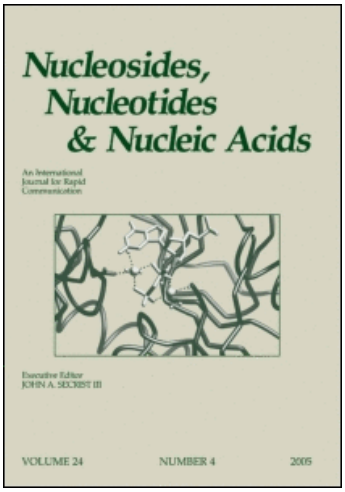

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PURINE NUCLEOTIDE CATABOLISM IN RAT LIVER. CERTAIN PRELIMINARY ASPECTS OF URICASE REACTION

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□ *We investigated the mechanism of action of uricase, which oxidizes uric acid to allantoin, in the rat. Allantoin may decompose chemically to urea and hydantoin, containing the carbons in positions 2 and 8 of the purine ring, respectively. These carbons are derived by formylation, catalyzed by formyltransferase, in two reactions of de novo synthesis. Since uric acid and allantoin are represented in equivalent amounts in the liver, we expected to find identical incorporation of radioactivity in C_2 and C_8 of both compounds after administration of ^{14}C-formate. In the case of ^{14}C-allantoin, this was true, but not for ^{14}C-uric acid extracted from rat liver. We interpret these results through a series of experiments and considerations.*

Keywords Allantoin; uric acid; uricase

INTRODUCTION

The catabolism and synthesis of purine nucleotides has been extensively studied by many authors.[1–5] Through a series of very well known steps (dephosphorylation, deamination and phosphorolytic splitting), free bases, adenine and guanine, are formed. Deamination of adenine leads to hypoxanthine.[6,7] Hypoxanthine is the key compound for the final steps of nucleotide breakdown, which occurs through the sequence: hypoxanthine → xanthine → uric acid → allantoin. Xanthine oxidase regulates the sequence hypoxanthine → uric acid (UA) and uricase the oxidative reaction leading to allantoin (ALL). ALL is the terminal compound in most mammals, whereas humans, arthropod apes and some monkeys lack this

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enzyme, and as a consequence excrete UA. The purine metabolic pathway is commonly followed using labeled precursors as markers.[8]

We considered specifically the incorporation of ^{14}C-formate. Since UA and ALL are represented in equivalent amounts in the liver, we expected to find similar incorporation of radioactivity in both compounds after administration of ^{14}C-formate. On the contrary, we found that labeling of ALL was much higher at short times after administration, and that the phenomenon was specific to liver.[9]

In the frame work of this research, we investigated the mechanism of action of the peroxisomal enzyme uricase (E.C. 1.7.3.3.). Uricase shows different properties in certain species. The mechanism of the urate oxidase reaction has not been clearly established, but involvement of radical intermediates has been hypothesized. The most widely accepted mechanism[10–12] show that the reaction occurs through formation of an intermediate and that C2 of UA is distributed equally among both hydantoin and the ureido moiety of ALL, when the urate is degraded enzymatically in the absence of borate ions. This hypothesis is belied by Bongaerts and Vogels[13] that propose the maintenance of a high degree of positional and stereochemical specificity, in the enzymatic oxidation of UA, in all steps. Kahn and Tripton[14] observed two discrete enzyme-bound intermediates and we carried out a study using EPR spectroscopy and spin trapping of radical intermediates and for the first time, the presence of a radical intermediate in the uricase reaction was proven experimentally.[15] Gabison et al.[16] characterize the active site of urate oxidase from *Aspergillus flavus*, analyzing the x-ray structure. The urate oxidase illustrates an unexpected plasticity of the active site; it can accommodate not only the well known cyclic purine NH-CO-NH-CO motif, but also open ring compounds like ALL. In this complex, all the N and O atoms are involved in strong polar interactions toward either the protein and/or water relays. The authors conclude, with two hypotheses, that the uricase reaction lead to an optically active [S]-allantoin. Now, it can be concluded that the catalytic mechanism of UA oxidation by urate oxidase is complex. Many studies have been done and are still on going to understand how this oxidase works without any cofactor or metal ion.

For a better comprension of this mechanism, in the present study we investigated certain aspects of the uricase reaction: labeling of C_2 and C_8 and tautomerization. We followed the formation of labeled urea and hydantoin from labeled ALL which was purified from liver of rats previously treated with ^{14}C-formate or $^{14}C_8$-urate. ALL was also produced from labeled UA treated in vitro with uricase. In Scheme 1 is represented the intermediate compound A, which undergoes tautomerization to form two differently ^{14}C-labeled ALL, which can be split chemically, not enzymatically, to urea and hydantoin. From the same scheme, it is evident that both labeled carbonyl groups of urea and hydantoin may derived, respectively, from C_2

and C_8 of the intermediate A. Really, in intermediate A the C of our interest there is not in eight position, but to indicate that this C derived from C_8-UA, we mark as C_{8*}. In conclusion, the labeling of urea and hydantoin should reflect the labeling of C_2 and C_{8*} of ALL, due to the original labeling of UA and to the efficiency of tautomerization process.

Our results provide interesting indications on the formation of the intermediate compound reported in Scheme 1, under different conditions, and, thus on the mechanism of action of uricase in vitro and in vivo.

MATERIALS AND METHODS

Materials

Allantoin, uric acid, trichloroacetic acid, hydantoin, urea, red phosphorus, hydroiodic acid, and uricase from Bacillus fastidious (15 units/mg protein) were purchased from Sigma Aldrich Corporation (St. Louis, MO, USA). Urease was obtained from Sclavo (Italy). Anhydrous acetic acid was obtained from Farmitalia Carlo Erba (Milan, Italy). Methanol (HPLC grade) was obtained from Baker (Phillisburg, NJ, USA), ^{14}C-formate (54.8 mCi/mmole) from Amersham Pharmacia Biotech Europe (Freiburg, Germany) and $^{14}C_8$-uric acid (50 mCi/mmole) from American Radiolabeled Chemicals Inc. (St Louis, MO, USA).

Animals

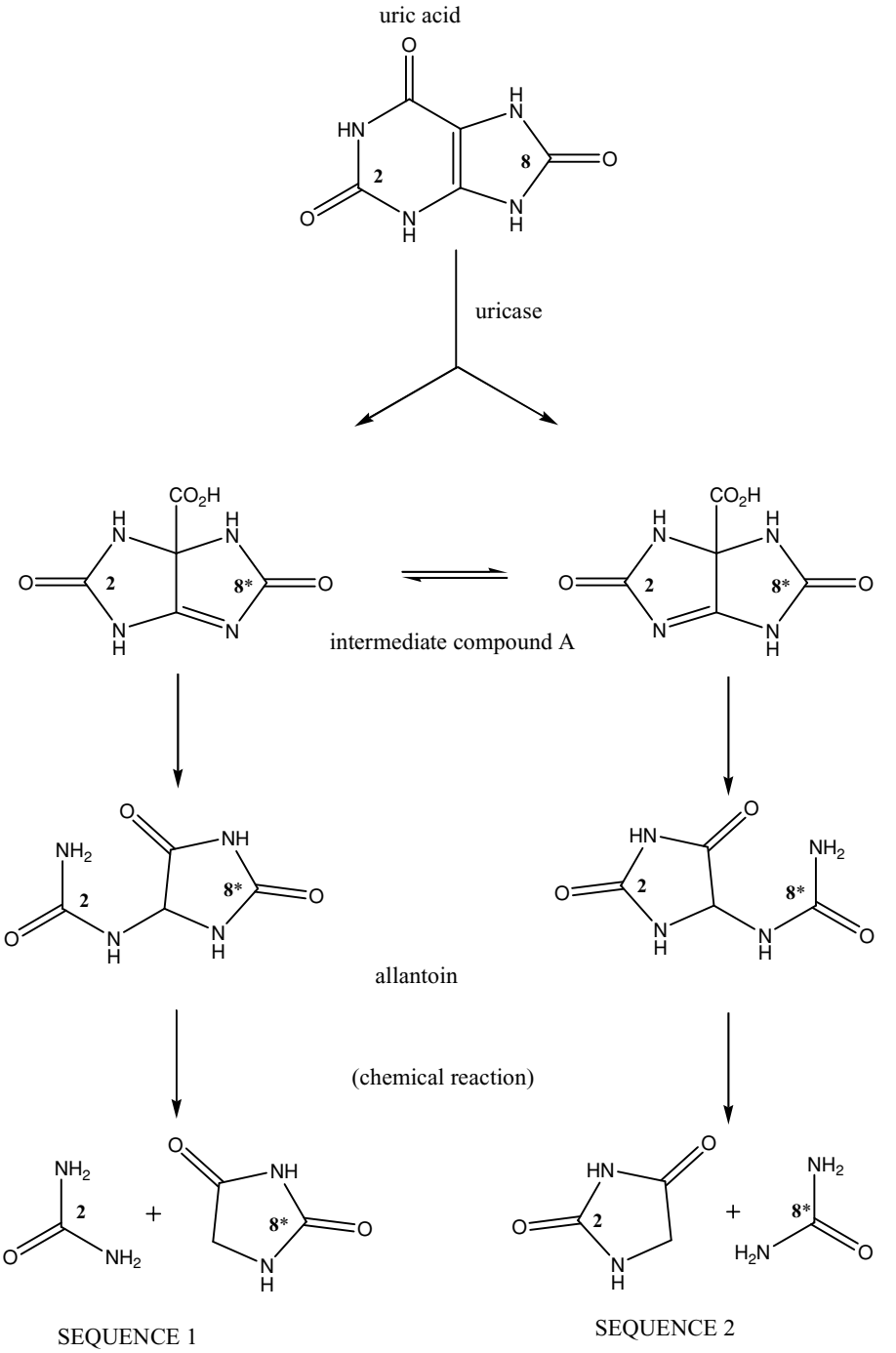
Male Wistar rats, weighing 250 g, previously kept on a standard diet, were used. All animals were fasted for 12 hours before sacrifice. They were housed three to four animals per clear plastic cages in a room kept at a constant temperature ($24 \pm 1^{\circ}C$) and humidity ($45\% \pm 5\%$) under the illumination of a 12:12-hour light/dark cycle. Food and water were available ad libitum.

Treatment with ^{14}C-formate or $^{14}C_8$-UA

^{14}C-formate or $^{14}C_8$-uric acid precursor were injected intraperitoneally at a dose of 10 μCi/100 g b.w. Rats were killed 15′ after administration. The livers were rapidly excised, washed in normal saline, homogenized in 5% trichloacetic acid (TCA) and centrifuged to obtain the supernatant.

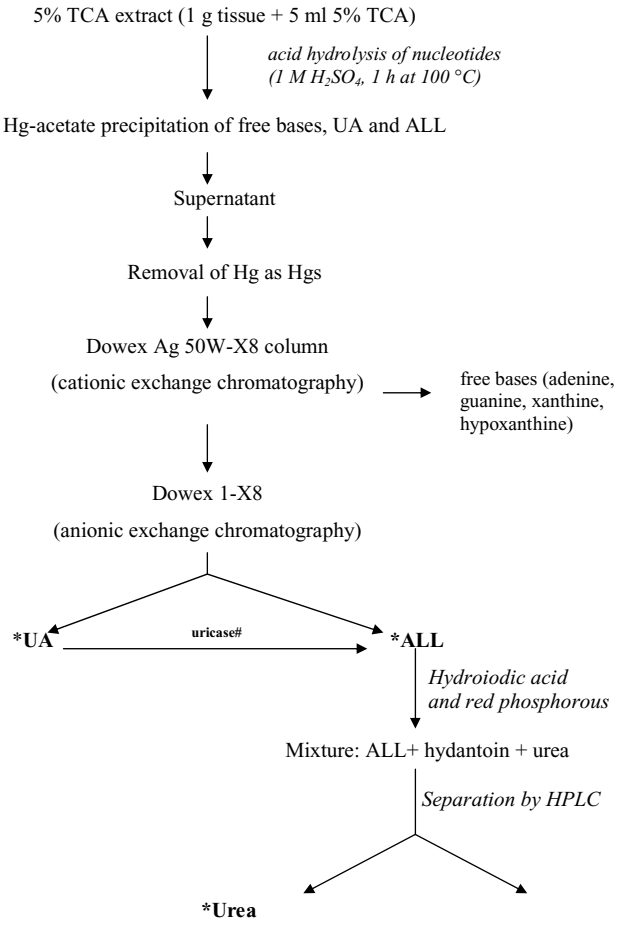
Purification of UA and ALL

^{14}C–UA and ^{14}C-ALL were purified from supernatant by our previously reported procedure.[17] UA was determined by the enzymic procedure



SCHEME 1 Uric acid treatment with uricase, formation of allantoin and subsequent chemical reaction to obtain urea and hydantoin.

of Praetorious and Poulsen.[18] ALL was assayed by the Rimini-Schryver reaction.[19] The procedure is summarized in Scheme 2.



***Hydantoin**

#UA was transformed into ALL by treatment:

- with uricase from *Bacillus Fastidiosus*
- with uricase from rat liver peroxysomes

SCHEME 2 Purification of uric acid and allantoin obtained of rat liver and chemical degradation of allantoin.

Preparation of Rat Liver Peroxysomes

We obtained rat liver uricase from crude tissue preparation. The rat liver was excised and minced in homogenization buffer (5 mM Mops pH 7.4, 250 mM sucrose, 1 mM EDTA, 0.1% ethanol). Five grams of liver was homogenized for 2 minutes at 1000 rpm in 20 ml of ice-cold buffer, hold on ice, using a Potter-Elvehejm homogenizer. The homogenate was centrifuged

at 2000 g for 10 minutes and the precipitate resuspended with phosphate buffer pH 7.5. After dialysis against double-distilled water at 0°C, the final suspension (suspension A), containing the peroxysomes, was used for UA treatment.

Treatment of UA with Uricase from Bacillus fastidiosus or Rat Liver Peroxisomes

To a solution containing 0.05 μmol/ml UA we added: 0.1 ml *Bacillus fastidiosus* uricase and 0.02 ml catalase; and 1 ml fresh suspension A containing liver peroxysomes.

The mixture was incubated at 37°C for 1 hour. TCA 12% was added up to pH 2.0 and the mixture centrifuged at 10000 g for 15 minutes. ALL was separated from UA by anionic exchange chromatography (Dowex 1-X8).

Chemical Degradation of ALL to Hydantoin and Urea

The chemical degradation of ALL to urea and hydantoin was carried out as previously described[20] and shown in Scheme 2. Urea was determined by a colorimetric enzyme method[21] and hydantoin by HPLC.

Mass Spectrum of Allantoin

ALL obtained from rat liver or from UA transformed into ALL by *Bacillus fastidiosus* or rat liver peroxysomes was purified by HPLC and derivatized with N-methyl-N-(tert-butyldimethylsilyl) trifluoroacetamide (MTBSTFA) in pyridine at 90°C for 24 hours.[22] The compound was then analyzed by ion trap mass spectrometry (ITMS, Finnigan, St. Jose, CA, USA). Collision experiments were performed selecting parent ions by the two-step procedure, at a q_2 of 0.3. A supplementary AC "tickle" voltage applied to the two endcaps was used to excite the ions. Typical tickle voltage and tickle time values were in the range 150–200 mV and 15–35 msec, respectively.

Determination of Radioactivity

This was carried out on a Nuclear Chicago Delta Scintillation Counter, using Instagel as liquid scintillation cocktail.

RESULTS

a) In a first set of experiments we focused attention on $^{14}C_8$-uric acid obtained from a commercial source, treating it first with uricase from *Bacillus fastidiosus*, and then chemically degrading it. The procedure

TABLE 1 Specific radioactivity of urea and hydantoin obtained by commercial $^{14}C_8$-uric acid trasformed in $^{14}C_8$-allantoin by uricase of *Bacillus Fastidiosus* or rat liver peroxisomes

Experiments	Specific radioactivity (dpm/μmoles)					
	Uric acid	Allantoin	Urea	% C_2	Hydantoin	% C_8
			Bacillus Fastidiosus uricase			
1	13444954	13527536	3000004	22	10869375	78
2	17555823	17767376	7102012	40	10479931	60
3	15168738	15222935	6283540	41	9067449	59
4	21706900	20170929	6799924	37	13076621	63
			Rat peroxisomal uricase			
1	10324472	10169134	16222	0.1	10191500	99.9
2	13235831	13052459	11584	0.1	12901255	99.9

showed that urea and hydantoin were both labeled, though hydantoin accumulated more ^{14}C than urea. When $^{14}C_8$-uric acid was first degraded by uricase from peroxysomes, and then underwent further chemical degradation, only hydantoin was strongly labeled, as predictable from Sequence 1 Scheme 1 (Table 1). Experiments in which $^{14}C_8$-uric acid obtained from rat liver after injection of commercial $^{14}C_8$-uric acid was treated with uricase of *Bacillus fastidiosus* or rat liver peroxysomes after injection of commercial $^{14}C_8$-uric acid, are reported in Table 2. It was evident that hydantoin was more strongly labeled than urea, indeed about 100% after treatment with peroxysomal enzyme.

b) Treatment of ^{14}C-UA obtained from rat liver after administration of ^{14}C-formate are reported in Table 3. ^{14}C-UA was then transformed into ^{14}C-ALL by uricase of *Bacillus fastidiosus* or rat liver peroxysomes. Different results were obtained in the two experiments: in the case of *Bacillus fastidiosus* enzyme high variability was evident, though labeling

TABLE 2 Specific radioactivity of urea and hydantoin obtained by liver $^{14}C_8$-uric acid[a] trasformed in $^{14}C_8$-allantoin by uricase of *Bacillus Fastidiosus* or rat liver peroxisomes

Experiments	Specific radioactivity (dpm/μmoles)					
	Uric acid	Allantoin	Urea	% C_2	Hydantoin	% C_8
			Bacillus fastidiosus uricase			
1	227441	228833	52016	23	174142	77
2	335339	329005	58940	18	268508	82
			Rat peroxisomal uricase			
1	301566	300798	20999	7	278652	93
2	279561	280016	17587	6	264338	94

[a] $^{14}C_8$-uric acid was obtained from rat treated with commercial $^{14}C_8$-uric acid.

TABLE 3 Specific radioactivity of urea and hydantoin obtained by liver ^{14}C-uric acid[a] trasformed in ^{14}C-allantoin by uricase of *Bacillus Fastidiosus* or rat liver peroxisomes

	Specific radioactivity (dpm/μmoles)					
Experiments	Uric acid	Allantoin	Urea	% C_2	Hydantoin	% C_8
			Bacillus Fastidiosus uricase			
1	3300	3471	2875	78	806	22
2	3520	3543	2760	73	1011	27
3	3146	2981	2017	67	993	33
4	2820	2701	915	34	1800	66
5	7,026	7,052	4296	61	2683	39
6	10100	10463	4977	48	5462	52
7	2180	2237	449	20	1745	80
			Rat liver peroxisomal uricase			
1	10600	9580	8381	85	1479	15
2	15425	15932	12694	79	3375	21

[a]^{14}C-uric acid was obtained from rat treated with ^{14}C-formate.

was prevalently of urea. In the case of treatment with rat peroxysomal enzyme, urea was labeled 80%.

c) When we considered the formation of ^{14}C-urea and ^{14}C-hydantoin from ^{14}C-ALL, purified from the livers of rats treated with ^{14}C-formate, the results were in perfect agreement from they and showed that rat liver allantoin, obtained in this way, contained equally labeled C_2 and C_{8*} (Table 4).

In determining whether ALL molecules were identical, irrespective of the source of uricase (from *Bacillus fastidiosus* or rat liver), we purified ALL by HPLC and derivatized it for analysis by mass spectrometry. In all cases we confirmed that the molecules of ALL obtained are identical.

TABLE 4 Specific radioactivity of urea and hydantoin obtained by liver ^{14}C-allantoin[a]

	Specific radioactivity (dpm/μmoles)				
Experiments	Allantoin	Urea	% C_2	Hydantoin	% C_8
1	23000	10250	49	10750	51
2	24293	12416	50	12525	50
3	17000	8030	49	8406	51
4	15250	7062	48	7620	52
5	28999	14876	51	14174	49

[a]^{14}C-allantoin was obtained from rat treated with ^{14}C-formate.

DISCUSSION

a) Experiment In Vitro Using $^{14}C_8$-Uric Acid From a Commercial Source or Obtained After Injection In Vivo

In this case, when UA was treated with uricase from *Bacillus fastidiosus*, urea and hydantoin were both differently labeled. This may be due to variable tautomerization degree of the intermediate compound probably occurring in the medium not to the enzyme. When we use the peroxysomal enzyme, the result was cleared: most radioactivity appeared in hydantoin. In this case, the rate of tautomerization is slower than the rate of formation of ALL, leading to only ^{14}C-labeled hydantoin.

The conclusion is that the bacterial enzyme can only be used to obtain approximate indications, leading to mixing up of C_2/C_{8*} labeling, due to an efficient tautomerization (Scheme 1, Sequence 1 and 2), whereas that of peroxysomes is very specific and acts as shown in Scheme 1, Sequence 1, according to which hydantoin is derived from C_{8*}.

b) Experiment in Which of ^{14}C-formate was Administered and ^{14}C-UA was Obtained From Rat Liver

In this case when UA is treated with uricase, a clearly prevalent labeling of urea is obtained, especially when peroxysomal enzyme is used. In line with the above conclusion, that the mechanism of action of peroxysomal enzyme in according to Sequence 1 of Scheme 1, we may conclude that in Experiment B UA was labeled at C_2, which is the source of urea.

c) Experiment in Which of ^{14}C-formate was Administered and ^{14}C-ALL was Obtained From Rat Liver

ALL obtained in vivo after ^{14}C-formate injection was equally labeled at C_2 and C_{8*}, which can be explained by assuming that it is derived from UA containing equally C_2 and C_{8*} labeled, or that it is formed by perfectly symmetrical tautomerization (50%).

The results of Experiments B and C seem in disagreement, because ALL is derived from UA, which is formed during de novo nucleotide synthesis and catabolism. However, during de novo synthesis of the purine ring, from which UA and ALL are derived, formylation of the two carbons in the well known reactions of formation of 5′-phosphoribosyl-N-formylglycinammide and 5′-phosphoribosyl-4-carboxamide-5-formamidoimidazole, catalyzed by formyltransferase, produces two carbon atoms which could be labeled at the same extent. If we consider our results for ^{14}C-ALL, this seems true, but it is not true for ^{14}C-UA extracted from rat liver: unlike ALL, all UA is not equally labeled at carbons 2 and 8*.

Presumably, there are two possible explanations for this. A reasonable interpretation is that not all UA formed in vivo, in different cells and different cell compartments is equally labeled at carbons 2 and 8. This can only occur in the presence of saturating pools of formyltetrahydrofolate, which are not necessarily distributed in all tissues, and can be found only in some cells and cell districts, possibly only in liver cells. Only this uric acid is transformed into allantoin. It does not accumulate, but is attacked very rapidly by liver peroxysomes, producing allantoin, equally labeled at Carbons 2 and 8*.

Another pool of UA, present in liver, or derived from other tissues, is labeled at C_2, it is not attacked by liver peroxysomal uricase, and is eliminated as such in urine.

Another interpretation of our results could be that in vivo most UA is labeled prevalently at Carbon 2, but the action of peroxysomal uricase, is different from that in our experimental preparations. The structure, linkages and organization of the enzyme in cells are different from those of free detached peroxysomes and have a different mechanism of action. They trigger a very fast tautomerization, producing both tautomers in equivalent amounts. This mean that labeling of Carbon 2 and Carbon 8 in the ALL formed.

To confirm our hypotheses additional experiments should be performed, also including the use of rat recombinant enzyme.

REFERENCES

1. Carlucci, F.; Rosi, F.; Di Pietro, M.C.; Marinello, E.; Pizzichini, M.; Tabucchi, A. Purine nucleotide metabolism: specific aspects in chronic lymphocytic leukemia lymphocytes. *Biochim. Biophys. Acta* **1997**, 1360, 203–210.
2. Carlucci, F.; Tabucchi, A.; Pagani, R.; Marinello, E. Synthesis of adenine and guanine nucleotides at the ''inosinic branch point'' in lymphocytes of leukemia patients. *Biochim. Biophys. Acta* **1999**, 1454, 106–114.
3. Christjanson, L.J.; Middlemiss, P.J.; Rathbone, M.P. Stimulation of astrocyte proliferation by purine and pyrimidine nucleotides and nucleosides. *Glia* **1993**, 7, 176–182.
4. Hershfield, M.S.; Mitchell, B.S. Immunodeficiency diseases caused by adenosine deaminase deficiency and purine nucleoside phosphorylase deficiency, in *The Metabolic and Molecular Basis of Inherited Disease*, eds. C.R. Scriver, A.L. Beauded, W.S. Sly, D. Valle, McGraw-Hill Inc, New York. 1995, pp. 1725–1768.
5. Moriwaki, Y.; Yamamoto, T.; Higashino, K. Enzymes involved in purine metabolism—A review of histochemical localization and functional implications. *Histology and Histopathology* **1999**, 14, 1321–1340.
6. Zimmermann, H. 5′-nucleotidase: molecular structure and functional aspects. *Biochem. J.* **1992**, 285, 345–365.
7. Zimmermann, H. Biochemistry, localization and functional roles of ecto-nucleotidases in the nervous system. *Prog. Neurobiol.* **1996**, 49, 589–618.
8. Zoref-Shani, E.; Kessler-Iceksonm, G.; Wassermanm, L.; Sperling, O. Characterization of purine nucleotide metabolism in primary rat cardiomyocyte cultures. *Biochim. Biophys. Acta* **1984**, 804, 161–168.
9. Pizzichini, M.; Pandolfi, M.L.; Arezzini, L.; Terzuoli, L.; Fè, L.; Bontemps, F.; Van Den Berghe, G.; Marinello, E. Labelling of uric acid and allantoin in different purine organs and urine of rat. *Life Sci.* 1996, 59, 893–899.

10. Canellakis, E.S.; Cohen, P.P. The end-products and intermediates of uric acid oxidation by uricase. *J. Biol. Chem.* **1955**, 213, 385–395.
11. Mahler, H.R. Uricase, in *The enzymes* eds. P. Boyer, H. Lardy, and K. Myrback, Academic Press, New York, 1963. 3rd ed., vol. 8, pp. 285–296.
12. Pitts, O.M.; Priest, D.G. Uricase reaction intermediate. Mechanism of borate and hydroxide ion catalysis. *Biochemistry* **1973**, 12, 1358–1363.
13. Bongaerts, G.P.A.; Vogels, G.D. Mechanism of uricase action. *Biochim. Biophys. Acta* **1979**, 567, 295–308.
14. Kahn, K.; Tripton, P.A. Spectroscopic characterization of intermediates in urate oxidase reaction. *Biochemistry* **1998**, 37, 11651–11659.
15. Busi, E.; Sinicropi, A.; Terzuoli, L.; Marinello, E.; Basosi, R. Identification and structural characterization of a transient radical species in the uricase reaction mechanism. *Appl. Magn. Reson.* **2007**, 31, 471–482.
16. Gabison, L.; Chiadmi, M.; Colloc'h, N.; Castro, B.; El Hajji, M.; Pragé, T. Recapture of [S]-allantoin, the product of the two-step degradation of uric acid, by urate oxidase. *FEBS Lett.* **2006**, 580, 2087–2091.
17. Di Stefano, A.; Pizzichini, M.; Leoncini, R.; Vannoni, D.; Pagani, R.; Marinello, E. Quantitative separation of uric acid and allantoin from rat liver tissue. *Biochim. Biophys. Acta* **1992**, 1117, 1–6.
18. Praetorius, E.; Poulsen, H. Enzymatic determination of uric acid. *Scand. J. Clin. Lab. Invest.*, **1953**, 5, 273–280.
19. Young, E.G.; Conway, C.F. On the estimation of allantoin by the Rimini-Schryver reaction. *J. Biol. Chem.* **1942**, 142, 839.
20. Terzuoli, L.; Pizzichini, M.; Arezzini, L.; Pandolfi, M.L.; Marinello, E.; Pagani, R. Separation and determination of allantoin, uric acid, hydantoin and urea. *J. Chromatogr. A* 1994, 684, 350–353.
21. Fawcett, J.K.; Scott, J.E. A rapid and precise method for the determination of urea. *J. Clin. Pathol.* **1960**, 13, 156–159.
22. Hagiwara, T.; Watanabe, Y. Determination of allantoin in *Discoreae Rhizoma* by capillary gas chromatography as tert-butyldimethyl silyl derivatives. *Annual Reports Tokyo Metr Research Laboratory* **1992**, 43, 51–55.