The pH Dependence of Red Cell Membrane Transport of Titratable Anions Studied by NMR Spectroscopy*

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The effects of varying extracellular pH on the rates of uptake of titratable anions by human erythrocytes under conditions of constant intracellular pH have been determined for a series of highly related anions, the phosphate "analogues." These compounds are simply substituted phosphorus oxyacids, differing in the number and acidity of titratable protons: phosphate (HPO\(_4^{2-}\), \(pK_a 6.8\)); phosphate (HPO\(_4^{3-}\), \(pK_a 6.4\)); hypophosphate (\(\text{H}_2\text{PO}_4^{-}\)); methylphosphonate ((CH\(_3\))\(_2\text{PO}_2^{-}\), \(pK_a 7.4\)); dimethylphosphite ((CH\(_3\))\(_2\text{P}O\))\(_{-}\); fluoro-phosphate (PO\(_4^{3-}\), \(pK_a 4.7\)); and thiophosphate (HSPO\(_3^{-}\), \(pK_a 5.5\)). Suspensions of intact, Cl\(^{-}\)-loaded erythrocytes (intracellular pH, 7.2) were incubated at 37°C in isotonic buffers (pH 4–9) containing 60 mM phosphate analog for specified time intervals, whereupon influx was halted by the addition of 1 mM 4-acetamido-4′-isothiocyanatostilbene-2,2′-disulfonic acid (SITS), an inhibitor of anion exchange. The intracellular anion concentrations were determined from \(^{31}\text{P}\) or \(^{31}\text{F}\) nuclear magnetic resonance spectra from the erythrocyte suspensions. The influx rates for the titratable phosphate analogs exhibited bimodal pH dependence, reaching maximal levels at pH values that increased with increasing anion \(pK\). This pH-dependent behavior is consistent with a transport channel that contains a titratable regulatory site which interacts with the translocated anion. Based upon the Henderson-Hasselbalch equation, the probability that a titratable anion will have an electric charge of equal magnitude to that of the titratable carrier is highest at a pH value exactly midway between the \(pK\) of the regulatory site and that of the anion. The pH maxima observed for the phosphate analogs indicate a \(pK\) for this site of 5.5 at 37°C. Intracellular pH changes associated with transport of slow anions were predominantly determined by partial ionic equilibrium effects and did not indicate the ionization state of the transported anion.

Anion transport in the human erythrocyte is accomplished by a specific transport protein (Band 3), which has been shown to mediate the one-for-one exchange of anions on opposite sides of the membrane, in a sequential or "ping-pong" fashion (1, 2). However, the structure of the transport site and the nature of the anion-transporter complex that induces the conformational change in the protein and affects the translocation of the anion have not yet been determined and remain a subject of great interest.

Knowledge of how the chemical properties of a substrate anion (such as molecular size and structure, net charge, and charge distribution) affect the transport rate for that anion may provide insight into the structure and mechanism of action of the transport site. For example, because the red cell is significantly more permeable to anions than to cations, the transport channel has long been suspected of containing a positively charged determinant (3). Moreover, the pH dependence of anion transport has been found to be chargedependent. The transport of monovalent anions such as Cl\(^{-}\) transport increases with pH and plateaus above pH 7.0 (4, 5) until very high pH values are reached, while the transport of divalent anions such as SO\(_4^{2-}\) has a definite peak around pH 6.3–6.5 (6). The titratable carrier model for anion transport, proposed in 1973 by Gunn (7), originally hypothesized that a titratable positive charge on the carrier would convert it from a carrier for divalent anions to one for monovalent anions as the pH is raised through the \(pK\) of the carrier. However, sulfate transport has been observed to be linked to the cotransport of a proton (8). This observation should lead to a monotonic decrease in divalent anion transport with increasing pH, due to decreasing availability of protons. Such proton activation of SO\(_4^{2-}\) influx was observed by Milanick and Gunn (9) at fixed values for intracellular pH (pHi) and intracellular Cl\(^{-}\) concentration, so that sulfate influx was not limited by eflux half-cycle reactions. Indeed, they found that SO\(_4^{2-}\) influx decreased with increasing extracellular pH (pH\(_o\)) in a sigmoidal fashion, with an apparent dissociation constant of pK 5.9 at 22°C.

If the carrier indeed contains an essential regulatory group that undergoes deprotonation at this pH, the pH dependence of the transport of titratable anions will be considerably different than that for monovalent or divalent anions, because the charge on the anion will also be pH dependent. Anion transport may be favored at the pH value at which the anion and the carrier are most likely to have electric charges of the same magnitude. Therefore, a systematic study of the pH dependence of titratable anion influxes will help to determine the role for electric charge in substrate specificity.

In a previous study (10) we introduced the "phosphate

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†The abbreviations used are: pHi, intracellular pH; HEPES, N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid; pH\(_o\), extracellular pH; PO\(_4\), hypophosphate; PO\(_4\), phosphate; PO\(_4\), phosphate; SITS, 4-acetamido-4′-isothiocyanatoethyl-2,2′-disulfonic acid.
To improve our ability to measure the influx of rapidly exchanging anions (fluorophosphate, phosphate, and hypophosphate), we developed a continuous flow apparatus that allows measurements of anion influx in a quasi-continuous fashion, as a function of increasing pH. A pH gradient is produced by a gravity-driven gradient mixer containing the solution at pH 4 in one cylinder and the pH 8 solution in the other cylinder. By pumping the solution through a tubing that allows mixture with red cells at a “Y” junction. The mixture flows through the tubing until another Y is reached, where the SITS-containing stopping buffer inhibits further influx. The suspension is then delivered to a fraction collector, and the fractions are analyzed for the intracellular and extracellular anion concentrations. This method provides much more accurate and reliable determinations of influx rates for rapidly exchanging anions, because the incubation times are highly consistent and dependent in a predictable manner on the length and diameter of the tubing and the flow rates. The ratios of cells:hypophosphate analog solution and of incubation mixture:stopping solution were similar to the discrete incubations described in the preceding paragraph. The incubation times were: phosphate, 3–4 s; fluorophosphate and hypophosphate, 4–5 s.

**NMR Methods**—The spectrometers were a Nicolet 360 WB, using a 10-mm broadband probe, operating at 146 MHz for 31P and a Nicolet 200 WB, using a 5-mm 31P probe, operating at 188.23 MHz for 31P. Spectra were collected into 8k data points, using quadrature detection, signal averaging, and Fourier transform capabilities. Simple 30°-acquisition time pulse sequences were employed, with recycle delay times of 3 s, which allowed for approximately 90% magnetization relaxation between successive free-induction decays. Approximately 64–128 transients were accumulated in 3–6 min. Samples were spun at 10–20 Hz. These conditions provided the best compromise between signal-noise and signal saturation, to allow quantitation of phosphorus signals, while minimizing settling of cells during the NMR experiment. Methylphosphonate data were obtained using broad-band heteronuclear proton decoupling. 31P chemical shifts are referenced to 85% phosphoric acid and 31P shifts to trifluoroacetic acid, with upfield shifts considered positive. Assignments of intracellular and extracellular chemical shifts were based upon previously published methods (11). Influx rates were calculated from the concentration of intracellular analog, the initial volume of cells added, and the elapsed time of incubation before the addition of SITS. Influx rates are expressed in terms of the initial volume of cells added.

**RESULTS**

**Description of Phosphate Analog**—The phosphate analogs are a series of singly or doubly substituted phosphorus oxyacids, with each substitution covalently bonded to the phosphorus atom, resulting in differences in the number and acidity of titratable protons among these analogs (Table I). Hypophosphite (H2PO2−) is a singly ionizable strong acid with a pK of 1. The other phosphate analogs have two or three ionizable protons, titrating between the mono- and di-ionized species near the physiologic pH range, with the pK values of ionization of their second protons in order of decreasing acidity being: fluorophosphate (PO3F2−), 4.7; thiophosphate (HSPO3−), 5.5; phosphate (HPO42−), 6.4; phosphate (H2PO4−), 6.8; and methylphosphonate [(CH3)PO2−], 7.4. The 31P and 31P NMR chemical shifts and their dependence on pH have been reported previously (10).

**pH Activation of Hypophosphite Transport**—Influx of the monoanion hypophosphate at 1 mm extracellular concentration (Fig. 1) was found to increase monotonically with pH over the pH range of 4–8, plateauing above pH 7. This pH dependence is sigmoidal in shape and is suggestive of a single titration site associated with hypophosphite influx. The solid curve depicted in Fig. 1 is that for a titration with a pK of 5.5 and maximum influx rate of 380 mmol/liter/min, and approximates the observed pH dependence reasonably well. This behavior is similar to the pH dependence of Cl− transport reported by Funder and Wieth (5), although they found an apparent pK of about 6.0 at 37°C.

**pH Dependence of Titratable Anion Influx**—The influx rates
NMR Study of Red Cell Membrane Transport

### TABLE I

The pH dependence of titratable anion influx

<table>
<thead>
<tr>
<th>Compound</th>
<th>Formula</th>
<th>pH</th>
<th>Peak pH</th>
<th>Peak rate</th>
<th>Predicted pK of carrier</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fluorophosphate</td>
<td>PO₄F⁻</td>
<td>4.7</td>
<td>5.1</td>
<td>450</td>
<td>5.5</td>
</tr>
<tr>
<td>Thiophosphate</td>
<td>HSPO₄⁻</td>
<td>5.5</td>
<td>5.2</td>
<td>3.9</td>
<td>5.35</td>
</tr>
<tr>
<td>Phosphate</td>
<td>HPO₃⁻</td>
<td>6.4</td>
<td>5.9</td>
<td>650</td>
<td>5.5</td>
</tr>
<tr>
<td>Phosphite</td>
<td>HPO₂⁻</td>
<td>6.8</td>
<td>6.2</td>
<td>5.1</td>
<td>5.6</td>
</tr>
<tr>
<td>Methylphosphonate</td>
<td>CH₃PO₂⁻</td>
<td>7.4</td>
<td>6.5</td>
<td>3.5</td>
<td>5.6</td>
</tr>
<tr>
<td>Hypophosphite</td>
<td>HPO₁⁻</td>
<td>&gt;8</td>
<td>380</td>
<td></td>
<td>5.5</td>
</tr>
</tbody>
</table>

* Derived from Henderson-Hasselbalch equation (see text).

* No second ionizable proton.

### FIG. 1

**pH activation of hypophosphite influx.** The influx rates for monovalent hypophosphite (○) were determined over a pH range of 4-8. The solid curve represents the titration of a functional group with a pK of 5.5 and a maximum rate of 380 mmol/liter/min.

For the other phosphate analogs were also measured at a concentration of 60 mM, over a pH range of 4.0-8.0, and are depicted in Fig. 2. Because the rates were found to range over nearly 4 orders of magnitude, the results are shown on two logarithmic plots. For reference, the hypophosphite influx data are also included (Fig. 2A). In contrast to monovalent anions, the pH dependence of each of the analogs with a titratable second proton was biphasic. The pH value where influx peaked was found to increase with increasing anion pK, as summarized in Table I. However, these peak pH values did not occur at the pK of each anion. The peak influx for fluorophosphate (pH 5.1) occurred above its pK of 4.7, while the pH values for peak influx of the other titratable analogs occurred below their respective pK values. The magnitudes of the peak influx rates for the phosphate analogs fell into two groups. The “fast” anions, phosphate, fluorophosphate, and hypophosphite (Fig. 2A), had peak influx rates of 650, 450, and 380 mmol/liter of red blood cells/min, respectively. The “slow” anions, phosphate, thiophosphate, and methylphosphonate (Fig. 2B), had peak influx rates of 5.1, 3.9, and 3.5 mmol/liter of red blood cells/min. There was no correlation between the magnitude of the peak influx rates and the pK values of these anions.

**Predicted pH Behavior of Titratable Anions—** At all pH values for which it has been studied, the anion transport system behaves as if it is positively charged, i.e. an anion carrier. However, if one hypothesizes that the anion binding site of the carrier contains at least one additional titratable proton responsible for the activation of hypophosphite and

### FIG. 2

**pH dependence of phosphate analog influx.** A, fast analogs. Fluorophosphate (FP; ○) and PO₃⁻ (●) influxes were biphasic, peaking at pH 5.1 and 5.9, respectively; PO₂⁻ (▲) influx plateaued above pH 7. B, slow analogs. Thiophosphate (SP; ■), PO₄⁻ (□), and methylphosphonate (MeP; ▼) were also biphasic, peaking at pH 5.2, 6.2, and 6.5, respectively. RBC, red blood cell.
CL\(^-\) influx as pH increases, then it is likely that transport is favored when the charge of the anion matches that of the carrier. Therefore, the pH dependence of titratable anion transport might be expected to peak at the pH value at which the anion and carrier have the highest probability of having charges of the same magnitude. The Henderson-Hasselbalch equation predicts for both the anion and the carrier the relative fractions of monoionized and diionized species.

\[
\begin{align*}
\text{pH} &= pK_a + \log(A^-/HA^-) \quad \text{(anion)} \\
\text{pH} &= pK_c + \log(C^-/HC^-) \quad \text{(carrier)}
\end{align*}
\]

Letting \(Ra = (A^-/HA^-)\) and \(Rc = (C^-/HC^-)\), the fraction of anion singly charged \((Fa^-)\) is equal to \(1/(1 + Ra)\), and that fraction doubly charged \((Fa^{2-})\) is \(Ra/(1 + Ra)\). The fraction of carrier singly charged \((Fc^+)\) is \(Rc/(1 + Rc)\) and that fraction doubly charged \((Fc^{2+})\) is \(1/(1 + Rc)\). Setting the sum of all possible combinations of anion and carrier charge equal to unit probability:

\[
1 = (Fa^-Fc^+) + Fa^{2-}Fc^{2+} + Fa^-Fc^{2+} + Fa^{2-}Fc^+.
\]

The term in parentheses represents the two cases where the anion and carrier have equal charge, in which transport may be favored. The probability of equal charge, expressed in terms of the ratios derived from the Henderson-Hasselbalch equation is then

\[
\text{Probability} = (Ra + Rc)/(1 + Ra)(1 + Rc).
\]

Fig. 3 illustrates the probability curves predicted by Equation 4 for anions having \(pK\) values that correspond to those of the phosphate analogs, with a carrier that titrates with a \(pK\) of 5.5. For the monovalent anion \(PO_4\), Equation 4 reduces to the probability that the carrier will have a single cationic charge. This curve is monotonic, with increasing probability as the pH is increased. In contrast, the divalent anions show biphasic probability curves that peak at pH values intermediate between the \(pK\) of the carrier and those of the anions. Although not shown, the curve for a fully divalent anion would be the mirror image of the hypophosphite curve, showing a monotonic decrease with increasing pH. A general equation for predicting the pH values at which these probability curves peak can be derived by differentiating Equation 4:

\[
\frac{d(\text{Probability})}{d(pH)} = 0.
\]

This equation has an exact solution.

\[
\text{pH}_{\text{max}} = (pK_a + pK_c)/2
\]

Therefore, the pH at which there is maximum probability that the anion and the carrier have electric charges of the same magnitude is predicted to be exactly halfway between the \(pK\) of the anion and that of the regulatory site. Table I lists the “predicted” \(pK\) of the titratable carrier site that would give rise to the observed pH peaks for the titratable anions. The predicted \(pK\) lies between 5.35 and 5.6.

**Intracellular pH Changes Associated with Influx**—To determine the intracellular pH response to the influx of the titratable anions, the pH values were measured at the end of the influx incubation periods (“final” pH\(_i\)) and plotted against initial pH\(_i\) (Fig. 4) for several of the phosphate analogs. In each experiment, the initial pH\(_i\) of the packed erythrocytes averaged pH 7.23. The final pH\(_i\) was determined from the \(^31\)P chemical shifts of intracellular phosphate or methylphosphonate, as reported previously (11, 12). Not unexpectedly, influx of the monovalent anion hypophosphite was associated with very minimal changes in pH\(_i\), which increased only slightly at pH\(_i\) values above pH 7. Titratable anion influx was associated with more significant changes in pH\(_i\). Transport of the slow anions, thiophosphate and methylphosphonate, resulted in final pH\(_i\) values that increasedmonotonically with increasing pH\(_i\), with final pH\(_i\) becoming greater than pH\(_i\) above pH 7.5. Transport of the fast anion phosphite resulted in a biphasic pH\(_i\) response, with final pH\(_i\) decreasing as pH\(_i\) increased from 4 to 6.8 and rising as pH\(_i\) increased further. Again, final pH\(_i\) was less than pH\(_i\) above pH 7.4. Although the pH\(_i\) changes observed in Fig. 4 for phosphate influx are generally of the same magnitude as those for the slow anions, thiophosphate and methylphosphonate, the time frame over which the pH changes for phosphate and hypophosphite occurred is approximately 2 orders of magnitude smaller than

![FIG. 3. Probability that anion and carrier have equal charge. These curves depict as a function of pH the probabilities that a carrier having pK 5.5 will have electric charge of the same magnitude as each of the phosphate analogs, as given by Equation 4 of the text. From left to right, the curves correspond to anion pK values of 4.7 (fluorophosphate (FP)); 5.5 (thiophosphate (SP)); 6.4 (phosphate (PO\(_4\))); 6.8 (phosphate (PO\(_4\))) 7.4 (methylphosphonate (MEP)); and a monovalent anion (hypophosphite (PO\(_3\))). The pH corresponding to peak probability is given for each curve and occurs at a pH value midway between the pK of the carrier and the pK of the anion, except for the monovalent hypophosphite, whose probability curve increases monotonically with pH.

![FIG. 4. Intracellular pH changes accompanying influx. Final pH\(_i\) is compared to initial pH\(_i\) for several of the phosphate analog influx studies. Initial pH\(_i\) was 7.2 in each case. The final pH\(_i\) was least affected by hypophosphite influx (■). Methylphosphonate (○), phosphate (△), and thiophosphate (•) influx were associated with a fall in pH\(_i\). at low pH\(_i\), and a rise in pH\(_i\), at high pH\(_i\), values. The dashed line is for pH\(_i\) = pH\(_i\).]
that for thiophosphate and methylphosphonate. Therefore, different factors may operate over the fast and slow time domains in producing the pH changes.

To interpret these pH changes, we sought to relate amount of anion transported to the proton fluxes required to produce the observed final pH, (Figs. 5 and 6). Over the time frame of the influx studies, essentially all of the proton transport across the membrane is linked to anion exchange (13). For a non-titratable anion, the number of protons transported can simply be expressed in terms of the buffering capacity (B) of the intracellular milieu, where

\[
d[H^+] = B \times d(pH).
\]

The net proton gain per millimolar anion transported becomes

\[
d[H^+] / d[A] = B \times d(pH) / d[A]
\]

For fast anions, hypophosphite and phosphite. Over the pH, range of 4–8, the net proton influx per millimolar anion influx was calculated from the final pH, following the influx period, using measured values for intracellular buffering capacity and the pKₐ of the anion studied. The *horizontal dashed line* at 1 meq of H⁺/mM corresponds to a 1:1 ratio of H⁺ to anion, as expected for transport of the monoionized form of a titratable anion. The *horizontal dashed line* at 0 meq of H⁺/mM corresponds to no proton influx, as expected for a monovalent anion or for transport of the diionized form of a titratable anion.

Thus, transport of the anion as the monoionized species should result in a gain of 1 meq of H⁺/mmol of anion, with a fall in pH, of a magnitude that will increase as pH, increases in relation to the pKₐ of the anion. Transport of the anion in diionized form should result in a gain of 0 meq of H⁺/mmol and a rise in pH, that will increase as pH, decreases relative to the anion pKₐ.

The buffering capacity of fresh erythrocytes was determined experimentally by lysing washed, packed erythrocytes in water (cells:water, 1:2) to form a dilute hemolysate (1.62 mM [Hb]). The pH was recorded as small, stepwise additions of 50 mM HCl were added very slowly to prevent denaturation of hemoglobin. Over the pH range 7.4–6.4, the buffering capacity expressed in terms of the [Hb] was 11.7 meq of H⁺/pH/mM, which was equivalent to 58.5 meq of H⁺/pH in the intact cell.

Fig. 5 portrays the net proton change accompanying influx of the fast anions, hypophosphite and phosphite, over the pH, range of 4–8. For hypophosphite, there is very little proton transport observed for pH, values below 6.5. Above this pH, there is an increasing net loss of protons. Because hypophosphite/Cl⁻ exchange does not result in the transfer of protons across the membrane, this loss of H⁺ at higher pH, must result from the exchange of intracellular Cl⁻ for OH⁻ or trace amounts of HCO₃⁻, both of which will be present at higher concentrations as pH, increases. In contrast to hypophosphite, phosphite influx is associated with a net influx of protons, with a magnitude of 0.93 meq of H⁺/mM at pH 4, decreasing very gradually to 0.68 meq/mM at 7.06, and abruptly falling to a net proton loss above pH 7.5. If phosphite influx were completely linked to Cl⁻ efflux, then the observed proton influx would indicate that, at pH 4, 93% of the phosphite is transported in monoionized form, gradually decreasing to 68% at pH 7 and rapidly falling to 0 at about pH 7.5. However, as the hypophosphite curve depicts, there is a net charge transfer not associated with the transport of titratable anions, which increases dramatically above pH 7. The hypophosphate and phosphite curves show a striking parallel dependence on pH, which is maintained almost throughout the entire range of pH, values, so that these two curves are separated by nearly 0.8–1.0 meq of H⁺/mm anion. Because the hypophosphate and phosphite data were accumulated over similar influx time domains (4 s) and their influx rates are of similar magnitude, this parallel behavior suggests that, over the range of pH, values studied, each phosphite anion transported results in a gain of one H⁺ more than the equivalent transport of hypophosphate. Therefore, phosphite is transported predominantly in monoionized form over this entire pH range. The separation between the hypophosphate and phosphate curves does diminish at very high pH, most likely because phosphate influx is decreasing above pH 7 while hypophosphate influx remains constant. Therefore, Cl⁻/OH⁻ exchange is expected to have a greater influence on the
phosphate system than the hypophosphite system as pH, approaches 8.

The pH, changes associated with the slow anions, thiophosphate and methylphosphonate, are remarkably different than those associated with fast anion exchange. As Fig. 6 depicts, during methylphosphonate influx there is an extraordinarily large H+ influx of 5.3 meq/mM at pH 4.2, which decreases monotonically as pH, increases, so that above pH, 7.3 there is a net proton loss. Thiophosphate similarly showed a large proton gain of 3.4 meq/mM at low pH, which decreased monotonically in a fashion parallel to methylphosphonate, so that above pH, 6.5 there is a net loss of protons. At any given pH value, the net proton influx for methylphosphonate was larger than that for thiophosphate. This may reflect the fact that the pK, of methylphosphonate is considerably higher than that for thiophosphate, so that at any given pH methylphosphonate will be protonated to a greater extent than thiophosphate.

There are two major factors which indicate that the analysis applied to the proton changes accompanying fast anion transport does not apply to slow anions. First, the time domain is approximately 2 orders of magnitude slower (3–5 min for methylphosphonate and 5–20 min for thiophosphate); and second, the observed proton gains (and losses) are far greater than can be accounted for by transport of the titratable anions alone. At high pH (above 7.3 for methylphosphonate and above 6.5 for thiophosphate), there is a net loss of protons (or gain in OH–) from the cell. Jennings (8) has observed that during the exchange of intracellular Cl– for the slow anion SO42–, there is a rapid (seconds) drop in pH, (and presumably rise in pH,) followed by a slow rise toward the equilibrium value. He attributed the rapid component of this pH behavior to two factors. First, the suspension of Cl–-containing cells in a Cl–-free medium results in a large positive membrane Cl– potential, which will tend to drive OH– into the cells. Second, the exchange of monovalent anions such as OH– and HCO3– is extremely rapid compared to divalent anions, resulting in a “partial ionic equilibrium” (14), in which [Cl–]o/[Cl–]i = 1. This ratio will approach [OH–]o/[OH–]i on a rapid time scale during which the divalent anion is relatively impermeant. By careful exclusion of CO2, he found that HCO3– was a powerful catalyst of the transient pH, drop. In an unbuffered SO42– solution at pH, 6.85, the pH drop was quite fast but slowed considerably as pH, decreased to 6.05, well before partial ionic equilibrium was reached, due to the low concentration of OH– and HCO3– at this pH. In the absence of CO2, the transient pH, drop was not observed. Instead, a gradual rise in pH, accompanied the slow SO42– influx, due to proton cotransport. In our studies of these slow phosphate analogs, the observed proton egress at higher pH values suggests strongly that partial ionic equilibrium has shifted the final pH, to higher values at the time influx was arrested. At low pH (below 6.0 for methylphosphonate and below 5.6 for thiophosphate), net proton influx exceeds influx of the titratable anion. Because the extracellular [OH–] has become exceedingly low compared to intracellular [OH–], and because Cl– transport decreases at low pH (5), partial ionic equilibrium may result in net OH– efflux, contributing to the intracellular protein gain. Small amounts of CO2 may catalyze this proton flux in the reverse direction of that observed at higher pH by Jennings (8) by diffusing into the cell, and being transported out as HCO3–, where reversion to CO2 would be favored by low pH, Therefore, unlike the fast analogs, the H+ fluxes associated with transport of the slow anions are due in part to partial ionic equilibrium effects and cannot be directly related to the charge on the transported anion under the conditions we used to study influx rates.

The phosphate analogs provide an ideal system for the study of the transport of small anions across the erythrocyte membrane. For example, we have previously found that the influx rates for these compounds vary over 3 orders of magnitude (10). Relative to phosphate at pH, 7, these rates were: phosphite, 306; hypophosphite, 170; fluorophosphate, 17; methylphosphonate, 1.45; phosphate, 1.0; thiophosphate, 0.32; and dimethylphosphonate ((CH3)2PO3–), 0.24. Structural studies indicate that methylphosphonate, all of these anions consist of a five-valent phosphorus atom covalently bonded to four atoms. In each case, these compounds are tetrahedral, with small changes in bond angle or size of the bonded groups. That minor substitutions lead to profound differences in transport rates suggests that either these anions (including their hydration spheres?) are at the limit of the size which can be accommodated by the transport channel or that the channel has extremely high selectivity toward the structure of the anion. The latter possibility is particularly intriguing in that the removal of an oxygen atom from phosphate to form phosphite is accompanied by a 300-fold increase in transport rate, while removal of another oxygen (hypophosphite) has little further effect. Phosphate is the phosphate analog that chemically most closely resembles HCO3–, a natural substrate for the anion exchange channel. Anions with three oxygen atoms may be selectively transported over those with four oxygens.

In the present study, the dependence of the phosphate analog influx rates on pH, was studied under conditions of nearly constant pH,. We found that monovalent hypophosphite influx titrated with an apparent pK of 5.5. Moreover, the titratable anions showed a pH dependence which peaked at pH values that increased as anion pK increased, yet at pH values that were not identical to those pK values. By using the Henderson-Hasselbalch equation to predict the electric charge on the anion and a titratable functional group on the carrier, we found that the pH dependence of titratable anion influx was also consistent with titration of a functional group at about pK 5.5. Therefore, transport of each of these small titratable anions appears to be regulated by the same functional group in the transporter, despite the large differences in peak transport rates among the phosphate analogs.

Several limitations must be considered in the interpretation of these experiments. First, although we attempted to maintain relatively constant pH, by arresting influx at the earliest possible times consistent with our ability to measure the intracellular compounds, we have not attempted to include the changes in pH, in the equations predicting peak transport pH. Therefore, the role for the efflux half-cycle in this experiment has not been explored. This may explain the fact that the pH dependencies of the influxes of these anions do not show the degree of symmetry implied by the curves depicted in Fig. 3. This asymmetry may also be due to the fact that the efflux of Cl–, although not rate-limiting compared with phosphate analog influx rates, is pH-dependent. Next, Equation 4 cannot be used to determine whether the titratable anions are transported exclusively in the monoionized form, diionized form, or both. Equation 4 can be separated into two equations, one describing the probability of the anion carrier both being singly charged and the other describing their doubly charged counterparts.

\[
\text{Probability}(+/-) = \frac{(Ra)}{(1 + Ra)(1 + Rc)} \tag{11}
\]

\[
\text{Probability}(2+/2-) = \frac{(Ra)}{(1 + Ra)(1 + Rc)} \tag{12}
\]

Although the probability at any given pH that the anion and the carrier are both singly charged is not the same as the probability that they are both doubly charged, both Equations...
11 and 12 predict that these probabilities peak at the midpoint between the pH of the anion and that of the carrier, as given by Equation 6. Therefore, this model will not discriminate between transport of titratable anions in the monooxonized and diionized states. The only requirement is that transport is favored when the anion and carrier have matching charges. Divalent ion transport, such as SO$_4^{2-}$, has been shown to be linked to proton cotransport (8), and Brauer et al. (15) have reported from the study of the pH changes that accompany phosphate transport that this anion is transported largely in the monooxonized state. Our study of the pH, changes accompanying transport of the fast anions have shown that phosphate is predominantly transported in monooxonized form for pH values below 7. Above this pH, concurrent Cl$^-$/OH$^-$ exchange renders the pH, changes accompanying phosphate transport more difficult to interpret. However, by comparison with the proton fluxes associated with transport of the monooxonated anion hypophosphite, it is evident that phosphate is transported in monooxonized form over the entire pH range studied.

Under our experimental conditions we could not determine from the proton fluxes associated with the influx of the slow anions, methylphosphonate and thio-phosphate, the ionic state of the transported anion. The longer incubation times required for sufficient anion accumulation to be detected spectroscopically resulted in proton fluxes that exceeded anion transport rates, due to partial ionic equilibrium. It may be that all anion transport results in the transfer of a single negative charge across the membrane, but this has yet to be determined. Indeed, when a titratable anion in diionized form is presented to an outward-facing carrier site that is currently protonated, it may be possible for the proton to be transferred to the anion during transport so that only a single negative charge is transferred to the intracellular milieu. Kaufmann et al. (16) have found that a simple diprotic anion transport model, in which the charge on the carrier determines the charge of the transported species, may display a variety of transport characteristics: anion-proton cotransport, anion-proton countertransport, tightly coupled anion exchange, or simple proton transport.

Because our studies of the pH dependence of the transport of the phosphate analogs were conducted at a single anion concentration, this study does not determine whether the observed pH maxima represent primarily the influence of pH on saturating transport rates (V$_{max}$) or on the concentration dependence (K_a). We have previously found (10) that the K_a for phosphate analog influx at pH 7 depended not upon the net charge on the anion but its chemical structure. For example, the K_a for the divalent phosphorus oxyacids phosphate and phosphite were similar to each other (62 and 59 mM, respectively), yet different from analogs containing P-C bonds (methylphosphonate, 123 mM; dimethylphosphinate, 124 mM). Milianick and Gunn (9) have found that the pH dependence of sulfate-proton cotransport is primarily a V$_{max}$ effect, governed by the protonation state of the carrier, rather than by the small but measurable concentration of the protonated form of sulfate HSO$_4^-$. Therefore, the pH dependence of titratable anion influx may similarly be a V$_{max}$ effect. However, the titratable phosphate analogs are considerably weaker acids than sulfate, and thus these compounds are more protonated than sulfate at any given pH. Further experimentation is needed to determine whether there is a pH dependence of the K_a of the phosphate analogs.

It is interesting to speculate on the nature of the functional group that titrates with an apparent pK of 5.5. Matsuyama et al. (17) have studied the pH dependence of phosphoenolpyruvate transport, under conditions of constant or varying pH, in red cell ghosts. They found that at constant intracellular pH influx increased as pH decreased, whereas in the absence of a transmembrane pH gradient the pH influx curve was bell-shaped. They concluded that a histidyl residue at the inner membrane surface of the anion exchange protein could explain this result. Wieth et al. (18, 19) have found evidence for functional arginyl residues that are deprotonated at pH 12, leading to decrease in Cl$^-$ transport above this pH. At least 2 lysyl residues are involved in the covalent binding of stilbenes that inhibit anion transport (20). Jennings et al. (21) have shown that these lysines are not responsible for translocation of the anion, but at least one lysine is necessary for anion binding. Dansylation of the transport site has been shown to completely eliminate the discrimination between monovalent and divalent anions (22, 23) but apparently does not act at these lysyl residues. In an elegant study using the spin-label stilbene probe NDS-TEMPO, Kaufmann et al. (16) have demonstrated that the substrate site for transport is distinct from the ionizable regulator site. Kopito and Lodish (24) have isolated the cDNA clone encoding the murine anion transport protein and determined the complete nucleotide sequence. From the deduced amino acid sequence, they then found that there are 12 membrane-spanning domains that could potentially form one or two hydrophilic channels. Seven of these helices contain charged and polar groups that may line such channels, although it is not clear whether any histidyl residues are present in these regions. With the known functional arginyl and lysyl residues in these regions, it may be that the functional group that titrates at pK 5.5 is not cationic but rather anionic, such as a carboxyl side group. Such a group may form a salt bridge with cationic residues, and the transport action of the anion exchange protein may be initiated by the breaking of this salt bridge by the approaching anion. This concept has been proposed in recent models of anion transport (19, 25, 26), and Bjerrum (27) has found participation of a carboxyl group in anion transport.

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REFERENCES

NMR Study of Red Cell Membrane Transport