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^{32}P-labelling anomalies in human erythrocytes

Is there more than one pool of cellular P_i?

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1. Human erythrocytes were incubated in autologous plasma containing [^{32}P]P_i, and sampled by a method which avoids washing the cells. 2. In experiments of up to 3 h duration, the specific radioactivity of cellular P_i stabilized at a value below that of extracellular P_i. This can be explained on the basis of a single cellular P_i pool exchanging with a large unlabelled pool of cellular organic phosphates. 3. However, a rapid initial phase of labelling, occurring within 30 s, was inconsistent with the situation described in point 2. A possible explanation is that about $\frac{1}{4}$ of cellular P_i occurs in a separate, fast-labelling pool. 4. When the extracellular P_i concentration was doubled, most of the corresponding increase in the steady-state cellular P_i concentration was accounted for by the apparent fast-labelling P_i pool, which also doubled. 5. The observed initial rate of labelling of cellular organic phosphates [which probably occurs through the reaction catalysed by glyceraldehyde-3-phosphate dehydrogenase (E.C. 1.2.1.12)] was considerably lower than that predicted from the flux through the Embden–Meyerhof pathway. This implies that the enzyme is exposed to P_i whose specific radioactivity is lower than the mean specific radioactivity of cellular P_i, and fails to support earlier suggestions that this enzyme uses extracellular P_i. 6. In 3 h incubations, the rate of organic phosphate labelling was roughly constant throughout, even though the specific radioactivity of cellular P_i had risen slowly to a plateau. Viewed in conjunction with point 5, this again suggests some inhomogeneity in cellular P_i. 7. Cellular P_i and extracellular P_i only reached isotopic steady state after 2 days. At this stage some organic phosphates were probably still incompletely labelled. 8. We conclude that, whatever their physical or technical reasons, such labelling inhomogeneities and slow attainment of isotopic steady state may cause serious misinterpretation of results if ignored during ^{32}P-labelling of intact cells.

INTRODUCTION

There is currently considerable interest in the [^{32}P]P_i labelling of mammalian cells, for two reasons. Firstly, there is growing recognition of the importance of P_i in regulating cell function [1,2], even though there is uncertainty over whether P_i transport can be explained exclusively by direct transport through the membrane [3–6] or by an additional group translocation mechanism by glyceraldehyde-3-phosphate dehydrogenase (G3PDH; EC 1.2.1.12), incorporating extracellular P_i directly into cellular ATP [7–10]. Secondly, [^{32}P]P_i labelling of intact cells is widely used in studying labelling of phosphoproteins and phospholipids (notably phosphoinositides). A recent important study [11] demonstrated that, in human erythrocytes, studies of phosphoinositides may be complicated by the existence of multiple metabolic pools of phosphoinositides and phosphatidate. Such studies depend critically on the specific radioactivities of organic phosphates (e.g. the γ-terminal phosphate group of ATP). These, in their turn, are determined by the specific radioactivity of P_i. Therefore, in the present study, we have re-examined cellular [^{32}P]P_i labelling in the supposedly simple case of human erythrocytes, to see whether such experiments are further complicated by heterogeneity in the labelling of cellular P_i.

Parts of this work have been described in preliminary reports [12–14].

MATERIALS AND METHODS

Notation and method of presentation of results

Concentrations of phosphorus metabolites (C) are expressed as mmol/l of cells or of extracellular medium. ^{32}P Radioactivities (A) are expressed as c.p.m./l, and specific radioactivities ($a = A/C$) are expressed as c.p.m./mmol of phosphorus. Subscripts $_{1,2,2f,2s,3}$ denote phosphorus pools defined in the theoretical models below. Superscripts ^{0,3} denote values 0 or 3 min after addition of [^{32}P]P_i to the cell suspension.

As a result of [^{32}P]P_i entering the cell, the extracellular radioactivity (A_1) or specific radioactivity (a_1) of [^{32}P]P_i gradually declined (e.g. see Fig. 1c). Therefore for conceptual clarity, cellular labelling is generally expressed as ratios (e.g. a_2/a_1 or A_3/a_1).

Abbreviations used: 2,3-BPG, 2,3-bisphosphoglycerate; G3PDH, glyceraldehyde-3-phosphate dehydrogenase (EC 1.2.1.12); DBP, di-n-butyl phthalate; C, A and a are concentration (mmol/l), radioactivity (c.p.m./l) and specific radioactivity (c.p.m./mmol) respectively (see the Materials and methods section); n, number of independent replicate experiments.

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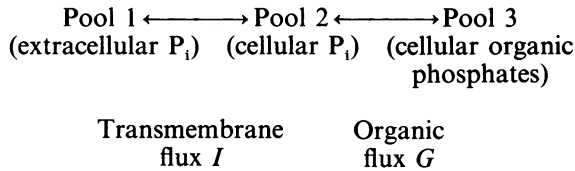
Theoretical models

(A) One cellular P_i pool. Cellular phosphate metabolism can be described by a simple 'catenary' model [15] as follows:



All metabolites are at steady state. Extracellular P_i (pool 1) exchanges with a single cellular P_i pool (pool 2) at a rate I mmol/h per l of cells (the 'transmembrane flux'). Similarly, cellular P_i exchanges with cellular organic phosphates (pool 3) at a rate G (mmol/h per l of cells; the 'organic flux'). If, at time $t = 0$, $[^{32}P]P_i$ is added to the extracellular medium, ^{32}P is transferred to cellular P_i and then to organic phosphates. The specific radioactivity of cellular P_i should ultimately rise until it equals that of extracellular P_i ($a_1 = a_2$ at 'isotopic steady state'). However, if the concentration of exchangeable organic phosphate groups (C_3) is much greater than the cellular P_i concentration (C_2) (as in e.g. [16]), the specific radioactivity of the organic phosphates (a_3) should be negligible in short experiments. Consequently, the phosphorus in the back-flux from organic phosphates to cellular P_i is essentially unlabelled and, if the organic flux (G) is at least of the same order as the transmembrane flux (I), the specific radioactivity of cellular P_i will be maintained below that of extracellular P_i. Consequently, the ratio a_2/a_1 rises at an initial rate I/C_2 and temporarily stabilizes at a plateau value [approx. $I/(I+G)$], which is considerably below unity, the value expected at isotopic steady state.

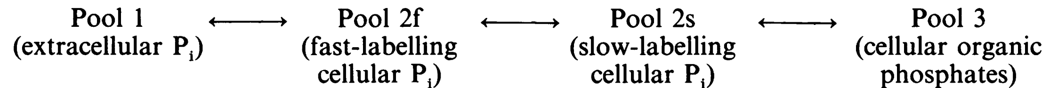
Long incubations with $[^{32}P]P_i$. In longer incubations, the specific radioactivity of cellular organic phosphates (a_3) can no longer be neglected, so there will be a significant back-flux of ^{32}P from organic phosphates to cellular P_i, whose specific radioactivity will slowly rise, eventually reaching that of extracellular P_i (a_1). The corresponding rate of increase of organic phosphate labelling will slacken and will be zero when all phosphorus pools reach isotopic steady state ($a_1 = a_2 = a_3$). At this stage, $A_3/a_1 = C_3$, the total concentration of exchangeable organic phosphate groups.

Organic phosphate labelling and glycolysis. In short experiments, the mean rate of labelling of organic phosphates is given by the product of the mean specific radioactivity of cellular P_i and the organic flux, G. The time course of organic phosphate labelling, expressed as A_3/a_1, should be linear once a_2/a_1 has reached its plateau value (see above), with slope $S = d(A_3/a_1)/dt = GI/(G+I)$.

In human erythrocytes, flux from cellular P_i to organic phosphates occurs through the reaction catalysed by G3PDH in the Embden–Meyerhof pathway [7–10]. From the stoichiometry of this pathway, if this reaction were far from equilibrium, 1 mol of P_i should be incorporated per mol of lactate generated. We would therefore predict that $G = L$, the lactate production rate. However, because the reaction may not be far from equilibrium so that incorporation of label can occur without net lactate generation [17,18], and because other (unknown) pathways might contribute to G, we can write $G \geqslant L$ [15].

As $S = G \times (a_2/a_1)$ (see above), we therefore predict that $S/L \geqslant (a_2/a_1)_m$ where m denotes the mean measured value over the period in which S is measured.

(B) Two cellular P_i pools. Consider a modification to Model A as follows:



Assume that the fast-labelling pool (2f), which is a fraction F of total cellular P_i, exchanges so rapidly with extracellular P_i that it attains the same specific radioactivity as extracellular P_i before the cell suspension can be sampled. The slower-labelling P_i pool (2s) exchanges with pool 2f (or, indistinguishably, with extracellular P_i) and with organic phosphates (pool 3), consequently behaving just like the single cellular P_i pool in Model A.

The first measurable value of the relative specific radioactivity (a_2/a_1) represents approximately complete labelling of pool 2f alone, and is an estimate of F. The relative specific radioactivity of the slower-labelling pool (2s) at any given time (a_{2s}/a_1) can be calculated as $[(a_2/a_1)-F]/(1-F)$.

Choice of incubation conditions

Cells were incubated in autologous plasma at 37 °C under humidified O_2/CO_2 (19:1), and for comparison at 37 °C under air in a Hepes-buffered Ringer solution ('Hepes/Ringer solution') of the following composition: NaCl, 120 mM; Hepes acid, 25 mM; KCl, 5.6 mM; glucose, 5 mM; $MgCl_2$, 2 mM and $CaCl_2$, 2 mM, titrated to pH 7.4 at 37 °C with NaOH. Unless otherwise stated, Na_2HPO_4 (1 mM) was also present. In both media, cellular concentrations of P_i and 2,3-bisphosphoglycerate (2,3-BPG) are stable for 48 h [19,20].

Incubation with concentrated suspensions

Heparinized whole blood was obtained by venipuncture from non-fasted healthy volunteers. For experiments in Hepes/Ringer solution, the cells were centrifuged at 1000 **g** and 4 °C for 5 min. They were resuspended in Hepes/Ringer solution to a haematocrit of approx. 30% and the centrifugation was repeated. The cells were then resuspended in fresh medium to give a final haematocrit of 45% and were incubated at 37 °C for 15–30 min before adding $[^{32}P]P_i$.

$[^{32}P]P_i$ (37 kBq or 1 μCi) was added to each 1 ml of whole blood or cell suspension at $t = 0$ and mixed by gentle vortexing for 2 s. At timed intervals from $t = \frac{1}{2}$ min onwards, 60 μl aliquots of suspension were sampled and immediately deproteinized with 660 μl of ice-cold 0.73 M-$HClO_4$. A further 350 μl of suspension was expelled into ice-cold di-*n*-butyl phthalate (DBP) and immediately centrifuged at 1700 **g** for 2 min at 2 °C, so that the cells were rapidly chilled, thus slowing transmembrane P_i movement, and sank through the DBP. The extracellular medium was left floating above the DBP, and a 60 μl aliquot was deproteinized as above.

The perchlorate extracts of suspension or medium were then centrifuged at 3000 **g** for 10 min at 2 °C to remove precipitated protein. The supernatants were placed on ice and, within 1 h of adding the $HClO_4$, were adjusted to pH 6.5–7.5 by titrating in 4.3 M-KOH containing 0.6 M-imidazole. To assist neutralization, a wide-range pH indicator (BDH 21072) was added (final concentration 2 %, v/v). Neutralized extracts were stored at −20 °C.

Cellular concentrations of P_i (in mmol/l of cells) and ^{32}P (in c.p.m./l of cells) were calculated [21] from values measured in suspension and medium, using the haematocrit value.

Incubations up to 48 h in dilute suspensions

Heparinized blood was centrifuged at 1000 **g** at 2 °C for 10 min to sediment the cells, and the plasma was removed. The loosely packed cells were added to plasma or Hepes/Ringer solution to give a suspension of approx. 3 % haematocrit. [^{3}H]Water was added to give 15 kBq (0.4 μCi) per ml of suspension. At $t = 0$, 37 kBq (1 μCi) of [^{32}P]P_i was added to each 1 ml of suspension and rapidly mixed. At timed intervals, 1 ml aliquots were withdrawn into a water-jacketed syringe at 37 °C and injected through a silicone rubber seal into a polypropylene tube containing 2.5 ml of DBP over a layer of 400 μl of 1 M-$HClO_4$. This was immediately centrifuged at 3000 **g** at room temperature for 1 min, and as rapidly as possible the tubes were placed on ice and the $HClO_4$ cell extract was removed and neutralized (with KOH and imidazole as described above). During this 1 min centrifugation, the cells are concentrated into a pellet of 70–80 % haematocrit which sinks through the DBP into the $HClO_4$. The haematocrit of the cell pellet was determined by sampling a parallel incubation containing 15 kBq (0.4 μCi) of [^{3}H]water and 15 kBq (0.4 μCi) of inulin[^{14}C]carboxylic acid per ml of suspension, but no [^{32}P]P_i. The inulin (unlike [^{3}H]water) is excluded from the cells so that the relative radioactivities of ^{3}H and ^{14}C in the extracellular medium and in the cell pellet can be used to calculate the haematocrit of the pellet [22].

Concentrations of P_i and radioactivities of ^{32}P were calculated per litre of cellular or extracellular water and, to facilitate comparison with concentrated suspensions, were converted to mmol/l of cells or plasma, assuming that erythrocytes are 72 % [23] and plasma is 93 % [24] water by volume.

For incubations of more than 3 h duration, aseptic technique was used.

Analytical methods

For measurement of P_i [21] ammonium molybdate [100 μl of a 3.35 % (w/v) solution of $(NH_4)_6Mo_7O_{24}$,$4H_2O$ in 2.4 M-HCl] was added to 200 μl of neutralized deproteinized sample on ice, and immediately 150 μl of a 4:1 (v/v) mixture of 2-methyl propan-1-ol and light petroleum (boiling range 80–100 °C) was added and vortexed. P_i is selectively extracted as phosphomolybdate into the organic solvent phase. An aliquot of the organic phase (80 μl) was mixed at room temperature with 160 μl of ethanol. To this was added 20 μl of stannous chloride [freshly made 4 % (w/v) $SnCl_2$, $2H_2O$ in 1.65 M-HCl] which reduced phosphomolybdate to a coloured species whose absorbance was measured at 725 nm after approx. 30 min.

This solvent extraction was also employed to separate [^{32}P]P_i (in the organic phase) from ^{32}P in organic phosphates (measured in the aqueous phase after neutralization with 10 M-NaOH). Small variations in recovery were corrected for by comparing the ^{32}P radioactivity measured directly in medium containing only [^{32}P]P_i with that measured in an organic solvent extract (prepared as described above) of a deproteinized sample of the same medium.

Lactate was assayed as described in [25]. Glucose was measured colorimetrically with a commercial kit (Sigma 510). The pH of the cell suspensions and Hepes/Ringer solution was determined by using a glass electrode calibrated at 37 °C.

Results are expressed as means ± S.E.M. The statistical significance of differences was assessed by Student's t analysis. Mean rates of change were calculated by linear regression. Half-times were calculated from the slopes of appropriate semilogarithmic plots.

Materials

Hepes acid was obtained from Sigma (H-3375). All other reagents were from BDH and, where available, were of AnalaR grade.

Radioisotopes (carrier-free [^{32}P]P_i, PBS 11; [^{3}H]water, TRS 3; and inulin[^{14}C]carboxylic acid, CFA 399) were obtained from Amersham.

RESULTS

Labelling of cellular P_i

When erythrocytes were incubated for up to 3 h with [^{32}P]P_i (Figs. 1*a* and 2*a*), the ratio of the specific radioactivity of cellular P_i to that of extracellular P_i (a_2/a_1) stabilized at less than unity, suggesting that cellular P_i was exchanging rapidly with organic phosphates (see the Materials and methods section, Model A). However, under all conditions studied, this ratio was significantly greater than zero at the first time point (3 min) and was not detectably lower in separate experiments at $t = \frac{1}{2}$ min (Table 1*a*). The rate of increase of a_2/a_1 over the first $\frac{1}{2}$ min must therefore have been at least 30 h^{-1} in cells in plasma and 20 h^{-1} in Hepes/Ringer solution. The existence of this rapidly exchanging cell-associated P_i was confirmed by measuring depletion of extracellular [^{32}P]P_i (Table 1*b*) and by demonstrating rapid elution of ^{32}P from prelabelled cells (Table 1*c*).

If this rapidly exchanging P_i constitutes a separate cellular pool, the relative specific radioactivity of the residual slow-labelling pool (a_{2s}/a_1) can be calculated (Materials and methods section, Model B), and is plotted in Figs. 1(*b*) and 2(*b*).

Effect of extracellular P_i concentration on cellular P_i handling

To examine the effect of extracellular P_i loading on the apparent fast- and slow-labelling cellular P_i pools (Materials and methods section, Model B), cells were preincubated for 24 h with added P_i to achieve a new steady-state transmembrane P_i distribution [20] before addition of [^{32}P]P_i (Table 2). Doubling the extracellular P_i concentration doubled the size of the apparent fast-labelling P_i pool but had little effect on the slow-labelling P_i pool.

Labelling of organic phosphates

After 3 min of incubation with [^{32}P]P_i, the labelling of the organic phosphates, expressed as A_3/a_1 (measured

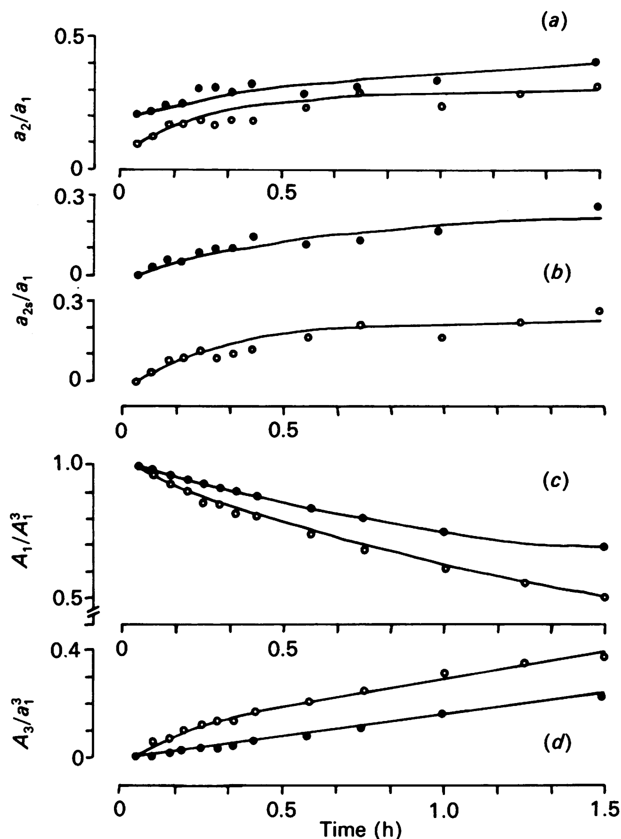


Fig. 1. Time course of ^{32}P-labelling of concentrated suspensions of human erythrocytes

Cells were incubated at 37 °C, pH 7.4 at approx. 45% haematocrit, in autologous plasma ($n = 7$) containing 0.9 mM-P_i under O_2/CO_2 (19:1) (●), or in Hepes/buffered Ringer solution ($n = 6$) containing 1 mM-P_i under air (○). (*a*) Specific radioactivity of cellular P_i (a_2) expressed relative to the specific radioactivity of extracellular P_i (a_1) at each time point. At $t = 3$ min, a_2/a_1 was 0.21 ± 0.02 in plasma and 0.10 ± 0.04 in Hepes/Ringer solution. (*b*) Specific radioactivity of the apparent slow-labelling cellular P_i pool (a_{2s}) expressed relative to a_1 at each time point, calculated as described in Model B (see the Materials and methods section). (*c*) Radioactivity of ^{32}P in extracellular medium (A_1) expressed relative to the value of A_1 at $t = 3$ min (A_1^3). (*d*) Radioactivity of ^{32}P in cellular organic phosphates (A_3) expressed relative to the value of a_1 at $t = 3$ min (a_1^3).

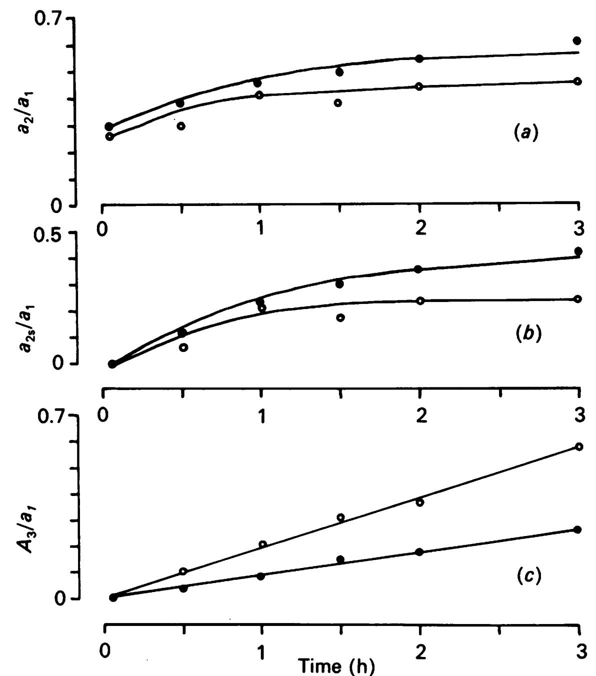


Fig. 2. Time course of ^{32}P-labelling of dilute suspensions of human erythrocytes

Cells were incubated at 37 °C, pH 7.4 at approx. 3% haematocrit in autologous plasma ($n = 7$) containing 1 mM-P_i under O_2/CO_2 (19:1) (●) or Hepes/Ringer solution ($n = 6$) containing 0.9 mM-P_i under air (○). a_1 did not change significantly with time. (*a*) Specific radioactivity of cellular P_i (a_2) expressed relative to specific radioactivity of extracellular P_i (a_1). At $t = 3$ min, a_2/a_1 was 0.34 ± 0.04 in plasma and 0.25 ± 0.04 in Hepes/Ringer solution. (*b*) Specific radioactivity of the apparent slow-labelling cellular P_i pool (a_{2s}) expressed relative to a_1 at each time point, calculated as described in Model B (see the Materials and methods section). At $t = 3$ h, a_{2s}/a_1 was 0.44 ± 0.07 in plasma and 0.25 ± 0.07 in Hepes/Ringer solution. (*c*) Radioactivity of ^{32}P in cellular organic phosphates (A_3) expressed relative to specific radioactivity of extracellular P_i (a_1).

directly or by subtracting A_2 from total cellular ^{32}P), was not significantly different from zero under all incubation conditions studied. The subsequent organic phosphate labelling (Figs. 1*d* and 2*c*) was approximately linear up to 3 h, even though the specific radioactivity of the cellular P_i supposedly responsible for labelling the organic phosphates was increasing towards a plateau (Figs. 1*a* and 1*b* and Figs. 2*a* and 2*b*).

Glycolysis and organic phosphate labelling

Even though the ratio of the organic phosphate labelling rate to the lactate production rate (S/L) was predicted to be greater than or equal to the mean relative specific radioactivity of cellular P_i (a_2/a_1) (Materials and methods section, Model A), the reverse was found in incubations in plasma (Table 3). A possible explanation is that the specific radioactivity of the P_i used for organic phosphate labelling might be considerably lower than the overall values plotted in Figs. 1(*a*) and 2(*a*).

This discrepancy was not as marked in Hepes/Ringer solution (Table 3), because, even though the lactate production rate was much lower than in plasma (Table 3), the organic phosphate labelling rate was, if anything, higher (Figs. 1*d* and 2*c*). Nevertheless, lactate production probably still reflects glycolytic flux here, as the molar ratio of lactate production to glucose consumption was 2.0 ± 0.2 ($n = 8$), the theoretical value for the Embden–Meyerhof pathway.

Prolonged incubations with [^{32}P]P_i

The specific radioactivity of cellular P_i only approached that of extracellular P_i after 48 h (Fig. 3*a*). However, even after 48 h, labelling of organic phosphates was probably incomplete as A_3/a_3 still seemed to be rising (Fig. 3*b*), and had only reached a value of 2–3 mmol of phosphorus/l of cells, much less than the total concentration of organic phosphate in erythrocytes, which is

Table 1. Comparison of three methods for studying the apparent fast phase of labelling of erythrocyte P_i

All incubations with $[^{32}P]P_i$ were performed at approx. 45% haematocrit. All values are significantly greater than zero ($P < 0.05$). Differences between estimates of A_2/A_1 by different methods are not significant. Method (*b*): loss of ^{32}P from extracellular medium. $[^{32}P]P_i$ (19 kBq or 0.5 μCi) was added to 500 μl of a cell suspension at about 45% haematocrit, or to 500 μl of cell-free medium. At $t = \frac{1}{2}$ min, samples of medium were recovered (as described in the Materials and methods section) from both incubations. The difference between their ^{32}P radioactivities (A_1, c.p.m./l) yields an estimate of cellular $[^{32}P]P_i$ radioactivity (A_2, c.p.m./l of cells) without direct measurements on the cells, as labelling of cellular organic phosphate is negligible at this point (Figs. 1*d* and 2*c*). Method (*c*): rapid elution of ^{32}P. $[^{32}P]P_i$ was added to a cell suspension as in *b* above. Immediately, 300 μl of this suspension was added to an equal volume of unlabelled medium. At $t = \frac{1}{2}$ min, extracellular medium was recovered (as described in the Materials and methods section) from both the original and diluted suspensions. The difference between the radioactivity (A_1, c.p.m./l) in the two samples of medium yields an estimate of the ^{32}P eluted (A_2, c.p.m./l of cells) from the cells during the rapid dilution.

Method	Parameter estimated*	Ratio			
		In plasma	*n*	In Hepes/Ringer solution	*n*
(*a*) From direct measurements $\frac{1}{2}$ min after adding $[^{32}P]P_i$ to the suspension (see Materials and methods section)	a_2/a_1	0.26±0.05	8	0.14±0.04	8
	A_2/A_1	0.18±0.03	12	0.10±0.02	15
(*b*) From loss of ^{32}P from extracellular medium in first $\frac{1}{2}$ min after adding $[^{32}P]P_i$ to the suspension	A_2/A_1	0.13±0.03	20	0.13±0.05	9
(*c*) From elution of ^{32}P from prelabelled cells into unlabelled medium	A_2/A_1	—	—	0.07±0.01	5

*See 'Notation and method of presentation of results' in the Materials and methods section.

Table 2. Effect of extracellular P_i concentration on the size of the apparent fast- and slow-labelling cellular P_i pools

Test cells were preincubated in plasma with added Na_2HPO_4 for 24 h to allow the cellular P_i concentration to reach a new steady state. Control cells were incubated for the same time in plasma containing only endogenous P_i (1 mM). $[^{32}P]P_i$ was then added to allow measurement of a_2/a_1, the ratio of cellular to extracellular specific radioactivities of P_i at $t = 3$ min, as an estimate of *F*, the fraction of cellular P_i in the apparent fast-labelling P_i pool (see the Materials and methods section, Model B). The fractional increase in P_i concentration (mmol/l of cells or plasma) is expressed as test/control ($n = 12$). *Significantly less than the fractional increase in extracellular P_i ($P < 0.05$); **Significantly less than the fractional increase in fast-labelling cellular P_i ($P < 0.05$)

P_i pool	Fractional increase in P_i concn. (test/control)
Extracellular P_i	2.2±0.1
Total cellular P_i	1.6±0.1*
Fast-labelling cellular P_i	2.4±0.3
Slow-labelling cellular P_i	1.3±0.1**

at least 15 mmol/l of cells [16]. Of this, about 10 mmol/l of cells is in the form of 2,3-BPG which is stable for at least 48 h under these incubation conditions [20], suggesting that most of the 2,3-BPG is unlabelled after 24–48 h with $[^{32}P]P_i$. Furthermore, even though incubation in plasma for 24 h at alkaline pH markedly increases the concentration of 2,3-BPG [20], this failed to increase A_3/a_1: indeed, a decrease was observed relative to controls at pH 7.4 ($P < 0.05$, $n = 5$) (Fig. 3*b*).

DISCUSSION

Labelling of cellular P_i

The initial rapid rise in the relative specific radioactivity of cellular P_i (a_2/a_1) followed by levelling off at a value much less than unity (Figs. 1*a* and 2*a*) is difficult to explain in terms of a single cellular P_i pool (Materials and methods section, Model A). For example, in incubations in plasma (Fig. 1*a*), a_2/a_1 stabilized at about 0.3. As the rate of organic phosphate labelling (Fig. 1*c*) was 0.17 mmol/h per l of cells, the organic phosphate exchange flux *G* can be calculated from Model A [15] as 0.17/0.3 = 0.6 mmol/h per l of cells. Model A requires that the plateau value of $a_2/a_1 = 0.3 = I/(I+G)$, so that transmembrane flux *I* must be 0.3 mmol/h per l of cells. However, *I* can be separately estimated in Model A [15] as the initial rate of increase of a_2/a_1, which was at least 30 h^{-1} (see the Results section), multiplied by the cellular P_i concentration (C_2), which is about 0.6 mmol/l of cells. This gives $I \geqslant 15$ mmol/h per l of cells. This striking lack of self-consistency between estimates of *I* (and other anomalies; see below) implies that Model A does not adequately explain the results.

The results seem more consistent with Model B (see the Materials and methods section) in which there is a second, fast-labelling cellular P_i pool. To account for the earliest value of a_2/a_1 in cells in plasma, this pool would need to contain $\frac{1}{4}$ of the chemically detectable cellular P_i. It has frequently been suggested that in erythrocytes there is compartmentation of nucleotides [6,8,10,26], glycolytic intermediates [27,28] and glycolytic enzymes [6], but nothing akin to the fast-labelling P_i pool has been reported. However, in many earlier studies, cells have been washed before P_i and ^{32}P were measured [6,9,10,17,26]. In view of the rapid elution of the fast-labelling P_i pool (Table 1*c*), this may explain the discrepancy. In support of this, when the estimated con-

Table 3. Comparison of the measured specific radioactivity of cellular P_i with that predicted from the rates of lactate production and organic phosphate labelling

If the specific radioactivity of cellular P_i (a_2) expressed relative to that of extracellular P_i (a_1) is a true reflection of that in the P_i pool used for organic phosphate labelling, then $S/L \geqslant (a_2/a_1)_m$ (see the Materials and methods section, Model A). Pooled data from n independent experiments ($n = 6$ in Hepes/Ringer solution; $n = 7$ in plasma).

Incubation conditions	Organic phosphate labelling rate, S (mmol/h per l of cells)		Lactate production rate, L (mmol/h per l of cells)	S/L	Measured $(a_2/a_1)_m$	
Plasma at 45% haematocrit	0.17±0.02	Fig. 1(*d*)	3.2±0.2	0.053	0.3	Fig. 1(*a*)
Hepes/Ringer solution at 45% haematocrit*	0.19±0.02*		1.5±0.1	0.13	0.2	
Plasma at 3% haematocrit	0.10±0.01	Fig. 2(*c*)	Assume 3.2†	0.031	0.5	Fig. 2(*a*)
Hepes/Ringer solution at 3% haematocrit	0.19±0.01		Assume 1.5†	0.13	0.4	

* Measured over the linear part of the time course from 20 min onwards (see Fig. 1*d*).

† In dilute cell suspensions, lactate accumulation is too small to measure reliably. We have therefore used the values from the experiments at high haematocrit.

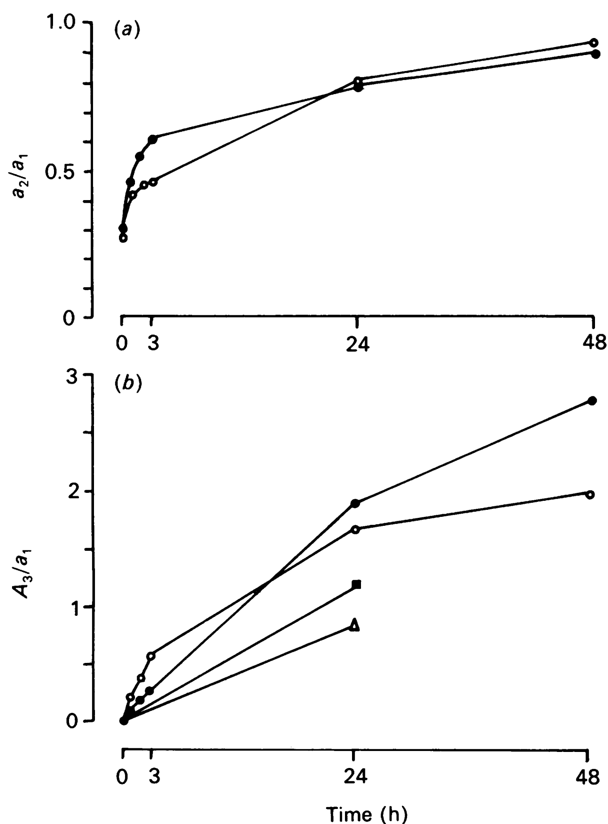


Fig. 3. ^{32}P-labelling of human erythrocytes incubated for up to 48 h at 37 °C and pH 7.4

Cells were incubated at 1 mM extracellular P_i at approx. 2% haematocrit in autologous plasma (24 h, $n = 13$; 48 h, $n = 11$) under O_2/CO_2 (19:1) (●) or in Hepes/Ringer solution (24 h, $n = 9$; 48 h, $n = 10$) under air (○). Values shown up to 3 h are from experiments shown in Fig. 2. Also shown are results from cells incubated for 24 h in plasma as above, but at pH 8.1±0.1 ($n = 5$) (□) and pH 7.1±0.1 ($n = 5$) (△). (*a*) Specific radioactivity of cellular P_i (a_2) expressed relative to specific radioactivity of extracellular P_i (a_1). Approximate half-times of change from t = 3–48 h are 21±1 h (plasma) and 14±1 h (Hepes/Ringer solution). (*b*) Radioactivity of ^{32}P in cellular organic phosphates (A_3) expressed relative to a_1.

tribution from the fast-labelling P_i pool is removed (Materials and methods section, Model B), the residual slow labelling of erythrocyte P_i (Figs. 1*b* and 2*b*) strikingly resembles time courses reported in washed cells [9,10,26]. Furthermore, a recent study of renal epithelial cells, with no washing step, has demonstrated a rapid phase of labelling at low temperature, which was not attributed to trapped extracellular medium [29].

Is the fast-labelling P_i pool extracellular?

Even though the contribution from trapped extracellular medium, which contains 1 mM-P_i, was subtracted here in all measurements, the fast-labelling P_i pool could be bound to the cell surface, although the binding would need to be weak (K_m approx. millimolar) to account for the doubling of the pool when the extracellular P_i concentration was doubled (Table 2 and [29]). Such binding would be surprising in view of the net negative charge on the membrane, but a P_i pool associated with the cytosolic face of the plasma membrane has been reported in pancreatic β-cells [30].

Regulation of cellular P_i concentration

We have shown previously that erythrocytes [31] and other cell types [32] seem able to regulate their steady-state P_i concentration in the face of changes in the extracellular P_i concentration. In the present study, this seemed to occur in the apparent slow-labelling P_i pool,

but not in the fast-labelling pool (Table 2). If the fast pool is not truly intracellular (see above), this may mean that erythrocytes can regulate their internal P_i concentration even more efficiently than was previously thought.

Is the slow-labelling P_i pool a hydrolysis artifact?

An alternative explanation for the low specific radioactivity of cellular P_i during the first 3 h after [^{32}P]P_i addition (Figs. 1*a* and 2*a*) is that, during extraction and measurement of P_i, hydrolysis of unlabelled organic phosphates had occurred, generating a spurious unlabelled contribution to cellular P_i. However, the procedures used here are designed to minimize such artifacts [21], and good evidence for their reliability is the observation that a_2/a_1 does approach its theoretical steady-state value of unity after 48 h, even though most of the potentially hydrolysable organic phosphate is still unlabelled at this stage (Fig. 3). It has also been shown that these methods detect negligible amounts of P_i in alkalotic erythrocytes which accumulate organic phosphates at the expense of cellular P_i [33]. This would not be expected if organic phosphate hydrolysis were a significant contributor to cellular P_i.

Are the fast- and slow-labelling P_i pools in separate cell populations?

In explaining apparent metabolic compartmentation, heterogeneity of the cell population must be considered. However, such a striking difference in the labelling kinetics of cellular P_i (and hence of ATP) between two hypothetical erythrocyte subpopulations seems unlikely in view of an earlier study which failed to detect any differences in ATP labelling between subpopulations [11]. Furthermore, the P_i content of leucocytes and platelets is too low [34] to explain these anomalies.

Which P_i pool is used by G3PDH?

Labelling of cell-associated P_i preceded detectable labelling of organic phosphates (Figs. 1 and 2). This suggests, contrary to earlier reports [7–10], that G3PDH is not performing group translocation of P_i across the cell membrane by selectively incorporating P_i of high specific radioactivity from the extracellular medium directly into cellular ATP. Indeed, the specific radioactivity of P_i used by G3PDH seems anomalously low. In the plasma incubations in Fig. 2(*b*), even the specific radioactivity of the slow-labelling P_i pool is too high to account for the slow rate of organic phosphate labelling (Fig. 2*c* and Table 3). Furthermore, the near-constant rate of organic phosphate labelling (Figs. 1*d* and 2*c*), in spite of the fact that the relative specific radioactivity of cellular P_i (a_2/a_1) had slowly risen to a plateau (Figs. 1*a* and 1*b* and Figs. 2*a* and 2*b*), also implies that a_2 and even a_{2s}, the specific radioactivity of the slow-labelling P_i pool, are not an accurate reflection of the specific radioactivity of P_i in the vicinity of G3PDH. Possibly, in the cell, G3PDH and a phosphatase are juxtaposed so that, in short incubations with [^{32}P]P_i, G3PDH receives P_i of low specific radioactivity liberated from organic phosphate(s) by the phosphatase. In view of its large size [16,20], slow turnover [35] and slow labelling [6,9,26], the 2,3-BPG pool of the erythrocyte is clearly a good candidate for this organic phosphate.

In any case, it seems hazardous to try to estimate the phosphate environment of an enzyme *in situ* from phosphate parameters measured over the whole cell.

Practical implications

It is common practice for phosphorus metabolites to be labelled by incubating cells with [^{32}P]P_i until no further incorporation is detectable, and then assume that changes in the amount of label in each metabolite reflect changes in the metabolite's concentration. The specific radioactivity measurements in this paper suggest that this is an unwarranted assumption even for P_i itself in a simple cell like the erythrocyte. The amount of label incorporated into cellular P_i may transiently stabilize long before isotopic steady state is reached (Figs. 1*a* and 2*a*), and interpretation may be further complicated by multiple metabolic pools. Isotopic steady state may take up to 48 h to attain (Fig. 3*a*), although the use of chloride-free incubation media may accelerate this [11]. For labelling of organic phosphates, isotopic steady state may not be reached even in 48 h and, as shown in Fig. 3, increasing the concentration of the main organic phosphate pool (2,3-BPG) by raising the pH for 24 h fails to give the predicted increase in organic phosphate labelling (Fig. 3*b*), although this incubation period is substantially longer than the half-lives of labelling [6,9,26] and turnover [35] of 2,3-BPG.

Preliminary studies of this type in cultured multicompartment cells suggest that such problems are not confined to the erythrocyte [36].

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