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Purine and Pyrimidine Salvage in Whole Rat Brain

UTILIZATION OF ATP-DERIVED RIBOSE-1-PHOSPHATE AND 5-PHOSPHORIBOSYL-1-PYROPHOSPHATE GENERATED IN EXPERIMENTS WITH DIALYZED CELL-FREE EXTRACTS*

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The object of this work stems from our previous studies on the mechanisms responsible of ribose-1-phosphate- and 5-phosphoribosyl-1-pyrophosphate-mediated nucleobase salvage and 5-fluorouracil activation in rat brain (Mascia, L., Cappiello M., Cherri, S., and Ipata, P. L. (2000) *Biochim. Biophys. Acta* 1474, 70–74; Mascia, L., Cotrufo, T., Cappiello, M., and Ipata, P. L. (1999) *Biochim. Biophys. Acta* 1472, 93–98). Here we show that when ATP at "physiological concentration" is added to dialyzed extracts of rat brain in the presence of natural nucleobases or 5-fluorouracil, adenine-, hypoxanthine-, guanine-, uracil-, and 5-fluorouracil-ribonucleotides are synthesized. The molecular mechanism of this peculiar nucleotide synthesis relies on the capacity of rat brain to salvage purine and pyrimidine bases by deriving ribose-1-phosphate and 5-phosphoribosyl-1-pyrophosphate from ATP even in the absence of added pentose or pentose phosphates. The levels of the two sugar phosphates formed are compatible with those of synthesized nucleotides. We propose that the ATP-mediated 5-phosphoribosyl-1-pyrophosphate synthesis occurs through the action of purine nucleoside phosphorylase, phosphopentomutase, and 5-phosphoribosyl-1-pyrophosphate synthetase. Furthering our previous observations on the effect of ATP in the 5-phosphoribosyl-1-pyrophosphate-mediated 5-fluorouracil activation in rat liver (Mascia, L., and Ipata, P. L. (2001) *Biochem. Pharmacol.* 62, 213–218), we now show that the ratio [5-phosphoribosyl-1-pyrophosphate]/[ATP] plays a major role in modulating adenine salvage in rat brain. On the basis of our *in vitro* results, we suggest that massive ATP degradation, as it occurs in brain during ischemia, might lead to an increase of the intracellular 5-phosphoribosyl-1-pyrophosphate and ribose-1-phosphate pools, to be utilized for nucleotide resynthesis during reperfusion.

It is well established that inosine and other ribonucleosides are transported into mammalian cells (1, 2) where they modulate the processes of nucleobase salvage and 5-fluorouracil (5-FUra)[1] activation (3–8). The molecular mechanism relies on the enhancement of the cytosolic ribose-1-phosphate (Rib1-P) level, stemming from nucleoside phosphorolysis and the subsequent synthesis of 5-phosphoribosyl-1-pyrophosphate (PRPP). Alternatively, Rib1-P may be converted into phosphorylated glycolytic intermediates via the pentose pathway (9). In previous papers, we have followed the time course of purine and pyrimidine ribonucleotides and 5-FUra-ribonucleotides biosynthesis in extracts of rat brain incubated with ATP and a nucleobase in the presence of Rib1-P or a Rib1-P-generating system such as inosine and P_i (10–13). During these studies we became aware that, after prolonged incubation, a small, but definite amount of nucleobases could be salvaged into their respective ribonucleotides even in the absence of Rib1-P or inosine (14). A related observation was the marked effect exerted by ATP in modulating the PRPP-mediated 5-FUra activation to cytotoxic 5-FUra-nucleotides (12) as catalyzed by rat liver orotate phosphoribosyltransferase (the PRPP pathway) (14, 15). Taken together, these observations prompted us to undertake a study on the capacity of rat brain to salvage purine and pyrimidine bases by deriving Rib1-P and PRPP from ATP and to ascertain whether the salvage of purine bases might be modulated by ATP. In this paper, we show that: (i) massive ATP breakdown *per se*, as it probably occurs in rat brain during ischemia (16), may provide at least part of the Rib 1-P and of the PRPP needed to salvage uracil and purine bases, respectively; (ii) the [PRPP]/[ATP] ratio modulates the process of adenine salvage in rat brain extracts. We recall that in rat brain, which does not possess the *de novo* nucleotide synthesis, purine and pyrimidine salvage is particularly active (3, 13, 17).

EXPERIMENTAL PROCEDURES

Materials—[8-^{14}C]Adenine (55 mCi/mmol), [8-^{14}C]hypoxanthine (54 mCi/mmol), [8-^{14}C]guanine (53.3 mCi/mmol), [2-^{14}C]cytosine (51 mCi/mmol), [2-^{14}C]uracil (54 mCi/mmol), [2-^{14}C]5-FUra (55 mCi/mmol), adenosine deaminase, hypoxanthine-guanine phosphoribosyltransferase (HGPRT), PRPP, dithiothreitol, nucleobases, nucleosides, and nucleotides were from Sigma. Adenosine deaminase and HGPRT were diluted with 5 mM Tris-HCl buffer before use to contain 0.63 units/ml and 10 units/ml, respectively. *Bacillus cereus* adenosine phosphorylase (0.14 units/ml) was prepared as described previously (18). Hi Safe II scintillation liquid was purchased from Wallac. Polyethyleneimine (PEI)-cellulose precoated thin-layer plastic sheets (0.1-mm thick) were purchased from Merck and prewashed once with 10% NaCl and three times with deionized water before use. All other chemicals were of reagent grade. Three-month-old male Sprague-Dawley rats were killed according to the "Guiding Principles in the Care and Use of Animals" (DHWEW, publication no. NIH 86-23). The brain was removed and kept frozen at −80 °C until needed. Storage time did not exceed 2 months.

Preparation of Rat Brain Extracts—Rat brain was cut in small

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[1] The abbreviations used are: 5-FUra, 5-fluorouracil; Rib1-P, ribose-1-phosphate; PRPP, 5-phosphoribosyl-1-pyrophosphate; HGPRT, hypoxanthine-guanine phosphoribosyltransferase; PEI, polyethyleneimine.

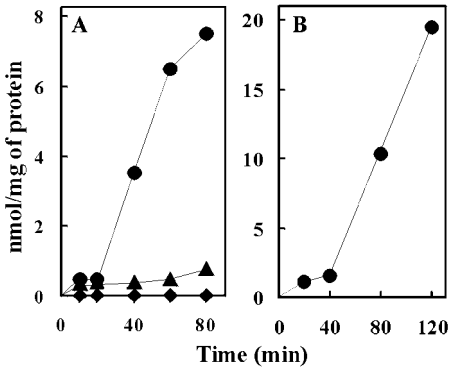

FIG. 1. **Time courses of ATP-mediated adenine salvage (*A*) and PRPP formation (*B*) catalyzed by rat brain crude extracts.** *A*, the incubation mixture contained, in a final volume of 70 μl, 100–150 μg of protein, 70 nmol of [8-^{14}C]adenine, 252 nmol of ATP, and 581 nmol of $MgCl_2$, in 5 mM Tris-HCl buffer, pH 7.4. ♦, adenosine; ▲, AMP; ●, ADP + ATP. *B*, [8-^{14}C]Adenine was omitted in the reaction mixture, and PRPP was determined as described under "Experimental Procedures." ●, PRPP. A bidimensional chromatographic analysis of the reaction mixture incubated for 60 min gave an ATP/ADP ratio of about 9:1.

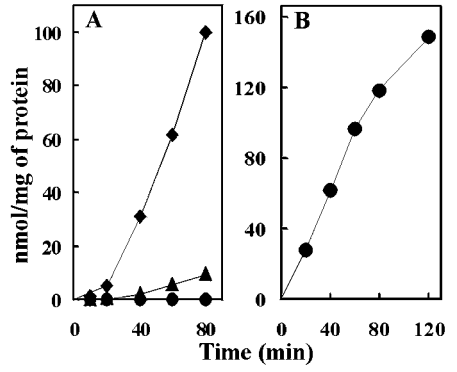

FIG. 2. **Time courses of ATP-mediated hypoxanthine salvage (*A*) and Rib1-P formation (*B*) catalyzed by rat brain crude extracts.** *A*, the incubation mixture was as in Fig. 1 with the exception of [8-^{14}C]hypoxanthine (70 nmol), which substituted [8-^{14}C]adenine. ♦, inosine; ▲, IMP; ●, IDP + ITP. *B*, [8-^{14}C]Hypoxanthine was omitted, and Rib1-P was determined as described under "Experimental Procedures." ●, Rib1-P.

pieces, washed with cold saline, and gently homogenized with a hand-driven Potter homogenizer in 3 volumes of 100 mM Tris-HCl buffer, pH 7.4, with 20 mM KCl and 1 mM dithiothreitol. The homogenate was centrifuged at 4 °C at 40,000 × *g* for 1 h. The supernatant fluid obtained was dialyzed overnight at 4 °C in dialysis bags against 10 mM Tris-HCl buffer, pH 7.4, supplemented with 1 mM dithiothreitol and is referred to as crude extract. The levels of the purine and pyrimidine salvage enzymes present in crude extract are reported in a previous paper (13).

Incubation Procedures—Crude extracts, containing 50–150 μg of protein, were incubated in a total reaction volume of 70 μl containing 5 mM Tris-HCl buffer, pH 7.4, 1 mM [8-^{14}C]adenine (8,000 dpm/nmol), [8-^{14}C]hypoxanthine (8,000 dpm/nmol), [8-^{14}C]guanine (25,000 dpm/nmol), [2-^{14}C]cytosine (10,000 dpm/nmol), [2-^{14}C]uracil (12,000 dpm/nmol), or [2-^{14}C]5-FUra (12,000 dpm/nmol), 3.6 mM ATP, and 8.3 mM $MgCl_2$. Modifications of the standard incubation mixture are indicated in the figure legends. The reaction was started by the addition of crude extract. At different time intervals, the reaction was stopped by rapidly drying portions of 10 μl of the incubation mixture on PEI-cellulose precoated thin-layer plastic sheets, and a chromatogram was developed in *n*-butanol/glacial acetic acid/H_2O (4:2:1, v/v) to separate adenosine and adenine-nucleotides, and cytidine and cytosine-nucleotides, or with *n*-propanol/NH_3/trichloroacetic acid (100%)/H_2O (75:0.7:5:20, v/v) to separate inosine and hypoxanthine-nucleotides, guanosine and guanine-nucleotides, uridine and uracil-nucleotides, and 5-fluorouridine and 5-FUra-nucleotides. 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.

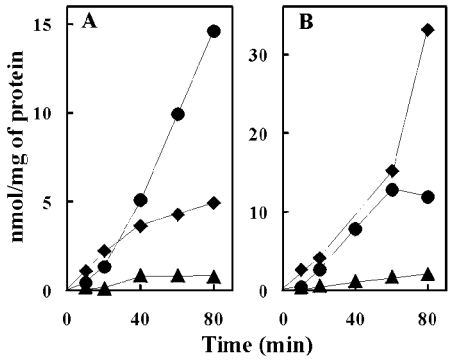

FIG. 3. **Time courses of ATP-mediated uracil salvage (*A*) and 5-FUra activation (*B*) catalyzed by rat brain crude extracts.** *A*, the incubation mixture was as described in Fig. 1 with the exception of [2-^{14}C]uracil (70 nmol), which substituted [8-^{14}C]adenine. ♦, uridine; ▲, UMP; ●, UDP + UTP. *B*, [2-^{14}C]Uracil was substituted with 70 nmol of [2-^{14}C]5-FUra. ♦, 5-fluorouridine; ▲, 5-fluorouridine 5′-monophosphate; ●, 5-fluorouridine 5′-diphosphate + 5-fluorouridine 5′-triphosphate.

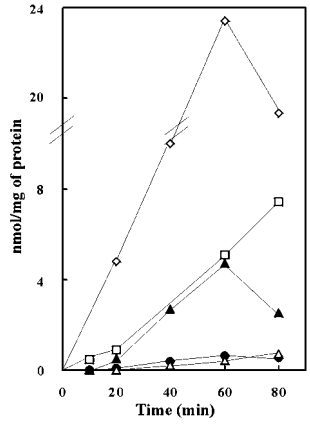

FIG. 4. **Time course of ATP-mediated guanine salvage catalyzed by rat brain crude extracts.** *A*, the incubation mixture was as described in the legend for Fig. 1 with the exception of [8-^{14}C]guanine (70 nmol), which substituted [8-^{14}C]adenine. ▲, GMP; ●, GDP + GTP; □, xanthine; ◇, xanthosine; △, xanthosine 5′-monophosphate.

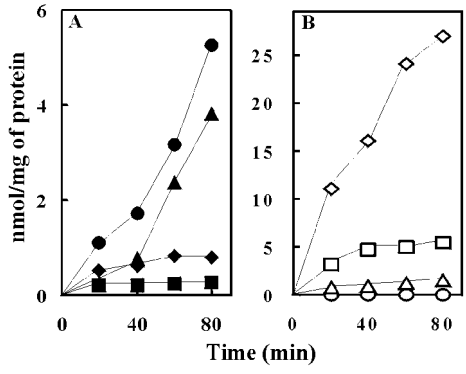

FIG. 5. **Effect of nucleobases on ATP-mediated purine and pyrimidine ribonucleotides (*A*) and ribonucleosides (*B*) synthesis in rat brain crude extracts.** The standard incubation mixture contained, in a final volume of 60 μl, 100–150 μg of protein, 12 nmol each of [8-^{14}C]adenine, [8-^{14}C]hypoxanthine, [8-^{14}C]guanine, [2-^{14}C]cytosine, or [2-^{14}C]uracil, 216 nmol of ATP, 498 nmol of $MgCl_2$ in 5 mM Tris-HCl buffer, pH 7.4, in the presence of a mixture of 12 nmol each of the other four cold nucleobases. *A*, ●, total adenine nucleotides; ▲, total uracil nucleotides; ♦, total hypoxanthine nucleotides; ■, total guanine nucleotides. *B*, ○, adenosine; △, uridine; ◇, inosine; □, xanthosine.

Determination of ATP-derived Rib1-P and PRPP—Crude extracts, containing 1100 μg of protein, were incubated in a total reaction volume of 500 μl in the presence of 3.6 mM ATP, 8.3 $MgCl_2$, 5 mM Tris-HCl

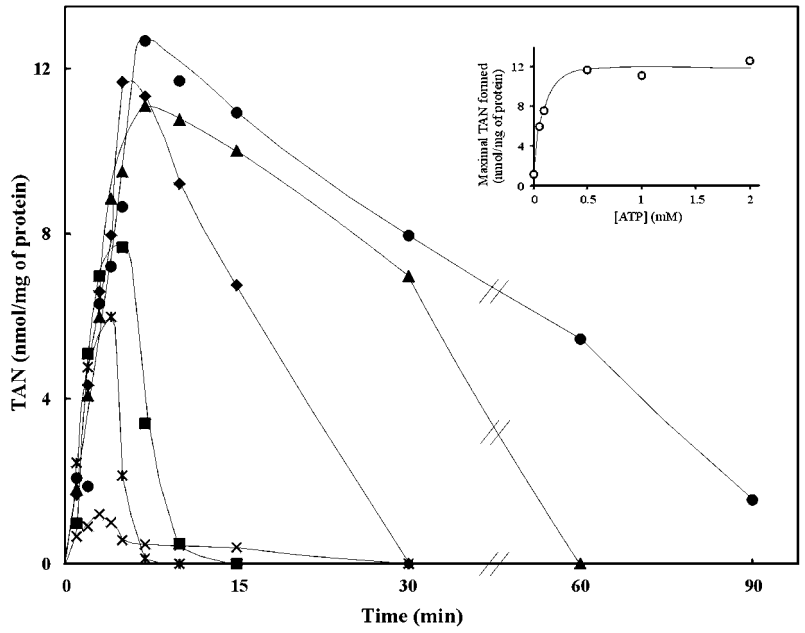

FIG. 6. **Effect of varying the concentration of ATP on adenine salvage at fixed PRPP concentration.** The incubation mixture contained, in a final volume of 60 μl, 100–150 μg of protein, 60 nmol of [8-^{14}C]adenine, 6 nmol of PRPP, 276 nmol of $MgCl_2$, and 120 nmol (●), 60 nmol (▲), 30 nmol (♦), 6 nmol (■), 3 nmol (*), or 0 nmol (×) of ATP and 276 nmol of $MgCl_2$, in 5 mM Tris-HCl buffer, pH 7.4. The *inset* shows the maximal amount of total adenine nucleotides (*TAN*) synthesized as a function of ATP concentration.

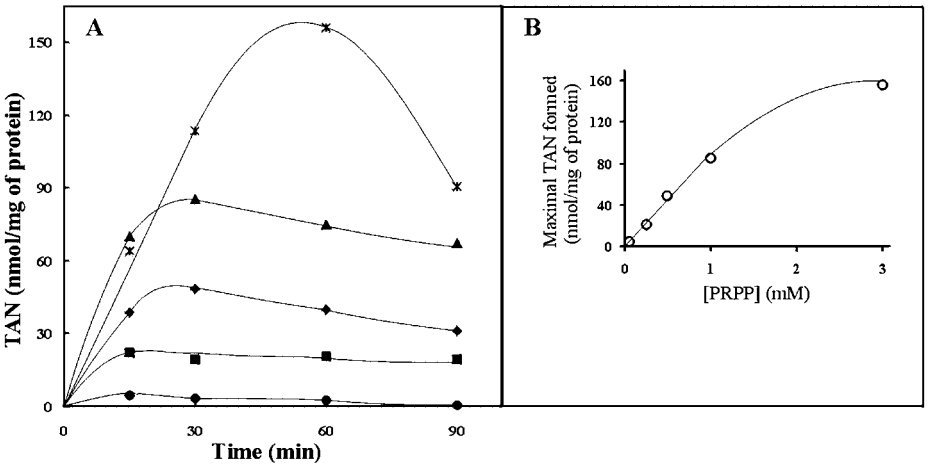

FIG. 7. **Effect of varying the concentration of PRPP on adenine salvage at fixed ATP concentration.** *A*, the incubation mixture contained, in a final volume of 30 μl, 70–80 μg of protein, 30 nmol of [8-^{14}C]adenine, 108 nmol of ATP, 249 nmol of $MgCl_2$, and 90 nmol (*),30 nmol (▲), 15 nmol (♦), 7.5 nmol (■, or 1.5 nmol of PRPP (●) in 5 mM Tris-HCl buffer, pH 7.4. *Panel B* shows the maximal amount of total adenine nucleotides (*TAN*) synthesized as a function of PRPP concentration.

buffer, pH 7.4. The incubation was carried out at 37 °C. At different time intervals, 70-μl portions were withdrawn, heated for 90 s at 100 °C, and centrifuged. The supernatant fluid was used to determine Rib 1-P and PRPP.

Rib1-P was determined according to Ipata and Camici (18). Briefly, 25 μl of the above supernatant fluid was added to 20 μl of 10 mM [8-^{14}C]adenine (8,000 dpm/nmol) in 5 mM Tris-HCl buffer, pH 7.4, 10 μl of diluted commercial adenosine deaminase, and 5 μl of the adenosine phosphorylase preparation. The reaction mixtures were incubated at 37 °C for 2 h. Then, 10-μl portions were applied to a PEI-cellulose precoated thin-layer plastic sheet and developed with deionized water. The marker compounds were detected with a UV lamp. The zones corresponding to inosine were excised and counted for radioactivity with 8 ml of scintillation liquid.

PRPP was determined according to King *et al.* (19), with minor modifications. Briefly, 25 μl of the above supernatant fluid was added to 10 μl of 10 mM [8-^{14}C]hypoxanthine (8,000 dpm/nmol) in 5 mM Tris-HCl buffer, pH 7.4, 2 μl of the diluted HGPRT, 1 μl of 100 mM dithiothreitol, 2 μl of 2 mM $MgCl_2$, and 10 μl of Tris-HCl, pH 7.4. The reaction mixtures were incubated at 37 °C for 10 min. Then 10 μl was applied to a PEI-cellulose precoated thin-layer plastic sheet and developed with 1.4 M LiCl. The marker compounds were detected with a UV lamp. The zones corresponding to IMP were excised and counted for radioactivity with 8 ml of scintillation liquid.

Protein Concentration—Protein concentration was determined by the Coomassie Blue binding assay using bovine serum albumin as standard (20).

RESULTS AND DISCUSSION

It is a widely accepted tenet in neurobiology that under normal conditions intracellular ATP levels are rigorously protected. However, in such events as ischemia or anoxia, massive ATP breakdown occurs. Many reports have demonstrated a substantial recovery of rat brain adenine nucleotides levels following ischemic episodes (16, 21–27). Moreover, a considerable increase in intracellular nucleosides and nucleobases, important precursors of nucleotide resynthesis following reperfusion, has been reported (16, 28). This paper deals with two different, although related, aspects of ATP metabolism in rat brain. First, we have shown that PRPP, in addition to Rib1-P, may be synthesized during massive ATP breakdown and utilized to salvage purine bases (Figs. 1-5). Second, we have shown that the [PRPP]/[ATP] ratio is an important factor in modulating the purine salvage process in rat brain (Figs. 6 and 7). Essentially, the rational of our experimental approach was the following. Because in rat brain adenine, hypoxanthine and guanine salvage are PRPP-dependent processes (3), we reasoned that any ATP-mediated purine ribonucleotide biosynthesis would necessarily imply that PRPP was synthesized during ATP breakdown. Likewise, because uracil salvage and 5-FUra activation are Rib 1-P-dependent processes in rat brain (12,

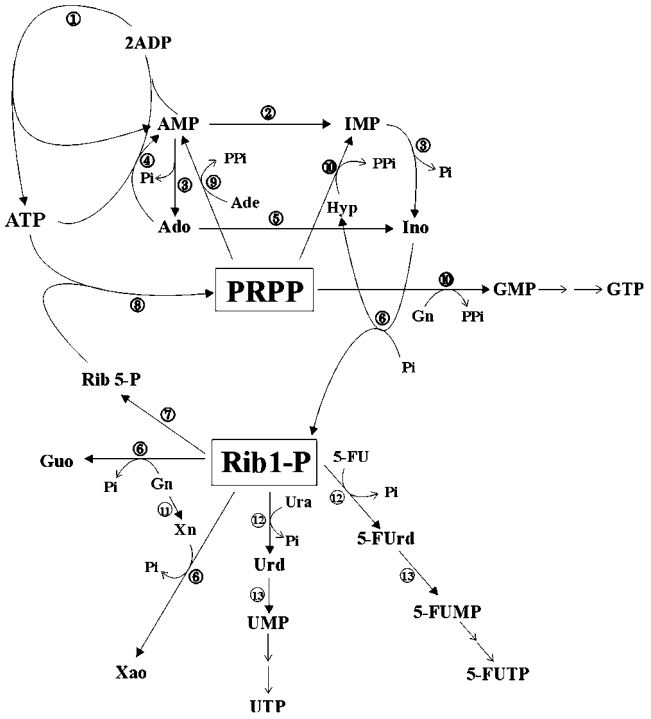

FIG. 8. **Pathways for ATP-mediated Rib1-P and PRPP formation in rat brain extract.** The *upper part* of the scheme is taken from Torrecilla *et al.* (39) (slightly modified). The enzymes participating in these pathways are: *1*, adenylate kinase; *2*, adenylate deaminase; *3*, 5′-nucleotidase; *4*, adenosine kinase; *5*, adenosine deaminase; *6*, purine nucleoside phosphorylase; *7*, phosphopentomutase; *8*, PRPP-synthetase; *9*, adenine-phosphoribosyltransferase; *10*, HGPRT; *11*, guanine deaminase; *12*, uridine phosphorylase; and *13*, uridine kinase. *Ade*, adenine; *Ado*, adenosine; *Hyp*, hypoxanthine; *Ino*, inosine; *Urd*, uridine; *Guo*, guanosine; *Gn*, guanine; *Xn*, xanthine; *Xao*, xanthosine.

13), any ATP-mediated uridine- and 5-FUra-ribonucleotide biosynthesis would necessarily imply that Rib1-P was formed during ATP breakdown. The results will be discussed with the aid of the scheme presented in Fig. 8.

ATP-mediated Adenine Salvage and PRPP Formation—Fig. 1*A* shows the time course of radioactive adenosine and adenine-nucleotide formation as catalyzed by crude extract of rat brain incubated in the presence of [^{14}C]adenine and ATP. A striking observation here was the absence of any [^{14}C]adenosine formation. It is evident that any AMP synthesized by the PRPP-mediated phosphoribosylation of adenine was further phosphorylated rather then being hydrolyzed to adenosine. Fig. 1*B* gives direct evidence of free PRPP formation in incubation carried out in the absence of adenine. The most obvious pathway for PRPP synthesis during ATP breakdown is that composed of purine nucleoside phosphorylase, phosphopentomutase and PRPP synthetase (*reactions 6, 7*, and *8* in Fig. 8). Because the latter enzyme has an absolute requirement of P_i for its activity (29), it is conceivable that some endogenous P_i, as well as the release of P_i from added ATP, might have been responsible for the PRPP synthetase activation. Interestingly, the amount of PRPP synthesized after 80 min of incubation (Fig. 1*B*) was in good correlation with that of total adenine nucleotides present in the reaction mixture after the same incubation time.

ATP-mediated Hypoxanthine Salvage and Rib1-P Formation—Fig. 2*A* shows the time course of [^{14}C]hypoxanthine compounds formed when crude extract of rat brain was incubated in the presence of [^{14}C]hypoxanthine and ATP. Both inosine and IMP were synthesized. The latter, however, was not further phosphorylated. Most likely Rib1-P, formed during ATP breakdown, was utilized to ribosylate hypoxanthine, whereas PRPP was utilized to phosphoribosylate hypoxanthine through the action of purine nucleoside phosphorylase and HGPRT activities present in rat brain, respectively. Because no inosine kinase activity is present in mammalian tissues (30), phosphorylation of inosine can be excluded *a priori*. We cannot exclude the possibility that a small portion of IMP might have arisen by the action of the phosphotransferase activity of cytosolic 5′-nucleotidase (31) as well as that some inosine might have arisen from IMP dephosphorylation. We noticed, however, that the amount of free Rib1-P formed in the absence of added nucleobase (Fig. 2*B*) was in good correlation with that of total inosine synthesized (Fig. 2*A*). A chromatographic analysis of the UV absorbing products of ATP breakdown clearly showed that IMP was formed before adenosine. Thus, IMP was already present in the reaction mixture after 10 min of incubation, whereas adenosine and hypoxanthine appeared after 20 and 60 min, respectively. ATP and ADP completely disappeared only after 2 h of incubation.

ATP-mediated Uracil Salvage and 5-FUra Activation—When crude extract of rat brain was incubated in the presence of either [^{14}C]uracil (Fig. 3*A*) or [^{14}C]5-FUra (Fig. 3*B*) and ATP, uridine and 5-fluorouridine were synthesized as intermediates of uracil- and 5-FUra-ribonucleotides synthesis, respectively (*reactions 12* and *13* in Fig. 8) in accordance with the existence of high uridine phosphorylase and uridine kinase activities in rat brain (13) and with our previous results showing that 5-FUra activation occurs through the Rib1-P-mediated ribosylation of the pyrimidine analog followed by multiple phosphorylation steps ("the Rib1-P pathway") (12).

ATP-mediated Guanine Salvage—Utilization of [^{14}C]guanine during ATP breakdown appears to be rather complex. Fig. 4 shows that a series of radioactive compounds was formed including guanine-nucleotides, xanthine, xanthosine, and XMP. Changes in the specific radioactivities of metabolites to ascertain their order of formation have not been analyzed. Such experiments would be helpful in the future. The appearance of large amounts of xanthine and xanthosine in the course of reaction is indicative of strong guanine deaminase and purine nucleoside phosphorylase activities and of recycling of Rib1-P in the nucleoside interconversion (32). The activity of xanthine oxidase in rat brain is very low (33). Because xanthine is not used as substrate by mammalian HGPRT (34), and we are not aware of the existence of any nucleoside kinase acting on xanthosine, the small amount of XMP formed might be ascribed to the phosphotransferase activity of rat brain cytosolic 5′-nucleotidase (35).

ATP-mediated Cytosine Salvage—Strikingly, cytosine was not utilized at all during ATP breakdown (results not shown), showing that the base is not deaminated to uracil and confirming the absence of cytidine phosphorylase and cytosine phosphoribosyltransferase activities in rat brain (13).

As shown in Fig. 5, the patterns of the ATP-mediated salvage of each radioactive nucleobase is not significantly altered by the presence of all other cold nucleobases.

Modulation of Adenine Salvage by the [PRPP]/[ATP] Ratio—The intracellular ATP level in mammalian tissues is in the mM range (*e.g.* 3.3 mM in rat brain (36)), whereas that of PRPP in mammalian tissues is in the μM range (37). Most likely, the [PRPP]/[ATP] ratio must be maintained at low values to avoid excessive *de novo* or "salvage" purine nucleotide synthesis. And in fact PRPP synthetase superactivity, a rare abnormality in man, leads to purine overproduction, gout, and urolithiasis (38). The results presented in Fig. 6 and 7 suggest that the [PRPP]/[ATP] ratio might modulate the process of adenine salvage. The maximal amount of adenine salvaged at a fixed

initial PRPP concentration was a function of ATP levels (Fig. 6). Moreover, the rate of salvaged adenine nucleotides degradation observed after prolonged incubation increased sharply by decreasing the initial ATP concentration. These observations further suggest that the ATP level might be an important factor in modulating the PRPP-mediated process of purine base salvage. As expected, at fixed "physiological" rat brain ATP concentration (36), the maximal amount of adenine salvaged was a function of initial PRPP levels (Fig. 7).

An artifactual presence of ecto-5′-nucleotidase in our brain extract cannot be excluded *a priori*. In our view, the presence of this enzyme does not invalidate the results presented here, which can be extrapolated to an *in vivo* situation because, as pointed out by Torrecilla *et al.* (39), in the presence of an ATP concentration as low as 10 μM, this enzyme would not have functional importance related to the metabolism of AMP (40, 41).

In conclusion, our results suggest that rat brain has the capacity to salvage the main natural purine and pyrimidine bases, except cytosine, and to activate 5-FUra by deriving Rib1-P and PRPP from ATP. In our opinion, this process represents the real salvage of nucleobases and might explain the capacity of the brain to achieve reperfusion-recovery of certain nucleotide pools lost during hypoxia. In addition, our results suggest that the ratio [PRPP]/[ATP] plays a major role in modulating the salvage process of purine nucleotides.

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