Specialized transport proteins are required for the movement of numerous nonlipophilic compounds across cell membranes and are likely to have evolved soon after the appearance of the lipid-based biological membrane. Any substance that must enter or leave a eukaryotic or prokaryotic cell and that is not soluble in lipid or small enough to diffuse between the constituent molecules that make up a membrane requires a specialized transport mechanism such as a transport protein. Thus transporters are essential components of cellular physiology, allowing access of a wide variety of compounds, such as nutrients and substrates, and facilitating release of compounds, such as signaling molecules. For nucleosides and nucleobases involved in myriad physiological processes, the specialized transport proteins responsible are currently accepted to be primarily the members of the gene families SLC28 and SLC29 (although the existence of additional or novel, previously unidentified transport proteins cannot be ruled out). Here, we discuss various aspects of the physiology of NTs in mammalian systems, particularly noting tissues and cells where there has been little recent research. Our central thesis is that reference back to some of the older literature, combined with current findings, will provide direction for future research into NT physiology that will lead to a fuller understanding of the role of these intriguing proteins in the everyday lives of cells, tissues, organs, and whole animals.

Nucleosides: Metabolites and Drugs

To fully understand the physiology of NTs, we need to understand the physiological relevance of their substrates. This takes us back almost a century, when nucleosides were first identified as physiologically important molecules. For instance, as early as 1927, the metabolism of pyrimidine nucleosides was studied (15, 28), whereas the physiological effects of purinergic compounds, including adenosine, on the heart were reported in a landmark paper in 1929 (26). Thus the early history of the study of nucleosides reflects their physiological roles as nutrients and metabolites within cells, tissues, and whole animals. During the mid-20th century, research on the physiology of nucleosides continued (e.g., Ref. 72), with growing interest in the potential therapeutic role of adenosine (and adenosinergic compounds) in the cardiovascular system (e.g., Ref. 41). However, interest in the metabolism of nucleic acids, combined with the discovery that the structure of DNA held the key to how information could be effectively replicated within cells, propelled nucleosides, and their analogs, rapidly into the world of anti-metabolite therapeutic agents. Thereafter, drugs derived from nucleosides became critical in the treatment of previously intractable diseases such as cancer (e.g., Ref. 14, 45, 81). The significance of this leap forward in therapeutic approaches to human disease is reflected in the award of the Nobel Prize in Physiology or Medicine to Gertrude Elion and George Hitchings in 1988 (shared with Sir James Black) for their development of key nucleoside analog drugs such 6-mercaptopurine (still a central treatment for acute lymphoblastic leukemia). The research by Elion and Hitchings, as well as many others, laid the foundation for an explosion of interest in the late 20th century in
nucleoside analog drugs as anti-metabolites (e.g., Refs. 57, 98).

In contrast to the intense research on modified nucleosides as therapeutic agents, our understanding of the role of endogenous nucleosides is incomplete with the significant exception of the roles of the purine nucleotides (such as ATP) and their metabolites (such as adenosine). There is now a huge body of literature on purinergic signaling, dating back almost 100 years, which has clearly established adenosine as an important paracrine and autocrine molecule (for review, see Refs. 13, 33). Adenosine and ATP are found both intracellularly and extracellularly. ATP is the universal currency of chemical energy in the cell and, like adenosine, acts as a signaling molecule through purinoceptors found in the cell membrane. Although much work has been done on the role of adenosine in various systems, the other ATP metabolites, such as ADP, AMP, inosine, and hypoxanthine, have significant but not fully described physiological roles, both inside and outside the cell. For instance, AMP activates the intracellular kinase AMPK, whereas inosine is known to have inotropic, vasodilatory, and anti-inflammatory effects (43, 52, 86). Inosine may exert its effects via inhibition of poly ADP-ribose polymerase (PARP), which is a final effector in tissue injury caused by circulatory shock and ischemia/reperfusion (96). Research has found that blocking PARP pathway protects against oxygen radicals and nitric oxide toxicity, attenuates injury, and preserves cellular ATP by improving mitochondrial respiration (92). Interestingly, other purine nucleosides may act as anti-mutagens and natural antioxidants capable of protecting against reactive oxygen species (ROS), which arise due to inflammation, ionizing radiation, or aerobic cell metabolism (38, 40, 67). In particular, guanosine and inosine have been found to prevent oxidative damage to DNA, decrease production of ROS, and protect against radiation cell death (38). Mice injected with a lethal dose of radiation were found to have increased survival when treated with guanosine and inosine (12, 38). The protective nature of guanosine and inosine may be due to their easily oxidized states (12) and suggest a role for these purine nucleosides in the DNA repair process and cellular protection.

Although most data suggest that purine nucleosides are endogenous therapeutic compounds involved in cytoprotective or anti-inflammatory roles, it is also increasingly clear that, for example, adenosine can also have pro-apoptotic and pro-inflammatory functions (85, 99). Thus it is likely to be critically important that cells and tissues control endogenous levels of nucleosides to promote homeostatic physiological responses.

In summary, nucleosides and nucleobases play diverse roles inside and outside of cells, and their flux across cell membranes is dependent on nucleoside transport proteins, which are thus likely to modulate, enhance, and limit their physiological effects (FIGURE 1).

### Nucleoside Transporters

Initial reports on mediated uptake of nucleosides date back many years (e.g., Refs. 68, 72, 73, 97), but it was not until almost the end of the 20th century when the cDNAs and proteins responsible for both facilitated and concentrative nucleoside transport activities were identified (37, 50). In the decade since the cloning of rat concentrative nucleoside transporter 1 (rCNT1) (50) and human equilibrative nucleoside transporter 1 (hENT1) (37), much progress has been made in our understanding of the role of the nucleoside transporters in mediating the effects of various nucleoside analog drugs (for review, see Ref. 22). We now know that nucleoside transporter proteins are encoded by two nonhomologous gene families: SLC28, the concentrative nucleoside transporters, consisting of three isoforms (CNT1–3), which are Na⁺–dependent symporters, and SLC29, the equilibrative nucleoside transporters consisting of four isoforms (ENT1–4), which are Na⁺–independent diffusion-limited channels (8, 9, 35, 36, 56, 58, 71). A number of excellent recent reviews provide more details on the structure and pharmacology of the cloned NTs (56, 61, 98). Some of the first observations of physiological relevance were those related to the broad but differential distribution of NTs throughout the body (3, 51, 62). These early studies on NT isoform tissue distributions were among the first to speculate on specific physiological roles for the various NT types (e.g., Ref. 75). At the cellular level, the presence of CNT on apical membranes and ENTs on basolateral membranes of polarized cells provided a plausible model for the vectorial flow of physiologically relevant molecules in tissue absorptive epithelia such as the intestine and kidney (e.g., Ref. 71). Building on the research at the molecular and cellular
level, more recent studies are beginning to investigate the role of NTs in systems [such as the cardiovascular system (CVS) and the central nervous system (CNS)], and new findings suggest a central role for NTs in many nucleoside-dependent pathways in a variety of tissues (FIGURE 2), such as those outlined below.

Central Nervous System

NTs are located throughout the central nervous system, although there appears to be significant differential tissue distribution that may be species and perhaps even gender specific (2, 51, 62, 69). Whether these studies reflect differences in technical approaches (e.g., PCR, regional immunoblotting, in situ hybridization) remains to be determined, but such variability currently limits our ability to develop unifying models of the role of NTs in nucleoside physiology within the central nervous system. In the human brain, hENT1 protein appears to be distributed in a pattern that correlates with the existence of $\alpha_1$ adenosine receptors and that is distinct from the distribution of hENT2. These data suggest a physiological relationship between hENT1 and $\alpha_1$ receptor signaling such that the transporter can modulate the response of the receptor. This places NTs (possibly primarily hENT1) in a potentially pivotal role within the brain, since they may be involved in modulating synaptic levels of purine nucleosides (typically adenosine) involved in sleep, arousal, sensation of pain, anxiety behavior, and drug and alcohol dependence (3, 19, 20, 39, 51, 56, 80). Moreover, NTs are likely to provide a route of efflux or reuptake of adenosine produced following ischemia or hypoxia and can thus modulate its activity as an endogenous neuroprotectant (e.g., Ref. 91).

The physiological role of ENT1 and the putative relationship between ENT1 and the $\alpha_1$ adenosine receptor were further revealed by recent studies using one of the first transgenic NT models, the mENT1 knockout mouse (20). These mice show decreased anxiety (19) and consume more alcohol (20) than wild-type littermates. The loss of ENT1 results in alterations in $\alpha_1$ adenosine receptor-dependent signaling, which has been shown to be involved in both ethanol effects and anxiety behaviors. Thus, in wild-type mice, ethanol inhibition of adenosine uptake via ENT1 leads to activation of $\alpha_1$ receptors, which contributes to the intoxicating and addictive effects of ethanol (20). The relationship between ENT1 and the $\alpha_1$ receptor was confirmed by treatment of knockout mice with $\alpha_1$ receptor-specific agonist, which returned their response to ethanol to that of wild-type mice (20). The mENT1 knockout mice also lacked anxiety-like behavior, presumably because of elevated extracellular adenosine (as a consequence of the loss of ENT1) and enhanced $\alpha_1$-dependent signaling, specifically in the amygdala (19).

Current data on the role of NTs in the central nervous system is primarily based on rodent models with very little information on the distribution of NTs in the human brain or their contribution to purine (or other) nucleoside physiology. This is a challenge for the field in the future, given the variability observed between rodent models and the difficulties of extrapolating to human systems. Despite these challenges, the knockout

![FIGURE 2. Tissues in which NTs have been described and some physiological roles elucidated](http://physiologyonline.physiology.org/)
mouse studies are among the first to link a specific NT with whole animal physiological responses, and the development of more transgenic models will significantly assist in future studies on the physiology of NTs.

**Cardiovascular System**

Given the importance of purinergic signaling in the cardiovascular, there is much interest in the distribution and relative roles of the different NTs. ENT isoforms 1–4 are expressed in cardiomycocytes (10, 62), with ENT1 appearing to be the predominant transporter responsible for nucleoside flux (including in cell culture models) (16, 17, 18, 60). In rat cardiac fibroblasts, ENT1, ENT2, CNT1, and CNT2 were found to be present, and expression of ENT2, CNT1, and CNT2 was influenced by insulin (suppressing CNT1 and CNT2 but increasing ENT2 expression) (79). These data correlate with insulin-dependent changes in NT expression observed in rat lymphocytes and a reduced adenosine release by diabetic rat cardiac fibroblasts due to changes in NT expression (78, 79). Whether changes in expression levels correlate with changes in protein levels and functional transport remains to be determined, and therefore the physiological relevance of these observations is not clear.

The role of purine nucleosides, and especially adenosine, in the regulation of cardiovascular function, particularly coronary blood flow, vasodilation, and cardioprotection, is well known (e.g., Ref. 74), and the physiological role of NTs in the endothelium is of intense interest (see Ref. 61 for extensive review). Briefly, endothelial NTs appear to act in a homeostatic manner to modulate nucleoside levels, primarily adenosine, and promote vascular integrity under conditions of inflammation or hypoxia. ENT1 and ENT2 are the main NTs in the endothelium (4), and ENT1 shows hypoxia-dependent repression of transcriptional activity via HIF-1α (18, 27) and via PKCε (17, 18). Reduced NTs at the cell membrane are likely to enhance extracellular adenosine levels and thus adenosine receptor signaling, but may also lead to retention of valuable pools of purinergic metabolites (18, 27). In human umbilical vascular endothelial cells, high glucose levels (as occurs in diabetes) lead to reduced ENT1 and ENT2 mRNA expression, whereas insulin can block this effect and enhance adenosine uptake (1, 30). In obstetric situations, altered extracellular adenosine levels could alter the blood flow from placenta to fetus, affecting fetus growth and development (65). In contrast, glucose increases ENT1-dependent adenosine transport in smooth muscle cells (59), suggesting alternate pathways of regulation depending on cellular context. A future challenge is to build on studies in isolated tissue or cell culture models to research that identifies the role of vascular NTs, which sit at the critically important blood-tissue interface, in modulating vascular nucleoside levels. Since adenosine receptors are also broadly distributed, the influence of the vascular NTs is likely to be significant in many systems.

The association between NTs and adenosine receptor signaling is also apparent in cardiomyocytes, which can be preconditioned by exposure to a brief period of decreased oxygen (18, 44, 64). Preconditioning can be induced pharmacologically via adenosine receptor and/or PKCε activation (74) or by manipulation of ENT1 (16, 17).

Although ENT1 appears to be predominantly expressed in cardiomycocytes and is the major contributor in the cardioprotection afforded by adenosine efflux, ENT2, which is capable of transporting both adenosine and inosine (produced by deamination of adenosine), is equally effective at PC as adenosine (66). ENT2 may be responsible for the re-uptake of extracellular inosine present after hypoxic challenge (16, 21).

**Reno-Hepato-Gastrointestinal System**

NTs are widely distributed within the reno-hepato-gastrointestinal system where the presence of polarized absorptive epithelial cells requires a perhaps more complex interplay between NT types. A recent extensive study in human tissue reports the presence of CNT1, CNT2, ENT1, and ENT2 in the human intestine, liver, and kidneys (34). Another study reports the presence of CNT3 in the human kidney (23). In combination with a significant body of previous findings, these more recent studies support the paradigm of CNTs and ENTs being expressed on apical and basolateral membrane, respectively (for an excellent review, see Ref. 71). Dietary nucleotides (and presumably nucleosides) have been reported to be important in the intestine (84, 95), although this aspect of their physiology has not been widely studied. Other data suggest that adenosine is involved in regulating intestinal motility, secretion, and glucose uptake (via A2 receptor-coupled signaling) in the mouse intestine. Thus it is likely that NTs are involved in modulating the activities of adenosine, and CNT2 has been proposed as being the predominant NT responsible (55, 62), although variability clearly exists between mammalian models.

A particularly interesting observation from recent studies in the intestine suggests that cellular differentiation (for instance, to enterocytes) correlates with the presence of CNTs, whereas proliferative tissue favors ENTs (5).

Within the liver, CNT2 has been reported as the predominant CNT in rodent models (6, 24, 70) in contrast to the distribution in human liver where CNT1, CNT2, ENT1, and ENT2 are all found at approximately equal levels (31, 34). Rat liver parenchymal cells have proved a useful model, however, in demonstrating an interesting, physiologically relevant relationship between CNT2 and the important intracellular signaling molecule AMP kinase (7). Inhibition of adenosine transport.
via CNT2 in these cells abolished adenosine-mediated AMPK signaling and phosphorylation of acetyl-CoA carboxylase (ACC) (7). A complementary study revealed that bile acids are capable of increasing CNT2 translocation activity by increasing CNT2 translocation to the plasma membrane (31), revealing the innate capacity of the liver to modulate its nucleoside transport and salvage pathways (31).

Nucleoside reabsorption and clearance in the kidney is modulated by NT function and is an area of intense research (71). Variations in expression levels and distribution between mammalian models and even genders (23, 29, 62, 83) have been described, but in general it would appear that CNTs are critically involved in active reabsorption of nucleosides from the tubular lumen (71). The complex interplay between the many cell types, regulatory factors, and various NTs in renal homeostasis remains a particularly challenging area of future study.

**Skeletal Muscle**

Skeletal muscle is one of the tissues where our understanding of the role of NTs is particularly limited, despite the fact that this tissue has been reported to possess high levels of expression of ENT1 and ENT2 (21, 63, 75) and is metabolically active with a vigorous turnover of purine nucleotides and the need for vasodilation to accommodate the demands of exercise (46, 47). Thus, as in other tissues, adenosine (and possibly inosine) may be important in the regulation of blood flow to, and glucose use in, skeletal muscle bundles (42). Extracellular levels of adenosine increase during contraction (in concert with changes in intracellular Ca²⁺ levels) and can be mimicked by electrical stimulation (48, 63). The source of the adenosine is typically considered to be extracellular due to the activity of an ecto-AMP 5’ nucleotidase, (11, 48, 82, 94), which would suggest that the major role of NTs in this tissue is in the salvage of purine nucleosides and their metabolites, presumably for recycling. As in other tissues, extracellular adenosine appears to act via A₁ receptors, which signal to increase, at least in part, insulin-stimulated glucose uptake (93). The role of NTs in modulating these effects has not been investigated.

**Adipose Tissue**

Adipose tissue is now recognized as a highly active and important endocrine tissue in normal physiology and the pathophysiology associated with diseases such as diabetes. Although little is known about NTs in skeletal muscle, almost nothing is known about the presence or role of NTs in adipose tissue. Adenosine has been reported to be present in adipose tissue under normal physiological conditions, and older literature reports that adenosine can inhibit lipolysis (25, 32), suggesting that adenosine could be a physiological regulator in this tissue. Interestingly, later studies demonstrated that the classic NT inhibitors dipyridamole, NTI, and NBTG (which are now known to inhibit ENT1 or ENT1/2) were effective at inhibiting basal and insulin-stimulated glucose uptake (89), although whether the mechanism was a direct inhibition of the glucose transporter in the case of dipyridamole (88) or an enhanced extracellular adenosine level and subsequent activation of adenosine-receptor signaling is not known. Given the physiological importance of adipose tissue to both normal physiology and pathophysiology, combined with the current molecular tools for study of NTs, it is time to more fully investigate the role of the NTs in purine nucleoside physiology in adipose tissue.

**Other Tissues**

There are numerous other tissues where very little is known about the presence, role, or physiology of NTs. Examples include the lung, where adenosine is critically important in stimulating mucociliary clearance but where excess levels can lead to inflammation, suggesting that modulation of extracellular adenosine is physiologically critical. Recent data in human primary culture models demonstrate that CNT2 and CNT3 are responsible for apical adenosine uptake on human airway epithelia (49).

Many other tissues have been shown to possess mRNA for NTs or to have NT-like transport activities (e.g., Refs. 53, 54, 62, 76, 77), but much more research is needed in these tissues to determine the physiological role of the NTs.

**Future Challenges**

As the research literature on the role of NTs in nucleoside physiology builds, a major challenge for the field in the future will be to develop models that place the NTs within the complex network of other proteins involved in nucleoside physiology, taking into account different mammalian systems. Models explaining the fundamental roles of NTs in cellular, organ-specific, and whole animal physiology will result in a broader and deeper understanding of the role of nucleosides beyond their basis as therapeutic agents or templates for analog drugs. The physiological relevance of the existence of multiple isoforms, from two families of transporters with varying affinities and overlapping substrate preferences broadly distributed within the mammalian system, continues to be elusive. One of the most challenging but exciting areas of future research will be un unraveling the complex network of cross-talk and interaction between NTs, adenosine receptors (and possibly other unidentified purinergic receptors), metabolic enzymes, and signaling pathways.
Conclusion

We anticipate the 21st century to be the golden era for membrane proteins such as NTs. Thus, by going back to asking fundamental questions about the physiology of nucleosides, we are likely to see future breakthroughs in understanding the physiology of NTs.

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