ENZYMATIC MEASUREMENT OF ADENINE AND GUANINE(PLUS INOSINE) TRIPHOSPHATES AND DIPHOSPHATES IN ISOLATED CELLS AND THE MITOCHONDRIAL MATRIX COMPARTMENT OBTAINED FROM RAT LIVER

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1. Introduction

The development of methods for subcellular fractionation of liver cells (reviewed in [1]) has reached a stage that provides useful information on the metabolic interaction of mitochondrial and cytosolic systems (reviewed in [2]). In particular, the phosphorylation potentials of the adenine nucleotides in these two compartments have attracted interest [3–6], but it has increasingly become apparent that also the guanine nucleotide phosphorylation potentials are of importance for a number of cell-physiological problems. However, comparatively little is known on subcellular GTP/GDP ratios, partly due to methodological difficulties.

Although it is known that non-adenine nucleotides are present in liver, it has been common practice to employ assay systems using enzymes which may react also with these non-adenine nucleotides [7]. Consequently, overestimated values for the adenine nucleotides have generally been reported; in particular, this concerns ATP/ADP ratios since triphosphates and diphosphates are subject to different extents of overestimation.

Recently, it was found that phosphoglycerate kinase from the blue–green alga *Spirulina platensis* (PGK-A) is specific for ATP [8] in contrast to the yeast enzyme (PGK-Y) which also reacts with GTP and ITP [9], so that a new specific determination of ATP and of GTP + ITP was rendered possible [8], an advance over previous enzymatic procedures [10,11].

This paper describes the specific determination of the diphosphates, ADP and GDP + IDP, also using the two types of phosphoglycerate kinase. The application of these new assays with tissue extracts is demonstrated, and the results of measurements of adenine and guanine nucleotides in isolated rat hepatocytes are presented and compared to the results obtained by conventional assays. Further, employing the digitonin subfractionation procedure [3], the subcellular distribution was studied. It was found that ~10% of the total cellular GTP + ITP and 55% of the total cellular GDP + IDP is located in the mitochondrial compartment. The GTP/GDP ratio in the mitochondrial compartment was lower than the corresponding ATP/ADP ratio, whereas the guanine and adenine triphosphate/diphosphate (T/D) values in the cytosolic compartment were similar.

2. Methods

2.1. *Isolation, incubation and sampling of hepatocytes*

Hepatocytes were isolated from male Wistar rats, fed on Altromin stock diet, weighing 150–200 g, as in [12]. The isolated hepatocytes were incubated in a bicarbonate-buffered medium equilibrated with O_2/CO_2 (95/5, v/v) in the presence of glucose (10 mM), L-lactate (2.1 mM), pyruvate (0.3 mM), D,L-β-hydroxybutyrate (0.6 mM) and acetoacetate (0.3 mM) and subfractionated exactly as in [13]. The perchloric acid extracts were neutralized with KOH (4 M) and MOPS (0.4 M) to pH 7.5.

2.2. *Assays*

ATP was measured using hexokinase and glucose-6-phosphate dehydrogenase according to [14], or ATP and GTP(+ITP) were determined separately in the same cuvette using PGK-A and PGK-Y [8].

ADP and GDP(+IDP) were determined by difference after pretreatment of different aliquots of one sample either with PGK-A to specifically convert ADP, or with PGK-Y to convert ADP plus GDP(+IDP). After pretreatment, the aliquots were analysed for XDP according to the standard pyruvate kinase/lactate dehydrogenase method [15] and compared to a blank-treated aliquot. The pretreatment was as follows: Three reaction cups (1.5 ml) were each supplied with 200 μl neutralized sample; 20 μl of a mixture consisting of K-phosphate (pH 7.5) 0.1 M, $MgCl_2$ 20 mM, NAD^+ 5 mM, D,L-glyceraldehyde-3-phosphate 5 mM, and pyruvate 2 mM; and 10 μl lactate dehydrogenase (0.3 U). The 10 min preincubation was started by adding 10 μl glyceraldehyde-3-phosphate dehydrogenase (0.15 U) to each cup, followed by 10 μl H_2O (cup 1), PGK-A (0.15 U) (cup 2) or PGK-Y (0.9 U) (cup 3). Reactions were stopped either by addition of perchloric acid (and subsequent neutralization) or, for convenience, by addition of 0.1 ml of phenol/chloroform/isoamyl-alcohol (38/24/1) under thorough mixing and subsequent centrifugation. The supernatants were analyzed for XDP. Cup 1 contains all XDP, cup 2 is devoid of ADP, and cup 3 is devoid of ADP and GDP(+IDP). Separate values for ADP and for GDP(+IDP) are obtained by difference (see below and fig.2).

3. Results

3.1. *Specificity of phosphoglycerate kinase from* Spirulina platensis *(PGK-A)*

The specificity of PGK-A for the triphosphates has been tested [8], with the rates for GTP or ITP being <1% of the rates observed with ATP, and those for UTP or CTP being below detection limits. Here we have assayed the specificity of PGK-A for the diphosphates, using glyceraldehyde-3-phosphate dehydrogenase as a pre-coupled indicator reaction [16]. The pattern of specificity was found to be essentially similar (not shown); therefore, an assay system for the diphosphates was rendered possible.

3.2. *An assay for ADP and GDP(+IDP) applicable for measurements in tissue extracts*

The direct measurement of ADP and GDP(+IDP) in the tissue extracts by successive addition of the two types of phosphoglycerate kinase, analogous to the direct determination of ATP and GTP(+ITP) [8], proved not to be practicable for reasons such as drifts and stoichiometry problems related to the pre-coupled indicator reaction. Therefore, the assay system described in section 2 was elaborated, and an example is shown in fig.1 illustrating specificity and in fig.2 illustrating feasibility with an hepatocyte extract.

The three panels in fig.1 show the assay of ADP, GDP and UDP without (upper trace) and with (lower trace) pretreatment with PGK-A using low (4 μM) concentrations of these nucleoside diphosphates. Whereas ADP is completely eliminated by the pretreatment (top), the amount of GDP is unaltered (center). Due to the slow reaction rate of pyruvate kinase with UDP, the endpoint of reaction is not reached for UDP within the time indicated;

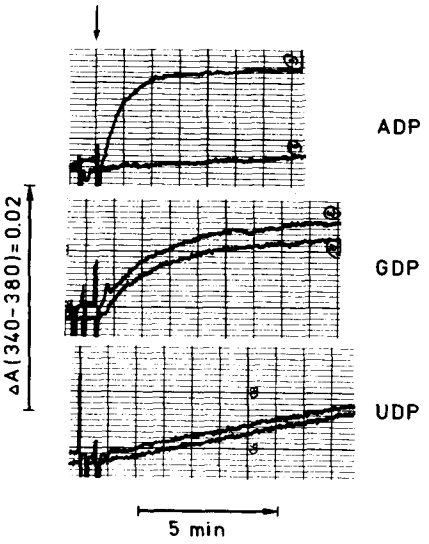

Fig.1. Enzymatic assay of nucleoside diphosphates using the pyruvate kinase/lactate dehydrogenase system, with and without pretreatment with phosphoglycerate kinase from the blue–green alga, *Spirulina platensis* (PGK-A). Top: ADP (4 μM) was preincubated in the absence (upper trace) or presence (lower trace) of PGK-A as in section 2. Center: same for GDP (or for IDP, not shown). Bottom: same for UDP (or for CDP, not shown).

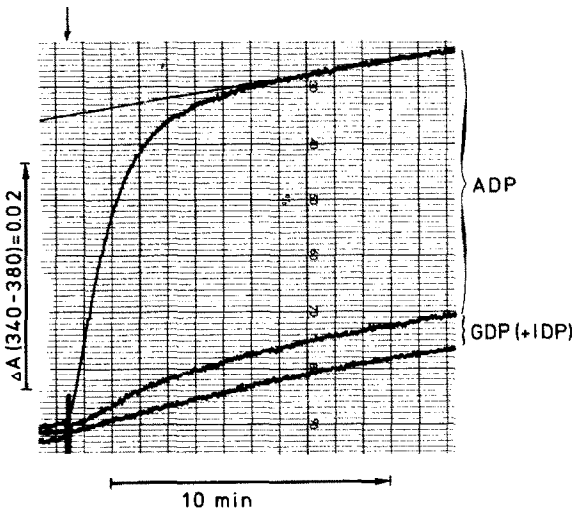

Fig.2. Enzymatic assay of nucleoside disphosphates in an extract of rat hepatocytes. The cells were incubated and extracted as in section 2. Aliquots of the extract were pretreated without (upper trace) or with PGK-A (center trace) or with PKG-Y (lower trace). The differences represent ADP and GDP(+IDP) as indicated. The line for back-extrapolation, conventionally used for ADP assays, is also shown to illustrate the extent of usual over-estimation, 17% in this case.

however, no difference by pretreatment could be detected for this nucleotide (bottom). Inosine diphosphate behaved like the guanine diphosphate whereas the diphosphates of uridine and cytosine behaved similar as well (not shown). The latter explain the 'drift' in the conventional ADP-assay, so that a back-extrapolation is not justified (see [15]).

The application of the assay with an extract obtained from an incubation of hepatocytes is presented in fig.2. The three traces represent, from top to bottom, the results after pretreatment corresponding to cups 1, 2 and 3 as mentioned in section 2.2. The evaluation for ADP and for GDP+IDP is shown on the right-hand side. Since the conventional evaluation in ADP assays (upper trace) is performed by back-extrapolation as is also indicated in fig.2, the conventional evaluation would lead to an overestimation of the ADP content of 17% in this case. As the contribution of non-adenine nucleotides may vary under different metabolic conditions, this error may not be a constant.

3.3. *Adenine and guanine triphosphates and diphosphates in isolated hepatocytes and in subcellular fractions derived therefrom*

The data in the literature, obtained by chromatographic [17–19] or enzymatic [20] procedures, indicate that the sum of GTP + GDP amounts to 8–15% of the sum of ATP + ADP in rat liver, whereas ITP + IDP was not detected in rat liver [18] nor in isolated rat liver mitochondria [21,22]. Therefore, in the following we will present the data for the guanosine + inosine nucleotides solely as guanosine, although the assay system would also measure inosine nucleotides.

The adenosine triphosphate and diphosphate values obtained by the conventional methods of back-extrapolation in the hexokinase/glucose-6-phosphate dehydrogenase and pyruvate kinase/lactate dehydrogenase systems, respectively, and those found with the specific methods described in this paper are

Table 1
Adenine and guanine nucleotides in isolated hepatocytes (total, T) and the particulate fraction (mitochondrial, M) obtained after digitonin treatment

	Total		Mitochondrial		Cytosolic (T–M)	
	ATP	ADP	ATP	ADP	ATP	ADP
Conventional	9.45 ± 0.34	1.62 ± 0.07	1.66 ± 0.14	0.77 ± 0.07	7.80 ± 0.25	0.85 ± 0.07
Specific	9.15 ± 0.42	1.34 ± 0.07	1.54 ± 0.12	0.63 ± 0.07	7.60 ± 0.34	0.71 ± 0.08
	GTP	GDP	GTP	GDP	GTP	GDP
Specific	1.06 ± 0.05	0.20 ± 0.02	0.11 ± 0.01	0.11 ± 0.01	0.95 ± 0.04	0.09 ± 0.03

Cytosolic values were obtained by difference (T–M). Data are given as means ± SEM (n = 4-5), in μmol/g dry wt

Table 2
ATP/ADP and GTP/GDP ratios in isolated hepatocytes (total) and subcellular fractions obtained after digitonin treatment

	Total	Mitochondrial	Cytosolic (T–M)
	ATP/ADP	ATP/ADP	ATP/ADP
Conventional	5.83	2.16	9.18
Specific	6.83	2.44	10.70
	GTP/GDP	GTP/GDP	GTP/GDP
Specific	5.41	1.06	10.67

Data from table 1

shown in table 1. The difference between the conventional and the specific methods is more pronounced for the diphosphate. The GTP and GDP values for the hepatocytes are shown in table 1 as well.

Subcellular fractionation by the digitonin procedure led to values for the mitochondrial and cytosolic (total minus mitochondrial) compartments. For the guanine nucleotides 10% of the cellular GTP and 55% of the cellular GDP is located in the mitochondrial compartment, the corresponding values for the adenine nucleotides being 17% and 47%, respectively. The latter values are similar to the results in [3–5].

The triphosphate/diphosphate (T/D) ratios calculated from these data are shown in table 2. The mitochondrial GTP/GDP ratio is ~50% of the mitochondrial ATP/ADP ratio, whereas the cytosolic T/D values are similar.

4. Discussion

The elaboration of an enzymatic assay for the selective measurement of adenosine triphosphates and diphosphates as well as of guanosine triphosphates and diphosphates in tissue extracts, based on the specificities of PGK-A and PGK-Y, may make possible a more detailed examination of the role of the guanine nucleotide system in different cellular compartments and in various metabolic functions. So far, such problems have been formulated but not fully resolved (e.g., [23–26]).

Published values obtained with liver extracts by chromatographic procedures are in good agreement with the results reported for isolated hepatocytes, particularly if the ratio of the guanine over the adenine nucleotide T/D values is considered. The equilibrium value for this nucleoside diphosphokinase mass action ratio is ~1 [27]. Subcellularly, the cytosolic value reported here is 1.0 (table 2). The mitochondrial value is 0.43. This is much lower than the calculated value of 70 as obtained from the succinate thiokinase indicator system in isolated mitochondria [24].

Such discrepancies are related to thermodynamic problems and remain to be elucidated. Because of the low level of guanine nucleotides, the binding of these nucleotides to specific binding sites, e.g., on the glutamate dehydrogenase [28,29], may have an influence on the phosphorylation potential. The mitochondrial and cytosolic concentrations may be calculated by referring the contents shown in table 1 to the water contents of 0.21 and 2.0 ml/g dry wt, respectively [30]. The mitochondrial matrix GTP and GDP concentrations thus are calculated to be 0.52 mM, whereas the cytosolic values are 0.48 mM for GTP and 0.05 mM for GDP.

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Note added in proof

Recently, other groups have reported measurements of guanine nucleotides using enzymatic procedures different from the one in this paper, and the applications were on guanine nucleotide compartmentation in isolated rat hepatocytes [31] and on total extracts from a variety of tissues [32].

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