The functional logic of cytosolic 5'-nucleotidases

Piero L. Ipata*, Francesco Balestri

Department of Biology, Unit of Biochemistry, University of Pisa, Via San Zeno 51, Pisa, Italy

Correspondence*: Prof. Piero Luigi Ipata, Department of Biology, Unit of Biochemistry - University of Pisa, Via San Zeno 51, 56127 Pisa, Italy. Phone: +390502211462, Fax: +390502211460, email: plipata@biologia.unipi.it

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Abstract

Adenosine- and uridine-cytidine kinases, purine-nucleoside phosphorylase, hypoxanthine-guanine phosphoribosyltransferase, and several related enzymes, are components of the salvage pathways which reduce the loss of intracellular purine and pyrimidine rings. Although this could explain the role of these enzymes, it poses a problem of the role of the cytosolic 5'-nucleotidase. Why are nucleosides produced from nucleoside-monophosphates, only to be converted back to the same compounds? To date, it is well established that a cross talk exists between the extracellular and intracellular nucleoside metabolism. In districts, such as brain, which are dependent on salvage nucleotide synthesis, nucleosides are produced through the action of the ecto-5'-nucleotidase, the last component a series of plasma-membrane bound enzyme proteins, catalyzing the successive dephosphorylation of released nucleoside-triphosphates. Both nucleoside-triphosphates (mainly ATP and UTP) and nucleosides (mainly adenosine), act as extracellular signals. Once transported into cell cytosol, all nucleosides are salvaged back to nucleoside-triphosphates, with the exception of inosine, whose salvage is limited to IMP. Intracellular balance of nucleosides is maintained by the action of several enzymes, such as adenosine deaminase, uridine phosphorylase and cytidine deaminase, and by at least three 5'-nucleotidases, the ADP activated AMP preferring cN-IA, the ATP-ADP activated IMP-GMP preferring cN-II, and the UMP-CMP preferring cN-III. Here we are reviewing the mechanisms whereby cytosolic 5’-nucleotidases control changes in nucleoside and nucleotide concentration, with the aim to provide a common basis for the study of the relationship between biochemistry and other related disciplines, such as physiology and pharmacology.

Key words: 5’-nucleotidases, cN-IA; cN-II, cN-III, nucleoside utilization, nucleoside recycling

HISTORICAL HINTS AND A BRIEF OVERLOOK

Changes in the concentration of intra- and extracellular nucleosides (NSs) regulate a number of physiological processes in man (e.g. neurotransmission, blood flow, lipolysis [1-6], membrane phospholipid synthesis and synapse generation [7-10]), and brain energetics [11-14]. In most tissues NSs are produced by nucleoside-5'-monophosphates (NMPs) dephosphorylation, an enzyme activity first reported in 1934 by Reis to dephosphorylate AMP and IMP in heart and skeletal muscle [15]. It has long been assumed that, in order to exert their functions, nucleosides should be released from the cells. For instance, adenosine is produced intracellularly by 5'-nuceotidase, and is then transported across the cell membrane by the equilibrative nucleoside transport (ENT) system according to its concentration gradient. However, as discussed by Arch and Newsholme in 1978 in their seminal paper on adenosine metabolism [16], the problem with this hypothesis is the localization of 5'-nucleotidase. Indeed, cytochemical, histochemical, and cell fractionation studies have unequivocally shown that 5'-nucleotidase activity is associated with plasma membrane in many tissues [16-18], and acts as an ecto-enzyme. It was then hypothesized that the nucleotidase hydrolyzes exclusively extracellular NMPs, and NSs are then transported into the cell. However, in 1988 Truong et al. described in rat heart a *cytosolic* 5'-nucleotidase, whose kinetic and regulatory properties differ markedly from those of the plasma membrane associated 5'-nucleotidase [19]. This nucleotidase is highly specific for AMP, with a K_m value in the mM range (vs. the broad specificity and μM K_m range of the membrane bound enzyme) and is *activated* by ADP, but not by ATP [20, 21]. On the contrary the membrane bound enzyme is strongly *inhibited* by both ADP and ATP [22-25]. It has been hypothesized that AMP specific 5'-nucleotidase has a physiological function in adenosine generation during ischemia, when ATP is actively broken down, and AMP accumulates [23-25]. Another important cytoplasmic 5'-nucleotidase, first identified by Itoh in 1967 [26], preferentially dephosphorylates IMP and GMP, and is highly sensitive to ATP *activation* [27-29]. A pyrimidine 5'-nucleotidase, specific for UMP and CMP, is highly expressed in reticulocytes, and is assumed to participate in the process of erythrocyte maturation [30,31]. Other 5'-nucleotidases, with different specificity for natural nucleoside- and deoxynucleoside-5'-monophosphates, have been described in the cytosol and in mitochondria of diverse tissues and organs. As discussed by Bianchi and Spychala [32], "the presence in human genome of at least seven genes for 5'-nucleotidases suggests that these enzymes perform important metabolic functions".

To date it is well established that the plasma membrane bound ecto-5'-nucleotidase is the last component of a signaling cascade that hydrolyses extracellular nucleoside tri-phosphates (NTPs) to NSs [23-25]. Here we are reviewing the mechanisms whereby cytosolic 5'-nucleotidases

control changes in NSs concentration, with the aim to provide a common basis for the study of the relationship between biochemistry and other related disciplines, such as physiology and pharmacology. The nomenclature of cytosolic 5'-nucleotidases reported by Bianchi and Spychala [32] will be used throughout this review.

AMP ACCUMULATES FROM ATP BREAKDOWN AS A SIGNAL OF ISCHEMIC CONDITION, AND IS DEPHOSPHORYLATED BY cN-IA NUCLEOTIDASE

As diagrammed in Fig. (**1**), the purine and the pyrimidine de novo pathway lead to the production of the four main NMPs, AMP, GMP, UMP, and CMP. Interestingly, the main NSs, adenosine (Ado), guanosine (Guo), uridine (Urd), cytidine (Cyd), and inosine (Ino), are neither precursors, nor intermediates, of de novo pathways [33]. To date there is general agreement that dephosphorylation of NMPs, synthesized de novo, is the main source of cytosolic NSs in the liver. NSs are then delivered to extrahepatic tissues, such as brain, that has a very limited de novo synthesis [34-36] and rely on preformed purine and pyrimidine rings, mainly in the form of NSs [37]. Indirect arguments, and the analysis of the kinetics and regulatory properties of AMP preferring cytosolic 5'-nucleotidase (cN-IA) [21, 32, 38-40] suggest that it plays a major role in intracellular Ado formation *at ischemic conditions,* when massive ATP breakdown occurs [23, 41-45].

Figure 1

In normoxic conditions most of the ADP generated by AMP phosphorylation, catalyzed by the highly specific adenylate kinase:

AMP + ATP $\rightarrow$ 2 ADP *adenylate kinase*

and by the relatively specific nucleoside mono- and diphosphate kinases:

(GMP, UMP, CMP) + ATP $\rightarrow$ (GDP, UDP, CDP) + ADP *nucleoside-monophosphate kinase*

(GDP, UDP, CDP) + ATP $\rightarrow$ (GTP, UTP, CTP) + ADP *nucleoside-diphosphate kinase*

enters the mitochondria, to fuel oxidative phosphorylation, thus contributing in maintaining a relatively constant normoxic ATP concentration (~ 5 mM) [16]. The time courses of ATP breakdown and of ATP catabolites formation in "post-mitochondrial" extracts of rat brain [23] (Fig.

2A) strongly suggest that at normoxic ATP levels AMP is catabolized to Ino and hypoxanthine (Hyp) through the successive action of two ATP activated enzyme proteins, AMP deaminase (AMPD) and IMP-GMP preferring 5'-nucleotidase (cN-II), followed by purine nucleoside phosphorylase (PNP) (the so called "IMP pathway" ATP → ADP → AMP → IMP → Ino → Hyp), rather than being dephosphorylated to Ado. The inset of Fig. **(2A)** shows that the production of Ino and Hyp precedes that of the minute amount of Ado, thus excluding a precursor-product relationship between Ado and Ino plus Hyp formation. Moreover, Ado started to be formed only at ATP concentration close to the ischemic levels. It can be hypothesized that in normoxic condition the AMP preferring cN-IA remains silent. At ischemic ATP initial concentrations (Fig. **2B**) we are faced with a different scenario: AMP is catabolized to Ino and hypoxanthine through the successive actions of the ADP activated cN-IA, adenosine deaminase (AdoD), and of PNP (the so called "adenosine pathway" ATP → ADP → AMP → Ado → Ino → Hyp), rather than being deaminated to IMP. It can be hypothesized that in ischemic condition both AMPD and cN-II are de-activated by the low ATP level. In addition, both enzymes are strongly inhibited by the P_i level, reaching a value of 82 mM in ischemic condition [41].

Figure 2

In anoxic conditions the impaired oxidative phosphorylation process, caused by the low pO_2 value, prevents the full recycling of ADP into ATP. ATP is not massively degraded as in no-flow ischemia, and both the IMP pathway and the adenosine pathway are operative, although the amount of IMP formed is considerably higher than that of Ado [23]. And in fact in anoxic heart the contribution of the IMP pathway to ATP breakdown is about threefold higher than that of the adenosine pathway [46].

AMP MODULATES THE ENERGY METABOLISM IN MAN

In 1963 Hans Krebs pointed out that the cellular AMP concentrations change more dramatically than those of ADP and ATP [47]. For a discussion on this important issue see Ref. [48]. One year later Ramaiah, Hathaway, and Atkinson showed that AMP (or the [AMP]/[ATP] ratio) is the main metabolic regulator of yeast phosphofructokinase [49], an enzyme quite similar to those from various animal sources. To date the [AMP]/[ATP] ratio is considered to be a signal of compromised energy status of the cell [48]. AMP "senses" this abnormal condition, and exerts a pivotal effect on the regulation of cellular and whole body metabolism. AMP interacts either

directly with key catabolic regulatory enzyme proteins, or indirectly through an ATP activated protein kinase, named AMPK. AMP binds to the allosteric sites of muscle glycogen phosphorylase, and stabilizes its active conformation [50-52]. The activity of muscle 6-phosphofructokinase is modulated by a number of allosteric regulators, including AMP and ATP. Adenine nucleotides implement their allosteric inhibitory (ATP) and activating (AMP) effects by binding to different allosteric sites [53-56]. During cellular stresses that deplete ATP, and consequently elevate AMP concentration, AMP specifically binds to one of the subunits of AMPK and switches off ATP consuming anabolic pathways, while switching on the ATP producing catabolic pathways, thus restoring the physiological ATP level [57-61] (Fig. **3**).

Figure 3

It may be speculated that fluctuations of AMP concentrations in cell cytosol, modulated by cN-IA and AMPD, may influence AMPK activity [62, 63]. Accordingly, silencing cN-IA in human and mouse muscle increases the [AMP]/[ATP] ratio, activates AMPK and stimulates AMP downstream signalling [64]. Conversely, overexpression of cN-IA decreases AMPK activation [65]. In no-flow ischemia the increased intracellular AMP concentration, due to the massive ATP breakdown and to the inhibition of cN-IA at pH 6.3 activated AMPK. On reflow the physiological pH relieves the inhibition, and physiological low AMP concentration is restored [62]. Other abnormal, pathological stresses, including heat shock, metabolic poisons, glucose deprivation [66, 67], activate AMPK by interfering with decreased ATP *production* from AMP. Conversely physical exercise, a physiological stress [68], activates AMPK by interfering with increased ATP *consumption*, a condition leading to AMP accumulation [48]. Finally, Metformin, the most widely used drug for type II diabetes [69], activates AMPK through inhibition of AMP deaminase [70]. Taken together, these observations suggest that cN-IA and AMPD, two key enzymes of the AMP catabolic pathways, may effectively modulate the energy metabolism in man.

THE IMP-GMP PREFERRING 5'-NUCLEOTIDASE cN-II

The physiological role of cN-II, an ubiquitous enzyme [71-73], has not been clearly defined yet. As a catabolic enzyme, cN-II, as well as cN-III, generate free diffusible nucleosides in the liver, to be delivered to other organs, such as brain, which depend on preformed purine and pyrimidine rings for the synthesis of nucleotides [37]. In early 1980 Guha and Rose showed that brain glucose-1,6-*bis*phosphatase (later shown to correspond to the previously described enzyme phosphomannomutase [74]) is highly sensitive to activation by IMP [75,76]. Glucose-1,6-*bis*phosphate has been identified as a modulator of rate limiting enzymes of glycolysis [75,76].

Thus, it might be hypothesized that fluctuations of IMP levels, modulated by cN-II, might play a role in the control of ATP concentration. Convincing evidence on the role of cN-II in cell viability has been obtained [77]: its wide distribution, its intricate regulatory properties [73, 78-80] and its capacity to transfer the phosphate group of the substrate IMP not only to water (the 5'-nucleotidase activity), but also to the 5' or 3'-OH group of a nucleoside acceptor (the phosphotransferase activity [81, 82]), strongly suggest that cN-II might contribute in maintaining the qualitative and quantitative balance of intracellular purine rings.

THE PURINE NUCLEOTIDE CYCLE AND THE OXYPURINE CYCLE

The purine nucleotide cycle (PNC), first described by Goodman and Lowenstein in 1977 [83] is composed of three reactions, catalyzed by cytosolic adenylate deaminase, adenylosuccinate synthase, and adenylosuccinate lyase (enzymes 4, 5, and 6 of Fig. (**4**)):

$$\text{AMP} + \text{H}^+ + \text{H}_2\text{O} \rightarrow \text{IMP} + \text{NH}_4^+ \qquad \textit{adenylate deaminase}$$

$$\text{IMP} + \text{GTP} + \text{aspartate} \rightarrow \text{S-AMP} + \text{GDP} + \text{Pi} + 2\,\text{H}^+ \qquad \textit{adenylosuccinate synthase}$$

$$\text{S-AMP} \rightarrow \text{AMP} + \text{fumarate} \qquad \textit{adenylosuccinate lyase}$$

The summary equation:

$$\text{GTP} + \text{aspartate} + \text{H}_2\text{O} \rightarrow \text{GDP} + \text{Pi} + \text{fumarate} + \text{NH}_4^+ + \text{H}^+$$

shows that the cycle turns over at the expense of GTP and aspartate.

The PNC is particularly active in skeletal muscle, under conditions associated with increased rate of glycolysis [83, 84]. During exercise, when the velocity of myosin ATPase does not match the capacity of contracting muscle to phosphorylate ADP by glycolysis or oxidative phosphorylation, the concentrations of AMP rise (Fig. **4**), leading to deamination of AMP to IMP, thus to activation of the PNC. Since PNC intermediates, AMP, IMP, and adenylosuccinate (S-AMP) are non-diffusible charged purines, the PNC favours a rapid return of the ATP stores to the resting level after a short oxygen independent bout, e.g., a 100-m sprint [85]. Deficiency of AMPD, one of the enzymes of PNC, is present in 2% of healthy population [86], and is associated with lower exercise performance [87]. During sustained oxygen independent exercise, e.g., a 400-m sprint, or during repeated short-sprint bouts, when the velocity of ATP hydrolysis by myosin ATPase markedly exceeds that of ATP re-synthesis [85, 88-93], IMP accumulates, thus increasing the rate of IMP dephosphorylation by cN-II, and, consequently, the rate of Ino and Hyp production,

which are transported into the bloodstream to be either excreted by the urine or imported into liver, where they are oxidized to urate [94].

Alternatively, IMP may enter an additional purine cycle, named oxypurine cycle (OPC), first described by Barankiewitz in mononuclear cells and fibroblasts in 1982 [95]. The OPC is composed of the following three reactions catalyzed by cN-II, PNP and hypoxanthine-guaninyl phosphoribosyltranserase (HPRT) (enzymes 7, 8, and 9 of Fig. **4**):

$$\text{IMP} + H_2O \rightarrow \text{inosine} + P_i \qquad \textit{cN-II}$$

$$\text{inosine} + \text{Pi} \rightarrow \text{hypoxanthine} + \text{Rib 1-P} \qquad \textit{PNP}$$

$$\text{hypoxanthine} + \text{PRPP} \rightarrow \text{IMP} + PP_i \qquad \textit{HPRT}$$

The summary equation:

$$\text{PRPP} + H_2O \rightarrow \text{Rib-1-P} + PP_i$$

shows that the cycle turns over at the expense of PRPP, a high energy sugar phosphate with a high potential of 5-phosphorybosyl transfer. A widely accepted tenet is that the PRPP pool is maintained at a low level, to avoid excessive and unbalanced nucleotide synthesis. Because the net reaction of the OPC is the hydrolysis of PRPP, OPC has been proposed to play a role in the metabolic regulation of PRPP levels [95-97] (Figure **4**).

Figure 4

As shown in Fig. **5**, while PRPP is being consumed, IMP, Ino, and Hyp attain a steady state level, and are continuously recycled. But as soon as PRPP disappears, HPRT becomes inactive, and the cycle collapses, causing IMP degradation and Hyp accumulation. The cycle has been reconstructed "in vitro" [97], by using commercial HPRT, PNP, and a highly purified preparation of cN-II, cloned and expressed in *Escherichia coli*. The kinetics was strikingly similar to that observed when crude extracts of rat brain were used as an enzyme source (Fig. **5**).

Figure 5

Although the OPC might contribute in maintaining the cytoplasmic concentration of IMP at the expense PRPP, the two unphosphorylated intermediates, Ino and Hyp, are easily transported into the blood stream. Similar cycles, involving AMP, Ado and adenine, or IMP, Ino and Hyp, cannot be

operative in man, owing to the absence of adenosine phosphorylase [98] and inosine kinase [99] in mammals. Finally, it should be noted that the PNC and the OPC are interconnected by IMP, a common intermediate acting as a substrate of S-AMP synthase and of cN-II in the PNC and the OPC, respectively. However, to our knowledge the metabolic features of this issue have not been investigated.

THE FUNCTION OF PYRIMIDINE NMP PREFERRING CYTOSOLIC 5'-NUCLEOTIDASE

The dephosphorylation of cytosolic pyrimidine NMPs to their corresponding nucleosides is catalyzed by pyrimidine 5'-nucleotidase cN-III [100-103]. The enzyme preferentially catalyzes the dephosphorylation of UMP and CMP, although it can act on pyrimidine antineoplastic agents as 5'-AZTMP and 5'-Ara-CMP. A cytosolic isozyme of cN-III (P5' N-II or PN-2, or dNT-1) hydrolyzes the deoxy counterparts of UMP and CMP [104, 105]. The activity of cN-III increases concomitantly with erythrocyte maturation, and is followed by a further continuous decline throughout the ageing of the cell [106], suggesting that it plays a major role in the elimination of pyrimidine nucleosides formed from RNA degradation during erythropoiesis [107]. This hypothesis is in accord with the observed accumulation of pyrimidine nucleotides within the erythrocyte stroma of patients affected by haemolytic anemia caused by cN-III deficiency [108-110]. Human erythrocyte cN-III is identical to p36, an interferon-α-induced protein associated with lupus inclusions [111, 112]. The significance of the identity of the two proteins is at the moment obscure. The mRNA turnover releases, in addition to the four regular nucleoside monophosphates, the methylated cap nucleotide in the form of 7-methylguanosine monophosphate (m(7)GMP) or diphosphate (m(7)GDP). A novel 5'-nucleotidase recently identified in Drosophila and in man preferentially cleaves UMP and m(7)GMP to uridine and 7-methylguanosine, respectively [113]. cN-III plays an important role also in the balance of Urd. At least in neuronal and glial cells Urd homeostasis is maintained by the concerted action of UrdK, UPase, and cN-III. The rationale of the underlying molecular mechanism is the following. Urd enters neuronal and glial cells via the Na^+-dependent, high affinity, concentrative nucleoside transporter (CNT) proteins or the equilibrative nucleoside transporter (ENT) proteins [114]. The balance between its anabolism and catabolism is maintained by UrdK, catalyzing the first step of UTP and CTP salvage synthesis, UPase, catalyzing the first step of uridine degradation, and by the inhibition exerted on UrdK by high levels of UTP and CTP, a signal of pyrimidine sufficiency [115]. The simultaneous activities of cN-III and uridine kinase might

generate a substrate cycle between UMP and uridine, similar to the AMP-adenosine cycle [16, 116, 117] (Fig. **6**).

Figure 6

NUCLEOSIDE RECYCLING IN THE BRAIN

The rate limiting reactions of nucleotide synthesis are modulated by intracellular fluctuations of NMP, NDP, and NTP concentrations. This topic has been mostly studied at the level of the de novo nucleotide synthesis from simple precursors, mainly in liver [34]. However, there are districts, such as brain, which rely more heavily on the salvage of preformed purine and pyrimidine rings, in the form of nucleosides [37]. This raises the following question: how does brain maintain the right balance between the purine and pyrimidine pools? We believe that it is now safe to state that a cross talk exists between the extra- and intracellular metabolism of purine (Fig. **7**) and pyrimidine NSs (Fig. **8**) in the brain. This interplay occurs between the extracellular milieu and the cytosol of a single cell, or between adjacent cells, e.g. between two astrocytes [118] or between an astrocyte and a neuron [119]. ATP and UTP, two important signalling molecules, are present in very small cytosolic vesicles and/or granules, at a concentration much higher than in the surrounding cytosol [25]. The low specificity of the vesicular nucleoside transporters may explain the presence of additional nucleoside triphosphates, such as GTP [25] and possibly CTP [120] for which a physiological function as extracellular messengers has not been identified.

Figure 7

Figure 8

Extracellular nucleoside generation. The actions of ATP and UTP at their respective receptors are terminated by rapid degradation, catalyzed by the ecto-enzyme cascade system, whose last products are Ado and Urd, respectively (Figs. **7** and **8**). The time courses of ATP breakdown and ATP catabolites formation, as well as of other NTPs, catalyzed by purified striatal cholinergic synaptosomes [121] and by brain plasma membrane preparation [23, 122] showed that the main regulatory site of extracellular NS generation is the ecto-5'-nucleotidase, the last enzyme of the ectonucleotidase cascade system. This enzyme is highly sensitive to the "feed-forward" competitive inhibition exerted by NTPs, the initial substrates [22, 23, 25]. It might be speculated that at elevated extracellular nucleoside triphosphate concentration, a signal of purine and pyrimidine sufficiency,

extracellular NMPs accumulate, rather than NSs. NSs become available to be transported into astrocytes or neurons by the ENT and CNT systems, and/or delivered into blood stream, when a sufficiently low NTPs concentration is reached, to relieve the inhibition of the ecto-5'-nucleotidase.

Intracellular nucleoside metabolic network: *adenosine*. Brain cytosol almost quantitatively anabolyzes Ado at 1 μM initial concentration to AMP, ADP, and ATP, while 100 μM Ado is mainly catabolized to Ino and Hyp [23]. Most likely Ado is maintained in the lowest μM level among the other nucleosides [123] by the relative extent of saturation of AdoK (enzyme 3 of Fig. (**7**)) and AdoD (enzyme 1 of Fig. **7**), whose K_m values for Ado are about 0.2 μM and 50 μM, respectively. Thus, the kinase becomes saturated with Ado at low micromolar concentration, and phosphorylates Ado at its V_{max}. Any further increase of uptaken Ado is irreversibly deaminated to Ino [37].

Guanosine and inosine. Contrary to Ado, Guo and Ino cannot be phosphorylated at their 5'-position by specific kinases, which are absent in the cytosolic compartment of mammals. They undergo prior phosphorolysis to Hyp and Gua before being converted to their respective NMPs by HPRT (enzyme 12 of Fig. **7**).

Uridine and cytidine. Urd homeostasis is maintained by UrdK (enzyme 1 of Fig. **8**), UPase (enzyme 10 of Fig. **8**), and by cN-III (enzyme 4 of Fig. **8**), which counteracts the activity of UrdK. Cyd is salvaged as such via UrdK, or as Urd, after deamination by Cyd deaminase (9).

CONCLUSIONS

In most textbooks cytosolic 5'-nucleotidases are considered as pure catabolic enzymes, catalyzing the entry step of purine and pyrimidine nucleotide degradation. New insights into their importance in human metabolism followed the recognition of cN-II hyperactivity in the erythrocytes of Lesch-Nyhan syndrome patients [124]. More recently, it has been shown that activating mutations in cN-II gene *NT5C2* are associated with increased nucleoside-analog metabolism and chemotherapy resistance in acute lymphoblastic leukemia [125]. Finally, it should be emphasized that cytosolic 5'-nucleotidases may also have a role in the formation of the age-and gender-modulated, regionally different nucleoside level in the brain, with implications for the therapy of many metabolic disorders, in which nucleosides are involved [126]. We hope that the studies described in this review, which is mainly devoted to non experts in the field, will convince the reader that cytosolic 5'-nucleotidases play a major role in maintaining the qualitative and quantitative balance of

intracellular purine and pyrimidine rings, in nucleotide salvage synthesis, and nucleoside homeostasis and recycling.

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ABBREVIATIONS

Ado	=	Adenosine
AdoD	=	Adenosine deaminase
AdoK	=	Adenosine kinase
AMPD	=	AMP deaminase
AMPK	=	AMP activated protein kinase
5'-Ara-CMP	=	5'-ArabinofuranolylCMP
5'-AZTMP	=	5'-AzidoTMP
cN-IA	=	AMP preferring 5'-nuclotidase
cN-II	=	IMP-GMP preferring 5'-nucleotidse
cN-III	=	Uridine-Cytidine preferring 5'-nucleotidase
CNT	=	Concentrative nucleoside transporters
Cyd	=	Cytidine
ENT	=	Equilibrative nucleoside transporters
Gua	=	Guanine
Guo	=	Guanosine

Hyp	=	Hypoxanthine
HPRT	=	Hypoxanthine-guanine phosphoribosyltransferase
Ino	=	Inosine
m(7)GMP	=	7-Methylguanosine monophosphate
NDP	=	NucleosideDiPhosphate
NMP	=	NucleosideMonoPhosphate
NTP	=	NucleosideTriPhosphate
NS	=	Nucleosides
OPC	=	OxyPurine Cycle
PNC	=	Purine Nucleotide Cycle
UK	=	Uridine-Cytidine kinase
UPase	=	Uridine phosphorylase
Ura	=	Uracil
Urd	=	Uridine
UrdK	=	Uridine kinase

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Figure **1**. Origin, utilization, and recycling of nucleosides. The dephosphorylation by liver cytosolic 5’-nucleotidases of nucleoside-monophosphates, synthesized de novo in the liver, is the main source of diffusible free nucleosides. Once taken up from the blood into several cell types, including neurons, glial cells, and erythrocytes, that rely on circulating preformed purine and pyrimidine rings rather than de novo synthesis from simple precursors, nucleosides are salvaged to their respective nucleoside-triphosphates by three successive phosphorylation steps, catalyzed by relatively specific kinases. Several allosterically modulated cytosolic 5'-nucleotidases counteract the activity of nucleoside kinases, thus contributing in maintaining AMP, GMP, UMP, and CMP in the proper quantitative and qualitative balance. Finally, nucleosides are generated extracellularly, by the action of an ecto-5'-nucleotidase, the last enzyme of an ecto-enzyme cascade system on exported nucleoside triphosphates. 5’-nulceotidase (1), nucleoside kinase (2), nucleoside monophosphokinase (3), nucleoside diphosphokinase (4), ecto-NTP diphosphohydrolase (5 and 6); ecto-5′-nucleotidase (7).

Figure **2**. Pathways of intracellular ATP catabolism and stack plots of the time courses of ATP breakdown and ATP catabolites formation in rat brain post mitochondrial extracts. (A): at normoxic condition, the strong activation exerted by the high ATP level on adenylate deaminase and cN-II favours the so called IMP pathway: IMP, rather than AMP accumulates. Moreover, as shown in the inset, any Ado formation is preceded by Ino and Hyp formation, thus excluding a precursor-product relationship between Ado and Ino plus Hyp. (B) At ischemic ATP level, the impaired oxidative phosphorylation favours the activation of cN-IA by ADP, and thus the so called adenosine pathway: AMP, rather than IMP accumulates. The enzymes participating in these pathways are: 1, ATPase; 2, adenylate kinase; 3, AMPD; 4, cN-II; 5, PNP; 6, cN-IA; 7 AdoD. Modified from Barsotti and Ipata [23].

Figure **3**. Model for the action of cN-IA and AMPD in regulating energy metabolism. Normally, energy demand increases ADP concentration (1), and consequently the flow of adenylate kinase towards AMP (2). Binding of AMP activates muscle glycogen phosphorylase (3), muscle and liver 6-phosphofructokinase (4), and AMPK-P (5), the phosphorylated form of AMPK. Phosphorylation and dephosphorylation of AMPK are catalyzed by LKB1 (6) and AMPK-P phosphatase (7), respectively. cN-IA (8), and AMPD (9) modulate the level of intracellular AMP, and thus the energetic cell metabolism.

Figure **4**. The role of PNC and OPC in energy metabolism of the cell. In A, the rate of ADP phosphorylation by glycolysis or oxidative phosphorylation (2) matches the rate of ATP

consumption by ATPase (1) (as in mildly contracting muscle). As a consequence, the intracellular ATP concentration remains constant. In B, an aliquot of the ADP pool is not tightly recycled (as in sustained contraction), and is degraded to IMP by the successive action of adenylate kinase (3) and AMPD (4). The PNC, composed of AMPD, S-AMP synthetase (5), and S-AMP lyase (6) maintains the purine ring in phosphorylated non diffusible forms, thus favouring a rapid ATP replenishment at rest, during recovery. In C, an IMP aliquot, which is not recycled by the PNC (as in strenuous contraction), enters the OPC, composed of cN-II (7), PNP (8) and HPRT (9), and is broken down to non phosphorylated purines. An aliquot of the two diffusible non phosphorylated purines, Hyp and Ino, enters the blood stream and is excreted by urine.

Figure **5**. The OPC in action. The initial reaction mixture contained 100 µM Ino, 5 mM Pi, 2 mM PRPP, 8.3 mM Mg^{2+}, and 1.15 mg/ml proteins of cytosolic brain extract, at pH 7.4. During PRPP consumption (dotted line), the three components of the cycle, Ino, Hyp, and IMP, attain a steady-state level, suggesting that they are continuously recycled. However, after PRPP is massively consumed, the cycle collapses, IMP is broken down, and Ino and Hyp accumulate.
Modified from Ref. 97

Figure **6**. The role of the UPase-UK-cN-III system on the reciprocal regulation of purine and pyrimidine salvage synthesis in brain. The rationale stands i) on the widely accepted idea that pyrimidine salvage occurs at the nucleoside level (mostly Urd), while purine salvage is a PRPP-mediated process, occurring at the nucleobase level, and ii) on the modulation exerted by UTP and CTP, the final products of the pyrimidine salvage synthesis, on UK. Relatively high cytosolic UTP and CTP levels, a signal of pyrimidine sufficiency, inhibit UK, shifting the equilibrium of UPase towards Urd phosphorolysis. The substrate cycle UMP-Urd-UMP is no longer operative, and any UMP is catabolized to uracil, rather than being recycled. Rib-1-P is then transformed into PRPP, which is used for purine salvage synthesis. On the contrary, at relatively low UTP and CTP levels, uptaken Urd is mainly anabolized to uracil- and cytosine-nucleotides. cN-III counteracts the activity of UK, thus contributing to the homeostatic regulation of Urd. The enzymes participating in the pathway are: UK (1); UPase (2); NS monophosphokinase (3); NS diphosphokinase (4); CTP synthetase (5); cN-III (6); phosphoribomutase (7); PRPP synthetase (8); HPRT and APRT (9 and 10), adenylate kinase (11).

Figure **7**. Cross talk between extra- and intracellular milieu for purine nucleoside recycling in brain. ATP and GTP are released from cytosolic vesicles into the extracellular space and broken down to Ado and Guo, respectively, by ectonucleotidase. Ado, once taken up, is phosphorylated at its 5′

position via AdoK. The AMP product is phosphorylated by adenylate kinase. Any excess Ado is deaminated by AdoD. Guo is salvaged as Gn via PRPP-mediated HPRT. Two relatively specific kinases, NS monophosphokinase and NS diphosphokinase, catalyze the successive phosphorylation of GMP to GTP. The enzymes participating in these pathways are: AdoD (1); PNP (2); AdoK (3); adenylate kinase (4); AMP preferring 5'-nucleotidase cN-IA (5); ecto-NTP diphosphohydrolase (6 and 8); ecto- NS pyrophosphatase diphosphohydrolase, (7); ecto-5′-nucleotidase (9); phosphoribomutase (10); PRPP synthetase (11); HPRT (12); NS monophosphokinase (13); NS diphosphokinase (14); IMP-GMP preferring 5'-nucleotidase cN-II (15). Cylinders represent the NS transporters CNT or ENT. $P2Y_2$ $P2Y_4$, $P2X_7$ and $P2Y_{11}$ represent subtypes of the P2Y and P2X family receptors.

Figure **8**. Cross talk between extra- and intracellular milieu for pyrimidine nucleoside recycling in brain. UTP and CTP are released from cytosolic vesicles into the extracellular space and broken down to Urd and Cyd, respectively, by ecto-nucleotidase. Once taken up, Urd and Cyd are phosphorylated at their 5′ position via Urd-Cyt kinase. UMP and CMP are either converted back to Urd and Cyd, or phosphorylated by NS monophosphokinase and NS diphosphokinase, respectively. Cyd, which is transported less efficiently than Urd, may be salvaged as Urd after deamination by Cyd deaminase. Any excess Urd undergoes phosphorolysis by Urd phosphorylase.The enzymes participating in these pathways are : Urd-Cyt kinase (1); NS monophosphokinase (2); NS diphosphokinase (3), UMP-CMP preferring 5'-nucleotidase (cN-III) (4); ecto-NTP diphosphohydrolase (5 and 7), ecto-NS pyrophosphatase diphosphohydrolase (6); ecto-5′-nucleotidase (8); Cyd deaminase (9); Urd phosphorylase (10); UDPG pyrophosphorylase (11). Cylinders represent the NS transporters CNT or ENT. UDPG, Urd diphosphoglucose; G 1-P, glucose-1-P. $P2Y_2$ $P2Y_4$, $P2Y_6$ represent subtypes of the P2Y family receptors.

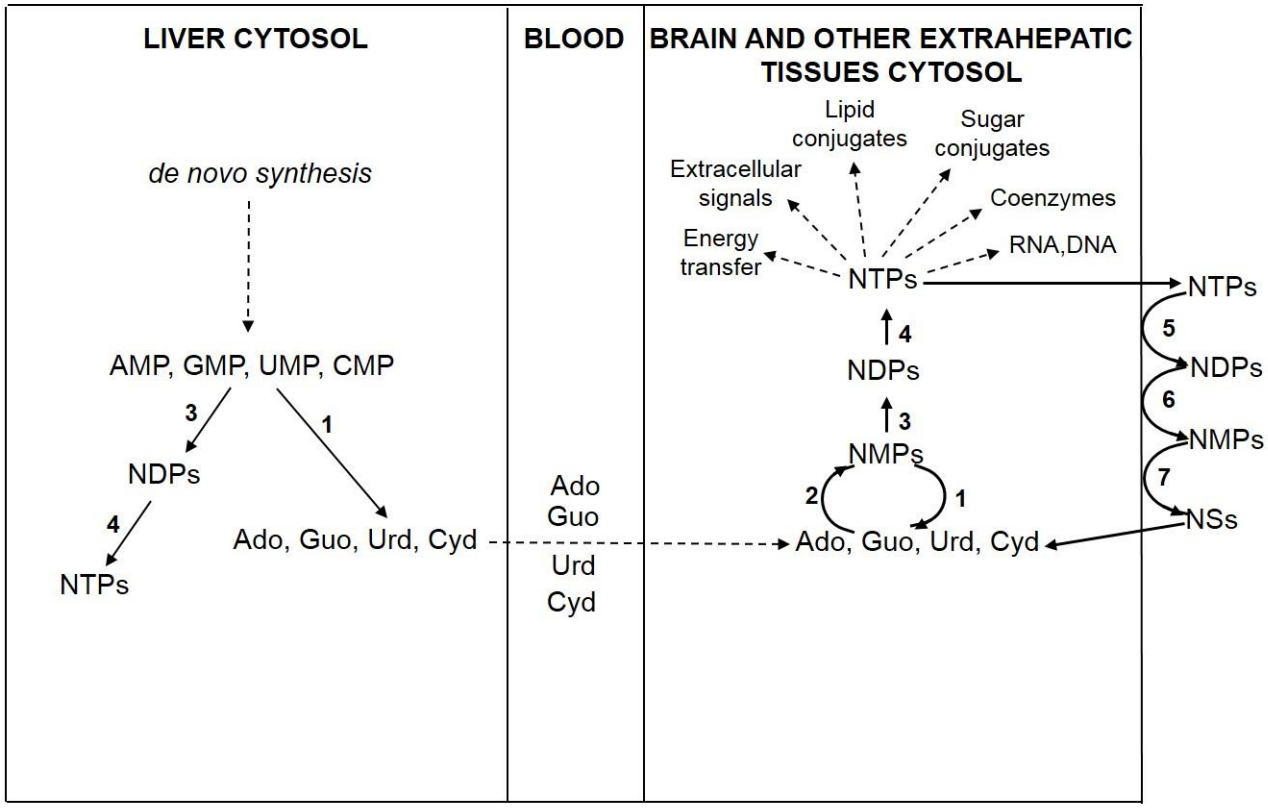

LIVER CYTOSOL
BLOOD
BRAIN AND OTHER EXTRAHEPATIC TISSUES CYTOSOL
de novo synthesis
AMP, GMP, UMP, CMP
3
1
NDPs
4
NTPs
Ado, Guo, Urd, Cyd
Ado
Guo
Urd
Cyd
Lipid conjugates
Sugar conjugates
Extracellular signals
Coenzymes
Energy transfer
RNA,DNA
NTPs
4
NDPs
3
NMPs
2
1
Ado, Guo, Urd, Cyd
NTPs
5
NDPs
6
NMPs
7
NSs

Figure 1

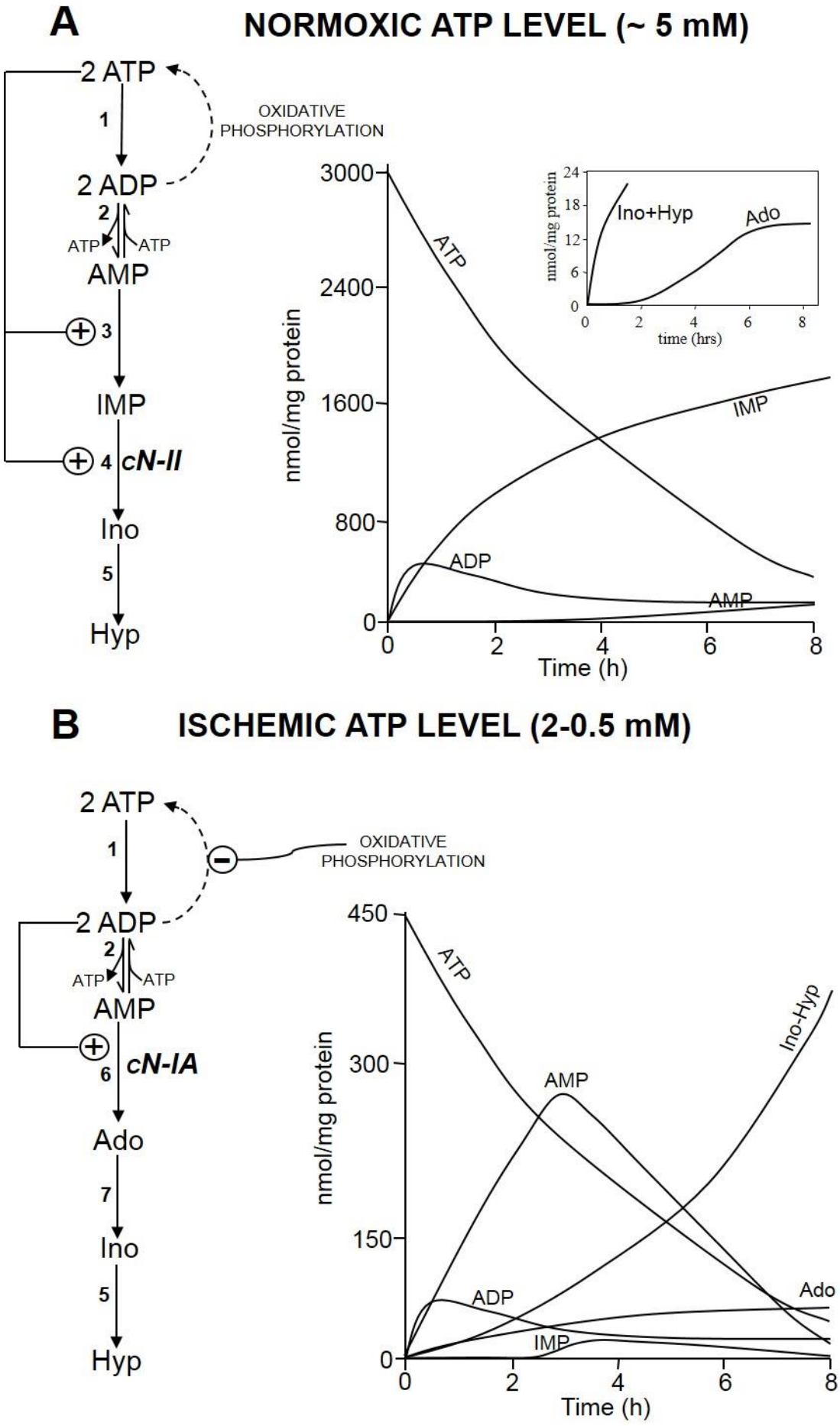

A
NORMOXIC ATP LEVEL (~ 5 mM)
2 ATP
1
OXIDATIVE
PHOSPHORYLATION
2 ADP
2
ATP
ATP
AMP
3
IMP
4
cN-II
Ino
5
Hyp
nmol/mg protein
Time (h)
ATP
IMP
ADP
AMP
Ino+Hyp
Ado
time (hrs)
B
ISCHEMIC ATP LEVEL (2-0.5 mM)
2 ATP
1
OXIDATIVE
PHOSPHORYLATION
2 ADP
2
ATP
ATP
AMP
6
cN-IA
Ado
7
Ino
5
Hyp
nmol/mg protein
Time (h)
ATP
AMP
Ino-Hyp
ADP
Ado
IMP

Figure 2

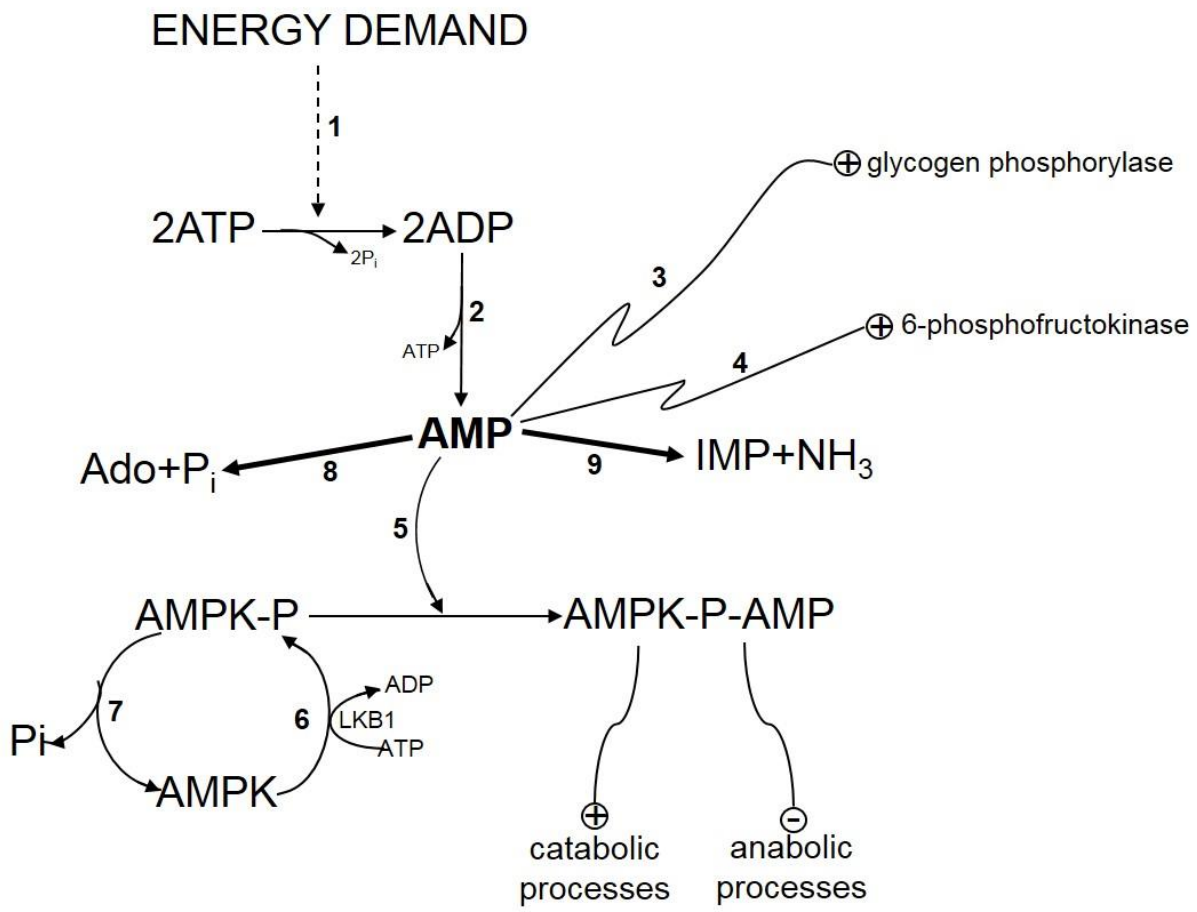

ENERGY DEMAND
1
2ATP
2Pi
2ADP
2
ATP
AMP
3
glycogen phosphorylase
4
6-phosphofructokinase
Ado+Pi
8
9
IMP+NH3
5
AMPK-P
AMPK-P-AMP
7
Pi
6
ADP
LKB1
ATP
AMPK
catabolic processes
anabolic processes

Figure 3

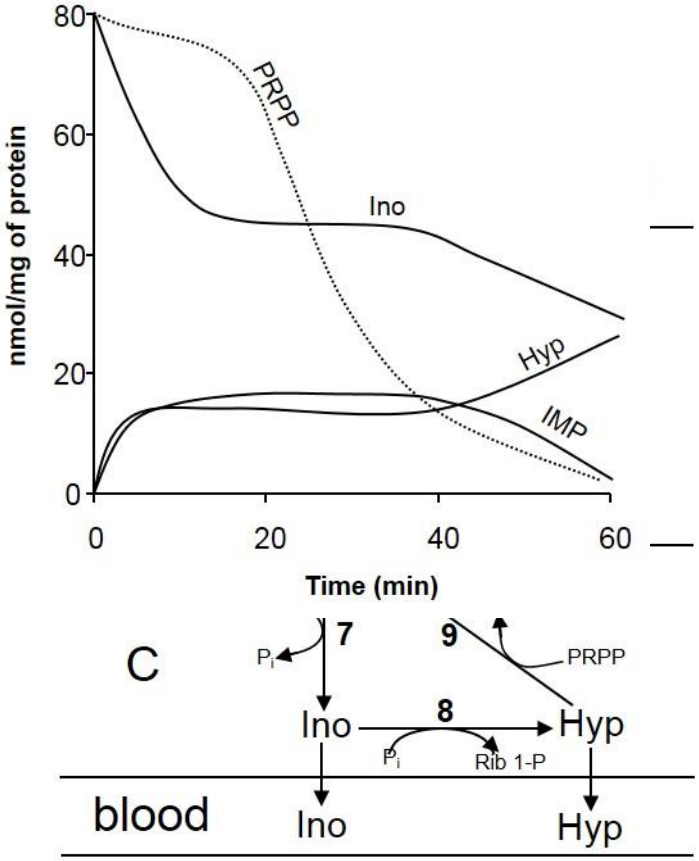

80
60
40
20
0
nmol/mg of protein
PRPP
Ino
Hyp
IMP
0
20
40
60
Time (min)
C
7
9
PRPP
Ino
8
Hyp
Rib 1-P
blood
Ino
Hyp

Figure 4

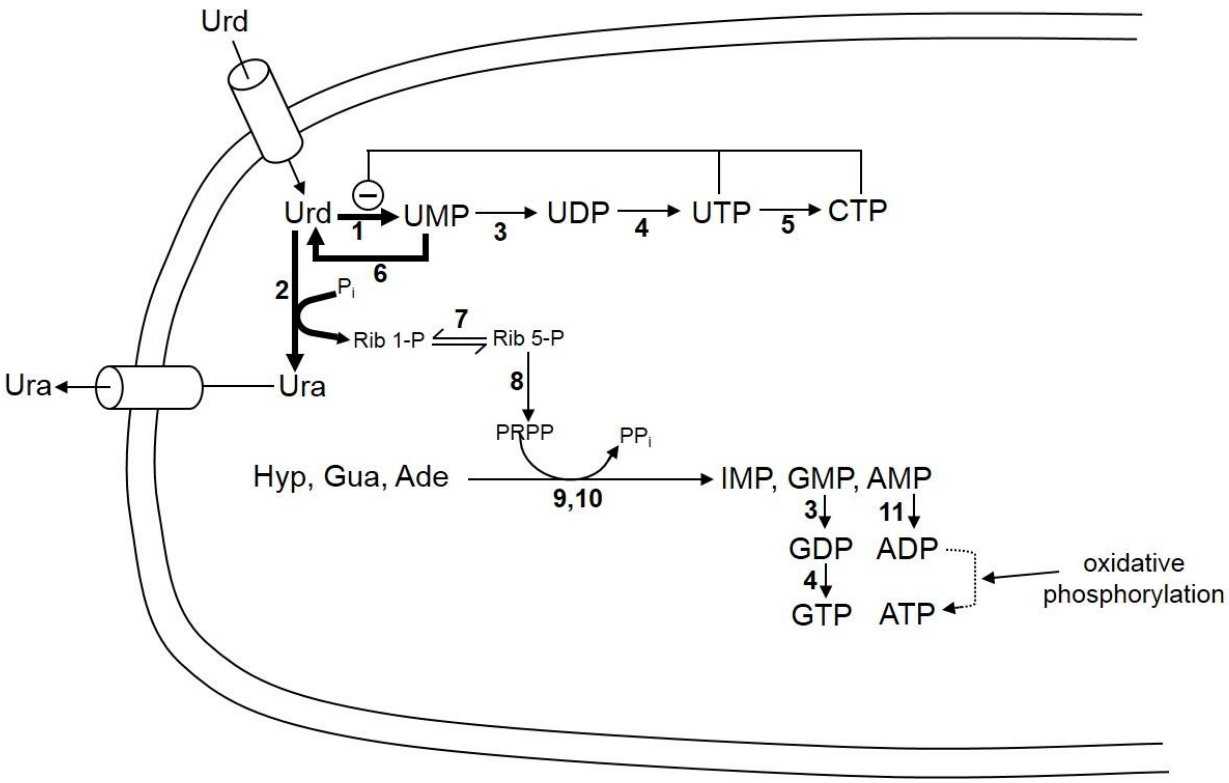
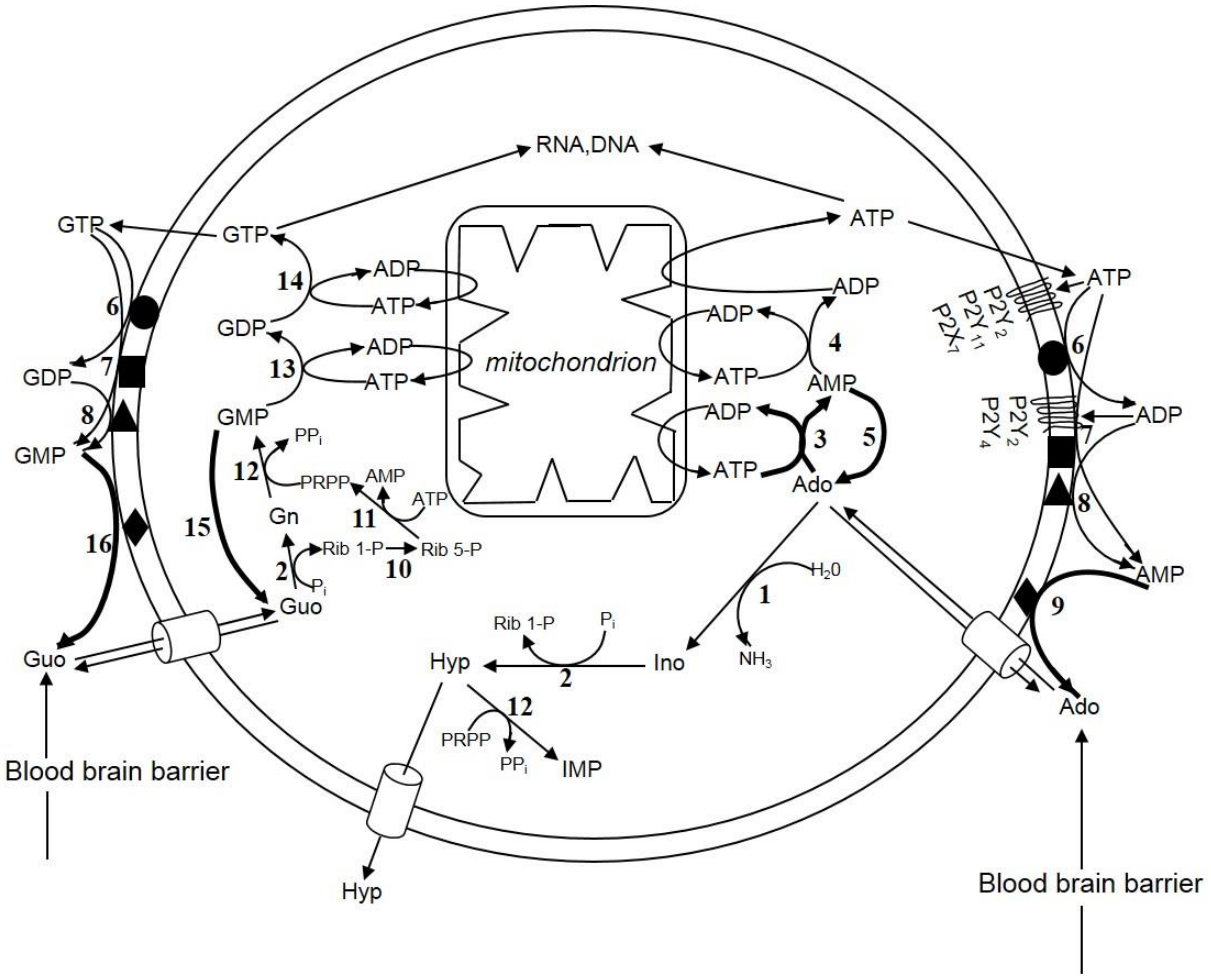

Urd
Urd
UMP
UDP
UTP
CTP
1
3
4
5
6
2
Pi
Rib 1-P
7
Rib 5-P
8
Ura
Ura
PRPP
PPi
Hyp, Gua, Ade
9,10
IMP, GMP, AMP
3
11
GDP
ADP
4
GTP
ATP
oxidative
phosphorylation

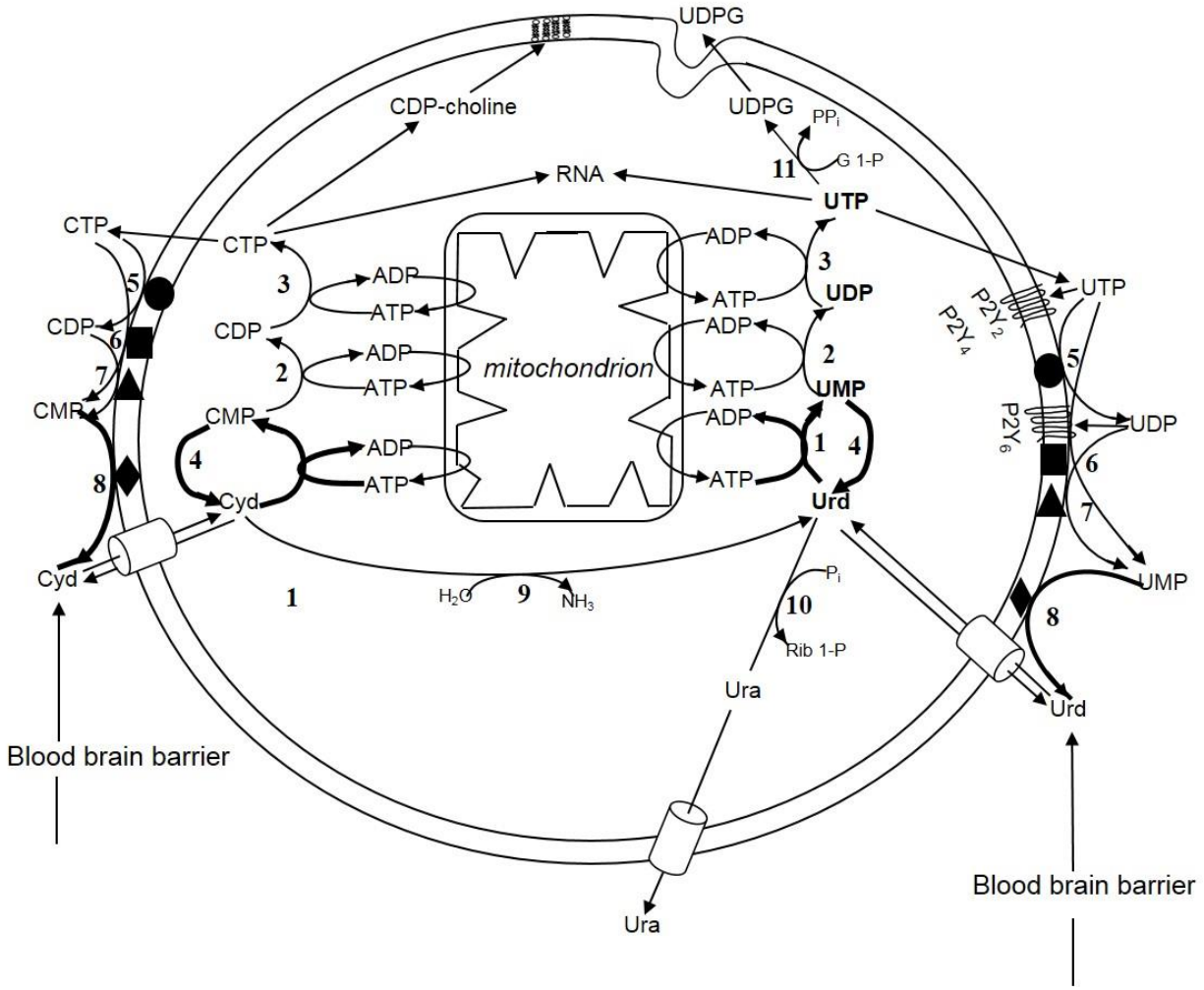
Figure 5

Figure 6

Figure 7


RNA,DNA
ATP
GTP
GTP
ADP
ATP
14
GDP
ADP
ATP
13
mitochondrion
ADP
ADP
ATP
4
AMP
ADP
3
5
ATP
Ado
GMP
PPi
12
PRPP
AMP
ATP
11
Gn
Rib 1-P
Rib 5-P
10
2
Pi
Guo
15
16
6
7
8
GDP
GMP
Guo
Blood brain barrier
Rib 1-P
Pi
Hyp
2
Ino
12
PRPP
PPi
IMP
Hyp
H20
1
NH3
ATP
P2Y2
P2Y11
P2X1
6
P2Y2
P2Y4
7
ADP
8
AMP
9
Ado
Blood brain barrier

1


UDPG
CDP-choline
UDPG
PPi
G 1-P
11
RNA
UTP
CTP
CTP
ADP
3
ADP
ATP
ATP
UDP
CDP
CDP
ADP
ADP
2
ATP
mitochondrion
ATP
UMP
CMP
CMP
ADP
ADP
1
4
4
ADP
ATP
ATP
Urd
Cyd
Cyd
1
H2O
9
NH3
Pi
10
Rib 1-P
Ura
Ura
UTP
P2Y2
P2Y4
UDP
P2Y6
UMP
Urd
Blood brain barrier
Blood brain barrier

2

3 Figure 8