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INORGANIC PHOSPHATE IN EHRLICH ASCITES TUMOR CELLS AND ITS DISTRIBUTION ACROSS THE CELL MEMBRANE

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Summary

A regulatory function of the cell membrane in controlling the cytoplasmic level of P_i has been proposed, and in Ehrlich ascites tumor cells an active influx of primary phosphate has been reported in the literature.

In the present study, Ehrlich cells were incubated at 1.5—50 mM extracellular P_i at pH 7.4 (P_i mainly secondary phosphate) and at pH 6.0 (mainly primary phosphate), and the measured cell P_i was compared with the value expected from a passive distribution of P_i. At a low extracellular P_i concentration the cell P_i was 3—6 μmol/g or even more. It is suggested that a major part of this cell P_i can be accounted for by enzymic release of Pi during the sampling procedure. If this interpretation is correct, the present results show that both ionic species of P_i are in electrochemical equilibrium across the cell membrane at steady state. Moreover, in vivo the concentration of free P_i in the cytosol will presumably be maintained at a steady-state level of about 0.4 mM, one order of magnitude below the directly measured values. This implies that the ratio $[ATP]/[ADP][P_i]$ which is important in the regulation of energy metabolism, is higher than reported in the literature.

Introduction

The concentration of free inorganic orthophosphate (P_i) in the cytosol is of considerable physiological significance. P_i is an essential reactant in oxidative and glycolytic phosphorylations, and cell P_i has been assigned an important role in the control of energy metabolism [1]. Recently it has been established that the phosphorylation state of the cytoplasmic adenine nucleotide system (i.e. the ratio $[ATP]/[ADP][P_i]$) controls the rate of mitochondrial

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respiration and cytoplasmic phosphorylation [2—4]. Furthermore, P_i is essential, being the major physiological counter-anion for the energy-dependent Ca^{2+} uptake into the mitochondria [5]. A regulatory function of the cell membrane in controlling the cytoplasmic level of P_i has been proposed [6]. In Ehrlich ascites tumor cells Levinson [7] has reported an active influx of primary phosphate but a passive transport of secondary phosphate. On this basis Koobs [8] has suggested that the respiratory chain and, possibly, specific glycolytic enzymes have a preference for primary phosphate.

In Ehrlich cells and various other cells and tissues the measured cell P_i has been found to be considerably higher than expected for P_i to exert metabolic control. Based on estimates of the free cell P_i concentration from determination of the phosphorylation state a compartmentation of cell P_i has been proposed [9].

In this paper we propose as the most likely explanation of te experimental findings that, (i) a major part of the measured cell P_i can be accounted for by enzymic release of P_i during the sampling procedure from organic phosphate compounds with rapid metabolic turn-over; (ii) both primary and secondary phosphate ions at steady state are in electrochemical equilibrium across the cell membrane. This interpretation implies that the concentration of free P_i in the cytosol is determined solely by the extracellular concentration and the membrane potential. A preliminary report of this study has been presented [10].

Materials and Methods

The experiments were performed with Ehrlich ascites tumor cells (hyperdiploid strain) maintained by weekly transplantations into white female mice (NMRI) weighing 15—18 g. The cells were harvested 6—8 days after transplantation, discarding mice with bloody ascites, in 10—20 vols. of ice-cold Ringer solution (pH 7.4 or 6.0) containing heparin (2 I.U./ml). The Ringer's contained: 150 mM Na^+, 5.5 mM K^+, 1.0 mM Mg^{2+}, 0.01 mM Ca^{2+}, 155 mM Cl^-, 1 mM SO_4^{2-}, and 1.5 mM P_i, buffered with 10 mM *N*-tris-(hydroxymethyl)-methyl-2-aminoethanesulphonic acid (pH 7.4) or 2-(*N*-morpholino)ethanesulphonic acid (pH 6.0). The cells were washed twice and the cytocrit adjusted to 8%.

In order to investigate the dependence of the intracellular P_i concentration on the extracellular concentration of P_i the cells were incubated in Ringer solutions (see above) in which the P_i concentration was varied by isosmotic substitution of NaCl with mixtures of primary and secondary phosphate, taking pH and the osmotic coefficients into consideration [11]. The incubation medium contained 1.5, 10, 25, and 50 mM P_i. The cell batch was subdivided into four parts, each part being washed twice in the appropriate incubation medium and incubated at a cytocrit of 8% at 37°C in a Dubnoff shaking incubator. [^{3}H]Inulin ($5 \cdot 10^6$ dpm/ml, Radiochemical Center, Amersham, U.K.) was added to the cell suspensions as a marker of extracellular space. The osmolarity of the incubation media was in the range 287—305 mosM (Advanced Laboratory Osmometer, model 3 L, Advanced Instruments, Mass., U.S.A.). The ionic strength varied with the P_i concentration in the range 0.16—0.24 mol/kg.

Preliminary experiments at high P_i concentration showed that the cell P_i increases slowly with time, reaching a steady state after about 40 min incubation, in agreement with the findings of Levinson [7]. In the present experiments the cells were incubated for 40—60 min before samples were taken for determination of the cellular P_i concentration.

From each cell suspension two 500 μl samples were collected in preweighed vials, quickly cooled to 2°C on a cold-bath (—20°C) while being stirred, and centrifuged at 0°C (18 000 × *g*, 60 s). The packed cells were then kept on ice-bath. The supernatant was carefully removed for subsequent pH determination. In order to minimize hydrolysis of labile phosphate esters the packed cells were immediately deproteinized by addition of 450 μl ice-cold 0.9 M $HClO_4$ and centrifuged as before. 150 μl of the clear supernatant was neutralized to pH 7.2 by addition of 50 μl 0.4 M 3-(*N*-morpholino)propanesulphonic acid in 2 M KOH. After centrifugation the neutralized supernatants were immediately analyzed for P_i and ATP, and subsequently for ^3H activity and Cl^- concentration. 50-μl samples of the extracellular phase were processed in parallel with the packed cells.

The wet weight of the packet cells was determined after the deproteinization step, correcting for the weight of the $HClO_4$ added and the 150 μl sample of the supernatant removed. The dry weight of the packed cells was determined on two separate samples from each cell suspension by drying at 90°C for 48 h. The cellular concentrations are given as μmol/ml cell water.

Inorganic orthophosphate was determined by the solvent extraction procedure of Vestergaard-Bogind [12] and, in some experiments, by the method of Baginski et al. [13]. In control experiments these methods were compared with the methods of Lowry and Lopez [14] and of Wahler and Wollenberger [15] which use low molybdate concentrations and low acid strength in the reaction mixture in order to minimize acid-molybdate-catalyzed hydrolysis of labile phosphate esters. By applying these different methods of P_i analysis on the same cell batch essentially identical results were obtained.

Chloride was determined by automatic, coulometric titration with potentiometric end-point detection (CMT 10 Chloride Titrator, Radiometer, Copenhagen, Denmark).

ATP concentration in the neutralized cell lysate was measured by the luciferin-luciferase method (see ref. 16). In some experiments ADP was determined from the increase in ATP concentration following quantitative conversion to ATP by addition of phosphoenolpyruvate, puruvate kinase, lactate dehydrogenase, and NADH.

pH measurements on 5—10 μl samples were made with a micro system (BMS 1 and PHM 72, Radiometer, Copenhagen, Denmark).

^3H activity was measured by liquid scintillation counting (Packard Tri-Carb, model 333045) using 50-μl samples of neutralized supernatant in a gel consisting of 10 ml Insta-gel (Packard) and 4 ml distilled water. Following this procedure no instability of [^3H]inulin in the counting vials could be detected (cf. ref. 17).

From each cell suspension two samples were collected and processed in parallel. All measurements were made in duplicate. From the deviations between the duplicate samples the overall standard deviation (S.D.) and coeffi-

cient of variation was calculated [18]. The coefficient of variation was 3% for cells and 2% for media in the measurements of both P_i and Cl^-, and 8% in the measurements of cell ATP. The pH measurements of the media showed a S.D. of 0.015 pH units.

Results

Ehrlich cells were incubated at varying extracellular P_i concentration at pH 7.4 and at pH 6.0 and the P_i concentration in the cell water was measured. Figs. 1A and 2A show that a linear relation was found between the measured cell P_i and the extracellular P_i concentration at both pH values. When the curves are extrapolated to zero extracellular P_i concentration they intersect the ordinate corresponding to a cell P_i of approx. 22 mM at pH 7.4 and approx. 5 mM at pH 6.0.

In each sample the measured cell P_i was compared with the value expected assuming a passive distribution of primary as well as secondary phosphate. Between pH 6.0 and 7.4 P_i is essentially a mixture of primary and secondary phosphate. Assuming a passive distribution of Cl^- across the cell membrane at steady state, as demonstrated in the study of Lassen et al. [19], and equal activity coefficients in cell water and in the extracellular medium, and neglecting intracellular sequestration of Cl^- [20], the distribution ratio for monovalent anions in electrochemical equilibrium will equal the distribution ratio for Cl^-, (r_{Cl^-}), and for divalent anions will equal $(r_{Cl^-})^2$. Therefore, the cell P_i expected from a passive distribution of both ionic species of P_i, $(P_i)_c^{calc}$, can be calculated from the extracellular concentration of primary phosphate, $(P_i^-)_m$, and secondary phosphate, $(P_i^{2-})_m$:

$$(P_i)_c^{calc} = (P_i^-)_m \cdot r_{Cl^-} + (P_i^{2-})_m \cdot (r_{Cl^-})^2$$

Dividing by the P_i concentration in the medium, $(P_i)_m$, the distribution

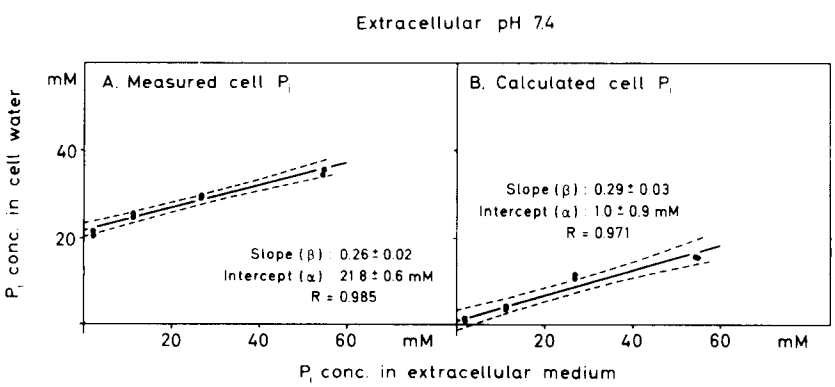

Fig. 1. Measured and calculated P_i concentration in cell water at steady state as a function of the extracellular P_i concentration at pH 7.4. Panel A shows a plot of the measured P_i concentration in cell water vs. the P_i concentration in the extracellular medium. The expected P_i concentration in cell water was calculated, assuming passive distribution of both primary and secondary phosphate (for details see text). In panel B these calculated P_i concentrations in cell water are plotted against the P_i concentration in the medium. The regression line is shown on the figure with the 95% confidence limits indicated (dotted lines). The intercept (α) and slope (β) is given ±S.D. R is the coefficient of correlation.

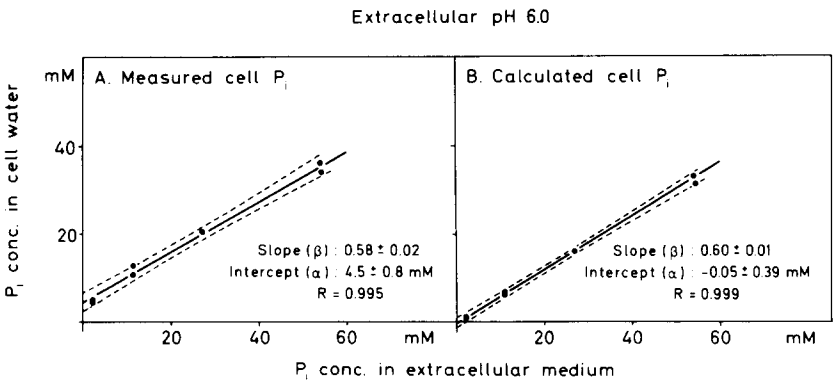

Fig. 2. Measured and calculated P_i concentration in cell water at steady state as a function of the P_i concentration in the extracellular medium at pH 6.0. Details in Fig. 1.

ratio for P_i (r_{P_i}) corresponding to a passive distribution of primary and secondary phosphate can be expressed as a weighted average between r_{Cl^-} and $(r_{Cl^-})^2$:

$$r_{P_i} = (P_i)_c^{calc}/(P_i)_m = f_{P^-} \cdot r_{Cl^-} + f_{P^{2-}} \cdot (r_{Cl^-})^2$$

The fraction of the total P_i in the extracellular medium present as primary phosphate (f_{P^-}) and as secondary phosphate ($f_{P^{2-}}$) was calculated from the extracellular pH of the sample and the acid dissociation constant of primary phosphate [21], $K_{ac\,A}H_2PO_4^-$:

$$pH = pK_{ac\,A}H_2PO_4^- + \log\{(f_{P^{2-}})/(f_{P^-})\}$$

$pK_{ac\,A}H_2PO_4^-$ was estimated from the following equations [21], which is a good approximation for ionic strengths (I) between 0.10 and 0.17 mol/kg:

$$pK_{ac\,A}H_2PO_4^- = 7.029 - 0.595 \cdot \sqrt{I} - 0.0013 \cdot \Delta T + 0.00006 \cdot (\Delta T)^2$$

where $\Delta T = (T - 310.15)$ K. The ionic strength of the extracellular medium was in the range 0.16—0.24 mol/kg, and the calculated $pK_{ac\,A}H_2PO_4^-$ in the range 6.79—6.74. At pH 7.4 primary phosphate only amounts to about 15% of the total P_i, whereas at pH 6.0 P_i is present predominantly (about 80%) as primary phosphate.

Using the above equations, $(P_i)_c^{calc}$ was calculated in each sample from the measured values of $(P_i)_m$, the pH of the medium, and r_{Cl^-}. The calculated cell P_i at pH 7.4 and 6.0 is plotted against the extracellular P_i concentration in Figs. 1B and 2B. These plots show a linear relationship indicating only minor variations in r_{Cl^-} and pH between the cell suspensions from the same cell batch. The slopes do not differ significantly from the slopes in Figs. 1A and 2A. In Fig. 3 the measured cell P_i in each sample is plotted against the calculated cell P_i in the same sample for the experiments shown in Figs. 1 and 2. At both pH values the slope calculated by regression analysis is close to unity. Table I summarizes the data from similar plots in five experiments.

In order to interpret these findings it is essential to consider the high P_i content found in cells incubated at low extracellular P_i (see Figs. 1A, 2A). If this measured cell P_i is present as free orthophosphate in the cytosol, the cell

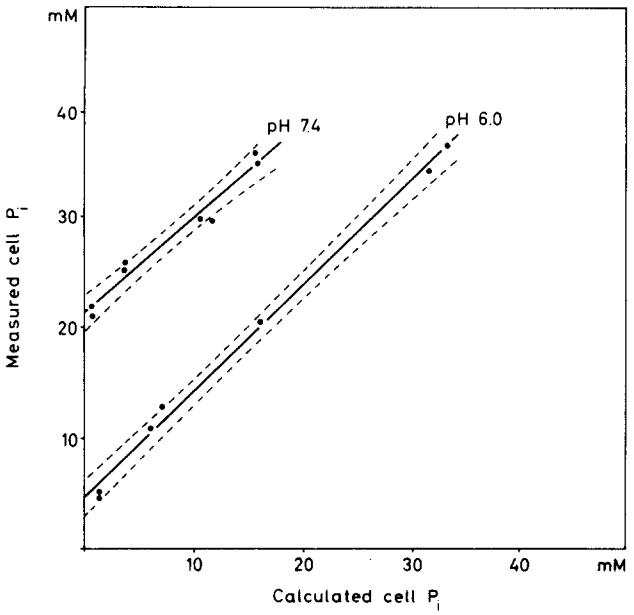

Fig. 3. Measured P_i concentration in cell water as a function of the cell P_i calculated assuming passive distribution of P_i at pH 7.4 and 6.0. At pH 7.4 the parameters of the regression line are: slope, $\beta = 0.86 \pm 0.07$; intercept, $\alpha = 21.1 \pm 0.7$ mM; coefficient of correlation, $R = 0.983$. At pH 6.0 the parameters are: $\beta = 0.96 \pm 0.03$; $\alpha = 4.6 \pm 0.6$ mM; $R = 0.997$. The 95% confidence limits are indicated (dotted lines).

P_i is maintained at a level above that corresponding to electrochemical equilibrium, implying an active influx of P_i. If however, P_i is passively distributed across the cell membrane the major part of the cell P_i measured at low extracellular P_i must either be present in the cell in sequestered form or alternatively arise from hydrolysis of labile organic phosphate compounds in the cell ("apparent P_i").

In separate experiments at pH 7.4 and 6.0 (see Table II) cellular P_i contents comparable to the intercepts on the ordinate in Figs. 1A and 2A were found in cells incubated at low extracellular P_i concentration. The P_i content was

TABLE I

CORRELATION BETWEEN MEASURED CELL P_i AND CELL P_i EXPECTED FROM A PASSIVE DISTRIBUTION OF PRIMARY AND SECONDARY PHOSPHATE

Ehrlich cells were incubated at pH and temperature given in the table. The measured cell P_i at steady state was plotted against cell P_i calculated assuming passive distribution of P_i (see Fig. 3). These plots gave straight lines with the slope (β) and coefficient of correlation (R) calculated by regression analysis. The last column gives the probability (P) of $\beta = 1$, calculated from Student's t-test.

pH	Temperature (°C)	Slope (β) ±S.D.	R	P ($\beta = 1$) (%)
7.4	37	0.86 ± 0.07	0.983	9
7.4	37	0.88 ± 0.09	0.968	25—30
7.4	25	1.06 ± 0.14	0.953	60—70
6.0	37	0.96 ± 0.03	0.997	25—30
6.0	25	0.95 ± 0.04	0.992	30

TABLE II

CELLULAR P_i AND ATP CONCENTRATIONS AT pH 7.4 AND 6.0 IN P_i-FREE MEDIUM AND AFTER ADDITION OF 2-DEOXYGLUCOSE

The cells were incubated at pH 7.4 and 6.0 at 37°C for 60—80 min in medium containing 1.5 mM P_i (control) or in P_i-free medium. 2-Deoxyglucose (20 mM) was added to cell suspension in 1.5 mM P_i medium 30 min before sampling. * The values given are the mean of duplicate.

Experimental condition	pH 7.4		pH 6.0	
	P_i (mM)	ATP (mM)	P_i (mM)	ATP (mM)
Control (1.5 mM P_i Ringer's)	17.1	4.0	6.4	5.4
P_i-free Ringer's	11.6	4.0	4.4	5.8
1.5 mM P_i Ringer's with 20 mM 2-deoxyglucose	2.6	0.3	3.5	0.5

* In the control experiment the ATP/ADP ratio was approx. 4.5 at pH 7.4 and approx. 7.4 at pH 6.0.

only slightly reduced upon incubation in P_i-free media for more than one hour, whereas addition of 2-deoxyglucose, which irreversibly depletes the adenine nucleotide pool of the cells [22], was followed by a decrease of the cellular P_i content to about 3 mM. These findings are in accordance with other reports [22,23] and taken together they suggest that the measured cell P_i does not represent free P_i in the cytosol, but rather represents apparent P_i, i.e. P_i arising from hydrolysis of labile organic phosphate compounds during the sampling and deproteinization procedure or during the P_i determination.

Control experiments (data not shown) gave no indication of acid-molybdate catalyzed hydrolysis of labile phosphate esters during the P_i determination: (i) addition of ATP to the cell samples revealed no hydrolysis of ATP during processing of the sample; (ii) the measured P_i content of the samples was unchanged following adsorption of the nucleotides by treatment with charcoal [24]; (iii) the measured cell P_i was found to be invariant with the P_i analyses used, although acid strength and molybdate concentrations in the reaction mixture are different (see Materials and Methods); furthermore, the same cell P_i values were obtained in preliminary experiments with enzymic P_i determination [16]; (iv) in the analysis of Lowry and Lopez [14] the time course of the colour development was identical for cell samples and P_i standards; (v) the measured P_i content of the cell samples was reduced to zero following specific precipitation of P_i with uranyl acetate [25]. In addition, samples deproteinized following the procedure of Somogyi [26] which also precipitates P_i were found to be P_i free.

When Ehrlich cells are incubated in P_i-free media the cell P_i can be determined from the difference between the P_i concentration of the cell suspension and the P_i concentration of the extracellular medium. In these experiments (at pH 7.4) various deproteinization procedures were compared: (i) the standard procedure using 0.9 M $HClO_4$; (ii) low $HClO_4$ concentration (0.4 M); (iii) neutral deproteinization with organic solvents (1 vol. chloroform/methanol, 1 : 1, v/v). The cell P_i was found to be invariant with the deproteinization procedure used. Also in these samples the measured cell P_i was demonstrated to be present as P_i: (i) the various methods of P_i analysis [12—14] gave essentially identical results; (ii) there was no indication of the presence of

labile organic phosphates [14]; and (iii) the phosphate content was quantitatively precipitated with uranyl acetate [25].

However, in the above experiments where the cell suspensions were incubated in P_i-free media at pH 7.4 and deproteinized directly without prior centrifugation, the cell P_i was reduced to 4—7 mM (6 experiments), significantly lower than the values obtained in experiments with a large number of samples and using the normal sampling procedure with packing of the cells before deproteinization (see Table II and Fig. 1A). This finding is taken to indicate that enzymic release of P_i by hydrolysis of organic phosphate compounds during the normal sampling procedure contributes significantly to the measured cell P_i.

Discussion

In the present experiments a linear relation with a slope of unity is found in plots of the measured cell P_i against the cell P_i calculated assuming a passive distribution across the cell membrane of both primary and secondary phosphate (Fig. 3 and Table I). This is the case both at pH 7.4, where only about 15% of P_i is present as primary phosphate, and at pH 6.0 where primary phosphate amounts to about 80% of the total P_i. This finding is in conflict with the results obtained at pH 7.4 by Levinson [7] who concluded that secondary phosphate is passively distributed across the cell membrane, whereas primary phosphate in the cell is maintained at a level above that corresponding to electrochemical equilibrium, implying an active influx of primary phosphate. A passive distribution of both primary and secondary phosphate implies, as discussed by Harris [27], that the hydrogen ions are also in electrochemical equilibrium across the membrane. This is in accordance with the results of Levinson [7] based on pH measurements on cell lysate, but in contrast to the results of Poole et al. [28] based on the distribution of 5,5-dimethyl-oxazolidine-2,4-dione.

The finding of a high cell P_i content in cells incubated at low extracellular P_i concentration (Figs. 1A, 2A and Table II) is in accordance with the findings of Levinson [7] and with several other reports in the literature (e.g. refs. 22, 29 and 30). This high cell P_i might be present in the cell in vivo as: (i) free orthophosphate in the cytosol or as (ii) sequestered P_i or (iii) arise during the sampling procedure by enzymic release from labile organic phosphate compounds in the cell.

The first possibility would imply an active influx of P_i. However, such an active influx must saturate at a very low extracellular P_i concentration as the plots of cell P_i vs. extracellular P_i (Figs. 1A, 2A) show a linear relation in the range 1.5—50 mM P_i with a slope corresponding to a passive distribution of P_i across the cell membrane. The results of incubation in P_i-free media and of addition of 2-deoxyglucose (Table II) are considered evidence against the presence of a high concentration of free P_i in the cytosol at low extracellular P_i concentration. In Ehrlich cells an active influx of P_i has been reported based on the finding that the uptake of ^{32}P-labelled P_i is sensitive to metabolic inhibition [31]. Recently, a passive transport of P_i across the cell membrane and an energy-dependent uptake in the mitochondria has been suggested [30].

Levinson [7] has suggested intracellular binding to account for the high

cell P_i at low extracellular P_i concentration. However, from available evidence, sequestered P_i is not likely to account for a major part of the high cell P_i. Sequestered P_i is present in the mitochondria which can accumulate impressive amounts of both calcium and phosphate as insoluble, but readily exchangeable, calcium phosphate complexes: in Ehrlich cells more than 1000 nmol calcium per mg mitochondrial protein, corresponding to 10 μmol/g cells [32]. However, freshly isolated mitochondria from Ehrlich cells are reported to contain only 20 nmol/mg protein, corresponding to about 0.2 μmol/g cells [32]. The endogenous content of P_i in mitochondria is of similar magnitude (10 nmol/mg protein [33]). From these data the mitochondrial P_i content can only account for a minor part of the measured cell P_i. A high content of P_i in the nucleus has been reported, but these findings have recently been questioned [34]. Furthermore, cell P_i has been demonstrated to exchange extremely rapidly with extracellular ^{32}P-labelled P_i and shows no indication of compartmentation [31].

The concentration of free P_i in the cytosol can be estimated from the intracellular concentration of metabolic intermediates. Recent evidence indicates that a near-equilibrium exists between the phosphorylation state, $[ATP]/[ADP][HPO_4^{2-}]$, of the cytoplasmic adenine nucleotide system, the "redox state" of the cytoplasmic NAD couple, and the mitochondrial respiratory chain [2,3]. Based on the intracellular concentrations of glycolytic intermediates in Ehrlich cells given by Coe (Table IV in ref. 35) the phosphorylation state can be calculated (cf. ref. 3) to 4300 M^{-1}, and from the ATP/ADP ratio given the concentration of free P_i in the cytosol for cells incubated in 2 mM extracellular P_i can be estimated to 1.7 mM. This suggests that the concentration of free cell P_i in vivo is indeed low.

A similar situation may exist in several other cells and tissues where the cell P_i in vivo and in vitro has been reported to be considerably higher than the extracellular P_i concentration (e.g. refs. 2, 14, 36–38). However, in human red blood cells, P_i is found to be passively distributed across the cell membrane with the cell P_i being proportional to the extracellular P_i concentration [39].

Based on the above considerations we suggest as the most straightforward explanation that a major part of the measured cell P_i at low extracellular P_i concentration can be accounted for by enzymic release of P_i during the sampling procedure from organic phosphates in the cell. In support of this explanation Ehrlich cells have been found to contain a high concentration of organic phosphate compounds (e.g. see ref. 22) with an extremely rapid metabolic turn-over [31]. If this interpretation is correct, the finding of a slope of unity in the plots of measured vs. calculated cell P_i (Fig. 3 and Table I) will indicate a passive distribution of P_i across the cell membrane. A passive distribution of P_i is consistent with the demonstration of a carrier-mediated transport of P_i in Ehrlich cells [40].

From the concentration of P_i and the ATP/ADP ratio the phosphorylation state can be calculated and the ΔG for ATP hydrolysis estimated from the equation:

$$\Delta G_{ATP} = \Delta G'_{0ATP} + RT \ln\{[ADP][HPO_4^{2-}]/[ATP]\}$$

where $\Delta G'_{0ATP}$, the standard free energy for hydrolysis of ATP at pH 7.0 and

1 mM free Mg^{2+}, is −31.9 kJ/mol (cf. ref. 2). From the values measured at 1.5 mM extracellular P_i and pH 7.4 (see Table II) the phosphorylation state is calculated to 393 M^{-1} and ΔG_{ATP} to −47.2 kJ/mol, in reasonable agreement with the values reported in, e.g., liver cells and ascites tumor cells [2,3]. This calculation is based on the direct measured values and the assumption that all three reactants are free in the cytosol. However, if the above interpretation is correct, the free inorganic phosphate ions at steady state are in electrochemical equilibrium across the cell membrane, and calculated from the Cl^- distribution ratio and an extracellular P_i concentration of 1.5 mM, the free P_i concentration in the cell is only about 0.4 mM, more than one order of magnitude below the directly measured concentration.

In vivo the P_i concentration in plasma and in the extracellular fluid is by homeostatic regulation maintained at a rather constant level of about 1.2 mM. Consequently, under the above assumptions the intracellular concentration of free P_i in Ehrlich cells will normally be maintained at a rather constant steady-state level of about 0.4 mM. Under conditions with decreased serum P_i level, the free cell P_i will be correspondingly reduced. This may be significant in relation to the cellular dysfunctions associated with hypophosphatemia [41]. Furthermore, during transient metabolic states as seen under the conditions of the Crabtree effect [1], free cell P_i can conceivably be reduced to much lower values.

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