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Characteristics of CO_2-Independent pH Equilibration in Human Red Blood Cells

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Summary. The rate of dissipation of a pH gradient across the red cell membrane has been measured at pH 5.7–7.5 in a medium free of CO_2 and other penetrating acids or bases. The measured rates and extents of pH movements are influenced only slightly by valinomycin-induced changes in the membrane potential. This indicates that the primary process involved is electrically silent OH^-/Cl^- exchange or H^+/Cl^- cotransport. This electrically silent pH equilibration has several characteristics which suggest the involvement of the red cell anion exchange protein.

1. It is strongly inhibited by phloretin and DIDS (4,4′-diisothiocyano-2,2′-stilbenedisulfonic acid).

2. The rates of pH equilibration depend on the halide present in the medium, the relative rates being 100, 18, and 2 in NaCl, NaBr, and NaI media, respectively.

3. The pH equilibration has apparent activation energies of 27 kcal/mol at $T < 13\,^\circ C$ and 16 kcal/mol at $T > 13\,^\circ C$.

The pH dependence of the equilibration rate, however, is much more consistent with H^+/Cl^- cotransport than with OH^-/Cl^- exchange; the rate increases steeply with the H^+, rather than the OH^- concentration. It is suggested therefore that the transport event is H^+/Cl^- cotransport, but that this transport is mediated by the membrane protein that catalyzes anion exchange.

The early work of Warburg (1922) and Van Slyke, Wu and McLean (1923), and subsequent studies in several laboratories (*see* Funder & Wieth, 1966), have established that hydrogen ions and small anions such as Cl^-, HCO_3^-, and OH^- are distributed passively across the red cell membrane. That is, the intra- and extracellular concentrations of these ions conform very nearly to a Gibbs-Donnan equilibrium distribution[1]:

$$Cl_i^-/Cl_o^- = HCO_{3i}^-/HCO_{3o}^- = OH_i^-/OH_o^- = H_o^+/H_i^+. \qquad (1)$$

1 In the notation used here the subscripts i and o denote, respectively, intracellular and extracellular.

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The red cell as a whole, of course, is not at equilibrium with its surrounding medium; for example, the maintenance of a steady distribution of Na^+ and K^+ requires metabolic energy. However, for H^+ and small anions, the relationship (1) is applicable, independent of the availability of metabolic energy. The red cell may be said, therefore, to be at "pH equilibrium".

Although the equilibrium status of H^+ and OH^- in red cells is well documented, much less is known about the rate of transport of these ions. A conventional tracer flux measurement, under conditions of no net change in cell contents, is impossible for these ions because of rapid exchanges of tritium among H_3O^+, OH^-, and H_2O. To attempt to study OH^- and/or H^+ transport, therefore, one must perform a net flux experiment. For example, the equilibrium represented by Eq. (1) may be perturbed by an extracellular acid addition, and the rate of establishment of a new equilibrium measured. If the system is free of CO_2 and other penetrating acids and bases, this pH equilibration must result from OH^- transport, H^+ transport, or some combination of them both.

The only extensive study of red cell pH equilibration in a low CO_2 medium was performed by Crandall, Klocke and Forster (1971), who assumed that OH^- is the transported species and that the OH^- flux may be described by a constant field treatment. Since the time of the Crandall *et al.* experiments, however, it has been established that inorganic anions cross the red cell membrane mainly by way of an electrically silent exchange mechanism (*see* Sachs, Knauf & Dunham, 1975). It is not clear, therefore, that the constant field equation provides an adequate description of OH^-/Cl^- exchange.

The present paper reexamines CO_2-free pH equilibration in human red cells with the goal of addressing the following two questions:

1. Is the pH equilibration the result of current-carrying ion transport or an electrically silent pathway?

2. Is the pH equilibration catalyzed by the red cell anion exchange protein (Cabantchik & Rothstein, 1974; Passow *et al.*, 1974/1975)?

Finally, the possibility that the transported species is H^+, rather than OH^-, is discussed.

Materials and Methods

Acid and Base Pulse Procedure

Human blood drawn into heparin or acid-citrate-dextrose was used after at most 4 days of storage at 4 °C. No significant effect of length of storage was observed. Red cells

were washed four or five times at room temperature in three or more volumes of one of the following media: 154 mM NaCl, 154 mM NaBr, 155 mM NaI. Cells were then suspended in 60–75 ml of the washing medium at a final hematocrit of 0.6–2.4%. The medium was buffered with a mixture of glycyl glycine, histidine, and citrate, in proportions chosen by trial and error to minimize the pH dependence of the buffer capacity over the pH range of a given set of experiments (concentration range of each is 30–1000 μM). In some experiments a red cell lysate, prepared by freezing and thawing red cells washed thoroughly in unbuffered NaCl, was used as the buffer, with results indistinguishable from those with the nonlysate buffers. Prior to the addition of the cells, the medium had been purged of CO_2 by vigorous bubbling of N_2 at low pH (pH 5.8–6.5) for at least 15 min. This treatment removes at least 99% of the atmospheric CO_2, based on pH changes in an unbuffered NaCl solution (Jennings, 1976). The residual total CO_2 content was not measured but is believed to be less than 1 μM. After addition of the cells, nitrogen was bubbled gently through the suspension for at least 30 min before the start of the first experiment. Subsequently, either the N_2 bubbling was continued, the chamber was closed, or a steady stream of N_2 over the suspension was maintained. Some lysis (usually <2%) resulted from the N_2 treatment, but the lysis had no effect on the transport other than to increase slightly the extracellular buffer capacity.

The chamber containing the cell suspension was constructed of polymethylmethacrylate (Lucite) and was surrounded by a jacket for temperature control. The top of the chamber was equipped with holes for N_2 inlet and outlet, pH electrode, and thermometer. The pH electrode was either a Corning combination electrode 476050 or a Radiometer combination electrode GK2321C. The time required for 90% response to a step change in pH was 3–5 sec. A single experiment consisted of adding a 2–10 μl pulse of 1 N NaOH or HCl (H_2SO_4 was used in the NaBr and NaI media), with continuous measurement of the extracellular pH.

Determination of Buffer Capacities

The intra- and extracellular buffer capacities were determined as follows. The pH trace was extrapolated to the time of the acid or base addition. The extrapolated pH was subtracted from the initial pH to obtain the change in extracellular pH resulting from the known amount of acid or base added. The extracellular buffer capacity (μeq/pH unit) so measured was consistently 2–10% greater than the sum of that of the suspending medium plus that resulting from the small amount of lysis. The small discrepancy may result from buffering by lactate and phosphate leaking from the cells (Crandall *et al.*, 1971) or from extrapolation errors resulting from the finite response time of the system. In any case, errors in the extracellular buffer capacity are at most 10%. The intracellular buffer capacity was estimated from the difference in the equilibrium extracellular pH before and after the acid or base addition, assuming that for each 0.1 unit change in the equilibrium extracellular pH, the equilibrium intracellular pH changes by 0.09 units (Duhm, 1972, 1976). The intracellular buffer capacity B_i is then calculated from the intracellular pH change and the known amount of acid or base which has entered the cells. Errors in B_i are about 5–10% but have only a very minor effect on the normalized rate constants since B_i is much larger than B_o and the buffer capacities add in the reciprocal (*see Results*). In the normalization of the rate constants, the "number of ml red cells", N, does not refer to the actual volume of the cells, which is pH dependent, but rather the dry weight of the cells divided by the dry weight of 1 ml red cells of normal volume. To calculate the parameter k_{OH}, the intracellular Cl^- concentration was calculated from the Cl^- ratios determined by Gunn, Dalmark, Tosteson and Wieth (1973), corrected to 22 °C by shifting the pH axis 0.4 units (Dalmark, 1976*a*).

Inhibitors and Valinomycin

In the phloretin experiments, the phloretin (K & K Laboratories, Plainview, N.Y.) was added from a 100 mM stock in 1 N NaOH (prepared immediately prior to use). The pH was allowed to equilibrate after the phloretin addition, before an acid pulse was applied. The cells treated with DIDS (a generous gift from Dr. P.A. Knauf) were incubated 30 min at pH 7.4, 37 °C, with 10 μM DIDS in HEPES-NaCl buffer, and washed twice in the same buffer, containing 0.5% albumin to remove the unreacted DIDS. The valinomycin (Calbiochem) was added from an ethanol stock solution to a final concentration of 0.45 μM and 0.1% ethanol. Cellular K^+ contents were determined by flame photometry.

Chloride Gradient Experiments

Red cells were washed 4 times in 154 mM NaCl, titrated with 1 N HCl after the first wash to a desired equilibrium extracellular pH, and then CO_2 was removed by bubbling nitrogen through a 10–20% suspension for 30 min. The extracellular pH of this suspension was then measured under N_2, the cells centrifuged, and the supernatant removed. The cells were resuspended at a 0.5–2.0% hematocrit in a CO_2-free medium of lowered ionic strength, e.g., 50 mM KCl, 200 mM sucrose. The medium was weakly buffered with citrate-histidine-glycyl glycine mixtures at the equilibrium pH of the cells in the 154 mM NaCl. The medium also contained 0.1% ethanol, with or without 0.45 μM valinomycin. Ethanol-free controls showed that 0.1% ethanol has no significant effect on the transport. The extracellular pH of the medium was monitored continuously before and after the addition of the cells. The membrane potentials in the presence of valinomycin were calculated from the constant field equation, using $P_K/P_{Cl}=20$ (from interpolation of data in Knauf *et al.*, 1977, and extrapolation of data of Kaplan and Passow, 1974, to higher valinomycin concentrations).

Results

Figure 1 (left) shows the time course of the extracellular pH during typical acid and base pulse experiments. In both cases, red cells are initially at Donnan equilibrium ($dpH/dt<0.02$/hr) with a weakly buffered NaCl medium, CO_2-free, at the indicated pH. The buffer capacity of the cell contents is about 5 times that of the medium. The addition at $t=0$ of 8 μmoles HCl or NaOH changes the extracellular pH by about 0.5 units, thus creating a pH gradient across the membrane. As this gradient is dissipated, the extracellular pH returns to a new equilibrium value, which differs from the original equilibrium pH by about 0.1 units.

The shape of the pH traces cannot be described by a simple mathematical equation. This is not surprising, since in the buffered medium the relation between the net flux of OH^- or H^+ and the rate of change of OH^- and H^+ concentration is logarithmic rather than linear. However, once the pH is within about 0.1 units of the final value, the pH trace is

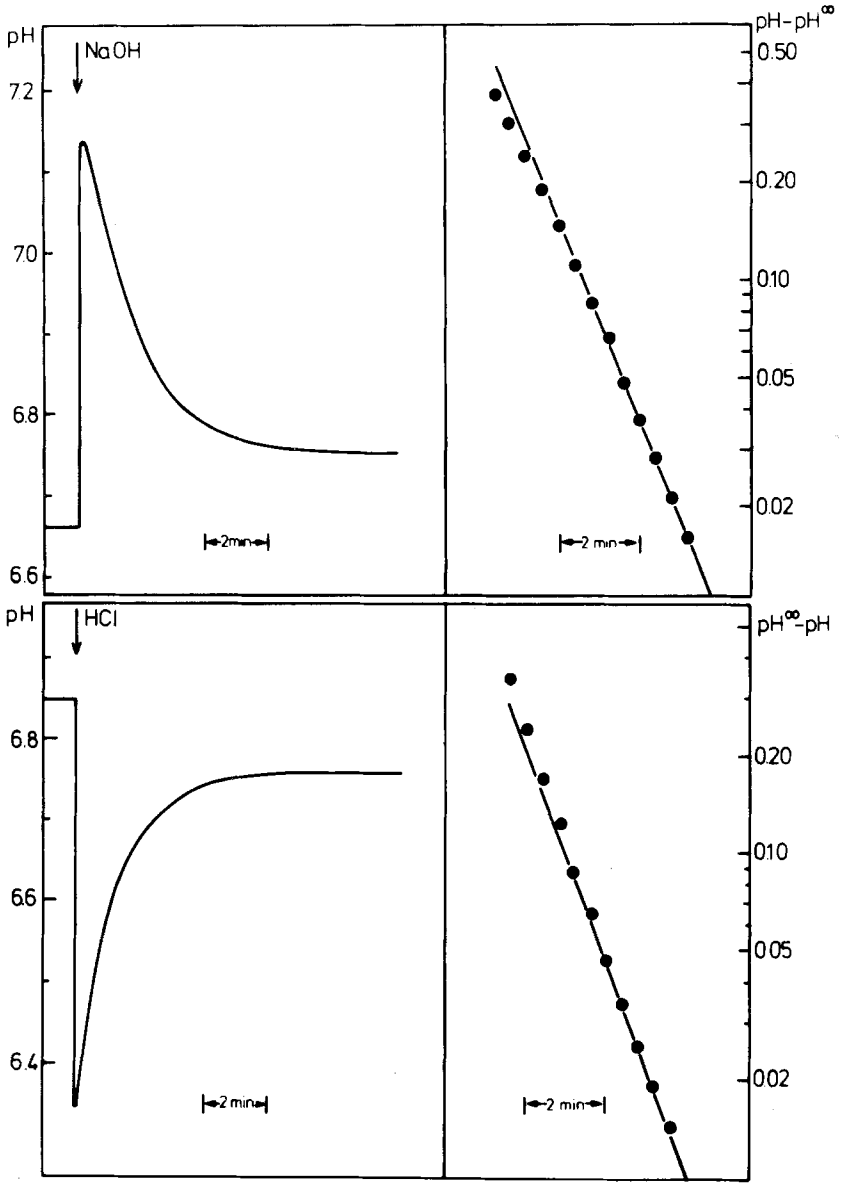

Fig. 1. *Left:* Time course of the extracellular pH for red cells in a CO_2-free 154 mM NaCl medium, hematocrit 2%, 22 °C. The medium is buffered with 200 μM histidine, 100 μM citrate, and 800 μM glycyl glycine. At the indicated times, 8 μl 1 NaOH *(above)* or 1 N HCl *(below)* were added to 75 ml of suspension. *Right:* A plot of $|pH - pH^{\infty}|$ *vs.* time in semi-log coordinates, using pH values obtained by eye from the traces on the left

not distinguishable from a single exponential (Fig. 1, right). Results of a given experiment, near equilibrium, may then be characterized by a single rate constant (sec^{-1}), obtained from semi-log plots such as those in Fig. 1, for pH values between 0.08 and 0.01 units from equilibrium.

The rate constant for the final approach to equilibrium has been chosen as the measured parameter because it may be measured more accurately than the time-dependent flux of OH^- or H^+ at a given extracellular pH. The real information of interest, however, is the relationship between the net flux of OH^- (or H^+) and the driving force for the transport. It is necessary, therefore, to derive the relationship among the measured rate constant, OH^- flux and driving force for OH^- transport. (Since red cell anion exchange is several orders of magnitude faster than cation transport, the working hypothesis in the following development is that OH^- rather than H^+ is the transported species.)

The most general approach to the problem of driving forces is by way of nonequilibrium thermodynamics. A nonequilibrium thermodynamic treatment of OH^-/Cl^- exchange *(Appendix)* shows that the net flux (μeq/ml cells · sec) of OH^- may be written:

$$J_{OH} = k_{OH}(OH_i^- Cl_o^- - OH_o^- Cl_i^-) \tag{2}$$

where k_{OH} is a proportionality factor defined in a way analogous to conventional definitions of permeability coefficients (units of k_{OH} are (μeq/ml cells · sec) $(mM)^{-2}$ when the concentration units are mM). Equation (2) says simply that there is zero net flux when $OH_o^- Cl_i^- = OH_i^- Cl_o^-$, and that the OH^- flux should increase as the OH^- concentration gradient increases. The exact meaning of k_{OH} depends on the nature of the transport. If OH^- is transported as a charged species, the constant field equation for J_{OH}, with the assumption that $V_m = (RT/F)\log(Cl_i^-/Cl_o^-)$, may be written in the form of Eq. (2), with $k_{OH} = P_{OH} A \log(Cl_i^-/Cl_o^-)/(Cl_i^- - Cl_o^-)$, where P_{OH} is the OH^- ionic permeability (cm/sec) and A is the membrane area (cm^2/ml cells). If the transport is by way of an electrically silent "carrier" system, the factor k_{OH} will be proportional to the number of carriers/ml cells times a turnover number for the OH^- transport event. Further, k_{OH} will depend on the affinity of sites on the carrier for OH^-, and also on the Cl^- concentration and affinity. Of course, k_{OH} (or P_{OH}) may also depend on the pH. However, over the limited pH range in which the rate constant is measured, k_{OH} should be fairly constant.

The empirically measured rate constant k is a function of k_{OH}, the number of cells present, and the intra- and extracellular buffer capacities

(Appendix):

$$k = 2.3\, k_{OH} Cl_i^{\infty} OH_o^{\infty} N(1/B_i + 1/B_o), \tag{3}$$

where the superscripts ∞ refer to the equilibrium concentrations, N is the number of ml cells present and B_i and B_o are, respectively, the intra- and extracellular buffer capacities (μeq/pH). At a given pH and Cl^- concentration, the rate constant is proportional to $N(1/B_i + 1/B_o)$, as shown in Fig. 2. That the expected relationship holds indicates that:

1. The buffer, in this case a mixture of glycyl glycine (200–1065 μM), histidine (100–533 μM), and citrate (25–133 μM), neither accelerates nor inhibits the transport processes responsible for pH equilibration.

2. The system behaves as a simple two-compartment (cells and medium) system.

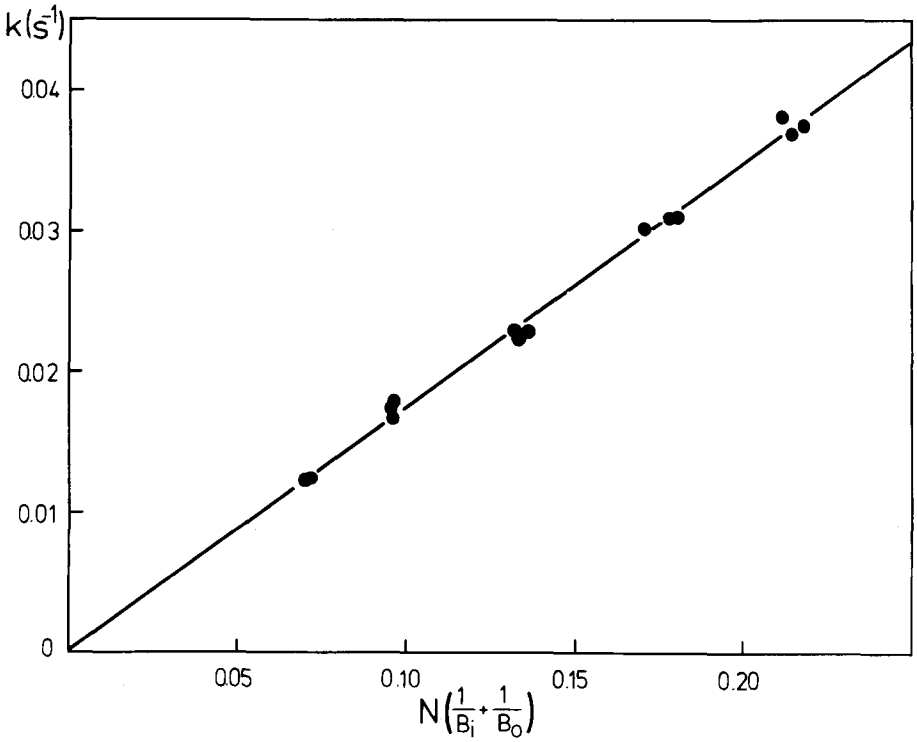

Fig. 2. *Abscissa:* $N(1/B_i + 1/B_o)$, where N is the number of ml red cells present and B_i and B_o are, respectively, the intracellular and extracellular buffer capacities (μeq/pH unit). *Ordinate:* The measured rate constant (sec^{-1}) for equilibration to pH 6.33 after a base pulse in CO_2-free 154 mM NaCl, 22 °C, 2% hematocrit. The extracellular buffer capacity was varied by adding increments of a concentrated 8:4:1 mixture of glycyl glycine, histidine, and citrate. The regression line (shown) is $k = 0.00011 + 0.174\, N(1/B_i + 1/B_o)$

In order to present the results in a way that is independent of hematocrit and buffer capacity, the measured rate constant k is divided by $N(1/B_i+1/B_o)$. This quantity $\left[\frac{k}{N(1/B_i+1/B_o)}\right]$ is referred to below as the "normalized rate constant" for equilibration to a particular extracellular pH, and is equal to the flux (μeq/ml cells·sec) divided by the total "distance from pH equilibration": $(pH_o-pH_o^\infty)-(pH_i-pH_i^\infty)$, for small departures from equilibrium.

Dependence of the Rate on pH; Demonstration of CO_2-Independence

Figure 3 is a plot of the normalized rate constant

$$\frac{k}{N(1/B_i+1/B_o)}$$

vs. the equilibrium extracellular pH, for red cells in a CO_2-free Cl^- medium, 22 °C. The rate is a sharply decreasing function of pH. In the pH range 6.5–7.4, the normalized rate constant decreases by a factor of

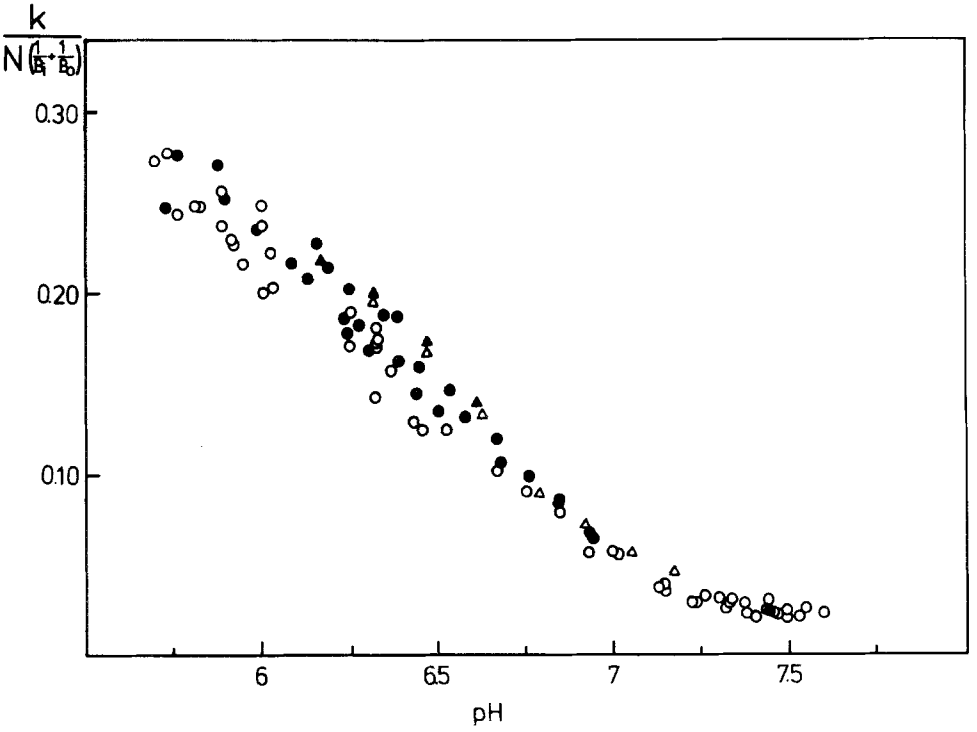

Fig. 3. Plot of normalized rate constant (μmoles/ml cells·sec·pH unit) *vs.* the equilibrium extracellular pH in 154 mM NaCl, 22 °C. *Open symbols:* base pulse. *Closed symbols:* acid pulse. The triangles refer to experiments conducted in a valinomycin-containing 154 mM KCl medium *(see text)*

nearly two for each 0.3 unit increase in the equilibrium pH. Below 6.5, the pH dependence is not as steep; the rate is roughly twofold larger at pH 5.7 than at 6.5. For a given equilibrium pH, the rate is quite similar for acid (closed symbols) and base (open symbols) pulse experiments. This indicates that, near equilibrium, the pH equilibration pathway shows no major rectification.

It is important to demonstrate that the pH equilibration measured here is not influenced by traces of CO_2, since CO_2 is an efficient carrier of OH^- by way of the Jacobs-Stewart cycle (Jacobs & Stewart, 1942; Scarpa, Cecchetto & Azzone, 1970; Cousin, Motais & Sola, 1975; Deuticke, 1972). The "CO_2-free" suspensions have a calculated total CO_2 content less than 1 μM, but even 1 μM is comparable to or larger than the H^+ and OH^- concentrations. However, the following facts argue that the "CO_2-free" pH equilibration experiments are indeed independent of CO_2 and HCO_3^-:

1. The residual CO_2 content should be an increasing function of pH. The rate constant, however, decreases sharply with pH (Fig. 3); the rate is smallest when the CO_2 contamination is expected to be largest.

2. The normalized rate constants are reasonably reproducible (extent of data at a given pH is at *most* ±20%), independent of the length of time of N_2 treatment, beyond the minimum described in Methods. If the remaining CO_2 were an important factor, the results would be expected to vary with time of N_2 treatment.

3. The normalized rate constants do not depend significantly on the hematocrit, over the fourfold range (0.6–2.4%) studied, even though residual CO_2 likely increases with increasing hematocrit, because of CO_2 added with the cells. The lack of dependence on hematocrit also indicates that the transport of other intermediary metabolites (e.g., lactate, phosphate) does not contribute measurably to the rate, even though these metabolites may contribute slightly to the extracellular buffering (Methods).

It may be concluded that the experiments in Fig. 3 are related to OH^- and/or H^+ transport, rather than CO_2, HCO_3^-, other metabolites, or the buffers.

Role of the Membrane Potential

The pH equilibration could result from OH^- (or H^+) ionic transport, or from an electrically silent system (OH^-/Cl^- countertransport or

H^+/Cl^- cotransport). In the former case, the flux of OH^- or H^+ is determined by the intra- and extracellular activities of these ions and by the membrane potential. In the case of electrically silent transport, the flux should depend mainly on the OH^-, H^+, and Cl^- activities; any effect of the membrane potential would be expected to be indirect, and relatively minor. Electrically silent transport may be distinguished from current-carrying transport by comparing experiments in which the pH and Cl^- gradients are the same, but in which the membrane potential is altered with valinomycin. In the presence of as little as 10^{-7} M valinomycin the membrane K^+ conductance is considerably larger than the Cl^- conductance (Kaplan & Passow, 1974). As a result, the membrane potential approximates the K^+ equilibrium potential, at least for extracellular K^+ concentrations greater than about 25 mM (Hoffman & Laris, 1974). By varying the extracellular K^+ concentration in the presence of valinomycin, it is therefore possible to produce large variations in the membrane potential.

Figure 3 (triangles) contains the results of several acid and base pulse experiments in a CO_2-free 154 mM KCl, 30 mM sucrose medium, plus 0.45 μM valinomycin. Under these conditions the membrane potential is roughly 5–10 mV (inside positive), since the extracellular K^+ slightly exceeds the intracellular K^+. Clamping the membrane potential at this value clearly has no effect on the rate constants for pH equilibration.

Figure 4 (upper) is a continuous record of the extracellular pH in a lower K^+ medium for two acid and two base pulse experiments, one each in the presence and absence of valinomycin. The medium is weakly buffered 123 mM NaCl, 31 mM KCl, CO_2-free. Initially, the pH is stable at 7.00; an acid and then a base pulse are given, with the usual results. At the time indicated, 0.45 μM valinomycin is added, and K^+ and Cl^- begin to leave the cells (Fig. 4, lower), the K^+ as a charged complex with valinomycin, the Cl^- by way of the "conductance pathway" (*see* Sachs *et al.*, 1975). The valinomycin addition changes V_m from about -4 to -26 mV, and the new "equilibrium" extracellular pH defined by the membrane potential is roughly 7.35, since at $pH_o = 7.35$, the condition $(RT/F)\log(H_o^+/H_i^+) = V_m$ would obtain. In the early stages of the Cl^- loss, K^+, Cl^-, and water leave the cells at about the same rate, so the Cl^- concentration ratio is changed only slightly by the valinomycin. The "equilibrium pH" defined by the condition $Cl_i^-/Cl_o^- = OH_i^-/OH_o^-$ is therefore still about 7.0. After an acid pulse, the pH rapidly returns toward 7.0, just as before the valinomycin addition, even though at pH 7 there is still a large *electrical* driving force for a further pH rise. After the

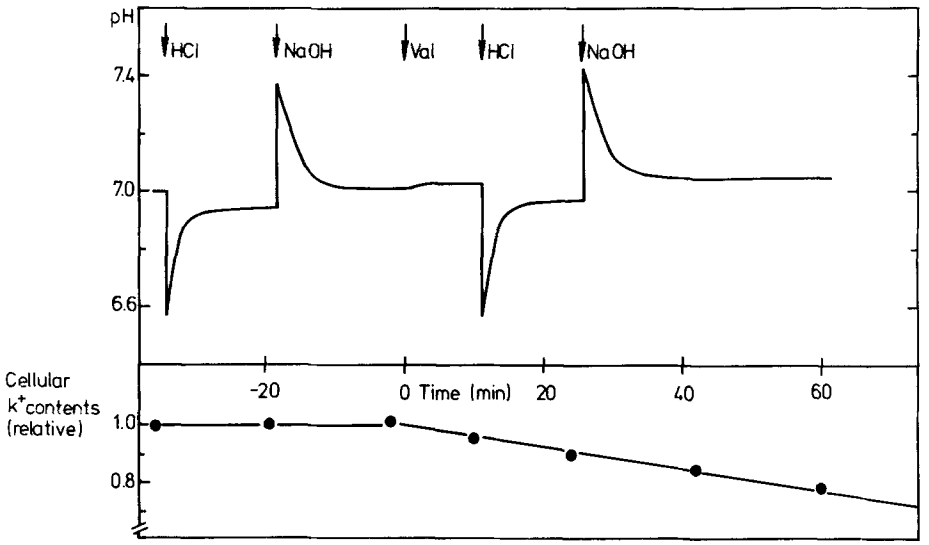

Fig. 4. *Upper:* pH trace for acid and base experiments before and after valinomycin addition. Valinomycin: 0.45 μM, ethanol: 0.1 %, 123 mM NaCl, 31 mM KCl, 660 μM glycyl glycine, 200 μM histidine, 100 μM citrate, 22 °C, 2 % hematocrit, CO_2-free. *Lower:* Cellular K^+ contents (relative to that before the valinomycin) for 1-ml samples of the suspension

base pulse, when the pH is 7.35, there is zero electrochemical driving force for pH drop because at this pH the calculated membrane potential equals the equilibrium potential for OH^- and H^+. Still, the pH drops toward 7.0, with a time course very similar to the corresponding experiment in the absence of valinomycin. The cells and medium, therefore, tend toward the situation $Cl_i^-/Cl_o^- = OH_i^-/OH_o^-$, with little effect of the membrane potential. In thirteen acid and base pulses in low K^+ (15–30 mM), valinomycin-containing media, pH 6.18–7.05, the normalized rate constants are indistinguishable from those in Fig. 3. The rate and extent of extracellular pH changes therefore are determined primarily by the pH gradient and Cl^- concentration ratio; the membrane potential has only a minor effect.

The relative importance of Cl_i^-/Cl_o^- and of V_m in determining OH^- or H^+ movements in red cells may be examined further by an experiment in which a Cl^- gradient, rather than a pH gradient, is imposed. Red cells are washed and equilibrated with 154 mM NaCl, CO_2-free at a given extracellular pH. These cells are resuspended in a low Cl^- medium (e.g., 50 mM KCl, 200 mM sucrose), buffered weakly at the same pH as the 154 mM NaCl medium. Initially, there is very little pH gradient, but a sizable Cl^- gradient. This Cl^- gradient produces an extracellular pH

drop, the extent of which is determined by the size of the Cl^- gradient. The rate of pH drop, for a given extracellular Cl^-, number of cells, and buffer capacity, is about 3.5-fold larger at pH 6.15 than at pH 7.1, i.e., the response to a Cl^- gradient shows very nearly the same pH dependence as the response to a pH gradient (Fig. 3).

The effect of the membrane potential on this Cl^- gradient-induced pH drop is quite small, as shown in Fig. 5. In this case, cells were initially at equilibrium with 154 mM NaCl, pH 6.96, CO_2-free, and are resuspended in weakly buffered, CO_2-free, 50 mM KCl, 200 mM sucrose, pH 6.96, in the presence (right) and absence (left) of 0.45 μM valinomycin. In the absence of valinomycin, if the membrane potential is given by the Cl^- gradient, V_m is about +24 mV (inside positive). In the presence of valinomycin, V_m is calculated to be about −15 mV, so if the pH movements resulted from ionic OH^- or H^+ transport, the pH should rise when the cells are added. However, not only does the pH drop, but the initial rate of change of pH and the extent of the pH drop are only slightly smaller in the presence of valinomycin. Therefore, only a very small fraction of the OH^- or H^+ flux which causes the pH drop involves charge transport across the membrane. In twelve pairs of Cl^- gradient experiments, pH 6.0–7.1, at extracellular K^+ between 0 and 50 mM,

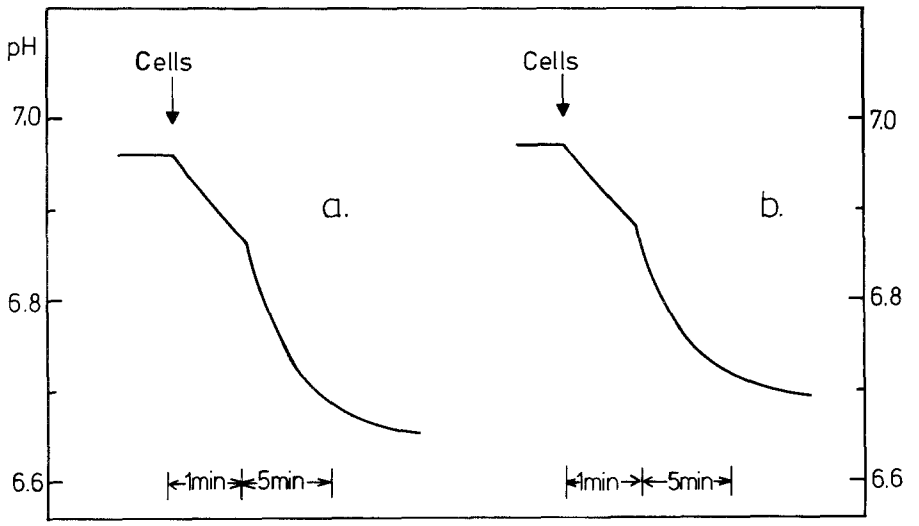

Fig. 5. Time course of extracellular pH of red cells, originally equilibrated with CO_2-free 154 mM NaCl, pH 6.96, and resuspended (at time indicated) in 50 mM KCl, 200 mM sucrose, 0.1 % ethanol, CO_2-free, pH 6.96, buffered as in Fig. 4. Valinomycin (0.45 μM) is present in the experiment on the right. Final hematocrit 1.2 %, 22 °C. The break in each trace (roughly 1 min after the cell addition) resulted from changing the chart speed

valinomycin reduces the initial rate of pH change by 0–21% (average 9%). Although this effect is significant, it is difficult to calculate with any precision the fraction of the pH equilibrating OH^- or H^+ flux which carries current, since such a calculation depends on difference formation. However, a conservative upper limit of this fraction is 10%, in the pH range studied. Therefore, over 90% of the OH^- or H^+ flux is by way of an electrically silent pathway which tends to make $OH_i^-/OH_o^- = Cl_i^-/Cl_o^-$.

Effect of Phloretin and DIDS on CO_2-free pH Equilibration

The electrically silent pH equilibration pathway is strongly inhibited by both phloretin and DIDS, as shown in Fig. 6. The effect of phloretin, measured at pH 6.2, on the pH equilibration rate is half maximal at about 3.3 μM. A double reciprocal plot of inhibition *vs.* phloretin concentration (not shown) is a straight line, with maximal inhibition not distinguishable from 100%. The CO_2-free pH equilibration therefore has a common feature with several "facilitated transport" pathways in the red cell membrane, in that they are all inhibited by low phloretin concentrations. These include the pathways for Cl^- self-exchange (Wieth *et al.*, 1973), glucose (LeFevre, 1961) and urea (Macey & Farmer, 1970).

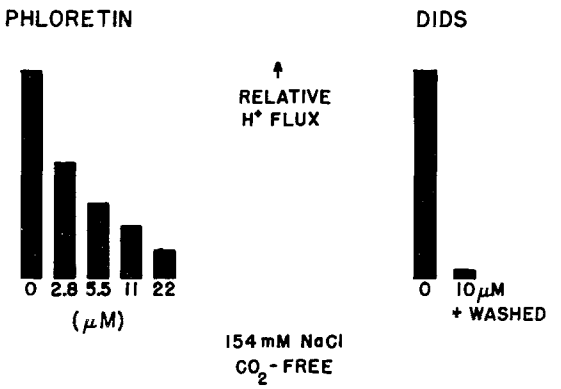

Fig. 6. *Left:* Relative rate of CO_2-free pH equilibration for red cells in the presence of varying concentrations of phloretin, 154 mM NaCl, 22 °C, lysate buffered. *Right:* Relative rate of pH equilibration after an acid pulse for cells treated with DIDS, compared with control cells. The extent of inhibition of H^+ transport was estimated from the slopes of the pH traces (2 experiments) at extracellular pH values of 5.8–6.5, intracellular pH near 7.2. 154 mM NaCl, 22 ± 2 °C, unbuffered, CO_2-free

DIDS, a potent, specific inhibitor of red cell anion exchange (Cabantchik & Rothstein, 1974) is also a potent, irreversible inhibitor of the CO_2-free pH equilibration (Fig. 6, right). The equilibration rate is so slow for DIDS-treated (10 μM, 30 min, 37 °C, then albumin washed) cells that accurate rate constants could not be obtained. It is estimated that the inhibition of pH equilibration by DIDS is greater than 96%. The effects of DIDS and phloretin are consistent with the involvement of the red cell anion exchange protein in the CO_2-free pH equilibration.

Halide Dependence of pH Equilibration

The rate of CO_2-free red cell pH equilibration is strongly influenced by the halide present in the cells and medium. If Br^- replaces Cl^- both inside and outside the cells, the rate constant for equilibration is much slower. The rate constant has roughly the same pH dependence as that in the Cl^- medium, although a more limited range was examined. At pH 6.1–6.3, the rates are 16–19% (range, 9 experiments), and at pH 6.7–6.93, the rates are 18–23.5% (range, 8 experiments) of those in the NaCl medium. In a NaI medium, the rates are slower still, and accurate rate constants could not be obtained at 22 °C. At 35 °C, the pH dependence (data not shown) of the rate constant is similar, but not quite as steep as for NaCl and NaBr, and the rate constants are only 2–3% of those in NaCl at 35 °C. It is interesting to note that the relative rates of pH equilibration in Cl^-, Br^-, and I^- media (100:18:2) are approximately the same as the relative rates of self-exchange of Cl^-, Br^-, and I^- (comparing data in Tosteson, 1959; Dalmark & Wieth, 1972; Brahm, 1977).

Temperature Dependence

Figure 7 is a plot of the natural logarithm of the normalized rate constant for pH equilibration *vs.* $1/RT$, for equilibrium pH 5.9 ± 0.1. The plot is not a straight line. One interpretation of the data is that there are two straight line regions, with a discontinuity in the slope at about 13 °C. Above this temperature $E_A = 16$ kcal/mole, and below 13 °C, $E_A = 27$ kcal/mole. The quality of the data, however, is not sufficient to distinguish between an abrupt break in the plot at 13 °C, or a continuous change in slope between 5 and 15 °C. In any case, it is clear that the

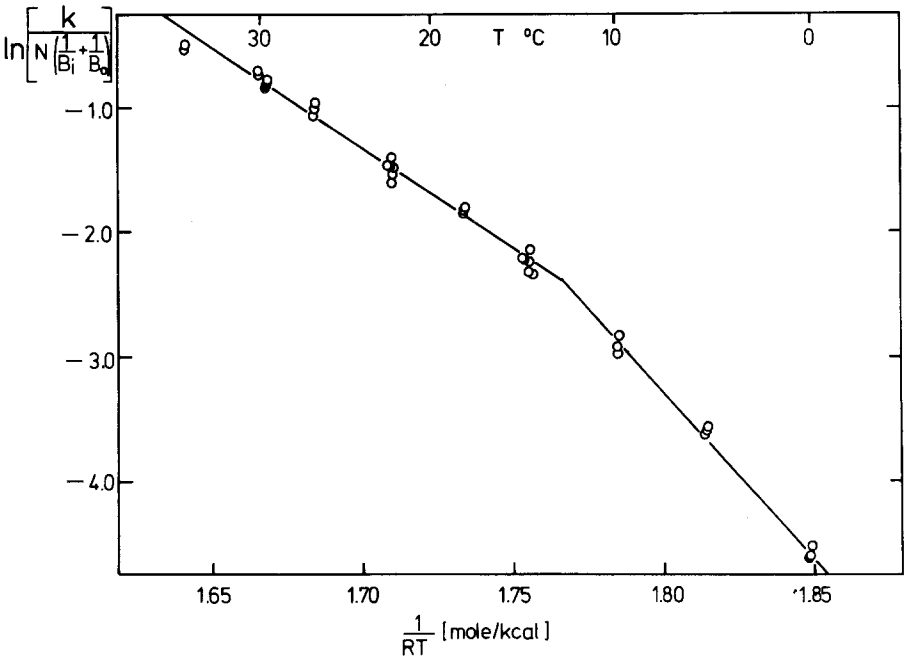

Fig. 7. Arrhenius plot of red cell pH equilibration rate. *Ordinate:* Natural logarithm of the normalized rate constant for equilibration to pH 5.9 ± 0.1 after a base pulse. *Abscissa:* $1/RT$ where R is the gas constant and T is the absolute temperature. Medium is CO_2-free, 154 mM NaCl, buffered weakly with histidine and citrate. The two lines, drawn by eye, have slopes of 16 and 27 kcal/mole

activation energy is quite high at low temperatures and more moderate at $T > 15\,°C$. This temperature dependence is very similar to that recently reported by Brahm (1977) for Cl^- self-exchange, and by Chow, Crandall and Forster (1976) for HCO_3^-/Cl^- exchange in human red cells.

Discussion

Is the Transport Event OH^-/Cl^- Exchange or H^+/Cl^- Cotransport?

The above described experiments show that CO_2-free pH equilibration in human red cells has several characteristics in common with the anion exchange pathway:

1. Both processes are primarily electrically silent.
2. Both are strongly inhibited by phloretin and DIDS.

3. The relative rates of pH equilibration in Cl^-, Br^-, and I^- media parallel the relative rates of Cl^-, Br^-, and I^- self-exchange.

4. The temperature dependence of pH equilibration in a NaCl medium is quite similar to that of Cl^- self-exchange.

These similarities suggest that the pH equilibration is catalyzed by the membrane protein that is believed to be involved in red cell anion exchange (Cabantchik & Rothstein, 1974; Passow *et al.*, 1974/5; Ho & Guidotti, 1975). It is therefore reasonable to propose that the equilibration indeed results from an anion exchange, i.e., OH^-/Cl^- exchange, rather than H^+/Cl^- cotransport. It is of interest to examine whether such a proposal is consistent with the experimental data.

The data in Fig. 3 show that pH equilibration is over 12 times faster at pH 5.8 than at 7.5. That is, for a given small pH gradient, e.g., 0.1 units from equilibrium, the apparent OH^-/Cl^- exchange flux is over 12 times *larger* at pH 5.8 than at pH 7.5. However, for the same pH gradient, the OH^- gradient ($OH_o^- Cl_i^- - OH_i^- Cl_o^-$) is nearly 40 times *smaller* at pH 5.8 than 7.5. In terms of Eq. (2), this means that the parameter k_{OH} must be almost 500 times larger at pH 5.8 than at 7.5, if OH^-/Cl^- exchange is responsible for the transport. This pH dependence is not only extremely large, but is also in the *opposite direction* from that of the self-exchange of other monovalent anions in human red cells (Gunn *et al.*, 1973; Passow & Wood, 1974). For example, the Cl^- self-exchange flux at 0 °C is about fivefold smaller at pH 5.8 than at 7.5 (Gunn *et al.*, 1973). There is no reason to expect that k_{OH} should not depend on pH. However, a 500-fold, reversible, increase in k_{OH} over only 1.7 pH units is very difficult to explain, especially given what is known about red cell monovalent anion exchange.

An alternative hypothesis is that the transport event is H^+/Cl^- cotransport, rather than OH^-/Cl^- exchange. A reasonable driving force for such transport, by analogy to Eq. (2) for OH^-/Cl^- exchange, should contain the factor $H_i^+ Cl_i^- - H_o^+ Cl_o^-$. The H^+/Cl^- cotransport flux is then written:

$$J_{H/Cl} = k_H(H_i^+ Cl_i^- - H_o^+ Cl_o^-), \tag{4}$$

with k_H analogous to k_{OH} in Eq. (2). The parameter k_H is a function of the number of postulated H^+/Cl^- transport systems, affinities for H^+ and Cl^-, and turnover number for the transport event. The flux is zero when $H_i^+ Cl_i^- = H_o^+ Cl_o^-$ and is an increasing function of the H^+ concentration. By a derivation strictly analogous to that for OH^-/Cl^- exchange, it may

be shown that the rate constant k for pH equilibration by H^+/Cl^- cotransport is given by:

$$k = 2.3\, k_H Cl_o^\infty H_o^\infty N(1/B_i + 1/B_o). \tag{5}$$

Therefore, if k_H does not depend on pH, one would expect the rate of equilibration to be directly proportional to the equilibrium H^+ concentration. Inspection of Fig. 3 shows that this is very nearly the case between pH 6.5 and 7.5, i.e., the rate of equilibration increases rapidly with the H^+ concentration, as one would expect if H^+ rather than OH^- were the transported species.

From the data in Fig. 3 and Eqs. (3) and (5), the parameters k_H and k_{OH} may be calculated, assuming in the one case that H^+/Cl^- cotransport is entirely responsible for the equilibration, and in the other that OH^-/Cl^- exchange is responsible. The relations used in the calculation, from Eqs. (3) and (5), are $\dfrac{k}{N(1/B_i + 1/B_o)} = 2.3\, k_{OH} Cl_i^\infty OH_o^\infty = 2.3\, k_H Cl_o^\infty H_o^\infty$. The results are presented in Fig. 8, plotted as k_{OH} (above) or k_H (below) *vs.* pH. As discussed above, k_{OH} decreases by nearly three orders of magnitude between pH 5.8 and 7.5. In contrast, the changes in k_H are quite moderate; there is a fivefold increase between 5.8 and 7.5. Therefore, H^+/Cl^- cotransport can explain the experimental data quite adequately if the proportionality constant k_H changes by a factor of only 5 between pH 5.8 and 7.5. Such a pH dependence is quite reasonable and could reflect either saturation of transport sites for H^+ or titration of inhibitory H^+ sites at low pH.

The suggestion that H^+/Cl^- cotransport rather than OH^-/Cl^- exchange is the transport event is based on the defined parameters k_{OH} and k_H. Since k_{OH} was found to have an enormous dependence on pH, and k_H has only a moderate dependence, it is argued that H^+/Cl^- cotransport is a more attractive explanation than OH^-/Cl^- exchange. This argument is, of course, an opinion, based on what is in the author's view an unreasonable pH dependence of k_{OH}. Equations (2) and (4) are not the only ways in which one may represent the relation between the fluxes and the OH^-, H^+, and Cl^- concentrations. However, no matter what representation is used, the OH^- concentrations must appear in the numerator of the expression for the OH^- flux. If the OH^- concentration is also an important term in the denominator, as could be the case for a saturable carrier mechanism, it would mean that OH^- transport shows saturation or self-inhibition at concentrations over one millionfold smal-

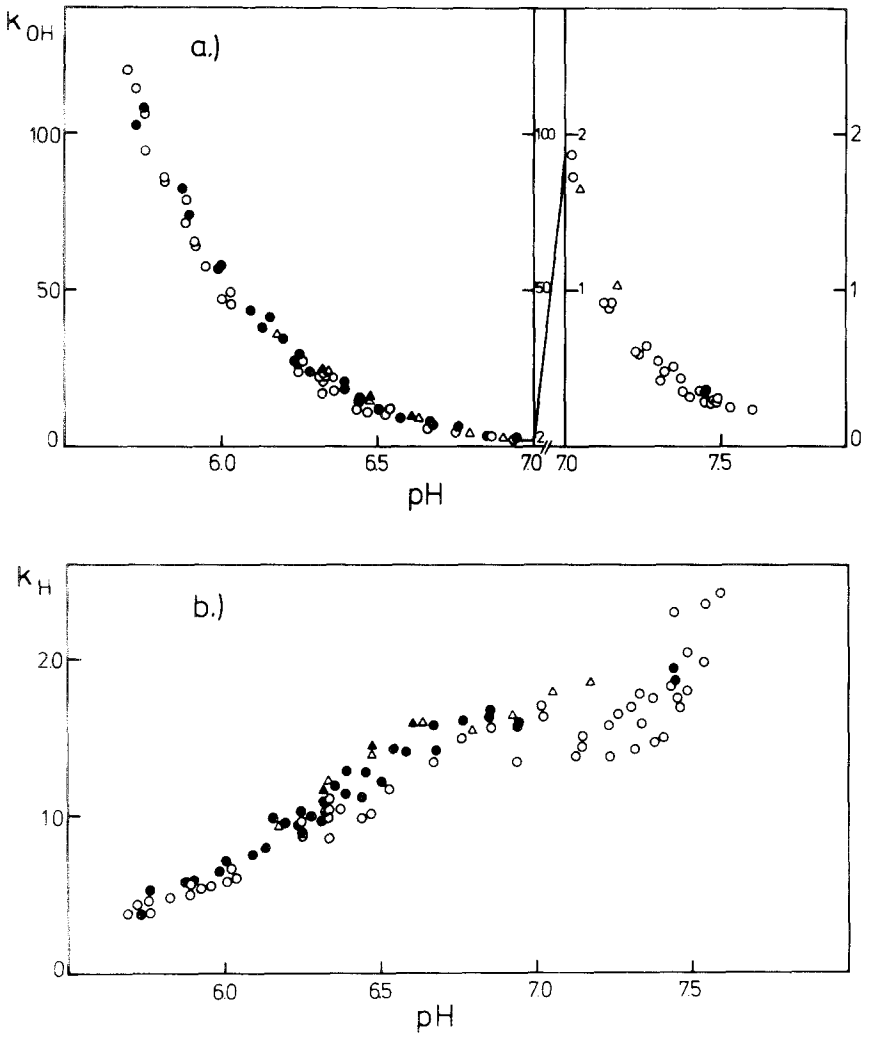

Fig. 8. Calculated parameters k_{OH} (*a*) and k_H (*b*) from Eqs. (3) and (5), respectively, using the rate constant data in Fig. 3. The various symbols have the same meaning as in Fig. 3. The units of k_{OH} and k_H are [μeq/ml cells · sec][mM]$^{-2}$. Two different vertical scales are used for k_{OH} to accommodate the large changes in k_{OH}

ler than for all other inorganic anions tested (Dalmark, 1975; Schnell, Gerhardt & Schöppe-Fredenburg, 1977). It is difficult to imagine any model of OH^-/Cl^- exchange in which the OH^- fluxes for a given pH gradient are largest at pH values where the OH^- concentrations are smallest. On the other hand, for a variety of models for H^+/Cl^- cotransport, one would expect the H^+ flux to be an increasing function of the H^+ gradient, as observed.

Relation to Models of Anion Exchange

The data presented here show that pH equilibration has several strong similarities with the anion exchange pathway. However, the system seems to behave kinetically more like H^+/Cl^- cotransport than OH^-/Cl^- exchange. A hypothesis that could account for these seemingly conflicting findings is that the red cell anion exchange protein may catalyze net H^+/Cl^- cotransport. In this regard, it would be of interest to compare the Cl^- self-exchange flux with the apparent H^+/Cl^- cotransport fluxes. Unfortunately, it is difficult to make this comparison, since the rate constants defined by the two types of experiments are not directly comparable. Nevertheless, it should be noted that at the largest H^+ gradients used here, the net H^+/Cl^- flux is only about 1/1000 of the Cl^- self-exchange flux at pH 7.2, at the same temperature (Tosteson, 1959). Further, although the parameter k_H varies with pH in a way very similar to the Cl^- self-exchange flux (Gunn *et al.*, 1973; Dalmark, 1975), the H^+ flux itself and the Cl^- self-exchange flux have opposite pH dependences. The pH equilibration pathway therefore differs from the halide self-exchange pathway in both the magnitude and pH dependences of the fluxes.

The possibility of H^+ transport in red cells is of little physiological relevance, because in the blood, the CO_2/HCO_3^- system operates much more rapidly than the CO_2 independent transport. The main significance of the H^+ transport lies in what it would imply about the anion exchange protein, and it is appropriate to discuss the possibility of H^+/Cl^- cotransport in the context of models of anion exchange. The only current model which postulates H^+ binding at a transport site is the titratable carrier model (Gunn, 1972, 1973). According to this model, by the addition of an H^+ ion, a site on the transport protein can be converted from an uncharged form C_o to a singly charged form (pK of 8.8) which is capable of binding a chloride ion:

$$C_o + H^+ \rightleftharpoons C_1^+; \quad C_1^+ + Cl^- \rightleftharpoons C_1Cl$$

This titration is not likely to be involved in H^+/Cl^- cotransport because the apparent H^+ flux increases at pH values much lower than the postulated pK. Furthermore, recent work by Funder and Wieth (1976) has shown that no functionally important titration of the Cl^- transport system takes place with pK between pH 7 and 11 (0 °C).

In the pH range over which the apparent H^+ flux increases steeply with the H^+ concentration, the titratable carrier model postulates an

additional titration which converts the site from a singly positive to a doubly positive form:

$$C_1^+ + H^+ \rightleftharpoons C_2^{++}.$$

If one assumes that C_1^+ binds one Cl^- ion and C_2^{++} binds two, then an exchange of a C_2Cl_2 for a C_1Cl could explain an electrically silent H^+/Cl^- cotransport. Since in the titratable carrier hypothesis the rate of transport of C_1Cl must be much more rapid than that of C_2Cl_2, the net H^+/Cl^- flux should be much smaller than the Cl^- self-exchange flux, as observed. The decrease of k_H as the pH is lowered (Fig. 8) could result from the progressive saturation of the $C_1^+ \rightharpoonup C_2^{++}$ titration. A further titration at low pH from C_2^{++} to C_3^{+++}, a form of the site that is not transported (Gunn, 1973), may also contribute to the fall in k_H with pH, although the quality of the data does not allow one to distinguish between saturation and inhibitory H^+ binding. Thus the titratable carrier model can explain the phenomenon of H^+/Cl^- transport, but only if the assumption is made that the C_2^{++} form of the site can bind and transport two Cl^- ions.

In a previous publication (Jennings, 1976) it was shown that a proton is cotransported with $SO_4^=$ during $SO_4^=/Cl^-$ exchange, with a 1:1 stoichiometry. According to the titratable carrier model, the same titratable group is involved in this process and in H^+/Cl^- cotransport, but from the available data, it is not possible to tell if this is actually the case. In addition, there are H^+ binding sites which inhibit both Cl^- and $SO_4^=$ self-exchange (Dalmark, 1976*b*; Schnell *et al.*, 1977); this H^+ binding may or may not be related to an H^+ binding site involved in H^+/Cl^- cotransport. The present results are, however, consistent with the hypothesis that protonation of a hydrogen ion titratable group ($pK < 7$) on the red cell anion exchange protein can result in net H^+/Cl^- cotransport by some of the same steps that are involved in Cl^-/Cl^- exchange.

Relation to Previous Work

The above argument that it may be possible to distinguish H^+ transport from that of OH^- is essentially that presented in 1932 by Jacobs and Parpart, whose experimental results were more consistent with OH^- than with H^+ transport. However, these experiments were performed on diluted whole blood, and the results were very likely influenced by CO_2 and HCO_3^-. Further, very low extracellular pH values

were employed, and it is not clear what relation these measurements have to those in the pH range nearer neutrality.

Crandall *et al.*, (1971) performed an extensive set of red cell pH equilibration experiments in a low CO_2 medium. They found that OH^- and H^+ transport could explain the data equally well. The calculated ionic permeabilities P_{OH} and P_H had pH dependences in the same direction as the parameters k_{OH} and k_H, respectively (Fig. 8), but the magnitudes of the changes in P_{OH} were comparable to those in P_H, in contrast to the very large changes in k_{OH} and small changes in k_H. One explanation of the difference between the present results and those of Crandall *et al.* may be that in the latter experiments, a trace of HCO_3^- accelerated the pH equilibration at alkaline pH values[2]. This would lessen the calculated pH dependence of P_{OH} and exaggerate the pH dependence of P_H, resulting in comparable magnitudes of the pH dependences of P_{OH} and P_H. Another reason for the difference between the two sets of experiments may lie simply in the methods of data analysis. Crandall *et al.* used the constant field equation, which, while the most reasonable approach at the time, has been shown here not to be applicable. Because of the differences in the methods of data analysis, it is unfortunately impossible to compare quantitatively the data from the two laboratories.

The finding that CO_2-free pH equilibration is mainly an electrically silent process is in agreement with the work of Tosteson, Gunn and Wieth (1973) and of Hladky and Rink (1976), who showed that large valinomycin-induced changes in the membrane potential produce only minor pH movements in red cells. The present experiments extend these findings in that it is shown that not only does the majority of the flux involve no net charge transport but also that the rate of the process is

2 Forster (1972) addressed this problem and presented evidence that HCO_3^- was not a significant factor at either acid or alkaline pH values. He found that either degassing the solutions *in vacuo* or adding 50 μM total CO_2 caused no significant change in the calculated P_{OH}. In ten experiments (data not shown), we have corroborated this low sensitivity to CO_2 at pH 5.9–6.20, where 100 μM added total CO_2 causes only a 50–80% increase in the equilibration rate. However, at pH 7.3–7.5, 100 μM total CO_2 increased the rate 10- to 14-fold (5 experiments), at levels of extracellular carbonic anhydrase (estimated from supernatant Hb) slightly smaller than those in the Crandall *et al.* (1971) experiments. Indeed, there is abundant evidence in the literature that 100 μM or less total CO_2 has a strong accelerating effect on red cell pH equilibration at neutral pH (Scarpa *et al.*, 1970; Deuticke, 1972; Cousin *et al.*, 1975; Jennings, 1976). If some HCO_3^- were initially present in the Crandall *et al.* experiments, the relative effect of added HCO_3^- would, of course, be smaller. The reason that Forster (1972) found no large effect of degassing the high pH stock solution may be that dissolved CO_2 constituted only a very small fraction of the total ($CO_2 + HCO_3^-$) present. (In the present experiments the solutions were N_2-pretreated at pH < 6.5.)

not influenced strongly by the membrane potential. Even an electrically silent system might be affected by the membrane potential, but any such effect is not large for pH equilibration. It should be pointed out that the estimate that over 90% of the CO_2-free pH equilibration is electrically silent applies only to the pH range (6–7.1) in which the effect of V_m was studied. It is possible that at higher pH, where the electrically silent system is slow, current-carrying OH^- transport could contribute more substantially, since the ionic OH^- permeability of the membrane appears to be considerably higher than that of Cl^- (Tosteson *et al.*, 1973; Knauf *et al.*, 1977).

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Preliminary accounts of this work have been presented at the First International Congress on Cell Biology, Boston, Massachusetts, September 1976 (*J. Cell. Biol.* 70:85*a*) and the XXVIIth International Congress of Physiological Sciences, Paris, July 1977.

Appendix

Nonequilibrium Thermodynamics of OH^-/Cl^- Exchange

For the passive exchange of Cl^- for OH^-, there are two flows, J_{Cl} and J_{OH} (μeq/ml cells · sec). (The very small osmotic water flow may for the present purposes be ignored.) The driving forces corresponding to these flows are the electrochemical potential differences $\Delta\bar{\mu}_{Cl}$ and $\Delta\bar{\mu}_{OH}$. The dissipation function (*see* Katchalsky, 1967, and references therein) per ml red cells for the system is then:

$$\Phi = J_{Cl}\Delta\bar{\mu}_{Cl} + J_{OH}\Delta\bar{\mu}_{OH}. \tag{A1}$$

Close to equilibrium, which is the domain considered experimentally, the flows are linear combinations of the driving forces:

$$\begin{aligned} J_{Cl} &= L_{11}\Delta\bar{\mu}_{Cl} + L_{12}\Delta\bar{\mu}_{OH} \\ J_{OH} &= L_{12}\Delta\bar{\mu}_{Cl} + L_{22}\Delta\bar{\mu}_{OH} \end{aligned} \tag{A2}$$

where L_{11}, L_{12} and L_{22} are the phenomenological coefficients. The cross coefficients L_{12} are equal by virtue of Onsager symmetry. No matter

what is the nature of the transport (current-carrying or electrically silent), J_{OH} must equal $-J_{Cl}$. This implies that:

$$0=[L_{11}+L_{12}][\Delta\bar{\mu}_{Cl}]+[L_{22}+L_{12}][\Delta\bar{\mu}_{OH}]. \tag{A3}$$

But since $\Delta\bar{\mu}_{Cl}$ and $\Delta\bar{\mu}_{OH}$ can be varied independently, the coefficients of each must be 0, i.e., $L_{11}=L_{22}=-L_{12}\equiv L$. Equations (A2) then become the single equation:

$$J_{OH}=-J_{Cl}=L[\Delta\bar{\mu}_{OH}-\Delta\bar{\mu}_{Cl}]. \tag{A4}$$

The electrochemical potential gradients $\Delta\bar{\mu}_{Cl}$ and $\Delta\bar{\mu}_{OH}$ are:

$$\begin{aligned}\Delta\bar{\mu}_{Cl}&=RT\ln\frac{Cl_i}{Cl_o}-FV_m\\ \Delta\bar{\mu}_{OH}&=RT\ln\frac{OH_i}{OH_o}-FV_m,\end{aligned} \tag{A5}$$

where F is the Faraday constant and V_m is the membrane potential. With (A5), Eq. (A4) is:

$$J_{OH}=-J_{Cl}=LRT\ln\frac{Cl_oOH_i}{Cl_iOH_o}. \tag{A6}$$

At this point it is useful to define the average concentrations of Cl^- and OH^-. This may be done in a variety of ways: arithmetic mean, geometric mean, or the Kedem-Katchalsky (1958) average, e.g.,

$$\overline{Cl}=\frac{Cl_i-Cl_o}{\ln\frac{Cl_i}{Cl_o}}.$$

In the present case, rather than defining the individual average concentrations $\overline{Cl}$ and $\overline{OH}$, one can define the average product of the concentrations $\overline{Cl\cdot OH}\equiv\frac{Cl_iOH_o+Cl_oOH_i}{2}$. With this average, and the approximation $\ln(1+X)\cong X$ for small values of X, (A6) becomes:

$$J_{OH}=\frac{LRT}{\overline{Cl\cdot OH}}[Cl_oOH_i-Cl_iOH_o]. \tag{A7}$$

An expression of the form (A7) may also be derived using the individual average concentrations $\overline{Cl}$ and $\overline{OH}$, but the approximations used are valid over a smaller range.

Equation (A7) may be rewritten as:

$$J_{OH} = k_{OH}[Cl_o OH_i - Cl_i OH_o], \tag{A8}$$

with $k_{OH} \equiv \frac{LRT}{\overline{Cl \cdot OH}}$. The proportionality factor k_{OH} is analogous to a permeability coefficient. For example, for the noncoupled transport of a single nonelectrolyte S, the flux $J_S = P_S[S_i - S_o]$, with $P_S = \frac{L_S RT}{\bar{S}}$, where P_S is the permeability coefficient, L_S is the phenomenological coefficient and $\bar{S}$ is an average concentration of S. The difference between k_{OH} and a conventional permeability coefficient is that since both Cl and OH are involved, LRT is divided by $\overline{Cl \cdot OH}$, rather than a single average substrate concentration. The factor k_{OH} is equal to the flux of OH divided by the effective concentration gradient of OH: $Cl_o OH_i - Cl_i OH_o$.

Time Course of pH Equilibration

Equation (A8), written in terms of pH is:

$$J_{OH} = k_{OH}[10^{-14+pH_i} Cl_o - 10^{-14+pH_o} Cl_i]. \tag{A9}$$

The "departure from equilibrium" for the extracellular pH is defined as:

$$X = pH_o^\infty - pH_o, \tag{A10}$$

where pH_o is the instantaneous pH, and pH_o^∞ is the equilibrium value. For an extracellular pH change of X, there must be an intracellular pH change of $-XB_o/B_i$, where B_o and B_i are respectively the extra- and intracellular buffer capacities (μeq/pH). Therefore,

$$-XB_o/B_i = pH_i^\infty - pH_i. \tag{A11}$$

Combining Equations (A9), (A10), and (A11) gives:

$$J_{OH} = k_{OH}[10^{(-14+pH_i^\infty + XB_o/B_i)} Cl_o^- - 10^{(-14+pH_o^\infty - X)} Cl_i^-]. \tag{A12}$$

But, $Cl_o^- = Cl_o^\infty$, and near equilibrium, Cl_i^- differs from Cl_i^∞ by at most 1%, so a factor of $Cl_o^\infty OH_i^\infty (= Cl_i^\infty OH_o^\infty)$ may be removed from the brackets in Eq. (A12):

$$J_{OH} = k_{OH} Cl_i^\infty OH_o^\infty [10^{XB_o/B_i} - 10^{-X}]. \tag{A13}$$

For small values of X,

$$10^{-X} \cong 1 - 2.3\,X$$

$$10^{XB_o/B_i} \cong 1 + 2.3\,X\,B_o/B_i,$$

and Eq. (A13) becomes:

$$J_{OH} = 2.3\,k_{OH}\,Cl_i^{\infty}\,OH_o^{\infty}\,X[1 + [B_o/B_i]]. \tag{A14}$$

The relation between J_{OH} and dpH_o/dt is:

$$dpH_o/dt = -dX/dt = NJ_{OH}/B_o, \tag{A15}$$

where N is the number of ml red cells present. Eqs. (A14) and (A15) may be combined to produce a first order linear differential equation in X:

$$dX/dt = -2.3\,k_{OH}\,Cl_i^{\infty}\,OH_o^{\infty}\,N[1/B_i + 1/B_o]\,X,$$

and the rate constant k for the exponential decay of X is:

$$k = 2.3\,k_{OH}\,Cl_i^{\infty}\,OH_o^{\infty}\,N[1/B_i + 1/B_o]. \tag{A16}$$

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