

Sodium-Phosphate Cotransport in Human Red Blood Cells

Kinetics and Role in Membrane Metabolism

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ABSTRACT Orthophosphate (P_i) uptake was examined in human red blood cells at 37°C in media containing physiological concentrations of P_i (1.0–1.5 mM). Cells were shown to transport P_i by a 4,4'-dinitro stilbene-2,2'-disulfonate (DNDS)-sensitive pathway (75%), a newly discovered sodium-phosphate (Na/P_i) cotransport pathway (20%), and a pathway linearly dependent on an extracellular phosphate concentration of up to 2.0 mM (5%). Kinetic evaluation of the Na/P_i cotransport pathway determined the $K_{1/2}$ for activation by extracellular P_i ($[Na]_o = 140$ mM) and extracellular Na ($[P_i]_o = 1.0$ mM) to be 304 ± 24 μ M and 139 ± 8 mM, respectively. The phosphate influx via the cotransport pathway exhibited a V_{max} of 0.63 ± 0.05 mmol P_i (kg Hb) $^{-1}$ (h) $^{-1}$ at 140 mM Na_o . Activation of P_i uptake by Na_o gave Hill coefficients that came close to a value of 1.0. The V_{max} of the Na/P_i cotransport varied threefold over the examined pH range (6.90–7.75); however, the Na/P_i stoichiometry of 1.73 ± 0.15 was constant. The membrane transport inhibitors ouabain, bumetanide, and arsenate had no effect on the magnitude of the Na/P_i cotransport pathway. No difference was found between the rate of incorporation of extracellular P_i into cytosolic orthophosphate and the rate of incorporation into cytosolic nucleotide phosphates, but the rate of incorporation into other cytosolic organic phosphates was significantly slower. Depletion of intracellular total phosphorus inhibited the incorporation of extracellular P_i into the cytosolic nucleotide compartment; and this inhibition was not reversed by repletion of phosphorus to 75% of control levels. Extracellular $^{32}P_i$ labeled the membrane-associated compounds that migrate on thin-layer chromatography (TLC) with the R_f values of ATP and ADP, but not those of 2,3-bisphosphoglycerate (2,3-DPG), AMP, or P_i . DNDS had no effect on the level of extracellular phosphate incorporation or on the TLC distribution of P_i in the membrane; however, substitution of extracellular sodium with *N*-methyl-D-glucamine inhibited phosphorylation of the membranes by 90% and markedly altered the chromatographic pattern of the membrane-associated phosphate. These results demonstrate the existence of a Na/P_i cotransport system in the red cell membrane that is important in

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the delivery of extracellular phosphate to the membrane compartment of human red cells.

INTRODUCTION

Orthophosphate (P_i) transport across cell membranes occurs by several different pathways. These transport pathways provide extracellular phosphate to the intracellular metabolic reactions. Regulation of plasma phosphate concentration is closely controlled by the proximal tubule cells of the kidney, which reabsorb over 90% of the filtered phosphate, and by other cell membranes whose transport pathways mediate equilibration of phosphate. There are two well-characterized phosphate transport pathways: the anion transport protein, band 3, of the human red blood cell (Gruber and Deuticke, 1973; Schnell and Besl, 1984; Runyan and Gunn, 1984), and the sodium-phosphate cotransport system (Na/P_i cotransport) of renal (Hoffmann et al., 1976; Amstutz et al., 1985) and duodenal (Danisi et al., 1984) brush border membranes. This paper reports for the first time the presence of a Na/P_i cotransport pathway in the membrane of the human red cell that carries 20% of the phosphate influx in the presence of physiological concentrations of extracellular sodium.

In the human red cell, there are several metabolic reactions and membrane enzymes that have P_i as a reactant or a product. Phosphate is used by glyceraldehyde-3-phosphate dehydrogenase (GAPDH) in the formation of 1,3-bisphosphoglycerate (1,3-DPG), by purine nucleoside phosphorylase in the formation of ribose-1-phosphate, and is liberated by the hydrolysis of 2,3-DPG via DPG phosphatase. In addition, phosphate is produced continually at the membrane by kinase (Babitch et al., 1984; Palfrey and Waseem, 1985), and Mg-ATPase activities (Hoffman, 1980; Patel and Fairbanks, 1986), actin polymerization (Oosawa and Kasai, 1971; Pinder and Gratzner, 1983), as well as by ATPases that are operating to maintain transmembrane ion gradients (Glynn and Karlish, 1975; Schatzmann, 1975). Therefore, the transport of phosphate into and out of the cell must be of sufficient magnitude to meet the different rates of phosphate consumption and production by the cell during different physiological conditions.

The compartmentation of glycolytic metabolism in human red cells has been reported by a number of laboratories (Parker and Hoffman, 1967; Okonkwo et al., 1975; Mercer and Dunham, 1981*a, b*). The evidence for the compartmentation of metabolism resides primarily in the existence of a membrane-associated pool of ATP that can be used preferentially to phosphorylate the Na/K pump (Proverbio and Hoffman, 1977) and to mediate cardiac glycoside-sensitive sodium transport (Mercer and Dunham, 1981*b*). It has also been shown that the human red cell membrane contains substantial amounts of ADP as well as ATP (Shoemaker and Hoffman, 1985). Together with the glycolytic enzymes that have been localized to the red cell membrane (Tillman et al., 1975; De and Kirtley, 1977; Strapazon and Steck, 1977; Tsai et al., 1982), these membrane-bound nucleotides form a separate glycolytic compartment that may provide energy for membrane-associated ATPase reactions. The association of GAPDH with the NH_2 -terminus of the band 3 protein has been shown to inhibit the glycolytic enzyme's activity (Tsai et al., 1982). This binding may serve to keep the GAPDH in proximity with the membrane rather than to promote enzyme-substrate-enzyme coupling between band 3 and GAPDH of the type

described previously for GAPDH and phosphoglycerate kinase (PGK) (Srivastava and Bernhard, 1986).

Several studies have attempted to investigate the role of extracellular phosphate in these membrane-associated glycolytic reactions by examining the rate of incorporation of extracellular phosphate into the three different cytosolic phosphate pools: P_i, nucleotide phosphates, and other organic phosphates (Gerlach et al., 1958; Bartlett, 1958; Chedru and Cartier, 1966; Reed and Young, 1967; Tenenhouse and Scriver, 1975). There is a discrepancy in these reports as to whether extracellular phosphate is first equilibrated into the cytosolic P_i pool and subsequently into cytosolic nucleotides, or whether the cytosolic nucleotide pools reach isotopic equilibrium with extracellular phosphate before the cytosolic P_i. Initial reports indicated that the rate of incorporation of extracellular ³²P_i into cytosolic nucleotides exceeded the rate of incorporation into cytosolic P_i (Gerlach et al., 1958; Bartlett, 1958). However, a subsequent study (Tenenhouse and Scriver, 1975) explained this result on the basis of the different cytosolic pool sizes of nucleotides and P_i. Tenenhouse and Scriver calculated the specific activity of the ³²P in these respective pools and found that the specific activities in P_i and ATP increase at equivalent rates. This result argues against the compartmentation of phosphate metabolism in the human red cell cytosol.

The nucleotide pool associated with the human red cell membrane, however, has not previously been examined for the kinetics of equilibration with extracellular ³²P_i, but early studies report the existence of a membrane-associated phosphate-acceptor complex that achieves a specific activity higher than that of cytosolic ATP (Schauer and Hillmann, 1961). In addition, Mercer and Dunham (1981b) have demonstrated that it is possible to incorporate P_i into membrane-compartmentalized ATP in inside-out vesicles using the glycolytic substrates of the membrane-associated GAPDH and PGK reactions together with extravesicular (intracellular) ³²P_i. However, the physiological function played by this membrane-compartmentalized pool of ATP has not yet been demonstrated in the intact red cell.

The present work characterizes the kinetics of the Na/P_i cotransport system in the membrane of the human red cell and the kinetics of extracellular phosphate incorporation into the three cytosolic phosphate pools (P_i, nucleotide phosphates, and other organic phosphates) as well as into the membrane phosphate compartment. We also examine the role played by each of the two major phosphate transport pathways, band 3 and Na/P_i cotransport, in providing the different phosphate pools with extracellular phosphate, and we investigate the metabolic dependence of this process. We show that the Na/P_i cotransport is the transport pathway primarily responsible for importing phosphate to the rapidly labeled membrane-bound nucleotide compartment. Preliminary reports of these findings have been published previously (Shoemaker and Gunn, 1986; Shoemaker et al., 1987).

METHODS

Preparation of Cells

Whole blood was drawn from adult humans into heparinized tubes and spun at 12,000 *g* in a refrigerated centrifuge (RC-5B; DuPont-Sorvall Instruments Div., Newton, CT) at 4°C for 10 min. Buffy coat and plasma were aspirated and the remaining cells were washed three times at

20% hematocrit in 165 mM NaCl. Cells were then prewashed twice at 10% hematocrit in media identical to that used in the influx and phosphorylation incubations, but without phosphate. In experiments conducted at pH 7.75, cell suspensions were titrated to the appropriate pH in the final prewash medium with either 1.0 N NaOH or KOH. Cell suspensions were similarly adjusted to pH 6.90 with 1.0 N HCl. After the prewash, the packed red cells were added directly to the flasks to initiate the influx or phosphorylation incubations. Phosphorus-depleted cells were prepared by incubation for 24 h in (in millimolar): 140 NaCl, 20 HEPES, 100 $\mu\text{g/ml}$ penicillin G, and 100 $\mu\text{g/ml}$ streptomycin; pH = 7.4 at 37°C. ATP-depleted cells were prepared by being incubated for 2 h in (in millimolar): 130 NaCl, 10 KCl, 10 2-deoxy-D-glucose, and 10 $\text{Na}_2\text{HPO}_4/\text{NaH}_2\text{PO}_4$; pH = 7.4 at 37°C. Repletion of phosphorus was achieved by incubation at 37°C for a minimum of 2 h in (in millimolar): 140 NaCl, 10 glucose, 5 inosine, 2 adenosine, and 10 $\text{Na}_2\text{HPO}_4/\text{NaH}_2\text{PO}_4$; pH = 7.4. Depleted and repleted cells were washed four times at 37°C in phosphate-free media before the influx measurement to lower intracellular phosphate to levels comparable to control cells.

Influx and Phosphorylation Studies

For the majority of fluxes conducted in this study, a 0.75-ml volume of packed red cells was added to 3.0 ml of temperature-equilibrated medium containing (in millimolar) 150 NaCl, 0.83 Na_2HPO_4 , 0.17 NaH_2PO_4 , and 10 HEPES; pH = 7.4 at 37°C. When required, the sodium was replaced with equal concentrations of *N*-methyl-D-glucamine (NMDG) or potassium, and 10 mM glucose was added to activate the metabolism. The specific activity of the $^{32}\text{PO}_4$ was 4.0 mCi/mmol for the flux experiments and 40 mCi/mmol for membrane phosphate incorporation studies. The specific activity for ^{22}Na in the flux experiments was 0.15 $\mu\text{Ci/mmol}$. At a minimum of five designated times (usually 6, 12, 18, 24, and 30 min), a 0.5-ml sample was removed from the incubation flask and added to 10 ml of an ice-cold stop solution containing (in millimolar) 150 KCl, and 10 HEPES, pH = 7.4 at 0°C. Sample tubes were spun at 3,000 *g* and the supernatant was aspirated. Cells were washed three times with 8.0 ml stop solution, and the final cell pellet was hemolyzed in 1.0 ml of ice-cold 10 mM HEPES titrated with 1 M KOH to pH 7.6 at 0°C (K/HEPES). From the hemolysate, duplicate 50- μl samples were added to 2.0 ml of Drabkin's reagent (van Kampen and Zijlstra, 1961) and the optical density, from which the hemoglobin (Hb) concentration was calculated, was read at 540 nm on a Gilford Stasar III spectrophotometer (Gilford Instrument Laboratories, Inc., Oberlin, OH). Subsequently, 0.5 ml hemolysate samples were added to 1.0 ml 7% perchloric acid (PCA) in 1.9-ml S/P microcentrifuge tubes (American Scientific Products, McGaw Park, IL), and spun at 6,000 *g* for 2 min in a Heraeus Biofuge B centrifuge (Heraeus-Amersil, Inc., Sayreville, NJ). The supernatant was assayed for radioactivity, by first diluting 100- or 200- μl aliquots into 3.0 ml of Ecoscint (National Diagnostics, Inc., Somerville, NJ) scintillation fluid, and then by counting the duplicates in a liquid scintillation counter (Packard Instrument Co., Downers Grove, IL). For analysis of the $^{32}\text{P}_i$ incorporated into nucleotides, 300- μl aliquots of PCA extract were added to 1.2 ml of 0.2 g/ml Norit A decolorizing carbon in 7% PCA, and then spun at 6,000 *g* for 2 min; afterwards 200- μl aliquots of the supernatant were taken for scintillation counting. The amount of ^{32}P incorporated into nucleotides (primarily ATP and ADP) was then determined by the difference between the ^{32}P counts in the PCA extract and those remaining in the Norit A supernatant (Crane and Lipmann, 1953). The fraction of radioactivity contained in P_i was determined by organic extraction of the orthophosphomolybdate complex from the neutralized Norit A extract (Martin and Doty, 1949). When used, inhibitors of transport processes were present at the following concentrations (in millimolar): 0.2 DNDS, 0.1 bumetanide, 1.0 amiloride, 0.1 ouabain, and 20 arsenate. Uptake rates into the various phosphate compartments were judged to be initial rates by virtue of the linearity of the plot of counts per minute (kg Hb) $^{-1}$ versus time for the influx duration (usually 30–40 min). Fluxes were always performed in duplicate.

Membrane Preparation

Membranes were prepared from the hemolysate after aliquots were taken for hemoglobinometry and PCA extracts. Hemolysates were diluted to 8.0 ml with ice-cold K/HEPES. The membrane suspensions were spun at 30,000 *g* for 10 min and the supernatant was aspirated. The membranes were washed twice more, resuspended in 0.5 ml K/HEPES, and frozen at -20°C until analyzed for protein concentration, for radioactivity, and by thin-layer chromatography (TLC).

Assays

Total intracellular phosphorus (Ames and Dubin, 1960), extracellular P_i (Forbush, 1983; Black and Jones, 1983), and intracellular orthophosphate (Vestergaard-Bogind, 1964; Parvin and Smith, 1969) were measured by previously published methods. Adenosine triphosphate was determined in 5.62 M K_2CO_3 -neutralized PCA extracts by the method of Ellis and Gardner (1980), and in boiled membrane extracts by the method of Kimmich et al. (1975), which employed purified luciferin-luciferase (L 0633; Sigma Chemical Co., St. Louis, MO). Protein concentrations were determined on membrane samples by the method of Lowry et al. (1951), and membrane concentrations and radioactivity were expressed per milligram of membrane protein. TLC was performed on thawed membrane samples or boiled membrane extracts (for 5 min at 100°C) with the same results. We used the method of Randerath and Randerath (1967), using a 1 M CH_3COOH :4 M LiCl (4:1, vol/vol)-running buffer on poly(ethyleneimine)-cellulose plates. Nucleotide standards were visualized with short-wave ultraviolet radiation using a Mineralight model UVGL-25 lamp (UVP, Inc., San Gabriel, CA) and acid labile phosphorus compounds by the method of Rosenberg (1959). TLC plates were cut in 2.0×0.5 cm regions and counted with 3 ml of scintillation fluid. Organic extraction of the membrane was performed by the method of Ferrell and Huestis (1984) and inositol phosphate ion chromatography by the method of Berridge et al. (1983).

RESULTS

Characterization of Phosphate Influx Pathways

Phosphate uptake into human red cells was examined in a medium containing physiological concentrations of sodium (150 mM) and phosphate (1.0–1.5 mM). As shown in Fig. 1, the majority of the phosphate entering the cell under these conditions (75%) was through a 4,4'-dinitro stilbene-2,2'-disulfonate (DNDS) -inhibitable pathway, presumably mediated by band 3, the anion transport protein; however, there was a substantial phosphate uptake (20%) mediated by a sodium-dependent pathway. The sodium-dependent portion of the influx was equivalent in the presence or absence of DNDS when sodium was replaced isoionically with *Nmdg*. When potassium was the replacement cation, however, the magnitude of the sodium-dependent flux in the absence of DNDS was always significantly less than its magnitude in the presence of DNDS. In addition, there was a DNDS-insensitive, sodium-independent residual portion (5% at 1.0 mM $[\text{P}_i]_o$) of the influx whose magnitude was linear with phosphate concentrations of up to 2.0 mM P_i (data not shown). The sodium-dependent pathway was only slightly elevated in the top 15% of the cells that were separated by density using the Murphy technique (1973), relative to the bottom 15% (data not shown), which indicates that the measured influx was not simply the expression of a very active reticulocyte transport pathway. The DNDS-sensitive flux, the sodium-dependent flux in the presence of DNDS, and the residual flux in

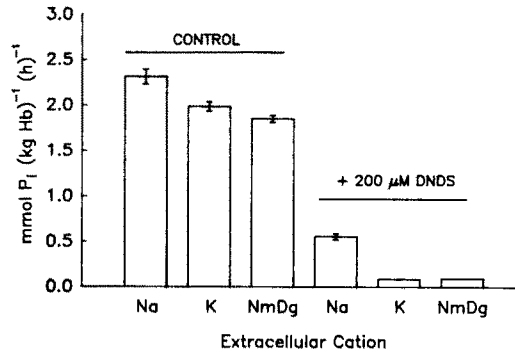


FIGURE 1. Initial rate of orthophosphate influx into human red cells in the absence and presence of the anion transport inhibitor, DNDS. Phosphate influx was measured in media containing (in millimolar): 150 NaCl, KCl, or NmDg; 0.83 Na₂HPO₄ or K₂HPO₄; 0.17 NaH₂PO₄ or KH₂PO₄; and 10 HEPES; pH 7.4 at 37°C. Influx values are the means of at least three determinations. The error bars indicate the SE of the mean. The error bars for the two rightmost bars are contained within the width of the line.

the presence of DNDS and the absence of sodium define for us three components of the phosphate influx into human red cells.

Inhibition Studies

The effect of a few inhibitors of membrane transport processes on the uptake of P_i ([P_i]_o = 1.0 mM) can be seen in Fig. 2. Ouabain, an inhibitor of the Na/K pump, and bumetanide, an inhibitor of Na/K/Cl cotransport, had no significant effect on phosphate influx. DNDS and the phosphate analogue arsenate (As_i) significantly inhibited the uptake. As_i has previously been shown to be a competitive inhibitor of glyceraldehyde-3-phosphate dehydrogenase (Needham and Pillai, 1937), as well as of the Na/P_i cotransport found in renal brush border membranes (Hoffmann et al., 1976; Rabito, 1983). Surprisingly, the partial inhibition observed was limited to DNDS-sensitive phosphate uptake, presumably mediated by the band 3 protein. As_i had no effect on phosphate uptake in the presence of DNDS at the concentration of phosphate employed.

Phosphate Activation of Sodium-dependent Phosphate Transport

The activation of the sodium-dependent phosphate influx by extracellular phosphate is depicted in Fig. 3. The phosphate influx in the absence of sodium was sub-

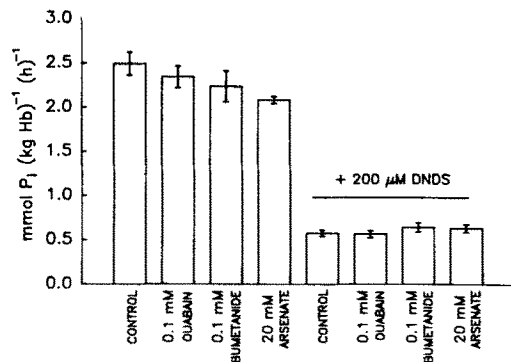
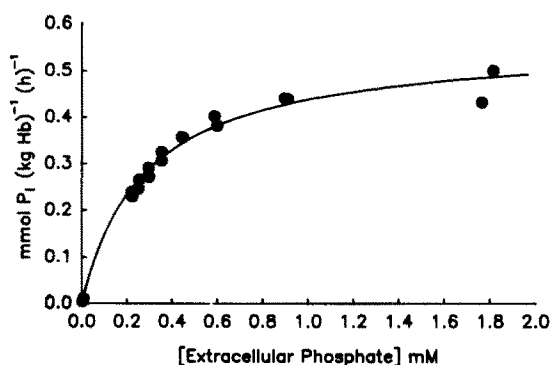


FIGURE 2. The effect of transport inhibitors on P_i influx. Phosphate uptake was measured in a media containing (in millimolar): 155 NaCl, 5.0 KCl, 0.83 Na₂HPO₄, 0.17 NaHPO₄, and 10 HEPES; pH 7.4 at 37°C. Sodium chloride was replaced isosmotically with NaAs_i such that the sodium concentration was 162 mM at 20 mM Na₂HAsO₄/NaH₂AsO₄. The error bars indicate the SE of the mean. The inhibition by As_i in the absence of DNDS was significant at the $P \leq 0.05$ level.

FIGURE 3. Phosphate concentration dependence of phosphate influx in the presence of 200 μM DNDS. The data points represent duplicate measurements of the differences between the phosphate influx in 140 mM NaCl and 25 mM HEPES; pH 7.4 at 37°C; and 140 mM KCl and 25 mM HEPES; pH 7.4 at 37°C. Potassium phosphate replaced sodium phosphate and no replacement anion for the phosphate was used. The data are representative of three similar experiments. The kinetic constants were derived from a line fit to the data using a nonlinear least square regression analysis by the method of Wilkinson (1961). $K_{1/2}$, 0.28 ± 0.03 mM; V_{max} , 0.56 ± 0.02 mmol P_i (kg Hb)⁻¹ (h)⁻¹; [Na]_o, 140 mM.



tracted at each phosphate concentration used. All fluxes were performed in the presence of DNDS. The phosphate concentration–dependence exhibited a $K_{1/2}$ for activation ($K_{1/2}^{\text{P}_i}$) of 304 ± 14 μM total phosphate, which represented 69 μM H_2PO_4^- and 235 μM HPO_4^{2-} at pH = 7.4 (monovalent phosphate has a pK_a of 6.87 at physiological ionic strength). The V_{max} that was determined from the analysis is 0.63 ± 0.05 mmol P_i (kg Hb)⁻¹ (h)⁻¹ at physiological concentrations of extracellular sodium.

Analogous experiments were carried out at pH 6.90 and 7.75, and the data are presented, along with the data obtained at pH 7.40, in Table I. The pH range was kept small to minimize effects on the transport protein per se as reported previously for the rabbit proximal tubule transporter (Cheng and Sacktor, 1981). Upon initial inspection, the $K_{1/2}$ values obtained at the three different pH values appeared to reflect specific activation of the transport system by monovalent phosphate anion (due to the relative constancy of $K_{1/2}$ for H_2PO_4^-). The marked effect of pH on V_{max} of the transport system, however, indicates that one cannot simply explain the results in terms of the variation in the $\text{HPO}_4^{2-}/\text{H}_2\text{PO}_4^-$ ratio. The data were there-

TABLE I
Effect of pH on Phosphate Activation of Sodium-dependent Phosphate Influx

Kinetic constant	Phosphate species	pH		
		6.90	7.40	7.75
$K_{1/2}$ (μM)	Total phosphate	$168 \pm 76^*$	304 ± 14	831 ± 114
	H_2PO_4^-	81 ± 37	69 ± 3	97 ± 9
	HPO_4^{2-}	87 ± 39	235 ± 11	734 ± 105
V_{max} (mmol P _i (kg Hb) ⁻¹ (h) ⁻¹)		0.33 ± 0.03	0.63 ± 0.05	0.94 ± 0.14

Effect of pH on the kinetic constants of phosphate activation of sodium-dependent phosphate influx. Data for pH 7.40 includes the data in Fig. 3 together with two additional experiments. Data at pH 6.90 and 7.75 represents the results from two experiments at each pH value. Values are presented for the $K_{1/2}$ for phosphate activation by total phosphate together with the calculated $K_{1/2}$ values for H_2PO_4^- and HPO_4^{2-} at each of the respective pH values using a pK_a of 6.87 for the dissociation of monovalent phosphate. *SEM.

fore inconsistent with simple Michaelis-Menten activation of the transport by a single species of phosphate, and a more complex scheme must be invoked to explain the observed results.

Sodium Activation of Sodium-dependent Phosphate Transport

Extracellular sodium activated the DNDS-insensitive influx of phosphate. Both potassium and *Nmdg* were used as sodium replacements, and similar activation was obtained. The phosphate influx in zero sodium was subtracted from the influx at each of the sodium concentrations used. This activation could be fit by a Michaelis-Menten function as shown in Fig. 4 A. No sigmoidicity was observed in any of the

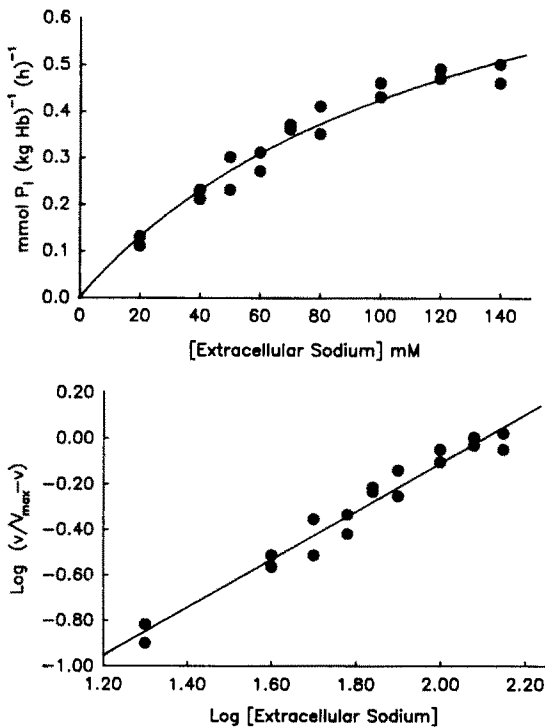


FIGURE 4. (A) Sodium concentration dependence of the phosphate influx in the presence of 200 μ M DNDS. The influx media consisted of (in millimolar): X NaCl, 140-X *Nmdg*Cl, 0.83 Na₂HPO₄, 0.17 NaH₂PO₄, 5.0 KCl, 1.0 MgCl₂, 10 dextrose, and 10 HEPES; pH 7.4 at 37°C. Data points are duplicate measurements on independent incubations. This figure is representative of results obtained in three similar experiments. Potassium was also used to replace sodium with similar results being obtained. Kinetic constants are derived from the nonlinear least squares regression analysis. $K_{1/2}$, 131 ± 25 mM; V_{\max} , 0.98 ± 0.11 mmol P_i (kg Hb)⁻¹ (h)⁻¹; [P_i]_o, 1.0 mM. (B) Hill plot of the data obtained in A. The slope was 1.05. The Hill coefficients from three experiments at 1.0 mM extracellular P_i were 1.02, 1.05, and 1.28.

three sodium activation experiments. The nonlinear regression analysis of the data gave a $K_{1/2}$ for sodium activation ($K_{1/2}^{\text{Na}}$) of 139 ± 8 mM. the V_{\max} value obtained for the sodium activation of phosphate influx was almost twice that seen for the phosphate activation because the sodium concentration used in the $K_{1/2}^{\text{P}_i}$ experiments was equal to the $K_{1/2}^{\text{Na}}$ (≈ 140 mM) of the process. For the experiments of sodium activation at 1.0 mM extracellular phosphate, the value of the Hill coefficient was 1.14 ± 0.08 (Fig. 4 B). The Hill coefficient indicates the minimum number of sodium ions required to activate phosphate transport across the membrane.

Demonstration of Cotransport of Sodium with Phosphate

Experiments were also carried out to examine the possibility of the coupled transport of sodium with phosphate, as well as to further characterize the pH dependence of the sodium-dependent phosphate transport. The ²²Na tracer influxes were measured in incubation media with and without 1.0 mM P_i (no anion replacement), together with ³²P_i tracer influxes with and without 150 mM Na (isoionic replacement with NMDg). Our results are summarized in Table II. Varying the pH_o ≈ pH_i (the pH_i was calculated to be 6.91, 7.34, and 7.63 at pH_o values of 6.90, 7.40, and 7.75, respectively [Gunn et al., 1973]) had a slight effect on the sodium-dependent phosphate influx. The magnitudes of the P_i-stimulated sodium influxes remained statistically indistinguishable at all pH values (¹M_{Na} = 0.73 ± 0.04 mmol P_i (kg Hb)⁻¹ (h)⁻¹). The sodium-stimulated P_i influx at pH 6.90 was found to be significantly different from the influx at pH 7.75, and presumably reflects the differences obtained for the V_{max} values for the transport system at these respective pH values (Table I).

TABLE II
Effect of pH on Na and P_i Influx

pH	Na influx plus P _i	Na influx minus P _i *	P _i -stimulated Na influx	P _i influx plus Na	P _i influx minus Na [†]	Na-stimulated P _i influx	Na/P _i ratio
6.90 (n = 6)	3.97 ± 0.26 [‡]	3.31 ± 0.29	0.66 ± 0.39	0.72 ± 0.04	0.34 ± 0.02	0.38 ± 0.04	1.74 ± 0.39
7.40 (n = 4)	3.99 ± 0.05	3.25 ± 0.08	0.74 ± 0.09	0.52 ± 0.05	0.09 ± 0.06	0.43 ± 0.08	1.72 ± 0.12
7.75 (n = 3)	4.80 ± 0.12	4.01 ± 0.16	0.79 ± 0.20	0.52 ± 0.02	0.06 ± 0.01	0.46 ± 0.02	1.72 ± 0.20

Effect of pH on Na-stimulated phosphate influx and P_i-stimulated sodium influx. The P_i-stimulated Na influxes were not significantly different at *P* > 0.500. The Na-stimulated P_i influxes at pH 6.9 and 7.4 (*P* ≤ 0.400) and at 7.4 & 7.75 (*P* ≤ 0.500) were not significantly different, however, there was a significant difference between the influxes at pH 6.9 and 7.75 (*P* ≤ 0.025). * ≤ 25 μM; † ≤ 0.5 mM; ‡ SEM

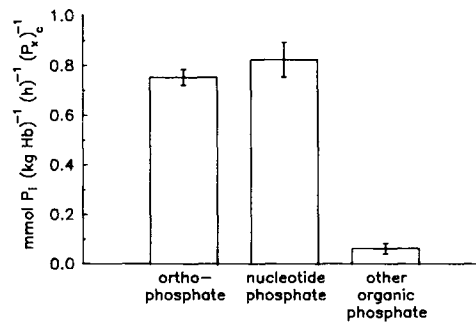
A stoichiometry of 1.70–1.75 mol of sodium transported/mol of phosphate transported was obtained in all cases for this mutually ion-dependent pathway. This was statistically different from 2.0 at pH 7.4 and 7.75, but as yet we have no explanation for the difference (see the Discussion). At this stage in the characterization of the transport pathway, we cannot rule out the possibility of other sodium-dependent transport pathways of differing stoichiometries in the red cell membrane. The sodium-independent phosphate influx was the same at pH 7.40 and 7.75, but was elevated at 6.90. In contrast with this finding, the phosphate-independent sodium influx was equal at the two lower pH values and increased at pH 7.75. Therefore the magnitude of the Na-P_i cotransport system remained relatively constant in the face of the asymmetric behavior of the co-ion-independent fluxes. The relatively constant value for Na/P_i influx over the pH range examined could be due to a variety of different factors. The direct relationship between the K_{1/2}^{P_i} and V_{max} values (Table I) at the different pH values would explain the findings if the transporter carried HPO₄⁻², or did not distinguish between HPO₄⁻² and H₂PO₄⁻. The inverse relationship between [H₂PO₄⁻] (as the pH is raised from 6.90 to 7.75 the [H₂PO₄⁻]

falls) and V_{\max} , in the presence of a constant $K_{1/2}$ for H_2PO_4^- , would also explain the findings if H_2PO_4^- was the transported species.

Intracellular Distribution of Imported Phosphate

The fate of this extracellular phosphate upon entry into the red cell was examined by determining the rate of influx of extracellular $^{32}\text{P}_i$ into the three cytosolic pools of phosphate: P_i , adenosine nucleotides, and all other organic phosphates. Extracellular $^{32}\text{P}_i$ accumulated twice as fast in the nucleotide phosphate pool as in the P_i pool (data not shown); however, the cytosolic concentration of adenosine nucleotides (≈ 1.7 mM) in these experiments was roughly twice that of the intracellular P_i concentration (≈ 0.8 mM), and consequently the rate of increase in the specific activity of these two cytosolic phosphate pools was not different (Fig. 5). The other organic phosphates (primarily, 2,3-DPG) were labeled at a significantly slower rate due primarily to the relatively low rates of the 2,3-DPG shunt (only 10–15% of glycolysis)

FIGURE 5. Distribution of extracellular phosphate once it has entered the red cell. Incubation media was (in millimolar): 140 NaCl, 0.83 Na_2HPO_4 , 0.17 NaH_2PO_4 , 5.0 KCl, 1.0 MgCl_2 , 10 dextrose, and 10 HEPES; pH 7.4 at 37°C. Phosphorus incorporation into the nucleotide fraction was determined by adsorption to Norit-A. Nonnucleotide organophosphorus was determined by subtracting the inorganic phosphate plus the Norit-A adsorbable phosphate from the total phosphate. Ordinate was determined by dividing the influx by the concentration of the intracellular pool of exchangeable phosphate. Therefore P_x was intracellular $[\text{P}_i]$ (0.81 ± 0.32 mM) for P_i , $[\text{ATP}] + [\text{ADP}] + [\text{AMP}]$ (1.71 ± 0.41 mM) for nucleotide phosphates, and the total concentration of the remaining exchangeable cytosolic organic phosphate (9.6 ± 1.1 mM) for other organic phosphates.



and the pentose phosphate cycle (10%), but also to the heterogeneous pool of phosphorus compounds that required more than one enzyme reaction to label some compounds. The relative rate of incorporation of inorganic phosphate into these three pools (inorganic, nucleotide, and nonnucleotide [Norit-A nonadsorbable] organic) was not affected by the presence of DNDS or the removal of sodium from the medium.

Metabolic State Dependence of Phosphate Transport and Intracellular Distribution

P_i is an essential metabolite in the glycolyzing human red cell, acting as a substrate for the GAPDH and the purine nucleoside phosphorylase reactions. We therefore examined what effect the metabolic rate of the red cell had on the requirement for extracellular phosphate. Initial experiments indicated the rate of phosphate uptake via all pathways was equivalent whether in the presence or absence of glucose; how-

ever, glycolysis was presumably still operating in the absence of glucose by using galactose as the primary substrate. The metabolic dependence of the red cell's phosphorus requirements can better be examined by placing the cell in a phosphorus-depleted metabolic state. By incubating red cells for 24 h at 37°C in the absence of phosphate or glucose, it was possible to reduce total intracellular phosphorus from 26.4 ± 0.8 to 5.5 ± 0.3 mM. When red cells were phosphorus-depleted in this manner, there was a marked effect on the rate of phosphate influx (Fig. 6). The DNDS-sensitive and the Na/P_i cotransport influx pathways were both markedly inhibited but there was no observed effect of phosphorus-depletion on the magnitude of the phosphate influx carried by the DNDS-insensitive, sodium-independent pathway.

We therefore examined the rate of phosphate incorporation into the P_i nucleotide, and nonnucleotide organic phosphate pools in fresh, phosphorus-depleted, and phosphorus-repleted red cells in an effort to determine the effect of phosphorus depletion on the mechanism of cytosolic phosphate distribution (Table III). These experiments indicate that the fraction of extracellular phosphate entering the cell (mediated entirely by the DNDS-sensitive pathway) that remained as intracellu-

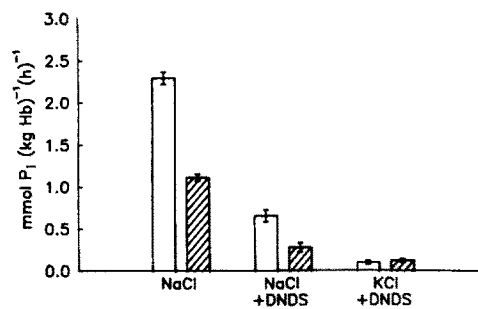


FIGURE 6. One representative experiment demonstrating the effect of phosphorus depletion on phosphate transport in human red cells. Phosphorus depletion was carried out by incubating the cells for 24 h in 150 mM NaCl and 10 mM HEPES; pH 7.4 at 37°C. Influx media consisted of (in millimolar): 140 NaCl or KCl; 0.83 Na₂HPO₄ or K₂HPO₄; 0.17 NaH₂PO₄ or KH₂PO₄; 5.0 KCl; 1.0 MgCl₂; 10 dextrose; and 10 HEPES; pH 7.4 at 37°C. The concentration of DNDS was 200 μM. □, fresh cells; ▨, depleted cells.

lar P_i, was increased in both depleted and repleted cells. In addition, both the experimental cell types incorporated significantly less extracellular phosphate into cytosolic nucleotides during the time course of the influx (30 min). Finally, the influx of extracellular phosphate into the nonnucleotide organic phosphate species was equivalent in control and depleted cells, and if anything slightly stimulated in the depleted cells. The lack of incorporation of ³²P_i into cytosolic nucleotides in the depleted cells was probably secondary to the depletion of adenosine nucleotides, since *de novo* synthesis of the nucleotides does not occur in the absence of added organic substrates. In the repleted cells the level of ATP was restored to approximately one-tenth the level of fresh cells, and the rate of phosphate incorporation into nucleotides was still inhibited by >90%. To examine if the decreased ATP concentration per se was responsible for the inhibition of phosphate transport and the lack of incorporation of phosphate into nucleotides, we depleted the cells of their nucleotides by an alternative method. After the cells were incubated in the presence of 2-deoxy-D-glucose for 2 h, the ATP levels were reduced to 0.15 ± 0.03 mmol (1

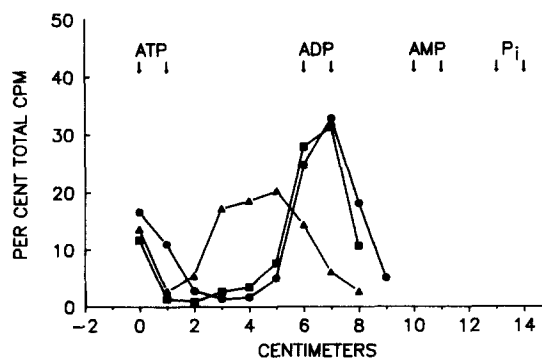
TABLE III
Rate of Incorporation of Extracellular Phosphate

Phosphate compartment	Influx pathway	Control	Depleted	Repleted
<i>(mmol P_i(kg Hb)⁻¹(h)⁻¹)</i>				
Total phosphate	DNDS-sensitive	1.60 ± 0.10	1.00 ± 0.09	1.20 ± 0.08
	Na ⁺ -dependent	0.58 ± 0.05	0.16 ± 0.02	0.18 ± 0.05
Orthophosphate	DNDS-sensitive	0.33 ± 0.05	0.89 ± 0.09	0.88 ± 0.07
	Na ⁺ -dependent	0.19 ± 0.04	0.16 ± 0.04	0.22 ± 0.04
Nucleotide phosphate	DNDS-sensitive	1.17 ± 0.09	0.0	0.12 ± 0.03
	Na ⁺ -dependent	0.35 ± 0.05	0.0	0.0
Other organic phosphates	DNDS-sensitive	0.10 ± 0.02	0.14 ± 0.02	0.21 ± 0.03
	Na ⁺ -dependent	0.04 ± 0.01	0.0	0.0

Effect of phosphorus depletion and repletion on the DNDS-sensitive and sodium-dependent incorporation of extracellular phosphate in human red cells. Depletion was carried out by incubating the cells for 24 h in 150 mM NaCl and 10 mM HEPES; pH 7.4 at 37°C. Repletion was carried out for 2 h at 37°C in (in millimolar): 130 NaCl; 10 Na₂HPO₄, 10 dextrose, 5.0 inosine, 2.0 adenosine, and 10 HEPES; pH 7.4 at 37°C. The concentration of intracellular P_i was 0.7 ± 0.2, 2.7 ± 0.6, and 0.8 ± 0.3 mmol (1 cells)⁻¹ for control, depleted, and repleted cells, respectively, immediately before the initiation of the influx incubation. Intracellular ATP was 1.6 ± 0.1, 0.010 ± 0.005, and 0.14 ± 0.03 mmol (1 cells)⁻¹ for control, depleted, and repleted cells, respectively. The intracellular total phosphate was 16.4 ± 0.5, 3.4 ± 0.2, and 12.2 ± 0.6 mmol (cells)⁻¹ for control, depleted, and repleted cells, respectively. Influx media consisted of (in millimolar): 140 NaCl or NMDgCl, 0.83 Na₂HPO₄ or K₂HPO₄, 0.17 NaH₂PO₄ or KH₂PO₄, 5.0 KCl, 1.0 MgCl₂, 10 dextrose, 10 HEPES, and ±250 μM DNDS; pH 7.4 at 37°C.

cells)⁻¹ (about the same levels as those found in the repleted cells). Cells depleted in this manner were not different from fresh cells or controls incubated for 2 h in the presence of glucose with regard to their rate of phosphate uptake or phosphate incorporation into nucleotides. Therefore, it does not appear to be the decreased concentration of ATP that is responsible for the persistent inhibition of phosphate transport and lack of phosphate incorporation into nucleotides in the cells that were phosphorus-repleted after incubation for 24 h in the absence of substrate.

FIGURE 7. Thin layer chromatogram of human red cell membranes prepared from red cells incubated in the presence of extracellular ³²P_i for 15 s. Membranes were prepared by hypotonic hemolysis of human red cells and by washing them in 10 mM Na/HEPES, pH 7.6 at 0°C. Membranes or boiled membrane (5 min) supernatants were applied to the thin layer plate with similar results.



The incubation medium was (in millimolar): 140 NaCl or NMDgCl (sodium free); 0.83 Na₂HPO₄ or K₂HPO₄; 0.17 NaH₂PO₄ or KH₂PO₄; 5.0 KCl; 10 dextrose; and 10 Na or K/HEPES; pH 7.4 at 37°C. ●, control; ■, DNDS; ▲, sodium free.

Incorporation of Extracellular Phosphate into Membrane-associated Nucleotides

In fresh red cells extracellular P_i was also incorporated into a membrane-associated pool of phosphate that migrates on thin-layer chromatograms with R_f values that approximated those of the nucleotides ADP and ATP (Fig. 7). The human red cell is known to have membrane-associated ATP (Mercer and Dunham, 1981b; Shoemaker and Hoffman, 1985) and presumably it is into this same pool of ATP that the extracellular ³²P_i was incorporated during these experiments. The higher level of incorporation of ³²P_i into ADP than into ATP on the chromatograms is consistent with the higher concentration of ADP ($2.6 \pm 0.8 \times 10^{-9}$ mol ADP [mg membrane protein]⁻¹) than ATP ($0.26 \pm 0.11 \times 10^{-9}$ mol ATP [mg membrane protein]⁻¹) in the membrane of human red cells (Shoemaker and Hoffman, 1985; Shoemaker, unpublished observations). There was insignificant radioactivity corresponding to the R_f values for P_i, AMP, ribose-1-phosphate, and ribose-5-phosphate, all of which are likely candidates to have incorporated extracellular phosphate with a rapid time

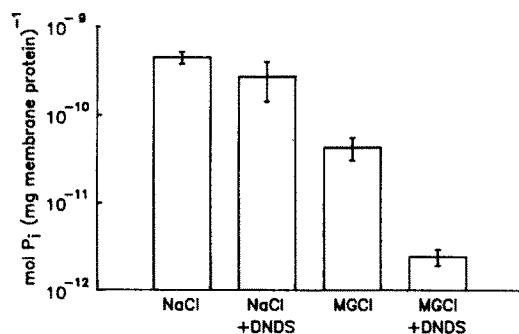


FIGURE 8. Phosphorylation levels of human red cell membranes exposed to extracellular phosphate in the exact same protocol as described in Fig. 7. Note the logarithmic scale of the ordinate. The sodium-dependent phosphorylation was $4.1 \pm 0.7 \times 10^{-10}$ and $2.7 \pm 1.3 \times 10^{-10}$ mol P_i (mg membrane protein)⁻¹ in the absence and presence of DNDS, respectively. The addition of DNDS has no effect on the phosphorylation level in the presence of extracellular sodium but causes a significant reduction in the level of phosphorylation in the absence of extracellular sodium. MGCl is *N*mdg Cl.

course. The compound 2,3-DPG did not migrate from the origin in the experimental solvent system and therefore was often difficult to resolve from the ATP peak. However, in chromatograms where the two compounds were resolved, the counts per minute in the ATP peak were sixfold higher than the origin. This finding, coupled with the fact that there are no known reports of membrane-associated 2,3-DPG, makes ATP the most likely molecular candidate to have incorporated the extracellular phosphate. The R_f value for 1,3-DPG could not be determined because the compound is not available commercially, but would presumably be equivalent to 2,3-DPG. Organic extraction failed to remove significant amounts of the radioactivity present in the membranes. The inositol phosphates were also likely candidates to have incorporated phosphate with a rapid time course under the conditions of the present experiments. Application of the ³²P_i-labeled aqueous red cell membrane fraction to a Dowex-1 anion-exchange column equilibrated with 3.0 M ammonium formate, produced 82% of the recovered radioactivity in the initial deionized H₂O

wash. The highest recovery of the radioactivity in an inositol-phosphate fraction was 11.4% in the peak coeluting with inositol (1,4,5) P_3 .

The incorporation of extracellular $^{32}P_i$ into red cell membranes was not significantly affected by the presence of DNDS in the incubation media; however, replacement of extracellular sodium with Nmdg inhibited incorporation by 90% (Fig. 8) and the residual radioactivity displayed a different chromatographic profile (Fig. 7). The incorporation of extracellular $^{32}P_i$ in the absence of sodium was still confined to the aqueous phase, so the most likely candidates for the residual phosphate incorporation continued to be those compounds listed in the previous paragraph. However, the peak did not correspond to the R_f value of any of these compounds, and since the integral under the sodium-free curve represented only 10% of the control incorporation, the importance of the new peak was perhaps of little significance.

These data indicate that the Na/ P_i cotransport was principally responsible for providing the membrane-bound nucleotide with extracellular phosphate. The rate of incorporation of phosphate into this membrane-nucleotide pool was very fast. The pool equilibrated within 6 s of the addition of the cells to the isotope. This result indicates that the extracellular phosphate that labels the membrane nucleotides does not first equilibrate with the intracellular cytosol, as the specific activity of the cytosolic P_i compartment continues to increase over 1 h, while the specific activity of the membrane compartment does not change significantly during the time course of the incubation after 6 s.

Comparison of the amount of sodium-dependent P_i incorporated into the membranes ($0.41 \pm 0.05 \times 10^{-9}$ mol P_i [mg membrane protein] $^{-1}$) with the amount of bound ATP and ADP ($2.9 \pm 1.1 \times 10^{-9}$ mol [mg membrane protein] $^{-1}$) indicated that only 14% of the membrane-bound nucleotide pool was equilibrated with the $^{32}P_i$ derived from the extracellular solution. The current results are therefore consistent with a role for the Na- P_i cotransport pathway in providing extracellular phosphate for the synthesis of a significant fraction of the membrane-associated nucleotide.

DISCUSSION

The results of this study have demonstrated the presence of a Na/ P_i cotransport system in the membrane of the human red cell. This transport system was shown to be responsible for 20% of the influx of phosphate under the experimental conditions employed. The Na/ P_i cotransport system of the red cell shares some properties with the well-characterized cotransporter found in renal and intestinal brush border membranes, but also has some distinct differences. One physiological function of the Na/ P_i cotransport system in the red cell appears to be to deliver the majority of extracellular phosphate that can be used by the GAPDH/PGK reactions to label membrane-associated nucleotides rapidly.

Characterization of Phosphate Influx Pathways

The phosphate uptake into the cell can be characterized by a DNDS-sensitive pathway, a Na/ P_i cotransport pathway, and a "leak" pathway that was not inhibited by any of the compounds tested, and whose magnitude was linearly dependent on

phosphate concentration of up to 2.0 mM. Phosphate uptake into human red cells in high-sodium media was inhibited by DNDS and As_i , while ouabain, an inhibitor of the Na/K pump, and bumetanide, an inhibitor of the Na/K/Cl cotransporter, were without effect. The 70% inhibition of the phosphate influx by DNDS at 37°C was significantly less than the 95% inhibition reported by Runyan and Gunn (1984) in a high-potassium (sodium-free) medium at 20°C. The reason for this discrepancy was the use of higher concentrations of phosphate in the former study (125 mM) and the operation of the DNDS-insensitive Na/P_i cotransport pathway in the current study. The higher concentration of phosphate used in the previous study increases the fraction of the DNDS-sensitive phosphate flux for two reasons: (a) the higher concentration of phosphate relative to its K_D (15 mM) on band 3 (R. B. Gunn, unpublished observations), and (b) the replacement of chloride by phosphate decreases the competitive inhibition by chloride.

Maximum inhibition of band 3-mediated phosphate transport was not achieved until relatively high concentrations of DNDS (200 μ M) were used. This can be simply explained by the known competition between DNDS and chloride (Fröhlich, 1982) at the external anion binding site of band 3, and the high chloride concentration of the medium in the phosphate influx experiments. Under the conditions of the experiments conducted in the current work, chloride as well as DNDS were acting as competitive inhibitors of phosphate transport via band 3. DNDS was the more effective inhibitor at the concentrations used because of its higher affinity ($K_1 = 90$ nM) for the external anion binding site compared with that of chloride ($K_{1/2} = 4$ mM).

By virtue of its structural homology with phosphate, As_i was thought to be an excellent candidate for a specific inhibitor of phosphate influx in light of its inhibition of other phosphate-utilizing systems: GAPDH (Needham and Pillai, 1937) and the renal brush border Na/P_i cotransport system (Hoffmann et al., 1976). The studies of Na/P_i cotransport in renal brush border membrane vesicles (Hoffmann et al., 1976) and in pig-kidney-derived LLC-PK₁ cells (Rabito, 1983) indicate that As_i has a K_1 of 1 mM, which is an order of magnitude lower in affinity for the transport pathway than phosphate's. In human red cell ghosts the Na/K pump and anion transport protein have been shown to use As_i as a substrate in place of intracellular phosphate (Kenney and Kaplan, 1988), but a previous report on the inability of extracellular As_i to inhibit phosphate influx or accelerate phosphate efflux has also appeared (Schrier, 1970). A comparison of the red cell data on the anion transport protein is complicated by the presence of chloride in all experiments. Since chloride is also a competitive inhibitor of phosphate flux, the As_i effects are no doubt attenuated to a large extent due to the high chloride concentrations, and depend on the affinity of all the anions (P_i , As_i , Cl, and DNDS) for the anion transport protein at one or both sides of the membrane. We found 20 mM As_i caused only partial inhibition of total phosphate uptake ($[P_i]_o = 1.0$ mM) and no inhibition of phosphate uptake in the presence of DNDS, as phosphate uptake was mediated primarily by the Na/P_i cotransport pathway. These results may well be consistent with the previous data existing on the interaction of As_i with the anion transport protein, once the affinities for all the anionic species are known. The results do, however, indicate a significantly different interaction of As_i with the phosphate uptake mediated by the

Na/P_i cotransport of the human red cell than what has been noted previously in epithelial tissue, as a concentration of 20 mM As_i should be sufficient to cause 86% inhibition of transport if the K_i for As_i is 1 mM. Even a sixfold increase in the K_i for As_i would still yield >50% inhibition assuming there is purely competitive inhibition.

The lack of significant inhibition of transport by ouabain or bumetanide indicates the specificity of the phosphate transporters in the red cell. The results presented here are in agreement with the inability of ouabain to inhibit the transport of phosphate unless it is derived from the gamma phosphate of intracellular ATP while the Na/K pump is operating in the uncoupled mode of transport (Marin and Hoffman, 1988). Also, phosphate has not been previously shown to be capable of substituting for any of the chloride-dependent, bumetanide-sensitive cotransport pathways, so the lack of significant inhibition by bumetanide (which has been shown to bind to a chloride site on the Na:K:2Cl cotransport system [Haas and McManus, 1983]) was consistent with previous findings.

In the presence of DNDS, the sodium-phosphate cotransport system of the red cell membrane has a requirement for sodium that was not satisfied by either potassium or NMDg. Previous reports indicate an absolute requirement for sodium in the renal transport system (Hoffmann et al., 1976; Brown et al., 1983). In the absence of DNDS, however, phosphate influx in potassium was always significantly greater than the corresponding influx in NMDg. When the extracellular phosphate concentration was raised to 1.5 mM, there was no longer a difference in the rate of phosphate influx when sodium was replaced with potassium. The sodium-dependent influx was still evident when NMDg was the substitute cation, however, indicating that potassium and NMDg interact differently with the phosphate influx pathways.

Characterization of Na/P_i Cotransport

The activation of the Na/P_i cotransport system by extracellular phosphate in human red cells appeared to have a higher $K_{1/2}^P$ than renal membranes. Renal brush border vesicles have a $K_{1/2}^P$ of 50–100 μ M total phosphate in the presence of high external sodium concentrations (Hoffmann et al., 1976; Cheng and Sacktor, 1981), while LLC-PK₁ cell membranes have an equivalent or lower $K_{1/2}^P$ (Rabito, 1983; Biber et al., 1983). Brush border membrane vesicles from rabbit duodenum, however, have a $K_{1/2}^P \approx 200 \mu$ M (Danisi et al., 1984) which is intermediate between that found in the kidney and that found here for red cells (300 μ M; Fig. 3). The differences in these values are significant but probably should not be used as evidence for nonidentical transport mechanisms in the three systems, since the ion gradients and temperatures of these studies were different.

The $K_{1/2}^{Na}$ of the red cell transporter in the presence of a sodium gradient was high (140 mM) in comparison with the value of 50–100 mM seen in the majority of vesicle studies from different tissue sources in the presence of a sodium gradient (Hoffmann et al., 1976; Biber et al., 1983; Danisi et al., 1984; Amstutz et al., 1985). The $K_{1/2}^{Na}$ appears to increase, however, in the absence of a sodium gradient (Cheng and Sacktor, 1981) or at acidic pH values (Hoffmann et al., 1976; Amstutz et al., 1985). The sodium gradient in our experiments was substantial ($[Na]_o/[Na]_i = 15$) so one would not expect large differences in the kinetic constants on this basis. The renal

studies were done with sodium-free vesicles, however, so it is conceivable that the 10 mM Na inside the red cell alters the kinetic properties of the transport pathway.

A more striking difference is the unanimous value of 2.0 found for the Hill coefficient of sodium activation in previous studies and the value of 1.0 found in the present work. The Hill coefficients of 2.0 were consistent with at least two sodium ions being transported per phosphate ion (Hoffmann et al., 1976; Burckhardt et al., 1981; Amstutz et al., 1985). Investigations in proximal-tubule-derived LLC-PK₁ cells (Rabito, 1983), as well as duodenal brush border membrane vesicles (Danisi et al., 1984), have also found Hill coefficients of 2.0 for the Na/P_i cotransport present in these membranes. The simplest interpretation of the data obtained in this study was that more than one sodium ion was required to activate transport, and that these activating sodium ions were cotransported with phosphate (Table II). However, the Hill coefficient of 1.0 obtained in the red cell makes it necessary to invoke nonidentical sodium sites on a single transporter or multiple transporters to explain the data (see below).

By varying the pH of the intracellular and extracellular medium at physiological concentrations of sodium and phosphate, we were able to examine the response of the transporter to varied proton/hydroxide concentrations ($\text{pH}_i \approx \text{pH}_o$), as well as the stoichiometry of the transporter. It was clear that at the three pH values examined the sodium influx stimulated by P_i was 1.7 times the phosphate influx stimulated by sodium (and significantly different from 2.0 at pH = 7.4 and 7.75). This value represents the first time the stoichiometry of a Na/P_i cotransport system has been determined by simultaneously measuring the coupled ³²P_i and ²²Na fluxes in a given preparation. The stoichiometry obtained in this study is in close agreement with the previously reported values obtained in renal vesicles (Hoffmann et al., 1976), LLC-PK₁ cells (Biber et al., 1983), and duodenal vesicles (Danisi et al., 1984). However, these stoichiometries were all estimated by Hill analysis of sodium activation of phosphate transport. The interpretation of the Hill analysis limits us to saying that at least two sodium ions are transported for each phosphate crossing the membrane. Thus it only provides a lower boundary on the stoichiometry and not a measured value.

The pH dependence of the V_{max} of the Na/P_i cotransport we observed in red cells (Table I) was consistent with the data reported by most investigators of other systems, who found a stimulation of transport at more alkaline pH values (Hoffmann et al., 1976; Cheng and Sacktor, 1981; Rabito, 1983; Amstutz et al., 1985). These investigators have used this alkaline stimulation to suggest the possibility that the divalent species is the transported anion, reasoning that the flux increases at more alkaline pH values due to the increment in the divalent anion concentration. However, the possibility of pH having additional effects on the transport, besides simply modulating the monovalent/divalent phosphate ratio, has been addressed as well.

Cheng and Sacktor (1981) showed that the pH causes not only a change in the proportion of monovalent and divalent species but also has effects on the transport system per se. They demonstrated an increase in the phosphate uptake into renal brush border vesicles with increasing pH whether the divalent anion species was allowed to increase with increasing pH or if it was kept constant by decreasing total phosphate correspondingly. It was therefore impossible for these authors to come

to any conclusions regarding the species of phosphate that was transported. However, they found no electrical potential dependence of Na/P_i cotransport and so concluded that if either of the two phosphate species is preferred, the divalent species is transported together with the two sodium ions, which is consistent with the Hill analysis.

Concomitantly with the variation in the H₂PO₄⁻/HPO₄⁻² ratio, any change in pH can theoretically affect the affinities of the transport system for sodium or phosphate as well as the rate coefficient for translocation by titration of groups on the transport protein. The data contained in Table I indicate that varying the pH from 6.9 to 7.75 causes profound effects on the V_{max} of the transport system. The study of Amstutz et al. (1985) found that protons had a marked effect on the affinity of the transport system for sodium, the K_{1/2} for sodium increasing two to threefold as the pH was lowered from 7.4 to 6.4. It may be this sort of interaction of protons with the sodium transport site in the red cell Na/P_i cotransport pathway that contributed to the variation in V_{max} with pH. The results contained in Table I also indicate that the phosphate species whose affinity remains constant over the pH range examined was H₂PO₄⁻. Therefore, from the K_{1/2} data alone it seems most likely that the transporter does not transport the two phosphate species indiscriminately, or the divalent species as is found in epithelial brush borders, but rather prefers the monovalent species. In the face of such large effects of pH on the V_{max} of the system, however, interpreting the K_{1/2} data independently seems rather improvident. Nevertheless, if the monovalent species was transported, then the Na/P_i cotransport stoichiometry would have to be more complex than the generally accepted 2 Na⁺:1 HPO₄⁻² of epithelial tissue (see the succeeding paragraph), or the transporter must necessarily be electrogenic (2.0 Na⁺:1.0 H₂PO₄⁻).

In the vesicle preparations it is generally assumed that two sodium ions are cotransported with divalent phosphate so there is zero current. Our stoichiometry in the red cell system was 1.7 Na:1.0 P_i, and is approximated by a model of transport that includes two sodium binding sites of markedly different affinities. Two sodium sites with binding constants of 10 and 140 mM will give an apparent K_m of 140 mM with a Hill coefficient of 1.0. The difference in stoichiometry of 1.7 Na:1.0 P_i from the theoretical 2.0 Na:1.0 P_i may be explained by a systematic experimental error, though no basis for one could be found. Also, no indication of sigmoidal activation at micromolar sodium concentrations was observed. Therefore, an alternative model with a single transporter that reconciles both the Hill coefficient of 1.0 and the stoichiometry of 1.7 Na:1.0 P_i is one in which the NaHPO₄⁻ ion pair is transported, and sodium is capable of being cotransported either with H₂PO₄⁻ or NaHPO₄⁻. At physiological concentrations of sodium, there is a reasonable possibility that the NaHPO₄⁻ ion pair could exist (Smith and Alberty, 1956). If Na⁺ and H₂PO₄⁻ makes up 30% of the cotransported species and Na⁺ and NaHPO₄⁻ makes up the remaining 70%, then a stoichiometry of 1.7 will be obtained. The K_{1/2}^{Na} of 140 mM for sodium would then reflect the binding of the ionized sodium to the H₂PO₄⁻- or NaHPO₄⁻-loaded system, and the electroneutrality of the transport mechanism would be preserved. Of course, more complicated schemes with multiple transporters could explain the data. There are several reports in the literature of multiple

Na/P_i cotransport systems in the proximal-tubule brush border membrane (Brunette et al., 1984; Walker et al., 1987; Bindels et al., 1987).

It has been demonstrated (Cheng et al., 1983) that a high phosphorus diet will induce a lower affinity sodium-dependent phosphate uptake in rabbit brush border vesicles. The data presented in Fig. 3 of that paper give a $K_{1/2}^{Na}$ of 135 ± 17 mM and a Hill coefficient of 0.99 ± 0.16 , values not significantly different from the ones obtained in this study. In addition, the magnitude of the pathway shows little dependence on varying the pH from 6.5 to 8.5. However, the phosphate affinity remained similar to control values, in the range of 50–100 μ M, significantly different from the value in this study. Nevertheless, the red cell Na/P_i cotransport characteristics were much more closely related to the properties of this inducible form of the transporter in the kidney than that form which is dominant in control animals.

Intracellular Distribution of Imported Phosphate

Newly imported P_i can be divided generally into three cytosolic pools: inorganic phosphate, adenosine nucleotides, and other organic phosphates (Tenenhouse and Scriver, 1975). The rates of incorporation of ³²P_i into the P_i pool and the nucleotide pool were comparable, while the rate of incorporation into the remaining organophosphorus compounds was slower. These results are in agreement with the findings of Tenenhouse and Scriver (1975), while differing from those obtained by Gerlach et al. (1958) and Bartlett (1958). The reason for this discrepancy is the failure of the latter authors to account for the intracellular concentration of P_i and ATP. When these pool sizes are considered, the rates of incorporation of extracellular phosphate into intracellular P_i and ATP are not significantly different. The retarded rate of incorporation of extracellular phosphate into the nonnucleotide organophosphorus compounds reflects the fact that a precursor for many of these reactions was ATP, which was approaching isotopic equilibrium during the course of the incubation, and the number of sequential reactions that must be followed to equilibrate the differing phosphate-containing organic species. In addition, the phosphorylation rate for both the 2,3-DPG shunt and the pentose phosphate cycle proceed at a rate roughly an order of magnitude slower than the glycolytic rate. There was no significant difference in the cytosolic distribution of the transported phosphate in the presence of DNDS in the presence or absence of sodium, indicating that each of the three transport pathways delivers extracellular phosphate to a common intracellular pool, from which it was dispensed to the cytosolic phosphorus-containing compounds.

Metabolic State Dependence of Phosphate Transport and Intracellular Distribution

A much different observation was made when the distribution of imported phosphate was studied in red cells in which the total phosphorus concentration has been reduced by incubating them overnight in the absence of phosphorus and substrates. These cells no longer incorporate extracellular ³²P_i into the nucleotides, and as a result the rate of incorporation into P_i was accelerated. This result can be explained since the incorporation of inorganic phosphate into nucleotides would not be possi-

ble until the adenosine diphosphate level was reestablished at a sufficient level. However, in repleted cells the ATP levels were partially restored and yet there was still minimal incorporation of extracellular P_i into nucleotides. This failure of the repleted cells to incorporate extracellular phosphate into nucleotides was not simply due to the depressed ATP level. Reducing the ATP to comparable levels by incubating the cells with 2-deoxy-D-glucose failed to alter control rates of phosphate influx or its incorporation into nucleotides. Therefore, the explanation for the persistent inhibition of phosphate influx and incorporation cannot be attributed to the ATP concentration alone, but rather to something resulting from the prolonged 24-h incubation in the absence of substrates.

The irreversible nature of the depletion-induced inhibition of phosphate transport was distinct from the reversible inhibition that has been seen previously for other cotransport systems in red cells depleted of ATP (Palfrey, 1983; Adragna et al., 1985). Our irreversible inhibition of phosphate incorporation into nucleotides was true whether the phosphate was delivered to the cytosol via the Na/P_i cotransport pathway or the leak pathway. Only partial restoration of the control rate of incorporation into nucleotides was observed (<10%) via the DNDS-sensitive pathway. This low rate of incorporation of extracellular P_i into nucleotides corresponded with the low ATP levels (0.15 mM) measured in these cells. Peculiarly, all the repleted cells still demonstrated lactate production rates that did not differ significantly from control cells.

One possibility to explain the lack of incorporation of extracellular phosphate into the intracellular nucleotide in repleted cells is that the PGK reaction is not participating in the phosphorylation of ATP. The increased flux through the 2,3-DPG shunt (whose existence is necessary to explain the control glycolytic rate in the repleted cells) may have contributed to the lowered levels of ATP. One might also expect phosphate to be incorporated into nucleotides via the pathway used to replete the cells. The inosine used to replete the ATP levels was still present in the cells due to the relatively slow rate of nucleoside transport (Jarvis et al., 1982). The phosphate incorporated with inosine into ribose-1-phosphate via the nucleoside phosphorylase reaction must pass through the pentose phosphate cycle before the phosphate can be distributed to ATP via the pyruvate kinase reaction of the Embden-Meyerhof pathway. Due to the length of the reaction pathway and the relative inactivity of the pentose phosphate shunt relative to the Embden-Meyerhof pathway (11%), this labeled phosphate would not be apparent in the nucleotides during the 30-min incubation periods used in this study.

Incorporation of Extracellular Phosphate into Membrane-associated Nucleotides

The membrane compartment of nucleotide in the red cell was shown to reach steady state with extracellular P_i within 6 s. This rate of equilibration was much faster than that found for the cytosolic compartments of phosphate, which did not reach steady state after 60 min under the conditions employed. The phosphorylation of glyceraldehyde-3-phosphate by inorganic phosphate via the GAPDH reaction is the first step in the synthesis of these membrane-associated nucleotides. The 1,3-DPG thus formed donates the ^{32}P to ADP via the phosphoglycerate-kinase reaction, with the ^{32}P being distributed to ADP by the high adenylate kinase activity of red cells (Nils-

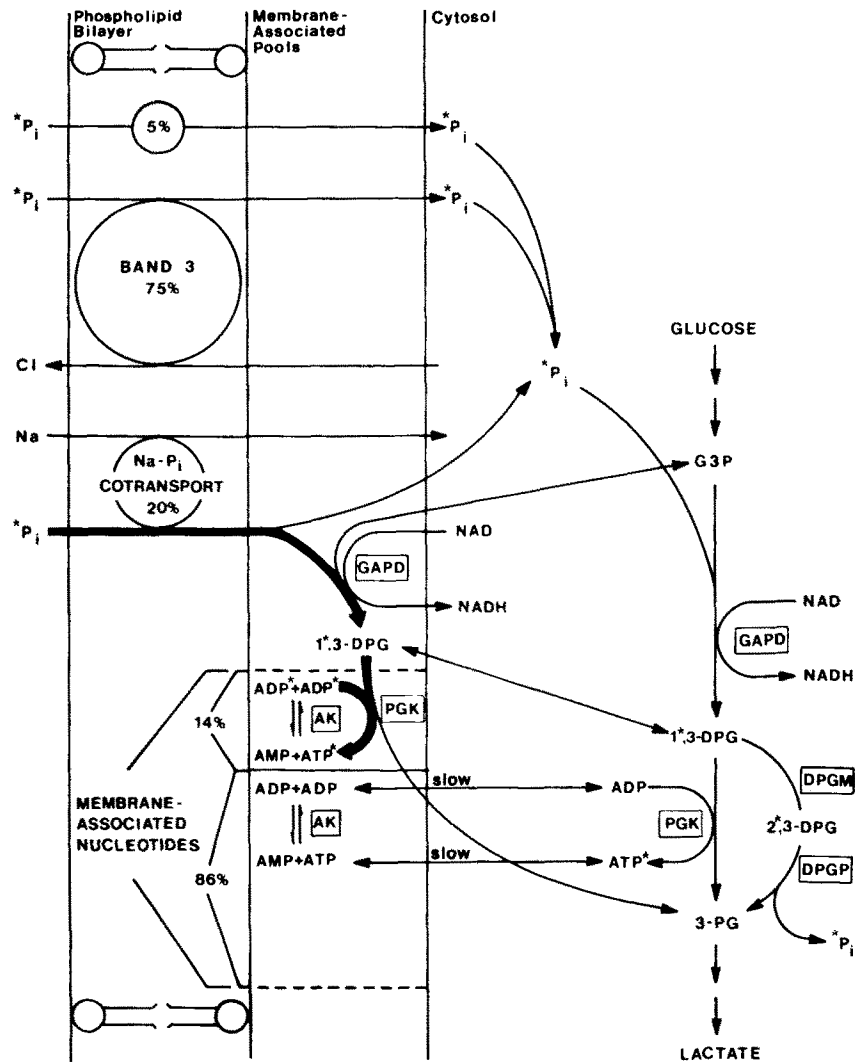


FIGURE 9. Functional organization of the pathways followed by extracellular phosphate during its incorporation into cytosolic- and membrane-associated nucleotides. The primary sodium-dependent pathway of phosphate incorporation into the membrane is indicated by the bolder arrows. *AMP*, *ADP*, and *ATP*, adenosine mono-, di-, and triphosphate; *AK*, adenylate kinase; *G3P*, glyceraldehyde-3-phosphate; *NAD* and *NADH*, oxidized and reduced nicotinamide adenine dinucleotide; *GAPD*, glyceraldehyde-3-phosphate dehydrogenase; *1,3-DPG*, 1,3-bisphosphoglycerate; *PGK*, phosphoglycerate kinase; *DPGM*, diphosphoglycerate mutase; *2,3-DPG*, 2,3-bisphosphoglycerate; *DPGP*, diphosphoglycerate phosphatase; *3-PG*, 3-phosphoglycerate.

son and Ronquist, 1969). The apparent access of adenylate kinase to the membrane-compartmentalized nucleotide was surprising in light of the finding of Mercer and Dunham (1981*b*) that the compartmentalized ATP fueling the Na/K pump is inaccessible to hexokinase. The higher level of incorporation of ^{32}P into ADP than into ATP on the chromatograms was consistent with the higher concentration of ADP than ATP in the membrane of human red cells (Shoemaker and Hoffman, 1985; Shoemaker, unpublished observations). The presence of DNDS had no effect on the rate of incorporation or the distribution of ^{32}P in the membrane fraction of the human red cell; however, the removal of sodium decreases the level of incorporation by 90%, which indicates the requirement of the Na/P_i cotransport system for mediating the delivery of extracellular phosphate to the rapidly labeled membrane pool of nucleotides. This further indicates the compartmentation of phosphate metabolism in the human red cell, in that only 20% of the phosphate entering the cell under physiologic conditions was carried by the sodium phosphate cotransport system; and yet it accounts for 90% of the extracellular phosphate incorporated into the membrane-bound nucleotides.

A diagram illustrating the major routes of extracellular phosphate transport and metabolism is shown in Fig. 9. Phosphate enters the cell by one of these pathways, band 3-mediated, Na/P_i cotransport, or DNDS-insensitive, sodium-independent transport. These three pathways deliver phosphate to the cytosol in the percentages shown. The Na/P_i cotransport is the pathway primarily responsible for the delivery to the membrane-associated nucleotide capable of being labeled rapidly. Adenylate kinase, known to be associated with red cell membranes (Nilsson and Ronquist, 1969), is postulated here to have access to the membrane-associated nucleotides by virtue of the rapid distribution of extracellular $^{32}\text{P}_i$ into ADP. A slow exchange of cytosolic nucleotides with the membrane-associated nucleotides would explain the negligible rate of incorporation of extracellular $^{32}\text{P}_i$ into the membrane compartment in the absence of extracellular sodium. The fraction of the membrane nucleotide pool rapidly accessed through the sodium-dependent transport of extracellular phosphate makes up 14% of the total membrane-bound nucleotide. The physiological function of this compartment of membrane nucleotide has yet to be demonstrated in the intact red cell.

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