine catabolic enzymes, with dramatic increases in the expression of each enzyme occurring during postnatal maturation of the gastrointestinal tract; in light of these high levels of purine catabolism, the authors presume that exogenous purine NT are probably not nutritionally significant. In addition, nucleic acids and their components are released by the cellular turnover of the intestinal mucosa, yielding daily up to 2 g of purine bases in adult humans [11]. The metabolic fate of these endogenously released NT is not known.

Despite extensive catabolism and a steady supply of NT from cell turnover, tracer studies in animals indicate that two to five percent (2-5%) of dietary NT are incorporated into tissue pools, primarily within the small intestine, liver, and skeletal muscle [6, 8]. With an increase in the dose of dietary RNA, the amount of purine is increased several times, particularly in the small intestine [3]. Roll et al. [12] reported that approximately 1% of nucleic acids of the combined viscera were

![Fig. 2: Digestion and absorption of nucleic acids and their related products. Nucleosides are mainly absorbed as such through the microvillous membrane of enterocytes. A fraction of nucleotides and some purine and pyrimidine bases are also absorbed.](image-url)
derived from $^{15}$N-labeled RNA fed to rats. Incorporation into tissues reportedly was increased at younger ages. Gross et al. [13] also demonstrated significantly increased salvage and retention, and decreased catabolism, of orally administered bases and NS in the fasted versus the fed state. Decreased catabolism may be due to a fasting-related decrease in xanthine oxidase activity. Extensive salvage of purines and pyrimidines NT has been demonstrated in intestinal tissues; however, the capacity for de novo synthesis remains unclear. Investigators have reported both the presence, the absence and limited de novo NT synthesis within intestinal tissues [6].

Finally, LeLeiko et al. [11] reported that dietary NT affect gene expression of intestinal enzymes. Feeding a purine and pyrimidine free diet to adult rats resulted in a highly significant decrease in total RNA and protein in the small intestine and colon, suggesting a mechanism by which dietary components differentially control the synthesis of specific proteins synthesized in the body. In vitro, exogenous NT may increase the normal growth and maturation of enterocytes, as well as reduce their dependence upon exogenous glutamine [14].

In summary, most orally administered nucleic acids, NT, NS and bases are readily catabolized and excreted. However, tissue retention is increased during periods of rapid growth and limited food intake. The de novo synthetic capacity of mammalian gastrointestinal tissues remains unclear. Further studies are needed to characterize the metabolism of NT and NT derivatives in human gastrointestinal tissues, and the impact of conditions such as immaturity, mucosal injury and limited nutrient intake.

**Human milk nucleotides**

Nucleotides are a component of the non-protein nitrogen fraction of human milk. Non-protein nitrogen accounts for as much as 25% of the total nitrogen in human milk [15], and includes compounds such as amino sugars and carnitine, which play specific roles in neonatal development. In contrast, non-protein nitrogen accounts for only 2% of total nitrogen in cow’s milk, and less than 20% in most cow’s milk-based infant formulae [15]. Many of the non-protein nitrogen components of human milk are present in significantly lower quantities in cow’s milk and cow’s milk-derived infant formulae [16].

NT are reported to account for 2-5% of human milk non-protein nitrogen. NT nitrogen may contribute to the more efficient protein utilization of the human milk-fed infant, who receives a relatively low protein intake compared with the formula-fed infant [17].

A wide range of NT concentrations, from 0.4 to over 7 mg/dl has been reported for human milk [17-24]. In several studies, only the free NT content of human milk was measured. However, human milk contains free NT, NS, bases, nucleic acids and adducts (e.g. uridine diphosphate galactose). Most of these constituents probably are contributed by human milk cells. The metabolic fate of NT and related compounds consumed by the breast-fed infant is not known. Leach et al. [24] used enzymatic hydrolysis to simulate in vivo digestion in milk samples collected from a large, culturally heterogeneous sample of women to estimate total potentially available nucleosides (TPAN). Polymeric NT, monomeric NT, NS and NT adducts accounted for 48%, 36%, 8% and 9%, respectively, of TPAN. There was no consistent relationship between TPAN, stage of lactation or country in which the women resided.

Relative quantities of the individual NT vary in human milk, although cytidine is usually reported to be present in the highest concentrations. Most investigators do not report the presence of inosine in human milk. Leach et al. [24] speculate that the presence of inosine may be an artifact of adenosine deaminase action upon human milk adenosine. Inosine and its metabolites enhance iron absorption in the rat by increasing the activity of intestinal xanthine oxidase. Xanthine oxidase, present in human milk, catalyzes the reduction of ferric iron to ferrous iron, and thus increases its bioavailability [6].

Orotate, the major NT of cow’s milk, is present in significant quantities in cow’s milk-based infant formulae [17, 22], but not in human milk. High levels of dietary orotic acid cause hepatic lipid accumulation; this effect is however unique to the rat [25].
Human milk is generally considered to be the “gold standard” for infant feeding, and infant formulae are usually manufactured to be as similar to human milk as possible. Nevertheless, additional studies utilizing standardized methodology for collection and analysis are needed for accurate determination of human milk NT content. NT interaction with other human milk components which may affect NT bioavailability and biological action should also be considered in the design of NT supplemented infant formulae.

**Gastrointestinal effects**

The intestinal epithelium is a rapidly proliferating tissue with a high cell turnover rate. The complete cell cycle in humans is 24 hours, with replacement of the entire enteric epithelium occurring in 3 to 6 days. Under most conditions, endogenous NT are made available to the gastrointestinal mucosa via hepatic supply and cell turnover [11]. Nevertheless, dietary NT are reported to play a role in growth and differentiation of the gastrointestinal tract. Uauy et al. found increased mucosal protein, DNA, villus height, and disaccharidase activities in the intestine of weanling rats fed diet supplemented with 0.8% w/w dietary NT; feeding 0.21% w/w dietary NT to weanling mice was associated with an increase in small bowel weight (as % body weight) and weight/unit length; however, disaccharidase activities were not affected [26]. Supplementation with AMP alone significantly increased jejunal wall thickness, protein, and villus cell number [27]. Other investigators report increased mucosal growth, maturity and crypt cell proliferation in rats administered a NS/NT supplemented TPN solution [28, 29].

Dietary NT also may be beneficial following intestinal injury. The intestinal tissue content of DNA, and disaccharidase activities were higher [30], and intestinal histology and ultrastructure were improved [31] by feeding NT-supplemented versus NT-free diet to rats following chronic diarrhea. Quan et al. [32] reported that dietary NT supplementation decreased mortality and intestinal inflammation, and increased disaccharidase activities in rats following radiation-induced intestinal injury. Bustamante et al. [33] subjected isolated loops of piglet ileum to ischemia and reperfusion. Luminal infusion of a NT mixture was associated with reduced leukocyte accumulation, protein leak and nitrite production, and with intestinal hyperemia, particularly in younger animals. Effects were not changed significantly in the presence of an adenosine antagonist, although purine receptor specificity of the agent was not evident. Hypoxanthine was not increased in the intestinal mucosa in the presence of NT. The latter observation is significant, since xanthine oxidase catalyzes the degradation of adenine NT to hypoxanthine, xanthine and uric acid during ischemia. Xanthine oxidase is a potential source of oxygen free radicals, and may play a role in the development of reperfusion damage to tissues [6].

A limited number of studies are available regarding dietary NT effects upon the gastrointestinal system in infants. Brunser et al. [34] studied dietary NT effects upon diarrhoeal disease in infants living in a relatively contaminated environment in urban Chile. NT supplemented infant formula was fed to 141 infants, and unsupplemented formula to 148 infants. Those who received supplemented formula experienced fewer episodes of diarrhea (109 versus 140), although clinical characteristics of the episodes, and the pattern of enteropathogens isolated were not affected. Gil et al. [35] report that the addition of NT to infant formula resulted in a microbial pattern in the stool which was more similar to that of the breast-fed infant, i.e. increased percentages of bifidobacteria and enterobacteria. Balmer et al. [36], however, found the opposite effect of feeding NT supplemented formula.

Özkan et al. [37] measured blood flow of the superior mesenteric artery of healthy term infants fed either human milk, NT-supplemented or non-supplemented formula. Infants fed NT supplemented formula had significantly higher postprandial blood flow velocity and volume flow compared with infants fed human milk or non-NT supplemented formula.

Due to rapid turnover, tissues of the gastrointestinal tract require increased levels of NT as precursors for nucleic acid synthesis. An exogenous source of NT may optimize tissue function, particularly during periods of accelerated growth, and during recovery from mucosal injury when the
endogenous supply may limit nucleic acid synthesis. Dietary NT also may contribute to intestinal hyperemia. It is not known if dietary NT effects are due to direct incorporation of NT into gastrointestinal tissue nucleic acid, and/or to other biological mediator effects of NT such as modulation of small intestinal blood flow.

**Immunologic effects**

Dietary NT are reported to play a role in maintenance of immune responses. While the mechanism is unknown, data suggest that exogenous NT supplied by the diet may contribute to the pool of NT available to stimulated leukocytes, which rapidly turn over and thus have increased NT requirements. Activation of lymphocytes causes a rapid increase in the synthesis of NT, which are required first for the increase in energy metabolism, and later as precursors for nucleic acid synthesis. Induction of lymphocyte proliferation is accompanied by a dramatic increase in intracellular NT pools, and the expression of large numbers of transmembrane NS transporters [38].

Cohen et al. [39] demonstrated that de novo purine biosynthetic activity is present in S-phase thymic lymphocytes; G1 phase lymphocytes, however, may have only salvage pathways to maintain their purine NT pools. Pérgnon et al. [40] found limited capacity of lymphocytes to salvage pyrimidines, while Marijnen et al. [41] suggested that the NT salvage pathway may not be capable of providing sufficient purine NT for proliferating lymphocytes. These studies suggest that proliferating lymphocytes require an exogenous supply of NT for optimum function.

Feeding a NT-supplemented versus a NT-free diet to mice has been associated with increases in the following immune parameters; 1) graft versus host disease mortality; 2) rejection of allogeneic grafts; 3) delayed cutaneous hypersensitivity; 4) alloantigen-induced lymphoproliferation; 5) reversal of malnutrition and starvation-induced immunosuppression; 6) natural killer cell activity and macrophage activation; 7) resistance to challenge with *Staphylococcus aureus* and *Candida albicans*; 8) macrophage phagocytic capacity; 9) spleen cell production of interleukin-2 and expression of interleukin-2 receptors and lyt-1 surface markers [42, 43]; and 10) T-cell dependent immunoglobulin production [44]. Moreover, the feeding of a nucleotide/nucleoside mixture was reported to increase bone marrow cell and peripheral neutrophil numbers following *Staphylococcus aureus* infection or neutropenia [45, 46] and to inhibit endotoxin-induced bacterial translocation [47].

Limited data, however, are available regarding dietary NT effects in infants. Pickering et al. [48] investigated antibody response to vaccination in infants fed for 12 months either: 1) human milk exclusively for 2 months and then human milk or Similac with iron® (n=103); 2) Similac with iron (n=107); or 3) Similac with iron supplemented with 7.2 mg NT/dl (n=101). Infants followed identical immunization schedules. At 7 months of age, infants fed the NT-supplemented formula had higher serum titers to *Haemophilus influenzae* B compared with infants fed human milk or unsupplemented formula, and a higher diphtheria titer compared with infants fed human milk; the differences in haemophilus titers remained significant at 12 months of age. Carver et al. [49] reported increased natural killer cell activity and interleukin-2 production in infants fed NT-supplemented formula or human milk compared with infants fed non-supplemented formula. These studies suggest that NT in human milk may contribute to the enhanced immunity of the breast fed infant.

Van Buren et al. [50] proposed that dietary NT exert effects upon immune responsiveness by acting upon the T-helper/inducer population, with the predominant effect upon the initial phase of antigen processing and lymphocyte proliferation. The presumed mechanism is suppression of uncommitted T lymphocyte responses, as demonstrated by higher levels of a specific intracellular marker for undifferentiated lymphocytes in primary lymphoid organs in mice fed a NT-free diet [51]. A regulatory role of dietary NT in immunohematopoiesis also has been proposed [52]. Rudolph et al. [53] suggest that dietary NT effects upon immunity were not observed previously, since they are only evident under conditions of stress, such as immune challenge.

Gut associated lymphoid tissue (GALT) also can initiate and regulate T-cell development, and may act as a thymus analogue. Dietary NT effects upon
peripheral immunity may be mediated in part via effects upon this important, but poorly understood, immune tissue [54].

Hepatic effects

The liver plays a major role in meeting the body’s NT requirements through active synthesis and release of NT for use by other tissues. Extracellular NT and NS also modulate hepatocyte growth and regeneration [55]. Parenteral administration of a NT/NS mixture is reported to improve hepatic function and promote earlier restoration of nitrogen balance following liver injury [56] or partial hepatectomy [57] in rats. Orally administered NT also may have effects upon the liver. Novak et al. [58] report that weanling mice fed NT-free diet had increased hepatic cholesterol, lipid phosphorous, and serum bilirubin, and decreased liver weight (as % body wt) and glycogen when compared with animals fed 0.21% w/w NT. Animals fed diets supplemented with AMP alone represented a greater contrast to animals fed NT-free diet than did animals fed a NT mixture. Dietary AMP effects may relate to the increased hepatic incorporation of dietary adenine versus other purines, or to adenosine’s role in increasing hepatic blood flow [6].

These studies suggest that exogenously administered NT may affect hepatic composition, function and repair. Dietary NT may be especially important in meeting NT needs when the liver’s capacity to supply pre-formed NT is diminished due to disease or injury.

Effects on blood lipids

Feeding a NT supplemented formula has been associated with increases in long chain polyunsaturated fatty acids (LCP) in the blood of term [59, 60] and preterm [61] infants, and rats [62-64]. An increase in plasma arachidonic acid in rats was associated with increased thromboxane B2 levels [62]. In most studies, absolute levels, and not percentages, of very long chain fatty acids were increased with NT supplementation. These data suggest that dietary NT play a role in the conversion of 18C essential fatty acids to 20-22C very long chain polyunsaturated fatty acids. Other investigators, however, found no effect of dietary NT upon long chain polyunsaturated fatty acids in mice livers (58) and in erythrocytes of infants [65, 66, unpublished observations].

Sanchez-Pozo et al. [67] report that term infants fed NT-supplemented formula or human milk had lower VLDL and higher HDL levels at one month of age compared with infants fed unsupplemented formula. NT-supplemented formula fed to preterm infants was associated with increased levels of several plasma lipoproteins [22] and with enhanced plasma lecithin:cholesterol acyltransferase (LCAT) activity [68]. The authors speculated that dietary NT enhance lipoprotein synthesis, particularly in the intestine.

Effects of individual nucleotides

Data regarding the effects of feeding individual NT are limited. Orally administered purines, particularly adenine NT, have been the most extensively studied due to their effects upon gout. Despite the biochemical similarity of various purines, they produce different effects upon uric acid metabolism. Oral hypoxanthine, adenosine monophosphate (AMP), guanosine monophosphate (GMP) inosine monophosphate (IMP) and adenine, but not guanine and xanthine, elevate serum uric acid levels. The metabolism of dietary adenine is different from that of other purines in that a greater portion is absorbed and incorporated into tissues. Adenine may be absorbed with minimum alteration, and is the most extensively re-utilized purine, in contrast to other purines which are degraded extensively to uric acid in the gut [6, 11]. Further, up to 20% of orally administered adenine may be recovered unmetabolized in the portal vasculature [69]. Kolassa et al. [70] reported that adenosine uptake by intestinal epithelium is faster than that of other purines, and suggested that adenosine is the most important source for maintenance of purine NT in intestinal epithelium. Excessive intake of adenine reduces growth rates in animals; however, these effects are seen only when adenine is fed in the free form and not as the NS or NT [6].
Adenosine is a potent vasodilator, and both intra-arterial and intra-luminal infusion of adenosine increase small intestinal blood flow in animals [6]. The vasodilatory action of adenosine may be responsible for the increased intestinal blood flow in neonates [37] and piglets [33] fed NT mixtures, and for the gastrointestinal and hepatic effects in animals fed diets supplemented with AMP alone [27].

Although dietary purines have been studied more extensively and are known to be important biomediators, several studies of NT supplementation in mice suggest that oral administration of the pyrimidine uracil enhanced immunity while adenine did not. These effects may relate to the limited capacity of lymphocytes to salvage pyrimidines, and/or to the potentially greater need of dividing lymphoblasts for pyrimidine NT [40]. The specific biologic effects of feeding individual NT and their related metabolic products require further investigation.

Conclusions

Human milk contains significantly higher levels of NT and NT derivatives compared with infant formulae; however, their metabolic fate and the role they play in the health of the breast-fed infant is not known. Studies in animals and infants suggest that dietary NT have significant effects upon the immune system, gastrointestinal growth and differentiation, hepatic function and lipid metabolism. It has been suggested that performed NT supplied by the diet may contribute to tissue NT pools, and thus optimize the metabolic function of rapidly dividing tissues such as those of the gastrointestinal and immune systems. An exogenous supply of NT may be important during infancy, which is characterized by rapid tissue growth, and increased NT requirements for the synthesis of nucleic acids. This may be especially important for infants born prematurely, since preterm birth is associated with limitations of many metabolic functions, and limited opportunities for breast-feeding.

In addition to serving as nucleic acid precursors, NT also plays roles as inter- and intracellular biological mediators. The purine adenosine is a potent vasodilator, and feeding NT mixtures has been associated with intestinal hyperemia. Studies in animals, however, suggest that dietary pyrimidines may be more effective in enhancing immune function.

Although NT supplementation of infant formulae remains controversial, formulae supplemented with NT at levels similar to those reported for human milk are marketed currently in several countries. Additional studies are needed to clarify the role of dietary NT in infant nutrition and to identify the mechanism of dietary NT effects. Areas for future study include: 1) the absorption and metabolism of NT and related metabolic products in infants; 2) dietary NT effects upon gut associated lymphoid tissues; 3) the relative contribution of individual nucleic acid, NT, NS and free bases to observed biologic effects; 4) identification of the levels of dietary NT which will provide optimum effects; and 5) dietary NT effects in preterm versus term infants.

References


