Evaluation of an Electrochemical Model of Erythrocyte pH Buffering Using $^{31}$P Nuclear Magnetic Resonance Data

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ABSTRACT When erythrocytes are suspended in a solution of known composition the resultant values of such basic cell parameters as volume and pH are difficult to predict. To facilitate such predictions, we developed a mathematical model describing the passive transmembrane distribution of permeant species; three simultaneous equations were produced. Certain essential data required for the model were determined experimentally; these included the pH dependence of the charge on the hemoglobin molecule and the variation of the osmotic coefficient of hemoglobin with cell volume. Finally, cells were added to various solutions, and then titrated to produce a wide pH range (pH 6–8). We measured the resultant cell volume, cellular and extracellular pH using both conventional and $^{31}$P NMR methods. The expected equilibrium values of these electrochemical parameters were also calculated by solving (numerically) the three model equations. The accuracy of the model simulations was evaluated by direct comparison of calculated and experimentally determined values.

INTRODUCTION

Gas exchange in erythrocytes is modulated by cellular pH variation while the buffering function of erythrocytes is essential for general homeostasis. Although erythrocyte pH is not actively regulated, pH control is complicated by the multiplicity of buffering species within the erythrocyte and the plasma, the interconnection of these two compartments via the Jacobs-Stewart cycle, and the equilibration of the blood with the contiguous gas phase (Hladky and Rink, 1977). Obviously, a quantitative description of each of the effectors of cellular pH, and their interactions, is necessary for an understanding of such a complex system. For this purpose equilibrium models, based on the maintenance of electroneutrality in both compartments, and on the inability of the red cell membrane to sustain a significant hydrostatic pressure, have been developed (Freedman and Hoffman, 1979; Rodeau and Malan, 1979; Wolf, 1982; Raftos et al., 1986). Such osmotic models oversimplify the biological system they describe and are relevant only when the complete equilibration...
of permeant solutes and water across the erythrocyte membrane is approximated. Nevertheless, these models are not only valuable tools for the understanding, and even the prediction, of the complex interaction between cells and their suspending solutions, they are also essential constituents of more elaborate modeling systems that integrate several aspects of erythrocyte function and can potentially provide estimates of transient changes in an erythrocyte suspension (Werner and Heinrich, 1985; Lew and Bookchin, 1986). If either type of model is to be used for quantitative simulation of induced changes in erythrocyte parameters, the validity of their basic assumptions and the accuracy of the input data must be ensured.

One inadequacy of most previous osmotic models (Brumen et al., 1979; Freedman and Hoffman, 1979; Rodeau and Malan, 1979; Wolf, 1982; Werner and Heinrich, 1985; Lew and Bookchin, 1986; Raftos et al., 1986) is that the term describing the osmotic coefficient of hemoglobin is dependent solely on its concentration; the contribution of ionic strength and other features of the intracellular environment that are thought to influence its osmotic contribution (Hladky and Rink, 1978; Solomon et al., 1986) are generally neglected.

As hemoglobin is the dominant noncarbonate buffering species in the blood, any mathematical description of the pH buffering function of the erythrocyte requires an accurate titration curve for this protein. Although the buffering characteristics of hemoglobin have been studied extensively, both theoretically (Mathew et al., 1979) and by titration of purified hemoglobin (Antonini et al., 1965; Rollema et al., 1975), few data obtained at 37°C are available. Due to extensive variation in the molecular environment of the titrating histidine residues (Mathew et al., 1979), the effect of temperature on their apparent dissociation constants, and so on the hemoglobin titration curve, is difficult to predict. A second important source of error in model simulations then is uncertainty in the calculation of the pH-dependent charge on hemoglobin. This uncertainty is reflected in the variation of such charge terms amongst published models (Freedman and Hoffman, 1979; Rodeau and Malan, 1979; Wolf, 1982; Raftos et al., 1986).

To overcome these perceived deficiencies of previous osmotic models, we have measured the effect of cell volume change on the total osmolality of whole cells and also the dependence of carboxyhemoglobin charge on pH, in a cell lysate, at 37°C. These empirical data were incorporated during the derivation of three simultaneous equations that comprised our electrochemical model. Solving these nonlinear equations, using a contemporary iterative technique, returned the equilibrium values for cell volume, cellular pH (pHi), and extracellular pH (pHe) for whole blood as well as for erythrocytes suspended in media of various compositions, buffering capacity, and pH.

Despite the considerable areas of uncertainty still associated with osmotic models, they have been used recently to predict the pH and associated volume changes in physiological, cell storage, and experimental situations (Rodeau and Malan, 1979; Wolf, 1982; Werner and Heinrich, 1985; Lew and Bookchin, 1986). However, quantitative validation of the predictions of these models is still extremely limited. When pH buffering is considered, direct comparison of experimental and calculated results is available for whole blood only, over a restricted pH range (or even for a single pH value) and then by comparison of relative (i.e., pHi compared with
pH changes rather than the absolute pH change on titration (Siggaard-Andersen, 1974; Rodeau and Malan, 1979). Therefore we thought it essential to pursue a rigorous and extensive comparison of expected pH and cell volume results, calculated using model equations, with experimental data obtained by titrating cells suspended in various artificial media, with strong acid or base. Measurements were made using both standard and $^{31}$P nuclear magnetic resonance (NMR) techniques, with the latter allowing simultaneous determination of pH, pHe, and cell volume in a single, undisrupted sample of the cell suspension. Such an evaluation of the errors associated with the model predictions was considered a prerequisite for the future inclusion of this electrochemical model into a more extensive and flexible mathematical system including the terms for the transport, metabolic, and oxygen dissociation functions of the erythrocyte.

**METHODS**

**Cell Preparation**

The erythrocytes were obtained from the New South Wales Red Cross Blood Transfusion Service, Sydney and were stored for less than 24 h at 4°C. All the erythrocytes used were washed three times in five volumes of isotonic saline (154 mM NaCl) at 4°C and then gassed for 20 min with carbon monoxide.

**Titration Experiments**

After washing the suspension, its pH was measured at 37°C using a capillary electrode in a temperature-controlled microelectrode unit (Radiometer, Copenhagen, Denmark) before the cells were concentrated to a hematocrit (Hct) of 0.80. The erythrocytes were then diluted with one of three test solutions. All the solutions contained 47 mM NaCl buffered with 8.5 mM NaH$_2$PO$_4$/Na$_2$HPO$_4$ and 8.5 mM sodium methylphosphonate (MeP), and either extra NaCl (86 mM), sucrose (150 mM), or sodium citrate (NaCit) (58 mM) to give a final pH (37°C) of 7.40, and an osmolality of 278 mosmol/kg H$_2$O measured with a vapor pressure osmometer (Wescor, Logan, UT).

To produce a range of pH values a standard volume of acid (290 mM HCl) or base (290 mM carbonate-free NaOH) or a mixture of both was added to the cell suspension at a rate of only 7 μl/ml per min (Recording Titration System, Radiometer), to avoid pH overshoot and possible cell damage. The suspensions were then incubated for 45 min at 37°C before measurement of the Hct (with a microhematocrit centrifuge) and the extracellular pH. Simultaneously, samples were frozen in liquid nitrogen for the subsequent determination of lactate (Lac) (Stat-Pack Rapid Lactate Test, Behring Diagnostics, La Jolla, CA), 2,3-bisphosphoglycerate (DPG) using 2,3-Diphosphoglyceric Acid Test Kits (Sigma Chemical Co., St Louis, MO) and ATP according to the method of Beutler (1984). These assays were all adapted for use on a Cobas Fara centrifugal analyzer (Roche Diagnostica, Basel, Switzerland). A further sample was immediately centrifuged (12,000 rpm, 30 s) and all the supernatant was removed before the cells were frozen in liquid nitrogen. To ensure complete lysis of these cells, they were thawed and then refrozen before the lysate was warmed to 37°C for pH measurement. Total hemoglobin (Hb) was measured spectrophotometrically as hemoglobin cyanide (ICSH, 1987) after an additional incubation step (3 min, 56°C) to ensure total conversion of carboxyhemoglobin (Rice, 1967). The cells were counted electronically (Coulter Counter, Coulter Electronics Ltd., Luton, UK).
Nuclear Magnetic Resonance Spectroscopy

For pH and cell volume measurement by $^{31}$P NMR spectroscopy an XL/VXR 400 instrument (Varian, Palo Alto, CA) was used, operating at 162 MHz in the Fourier transform mode with broad-band proton decoupling. Dimethyl methylphosphonate, the probe molecule for volume measurement (Kirk and Kuchel, 1985), was added to the samples to a final concentration of 10 mM. Each spectrum was the average of 64 transients acquired at a rate of one per second, and the sample temperature was 37°C.

The mean cell volume was determined from the separation between the resonances arising from the $^{31}$P nuclei of the dimethyl methylphosphonate molecules located inside the cells and those in the external solution (Kirk and Kuchel, 1985 and Fig. 1). Calibration experiments were performed as previously (Raftos et al., 1988) to determine the value of $m$, the calibration parameter in each of the three suspending solutions. Experimental values for the mean cell volume (MCV, in femtoliters) were then calculated from the chemical shift difference between the two dimethyl methylphosphonate peaks ($\delta$DMMP, in Hertz) measured in each sample after pH titration. The expression used was (Raftos et al., 1988):

$$\text{MCV} = \frac{\text{MCH}}{[\text{MCHC}_o - (\delta\text{DMMP}_i - \delta\text{DMMP})/m]}$$

where $\text{MCH}$ (the mean cell hemoglobin content in picograms), $\text{MCHC}_o$ (the mean cell hemoglobin concentration in grams per liter cell), and $\delta$DMMP, values had all been measured (at one MCV only) for a sample of cells from the particular blood donation used in the experi-

![Figure 1. $^{31}$P NMR spectra of red cell suspensions acquired for pH and cell volume determination. The resonances were assigned as follows: a, dimethyl methylphosphonate; b, MeP; c, Pi. The subscripts indicate whether the resonance was due to solute located inside (i) or outside (o) the cell. The chemical shift differences, $a_i - a_o$, $b_i - b_o$, and $c_i - c_o$, were used as a measure of the cell volume, $\text{pH}_i$, and $\text{pH}_o$, respectively. The cells were suspended in phosphate-buffered saline with sucrose and the pH was adjusted with 290 mM NaOH or HCl. The cell suspension used for spectrum A had a mean cell volume of 78.1 fl, pH$_i$ was 7.06, and pH$_o$ was 7.11 while for B the respective values were 94.8 fl, 6.16, and 5.88.](image)
For Hct determinations,

\[ \text{Hct} = \frac{\text{Hb}}{[\text{MCHC} - (\delta \text{DMMP}_1 - \delta \text{DMMP})/m]} \quad (2) \]

where Hb is the suspension hemoglobin concentration (in grams per liter), which was constant in all samples.

The intracellular pH was determined from the frequency separation of the resonances due to inorganic phosphate (Pi) and MeP molecules located inside the cells, while the extracellular pH was determined from the separation of the resonances arising from the extracellular molecules of the same species (Fig. 1). When required, 5 μl of MnCl₂ (15 mM) in an albumin solution (20% wt/vol) was added to the 2-ml samples, to suppress totally the extracellular Pi and MeP peaks. This allowed positive identification of the intracellular peaks, and the determination of their chemical shift position, when their separation from the external peaks was small. Standard parameters for the calculation of cellular and extracellular pH were obtained by the titration of concentrated lysates and the experimental buffers, respectively (Stewart et al., 1986).

**Charge on the Hemoglobin Molecule**

Saline-washed cells were concentrated to a Hct of 0.80 and lysed by sonication. The hemolysate was titrated with 290 mM HCl or NaOH to produce samples with a pH range of 5.9–7.9, while representative samples were removed for determination of hemoglobin, lactate, DPG, ATP, and Pi (Phosphorus, Inorganic, Sigma Chemical Co.) concentrations. The charge on the hemoglobin molecule at each measured pH value was then calculated using the rearranged electroneutrality equation,

\[
z_{\text{HB}} = (z_{\text{Pi}}[\text{DPG}] + z_{\text{ATP}}[\text{ATP}] + z_{\text{GSH}}[\text{GSH}] + z_{\text{Pi}}[\text{Pi}]
+ [\delta] + 2[\text{de}] - [\text{Cl}^-] - [\text{Lac}] - [\text{HCO}_3^-] - 2[\text{CO}_3^{2-}])/[\text{Hb}] \quad (3)\]

where the square brackets denote concentration (in millimoles per liter suspension) and z, the proton charge per molecule. Solute symbols are defined in Table I. Solute concentrations that were not assayed as described above were taken to be normal cellular levels (Table I), while the additional contributions of Na and Cl from the saline washing solution, HCl, and NaOH, were included. The charge terms (z) and the charge contribution by total carbonate were evaluated as indicated in Table II.

**The Osmotic Coefficient of Hemoglobin**

The Hct of washed cells was adjusted to 0.80 and the supernatant was retained. The total water content of this initial suspension \((V_{\text{tot}}\text{, liter } \text{H}_2\text{O/liter suspension})\) was determined by complete dehydration of the sample at 95°C (Cook, 1967). A volume of the wash-supernatant equal to the total water content of 4 ml of the suspension was then added to 1.0 ml of one of 12 NaCl solutions, which varied in concentration from 6 to 600 mM and in water content \((V_{\text{NaCl}})\) from 0.998 to 0.988 liter H₂O/liter solution. The measured osmolality of the resultant solution was denoted by Eosmol, the "expected" osmolality. 4 ml of the cell suspension was added to a separate 1.0-ml sample of the anisotonic saline and at this stage the water content of the suspension \((V_{\text{tot}}\text{, suspension})\) was:

\[ V_{\text{tot}} = (4 \cdot V_{\text{tot}} + V_{\text{NaCl}})/5 \quad (4) \]

After 10 min, this suspension was centrifuged, the supernatant removed and its osmolality measured \((\text{Sosmol})\). If it is assumed that the difference between the osmolality measured in the cell-free sample \((\text{Eosmol})\) and in the supernatant \((\text{Sosmol})\) from the cell suspension, is due
### TABLE I

The Whole-Blood Solute Concentrations Used in the Model Calculations

| Solute (symbol) | Concentration (normal range) | Ref. |
|-----------------|------------------------------|------|
| Hemoglobin (Hb) | 5.13 (4.8-5.4) | 1    |
| Albumin (Alb)   | 0.70 (0.54-0.74) | 2    |
| DPG             | 5.10 (4.2-7.9) | 3    |
| ATP             | 1.80 (1.0-2.5) | 3    |
| GSH             | 2.23 (2.1-2.6) | 2    |
| Na and K ions (b) | 103 (95-109) | 2    |
| Uncharged solutes (us) | 1.08 | 2    |
|椅 | 49.9 (48-55) | 2    |
| Lactate ions (Lac) | 0.62 | 2    |
| HCO₃⁻ | 11.6 | 2    |
| Pi | 0.02 | 2    |
| Uncharged permeant solutes | 9.03 | 2    |

The normal ranges were obtained from the cited references: 1, Dacie and Lewis (1975); 2, Henry et al. (1974); 3, Grimes (1980). Solutes not included above but present in media used in the titration experiments were MeP, citrate (cit), and the sodium added as counter ions with citrate (ncit). When present, sucrose was included as an uncharged, impermeant solute (us) in the extracellular compartment.

The standard conditions prevailing were Hct, 0.445; cell water content, 0.717; plasma water content, 0.395; pH₁, 7.20; pHᵥ, 7.40; temperature, 37°C; pCO₂, 40 mmHg; solubility of CO₂ in water, 0.0329 mmol/liter H₂O/mmHg (Siggaard-Andersen, 1974).

### TABLE II

Charge Terms and Dissociation Constants for the Cellular and Extracellular Buffers Included in the Model Equations

| Solute       | Apparent pK₅ | Ref. | Proton charge per molecule |
|--------------|--------------|------|----------------------------|
| Hemoglobin   | 6.69, 7.89   | Eq. 37 | zₕb = 15.6 - 23hᵦ/(1 + bᵦ) - 4hᵦ/(1 + bᵦ) |
| Albumin      | 6.76         | 1    | zₕₘₐₜ = -1.16 - 16hᵦ/(1 + bᵦ) |
| DPG          | 7.56, 7.32   | 2    | zₕₐₜ = -1 - 1.16/(1 + bᵦ) |
| ATP          | 6.80         | 2    | zₚₚₚ = -5 - 9.16/(1 + bᵦ) |
| GSH          | 8.54, 9.42   | 3    | zₕₐₜ = -1 - 9.16/(1 + bᵦ) |
| Pi           | 6.87         | 2    | zₚₚₚ = -1 - 9.16/(1 + bᵦ) |
| MeP          | 7.53         | 2    | zₕₐₜ = -1 - 1.16/(1 + bᵦ) |
| Citrate      | 5.49         | 1    | zₕₐₜ = -1 - 1.16/(1 + bᵦ) |
| H₂CO₃, HCO₃⁻ | 6.11, 10.2   | 4    | zₕₐₜ = -1 - 1.16/(1 + bᵦ) |

For a particular solute, c, b = 10ᵖK₅, or when the pK₅ values (pK₅₁ and pK₅₂) of two titrating groups (c₁ and c₂) on the one molecule can be specified separately, bᵦ = 10ᵖK₅₋pK₅₁ and bᵦ = 10ᵖK₅₋pK₅₂. The pK₅ values listed apply for physiological conditions of temperature and ionic strength and were obtained from the references cited: 1, Wolf (1982); 2, Stewart et al. (1986); 3, Rabenstein et al. (1979); 4, Rispens et al. (1968).

*The charge arising from each molecule of dissolved CO₂ by hydration and dissociation (see Eq. 29).
solely to a change in the osmotic coefficient of hemoglobin then:

$$\Phi_{\text{Hb}} \cdot \frac{\text{Hb}}{V_i} - \Phi_{\text{Hb0}} \cdot \frac{\text{Hb}}{V_i} = (\text{Sosmol} - \text{Eosmol}) \cdot \frac{V_{\text{wat}}}{V_i}$$

(5)

In this expression $\Phi_{\text{Hb}}$ and $\Phi_{\text{Hb0}}$ are, respectively, the osmotic coefficients of hemoglobin in the initial sample and after cell volume change in the anisotonic saline, while $V_i$ (liter $\text{H}_2\text{O}$/liter suspension) is the cell water volume after cell volume change. $\text{Hb}$ is the amount of hemoglobin (in millimoles) present, this value being constant for all the samples in a particular experiment. A value of 2.69 was assumed for $\Phi_{\text{Hb0}}$ (Freedman and Hoffman, 1979), so that,

$$\Phi_{\text{Hb}} = (\text{Sosmol} - \text{Eosmol}) \cdot \frac{V_{\text{wat}}}{\text{Hb}} + 2.69$$

(6)

In a second group of experiments cell volume was varied by using valinomycin. A saline-washed cell suspension, with a Hct of 0.70 and containing glucose (2 mM) was used. To half the suspension (16 ml), 30 μl of 5 mM valinomycin, dissolved in a mixture of equal proportions of ethanol and dimethylsulfoxide, was added, while the control sample received only the solvents. The suspensions were incubated at 25°C for 4 h, with regular removal of samples which were immediately centrifuged and the supernatant was removed for the measurement of osmolality. Whole samples were frozen for the subsequent assay of lactate, ATP, and DPG. Again, Eq. 6 was used to calculate the change in the osmotic coefficient of hemoglobin but in this instance Eosmol was the osmolality of the control suspension, and Sosmol was that of the valinomycin-treated suspension.

Development of the Electrochemical Model

The model equations were developed to calculate the steady-state pH, cell volume, and transmembrane solute distribution achieved by erythrocytes in plasma under physiological conditions or when suspended in artificial media. The requirement of electroneutrality in both intra- and extracellular compartments and the negligible difference in osmotic pressure across the cell membrane that exists once electrochemical equilibrium is achieved (Hladky and Rink, 1977) are the basis of the equations. It is also essential that after mixing the cells and media, and during equilibration, the total water volume and the amounts of all solutes (except carbonates) per liter of suspension are conserved.

Water Volumes

$V_{\text{wat}}$ is the total water volume in one liter of suspension and

$$V_{\text{wat}} = V_i + V_o$$

(7)

where $V_i$ and $V_o$ are the equilibrium cellular and extracellular water volumes (liter $\text{H}_2\text{O}$/liter suspension), respectively. Therefore, any change in extracellular water volume due to water equilibration across the cell membrane would be quantified by the factor

$$V_o/(V_{\text{wat}} - V_i)$$

(8)

with $V_i$ as the initial supernatant water volume after exposure of the cells to the new external solution, but before osmotic alterations.

Solute Terms

In all further equations solute symbols (see Table I for a list of these symbols) alone, or with a subscript indicating their location, denote the millimolar concentration of that particular species in the total suspension (e.g., $c$ denotes millimoles of $c$/liter suspension), while square brackets indicate its millimolar concentration in the compartment specified by the subscript (e.g., $[c]_i$ denotes millimoles of $c$/liter cell $\text{H}_2\text{O}$; $[c]_o$ denotes millimoles of $c$/liter extracellular
H₂O). If the solute appears in only one compartment its location is not specified by a subscript.

**Calculation of Total Osmolality in Each Compartment**

To calculate the total osmolality in each compartment, it was necessary to develop expressions for, or specify the value of, the molal concentrations of the principal solutes in each compartment and their osmotic coefficients. The effect of possible heterogeneous solute-solute interactions on the value of the osmotic coefficients was not considered.

**Osmotic Coefficients**

The osmotic coefficient of all the uncharged solutes was specified as 1.00, for trisodium citrate as 0.72, and for the other electrolytes as 0.93. The osmotic coefficient of hemoglobin (Φ_{Hb}) was determined empirically (see Results) as

$$\Phi_{\text{Hb}} = 1 + 0.115 \cdot \text{Hb}/V + 0.0256 \cdot (\text{Hb}/V)^2$$

**Impermeant Solutes**

The intracellular impermeant solutes included in the calculations are listed in Table I. Na and K permeability comprises passive diffusion, direct active transport mediated by the membrane Na/K ATPase, and flux through a variety of transporters (e.g., the Na/K/Cl and the K/Cl cotransport systems). Nevertheless, the rate of cation flux is so low relative to the rate of redistribution of monovalent anions, that the effect of cation transport on rapid changes (time scale < 1 h) in pH and cell volume can be assumed to be negligible (Lew and Bookchin, 1986). Therefore, Na and K ions are considered to be impermeant for the purposes of this model. For the suspending solutions investigated (i.e., plasma and phosphate-buffered saline, sucrose, and citrate) the impermeant extracellular species were monovalent and divalent cations, and were albumin, sucrose, and citrate. For the model simulations, any changes in the concentration of the impermeant solutes in either compartment were a direct result of water movement across the membrane.

**Permeant Ions**

At equilibrium the permeant anions are passively distributed across the cell membrane so that their chemical and electrical gradients are balanced as described by the Nernst equation (Hladky and Rink, 1977):

$$E_m = \frac{RT}{zF} \ln \left( \frac{\alpha_a[c]_o}{\alpha_a[c]_i} \right)$$

where $E_m$ is the membrane potential, $R$ the gas constant, $F$ the Faraday constant, and $z$ the proton charge per molecule of the solute. The cellular ($\alpha_a$), and external ($\alpha_a$) activity coefficients of each solute are assumed to be equal (Freedman and Hoffman, 1979) and cancel in Eq. 10. Protons are effectively permeant via the Jacobs-Stewart cycle which involves the rapid equilibration of carbon dioxide across the cell membrane and its hydration, catalyzed within the cell, by carbonic anhydrase. Protons and bicarbonate ions are released and the latter redistribute across the membrane in exchange for other anions, principally chloride, through the anion exchanger band 3 (Hladky and Rink, 1977). So for the monovalent anions (Cl⁻, HCO₃⁻, H₂PO₄⁻, MeP⁻, and lactate) and for protons:

$$[a^-]/[a^-]_o = [H^+]/[H^+]_o = \Phi$$

(11)
when $a^-$ represents any of the anions listed above and $\phi$ is the Donnan ratio. Similarly for the
divalent anions (HPO$_4^{2-}$ and MeP$_2^{-}$):

$$\frac{[a^{2-}]}{[a^{2-}]}_o = \phi^2$$

(12)

Although the transmembrane distribution of the permeant monovalent anions ($a$) may alter
during equilibration, the total suspension concentration ($a_{\text{tot}}$) of each species (except carbonate)
is maintained and (omitting the superscript negative symbols)

$$a_{\text{tot}} = [a]_i V_i + [a]_o V_o$$

(13)

Substituting Eqs. 8 and 11 into 12, followed by some rearrangement gives:

$$[a]_i - a_{\text{tot}} \phi / [V_{\text{tot}} + V_i (\phi - 1)]$$

(14)

and from Eq. 11:

$$\phi = 10^{pH_{i} - pH_{o}}$$

(15)

then:

$$[a]_i = a_{\text{tot}} 10^{pH_{i} - pH_{o}} / [V_{\text{tot}} + V_i (10^{pH_{i} - pH_{o}} - 1)]$$

(16)

and similarly:

$$[a]_o = a_{\text{tot}} / [V_{\text{tot}} + V_i (10^{pH_{i} - pH_{o}} - 1)]$$

(17)

For permeant anions that titrate between pH 6 and 8, (i.e., Pi and MeP) the concentrations in
each compartment can be calculated using the Donnan ratio and the Henderson-Hasselbalch
equation, if the apparent dissociation constants ($pK_a$) are assumed to be equal for the same
solute type on both sides of the cell membrane. The concentration of each of these solutes in
the total suspension is given by:

$$(c + c^-)_{\text{tot}} = V_i [\phi [c]_o + \phi^2 [c^-]_o] + (V_{\text{tot}} - V_i) ([c]_o + [c^-]_o)$$

(18)

where $c$ and $c^-$ represent the protonated and deprotonated forms of the solute, respectively,
and the intracellular concentrations have been eliminated using Eqs. 11 and 12. When the
Henderson-Hasselbalch equation is applied to the external compartment, with $pK$, denoting
the apparent dissociation constant of the solute, $c$, then:

$$[c^-]_o = 10^{pH_{o} - pK_c} [c]_o$$

(19)

and substitution into Eq. 18 gives:

$$[c]_o - (c + c^-)_{\text{tot}} / [V_i (\phi (1 + 10^{pH_{i} - pK_c}) - (1 + 10^{pH_{i} - pK_c})) + V_{\text{tot}} (1 + 10^{pH_{i} - pK_c})]$$

(20)

If Eq. 19 is then used to obtain an expression for $[c^-]_o$, from Eq. 20, then the total concentra-
tion of the species in the extracellular compartment is:

$$[c + c^-]_o = (c + c^-)_{\text{tot}} (1 + 10^{pH_{i} - pK_c}) / [V_i (10^{pH_{i} - pK_c} (1 + 10^{pH_{i} - pK_c}) - (1 + 10^{pH_{i} - pK_c})) + V_{\text{tot}} (1 + 10^{pH_{i} - pK_c})]$$

(21)

after the elimination of $\phi$ (Eq. 15). A similar expression was derived for the concentration of
the species inside the cell:

$$[c + c^-]_i = 10^{pH_{i} - pK_c} / (c + c^-)_{\text{tot}} (1 + 10^{pH_{i} - pK_c}) / [V_i (10^{pH_{i} - pK_c} (1 + 10^{pH_{i} - pK_c}) - (1 + 10^{pH_{i} - pK_c})) + V_{\text{tot}} (10^{pH_{i} - pK_c} + 10^{pH_{i} - pK_c})]$$

(22)
The cell suspension was considered to be in equilibrium with the vapor phase to which it was exposed (e.g., alveolar or atmospheric air) so that Henry's law applies:

$$[\text{CO}_2] = S \cdot \text{pCO}_2$$  \hspace{1cm} (23)

where $S$ is the solubility coefficient of carbon dioxide in water, pCO$_2$ is its partial pressure in the relevant gas mixture, and $[\text{CO}_2]$ is its molal concentration, which is assumed to be equal throughout the cell suspension because of the rapid diffusion of carbon dioxide across the cell membrane. The reversible hydration of carbon dioxide:

$$\text{CO}_2 + 2\text{H}_2\text{O} \rightleftharpoons \text{HCO}_3^- + \text{H}_3\text{O}^+$$

and

$$\text{HCO}_3^- + \text{H}_2\text{O} \rightleftharpoons \text{CO}_3^{2-} + \text{H}_3\text{O}^+$$

occurs extremely rapidly via carbonic anhydrase within erythrocytes. The dissociation constants for these reactions ($K_{\text{carb}}$ and $K_{\text{carb2}}$, respectively) were assumed to be equal in both compartments so that inside the cells

$$[\text{HCO}_3^-] = S \cdot \text{pCO}_2 \cdot 10^{p\text{Hi}-pK_{\text{carb}}}$$  \hspace{1cm} (24)

and

$$[\text{CO}_3^{2-}] = S \cdot \text{pCO}_2 \cdot 10^{p\text{Hi}-pK_{\text{carb2}}-pK_{\text{carb}}}$$  \hspace{1cm} (25)

while analogous functions of $S \cdot \text{pCO}_2$, $p\text{Hi}$, and the same dissociation constants give the extracellular concentrations. In the pH range of interest ($p\text{H} 6$–$8$), the concentration of carbonate ions ($\text{CO}_3^{2-}$) is too low to contribute significantly to the osmotic pressure.

**The Osmotic Balance Equation**

When the cell is in equilibrium with its suspending solution, the total osmotic contribution of cellular and extracellular solutes is the same, so:

$$(1/V_v) \left[ ([\lambda] + \text{DPG} + \text{ATP} + \text{GSH} + \text{ac}) 0.93 + \Phi_{\text{Hb}} \cdot \text{Hb} + \mu_3 \right] + 0.93 ([\text{Lac}] + [\text{Pi}] + [\text{MeP}] + [\text{HCO}_3^-]) =$$

$$[V_v/(V_v - V_o)] ([\lambda]_o + [\text{ac}]_o) 0.93 + ([\text{Lac}]_o + [\text{Pi}]_o + [\text{MeP}]_o + [\text{HCO}_3^-])_o$$

$$+ 0.93 ([\text{Cl}^-]_o + [\text{Lac}]_o + [\text{Pi}]_o + [\text{MeP}]_o + [\text{HCO}_3^-])_o$$  \hspace{1cm} (26)

with all the included terms defined by Eqs. 7–25. As the (molal) concentrations of uncharged permeant solutes such as glucose, urea, and carbon dioxide are equal in both compartments, the terms for these solutes were not included in Eq. 26.

**Electroneutrality**

The electroneutrality condition requires that the sum of the charges on the solutes in a particular compartment is equal to zero.

**Variable Charge Terms**

Certain solute molecules (Table II) have both a variable charge component at pH values 6–8, due to ionizable groups with dissociation constants within or close to this pH range, and a fixed charge component from charged groups that do not titrate in this pH range. The total charge on each of these molecules is given by:

$$\zeta = \zeta_0 - [\zeta_1]/([\zeta_1] + [\zeta_1]) - ... - [\zeta_n]/([\zeta_n] + [\zeta_n])$$  \hspace{1cm} (27)

where $\zeta_1$ to $\zeta_n$ denote different titrating groups on the molecule; the superscript (-) indicates the deprotonated form, and $\zeta_0$ is defined as the proton charge on the molecule at a pH just
below the value where the groups $c_i$ to $c_s$ titrate. From Eq. 19:

$$10^{\text{pH}-pK_{c_i}} = [c_i]/[c_s]$$

and so

$$z_c = z_s - 10^{\text{pH}-pK_{c_i}}/(1 + 10^{\text{pH}-pK_{c_s}}) - \ldots - 10^{\text{pH}-pK_{c_n}}/(1 + 10^{\text{pH}-pK_{c_m}})$$

(28)

Table II lists the expressions used in the calculation of molecular charge as a function of the pH in the appropriate compartment and the values of the apparent dissociation constants at 37°C and physiological ionic strength.

The concentration of negative charges due to the total carbonates was calculated using Eqs. 24 and 25 as:

$$[\text{HCO}_3^-] + 2[\text{CO}_2^-] = S_p\text{CO}_2 \cdot 10^{\text{pH}-pK_{\text{ext}}}(1 + 2 \cdot 10^{\text{pH}-pK_{\text{ext}}})$$

(29)

The Electroneutrality Equations

For electroneutrality inside the cell:

$$b_i + z_{\text{Hb}} \text{Hb} + z_{\text{DPG}} \text{DPG} + z_{\text{ATP}} \text{ATP} + z_{\text{GSH}} \text{GSH} + 2d_i$$

$$- V_i([\text{Cl}^-], + [\text{Lac}], - z_p[\text{Pi}], - z_{\text{MeP}}[\text{MeP}], + [\text{HCO}_3^-], + 2[\text{CO}_2^-]) = 0, (30)$$

and for the extracellular solution:

$$V_o([\text{Cl}^-], + [\text{cit}], + 2[\text{cit}], + z_{\text{MeP}}[\text{Alb}] + z_{\text{cit}}[\text{cit}])$$

$$- (V_o - V_i) ([\text{Cl}^-], + [\text{Lac}], - z_p[\text{Pi}], - z_{\text{MeP}}[\text{MeP}], + [\text{HCO}_3^-], + 2[\text{CO}_2^-]) = 0 \quad (31)$$

Solving the Equations

The Broyden algorithm (Broyden, 1969), an iterative method, was selected to solve the three equations (26, 30, and 31) for $pH_i$, $pH_o$, and $V_o$, thus allowing the direct calculation of the equilibrium concentrations of all the solutes in each compartment.

Application to Whole Blood

Our experimental approach was to suspend cells in various artificial media and measure the resultant change in cell volume and pH. To simulate these changes using the model equations, the electrochemical state of the cells and the medium before equilibration had to be defined. The necessary data were the solute and solvent concentrations in both compartments, the ratio in which they were mixed, the osmotic coefficients and dissociation constants of the relevant solutes, and the partial pressure of carbon dioxide to which the suspension was exposed.

This information was most easily obtained for fresh whole blood (Table I). As the effective osmolality of normal blood is 273 mosmol/kg H$_2$O (Gennari, 1984), the total osmotic contribution in each compartment must equal this value, and so Eq. 26 was separated into two equations. The pH and cell water volume of fresh blood were also specified and included with the other standard data listed in Tables I and II. The four equations (30, 31, and two from 26) were then solved with these data to return values for the total chloride concentration (and therefore $[\text{Cl}^-]$) and $[\text{Cl}]$, using Eqs. 19 and 20, the cell and plasma monovalent cation concentrations, and the concentration of intracellular uncharged solutes. These values completed the data set of 33 parameters required to define the electrochemical state of whole blood.
Simulating the Titration Experiments

Modification of the whole blood data set was essential, as the erythrocytes used in these experiments were collected into anticoagulant containing citrate and phosphate, stored for up to 24 h, and then washed in saline. This treatment resulted in an increase in intracellular Pi, a decrease in total carbonates as the cells equilibrated with air (a process facilitated by washing), and a decrease in pH, due to buffering by the anticoagulant (pH 6.0) and continuing anaerobic glycolysis.

When simulating the titration experiments, mixing the washed cells with the experimental buffer and the addition of varying concentrations of NaOH or HCl was considered to be a single step. The concentration of each ion and uncharged species in the mixture of buffer, acid, and base was included in the data file, as was the measured lactate concentration of the suspension. Using eight different data files (i.e., one for each different NaOH/HCl concentration), eight estimates of pH_i, pH_o, and V_i were made for each titration experiment.

The Hct attained after equilibration of the cells with a particular solution was calculated as follows:

$$Hct = V_{i} + \text{Hct}'(1 - CW')$$  \hspace{1cm} (32)

where CW' is the initial cell water content (liter H_2O/liter cell) and Hct' is the Hct after addition of cells to the solution but before any transport across the cell membrane.

RESULTS

Titration Experiments

Erythrocytes suspended in phosphate-buffered saline with or without sucrose (150 mM) or citrate (58 mM), were titrated using a strong acid and base through a pH range of 6.0-7.6 while pH_o, pH_i, and cell volume were measured using conventional or ^31P NMR techniques. On one occasion, the pH and volume of 24 samples were determined using both techniques, and the results compared by linear regression analysis; this gave the following expression for pH:

$$pH_{\text{meter}} = pH_{\text{NMR}} \cdot 1.070 - 0.450, \ r = 0.9974$$  \hspace{1cm} (33)

and

$$pH_{\text{NMR}} = pH_{\text{meter}} \cdot 0.993 + 0.056, \ r = 0.9996$$  \hspace{1cm} (34)

and for mean cell volume (MCV, in femtoliters):

$$MCV_{\text{hematological}} = MCV_{\text{NMR}} \cdot 0.961 + 3.170, \ r = 0.9822$$  \hspace{1cm} (35)

where the method used is indicated by the superscript. Because of the close agreement between these two sets of measurements, the results obtained using the different methods were pooled for subsequent analysis (Figs. 2 and 3).

Comparison of Model Calculations and Experimental Results

Equilibrium values of pH_o, pH_i, and V_i were calculated for the various suspensions investigated in the titration experiments. In Figs. 2 and 3 logarithmic functions were fitted onto the calculated values to facilitate the comparison of these values with the
experimental measurements. In each of the three artificial media, and for a wide range of pH values, good agreement was seen between the calculated and experimental values for the absolute pH$_i$ and pH$_o$, the transmembrane pH differences and also the buffering capacity of both compartments (Fig. 2). The dependence of cell volume on pH was also accurately predicted (Fig. 3).

**Figure 2.** Comparison of experimental data and model simulations of equilibrium pH$_i$ and pH$_o$ values produced when red cells in artificial media were titrated with 290 mM NaOH or HCl. The experimentally determined pH values are denoted by the symbols: open symbols, pH$_i$; closed symbols, pH$_o$; triangles, $^3$P NMR measurements; squares, estimates made using a pH meter. The standard deviations of the estimates were less than the size of the symbols. The lines were determined by nonlinear least-squares fitting of the equation:

$$\text{pH} = P_1 + \ln \left\{ \frac{(P_2 - x)/x}{P_3} \right\}$$

onto pH values calculated using the model equations. Each panel presents data obtained from erythrocytes suspended in a different medium (see Methods): A, phosphate-buffered saline; B, phosphate-buffered saline with sucrose; C, phosphate-buffered saline with citrate. The parameters values ($P_1$, $P_2$, and $P_3$) were, respectively: (A) pH$_i$, 8.17, 43.1, and −178.3; (A) pH$_o$, 7.59, 43.2, and −90.1; (B) pH$_i$, 8.26, 43.2, and −187.1; (B) pH$_o$, 7.39, 42.0, and −84.9; (C) pH$_i$, 7.12, 73.8, and −99.2; (C) pH$_o$, 6.67, 69.9, and −66.6.

**Charge on Hemoglobin**

We measured the noncarbonate buffering capacity, at 37°C, of CO-saturated hemolysates, by titrating them with strong acid or base at low CO$_2$ tension (0.25 mmHg). An equation was written, which did not restrict the number of titrating groups ($P_2 + P_3$) present in the lysate, but allowed them only two different pK$_a$ values (pK$_1$ and pK$_2$) i.e.:

$$Y = P_1 - P_2 \cdot 10^{pH - pK_1} / (1 + 10^{pH - pK_1}) - P_3 \cdot 10^{pH - pK_2} / (1 + 10^{pH - pK_2})$$

(36)
where \( Y \) represented the amount of acid added (mol/mol Hb). This equation adequately fitted the experimental data (Fig. 4) and nonlinear least-squares analysis returned values for the five parameters \( P_1, P_2, P_3, pK_1, \) and \( pK_2 \) of 15.6, 14.8, 17.7, 6.13, and 7.14, respectively.

An equation identical to Eq. 36, except that \( P_3 \) was specified as 4, was used to fit the hemoglobin titration data (Fig. 4);

\[
\alpha_{Hb} = P_1 - P_2 \cdot 10^{pH-pK_1)/(1 + 10^{pH-pK_1}) - 4 \cdot 10^{pH-pK_2)/(1 + 10^{pH-pK_2})} \quad (37)
\]

returning values of 6.69 for \( pK_1 \), 7.89 for \( pK_2 \), 15.6 for \( P_1 \), and 23.0 for \( P_2 \). Differentiation of Eqs. 36 and 37 yielded expressions describing the buffering capacity of the lysate \(-d(\text{acid})/dpH\) and of hemoglobin \(-d\alpha_{Hb}/dpH\), respectively.

**Figure 3.** The dependence of cell volume on pH; comparison of experimental data and model simulations. pH was varied by titration with 290 mM HCl or NaOH or both. As the cell count was the same in each sample, the mean cell volume was directly proportional to the Hct. The symbols denote data points defined by measurement of both pH and Hct: open symbols, measurements made using \( ^{31} \text{P} \) NMR; closed symbols, measurements made using conventional methods; squares, cells suspended in phosphate-buffered saline; triangles, in phosphate-buffered saline with sucrose; circles, in phosphate-buffered saline with citrate. The error bars represent ±1 SD of the Hct values estimated by the NMR method and for clarity are only drawn for selected points. The lines were determined by nonlinear least-squares fitting of the equation:

\[
\text{Hct} = \frac{P_1 + P_2/(1 + 10^{pH-pK_3})}{1 + 10^{pH-pK_1}}
\]

onto data points (not shown) for which both pH and Hct were calculated using the model equations. In A, the upper line represents simulated data for cells in phosphate-buffered saline (\( P_1 = 0.339, P_2 = 0.130, \) and \( P_3 = 6.83 \)), and the lower line for cells in phosphate-buffered saline with sucrose or with citrate (\( P_1 = 0.340, P_2 = 0.126, \) and \( P_3 = 6.57 \)). In B the relationship between calculated pH, and Hct data for cells in all three media were so similar as to be represented by a single line (\( P_1 = 0.324, P_2 = 0.149, \) and \( P_3 = 6.78 \)).
RAFTOS ET AL.  Electrochemical Model of Erythrocyte Buffering

**Figure 4.** Titration of hemolysate and hemoglobin with 290 mM HCl or NaOH at pCO₂ of 0.25 mmHg and 37°C. Open symbols represent hemolysate data and refer to the ordinate scale on the left. The hemoglobin data (closed symbols) were plotted against the right-hand scale. The circles and squares indicate data obtained in two separate experiments. The lines fitted to the lysate and hemoglobin data were defined by Eqs. 36 and 37, respectively.

**Osmotic Coefficient of Hemoglobin**

When cells were swollen by suspension in anisotonic saline, the supernatant osmolality was less than the expected osmolality, but for shrunken cells the osmolality was significantly higher than expected. As an example, at the highest cell volume (105 fl, 6.09 mmol Hb/liter cell H₂O) the supernatant osmolality was 243 ± 1 mosmol/kg H₂O while the expected osmolality was 246 ± 1 mosmol/kg H₂O. When the cells were at the minimum volume achieved (61.3 fl, 13.6 mmol Hb/liter cell H₂O), the supernatant and expected osmolalities were 708 ± 2 and 676 ± 1, respectively, with the difference representing 4.6% of the measured osmolality.

When valinomycin was added to cells suspended in normal saline, they shrank producing a typical alteration in the supernatant osmolality for the test suspension of 288 ± 1 (cell volume 102 fl, 6.49 mmol Hb/liter cell H₂O) to 299 ± 1 mosmol/kg H₂O.

**Figure 5.** The osmotic coefficient of hemoglobin estimated in electrolyte-rich and electrolyte-depleted whole cells. The squares denote estimates obtained when the cellular hemoglobin concentration was varied by exposing the cells to anisotonic saline, and the triangles denote data from valinomycin-treated cells. The two solid lines represent Eqs. 9 and 38, which were fitted onto the valinomycin data and anisotonic saline data, respectively. The dotted line represents the osmotic coefficient term used by Freedman and Hoffman (1979).
kg H₂O (cell volume 70.8 fl, 11.0 mmol Hb/liter cell H₂O), while the volume and
the osmolality (290 ± 1 mosmol/kg H₂O) of the control suspension remained con-
stant. Assay results showed constant ATP levels in each suspension throughout the
incubation. DPG levels declined at a constant rate (valinomycin sample, −227 ± 7
μmol/liter per h, and the control, −226 ± 6 μmol/liter per h) while a linear accu-
mulation of lactate was observed in both suspensions (valinomycin sample, 306 ± 8
μmol/liter per h, and control, 256 ± 10 μmol/liter per h).

Values of the osmotic coefficient, calculated using Eq. 6, appeared to depend on
the method used to vary the cell volume (Fig. 5). Eq. 9 was fitted to the valinomycin
data. The equation that best fitted the saline data but which ensured that Φ_Hb
approached unity as [Hb] approached zero was

$$\Phi_{Hb} = 1 - 0.181 \cdot [Hb] + 0.0683 \cdot [Hb]^2$$ (38)

For reasons discussed below, Eq. 9 rather than Eq. 38 was incorporated into the
model.

DISCUSSION

Our aim was to measure the equilibrium cell volume, pHᵢ, and pHₑ achieved when
erthrocytes were exposed to various suspending solutions and then to compare
these results with the values calculated using our electrochemical model. The experi-
mental measurements were made using either conventional methods or recently
developed ³¹P NMR techniques (Stewart et al., 1986; Raftos et al., 1988). Results
obtained by both methods showed good agreement and were plotted together in
Figs. 2 and 3.

Drawbacks to the use of NMR in experiments of this type included the need to
calibrate the NMR methods against measurements obtained using the standard
techniques. Also, the probe molecules MeP and dimethyl methylphosphonate had to
be introduced into the suspension at relatively high concentrations (i.e., 5 and 10
mM, respectively). As MeP is known to distribute across the cell membrane accord-
ing to the Donnan ratio (Stewart et al., 1986), terms describing its concentration
and charge (Table II) were included in the model equations. In contrast, dimethyl
methylphosphonate is a neutral molecule that rapidly equilibrates across the cell
membrane (Kirk and Kuchel, 1986), and therefore it does not influence the effec-
tive osmolality or the charge distribution.

The principal benefit of using ³¹P NMR lay in its ability to provide estimates of
pHᵢ, pHₑ, and cell volume simultaneously in a single suspension of undisrupted cells
(Fig. 1). Possible errors arising in the measurement of transmembrane pH differ-
ence due to insufficient control of temperature and the partial pressure of CO₂
during the separation of cells and extracellular fluid (Siggaard-Andersen, 1974)
were completely avoided. A further advantage is that from each ³¹P NMR spectrum
acquired for pH and cell volume determination, the concentration of the metabo-
lites ATP and DPG inside the cell (Chapman et al., 1985), and of Pi and MeP in both
compartments (Stewart et al., 1986), could also be estimated. The proven ability of
NMR to follow the time course of metabolic and transport-induced changes in the
concentration of numerous solutes at one time (Chapman et al., 1985), combined
with the measurement of the basic cellular parameters of volume and pH, make
NMR an extremely powerful tool for the development and verification of even the most complex models of erythrocyte function.

Instead of developing separate expressions for the titration of each of the noncarbonate cellular buffers several authors have included in their model equations estimates of the isoelectric point and buffering capacity of the complete intracellular milieu (Dalmark, 1975; Freedman and Hoffman, 1979; Lew and Bookchin, 1986). Clearly this approach limits the flexibility of the model equations as the combined buffering term is only accurate for cells containing standard amounts of all the buffers. Also, the ability to quantify the influence of an individual buffering species on an observed pH change is precluded.

To determine the buffering capacity of the cellular solution, hemolysates at 37°C and with pCO₂ of ~0.25 mmHg were titrated with strong acid or base. The noncarbonate buffering capacity, evaluated using the differentiated form of the equation (Eq. 36) fitted to the experimental data (Fig. 4) increased from 10.8 ± 0.30 mol/pH unit per mol Hb at pH 6.0, to a maximum of 13.8 ± 0.18 mol/pH unit per mol Hb at pH 6.8, then decreased rapidly to 7.8 ± 0.62 at pH 7.7. These values are almost identical to results obtained by previous authors (Siggaard-Andersen, 1974; Duhm, 1976) using the same conditions of CO₂ partial pressure and temperature. However Dalmark (1975) estimated the noncarbonate buffering capacity in cation-permeabilized whole cells at 4°C and found it to be constant over 2–3 pH units and equal to 10 mol/pH unit per mol Hb.

In order to extract the buffering component due solely to hemoglobin from our lysate data, the calculated charge contribution of all the other solutes was summed for each pH value measured. The remaining charge required to ensure electroneutrality was attributed to hemoglobin, and the data were plotted in Fig. 4.

For the pH range 5–9 at 25°C, the variation of the proton charge on each molecule of carboxyhemoglobin is due to the titration of 20 exposed histidine residues and four terminal valines on each tetramer (Mathew et al., 1979). For the 10 distinct histidine residues, the ionization depends on the position of the group within the molecule and its proximity to other charged groups; the range of their pK'ₐ values is from 6.31 to 8.21 (Mathew et al., 1979). We constructed an equation that included 24 titrating groups, but allowed only a single pK'ₐ value for each type of residue. The fitted curve underestimated \( -\frac{dz_{mb}}{d\text{pH}} \) as pH approached both 6 and 8. Thus to achieve a good fit it was necessary to increase the number of titrating groups from 24 to 27 (see Fig. 4 and Eq. 37), but two pK'ₐ values were sufficient. When Eq. 37 was solved for pH with \( z_{mb} \) equal to zero the value of the isoelectric point of hemoglobin was calculated at 6.98 at 37°C, which is slightly lower than the value of 7.07 obtained after temperature correction (Reeves, 1976) of measurements made for stripped carboxyhemoglobin at 25°C using exchange chromatography (Rollema et al., 1975).

It is usually assumed that although the isoelectric point is highly temperature dependent (Reeves, 1976), the overall shape of the hemoglobin titration curve is unaffected by temperature (Dalmark, 1975; Rodeau and Malan, 1979; Wolf, 1982). However, titration data for purified oxyhemoglobin obtained by Antonini et al. (1965) clearly indicate an increase in the absolute value of \( -\frac{dz_{mb}}{d\text{pH}} \) with temperature (for pH values <7.2) that is not fully accounted for by transposition of the titration curve on the pH scale with temperature correction for the isoelectric point.
An increase in temperature has also been reported to increase the apparent noncarbonate buffering of whole blood (Castaing and Pocidalo, 1979) due to a proposed reduction in the stability of tertiary and quaternary protein structures, resulting in the exposure of more residues capable of ionization. Therefore, it is not unreasonable that the expression that best fits our titration data for hemoglobin at 37°C (Eq. 37) requires the deprotonation of 27 groups, while only 24 are available for ionization at 25°C. In their variable temperature model, Rodeau and Malan (1979) adjusted the mean histidine pH for changes in temperature but the number of titrating groups was held constant at 28.

Differentiation of the expression for the hemoglobin titration curve (Eq. 37) allows evaluation of the buffering capacity of hemoglobin as a function of pH, and at pH 7.2 $-\frac{d\log pCO_2}{dpH}$ was calculated as 10.8 proton charges/pH unit per tetramer. Available estimates of the buffering capacity of purified carboxyhemoglobin at this pH are 9.8 at 25°C (Rollema et al., 1975), 10 at 0°C (Gary-Bobo and Solomon, 1968), and 12.7 at 40°C (Antonini et al., 1965). By estimating hemoglobin buffering in a hemolysate at 37°C, we have more closely approximated the physiological situation than titration experiments using highly diluted (0.15 mM) hemoglobin in NaCl or KCl solutions.

To test the accuracy of the expressions for the titration of the noncarbonate buffers (Table II), the model equations were used to calculate the pH levels expected when fresh whole blood was equilibrated with CO$_2$ at partial pressures from 16 to 114 mmHg at 37°C. The resulting pH values ranged from 7.09 to 7.63 and within this range $-\frac{d(\log pCO_2)}{dpH}$ was constant and equal to 1.56 ± 0.02, which was in agreement with a value of 1.58 determined experimentally at 38°C by Siggaard-Andersen (1962).

A more rigorous assessment of the ability of the model equations to approximate the buffering capabilities of erythrocytes over a wide range of pH, was by the comparison of experimental and simulated pH values produced by titrating erythrocytes in artificial media with strong acid or base (Fig. 2). The average of the difference between calculated and experimental results for pH$_i$ was 0.036 and for pH$_o$ it was 0.046 with 95% of the simulated values falling within ±0.07 and ±0.09 of the experimental values for pH$_i$ and pH$_o$, respectively.

During the standardization procedure used for the present model, the equations were solved for whole blood with osmolality, cell volume, and pH fixed at standard values while the variables were the concentrations of selected solutes (Table I). The returned values were well within normal ranges except for the concentration of uncharged, impermeant solutes inside the cell, which was 16.5 mmol/liter cell. This value does not represent the osmotic effect of any known cellular solute but rather it is a measure of an apparent osmotic deficit inside the cell. Similarly, to standardize their model for whole blood, Rodeau and Malan (1979) required an abnormally high, intracellular concentration of monovalent cations (130 mmol/liter cell). Other authors (Wolf, 1982; Lew and Bookchin, 1986) used concentrations of nonprotein, charged intracellular solutes (e.g., ATP, DPG, and GSH) of up to 20 mmol/liter cell (see Table I for normal levels), while Freedman and Hoffman (1979) assumed a normal internal osmolality of only 96% of the normal plasma value.

Approximations of the cellular concentrations (millimoles per liter cell) of osmotically effective solutes not included in our model are ~1.0 for metabolites (Pennell,
1974; Beutler, 1984), 2.6 for free amino acids (Levy and Barkin, 1971), and 0.8 for the combined concentration of creatine, creatinine, ergothioneine, ethanolamine, and choline (Pennell, 1974). Although these additional solutes only account for 27% of the calculated deficit, the profusion of other chemical species inside the erythrocyte could conceivably provide the outstanding cellular osmolality. Alternatively the deficit in intracellular osmolality may be due to an underestimation of the osmotic coefficients of organic solutes inside the cell.

The changes in intracellular osmolality that occur as a result of water movement across the cell membrane differ significantly from those produced by diluting or concentrating a protein-free solution by the same factor (Gary-Bobo and Solomon, 1968). As yet a theoretical calculation of solute activity in complex solutions is not possible and instead empirically derived osmotic coefficients must be included in expressions developed to estimate intracellular osmolality. When cell volume changes occur at constant ionic strength, the osmotic coefficients of NaCl and KCl remain equal on both sides of the membrane (Freedman and Hoffman, 1979) and so deviations in erythrocyte behavior from that of a perfect osmometer are attributed to the strong concentration dependence of the osmotic coefficient of hemoglobin. The expressions commonly used to calculate the osmotic coefficient are obtained by fitting data obtained from purified hemoglobin and they do not consider the known dependence of the coefficient on ionic strength (Solomon et al., 1986), or the possibility of gel formation by hemoglobin within red cells (Hladky and Rink, 1978). It is obviously more appropriate to estimate the osmotic coefficient of hemoglobin inside whole cells.

As they shrink, valinomycin-treated cells suspended in normal saline lose KCl and water as a hypertonic solution (Lew and Bookchin, 1986). The time-dependent changes in the external osmolality of a suspension of valinomycin-treated whole cells could not be accounted for by metabolic activity and so were attributed to the increase in the osmotic coefficient of hemoglobin with its concentration. The virial equation (Eq. 9) fitted to these results did not differ greatly from that used by Freedman and Hoffman (1979) in their calculation of volume changes at constant ionic strength (Fig. 5).

When the cell volume was varied by suspending cells in anisotonic saline, the concentration of intracellular electrolytes and hemoglobin was altered by the same factor while cell shrinkage due to valinomycin treatment was accompanied by a slight decrease in intracellular ionic strength. It is not surprising then that the estimates of hemoglobin osmotic coefficients obtained using anisotonic saline appear to form a separate population to those determined using valinomycin (Fig. 5). When both the ionic strength and the hemoglobin concentrations were increased the value of the osmotic coefficient of hemoglobin increased more steeply. McConaghy and Maizels (1961) also reported an indication of higher values in cells with increased ionic strength.

When 290 mM HCl or NaOH is added to an erythrocyte suspension at normal osmolality, to produce pH values between 6 and 8, the osmolality of the suspension is virtually unchanged because all but a minute fraction of the protons or hydroxide ions are bound by buffers. Therefore changes in the osmotic coefficient of hemoglobin that occur as a result of volume change on pH titration closely resemble those in valinomycin treated cells and are best described by Eq. 9.
Although variation in the osmotic coefficient of hemoglobin influences the equilibrium volume achieved by erythrocytes in response to pH change, the volume changes are principally determined by the redistribution, between compartments, of permeant anions followed by osmotic water movement. The net amount of permeant anions in the cell depends essentially on the equilibrium pH, as pH specifies the proton charge on the impermeant intracellular buffers, which must be balanced by these permeant anions.

The single population of experimental data points shown in Fig. 3B demonstrated that the volumes achieved by erythrocytes suspended in solutions, of varying composition but identical osmolality, were dependent only on the pH and not on the solute composition. The relationship between pH and cell volume, however, is strongly influenced by the concentration of permeant anions in the suspending solution (Fig. 3A) because the extracellular concentration of these ions profoundly affects the transmembrane pH difference (Fig. 2). The difference in the relationship between the pH of the two compartments and cell volume emphasizes the difficulties that can arise when basic red cell properties or behavior are related to pH, which is easily measured, rather than the more appropriate pH.

As previously described, the model equations were solved to return estimates of the equilibrium pH, pH, and volume for cells that were suspended in phosphate-buffered solutions and then pH titrated. At each addition of acid or base, Hct values (liter cell/liter suspension) obtained by direct measurement and by calculation were compared (results not shown). The range of Hcts produced was from 0.35 to 0.46 and the mean difference between the theoretically and experimentally determined values was 0.0046, which is less than the error involved in microhematocrit measurements (+0.005); 95% of the calculated values fell within ±0.0110 of the experimental estimates. The model calculations also accurately predicted the magnitude of the decrease in cell volume with increasing pH, as well as the dependence of position of the pH vs. Hct curves on the type of suspending solution (Fig. 3).

In conclusion, in order to calculate the equilibrium values of pH and cell volume that occur after perturbation of extracellular pH, we have elaborated an electrochemical model. Our aim was to maximize the accuracy of the estimates, which were calculated using the model equations, by improving the input data concerning the pH titration and osmotic contribution of hemoglobin. The direct comparison of the results of pH titration experiments, using both whole blood and erythrocytes in artificial media, with model calculations has not been attempted previously, and we have been able to confirm the ability of our equations to predict the resultant pH and pH to within ±0.09 over a pH range of 6.0–7.8, while accompanying changes in cell volume were estimated to within experimental error. Although this model is restricted to situations in which permeant species are fully equilibrated across the cell membrane, and in that it neglects the metabolic, transport, and oxygen dissociation functions of the cell, the close agreement between calculated and experimental results indicates that other effects are secondary. Potential applications of the model are numerous and include estimation of the pH and volume outcome after concurrent perturbation of several effectors, and the design of experimental suspending solutions to produce a desired cellular pH or solute concentration.

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