BBA 75848

TEMPERATURE DEPENDENCE OF PHOSPHATE ENTRY INTO STORED HUMAN ERYTHROCYTES

STEPHEN C. PETERSON

Division of Cell Studies, Faculty of Medicine, Memorial University of Newfoundland, St. John's, Newfoundland (Canada)

(Received August 23rd, 1971)

ABSTRACT

The kinetics of phosphate entry into 3–4-week-old, cold stored human erythrocytes incubated in the absence of metabolic substrate at 37 °C is similar to uptake by fresh cells. At temperatures below 37 °C an apparent activation energy of 20 kcal/mole was measured. At temperatures above 37 °C the apparent activation energy was 7 kcal/mole. A model for phosphate entry into the stored erythrocyte is presented.

INTRODUCTION

The experiments reported in this paper were designed to investigate the high apparent activation energy of phosphate ion (P_i) transfer across the human erythrocyte membrane. Activation energies between 16 and 20 kcal/mole have been observed for phosphate ion entry into erythrocytes[1,2]. Measurements of the specific activity of various glycolytic intermediates have suggested that either ATP or 2,3-diphosphoglycerate was involved in the translocation of phosphate ion[2,3]. Recent work however seems to indicate that intracellular inorganic phosphate had a specific activity greater than any other glycolytic intermediate indicating the direct translocation of P_i[4]. Kinetic studies of P_i uptake fail to show a saturable system[5–8]. Recently evidence has been accumulating to support a fixed charge concept of anion permeability[9]. This theory proposes that there are positively charged regions in the cell membrane through which anions diffuse. The charged "pore" theory explains the membranes discrimination between cations and anions but fails to account for the high apparent energy of activation observed for phosphate, and sulfate. According to this model, activation energies for PO_4^{3-} and SO_4^{2-} should be similar to those observed in ion exchange resins (approx. 8 kcal/mole). Cl^- and HCO_3^-, however, exhibit very low temperature coefficients (in the temperature range 24–38 °C) corresponding to low activation energies, a point which is consistent with the fixed charge theory[10]. One possible explanation for the high apparent energy of activation is that in fresh human erythrocytes only about 6 % of the trichloroacetic acid soluble phosphate is in the form of P_i[11], thus if the rate limiting step is not translocation but some step involved in the incorporation of P_i into glycolytic intermediate, one would be measuring the activation energy of this process rather than the translocation itself. In order to optimize the possibility of measuring the temperature coefficient of translocation it

would be desirable to have erythrocytes with high intracellular P_i concentration as well as with little or no metabolism. Outdated blood bank blood incubated without metabolic substrate seems to fulfill these conditions. Human erythrocytes after three weeks of storage in acid–citrate–dextrose at 4° C have enhanced levels of P_i and reduced amounts of ATP and 2,3-diphosphoglycerate[11]. It therefore seemed desirable to measure the activation energy of P_i entry into stored cells.

METHODS

Outdated human blood was used between 3 and 4 weeks after collection*. All operations with the cells except the actual incubations were done at between 0 and 4°C. The cells were washed three times in the appropriate medium** and the buffy coat aspirated after each centrifugation. The cells were resuspended in cold medium to a cell fraction of about 0.35 and incubated in a temperature controlled shaker-water bath. After a 5-min preincubation to allow for temperature equilibration, carrier free $H_3{}^{32}PO_4$ was added.

Phosphate uptake

At various times 1-ml samples were removed and pipetted into 25 ml of ice cold incubation medium. Cells were sedimented at 30000 × *g* for 2 min and 0.5 ml of the supernatant was removed and plated for counting.

The counts/min in the supernatant decreased with time indicating influx of phosphate. Preliminary experiments showed that at the specific activities used no measurable radioactivity adhered to the glassware. A least square fit of supernatant counts/min vs time was used to obtain the initial (time = 0) counts/min. If correlation coefficients for linear fits were less than 0.85 the experiment was considered unreliable and discarded. Failure to satisfy this criteria seemed to be due to variation rather than curvilinearity. Initial counts/min and the initial phosphate concentration in the incubation medium was used to compute the specific activity of the phosphate. The counts/min entering the cells was the difference between the initial counts/min and the counts/min in the supernatant at various times. From these data least square fitting was used to compute the time rate of change of counts/min in the cells. Dividing the time rate of change by the specific activity yields the influx rate of phosphate. All values were normalized to a per ml of packed cells value by using the mean cell fraction. Cell fraction measurements made just prior to and just after incubation agreed within 5 % indicating little or no hemolysis during the 12-min incubation periods at all temperatures used.

Measurement of intracellular P_i

5 ml of the incubation mixture was pipetted into 25 ml of ice cold isotonic NaCl and washed three times by centrifugation in ice cold NaCl. The pellet was precipitated by 2 vol. of 10 % trichloroacetic acid. The supernatant was removed and divided into aliquots. One group of aliquots was analyzed for total phosphorus using the method of FISKE AND SUBBAROW[12]. The other group was analyzed for inorganic

* The author wishes to acknowledge the kind cooperation of the Canadian Red Cross.

** Composition of incubation medium: NaH_2PO_4 to concentration desired, KCl 4.6 mM, Tris 2.64 g/l, HCl or NaOH to pH 7.4, NaCl to make a final osmolarity of 300 mosM.

phosphate using the method of MARTIN AND DOTY[13] as modified by WEIL-MALHERBE AND GREEN[14].

Separation of glycolytic intermediates

Separation of glycolytic intermediates was by a modification of the method of BARTLETT[11]. 5 ml samples of the incubated erythrocytes were washed three times in cold isotonic saline. Cells were precipitated by 2 vol. of 10 % trichloroacetic acid and centrifuging. The supernatant was washed four times with 2 vol. of diethylether to remove the trichloroacetic acid. The washed supernatant was stored frozen overnight and then chromatographed on 0.4 mm × 40 mm Dowex AG 1- X 2, 200–400 mesh columns, formate form. Elution was with 50 ml of ammonium formate buffer (pH 3.0) in a linear gradient (0–4 M) at a flow rate of 0.4 ml/min. Phosphate was analyzed using the Bartlett method after perchloric acid digestion. ATP was measured after evaporation of the formate in a vacuum oven at 80°C by absorption of 260 nm ultraviolet light.

RESULTS

First it was necessary to characterize the preparation so that the results could be compared to those of other workers. Fig. 1 shows that the uptake of ^{32}P by the cold stored erythrocytes is linear with time. This observation indicates that during the period of incubation used in the following experiments, there was no significant efflux of tracer and hence the uptake of isotope by the erythrocytes is a true measure of influx. Similar linearities (the correlation coefficient (r) > 0.85) were observed at all temperatures and concentrations of P_i used in the following experiments.

One of the objectives of this study was to increase the concentration of inorganic phosphate inside the cell and at the same time minimize metabolism. The amount of P_i in the stored cells was measured and compared to the total amount of trichloroacetic acid soluble phosphate. In this preparation 58 % of the trichloroacetic acid soluble phosphate was in the form of P_i. Intracellular phosphate concentration at the beginning of the 20 mM experiments (after the preincubation) averaged 7.6 μmoles/ml

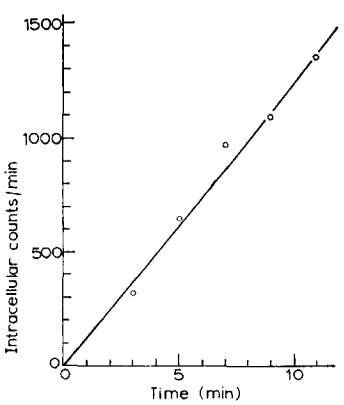


Fig. 1. ^{32}P uptake into red cells incubated in 80 mM phosphate medium. Influx rate in this experiment was 1.51 μmoles·min^{-1}·ml^{-1} erythrocytes. The correlation coefficient is 0.985. Temp. 37°C.

erythrocytes. The concentration of internal phosphate divided by concentration of external phosphate gives a value of 0.38.

Inorganic phosphate concentration has been increased from about 6 % to about 60 % of labile phosphate. Phosphate distribution ratios approach those reported by Deuticke indicating that PO_4^{3-} is probably at or near equilibrium. The fact that there was no substrate present in the incubation medium minimized the metabolism of the cell. Chromatographic separation of glycolytic intermediates failed to show any significant radioactivity in any compound except P_i even after 20 min. of incubation. Zero time samples contained essentially no ^{32}P in any fraction indicating that the washing procedures were adequate to remove extracellular phosphate.

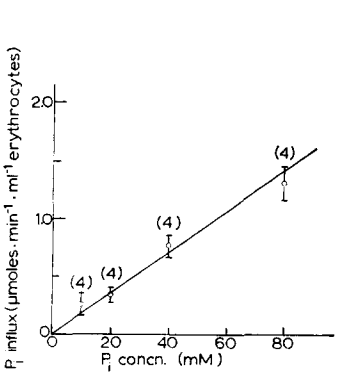
ATP concentration was markedly reduced in this system, incubated in 20 mM phosphate medium, as is shown in Table I.

ATP concentration at the beginning of the experiment represents only 8 % of that found in fresh cells[11]. At the end of a total of 10 min of incubation ATP has been reduced to only 2 % of that found in fresh blood indicating that hydrolysis of ATP exceeds synthesis during the period when influx is measured.

Fig. 2 shows the effect of varying external phosphate concentration on phosphate influx. The influx rates into these stored cells at various temperatures were measured (Fig. 3). For temperatures at or below 37°C an apparent activation energy of 20000 cal/mole was measured. At 37°C there appeared a relatively sharp break in the line

TABLE I

Incubation time at 37° (min)	*μmole ATP per ml erythrocyte*
5	0.19
10	0.047
25	0.046



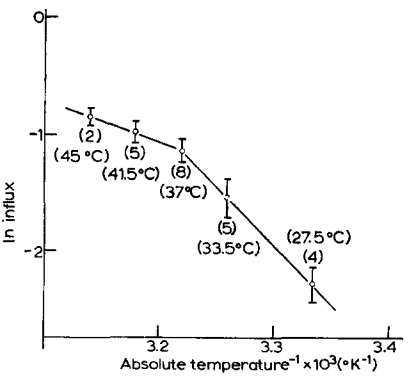


Fig. 2. Phosphate influx as a function of external phosphate concentration. The bars represent the standard error and the number in parenthesis is the number of observations. Temp., 37°C.

Fig. 3. ln of influx rate as a function of the reciprocal of absolute temperature. The slope of the line times —1 times the gas constant (R) equals the apparent activation energy. Extracellular phosphate concentration is 20 mM. The temperature in °C of each determination is also given.

and at temperatures above 37°C a value of 7000 cal/mole was obtained for the activation energy.

DISCUSSION

Inorganic phosphate uptake into 3–4-week-old cold stored erythrocytes occurs at rates comparable to those for fresh cells[7] and by resealed ghosts[8] although the kinetic details appear to be different. Phosphate influx at pH 7.4 into fresh cells increases faster than external phosphate concentration while influx into resealed ghosts at pH 7.4 increases slower than external phosphate concentration. SCHRIER finds, however, a proportional increase in influx with increases in external phosphate at pH 8.0, a result which is comparable to that obtained at pH 7.4 in this study. These changes in the uptake kinetics can in part be ascribed to interaction of the membrane with other anions, as DEUTICKE's work suggests and pH as SCHRIER's work indicates. Other factors may be involved if one compares the results reported here with those at the same pH and similar ionic conditions within the cell. In fresh cells ATP levels are quite high, in cold stored erythrocytes ATP concentration is low but measurable and in the ghosts used by SCHRIER there was no ATP present. Variation in ATP concentration correlates with a curvilinear upward to curvilinear downward change in uptake kinetics. Evidence is accumulating that some of the phospholipids in red cell membranes are actively turning over and the maintenance of these lipids in the membrane is dependent on metabolism[19]. Loss of phospholipid is also known to occur during the preparation of red cell ghosts[20]. It could well be that changes in the phospholipid composition of the membranes of the various preparations have occurred and hence alterations in the fixed charge of the membrane.

The temperature dependence of the uptake process at or below 37°C in stored cells is similar to that previously reported[1,3]. Enhanced levels of intracellular phosphate do not seem to alter the apparent activation energy. This observation suggests that the high activation energy arises during the actual translocation step. The apparently sharp change in the activation energy at 37°C was an unexpected result. The value of 7 kcal/mole at temperatures above 37°C is similar to the activation energy for the self diffusion of phosphate ion in artificial ion exchange resins[15]. This change in activation energy may well represent a phase change of the phospholipid portion of the membrane. Such phase changes have been observed in phospholipid liposomes at temperatures of about 40°C[16,17]. In fact, although data does not always overlap, the similarities between phosphatidylcholine liposomes and red cell membranes are striking. Both have relatively low temperature coefficients for Cl^-, both exhibit phase changes at common temperatures, both can discriminate between anions and cations, both have high activation energies for the passive movement of Na^+ and K^+ (refs. 16, 18) to list a few. At temperatures above 37°C one or more of the bulk phospholipids, for example phosphatidylcholine or phosphatidyl ethanolamine, may undergo a phase change in stored erythrocytes. One reason this may have not been observed in fresh cells could be that metabolism stabilizes the membrane preventing the phase change. In cold stored cells metabolism is minimal and the membrane may be degenerating with respect to phase stability. In order to explain the high activation energy for P_i at temperatures below 37° C in stored cells the following hypothesis is presented. Cl^- movement across the cell membrane has a low temperature

coefficient. This observation is explained if there exist positively charged pores which are more or less permanent membrane structures (approx. 2 Å diameter), due to the structural arrangement of the phospholipid in the membrane. Suppose that there are larger, between 2 and 8 Å diameter, pores that are formed for short times due to the thermal motion of the phospholipid. These thermally generated pores would have a high temperature dependence and this gives rise to the high activation energies observed for the "large" anions. To maintain cation-anion discrimination these pores would have to be positively charged. As the temperature increases the probability of a pore being formed is higher and the influx of ion increases. At the temperature where the phase change occurs the phospholipid undergoes rearrangement producing relatively large, temperature independent, positively charged pores in which the activation energy decreases to that for ion diffusion in a large charged pore or artificial ion exchanger. This hypothesis suggests many experimental tests some of which are being carried out in this laboratory.

ACKNOWLEDGEMENTS

This research was supported by a grant (MA-4144) from the Medical Research Council.

REFERENCES

1 R. H. GOURLEY AND C. L. GEMMILL, *J. Cell. Comp. Physiol.*, 35 (1950) 341.
2 R. H. GOURLEY, *Arch. Biochem.*, 40 (1952) 13.
3 T. A. J. PRANKERD AND K. I. ALTMAN, *Biochem. J.*, 58 (1954) 622.
4 J. CHEDRU AND P. CARTIER, *Biochim. Biophys. Acta*, 126 (1966) 500.
5 A. ZIPURSKY AND L. G. ISRAELS, *Nature*, 189 (1961) 1013.
6 E. GERLACH, B. DEUTICKE AND J. DUHM, *Pflügers Archiv*, 280 (1964) 243.
7 B. DEUTICKE, *Pflügers Archiv*, 296 (1967) 21.
8 S. L. SCHRIER, *J. Lab. Clin. Med.*, 75 (1970) 422.
9 H. PASSOW, *Prog. Biophys.*, 19(2) (1969) 425.
10 H. LUCKNER, *Pflügers Archiv*, 250 (1948) 303.
11 G. R. BARTLETT in GEO. BREWER, *Red Cell Metabolism and Function*, Plenum Press, New York, 1970, p. 245.
12 C. H. FISKE AND Y. SUBBAROW, *J. Biol. Chem.*, 66 (1925) 375.
13 J. B. MARTIN AND D. M. DOTY, *Anal. Chem.*, 21 (1949) 965.
14 H. WEIL-MALHERBE AND R. H. GREEN, *Biochem.*, 49 (1951) 286.
15 B. A. SOLDANO AND G. E. BOYD, *J. Am. Chem. Soc.*, 75 (1953) 6099.
16 D. PAPAHADJOPOULOS AND J. C. WATKINS, *Biochim. Biophys. Acta*, 135 (1967) 639.
17 D. DE GIER, J. G. MANDERSLOOT AND L. L. M. VAN DEENEN, *Biochim. Biophys. Acta*, 150 (1968) 666.
18 I. M. GLYNN, *J. Physiol.*, 134 (1956) 278.
19 S. C. PETERSON AND L. B. KIRSCHNER, *Biochim. Biophys. Acta*, 202 (1970) 295.
20 R. M. C. DAWSON, N. HEMINGTON AND D. B. LINDSAY, *Biochem. J.*, 77 (1960) 226.