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# The comparative content of the mitochondrial phosphoglyceroyl-ATP-containing polymer, purinogen, in rat tissues

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**Abstract**

Here, we describe an assay for the tissue content of purinogen, a highly phosphorylated labile polymer containing ATP and phosphoglycerate found in the mitochondrial intermembrane space. We report the purinogen content (as adenine nucleotide equivalents) of rat heart and, for the first time, of rat liver, kidney, brain and mixed skeletal muscle. The findings show that purinogen contains very significant proportions of cell adenine nucleotides ranging from 25% of the free pool in brain and skeletal muscle to 135% of it in kidney. The evidence that purinogen may form a controlled intracellular reservoir of inorganic phosphate is briefly discussed. 

*Keywords:* Brain purinogen; Heart purinogen; Kidney purinogen; Liver purinogen; Muscle purinogen; ATP polymer

## 1. Introduction

Rapid changes in adenine content of perfused hearts led us to propose that some storage form must exist [1–3] and in subsequent studies, we located a very labile rapidly turning-over oligomer of alternating units of ATP and 3-phosphoglyceric acid in rat heart [4], kidney [5] and liver [6]. Selective digestion and chromatography along with $^{31}P$ nuclear magnetic resonance (NMR) and mass spectrometry studies led us to propose a structure of 3-phospho-[glyceroyl-γ-triphospho-5′-adenosine-3′-3-phospho]$_n$-glyceroyl-γ-triphospho-5′-adenosine (PG-ATP)$_n$ for this compound for which we suggested the name oligophosphoglyceroyl-ATP [7]. In subsequent work, we have identified in liver and heart a (PG-ATP)$_n$-specific 3′-phosphodiesterase which is found only in the mitochondrial intermembrane space [8] tightly complexed with its substrate [9], suggesting that (PG-ATP)$_n$ may be exclusively located there too.

Attempts to quantitate (PG-ATP)$_n$, which rapidly reaches specific radioactivity equilibrium with cell [$^{14}C$]ATP, led to the recognition that the (PG-ATP)$_n$ oligomers appear to be the rapidly labelled end-chains of a more complex polymer whose core included additional nucleotide components [10]. We have provisionally called this as yet incompletely characterised polymer purinogen and devised a quantitative assay which involves deliberate destabilisa-

Abbreviations: (PG-ATP)$_n$ or oligo(phosphoglyceroyl-adenosine triphosphate), 3-phospho[glyceroyl-γ-triphospho(5′)adenosine(3′)-3-phospho]$_n$-glyceroyl-γ-triphospho(5′)adenosine; PG-ATP, 3-phosphoglyceroyl-γ-triphospho(5′)adenosine; PPPAdoPGri, triphospho(5′)adenosine(3′)-3-phosphoglycerate

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tion of the precipitated substance and measurement of the breakdown products by high-performance liquid chromatography (HPLC). Using this assay, we have been able to demonstrate the quantitative transfer of adenosine units between free ATP and purinogen in perfused rat hearts [10,11]. However, the findings have shown that purinogen does not act as a storage reservoir for ATP in either moderate or severe ischaemia [11]. Conversely, they support a proposal that $(PG\text{-}ATP)_n$ may act as a rapidly exchangeable pool of intracellular inorganic phosphate where transsarcolemmal phosphate exchange is relatively slow [10,11]. Moreover, its properties are consistent with those expected of a labile sequestered form of inorganic phosphate which discrepant findings from $^{31}P$ NMR and extraction studies have suggested may exist in liver and heart (see [11]).

Here, we describe the technique we have devised to quantify tissue purinogen content, report that it can be found additionally in brain and skeletal muscle and provide comparative measurements for the amounts present in some rat tissues.

## 2. Materials and methods

[8-$^{14}$C]Adenosine (57 Ci/mol) was supplied by Amersham International, Little Chalfont, Bucks, UK. All other materials were as described previously [1,3,5,10,11].

### 2.1. Tissue sampling and extraction

Rats (220–250 g Sprague–Dawley strain bred at UCL) were killed by cervical dislocation. The brain was immediately removed, rinsed in modified 4°C Krebs bicarbonate Ringer solution (4.8 mM KCl, 1.2 mM $KH_2PO_4$, 0.6 mM $MgSO_4$, 1.3 mM $CaCl_2$, 120 mM NaCl, 235.3 mM $NaHCO_3$), pH 7, and freeze-clamped with liquid nitrogen-cooled tongs. In turn, the heart, liver, kidneys and hind-leg skeletal muscle were treated in the same way: in total, the time from tissue removal to freeze-clamping took no more than 60 s. All subsequent operations were carried out with sterile media and apparatus.

The freeze-clamped tissue was then pulverised in a mortar pre-cooled with liquid nitrogen and subsequently homogenised in 3 ml of cold (−10°C) 10% (mass/volume) trichloroacetic acid (TCA)/25% (by volume) methanol/10 mM $MgCl_2$ using an Ultra Turrax homogeniser TP 18 (Janke&Kunkel). The probe was washed with a further 3 ml of TCA/methanol/$MgCl_2$ and the combined homogenate centrifuged in a Sorvall RC-5B at 17 000 rev./min (SM-24 rotor; $g_{av}$ 35 600). The supernatant containing the soluble purine nucleotides was stored at −70°C and assayed by HPLC on a Waters Millenium System using an APS-Hypersil column (15 cm×4 mm) developed with a linear gradient (5–400 mM) of $KH_2PO_4$ pH 3.0. The column was calibrated using standard solutions of the adenine nucleotides.

### 2.2. Estimation of purinogen

The pelletted TCA/methanol/$MgCl_2$-insoluble material was washed once with 10 ml cold (−10°C) TCA/methanol/$MgCl_2$, once with 10 ml acetone and three times with 10 ml diethyl ether before being dried under a stream of $N_2$ gas. At each wash, the pellets were resedimented by centrifugation at 10 000 rev./min in a Sorvall RC-5B (SM-24 rotor; 9774 $g_{av}$). Between 0.1 g and 0.15 g of the weighed dried extract was resuspended in 1 ml 4 M KOH/10 mM EDTA and placed in a water-filled sonic cleaning bath (Cole-Parmer 8850; output 88 W) at room temperature and agitated by sonication for 1 min and left for 15 min with repeated sonication periods at 5 min and 10 min. The samples were then centrifuged at 10 000 rev./min in a Sorvall RC-5B (SM-24 rotor; 9774 $g_{av}$) for 10 min at 4°C. The supernatant was neutralised with 2 M HCl and the precipitate removed by centrifugation for 2 min in an Eppendorf Zentrifuge 3200 (8000 $g_{av}$). The pellet recovered from the Sorvall was resuspended in 0.5 ml KOH/EDTA and the above procedure repeated. The combined supernatants were stored at −70°C. Before HPLC analysis of the released purine derivatives, 200 μl of supernatant was filtered through a 0.45 μm Millipore filter. The quantitative extraction of purinogen was followed and purinogen breakdown products identified using extracts from hearts previously perfused with [8-$^{14}$C]adenosine to label the purinogen [10]. For radiochemical estimation, the HPLC column effluent was collected in 750 μl fractions and 200 μl samples of these were added to 4 ml of Ecosint A scintillation cocktail (National Diagnostics) and as-

sayed in a Packard Tri-Carb 900CA liquid scintillation counter.

## 3. Results and discussion

### 3.1. Devising a quantitative assay for purinogen

In order to study the metabolic role of the very labile rapidly turning-over $(PG\text{-}ATP)_n$, we needed a quantitative assay. Since we had shown in both incorporation experiments [4] and by direct extraction and purification [7] that this compound rapidly appeared to reach specific activity equilibrium with [$^{14}$C]ATP, the obvious solution was to use radioactivity measurements after radiolabelling. However, with perfused hearts subjected to a variety of metabolic stimuli, we were able to show convincingly that the two pools were not at radioactivity equilibrium and that the stimuli led to changes in the specific activity of the sequestered material [10].

Attempts to base an assay on the specific phosphodiesterase which we had found to be located in the mitochondrial intermembrane space [8] foundered, not only because we were unsuccessful in removing its tightly bound substrate while keeping it native, but also because, probably as a result of this tight association with protein, we were unable to resolubilise completely the radioactivity in the TCA/methanol-precipitated material in order to make it available as substrate. We therefore devised a procedure which depended on conditions which promoted the breakdown of purinogen but not that of more stable polynucleotide species also present in washed TCA/methanol extracts: the breakdown products could then be separated by HPLC and measured by their absorbance at 259 nm as ATP equivalents (see [10]). Initial extraction using alkali/EDTA only solubilised about 50% of the precipitated radioactivity, mostly as ATP, ADP and triphospho(5′)adenosine(3′)-3-phosphoglycerate (PPPAdoGri) (the product of $(PG\text{-}ATP)_n$ cleavage at its labile mixed anhydride bond): repeated extraction using sonication to aid dispersal of the insoluble pellet solubilised most of the remaining radioactivity but provided a much increased quantity of PPPAdoGri of much lower specific radioactivity (see [10]). It yielded moreover several labelled peaks eluting before PPPAdoGri in the HPLC. These 'unknowns' have as yet not been definitively identified but appear to be oligomers which break down further on storage in the absence of $Mg^{2+}$ to give products that co-chromatograph with ATP, adenosine, inosine and glyceric acid (M. Sarcina, unpublished findings). While these unknowns could derive from a separate rapidly labelled labile precipitable species, these findings suggest that they are closely associated with $(PG\text{-}ATP)_n$ although their phosphate:purine ratio is somewhat lower at around 2:1. For these reasons, we have suggested that the more highly labelled $(PG\text{-}ATP)_n$ may form the end-chains of a more complex polymer of adenine nucleotide, phosphate and glycerate for which we have suggested the name purinogen [10].

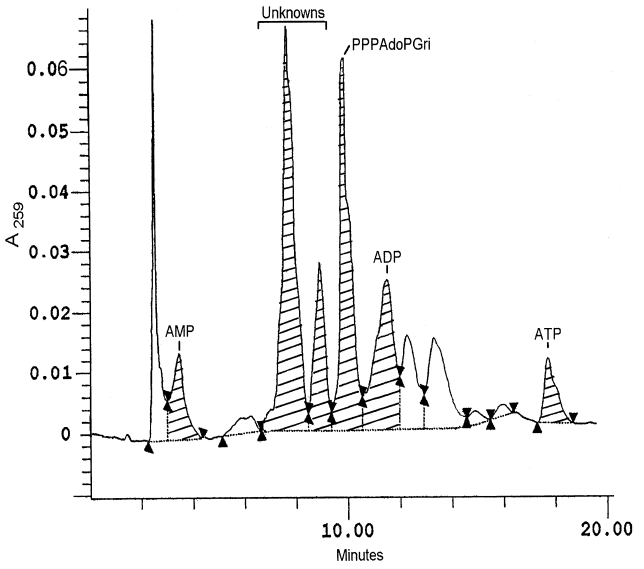


Fig. 1. The TCA/methanol/$MgCl_2$-insoluble extract from a heart was incubated with KOH/EDTA as detailed in Section 2.2. Fifty μl of the solubilised filtrate was analysed by HPLC on APS-Hypersil as detailed in Section 2.1. The triangles define the beginning and end of each peak whose computed area is bounded by the solid and dotted lines. The known peaks were identified by co-chromatography of standards. Those products rapidly labelled by prior perfusion with [8-$^{14}$C]adenosine [10] are shaded: purinogen content is estimated as the sum of these peaks using the molar absorbance of ATP as standard.

Fig. 1 gives an example of the HPLC profile of purinogen breakdown products obtained from a rat heart which had previously been perfused for 10 min with [8-$^{14}$C]adenosine to label the tissue adenine nucleotides [4]. Using the extraction technique de-

scribed here (Section 2.2), solubilisation of 98–100% of the precipitated radioactivity was obtained. Fig. 1 shows too that the breakdown products also contain species absorbing at 259 nm which are unlabelled by our perfusion regime and are therefore not rapidly exchanging with free [$^{14}$C]ATP as is purinogen. We have therefore chosen to ignore them and define purinogen content as the sum of the absorbances of the labelled species. The validity of this definition is supported by the observation of the quantitative transfer of ATP equivalents between purinogen and soluble adenine nucleotides in perfused hearts subjected to two different conditions which might be expected to alter the demand for intracellular inorganic phosphate [11].

### 3.2. The purinogen content in rat tissues

A number of rat tissues were rapidly removed, freeze-clamped and the extracts assayed for purinogen and the soluble adenine nucleotides as described above and in Section 2. As might be expected, there is some variation in the proportions of the purinogen breakdown products from different preparations; the sum of the adenine nucleotides represented between 19 and 32%, PPPAdoGri 16 and 27% and the unknowns 43 and 55% of the total. However, the HPLC traces all conformed to the typical pattern shown in Fig. 1 and were thus readily identified by comparison with those from labelled hearts. Radioactive perfusion studies similar to those with hearts had previously shown the presence of purinogen in kidney [5] and liver [6] but not its quantity. These extracts showed that purinogen was also readily detectable in brain and skeletal muscle and Table 1 sets out the amounts measured in tissues from four rats relative to the tissue protein [12] and DNA [13] content.

The use of a number of tissues from the same animal and the consequent time difference in freeze-clamping between different tissues, however slickly the sampling was organised, raises the prospect of nucleotide metabolism in the brief ischemic interval. Previous studies with ischemic hearts, livers and kidneys [5,6,10] have shown that in brief ischemic periods, there is some net conversion of ATP to ADP and AMP but no change in the sum of these nucleotides. In the experiments reported here, with one exception, ATP represented between 57% and 77% of the adenine nucleotide content (data not given): the exception was brain which, although sampled first, had equal amounts of ATP and AMP at 40% of the total. In the case of purinogen, we have shown with perfused hearts that neither severe reversible (20 min) or irreversible (40 min) ischemia causes any change in the measured purinogen content from that in controls [11] and in line with this, the heart purinogen content in Table 1 compares well with previous estimates [10,11] using a mean protein content of 140 mg/g wet tissue. It seems reasonable therefore, at least initially, to expect that a brief period of ischemia will have no effect on the purinogen content of other rat tissues.

There is no obvious correlation between the contents of free nucleotides and those in purinogen in the different tissues. The findings do show, however, that substantial proportions of tissue nucleotides are bound up in the polymer. In brain and mixed skeletal muscle, the purinogen nucleotide content amounts to a quarter of that in free adenine nucleotide, while in heart it is 60% and in liver equal to that of the free adenine nucleotides. Relatively the greatest propor-

Table 1
The contents of purinogen and of free adenine nucleotides in some rat tissues

| | Purinogen (nmol/mg protein) | Σ AN | Purinogen (nmol/mg DNA) | Σ AN |
|---|---|---|---|---|
| Liver | 14.4 ± 1.5 | 14.9 ± 3.5 | 1 204 ± 74 | 1 209 ± 281 |
| Heart | 10.96 ± 0.55 | 18.9 ± 1.2 | 647 ± 33 | 1 115 ± 73 |
| Brain | 5.69 ± 0.78 | 23.5 ± 5.1 | 677 ± 62 | 2 769 ± 439 |
| Kidney | 19.97 ± 0.99 | 14.5 ± 1.7 | 951 ± 110 | 726 ± 62 |
| Skeletal muscle | 11.2 ± 1.8 | 46.2 ± 2.8 | 3 571 ± 574 | 16 600 ± 1 100 |

Σ AN denotes the sum of the contents of ATP+ADP+AMP in the soluble tissue extract.

tion is in kidney which has 35% more nucleotide in purinogen than in the free state. For comparison, the adenine nucleotide content of rat heart total RNA is about 30% of that in free nucleotides [1,14].

From these findings, the potential for rapidly turning-over purinogen to augment the adenine nucleotide pool would appear to be considerable: and its failure to do so in severely ischemic rat hearts when there is substantial conversion of ATP to oxidised purines [11] is all the more surprising. By contrast, we have found that removing inorganic phosphate from heart perfusion buffer and providing glucose to a heart previously perfused without nutrient to deplete endogenous supplies of glycogen and fatty acid caused net transfer of nucleotide between free ATP and purinogen [10,11]. These treatments might be expected to alter the demand for intracellular inorganic phosphate and have led to the suggestion [11] that the four phosphates per purine base present in $(PG\text{-}ATP)_n$ may provide a controlled reservoir of intracellular phosphate, which a number of $^{31}P$ NMR studies have suggested may exist (see [11] for further discussion). Further investigation is needed to examine this proposition; but it could be consistent with the presence of a relatively large purinogen content in kidney which has a major role in the control and transport of body inorganic phosphate.

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