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“ROLE OF PENTOSE PHOSPHATES IN NUCLEOSIDE CATABOLISM AND INTERCONVERSION”

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ABSTRACT

Purine and pyrimidine are the basic constituents of the polynucleotides DNA and RNA; they are considered of predominant importance also as information molecules, energy transducers, antioxidants and they have also important roles in cellular signalling processes. Reports suggest protective roles for purines and pyrimidines in various pathological conditions ranging from cancer, to ischemia-associated injury, traumatic tissue damage, bone resorption, stress and haemorrhagic shock. Mitochondrial oxidative phosphorylation, along with glycolysis, is essential for the maintenance of brain ATP levels. The depletion of cellular energy sources and reduction of ATP synthesis during ischemia or brain insults cause the loss of cellular homeostasis, which eventually leads to irreversible cellular damage. Some papers have shown that adenosine and inosine protect neural cells during hypoxia/ischemia *in vivo* and *in vitro*. While in some cases the action of adenosine is receptor-mediated, to explain the effect of its deamination product, inosine, the contribution of hitherto unknown specific receptors has been invoked. On the other hand, several papers report a receptor-independent mechanism of nucleoside action, which indicates that nucleosides donate their ribose moiety to the pentose phosphate pathway, where it is converted to intermediates for entry into the glycolytic pathway for anaerobic production of ATP.

To shed light on the mechanism underlying the protective role of purine and pyrimidine during ischemia or brain insults, we have used a human astrocytoma cell line (ADF) which has been subjected to metabolic stress conditions by exclusion of glucose and pre-incubation with oligomycin (an inhibitor of oxidative phosphorylation). This treatment brings about a depletion of the ATP pool, with a concomitant increase in the AMP levels, which results in a significant decrease of the adenylate energy charge. The presence of purine nucleosides in the culture medium preserves the adenylate energy charge, and improves cell viability. Besides purine nucleosides, also pyrimidine nucleosides, such as uridine and, to a lesser extent, cytidine, are able to preserve the ATP pool. The determination of lactate in the incubation medium indicates that nucleosides can preserve the ATP pool through anaerobic glycolysis, thus pointing to a relevant role of the phosphorolytic cleavage of the N-glycosidic bond of nucleosides which generates, without energy expense, the phosphorylated pentose, which through the pentose phosphate pathway and glycolysis can be converted to energetic intermediates also in the absence of oxygen. In fact, ADF cells possess both purine nucleoside phosphorylase and uridine phosphorylase activities. The results of this research line have been published in Neurochem. Int. 50: 517-523 (2007) (see attached).

Additionally, the phosphorylated ribose moiety of nucleosides may be used itself for the salvage of pyrimidine nucleosides or can be converted by 5-phosphoribosyl-1-pyrophoshate (PRPP) synthetase, into PRPP which is an essential compound for the salvage pathway of purines.

In fact, adenine and hypoxanthine, in the presence of PRPP, are substrates of adenine phosphoribosyltransferase (APRT) and hypoxanthine-guanine phosphoribosyltransferase (HPRT), which catalyze the formation of adenosine and inosine monophosphate, respectively (purine salvage pathway).

On the other hand, uridine can be both phosphorolytically cleaved by uridine phosphorylase (UPase) to ribose-1-phosphate (Rib-1-P) and the uracil base, or can be converted by uridine kinase (UK) into uridine nucleotides (pyrimidine salvage pathway). It has been shown that disruption of uridine homeostasis by deletion of rat UPase gene leads to disorders of both pyrimidine and purine nucleotide synthesis, thus suggesting a linkage between the two metabolic processes. We have hypothesized that the UPase–UK enzyme system, which maintains uridine homeostasis, regulates the processes of both purine and pyrimidine salvage. Exogenous uridine and, to a lesser extent inosine, activate the salvage of exogenous adenine in human astrocytoma cells in a concentration-dependent manner. Moreover, uridine is also able to activate the salvage of exogenous hypoxanthine. When uridine and inosine are present, more Rib-1-P becomes available, through the action of UPase and purine nucleoside phosphorylase (PNP), for the PRPP-mediated adenine and hypoxanthine salvage. Exogenously added adenine partially inhibits uridine salvage; in fact, the increased utilization of PRPP exerted by added adenine, might further favour uridine phosphorolysis, thus subtracting the pyrimidine nucleoside to the UK action. Moreover, exogenous inosine favours not only uracil salvage but also 5-FU activation through a Rib-1-P-mediated process. The pre-treatment of the cells with cytidine brings about an inhibition of the pyrimidine salvage and an activation of the purine salvage in the presence of uridine as ribose phosphate donor. In fact, cytidine enters the cells and is converted into CTP which inhibits UK and this inhibition causes a shift of the equilibrium of the reversible UPase reaction towards uridine phosphorolysis. The Rib-1-P formed is then converted into PRPP, which is used for the purine salvage synthesis. Conversely, when the concentration of CTP is relatively low, the fully active UK, which catalyzes a virtual irreversible reaction, drives uridine towards uridine nucleotide formation, thus lowering the rate of purine synthesis. Therefore, the ribose phosphate stemming from the phosphorolysis of purine and pyrimidine nucleosides not only can be converted into energetic intermediates in order to restore the ATP pool during cellular stress but it can be considered a link between the purine and pyrimidine salvage and these two processes are regulated at the level of UPase–UK enzyme system by the relative pyrimidine nucleoside triphosphate concentration. The results of this research line have been published in Neurochem. Int. 51: 517-523 (2007) (see attached).

ABBREVIATIONS

5-(phenylthio) acyclouridine (PTAU)

5-phosphoribosyl-1-pyrohosphate (PRPP)

5- fluorouracil (5-FU)

8-cyclopentenyl-1,3-dipropylxanthine (DPCPX)

Adenosine (Ado)

Adenine (Ade)

Adenine phosphorybosiltransferase (APRT)

Adenosine 5’-diphosphate (ADP)

Adenosine 5’-monophosphate (AMP)

Adenosine 5'-triphosphate (ATP)

Adenosine deaminase (ADA)

Adenosine kinase (AK).

Adenosine phosphoribosyltransferase (APRT)

Adenosine receptors (ARs)

Astrocyte-neuron-lactate shuttle (ANLSH)

Blood-brain barrier (BBB)

Carbamoyl phosphate II (CPII)

Central nervous system (CNS)

Citric acid cycle (TCA)

Cyclic AMP (cAMP)

Cyclic GMP (cGMP)

Cytidine (Ctd)

Cytosine (Cyt)

Cytidine 5’-monophosphate (CMP)

Cytidine 5’-diphosphate (CDP)

Cytidine 5’-triphosphate (CTP)

Cytidine kinase (CK)

Cytosolic 5’-nucleotidase type II (cN-II)

Concentrative nucleoside transporters (CNT)

Deoxyadenosine (dAdo)

Deoxycytidine monophosphate (dCMP)

Deoxycoformycin (dCF)

Deoxyinosine (dIno)

Deoxyribonucleic acid (DNA)

Deoxythimidine monophosphate (dTMP)

Dihydropyrimidine dehydrogenase (DPD)

Dopamine receptors (DRs)

Dulbecco's modified Eagle medium without glucose (DMEM)

Dulbecco's phosphate buffered saline (PBS)

Ecto-nucleoside triphosphatase (E-NTPase)

Equilibrative nucleoside transporters (ENT)

Flavin adenin dinucleotide (FAD)

Fibroblast growth factor (FGF)

Foetal bovine serum (FBS)

Glial-derived neurotrophic factor (GDNF)

Glyceraldehyde 3-P (Gly 3-P)

Glucose (Glc)

Glucose transporter (GLUT)

Glutathione (GSH)

G protein-coupled membrane receptors (GPCRs)

Guanine (Gua)

Guanosine (Guo)

Guanosine 5'-monophosphate (GMP)

Guanosine 5'-triphosphate (GTP)

High Pressure Liquid Chromatography (HPLC)

Hypoxanthine (Hyp)

Hypoxanthine-guanine phosphoribosyl transferase (HGPRT)

Human astrocytoma cell line (ADF)

Inosine (Ino)

Inosine monophosphate (IMP)

Inosine nucleoside cycle (INC)

Interleukin-6 (IL-6)
Lactate dehydrogenase (LDH)
LDH-5 (lactate dehydrogenase-5)
Lesch-Nyhan syndrome (LNS)
Mitochondrial neurogastrointestinal encephalomyopathy (MNGIE)
Mitogen-activated protein kinases (MAPK)
Monocarboxilate transporter (MCT)
Nerve growth factor (NGF)
Neurite growth-promoting factors 1 (NEGF1)
Nicotinamide adenine dinucleotide (NAD)
Nicotinamide adenine dinucleotide phosphate (NADP)
Nitric oxide (NO)
Nitric oxide synthase (iNOS)
Nitrobenzylthioinosine (NBTI)
N-methyl D-aspartate (NMDA)
Orotidine 5'-monophosphate (OMP)
Phosphatidylcholine (PC)
Phosphatidylethanolamine (PE)
Phosphoribosyltransferase (OPRT)
Protein kinase A (PKA)
Protein kinase C (PKC)
Purine nucleoside phosphorylase (PNP)
Purinergic receptors (P receptors)
Ribonucleic acid (RNA)
Ribose (Rib)
Ribose-1-phosphate (Rib1P)
Ribose 5-phosphate (Rib5P)
Reactive oxygen species (ROS)
Thymidine 5'-monophosphate (TMP)
Thymidine phosphorylase (TP)
Total adenine nucleotide (TAN)
Total uracil nucleotide (TUN)

Tumor necrosis factor (TNF)

Uracil (Ura)

Uridine (Urd)

Uridine diphosphoglucose (UDPG)

Uridine 5'-diphosphate (UDP)

Uridine 5'-monophosphate (UMP)

Uridine 5'-triphosphate (UTP)

Uridine monophosphate hydrolase (UMPH)

Uridine monophosphate synthase (UMPS)

Uridine kinase (UK)

Uridine phosphorylase (UPase)

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CHAPTER 1

INTRODUCTION

1 Astrocyte cells: morphology, physiology and metabolism

1.1 Cells types in the central nervous system

The central nervous system (CNS) is composed of a variety of different cell types, which depend on each other for survival and functional integrity, and have the capacity for intercellular communication.

The mammalian CNS consists of two main cell types, neurons and glia. Neurons are directly involved in electrical transmission and information processing, and glia carry out many indispensable functions, both in development and during the normal function of the mature CNS.

Glial cells are numerically the dominant cell type in the central and peripheral nervous system. They are intermixed with the nerve cells and are found in intimate contacts with neuronal cell bodies, dendrites, axons and synaptic contacts. Like neurons, glial cells are a heterogeneous population of cells that differ in developmental origin, molecular composition, structure and activity. When these cells were first discovered some 150 years ago, they were viewed as a type of connective tissue support for nerve cells. They were considered to assume a passive role of structural support for neurons and to provide a preferential, but metabolically inert, route for the passage of glucose from the circulation to neurons. More recent reports, however suggest that they have much more important and dynamic functions in the brain and other parts of the nervous system: they respond to synaptic activity and they play an important role in the communication network within the central nervous system (Voutsinos-Porche *et al.,* 2003; Jessen *et al.*, 2006).

Glial cells in the CNS are represented by three main types: macroglia (astroglia, oligodendroglia) and microglia. The macroglial cells are of ectodermal origin, whereas microglia stem from the mesoderm; the functions of oligodendrocytes and microglial cells are rather well defined: the oligodendrocytes are responsible for myelination and metabolic support of axons, whereas the microglial cells are involved in brain immune reactivity and defence. In particular,microglia phagocytize foreign particles and cellular debris, and produce many immune factors.

1.2 Astrocyte cells: morphology

Astrocytes (Fig.1) are the most abundant type of glial cell, and in contrast with microglia, are much more intimately involved in the formation of CNS cellular circuits and in information processing in the brain (Verkhratsky *et al.*, 2006).

There are generally two types of astrocytes, protoplasmic and fibrous, similar in function but distinct in morphology and distribution. Protoplasmic astrocytes have short, thick, highly branched processes and are typically found in grey matter.

Fibrous astrocytes have long, thin, less branched processes and are more commonly found in white matter.

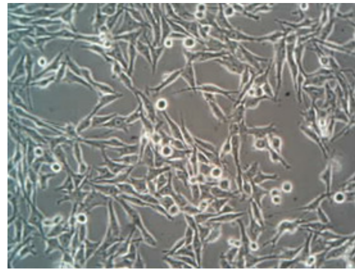


Fig.1 Culture of astrocyte cells

Astrocytes participate in numerous functions including structural and metabolic support for neurons, homeostatic and signalling functions in the brain including synthesis of the citric acid cycle (TCA) intermediates, regulation of extracellular glutamate and cation levels, energy storage of glycogen and regulation of blood flow (Mulligan *et al.*, 2004).

Neuronal networks are physically discontinuous, with neurons being separate entities. The integration and communication within these neuronal networks is provided by specialised structures, the synapses, which are considered the substrates of chemical neurotransmission (Verkhratsky *et al.*, 2006).

Because of their capacity to detect and respond to neuronal activity, glial cells, and in particular astrocytes, may be an integral part of the communication network within the central nervous system. Among their new emerging roles, glial cells have been shown to enhance synapse number and spontaneous synaptic activity between developing neurons in culture and to play a role in the stabilization and maintenance of synapses (Voutsinos-Porche *et al.*, 2003). The modulation of synaptic transmission by glial cells has been also described at the neuromuscular junction, in hippocampal cultures, and in the retina (Voutsinos-Porche *et al.*, 2003).

Astroglia divide the grey matter into distinct compartments, and within each of these compartments, a single astrocyte forms contacts with all neuronal membranes and synapses residing within its confines. These contacts are created by fine astroglial processes, called filopodia and lamellipodia. These are highly dynamic structures: lamellipodia being able to glide along neuronal surfaces, whereas filopodia are rapidly extended from the astroglial processes and surround synapses (Verkhratsky *et al.*, 2006). Mitochondria are too large to fit within fine processes, and the filopodia are, therefore, likely to be highly dependent on glycolytic metabolism of glucose and glycogen as local fuel sources. Thus, glycolysis is expected to predominate in astrocytic peripheral fine processes that are too small for mitochondria, whereas oxidative metabolism would be carried out in the soma, larger processes and end-feet (Dienel *et al.*, 2005).

Astrocyte cells not only form interactions with neurons but they also provide a link between neurons and blood capillaries. In fact the end-feet of several astrocytes cover the capillary wall in order to form a glial-vascular interface which is a part of the blood-brain barrier (BBB) (Verkhratsky *et al.*, 2006). These processes express glucose transporters suggesting that astrocytes may constitute a privileged site of glucose uptake as it penetrates within the brain parenchyma (Morgello *et al.*, 1995).

Some astrocyte processes contact blood–brain barrier, also in order to protect the CNS from being exposed to endogenous neurotoxins present in the general circulation (Alberdi *et al.*, 2005).

1.3 Astrocytoma cells

Astrocytomas are primary intracranial tumours derived from astrocyte cells of the brain. They may arise in the cerebral hemispheres, in the posterior fossa, in the optic nerve, and rarely, the spinal cord (Mangiardi *et al.*, 1990). Well-differentiated astrocytomas constitute about 25 to 30% of cerebral gliomas. They have a predilection for the cerebrum, cerebellum, hypothalamus, pons, and optic nerve and chiasm.

Astrocytic tumours are the most common type of gliomas. They can occur in most parts of the brain and occasionally in the spinal cord. However, they most commonly grow in the main part of the brain, the cerebrum (Mangiardi *et al.*, 1990). People of any age can develop an astrocytic tumour, but those in the cerebrum are more common in adults, and the chance of developing one increases as people get older. Astrocytic tumours in the cerebellum are more common in children or young people (Mangiardi *et al.*, 1990). Regional effects of astrocytomas include compression, invasion, and destruction of brain parenchyma. Arterial and venous hypoxia, competition for nutrients, release of metabolic end products (eg, free radicals, altered electrolytes, neurotransmitters), and release and recruitment of cellular mediators (eg, cytokines) disrupt normal parenchymal function (Mangiardi *et al.*, 1990). The astrocytoma cells prefer to produce ATP through glycolysis rather than oxidative phosphorylation and the cells continue with the lactate production also when oxygen is present (Mangiardi *et al.*, 1990). In fact in these cells the Krebs cycle and the oxidative phosphorylation seem to be partially blocked; moreover in the astrocytoma cells there is a decrease of the mitochondrial number in comparison with normal cells (Mangiardi *et al.,* 1990). On the other hand in these cells the pentose phosphate pathway is more active compared to normal cells; therefore these cells are able to produce a great amount of ribose 5-P which is absolutely necessary for 5-phosphoribosyl-1-pyrohosphate (PRPP) production which is a common substrate for the purine and pyrimidine synthesis (Mangiardi *et al.*,1990). Astrocytoma cells show a relatively low activity of purine metabolism when compared with normal brain and non-glial brain neoplasms (Bardot *et al.*, 1994).

2. Energetic metabolism in the central nervous system

2.1 Glucose

Glucose (Glc), a monosaccharide, is the most important carbohydrate in biology. The cell uses it as a source of energy and metabolic intermediates. Glucose is one of the main products of photosynthesis and starts cellular respiration in both prokaryotes and eukaryotes. Glucose is considered the major if not exclusive energy source for mammalian brain and a continuous supply of this substrate is essential to maintain normal cerebral function. This indicates that oxidative phosphorylation is the most important process used to generate from glucose the energy necessary to sustain the brain functions (Ioudina *et al.*, 2004). Glucose crosses the blood-brain barrier and enters neurons or glial cells via specific glucose transporters and is metabolized via glycolysis and the Krebs cycle in order to produce ATP. The brain is able to maintain cerebral energy metabolism due, in part, to the relatively high glucose concentration in the plasma and the presence of glucose transporters in the capillary endothelial cell, glial cells and neurons. In the rat brain, extracellular glucose concentration ranges from 0.7 to 2.5 mM, corresponding to plasma glucose levels. The total glucose deprivation leads to neuronal degeneration while the effects of a moderate deprivation of glucose are not well understood (Ioudina *et al.*, 2004). In the cells grown in a low glucose media there is a decrease in the cellular ATP and cell viability and an increase of caspase-3 activity which leads to apoptosis and therefore the glucose deprivation could be a serious risk factor that potentiates the pathophysiological consequences of certain neurodegenerative diseases (Ioudina *et al.*, 2004).

During the physiologic brain activity, glucose crosses the blood-brain barrier via facilitative transporters named glucose transporter 1 (GLUT1). Once within the brain parenchyma, it is taken up by both neurons and astrocytes via glucose transporter 3 (GLUT3) and GLUT1, respectively. In particular, about half of the glucose leaving the capillaries crosses the extracellular space and directly enters neurons; the other half is taken up by astrocytes (Nehlig *et al.*, 2007). GLUT 1 is expressed in all cell types, and it is the only glucose transporter identified in the brain. In the brain it is present at a high concentration at the blood-brain barrier as well as in parenchymal cells, most likely in astrocytes. In particular, GLUT 1 is detected in astrocytic end-feet around blood vessels, and in astrocytic cell bodies and processes in both grey and white matter. GLUT 3, in the brain, is expressed in neurons; in particular is located primarily in pre- and postsynaptic nerve endings and in small neuronal processes (Maher *et al.*, 1994).

In each cell type, glucose is metabolized via glycolysis into pyruvate, leading to the formation of 2 ATP. Then, pyruvate is further metabolized via the TCA cycle, and in conjunction with oxidative phosphorylation using oxygen provided by the blood circulation, it generates 30 ATP (Maher *et al.*, 1994). However calculations suggest that neurons consume more energy than do astrocytes, implying that astrocytes transfer an intermediate substrate to neurons.

Experimental approaches *in vitro* on the honeybee drone retina and on the isolated vagus nerve also point to a continuous transfer of brain intermediate metabolites *in vivo* and *in vitro* as is well documented in the ANLSH theory (Nehlig *et al.*, 2007).

2.2 Astrocyte-neuron-lactate shuttle theory (ANLSH)

There is controversy at present concerning the metabolic changes that accompany neuronal activation and the energy substrate used by the activated neurons. The traditional theory considers the glucose as the exclusive compound involved in the production of energy necessary for neuronal and astrocyte survival. As already described, glucose is utilized by both neurons and astrocytes in order to produce pyruvate which can enters the TCA cycle and oxidative phosphorylation for ATP production.

Another theory concerning the glucose utilization by neuronal and astrocyte cells is the astrocyte-neuron-lactate shuttle (ANLSH), revealed for the first time by Pellerin and Magistretti (1999). The ANLSH model postulates that activated neurons use lactate which is provided by astrocytes (Fig.2). It is well known that the major amount of energy consumption (90-95%) can be attributed to neurons whereas glial cells contribute only in a small fraction (5-10%) under normal level of activity; on the other hand, several studies reveal that 80% of glucose utilization is taking place in glial cells. And so, to fulfil the greater energy needs of neurons, glial cells must release a metabolic intermediate from glucose that will be eventually taken up and oxidized by neurons (Pellerin and Magistretti, 1999, 2004).

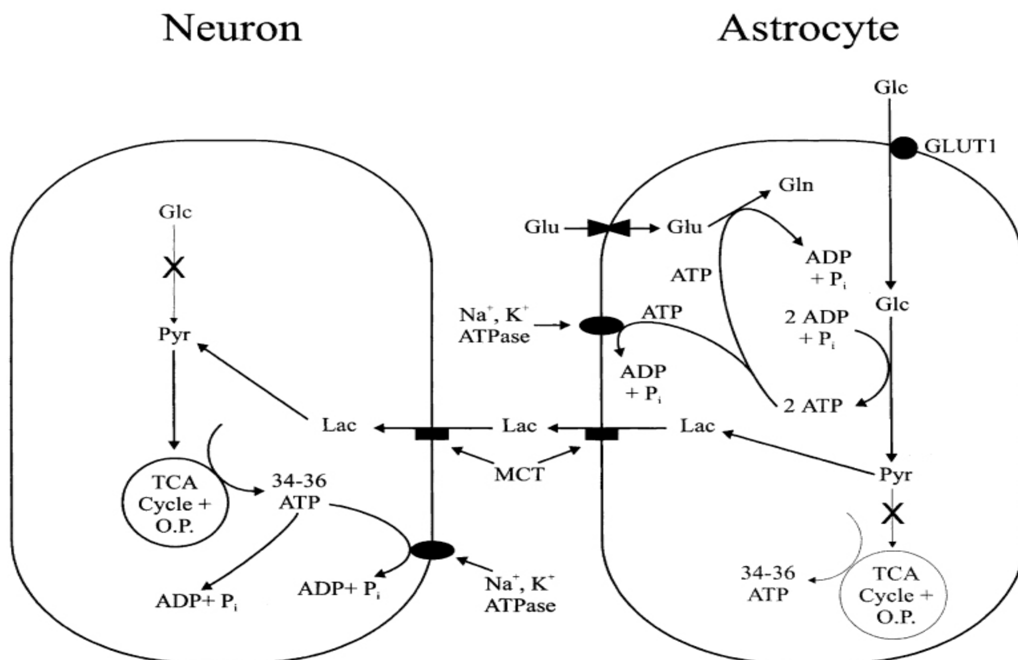


Fig.2 Schematic illustration of the astrocyte-neuron-lactate shuttle (ANLSH) (Image taken from www.nature.com)

It has been known that glial cells and in particular astrocytes are able to produce a large amount of lactate in the presence of normal oxygen levels; this lactate production is enhanced during a neuronal synaptic activity at glutamatergic synapses: after this stimulation there is a significant increase of the extracellular neurotransmitter glutamate which, via glutamate transporters (GLAST or GLT1), enters astrocyte cells and causes an increase in both glucose utilization and lactate production. In addition, glutamate causes a rapid stimulation of glucose transport in astrocytes and it is considered the fastest glucose transport response ever recorded in any other cell type. Lines of evidence of this mechanism have been provided *in vivo*: in knockout mice for either GLAST or GLT1 transporters, a significant reduction of the glucose utilization during a neuronal stimulation has been observed (Pellerin and Magistretti, 1999, 2004).

Glutamate transporters operate by coupling glutamate transport with the Na^+ gradient. For each glutamate molecule transported within the cell, three Na^+ (or two Na^+ and one H^+) ions are cotransported together with one proton while one K^+ ion and one OH^- is extruded. As a consequence, a significant Na^+ influx occurs in astrocytes, with an accumulation of Na^+ inside the cell accompanied by an intracellular acidification and extracellular alkalinization (Danbolt *et al.*, 2001).

Changes in the intracellular sodium concentration modulate the activity of the α_2 isoform of the Na^+, K^+-ATPase pump and this correlation is demonstrated in several cell types and also in the astrocyte cells (Pellerin and Magistretti, 1999, 2004). The Na^+ influx not only activates the Na^+-K^+ ATPase but also the enzyme glutamine synthase which leads to the production of glutamine with a concomitant increase of ADP, Pi and AMP levels and a decrease of levels of ATP. As a consequence, an activation of anaerobic glycolysis, which generates lactate from glucose by LDH-5 (lactate dehydrogenase-5) and restores the ATP levels, is observed (Pellerin and Magistretti, 1999, 2004).

The lactate is transported across the glial membrane and leaves the astrocytes by a specific monocarboxylate transporter (MCT1); once in the extracellular space, the increased concentration gradient leads to its net transport into neurons by its own specific monocarboxylate transporter MCT2. The increased lactate in the neurons is converted to pyruvate via LDH-1 (lactate dehydrogenase-1), which enters the TCA cycle and oxidative phosphorylation with the final ATP production (Pellerin and Magistretti, 1999, 2004). The generation of ATP serves also for the production of the neurotransmitter glutamate. Therefore, in this theory there is a metabolic coupling between astrocytes and neurons in brain tissue and the neurons should prefer lactate to glucose as substrate, particularly during periods of intense neuronal activity (Pellerin and Magistretti, 1999, 2004).

2.3 Glycogen

Glycogen is a polysaccharide that is the principal storage form of glucose in animal cells. Glycogen energy stores in the brain are made exclusively by astrocytes which express the gluconeogenesis and glycogenolysis enzymes while neurons do not have these enzymes (Maher *et al.*, 1994). Glycogen is a fuel in the normal astrocytic functions during brain activation, in fact glycogen is located mainly in astrocytes and widely distributed throughout brain, and glycogen phosphorylase is present in the soma, end-feet and fine processes (Dienel *et al.*, 2006). Glycogen levels change during stress conditions: during physiological stimulation the rate of glycogenolysis in brain rises 50-fold above that in the resting state, whereas it increases more than 200-fold during an energy crisis when oxygen or glucose supply is inadequate or eliminated (Magistretti, 1993).

Neurons are more sensitive to ATP depletion than astrocytes. In fact astrocytes, but not neurons, are capable of storing glycogen and glycogenolysis is another mechanism by which astrocytes can resist ischemia-like conditions (Parkinson and Xiong, 2004). Glycogenolysis produces glucose-6-P but the metabolic fate of this compound derived from glycogen *in vivo* is not well known, moreover the activity glucose-6-phosphatase which produces glucose from glucose-6-P in brain and cultured astrocytes is very low and therefore, the glycogen metabolism generates lactate, which is released to the tissue culture medium (Dienel *et al.*, 2006). On the contrary, the plasma lactate cannot fully substitute for glucose as a metabolic substrate for brain because of its limited permeability across the blood-brain barrier (Dienel *et al.*, 2006).

In response to an acute hypoxic challenge, astrocytes respond to hypoxia by mobilizing their glycogen stores and by increasing their glucose utilization and lactate release for their own survival as well as for the neighbouring cells. In fact in the astrocyte cells, during hypoxia, there is an enhancement of the expression of the sodium-dependent and independent glucose transporters and also of the astrocytic monocarboxylate transporters isoform MCT1 (Vega *et al.*, 2006; Brown *et al.*, 2007).

Astrocyte glycogen, therefore, offers some protection against hypoglycemic neural injury and ensures that neurons and axons can maintain their function during very intense periods of activation. These emerging principles about the roles of astrocyte glycogen contradict the long held belief that this metabolic pool has little or no functional significance (Brown *et al.*, 2007). After a prolonged hypoxia exposure, astrocytes decrease their glucose uptake and the lactate release, conserving lactate to ensure their own survival to the detriment of surrounding cells that could use the released lactate as a source of energy (Vega *et al.*, 2006). These findings could explain the higher susceptibility of neurons to hypoxia and their earlier cell death compared with astrocytes when exposed to long-term hypoxic challenges (Vega *et al.*, 2006).

2.4 Lactate

Lactic acid is a chemical compound that plays a role in several biochemical processes. In animals, L-lactate is constantly produced from pyruvate via the enzyme lactate dehydrogenase (LDH) in a process of fermentation during normal metabolism and exercise. A number of recent observations have challenged the canonical view of glucose as the sole energy substrate delivered directly to neurons via the blood supply to sustain their activity: many lines of evidence reveal that monocarboxylates, and in particular lactate, might play useful roles in the adult central nervous system (Parkinson and Xiong, 2004).

Neurons, astrocytes, and oligodendrocytes use lactate as a preferential substrate for both energy purposes and as precursor of lipids (Parkinson and Xiong, 2004). Astrocytes use lactate and other metabolic substrates for the synthesis of oleic acid a fatty acid which is able to increase the fluidity of the neurite bases suggesting that increased fluidity is required at the sites of newly emerging axons and dendrites. Oligodendrocytes mainly use lactate as precursor of lipids, presumably those used to synthesize myelin. Neurons use lactate as a source of energy and as precursor of lipids (Smith *et al.*, 2003).

Lactate produced by muscle during strenuous exercise could be used as a fuel source by the brain (Smith D. *et al.*, 2003). During the perinatal period, neurons may use blood lactate directly to meet the need for the energy and carbon skeletons required for proliferation and differentiation (Medina and Tabernero, 2005). Lactate is a hydrophilic compound which requires transporters to cross cellular membranes. A family of proton-linked monocarboxylate transporters has been described. It contains 14 members sharing sequence homologies and identified as MCT1 to 9 and MCT11 to 14 as well as another member known as TAT1.

A distribution of specific MCTs between different cell types is consistent with a role in mediating a transfer of energy substrates from astrocytes to neurons. It is also correlated with a differential distribution of lactate dehydrogenase (LDH) isoforms: LDH1 appears to be the predominant isoform in neurons, whereas LDH5 is found predominantly in astrocytes (Laughton *et al.*, 2000).

Lactate dehydrogenase is the enzyme catalysing the interconversion of pyruvate and lactate, and is thus essential for both production and utilization of lactate. It is a tetrameric enzyme which can consist of three types of subunit arising from three distinct genes: LDH-A, LDH-B and LDH-C The product of the LDH-C gene is found exclusively in the testis, where it forms a homogeneous tetramer (C4). The LDH-A and LDH-B gene products are more widely expressed, and both are found in the CNS (Laughton *et al.*, 2000; Medina and Tabernero, 2005).

Hypoxia is the oxygen deprivation which provokes a mitochondrial dysfunction and a diminution in the levels of the intracellular energy metabolites which leads to a drastic reduction of ATP production with a concomitant neurodegeneration through both apoptotic and necrotic mechanisms (Cater *et al.*, 2003). In terms of energy metabolism, during hypoxia, there is a rapid switch from oxidative phosphorylation to anaerobic glycolysis, in order to maintain the cellular energy charge. Under normal physiological conditions the NAD^+ molecules necessary to drive the glycolytic reactions are regenerated via the TCA cycle.

During anaerobic conditions, the TCA cycle cannot operate in the absence of oxygen so an alternative source of NAD^+ is provided through the conversion of pyruvate, derived from glucose, to lactate (Cater *et al.*, 2003).

Therefore, during oxygen deprivation in nervous tissue, a significant rise of the lactate concentrations is observed which could lead to acidosis and for this reason, lactate for a long period was considered the major factor in the resultant neuronal degeneration (Cater *et al.*, 2003). The amount of acidosis differs between animal models, yet intracellular pH typically acidifies to the range of pH 6.5–6.7 (Cater *et al.*, 2003).

A research *in vitro* suggests that the role of lactate during neuronal stress may, on the contrary, be beneficial under certain circumstances (Cater *et al.*, 2003). There are also some lines of evidence *in vivo* that lactate is neuroprotective in a number of ischemic/excitotoxic models as well as following severe insulin-induced hypoglycemia (Schurr *et al.*, 2001).

3 Biochemistry of the purine and pyrimidine nucleotide compounds

3.1 General aspects

A nucleotide is a chemical compound that consists of a heterocyclic base, a sugar, and one or more phosphate groups. In the most common nucleotides the base is a derivative of purine or pyrimidine, and the sugar is the pentose (five-carbon sugar) deoxyribose or ribose. All types of cells (mammalian, bacterial and plant) contain a wide variety of nucleotides and their derivatives. In normal cells the total concentrations of the nucleotides are fixed within rather narrow limits, although the concentration of the individual components can vary. The total concentration of adenine nucleotides (AMP, ADP and ATP) is constant, although there is a variation in the ratio of ATP to AMP+ADP, depending on the energy state of the cells.

Deoxyribonucleotides are formed by the direct reduction of the 2' position of the corresponding ribonucleotides. This reaction occurs at the level of the nucleoside diphosphates and is catalyzed by nucleoside diphosphate reductase. It is generally accepted that only liver and kidney maintain the *de novo* pyrimidine and purine synthesis and supply other tissues and organs, including brain, with preformed pyrimidine nucleosides and purine nucleosides and bases.

In the central nervous system, most of the enzymes involved in the purine nucleotide metabolism (except for the *de novo* pathway and of AMP-5'-nucleotidase), exhibits a higher activity in the astroglia, in comparison to the neurons. Thus, as for other metabolic pathways, the glia appears to be more active than the neurons (Zoref-Shani, *et al.*, 1995).

The functions exerted by nucleotides are:

1. role in energy metabolism: ATP is the main form of chemical energy available to the cell and is generated by oxidative phosphorylation and substrate-level phosphorylation. The ATP is utilized to drive metabolic reactions as a phosphorylating agent and is involved in several processes as muscle contraction, active transport and maintenance of cell membrane integrity. ATP donates the phosphate also for the generation of the other nucleoside 5'-triphosphates (GTP, UTP and CTP). GTP is considered an important non-protein organic cofactor and enzyme regulator present in all cells. GTP produces energy for the assemblage of ribosome, kinesins and myosines.
2. monomeric units of nucleic acids DNA and RNA.
3. mediators of key metabolic processes. (es. cyclicAMP, involved in the molecular signal evoked by hormones)
4. components of coenzymes (NAD, NADP, FAD and CoA) which are involved in many metabolic pathways.
5. allosteric effectors; in fact, many of the regulated steps of the metabolic pathways are controlled by the intracellular concentrations of nucleotides.

3.2 Metabolic pathways of purines and pyrimidines

3.2.1 De novo purine synthesis

Purine nucleotides may be formed *de novo,* from small molecules, or by salvage, from preformed purines. The former pathway is associated mainly with cell proliferation, requiring formation of new nucleic acids, whereas the latter pathway is associated with maintenance of cellular purine pool size (Zoref-Shani *et al.*, 1995). The *de novo* synthesis of purine nucleotides in which these precursors are incorporated into the purine ring, proceeds by a 10 step pathway to the final point intermediate IMP, the nucleotide of the base hypoxanthine and AMP and GMP are subsequently synthesized from this intermediate via separate, two step each, pathways. Thus purine moieties are initially formed as part of the ribonucleotides rather than as free bases. The basal precursor for the *de novo* synthesis of purine (and pyrimidine) is 5-phosphoribosyl-1-pyrohosphate (PRPP). PRPP is formed by ribose 5-phosphate and ATP by a reaction catalyzed by PRPP synthetase.

The purine ring atoms is built using a variety of different precursors: N1 of purines arises from the amine group of aspartate; C2 and C8 originate from N10-formyltetrafolate; N3 and N9 are contributed by the amide group of glutamine; C4, C5 and N7 are derived from glycine; C6 comes from HCO_3^- (CO_2). The *de novo* synthesis requires directly four ATP molecules.

In the whole brain, there is a maturation-dependent decrease in the capacity to produce purines *de novo*, and this particular trend reflects the trend observed in the astroglia, rather than that in the neurons (Zoref-Shani *et al.,* 1995).

3.2.2 Purine catabolism

The end product of purine catabolism in man is uric acid. Other mammals have the enzyme urate oxidase and excrete the more soluble allantoin as the end product. Man does not have this enzyme so urate is the end product. Uric acid is formed primarily in the liver and excreted by the kidney into the urine (Fig.3).

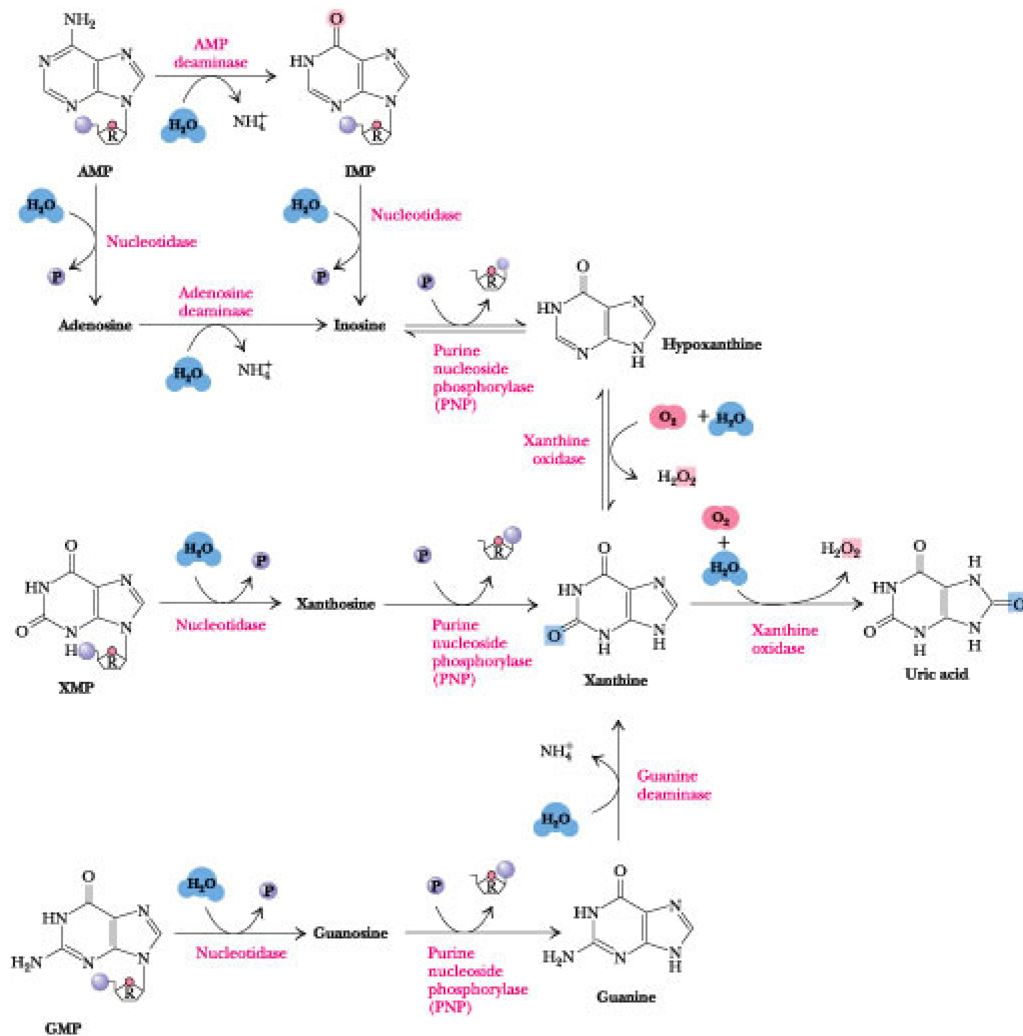


Fig.3 Schematic illustration of purine catabolism (Image taken from web.virginia.edu)

Nucleotides are hydrolyzed to nucleosides by the enzyme family of 5'-nucleotidase (5NT) which have a different subcellular location and a remarkable low sequence homology; 5'-nucleotidases hydrolyze the phosphate esterified at the 5' position of purine and pyrimidine nucleoside monophosphates (Allegrini *et al.*, 1997, 2004; Pesi *et al.*, 2004).

ATP degradation proceeds via AMP, which can be either dephosphorylated to adenosine, catalyzed by AMP-5'-nucleotidase, or deaminated to IMP, catalyzed by the enzyme adenylate (AMP) deaminase. The reaction catalyzed by AMP deaminase is the rate-limiting step in the operation of the purine nucleotide cycle (AMP →IMP → adenylosuccinic acid →AMP). This cycle has a major role in the maintenance of the normal energy charge in the cell, as well as in the preservation of cellular purine nucleotide content. Guanine nucleotides are hydrolyzed to the nucleoside guanosine which undergoes phosphorolysis to guanine and ribose 1-P.

Rather, AMP is deaminated by the enzyme AMP deaminase to IMP. AMP deaminase is activated by K^+ and ATP and is inhibited by P_i, GDP and GTP.

In the catabolism of purine nucleotides, IMP is further degraded by hydrolysis with nucleotidase to inosine and then phosphorolysis to hypoxanthine in a reaction catalyzed by purine nucleoside phosphorylase (PNP), which acts also on guanosine and xanthosine in order to produce guanine and xanthine and Rib1-P. Based on their structural properties, nucleoside phosphorylases have been classified into two families: NP-I and NP-II. The NP-I family includes homotrimeric and homohexameric enzymes from both prokaryotes and eukaryotes acting on inosine, guanosine, adenosine and uridine. The NP-II family includes homodimeric proteins structurally unrelated to the NP-I family, such as bacterial pyrimidine phosphorylases and eukaryotic thymidine phosphorylase (Tozzi *et al.*, 2006). It has been shown that the activity of purified PNP from bovine spleen may be inhibited by the accumulation of its metabolic products (hypoxanthine and guanine), but it is also inhibited by a negative control exerted by the respective substrates, inosine and guanosine, especially when their concentrations are elevated, to the micromolar range (Tozzi *et al.*, 2006).

The equilibrium of PNP-catalysed reactions is thermodynamically in favour of nucleoside synthesis. Nevertheless, *in vivo* the equilibrium of the PNP reaction is shifted towards Rib-1-P accumulation because the intracellular concentration of Pi is higher than that of nucleosides inosine and guanosine and also because hypoxanthine and guanine are consumed by hypoxanthine-guanine phosphoribosyltransferase (HPRT) and, in certain tissues, by xanthine oxidase or guanase, respectively (Tozzi *et al.*, 2006). The phosphorolysis of inosine and guanosine is favoured also because mammals lack the kinase acting on inosine and guanosine (Tozzi *et al.*, 2006). PNP is generally considered as an intracellular enzyme. In fact, the presence of this enzyme is not demonstrated extracellularly (Tozzi *et al.*, 2006).

Adenosine is deaminated to inosine by an adenosine deaminase. Both adenine and guanine nucleotides converge at the common intermediate xanthine. Hypoxanthine is oxidized to xanthine by the enzyme xanthine oxidase. Guanine is deaminated, with the amino group released as ammonia, to xanthine. Xanthine, like hypoxanthine, is oxidized by oxygen and xanthine oxidase with the production of hydrogen peroxide. In man, the urate is excreted and the hydrogen peroxide is degraded by catalase. Xanthine oxidase is present in significant concentration only in liver and intestine. The pathway to the nucleosides, possibly to the free bases, is present in many tissues. The key enzyme for the catabolism of the pentose moiety of deoxyribonucleosides is deoxyriboaldolase, which cleaves deoxyRib-5-P into acetaldehyde and glyceraldehyde 3-P.

3.2.3 Purine salvage

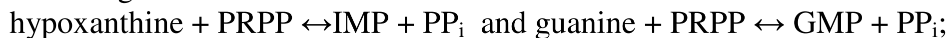
Whereas some organs such as kidney and liver or some cell types such as hepatocytes can synthesize purine and pyrimidine nucleotides *de novo*, others, in particular protozoan parasites and cells in the brain and bone marrow, rely on salvage pathways for purine and pyrimidine nucleotide synthesis (King *et al.*, 2006). In the cultured astroglia, *de novo* purine synthesis is found to decrease with aging, but the activity of the salvage enzyme HGPRT, is found to increase (Zoref-Shani *et al.*, 1995).

In contrast to the glia, in the cultured neurons, the rate of *de novo* purine synthesis is found to increase with maturation (Zoref-Shani *et al.*, 1995). The finding of the age-related increase in the rate of *de novo* purine synthesis in the non-dividing neurons, may be interpreted to indicate that the functioning mature neurons, more than the glia, have to cope with loss of purines from the cells (Zoref-Shani *et al.*, 1995).

The *de novo* synthesis of purine and pyrimidine requires a relatively high input of energy in the form of ATP. To compensate for this, most cells have developed a very efficient salvage pathway, by which the preformed purine and pyrimidine bases can be reutilized and so, the cell obtains a considerable energy saving for itself. The synthesis of nucleotides from the purine bases and purine nucleosides takes place in a series of steps known as the salvage pathways. The free purine bases, adenine, guanine, and hypoxanthine, can be reconverted to their corresponding nucleotides by phosphoribosylation. Two key transferase enzymes are involved in the salvage of purines: adenosine phosphoribosyltransferase (APRT), which catalyzes the following reaction:

adenine + PRPP ↔AMP + PP_i

and hypoxanthine-guanine phosphoribosyltransferase (HGPRT), which catalyzes the following reactions:



hypoxanthine + PRPP ↔IMP + PP_i and guanine + PRPP ↔ GMP + PP_i;

these reactions are important not only because they conserve energy, but also because they permit cell such as erythrocytes, neurons and astrocytes (which do not possess the *de novo* purine synthesis) to form nucleotides from the bases. AMP is an inhibitor of APRT and IMP and GMP regulates the HGPRT activity.

PRPP may be considered as a high-energy sugar phosphate, with a high potential of 5-phosphoribosyl transfer; PRPP is also the common precursor of both *de novo* and salvage synthesis of nucleotides. During physiological cell conditions, the PRPP pool is maintained at low level, to avoid excessive and unbalanced nucleotide synthesis. PRPP level is regulated by PRPP synthetase and by phosphoribosyl transferases which are the major factors maintaining the low intracellular level of PRPP. PRPP level is also regulated by the inosine nucleoside cycle (INC) constituted by HGPRT (which forms IMP from hypoxanthine), PNP (which forms inosine from hypoxanthine and vice versa) and cN-II (which forms inosine from IMP). Some experiments exerted in rat brain extracts reveal that when PRPP is present, the intermediates of the INC are continuously recycled but as soon as PRPP disappears, HGPRT becomes inactive and the cycle is interrupted causing IMP degradation by cN-II, and hypoxanthine accumulation. The [Pi]/[ATP] ratio regulates the inosine cell cycle and the PRPP levels: in fact, the allosteric activators of cN-II are 2,3-bisphosphoglycerate (BPG),and ATP while Pi is an inhibitor of the enzyme and at the normal low [Pi]/[ATP] ratio, as found in the well-oxygenated cells, cN-II is fully active and the velocity of the cycle is maximal and the PRPP pool is maintained at a low level (Barsotti *et al.*, 2002). On the other hand, during ischemia the [Pi]/[ATP] ratio raises drastically and cN-II is inhibited while PRPP synthetase is activated: as consequence the PRPP level increases and PRPP is made available for purine nucleotide salvage synthesis (Barsotti *et al.*, 2002).

3.2.4 De novo pyrimidine synthesis

The pyrimidine ring is synthesized de novo in mammalian cells utilizing amino acids as carbon and nitrogen donors and CO_2 as a carbon donor. In particular the N1, C4 and C6 of the pyrimidine ring derive from aspartate, while the N3 from glutamine and C2 from CO_2. As is true with purine nucleotides, the sugar phosphate portion of the molecule is supplied by PRPP. Pyrimidine synthesis begins with carbamoyl phosphate II (CPII) synthesized in the cytosol of those tissues capable of making pyrimidines (highest in spleen, thymus and testes). Carbamoyl phosphate condenses with aspartate in the presence of aspartate carbamoyl transferase to yield N-carbamylaspartate which is then converted to dihydroorotate by dihydroorotase and subsequently, oxidated into orotate by dihydroorotate dehydrogenase. Orotate is converted to its nucleotide, orotidine 5'-monophosphate (OMP) with PRPP, which is the ribose-5-phosphate donor. This reaction is catalyzed by orotate phosphoribosyltransferase. In the last step pathway, OMP is decarboxylated into UMP by OMP-decarboxylase. After conversion of UMP to the triphosphate, the amide of glutamine is added, at the expense of ATP, to form CTP by CTP synthetase. The de novo synthesis of purine differs by the de novo synthesis of pyrimidine in two major respects. First, in purine nucleotide synthesis, the N-glycosidic bond is formed in the first committed step of the pathway. In the pyrimidine pathway, the first step is the formation of the ring and then the phosphate is added. Second, all the enzymes of the purine nucleotide pathway are in the cytosol, while in the case of the pyrimidine synthesis, the enzyme dihydroorotate dehydrogenase is localized in the mitochondria.

3.2.5 Pyrimidine catabolism

In contrast to purines, pyrimidines undergo ring cleavage and the usual end products of catabolism are β amino acids plus ammonia and carbon dioxide. Pyrimidines from nucleic acids or the energy pool are acted upon by nucleotidases and pyrimidine nucleoside phosphorylase to generate the free bases. Cytidine and deoxycytidine are deaminated to uridine and deoxyuridine, by the nucleoside deaminase. Uridine phosphorylase catalyzes the phosphorolysis of uridine, deoxyuridine and deoxythimidine. As already described, the equilibrium of the reaction catalyzed by PNP is shifted towards Rib-1-P accumulation even if the reaction is thermodynamically in favour of nucleoside synthesis. On the other hand, some *in vitro* experiments reveal that uridine phosphorylase may catalyse the Rib-1-P-mediated rybosilation of 5-fluorouracil and uracil, even in the presence of excess of Pi (Mascia *et al.*, 1999, 2001). Uridine phosphorylase plays an important role in the homeostatic regulation of uridine concentration in plasma and tissues (Pizzorno *et al.*, 2002).

3.2.6 Pyrimidine salvage

Pyrimidine nucleotide salvage is important for the synthesis of RNA and DNA. Moreover, salvage pyrimidine synthesis is used also for phosphatidylcholine and phosphatidylinositol synthesis in lymphoid cells. In particular, in CNS the continued biosynthesis of phospholipids needs specific activated pyrimidine nucleotides. Even the conversion of uracil to L-alanine is important in neuronal cells since L-alanine, which is produced and exported by liver, enters the CNS and may act as a neurotransmitter, as well as a building block for various dipeptides (Mascia *et al.*, 1999).

A crucial difference between purine and pyrimidine metabolism is that purines are recycled from their bases while pyrimidines are salvaged from their nucleosides, particularly uridine. In fact, in patients with deficient pyrimidine biosynthesis, only uridine is able to overcome this pathological manifestation but uracil is not (Pizzorno *et al.*, 2002).

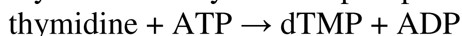
Uracil can be salvaged to form UMP through the concerted action of uridine phosphorylase and uridine kinase, as indicated:

uracil + ribose-1-phosphate $\leftrightarrow$ uridine + P_i

uridine + ATP $\rightarrow$ UMP + ADP

uridine phosphorylase type 1 (UPase-1) activity has been reported in the homogenates of many human tissues and from tumours. However, several studies reveal that that functional UPase-1 is confined predominantly to the liver in humans (Loffler *et al.*, 2005). A second minor human uridine phosphorylase, UPase-2 is identified recently, and is expressed predominantly in the kidney (Loffler *et al.*, 2005). Deoxyuridine is also a substrate for uridine phosphorylase. The salvage of dTMP requires thymine phosphorylase and the previously encountered thymidine kinase:

thymine + deoxyribose-1-phosphate $\leftrightarrow$ thymidine + P_i


thymidine + ATP $\rightarrow$ dTMP + ADP

The salvage of deoxycytidine is catalyzed by deoxycytidine kinase

deoxycytidine + ATP $\leftrightarrow$ dCMP + ADP

Intracellularly, mitochondrial and cytosolic nucleoside kinases participate in the recycling of uridine, deoxycytidine and deoxythymidine arising from cell turnover or diet, thereby restricting the requirement for the energetically expensive *de novo* synthesis. Although deoxynucleotides are considered to be used exclusively in DNA replication and repair, deoxycytidine is also known to be salvaged as a precursor for phospholipid synthesis (Loffler *et al.*, 2005).

3.3 Metabolism of the pentose phosphate

Pentose phosphates are heterocyclic, five-membered, oxygen-containing phosphorylated ring structures, with ribose-5-phosphate and 2-deoxyribose-5-phosphate being basal structures of ribonucleotides and deoxyribonucleotides, respectively, and 5-phosphoribosyl-1-pyrophosphate (PRPP). There are two pathways of the pentose phosphate biosynthesis (Fig.4): the pentose phosphate pathway where Rib-5-P is generated from glucose-6-phosphate and the phosphorylase-mediated pathway where Rib-1-P and deoxRib-1-P are supplied by various nucleoside phosphorylases, such as thymidine phosphorylase, uridine phosphorylase and purine nucleoside phosphorylase. Rib-5-P can be also formed from free ribose by the action of ribokinase an enzyme well characterized in bacteria but less investigated in mammals. Phosphopentomutase catalyzes the reversible reaction between Rib-1-P and Rib-5-P and between deoxyRib-1-P and deoxyRib-5-P. This enzyme has been extensively studied in bacteria but has been purified also in human cell lines and tissues. The key enzyme for the catabolism of the pentose moiety of deoxyribonucleosides is deoxyriboaldolase, which cleaves deoxyRib-5-P into acetaldehyde and glyceraldehydes 3-P.

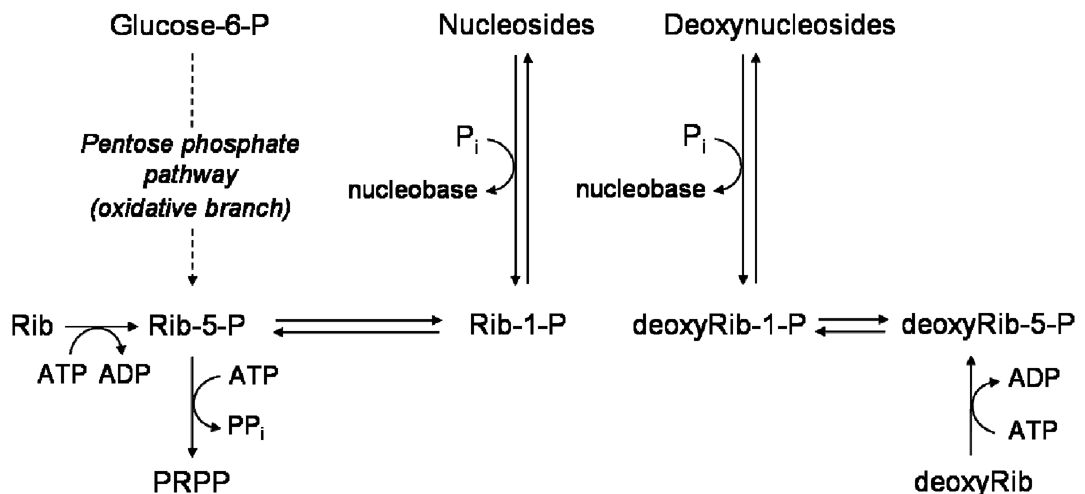


Fig.4 Pathways of the pentose phosphate biosynthesis.

Some experiments performed in rat brain extracts show that the activated ribose, originating from inosine and guanosine phosphorolysis as ribose 1- phosphate, can be transferred to uracil to synthesize uridine by uridine phosphorylase and the nucleoside formed is phosphorylated to UMP by uridine kinase, and hence to UDP and UTP (and possibly to CTP). The transfer of the ribose moiety occurs even in the presence of excess Pi, suggesting that at least in brain, uridine phosphorylase might function *in vivo* as an anabolic enzyme (Mascia *et al.*, 1999). CTP and UTP, the final products of the pyrimidine salvage, inhibit their production and this inhibition occurs at the level of uridine kinase, the enzyme which regulates the flux (Mascia *et al.*, 1999). The absence of any PRPP involvement in this metabolic pathway, is supported by the lack of uracil phosphoribosyltransferase activity.

The rate of this pyrimidine salvage pathway is about threefold higher in brain than in liver, possibly reflecting the need of CNS to synthesize pyrimidine nucleoside diphosphate sugars for myelin biosynthesis (Mascia *et al.*, 1999). The transfer of the ribose moiety stemming from the purine nucleoside to the uracil via uridine phosphorylase in order to produce uridine and then UMP, UDP and UTP, is also demonstrated in liver and PC12 cells (Mascia *et al.*, 2001). In addition, the ribose 1-P stemming from the purine nucleoside is also able to enter the purine salvage pathway and then produce the purine nucleotide compounds: the pentose phosphate is isomerized into ribose 5-P and then converted to PRPP by PRPP synthetase and PRPP can react with adenine, guanine and hypoxanthine in order to form AMP, GMP and IMP respectively through the action of APRT and HGPRT (Tozzi *et al.*, 2006). On the other hand, deoxyRib-5-P is not a substrate of PRPP synthetase and then the pentose phosphate stemming from the purine deoxynucleoside is not able to enter the purine salvage pathway. Thus the ribose 1-phosphate stemming from the purine nucleoside is able to enter also the pyrimidine salvage pathway in order to produce the nucleotide compounds establishing a metabolic link between purine and pyrimidine salvage (Mascia *et al.*, 1999, 2001; Tozzi *et al.*, 2006).
Ribose 1-phosphate formed from guanosine through the action of purine nucleoside phosphorylase acts as ribose donor also in the synthesis of xanthosine catalyzed by the same enzyme (Giorgelli *et al.*, 1997).

3.4 Adenosine metabolism in the central nervous system

Adenosine is an endogenous nucleoside consisting of the purine base, adenine, in glycosidic linkage with the sugar ribose. Adenosine is generated in the cells and in the extracellular space during normal metabolic activity and can exert its biological activity by ligating specific receptors linked to a variety of signalling systems (Hasko *et al.*, 2005). In the cells, adenosine is generated from AMP which is dephosphorylated by 5'-nucleotidase and from S-adenosylhomocysteine which is hydrolyzed into adenosine and homocysteine by S-adenosylhomocysteine hydrolase (SAH); the first reaction predominates during metabolically stressful conditions while the second predominates in the physiological conditions (Tozzi *et al.*, 2006).

There are two sources of extracellular adenosine: release of adenosine from the intracellular space via specialized bi-directional equilibrative transporters and extracellular conversion of released adenine nucleotides (ATP, ADP and AMP) by a cascade of ectoenzymes of the ecto-nucleoside triphosphatase (E-NTPase) family (Tozzi *et al.*, 2006). E-NTPases include the ecto-ATPase that preferentially transforms ATP into ADP; the ecto-ATP diphosphohydrolase, also named ectoapyrase, that hydrolyses either ATP or ADP, and the ecto-5'-nucleotidase, GPI-anchored plasma membrane protein, that catalyses the hydrolysis of nucleoside 5'-monophosphate to the nucleoside (Tozzi *et al.*, 2006).

The ectonucleotidases reduce the amount of ATP available for the activation of P2 receptors, limiting the excitatory effects of the nucleotide, and, at the same time, increase the extracellular levels of adenosine, which is also released per se from neurons and glial cells, and may activate A1 and/or A2 receptors (Rathbone *et al.*, 1999). Therefore ectonucleotidases could play an important role in regulating the extent and also the duration of the intracellular signal cascade pathway mediated by P1 and P2 receptors (Rathbone *et al.*, 1999).

The levels of extracellular adenosine also depend on the control of the cytoplasmic adenosine concentrations; intracellular levels of adenosine are kept low principally by its conversion to AMP by the salvage enzyme adenosine kinase, but adenosine may also be degraded to inosine by adenosine deaminase (ADA) (Hasko *et al.*, 2005). These enzymes are able to maintain adequate levels of adenosine available for nucleic acid and ATP synthesis. The reactions of phosphorylation predominate when adenosine occurs at a low physiological concentration (< 1 μM), whereas adenosine deaminase is activated at higher concentrations of the substrate ($>$ 10 μM) during ATP depletion (Borowiec *et al.*, 2006). ADA is mainly a cytosolic enzyme, thought to maintain a strict control of adenosine levels, preventing cytotoxic actions of adenosine and deoxyadenosine in peripheral tissues and CNS.

In the central nervous system, ADA activity is most prominent in glia, where its activity is at least five times that in peripheral ciliary ganglion neurons and nine-fold that in central neurons (Rathbone *et al.*,1999). ADA is also expressed in the external surface of the cells in the CNS. In particular, ADA has been found in the synaptic vesicles which contains also ecto-5'-nucleotidase and the combined effect of ecto-5'-nucleotidase, nucleoside transport and ADA may serve to regulate the effective local concentration of adenosine and therefore the extent of adenosine receptor activation (Rathbone *et al.*, 1999).

In the central nervous system adenosine is present at low concentrations in the extracellular space and its levels are greatly increased under metabolically stressful conditions (Hasko *et al.*, 2005). Under these conditions, the extracellular concentration of adenosine rapidly rises from nanomolar to micromolar levels (van Calker and Biber, 2005). When adenosine deriving from the intracellular degradation of ATP to adenosine by the metabolic enzyme 5'-nucleotidase, reaches high concentrations inside the neuronal cell, it is expelled into the extracellular space by bidirectional, equilibrative, nucleoside transporters, meaning that the net transport of adenosine either into or out of the cell depends upon the adenosine concentration gradient in both sides of the membrane. Inhibition of adenosine transport can, therefore, inhibits either adenosine release or adenosine uptake, depending upon the intra- and extracellular levels of adenosine (Hasko *et al.*, 2005). On the other hand, under ATP-depleting conditions, cultured astrocytes first release adenine nucleotides which are subsequently hydrolyzed to adenosine extracellularly by ecto-nucleotidases (Parkinson and Xiong, 2004).

3.5 Inosine and guanosine-based purine metabolism in the central nervous system

Also in CNS, inosine which derives from the deamination of adenosine, is phosphorolytically cleaved by a family of enzymes, the purine nucleoside phosphorylases (PNPs), in order to produce the base, hypoxanthine and the phosphorylated sugar. The base can be reconverted to IMP by phosphoribosylation and the activity of this nucleotide cycle, considered as the main salvage pathway of nucleoside, is demonstrated in the brain and dominates in neurons (Tozzi *et al.*, 2006).

The pathway for the degradation of IMP proceeds through inosine, hypoxanthine and xanthine to uric acid. These reactions are catalyzed by IMP-5'-nucleotidase, PNP and xanthine oxidase, respectively. Xanthine oxidase activity could not be detected in the cultured astroglia. Absence of xanthine oxidase activity is an advantage for the reutilization of hypoxanthine for IMP synthesis (Zoref-Shani *et al.*, 1995).

Inosine is degraded further to the stable end-product uric acid, which has anti-inflammatory properties and, as such, is a potential candidate agent for the treatment of multiple sclerosis (Mattle H.P. et al., 2004).

Guanine in the brain is formed by degradation of guanine nucleotides through the pathway: GMP → guanosine → guanine.

Guanine can be reconverted into GMP in the reaction catalyzed by HGPRT, or converted to xanthine in the reaction catalyzed by guanase; thus, the fate of guanine is determined by the relative activities of these enzymes, since the affinity of both enzymes for guanine is apparently similar (Zoref-Shani *et al.*, 1995). In the 45-day-old rat astroglia, the activity of guanase is four to five-fold that of HGPRT. This ratio indicates increased advantage for guanine degradation in the form of xanthine, over its reutilization for nucleotide synthesis. A similar situation occurs also in the neurons (Zoref-Shani *et al.*, 1995). However, in cultured astrocytes the activity of phosphoribosyltransferases and guanase increased markedly with age; thus, cultured astroglia are able to metabolise guanine more intensively than are neurons. Guanase is considered one of the major responsible for ammonia production in the brain (Tozzi *et al.*, 2006). Cells, including astrocytes, cannot accumulate deoxy purines, as they are toxic (Tozzi *et al.*, 2006).

3.6 Pyrimidine metabolism in the central nervous system

The circulating pyrimidines uridine and cytidine, besides being incorporated into nucleic acids, can serve as substrates for the salvage pathway of pyrimidine nucleotide synthesis (Cansev *et al.*, 2006).

Uridine and cytidine are also considered as precursors of the cytidine triphosphate (CTP) needed in the phosphatidylcholine (PC) and phosphatidylethanolamine (PE) biosynthetic pathway which are the two major membrane phospholipids and they are precursors for the UDP and UTP that activate brain P2Y receptors and that promote brain glycogen synthesis via UDPglucose (Cansev *et al.*, 2006). Some experiments reveal that an increase in neuronal cytidine and uridine levels augments CTP levels both *in vitro* and *in vivo*. In humans, the predominant circulating pyrimidine is uridine whereas in rats, it is cytidine. These variations probably reflect the species differences in cytidine deaminase, the enzyme that converts cytidine to uridine in the body (Cansev *et al.*, 2006).

Except for erythrocytes, liver and kidney, which maintain *de novo* pyrimidine biosynthesis and supply other tissues with uridine or cytidine for salvage, in humans most normal tissues in adults rely on the salvage of uridine and cytidine (Cao *et al.*, 2005). The homeostasis of uridine, which regulates several physiological and pathological processes, is maintained by the relative activities of two enzymes: the UTP-CTP inhibited uridine kinase and uridine phosphorylase. The first produces the uridilic nucleotide compounds by the nucleoside uridine whereas the second catalyzes the Rib-1-P-mediated ribosylation of uracil in order to produce the nucleoside (Tozzi *et al.*, 2006).

As already described, the reaction catalyzed by uridine phosphorylase acts anabolically and at least in rat brain, the anabolism of uridine mediated by the uridine phosphorylase enzyme, is favoured because the degradation of uracil to β-alanine, which would drive uridine phosphorolysis, is absent and also because multiple consecutive phosphorylations of uridine by the ubiquitous uridine kinase and nucleoside mono and diphosphokinases drive the phosphoribosylation of uracil catalysed by uridine phosphorylase. Moreover, the absence of uracil phosphoribosyltransferase (catalyzing the formation of UMP by uracil and PRPP) in mammals, further leads to the uracil phosphoribosylation (Tozzi *et al.*, 2006).

In rat brain, *de novo* pyrimidine synthesis, although at lower rates than those of liver, has been described and brain pyrimidine levels depend on *de novo* synthesis even if circulating cytidine and uridine are essential for maintaining various brain functions. Thus, pyrimidines have to be taken up from the circulation in order to maintain all brain functions. Hence, their plasma concentrations, as well as their transport proteins, will be important in modulating brain levels of cytidine and uridine (Cansev *et al.*, 2006).

The liver has been proposed as the major tissue for regulating plasma pyrimidine levels even if, in humans, the liver may not be the major catabolic tissue for pyrimidines; there may be multiple sites of uptake and degradation such as in erythrocytes (Cansev *et al.*, 2006).

4. Neurological disorders in purine and pyrimidine dismetabolism

Neurological disorders are disorders that affect the central nervous system (brain and spinal cord), the peripheral nervous system (peripheral nerves and cranial nerves included), or the autonomic nervous system. It is well established that there is a strong correlation between the alteration of purine and pyrimidine metabolism and the outbreak of neurological disorders. In particular, deficiencies of some enzymes of purine and pyrimidine metabolism have been known to be associated with severe neurological dysfunctions.

4.1 Alteration of purine metabolism

Lesch-Nyhan syndrome (LNS) is caused by the complete deficiency of the enzyme hypoxanthine-guanine phosphoribosyltransferase (HGPRT). In the absence of HGPRT, hypoxanthine and guanine are not utilized but are degraded to uric acid which is overproduced. Besides the absence of the recycling of purine bases there is a pronounced activation of purine synthesis via *de novo* pathway (Nyhan *et al.*, 2005). In this syndrome, the direct cause for the neurological deficit has been shown to be defective arborization of the dopaminergic neurons, but the relationship between a complete deficiency of HGPRT and the neurobehavioural abnormalities in Lesch-Nyhan disease remains an enigma (Nyhan *et al.*, 2005). These studies reveal several metabolic alterations, including excessive production and excretion of uric acid, which may be the link between HGPRT deficiency and the derangement in dopamine metabolism. The gene of HGPRT is located on the X chromosome and the expression is almost exclusively recessive, but a small number of females have been reported, most reflecting nonrandom inactivation of the normal X chromosome (Nyhan *et al.*, 2005).

The clinic picture combines neurologic features of retarded motor development, dystonia and involuntary movements, and self-injurious behaviour. The patient is unable to walk or to sit unassisted. Loss of tissue results from biting of lips or fingers or both. Patients are not insensitive to pain. Aggressive behaviour is also directed against others. Hyperuricemia causes a deposition of uric acid crystals in the kidneys, joints and subcutaneous tissues. These deposits may cause gouty arthritis, tophi, hematuria, nephrolithiasis, urinary tract infection, and renal failure (Nyhan *et al.*, 2005). Treatment with allopurinol, a xanthine oxidase enzyme inhibitor, is useful to prevent the hyperuricemia, but has no beneficial effect on the neurobehavioral features of the disease. In fibroblasts of patients affected by Lesch-Nyhan syndrome, a reduced cytidine/uridine salvage into UDP sugars is observed and the incorporation of uridine into CTP is minimal compared to the levels of cytidine metabolized to UTP, indicating that fibroblasts, unlike lymphoblasts, lack active CTP synthetase. Therefore, Lesch-Nyhan fibroblasts show a disturbed pyrimidine metabolism. Thus, aberrant pyrimidine nucleotide metabolism, could play a vital role in the pathophysiology of Lesch-Nyhan disease (Fairbanks *et al.*, 2002).

The other disorder that causes hyperuricemia results from variation in phosphoribosylpyrophosphate (PRPP) synthetase which catalyzes the conversion of the ribose5-P into PRPP. This disorder leads to greater than normal enzymatic activity. Complications include nephropathy, urinary tract calculi, and gouty arthritis (Nyhan *et al.*, 2005).

Another disorder of purine metabolism that leads to renal stone disease in children is adenine phosphoribosyltransferase (APRT) deficiency. This enzyme catalyzes the conversion of adenine to its mononucleotide (AMP). Deficiency of APRT leads to accumulation of adenine, which is oxidized in the presence of xanthine oxidase to 2,8-dihydroxyadenine. This compound is very insoluble (Nyhan *et al.*, 2005).

Adenosine deaminase (ADA) deficiency is the major metabolic cause of severe combined immunodeficiency disease (SCID) with mental and growth retardation; ADA catalyzes the irreversible deamination of adenosine to form inosine, and of deoxyadenosine to deoxyinosine (Nyhan *et al.*, 2005). The ADA-immunodeficiency syndrome results in the accumulation of toxic levels of adenosine and 2-deoxy-Ado with a failure of embryonic neural cell development (Nyhan *et al.*, 2005).

Purine nucleoside phosphorylase (PNP) catalyzes the reversible cleavage of inosine and guanosine to their respective bases hypoxanthine and guanine. Deoxyinosine and deoxyguanosine are also substrates. PNP deficiency causes a severe immune-deficiency syndrome associated with incomplete cerebral development and mental retardation which is SCID indistinguishable from that of ADA deficiency. In the PNP deficiency, the urinary excretion of uric acid is reduced (Nyhan *et al.*, 2005).

A prominent cause of hypouricemia is hereditary xanthinuria which results from deficiency of xanthine oxidase which catalyzes the conversion of hypoxanthine to xanthine and can further catalyze the oxidation of xanthine to uric acid. The xanthine oxidase deficiency causes a xanthine accumulation and this compound is very insoluble; so patients are also of risk for the renal complications of calculus formation. Crystals of xanthine may also be found in muscle in patients with muscular pains or cramps. Also the deficiency of molybdenum which is the xanthine oxidase cofactor, causes hypouricemia. It also causes sulfite oxidase deficiency, a much more devastating disease (Nyhan *et al.*, 2005).

AMP deaminase catalyzes the conversion of AMP to IMP and NH_3. The deficiency of this enzyme causes pain or cramps in muscle following exercise (Nyhan *et al.*, 2005).

Page et al. (1997) describe some unrelated patients in whom developmental delay, seizures, ataxia, recurrent infection, speech deficit, and an unusual behavioural phenotype are associated with highly elevated activity of a cytosolic 5’-nucleotidase. Moreover, also Lesch-Nyhan patients show cytosolic 5'-nucleotidase hyperactivity in erythrocytes (Pesi *et al.*, 2000). In the treatment of a form of autism with seizures, oral uridine administration has led to improvement in speech, behaviour and decreased frequency in seizures but the relationship between the partial recover of the patients and the action of uridine remains an enigma (Page *et al.*,1997).

4.2 Alteration of pyrimidine metabolism

In nature there are several inherited disorders affecting pyrimidine metabolism, the clinical signs of which often manifest as a serious disruption of normal neurological function. Patients with pyrimidine metabolic, or related, disorders, which lead to the excessive production of one or more pyrimidines, exhibit various neurological manifestations, for example, hereditary orotic aciduria, ornithine carbamoyl-transferase and dihydro pyrimidinase deficiencies. Conversely, a reduced synthesis of endogenous pyrimidines has been associated with symptoms of schizophrenia and maniac depression, and Huntington's diseases (Connelly *et al.*, 1996).

The classic disorder of pyrimidine biosynthesis *de novo* is orotic aciduria. The metabolic defect is in the enzyme uridine-5-monophosphate (UMP) synthase which contains in a single protein the activities of two enzymes, phosphoribosyltransferase (OPRT) and orotidine-5'-monophosphate (OMP) decarboxylase, which catalyze the last two steps of UMP synthesis. Orotic aciduria is a rare disease that is characterized by megaloblastic anemia. Crystals of orotic acid have caused urethral and ureteral obstructions hematuria and azotemia. Retardation of physical and intellectual development may be observed with or without hematologic abnormalities. The activities of OMP and OPRT are deficient in erythrocytes, leukocytes, and cultured fibroblasts (Nyhan *et al.*, 2005).

Uridine monophosphate synthase (UMPS) is the enzyme responsible for converting orotic acid to uridine monophosphate (UMP), which is an essential component of pyrimidine nucleotides. UMPS type-1 deficiency causes orotic aciduria, gross crystaluria and occasional ureteric obstruction (Nyhan *et al.*, 2005).

Uridine monophosphate hydrolase (UMPH) is the first degradative enzyme of the pyrimidine salvage cycle and catalyses the specific hydrolysis of the monophosphate nucleotides: uridine monophosphate (UMP) and cytidine monophosphate (CMP) to pyrimidines (uridine or cytidine) and Pi. Clinically, UMP hydrolase deficiency presents as haemolytic anaemia, with reticulocytosis and gross basophilic stippling, sometimes associated with learning difficulties, or as anaemia associated with lead poisoning (Nyhan *et al.*, 2005).

CDP-choline phosphotransferase catalyses the last step in the synthesis of phosphatidyl choline. A deficiency of this enzyme is proposed as the metabolic basis for the selective accumulation of CDP-choline in the erythrocytes of rare patients with an unusual form of haemolytic anaemia. In patients with this defect, which occurs mainly in children, CDP-choline and CDP-ethanolamine accumulate in erythrocytes to a similar degree as in UMPH-1 deficiency. A diagnosis of CDP-choline phosphotransferase deficiency should be considered when erythrocyte screening shows abnormal nucleotide levels but UMPH-1 activity is normal (Nyhan *et al.*, 2005).

Dihydropyrimidine dehydrogenase (DPD) is the initial and rate-limiting step in pyrimidine catabolism. DPD is the first of three enzymes catalysing the degradation of uracil or thymine to β-alanine or β-aminoisobutyric acid. DPD deficiency shows considerable genetic heterogeneity in clinical presentation, such as epilepsy, dysmorphic features and mental retardation (Nyhan *et al.*, 2005).

Mitochondrial neurogastrointestinal encephalomyopathy (MNGIE) is an autosomal recessive disorder caused by loss-of-function mutations in the gene encoding thymidine phosphorylase (TP) which catalyzes the reversible phosphorolysis of thymidine to thymine and 2-deoxy-D-ribose 1-phosphate. MNGIE is characterized clinically by onset between the second to fifth decades, apoptosis, progressive external ophthalmoplegia, gastrointestinal dysmotility, thin body habitus, peripheral neuropathy, myopathy, leukoencephalopathy, and lactic acidosis (Nishino *et al.*, 1999).

No defect in uridine phosphorylase, or four of the six synthetic enzymes, has been reported, suggesting that such defects are incompatible with life, or that current techniques are not sufficiently sensitive to detect these defects (Loffler *et al.*,2005).

The study of purine and pyrimidine metabolism and the pharmacologically induced inhibition of key enzymes involved in the synthetic pathways provided much useful information for new drug design, particularly in the field of antitumor and antiviral agents. Similar studies could also enlarge the knowledge of pathophysiology of neurological disorders.

5. Physiological activity of purine and pyrimidine compounds exerted through a receptor dependent mechanism

5.1 General aspects

Purines and pyrimidines are emerging as physiological regulators of a number of cellular functions such as cell growth, differentiation, cell death, release of hormones, neurotransmitters and cytokines (Fig.5).

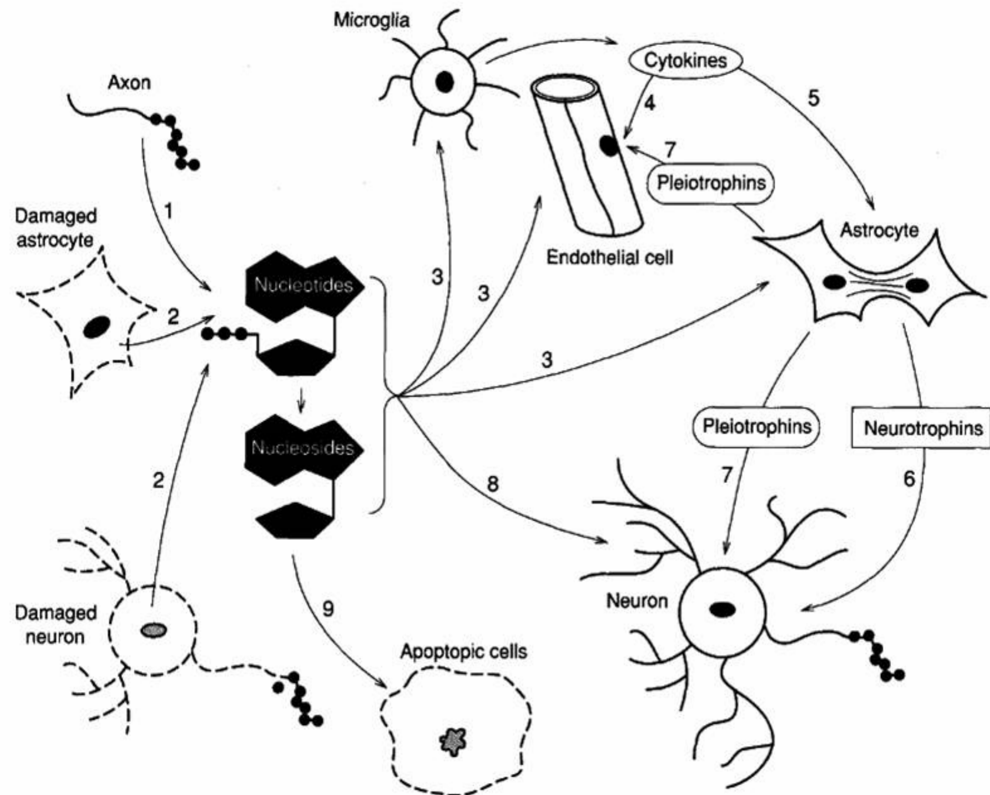


Fig.5. Action of purines and pyrimidines on cells in the CNS (Image taken from Abbracchio *et al.*, 1998)

Purines and pyrimidines can also both stimulate the progression of cells through the cell cycle and inhibit cell growth, simply depending upon their extracellular concentrations, the physiological state of target cells and/or the expression of specific receptors called P receptors (Abbracchio and Burnstock, 1998). There are two classes of purinergic receptors (P receptors), P_1 and P_2. P_1 receptors are mainly activated by adenosine while the P_2 are activated by ATP, ADP and UTP. The role of extracellular purines in intercellular communication is phylogenetically ancient and universal in plants and animals. Receptors for extracellular purine nucleosides are found on the surface of all higher animal and tissues (Neary *et al.*, 1996). Some of these receptors are linked to signalling mechanisms that have been implicated in mitogenesis and differentiation, such as Ca^{2+} influx and mobilization, polyphosphoinositide metabolism and protein phosphorylation (Neary *et al.*, 1996).

The principal release of nucleosides from glia is non-exocytotic, independent of nucleotide release and occurs through bi-directional nucleoside transport systems as has been observed in hippocampal slices and in brain synaptosomal preparations (Rathbone *et al.*, 1999). It is evident that alterations in the physiology of purinergic signalling may result in the development of a variety of pathologies, including immune system diseases, infiammation and pain, neurodegenerative disorders and osteoporosis (Abbracchio and Burnstock, 1998).

The intracellular levels of purine nucleotides and nucleosides also determine the amount of these substances that can be released by the cells upon appropriate stimulation. Thus, the inhibition of some key enzymes of purine metabolism dramatically alters both intracellular and extracellular purine levels (Abbracchio and Burnstock, 1998).

Purine and pyrimidine nucleotides and nucleosides act as trophic agents on the cells of the nervous system in several ways: they may target glial cells directly, inducing functional changes which modulate neuronal differentiation; they may stimulate the synthesis and the release of trophic factors from neuronal and non-neuronal cells; purines may enhance the effects of growth factors on their target cells; finally, purines may interact directly with neurons or neuronal precursors, eliciting neuritogenesis, biochemical differentiation, the maintenance of existing neuritis or enhancing neuronal survival. Purine nucleotides and nucleosides may also act as trophic agents through a combination of these actions (Rathbone *et al.*, 1999)

Neurotrophic factors are molecules that exert a variety of actions stimulating both the development and differentiation of neurons and the maintenance of cellular integrity; they are required for the survival and development of neurons throughout the life cycle of organisms. Generally, neurotrophic factors may be divided into two broad classes: neurotrophins and pleiotrophins. Neurotrophins constitute a large family of related proteins such as nerve growth factor (NGF), neurotrophins 3 and 4 (NT3, NT4) or glial-derived neurotrophic factor (GDNF).

These proteins are growth factors that encourage survival of nervous tissue after a brain injury by prohibiting the neuron from initiating apoptosis. Neurotrophins also induce differentiation of progenitor cells, to form neurons (Zafra *et al.*, 1992). Pleiotrophins or neurite growth-promoting factors 1 (NEGF1) are an important developmental cytokine and constitute a family of secreted heparin-binding proteins. Cytokines are a group of proteins and peptides that are used in organisms as signalling compounds. These chemical signals are similar to hormones and neurotransmitters and are used to allow one cell to communicate with another (van Calker and Biber, 2005). Cytokines act in order to stimulate and regulate immune cell proliferation and differentiation. A number of glial derived cytokines have also been described to have neuroprotective properties; IL-1a, IL-1b, IL-6, IL-10, TNF-a, TGF-b and the chemokine CCL2 (van Calker and Biber, 2005).

Some authors report that purines are able to influence the synthesis and release of neurotrophins (for example NGF or GDNF), pleiotrophins (for example FGF in neurons and astrocytes) and also cytokines. In particular purines (and also pyrimidines) released under some pathological conditions, serve as signal that acts in concert with polypeptide growth factors to initiate brain repair mechanisms, including astrogliosis, activation of microglial cells and regeneration of damaged neuronal axons (Abbracchio and Burnstock, 1998; Yamagata *et al.*, 2007).

It is also well established that neurotrophic factors are potential candidates for the pharmacological treatment of chronic neurological diseases or acute brain injuries; unfortunately, pharmacokinetic features such as the poor penetration across the blood-brain barrier limit their systemic administration, which also produces peripheral side effects. Therefore, purine and pyrimidines and their analogs can be used as pharmacological inductor of local production of these trophic factors (Ciccarelli *et al.*, 2001).

5.2 Trophic action of adenosine and classification of adenosine receptors in the central nervous system

When adenosine is released both in normoxic and pathological conditions, it behaves as an extracellular signal molecule influencing synaptic transmission without itself being a neurotransmitter. In fact, while ATP may function as a neurotransmitter in some brain areas, adenosine is neither stored nor released as a classical neurotransmitter since it does not accumulate in synaptic vesicles, being released from the cytoplasm into the extracellular space through a nucleoside transporter (Ribeiro *et al.*, 2002).

The subclassification of adenosine receptors is based on the effects which they cause over changing cAMP concentration in the cell and their affinity for the ligand (Fig.6) (Borowiec *et al.*, 2006). P_1 receptors have four subtypes: A_1, A_{2A}, A_{2B}, and A_3, all of which are G protein-coupled receptors; A_1 and A_3 receptors activate the G_i family of G proteins, whereas $A2_A$ and $A2_B$ receptors activate the Gs family. However, other G proteins can also be activated even though the physiological significance of this is unknown (Fredhlom *et al.*, 2001). A_{2A} receptors stimulate adenyl cyclase activity and therefore induce an increase of intracellular cAMP which activates protein kinase A. On the other hand, A_1 receptors may lead to inhibition of this enzyme. The activation of A_1 and A_3 receptors provokes the modification of phospholipase C activity and/or phosphoinositide turnover (Ribeiro *et al.*, 2002).

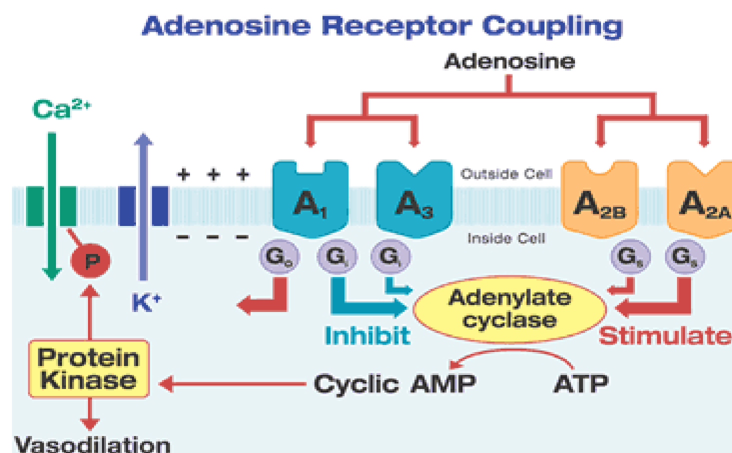


Fig.6 The subclassification of adenosine receptors (Image taken from www.aderis.com/science/programs.htm)

In the central nervous system, the adenosine A_1 receptor is highly expressed in brain cortex, cerebellum, hippocampus, and dorsal horn of spinal cord (Ribeiro *et al.*, 2002).

In the peripheral and central nervous system, adenosine is able to regulate nerve cell activity. Presynaptically, adenosine inhibits, via A_1 receptors, the release of several neurotransmitters such as acetylcholine, noradrenaline, serotonin, dopamine and excitatory amino acid including glutamate (Rathbone *et al.*, 1999; Martin *et al.*, 2007). Postsynaptically, adenosine, via A_1 receptors, causes hyperpolarization of the cell membrane, mainly by increasing K^+ conductance, by stabilization of the Mg^{2+} blockade of N-methyl D-aspartate (NMDA) receptors and by a reduction of Ca^{2+}-influx, probably caused by an inhibition of N-type voltage-dependent Ca^{2+} channels. Thus the sum of these pre- and postsynaptic effects leads to decrease of neuronal excitability (van Calker and Biber, 2005). The decrease of neuronal excitability is particularly important under pathological conditions such as ischemia, when excessive stimulation by excitatory amino acids such as glutamate can lead to death of neurons (van Calker and Biber, 2005).

Astrocytes are the main cerebral source of extracellular adenine- and guanine-based purines and express specific receptors for these substances. Adenine nucleotides stimulate both astrocyte proliferation and differentiation whereas adenine nucleosides inhibit cell proliferation and induce apoptosis cell death. The best characterised P receptors in astrocyte are those for adenine-based purines, belonging to both Ado P1 receptors (A_1, A_{2A}, A_{2B} and perhaps A_3 subtypes) and ATP P2 receptors (P2Y1, P2Y2 and P2X7 subtypes) (Neary *et al.*, 1996).

Adenosine acts at high affinity A_1 receptors to reduce astrocyte proliferation. Stimulation of A_1 receptors exerted by adenosine in cultured astrocytes results in hyperpolarization of the membrane potential, inhibition of adenylate cyclase and potentiation of the effects of metabotropic glutamate receptor-mediated (van Calker D. and Biber K., 2005).

The stimulation of A_{2A} receptor exerted by adenosine in astrocyte cells, inhibits the expression of inducible nitric oxide synthase (iNOS), and thus the production of nitric oxide (NO). The production of NO by iNOS in the brain seems to contribute to the pathophysiology of many CNS diseases, so the inhibition of NO formation by adenosine might be an important protective mechanism during inflammatory conditions in the brain (Hasko *et al.*, 2005). Antipsychotic drugs, potent dopamine receptor antagonists, are commonly used in the treatment of psychotic and affective illness. The discovery of antagonistic interactions between A_{2A} adenosine receptors (ARs) and D2 dopamine receptors (DRs) in the central nervous system suggests that the adenosine system may be involved in the pathogenesis of psychiatric and neurological disorders (Martini *et al.*, 2006). $A2_B$ possesses low levels of expression in the brain (Fredholm *et al.*, 2001). Stimulation of A_{2B} (and A_1) receptors elicits the release of the pro-inflammatory cytokine IL-6 from astrocytes (Hasko *et al.*, 2005; van Calker and Biber, 2005). Because IL-6 is neuroprotective against hypoxia and glutamate neurotoxicity, stimulation of A_{2B} receptors provides a damage-control mechanism during CNS injury (Hasko *et al.*, 2005; van Calker and Biber, 2005).

Low-affinity A_{2B} adenosine receptors, which are expressed in astrocytes, are mainly activated during brain hypoxia and ischaemia, when large amounts of adenosine are released and they are able to stimulate adenylate cyclase through Gs proteins (Trincavelli *et al.*, 2004). Cell treatment with tumor necrosis factor alpha increases A_{2B} functional response and receptor G protein, without any changes in receptor protein and mRNA levels (Trincavelli *et al.*, 2004). Moreover, in the presence of TNF-alpha, A_{2B} stimulation *in vitro* induced the elongation of astrocytic processes, a typical morphological hallmark of *in vivo* reactive astrogliosis (Trincavelli *et al.*, 2004).

5.3 Classification and properties of P2 receptors

P2 receptors are classified into P2X and P2Y receptors. P2X-receptors are ligand-gated ion channels (ionotropic), whereas P2Y-receptors belong to the superfamily of G-protein-coupled receptors (metabotropic).

P2X receptors are a family of cation-permeable ligand gated ion channels that open in response to extracellular adenosine 5'-triphosphate (ATP) and neither G-proteins nor second messenger cascades appear to be directly involved (Rathbone *et al.,* 1999). These ligand-gated channels show little selectivity for monovalent cations and are permeable to Na^+, K^+ and Ca^{2+} (Rathbone *et al.*, 1999). ATP binds to the extracellular loop of the P2X receptor, causing a conformational change in the structure of the ion channel which results in the opening of the ion-permeable pore. This allows cations such as Na^+, K^+ and Ca^{2+} to enter the cell, leading to depolarization of the cell membrane and the activation of various Ca^{2+}-sensitive intracellular processes. At least three ATP molecules are required to activate a P2X receptor, suggesting that ATP needs to bind to each of the three subunits in order to open the channel pore (Abbracchio and Burnstock, 1998). P2X receptors are expressed in cells from a wide variety of animal tissues.

On presynaptic and postsynaptic nerve terminals throughout the central, peripheral and autonomic nervous systems, P2X receptors have been shown to modulate synaptic transmission (Abbracchio and Burnstock,1998). Furthermore, P2X receptors are able to initiate contraction in cells of the heart muscle, skeletal muscle, and various smooth muscle tissues. P2X receptors are also expressed on leukocytes, including lymphocytes and macrophages, and are present on blood platelets (Abbracchio and Burnstock, 1998).

P2Y receptors are G protein-coupled receptors that are stimulated by nucleotides such as ATP ($P2Y_2$, $P2Y_{11}$), ADP, UTP ($P2Y_2$, $P2Y_4$), UDP ($P2Y_6$) and UDP-glucose. To date, 8 P2Y receptors have been cloned in humans: $P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$, $P2Y_{11}$, $P2Y_{12}$, $P2Y_{13}$ and $P2Y_{14}$. The P2Y receptors are present in almost all human tissues where they mediate slow responses through G-proteins that are usually coupled to phospholipase C and activate the inositol phosphate cascade and subsequently modulate intracellular calcium (Rathbone *et al.*, 1999). These receptors are involved in blood platelet aggregation, vasodilatation and neuromodulation and the biological effects of P2Y receptor activation depends on how they couple to downstream signalling pathways, either via G_i, G_q or G_s G proteins (Rathbone *et al.*, 1999; von Kugelgen, 2006).

5.4 Trophic effects of inosine and guanosine-based purines and their specific receptors

Inosine has been shown to produce inhibitory responses in rat cerebellar Purkinje cells, mouse spinal cord neuronal cultures, rat cortical neurons, and pig hippocampal slice. Inosine also appears to elicit a rapidly desensitizing excitatory response in mouse spinal cord neuronal cultures (Haun *et al.*, 1996). Inosine delays the time to contracture, and decreases infarct size in ischemic myocardium (Haun *et al.*, 1996).

Shen *et al.* (2005) demonstrate that inosine is able to reduce ischemic brain injury in rats; the authors suppose that the effect is not elicited by hypoxanthine and the lack of protection by hypoxanthine indicates that inosine-mediated protection is not through the production of active metabolite hypoxanthine or ATP *in vivo*. In this study, the authors find that A3 antagonist MRS1191 attenuated inosine-mediated protection, suggesting that the activation of A3 receptors contributes to these protective effects of inosine in the CNS (Shen *et al.*, 2005).

Inosine, guanosine and adenosine are able to improve neurite outgrowth and viability in cells subjected hypoxic stress through a strong activation of mitogen-activated protein (MAP) kinases (Tomaselli *et al.*, 2005)

There are some lines of evidence indicating that guanosine may also act as neurotransmitter or neuromodulator. There is no strong evidence that GTP is a neurotransmitter or neuromodulator. However, GTP is stored in synaptic vesicles with ATP. This indicates that GTP is co-released with ATP during physiological nerve activity (Rathbone *et al.*, 1999).

Indirect evidence indicates that certain neurons release guanosine following depolarization and that extracellular guanosine is excitatory to hippocampal neurons in slice preparations (Rathbone *et al.*, 1999). GTP is also found to stimulate voltage-gated Ca^{2+} channels to increase $[Ca^{2+}]_i$ (Rathbone *et al.*,1999). Guanosine, GTP and cGMP, promote neurite outgrowth from PC12 neuronal cells and also from primary cultures of hippocampal neurons (Rathbone *et al.*, 1999). The nucleoside transport inhibitors nitrobenzylthioinosine and dipyridamole fail to block this neuritogenic effect of guanosine, suggesting that this effect is mediated through cell-surface purine receptors and not directly through intracellular mechanisms requiring uptake (Rathbone *et al.*, 1999). Exogenous guanine nucleotides and nucleosides are very active in stimulating the proliferation rate of different types of cells, including cultured astrocytes derived either from avian or mammalian brain (Rathbone *et al.*, 1999). In fact astrocytes have various receptors for adenosine, but the presence of putative receptors or specific binding sites also for guanosine or GTP has been postulated in astrocytes (Ciccarelli *et al.*, 2001). Astrocytes release guanine nucleotides and guanosine in the resting state and this release is greatly increased by an anoxic-hypoglycemic insult (Rathbone *et al.,* 1999).

Guanine-based purines may increase the production of neurotrophins, such as NGF and neurotrophin- 3, and pleiotrophins, such as FGF or S100β protein from cultured astrocytes (Ciccarelli *et al.*, 2001). Guanosine activates also MAP kinase cascade in cultured astrocytes. This cascade is the primary mechanism by which NGF can induce differentiation and neurite outgrowth in a neuronal cell line (Ciccarelli *et al.*, 2001).

5.5 Trophic effects of pyrimidine compounds and their specific receptors

Uridine, a pyrimidine nucleoside, has a variety of roles in physiological states, such as the synthesis of DNA, RNA or biomembranes. Besides these, uridine has a lot of roles in biological processes, including vascular resistance, spermatogenesis, dopaminergic neurotransmission, and sleep-inducing. Uridine appears to be involved in the cellular responses in various tissues under ischemic-like conditions. High concentration of uridine is released from cold stored liver after reperfusion and is present in plasma after infarct. In addition, uridine perfusion restored myocardial ATP, glycogen and uridine diphosphoglucose (UDPG) which functions as a key in the transformation of glucose to other sugars (Pizzorno *et al.*, 2002; Choi *et al.*, 2006).

Uridine and its nucleotides have shown a complex effect in the regulation of vascular resistance, producing opposing effects in some tissues, either by acting directly on the smooth muscle cells or by stimulating the surrounding endothelial cells (Pizzorno *et al.*, 2002). Uridine is also present in the seminal fluid at millimolar concentrations, a level that is approximately three orders of magnitude higher compared to other body fluids or tissues. The presence of uridine at these high concentrations and its correlation with sperm motility suggests a role of uridine in spermatogenesis (Pizzorno *et al.*, 2002).

Uridine can regulate the output of neurites from differentiating neuronal cells and intact brain acting through cytidine triphosphate (the final product of the pathway uridine→UTP→CTP) as a precursor for phosphatidylcoline biosynthesis and acting through UTP as an agonist for P2Y receptors; in fact the stimulation of neurite outgrowth depends also on activation of P2Y receptors (Pooler *et al.*, 2005)

In the clinical setting, uridine is utilized in the treatment of autism with seizures and pyrimidine-deficient genetic diseases such as orotic aciduria (Cao *et al.*, 2004). Thus, uridine exerts several modulatory and regulatory functions even though no clear mechanism has been identified in order to modulate these physiological activities (Pizzorno *et al.*, 2002). In fact, specific receptor for uridine are not still discovered even if Kimura *et al.* (2001) postulate the presence of a specific receptor for uridine involved in sleep mechanism. On the other hand, extensive evidences indicate that there are specific receptors for pyrimidine nucleotides, including UTP, UDP and UDP-glucose.

Pyrimidine nucleotides are important signalling molecules which activate G protein-coupled membrane receptors (GPCRs) of the P2Y family. Uracil nucleotide-sensitive P2Y receptor subtypes may constitute future targets for the treatment of certain cancer types, vascular diseases, inflammatory diseases, and immunomodulatory intervention. They have also been proposed to play a role in neurodegenerative diseases (Brunschweiger *et al.*, 2006). Uracil nucleotides exert specific P2 receptor-mediated effects on midbrain-derived human mesencephalic neuronal stem/precursor cells and may be used to enhance both proliferation and dopaminergic differentiation (Milosevic *et al.*, 2006). In order to induce proliferation and dopaminergic differentiation, uracil nucleotides exert their action activating the MAPK proteins (Pooler *et al.*, 2005; Milosevic *et al.*, 2006).

This effect is abolished by apyrase, an enzyme that degrades nucleotides such as UTP and blocked by P2Y receptor antagonists, suggesting that uridine, in addition to increasing CTP synthesis, promotes neurite outgrowth by UTP mediated stimulation of a P2Y receptor-coupled signalling pathway (Pooler *et al.*, 2005).

6. Protective role of purine and pyrimidine compounds exerted through a receptor independent mechanism

The brain depends on both glycolysis and mitochondrial oxidative phosphorylation for maintenance of ATP levels. The substrate and oxygen deprivation associated with hypoxia or ischemia leads to reduced mitochondrial ATP synthesis and subsequent decline in cellular energy stores as ATP generation fails to meet cellular demands (Jurkowitz *et al.*, 1998). It is presumed that glucose deprivation induces depletion of ATP, membrane depolarization, extracellular accumulation of excitatory amino acids, excitotoxic calcium overload, loss of neuronal homeostasis and degeneration (Ciccarelli *et al.*, 2001). In particular, mitochondrial dysfunction and oxidative stress have been implicated in neurodegenerative disorders such as Alzheimer's, amyotrophic lateral sclerosis, Huntington's and Parkinson's disease (Jurkowitz *et al.*, 1998). Consequently, reactions requiring ATP are compromised, including those involved in synthesis of macromolecules, ion pumping, and protein phosphorylation (Jurkowitz *et al.*, 1998). The loss of homeostasis eventually leads to irreversible cell damage if high-energy phosphate reserves are not soon replenished. The level to which ATP falls during hypoxia or ischemia has been correlated with the extent of subsequent cellular damage and loss of function in both *in vitro* and *in vivo*.

The catabolism of ATP during hypoxia or ischemia results in increases in the levels of AMP, IMP, adenosine, and inosine in brain as well as in cultured glia and neurons. Adenosine and inosine are released to the extracellular space where concentrations may reach high micromolar level (Jurkowitz *et al.*, 1998). Haun et al (1996) demonstrate that the combined glucose-oxygen deprivation in rat astrocyte cultures, causes an astroglial injury; nevertheless, the somministration of adenosine, dramatically reduces cellular injury in the cultures and selective adenosine receptor agonists fail to exert a protective effect while adenosine receptor antagonist failed to reverse the protective effect of adenosine. Moreover, these authors have revealed that inosine is protective at concentrations nearly identical to those of adenosine, whereas hypoxanthine and ribose fail to provide protection during the combined glucose-oxygen deprivation. They also demonstrate that an adenosine deaminase inhibitor, reverses the protective effect of adenosine, suggesting that the glioprotective effect of adenosine requires degradation of adenosine to inosine. Finally, they demonstrated also that dipyridamole, a purine uptake inhibitor, blocked the protective effect of both adenosine and inosine, implying an intracellular mechanism (Haun. *et al.*, 1996). Therefore, adenosine must be metabolized to inosine to exert a protective effect without any involvement of adenosine receptor. In fact, adenosine deaminase is a cytosolic enzyme, therefore adenosine must enter the cells to be metabolized to inosine (Haun *et al.*, 1996). According to these authors, inosine could be phosphorolytically cleaved in order to produce the base, hypoxanthine which can be converted to inosine 5'-monophosphate, the precursor for adenine nucleotide synthesis, in the reaction catalyzed by hypoxanthine-guanine phosphoribosyl-transferase. However they observe that hypoxanthine has no effect on astroglial injury in their model, suggesting preservation of the adenine nucleotide pool is not the mechanism by which inosine protects.

Therefore, the authors suppose that inosine exerts its protective effect via an unknown second intracellular messenger system (Haun *et al.*, 1996).

Inosine has been previously shown to induce neurons to express a set of growth-associated proteins and to extend axons in culture and *in vivo*. In adult rats with unilateral cortical infarcts, inosine stimulated neurons on the undamaged side of the brain to extend new projections to denervated areas of the midbrain and spinal cord (Chen *et al.*, 2002). Other authors have used ROC-1 clonal cell line formed by a fusion between rat oligodendroglia and rat C6 astroglial tumor cells and these cells are subjected to metabolic stress conditions by glucose deprivation and mitochondrial inhibition with the respiratory chain amobarbital. Also these authors show that adenosine and inosine are able to protect these cells and for the first time, they demonstrate that also guanosine had this protective effect (Jurkowitz *et al.*, 1998). The effect of adenosine is blocked by coformicin, an inhibitor of adenosine deaminase and the protective effect of inosine and guanosine in viability and ATP does not occur in the presence of BCX-34, a potent inhibitor of nucleoside phosphorylase. Also these authors demonstrate that these compounds have to entry the cells where they are metabolized in order to exert the protective effect but they suppose that the protective action is not exerted by an intracellular messenger system but through the formation of the phosphorylated ribose stemming from the phosphorolysis of the nucleoside which is used as an energy source, alternative or supplementary to glucose (Fig.7). (Jurkowitz *et al.*,1998).The pronounced increase observed in lactate production in the presence of nucleosides suggests that ribose moieties derived from the nucleoside are fueling glycolysis. According to these data, they propose the following metabolic pathway: inosine is phosphorolyzed to ribose l-phosphate and hypoxanthine by purine nucleoside phosphorylase. Three ribose l-phosphates are isomerized to ribose 5-phosphate, and then converted to two glucose 6-phosphates and one glyceraldehydes 3-phosphate, via transaldolases and transketolases of the pentose phosphate pathway. These phosphorylated intermediates enter the glycolytic pathway, yielding a net production of eight molecules of ATP per three molecules of ribose 1-phosphate (Jurkowitz *et al.*, 1998). Therefore, the phosphorylated ribose stemming from the phosphorolysis of the nucleoside can be converted, without energy expense, into energetic intermediates and the cells, subjected to metabolic stress conditions, are able to produce ATP and preserve their energy charge.

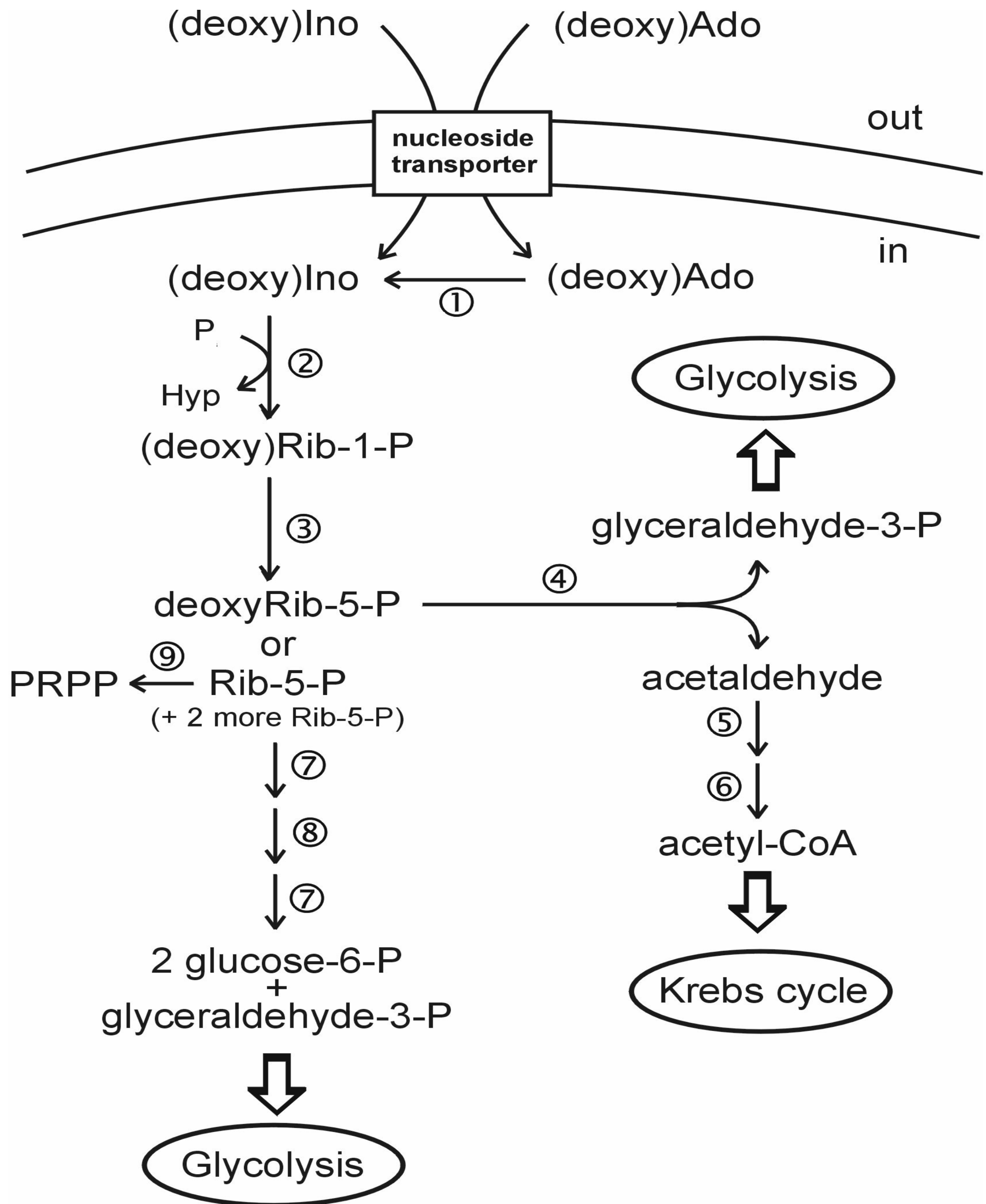


Fig.7. Schematic illustration of the uptake and utilization of (deoxy)nucleosides.
1: adenosine deaminase; 2: purine nucleoside phosphorylase; 3: phosphoribomutase; 4: deoxyRib-5-P aldolase; 5: aldehyde oxidase; 6: acetyl-CoA synthetase; 7: transketolase; 8: transaldolase; 9: PRPP synthetase.
(image taken from Giannecchini *et al.,* 2004)

Giannecchini et al. (2004) demonstrate that the phosphorylated ribose moiety of inosine can be used for energy repletion through anaerobic glycolysis in human carcinoma cell line subjected to metabolic stress conditions.

Shin *et al.* (2002) demonstrate that not only adenosine and inosine but also guanosine is able to protect the death in glucose-deprived immunostimolated astrocytes and this protective effect is blocked by the concomitant incubation with inhibitors of nucleoside transporter (NBTI and dypiridamole) thus confirming a receptor independent mechanism of nucleoside action.

Excessive generation of H_2O_2 has been implicated in the pathogenesis of several neurodegenerative diseases. In particular, activated astrocytes produce various reactive oxygen species (ROS) including H_2O_2. Excess H_2O_2 may cause deterioration of cellular energy metabolism and cellular redox homeostasis. In cortical neurons, H_2O_2 causes a severe loss of ATP and related adenine nucleotides. Depletion of cellular ATP level causes disruption of glutathione (GSH) redox homeostasis, because ATP is closely related to the synthesis of the nicotinamide nucleotides (NADPH), a critical source of GSH synthesis (Yoo *et al.*, 2005).

Yoo *et al.* (2004) demonstrate that adenosine and other purine nucleosides and nucleotides including ATP prevent the augmented cell death of astrocytes co-treated with H_2O_2 and glucose deprivation and this protective effect is made via a receptor independent mechanism.

Choi *et al.* (2006) demonstrate that also uridine is able to restore the intracellular ATP levels in the cells subjected to the ischemic conditions and this protective effect is attenuated when the cellular uptake of uridine is blocked using an inhibitor of nucleoside transporter (NBTI). These authors also demonstrate that the inhibition of uridine phosphorylase activity diminishes the uridine-derived preservation of cellular ATP levels and cell survival. These data indicate that uridine phosphorylase exists in astroglia and has a crucial role in the protective effect of uridine.

7. Nucleosides transporters

The intracellular concentration of nucleosides is determined by the enzymes involved in their metabolism which are regulated only by the availability of their substrates, but of relevant importance is also the efficiency of the nucleoside transporters (Giannecchini *et al.*, 2004). Nucleoside transporters are a family of proteins with different substrate affinity, tissue distribution, species specificity and sensitivity to blockade by pharmacological agents. They are classified as being equilibrative (ENT) and concentrative (CNT) nucleoside transporter systems (Fig.8) (King *et al.*, 2006).

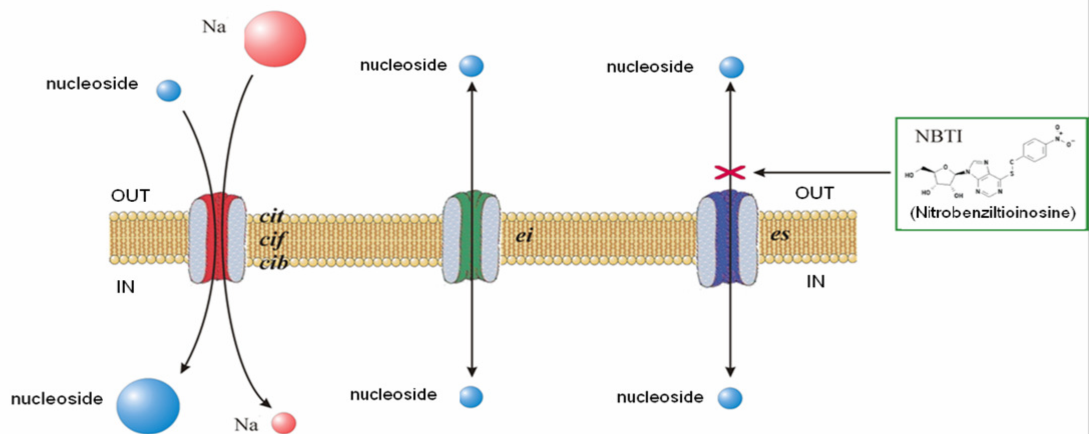


Fig.8. Equilibrative (ENT) and concentrative (CNT) nucleoside transporter systems

The ENT family is restricted to eukaryotes, whereas CNT family members are also found in eubacteria. Their names reflect the properties of the first members to be characterized from mammals: mammalian CNT proteins are cation symporters while ENT proteins are facilitated diffusion systems (King *et al.*, 2006).

7.1 Equilibrative transporters

The equilibrative transport system mediates nucleoside transport in both directions depending on the nucleoside concentration gradient across the plasma membrane. The equilibrative transporters are facilitative-diffusion carriers that display a broad permeant selectivity, accepting both purine and pyrimidine ribo- and deoxyribonucleosides as permeants, whereas the concentrative transporters are nucleoside-sodium symporters that are driven by transmembrane sodium gradients and exhibit relatively narrow permeant selectivity (King *et al.*, 2006). This class of equilibrative transport system is subdivided in two types (*es* and *ei*) based on their sensitivity to nitrobenzylthioinosine (NBTI). The *es* transport system is highly sensitive to inhibition by NBTI (Ki = 0.1 to 10 nM). In contrast, the *ei* transport system is resistant to NBTI up to 1 μM (Pinto-Duarte *et al.*, 2005). Four transporters (ENT1, ENT2, ENT3 and ENT4) belonging to the equilibrative transport system have been identified and cloned. ENT1 and ENT3 are susceptible to NBTI, whereas this compound does not inhibit ENT2. ENT4 has been identified only recently and its transporting characteristics have not been fully determined (Pinto-Duarte *et al.*, 2005). These four members of the equilibrative transport system are widely distributed among various cell types, but the number of molecules of each nucleoside transporter depends on the cell and tissue type. All transporters are able to transport adenosine, but they have different capacities to transport other nucleosides and nucleobases (Pinto-Duarte *et al.*, 2005; Nagasawa *et al.*, 2007).

Each member of the ENT family consists of 11 hydrophobic transmembrane (TM) α-helices arranged in the plasma membrane by such way that the N-terminus is cytoplasmic while the C-terminus is located in the extracellular space. These transporters are post-trasductionally modified by glycosylation (King *et al.*, 2006). The activity of ENTs is controlled by G proteins and protein kinases. Adenosine receptors, which are coupled to G proteins and protein kinase-dependent transducing systems, appear therefore to be good candidates to modify the levels of their endogenous ligand, adenosine (Pinto-Duarte *et al.*, 2005). In particular, A2A receptors are coupled to several G proteins and their activation influences at least the activities of two types of protein kinases, PKA and PKC.

The findings that a PKC inhibitor, but not a PKA inhibitor, prevents the enhancement of adenosine transport induced by the A2A receptor agonist, and that PKC activators *per se* influence adenosine transport, suggest that A2A receptors control ENTs activity in a PKC-dependent manner (Pinto-Duarte *et al.*, 2005).

It has been also demonstrated that insulin up-regulated the level of ENT2 with no involvement of MAP kinases. On the other hand, insulin-induced suppression of CNT2 expression was totally blocked by an inhibitor of MEK-1 (PD98059), indicating that insulin controls CNT2 level by signalling through the MAPK pathway (King *et al.*, 2006). An involvement of growth factors in the regulation of expression of nucleoside transporters is demonstrated in murine bone marrow-derived macrophages. These cells express both the equilibrative (ENT1, ENT2) and concentrative (CNT1, CNT2) nucleoside transporters.

Studies performed on human B cell lines have shown that differentiation factors such as phorbol esters (PMA) and LPS up-regulate concentrative transport systems, but down-regulate the equilibrative transporters in a PKC-dependent manner (King *et al.*,2006).

7.2 Concentrative transporters

The concentrative transport system is Na^+-dependent and the movement of the nucleoside regardless of its concentration gradient is coupled to that of the sodium ion (King *et al.*, 2006). Six functionally different concentrative nucleoside transport activities have been described in human cells and they have been named in two different ways. The first nomenclature is numerical and follows the order of discovery (N1–N6), and the second one is based on the substrate specificity (*cit, cif, cib, cit-like, cs, csg*) (King *et al.*, 2006). The N1-*cif* activity is purine-selective, the N2-*cit* transport system accepts pyrimidine nucleosides, the N3-*cib* system transports both purine and pyrimidine nucleosides, the N4-*cit-like* system is selective for pyrimidine nucleosides but also accepts adenosine and guanosine, the N5-*cs* system transports adenosine and its analogues, and the N6-*csg* activity is guanosine-selective. To date three different proteins responsible for the *cit* (CNT1), *cif* (CNT2), and *cib* (CNT3) activities have been identified. Proteins that demonstrate the N4-*cit-like*, N5-*cs* or N6-*csg* activities have not been identified yet (King *et al.*, 2006). A study performed on starved rats has demonstrated that food supply modulates the expression level of CNT1 in rat small intestine. It was showed that nucleotide-deficient diets increased the amount of CNT1 protein in jejunum brush border membranes. These findings suggest that the supply of nucleotides modulates CNT1 expression in a tissue-specific manner (King *et al.*, 2006) Hormonal regulation of nucleoside transport activity has been reported in several tissues and cell types. Exposition of cultured chromaffin cells to lymphocyte T3 cells, results in an increase in the number of nucleoside transporters and stimulation of adenosine transport. Moreover, a study on rat liver documents up-regulation of nucleoside transport by glucagon through a mechanism involving membrane hyperpolarization (King *et al.*, 2006).

A study performed on rats, shows that the mRNA levels of rENT1, rENT2, rCNT1, and CNT2 are significantly altered in diabetic heart, liver, and kidney. Experiments performed on cultured rat T and B lymphocytes demonstrate that nucleoside transporters expression levels are independently and differentially regulated by glucose and insulin.

It appears that in rat lymphocytes the expression level of rENT2 and rCNT2 highly depends on insulin but is not affected by changes in extracellular glucose level, whereas the expression level of rENT1 is sensitive to extracellular glucose level but not to insulin (King *et al.*, 2006). Insulin is also able to stimulate Na^+-dependent uridine uptake by a process consistent with *de novo* synthesis of the carrier protein (King *et al.*, 2006).

CHAPTER 2

MATERIALS

RPMI medium 1640, L-glutamine, antibiotics, trypsin (2.5%) and trypan blue stain (0.4 %) were from Cambrex Bio Science (Walkersville, MD, USA). Dulbecco's modified Eagle medium without glucose (DMEM) and foetal bovine serum (FBS) were from Gibco (Berlin, Germany). Dulbecco's phosphate buffered saline (PBS), oligomycin, nitrobenzylthioinosine (NBTI), 8-cyclopentenyl-1,3-dipropylxanthine (DPCPX), xanthine oxidase (EC 1.1.3.22), alcohol dehydrogenase (EC1.1.1.1) and lactic dehydrogenase (EC 1.1.1.27) were purchased from Sigma (St Louis, MO, USA). Mycoplasma removal agent was from ICN Biomedicals (Costa Mesa, CA, USA). Glutamate–pyruvate transaminase (EC 2.6.1.2) was from ICN Biomedicals (Irvine, CA, USA). [2-^{14}C]Uridine (53mCi/mmol) was from Moravek Biochemicals (Brea, CA, USA). [2-^{14}C]Uracil (54 mCi/mmol), [2-^{14}C] cytidine (53.8 mCi/mmol), [8-^{14}C] adenine (55 mCi/mmol), [8-^{14}C] hypoxanthine (49,5 mCi/mmol), [2-^{14}C]5-fluorouracil (5-FU) (53 mCi/mmol), bases, nucleosides, and nucleotides were purchased from Sigma (St. Louis, MO, USA). HiSafe II scintillation liquid was purchased from LKB Pharmacia (Uppsala, Sweden). Polyethyleneimmine (PEI)-cellulose precoated thin-layer plastic sheets (0.1 mm thick) were purchased from Merck (Darmstadt, Germany) and prewashed once with 10% NaCl and three times with deionized water before use. DE81 filter papers were from Whatman (Kent, UK). Human astrocytoma cells (ADF) were a kind gift of Dr. W. Malorni, Istituto Superiore di Sanita` (Roma, Italy). Deoxycoformycin (dCF) was provided by Dr. O. Sanfilippo (Milan, Italy). 5-(phenylthio) acyclouridine (PTAU) was kindly provided by Dr. Mahmoud H. el Kouni, University of Atlanta, USA. C18 ultrasphere ODS HPLC column was from Beckman Instruments (Fullerton, CA, USA). All other chemicals were of reagent grade.

METODS

.1.Treatment of cells

Human astrocytoma cells (ADF) were routinely grown in RPMI medium with 10% FBS and 2% antibiotics at 37°C in a humidified 5% CO_2/95% air atmosphere. The contamination of ADF cells by Mycoplasma, which is known to possess adenosine-phosphorylase activity (Hatanaka *et al.,* 1975), was tested through the assay of adenosine-phosphorylase (EC 2.4.2.1). Tests for Mycoplasma contamination were made in routinely cultured cells and in cells exposed for 3 passages to Mycoplasma removal agent (0.5 mg/ml). Overall, the results excluded any contamination by Mycoplasma.

The experiments were performed with cells (approximately 1,500,000) kept in 35-mm plates for 10 min in 0.5 mL of serum-free DMEM medium without glucose in the absence or presence of 0.1 µM oligomycin. Cells were washed twice with 2 mL cold physiological solution. Some plates were treated as described in section 9.2.2 for the extraction of nucleotides whereas 0.5 mL of DMEM medium either alone or containing the following metabolites were added to other plates: glucose (from 0.5 to 10 mM), inosine (from 0.25 to 10 mM), adenosine (from 0.25 to 10 mM), uridine (from 1 to 10 mM), guanosine, cytidine, hypoxantine, ribose, and deoxyinosine at a final concentration of 10 mM. When nitrobenzylthioinosine (NBTI) or 8-cyclopentenyl-1, 3-dipropylxanthine (DPCPX) were present, their final concentrations were 10 and 100 mM, respectively. When deoxycoformycin (dCF) and 5-(phenylthio) acyclouridine (PTAU) were present in the incubation mixture, their final concentrations were 1 and 50 µM, respectively. After 30 min incubation, medium was collected and kept at -20 °C for the determination of lactate; cells were then treated as described below for the extraction of nucleotides.

2. Extraction and measurement of intracellular adenine nucleotides

Cells were subjected to the treatment described in the previous section. After treatment, the incubation medium was removed, and cells were washed twice with 2 mL of cold physiological solution. For the extraction of nucleotides, 0.1 mL of ice-cold 0.6 M perchloric acid was added to each plate and kept for 15 min; cells were harvested with a cell scraper, and the suspension was centrifuged in an Eppendorf Microfuge. The supernatant is neutralized with 15 µL of 3.5 M K_2CO_3. After centrifugation in a Microfuge, the supernatant was analyzed by HPLC: the analysis was performed with a Beckman System Gold apparatus, consisting of 2 HPLC pumps, a mixing chamber, an injector valve, and a diode-array detector. An Ultrasphere C-18 (Beckman) column (4.6 mm × 25 cm, 5-µm particle size) was used as described in Micheli *et al.* (1999).

3. Determination of lactate

The culture medium, collected as described in section 8.2.1, was subjected to the spectrophotometric assay for the determination of lactate (Martı *et al.*, 1997). The reaction mixture contained in a final volume of 0.5 mL, 200 mM glycylglycine–40 mM glutamate (pH 10), 4 mM NAD^+, 27 U of lactic dehydrogenase (5248 U/mL), 7 U of glutamate–pyruvate transaminase (1773 U/ mL), 90 µL of culture medium, and 20 mM Tris–HCl pH 7.4.

4. Preparation of cell extracts and enzyme assays

In order to obtain the extracts for the determination of enzyme activities, the cells were treated with trypsin (0.25%) and incubated for 5' at 37°, after the incubation 4 ml of RPMI 1640 medium was added. The suspension was centrifuged for 5' at 900g and then the supernatant was removed while the pellet was washed with PBS and the cell suspension was centrifuged for 5' at 900g, washed with 100 mM Tris-HCl pH 7.4 and then subjected to ultrasonic treatment and centrifuged for 30 min at 4°C at 39,000g (Giorgelli *et al.,* 2000). All enzyme assays were performed at 37°C. For each sample, at least 2 determinations with different amounts of cell extract were carried out. Linearity with time and protein concentration was observed for all enzyme assays. A spectrophotometric method was applied for the assay of purine nucleoside phosphorylase (EC 2.4.2.1), adenosine deaminase (EC 3.5.4.4) and deoxyriboaldolase (EC 4.1.2.4). A radioenzymatic assay was applied to measure the activity of uridine phosphorylase (EC 2.4.2.3), cytidine deaminase (EC 3.5.4.5), adenosine-phosphorylase (EC 2.4.2.1), adenosine kinase (EC 2.7.1.20), cytosolic IMP/GMP-specific- 5'-nucleotidase (cN II) (EC 3.1.3.5), adenine phosphoribosyl transferase (EC 2.4.2.7), hypoxanthine phosphoribosyl transferase (EC 2.4.2.8), uridine-cytidine kinase (EC 2.7.1.48).

I. Purine nucleoside phosphorylase (PNP)
The reaction mixture (0.5 mL) contained 100 mM phosphate buffer, pH 7.4, 0.3 mM inosine, 0.02 U of xanthine oxidase (1.8 U/mL), and 30–70 µg of cell extract. The change in optical density at 293 nm was followed against a reference cuvette in which inosine was substituted by water.

II. Adenosine deaminase (ADA)
The reaction mixture (0.5 mL) contained 100 mM Tris–HCl, pH 7.4, 0.08 mM adenosine, and 30–70 µg of cell extract. The change in optical density at 265 nm was followed against a cuvette in which adenosine was substituted by water.

III. Deoxyriboaldolase
The reaction mixture (0.5 mL) contained 70 mM Tris–HCl, pH 7.4, 1.5 mM dithiothreitol, 0.22 mM NADH, 11 U of alcohol dehydrogenase (1098 U/mL), 2 mM deoxyRib-5-P, and from 0.3 to 0.6 µg of cell extract. The assay was performed spectrophotometrically, by monitoring the change in optical density at 340 nm, against a reference cuvette in which deoxyRib-5-P was substituted by water.

IV. Uridine phosphorylase (UPase)
The reaction mixture (50 µL) contained 5 mM phosphate buffer, pH 7.4, 1 mM [2-^{14}C] uridine (3720 DPM/nmol), from 75 to 150 µg of cell extract, and 30 mM Tris–HCl, pH 7.4. At 0, 10, 20, and 30 min incubation, 10 µL aliquots were withdrawn and spotted on a PEI-cellulose plate which was developed overnight with n-propanol/TCA(100% saturation)/NH_3/H_2O (75/0.7/5/20, v/v). The spots corresponding to uracil (for uridine phosphorylase) and uridine (for cytidine deaminase) were cut and the radioactivity counted, after addition of 8 mL of liquid scintillation counter.

V. Cytidine deaminase
The reaction mixture (50 µL) contained 60 mM Tris–HCl, pH 7.4, 3 mM dithiothreitol, 0.25 mM [2-^{14}C] cytidine (6072 DPM/nmol), 5 mM $ZnSO_4$, and from 10 to 20 µg of cell extract. The aliquots were treated as described for the UPase assay.

VI. Adenosine phosphorylase
The reaction mixture (50 µL) contained 0.5 mM [8-^{14}C] adenine (15,000 dpm/nmol), deoxyribose-1-phosphate 0.5 mM, dCF 6 µM and from 10 to 20 µg of cell extract. At 0, 10, 20, and 30 min incubation, 10 µL aliquots were withdrawn and spotted on a PEI-cellulose plate which was developed for three hours in 0.1M $(NH_4)_2SO_4$.

VII. Adenosine kinase (AK).
The reaction mixture (50 µL) contained 54µM [8-^{14}C] adenosine (10,000 dpm/nmol), 1 µM dCF, 1 mM ATP, 1.5 mM $MgCl_2$ and from 10 to 20 µg of protein. At 0, 10, 20, and 30 min incubation, 10 µL aliquots were withdrawn and spotted on a DE 81 filter paper which was washed 15 min in 1 M ammonium formate and 10 min in 2 changes of water. The disks were dried and counted for radioactivity.

VIII. Nucleoside phosphotransferase activity of cytosolic 5'-nucleotidase
The reaction mixture (50 µL) contained 1 mM [8-^{14}C] inosine (4,300 dpm/nmol), 2 mM IMP, 4 mM dithiothreitol (DTT), 5 mM ATP, 20 mM $MgCl_2$ and 15 to 30 ug of protein. The aliquots were treated as described for the AK assay.

IX. Hypoxanthine phosphoribosyl transferase (HPRT)
The reaction mixture (50 µL) contained 1 mM [8-^{14}C] hypoxanthine (9100 dpm/nmol), 2.6 mM 5-phospho-D-ribosyl-1-pyrophosphate, 2 mM $MgCl_2$, 2mM DTT, 50 mM Tris-HCl pH 7.4 and from 15 to 30 µg of protein. The aliquots were treated as described for the AK assay.

X. Adenine phosphoribosyl transferase (APRT).
The reaction mixture (50 µL) contained 0.25 mM [8-^{14}C] adenine (15,000 dpm/nmol), 2.5 mM PRPP, 10 mM $MgCl_2$, 5 mM DTT, 50 mM Tris-HCl pH 7.4 and from 20 to 40 µg of protein. The aliquots were treated as described for the AK assay.

XI. Uridine kinase (UK).
The reaction mixture (50 µL) contained 1 mM [2-^{14}C] uridine (3720 DPM/nmol), 10mM $MgCl_2$, 5mM ATP, 50 mM Tris-HCl pH 7.4 and from 30 to 40 µg of protein. The aliquots were treated as described for the AK assay.

XII. Cytidine kinase (CK).
The reaction mixture (50 µL) contained, 0.25 mM [2-^{14}C] cytidine (6072 DPM/nmol), 10 mM $MgCl_2$, 5 mM ATP, 50 mM Tris-HCl pH 7.4 and from 10 to 20 µg of protein. The aliquots were treated as described for the AK assay.

One unit of enzyme activity was the amount of enzyme which catalyzes the formation of 1 µmol of product/min under the adopted assay conditions

5.Incubation of human astrocytoma cells with purine and pyrimidine compounds

The experiments were performed with cells (approximately 1,500,000) kept in 35-mm plates for different times in 0.5 ml of serum-free DMEM medium. In order to test the adenine salvage synthesis, cells were incubated for different times with 50 µM [8-^{14}C] adenine (15,000 dpm/nmol) alone or in the presence of either 100 µM inosine, cytidine or deoxyinosine, or uridine (from 50 to 200 µM); when 5-(phenylthio)acyclouridine (PTAU) was present in the incubation mixture, its final concentrations was 50 µM. To test hypoxanthine salvage synthesis, cells were incubated with 50 µM [8-^{14}C] hypoxanthine alone or in the presence of 100 µM uridine. For pyrimidine salvage, cells were incubated with 50 µM [2-^{14}C] uracil (35,000 dpm/nmol) alone or in the presence of 100 µM inosine, or with 100 µM [2-^{14}C] uridine (5600 dpm/nmol) alone or in the presence of 50 µM adenine. The formation of 5-FU nucleotides was followed by incubating 50 µM [2-^{14}C] 5-flurouracil (5FU) (10,500 dpm/nmol) either alone or in the presence of 100 µM inosine. In order to test the cytidine effect on the purine salvage synthesis, cells were incubated for 30’ in the presence or in the absence of 0.25, 0.5 1mM cytidine; at the end of incubation the serum-free DMEM medium was removed and cells were incubated for the indicated times with 50 µM [8-^{14}C] adenine (15,000 dpm/nmol) alone or in the presence of uridine.

To test the cytidine effect on pyrimidine salvage, cells were incubated for 30’ in the presence or in the absence of 1 mM cytidine; at the end of incubation the serum-free DMEM medium was removed and cells were incubated for the indicated times with 50 µM [2-^{14}C]uridine (5600 dpm/nmol) alone. At the different times of incubation, the medium was removed, and cells were washed twice with 2 ml of cold physiological solution. For the extraction of purine and pyrimidine compounds, cells were treated as already described in section 9.2.2.

After centrifugation in a Microfuge, 50 µl of the supernatant was applied to a PEI-cellulose plate which was developed overnight with n-propanol/TCA (100% saturation)/NH_3/H_2O (75/0.7/5/20, v/v). The zones corresponding to radioactive AMP + ADP + ATP (TAN) (adenine salvage) or IMP (hypoxanthine salvage) and radioactive UMP + UDP + UTP (TUN) (uracil and uridine salvage) or FUMP+ F-UDP + F-UTP (5-FU activation) were cut and the radioactivity counted, after addition of 8 ml of liquid scintillation counter. In order to test the CTP formation in cells incubated for 30' with cytidine, the cells were incubated for 30' with 500 µL of serum-free DMEM medium in the presence or absence of cytidine.

After the incubation cells were treated for the extraction of purine and pyrimidine compounds and the CTP intracellular level was determined by HPLC technique as described in section 9.2.2.

6. Other methods

For the counting of cells adhering to the plate and for the trypan blue staining of cells subjected to different treatments, the medium was withdrawn, in order to remove detached cells, and cells remaining adherent to the plate were washed with cold physiological solution. A 0.5-mL portion of 0.025% trypsin is then added and kept for few minutes at 37 °C. To measure the number of adhering cells, the cell suspension is collected, diluted in an appropriate volume of RPMI medium, and cells counted under a microscope in a Bürker chamber. To measure the percentage of dead cells among those adhering to the plates, the cell suspension is centrifuged and then re-suspended in 2.5 mL of PBS. Cell suspensions (0.5 mL) are mixed with 0.5 mL of 0.4% trypan blue solution, and after 5 min, the stained non-viable cells are counted under a microscope in a Bürker chamber.

Proteins were determined according to Bradford (1976), using bovine serum albumin as standard. The statistical analyses are performed by using the unpaired t-test.

CHAPTER 3

RESULTS

1. Cell energy charge without and with treatment with oligomycin

To shed light on the protective role of purine nucleosides during ischemia or brain insults, we have used a human astrocytoma cell line (ADF) which has been subjected to metabolic stress conditions by exclusion of glucose and pre-incubation for 10 min with oligomycin (an inhibitor of oxidative phosphorylation). The determination of the intracellular adenylate pool indicates that treatment for 10 min with 0.1 μM oligomycin in a culture medium devoid of glucose brings about a decrease in the ADF cell energy charge ([ATP] + (1/2)[ADP]/[ATP] +[ADP] + [AMP]) from 0.89 ± 0.04 (n = 10) to 0.73 ± 0.09 ($p < 0.0001$, n = 22). After removal of the culture medium, a further incubation for 30 min with a medium devoid of glucose in the absence of oligomycin results in a further decrease in the cell energy charge up to 0.44 ± 0.15 ($p < 0.0001$, n = 22), thus indicating that, in these conditions, cells are not able to use the nutrients present in the medium (mainly aminoacids) as energy source, and suggesting that the inhibition exerted by oligomycin is not overcome by the removal of the inhibitor.

2. Effect of glucose and nucleosides on energy repletion after treatment with oligomycin

The adenylate pool was measured in cells previously subjected to a 10 min treatment with oligomycin and then suspended for 30 min in a medium containing different metabolites. Results indicate that glucose is able to counteract the fall in the ATP content which is observed when the culture medium was devoid of substrates metabolizable through fermentation (Fig. 9A). Concomitantly, a marked decrease in AMP levels is observed, thus resulting in a restoration of the cell adenylate charge to values similar to those found for untreated cells. The assay of lactate in the incubation medium indicates that the ATP pool is restored through anaerobic glycolysis (Fig. 9B). The addition of inosine in the incubation medium (in a range of concentrations from 10 to 0.25 mM) exerts a protective action comparable to that observed with glucose (Fig. 10A). Indeed, an enhancement of ATP content and a decrease in AMP levels are observed. While guanosine, being a carrier of the sugar moiety is able to exert a protective action, hypoxanthine and ribose are not. Also deoxyinosine has no effect on the preservation of the ATP pool. Again, anaerobic glycolysis appears to be the metabolic pathway through which cells are able to restore the ATP level (Fig. 10B). Also adenosine restores the ATP pool through anaerobic glycolysis, independently of the presence of DPCPX, which is an antagonist of adenosine A1, A2A, A2B, and, in the micromolar range, A3 receptors (Klotz, 2000) (Fig.11). Besides purine, also pyrimidine nucleosides, such as uridine, and to a lesser extent cytidine, when added to the incubation medium exert a protective action (Fig. 12A), which is related to their capability to restore the ATP pool through anaerobic glycolysis, as evidenced by the formation of lactate (Fig. 12B).

To address the question about the presence of a receptor-independent mechanism of nucleoside action, cells were treated with oligomycin both in the presence or absence of an inhibitor of nucleoside transporter (NBTI). The presence of 10 μM NBTI in the culture medium does not exert any significant effect on control cells and does not affect the protective action of glucose, but counteract significantly the efficacy of inosine and uridine (Fig.13). The number of cells adhering to the plates after 10 min treatment with oligomycin is not significantly different as compared to control untreated cells. A further incubation for 30 min in the absence of effectors brings about a significant decrease of the adhering cell number. The presence of glucose, inosine, and uridine exert a rescue effect, the number of adhering cells being comparable to that of untreated cells (Fig. 14A). Although not statistically significant (unless when inosine is present), in the incubation performed in the absence of effectors, also the percentage of dead cells appears to be consistently greater than in the presence of glucose, inosine and uridine (Fig. 14B)

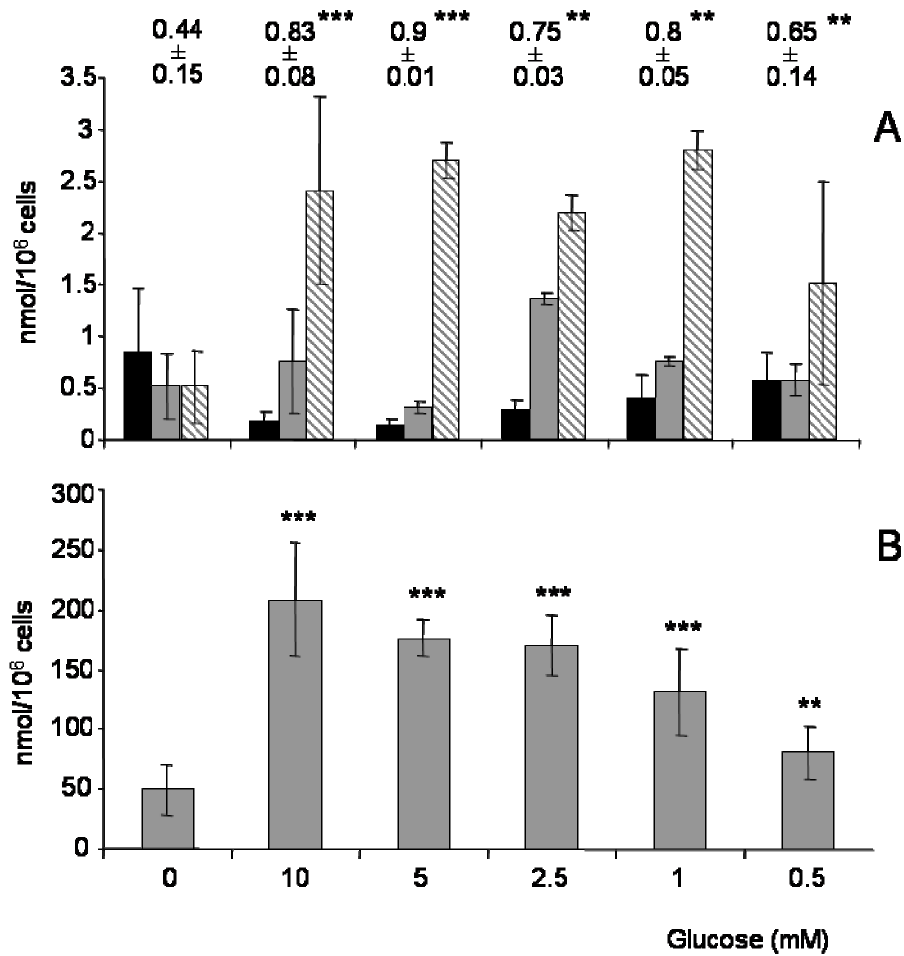


Fig.9. Effect of glucose on the adenylate energy charge and lactate production in astrocytoma cells subjected to ischemic conditions. Cells were previously treated for 10 min in DMEM medium with 0.1 μM oligomycin, then medium was removed, the cells were washed and DMEM medium was added (for 30 min) either with no addition or with the indicated amount of glucose. (Panel A) Histograms indicate the amount of AMP (black bar), ADP (grey bar) and ATP (dashed bar). The numbers indicate the adenylate energy charge (±S.D.). (Panel B) Histograms indicate the amount of lactate formed in the DMEM medium alone or supplemented with glucose. Number of experiments (n): n = 22 (0); n = 10 (0.5 mM); n = 6 (1 mM); n = 4 (2.5 mM); n = 4 (5 mM); n = 20 (10 mM). Statistical significance of cell adenylate energy charge and lactate content vs. cells with no addition: ***$p < 0.0001$; **$p < 0.001$;*$p < 0.05$.

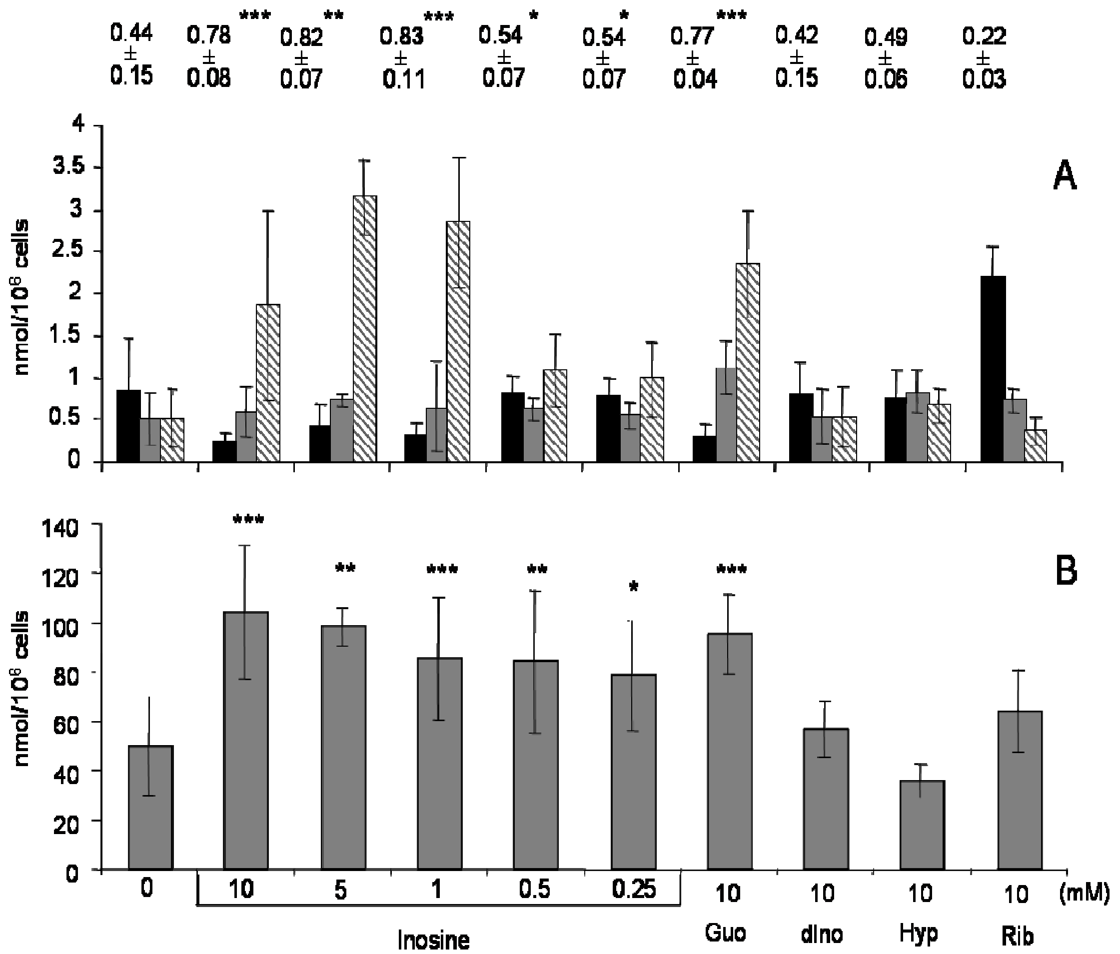


Fig.10. Effect of inosine, guanosine, deoxyinosine, ribose and hypoxanthine on the adenylate energy charge and lactate production in astrocytoma cells subjected to ischemic conditions. Cells were previously treated for 10 min in DMEM medium with 0.1 μM oligomycin, then medium was removed, the cells were washed and DMEM medium was added (for 30 min) either with no addition or with the indicated amount of inosine (Ino), guanosine (Guo), deoxyinosine (dIno), hypoxanthine (Hyp) and ribose (Rib). (Panel A) Histograms indicate the amount of AMP (black bar), ADP (grey bar) and ATP (dashed bar). The numbers indicate the adenylate energy charge (± SD). (Panel B) Histograms indicate the amount of lactate formed in the DMEM medium alone or supplemented with the indicated compound. Number of experiments (n): n = 22 (0); n = 12 (0.25 mM Ino); n = 11 (0.5 mM Ino); n = 6 (1 mM Ino); n = 4 (5 mM Ino); n = 14 (10 mM Ino); n = 6 (Guo); n = 6 (dIno); n = 4 (Hyp); n = 4 (Rib). Statistical significance of cell adenylate energy charge and lactate content vs. cells with no addition: ***$p < 0.0001$; **$p < 0.001$;*$p < 0.05$.

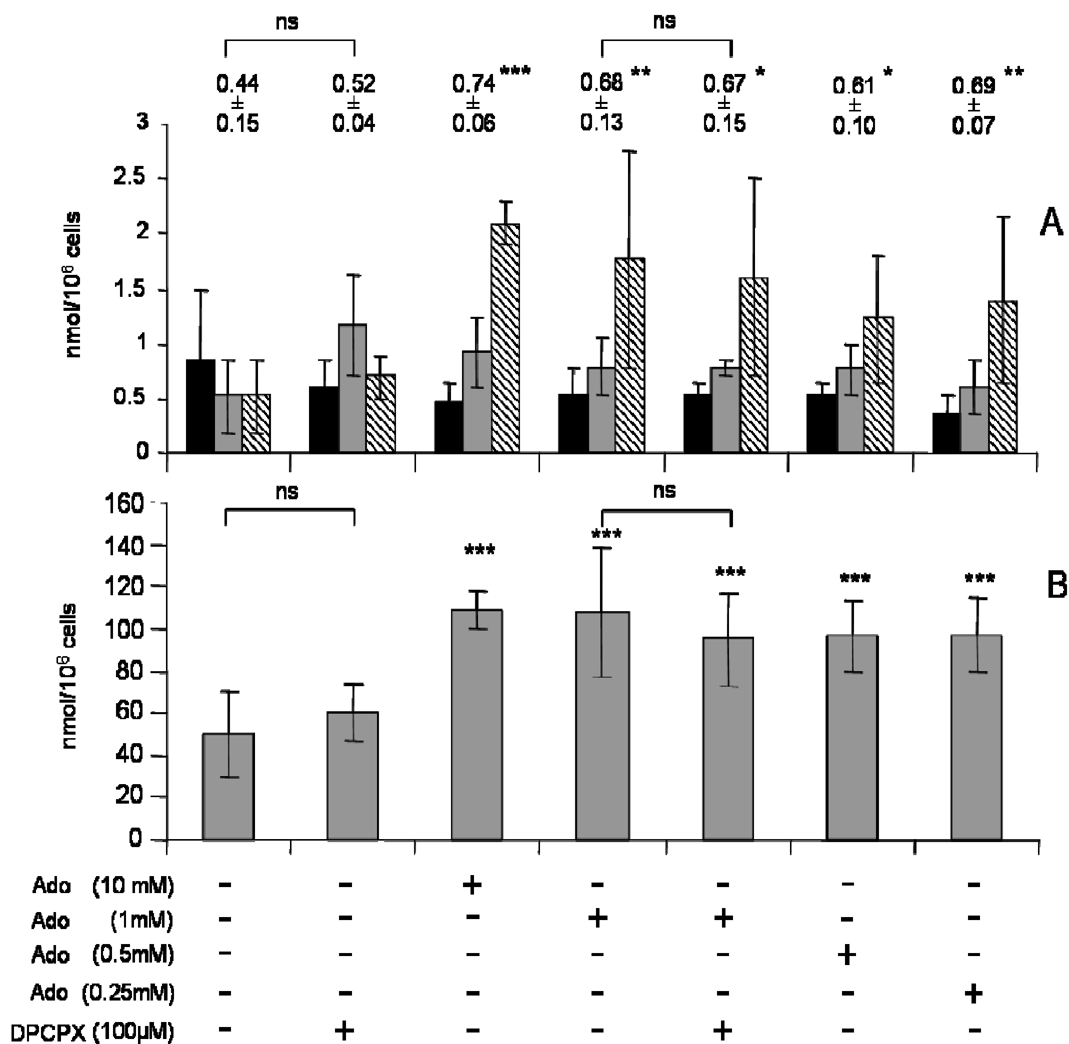


Fig.11. Effect of adenosine on adenylate energy charge and lactate production in astrocytoma cells subjected to ischemic conditions. Cells were previously treated for 10 min in DMEM medium with 0.1 µM oligomycin, then medium was removed, the cells were washed and DMEM medium was added (for 30 min) either alone or with 0.25, 0.5, 1 and 10 mM adenosine (Ado). Parallel incubations were also performed in which 100 µM DPCPX was added to the culture medium. (Panel A) Histograms indicate the amount of AMP (black bar), ADP (grey bar) and ATP (dashed bar). The numbers indicate the adenylate energy charge (± S.D.). (Panel B) Histograms indicate the amount of lactate formed in the DMEM medium alone or supplemented with adenosine. Number of experiments (n): n = 22 (without Ado); n = 6 (with Ado and/or DPCPX). Statistical significance of cell adenylate energy charge and lactate content vs. cells with no addition: ***$p < 0.0001$; **$p < 0.001$; *$p < 0.05$; ns = no significant difference was found in samples with and without DPCPX.

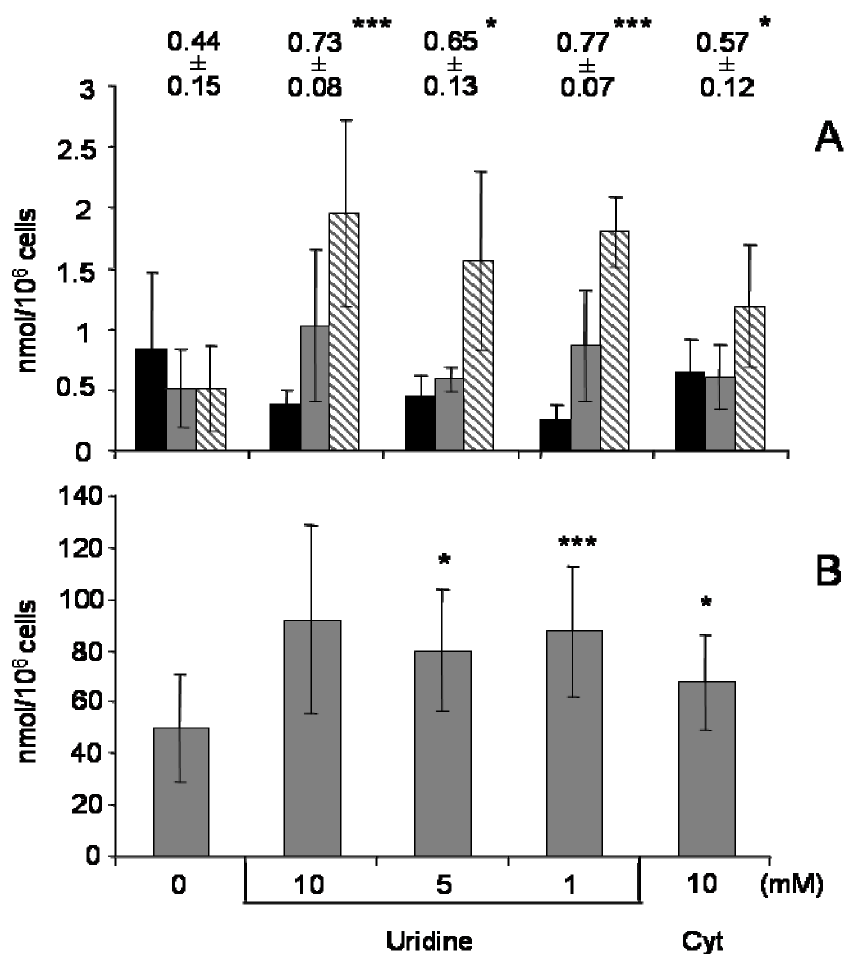


Fig.12. Effect of pyrimidine nucleosides on the adenylate energy charge and lactate production in astrocytoma cells subjected to ischemic conditions. Cells were previously treated for 10 min in DMEM medium with 0.1 mM oligomycin, then medium was removed, the cells were washed and DMEM medium was added (for 30 min) either with no addition or with the indicated amount of uridine (Urd) or cytidine (Cyt). (Panel A) Histograms indicate the amount of AMP (black bar), ADP (grey bar) and ATP (dashed bar). The numbers indicate the adenylate energy charge (±S.D.). (Panel B) Histograms indicate the amount of lactate formed in the DMEM medium alone or supplemented with the indicated compound. Number of experiments (n): n = 22 (0); n = 6 (1 mM Urd); n = 5 (5 mM Urd); n = 12 (10 mM Urd); n = 15 (Cyt). Statistical significance of cell adenylate energy charge and lactate content vs. cells with no addition: ***$p < 0.0001$; *$p < 0.05$.

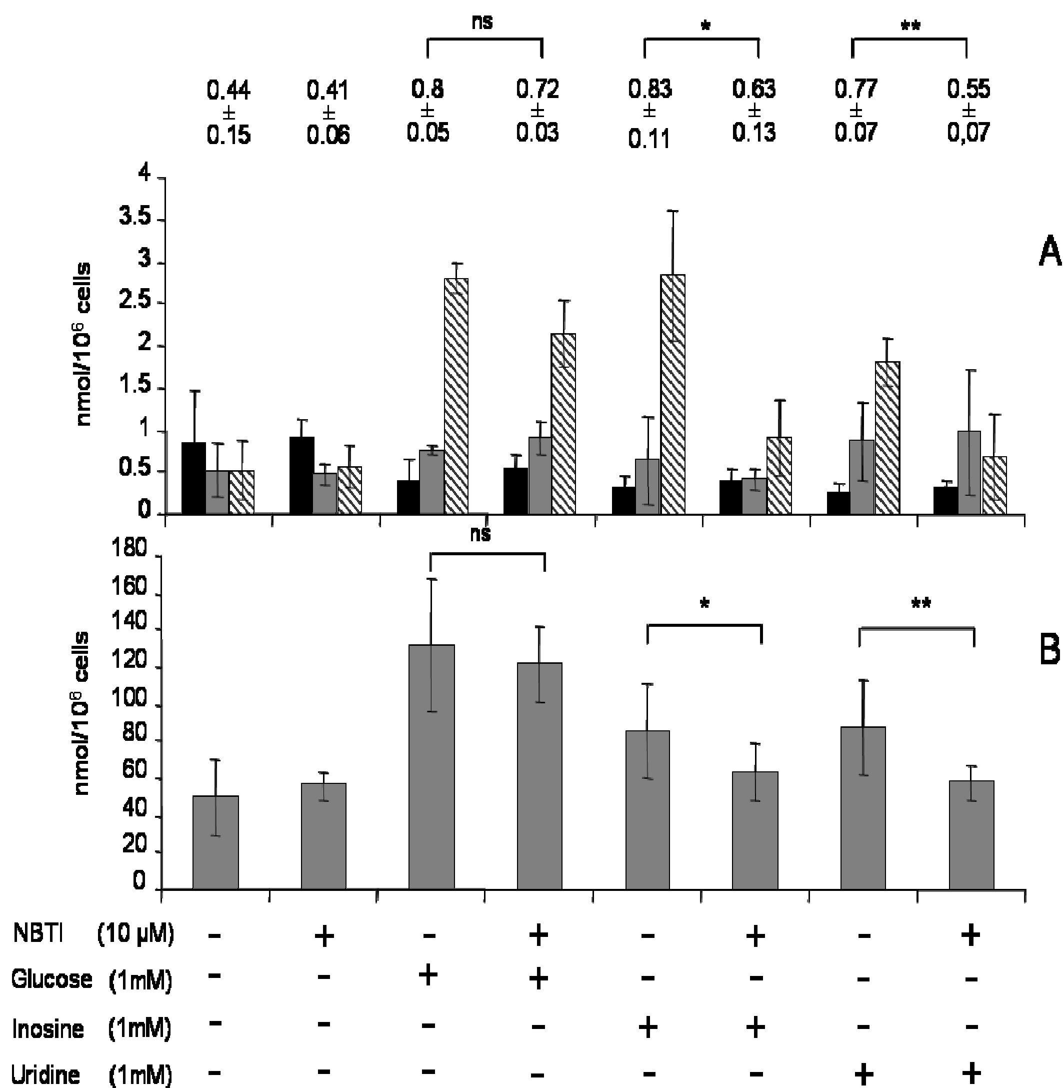


Fig.13. Adenosine, inosine and uridine must enter astrocytoma cells in order to preserve ATP pool. Cells were previously treated for 10 min in DMEM medium with 0.1 µM oligomycin, then medium was removed, the cells were washed and DMEM medium was added (for 30 min) either alone or with the indicated amount of adenosine, inosine and uridine. Parallel incubations were also performed in which 10 µM NBTI was added to the culture medium. (Panel A) Histograms indicate the amount of AMP (black bar), ADP (grey bar) and ATP (dashed bar). The numbers indicate the adenylate energy charge (±S.D.). (Panel B) Histograms indicate the amount of lactate formed in the DMEM medium alone or supplemented with the indicated compound. Number of experiments (n): n = 22 (0); n = 6 (Gluc); n = 6 (Ino); n = 6 (1 mM Urd); n = 9 (Gluc+ NBTI; Ino+ NBTI); n = 9 (Urd+ NBTI). Statistical significance of cell adenylate energy charge and lactate content in samples with and without NBTI: **$p < 0.001$; *$p < 0.05$. ns = not significant.

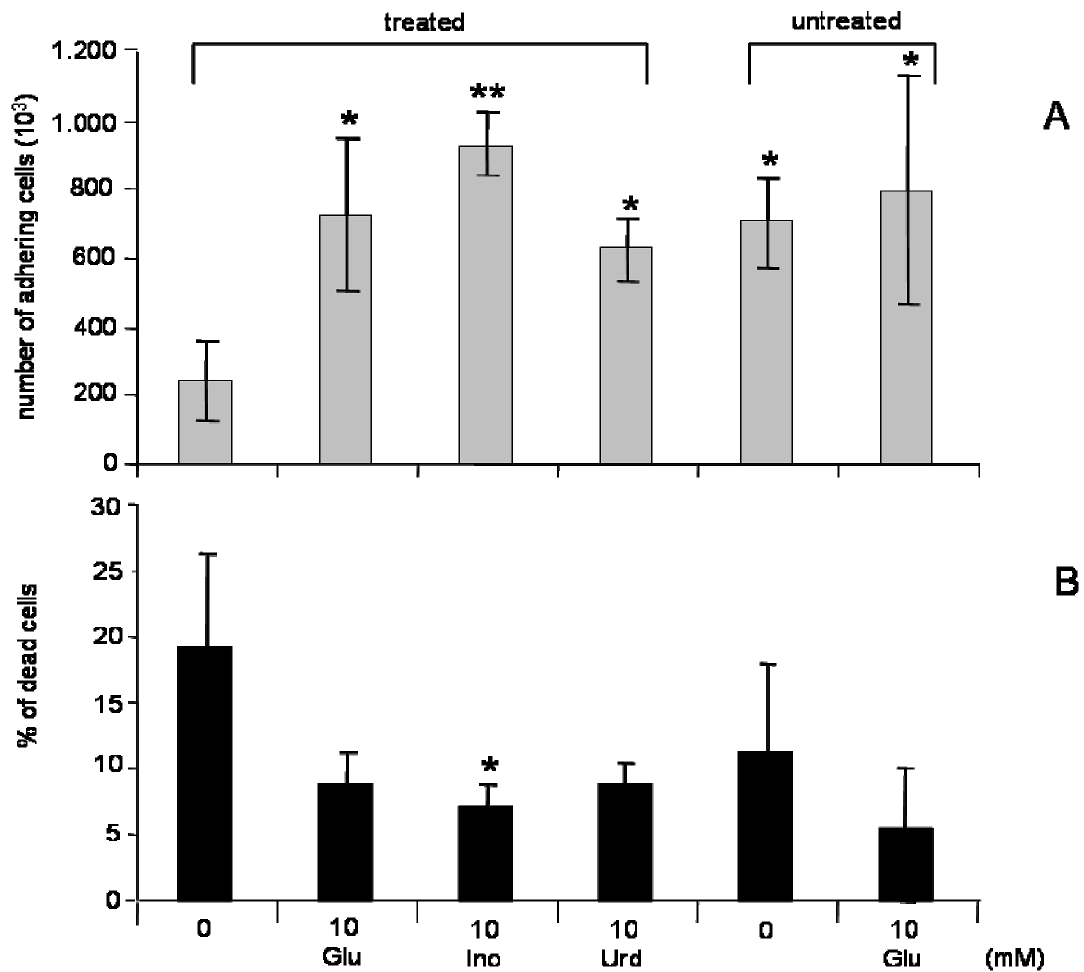


Fig.14. The presence of inosine and uridine improves viability of astrocytoma cells undergoing ischemic conditions. Cells were previously treated for 10 min in DMEM medium with (treated) or without (untreated) 0.1 μM oligomycin, then medium was removed, the cells were washed and DMEM medium was added (for 30 min) either alone or with glucose, inosine and uridine. The number of cells still adhering to the plates was counted (Panel A). The percentage of dead cells, evaluated by the trypan blue staining technique, is also reported (Panel B). For each bar n = 4. Statistical significance vs. cells treated with oligomycin with no addition: **$p < 0.001$; *$p < 0.05$.

3. Activity of enzymes involved in the catabolism of nucleosides

The activity of enzymes involved in the catabolism of nucleosides was measured (Table 1).

Table 1

Enzyme	Specific activity (U/mg)
Purine nucleoside phosphorylase (PNP)	21.1×10^{-3}
Adenosine deaminase (ADA)	7.1×10^{-3}
Deoxyriboaldolase	1.1×10^{-3}
Uridine phosphorylase (UPase)	4.3×10^{-3}
Cytidine deaminase	0.32×10^{-3}

The results indicate that deoxyinosine, inosine and guanosine can be phosphorolytically cleaved by purine nucleoside phosphorylase. Also uridine may be subjected to a phosphorolytic cleavage by the action of uridine phosphorylase. Although in a low amount, also cytidine deaminase and deoxyriboaldolase are present in ADF cells. The presence of adenosine deaminase indicates that adenosine may be deaminated to inosine.

To address the question about the intracellular destiny of the nucleoside compounds, cells were treated with oligomycin in the presence of a dCF and PTAU which are potent inhibitors of adenosine deaminase and uridine phosphorylase, respectively. The presence of 1 μM dCF and 50 μM PTAU, significantly affects the efficacy of adenosine and uridine, respectively (Fig.15).

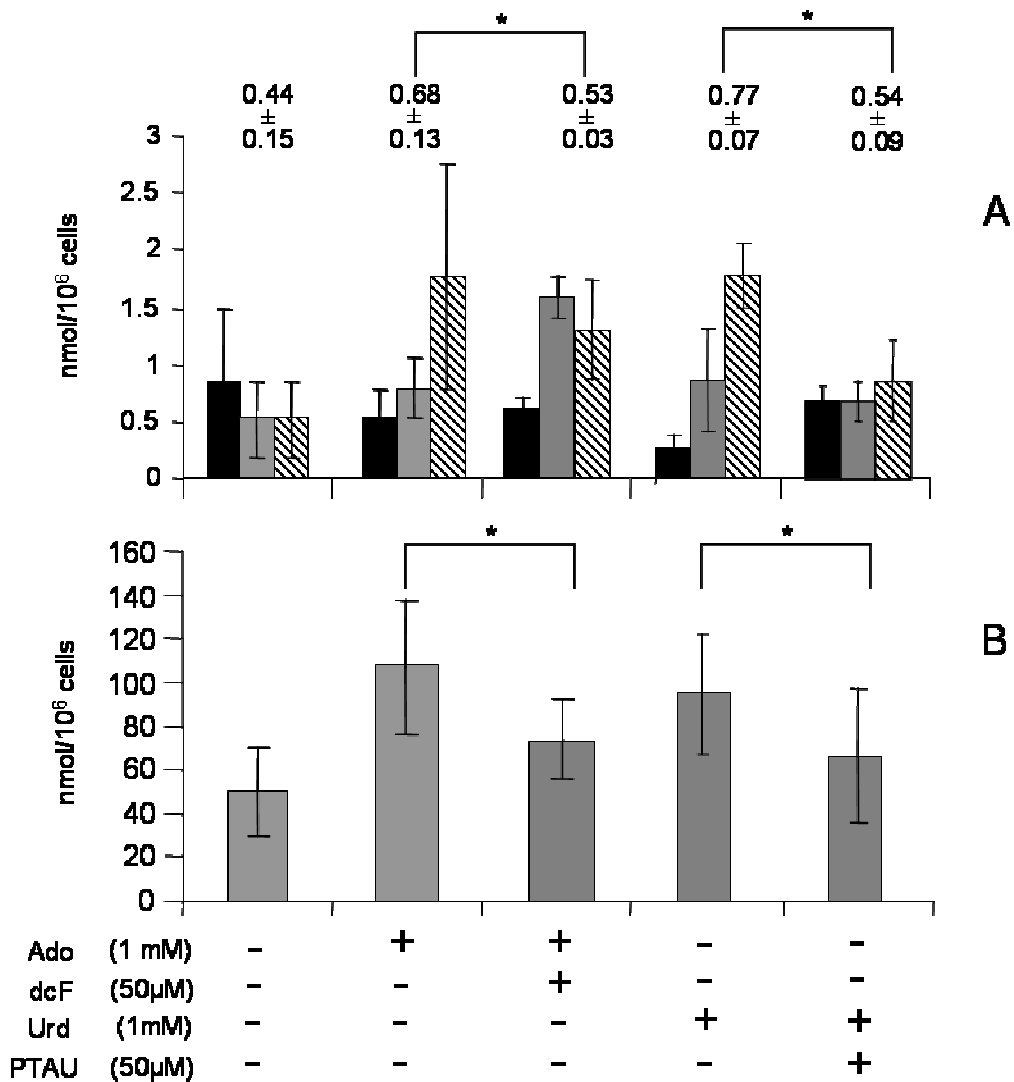


Fig.15. The presence of dCF and PTAU counteracts significantly the efficacy of adenosine and uridine, respectively. Cells were previously treated for 10 min in DMEM medium with 0.1 µM oligomycin, then medium was removed, the cells were washed and DMEM medium was added (for 30 min) either alone or with the indicated amount of adenosine and uridine. Parallel incubations were also performed in which 1 µM dCF and 50 µM PTAU were added to the culture medium. (Panel A) Histograms indicate the amount of AMP (black bar), ADP (grey bar) and ATP (dashed bar). The numbers indicate the adenylate energy charge (±S.D.). (Panel B) Histograms indicate the amount of lactate formed in the DMEM medium alone or supplemented with the indicated compounds. Number of experiments (n): n = 22 (0); n = 6 (Ado); n = 6 (Ado+ dCF); n = 6 (Urd); n = 6 (Urd+ PTAU). Statistical significance of cell adenylate energy charge and lactate content in samples with and without dCF (plus Ado) or PTAU (plus Urd): $*p < 0.05$.

4. Determination of purine and pyrimidine salvage in ADF cells.

To test the adenine salvage pathway, ADF cells were incubated at different times with radioactive adenine alone or in the presence of either inosine (100 μM), dIno (100 μM), or uridine (from 50 to 200 μM) (Fig.16). The time course of nucleotide formation was followed. Exogenous uridine and, to a lesser extent inosine, activate the salvage of exogenous adenine in ADF cells. The activation exerted by uridine is concentration-dependent. dIno is not able to activate the salvage of exogenous adenine. ADF were also incubated at different times with radioactive adenine in the presence of cytidine or with uridine in the presence of the inhibitor of uridine phosphorylase, PTAU. Cytidine has only a moderate effect on adenine salvage and the presence of PTAU significantly affects the activation of adenine salvage exerted by uridine (Fig.17)

To test hypoxanthine salvage synthesis, cells were incubated with radioactive hypoxanthine alone or in the presence of uridine (Fig.18); exogenous uridine activates the salvage of exogenous hypoxanthine in ADF cells.

To test the pyrimidine salvage pathway, ADF cells were incubated at different times with radioactive uracil and fluorouracil alone or in the presence of inosine or deoxyinosine, or with radioactive uridine alone or in the presence of adenine. Exogenous inosine favours 5-FU (antitumoral agent) activation and uracil salvage, while dIno not activate the salvage of exogenous uracil (Fig.19). Finally, fig.20 shows that the transformation of exogenous uridine into endogenous uracil nucleotides in ADF cells is consistently lowered by exogenously added adenine.

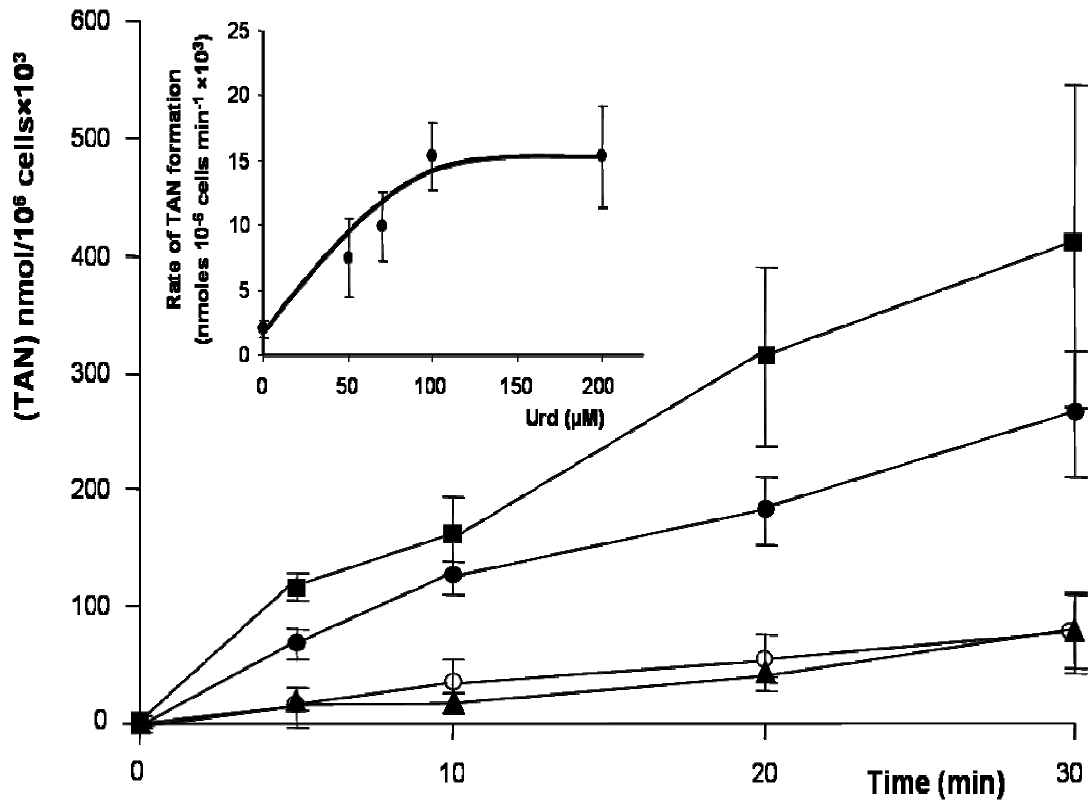


Fig.16. Time course of total adenine nucleotide (TAN) formation from adenine in cultured human astrocytoma cells. ADF cells were incubated with 50 µM [8-^{14}C] adenine (15,000 dpm/nmol) in the absence (○) and in the presence of 100 µM inosine (●), or 100 µM uridine (■), or 100 µM deoxyinosine (▲). The inset shows the rate of TAN formation from adenine at the indicated concentrations of uridine. Number of experiments (n): n = 22 (control); n = 6 (Urd); n=6 (Ino); n=3 (dIno).

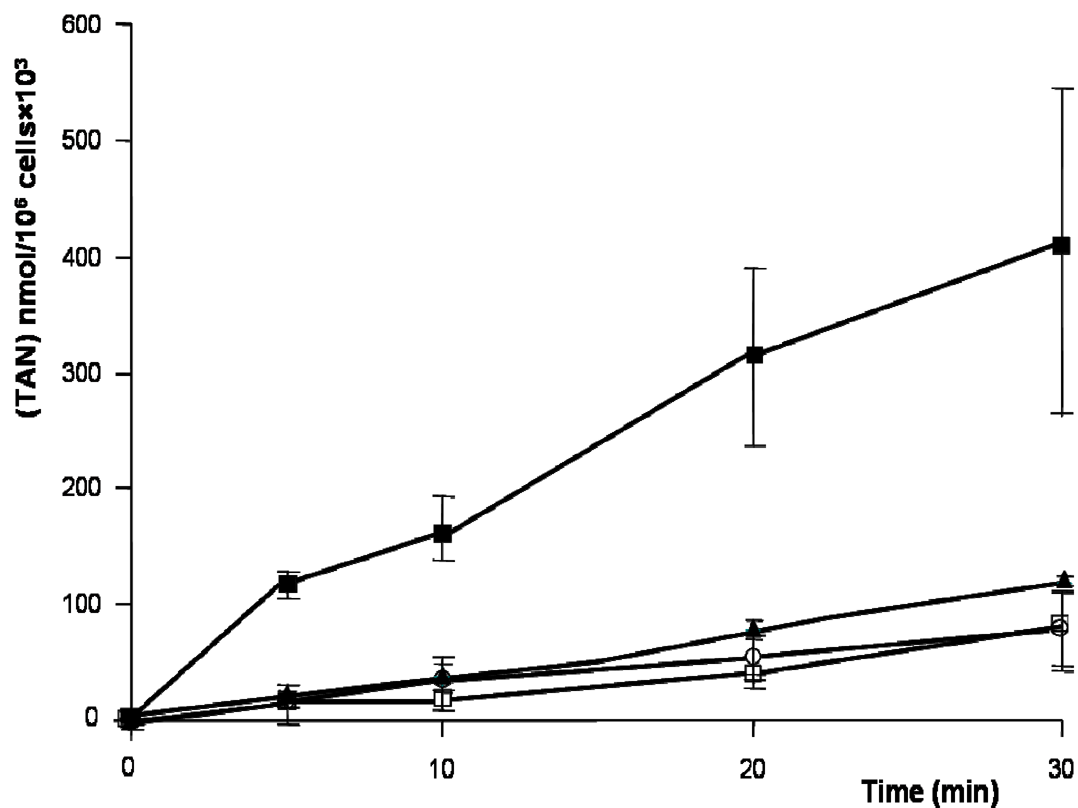


Fig.17. Time course of total adenine nucleotide (TAN) formation from adenine in cultured human astrocytoma cells. ADF cells were incubated with 50 μM [8-^{14}C] adenine (15,000 dpm/nmol) in the absence (o) and in the presence of either 100 μM cytidine (▲) or 100 μM uridine alone (■) and in the presence of 50 μM PTAU (□). Number of experiments (n): n= 3 (control); n = 6 (Urd); n = 3 (Urd+PTAU); n= 3 (Cyt).

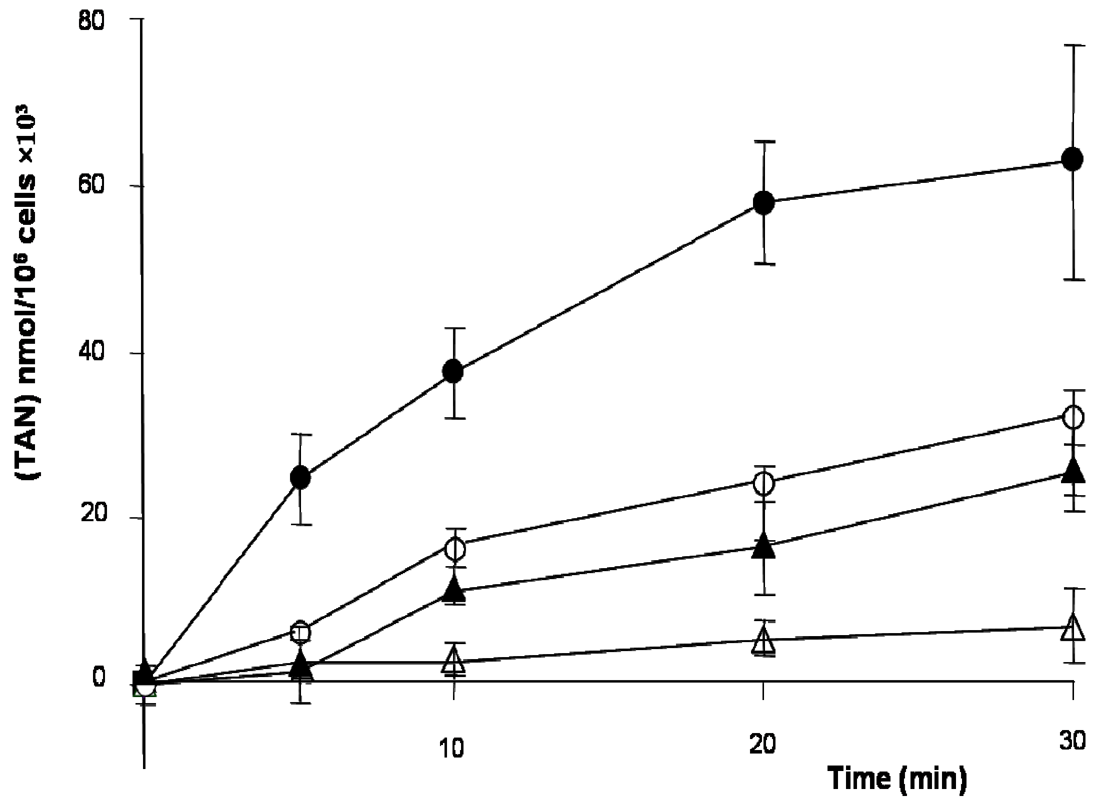


Fig.18. Time course of total adenine nucleotide (TAN) formation and IMP formation from hypoxanthine in cultured human astrocytoma cells. ADF cells are incubated with 50 µM [8-^{14}C] hypoxanthine (15,000 dpm/nmol) in the absence (open symbols) and in the presence (closed symbols) of 100 µM uridine. IMP (▲,Δ); AMP+ADP+ATP (○, ●). Number of experiments (n): n = 3 (Hyp); n = 3 (Hyp+Urd).

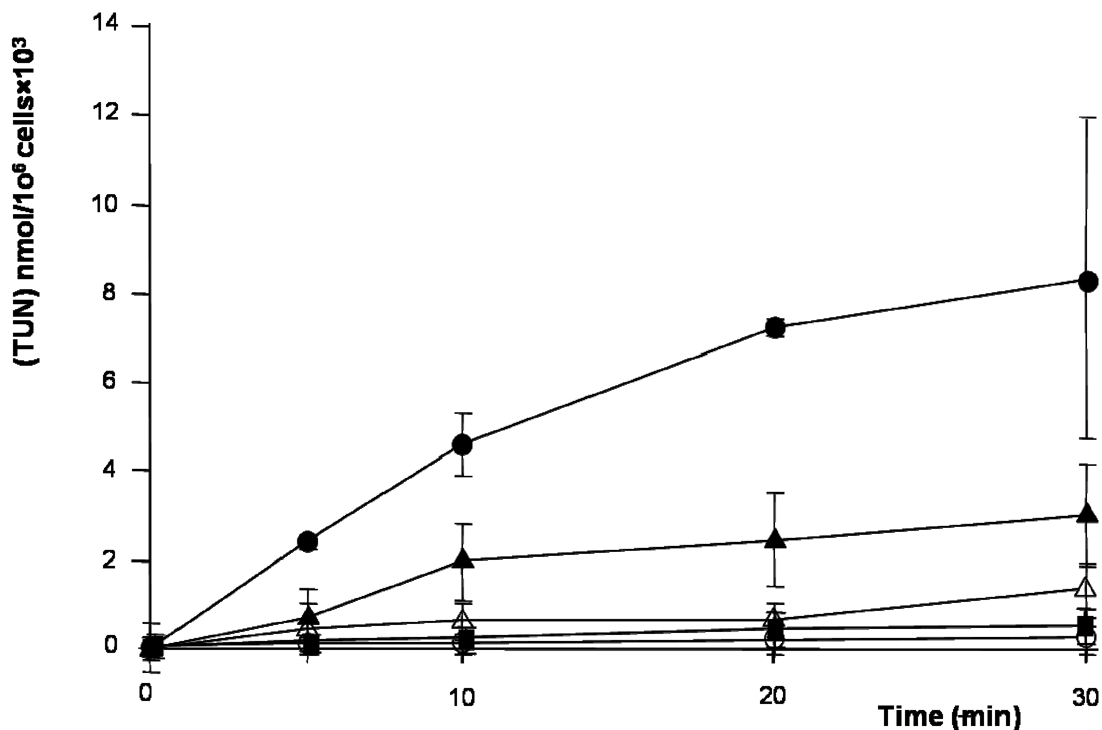


Fig.19. Time course of total uracil nucleotide (TUN) formation from uracil and of 5-FU nucleotide formation from 5-FU in cultured human astrocytoma cells. ADF cells were incubated with 50 μM [2-^{14}C] uracil (35,000 dpm/nmol) (○, ●, ■) or with 50 μM [2-^{14}C] 5-FU (10,500 dpm/nmol) (Δ, ▲) in the absence (○,Δ) and in the presence of 100 μM inosine (●, ▲) or 100 μM deoxyinosine (■). Number of experiments (n): n = 3 (Ura); n = 3 (5FU); n=3 (Ura+Ino); n=3 (5FU+Ino); n=3 (Ura+dIno).

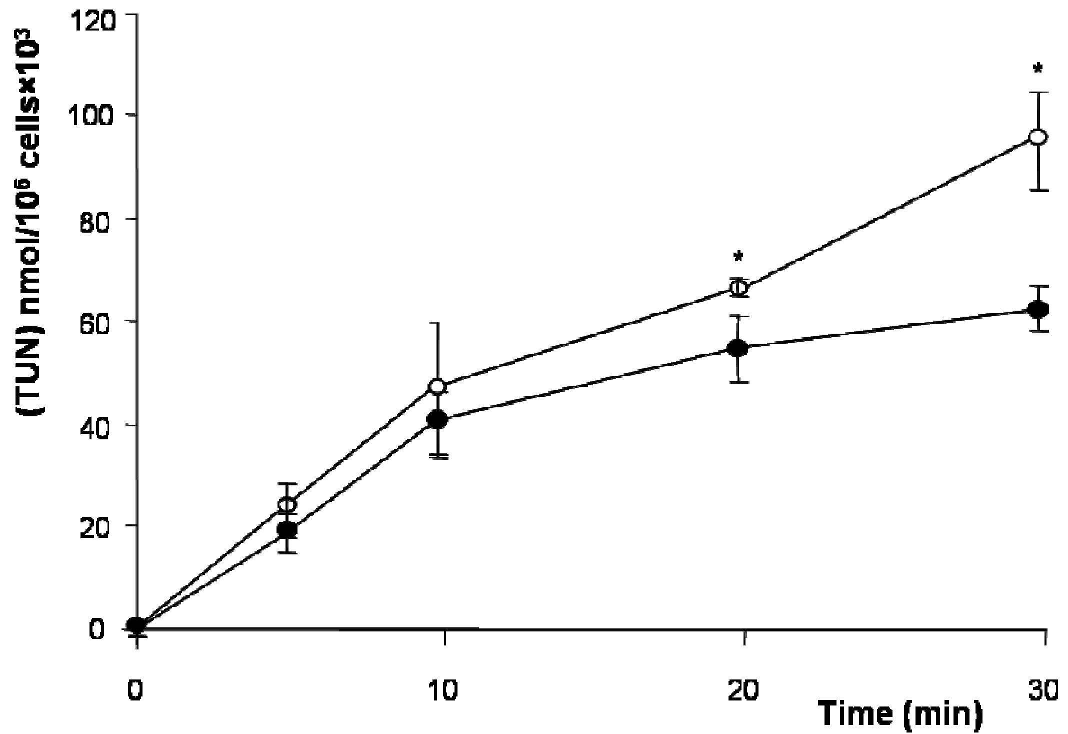


Fig.20. Time course of total uracil nucleotide (TUN) formation from uridine in cultured human astrocytoma cells. ADF cells are incubated with 100 µM [8-^{14}C] uridine (5,600 dpm/nmol) in the absence (o) and in the presence of 50 µM adenine (●). Number of experiments (n): n = 3 (Urd); n = 3 (Urd+Ade). Statistical significance vs cells not incubated with adenine: *$p < 0.05$.

5. Activity of enzymes involved in the purine and pyrimidine salvage.

The activity of enzymes involved in the purine and pyrimidine salvage was measured (Table 2).

Table 2

Enzyme	**Specific activity U/mg**
Uridine kinase (UK)	13.38×10^{-3}
Cytidine kinase (CK)	5.25×10^{-3}
Hypoxanthine phosphoribosyltransferase (HPRT)	5.35×10^{-3}
Adenine phosphoribosyltransferase (APRT)	9.39×10^{-3}

The results indicate that uridine and cytidine can be phosphorylated by their respective kinases in order to produce UMP and CMP. Adenine and hypoxanthine could be converted into IMP and AMP by their respective phosphorybosiltransferases.

6. Effect of CTP on the purine and pyrimidine salvage synthesis

In order to enrich the cells with CTP and to test the effect of the pyrimidine nucleotide on the purine salvage synthesis, cells were incubated for 30 min in the presence of cytidine at different concentrations; at the end of incubation the medium was removed and cells were incubated for the indicated times with radioactive adenine alone or in the presence of uridine. The pre-incubation with cytidine leads to an activation of the adenine salvage pathway in a concentration dependent manner, only in the presence of uridine as ribose phosphate donor (Fig.21). The intracellular concentration of CTP formed in cells incubated for 30 min with cytidine, was evaluated by HPLC analysis and a direct correlation between the extracellular cytidine added in the medium and the intracellular CTP formation was observed (Table 3).

To test the CTP effect on pyrimidine salvage, cells were incubated for 30 min in the presence of 1 mM cytidine; at the end of incubation the medium was removed and cells were incubated for the indicated times with radioactive uridine. The pre-incubation with cytidine leads to a strong inhibition of the uridine salvage pathway (Fig.22).

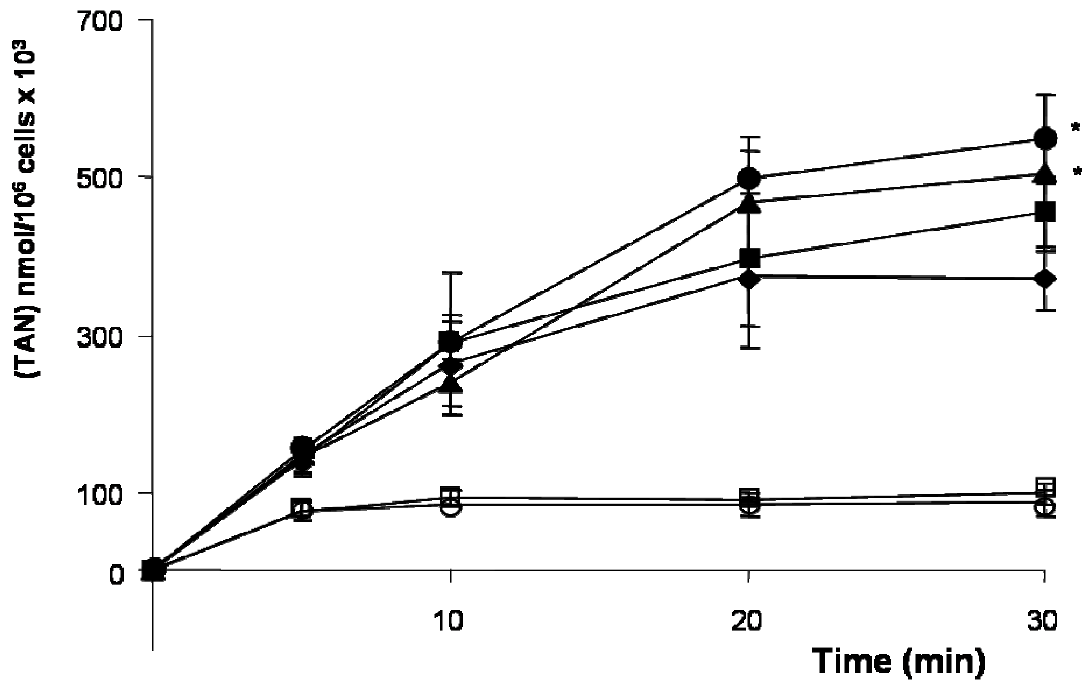


Fig.21. Time course of total adenine nucleotide (TAN) formation from adenine in cultured human astrocytoma cells pre-incubated with cytidine at different concentrations. ADF cells were pre-incubated for 30 min in the presence or absence of cytidine 1 (●, ♦), 0.5 (▲) and 0.25 mM (■) and incubated at different times with 50 μM [8-^{14}C] adenine (15,000 dpm/nmol) in the absence (open symbols) and presence of 100 μM cold uridine (closed symbols). Number of experiments (n): n = 3 (control); n = 3 (cyt 0.25 mM); n=3 (cyt 0.5 mM); n=6 (cyt 1 mM). Statistical significance vs cells not enriched with CTP: *$p < 0.05$.

Table 3. Correlation between the extracellular cytidine added in the medium and the intracellular CTP formation

Table 3

Extracellular cytidine (mM)	Intracellular CTP (nmol/10^6 cells)
0,25	0,35
0,5	0,68
1	0,81

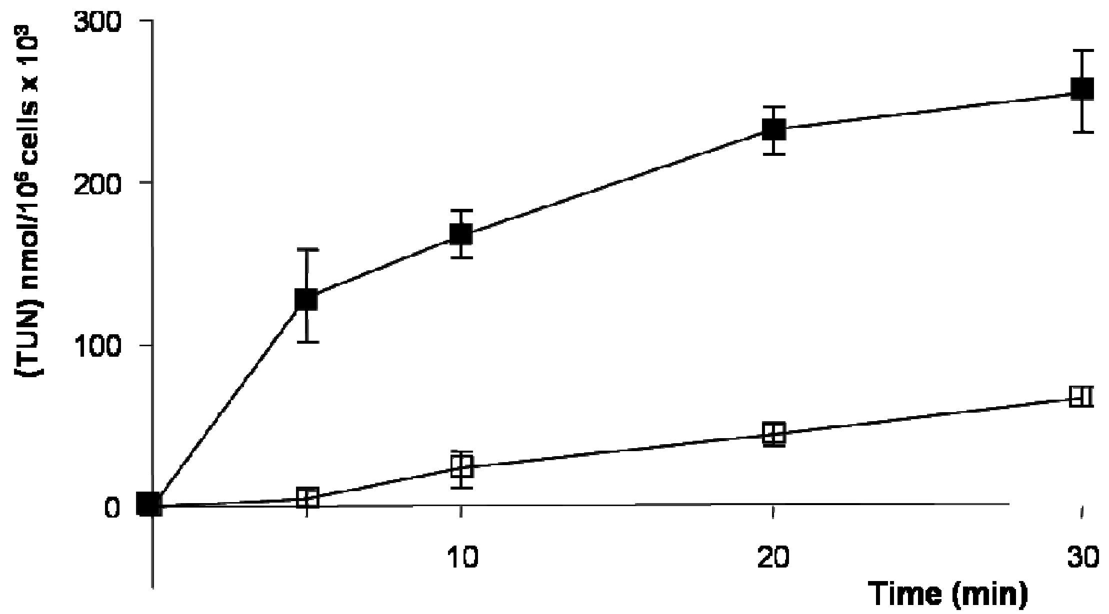


Fig.22. Time course of total uracil nucleotide formation from uridine in cultured human astrocytoma cells pre-incubated in the presence of cytidine 1 mM. ADF cells were pre-incubated for 30 min in the absence (open symbol) and presence (closed symbol) of cytidine 1 mM and incubated at different times with 50 μM [2-^{14}C] uridine (5,600 dpm/nmol). Number of experiments (n): n = 3 (control); n = 3 (cyt 1 mM).

CHAPTER 4

DISCUSSION

Purine and pyrimidines are emerging as physiological regulators of a number of cellular functions such as cell growth, differentiation and cell death, and release of hormones, neurotransmitters and cytokines. As a consequence of such a wide physiological involvement, it is also becoming evident that alterations in the physiology of purinergic signalling may result in the development of a variety of pathologies, including immune system diseases, infiammation and pain, neurodegenerative disorders and osteoporosis. There are reports suggesting protective roles for purines and pyrimidines in various pathological conditions ranging from cancer, to ischemia-associated injury, traumatic tissue damage, bone resorption, stress and haemorrhagic shock (Abbracchio and Burnstock, 1998; Ciccarelli *et al.*, 2001; Pizzorno *et al.*, 2002).

Mitochondrial oxidative phosphorylation, along with glycolysis, is essential for the maintenance of brain ATP levels. The depletion in cellular energy sources and reduction of ATP synthesis during ischemia or brain insults cause the loss of cellular homeostasis, which eventually leads to irreversible cellular damage. Growing lines of evidence indicate that purine nucleosides exert a neuroprotective effect. However, still matter of debate is whether their protective action is exerted through a receptor-dependent (Abbracchio and Burnstock, 1998; Rathbone *et al.*, 1999; Ciccarelli *et al.*, 2001; Shen *et al.*, 2005; Pizzorno *et al.*, 2002; Pooler *et al.*, 2005) or receptor-independent mechanism (Haun. *et al.*, 1996; Jurkowitz *et al.*, 1998; Yoo *et al.*, 2005; Giannecchini *et al.*, 2004; Choi *et al.,* 2006; Balestri *et al.,* 2007). Some papers have shown that adenosine protects neural cells during hypoxia/ischemia *in vivo* and *in vitro* (Ribeiro *et al.*, 2002; van Calker and Biber, 2005; Rathbone *et al.*, 1999). Many neuroprotective effects of adenosine appear to be mediated via adenosine A1 and A2 receptors, whereas other protective effects occur by receptor-independent mechanisms. In contrast with adenosine, there are very few studies on the effects of exogenous inosine and guanosine during hypoxia/ischemia in brain *in vitro* or *in vivo*.

In a receptor independent mechanism, nucleosides, that act after their entry into the cells, can be regarded as carriers of sugar that is made available through the action of nucleoside phosphorylases; in fact, without energy expense, a phosphorylated compound (Rib-1-P) is generated, which through the pentose phosphate pathway and glycolysis may be converted to energetic intermediates that can be utilized as energy source. In particular, purine or pyrimidine nucleosides could be phosphorolyzed to ribose l-phosphate and purine and pyrimidine base respectively, by purine/pyrimidine nucleoside phosphorylase. Three ribose l-phosphates are isomerized to ribose 5-phosphate, and then converted to two glucose 6-phosphates and one glyceraldehyde 3-phosphate, via transaldolases and transketolases of the pentose phosphate pathway. These phosphorylated intermediates enter the glycolytic pathway, yielding a net production of eight molecules of ATP per three molecules of ribose 1-phosphate (Fig.23) (Jurkowitz *et al.*, 1998).

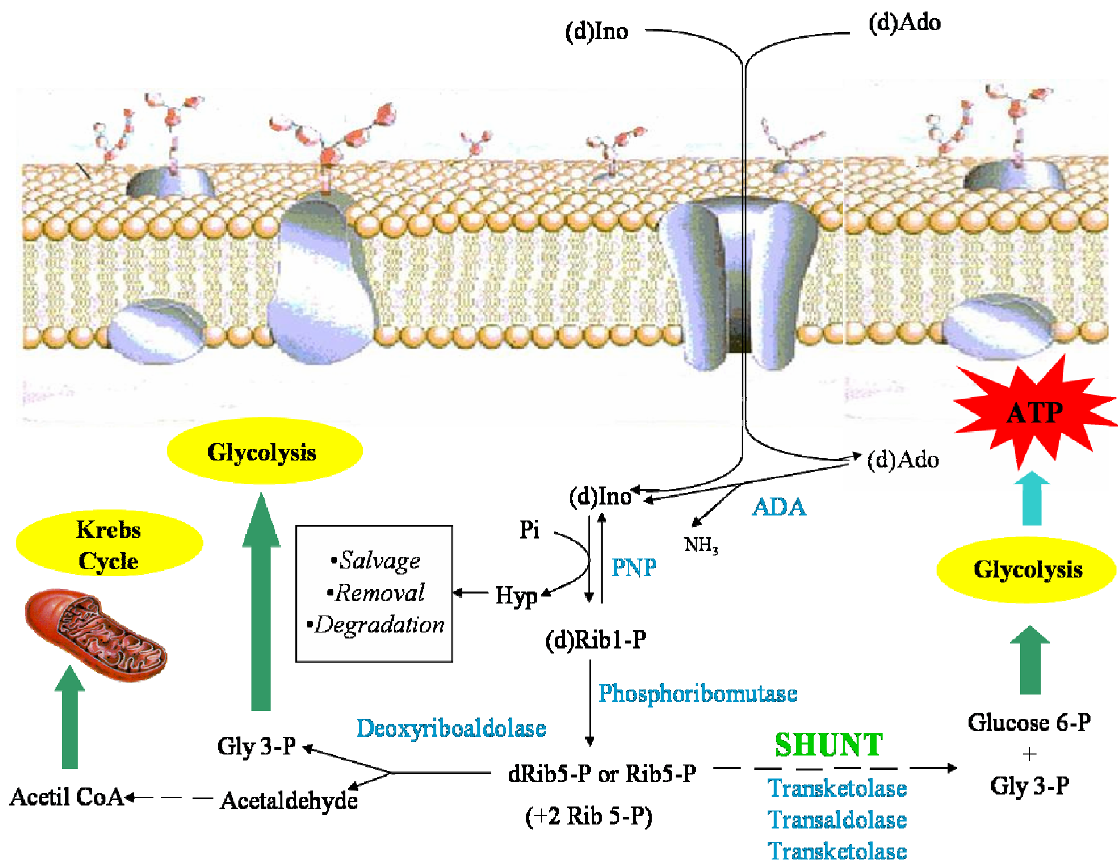


Fig.23. Schematic illustration of the putative metabolic fate of the pentose moiety of purine and pyrimidine nucleosides

In this regard, although glucose represents the major energy source for neuronal cells, when the glucose supply is lowered and the oxygen is not sufficient to support mitochondrial respiration (ischemic and/or hypoxic conditions), nucleosides may become one of the major energy sources for the cell. Indeed, the nucleic acids that are released by dead cells during hypoxic/ischemic conditions are subjected to degradation, and therefore might provide a discrete amount of purine and pyrimidine nucleosides.

The results reported in this research project clearly demonstrate that inosine, adenosine and guanosine are able to restore the ATP pool in ADF cells subjected to mitochondrial inhibition by oligomycin. In our experimental simil-ischemic conditions, the formation of lactate in the incubation medium indicates that purine nucleosides, like glucose, are utilized as energy source through anaerobic glycolysis. On the other hand, hypoxanthine and ribose are not able to restore the ATP levels, thus pointing to a relevant role of the phosphorolytic cleavage of nucleosides which generates, without energy expense, the phosphorylated pentose, which through the pentose phosphate pathway and glycolysis can be converted to energetic intermediates. ADF cells have high purine nucleoside phosphorylase activity (Fig. 23).

Several reports indicate that pyrimidines play an important role for the maintenance of normal activity of the central nervous system (Loffler *et al.*, 2005), and UTP has been demonstrated to act through pyrimidinergic receptors (Brunschweiger *et al.*, 2006). In this research project, we show that also pyrimidine nucleosides, such as uridine and, to a lesser extent, cytidine exert a rescuing effect on cells previously subjected to mitochondrial inhibition. In fact, ADF cells express uridine phosphorylase activity, that makes the phosphorylated ribose moiety of uridine available for energetic purposes. Owing to the lack in mammals of a phosphorolytic activity on cytidine (Barsotti *et al.*, 2002), this nucleoside must be deaminated to uridine, in order to make its ribose moiety available for energy repletion. Therefore, the low cytidine deaminase activity might account for the incomplete recovery when cytidine is present. Again, the formation of lactate indicates that pyrimidine nucleosides are utilized as energy source through anaerobic glycolysis. Our findings, which indicate the beneficial effect of pyrimidine nucleosides on ischemic neuronal cells, exerted through the restoration of the ATP pool, are in line with recent observations (Choi *et al.*, 2006), in which uridine has been reported to prevent the glucose deprivation induced death of immunostimulated astrocytes, and point to a relevant role of uridine phosphorolysis. In fact, while for purine nucleosides different from adenosine, several authors postulate that an interaction of these nucleosides with purinergic receptors might account for their effect (Hasko *et al.*, 2004; Shen *et al.*, 2005), a similar receptor-dependent mechanism is unlikely to occur for pyrimidine nucleosides. Indeed, although postulated (Connolly *et al.*, 1997), the presence of a receptor specific for uridine has never been demonstrated. Furthermore, our observation that PTAU, a potent inhibitor of uridine phosphorylase, significantly reverses the protective effect of uridine on ADF cells subjected to mitochondrial inhibition, is in agreement the relevant role exerted by this enzyme which produces the phosphorylated pentose, necessary for the restoration of ATP levels. Our hypothesis of a receptor-independent nucleoside effect is further supported by the finding that NBTI, an inhibitor of nucleoside transport, significantly reduces the protective action of inosine, adenosine, and uridine, while no effect is observed with glucose.

Under diverse ATP depleting conditions, such as ischemia, astrocytes have been reported to release ATP, which is subsequently extracellularly hydrolyzed to adenosine, which may exert its hormone-like action interacting with specific membrane receptors. On the other hand, the bidirectional equilibrative nucleoside transport supplies a system for a re-entry of adenosine into the cell, where the nucleoside can be readily metabolized to AMP by adenosine kinase or to inosine by adenosine deaminase. As adenosine kinase requires ATP as a phosphate donor, adenosine deaminase is likely the most important enzyme for adenosine metabolism during ischemia conditions, therefore generating inosine which, as indicated in this research project, may well contribute to the maintenance of the ATP pool, thus improving cell viability.

The results reported in this thesis indicate that the protective effect of adenosine appears to be receptor-independent, and therefore, conceivably related to its intracellular conversion into inosine through the action of adenosine deaminase (which is present in ADF cells), as previously reported in different experimental models (Haun *et al.*, 1996; Jurkowitz *et al.*, 1998; Shin *et al.*, 2002; Yoo *et al.*, 2005). In fact adenosine is able to restore the ATP pool independently of the presence of DPCPX which is an antagonist of adenosine receptors.

Furthermore, the lack of protective effect exerted by adenosine when ADF cells subjected to mitochondrial inhibition are incubated in the presence of deoxycoformycin, the inhibitor of adenosine deaminase, clearly supports the relevant role exerted by this enzyme. It is worth noticing that inosine concentrations near millimolar have been reported in human brain (Traut, 1994), and therefore, this nucleoside might be considered as a suitable energy source for neuronal cells. Furthermore, our observations indicate that in the experimental simil-ischemic conditions adopted in the present work, a correlation exists between the restoration of the ATP pool, operated by purine and pyrimidine nucleosides, and cell viability.

Deoxyinosine does not act as a neuroprotective compound, thus confirming a finding reported for a different experimental cell model (Giannecchini *et al.*, 2004). Since purine nucleoside phosphorylase acts equally well on both ribo- and deoxyribonucleosides, once deoxyinosine enters the cells, deoxyRib-1-P is generated, which is then converted to deoxyRib-5-P by phosphopentomutase. DeoxyRib-5-P, in turn, may be cleaved into acetaldehyde, that can be converted to acetyl-CoA, and therefore, be used as energetic substrate only in the presence of oxygen, and glyceraldehyde-3-P that through glycolysis may generate ATP also in anaerobic conditions (Fig. 23). The lack of effect of deoxyinosine on the preservation of the ATP pool may be explained by the low deoxyriboaldolase activity present in ADF cells, which is probably inadequate to provide an amount of glyceraldehyde-3-P sufficient for the maintenance of the ATP pool.

In conclusion, while indicating that both purine and pyrimidine nucleosides exert a neuroprotective action by preserving the ATP pool in astrocytoma cells subjected to ischemic conditions, our data also support the hypothesis that their action relies on a receptor-independent mechanism.

It is generally accepted that only liver and kidney maintain the *de novo* pyrimidine and purine synthesis and supply other tissues and organs, including brain, where the salvage synthesis is more active than the *de novo* synthesis, with preformed pyrimidine nucleosides (mainly uridine) and purine nucleosides and bases for nucleotide synthesis (Barsotti *et al.*, 2002; Cao *et al.*, 2005; Cansev, 2006). Therefore, another purpose of this research project is to study how do these districts, which rely more heavily on salvage synthesis, maintain the right balance between the purine and pyrimidine pools for the stability of genetic information. In this regard, we have hypothesized that the UPase–UK enzyme system, which maintains uridine homeostasis, could regulate the two processes of purine and pyrimidine salvage. Uridine kinase is the major entry step in the salvage of preformed uridine to UTP, and then to CTP, while the phosphorylase is the major entry step in the catabolism of uridine to β-alanine, a process considered to be restricted to the liver (Loffler *et al.*, 2005).

UK is inhibited by elevated UTP and CTP levels, a signal of pyrimidine sufficiency, and activated by ATP, a signal of purine sufficiency (Suzuki *et al.*, 2004). This kind of regulation is reminiscent of that exerted by UTP and CTP, acting as inhibitors, and ATP, acting as activator, on carbamoylphosphate synthetase and aspartate transcarbamylase, the committed steps of de *novo* pyrimidine biosynthesis in mammalian and bacterial cells, respectively (England and Herve*, 1994*). Uridine could represent a link between the purine and pyrimidine salvage synthesis (Cao *et al.*., 2005).

In fact, the phosphorylated pentose stemming from uridine phosphorolysis, is a substrate of PRPP synthase which, in the presence of ATP, can converted the sugar into PRPP. PRPP is an essential compound for the salvage pathway of purine which is realized at the purine base level or maybe used itself for the salvage of pyrimidine nucleosides which is realized at the nucleoside levels. Adenine and hypoxanthine, in the presence of PRPP, are substrates of adenine phosphoribosyltransferase (APRT) and hypoxanthine-guanine phosphoribosyltransferase (HPRT), which catalyze the formation of adenosine and inosine monophosphate, respectively (purine salvage pathway). On the other hand, uridine can be both phosphorolytically cleaved by uridine phosphorylase (UPase) to ribose-1-phosphate (Rib-1-P) and the uracil base, or can be converted by uridine kinase (UK) into uridine nucleotides (pyrimidine salvage pathway) (Fig. 24).

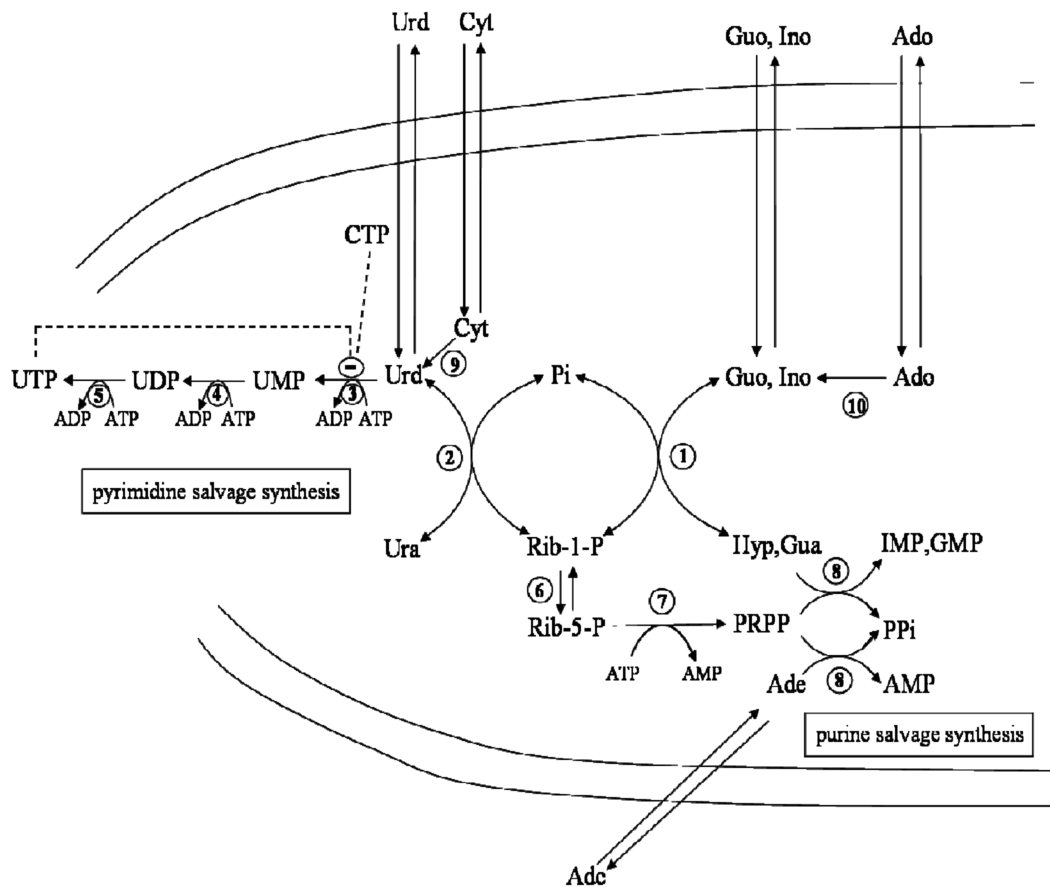


Fig.24. Interrelationship between purine and pyrimidine salvage. The enzymes participating in the pathway are: 1.purine nucleoside phosphorylase; 2.Upase; 3.UK; 4.nucleoside-monophosphate kinase; 5.nucleoside-diphosphate kinase; 6.phosphopentomutase; 7.PRPP synthetase; 8.phosphoribosyltransferases; 9.cytidine deaminase; 10.adenosine deaminase.

Previous experiments performed in this laboratory using rat brain extracts revealed that purine and pyrimidine salvage might be reciprocally regulated (Balestri *et al.*, 2007). In particular, when the concentration of UTP and CTP is relatively high, the inhibition of UK causes a shift of the equilibrium of the reversible UPase reaction towards uridine phosphorolysis. The Rib-1-P formed is then converted into PRPP, which is used in the salvage synthesis of purine nucleotides which is favoured over pyrimidine salvage. Conversely, when the concentration of UTP and CTP is relatively low, the fully active UK, which catalyzes a virtual irreversible reaction, drives uridine towards uridine nucleotide formation, thus pyrimidine salvage is favoured over purine salvage (Fig.25).

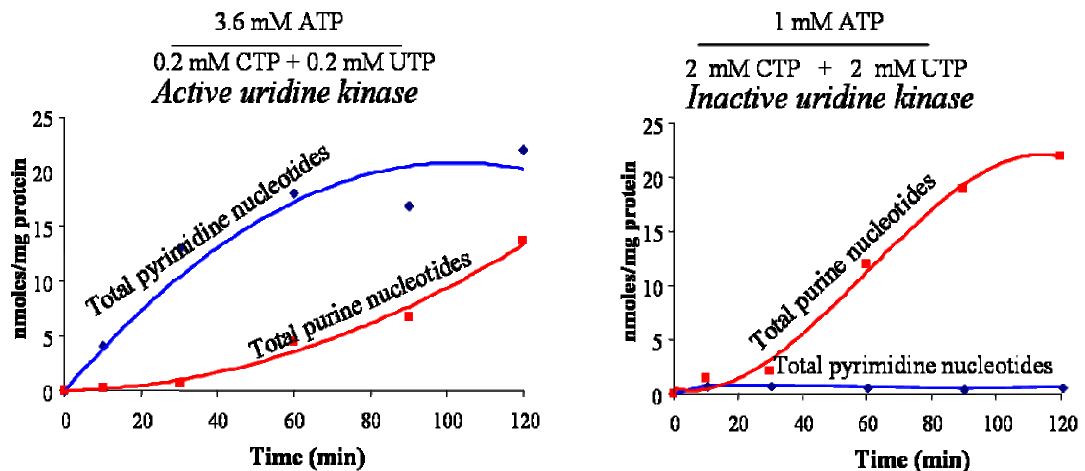


Fig.25 Total pyrimidine and purine nucleotides salvaged at two [ATP]/[UTP] + [CTP] ratios (from Balestri *et al.*, 2007).

The experiments performed in our cell model are in agreement with these previous observations and explain how extracellular added nucleoside could regulate the purine and pyrimidine nucleotides production (Fig.25).

In cultured ADF cells, exogenous uridine and, to a lesser extent inosine, are able to increase the total adenine nucleotide formation in a concentration-dependent manner as observed in the case of uridine. Moreover, uridine is also able to activate the salvage of exogenous hypoxanthine. When uridine accumulates, also in view of the inhibition exerted on UK by the raise of pyrimidine nucleotides, more Rib-1-P becomes available, through the action of UPase, for the PRPP-mediated adenine salvage. The fact that in the presence of PTAU, the inhibitor of uridine phosphorylase, the activation of adenine salvage by uridine is significantly lowered, confirms the relevant role exerted by the phosphorolysis of the nucleoside in order to donate the ribose moiety which can be converted by the cell, into PRPP. In this regard, also inosine, which, through purine nucleoside phosphorylase, acts as Rib- 1-P donor, appears to stimulate adenine salvage. The highest amount of adenylate pool formed is observed when uridine is present.

In fact, the phosphorolysis of inosine and guanosine, besides Rib-1 P, produces also hypoxanthine and guanine, respectively, which are converted, in the presence of PRPP, to IMP and GMP by hypoxanthine-guanine-phosphoribosyl transferase (Fig. 24). Therefore, a certain amount of PRPP which derives from inosine or guanosine catabolism, can be utilized in this reaction, while in the presence of uridine, the whole PRPP formed by the phosphorylated pentose derived from the uridine catabolism, is utilized to produce the adenine nucleotide pool. In the presence of cytidine only a slight activation of the adenine nucleotide production was observed. This result may be explained by the low cytidine deaminase activity measured in ADF cells: in mammals, cytidine phosphorylase is absent, therefore cytidine can donate the ribose moiety only after its conversion to uridine by cytidine deaminase.

We have also showed that dIno has no effect on adenine salvage. Since deoxyRib-5-P is not a substrate of PRPP synthetase, the lack of effect of deoxyinosine is a further indication that the salvage of the purine ring occurs at the nucleobase level, using PRPP as the donor of the phosphoribosyl moiety. In addition, the increased utilization of PRPP exerted by added adenine, might further favour uridine phosphorolysis, thus explaining the apparent inhibition exerted by adenine on exogenous uridine salvage to uracil nucleotides. We emphasize that adenine, even at 1 mM, does not inhibit uridine transport (Crawford *et al*., 1998).

On the other hand, we have also demonstrated that the purine inosine stimulates 5-FU activation and uracil salvage: most likely, this occurs through a Rib-1-P-mediated process, because uracil phosphoribosyltransferase is absent in mammals (Cappiello *et al.,* 1998). However, it cannot be excluded *a priori* that 5-FU activation might occur through the PRPP-mediated process, catalyzed by orotate phosphoribosyltransferase acting on 5-FU (Mascia and Ipata, 2001). Again, deoxyinosine has no effect on pyrimidine salvage.

In order to investigate the effect of pyrimidine nucleotide level variation on the adenine salvage, ADF cells were enriched with CTP by pre-incubation with cytidine. In fact, cytidine enters the cells and is converted into CTP (in a concentration-dependent manner) which inhibits UK. This inhibition causes a shift of the equilibrium of the reversible UPase reaction towards uridine phosphorolysis. The Rib-1-P formed is then converted into PRPP, which is used for the purine salvage (Fig.24). Therefore, an activation of adenine salvage is observed; conversely, when the concentration of CTP is relatively low, the fully active UK, which catalyzes a virtual irreversible reaction, drives uridine towards uridine nucleotide formation, thus lowering the rate of purine synthesis (Fig. 24).

In conclusion, the ribose phosphate stemming from the phosphorolysis of purine and pyrimidine nucleosides not only can be converted into energetic intermediates in order to restore the ATP pool during cellular stress but it can be considered a link between the purine and pyrimidine salvage; these two processes are regulated at the level of UPase–UK enzyme system by the relative pyrimidine nucleoside triphosphate concentration.

CHAPTER 5

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