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Uracil salvage pathway in PC12 cells

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Abstract

The salvage anabolism of uracil to pyrimidine ribonucleosides and ribonucleotides was investigated in PC12 cells. Pyrimidine base phosphoribosyl transferase is absent in PC12 cells. As a consequence any uracil or cytosine salvage must be a 5-phosphoribosyl 1-pyrophosphate-independent process. When PC12 cell extracts were incubated with ribose 1-phosphate, ATP and uracil they can readily catalyze the synthesis of uracil nucleotides, through a salvage pathway in which the ribose moiety of ribose 1-phosphate is transferred to uracil via uridine phosphorylase (acting anabolically), with subsequent uridine phosphorylation. This pathway is similar to that previously described by us in rat liver and brain extracts (Cappiello et al., Biochim. Biophys. Acta 1425 (1998) 273; Mascia et al., Biochim. Biophys. Acta 1472 (1999) 93). We show using intact PC12 cells that they can readily take up uracil from the external medium. The analysis of intracellular metabolites reveals that uracil taken up is salvaged into uracil nucleotides, with uridine as an intermediate. We propose that the ribose 1-phosphate-dependent uracil salvage shown by our in vitro studies, using tissues or cellular extracts, might also be operative in intact cells. Our results must be taken into consideration for the comprehension of novel chemotherapeutics' influence on pyrimidine neuronal metabolism.

Keywords: α-D-Ribose 1-phosphate; Pyrimidine salvage pathway; PC12 cell

1. Introduction

A great amount of information is available in the literature about the involvement of nucleotides and nucleosides in various brain functions. In the central, peripheral and enteric nervous system, extracellular purines have well-documented functions as neurotransmitters and neuromodulators [1–4]. Pyrimidine metabolism is not so well characterized. It is known that pyrimidine nucleotides have specific pyrimidinoceptors [5,6] even in cells of brain origin [7], but their function in the nervous system is still unknown.

In addition to their roles in neuromodulation and in neurotransmission [8–11], growing evidence suggests that nucleotides and nucleosides might also act as trophic factors in both the central and the peripheral nervous system. Extracellular purines and to a lesser extent pyrimidines can act as trophic factors with potential to regulate neuronal development, proliferation and apoptosis of glial and brain capillary endothelial cells, neural plasticity and the response of the nervous system to disease processes [12,13].

Purine and pyrimidine metabolism plays a crucial role in mature central nervous system, which lacks de novo synthesis [14]. Several of the inborn errors in purine and pyrimidine metabolism [15] are associated with neurological abnormalities. Moreover, during the past years the neurological side effects of immunosuppressants [16], anti-tumor [17] and anti-viral agents [18] highlighted the importance of studying purine and pyrimidine metabolism in brain.

We recently observed in in vitro conditions, using rat brain and liver extracts, that ribose 1-phosphate (Rib1-P) can play a key role in the process of uracil salvage [19] and of purine salvage [20]. Rib1-P, stemming from purine nucleoside phosphorolysis, can be used for the salvage of uracil to uracil nucleotides. The salvage process occurs even in the presence of excess inorganic phosphate suggesting that uridine phosphorylase, generally thought to catalyze nucleoside phosphorolysis rather than base ribosylation, might function also as an anabolic enzyme [21].

Abbreviations: PRPP, 5-phosphoribosyl 1-pyrophosphate; Rib-1P, α-D-ribose 1-phosphate

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In order to verify if the pathway is operative in intact cultured cells, which express neuronal properties, tumor cells originated from rat adrenal pheochromocytoma (PC12) [22] were used. Up to now, only a limited amount of information is available in the literature on nucleotide metabolism in cells derived from the neural crest. In this paper we clarify the uracil salvage pathway in PC12 cells. This could contribute to the comprehension of novel chemotherapeutics' influence on pyrimidine neuronal metabolism.

2. Materials and methods

[2-^{14}C]Uracil (50 mCi/mmol), [2-^{14}C]uridine (57 mCi/mmol), [2-^{14}C]cytosine (51 mCi/mmol), [2-^{14}C]cytidine (53.8 mCi/mmol), [2-^{14}C]thymidine (47.7 mCi/mmol), [8-^{14}C]adenine (51 mCi/mmol), [8-^{14}C]hypoxanthine (51.9 mCi/mmol), bases, nucleosides, nucleotides, RPMI 1640, L-glutamine, horse and fetal serum were from Sigma Chemical Co. Scintillation liquid HiSafeII was purchased from Wallac. Polyethyleneimine (PEI)-cellulose-precoated thin-layer plastic sheets (0.1 mm thick) were obtained from Merck and prewashed once with 10% NaCl and three times with deionized water before use. DE-81 chromatographic paper was from Whatman. All other chemicals were of reagent grade.

2.1. Cell line

Rat pheochromocytoma PC12 cells [16] were cultured in RPMI 1640, supplemented with 300 mg/l of L-glutamine, 5% fetal bovine serum, 10% horse serum heat-inactivated, penicillin (100 U/ml), streptomycin (100 mg/ml) and 30% conditioned medium to facilitate the propagation. The cells were incubated in plastic dishes at 37°C in humidified (96%) air containing 5% CO_2. The medium was changed every 3 days and cells were passed once a week. The number of cells was quantified with a hemocytometer.

2.2. Preparation of cell extracts

When cells were subconfluent the medium was aspirated and cells were washed twice with phosphate-buffered saline (PBS), scraped, collected and centrifuged at $8000 \times g$ for 3 min and then stored at −80°C until use. Cell pellets were resuspended in 2 vol of 50 mM Tris–HCl buffer (pH 7.4), subjected to ultrasonic treatment (four cycles, each of 10 s) and then centrifuged at $39\,000 \times g$ for 1 h at 4°C. The supernatant, referred to as cell extract, was kept at −80°C until use.

2.3. Standard incubation procedures

Cell extract, containing 20–50 μg of protein, was incubated in a total reaction volume of 30 μl containing 5 mM Tris–HCl buffer (pH 7.4), 8 mM $MgCl_2$, 0.98 mM [2-^{14}C]uracil (18 000 dpm/nmol), 3.6 mM ATP and 3 mM Rib-1P. Modifications of the standard incubating procedure are indicated in the figure and table legends. The reaction was started by addition of the cell extract. At different time intervals the reaction was stopped by rapidly drying portions of 5 μl of the incubation mixture on PEI-cellulose-precoated thin-layer plastic sheets and a chromatogram was developed in *n*-butanol/glacial acetic acid/water (4:2:1 v/v). In all separations appropriate standards were used and detected as ultraviolet absorbing areas, which were excised and counted for radioactivity with 8 ml of scintillation liquid.

2.4. Incorporation of uracil

Before starting the experiments of uracil incorporation the number of cells was quantified. Then the culture medium was removed, cells were washed twice with PBS and a serum-free medium was replaced. PC12 cells were incubated in triplicate with [2-^{14}C]uracil (15 000 dpm/nmol). After incubations the cells were washed twice with 0.9% NaCl, then 500 μl of 0.5 M TCA were added to each plate and the cells were scraped off. Cells were heated at 100°C for 5 min and then counted for radioactivity.

2.5. Analysis of intracellular metabolites

PC12 cells were incubated in triplicate with [2-^{14}C]uracil (28 000 dpm/nmol) in serum-free medium at different time intervals. After incubations the cells were washed twice with 0.9% NaCl and then 500 μl of 60% cold methanol were added to each plate, then cells were scraped off and subjected to ultrasonic treatment (three cycles each of 15 s). Cellular lysates were centrifuged in a Beckman Microfuge at $12\,000 \times g$ for 2 min. The supernatants, about 600 μl, were dried with a Speed-Vac evaporator and the resuspended in 20 μl of MilliQ water, which were all spotted on PEI-cellulose plates. Uridine, uracil and uracil nucleotides were separated as described in Section 2.3.

2.6. Purine and pyrimidine salvage enzymes

The following enzyme activities were assayed at 37°C in PC12 cell extracts. Hypoxanthine–guanine phosphoribosyltransferase, adenine phosphoribosyltransferase, uracil phosphoribosyltransferase, uridine phosphorylase, uridine kinase, cytidine kinase, thymidine kinase cytidine phosphorylase were assayed radiochemically as follows. The reaction mixtures for purine and pyrimidine bases phosphoribosyltransferase contained 5 mM Tris–HCl buffer (pH 7.4), 8 mM $MgCl_2$, 3 mM PRPP and 1 mM each of [8-^{14}C]hypoxanthine (8500 dpm/nmol), [8-^{14}C]adenine (8500 dpm/nmol), [2-^{14}C]uracil (18 000 dpm/nmol), or [2-^{14}C]cytosine (15 000 dpm/nmol), 20–50 μg of protein in a final volume of 60 μl. The reaction mixtures of py-

rimidine nucleosides kinase contained 5 mM Tris–HCl buffer (pH 7.4), 11 mM $MgCl_2$, 10 mM ATP and 1 mM each of [2-^{14}C]uridine (8000 dpm/nmol), [2-^{14}C]cytidine (23 000 dpm/nmol), or [2-^{14}C]thymidine (11 000 dpm/nmol), 20–50 μg of protein in a final volume of 60 μl. Portions of the incubations mixtures were withdrawn at appropriate times and immediately spotted on DE-81 paper disks. After one washing of 15 min in 1 mM ammonium formate and two washings in water (10 min each), the disks were dried and counted for radioactivity with 8 ml of scintillation liquid. The composition of the reaction mixture for uridine phosphorylase was the same as the standard incubation mixture except that ATP was omitted and the separation and detection of the reaction products were obtained as described in Section 2.3.

2.7. Other methods

Protein concentration was determined by the Coomassie Blue Binding assay [23], using bovine serum albumin as standard.

3. Results and discussion

3.1. PRPP-Dependent salvage of purine and pyrimidine in PC12 cell extracts

In PC12 cells, which are tumor cells originated from rat adrenal pheochromocytoma, salvage pathways are of great importance. Other authors [24] demonstrated that in PC12 cells a high flux through the pyrimidine salvage pathways exists compared to a low flux through the de novo pathways, as measured in situ in the cells. This result is also in agreement with results obtained for rat brain and cat brain cells, which possess a low-activity de novo pathway and which may have a special requirement for the salvage of pyrimidine. In this context we studied PRPP-dependent salvage of purine and pyrimidine in PC12 cell extracts. Fig. 1 shows the time course of labeled ribonucleotide formation when PC12 cell extract was incubated in the presence of labeled purine and pyrimidine bases and PRPP. No nucleotide formation was observed when PC12 cell extract was incubated with uracil or cytosine, while hypoxanthine and adenine salvage is a PRPP-dependent process. These observations are in line with our previous results obtained with rat brain extracts [19] even if in PC12 cells the PRPP-dependent salvage for adenine and hypoxanthine is higher by far than in rat brain. As a consequence any uracil conversion into uracil nucleotides must follow PRPP-independent salvage routes.

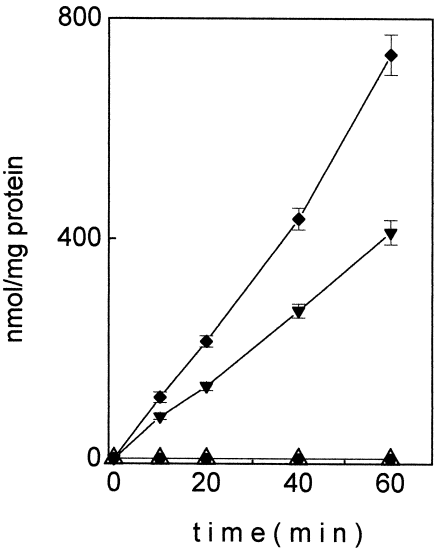


Fig. 1. Time course of PRPP-dependent salvage of purine and pyrimidine bases catalyzed by PC12 cell extract. The incubation mixture contained, in a final volume of 60 μl, 25–50 μg of protein, 500 nmol of $MgCl_2$, 80 nmol of PRPP and 60 nmol of [8-^{14}C]adenine (8500 dpm/nmol) (♦), [8-^{14}C]hypoxanthine (8500 dpm/nmol) (▼), [2-^{14}C]uracil (18 000 dpm/nmol) (●), or [2-^{14}C]cytosine (15 000 dpm/nmol) (△) in 50 mM Tris–HCl buffer, pH 7.4. Points represent mean ± S.D. of triplicate determinations.

Table 1
Specific activities of purine and pyrimidine salvage enzymes in PC12 cell extract[a]

Enzyme	PC12 cell extract
Thymidine kinase	0.06
Uridine kinase	0.86
Cytidine kinase	0.08
Adenine phosphoribosyltransferase	11.47
Uracil phosphoribosyltransferase	0
Cytidine phosphoribosyltransferase	0
Hypoxanthine–guanine phosphoribosyltransferase	6.0
Uridine phosphorylase	0.81

[a]The enzyme activities are expressed as nmol min^{-1} mg^{-1} of protein.

In Table 1 the enzyme pattern of purine and pyrimidine salvage is reported.

3.2. Rib-1P-, inosine- and guanosine-dependent salvage of [2-^{14}C]uracil in PC12 cell extracts

Fig. 2A shows the time-dependent formation of labeled uridine and of uracil nucleotides when PC12 cell extract was incubated in the presence of [2-^{14}C]uracil, Rib-1P and ATP. Fig. 2B and C show similar time courses of [2-^{14}C]-uridine and [2-^{14}C]uracil nucleotide formations when Rib-1P was substituted with inorganic phosphate and inosine or guanosine, respectively, as Rib-1P donor. From Fig. 2D it is evident that ATP is required for uracil conversion into the corresponding nucleotides: in the absence of the phosphate donor uridine accumulates, without any uracil nucleotide formation, suggesting a precursor–product relationship between uridine and uracil nucleotides. These results show that as in rat brain extracts [19], also in PC12 extracts Rib-1P is a key compound in the process of uracil salvage. Rib-1P, stemming from the phosphorolysis of inosine and guanosine, catalyzed by purine nucleo-

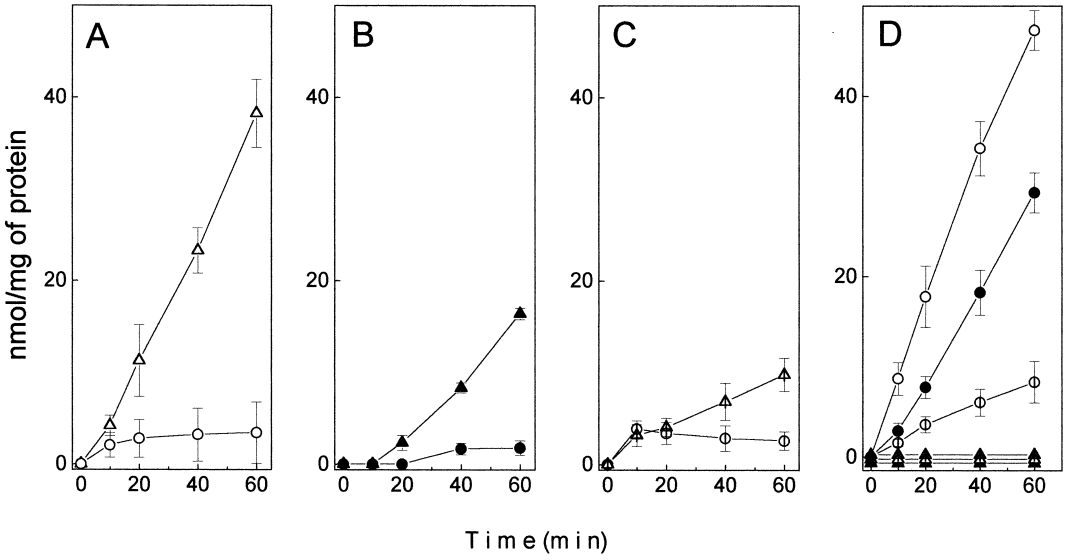
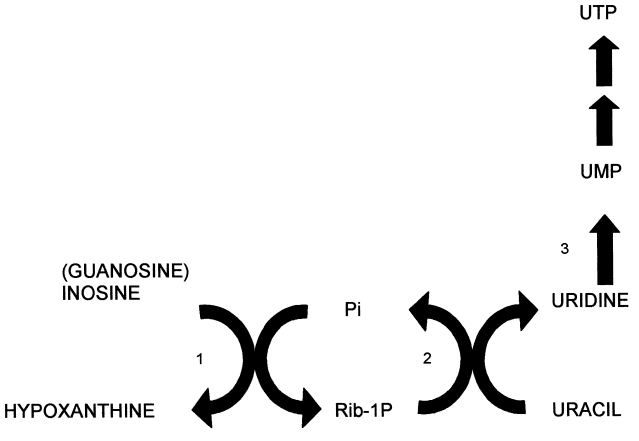


Fig. 2. Time course of Rib-1P-dependent uracil salvage synthesis catalyzed by PC12 cell extracts. (A) The incubation mixture contained, in a final volume of 30 μl, 25–50 μg of protein, 250 nmol of $MgCl_2$, 90 nmol of Rib-1P, 108 nmol of ATP and 30 nmol of [2-^{14}C]uracil (18 000 dpm/nmol), in 50 mM Tris–HCl buffer, pH 7.4. ○, uridine; △, uracil nucleotides. (B) Rib-1P was substituted with 200 nmol of inosine and 150 nmol of inorganic phosphate. ●, uridine; ▲, uracil nucleotides. (C) Rib-1P was substituted with 200 nmol of guanosine and 150 nmol of inorganic phosphate. I-center circles, uridine; I-center triangles, uracil nucleotides. (D) ATP was omitted. Symbols are those of A–C. Points represent mean ± S.D. of triplicate determinations.

side phosphorylase, is transferred to uracil to synthesize uridine, in the reaction catalyzed by uridine phosphorylase. Uridine is then converted to uracil nucleotides by multiple phosphorylation steps, catalyzed by appropriate kinase (Scheme 1). Compared to rat brain extracts only a limited intermediate uridine formation could be observed, most likely due to the higher uridine kinase activity of PC12 cell extracts with respect to rat brain extracts [19]. Fig. 3 shows that the presence of inorganic phosphate at 5 mM does not hinder uracil nucleotides and uridine formation; this result appears more remarkable if one considers that the relevant concentration of inorganic phosphate for the salvage pathways is that of cytosol and that its value is even lower than 5 mM [25]. The pathway described here could have a role during hypoxia or ischemia, when the accelerated catabolism of ATP produces AMP, IMP, adenosine and inosine in brain [26] as well as in cultured glia and neurons [27]. Recently Jurkowitz et al. [28] have shown that inosine, guanosine and adenosine



Scheme 1. Uracil salvage in PC12 cells. 1, purine nucleoside phosphorylase; 2, uridine phosphorylase; 3, uridine kinase.

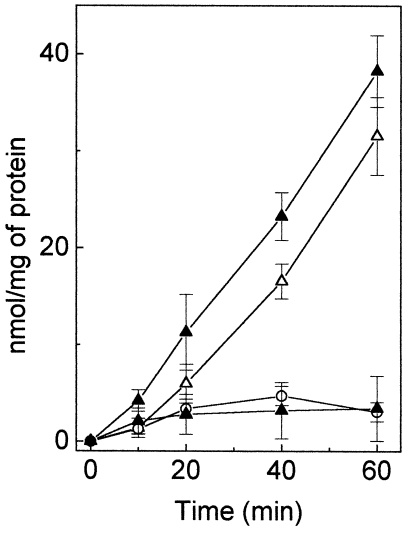


Fig. 3. Effect of inorganic phosphate on Rib-1P-dependent uracil salvage catalyzed by PC12 cell extracts. The incubation mixture contained, in a final volume of 30 μl, 25–50 μg of protein, 250 nmol of $MgCl_2$, 90 nmol of Rib-1P, 108 nmol of ATP, and 30 nmol of [2-^{14}C]uracil (18 000 dpm/nmol) either in the absence (closed symbols) or in the presence (open symbols) of 150 nmol of KH_2PO_4, in 50 mM Tris–HCl buffer, pH 7.4. ●,○, uridine; ▲,△, uracil nucleotides. Points represent means ± S.D. of triplicate determinations.

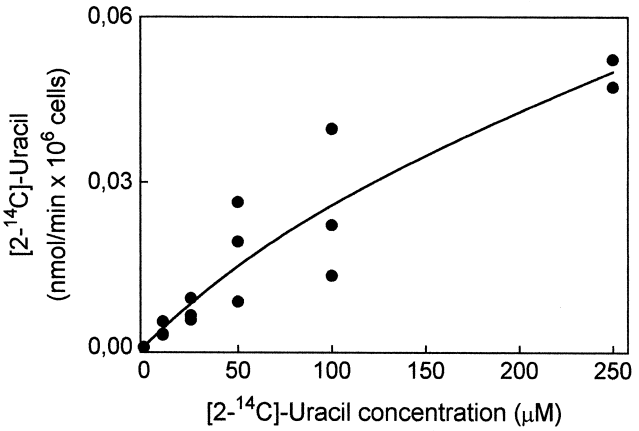


Fig. 4. Incorporation of [2-^{14}C]uracil at different concentrations (10, 25, 50, 100, 250 μM). PC12 intact cells were incubated in triplicate for 3 min with the indicated [2-^{14}C]uracil concentrations as described in Section 2.

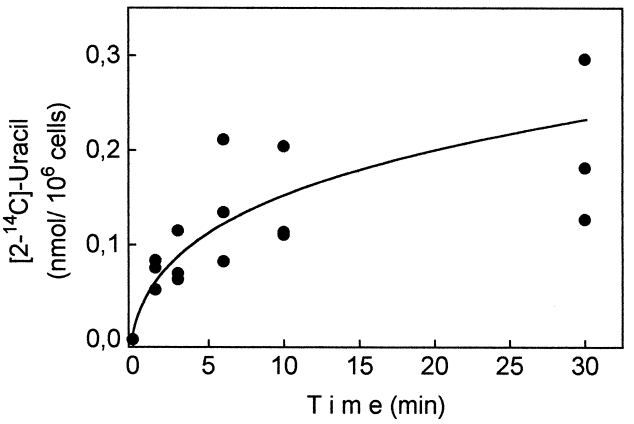


Fig. 5. [2-^{14}C]Uracil incorporation into PC12 cells at different time intervals. PC12 intact cells were incubated in triplicate with 200 μM [2-^{14}C]uracil at different time intervals as described in Section 2.

exert a protective effect on glial cell viability. The molecular mechanism involves the conversion of the ribose moiety of inosine and guanosine to phosphorylated glycolytic intermediates, via the pentose phosphate pathway and its subsequent catabolism in glycolysis, thus providing the ATP necessary for maintaining the plasmalemmal integrity.

These results suggest that as in rat brain [21], also in PC12 cells pyrimidine nucleoside phosphorylase has an anabolic rather than catabolic role. We propose that this result may reflect the need of nervous derived cells for specific activated pyrimidine nucleotides for the biosynthesis of phospholipid, continuously required for myelin production [29] or the production of derived compounds which have extracellularly roles as neuromodulators, neurotransmitters and as trophic factors [13]. In this aspect PC12 cells differ completely from other cultured cells such as lymphoid cells [30] and Novikoff hepatoma cells [31], which do not salvage pyrimidine bases and in which pyrimidine nucleoside phosphorylases, when they are present, may rather have a catabolic role.

3.3. Uracil incorporation into PC12 intact cells

The incubations with PC12 cells show that the uracil salvage pathway proposed can in fact work in cultivated cells. Figs. 4 and 5 show the effect of increasing externally added uracil concentration on the amount of uracil taken up and the time-dependent uracil uptake by intact PC12 cells, respectively. The results show that uracil can be transported by PC12 cells probably via an apparent facilitated mechanism. Uracil taken up by intact cells is in fact converted into uracil nucleotides, with uridine as an intermediate as just seen with PC12 cell extracts. The amount of uracil metabolites formed intracellularly after 30 min and 60 min incubation is reported in Table 2.

In conclusion, our results present clear evidence that intact PC12 cells are capable of taking up uracil from the external medium and of salvaging uracil via a pathway that depends on Rib-1P. Taken together with our previous results obtained with rat brain and liver extracts, the results here obtained with PC12 cells suggest that Rib-1P-dependent uracil salvage might be operative in intact cells.

Our results might be useful in understanding the process that regulates the intracellular nucleobase and nucleoside homeostasis, and will enhance our ability to manipulate salvage pathways for chemotherapy and signaling pathways for pharmacological effects [32–34]. Moreover, the uracil salvage pathway described here should be considered in studies on the side effects of 5-fluorouracil treatment, which have been sometimes described at the level of the central nervous system [35].

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Table 2
PC12 intracellular [2-^{14}C]uracil metabolites formed intracellularly after 30 or 60 min incubation

[2-^{14}C]Uracil intracellular metabolites	nmol/10^6 cells (after 30 min incubation)	nmol/10^6 cells (after 60 min incubation)
[2-^{14}C]Uridine	0.029 ± 0.006	0.026 ± 0.004
[2-^{14}C]Uracil nucleotides	0.034 ± 0.013	0.141 ± 0.002

Each incubation was repeated three times. Values are mean ± S.D.

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