INORGANIC PYROPHOSPHATE CONTENT IN ADULT GUINEA PIG LIVER

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1. Introduction

Reliable measurements of the concentration of inorganic pyrophosphate (PP_i) in biological material are not found in the literature. A very low intracellular concentration has to be expected, due to rapid hydrolysis by widely distributed inorganic pyrophosphatases. By simple hydrolysis the energy in PP_i is lost, but the possibility of maintaining a low concentration is thereby opened, and seems necessary to pull many synthetic processes, producing PP_i, in the right direction. On the other hand, recent papers of Nordlie et al. [1] on the phosphotransferase activity of glucose-6-phosphatase using PP_i as phosphate donor, raise the queation whether PP_i is involved in phosphorylating systems, thereby conserving some of the energy. However, if such systems are operating, a considerable PP_i concentration should be expected since K_m values for transferase activity are reported to be of the order of 10^{-3} M [1].

The present work was carried out in order to measure the metabolically active pool of PP_i in adult guinea pig liver. After intravenous injection of ^{32}P to guinea pigs, the specific activity (s.a.) of inorganic phosphate (P_i) and several nucleotides was determined in liver biopsies, taken from the anesthetized and artificially respired animals. The observation of a rapidly established isotope equilibrium between P_i and the pyrophosphate groups of the nucleotides opens up the possibility of calculating the s.a. of PP_i from measurements of that of P_i. This is based upon the assumption of an isotope equilibrium even between P_i and PP_i, the latter being derived directly from PP_i groups of the nucleotides. The experimental data confirm the validity of this assumption. PP_i was finally isolated from the biopsies with addition of known amounts of carrier. The sum of free and complex bound PP_i is found to be 24 ± 5.8 μM. Sources of error are investigated by altering the experimental procedure and are discussed in relation to the reliability of the method.

2. Materials and methods

Guinea pigs weighing about 300 g were anesthetized by intraperitoneal injection of Nembutal (30 mg/kg body weight) and were respired after tracheotomy using a Rodent Respirator (model 680, Harward Apparatus Co., Inc.). About 1 mC ^{32}P in phosphate buffer pH 7.4 was injected in the common iliac vein. Five to 8 biopsies weighing about 500 mg were taken from the same animal using the freezing clamp technique. The biopsies were weighed, homogenized in 10 ml icecold 1 M perchloric acid (PCA) with addition of 8.3 μmoles of carrier PP_i. After standing for 5 to 30 min (see discussion) at 0° with occasional stirring, the sediment was removed by centrifugation and the PCA neutralized with KOH. An aliquot was removed for determination of s.a. of P_i isolated as phosphomolybdate in benzene-isobutanol [2]. Another aliquot of the mixture was counted in a Tri-Carb liquid scintillation counter, and the P_i concentration was measured photometrically after reduction with stannous chloride. The remaining PCA extract was filtered through charcoal column until no more UV absorbing material could be detected in the effluent. Five g of Dovex 1 on formate form was added and the resin

was washed several times with water. This step was found necessary to remove impurities which would interfere with subsequent thin-layer chromatography. The material absorbed on the Dowex resin was eluted with 1.6 M ammonium formate pH 5 in the cold, and the salt was removed under reduced pressure. The residue was dissolved in 100 μl of water and anion exchange thin-layer chromatography on polyethylene-imide-cellulose covered PVC sheets was carried out using a LiCl gradient [3]. The pyrophosphate spot was detected on a guide strip cut through the chromatogram. After treatment of the strip with 10% ammonium molybdate in 5 M H_2SO_4 a marine blue color developed after irradiation under a short wave UV lamp. The PP_i spot was eluted with 2 M LiCl and the PP_i was precipitated as the manganese complex in 0.5 M acetate buffer pH 5 [4]. This step was included in order to eliminate traces, if any, of nucleotide triphosphate, which have R_f values close to that of PP_i. The precipitate was hydrolysed in 0.1 N HCl and the s.a. of P_i was determined as described above. The nucleotides adsorbed on charcoal in the start of the procedure were eluted with a mixture of pyridine-alcohol-water (10:40:50 by vol) and were separated by two dimensional anion exchange thin-layer chromatography on polyethylene-imide-cellulose for determination of their specific activity [3].

3. Results and discussion

Isotope equilibrium between P_i and the pyrophosphate groups in the nucleotides is achieved about 15 min after the injection of the tracer (fig. 1). Results of determination of PP_i in biopsies taken later than this are shown in table 1. No increase in the figures with increasing time after injection of the ^{32}P is observed, providing evidence for isotope equilibrium between P_i and PP_i in the liver. To disprove the possibility of PP_i formation through hydrolysis of the β-linkage in nucleotide triphosphates during the PCA extraction procedure, prolonged PCA treatment was employed. As seen from fig. 2 no increase in PP_i formation with increasing PCA treatment takes place, and the slope of the regression line calculated does not differ significantly from zero. The concentration of metabolically active PP_i in the liver is thus found to be 24 ± 5.8 μM. With the method used, complex

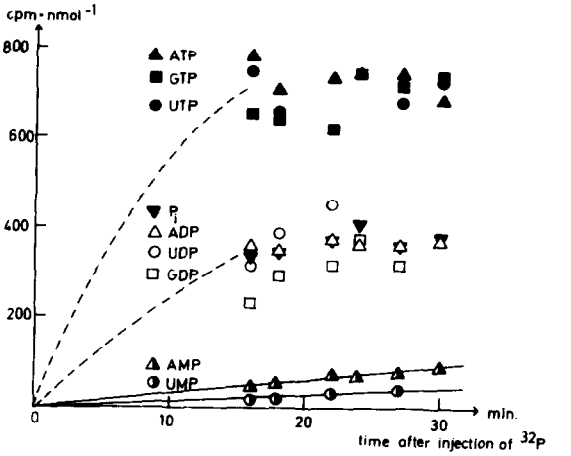

Fig. 1. Specific activity (s.a.) of inorganic phosphate (P_i) and some nucleotides isolated from biopsies taken at time intervals after injection of ^{32}P to animal no. 2. The di- and triphosphate values have been corrected for activity of the esterphosphate.

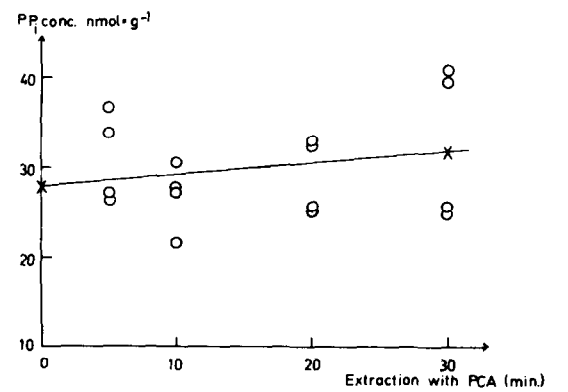

Fig. 2. PP_i concentration as a function of extraction time with PCA (animal no. 3). Slope of regression line 0.12 ± 0.22. No formation of PP_i through hydrolysis of the β-linkage in nucleotide triphosphates takes place.

bound PP_i is included, because PCA splits such complexes.

A rough estimate of ΔG for PP_i producing processes is now possible if data for the concentrations of the other reactants are available, and one assumes, that the activity coefficients tend to balance each other. Calculation of ΔG for the formation of UDPG from UTP and glucose-1-P at the following concentra-

Table 1

PP_i content in adult guinea pig liver, determined in biopsies taken from 3 animals injected with ^{32}P.

Animal No.	Time after injection of ^{32}P (min)	Weight of biopsies (g)	S.a. P_i (cpm $nmol^{-1}$)	S.a. P_i from PP_i isolated with carrier (cpm $nmol^{-1}$)	PP_i conc. calculated (nmol g^{-1})
1	11	0.663	680	1.26	18
	15	0.436	667	1.13	25
	20	2.433	640	5.54	23
2			439		
	16	0.557	482	0.577–0.592	18–18
			* 424		
			541		
	18	0.557	508	0.626–0.514	18–15
			474		
				0.985–0.989	28–28
			535		
	22	0.536	538	0.542–0.599	16–17
			541		
3	18	0.394	344	0.442–0.432	27–27
	18	0.358	317	0.385–0.418	28–30
	22	0.283	365	0.412–0.406	33–33
	22	0.301	372	0.531–0.561	39–42
	29	0.355	400	0.577–0.626	34–37
	29	0.415	394	0.546–0.423	28–22
	36	0.486	411	0.610–0.619	25–26
	36	0.446	404	0.554–0.532	26–25

8.3 μmole carrier PP_i has been used in all experiments. From the step, at which PP_i is applied for thin-layer chromatography duplicate analysis has been done. Standard deviation has been calculated from the 8 observations in animal no. 3.

* Determination of s.a. P_i in PCA extract treated with charcoal.

tions, UTP: 100 μM, UDPG: 500 μM, glucose-1-P: 60 μM [5] and the equilibrium constant 0.31 at pH 7.8 [6] gives about 1 kcal/mol. Apart from compartmentalization, and the fact, that the correct activity coefficients in vivo may be considerable different from those in the assay used to determine the equilibrium constant, the calculation indicates that processes, producing PP_i with standard affinities near zero, e.g. UDPG formation, proceed at concentrations close to equilibrium. Removal of PP_i is thus a critical step, and might serve as a regulatory device.

This phenomenon has been discussed in relation to development of neonatal jaundice [7]. Due to low activity of microsome glucose-6-phosphatase in the newborn liver [8], insufficient hydrolysis of PP_i by this enzyme [1] would cause an accumulation of PP_i, and inhibit the formation of UDPGA necessary for conjugation of bilirubin [9]. Inorganic pyrophosphatases from different animal sources have been shown to have the magnesium pyrophosphate complex as their true substrate [10,11]. K_m values for this substrate is reported to be in order of the total PP_i concentration found in the present work.

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