# A Phosphate-Analogue Probe of Red Cell pH Using Phosphorus-31 Nuclear Magnetic Resonance†

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ABSTRACT: In order to find a suitable marker of intraerythrocytic pH in a phosphorus-31 nuclear magnetic resonance ($^{31}$P NMR) system, analogues of inorganic phosphate were studied. Fluorophosphate ($pK_a$ = 4.7) and phosphite ($pK_a$ = 6.4) chemical shifts and coupling constants were found to be pH sensitive but at pH ranges too low to be useful for the study of fresh red cells. Methylphosphonate ($pK_a$ = 7.6) showed ideal characteristics for a pH probe: its chemical shift was far downfield for red cell phosphates and showed a large pH dependence near its $pK_a$ ($\Delta pH/\Delta\delta$ = 0.46 pH/ppm). Methylphosphonate readily entered red cells [influx 7.8 $\mu$M (mL of RBC)$^{-1}$ h$^{-1}$] and did not appear to alter glucose consumption or hemoglobin–oxygen affinity in intact cells. NMR spectra were obtained on eight samples of fresh red cells incubated for 30–60 min with methylphosphonate. Chemical shift differences between the extracellular and intracellular methylphosphonate signals were found to be predictive of the transmembrane pH gradient. The extracellular pH was 7.336 ± 0.031 (range 7.31–7.41), while the intracellular pH was 7.202 ± 0.034 (range 7.14–7.23), and the transmembrane pH gradient measured 0.129 ± 0.008 (range 0.11–0.14). Methylphosphonate is a useful probe of pH in the $^{31}$P NMR spectroscopic study of red cells.

In the study of erythrocyte metabolism, knowledge of the intracellular pH is of prime importance. Nuclear magnetic resonance (NMR)[1] spectroscopy has been shown to be a useful tool for the study of metabolism in intact normal (Henderson et al., 1974; Brown et al., 1977) and abnormal (Lam et al., 1979; Labotka & Honig, 1980; Tehrani et al., 1982; Swanson et al., 1983) red cells, other tissues (Burt et al., 1979; Radda & Seeley, 1979), and even living animals (Ackerman et al., 1980). The use of $^{31}$P NMR to measure intraerythrocytic pH was suggested by Moon & Richards (1973), when they measured chemical shifts of inorganic phosphate ($P_i$) and 2,3-diphosphoglycerate (2,3-DPG) as functions of pH.

We have found that in the study of fresh, intact red cells, $P_i$ and 2,3-DPG are not suitable markers of intracellular pH. The chemical shift ($\delta$) of the $P_i$ signal is often superimposed upon the 2-P signal of 2,3-DPG and of low intensity due to the low levels of $P_i$ in fresh cells (Labotka & Honig, 1980). The two 2,3-DPG chemical shifts and their relative separation are dependent not only on pH but also on the degree of association of this compound with hemoglobin (Huestis & Raftery, 1972; Costello et al., 1976). Therefore, we sought a "nonphysiologic" phosphate analogue that would be a suitable pH marker, when introduced into red cells. The desirable properties for a pH marker should include the following: (1) the $^{31}$P NMR chemical shift should vary predictably with pH near the "physiologic" pH range (pH 7.0–7.4), (2) the chemical shift should occur in a region of the spectrum not occupied by the red cell phosphate signals, (3) the compound must readily enter red cells, and (4) red cell metabolism and hemoglobin–oxygen affinity should be minimally affected by the compound. In this paper, we describe the selection of methylphosphonate (MeP) as an appropriate marker of intraerythrocytic pH by the above criteria and apply the probe in the study of the pH of fresh red cells after short-term incubation with MeP.

## Materials and Methods

*NMR Instrumentation.* The NMR instrument was a Bruker CXP-180, operating at 72.88 MHz for phosphorus (4.2 T); Fourier-transform technology, quadrature detection, and broad-band gated heteronuclear proton decoupling were employed. Samples were placed in 20 mm diameter NMR tubes, containing a concentric reference capillary (methylenediphosphonic acid, $\delta$ = 17.05 ppm). The ambient temperature was maintained at 37 °C. Samples were spun at 10 Hz and were capped with a Teflon plug to control gas exchange and prevent vortex formation. Unlike some investigators (Moon & Richards, 1973), we have found that spinning the samples inproved the signal resolution. At a rate of 10 Hz, the red cells are subject to a maximum of about 3.5*g* at the periphery of the tube and do not appear to pack significantly. On the other hand, without spinning, there is significant settling of the cells in the NMR tube, leading to sample inhomogeneity as well as artifactual changes in signal intensities. Typical NMR experiments consisted of 5–10-min signal accumulation into 8K data points with 45° pulses and 0.83-s signal acquisition times. All chemical shifts were referenced in parts per million (ppm) to 85% inorganic phosphoric acid. By convention, upfield chemical shifts were considered negative.

*Sample Preparation.* Phosphate analogues, fluorophosphate as the sodium salt and phosphite and methylphosphonate as the free acids, were obtained from Alfa Products, Davenport, MA. Other chemicals were purchased from standard sources. Stock solutions were made from each analogue at 150 mM concentration, with the pH adjusted to 7.40 with $NaHCO_3$

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[1] Abbreviations: ATP, adenosine 5-triphosphate; 2,3-DPG, 2,3-diphosphoglycerate; $\delta$, chemical shift; FP, fluorophosphate; *J*, coupling constant; MeP, methylphosphonate; NMR, nuclear magnetic resonance; NTP, nucleoside 5′-triphosphate; PCP, methylenediphosphonate; $P_i$, inorganic phosphate; ppm, parts per million; RBC, red cell; Bis-Tris, 2-[bis(2-hydroxyethyl)amino]-2-(hydroxymethyl)-1,3-propanediol.

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or HCl. Blood specimens were obtained from adult volunteers and consisted of 10–30-cm$^3$ donations, anticoagulated with sodium heparin, 10 units/cm$^3$. All red cell experiments were conducted with red cells washed and suspended in a Ringer's solution (Labotka et al., 1976a) buffered to pH 7.38–7.40 with either 24 mM Bis-Tris or 24 mM $NaHCO_3$ and containing 5 mM glucose.

*NMR–pH Titration.* The p$K_a$s of $P_i$, fluorophosphate, MeP, and phosphite were determined by titration of the respective free acid at 50 mM concentration with 0.5 M NaOH. pH measurements were performed on a Corning Model 130 digital pH meter. The pH behavior of the $^{31}$P NMR chemical shifts of $P_i$ and the phosphate analogues was determined simultaneously by obtaining spectra of solutions that contained $P_i$ (40 mM), MeP (20 mM), fluorophosphate (20 mM), and phosphite (50 mM). The differences in concentrations between compounds were selected to aid in identification of chemical shifts in succeeding spectra. The solutions were titrated to the desired pH values with NaOH and HCl.

*MeP Red Cell Studies.* MeP influx was measured in fresh red cells washed 2 times in Bis-Tris-containing buffer and resuspended to a packed cell volume of 45%. MeP was added to initial extracellular concentration of 20 mM. Aliquots were taken at incubation (37 °C) times from 0 to 3 h for NMR analysis, and extracellular MeP signals were suppressed with $10^{-4}$–$10^{-5}$ M $MnCl_2$ (Hamasaki et al., 1981). Glucose consumption was measured in fresh red cells, washed 2 times and suspended in bicarbonate-containing buffer to a packed cell volume of 40% and then divided into three aliquots. To each of two aliquots was added either 150 mM phosphate or 150 mM MeP, to an extracellular concentration of 10 mM. A control was prepared by adding an equivalent volume of 150 mM NaCl to the third aliquot. The aliquots were preincubated at 37 °C for 90 min in humidified air containing 5% $CO_2$, whereupon 5 mM additional glucose was added. Samples were then drawn for glucose analysis over a 4-h period. Red cell glucose consumption was determined by measuring the disappearance of glucose from plasma. Glucose determinations were performed on a Beckman glucose analyzer Model 6708 as well as by *o*-toluidine reduction (Henry et al., 1974). For hemoglobin-oxygenation studies, normal 2,3-DPG cells were prepared from fresh blood washed and suspended to a packed cell volume of 45% in Bis-Tris-containing buffer, pH 7.40, and incubated for 90 min at 37 °C with or without 10 mM MeP, fluorophosphate, or phosphite. DPG-depleted cells were similarly prepared from outdated bank blood. Hemoglobin-oxygenation curves were determined on a TCS Hemox analyzer. Red cell 2,3-DPG levels were measured enzymatically (Keitt, 1971).

*Red Cell pH Experiments.* Eight fresh red cell samples were washed 2 times and resuspended in bicarbonate-containing buffer to packed cell volumes of 45–50% and incubated with agitation at 37 °C in the presence of 10 mM (initial extracellular concentration) MeP for 30–60 min in a humidified gas mixture of air and 5% $CO_2$, to ensure full oxygenation and normal bicarbonate buffering of the cells. Blood gas measurements were performed on a Corning Model 168 blood gas analyzer. pH measurements were made on a Corning Model 130 digital pH meter equipped with a glass–calomel combination electrode. Intracellular pH was measured by freeze-thawing of the red cells (Purcell et al., 1961) packed by centrifugation.

## Results

*Selection of a pH Probe.* Figure 1 depicts typical NMR spectra of fresh washed red cells with phosphate analogues added. In the bottom spectrum, peaks arising from the major red cell phosphates are labeled. The very large signal at 17.05 ppm arises from an external methylenediphosphonate (PCP) capillary. The two peaks at 3.4 and 2.5 ppm are from the 3-P and 2-P, respectively, of 2,3-DPG. The $P_i$ signal ordinarily appears superimposed upon or just upfield of the 2-P peak (open arrow) and cannot be identified in this fresh sample. Smaller signals at −5.3, −10.4, and −19.2 ppm are due to the $\gamma$-, $\alpha$-, and $\beta$-phosphates, respectively, of nucleoside 5′-triphosphates (NTP), primarily ATP. The signal at 23.0 ppm arises from the added MeP and occurs in a region far downfield of the red cell phosphates.

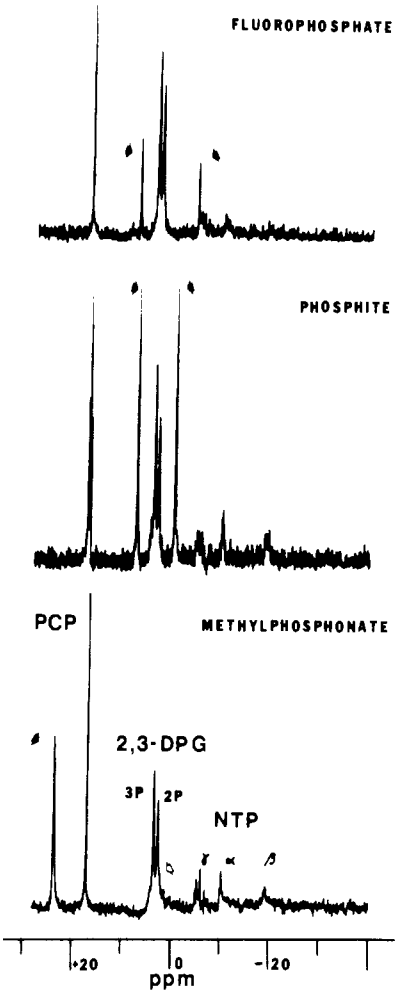


FIGURE 1: $^{31}$P NMR spectra of washed red cells with added phosphate analogues. (Top spectrum) (proton-decoupled) Fluorophosphate (arrows). The fluorophosphate signal is split into two widely separated peaks by the large (870-Hz) F–P coupling constant. (Middle spectrum) (proton-coupled) Phosphite. The phosphite signal (arrows) is also split into two widely separated peaks by a large (570-Hz) H–P coupling constant. (Botton spectrum) Methylphosphonate (proton decoupled). The methylphosphonate (~10 mM) peak (solid arrow) at 23.0 ppm is far downfield of the red cell phosphate signals. PCP represents an external methylenediphosphonate reference capillary. The 2,3-DPG and nucleoside 5′-triphosphates (NTP) are labeled. $P_i$ is very low in this sample and ordinarily would appear just upfield (open arrow) of the 2-P of 2,3-DPG. With incubation, an intracellular methylphosphonate peak would appear just upfield of the observed extracellular methylphosphonate peak (see Figure 7).

In contrast to the MeP signal, signals from fluorophosphate (arrows, top spectrum) and phosphite (arrows, middle spectrum) occur in the midst of the red cell spectrum. The signal from each of these two phosphate analogues is split into two widely separated peaks by intense spin coupling between the phosphorus and the directly bonded fluorine or proton. As

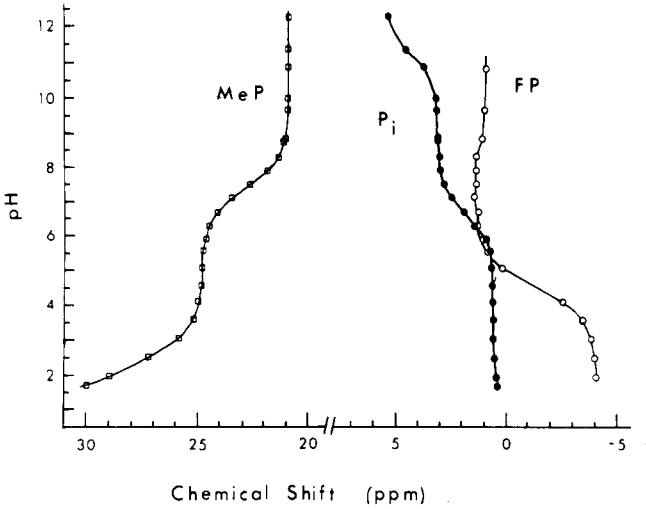


FIGURE 2: pH dependence of chemical shifts of phosphate ($P_i$, solid circles), methylphosphonate (MeP, open squares), and fluorophosphate (FP, open circles). $P_i$ undergoes a pH-dependent chemical shift transition of 2.45 ppm corresponding to a $pK_a$ of 6.8 and another transition with a $pK_a$ of about 12. FP has a 5.5 ppm transition corresponding to a $pK_a$ of 4.7. MeP undergoes a transition of larger magnitude (4.0 ppm) than, and in a direction opposite that of, $P_i$ corresponding to a $pK_a$ at pH 7.5 and another transition corresponding to a strong acid ($pK_a < 2.0$).

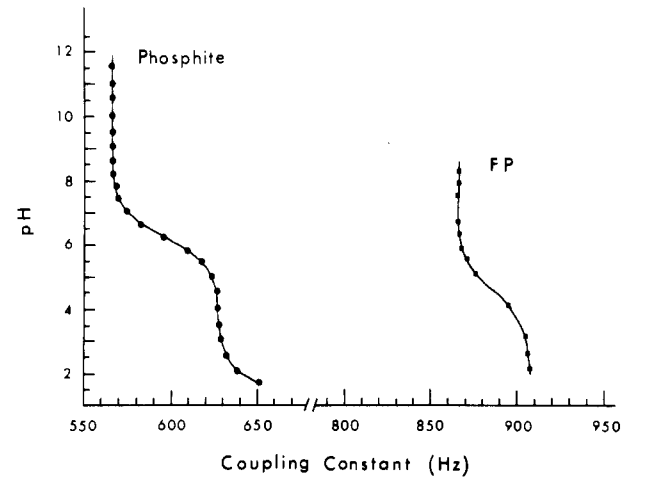


FIGURE 3: pH dependence of coupling constants of phosphite (solid circles) and fluorophosphate (solid squares). The very large F–P coupling constant of fluorophosphate decreases with decreasing acidity near its $pK_a$ of 4.7. The large H–P coupling constant of phosphite decreases near its $pK_a$ of 6.4. A transition corresponding to a strong acid ($pK_a < 2$) is also observed.

discussed below, the F–P and H–P coupling constants ($J$) are pH sensitive in the region of their respective $pK_a$s.

By acid titrations, the $pK_a$s of $P_i$ and its analogues were measured as follows (not shown): $P_i$, 6.8; fluorophosphate, 4.7; MeP, 7.6; phosphite, 6.6. The results of the NMR phosphate titrations are shown in Figures 2 and 3. Figure 2 depicts the chemical shifts of $P_i$, fluorophosphate, and MeP, as functions of pH. The chemical shift of $P_i$ undergoes a downfield transition from 0.55 ppm at pH 6.0 to 3.00 ppm at pH 8.0, corresponding to the ionization of its neutral proton at $pK_a = 6.8$. Another transition is seen at high pH due to the ionization of the basic proton at $pK_a = 12$. Fluorophosphate undergoes a downfield transition as pH is increased from 2 to 7, corresponding to an acid proton ionization at $pK_a = 4.7$. however, no pH-dependent behavior is noted in the physiologic range. In contrast, MeP experiences an upfield chemical shift transition from 24.80 ppm at pH 5 to 20.80 ppm

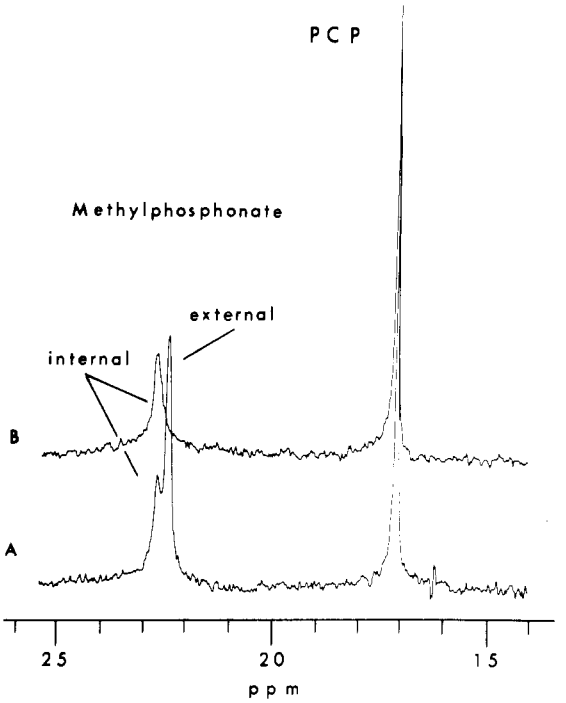


FIGURE 4: Expanded portion of NMR spectrum of a sample of washed red cells (Hct, 45%; extracellular pH 7.55) incubated for 30 min with methylphosphonate. The signal from an external PCP reference capillary is labeled. Spectrum A depicts two methylphosphonate signals: intracellular and extracellular. Spectrum B was obtained immediately after adding $Mn^{2+}$ to the same red cell suspension, suppressing the extracellular signal.

at pH 9, corresponding to a neutral proton ionization having a $pK_a$ at about 7.5. The direction of this transition is larger in magnitude than, and in the direction opposite of, that of $P_i$. Therefore, the chemical shift of MeP is quite sensitive to pH in the physiologic range ($\Delta pH/\Delta\delta = 0.460$ pH/ppm at 7.4). The chemical shift of phosphite (not shown) exhibited only minor changes with pH, from 3.18 ppm at pH 4.02 to 3.56 ppm at pH 7.45 and above.

The pH dependence of the coupling constants of fluorophosphate and phosphite are depicted in figure 3. The larger F–P coupling constant of fluorophosphate undergoes a transition from 908 Hz at pH 2 to 868 Hz at pH 7, corresponding to an acid proton ionization with a $pK_a$ of 4.7. The coupling constant is insensitive to pH above pH 6. Unlike the phosphite chemical shift, the large H–P coupling constant of phosphite has a transition from 627 Hz at pH 4 to 567 Hz at pH 9, corresponding to a neutral proton ionization at $pK_a = 6.4$. Unfortunately, only 23% of this transition occurs between pH 6.8 and 8 ($\Delta pH/\Delta J = 0.1$ pH/Hz), making the phosphite coupling constant relatively pH insensitive in this range. At the phosphorus frequency of 72.88 MHz, this pH dependence is equivalent to a 7.28 pH/ppm chemical shift change. At pH values lower than 7, however, the pH sensitivity is larger ($\Delta pH/\Delta J = 0.029$ pH/Hz, equivalent to 2.09 pH/ppm chemical shift at pH 6.4), making this compound potentially useful for pH measurements in this range.

Because MeP appeared to have ideal pH-dependent behavior in the physiologic range, this compound was chosen for further study as a possible red cell pH marker. When suspensions of red cells were incubated with MeP, their NMR spectra showed two closely spaced peaks due to methylphosphonate (Figure 4). That these two peaks were arising from two separate MeP domains was determined by the addition of $10^{-4}$–$10^{-5}$ M $Mn^{2+}$, which suppressed extracellular signals but left intracellular signals intact. The more downfield MeP signal was not sup-

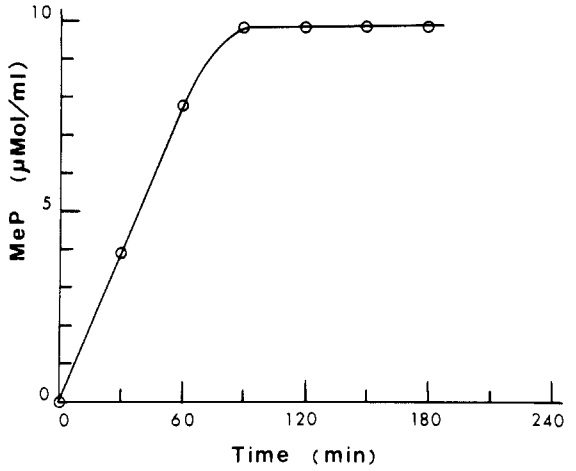


FIGURE 5: Methylphosphonate red cell influx. In a red cell suspension (Hct, 45%) was added 20 mM MeP (initial extracellular concentration) at time 0 min. Intracellular MeP was measured by NMR, with suppression of the extracellular signals with $Mn^{2+}$ (Hamasaki et al., 1981). The initial influx velocity was 7.8 $\mu$M (mL of RBC)$^{-1}$ with full equilibration achieved within 90–120 min.

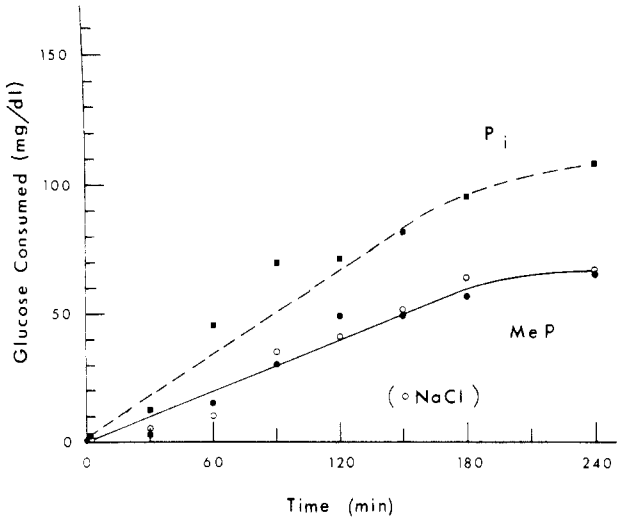


FIGURE 6: Glucose consumption by washed red cells (Hct, 40%) that had been preincubated for 90 min with 10 mM $P_i$ or 10 mM MeP. 5 mM glucose was then added, and the rate of disappearance of glucose from the suspension was measured. MeP-incubated cells (closed circles) consumed glucose at an hourly rate of 20 mg/dL of RBC, as did a saline control (open circles). In contrast, $P_i$-incubated cells (closed squares) consumed glucose at a higher rate of 38 mg/dL of RBC.

pressed by $Mn^{2+}$, confirming that MeP did enter erythrocytes. In order to determine the rate of influx, a fresh suspension of red cells was prepared in Bis-Tris-containing buffer (packed cell volume 45%), with an initial extracellular concentration of MeP of 20 mM. At appropiate intervals during incubation, aliquots of the suspension were removed, $Mn^{2+}$ was added, and NMR spectra were obtained. Figure 5 demonstrates that MeP rapidly entered red cells, reaching equilibration in 90–120 min, with an initial rate of influx of 7.8 $\mu$M (mL of RBC)$^{-1}$ h$^{-1}$.

Since erythrocyte energy metabolism is largely manifested by the utilization of glucose in the Embden–Meyerhof and hexosemonophosphate pathways (Bunn et al., 1971), the effect of MeP on red cell metabolism was estimated by measuring glucose consumption of fresh red cells (Figure 6). Red cells were incubated with 10 mM extracellular MeP for 90 min, after which 5 mM glucose was added. Glucose consumption in the presence of MeP was 20 mg (dL of RBC)$^{-1}$ h$^{-1}$, similar to a NaCl control. In contrast, 10 mM $P_i$ strongly stimulated

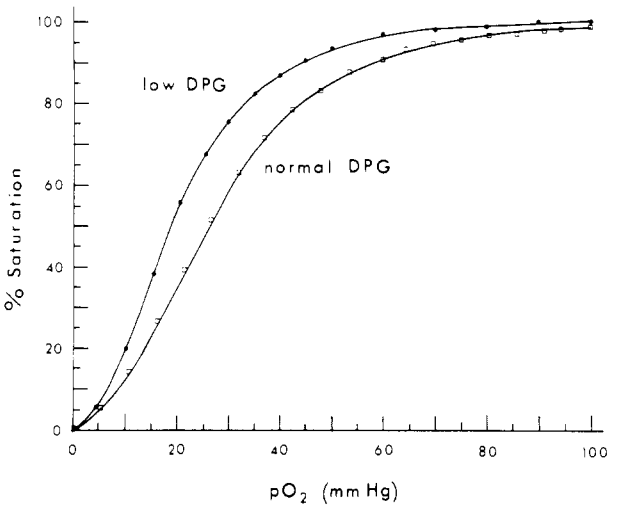


FIGURE 7: Hemoglobin–oxygen saturation curves of red cells. At low (<1 $\mu$M/mL of RBC) 2,3-DPG levels, the oxygen affinity of cells incubated with MeP (closed circles) was identical with that of cells without MeP (left solid line), with a $P_{50}$ $O_2$ of 19 mmHg. At normal levels of 2,3-DPG (4.6–4.9 $\mu$M/mL of RBC), the oxygen affinity of MeP-incubated cells (open squares; $P_{50}$ $O_2$ 25.5 mmHg) was essentially identical with that of cells without MeP (right solid line; $P_{50}$ $O_2$ 26 mmHg).

glucose consumption at 38 mg (dL of RBC)$^{-1}$ h$^{-1}$.

Organic phosphates such as 2,3-DPG bind strongly to hemoglobin and modify its oxygen affinity (Benesch et al., 1967; Oski & Gottlieb, 1971). Furthermore, small anions may bind to nonspecific sites on hemoglobin and lower its oxygen affinity in the absence of organic phosphates (Bunn, 1980). In order to determine whether MeP alters hemoglobin–oxygen affinity by competing with 2,3-DPG for its specific hemoglobin binding site or by other mechanisms, the oxygen-dissociation curves of red cells incubated at pH 7.4 in the presence of 10 mM extracellular MeP were obtained (Figure 7). At low levels of 2,3-DPG (<1 $\mu$M/mL of RBC), the hemoglobin–oxygen dissociation curve was unaffected by the presence of MeP ($P_{50}$ $O_2$ 19 mmHg). At normal levels of 2,3-DPG, the hemoglobin–oxygen dissociation curve in the presence of MeP ($P_{50}$ $O_2$ 25.5 mmHg; 2,3-DPG, 4.9 $\mu$M/mL of RBC) was essentially unchanged from the control ($P_{50}$ $O_2$ 26 mmHg; 2,3-DPG, 4.6 $\mu$M/mL of RBC). MeP did not appear to alter oxygen affinity in the absence of 2,3-DPG, nor did it appear alter the effect of 2,3-DPG on oxygen affinity.

*Intracellular pH.* NMR spectra were obtained on eight fresh red cell samples that were incubated for 30–60 min at 37 °C with methylphosphonate. By incubation in humidified air with 5% added $CO_2$, the samples were fully oxygenated and buffered by bicarbonate, to simulate in vivo conditions. As described earlier, separate signals from the extracellular and intracellular MeP compartments were observed. Assignment of the intracellular MeP signal was made by the addition of $Mn^{2+}$. The intracellular MeP signal appeared consistently downfield of the extracellular MeP signal, suggesting that the intracellular pH under these conditions was slightly lower than the extracellular pH. The extracellular pH ranged from 7.310 to 7.407, with a mean of 7.336 ± 0.031 (standard deviation). The intracellular pH as measured by freeze–thawing ranged from 7.140 to 7.230, with a mean of 7.202 ± 0.034.

In Figure 8, the extracellular MeP chemical shift and pH of each sample are plotted in relation to the intracellular MeP chemical shift and freeze–thaw pH of that sample. An expansion of the chemical shift–pH titration curve of MeP is also shown. Since the p$K_a$ of MeP is near the physiologic pH range,

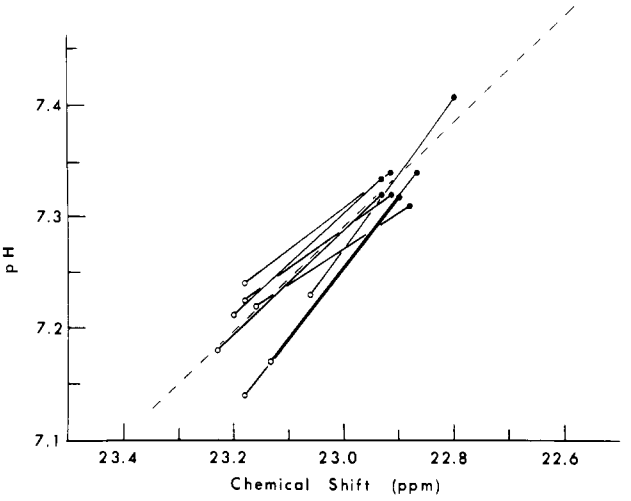


FIGURE 8: Plot of relationship between pH and methylphosphonate chemical shift in eight red cell suspensions (Hct, 45–50%), on a greatly expanded pH scale. Each solid circle represents the extracellular methylphosphonate chemical shift and independently measured pH of one sample. Each point is connected to an open circle representing the intracellular methylphosphonate chemical shift and the intracellular pH measured by freeze–thawing of that sample. The dotted line represents an expansion of the chemical shift–pH titration curve of methylphosphonate from Figure 2 (slope 0.460 pH/ppm). The slope of a regression line between the intracellular and extracellular points is 0.485 pH/ppm, with a correlation coefficient of 0.93.

the line is essentially linear in this greatly expanded plot and has a slope of 0.460 pH/ppm. Linear regression analysis of the eight pairs of points from the red cell spectra gives a line with a slope of 0.485 with a correlation coefficient of 0.93. That these points generate a line with a slope very similar to that of the MeP titration curve is strongly suggestive that the chemical shift difference between the simultaneously observed intracellular and extracellular MeP signals of each sample reflects the transmembrane pH difference and does not arise from other factors that might affect NMR chemical shifts: e.g., magnetic susceptibility of red cells or binding of MeP to intracellular substances other than hemoglobin. The mean transmembrane pH gradient of the eight samples, measured as the difference between the NMR chemical shifts of the internal and external MeP signals, was 0.129 ± 0.008 (range 0.11–0.14). This compares favorably to the mean pH difference of 0.128 ± 0.032 (range 0.09–0.17) measured by freeze–thawing.

## Discussion

Intracellular pH plays a major influential role on many of the biologic functions of the red cell such as hemoglobin–oxygen affinity (Oski & Gottlieb, 1971), hemoglobin–2,3-DPG interactions (Asakura et al., 1966; Duhm, 1971; Bunn et al., 1977), and glycolytic activity (Rona & Wilenko, 1914; Murphy, 1960; Bunn et al., 1977). Earlier methods of pH measurement such as the freeze–thaw technique (Funder & Wieth, 1966) have the disadvantage of requiring the destruction of the cell under study in order to measure it. Such profound disruptions may in some cases alter the property under investigation; for example, errors in freeze–thaw pH measurements may arise from alterations in $pCO_2$ introduced by freezing (Warth et al., 1977). Other methods of pH measurement have utilized the distribution of radioactive weak acids across the cell membrane (Waddell & Bates, 1969).

$^{31}P$ NMR potentially offers a simpler method of nondestructively determining pH in an intact cell system, and excellent reviews of such use have been published (Roos & Boron, 1981; Gillies et al., 1982). In biochemical experiments in which NMR is the primary study modality, pH data can be obtained simultaneously with other data. Moon & Richards (1973) first utilized the $^{31}P$ NMR chemical shifts of $P_i$ and 2,3-DPG in the measurement of red cell pH, while others (Lam et al., 1979; Tehrani et al., 1982) used the relationship between the chemical shifts of the 2,3-DPG phosphates in hemolysates and hemolysate pH to measure the pH of intact red cells. However, we have found that $P_i$ is unsuitable as a pH marker in fresh erythrocytes, since this compound is present in low quantity and its signal is obliterated by the nearby 2-P signal from 2,3-DPG (Labotka et al., 1976a). Moreover, the $pK_a$ of $P_i$ is sensitive to ionic strength effects (Gillies et al., 1982).

The 2,3-DPG chemical shifts are unsuitable for intracellular pH measurements for different reasons. As Moon & Richards (1973) and others (Huestis & Raftery, 1972; Costello et al., 1976; Marshall et al., 1977) have observed, the 2,3-DPG chemical shifts are sensitive to alterations in 2,3-DPG binding to hemoglobin. Such alterations accompany changes in intracellular 2,3-DPG concentration, hemoglobin concentration, or oxygen concentration, either between different samples or within the same sample during incubation studies. Moon & Richards suggested that the *separation* between the 2-P and 3-P signals may be a more reliable indicator of pH. Unfortunately, the 2-P and 3-P signals move in the same direction with pH changes, thus making this parameter relatively insensitive to pH changes ($\Delta pH/\Delta\delta$ = 1.9 pH/ppm). Moreover, the separation between the 3-P and 2-P signals appears also to depend on the degree of hemoglobin binding (Costello et al., 1976; Marshall et al., 1977).

The relative separation of the signals from two different intracellular phosphate compounds has also been related to pH. Swanson et al. (1983) found that the relative separation of the signals from the $\alpha$-P of ATP and the 3-P of 2,3-DPG is pH sensitive. The potential advantage of such a system is that the $\alpha$-P does not vary with pH (Cohn & Hughes, 1962; Labotka et al., 1976b), providing an internal chemical shift reference. Unfortunately, the ATP phosphates are sensitive to the state of binding of $Mg^{2+}$ (Cohn & Hughes, 1962; Costello et al., 1977). We have observed that the $\alpha$-, $\beta$-, and $\gamma$-P of ATP in aqueous solutions at pH 7.4 underwent downfield shifts of 0.8, 2.5, and 2.2 ppm, respectively, when $Mg^{2+}$/ATP ratios were increased from 0 to 1.0 (R. J. Labotka, unpublished observation). Gupta et al. (1978) have utilized changes in the chemical shifts of the ATP phosphates to measure the amount of free $Mg^{2+}$ in oxygenated and deoxygenated red cells. The sensitivity of the DPG chemical shifts to the degree of hemoglobin binding has already been discussed. Therefore, the intrinsic phosphates of the red cell are not suitable intracellular pH markers.

$^{19}F$ NMR has been used to study red cell transmembrane pH gradients (Taylor et al., 1981) but has the disadvantage that fluorine compounds are not involved in ordinary cell metabolism. Thus, pH measurements cannot be made in the same NMR spectrum used to make other biochemical measurements. Moreover, the fluorinated compound used was in rapid equilibrium across the cell membrane, requiring cooling the cells to 4 °C to slow exchange sufficiently to resolve intracellular and extracellular signals. Proton NMR of histidine residues of hemoglobin in solution has been used to measure the pH of hemoglobin solutions and intact red cells (Brown et al., 1976). However, the proton shifts are difficult to resolve in whole cell systems, due to the complexity of intact cell proton spectra. In addition, the histidyl residue shifts appear to depend on the degree of oxygenation of hemoglobin.

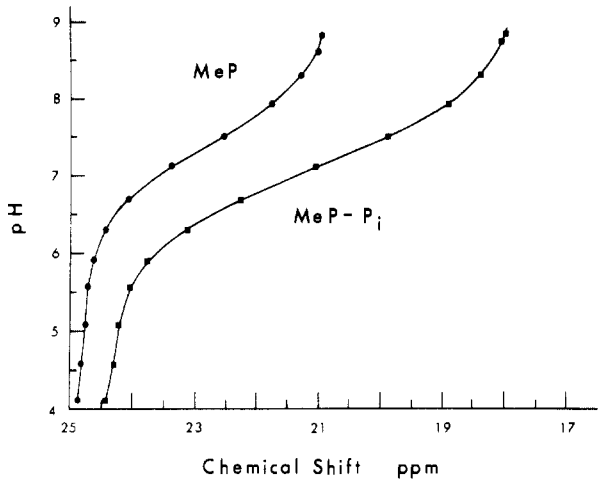


FIGURE 9: Expanded plot of pH sensitivity of MeP chemical shift (solid circles). Near the intraerythrocytic pH of red cells (pH 7.22), the MeP curve is nearly linear, with a slope of 0.460 pH/ppm. By subtraction of the $P_i$ chemical shift, the pH sensitivity of the difference curve (solid squares) is increased in magnitude and extended over a wider pH range, since the phosphate and the phosphonate transitions occur in opposite directions and at different $pK_a$s. The slope of this curve is 0.325 pH/ppm at pH 7.22.

Since the intrinsic phosphates of the red cell did not appear to be useful indicators of pH in fresh cells, we sought a nonphysiologic phosphate analogue that would have minimal metabolic effects on the red cell, in order to diminish the disadvantage of the addition of a foreign substance to our system. Therefore, the ideal pH probe was required to enter red cells readily, not to affect glucose consumption, and not to interfere with hemoglobin–oxygen affinity or hemoglobin–2,3-DPG interaction. Although the chemical shift of fluorophosphate and the coupling constants of fluorophosphate and phosphite were found to be sensitive to pH, this pH dependence occurred at pH ranges too low to be useful in the study of fresh red cells. These compounds may be useful, however, in the study of cellular or bacterial systems at low pH . The metabolic effects of these analogues on red cells have not yet been studied. Methylphosphonate has been used as a pH marker in the $^{31}P$ NMR study of bacteria (Slonczewski et al., 1981); however, the effects of MeP on red cells have not been previously studied.

Inorganic phosphorus is a potent stimulator of erythrocyte metabolism (Rose & Warms, 1964). We were concerned that a phosphate analogue might either stimulate metabolism by similar actions on control enzymes or inhibit metabolism by blocking the normal enzymatic reactions involving phosphate. The rates of glucose consumption in the presence of MeP and a control were comparable to rates reported for red cells having normal levels of $P_i$ (Rose, 1966), while glucose consumption was strongly simulated by added $P_i$. MeP appears to have no effect on glucose consumption during brief incubations with red cells.

We were also concerned whether MeP might bind hemoglobin at its high-affinity 2,3-DPG binding site (Bunn et al., 1977), since other organic phosphates such as ATP can bind at this location. Such binding might alter hemoglobin–oxygen affinity or displace 2,3-DPG from its hemoglobin-bound state. However, at low 2,3-DPG concentration, no effect on oxygen affinity of red cells containing MeP was seen, suggesting that MeP itself does not modify this affinity. Similarly, at normal DPG levels, no effect was observed on oxygen affinity, suggesting that MeP did not displace 2,3-DPG from hemoglobin. Therefore, MeP appeared to be relatively metabolically inert. The rate of influx of MeP was found to be sufficiently rapid that reasonable intracellular signals could be obtained after 30-min incubation. The rate of transmembrane exchange was low enough to produce distinct intracellular and extracellular resonances.

The use of a $^{31}P$ NMR methylphosphonate probe in oxygenated cells is straightforward. The differences between the extracellular and intracellular MeP chemical shifts were found to correlate well with the differences between extracellular and intracellular pH (measured by freeze–thawing). NMR measurement of red cell pH yields results comparable to values obtained by other methods of pH determination (Waddell & Bates, 1969; Roos & Boron, 1981). In our system, chemical shifts of red cell phosphates can be reliably measured to 0.03 ppm or better (corresponding to a pH sensitivity of 0.013 pH unit), with signal accumulation times of 5–10 min, and intracellular MeP concentrations of 2 mM, with a red cell mass corresponding to a hematocrit of 40–50%. Separation of the intracellular and extracellular signals by as little as 0.2 ppm or 0.09 pH unit difference could reliably be measured. These sensitivity limits are similar to those reported by others using superconducting NMR systems (Roos & Boron, 1981; Gillies et al., 1982). Unlike $P_i$, the $pK_a$ of MeP appears to be insensitive to ionic strength (Gillies et al., 1982), so that only as much compound as is necessary to obtain measurable intracellular signals need to present in the experiment, and the intracellular concentration need not be known to make valid observations. Therefore, our MeP probe could rapidly measure red cell pH without packing the cells, and NMR experiments of red cell metabolism can be carried out under reasonably physiologic conditions.

One method for improving the sensitivity of NMR pH measurements under selected circumstances may be to combine the data from $P_i$ and MeP chemical shifts. In cellular systems where the $P_i$ signal is readily visible and not changing in magnitude, the pH dependence of $P_i$ should be reliable. Since the pH dependence of the phosphate is opposite that of the phosphonate, the arithmetic difference between their chemical shifts has a much larger pH dependence than that of either compound alone (Figure 9). At pH 7.4, this difference curve gives a sensitvity of 0.325 pH/ppm. Moreover, since the $pK_a$'s differ by about 0.8 pH unit, the pH range over which this chemical shift sensitivity occurs is larger than that of either compound. An added advantage of the difference curve is that an absolute reference for chemical shift need not be present in the system. Furthermore, this difference curve may be independent of some environmental factors that might cause symmetric shifts in NMR signals, such as the presence of a paramagnetic compound. Methylphosphonate appears to be a useful probe of intraerythrocytic pH by $^{31}P$ NMR spectroscopy.

## Acknowledgments

We thank Dr. Akira Omachi for this generosity in allowing the use of his facility and Virginia Winecki for her invaluable assistance.

**Registry No.** Methylphosphonic acid, 993-13-5; sodium fluorophosphate, 7631-97-2; phosphite, 14901-63-4.

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