Oxypurine Cycle in Human Erythrocytes Regulated by pH, Inorganic Phosphate, and Oxygen

Peter A. Berman, Duncan A. Black, Lucille Human, and Eric H. Harley
Department of Chemical Pathology, University of Cape Town Medical School, Observatory, 7925, South Africa

Abstract

The effect of pH, Po_2, and inorganic phosphate on the uptake and metabolism of hypoxanthine by erythrocytes has been studied. Uptake of hypoxanthine and accumulation of inosine 5′-monophosphate (IMP) were markedly increased at acid pH, high external phosphate concentrations, and low Po_2. Release of accumulated IMP as hypoxanthine occurred at alkaline pH values and low external phosphate concentrations. Conditions favoring IMP accumulation gave rise, in the absence of hypoxanthine, to a corresponding increase in 5′-phosphoribosyl-1-pyrophosphate. Intracellular phosphate concentrations were markedly pH dependent and a model is presented whereby hypoxanthine uptake and release are controlled by intracellular concentrations of inorganic phosphate and 2,3-bisphosphoglycerate. These allosteric effectors influence, in opposing ways, two enzymes governing IMP accumulation, namely 5′-phosphoribosyl-1-pyrophosphate synthetase and 5′-nucleotidase. These metabolic properties suggest that the erythrocyte could play a role in the removal of hypoxanthine from anoxic tissue.

Introduction

Mature erythrocytes lack the enzymes for de novo purine synthesis (1), but possess the ability to take up and release purine bases and nucleosides (2–4). Hypoxanthine is rapidly transported across the red cell membrane by facilitated diffusion, so that equilibrium is reached within a minute even at millimolar concentrations of the oxypurine (5). The salvage of hypoxanthine to inosine 5′-monophosphate (IMP)[1] depends on the concentration of inorganic phosphate (Pi) in the medium (2–4, 6, 7). This is ascribed to stimulation by Pi of the synthesis of 5-phosphoribosyl-1-pyrophosphate (PRPP), a cosubstrate and rate limiting factor in the phosphoribosylation of hypoxanthine to IMP (4, 7). Whereas PRPP synthetase is activated by Pi, it is inhibited by ADP, GDP, and 2,3-bisphosphoglycerate (2,3-DPG) (8, 9). The low intrinsic rate of PRPP synthesis in intact cells, even under experimental conditions of high external Pi concentrations, has been attributed to allosteric inhibition by these compounds (4, 10).

Unlike other purine base substrates, which contribute to di- and triphosphate nucleotide pools, anabolism of hypoxanthine in the erythrocyte ceases at the level of IMP (2). A recently described cytosolic erythrocyte purine 5′-nucleotidase can catalyze the conversion of IMP to inosine (11, 12), which can then be degraded by purine nucleoside phosphorylase (PNP) to hypoxanthine.

There are conflicting reports on the effect of pH and oxygen tension (Po_2) on oxypurine metabolism in erythrocytes. Salerno has reported enhanced incorporation of hypoxanthine into IMP with decreasing pH and Po_2 (13), whereas Schrauf-statter failed to confirm these findings (14). Bontemps reported that alkalinization of the medium resulted in a 15-fold increase in the production of hypoxanthine by erythrocytes (11).

Here we have studied the uptake and release of hypoxanthine under conditions of varying Pi concentration, pH, and Po_2 and shown that uptake and conversion of hypoxanthine to IMP is enhanced by increasing Pi and decreasing pH and Po_2. These conditions are similar to those prevailing in anoxic tissue, in which pH is low due to lactic acidosis and Pi is high from hydrolysis of creatine phosphate and ATP. We have also shown that release of IMP as hypoxanthine is favored at low Pi concentration and high pH. The pH changes studied cover a narrow range of < 1 pH unit and reflect values seen in vivo in certain pathological states. These findings may be relevant to the clearance of hypoxanthine from anoxic tissues.

Methods

Metabolism of oxypurines by erythrocytes. Washed erythrocytes were incubated in medium consisting of 50 mM Hepes, 10 mM glucose, 10 mM Na_2PO_4, and 75 mM NaCl at a packed cell to medium ratio of 1:2 (vol/vol). pH was adjusted by the addition of 1 M HCl or 1 M NaOH. In some experiments, the medium also contained 100 μM [^{14}C]hypoxanthine, (1.2 Ci/mol) (Amersham Corp., Amersham, England) or ^{32}Pi (5 mCi/mol) (Amersham Corp.). To quantitate labeled purine species, an aliquot of suspension, typically 0.5 ml, was centrifuged through dibutylphthalate (E. Merck, Darmstadt, FRG), as described by Wohlhueter (15) in a 1.5-ml microfuge tube, and the supernatant medium was deproteinized with an equal volume of 0.6 M perchloric acid. The dibutylphthalate was aspirated and the red cell pellet was resuspended in an equal volume of isotonic saline, followed by 2 vol 0.6 M perchloric acid. After centrifugation, the clear supernatant was counted directly by scintillation photometry using an open window setting. From the specific activity, the uptake of purine or phosphate could be expressed in micromolar or millimolar, respectively. In some cases, aliquots were neutralized with 2.5 M K_2CO_3, and after centrifugation, the supernatant (typically 50 μl) was injected onto an anion exchange or reverse phase HPLC column (details of HPLC given below). Eluate fractions were counted and from the position of markers individual labeled purine species were identified and quanti-

Address reprint requests to Dr. Peter A. Berman, Department of Clinical Pathology, University of Cape Town Medical School, Falmouth Road, Observatory, 7925 Cape Town, South Africa.

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1. *Abbreviations used in this paper:* HPRT, hypoxanthine-guanine phosphoribosyl-transferase; IMP, inosine 5′-monophosphate; Pi, inorganic phosphate; PNP, purine nucleoside phosphorylase; PRPP, 5-phosphoribosyl-1-pyrophosphate; 2,3-DPG, 2,3-bisphosphoglycerate.

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tated. In experiments in which ^{32}Pi was used, the individual labeled ^{32}P species were separated in a similar manner, using anion-exchange HPLC. The Pi concentration of cells and medium was measured by the colorimetric method of Fiske and Subbarow (16). The pH of the red cell suspensions was determined to two decimal places using an ABL blood gas analyzer (Radiometer, Copenhagen, Denmark).

Incubation of red cells in whole blood was performed by addition of Na_2HPO_4, glucose, and [^{14}C]hypoxanthine to freshly drawn heparinized blood to give final concentrations of 10 and 10 mM and 100 μM, respectively without significant increase in volume. pH was reduced to ~ 7 by equilibration with 20% CO_2 in oxygen. Samples were removed before and after a 90-min incubation to determine hypoxanthine uptake. After incubation, erythrocytes were isolated by centrifugation through dibutylphthalate and resuspended in autologous fresh plasma. A baseline sample was removed at this stage. The pH of the suspension was increased by equilibration with air to displace CO_2. Samples of varying pH were removed during this procedure and incubated for 1 h to determine hypoxanthine release. The concentrations of Pi, total labeled purine, and in some cases individual purines were determined as described above for cells incubated in defined aqueous medium. Blood pH and P_{CO_2} were measured on an ABL blood gas analyzer (Radiometer). Plasma lactate was measured on the perchloric acid extracts by monitoring the formation of NADH in the presence of lactate dehydrogenase (17).

PRPP assay. The PRPP content of erythrocytes was measured by the method of Hershko et al. (4), in which [^{14}C]IMP formed in the presence of [^{14}C]hypoxanthine and hypoxanthine-guanine phosphoribosyltransferase (HPRT) reflects the PRPP originally present. Typically, 20 μl EDTA (10 mM) was added to 200-μl aliquots of red cell suspension in 1.5-ml microfuge tubes that were heated at 100°C for 1 min. The samples were chilled and mixed with 50 μl of a 10% suspension of activated charcoal. The charcoal adsorbs endogenous nucleotides and together with precipitated protein was deposited by centrifugation. 30 μl of clear supernatant was incubated for 3 h at 37°C with 20 μl of a combined reagent containing 0.1 M Tris HCl, pH 8.0, 30 mM $MgCl_2$, 300 μM [^{14}C]hypoxanthine (5 Ci/mol), and 40% vol/vol of a dialyzed hemolysate from normal erythrocytes as a source of HPRT. This hemolysate was prepared according to Steyn (18). 15-μl aliquots of the incubation mixture were spotted in duplicate onto 2-cm-diam discs marked with pencil on polyethyleneimine phosphocellulose thin layer plates (E. Merck) and washed exhaustively with distilled water to remove [^{14}C]hypoxanthine. The discs were then dried, cut out, and placed in counting vials with scintillant to quantitate the [^{14}C]IMP bound. The assay was standardized using solutions of 0, 50, 100, 150, and 200 μM PRPP in place of sample.

PRPP synthetase assay. A partially purified preparation of PRPP synthetase was prepared following the procedure of Hershko (4) and Hennessy (19). The assay of PRPP synthetase was performed according to the procedure of Hershko (4).

High pressure liquid chromatography. The separation of oxypurine metabolites was effected by HPLC (model 3500 B; Spectra-Physics Inc., Mountain View, CA) of neutralized perchloric acid extracts of erythrocytes using both anion-exchange and reverse-phase columns. Anion-exchange chromatography was performed on an APS HVP-2511 column (Hichrom Ltd., Reading, England), with a linear buffer gradient developed over 10 min from 5 mM KH_2PO_4 (Aristar grade; British Drug Houses, Poole, England), pH 2.50, to 500 mM KH_2PO_4 plus 912 mM KCl, pH 3.8 at a flow rate of 1.2 ml/min. Depending on the retention times of the metabolites studied, 30- or 60-s fractions of the effluent were collected, mixed with scintillation fluid (HIONIC-FLUOR; Packard Instruments, Downers Grove, IL), and counted in an LS 233 liquid scintillation system (Beckman Instruments, Fullerton, CA).

Reverse-phase chromatography was performed on a Hichrom S50 DS-3571 column (Hichrom) using 5% methanol in 1 mM KH_2PO_4 (vol/vol) as an isocratic buffer at a flow rate of 1.6 ml/min. Fractions were collected and counted as described above. In all cases, the absorbance of the effluent was monitored at 260 nm.

Effect of P_{O_2} on hypoxanthine uptake by erythrocytes. Fresh heparinized blood was centrifuged for 10 min at 1,700 *g* and the plasma, buffy coat, and top fifth of red cells were removed. The infranatant cells were washed twice with 3 vol of ice-cold saline and resuspended in 3 vol buffer containing, in final concentration, 50 mM Hepes; 1, 5, or 10 mM NaH_2PO_4 120 μM [^{14}C]hypoxanthine (5 Ci/mol), 10 mM glucose, and NaCl to provide a final osmolality of 290 mosm/kg. pH was adjusted to 7.1 with NaOH. After equilibration with air at room temperature, 0.7-ml samples of the erythrocyte suspension were removed and introduced under dibutylphthalate in a 1.5-ml microfuge tube, which was then closed and kept at room temperature. The pH and P_{O_2} of an aliquot of the erythrocyte suspension was measured. Nitrogen gas was then bubbled through the remaining suspension at room temperature and at appropriate intervals, 0.7-ml aliquots were removed and treated as described above, until the P_{O_2} had dropped to 2.7 kPa or below. All the microfuge tubes were then incubated at 37°C for 90 min with intermittent inversion to keep red cells suspended. After incubation, the erythrocytes were separated from the medium by centrifugation through the dibutylphthalate. 50-μl aliquots of the pelleted erythrocytes were then lysed with 100 μl H_2O and deproteinized with 150 μl 0.6 M perchloric acid. After centrifugation and neutralization of the supernatant fluid with K_2CO_3, the total red cell content of labeled oxypurine was determined by counting an aliquot of supernatant. The nature and quantity of individual hypoxanthine metabolites in the red cells was determined using both reverse-phase and anion-exchange chromatography.

All estimations were performed in duplicate. The difference between duplicates expressed as a percentage of the mean was found to be < 5% in most cases and < 10% in all cases.

Results

Effect of pH on oxypurine metabolism. Washed human erythrocytes were incubated at 37°C for 2 h in Hepes-buffered medium containing 10 mM Pi and 100 μM [^{14}C]hypoxanthine at varying pH. In a parallel experiment, cells preincubated at the lowest pH (7.1) for 2 h were alkalinized by the progressive addition of NaOH to give a second set of samples of varying pH, which were incubated for a further period of 2 h at 37°C. Cells from both the first and second incubations were separated from medium by centrifugation through dibutylphthalate and the distribution of labeled purine species was determined by anion-exchange and reverse-phase chromatography.

The results of the first 2-h incubation are given in Fig. 1 *A* and show that IMP is synthesized in progressively greater amounts with decreasing pH. Minimal quantities are present after 2 h above pH 7.5. When incubation of cells at pH 7.1 is continued at the same pH value (Fig. 1 *B*) for a further 2 h, the conversion of hypoxanthine to intracellular IMP becomes essentially complete. However, subsequent alkalinization of cells preincubated for an initial 2 h at pH 7.1 resulted in a reversal of this process, with depletion of intracellular IMP and reappearance of hypoxanthine. At pH values > 7.6, detectable quantities of inosine accumulated. Intracellular hypoxanthine concentrations were generally similar to those in the medium, irrespective of pH, consistent with the known rapid equilibration of hypoxanthine across red cell membranes (5).

Effect of inorganic phosphate on hypoxanthine metabolism. The effect of varying Pi concentrations on IMP formation in intact erythrocytes at a number of different pH values is shown in Figs. 2 and 3, and demonstrates how Pi and pH affect IMP accumulation. No IMP accumulates at any pH in the absence of Pi in the medium. In the presence of external Pi, IMP accumulation and corresponding medium hypoxanthine de-

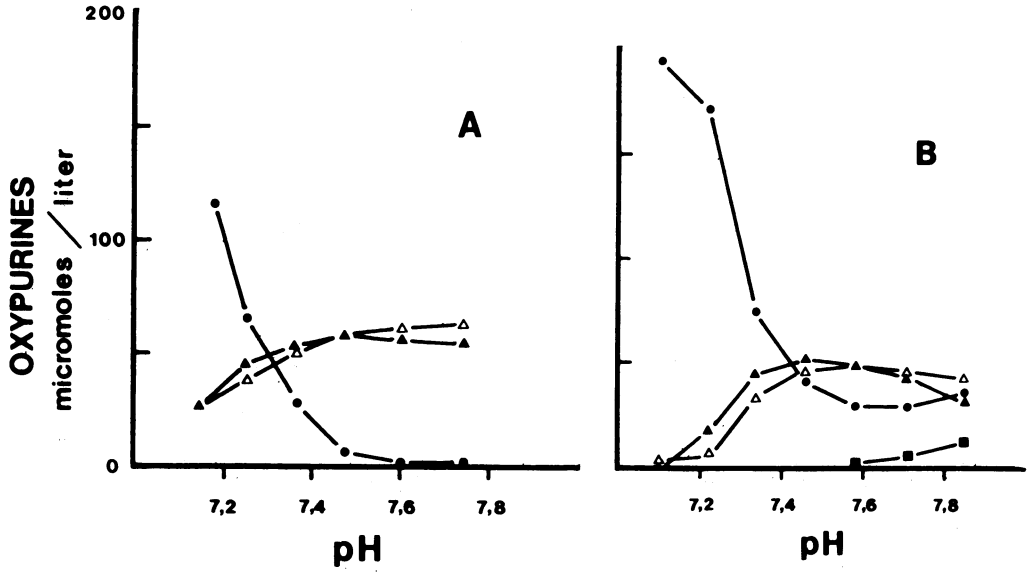

Figure 1. Variation with pH in uptake and metabolism of hypoxanthine by erythrocytes. (*A*) Incubation for 2 h at 37°C in 10 mM Pi and 100 μM [^{14}C]-hypoxanthine. (*B*) Preincubation for 2 h in 10 mM Pi and 100 μM [^{14}C]hypoxanthine, pH 7.15, followed by alkalinization and incubation for a further 2 h. — ● —, intracellular IMP; — ▲ —, intracellular hypoxanthine; — ■ — intracellular inosine; — △ —, medium hypoxanthine. Units on the abscissa are in micromoles/liter packed erythrocytes or micromoles/liter medium.

pletion (results not shown) depend on pH, so that at a given Pi concentration, decreasing pH results in a progressively greater IMP accumulation.

Effect of pH on PRPP formation. To investigate the mechanism whereby low pH enhances IMP synthesis, erythrocytes were incubated for varying times in medium similar to that used for the experiment illustrated in Fig. 1 *A*, but containing no hypoxanthine. The PRPP content of the red cells at varying times is shown as a function of pH in Fig. 4, and demonstrates that detectable accumulation of PRPP occurs only at acid pH, proceeding at a maximum rate of ~ 100 μmol/liter red cells per hour at pH 7.0. The initial content of PRPP is < 5 μmol/liter, reflecting the situation in circulating red cells, and remains unchanged at pH values > 7.6.

Effect of pH on phosphate uptake and metabolism. Since the Pi concentration has such a marked effect on PRPP synthesis, both in intact cells and cell-free hemolysates (8), the ingress of phosphate and the intracellular Pi concentration were measured in cells incubated in media containing 10 mM ^{32}P-labeled Pi at a number of different pH values. After incubation for 1 h, the cells and medium were harvested and the intracellular Pi concentration was measured colorimetrically. The total uptake of phosphate was determined by measurement of ^{32}P content of both cells and medium (Fig. 5 *A*). In a parallel experiment, cells incubated for 1 h at pH 6.8 under the conditions described above were subsequently divided into aliquots and the pH was adjusted to provide a second set of suspensions covering a pH range of 6.8 to 7.9. These were incubated for a further hour at 37°C and then processed in the same manner as the first set (Fig. 5 *B*). The results showed that acidification of cells suspended in a phosphate containing medium results in a steep, nonlinear increase in the intracellular Pi concentration. The influx of phosphate, measured by the intracellular accumulation of ^{32}P, also increases with decreasing pH but in a more linear manner and to a lesser extent, as shown by the shallower slope. This is reflected by a corresponding decrease in ^{32}P concentration in the medium. These results indicate that at higher pH values, the entry of Pi into erythrocytes is slower and that much of the phosphate that does enter is no longer measurable as free Pi. At lower pH

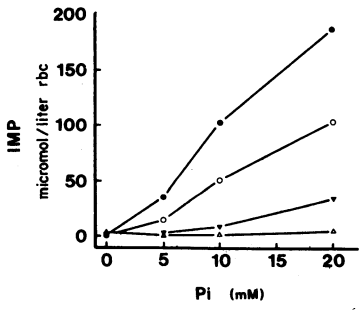

Figure 2. Effect of extracellular Pi and pH on the uptake and conversion of hypoxanthine to IMP by erythrocytes. Accumulation of IMP in erythrocytes after 1 h incubation at 37°C in 60 μM [^{14}C]-hypoxanthine (1.2 Ci/mol). — ● —, pH 7.11; — ○ —, pH 7.26; — ▼ —, pH 7.48; — △ —, pH 7.82. rbc, packed erythrocytes.

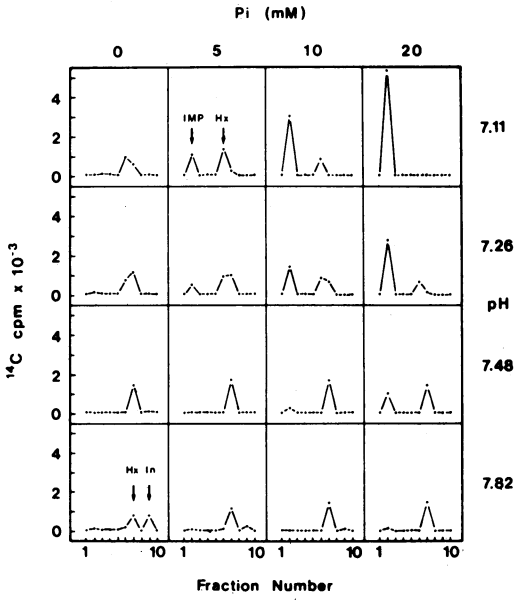

Figure 3. Reverse-phase HPLC of erythrocyte oxypurines. The erythrocytes were incubated with [^{14}C]hypoxanthine as described in Fig. 2, in media of varying pH and Pi concentration. The minimum number of fractions required for resolution of the three species was collected. (*Arrows*) Elution positions of unlabeled markers of IMP, inosine (In), and hypoxanthine (Hx).

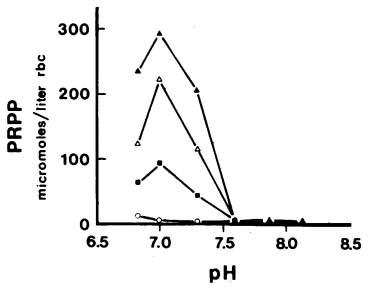

Figure 4. Variation with pH in PRPP accumulation in erythrocytes. Incubation at 37°C in 10 mM Pi. Aliquots for PRPP determination removed at hourly intervals as indicated; — ○ —, 0 h; — ■ —, 1 h; — △ —, 2 h; — ▲ —, 3 h.

values, phosphate entry is faster and is measurable as free Pi; in fact, the levels of intracellular Pi exceed those resulting from phosphate influx alone. In the second part of the experiment (Fig. 5 *B*), it can be seen that alkalinization of cells preloaded with phosphate at acid pH results in a fall, progressive with increasing pH, of intracellular free Pi concentration, with little change in total ^{32}P content. This is confirmed by anion-exchange HPLC of extracts from cells incubated at the extremes of pH (Fig. 6). A redistribution occurs in the relative amounts of ^{32}P-labeled species with increasing pH; ^{32}Pi falls, with a concomitant increase in more anionic ^{32}P-labeled compounds. In an experiment in which similarly incubated cells were transferred to a medium containing no phosphate for the second incubation step, efflux of inorganic phosphate from the cells occurred readily and was more pronounced at low pH values (data not shown).

Effect of pH on adenine nucleotide levels. Washed erythrocytes were incubated for 2 h at 37°C in Hepes-buffered medium containing 10 mM glucose and 10 mM Pi at varying pH. The neutralized perchloric acid extracts of the cells were chromatographed on anion-exchange HPLC, and ADP and ATP levels were measured relative to that of authentic standards of known concentration. The results are given in Table I and show that with increasing pH, there is an increase in the ADP, a fall in ATP, and increased lactate accumulation.

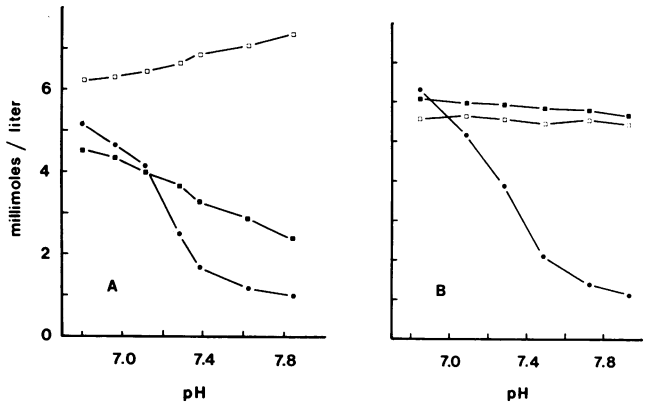

Figure 5. Variation with pH in the net influx of extracellular Pi and the free intracellular Pi concentration in erythrocytes. (*A*) Incubation of erythrocytes for 1 h at 37°C with 10 mM ^{32}P-labeled Pi. (*B*) Erythrocytes preincubated for 1 h at 37°C in 10 mM [^{32}P]Pi at pH 6.8 as described in *A*, then divided into aliquots, and the pH was adjusted to values between 6.8 and 7.9. These were incubated for a further hour at 37°C. — ● —, free intracellular Pi (millimoles/liter packed erythrocytes); — ■ —, total erythrocyte ^{32}P (amount of ^{32}Pi accumulated from the medium into both free and esterified ^{32}P phosphate) (millimoles/liter packed erythrocytes); — □ —, medium ^{32}Pi (millimolar).

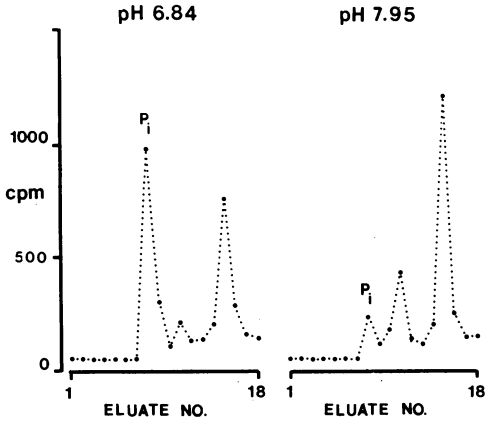

Figure 6. Anion-exchange HPLC of cell extracts corresponding to the most acid (pH 6.84) and alkaline (pH 7.95) incubation conditions described in Fig. 5 *B*. The distribution of ^{32}P-labeled compounds is shown, with the elution position of Pi marked. The two ^{32}P containing peaks eluting after Pi were not identified, but their position suggests they represent highly anionic organic phosphates such as 2,3 DPG.

Effect of pH on oxypurine metabolism in whole blood. To determine whether the accumulation of IMP and free Pi in erythrocytes showed the same pH dependence when suspended in plasma as in defined aqueous medium, whole blood equilibrated with CO_2 to achieve a pH of 7.0 was incubated for 90 min at 37°C in the presence of 10 mM Pi and 100 μM [^{14}C]hypoxanthine. HPLC analysis of cell extracts prepared from aliquots removed after 5 min (Fig. 7 *A*) and 90 min (Fig. 7 *B*) showed progressive accumulation of IMP and decrease in hypoxanthine. After incubation, cells were resuspended in fresh plasma equilibrated with CO_2 to maintain the pH at 7.0. Initially, there was a slight decrease in cellular hypoxanthine (because of efflux into the fresh plasma) but no significant change in IMP levels (Fig. 7 *C*). The resuspended erythrocytes were equilibrated with air for varying times to remove CO_2 and thus provide samples covering a pH range from 7.03 to 7.77. These suspensions were incubated for a further hour. Analysis of the plasma showed little release of radioactivity below pH 7.3 with a sharp increase above this pH (Table II). HPLC analysis of plasma radioactivity from the most alkaline aliquot (i.e., the one from which the most counts were released) showed that label was confined to hypoxanthine (Fig. 7 *F*).

Table I. Effect of pH on Adenine Nucleotide Levels and Lactate Production

pH	RBC[ADP]	RBC[ATP]	Medium lactate
	μmol/liter		*mM*
7.12	78	893	1.87
7.40	147	837	3.14
7.71	202	663	3.60

Erythrocytes were incubated at 37°C for 2 h in 10 mm Pi at the pH values indicated. ADP and ATP levels were determined by anion exchange HPLC of cell extracts and expressed as micromoles per liter of packed erythrocytes. L-Lactate in the medium was measured by the enzymatic method described.

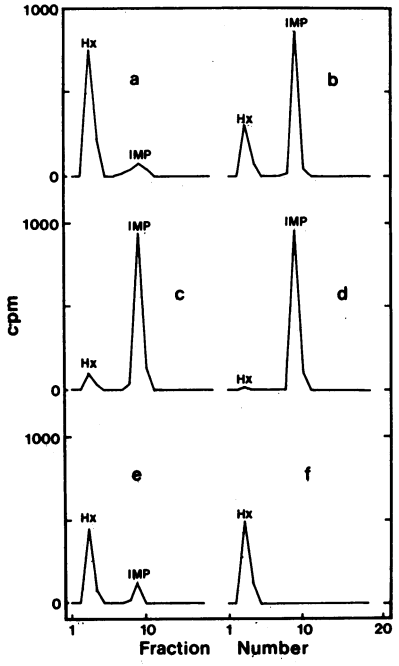

Figure 7. Anion-exchange HPLC of erythrocyte oxypurines. Whole blood incubated with 10 mM Pi and 100 μM [^{14}C]hypoxanthine at 37°C. pH adjusted to 7.0 by equilibration with 20% CO_2 in oxygen. HPLC analysis of intracellular purines was performed on aliquots removed after 5 (*A*) and 90 min (*B*). The cells were centrifuged and resuspended in fresh autologous plasma containing no added hypoxanthine or Pi. Aliquots of cells removed before (*C*) and after (*D*) a further 90-min incubation at pH 7.0. A portion of freshly resuspended cells was equilibrated with air to increase the pH to 7.8, then incubated in parallel for 90 min (*E*). The plasma from this alkaline suspension was also analyzed (*F*).

Analysis of the cells incubated at pH 7.03 and 7.77 revealed that at low pH, remaining hypoxanthine was quantitatively converted to IMP (Fig. 7 *D*), whereas at high pH, most of the IMP was degraded to hypoxanthine (Fig. 7 *E*) that then diffused into the plasma (Fig. 7 *F*). Table II also shows that there is a decline in intracellular Pi with increasing pH. This is not accompanied by a corresponding increase in plasma Pi, indicating that the fall in red cell Pi was not due to efflux, but rather to intracellular consumption. The rate of lactate production increased progressively with a rise in pH, reflecting a twofold enhancement of glycolysis over the observed pH range.

Table II. pH Effects on Red Cells and Plasma in Whole Blood Preincubated with Phosphate and Hypoxanthine

pH	RBC purine	Plasma Hx	RBC Pi	Plasma Pi	Plasma lactate
	mmol/liter	*μM*	*μmol/liter*	*μM*	*mM*
7.03	131	1.6	5.48	3.86	2.19
	127	1.6	5.67	3.94	2.40
7.20	130	1.9	4.94	3.51	2.49
	127	1.8	4.99	3.64	2.56
7.29	128	2.7	4.54	3.50	2.54
	132	2.6	4.54	3.51	2.09
7.47	106	22.3	3.06	3.33	3.44
	104	23.0	3.00	3.38	3.50
7.72	77	40.9	1.92	3.21	3.82
	79	40.8	1.66	3.22	3.96
7.77	71	47.1	1.53	3.42	4.40
	72	47.6	1.70	3.50	3.91

Whole blood at pH 7.0 was incubated for 90 min at 37°C in the presence of 10 mM inorganic phosphate and 100 μM labeled hypoxanthine. The erythrocytes were centrifuged, resuspended in fresh plasma, equilibrated with air for various times to give the range of pH values given in the table, and then incubated for 60 min at 37°C. Measurement of purine and phosphate are expressed per liter of packed erythrocytes.

Effect of P_{O_2} on oxypurine metabolism. To determine whether P_{O_2} affects hypoxanthine uptake, erythrocytes were incubated with [^{14}C]hypoxanthine at pH 7.1 under conditions of varying P_{O_2} in the presence of three different Pi concentrations (Fig. 8). IMP synthesis was increased at P_{O_2} values < 7 kPa at all Pi concentrations studied.

Discussion

The effects of pH in the range 7.0 to 7.8 on erythrocyte oxypurine metabolism shown in this study may be related to the pH-dependent changes in a number of intracellular compounds.

A fall in pH results in a rise in intracellular Pi. This may occur by two mechanisms. First, transport into the cells of divalent anions, including phosphate, by the band 3 membrane proteins is increased with decreasing pH, with the maximum rate at pH 6.4 (20). Secondly, prevailing pH affects the distribution of phosphate between phosphorylated glycolytic intermediates (predominantly 2,3 DPG) and Pi. A fall in pH results in a decrease in 2,3 DPG (21) and ADP, with a concomitant increase in intracellular Pi and ATP. These changes may be explained by the net conversion of 2,3 DPG to lactate. Since the production of lactate from 2,3 DPG yields twice as much ATP (2 mol/mol) as production from glucose (1 mol/mol), overall glycolytic flux falls as measured by lactate production (11, 22, 23) and there is an increase in the ATP/ADP ratio. The phosphate moieties of 2,3 DPG ultimately accumulate as Pi. Increase in pH reverses all these changes.

Levels of total erythrocyte 2,3 DPG fall with a decrease in pH (21). However, much more rapid changes in the concentration of free 2,3 DPG are caused by the binding of this compound to hemoglobin (24). 2,3 DPG binds preferentially to deoxyhemoglobin, and a 10-fold lower concentration of 2,3 DPG has been calculated by Bunn, Ransil, and Chao (25) to exist in fully deoxygenated as compared with fully oxygenated erythrocytes. By decreasing the affinity of hemoglobin for ox-

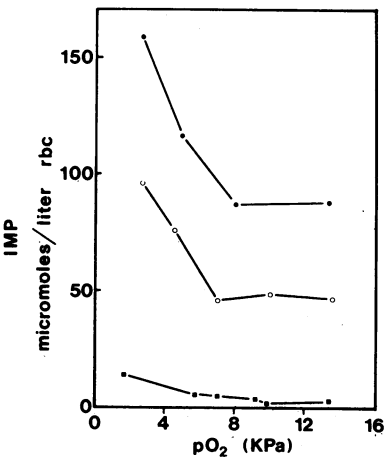

Figure 8. Variation with P_{O_2} in the metabolism of hypoxanthine to IMP in erythrocytes. Erythrocytes incubated for 90 min at 37°C with 120 μM [^{14}C]-hypoxanthine at pH 7.1 in the presence of varying concentrations of Pi: — ■ —, 1; — ○ —, 5; and — ● —, 10 mM. rbc, packed erythrocytes.

ygen (the Bohr effect), a fall in pH will therefore enhance the binding of 2,3 DPG to hemoglobin and also lower free 2,3 DPG levels.

It has been calculated that free intracellular magnesium ion (Mg^{2+}) concentration rises from 0.6 to 1.9 mM as fully oxygenated blood becomes deoxygenated (25). This is a result of the competition between deoxyhemoglobin and Mg^{2+} for the binding of organic phosphates, mainly 2,3 DPG and ATP.

The activity of two principle enzymes of purine metabolism are regulated by the compounds discussed above. These are PRPP synthetase and purine 5′ nucleotidase. Purified PRPP synthetase shows a broad pH optimum between 7.2 and 7.4 (compared with PRPP synthesis in intact cells) and an absolute requirement for Pi (8). Our results (data not shown) confirmed these findings. Mg^{2+} is required for activation of PRPP synthetase (8) although the low K_m for activation (0.2 mM) implies that the variation in intracellular free Mg^{2+} concentration described above will have little regulatory influence. In comparison to Pi and Mg^{2+}, ADP and 2,3 DPG are allosteric inhibitors of PRPP synthetase activity (4, 8, 9).

The purine 5′ nucleotidase of red cells is a cytosolic enzyme only recently described and characterized by Bontemps, van den Berghe, and Hers (12), who showed that the activity of this enzyme is inhibited by Pi and activated by 2,3 DPG, effects opposite to those on PRPP synthetase.

The two other enzymes involved in hypoxanthine and IMP metabolism in the erythrocyte are HPRT and PNP. Since both IMP and PRPP accumulation show a similar dependence on pH, it can be concluded that IMP synthesis in the presence of hypoxanthine is determined by PRPP availability, and that HPRT activity is not rate limiting. The rapid conversion of IMP to hypoxanthine at alkaline pH demonstrates that PNP is not usually rate limiting, except at high pH in the absence of external phosphate. Under these conditions, intracellular Pi concentration is at a minimum, and inosine becomes detectable, since Pi is required as cosubstrate for PNP. This Pi dependence of PNP explains the reduction in plasma inosine seen in patients with elevated plasma Pi due to renal failure (26).

A model for the regulation of oxypurine metabolism in the red cell is illustrated in Fig. 9 and proposes the following: hypoxanthine, IMP, and inosine are components of an oxypurine cycle in erythrocytes. Under conditions of low pH and high extracellular Pi, intracellular Pi concentrations rise and levels of ADP and 2,3 DPG fall, the latter effect augmented at low P_{O_2}. The consequent stimulation of PRPP synthesis favors the conversion of hypoxanthine to IMP, and the latter accumulates due to inhibition of purine 5′ nucleotidase. Enhanced synthesis of PRPP explains the increased levels of IMP and ATP (via salvage of adenine) found in erythrocytes of patients with chronic renal failure, in which blood pH is invariably decreased and plasma Pi concentration increased (26). On returning to conditions of neutral or alkaline pH, low extracellular Pi, and high P_{O_2}, intracellular Pi levels fall and 2,3 DPG and ADP levels rise. These conditions result in a concerted activation of purine 5′ nucleotidase and inhibition of PRPP synthetase, with net conversion of IMP to hypoxanthine. Efflux of the accumulating hypoxanthine is facilitated by rapid equilibration across the red cell membrane (5).

Low pH, high Pi, and low P_{O_2}, which favor the uptake of hypoxanthine by erythrocytes, are found in poorly oxygenated tissue and suggest that the erythrocyte oxypurine cycle might provide a mechanism for the transfer of hypoxanthine from poorly oxygenated to well oxygenated tissue in vivo. Although Pi levels studied are far in excess of physiological levels, a severalfold increase in Pi concentration has been shown in intact ischemic tissue by ^{31}P NMR (27, 28). The cycle might also play a role in a more pathophysiological context, since in frankly ischemic tissue hypoxanthine is known to accumulate (29–31) and has been implicated in the causation of reperfusion injury (32).

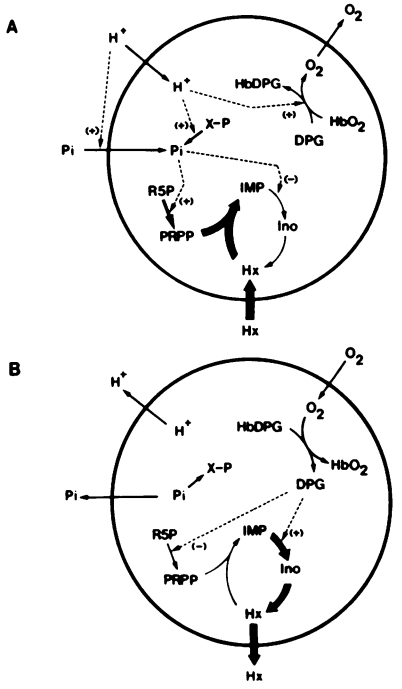

Figure 9. Model depicting the factors regulating IMP and hypoxanthine metabolism in (*A*) conditions of acid pH, low P_{O_2}, and high external Pi concentration, and (*B*) conditions of neutral or alkaline pH, high P_{O_2}, and low external Pi concentration. DPG, 2,3-biphosphoglycerate; Hb, hemoglobin; Hx, hypoxanthine; Ino, inosine; IMP, inosine 5′-monophosphate; Pi, inorganic phosphate; PRPP, 5-phosphoribosyl-1-pyrophosphate; R5P, ribose-5-phosphate; X-P, phosphorylated glycolytic intermediates, including 2,3 DPG.

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