HYPOPHOSPHITE ION AS A ^{31}P NUCLEAR MAGNETIC RESONANCE PROBE OF MEMBRANE POTENTIAL IN ERYTHROCYTE SUSPENSIONS

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ABSTRACT Hypophosphorus acid has a single pKa of 1.1 and at physiological pH values it is therefore present almost entirely as the univalent hypophospite ion. When added to a red cell suspension the ion crosses the cell membrane rapidly, via the anion exchange protein, and the intra- and extracellular populations of the ion give rise to separate ^{31}P NMR resonances. From a single ^{31}P NMR spectrum it was possible to determine the relative amounts of hypophosphite in the intra- and extracellular compartments and thereby estimate the corresponding concentrations. The ratio of intracellular to extracellular hypophosphite concentration was independent of the total hypophosphite concentration for cells suspended in NaCl solutions and was independent of hematocrit. The hypophosphite distribution ratio increased as extracellular NaCl was replaced iso-osmotically with citrate or sucrose, though it remained very similar to the corresponding hydrogen ion distribution ratio. Incorporation of the hypophosphite distribution ratio into the Nernst equation yielded an estimate of the membrane potential. For cells suspended in NaCl solutions the estimated potential was consistently around -10 mV.

INTRODUCTION

The estimation of membrane potential in mammalian erythrocytes is not straightforward. The small size and low membrane conductance of the red cell makes the use of microelectrodes impractical and inaccurate (1). Under most conditions the ratio of intracellular to extracellular chloride concentrations provides a measure of the membrane potential (1, 2), however the determination of the chloride concentration ratio requires the physical separation of the cells from the extracellular solution and the subsequent lysis of the cells (2). A number of fluorescent dyes have been utilized to report membrane potential in intact cell suspensions (3–6), but their use is fraught with possible sources of error. The method entails measuring the fluorescence of a low hematocrit cell suspension to which the dye has been added, yet it does not provide a direct estimate of the potential (except under very limited conditions [1, 3, 5]) and requires calibration. Quite apart from the obvious difficulties associated with producing a range of stable, accurately known potentials to enable the calibration, the relationship between the membrane potential and the measured fluorescence varies with intracellular pH (6) and hematocrit (1).

We present here a method in which the univalent hypophosphite ion is utilized as a ^{31}P NMR probe of red cell membrane potential. The method is noninvasive and enables, under most conditions, the calculation of membrane potential from a single ^{31}P NMR spectrum of cells suspended at physiological hematocrits.

MATERIALS AND METHODS

The Hypophosphite Ion

The hypophosphite ion is the conjugate base of hypophosphorus acid which was obtained from Aldrich-Chemie, Steinheim, FRG, as a 50% wt/vol aqueous solution. In all cases hypophosphite was added to cell suspensions as an isotonic solution (~115 mM) prepared by first adjusting the pH of the acid solution to ~7.3 with NaOH (10 M) then diluting the solution with distilled water to give an osmolality of ~300 mOsm kg^{-1}. Hypophosphorus acid has a pKa of 1.1 (7) and the concentration of the protonated species is therefore negligible at physiological pH values.

Isotonic, pH-adjusted solutions of hypophosphite were found (by ^{31}P NMR) to undergo slow oxidation to phosphite over a period of weeks (at ~4°C) and fresh solutions were therefore made up immediately before each experiment. Hypophosphite added to a fresh red cell lysate and incubated at 25°C showed no significant oxidation over a 5-h period.

Erythrocyte Suspensions

Erythrocyte suspensions were prepared from fresh blood obtained from the Red Cross Transfusion Service, New South Wales, Australia. For all NMR experiments the cells were washed five times in isotonic saline (154 mM NaCl). After the initial washes glucose was added (10 mM), then the cells were suspended at low hematocrit (H_t) and bubbled gently with carbon monoxide for 15 min. This served to convert intracellular hemoglobin to the stable diamagnetic form (carbonmonoxyhemoglobin) and thereby ensured maximum resolution of intra- and extracellular reso-

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nances in the ^{31}P NMR spectra (8). After carbonmonoxygenation the cells were packed by centrifugation ($H_t > 0.90$). Subsequent experimental manipulations are described in the appropriate figure legends.

Hematocrits were estimated using a microhematocrit centrifuge. A 2% correction was applied to the measured hematocrit to account for trapped extracellular solution (9). Mean cell volumes were calculated from the corrected hematocrit and cell count which was measured using a counter (model ZF, Coulter Electronics, Dunstable, UK). The fraction of the total cell volume that is accessible to intracellular water was determined gravimetrically as has been described previously (10).

pH was measured using a microcapillary electrode (Radiometer, Copenhagen, Denmark) equipped with a water jacket that maintained the sample temperature at 25°C. Extracellular pH (pH_o) was taken as that measured in whole cell suspensions (2). Intracellular pH (pH_i) was measured on packed cell lysates obtained by centrifuging cell suspensions (~6,000 *g*, 15 s), removing the supernatant solution and the top portion of cells, then repeatedly freezing the cell pellet in liquid nitrogen (−196°C) and thawing in a waterbath at 37°C (2).

Disodium 4,4'-diisothiocyanostilbene-2,2'-disulphonic acid (DIDS) was obtained from Sigma Chemical Co. (St. Louis, MO) and was used to inhibit the band 3 anion exchange protein as has been described previously (11).

NMR Methods

^{31}P NMR spectra of cell suspensions were acquired at 162 MHz using an XL400 spectrometer (Varian Associates, Inc., Palo Alto, CA) operated in the Fourier transform mode. Broadband proton decoupling was used throughout each NMR experiment and the temperature control was set to give 25°C in the sample. The large proton-phosphorus coupling constant in hypophosphite ($J_{H\text{-}P} \simeq 520$ Hz) gave rise to a number of small (residual coupling) resonances symmetrically distributed about the main ^{31}P NMR resonance. The first of these appeared ~0.5 ppm on either side of the main resonance and therefore did not interfere with estimates of the relative intra- and extracellular (main) signal intensities. These resonances were absent from the undecoupled spectrum (in which the hypophosphite signal takes the form of a triplet) and the possibility of their being due to "impurities" may therefore be discounted. Chemical shifts (δ) are quoted relative to that of 85% phosphoric acid.

Samples were spun at 15 Hz throughout the spectral acquisition period to improve signal resolution with minimal centrifugation effect (12). The longitudinal relaxation time (T_1) of the hypophosphite ion was determined using the inversion recovery technique (13) and was found to be ~6 s in isotonic saline. In all NMR experiments each transient consisted of a 90° excitation pulse followed by a delay of at least 30 s ($\geq 5T_1$) to allow complete longitudinal relaxation of magnetization. All spectra other than those giving rise to the time-courses of Fig. 2 were derived from averaging 16 transients. An exponential multiplication factor of 1.0 Hz was routinely applied to the NMR data before Fourier transformation.

Metabolic Experiments

Fresh cells were washed three times in isotonic, Hepes-buffered saline (144 mM NaCl, 7 mM glucose, 20 mM Hepes, pH 7.4). The cells were bubbled gently with carbon monoxide and the hematocrit was adjusted to ~0.5. The suspension was divided into two parts; hypophosphite was added to one so as to give a concentration of 15 mM (with respect to the total sample volume) and an equivalent volume of the washing solution was added to the other. Both suspensions were incubated at 25°C in a shaking waterbath for a 5-h period. At 30-min intervals, aliquots (2 ml) were withdrawn from each suspension and added to an ice-cold perchloric acid solution (2 ml, 10% vol/vol). The perchloric acid extracts were treated as described by Beutler (14) and used for the estimation of glucose, lactate, DPG, and ATP. The estimations were made using standard methods (14) adapted for use with a centrifugal analyzer (Cobas Fara, Roche, Basel, Switzerland).

RESULTS

Estimation of Intra- and Extracellular $H_2PO_2^-$ Concentrations

Fig. 1 depicts the proton-decoupled ^{31}P NMR signal of $H_2PO_2^-$ in the presence of whole cells. As has been shown previously for the fully coupled spectrum (15) the intra- and extracellular resonances are clearly resolved from one another. Addition of a Mn^{2+}-albumin complex (100 μM) to the extracellular solution eliminated the high frequency resonance, which was therefore identified as that corresponding to the extracellular hypophosphite population. The broader lower frequency resonance remained largely unaffected by the presence of extracellular Mn^{2+} and therefore corresponds to the intracellular hypophosphite population.

As is evident from Fig. 1 the intra- and extracellular signals are sufficiently far apart from one another to allow the estimation of their areas (I_i and I_o, respectively) using a standard peak integration procedure (Varian software, version 6.2). The relative areas of the two peaks (I_i/I_o) corresponds to the relative sizes of the intra- and extracellular hypophosphite populations. The ratio of intracellular volume to extracellular volume (V_i/V_o) in a cell suspension is given by

$$V_i/V_o = \alpha H_t/(1 - H_t), \quad (1)$$

where α is the fraction of the total cellular volume accessible to intracellular solute. The concentration distribution

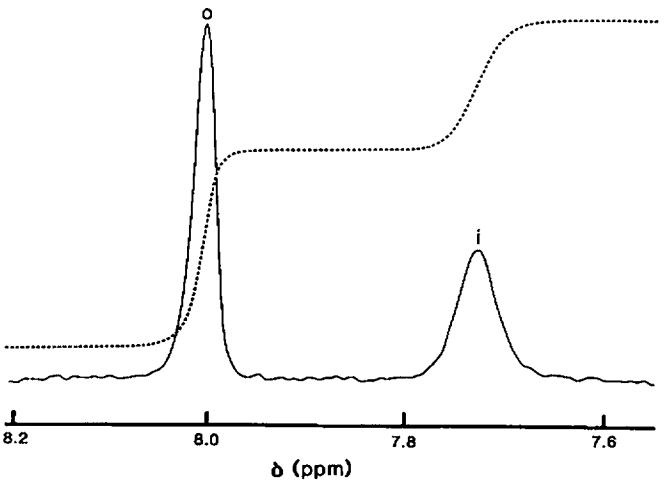

FIGURE 1 Proton-decoupled ^{31}P NMR spectrum of $H_2PO_2^-$ added to red cells suspended in isotonic saline. Fresh cells were prepared as described in Materials and Methods. $H_2PO_2^-$ was added to a concentration of 15 mM (with respect to the total sample volume). The final hematocrit of the suspension was 0.56. The high-frequency resonance (*o*) corresponds to the extracellular $H_2PO_2^-$ population; the broader low-frequency resonance (*i*) corresponds to the intracellular $H_2PO_2^-$ population. The dashed line is the integral of the area under the two resonances from which the relative sizes of the intra- and extracellular populations were evaluated.

ratio for the hypophosphite ion ($r(H_2PO_2^-) = [H_2PO_2^-]_i/[H_2PO_2^-]_o$) is therefore given by the expression

$$r(H_2PO_2^-) = (I_i/I_o)((1 - H_t)/\alpha H_t). \quad (2)$$

If it is assumed that all of the intracellular water acts as solvent for the intracellular hypophosphite ion population, then α is equivalent to the gravimetrically determined fractional water volume of the cell (16). For cells (from different donors) suspended in isotonic saline, the gravimetric method consistently yielded a value of 0.717 ± 0.005, which is in close agreement with that obtained in previous studies for cells at normal volume (10, 16). This value was therefore used in the calculation of $r(H_2PO_2^-)$ for all experiments in which cells were suspended in isotonic NaCl solution.

For cells from a single donor, suspended in isotonic saline, $r(H_2PO_2^-)$ was independent of the hypophosphite concentration over the range 10–50 mM ($r[H_2PO_2^-]$ = 0.69).

$H_2PO_2^-$ Transport

Fig. 2 shows the results of time-courses in which the transport of $H_2PO_2^-$ into intact cells was monitored using ^{31}P NMR spectroscopy. In the absence of a band 3 inhibitor the hypophosphite ion equilibrated across the cell membrane within the time necessary for the acquisition of the first spectrum (~2 min). In cells pretreated with DIDS the influx was very much slower.

Hematocrit Dependence of $H_2PO_2^-$ Distribution

Simple rearrangement of Eq. 2 yields an expression for I_i/I_o as a function of α, H_t, and $r(H_2PO_2^-)$. Fig. 3 shows the results of an experiment in which cells from a single donor were suspended in isotonic saline over a range of hematocrits. The rearranged form of Eq. 2 was fitted to the data to yield an estimate of $r(H_2PO_2^-)$ of 0.70 ± 0.01. The close fit infers that $r(H_2PO_2^-)$ is independent of hematocrit.

Variation of $H_2PO_2^-$ and H^+ Distribution with the Composition of the Extracellular Solution

Although $r(H_2PO_2^-)$ was independent of both hypophosphite concentration and hematocrit over the specified ranges it was found to vary with the composition of the extracellular solution. Fig. 4 shows the variation of the ^{31}P NMR spectrum of hypophosphite added to cells from a single donor as extracellular NaCl was replaced isoosmotically with Na_3 citrate. The mean cell volume was found to decrease with increasing external citrate concentration. The fractional water volume of the cells (α) was estimated for each sample and the hematocrits were

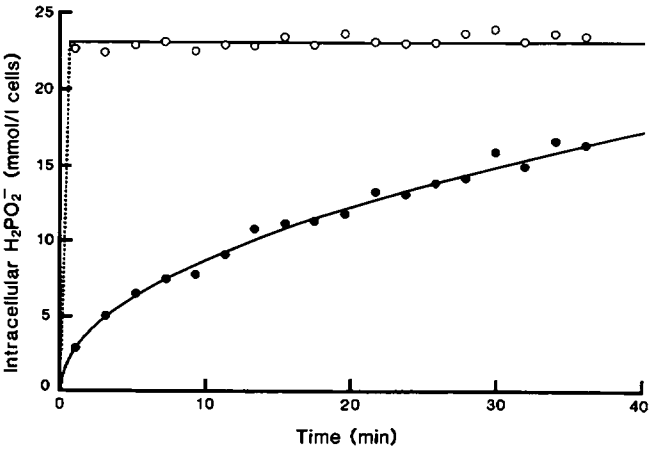

FIGURE 2 Influx of $H_2PO_2^-$ into intact erythrocytes pretreated (●) and untreated (○) with DIDS. At time = 0 min, isotonic hypophosphite solution was added to cells suspended in phosphate-buffered sucrose to yield a final hematocrit of 0.64 and a hypophosphite concentration of 30 mM (with respect to the total sample volume). Sequential ^{31}P NMR spectra were acquired (four transients, 30 s recycle time) and from each spectrum the intracellular hypophosphite concentration was calculated from the intracellular ^{31}P NMR resonance intensity. Each time point corresponds to the midpoint of the spectral acquisition period.

adjusted (before spectral acquisition) so that the ratio of intracellular volume to extracellular volume (V_i/V_o) was approximately the same in each case. Such an adjustment is not necessary for the analysis but enables a direct comparison to be made between the intra- and extracellular signal intensities in the four samples. The increasing external citrate concentration is seen to cause a depolarization of the membrance as indicated by the decreasing

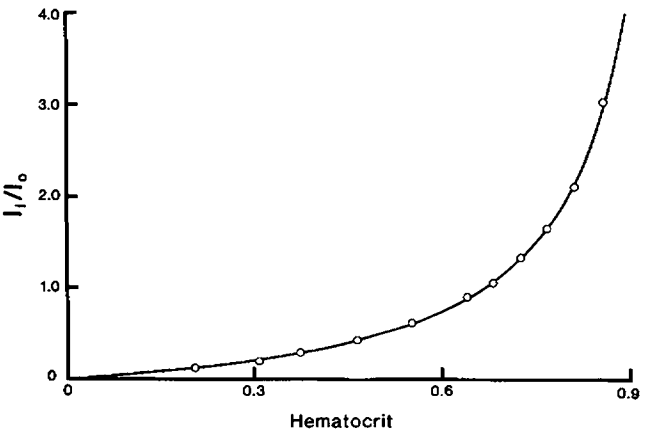

FIGURE 3 Variation of the ratio of the intensity of intra- and extracellular hypophosphite (I_i/I_o) with hematocrit. Hypophosphite (15 mM with respect to the total volume) was added to cells suspended in isotonic saline. The cells and extracellular solution were separated from one another by centrifugation then recombined in varying proportions to give a range of hematocrits. The ratio I_i/I_o was estimated from the relative intensities of the intra- and extracellular $H_2PO_2^-$ ^{31}P NMR resonances. The curve was drawn using Eq. 2 (appropriately rearranged) with α = 0.717 and $r(H_2PO_2^-)$ = 0.70.

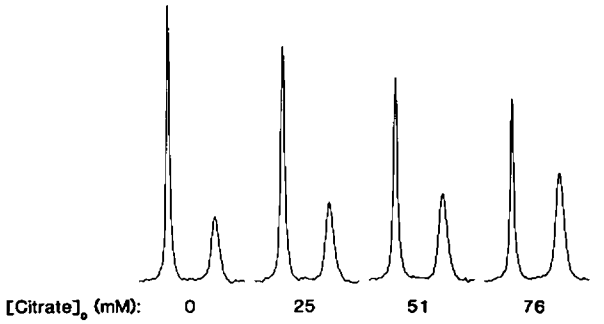

FIGURE 4. Variation of the ^{31}P NMR signal of $H_2PO_2^-$ added to cells suspended in solutions of increasing citrate concentration. Blood from a single donor was divided into two portions; one was washed (5×) in isotonic NaCl solution (154 mM), the other (5×) in isotonic Na_3 citrate solution (105 mM). After washing the two suspensions were blended together (in the proportions 1:0, 2:1, 1:2, and 0:1) so as to give four suspensions having extracellular citrate concentrations ranging from 0 to 105 mM. Hypophosphite was added to each suspension to a concentration of 15 mM (with respect to the total sample volume), thereby reducing the maximum extracellular citrate concentration to 76 mM. The increase in the final extracellular citrate concentration (from 0 to 76 mM) was observed to cause a decrease in the mean cell volume (from 85 to 74 fl) and a consequent decrease in α (from 0.714 to 0.675). The hematocrit of each sample was therefore adjusted before spectral acquisition so as to ensure that the ratio of intracellular to extracellular volume was approximately the same in each sample ($V_i/V_o \simeq 1.32$). Such an adjustment is not necessary for the analysis but enables a direct comparison to be made between the intra- and extracellular signal intensities in the four spectra.

extracellular signal intensity and the increasing intracellular signal intensity.

The distribution of hydrogen ions between the intra- and extracellular compartments is readily determined from the difference between the measured intra- and extracellular pH (2),

$$r(H^+) = 10^{(pH_i - pH_o)}. \quad (3)$$

It should be noted though that the pH is an expression of hydrogen ion activity and that $r(H^+)$ is therefore an activity ratio rather than a concentration ratio. The two are equivalent only if there is a negligible difference between the intra- and extracellular hydrogen ion activity coefficients (2).

Table I shows the effects of increasing extracellular citrate concentration on both $r(H^+)$ and $r(H_2PO_2^-)$. Very similar results were obtained when extracellular NaCl was replaced iso-osmotically with sucrose (data not shown).

Effects of $H_2PO_2^-$ on Metabolism

The addition of sodium hypophosphite (15 mM) to a cell suspension had no significant effect on the time dependence of glucose, lactate, ATP, or DPG concentrations at 25°C. In a representative experiment glucose was consumed at a rate of 0.26 ± 0.05 mmol (1 cells)$^{-1}$h^{-1} in the presence of $H_2PO_2^-$ and at a rate of 0.30 ± 0.04 mmol (1 cells)$^{-1}$h^{-1} in its absence. Lactate was produced at a rate of 0.64 ± 0.06 mmol (1 cells)$^{-1}$h^{-1} in the presence of $H_2PO_2^-$

TABLE I
COMPARISON OF $r(H^+)$ AND $r(H_2PO_2^-)$ FOR CELLS SUSPENDED IN SOLUTIONS OF INCREASING CITRATE CONCENTRATION ([Citrate]$_o$)

[Citrate]$_o$	pH_i	pH_o	$r(H^+)$*	$r(H_2PO_2^-)$‡
mM				
0	6.97 ± 0.02	7.11 ± 0.02	0.72 ± 0.02	0.65 ± 0.02
25	7.13 ± 0.02	7.19 ± 0.02	0.87 ± 0.02	0.79 ± 0.02
51	7.24 ± 0.02	7.22 ± 0.02	1.05 ± 0.02	1.00 ± 0.03
76	7.41 ± 0.02	7.21 ± 0.02	1.55 ± 0.05	1.59 ± 0.06

Samples were prepared as described in the legend to Fig. 4. pH measurements were made on each sample (as described in Materials and Methods) immediately after spectral acquisition. The values in the table represent the mean (±1 SD) of estimates from five samples for each of the extracellular citrate concentrations. *$r(H^+) = 10^{(pH_i - pH_o)}$. ‡$r(H_2PO_2^-) = (I_i/I_o)([1 - H_t]/\alpha H_t)$.

and at a rate of 0.61 ± 0.02 mmol (1 cells)$^{-1}$h^{-1} in its absence. In both suspensions ATP concentrations remained constant within experimental error throughout the 5-h duration of the experiment. DPG levels declined from 4.4 mmol (1 cells)$^{-1}$ at a rate of 0.26 ± 0.01 mmol (1 cells)$^{-1}$h^{-1} in the presence of $H_2PO_2^-$ and at a rate of 0.28 ± 0.01 mmol (1 cells)$^{-1}$h^{-1} in its absence.

DISCUSSION

The membrane potential (V_m) in red cells is determined by the activities and membrane permeabilities of the ions present in the suspension. If it is assumed that the intra- and extracellular activity coefficients are equivalent to one another and that the contribution from ions other than Na^+, K^+, and Cl^- may be ignored then the membrane potential is specified by the expression (17–19)

$$V_m = (RT/F) \ln \frac{P_K [K^+]_o + P_{Na} [Na^+]_o + P_{Cl} [Cl^-]_i}{P_K [K^+]_i + P_{Na} [Na^+]_i + P_{Cl} [Cl^-]_o}, \quad (4)$$

where R is the gas constant, T is the absolute temperature, F is the Faraday constant and P_K, P_{Na}, and P_{Cl} are the membrane permeability coefficients for K^+, Na^+, and Cl^-, respectively. In human erythrocytes P_{Cl} is normally much larger than either P_K or P_{Na} and Eq. 3 may therefore be simplified to (19)

$$V_m \simeq E_{Cl} = (RT/F) \ln r(Cl^-). \quad (5)$$

Eqs. 4 and 5 are applicable irrespective of whether or not the cells are in a steady state with respect to ion fluxes.

In red cells permeant anions are passively distributed across the membrane. Consequently, for cells in a steady state the transmembrane chloride distribution must be at equilibrium with the membrane potential. Under these conditions Eq. 4 becomes exact ($V_m = E_{Cl}$) and reverts to the Nernst equation (19).

It is evident from Fig. 2 that the hypophosphite ion permeates the red cell membrane primarily via the band 3 anion transport protein. The significant (though greatly

reduced) flux of $H_2PO_2^-$ into cells pretreated with DIDS is presumably a consequence of either the incomplete inhibition of the anion transporter or the existence of another pathway by which the ion is able to permeate the membrane. The principle function of the anion transport protein is to mediate the electrically neutral exchange of intra- and extracellular anions across the cell membrane, so it is likely that the hypophosphite ion gains rapid entry into the red cell by simply exchanging with intracellular anions (predominantly Cl^-). This being the case, hypophosphite ions added (as isotonic solution) to a cell suspension will assume (within minutes [15]) a transmembrane concentration distribution ratio, $r(H_2PO_2^-)$, equivalent to that of chloride, $r(Cl^-)$.

Thus, for cells in a steady state,

$$V_m = (RT/F) \ln r(H_2PO_2^-). \quad (6)$$

For cells not in a steady state Eq. 6 remains approximately true, providing that P_{Cl} is much larger than P_{Na} and P_K (and that $r[H_2PO_2^-] \simeq r[Cl^-]$).

For cells (from different donors) suspended in isotonic NaCl (as for Fig. 1) Eq. 6 consistently yielded a membrane potential estimate of around -10 mV ($r[H_2PO_2^-] = 0.70$). This value is in close agreement with that obtained by Rink and Hladky (5) who used a fluorescent dye (in conjunction with valinomycin and a range of extracellular K^+ concentrations) to estimate a resting potential of -9.3 mV for red cells suspended in an isotonic chloride medium.

When extracellular NaCl was replaced iso-osmotically with Na_3 citrate (Fig. 4) the membrane potential changed progressively from -11 mV ($r[H_2PO_2^-] = 0.65$) in the absence of citrate to $+12$ mV ($r[H_2PO_2^-] = 1.59$) at an extracellular citrate concentration of ~76 mM. The variation of the membrane potential with the composition of the suspending solution is readily understood in terms of Eqs. 4 and 5. The replacement of extracellular NaCl with either citrate or sucrose (both of which are largely impermeant to the membrane [2]) causes a reduction in $[Cl]_o$ which results in an increase in V_m.

It has long been believed that hydrogen ions are passively distributed across the red cell membrane in a Donnan equilibrium and that the hydrogen ion activity ratio ($r[H^+]$) is therefore directly related to the membrane potential by the Nernst equation (2, 20, 21). Table I shows a close similarity between $r(H^+)$ and $r(H_2PO_2^-)$ for cells suspended in solutions of varying composition. The results of Table I are similar to those of Funder and Wieth (2) who found that for cells in plasma to which varying concentrations of citrate had been added, $r(H^+)$ and $r(Cl^-)$ agreed to within 0.08 of one another, in spite of an inversion of the membrane potential.

The similarity between $r(H^+)$ and $r(H_2PO_2^-)$ raises the possibility of using $H_2PO_2^-$ distribution (in conjunction with a direct measure of the extracellular pH) as an alternative means of estimating the intracellular pH using ^{31}P NMR spectroscopy. Replacement of $r(H^+)$ with $r(H_2PO_2^-)$ in Eq. 3 leads to the following expression for the intracellular pH.

$$pH_i = pH_o + \log r(H_2PO_2^-). \quad (7)$$

Substituting the pH_o and $r(H_2PO_2^-)$ values of Table I into Eq. 7 yields intracellular pH estimates of 6.92, 7.09, 7.22, and 7.41 for the four samples (in order of increasing extracellular citrate concentration). The corresponding pH values measured by electrode in the cell lysates were 6.97, 7.13, 7.24, and 7.41 (Table I). Thus, the intracellular pH estimates calculated from Eq. 7 are within 0.05 pH units of those measured.

The established ^{31}P NMR method for the determination of intracellular pH entails measuring the chemical shift of the ^{31}P NMR signal arising from an intracellular compound that has a pK_a (and therefore titrates) in the physiological pH range. The methylphosphonate ion ($pK_a \simeq 7.6$) is currently favoured as a ^{31}P NMR probe of intracellular pH (11, 12, 22–24), and in the most recent study in which it was applied to red cell suspensions (23) the authors demonstrated that the intracellular pH estimate derived from the chemical shift of intracellular methylphosphonate differed from that obtained from the freeze-thaw pH electrode procedure by 0.053 pH units. It would therefore seem that the ^{31}P NMR estimate of the transmembrane hypophosphite ion distribution, in conjunction with a standard electrode measurement of the extracellular pH, provides as accurate an estimate of the intracellular pH as the established methylphosphonate procedure.

For an ion to be a suitable probe of cell membrane potential it should fulfill a number of criteria (25, 26). (*a*) It should equilibriate rapidly (and passively) across the cell membrane; (*b*) there should be available a ready means of measuring its transmembrane distribution; and (*c*) it should have negligible effect on the existing potential and on any cellular processes that contribute (either in the long or short term) to the membrane potential. The hypophosphite ion equilibrated across the membrane within 1–2 min via the anion exchange protein, and the observation of separate intra- and extracellular ^{31}P NMR resonances enabled the estimation of its transmembrane distribution from a single ^{31}P NMR spectrum. For cells suspended in NaCl solutions $r(H_2PO_2^-)$ was independent of hypophosphite concentration over the range 10–50 mM. Furthermore, $H_2PO_2^-$, at a concentration of 15 mM, had no significant effect on the rate of glycolysis (as measured by glucose consumption and lactate production), the rate of DPG decline, or the maintenance of ATP levels in fresh, metabolically active cells at 25°C. In the human erythrocyte, glycolysis is the sole source of ATP, and a significant proportion of the ATP produced is utilized by the membrane cation pumps (27). The observation that glycolysis proceeded at the same rate in the presence and absence of

$H_2PO_2^-$ and that ATP levels remained constant in both cases suggests that the hypophosphite ion has no significant effect on cation active transport. It should be noted though that the absence of any such effect is not of critical importance in red cells as under normal conditions the electrogenic contribution of the Na:K pump to the net membrane potential is negligible (28).

Although the hypophosphite ion seemingly meets the requirements for a suitable probe of transmembrane potential the method presented here has a number of limitations. The relative insensitivity of ^{31}P NMR spectroscopy as an analytical technique requires that $H_2PO_2^-$ concentrations in the millimolar range must be employed if an accurate estimate of $r(H_2PO_2^-)$ (and thereby V_m) is to be obtained within a reasonable time. Although $r(H_2PO_2^-)$ is independent of $H_2PO_2^-$ concentration for cells in chloride media the same will not be true of cells suspended in solutions of impermeant solutes (such as citrate or sucrose). The isotonic addition of a permeant anion to such suspensions will inevitably cause a decrease in $r(Cl^-)$ and, therefore, a decrease in V_m (Eq. 5). This criticism applies equally to the use of titratable phosphorus compounds as ^{31}P NMR probes of intracellular pH (11, 12, 22–24, 29).

A second limitation of the method is that for cells not in a steady state, $r(H_2PO_2^-)$ provides a reasonable estimate of V_m (via Eq. 6) only if $r(H_2PO_2^-) = r(Cl^-)$ and P_{Cl} is much larger than P_{Na} and P_K. Inhibition of the anion exchange protein (which mediates the net flux of chloride ions across the membrane in addition to their electrically neutral exchange [30]) prevents the equalization of the $H_2PO_2^-$ and Cl^- distribution ratios and furthermore reduces P_{Cl} so that the membrane potential is no longer approximated by the chloride potential (i.e., $V_m \neq E_{Cl}$). The same is true of cells to which ionophores such as valinomycin (5) or gramicidin (31) have been added. Such compounds increase the membrane permeability to cations (P_K and P_{Na}) and thus increase the relative contribution of the transmembrane cation distribution to V_m (Eq. 4).

Limitations arising from the hyphosphite ion traversing the cell membrane via a mediated (one for one) anion exchange process would not apply if the probe ion were able to diffuse through the lipid bilayer and distribute across the membrane in accordance with the Nernst equation (irrespective of the value of $r(Cl^-)$ and its relative contribution to the total potential). Current research is directed at understanding the physical basis for the separation of the intra- and extracellular ^{31}P NMR resonances of the hypophosphite ion. A similar (though smaller) effect has been noted for a number of other phosphorus oxyacids (15) as well as for the nonelectrolyte dimethyl methylphosphonate (32). The effect is due, in part, to the difference in the magnetic susceptibilities of the intra- and extracellular compartments that is primarily a consequence of the high intracellular hemoglobin concentration (33). Susceptibility effects alone do not, however, fully account for the observed transmembrane chemical shift differences. In the case of dimethyl methylphosphonate the additional transmembrane chemical shift difference has been attributed to the effect of intracellular hemoglobin on the formation of hydrogen bonds between solvent water and the phosphoryl oxygen of the phosphonate (34). A similar phenomenon most probably accounts for the hypophosphite chemical shift difference.

An understanding of the phenomenon should enable the rational selection of alternative ions that show the same separation of intra- and extracellular NMR resonances (thereby allowing the determination of intra- and extracellular concentrations from a single NMR spectrum) but which traverse the membrane rapidly by simple diffusion through the bilayer.

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