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A ^{31}P NMR STUDY OF PHOSPHOENOLPYRUVATE TRANSPORT ACROSS THE HUMAN ERYTHROCYTE MEMBRANE

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SUMMARY: Intracellular PEP signals were noted in ^{31}P NMR spectra obtained directly from intact erythrocytes when (i) red cells were suspended in 0.1 M citrate buffer (pH 6.0), (ii) glycolysis was inhibited with 10 mM NaF, (iii) extracellular PEP signals were broadened by Mn ion (1 mM), and (iv) a high medium PEP concentration (65 mM) was used to assure significant PEP uptake over short periods of signal accumulation. In the absence of NaF, PEP signals were not observed but 2,3-bisphosphoglycerate concentration increased at three times the PEP entry rate in NaF-poisoned cells. This study demonstrates that PEP transport into intact erythrocytes and associated metabolic sequelae can be monitored noninvasively with the aid of ^{31}P NMR.

Although it is generally believed that phosphorylated compounds are not transported across cell membranes, PEP** was metabolized to pyruvate and DPG when this compound was added to erythrocytes suspended in acid citrate or sucrose solutions (1,2). Net PEP accumulation was shown by inhibiting glycolysis with 10 mM NaF (3). PEP transport has also been shown to be related to the SITS-inhibitable anion transport system (3), the action of echinocytogenic agents (4), the improvement of DPG and ATP levels in cold-stored red cells (5) and a possible membrane defect in erythrocytes from patients with hereditary spherocytosis (6).

The aim of the present investigation was to determine whether PEP entry into intact erythrocytes can be demonstrated noninvasively with the aid of

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**Abbreviations: PEP, phosphoenolpyruvate; DPG, 2,3-bisphosphoglycerate; ^{31}P NMR, phosphorus-31 nuclear magnetic resonance; SITS, 4-acetamido-4'-isothiocyanostilbene-2,2'-disulfonic acid.

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^{31}P NMR spectroscopy. In recent years, this technique has been used increasingly for monitoring *in situ* metabolism of cells of various types (7-11). Membrane transport studies have also been made possible by the presence of paramagnetic agents in the medium which cause a difference in magnetic susceptibilities to be developed between external and internal compartments (12). The results of the present study indicate that cellular entry of PEP can be readily demonstrated from ^{31}P NMR spectra obtained directly from intact erythrocytes. The principal advantage of the NMR technique over the more traditional procedure (2,3) is the circumvention of certain methodological steps such as cell separation by centrifugation, removal of extracellular PEP by washing cells, and extraction of the PEP-loaded cells, all of which could lead to uncertainties.

METHODS

Erythrocytes collected in citrate-phosphate-dextrose medium (13) were washed three times with the medium in which the cells were ultimately suspended. A hematocrit of 35% was chosen for the final suspension in order to maximize the medium buffer capacity for stabilization of extracellular pH; 13% D_2O was also included in the medium for spectrometer stabilization. At the appropriate time, PEP powder (Sigma) was added to three ml of erythrocyte suspension contained in a 10 mm NMR tube. Other experimental details are described in the legend to Fig. 1. The spectrometer was a Bruker WP-80 operating at 32.37 MHz for ^{31}P and incorporating facilities for Fourier transform signal-averaging, quadrature detection and broad-band heteronuclear ^{1}H decoupling. Area measurements were obtained by computerized integration of the transformed spectra. The observed areas were adjusted for Overhauser enhancements by comparison with areas obtained with an erythrocyte suspension in which PEP and 2,3-DPG were analyzed enzymatically (14,15). Internal pH was determined from the chemical shift of the PEP signal (7), and external pH was measured with a glass electrode connected to a Beckman Expandomatic pH meter.

RESULTS

In order to demonstrate PEP entry into intact red cells, erythrocytes were suspended in 0.1 M citrate buffer (pH 6.0, 37°C) containing 10 mM NaF and 1 mM $MnCl_2$. After 15 min of a control recording (Expt. 1, Fig. 1A and Table 1), solid PEP was added to yield a final medium concentration of 65 mM. The enolase inhibition by NaF prevents the incorporated PEP from being meta-

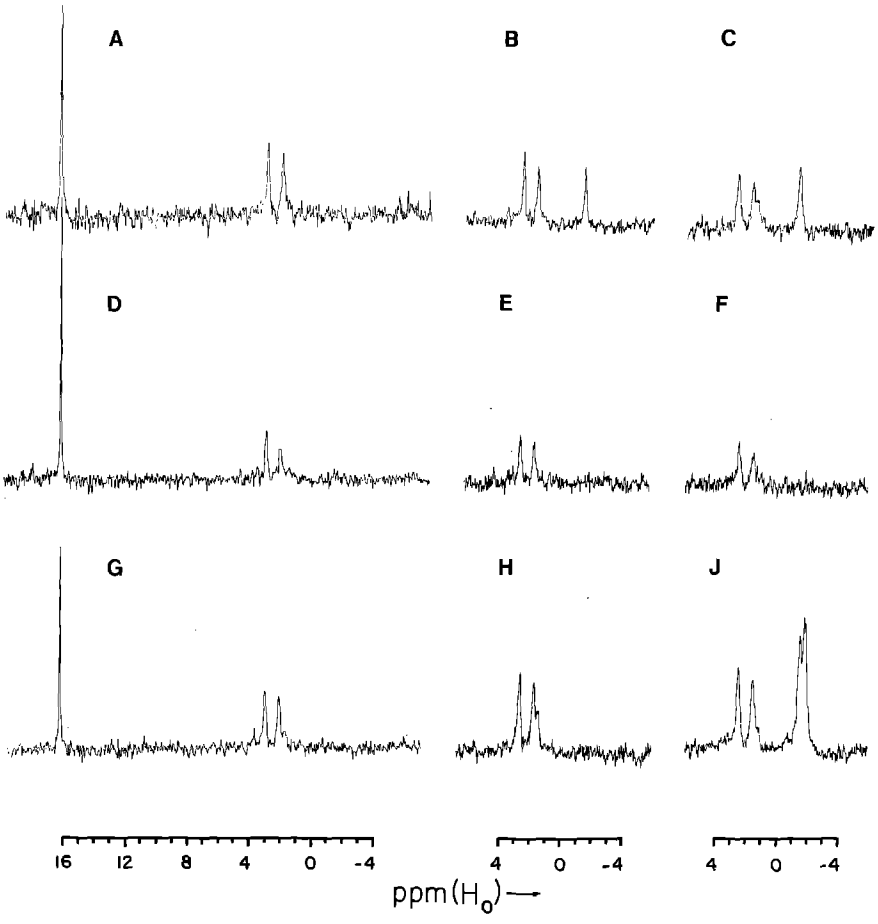

Fig. 1. ^{31}P NMR spectra of intact human erythrocytes incubated in 0.1 M citrate buffer (pH 6.0).

Spectra A-C: Experiment 1, with 65 mM PEP, 10 mM NaF and 1 mM $MnCl_2$. A was recorded before PEP addition; B and C were obtained 15-30 and 45-60 min after PEP addition. The signals observed are methylene diphosphonate in a reference capillary, 16.22 ppm; 3-phosphate of DPG, 2.70 ppm; 2-phosphate of DPG, 1.77 ppm; and PEP, -1.31 ppm.

Spectra D-F: Experiment 2, without PEP but with NaF and $MnCl_2$. D, E, and F were recorded 0-15, 15-30, and 45-60 min after the sample was placed in the NMR spectrometer.

Spectra G-H: Experiment 3, without NaF but with PEP and $MnCl_2$. G and H were recorded 0-15 and 45-60 min after PEP addition.

Spectrum J: Experiment 4, without $MnCl_2$ but with PEP and NaF. J was obtained 30-60 min after PEP addition. The extracellular PEP signal is seen immediately upfield to the intracellular PEP signal.

bolized to DPG; pyruvate formation from PEP is also precluded due to the absence of ADP formation in the phosphoglycerate kinase reaction, a step which would otherwise be coupled to the pyruvate kinase reaction through ADP (and ATP) in the non-poisoned cell. The absence of PEP catabolism by these

Table 1. Concentrations of PEP and DPG (μmol/ml of cells) calculated from NMR spectra following incubation of human red cells in citrate buffer containing PEP with and without NaF.

Expt. No.	NMR Signals	Medium PEP mM	10 mM NaF	Time Period, min					
				(-15)-0	0-15	15-30	30-45	45-60	60-75
1	PEP	65	+	0.00 (A)	1.76	5.25 (B)	5.93	7.48 (C)	7.64
1	DPG	65	+	4.71 (A)	4.78	5.43 (B)	4.99	6.31 (C)	5.51
2	DPG	0	+	--	3.94 (D)	4.42 (E)	3.96	4.06 (F)	--
3	DPG	65	0	4.06* (F)	7.65 (G)	9.70	9.78	9.26 (H)	11.08

The letters in parentheses refer to individual spectra shown in Fig. 1. The 2-P and 3-P signal areas were averaged in evaluating DPG concentration.
*The DPG value associated with (F) in Expt. 2 was used to represent the value present before PEP addition in Expt. 3, since the same refrigerated sample of packed erythrocytes was used in Expt. 3 within two hours of the (F) recording.

mechanisms permits the incorporated PEP to be accumulated and visualized. The paramagnetic Mn ion was added to broaden the extracellular PEP signal so as to effectively mask its presence while monitoring the intracellular PEP change. Fifteen min segments of signal accumulation (615 scans) were recorded and the intracellular PEP signal was observed to develop at a chemical shift of -1.31 ppm. This value was similar to one observed in an experiment conducted to establish the PEP chemical shift under these conditions, i.e. when PEP was added to a NaF-containing hemolysate at a comparable pH. Increases in signal area were evident during subsequent recording periods in Expt. 1 (Figs. 1B, 1C).

The PEP and DPG concentrations in μmol/ml of erythrocytes (Table 1) were determined from computer-integrated areas of the PEP and DPG signals and from the signal area of methylene diphosphonate, which was contained in a sealed glass capillary and served as an external standard. These data show more clearly the progressive increases in PEP concentration during successive recording periods (Table 1). They also show that DPG content may have been

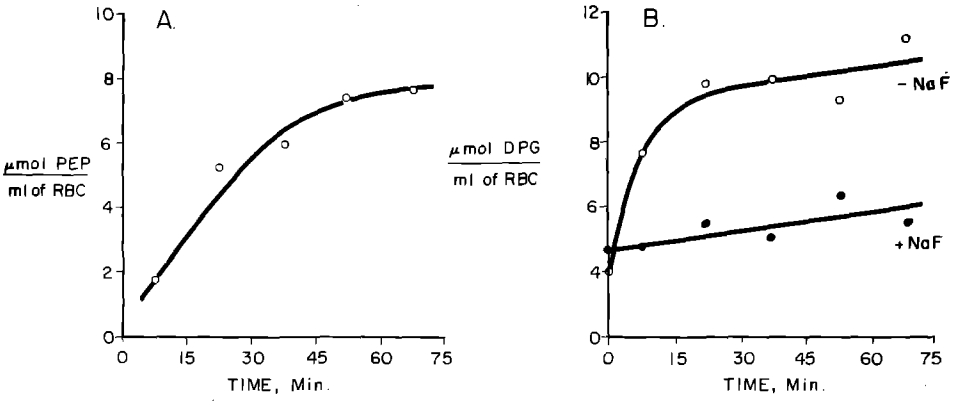

Fig. 2. Time course of PEP accumulation (A) and DPG formation (B) by intact human erythrocytes incubated in 0.1 M citrate buffer (pH 6.0) containing 65 mM PEP. The basic data for the curves in A and B are given in Table 1.

increasing slightly during the experiment (Fig. 2B), indicating that the NaF inhibition was not complete.

The initial PEP entry rate in our study was estimated from the initial slope to be 0.20 μmol/min per ml of erythrocyte (Fig. 2A) which may be contrasted with the value of 0.54 μmol/min per ml of erythrocyte reported previously (3). These values are not strictly comparable since the experimental conditions were different in the two cases. The lower uptake in the present study may be best explained by the absence of a constantly maintained extracellular pH of 6.0 in the present experiments, since there is a marked dependence of PEP uptake on pH (3).

The internal pH was estimated from the chemical shift of the PEP signal and a pH titration curve of PEP solutions to be 6.71 at the start of the experiment and 6.67 after 75 min of incubation (Table 2). This is similar to the result noted earlier when the pH change was evaluated from a ^{14}C-pyruvate concentration ratio (3). The chemical shifts of DPG also reflected a similar pH variation (Table 2). In this case, an altered degree of DPG binding to the hemoglobin due to the pH change could have possibly affected the chemical shifts; however, this did not appear to be a significant factor in this case since greater binding alone would have caused a downfield rather than an upfield variation (16).

Table 2. Chemical shifts (ppm) of DPG and PEP as well as calculated and measured pH values during incubation of human red cells in citrate buffer containing 65 mM PEP and 10 mM NaF.

Measurement	Time Period, min				
	0-15	15-30	30-45	45-60	60-75
Chemical shifts:					
3-P of DPG	2.77	2.68	2.64	2.62	2.64
2-P of DPG	1.82	1.75	1.71	1.62	1.56
PEP	-1.31	-1.29	-1.29	-1.38	-1.40
Intracellular pH (calc.)*	6.71	6.72	6.72	6.68	6.67
Extracellular pH (meas.)	6.17	--	--	--	6.52

*Calculated from the chemical shift of PEP.

In Fig. 1J, a recording obtained in the absence of Mn^{2+} displays both intra- and extracellular PEP signals. Since extracellular pH is more acid under the conditions of this study (3), the extracellular signal is located upfield from its intracellular counterpart. This was verified by Mn^{2+} addition before a final 15 min recording which obliterated the upfield signal (not shown).

An experiment conducted in the absence of added PEP demonstrated that intracellular PEP is undetectable in NaF-poisoned erythrocytes and that very little change in the size of the DPG signals is observed over a 60 min incubation period (Expt. 2, Figs. 1D-F and Table 1). A similar result was obtained in the presence of added PEP when 1.3 mM SITS, the anion transport inhibitor, was also present.

In an experiment conducted in the presence of PEP but in the absence of NaF, no PEP signals were visible since PEP levels are normally very low (14) and the incorporated PEP was apparently being converted rapidly to pyruvate and DPG (1). The DPG change was observed in ^{31}P NMR spectra (Expt. 3, Figs. 1G, 1H and Table 1). The time course of the DPG concentration change in these cells is shown in Fig. 2B. Five μmol of DPG was formed per ml of

erythrocyte during the first 15 min compared to 1.6 μmol/ml of erythrocyte noted in an earlier study (1). The greater rate was due probably to the six-fold greater medium PEP level that was employed in the present study. It had been noted earlier that PEP transported into the cells is metabolized about equally to DPG and pyruvate (1). Thus, the PEP entry rate that would account for the initial rate of change in DPG concentration in nonpoisoned cells appears to be three times the entry rate in NaF-poisoned erythrocytes. This may have been related to the internal PEP concentration increasing steadily in the poisoned system, while the concentration gradient in the nonpoisoned erythrocyte remained high due to the maintenance of a low intracellular PEP level, a consequence of the rapid metabolism of PEP to other compounds (1).

In addition to the PEP and DPG signals, a P_i signal was seen upfield (1.60 ppm) to the signal for the 2-P of DPG (1.92 ppm) within the first 15 minutes of recording of erythrocytes incubated with PEP in the absence of NaF (Fig. 1G). In the presence of NaF, the P_i signal appeared later (Fig. 1C, 1J). In Fig. 1G, a small peak was also observed downfield to the signal for the 3-P of DPG which could be that of 3-phosphoglycerate since its accumulation was noted previously under similar conditions (1) and the chemical shift (3.50 ppm) was close to that recorded previously for this compound (9).

DISCUSSION

This investigation is one of the few NMR studies to date in which rate measurements have been attempted in intact eythrocytes. The results of this study verify the earlier observation made by traditional procedures that PEP can penetrate the cell membrane of the intact erythrocyte suspended in an acidified (pH 6.0) sucrose solution (3). The NMR technique appears to be useful in PEP transport studies because (i) measurements are made under physiological conditions, (ii) the metabolic state of the cell may be assessed from the simultaneously recorded DPG and P_i signals, i.e. the more

favorable metabolic state is generally associated with higher DPG and lower P_i concentrations, (iii) intracellular pH may be distinguished at the same time from the chemical shifts of DPG, P_i or PEP, and (iv) technical errors associated with traditional analytical procedures may be avoided, as mentioned earlier. In our previous work (1-6), the standard transport methodology did not allow us to take advantage of the last three factors. One disadvantage of the NMR procedure is the relative insensitivity of the measurement so that large amounts of PEP need to be added in order to conduct the uptake study. Additional experiments that we have performed indicate that lower medium PEP concentrations (e.g. 10-20 mM) than the one employed in this study should yield significant data as well. Since it is a simpler procedure, the NMR technique could become the procedure of choice in certain instances such as in the routine testing of patient blood.

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