Measurement of Intracellular pH and Deoxyhemoglobin Concentration in Deoxygenated Erythrocytes by Phosphorus-31 Nuclear Magnetic Resonance†

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ABSTRACT: Deoxygenation of erythrocytes produced marked changes in their ^{31}P nuclear magnetic resonance spectra in the superconducting spectrometer. Most significantly, all intracellular and extracellular phosphates underwent downfield shifts. In fully deoxygenated blood the extracellular phosphates showed downfield shifts that were dependent upon packed cell volume, when added pyrophosphate was used as a measure of extracellular chemical shift behavior. This effect on extracellular signals was attributed to the paramagnetic contribution of deoxyhemoglobin to the "bulk" magnetic susceptibility of the red cell suspension. Line broadening was observed in deoxygenated whole cell systems but not in hemolysates, as a result of paramagnetic susceptibility gradients across the cell membrane. The degree of downfield shift upon deoxygenation was of different magnitude for each intracellular phosphate [2-P of 2,3-diphosphoglycerate (2,3-DPG) > 3-P of 2,3-DPG > inorganic phosphate > ATP phosphates], independent of packed cell volume but dependent on the degree of deoxygenation of hemoglobin. When deoxygenation shift effects in adult cells were compared to those of cord blood cells containing 70% fetal hemoglobin, it was found that 45% of the 2,3-DPG shift effects were attributable to binding of that compound to hemoglobin. By use of a nonphysiologic phosphate analogue, methylphosphonate, as an internal reference, it was found that an increase in pH of deoxy cells contributed to the downfield shift of inorganic phosphate. In hemolysates, the methylphosphonate – inorganic phosphate chemical shift difference was found to be pH dependent, with a sensitivity of (–) 0.39 pH unit/ppm, independent of the hemoglobin oxygenation state. Moreover, the paramagnetic shift effect on methylphosphonate was proportional to the degree of deoxygenation and was found to be identical with that on phosphate (1.0 ppm for fully deoxygenated hemolysates). By measurement of the methylphosphonate – phosphate shift difference in intact red cells, the intracellular pH was estimated to rise from 7.15 to 7.29 upon deoxygenation. In addition, the paramagnetic shifts of methylphosphonate and phosphate were predictive of the degree of hemoglobin–oxygen saturation in the intact cells.

Nuclear magnetic resonance (NMR)[1] spectroscopy is being extensively applied to the study of erythrocytes, because of its ability to provide important metabolic information about the cell nondestructively. One such metabolic parameter is the pH of the living cell. Since the landmark study of intact red cells by Moon & Richards (1973), numerous investigators have utilized ^{31}P NMR in the measurements of living biological systems (Roos & Boron, 1981; Gillies et al., 1982). However, as Fabry & San George (1983) and we (Labotka & Omachi, 1983) have observed, deoxygenation of red cell leads to profound changes in ^{31}P NMR spectra obtained on a superconducting spectrometer. Global changes in the NMR chemical shifts of the cell phosphates result from the appearance of a paramagnetic species, deoxyhemoglobin. In addition, line broadening occurs as a result of magnetic field gradients across the red cell membrane arising from differences in the paramagnetic susceptibilities of the intracellular and extracellular phases, as Fabry & San George (1983) have shown. Because of the alterations in the phosphate chemical shifts, previous measurements of red cell pH that relied on chemical shifts are no longer valid in the deoxygenated erythrocyte. Since several physiologic changes accompany the change in oxygenation state of hemoglobin, the alterations observed in the phosphate chemical shifts upon deoxygenation may not bear a simple relationship to the quantity of the paramagnetic species.

We have shown that reliable pH measurements can be made in the oxygenated, fresh red cell by the use of a nonphysiologic probe, methylphosphonate (MeP) (Labotka, & Kleps, 1983). MeP had several advantages over intrinsic red cell phosphates as markers of intracellular pH: its chemical shift was in a region separate from that of the red cell phosphate shifts; the pK_a (7.6) was near the physiologic pH range, and the MeP chemical shift was highly pH sensitive in this range; MeP readily entered red cells; MeP did not interact significantly with hemoglobin and did not affect red cell glucose consumption. Moreover, since phosphonate chemical shifts exhibit a pH dependence that is in the opposite direction of that of phosphates, the MeP – P_i chemical shift difference was found to be highly pH sensitive and independent of an absolute chemical shift reference. In the present study, MeP is used as an internal probe to examine three factors that contribute to the alterations in deoxygenated red cell spectra: paramagnetic shift effects, binding of 2,3-diphosphoglycerate (2,3-DPG) to deoxyhemoglobin, and intracellular pH. These factors can be exploited to provide intracellular pH mea-

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[1] Abbreviations: ATP, adenosine 5′-triphosphate; 2,3-DPG, 2,3-diphosphoglycerate; MeP, methylphosphonate; NMR, nuclear magnetic resonance; NTP, nucleoside triphosphate; PCP, methylenediphosphonate; PCV, packed cell volume; P_i, (inorganic) phosphate; POP, pyrophosphate; RBC, red blood cell.

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surements and to quantify deoxyhemoglobin in the deoxygenated red cell.

Materials and Methods

Instrumentation. The spectrometer was a Bruker CXP 180 superconducting spectrometer operating at a phosphorus frequency of 72.88 MHz and employing quadrature detection, broad-band heteronuclear gated proton decoupling, Fourier transformation, and signal accumulation technology. Samples of 8–10 mL volume were placed in 20-mm NMR tubes and capped with a Teflon plug. A concentric capillary contained methylenediphosphonate (PCP) which served as a chemical shift reference (17.05 ppm). Samples were spun at 10 Hz. Unlike others (Moon & Richards, 1973), we have observed that spinning red cell samples improves signal resolution, possibly by preventing settling of the cells during experiments. The ambient temperature was 37 °C. Typical red cell experiments consisted of a simple 45° signal aquisition sequence, with 500–1000 transients accumulated in a 7–15 min period. Chemical shifts are reported in parts per million (ppm) relative to 85% inorganic phosphoric acid, with upfield shifts considered negative. Hemoglobin–oxygen dissociation curves of the fresh red cells were determined on a TCS Hemox analyzer, and oxygen saturations of individual samples were measured on an American Optical oximeter. Red cell and hemolysate pO_2 was measured on a Corning Model 168 blood gas analyzer, and pH was determined on a Corning Model 130 digital pH meter equiped with a glass–calomel combination electrode.

Red Cell Studies. Experiments were performed on fresh, washed red cells, whole blood, or hemolysates. Donations for each experiment consisted of 20 mL of blood from three to five adult volunteers of identical ABO and Rh blood type, anticoagulated with heparin and pooled for incubation.

The effects of deoxygenation on the ^{31}P NMR spectrum of erythrocytes were studied by using fresh cells, washed twice and resuspended in an isotonic bicarbonate buffer previously described (Labotka & Kleps, 1983), containing 10 mM glucose and 5 mM MeP (extracellular pH 7.32). The packed cell volume (PCV) was 73%. Full oxygenation (pO_2 148 mmHg) was achieved by incubation in humidified air/5% CO_2, at 37 °C. Deoxygenation (1 mmHg) was achieved by similar incubation in N_2/5% CO_2 for 60 min. Fresh, similarly deoxygenated (pO_2 3.7–6.5) whole blood (pH 7.44–7.48) was used to study the effects of deoxygenation on extracellular phosphate signals. MeP at 150 mM was added to a final concentration of 5 mM, and 150 mM pyrophosphate (POP) was added as an extracellular phosphate probe, to a final extracellular concentration of 10 mM. Aliquots having packed cell volumes from 0% (plasma) to 82% were prepared for NMR analysis by centrifugation.

Washed red cells with added 5 mM MeP were used in measuring the dependence of intracellular phosphate chemical shifts on oxygen tension and on hemoglobin–oxygen saturation. Suspensions were incubated in mixtures of air and N_2 with 5% added CO_2, with aliquots taken for NMR analysis as the cells were progressively deoxygenated, to give sample pO_2 values ranging from 126 to 1 mmHg.

Hemolysates were used in the study of the dependence of MeP and P_i chemical shifts on intracellular pH and hemoglobin–oxygen saturation. These were prepared by centrifugation of fresh, whole blood in a Sorvall RC2-B centrifuge at 10000*g* for 10 min, followed by three cycles of rapid freezing in dry ice–ethanol of the samples in sealed tubes and thawing in a water bath at 37 °C; 150 mM MeP was added to a concentration of 3 mM. For pH titrations, the whole blood pH was adjusted within the range 6.8–7.8 by gently adding

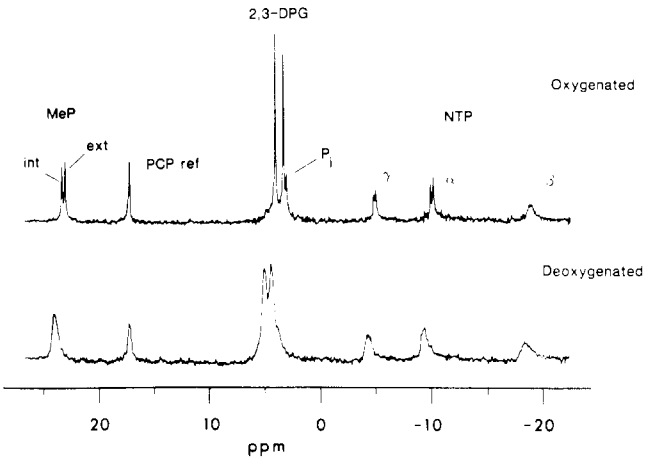

FIGURE 1: ^{31}P NMR spectra of fresh, washed erythrocytes (PVC = 73%; extracellular pH 7.32). Top spectrum: oxygenated (pO_2, 148 mm Hg). The major intrinsic phosphates are 2,3-DPG (3-P, 3.80 ppm; 2-P, 2.97 ppm), P_i (upfield shoulder at 2.70 ppm on the 2-P signal from 2,3-DPG), and nucleoside triphosphates (NTP), chiefly ATP (γ-P, −4.94 ppm; α-P, −10.4 ppm; β-P, −18.77 ppm). PCP is an external methylenediphosphonate capillary (17.05 ppm). Methylphosphonate has two signals, extracellular (22.93 ppm) and intracellular (23.26 ppm), corresponding to a transmembrane pH gradient of 0.15. Bottom spectrum: deoxygenated (pO_2 1 mmHg). Intracellular signals are broadened and shifted downfield. Because of broadening of MeP (23.75 ppm), the intracellular and extracellular phases are not separately resolved. P_i is difficult to separate from the 2,3-DPG signal. In decreasing order, the degrees of shifts of the intracellular compounds (in ppm) are the following: 2-P of 2,3-DPG, 1.36; 3-P, 1.10; P_i, 0.86; α-P of ATP, 0.76; γ-P, 0.69; β-P, 0.47.

1 M NaOH or 1 M HCl to the supernatant plasma of gently centrifuged blood and incubating before hemolysis. The true hemolysate pH was measured after lysis. For oxygenation dependence studies, hemolysates were prepared from fresh, unadjusted blood and incubated under conditions of progressive deoxygenation (pO_2 values ranging from 104 to 5 mmHg) similar to the intact cell experiments.

All experiments were concluded within 3 h of beginning incubation, and no significant changes were observed in the 2,3-DPG or ATP levels during incubation. When suppression of extracellular signals was required, 10^{-4} M $MnCl_2$ was added as an extracellular paramagnetic relaxation agent (Hamasaki et al., 1981), just prior to NMR analysis. In some deoxygenated cell experiments, 3 mM dysprosium tripolyphosphate (Fabry & San George, 1983) was added to decrease line broadening induced by paramagnetic susceptibility gradients across the cell membrane.

Results

Figure 1 illustrates the ^{31}P NMR spectra of both oxygenated (pO_2 148 mmHg) and deoxygenated (pO_2 1 mmHg) washed erythrocytes (extracellular pH of 7.32) having a packed cell volume of 73% and with added MeP (5 mM extracellular). In the oxygenated cell spectrum, the major intracellular phosphate signals arise from 2,3-DPG (3-P, 3.80 ppm; 2-P, 2.97 ppm) and nucleoside triphosphates (NTP), chiefly ATP (γ-P, −4.94 ppm; α-P, −10.04 ppm; β-P, −18.77 ppm). Inorganic phosphate is present in low concentration in fresh red cells and in this spectrum is represented by a small upfield shoulder (2.70 ppm) on the 2-P peak of 2,3-DPG. MeP appears as two signals: a downfield peak at 22.93 ppm arising from the extracellular phase and an upfield peak at 23.26 ppm from the intracellular phase. The separation between these peaks is consistent with a transmembrane pH gradient of 0.15 (Labotka & Kleps, 1983).

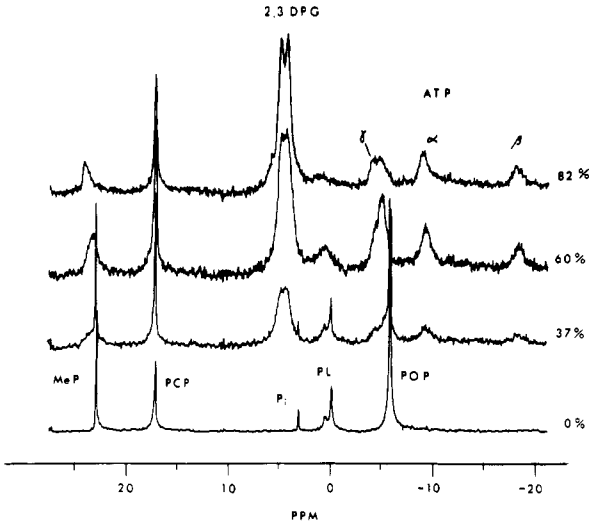

FIGURE 2: Effect of increasing packed cell volume on extracellular phosphate chemical shifts in whole blood. The bottom spectrum is of deoxygenated blood plasma with MeP (22.83 ppm) and pyrophosphate (POP) (−5.89 ppm) added. Pyrophosphate does not readily enter the intracellular phase. At a PCV of 37%, extracellular phosphates are minimally shifted. Intracellular MeP is represented by a broad base beneath the extracellular MeP signal. As the PCV increases, the extracellular phosphates broaden and shift progressively. At a PCV of 82%, extracellular POP has shifted by 0.87 ppm. In contrast, intracellular phosphate chemical shifts are little affected by PCV. The extracellular pH ranged from 7.44 to 7.48 in these spectra.

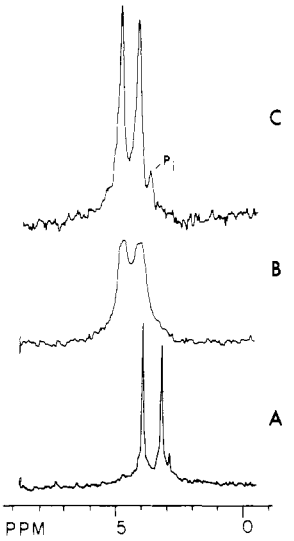

FIGURE 3: Use of dysprosium tripolyphosphate to resolve the inorganic phosphate signal from deoxygenated, intact red cells. (A) A portion of the spectrum from oxygenated cells, showing P_i as a small peak (2.88 ppm) upfield of the 2-P signal from 2,3-DPG (3.18 ppm). (B) The same cells deoxygenated, showing obliteration of the P_i signal by the broadened 2,3-DPG signals. (C) The same deoxygenated cells with added 3 mM dysprosium tripolyphosphate. Line broadening is significantly reduced, and the P_i signal is clearly resolved at 3.64 ppm.

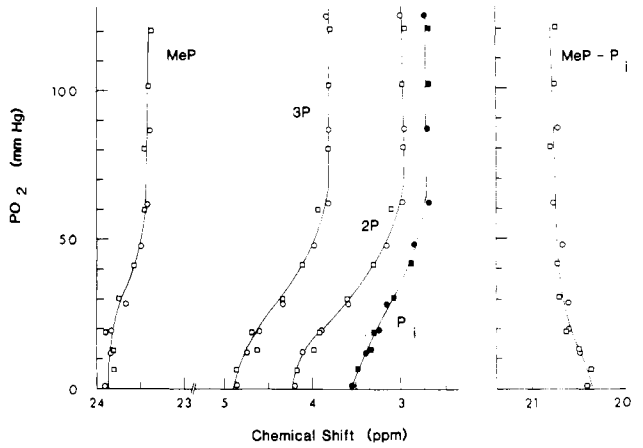

FIGURE 4: Dependence on oxygen tension of intracellular MeP, 2,3-DPG, and P_i chemical shifts from two sets of washed, fresh red cells (set 1, PCV = 45%; squares) (set 2, PCV = 73%; circles). With progressive deoxygenation, all these compounds exhibit nonlinear downfield shifts (2-P of 2,3-DPG > 3-P > P_i > MeP), slight at higher pO_2 and pronounced at lower pO_2. The right panel depicts a curve labeled "MeP − P_i", the arithmetic chemical shift difference between these two compounds, which reflects intracellular pH.

Two features distinguish the NMR spectrum of the deoxygenated cells from that of the oxygenated cells. In the deoxygenated cell spectrum, all of the intracellular phosphate signals are shifted downfield and are broadened compared to their counterparts in the oxygenated system. The magnitudes of the intracellular phosphate shifts (in decreasing order) are the following: 2-P of 2,3-DPG, 1.36 ppm; 3-P of 2,3-DPG, 1.10 ppm; P_i, 0.86 ppm; α-ATP, 0.76 ppm; γ-ATP, 0.69 ppm; β-ATP, 0.47 ppm. As a result of broadening of both the intracellular and extracellular MeP signals (23.75 ppm), identification of the separate contributions for this compound is not possible in this spectrum, in the absence of an extracellular paramagnetic relaxation agent. Subsequent studies included 10^{-4} M Mn^{2+} when suppression of extracellular signals was needed.

The paramagnetic effects of deoxyhemoglobin are not confined to the intracellular millieu. The effect of deoxyhemoglobin on the extracellular chemical shifts in whole blood was investigated by deoxygenating the blood (pO_2 3.7–6.5 mmHg) and preparing several samples having packed cell volumes between 0% and 82%. The extracellular pH ranged from 7.44 to 7.48. The NMR spectra of these samples are shown in Figure 2. The bottom spectrum consists only of blood plasma with added MeP (22.83 ppm) and POP (−5.89 ppm). Since incubation studies have shown POP not to enter red cells readily (A. Omachi, personal communication), it is used here as a marker for extracellular phosphate chemical shift behavior. The native plasma phosphates, P_i (2.92 ppm), and phospholipids (0.37 and −0.26 ppm) are also identified. At a PCV of 37%, slight changes are observed in the extracellular phosphates. The intracellular phosphates are characteristically broadened, and the intracellular MeP can be appreciated as the broad base out of which arises the extracellular MeP at 22.89 ppm. As the PCV increases to 60% and 82%, the extracellular POP and phospholipids become progressively broadened and shifted downfield. In contrast, the intracellular phosphate chemical shifts such as 2,3-DPG and ATP appear to be little affected by changes in PCV.

The observation that each intracellular phosphate is shifted to a different degree upon deoxygenation needs to be explained on the basis of the physiologic changes that accompany the deoxygenated state. However, the line broadening observed in the deoxygenated whole cell systems can interfere with accurate measurements of the intracellular chemical shifts. This is particularly important for inorganic phosphate, which is present in low concentration in fresh cells, and its signal can be obscured by the large 2,3-DPG peaks. The addition of a small amount of a strongly paramagnetic compound (3 mM dysprosium tripolyphosphate) to the extracellular space reduces the paramagnetic susceptibility gradient across the cell membrane and significantly improves signal resolution (Fabry & San George, 1983). As Figure 3 shows, this technique allows for accurate identification of a P_i signal that may otherwise be completely obscured in deoxygenated cells.

Figure 4 depicts the dependence of the chemical shifts of intracellular MeP, 2,3-DPG, and P_i on oxygen tension. Two sets of fresh, washed erythrocytes having differing packed cell volumes (45% and 73%), with an initial extracellular pH of 7.32–7.34, were progressively deoxygenated, and aliquots were taken for NMR analysis at pO_2's ranging from 126 to 1 mmHg. As expected, downfield chemical shifts were observed for all intracellular phosphates. Moreover, these shifts exhibited a sigmoidal dependence on oxygen tension, reminiscent of the behavior of the hemoglobin–oxygen saturation curve. In decreasing order, these shifts were the following: 2-P of 2,3-DPG, 1.30 ppm; 3-P of 2,3-DPG, 1.08 ppm; P_i, 0.85 ppm; MeP, 0.50 ppm. Since 2,3-DPG binds strongly to deoxyhemoglobin A, this binding may contribute to the large magnitude of the downfield chemical shifts observed for 2,3-DPG. This hypothesis was tested by comparing the degree of downfield shift in the 2,3-DPG phosphates in deoxygenated adult cells to that in similarly deoxygenated cord blood cells (extracellular pH 7.36). These cells contained 70% fetal hemoglobin (Hb F), which in its deoxygenated state has very little affinity for 2,3-DPG (De Verdier & Garby, 1969). Upon deoxygenation of the cord blood cells (pO_2 4 mmHg; oxygen saturation 4%), the 2-P of 2,3-DPG shifted downfield by 0.72 ppm and the 3-P by 0.60 ppm. That these shifts are only 55% of those observed in the adult cells containing Hb A indicates that the strong binding affinity of deoxygenated Hb A for 2,3-DPG is responsible for a significant portion of the shifts observed in the deoxygenated red cell spectrum.

With deoxygenation, the phosphonate MeP shifts to a smaller degree than does inorganic phosphate. In contrast to 2,3-DPG, neither P_i (Chanutin & Curnish, 1967; Hamasaki & Rose, 1974) nor MeP (Labotka & Kleps, 1983) interacts strongly with Hb A under normal intracellular conditions, and therefore, such an interaction cannot account for the differences observed in the chemical shift behavior of these two compounds. This behavior may result from an increase in intracellular pH with deoxygenation, which is consistent with the observation that deoxyhemoglobin is a stronger base than oxyhemoglobin (Oski & Gottlieb, 1971). Phosphonates shift upfield with increasing pH, whereas phosphates shift downfield. Increasing pH would therefore augment downfield paramagnetic shifts of phosphates and diminish paramagnetic shifts of phosphonates.

In order to measure the effects of deoxygenation on pH in erythrocytes, an intracellular pH marker was required. The Me – P_i chemical shift difference was selected as a possible marker. As discussed previously, the MeP – P_i shift difference was highly dependent on pH and independent of an absolute chemical shift reference in oxygenated systems. To determine whether this quantity was a valid measure of pH in deoxygenated cells, NMR spectra were obtained from aliquots of freeze–thaw hemolysates, where pH could be measured directly. Unlike the intact cell spectra, line broadening was not observed in hemolysates. Figure 5 depicts the results of two experiments designed to measure the relationship between the MeP – P_i chemical shift difference, oxygen tension, and the directly measured pH of hemolysates. The first experiment, represented by the dashed line, shows the pH dependence of the MeP – P_i shift difference in fully oxygenated hemolysates (individual data points omitted for clarity). These hemolysates were prepared from whole blood samples whose pH values ranged from 6.8 to 7.8. The chemical shift difference shows a linear dependence on pH in this pH range, with a pH sensitivity of (–) 0.39 ± 0.04 pH unit/ppm (95% confidence limits).

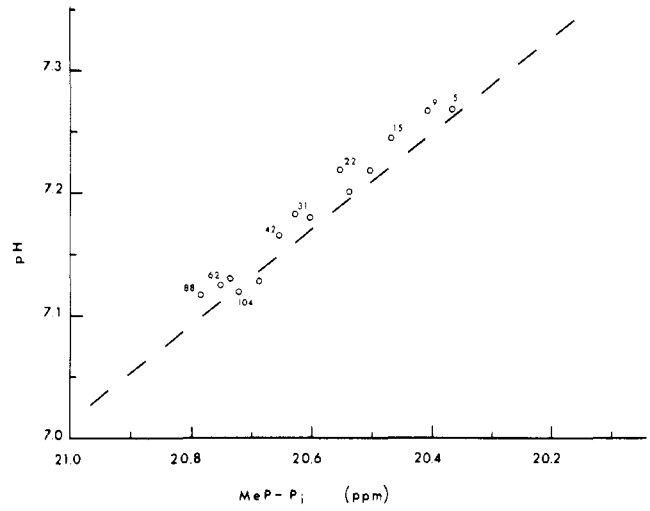

FIGURE 5: Relation between the MeP – P_i chemical shift difference, pH, and pO_2 in hemolysates. The dashed line depicts the pH titration curve of fully oxygenated hemolysates (individual points not shown). The open circles represent aliquots of hemolysates sampled under conditions of progressive deoxygenation. The pO_2's of a number of these aliquots are shown. The MeP – P_i chemical shift difference accurately reflects pH, independent of pO_2, with a sensitivity of (–) 0.39 pH unit/ppm.

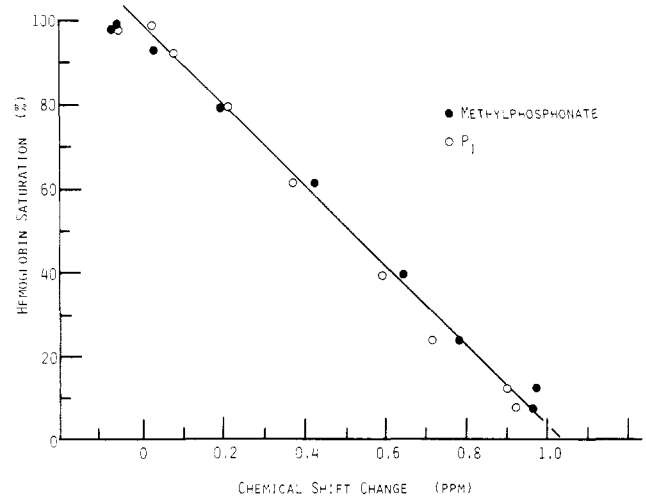

FIGURE 6: Paramagnetic shift effect on MeP and P_i in hemolysates. The chemical shift change of either compound is the difference between the actual chemical shift at a given hemoglobin saturation and the expected shift at the same pH at full oxygenation. MeP experiences the same degree of paramagnetic shift as does P_i.

In the second experiment, NMR spectra were obtained from aliquots of a hemolysate preparation incubated under conditions of progressive deoxygenation in a manner similar to the washed red cell experiments. The individual data points from this experiment are shown as open circles in Figure 5, with pO_2 values listed near representative points. The pH was observed to increase from 7.125 at full oxygenation (pO_2 104 mmHg) to 7.270 at full deoxygenation (pO_2 5 mmHg). There is a direct relationship between pH and the MeP – P_i shift difference, which is essentially identical with the relationship observed in the oxygenated hemolysates. By linear regression, this line (not shown) has a slope of (–) 0.39 ± 0.03 pH unit/ppm (correlation coefficient = 0.99). Therefore, the MeP – P_i chemical shift difference in hemolysates is independent of the state of hemoglobin–oxygen saturation and dependent only upon pH. The pO_2 independence of this relationship implies that the paramagnetic shift effects for MeP and P_i are the same.

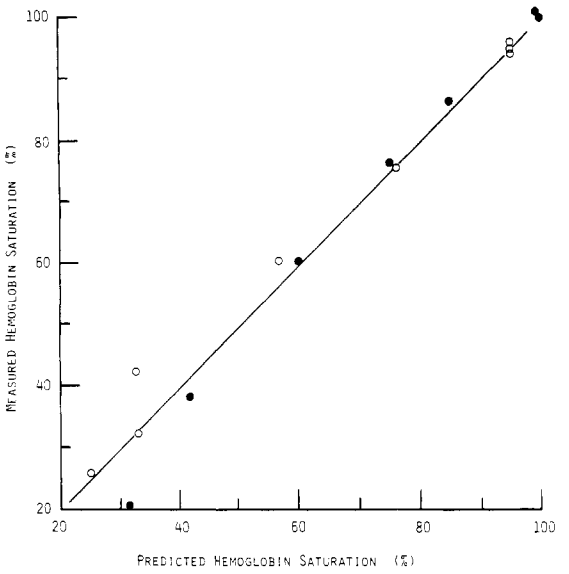

FIGURE 7: Comparison between predicted and measured values for hemoglobin–oxygen saturation for the two sets of red cells of Figure 4. The intracellular pH of each sample was estimated from the MeP – P_i chemical shift difference, and the saturation was predicted from the paramagnetic shifts of MeP and P_i in that sample.

The paramagnetic shift effects of deoxyhemoglobin on MeP and P_i are shown in Figure 6. This figure was obtained by measuring the difference between the chemical shifts of MeP and P_i in the progressively deoxygenated hemolysates and the expected shifts in oxygenated hemolysates having the same pH. The paramagnetic shifts are proportional to the concentration of deoxyhemoglobin and are identical for MeP and P_i. When the hemolysates were fully deoxygenated, both compounds shifted downfield by approximately 1.0 ppm. This finding confirms the pO_2 independence of the MeP – P_i chemical shift difference.

The effect of deoxygenation on the pH in intact red cells was estimated by using the relation between the MeP – P_i chemical shift difference and pH found in hemolysates. In Figure 4 the MeP – P_i shift difference in the intact cells showed a nonlinear decrease with deoxygenation, from 20.7 ppm at pO_2 121 mmHg to 20.34 ppm at 1 mmHg. This decrease corresponds to an increase in pH of from 7.15 to 7.29 upon deoxygenation. Furthermore, the estimated intracellular pH allowed measurement of the paramagnetic shifts of MeP and P_i in the intact cells. Since the paramagnetic shifts are proportional to the deoxyhemoglobin concentration, the state of hemoglobin oxygenation can be predicted in cells. Figure 7 compares the values for hemoglobin–oxygen saturation predicted for the red cells of Figure 4, with the values measured by oximetry. Good correlation was observed, which suggests that the relationships between MeP and P_i chemical shifts, deoxyhemoglobin concentration, and pH are preserved in intact cells.

Discussion

Knowledge of the intracellular pH is of prime importance in understanding the physiology of the erythrocyte. Rates of glycolysis (Minakami & Yoshikawa, 1966; Murphy, 1960), hexose monophosphate shunt (Murphy, 1960) activity, and hemoglobin–oxygen affinity (Bunn, 1977) are all pH-dependent processes. Following the study by Moon & Richards (1973) of intact red cells by ^{31}P NMR, numerous investigators have utilized NMR to measure blood cell pH. The pH in hemoglobin solutions and intact red cells has been related to the chemical shifts of histidine residues (Brown & Campbell, 1976; Brown et al., 1977) in proton NMR spectra. ^{19}F NMR of fluorinated amines and amino acids has been used as a probe of leukocyte pH (Deutsch et al., 1982) and red cell pH (Taylor et al., 1981). Other investigators have utilized the relationships between various phosphate resonances in ^{31}P NMR spectra to estimate pH (Kazimoto et al., 1978; Lam et al., 1979; Swanson et al., 1983) in normal and abnormal red cells.

In our ^{31}P NMR studies of red cell pH, we have found that, because of intermolecular interactions involving intracellular organic phosphates, and because of the low level of P_i in fresh cells, intrinsic red cell phosphates are not ideal indicators of pH (Labotka & Kleps, 1983). The nonphysiologic compound methylphosphonate proved to be a useful pH marker in oxygenated cells. One proposed advantage of the MeP probe relates to its nature as a phosphonate. Since the pH dependence of the phosphonate is in the opposite direction of that of (inorganic) phosphate and their respective pK_a values differ by about 0.8 pH unit, the pH sensitivity of the phosphonate – phosphate chemical shift difference is higher than that of either compound and extends over a wider pH range. In addition, the chemical shift difference is independent of an absolute chemical shift reference and was predicted to be independent of paramagnetic shift effects. Moon & Richards (1973) have suggested the use of the separation between the 2-P and 3-P phosphates of red cell 2,3-DPG as a measure of pH. However, since both phosphates shift in the same direction with pH, with similar pK_a values, this shift difference is of low pH sensitivity, about 1.9 pH unit/ppm at pH 7.2. Moreover, this shift difference is altered by 2,3-DPG binding to hemoglobin (Costello et al., 1976) and does not correlate with pH in our deoxygenated red cells.

Deoxyhemoglobin has a high-spin state Fe^{2+} ion which is paramagnetic. The paramagnetic shift effects of heme proteins have been employed extensively as structural probes of such proteins in proton NMR (Jardetzky & Roberts, 1981). However, paramagnetic shift effects have been considered negligible in metabolic studies on biologic systems and largely ignored. 2,3-DPG chemical shift changes in the ^{31}P NMR spectra of carboxy- and deoxyhemoglobin solutions were used by Huestis & Raftery (1972) to estimate hemoglobin–2,3-DPG binding. Others have related the 2,3-DPG chemical shift changes in oxy- and deoxyhemoglobin solutions (Costello et al., 1976) and in oxygenated and deoxygenated red cells (Marshall et al., 1977a) to hemoglobin–2,3-DPG binding. However, as Fabry & San George (1983) and we (Labotka & Omachi, 1983) have observed, deoxygenation of red cells leads to downfield shifts and line broadening in ^{31}P NMR signals in a superconducting spectrometer. Fabry and San George have nicely shown that the paramagnetic shift effects are proportional to the concentration of deoxyhemoglobin and highly dependent on magnetic field geometry. They found that, in electromagnetic systems where the cylindrical axis of the NMR tube is perpendicular to the field, paramagnetic shifts for deoxyhemoglobin are upfield and only half as large in magnitude as the downfield shifts observed in superconducting magnet systems (axis parallel to field). Therefore, these paramagnetic shift effects are smaller in electromagnet spectrometers but nonetheless are becoming more important as high-field, superconducting spectrometers are employed in the study of biological systems.

Not only are intracellular phosphates shifted downfield upon deoxygenation, but extracellular phosphates are shifted as well, and the extracellular phosphate shifts increase with increasing packed cell volume. This is expected, since the chemical shift of any compound is proportional to the applied magnetic field, and the magnetic field is dependent on the bulk magnetic

susceptibility of the medium (Becker, 1980). The red cell suspension consists of two phases: an intracellular medium with high paramagnetic susceptibility and an extracellular medium with low susceptibility. As the packed cell volume approaches 100%, the average proximity of the extracellular phosphates to the intracellular phase increases; hence, the paramagnetic shifts increase, and the bulk susceptibility of the entire sample approaches that of the intracellular space. The intracellular phosphorus nuclei, on the other hand, are in solution with the paramagnetic species, in high proximity, and consequently the intracellular shifts are not significantly affected by changes in PCV.

The changes that occur in the red cell spectrum upon deoxygenation are more complex than can be expected on the basis of paramagnetic shift effects alone, since important physiologic alterations exist in the deoxygenated cell: binding of organic phosphates to deoxyhemoglobin (Benesch & Benesch, 1962), pH increases due to the acid–base properties of deoxyhemoglobin (Van Slyke et al., 1922; Hastings et al., 1924), decreased binding of Mg^{2+} by ATP and increased binding of ATP to deoxyhemoglobin (Gupta et al., 1978), and alterations in cell glycolysis upon incubation (Oski et al., 1970). In order to determine whether intracellular pH could be measured and whether hemoglobin–oxygen saturation could be estimated in erythrocytes that were not fully oxygenated, three of these factors were studied: hemoglobin binding of 2,3-DPG, pH, and paramagnetic shift effects.

The chemical shift changes observed in the progressively deoxygenated red cell spectra were found not to be proportional to oxygen tension but rather to hemoglobin–oxygen saturation. This is appropriate, since paramagnetic shift effects, pH, and 2,3-DPG (and other organic phosphate) hemoglobin interactions are all dependent on deoxyhemoglobin concentration. The largest downfield shifts occurred in the 2,3-DPG phosphates, due to the strong hemoglobin–2,3-DPG binding in the deoxygenated system. There is some evidence for 2,3-DPG binding to hemoglobin in oxygenated cell systems (Diederich et al., 1970; Hamasaki & Rose, 1974; Marshall et al., 1977a); however, all observers have reported significantly higher binding in deoxygenated systems (Benesch & Benesch, 1967; Gerber et al., 1973; Hamasaki & Rose, 1974; Marshall et al., 1977a). Although deoxyhemoglobin binding of 2,3-DPG was not directly investigated in this work, that such binding was contributing to the large magnitude of the 2,3-DPG shifts was confirmed by studying deoxygenated cord blood cells containing mostly Hb F. Fetal hemoglobin has a binding affinity for 2,3-DPG considerably smaller than that of Hb A (De Verdier & Garby, 1969; Bunn & Briehl, 1970). Thus, the 2,3-DPG signals in the deoxygenated cord blood cells were shifted downfield by only 55% of the shifts seen in adult cells and were of only slightly greater magnitude than the ATP shifts.

The ATP phosphates shifted downfield to a smaller degree than other phosphates. ATP is a condensed phosphate, which has several properties that complicate interpretation of its behavior in cells: ATP chelates divalent ions such as intracellular Mg^{2+} (Cohn & Hughes, 1962); ATP can bind to deoxyhemoglobin (Chanutin & Curnish, 1967); the condensed phosphate chemical shifts respond differently to pH than do phosphate esters. Therefore, no direct relationship between ATP shifts, pH, and paramagnetic effects could be found.

Like the native red cell phosphates, MeP is also subject to paramagnetic shift effects. Therefore, a spectroscopic marker of intracellular pH independent of oxygenation status was needed. A chemical shift difference between MeP and another compound with a similar paramagnetic shift was expected to be independent of paramagnetic shift effects. Since the 2,3-DPG and ATP phosphate chemical shifts were not directly related to pH in deoxygenated cells, the only other native intracellular phosphate suitable for study was P_i. Although a second nonphysiologic phosphorus compound could be introduced intracellularly for this purpose, it was felt that this was not consistent with a minimally invasive pH measurement system. The aforementioned objection to the use of P_i as a part of a pH marker system is the low concentration of this compound in fresh cells, leading to difficulty in accurately measuring its chemical shift in the deoxygenated cell system because of line broadening. Thus, a means to overcome this difficulty was sought.

The broadening of the intracellular and extracellular signals in the deoxygenated cell systems is a result of the presence of a phase boundary across which a paramagnetic susceptibility gradient gives rise to magnetic field inhomogeneities. Deoxygenated plasma and deoxygenated hemolysates do not exhibit broadening, consistent with this concept. Fabry & San George (1983) have employed a novel approach to reduce line broadening: the addition of an extracellular paramagnetic reagent in sufficient quantity to render the extracellular magnetic susceptibility the same magnitude as that of the intracellular environment. They found dysprosium to be superior in this regard to manganese in that dysprosium had a much higher magnetic susceptibility and was effective in lower (3 mM) concentration. Mn^{2+}, although a good relaxation agent at the lower concentration (10^{-4} M) used to suppress extracellular signals in the present experiment, rapidly enters cells at the high concentration (18 mM) needed to match magnetic susceptibility with deoxyhemoglobin and degrades the intracellular signals. The use of dysprosium in the present experiment greatly aided in the measurement of the P_i chemical shifts in deoxygenated cells, and the use of the MeP – P_i shift difference as a potential pH probe was investigated further.

Neither P_i (Chanutin & Curnish, 1967; Hamasaki & Rose, 1974) nor MeP (Labotka & Kleps, 1983) binds hemoglobin significantly under normal intracellular conditions. Thus, it was expected that their respective signals should depend only on pH and paramagnetic shift effects. Since simple molecules in solution with a paramagnetic ion should experience similar paramagnetic shifts (Becker, 1980), the MeP–P_i chemical shift difference was expected to be free of paramagnetic shift effects and dependent only on pH. This was confirmed by studies in oxygenated and progressively deoxygenated hemolysates. The paramagnetic shift effects on MeP and P_i were directly measured in the hemolysates as the differences between the actual chemical shifts and the expected chemical shifts for those compounds in fully oxygenated hemolysates. Indeed, both compounds shifted proportionally to the concentration of deoxyhemoglobin, by an identical amount. The degree of shift was 1.0 ppm for fully deoxygenated cells, a value in good agreement with Fabry and San George's prediction for paramagnetic shifts at normal intracellular hemoglobin concentration in a superconducting system.

When applied to the intact cell system, the MeP – P_i chemical shift difference predicted a rise in pH in deoxygenated cells of 0.14 pH unit when compared to that of oxygenated cells. Deoxygenated hemoglobin is a stronger proton acceptor than oxyhemoglobin (Van Slyke et al., 1922; Hastings et al., 1924); hence, intracellular pH is expected to rise with deoxygenation. Previous measurements of the effects of deoxygenation on intracellular pH have been of two forms. The

first consists of comparisons of the intracellular pH of oxygenated cells to that of deoxygenated cells having the same final, extracellular pH. Such experiments have found intracellular pH to be 0.03–0.07 higher in deoxygenated cells (Duhm & Gerlach, 1971; Bauer & Schroder, 1972; Takano et al., 1976) at normal extracellular pH. A second approach is to measure the intracellular pH of individual blood samples before and after deoxygenation. By this method, the intracellular pH has been reported to increase by 0.1–0.15 upon deoxygenation (Marshall et al., 1977a,b; Grimes, 1980). These two kinds of experiments are fundamentally different in that the former kind actually measures the effect of deoxygenation on the transmembrane pH gradient. The present experiment, in which intracellular pH changes were measured during progressive deoxygenation of individual red cell samples, is of the second form, and the results are comparable to other studies of a similar nature.

Since intracellular pH was now measurable by the MeP – P_i difference, the deoxyhemoglobin concentration in intact cells could be predicted on the basis of the observed paramagnetic shift (actual minus expected chemical shift) for either compound. However, these paramagnetic shifts must be cautiously applied to cellular systems having hemoglobin concentrations significantly different from normal, since it is the actual concentration of deoxyhemoglobin that produces these shifts and not the fractional oxygen saturation. Nonetheless, the MeP – P_i difference will be independent of these shifts and should still be indicative of pH.

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Registry No. 2,3-DPG, 138-81-8; ATP, 56-65-5; HbF, 9034-63-3; HbA, 9034-51-9; phosphate, 14265-44-2; oxygen, 7782-44-7.

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