

# Inosine/pyruvate/phosphate medium but not adenosine/pyruvate/phosphate medium introduces millimolar amounts of 5-phosphoribosyl 1-pyrophosphate in human erythrocytes

## A <sup>31</sup>P-n.m.r. study

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Incubation of human erythrocytes in medium containing inosine (10 mM), pyruvate (10 mM), phosphate (50 mM) and NaCl (75 mM) at pH 6.6 leads to a more than 1000-fold increase in the concentration of 5-phosphoribosyl 1-pyrophosphate (PRPP), as identified and quantified by <sup>31</sup>P-n.m.r. spectroscopy. The accumulation is highly pH-dependent, with a maximum at extracellular pH 6.60, and the maximum value of 1.3–1.6 mmol/l of erythrocytes is attained within 1 h at 37 °C. PRPP was accumulated despite high concentrations of 2,3-bisphosphoglycerate (2,3-BPG), an inhibitor of PRPP synthetase. The concentration of PRPP correlated with the intracellular concentration of inorganic phosphate (P<sub>i</sub>). Substitution of either adenosine or adenosine plus inosine for inosine in the medium did not lead to <sup>31</sup>P-n.m.r.-detectable accumulation of PRPP. These results show that neither 2,3-BPG nor PRPP itself inhibits the synthesis of PRPP in the human erythrocyte. Adenosine, however, prevents the inosine-stimulated accumulation of PRPP.

## INTRODUCTION

5-Phosphoribosyl 1-pyrophosphate (PRPP) is a central metabolite in both the synthesis *de novo* and salvage pathway of purine nucleotides [1]. PRPP is synthesized by the enzyme PRPP synthetase (ribose-5-phosphate pyrophosphokinase, EC 2.7.6.1) utilizing ribose 5-phosphate and ATP [1–3]. The enzyme has an absolute requirement for Mg<sup>2+</sup> and phosphate (P<sub>i</sub>), which also markedly stimulates the enzyme [1–3]. In the human erythrocyte the concentration of PRPP is normally about 1–6 μM [4–6], and the rate of synthesis is reported to be regulated in a complex manner by the concentration of P<sub>i</sub> and the inhibitory actions of 2,3-bisphosphoglycerate (2,3-BPG) and nucleoside diphosphates and product inhibition by PRPP [1–3,7,8]. It had earlier been shown that incubation of human erythrocytes in media containing different concentrations of various purines, purine nucleosides and P<sub>i</sub> yields variable stimulation of PRPP synthesis [3,9–13]. Recently it was shown that adenine uptake in human erythrocytes, in contrast with hypoxanthine uptake [12], is independent of conditions known to stimulate PRPP synthesis such as low pH and high P<sub>i</sub> concentrations [14].

Inosine/pyruvate/phosphate medium (IPP medium) has previously been used to increase the concentration of 2,3-BPG to levels far above physiological and to replenish

2,3-BPG and ATP in stored erythrocytes [15–18]. In the present paper we show that fresh human erythrocytes incubated in IPP medium, but not in the corresponding adenosine medium, can result in the accumulation of more than 1 mM-PRPP, as detected by <sup>31</sup>P-n.m.r. spectroscopy.

## MATERIALS AND METHODS

### Erythrocytes

Venous blood was obtained from healthy donors and treated with 10 i.u. of heparin/ml as anticoagulant. The blood was centrifuged at 1000 g for 10 min at room temperature, the plasma and buffy coat were discarded and the cells were washed three times in an iso-osmotic Hepes-buffered saline [19]. After the final wash the erythrocytes were resuspended in IPP medium, APP medium or the combined A+IPP medium at the appropriate pH and at a haematocrit of approx. 5–10%. The IPP medium has the following composition: NaCl, 75 mM; inosine, 10 mM; pyruvate, 10 mM; P<sub>i</sub>, 50 mM. In the APP and A+IPP media adenosine (10 mM) and adenosine (10 mM) plus inosine (10 mM) respectively are substituted for inosine. The suspensions were incubated for various times in a shaking water bath at 37 °C, after which they were quickly centrifuged down (at 3500 g for

Abbreviations used: IPP medium, inosine/pyruvate/phosphate medium; APP medium, adenosine/pyruvate/phosphate medium; A+IPP medium, adenosine/inosine/pyruvate/phosphate medium; PRPP, 5-phosphoribosyl 1-pyrophosphate; 2,3-BPG, 2,3-bisphosphoglycerate; PEP, phosphoenolpyruvate; MDP, methylenediphosphonic acid.

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5 min); the supernatant of each was carefully sucked off and discarded before dilution of the pellet with more than 10 vol. of the Hepes-buffered saline. After a further wash in Hepes-buffered saline the erythrocytes were concentrated to a haematocrit of approx. 80% for n.m.r. analysis.

Boiled extracts of PRPP-loaded erythrocytes were prepared essentially according to the method of Tax & Veerkamp [5] and concentrated by freeze-drying. The freeze-dried extracts were resuspended in medium containing 10% (v/v)  $^2\text{H}_2\text{O}$ , 10 mM-EDTA and 100 mM-Tris/HCl buffer, pH 7.6, for  $^{31}\text{P}$ -n.m.r. measurements.

### $^{31}\text{P}$ -n.m.r. measurements

$^{31}\text{P}$ -n.m.r. measurements were performed at 101.3 MHz in 10 mm tubes in a standard probe on a Bruker AM-250 instrument. For intact cells a pulse angle of  $90^\circ$ , an acquisition time of 0.5 s and a relaxation delay of 6.5 s were used to ensure complete relaxation of the nucleotides and PRPP. The repetition time used partially saturated the peaks for 2,3-BPG and  $\text{P}_i$ , for which correction factors were obtained by accumulating spectra alternatively at a repetition time of 7 s and 20 s. Each spectrum was accumulated within 15–30 min.  $^{31}\text{P}$ -n.m.r. spectra of extracts were accumulated at  $25^\circ\text{C}$ , with a pulse angle of  $67^\circ$ , repetition time of 0.5 s and broadband proton decoupling with a variable number of scans.

Methylenediphosphonic acid (MDP) (125 mM) at pH 9.0 in 50 mM-Tris/HCl buffer served as external reference at 16.90 p.p.m. relative to 85%  $\text{H}_3\text{PO}_4$ . The area of the MDP reference was estimated to correspond to  $1.97 \pm 0.06$  mmol/l (mean  $\pm$  s.d.,  $n = 6$ ) in spectra accumulated with known amounts of  $\text{P}_i$ .

The pH was measured as previously described [19].

PRPP was obtained from Sigma Chemical Co. (catalogue no. P 8296).

## RESULTS

### Peak assignments

Fig. 1(a) shows a  $^{31}\text{P}$ -n.m.r. control spectrum of fresh human erythrocytes suspended in Hepes-buffered saline, and Figs. 1(b)–1(d) are spectra of erythrocytes preincubated in the IPP medium, APP medium and A + IPP medium respectively. As shown in Fig. 1(b), a new major resonance at  $-11$  p.p.m. has developed in erythrocytes preincubated in the IPP medium, but not in the APP and A + IPP media (Figs. 1c and 1d). There is also a small but distinct peak at  $-0.6$  p.p.m. (Figs. 1b and 1d). The doublet at  $-11$  p.p.m. together with the corresponding doublet at  $-5.4$  p.p.m. was assigned to PRPP on the basis of pH titrations in the range 6–8 both before and after addition of authentic PRPP to boiled erythrocyte extracts (Figs. 2a and 2c), as well as by the doublet split pattern of the  $\alpha$ -peak at  $-11$  p.p.m. in proton-coupled spectra (Fig. 2b). The peak at  $-0.6$  p.p.m. was similarly assigned to phosphoenolpyruvate (PEP). PEP is also detectable in spectra of erythrocytes preincubated in the APP and A + IPP media (Figs. 1c and 1d). The induction of high concentrations of PEP in the IPP medium has been noted previously [16] and is probably related to the very high concentration of pyruvate used in these studies (10 mM). Additionally, there is a complex peak downfield of the 3-phosphorus peak of 2,3-BPG (Figs. 1b–1d). In the extract spectrum with better resolution, this peak is composed of at least

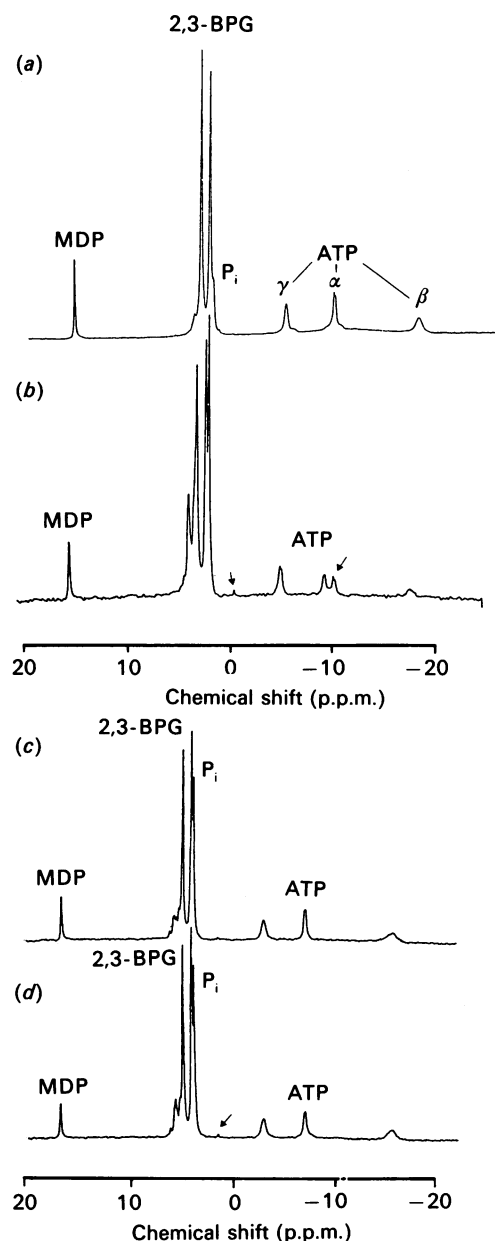


Fig. 1.  $^{31}\text{P}$ -n.m.r. spectra at 101.3 MHz (a) of human erythrocytes suspended in Hepes-buffered saline at a haematocrit of 72%, (b) of erythrocytes preincubated in IPP medium at pH 6.60 for 1 h at  $37^\circ\text{C}$ , (c) of erythrocytes preincubated in APP medium for 1 h at pH 6.70 and (d) of erythrocytes preincubated in A + IPP medium for 1 h at pH 6.60

The arrows in (b) and (d) point to peaks developed during incubation.

nine different resonances (Fig. 2a). The most prominent peak, at 4.3 p.p.m., in this region is tentatively assigned to IMP on the basis of the chemical shift and on the known synthesis of IMP in erythrocytes incubated in the IPP medium [20]. As for the other peaks in this region, they represent a complex mixture of sugar phosphates [21]. In a recent  $^{31}\text{P}$ -n.m.r. study, sugar phosphates in this region were assigned [21], although the lack of precise information on chemical shifts and the difference in pH makes it difficult to compare those assignments directly with the present study.

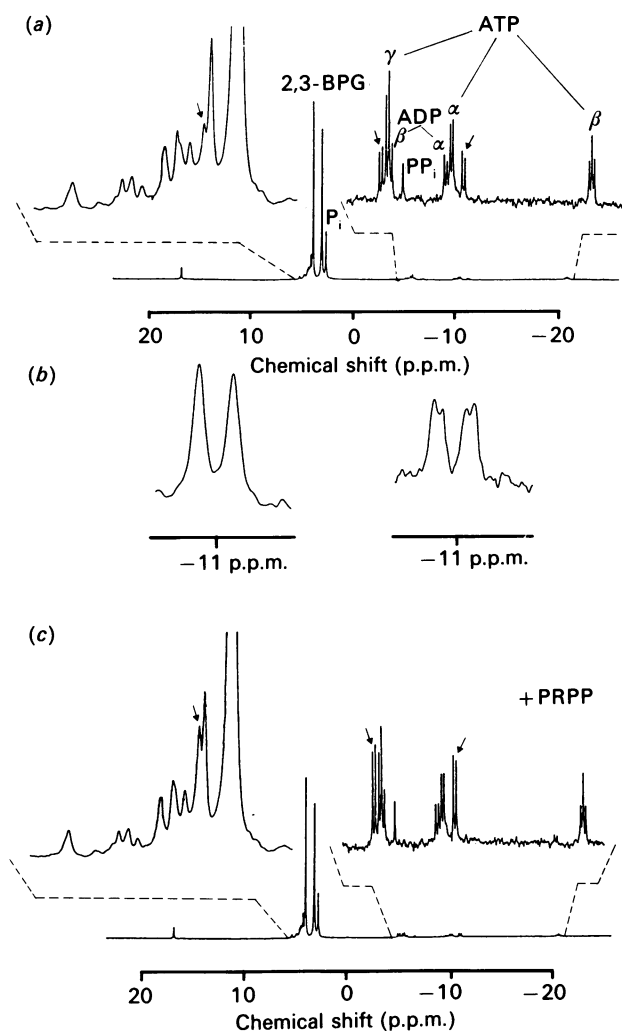


Fig. 2. <sup>31</sup>P-n.m.r. spectra at 101.3 MHz of an extract of erythrocytes preincubated in IPP medium at pH 6.6 (a) before and (c) after the addition of PRPP

The inset spectra in (a) and (c) are extensions of the phosphomonoester region and nucleotide region respectively. The arrows point to unidentified peaks. (b) shows the doublet at -11 p.p.m. recorded in (a) with and without proton decoupling.

#### Effect of incubation conditions on PRPP accumulation

The accumulation of PRPP in IPP medium was found to be highly pH-dependent, with an optimum at about pH 6.6 after 1 h of incubation giving rise to 1.65 mmol/l of erythrocytes (Fig. 3a). As recorded in Fig. 3, the intracellular concentration of P<sub>i</sub> is inversely related to pH, whereas 2,3-BPG shows a broad maximum at pH 7 (Fig. 3b).

The time-dependence of the accumulation of PRPP at pH 6.60 is shown in Fig. 4(a) and the related concentrations of P<sub>i</sub> and 2,3-BPG are shown in Fig. 4(b). Within 1 h of incubation there is a sharp increase in the concentration of PRPP, slowly declining thereafter. Simultaneously, the intracellular concentration of P<sub>i</sub> increases, reaching a plateau around 14 mmol/l of erythrocytes within 1 h. The concentration of 2,3-BPG

also rises quickly during the first hour, with a slower sustained increase to a final value of about 20 mmol/l of erythrocytes after 3 h (Fig. 4b). The concentration of nucleoside triphosphates is almost constant at about 1 mmol/l of erythrocytes during the first hour, jumping to a constant value of about 2 mmol/l during the second and third hours of incubation (results not shown). The concentration of PEP rises linearly to a maximum value of 0.24 mmol/l of erythrocytes after 90 min and declines linearly with a slightly lower rate to a value of about 0.06 mmol/l after 5 h (results not shown).

Incubation at several pH values in the range 6.6–7.4 failed to show any accumulation of PRPP in APP medium (Fig. 1c) or in the combined A + IPP medium (Fig. 1d). The concentration of nucleoside diphosphates under control conditions (Krebs–Ringer buffer) [22], in the IPP medium at pH 6.60 and in the APP or A + IPP media at pH 6.6–6.7 are given in Table 1. The concentration of PRPP in erythrocytes preincubated in IPP medium at pH 6.60 for 45–90 minutes is also shown in Table 1. Whereas the concentration of nucleoside diphosphates in erythrocytes preincubated in IPP medium is comparable with the value for erythrocytes in Krebs–Ringer buffer, the APP and A + IPP media caused almost a doubling (Table 1).

## DISCUSSION

### Identification of PRPP

It is well known that PRPP synthetase is markedly stimulated by P<sub>i</sub> [1–3], and that the reaction is inhibited by physiological concentrations of 2,3-BPG and nucleoside diphosphates, i.e. ADP in human erythrocytes [3,7,12]. Here we show by <sup>31</sup>P-n.m.r. spectroscopy, however, that the concentration of PRPP in human erythrocytes can be elevated 1000-fold by incubation in IPP medium at pH 6.6–6.8 (Figs. 3a and 4a), but that adenosine prevents this PRPP accumulation. That the peak at -11 p.p.m. in Fig. 1(b) was in fact due to the α-peak of the 1-pyrophosphate of PRPP was substantiated by the doublet in proton-coupled spectra (Fig. 2b) and by addition of authentic PRPP, and was confirmed by pH titrations both before and after addition of PRPP (Figs. 2a and 2c).

### Effect of incubation conditions on PRPP accumulation

The pH optimum for the accumulation of PRPP in the IPP medium was found to be 6.60 (Fig. 3a), corresponding to an intracellular pH of approx. 6.50 [23]. The increase in PRPP with decreasing pH correlated to an increase in the intracellular concentration of P<sub>i</sub> with decreasing pH (Fig. 3b), although the P<sub>i</sub> concentration continued to increase inversely with pH without an optimum. The increase in intracellular P<sub>i</sub> accompanying lowering of the extracellular pH is probably mediated by an enhanced transport of P<sub>i</sub> [24] and by the degradation of 2,3-BPG evident in Fig. 3(b). Thus the accumulation of PRPP could be the result of the stimulatory effect of P<sub>i</sub>, although the apparent coupling between the concentration of PRPP and P<sub>i</sub> is lost at lower pH values (Fig. 3b). A decrease in the inhibitory action of 2,3-BPG could also be involved [12], although this possibility seems more unlikely, as discussed below.

Erythrocytes incubated in IPP medium at pH 6.60 accumulates PRPP to a level of 1.3–1.6 mmol/l of erythrocytes within the first hour, and the concentration

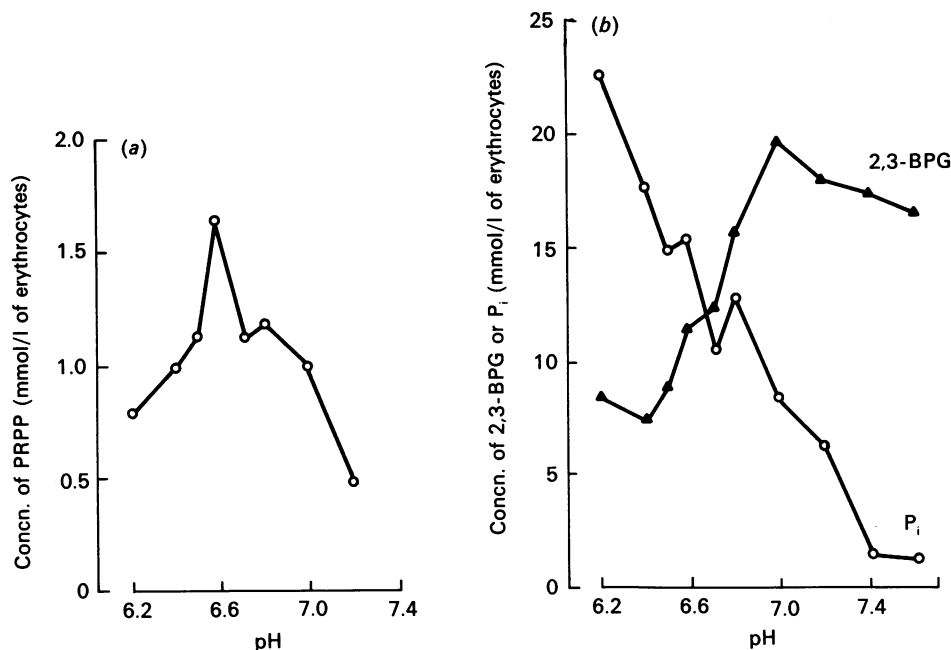


Fig. 3.  $^{31}\text{P}$ -n.m.r.-measured concentrations of (a) PRPP and (b) 2,3-BPG ( $\blacktriangle$ ) and  $P_i$  ( $\circ$ ) in erythrocytes preincubated for 1 h at  $37^\circ\text{C}$  in IPP medium at various pH values

At pH 6.60 the concentration of PRPP attained during 45–90 min of incubation was  $1.45 \pm 0.12$  mmol/l of erythrocytes (mean  $\pm$  S.E.M.,  $n = 6$ ) (Table 1). The corresponding concentration of  $P_i$  was  $15.08 \pm 0.55$  mmol/l of erythrocytes (mean  $\pm$  S.E.M.,  $n = 6$ ), and the concentration of 2,3-BPG was  $11.25 \pm 0.39$  mmol/l of erythrocytes (mean  $\pm$  S.E.M.,  $n = 6$ ).

then slowly decreases during the subsequent 4 h (Fig. 4a and Table 1). Despite accumulation of 2,3-BPG far above physiological concentrations, there is still significant synthesis of PRPP, as shown in Fig. 4. This indicates that the 2,3-BPG inhibition can be relieved

completely by  $P_i$  and not only partially as previously suggested [3,7]. Our results also question the inhibitory actions of 2,3-BPG under physiological conditions [1,3,7].

Product inhibition by PRPP with a  $K_i$  of 0.05–0.1 mM [7] also seems negligible under these circumstances.

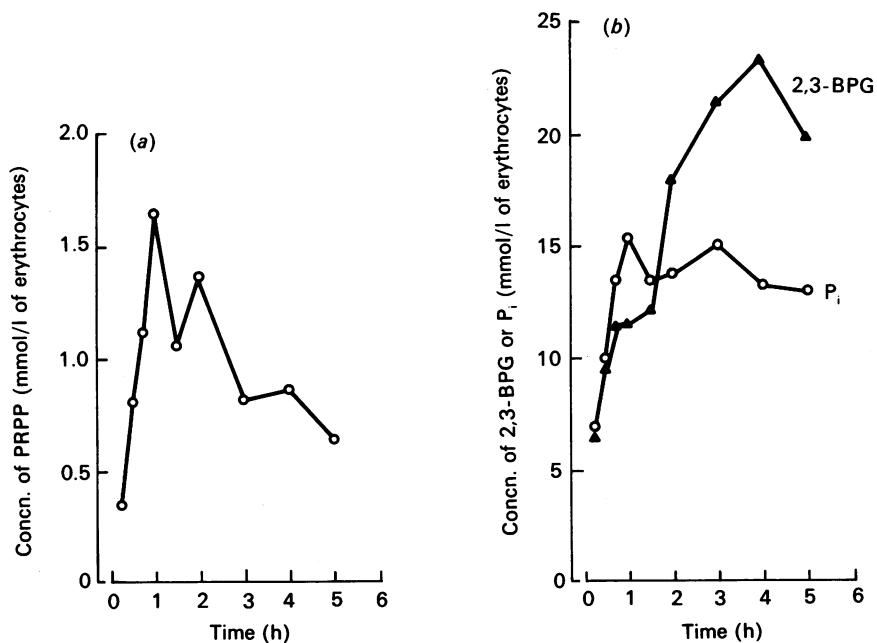


Fig. 4.  $^{31}\text{P}$ -n.m.r.-measured concentrations of (a) PRPP and (b) 2,3-BPG ( $\blacktriangle$ ) and  $P_i$  ( $\circ$ ) in erythrocytes preincubated at pH 6.60 and  $37^\circ\text{C}$  for various times in IPP medium

The Figure is representative of three separate experiments.

**Table 1. Concentrations of nucleoside diphosphates and PRPP in human erythrocytes**

The Table gives the <sup>31</sup>P-n.m.r.-measured values of nucleoside diphosphates (NDP) and PRPP in human erythrocytes in control solution (Krebs–Ringer buffer), erythrocytes preincubated in IPP medium, pH 6.60, at 37 °C and erythrocytes preincubated in either APP medium or the combined A + IPP medium, pH 6.60–6.70, regardless of incubation time. The value for PRPP in IPP medium was obtained from erythrocytes preincubated in the medium for 45–90 min. The nucleoside diphosphate value for erythrocytes in the control solution was taken from data published in ref. [22]. The results are expressed as means ± s.e.m. and the numbers of experiments are shown in parentheses. Abbreviation: N.D., not detected.

Medium	pH	Concn. of NDP (mmol/l of erythrocytes)	Concn. of PRPP (mmol/l of erythrocytes)
Krebs–Ringer buffer	7.40	0.32 ± 0.06 (6)	N.D.
IPP medium	6.60	0.39 ± 0.10 (9)	1.45 ± 0.12 (6)
APP medium or A + IPP medium	6.60–6.70	0.79 ± 0.09 (5)	N.D.

### Effect of adenosine

It has recently been shown that hypoxanthine uptake in human erythrocytes is dependent on experimental conditions favouring the formation of PRPP, but that adenine uptake is independent of the same conditions [12,14]. Using the nucleosides instead of the free bases in the present study, we show that there is not any n.m.r.-detectable accumulation (less than 0.1 mmol/l of erythrocytes) of PRPP on incubating the erythrocytes in APP medium or A + IPP medium in spite of increased intracellular P<sub>i</sub> (Figs. 1c and 1d). Thus the lack of accumulation of PRPP is not related to a lower intracellular concentration of P<sub>i</sub> as previously suggested [8]. In fact, intracellular P<sub>i</sub> concentration rose to approx. 15 mmol/l of erythrocytes after 1 h of incubation in the A + IPP medium (Fig. 1d). Furthermore the nucleoside triphosphate synthesized in the A + IPP medium is ATP, and not ITP [11,25,26] (results not shown). Inosine and/or adenosine is transported across the erythrocyte membrane and in the case of inosine cleaved to hypoxanthine and ribose 1-phosphate [20], which in turn is converted into ribose 5-phosphate, the substrate of PRPP synthetase [1]. The ribose moiety may also be utilized in glycolysis for generation of ATP. The human erythrocyte lacks the capacity of phosphorolytic cleavage of adenosine, which instead is either deaminated to inosine or phosphorylated directly to AMP [26,27]. One possible reason for the lack of accumulation of PRPP in both the APP and the A + IPP media may be that the direct phosphorylation of adenosine to AMP and the subsequent action of adenylate kinase increases the ADP concentration. Together with pyruvate and P<sub>i</sub>, the increased concentration of ADP (see Table 1) may lead to an acceleration of glycolysis [28], thereby diverting ribose 5-phosphate from PRPP synthesis to glycolysis. In addition, the higher ADP concentration might be inhibitory towards PRPP synthetase [1–3]. Although the concentration of ribose 5-phosphate and the rate of glycolysis were not measured in this study, the above interpretation is supported by the higher nucleoside diphosphate (mainly ADP) concentrations measured by <sup>31</sup>P-n.m.r. in APP and A + IPP media (0.79 mmol/l of erythrocytes) as opposed to the IPP medium (0.39 mmol/l of erythrocytes) (see Table 1). The higher ADP concentrations in erythrocytes incubated in the

APP medium as opposed to the IPP medium have also been confirmed at pH 7.40 (A. Petersen & B. Quistorff, unpublished work).

In conclusion, we have shown that the concentration of PRPP in human erythrocytes can be elevated 1000-fold by incubation in IPP medium at pH 6.6–6.8. This increase is related to the intracellular P<sub>i</sub> concentration and occurs despite a 2,3-BPG concentration that is 2–3 times above normal. Substituting adenosine or adenosine plus inosine for inosine abolishes this accumulation of PRPP, possibly caused by a higher ADP concentration induced in the adenosine-containing media.

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