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Determination of high-energy phosphate compounds and inorganic phosphate by reversed-phase high-performance liquid chromatography: evaluation of myocardial metabolic status in aerobically perfused and hypoxic mouse heart

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Abstract

The present paper describes a simple HPLC method designed for measuring high-energy phosphate (HEP) compounds in a single run and inorganic phosphate (P_i) in an other short run under the same HPLC conditions. Inorganic phosphate was estimated by using thymidine phosphorylase (EC 2.4.2.4) which catalyzes a reaction involving inorganic phosphate to produce 2-deoxyribose 1-phosphate and thymine. The thymine/P_i stoichiometry was 1. The method provides a reproducible instrument for evaluating myocardial high-energy metabolism under physiological and pathological conditions. © 2001 Elsevier Science B.V. All rights reserved.

Keywords: Inorganic phosphate; High-energy phosphate

1. Introduction

Determination of high-energy phosphate (HEP) compounds and inorganic phosphate (P_i) in cells, tissues and organs is of great importance in the evaluation of metabolic status. Assessment of changes in ATP, ADP, AMP, creatine, phosphocreatine and inorganic phosphate content is essential for the characterisation of ischemic and anoxic myocardial injury [1].

Recently, the phosphorus nuclear magnetic resonance (^{31}P-NMR) technique has been used to study dynamic metabolic aspects of these compounds in isolated, intact organs while normal functions were maintained [2,3]. A primary advantage of ^{31}P-NMR spectroscopy is the capability of detecting and quantifying sequential changes in the phosphate-containing compounds under different experimental conditions. Usually, changes are calculated as percentage of control values, but this approach is feasible only if the entire metabolite pool is detectable by NMR. Some authors reported that the ATP content in the heart is partially detectable by ^{31}P-NMR [4–6]. Similarly, Murphy et al. [7] reported that ATP in liver is not totally detectable by NMR. The presence of NMR-invisible ATP might depend on the fact that this compound is sequestered in mitochondria and bound to macromolecules such as myosin, the most abundant protein in myocytes.

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Some authors maintain that the NMR-invisible pool of ATP exists under all conditions [5,6] whereas others suggest that the NMR-invisible fraction of ATP is present only during ischemia [7] or during reperfusion after ischemia [4]. On the contrary, a good correlation was observed between the ATP content in liver [8] or in heart [9] as measured by ^{31}P-NMR and by biochemical assay. However, since the area of the phosphorus resonance is proportional to the amount of metabolites and not to their concentration, quantitative interpretation of ^{31}P-NMR spectra of tissue implies the determination of metabolite concentrations and their intracellular distribution, using other techniques.

High-performance liquid chromatography (HPLC) has been widely used for detection of HEP in several biological materials, e.g., blood [10,11], and various tissues including myocardium [12,13]. However, a limitation of the HPLC assay is that P_i cannot be detected using such technique.

Different methods have been described for measuring inorganic phosphate released from nucleotides or from phosphocreatine. The spectrophotometric assay is one of the most used and is based on the quantification of P_i as a coloured complex of molybdophosphoric acid [14]. Although the method is sensitive and feasible, it has some drawbacks due to the fact that colour intensity is time dependent, labile compounds are hydrolysed and various substances compete for free molybdate [15].

Therefore, it is necessary to provide a more accurate method for determination, in perchloric acid extracts, of HEP and P_i, which are directly related to cell energy level.

In the present paper we describe a method which combines a simple HPLC isocratic analysis for simultaneous determination of HEP compounds [16], with an inorganic phosphorus assay using the same HPLC procedures. The method has been applied to myocardial tissue from mice heart under normoxic and hypoxic conditions.

2. Materials and methods

2.1. Reagents

All chemicals were of the highest degree of purity. ATP, ADP, AMP, creatine, phosphocreatine, NAD^+, NADH, thymidine, thymine and thymidine phosphorylase from *Escherichia coli* (EC 2.4.2.4) were obtained from Sigma (St. Louis, MO, USA). Perchloric acid, acetonitrile, potassium dihydrogenphosphate and tetrabutylammonium hydrogen sulfate (TBAHS) were purchased from Fluka (Buchs, Switzerland). All water solutions were made using distilled water processed by filtration and reverse osmosis prior to particle absorption, ionic exchange and bacterial filtration (Milli-Q system; Millipore, USA).

2.2. HPLC system

The HPLC apparatus consisted of a pump (2150 LKB Pharmacia) coupled with a spectrophotometer (Uvicord SD 2152, LKB Pharmacia) equipped with a HPLC controller 2152 LKB. The data were recorded and analysed by a recording integrator (2220 LKB, Pharmacia). The compounds were separated by reversed-phase HPLC with isocratic elution. The analytical column, (Discovery Supelco C_{18}, 150×4.6 mm, 5 μm particles) protected by a Discovery Supelco (5 μm, 5×4.6 mm) guard column, was eluted with a mobile phase containing potassium dihydrogenphosphate (125 m*M*), TBAHS (2.3 m*M*) and acetonitrile (3.5%). The mobile phase adjusted to pH 6.25 with KOH was filtered through a 0.22-μm cellulose filter (Millipore, Molsheim, France) and degassed before use. Chromatography was carried out at room temperature at a flow-rate of 0.8 ml/min and the spectrophotometer was set at 206 nm.

2.3. Perfusion of isolated mouse hearts and myocardial tissue extracts

Hearts were isolated from 10 to 12-month-old mice (C57/BL10), weight range of 30–35 g, obtained from Jackson Lab. (Bar Harbour, ME, USA). The strain was bred in our own animal facilities under standard conditions in a 12 h light/dark cycle. All animal experiments were carried out in accordance with guidelines established by the Italian Council for animal care. Isolated hearts were perfused aerobically (95% O_2; 5% CO_2) with standard Krebs Henseleit hydrogencarbonate buffer containing 11.1 mmol/l glucose in the retrograde Langendorff [17]. Perfusion was carried out at 37°C: (a) for 30 min to

ensure normoxic conditions; (b) for 3 min, enough to wash out the blood but not enough to correct hypoxia. At the end of perfusion, the hearts were quickly frozen in isopentane chilled by liquid nitrogen and immediately freeze–dried for at least 20 h (Modulyo, High Vacuum International, Edwards, UK). Samples of dried hearts were homogenised with a mechanical homogeniser (Mixer Mill MM 200; Retsch, Germany) for 2 min [16]. The dried tissue powder (4–5 mg) was dissolved in 500 μl of 0.42 *M* perchloric acid and immediately stirred at 4°C for 10 min. After precipitation by 240–245 μl of 1 *M* KOH the extract was spun for 1 min in an Eppendorff centrifuge (15 000 *g*, 4°C).

2.4. Measurement of adenine nucleotides and creatine compounds

The acid supernatant, kept on ice, was diluted 1:10 with the mobile phase, and immediately injected (20 μl) into the HPLC system. Quantitative measurements were carried out by injection of pure standards dissolved into the mobile phase at known concentrations. The concentration of adenine compounds (ATP, ADP, AMP), NAD^+ and NADH were determined spectrophotometrically using the following extinction coefficients, $\epsilon = 13.8\ mM^{-1}\ cm^{-1}$, $\epsilon = 16.6\ mM^{-1}\ cm^{-1}$ and $\epsilon = 13.0\ mM^{-1}\ cm^{-1}$, respectively, at 254 nm.

2.5. Determination of inorganic phosphate

Inorganic phosphate was determined by the use of thymidine phosphorylase which catalyses the following reaction: thymidine+phosphate→thymine+2-deoxyribose 1-phosphate [18]. Thymine was quantified by fast HPLC separation and compared with a P_i standard curve (Fig. 5).

Thymidine phosphorylase (450 μl, 1200 U/ml) was dialysed for 24 h against 3 l of 10 m*M* Tris–HCl, pH 7.3. Aliquots of 50 μl (100 U/ml) were frozen at −20°C until use.

An 85-μl volume of 10 m*M* thymidine in Tris–HCl (10 m*M*) at pH 7.3, was added to 10 μl of tissue extract and the reaction was started by adding 5 μl of thymidine phosphorylase (0.5 U). After 5 min of incubation at room temperature, the enzyme was eliminated by denaturation at 98°C for 1 min [19]. The sample was centrifuged at 14 000 *g* for 2 min and the surnatant diluted 1:10 with mobile phase was injected (20 μl) into the HPLC system. For calibration, thymine standard solutions were prepared from 1 m*M* thymine stock solution. Thymine and thymidine concentrations were determined spectrophotometrically using the respective extinction coefficients, $\epsilon_{264.5} = 7.9 \cdot 10^{-3}\ mM^{-1}\ cm^{-1}$ (pH 7) and $\epsilon_{267} = 9.7 \cdot 10^{-3}\ mM^{-1}\ cm^{-1}$ (pH 7). P_i standard solutions were obtained by dilution of 1 m*M* NaH_2PO_4 stock solution. In order to minimise background absorbance and P_i contamination, plastic ware and distilled water were used.

2.6. Statistical analysis

Results are shown as mean±SD values and significant differences between samples were determined by the Student *t*-test. Correlation coefficients among samples were determined by linear regression and probability values of less than 0.05 were considered significant.

3. Results and discussion

Fig. 1a shows the chromatogram from a mixture of standard substances. All compounds were well resolved and separated within 20 min. The detection limit was less than 10 pmol and the linearity was tested for each compound up to 400 pmol without loss of resolution.

The precipitation of the perchloric ion by 1.0 *M* KOH in the tissue extracts had to be performed without any precise setting of the pH since the use of tested buffers (phosphate, Tris) led to considerable disturbances of the chromatogram. Neutralisation was practically impossible because of a very steep titration curve in the absence of buffer. pH values over 7.00 resulted in degradation of ADP and ATP associated with an increase in the AMP content. On the contrary, creatine and phosphocreatine were unaffected by pH variations. When the acid extract was precipitated with an amount of KOH sufficient for keeping an acidic pH (pH 5–6) the stability of purine compounds was good: on ice the decline of ATP and ADP concentrations was negligible for at least 3 h.

Fig. 1b and c are representative chromatograms

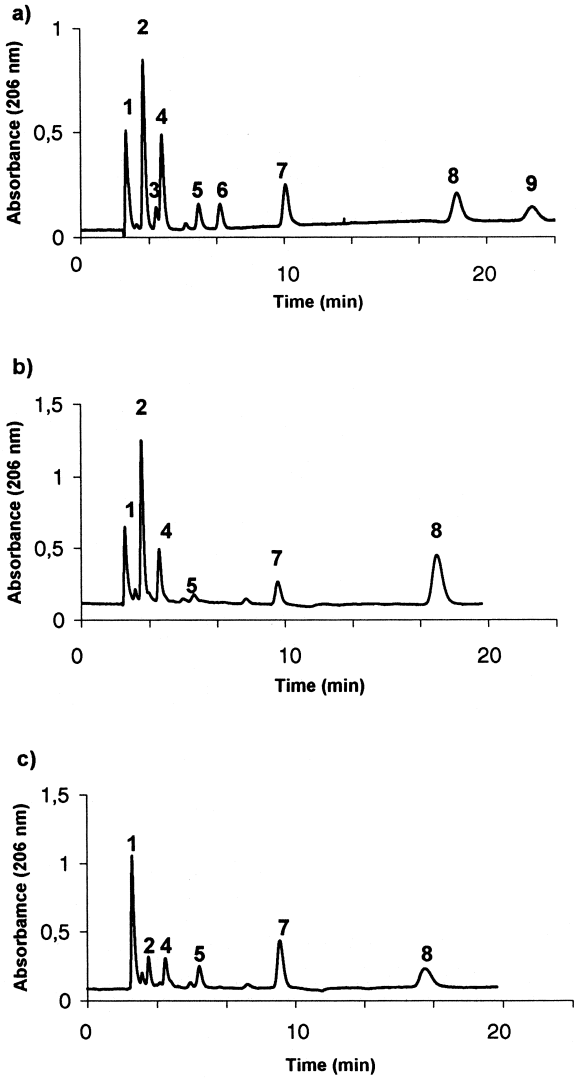

Fig. 1. Chromatograms of standard solutions and extracts from mouse cardiac tissue. (a) Chromatogram of a mixture of nine standards [20 μ*M* creatine (1), 20 μ*M* phosphocreatine (2), 2 μ*M* inosine (3), 5 μ*M* NAD^+ (4), 2 μ*M* AMP (5), 2 μ*M* adenosine (6), 5 μ*M* ADP (7), 5 μ*M* ATP (8), 5 μ*M* NADH (9)]. (b) Chromatogram of an extract from myocardial tissue with 30 min of aerobic perfusion (control). (c) Chromatogram of an extract from myocardial tissue perfused for 3 min (hypoxic status). Chromatographic conditions: Discovery Supelco C_{18} column (5 μm, 150×4.6 mm) protected by a Discovery Supelco (5 μm, 5×4.6 mm) guard column. Mobile phase: 125 m*M* NH_2PO_4 buffer, 3.5% (v/v) acetonitrile, pH 6.25. Injection volume of 20 μl. Isocratic elution at flow-rate of 0.8 ml/min.

obtained from mice heart tissue extracts under physiological conditions (b) and in hypoxia (c). Recovery studies were carried out by adding a mixture of different standards to a dried tissue

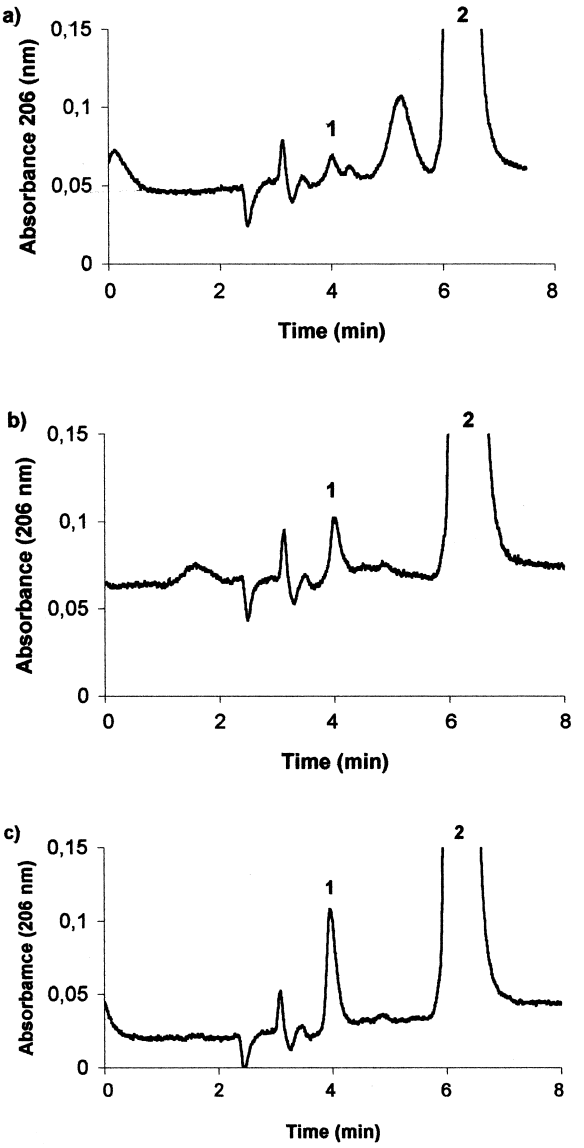

Fig. 2. HPLC estimation of P_i standards in the presence of 10 m*M* thymidine (2). Thymine (1) measured: (a) 0 μ*M* P_i, (b) 20 μ*M* P_i, (c) 100 μ*M* P_i. Chromatographic conditions: Discovery Supelco C_{18} column (5 μm, 150×4.6 mm) protected by a Discovery Supelco (5 μm, 5×4.6 mm) guard column. Mobile phase: 125 m*M* NH_2PO_4 buffer, 3.5% (v/v) acetonitrile, pH 6.25. Injection volume of 20 μl. Isocratic elution at flow-rate of 0.8 ml/min.

Table 1
Intra-/inter-day variability of HEP compound and P_i levels measured by HPLC–UV

Compound	Intra-day variability		Inter-day variability	
	Mean±SD	RSD (%)	Mean±SD	RSD (%)
Cr (μmol/g dry wt.)	31.9±1.4	5	29.3±2.9	10
PCr (μmol/g dry wt.)	41.0±1.4	3	38.2±2.8	7
PCr (%)	56±2	3	57±2	3
Cr sum[a] (μmol/g dry wt.)	72.8±0.33	0.5	67.5±2.7	8
NAD^+ (μmol/g dry wt.)	4.40±0.48	10	4.35±0.46	10
AMP (μmol/g dry wt.)	0.96±0.08	9	0.89±0.17	19
ADP (μmol/g dry wt.)	4.85±0.56	11	5.0±1.27	23
ATP (μmol/g dry wt.)	19.0±1.6	9	18.2±1.46	8
P_i (μmol/g dry wt.)	9.4±0.71	8	9.94±0.90	13
Ad sum[a] (μmol/g dry wt.)	28.1±3.1	11	26.2±2.7	10
Energy charge[a]	0.80±0.07	9	0.82±0.05	7

[a] Ad sum=ATP+ADP+AMP; Cr sum=Cr+PCr;energy charge $= \frac{1/2\text{ADP} + \text{ATP}}{\text{AMP} + \text{ADP} + \text{ATP}}$

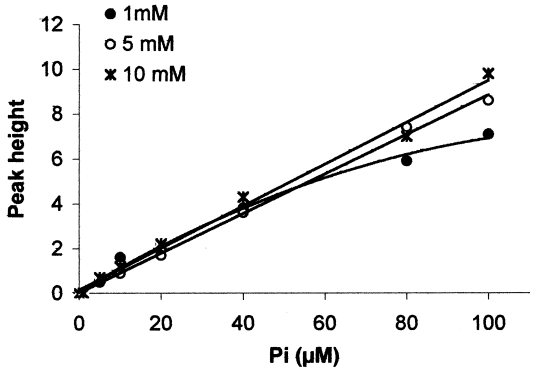

Fig. 3. Effect of various thymidine concentrations on thymine produced as a function of P_i. (●) 1 m*M* Thymidine, (○) 5 m*M* thymidine, (*) 10 m*M* thymidine.

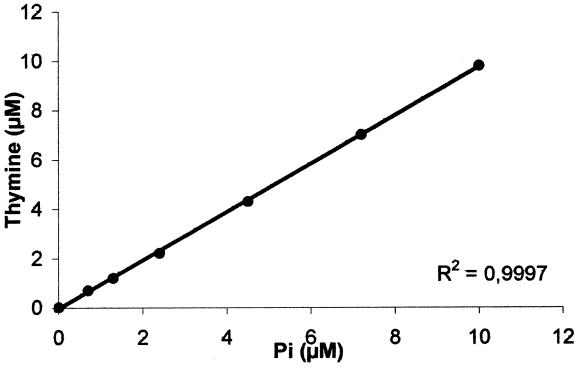

Fig. 5. Linear relationship between thymine and P_i concentrations.

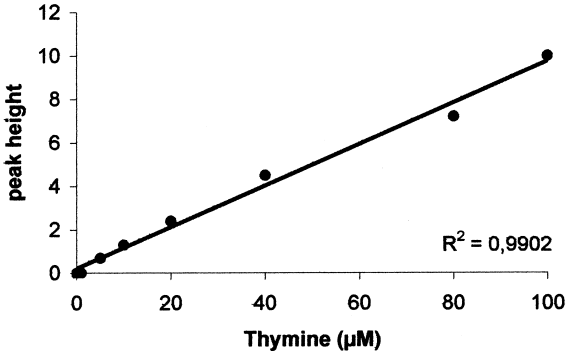

Fig. 4. Linear relationship between thymine concentration and thymine peak height.

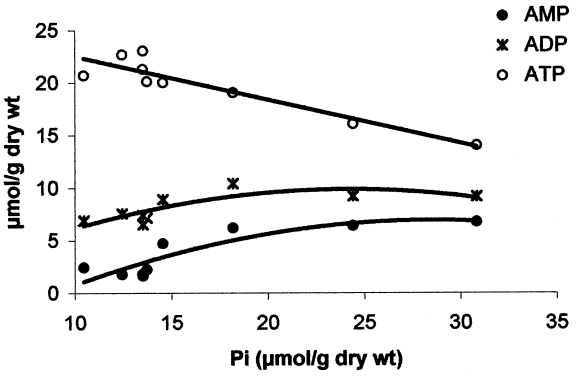

Fig. 6. Relationship of P_i versus (●) AMP, (*) ADP, (○) ATP concentrations.

Table 2
Concentrations of HEP compounds and energy charge in heart of mouse under normoxic or hypoxic conditions

Compound	Normoxic conditions	Hypoxic conditions
Cr (μmol/g dry wt.)	30.8±1.7	64.7±3.7*
PCr (μmol/g dry wt.)	36.1±6.5	5.0±5.0*
Cr sum[a] (μmol/g dry wt.)	67.0±6.1	69.6±2.0
PCr (%)	53.6±5.6	7.1±2.1*
NAD^+ (μmol/g dry wt.)	5.3±0.62	4.8±0.54
AMP (μmol/g dry wt.)	1.24±0.32	5.2±2.0*
ADP (μmol/g dry wt.)	5.8±0.5	9.0±1.0*
ATP (μmol/g dry wt.)	22.5±2.9	18.5±3.6
P_i (μmol/g dry wt.)	10.7±0.3	23.1±6.7*
Ad sum[a] (μmol/g dry wt.)	29.6±2.6	32.8±2.1
Energy charge[a]	0.83±0.03	0.70±0.08*
$P_i/PCr+P_i$	0.23±0.04	0.80±0.20*

[a] Ad sum = ATP + ADP + AMP; Cr sum = Cr + PCr; energy charge = $\frac{1/2ADP + ATP}{AMP + ADP + ATP}$
*$P < 0.01$.

powder before processing the sample. The recovery varied between 102 and 106% ($n=4$). Repeated injections of the extract from the same myocardial dried tissue gave almost identical levels. In order to assess the reproducibility and accuracy of the analytical procedure, intra- and inter-day variation analyses were made from 1 day injections of 10 consecutive tissue extractions and from repeated injections of tissue samples in different days over a period of 6 months. The intra- and inter-day variability, means±SD values and the relative standard deviations (RSDs) for each substance are summarised in Table 1.

Intra- and inter-day RSDs ranging from 3 to 10% and 7 to 15% showed a good reproducibility. Higher RSDs were observed for AMP, ADP (13–20%), probably due to fast degradation of ADP.

P_i estimation by thymine measurement after the phosphorolysis of thymidine phosphorylase is highly selective. In fact, the enzyme is specific for pyrimidine 2′-deoxyribosides and such specificity eliminates artefacts with HEP compounds, particularly with adenine nucleotides. The reaction was complete before 5 min of incubation (data not shown) and the thymine formed was stable. Thymine was separated and quantified by HPLC fixed at the same conditions used for the HEP compounds assay as described above. Fig. 2 shows a typical chromatogram of (a) 0 μ*M* P_i, (b) 20 μ*M* P_i and (c) 100 μ*M* P_i. The method shows an excellent reproducibility and a short analysis time (8 min), allowing an injection of a new sample immediately after thymidine elution.

As shown in Fig. 3, a good linearity between phosphate concentration and thymine formation was observed with 10 m*M* thymidine concentration in a range of 0–100 μ*M* phosphate. The products of the reaction (thymine and 2′-deoxyribose 1-phosphate) are believed to be inhibitors of the reaction [19]. The inhibition by the product was avoided by thymidine enzyme saturation. Peak height versus thymine concentration over the range from 1 to 100 μ*M* showed a linear correlation coefficient of $r=0.998$ (Fig. 4). The relationship between P_i and thymine concentrations is shown in Fig. 5. The intra- and inter-day

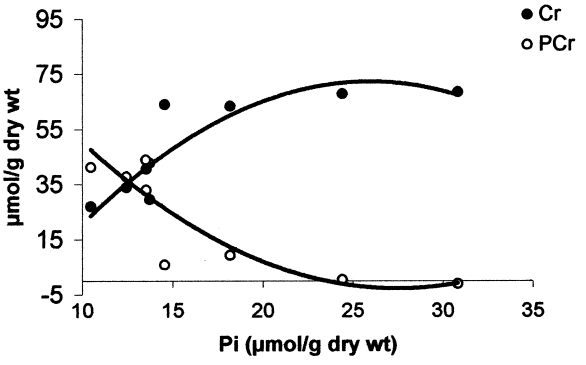

Fig. 7. Relationship of P_i versus (●) creatine, (○) phosphocreatine concentrations.

variability means±SD values and the RSDs, show the accuracy and precision of the analysis (Table 1).

Table 2 shows the values of HEP compounds in cardiac tissue undergoing normal aerobic perfusion and in hypoxic conditions. In aerobically perfused hearts (30 min), 54% of total creatine was present as phosphocreatine (PCr), adenine compounds were mostly present as ATP (72±4%), whereas ADP and AMP were present in percentages of 23±3% and 5±2%, respectively.

When the heart underwent hypoxia, a fall in creatine phosphate to 1% of control and a concomitant rise in creatine concentration were observed. Moreover, a decrease in ATP levels occurred with an increase in ADP and AMP concentrations (57±5%, 27±6% and 16±4%, respectively).

Hypoxia gave a characteristic picture of hearts in a low-energy state as compared to control (energy charge was 0.70±0.08 vs. 0.83±0.03, $P<0.01$, respectively).

Furthermore, loss in PCr and ATP concentrations provoked an increase in the P_i pool. A negative linear correlation was observed between P_i and ATP ($r=0.93$, $P<0.01$), whereas a positive correlation was observed for ADP and AMP ($r=0.63$ and $r=0.88$, $P<0.01$, respectively), Fig. 6. Degradation of PCr matched with an increase of creatine associated to release of P_i (Fig. 7).

A good negative correlation was observed between P_i and energy charge ($r=0.89$, $P<0.01$), Fig. 8.

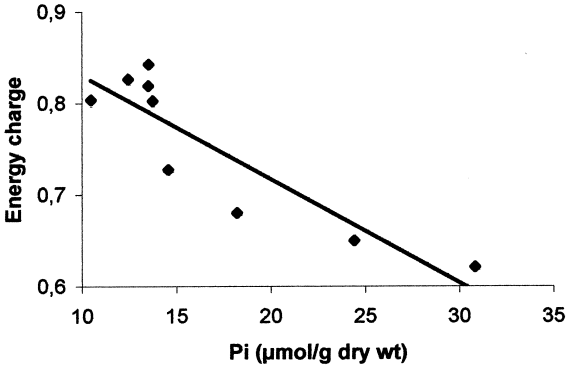

Fig. 8. Relationship of P_i concentration versus myocardial energy charge.

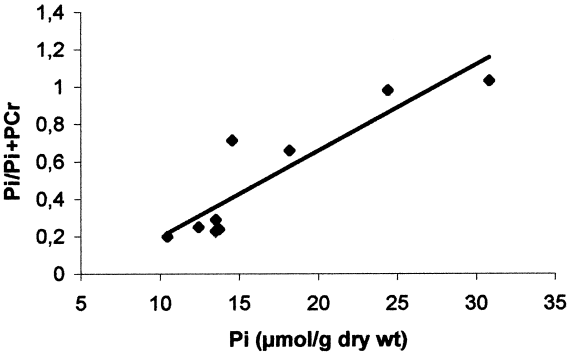

Fig. 9. Relationship of P_i concentration versus myocardial P_i/P_i+ PCr ratio.

On the contrary, an inverse correlation was found between P_i and P_i/P_i+PCr ($r=0.9$, $P<0.01$), Fig. 9.

In conclusion, an ion-pair reversed-phase HPLC method designed for measuring HEP compounds and P_i in biological samples has been developed. We are able to determine, in tissue acid extracts, nucleotides and nucleosides as well as creatine and creatine phosphate in a single run (20 min), and inorganic phosphate in a successive short run (8 min), using the same HPLC conditions. It appears that the present method gives a reproducible instrument in evaluating tissues high energy metabolism under physiological and pathological conditions.

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