Hydroxyl Ion Movements across the Human Erythrocyte Membrane

Measurement of rapid pH changes in red cell suspensions

E. D. CRANDALL, R. A. KLOCKE, and R. E. FORSTER

From the Department of Physiology, School of Medicine, University of Pennsylvania, Philadelphia, Pennsylvania 19104. Dr. Klocke's present address is Department of Medicine, State University of New York, Buffalo, New York 14215.

ABSTRACT A stopped flow rapid reaction apparatus capable of following changes of ±0.02 pH unit in 0.1 ml of solution in less than 0.005 sec has been developed, utilizing a commercially available pH-sensitive glass electrode. Using this instrument, extracellular pH at 37°C was followed from less than 0.025 sec to 300 sec after mixing equal volumes of the following CO_2-free solutions: (A) normal human red cells, washed three times and resuspended in 150 mM NaCl at pH 7.2 with a hematocrit of 18%; and, (B) 150 mM NaCl adjusted with HCl or NaOH to pH 2.1 to pH 10.3. A minimum of 2 ml of mixture had to flow through the electrode chamber to ensure complete washout. The mixing process produced a step change in the pH of the extracellular fluid, after which exchanges across the red cell membrane and buffering by intracellular hemoglobin caused it to return toward pH 7.2 with an approximately exponential time course. Under the assumption that pH changes after mixing represent exchanges of hydroxyl for chloride ions across the cell membrane, hydroxyl ion permeabilities (P_{OH^-} in cm/sec) were calculated and found to vary from 2×10^{-4} at pH 9 to 4×10^{-1} at pH 4 according to the empirical relationship $P_{OH^-} = 170 \exp(-1.51\ pH)$. The form of the dependence of P_{OH^-} on extracellular pH does not appear compatible with a simple fixed charge theory of membrane permselectivity.

INTRODUCTION

This paper reports estimates of the hydroxyl ion permeability (P_{OH^-}) of human erythrocytes calculated from observations of rapid pH changes in red cell suspensions under conditions where only chloride and hydroxyl ion fluxes are significant. The experimental design used is similar to procedures previously reported by Maizels (1934), Wilbrandt (1940), and others.

It is well-known that anion movements across the red cell membrane occur much faster than cation movements. For example, potassium ion permeability has been reported to be about 2.2×10^{-11} cm/sec at 23°C and low ionic strength (Donlon and Rothstein, 1969), while that for chloride ion calculated from the data of Tosteson (1959) is about 10^{-4} cm/sec at about 23°C. The half-time for this chloride exchange is about 0.2 sec, so rapid that special techniques had to be devised to follow its time course. The same holds true for the study of OH^- movements. Previous workers have used continuous flow (Dalziel, 1953) and stopped flow (Gibson and Roughton, 1955) rapid reaction instruments to follow fast pH changes by means of spectrophotometric measurements of indicator dyes. Unfortunately, spectrophotometric measurements are complicated by light scattering in cell suspensions as well as by the presence of hemoglobin. A pH-sensitive glass electrode has been used successfully in a continuous flow apparatus to follow rapid pH changes (Rossi-Bernardi and Berger, 1968) and rapid NH_3 exchanges (Klocke et al., 1968) in red cell suspensions. The fastest device of the pH electrode, stopped flow type previously reported appears to have had a response time of less than 0.3 sec (Sirs, 1958). An undocumented report of a system with a 60 msec response time has also appeared in the literature (Love, 1953). In the present work, a stopped flow rapid reaction apparatus has been developed, using a commercially available pH-sensitive glass electrode, which is capable of following changes of ± 0.02 pH unit in less than 0.005 sec in a fluid volume of 0.1 ml, and which requires reaction solution volumes on the order of 1 ml.

RAPID REACTION APPARATUS

Description of Apparatus

A schematic diagram of the stopped flow equipment is shown in Fig. 1. The reacting solutions in a pair of 3 ml syringes, *A* and *B*, are forced by manually activated hydraulic pressure into a four tangential jet mixing chamber (volume ~0.004 ml), out of which the mixture flows against the pH electrode in a 0.1 ml chamber and then out into the stop syringe (or a bypass). The mixing chamber and electrode assembly are made of Lucite and are attached to a Gibson-Milnes type instrument (Gibson and Milnes, 1964). The valves through which syringes *A* and *B* are filled are not shown. The apparatus within the dashed lines is in a constant temperature water bath. The Ag-AgCl reference electrode and the glass electrode both have maximal diameters of about 0.3 cm (commercially available as Leeds and Northrup No. 117233), with the pH-sensitive glass forming the conical tip of the glass electrode.

The bridge between the glass and reference electrode chambers is a 0.125 cm diameter channel about 1.1 cm long, filled with saturated KCl. At the glass electrode end of the channel is a snug fitting Teflon plug, across which lies a KCl-saturated absorbent cotton wick of negligible electrical resistance. The plug is necessary to prevent bulk mixing between the fluid whose pH is being determined and the saturated KCl solution.

The output from the glass electrode goes directly into the grid of an electrometer tube (Raytheon CK 5886), operated in the triode configuration and wired as a cathode follower. The circuit diagram is shown in Fig. 2. The gain of the cathode follower in its linear operating range is about 0.52, varied somewhat by the zero adjustment (not moved after calibration). The span was always kept at maximum output position. Minimum capacitance of the electrometer tube itself is about 2.0 $\mu\mu$F, and by keeping all electrical input wires as short as possible, the effective capacitance of the cathode follower input can be reduced to about 8.0 $\mu\mu$F.

The output from this high input impedance cathode follower is fed directly into a storage oscilloscope (Tektronix Type 564). It was possible to read the oscilloscope

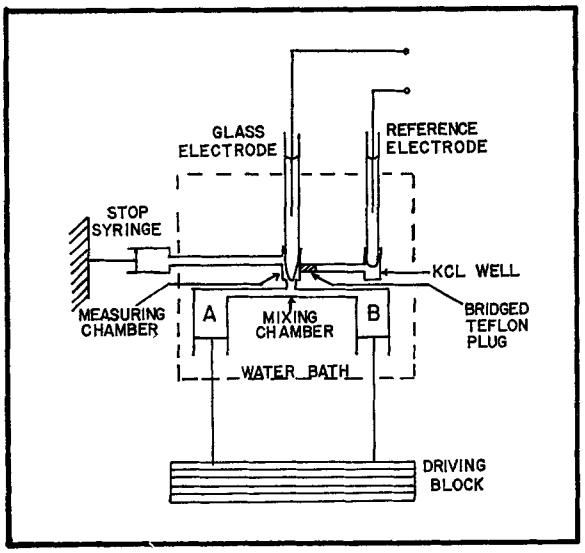

FIGURE 1

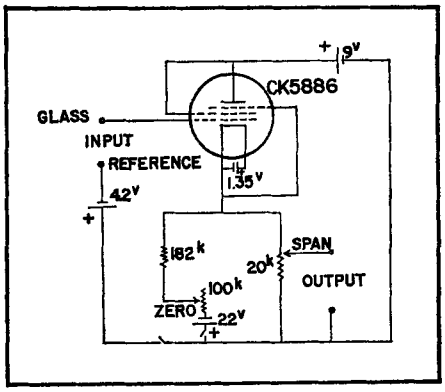

FIGURE 2

FIGURE 1. Schematic diagram of the rapid reaction apparatus.
FIGURE 2. Circuit diagram of the cathode follower.

output to about $\pm$0.01 pH unit, limited by a 60 cycle per sec noise level of about 0.002 v peak to peak. Considerable difficulty with grounding was encountered due to the fact that the reacting fluid is inevitably grounded through the water bath, as is the ground of the cathode follower through the oscilloscope. The most satisfactory solution found was to float the ground of the latter.

Response Characteristics

The pH electrode in the assembled apparatus was calibrated by flowing standard buffer solutions through the measuring chamber. The electrode output was linear with pH up to about 10.0, above which alkaline error became significant. The sensitivity of the electrode was usually about 56 mv/pH unit at 37°C.

When the apparatus is used to measure the pH of buffered solutions, the volume

needed to ensure complete washout of the mixing and measuring chambers is about 0.5–1.0 ml, which corresponds reasonably well with the expected amount of fluid necessary to wash out the volume of the mixing and electrode chambers. In the case of unbuffered solutions, however, a minimum of 2.0 ml has to flow through the measuring chamber, with an even greater volume required if the unbuffered solution is washing out a buffered solution. Necessary washout volume was reduced at lower flow velocities, presumably because of improved hydrodynamic characteristics.

In order to determine the speed of response of the instrument, a step change in the pH of the fluid around the electrode would be ideal, but it was not possible to do this experimentally in less than 0.030 sec, owing to the time required to wash out the electrode chamber. However, it is possible to estimate this speed using a ramp input, namely, the linear increase in pH produced by the dehydration of carbonic acid (Dalziel, 1953; Gibson and Roughton, 1955; Sirs, 1958; Edsall, 1969):

$$H^+ + HCO_3^- \underset{K_a}{\rightleftharpoons} H_2CO_3 \overset{k_v}{\underset{k_u}{\rightleftharpoons}} H_2O + CO_2 \qquad (1)$$

where K_a is the true equilibrium constant for the first reaction and k_v and k_u are the forward (dissociation) and backward (association) reaction rate constants for the second reaction.

The first reaction is a neutralization which for practical purposes can be considered instantaneous (Eigen and Hammes, 1963). When the reacting solutions (in syringes *A* and *B*) are chosen as 0.01 N HCl and 0.02 M $NaHCO_3$, the initial bicarbonate concentration after neutralization is approximately 0.01 M, the total pH change is from (approximately) initial 3.5 to final 6.0, and the total CO_2 formed is only a few per cent of the initial bicarbonate concentration (which can therefore be assumed constant). Under these conditions, it can be shown (Brinkman et al., 1933) that

$$k_v(t_2 - t_1) = \left(1 + \frac{K_a}{b - a}\right) \ln \frac{[H^+]_2}{[H^+]_1} \qquad (2)$$

where b is the total bicarbonate concentration and a is the total hydrogen ion concentration before neutralization. pH will therefore increase linearly with time, at least until the back reaction $H_2O + CO_2 \rightarrow H_2CO_3$ becomes important.

A representative curve obtained in our apparatus is shown in Fig. 3. The reaction rate constant k_v at 26°C was calculated from the initial slopes of this curve and others like it to be 24.1 sec^{-1} (SD = 1.6, n = 8), to be compared with 25.5 sec^{-1} at 25°C reported by Rossi-Bernardi and Berger (1968) and 20.0 sec^{-1} at 25°C reported by Khalifah (see Edsall, 1969 for an excellent review and discussion).

An estimate of the response time of the electrode system can be obtained from Fig. 3 by the following argument. In these experiments on the dehydration of carbonic acid, the average linear fluid velocity at the instant before stopping was at least 150 cm/sec. Stopping takes place in less than 0.001 sec, so that within this extremely brief period the pH of the fluid seen by the electrode changes from a steady value to a linearly increasing one. Any failure of the record to change abruptly indicates a

limitation of the time response of the system. Extrapolating linear slope backwards, it is estimated that the electrode system does respond to a sudden change in pH in 0.005 sec or less. This figure may be an overestimation owing to an imperfect stopping device, but the requirements of the present investigations did not warrant extensive refinement at this time.

This value for response time is independent of the lag time between initial mixing and impingement of the mixture on the electrode tip. A realistic estimate of lag time can be obtained by calculating the pH of the mixture immediately after neutralization and comparing this value with the observed pH at the instant of stopping. Lag time can then be determined from the difference between the two pH values and the linear change of pH with time observed after stopping. Unfortunately, the calculated

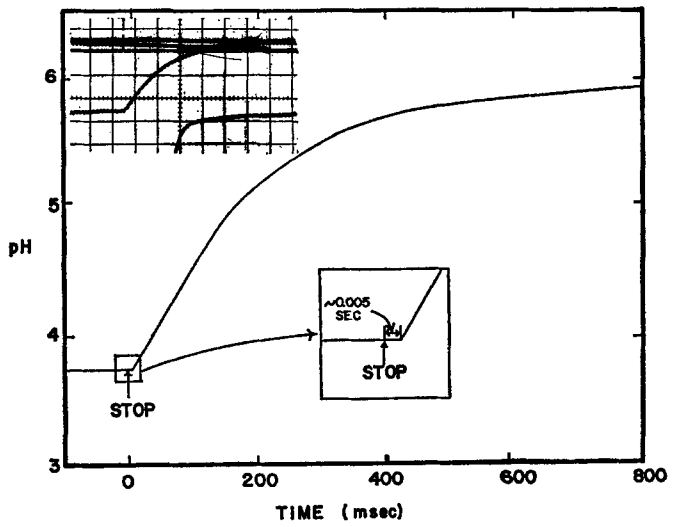

FIGURE 3. Time course of pH_o after mixing equal volumes of 0.01 N HCl and 0.02 M $NaHCO_3$ at 26°C. Included in the figure is the oscilloscope tracing from which the curve was drawn (in which one horizontal division = 100 msec, and one vertical division = 20 mv).

value of neutralized pH of the mixture is quite sensitive to the value used for K_a, varying from 3.51 to 3.82 as K_a goes from 3.5×10^{-4} (Roughton, 1964) to 1.7×10^{-4} (Wissbrun et al., 1954). An approximate value for the observed initial pH is 3.71; thus, an upper limit for lag time (using $K_a = 3.5 \times 10^{-4}$) can be estimated as 0.02 sec. Therefore, the recorded pH is in all cases that of the mixture no later than 0.025 sec after mixing, and this number is probably grossly overestimated.

The resistance of the glass electrode, measured at 26°C on a vibrating reed electrometer, is about 200 megohms. With an over-all input capacitance of 8.0 $\mu\mu$F in the cathode follower, this gives a time constant of 0.0016 sec. Indeed, the response time of the electrode system appears to be of this order of magnitude, pointing to the possibility that the capacitance of the electrode itself is very small and that the electrode is not the slowest responding unit in the system. However, we have no estimate of the actual speed of the electrode response (the speed of development of

boundary layers, movement of charge through the glass, etc.), and whether or not the response time of the present apparatus is in fact limited by the intrinsic characteristics of the electrode.

METHODS FOR PERMEABILITY EXPERIMENTS

Blood was drawn from five normal subjects (one female) into heparin-rinsed syringes and immediately centrifuged for 25 min at 2750 $\times g$. The cells were separated, washed with 10 times their volume of 150 mM NaCl, and recentrifuged, this procedure being repeated three times. Following the last wash, the cells were resuspended in 150 mM NaCl to a hematocrit of about 18% to form solution (or, more correctly, suspension) A, the pH of which was always about 7.2 at 37°C. Preparation of the solutions and the experiments required several hours, during which time the cells were kept mainly at room temperature. The longest period between venepuncture and experiment was 10 hr. The calculated P_{OH^-} was unchanged in experiments using suspensions kept for anywhere from 3 to 10 hr at room temperature. Temperature of the cells during preparation also did not appear to be critical, because the calculated P_{OH^-} was not significantly altered in those few experiments in which the cells were maintained at 37°C or at 10°C for as long as 4 hr between venepuncture and experiment.

Solution B was 150 mM NaCl with pH adjusted as desired to any value between 2.1 and 10.3 by the addition of small amounts of either HCl or NaOH. Both suspension A and solution B were CO_2-free ($[CO_2] < 0.02$ mM), as confirmed by the method of Van Slyke and Neill (1924).

Total hemoglobin was measured as cyanmethemoglobin at 540 mμ (Drabkin and Austin, 1932). Trace concentrations of hemoglobin, such as that in the extracellular fluid of suspension A, were measured using a modified benzidine method (Crosby and Furth, 1956), reading light absorption at 515 mμ. Hematocrit determinations were made in standard Wintrobe tubes after centrifugation at 2750 $\times$ g for 20 min. pH measurements, made independently using an anaerobic pH electrode (Instrumentation Laboratory, Watertown, Mass., Model 113), were performed on cell suspensions and on the lysate from packed cells (lysed by freezing and thawing).

The procedure for a specific experiment was as follows. Total hemoglobin, supernatant hemoglobin, hematocrit, pH, and cell lysate pH measurements were made on suspension A. The driving syringes, *A* and *B*, were then filled with about 2.5 ml of solutions A and B, respectively, the experiment carried out at 37°C, and recorded (from <0.025 sec to 300 sec after mixing), and the effluent mixture collected. The effluent mixture was analyzed for total hemoglobin, supernatant hemoglobin, hematocrit, and pH. The experiment was then repeated with supernatant from an aliquot of the same suspension A employed in the previous experiment in place of suspension A.

RESULTS

Experimental Results

The time course of pH after mixing equal volumes of suspension A with solution B at pH 10.26 is shown for two experiments in Fig. 4, while that for

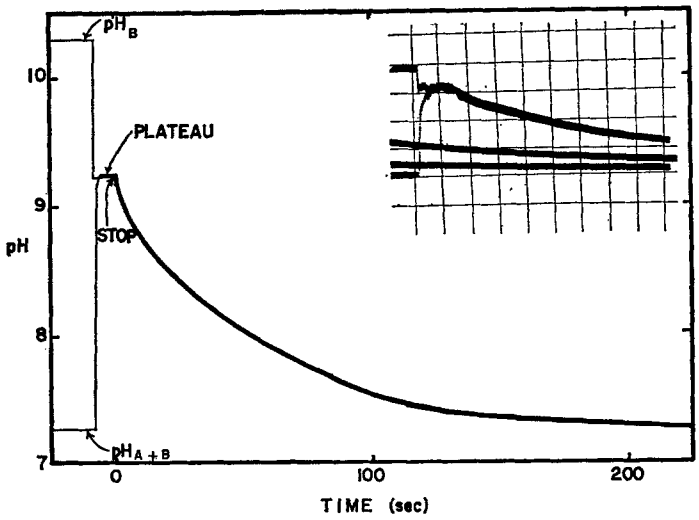

FIGURE 4. Time course of pH_o after mixing equal volumes of suspension A and solution B at pH 10.26. Donor, R. F. Included in the figure is the oscilloscope tracing from which the curves were drawn (in which one horizontal division = 5 sec, and one vertical division = 20 mv).

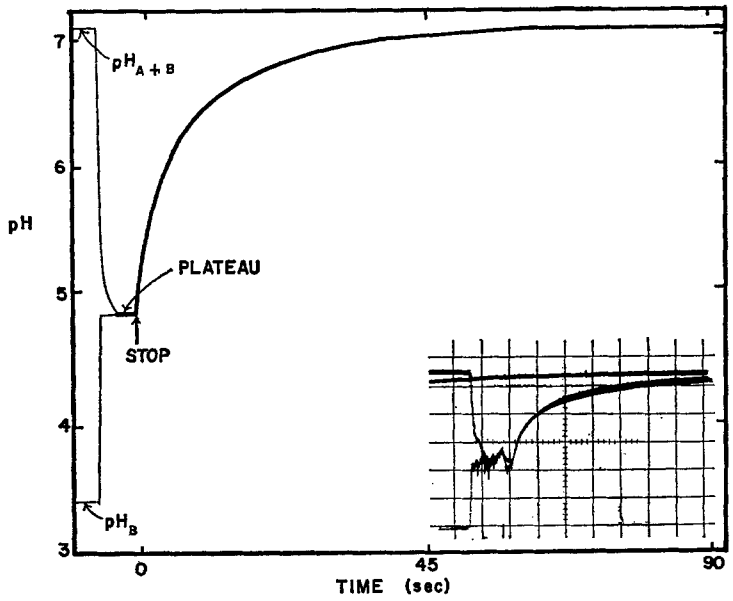

FIGURE 5. Time course of pH_o after mixing equal volumes of suspension A and solution B at pH 3.42. Donor, E. C. Included in the figure is the oscilloscope tracing from which the curves were drawn (in which one horizontal division = 5 sec, and one vertical division = 20 mv).

mixing suspension A with solution B at pH 3.42 is shown in Fig. 5. The pertinent data for these figures are given in Table I. Curves were in general very reproducible, to the extent that two time courses for which the pH of solution B (pH_B) is the same can be, for all practical purposes, superimposed from the time of stopping onward (as in Figs. 4 and 5).

The initial pH levels at the extreme left side of the records are those of the solutions in the electrode chamber at the start (ordinarily either the mixture (A + B) or solution B). Thus, in Fig. 4, the initial pH is 10.26 for one curve and 7.26 for the other. While flow occurs, the pH reaches a plateau at about 9.21, representing the essentially instantaneous neutralization of the suspending fluid in suspension A by solution B, plus that portion of the red cell exchange that has taken place in 0.020 sec (or less). The pH within this steady flow period is somewhat erratic since there is very little buffer present in the

TABLE I

COMPOSITION OF SUSPENSION A AND OF THE EFFLUENT MIXTURE (AT EQUILIBRIUM) FOR EXPERIMENTS WITH pH_B AT 10.26 (FIG. 4) AND pH_B AT 3.42 (FIG. 5)

Fig.	Solution	Total Hb	Supernatant Hb	pH	pH (lysed cells)	Hematocrit
		g%	*mg%*			%
4	A	5.91	4.8	7.21	6.98	17.2
4	Effluent mixture	2.71	41.1	7.26	—	8.0
5	A	5.03	8.6	7.19	6.99	16.0
5	Effluent mixture	2.66	28.6	7.13	—	7.8

extracellular fluid. (This problem was investigated in detail and is discussed below.) At time zero, stoppage occurred and exchanges across the red cell membrane continued, returning the external pH toward the pH (7.2) of the original suspension A (ending with a quasi-equilibrium pH about 7.26 in Fig. 4). In Fig. 5, the starting pH values were 3.42 and 7.13, the plateau pH 4.86, and the end pH 7.13. The curves were approximately exponential and a new quasi-equilibrium was reached in about 300 sec or less. There may be slower exchanges of cations occurring later still which were not investigated. It is of interest to note that the fact that the same plateau pH is reached (for a given pH of solution B, as in Figs. 4 and 5), regardless of the pH of the fluid in the electrode chamber before flow starts, indicates that complete washout has indeed taken place.

Many curves were also obtained after mixing solution B with supernatant from suspension A. These curves reached a plateau pH during flow, and the subsequent measured pH remained at this value and did not change with time after stoppage (as expected).

Theory of Computations

Under certain basic assumptions, movement of univalent ions across the erythrocyte membrane can be shown to be described by (Davson, 1964):

$$\phi = \frac{uE}{a}\frac{(C_o - C_i e^{-EF/RT})}{1 - e^{-EF/RT}} = \frac{-V_o}{A}\frac{dC_o}{dt} = \frac{V_i}{A}\frac{dC_i}{dt} \qquad (3)$$

where ϕ is flux (mmoles/cm²-sec), u is ion mobility implicitly including a partition coefficient (cm/sec(mv/cm)$^{-1}$), E is membrane potential (mv), a is membrane thickness (cm), C_o is external concentration (M), C_i is internal concentration (M), F is the Faraday (96500 coul/mole), R is the universal gas constant (8314.4 millijoules/mole–°K), T is temperature (°K), A is membrane area (cm²), V_o is extracellular solution space (cm³), and V_i is intracellular solution space (cm³). The assumptions underlying equation (3) include: (*a*) ions move under an electrochemical gradient; (*b*) the membrane is homogeneous; (*c*) potential drop across the membrane is linear (Goldman, 1943); and (*d*) there is one well-mixed internal compartment and one well-mixed external compartment. In the present experiments, the only diffusible ions present are chloride (Cl^-) and hydroxyl (OH^-), so that equation (3) can be written twice, once for each species. (The reasons for rejecting the possibility of significant hydrogen ion movement are discussed below.)

If it is also assumed that the flux of Cl^- is always equal and opposite to the flux of OH^- (after a very short transient period), then membrane potential can be calculated from a rearrangement of equations (3):

$$E = -\frac{RT}{F}\ln\frac{u_{OH^-}[OH^-]_o + u_{Cl^-}[Cl^-]_o}{u_{OH^-}[OH^-]_i + u_{Cl^-}[Cl^-]_i}. \qquad (4)$$

All concentrations in equations (3) and (4) should be read as activity, and all pH electrode measurements are in fact measurements of pa_H. The only correction necessary was to convert 150 mM (concentration) NaCl to 112.5 mM (activity) NaCl, assuming an activity coefficient of 0.75 (Robinson and Stokes, 1959).

Equations (3) are first-order nonlinear ordinary differential equations. If E and $[OH^-]_i$ are assumed constant, they can be solved analytically. However, in general these quantities are not constant, in addition to which complex buffering on both sides of the membrane lessens the utility of analytical solutions. Therefore, the following procedure was used to solve equation (4) simultaneously with equation (3) written for OH^-: (*a*) External chloride activity was known, and intracellular (lysate) and external pH (pH_o) were measured in suspension A. Internal chloride concentration was calculated on the basis of the Donnan equilibrium laws, assuming the same activity coeffi-

cient intra- and extracellularly. (*b*) Using these values for $[Cl^-]_o$, $[Cl^-]_i$, and $[OH^-]_i$, and the measured pH_o at time zero (after mixing), equation (4) was solved for E. A value for u_{Cl^-} calculated from the data of Tosteson (1959) was used (see discussion below), along with an assumed value for u_{OH^-}. (*c*) Equation (3) was solved for ϕ, which was assumed constant over an arbitrarily short time increment Δt (1 sec was used in these computations). The calculated value of E was used, and a was assumed to be 100 A. (*d*) Internal and external chloride and hydroxyl ion concentrations at 1 sec (Δt) after mixing were calculated using ϕ and appropriate mass balances. Intracellular and extracellular buffering systems were taken into account (see below). (*e*) The computations were repeated as above for the time increment 1–2 sec, then 2–3 sec, and so on. (*f*) At appropriate times (say, 10 sec after mixing), the computed pH_o was compared with the pH_o determined experimentally. If they agreed within an arbitrary value (0.04 pH unit was used here), the assumed value of u_{OH^-} was accepted for that time interval; if they did not agree, the assumed value of u_{OH^-} was corrected and the procedure started anew for that time interval (and repeated until an acceptable value of u_{OH^-} was found). (*g*) The above computations were repeated for the next time interval (say, 10–20 sec after mixing), using as starting data the numbers from the end of the previous interval. This process was continued until a complete time course of pH was computed that matched the experimental curve, resulting in a set of values for u_{OH^-} as a function of time (or pH_o). All computations were performed by a PDP-6 digital computer.

A definition for membrane permeability independent of any time-varying quantities is

$$P = \frac{uRT}{aF}. \tag{5}$$

With this definition, P_{OH^-} can be calculated from the hydroxyl mobility data computed as above. Also, from the data of Tosteson (1959), and assuming cell water to be 72% of cell volume (Savitz et al., 1964), P_{Cl^-} is about 10^{-4} cm/sec and u_{Cl^-} is 4.0×10^{-12} cm/sec(mv/cm)$^{-1}$, and this number was used in the computations. (The validity of using this value is discussed below.)

The internal and external buffering systems were included in the computations as follows. The important intracellular buffer is hemoglobin, and its buffer capacity under the conditions of these experiments was assumed to be 2.54 mM acid/mM Hb-pH (German and Wyman, 1937; Rossi-Bernardi and Roughton, 1967). (The computations were relatively insensitive to the numerical value used.) In one time increment, from t_1 to t_2, the change in intracellular pH was calculated from

$$\frac{[H^+]_{i2}}{[H^+]_{i1}} = \exp\left(-2.303[\phi_{OH^-}]A(t_2 - t_1]/2.54[Hb]_i V_i\right). \tag{6}$$

Extracellularly, hemoglobin concentrations are very low and other buffers (water, lactate, phosphate, etc.) become important (see discussion below). An empirical buffering curve for the extracellular fluid was therefore determined by plotting concentration of acid added vs. the plateau pH values. Then, the buffering power at any given extracellular pH was simply assumed to equal the slope (m) of the curve at that pH. Thus, in a time increment, extracellular pH change was calculated from

$$pH_2 - pH_1 = \frac{[\phi_{OH^-}]A(t_2 - t_1)}{mV_o}. \quad (7)$$

Although it was not necessary for solving the equations (unless solvent drag

TABLE II
HYDROXYL ION PERMEABILITIES (P_{OH^-}) CALCULATED FOR THE CURVES SHOWN IN FIGS. 4 AND 5

Fig.	Time segment	pH range	$\overline{pH}$	Hydroxyl ion permeability
	sec			*cm/sec*
4	0–5	9.42–8.99	9.21	2.14×10^{-4}
4	5–10	8.99–8.73	8.86	2.58×10^{-4}
4	10–20	8.73–8.45	8.59	2.90×10^{-4}
4	20–35	8.45–8.19	8.32	3.59×10^{-4}
4	35–60	8.19–7.91	8.05	4.57×10^{-4}
5	0–2	4.86–5.58	5.22	1.12×10^{-1}
5	2–5	5.58–6.05	5.82	4.92×10^{-2}
5	5–10	6.05–6.41	6.23	2.37×10^{-2}
5	10–20	6.41–6.73	6.57	1.61×10^{-2}
5	20–35	6.73–6.95	6.84	1.61×10^{-2}
5	35–60	6.95–7.06	7.01	1.61×10^{-2}

is considered a problem), net water movement was assumed to be zero to simplify the computations. Since all extracellular fluids were isotonic, this assumption should be very close to fact. Theoretically, the only factor which could cause water movement under true isotonic conditions would be a shift in the distribution of diffusible charges across the membrane, mediated by alterations in the charge density of intracellular hemoglobin. However, in several measurements, it was shown that the Donnan ratio for OH^- in the effluent mixture was approximately the same as that in suspension A. Furthermore, the calculated maximum intracellular pH change was, except in the extreme experiments reported below, always less than 0.13 pH unit, which produces a calculated change in cell volume of 2% or less. For these reasons, net water movement was assumed to be negligible.

P_{OH^-} values were determined for a total of 27 experiments. In every case, the permeability has a marked dependence on pH (and/or time after mixing). The P_{OH^-} values calculated for the experiments shown in Figs. 4 and 5 are given in Table II.

In Fig. 6, the results from 23 experiments ($pH_B > 3$) are plotted as $\log_{10} P_{OH^-}$ vs. average extracellular pH for the time interval in which that P_{OH^-} was computed. The larger symbols denote the P_{OH^-} values determined in the first time segment of each experiment. Also shown is the best straight line

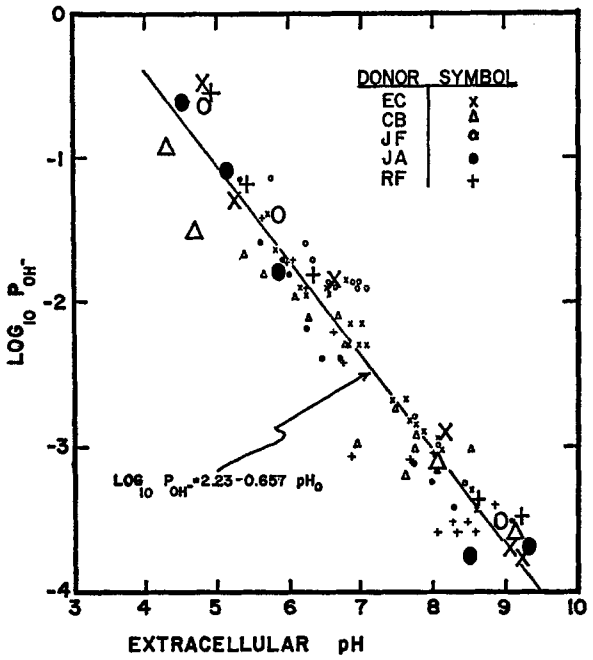

FIGURE 6. $\text{Log}_{10} P_{OH^-}$ as a function of extracellular pH from experiments in which $3.1 < pH_B < 10.3$. The line is the linear regression line for all these points (equation 8).

through all the points as determined by linear regression analysis. The equation for this line is

$$\log_{10} P_{OH^-} = 2.23 - 0.657\ pH_o \tag{8}$$

with a standard error of the estimate of 0.255 ($n = 95$). There were no important discernible differences in the dependence of P_{OH^-} on pH_o among individual bloods or between initial time segment values and all values. The relationship between P_{OH^-} and extracellular pH can be seen to be qualitatively logarithmic.

DISCUSSION

Hydroxyl Ion Permeabilities

The steady-state data obtained appear to support the generally accepted hypothesis that hydroxyl (and hydrogen) ions are distributed according to Donnan equilibrium laws across the red cell membrane, and therefore are not actively transported. The transmembrane potentials calculated from their distributions (8–15 mv) show reasonable agreement with recently measured values of 8 mv (Jay and Burton, 1969).

The computed magnitudes of hydroxyl ion permeability are not inconsistent with permeability data for other chemical species with respect to the erythrocyte membrane. The hydroxyl ion permeability at 37°C, pH 7.4, and an ionic strength of 0.150 can be seen to be about 2.2×10^{-3} cm/sec from Fig. 6. That for chloride at 23°C, pH 7.4, and similar ionic strength is about 10^{-4} cm/sec. In view of the dependence of P_{Cl^-} on temperature (Luckner, 1939; Wieth, 1970), it is probable that the two permeabilities are of the same order of magnitude or at least no greater than a factor of 10 apart. This is not particularly surprising since the two ions have the same charge and not dissimilar ionic radii and hydration energies (Bull, 1951) Bicarbonate ion permeability of the red cell membrane is equal to or greater than this number for chloride (Dirken and Mook, 1931; Luckner, 1939; Piiper, 1969). It has also been reported (Donlon and Rothstein, 1969) that potassium permeability at 23°C, pH 7.4, and low ionic strength is 2.2×10^{-11} cm/sec (about 10^{-8} the hydroxyl ion permeability). The rate of hydroxyl ion movement across the red cell membrane is roughly 10^{-4} its own mobility in water (Bull, 1951).

Dependence of P_{OH^-} on time after mixing can be eliminated as a possibility by the following observation: P_{OH^-} values computed from each experiment were approximately the same as those computed from the other experiments when pH_o was the same, but not when time after mixing was the same. This observation held whenever pH_o ranges overlapped between experiments. P_{OH^-} thus appears to be a function of only pH_o under the conditions of these experiments.

Relation to Fixed Charge Concept

According to the computation described above, the movement of an ion across the red cell membrane is governed by both its concentration gradient and the transmembrane potential. It has been suggested (Wilbrandt and Schatzman, 1960) that the membrane potential is one factor that, in addition to its direct effect on flux, is a determinant of ionic permeability. For cation movements over wide ranges of membrane potential, a relatively small dependence of permeability on potential has been demonstrated (LaCelle and Rothstein, 1966).

A seemingly much more important determinant of ion permeability is the concentration of fixed charges within the membrane. The evidence in support of a fixed cation hypothesis has recently been reviewed by Passow (1969). The data presented by Passow suggest that both anion and cation permeabilities are markedly affected by the concentration of these groups, perhaps amino groups of membrane protein components with a pK of 9 and a total concentration in the membrane of 2.5 M. The degree of dissociation of these groups (or charge density) would be expected to be dependent on temperature, on ionic strength, strongly on pH (internal and/or external), and perhaps on other factors. The reported experimental effects of these variables on both cation and anion permeabilities have in general been consistent with the hypothesis, although there are exceptions. Decreasing pH ($\sim 6.5 < \text{pH} < 8.0$) tends to increase the permeability to anions and decrease that to cations, presumably increasing the concentration of fixed positive (perhaps predominantly $R - NH_3^+$) groups in the membrane. Passow (1969) has also reviewed the evidence that the rate-limiting barrier for cation movements is the positive fixed groups themselves, while another site (perhaps negatively charged hydrophobic groups) limits anion movements.

Ideally, to investigate the fixed charge hypothesis, isotopic permeability measurements at steady-state conditions are to be desired, varying one of the variables from experiment to experiment while keeping all others constant. These conditions are difficult to meet in the study of anion movements, since they are so rapid, and are impossible to meet in studying hydroxyl ion movements which by their very nature involve a varying pH. Nonetheless, the experiments reported here can be used to determine the effects of external pH on hydroxyl ion permeability, since temperature and ionic strengths were maintained constant throughout all experiments, and the calculated internal pH never varied by more than 0.13 unit within an experiment, nor by more than 0.24 unit between experiments.

The effects of external pH on hydroxyl ion permeability have been shown in Fig. 6 and summarized in equation (8). There is an increase in P_{OH^-} with decreasing pH, as has been reported for other anions between about pH 6.5 and 8.0 (Passow, 1969), except that we find P_{OH^-} is an exponential rather than an S-shaped function of pH. The actual experimental data reported by Passow (1969) extend over a small range of pH and are not incompatible with our equation (8). Our results, however, do not fit the concept of a single pK for the fixed dissociable groups in the red cell membrane which determine permeability. It is, furthermore, difficult to reconcile them with the concept of a series of such dissociable groups of varying pK, if the titration curve of these groups resembles those of red cell ghosts (Passow, 1969) or complex proteins (e.g., hemoglobin), which are approximately linear with pH, while equation (8) would suggest that the titration curve should be an exponential

function of pH (which is conceivable but unlikely). It is, of course, always possible that P_{OH^-} does not vary in the same fashion with pH as do the other anions previously studied.

Hydrogen Ion Movement

Jacobs and Parpart (1932), in a very clever and elegantly simple experiment in which red cells were exposed to acid and the time of lysis observed, concluded that the mechanism for acidity changes across the membrane was hydroxyl ion movement, not hydrogen ion movement. However, there was an implicit assumption in the treatment of the data that the permeability of the cell membrane to these two ions was constant despite changes in pH and ionic strength. Our present work invalidates this assumption. Therefore, the conclusion of Jacobs and Parpart is in doubt.

Unfortunately, that paper is the only one of which we are aware that addresses itself specifically to the question of hydroxyl vs. hydrogen ion movement across the erythrocyte membrane. This situation is not surprising, since it is not possible to differentiate the effects of the two mechanisms; one will always be the mirror image of the other. The data presented in this paper go no further toward elucidating the true mechanism. If we assume that hydrogen ion moves (and that OH^- does not), the permeabilities thus computed turn out to be of the same order of magnitude as those presented for hydroxyl ion, but a mirror image in that P_{H^+} increases with increasing pH.

With this in mind, there are two reasons that all the data have been interpreted and presented here in terms of the movement of hydroxyl ions alone. The important consideration is the circumstantial evidence that anion movement across the erythrocyte membrane at normal temperatures is very much faster than cation movement for all ions studied to date; for example, chloride moves more than 1 million times faster than potassium ions. Thus it is reasonable to expect the mechanism for acidity changes to be one of hydroxyl (anion) movement, with hydrogen ion movement relatively unimportant. However, it should be noted that the hydrogen ion is a very unusual cation, so that applying to its behavior evidence obtained from other cations such as potassium and sodium must be viewed somewhat skeptically.

The other reason for interpreting the data on the basis of negligible hydrogen ion movement is that it makes no difference ultimately which mechanism actually holds, except for elucidation of membrane properties; that is, permeabilities calculated with either mechanism show, for example, the expected dependence upon pH. In that sense, it really makes no difference to the cells which mechanism is the actual one.

One other point must be noted, and that is the possibility that the data could be explained using significant movement of both hydrogen and hydroxyl ions at the same time, with one constant value of permeability for each

over the entire pH range. This point was considered but could not be made compatible with the experimental data; that is, even with movement of both ions, a variable permeability (with pH) had to be invoked for at least one ion in order to fit the experimental curves.

Chloride Permeability

The number used in the computations for P_{Cl^-} (10^{-4} cm/sec) was determined at 23°C and pH 7.4 (Tosteson, 1959). There is evidence, however, that it is somewhat temperature dependent (Luckner, 1939; Wieth, 1970), and it is probably pH dependent as well (Passow, 1964). Therefore the use of this value with data obtained at 37°C and at widely varying pH is suspect. Fortunately, the computations of P_{OH^-} are relatively insensitive to the value of P_{Cl^-} used, for changes in P_{Cl^-} from 10^{-6} to 10^{-1} cm/sec. The reasons for this lie in the "swamping" effects of the very high chloride concentrations relative to the hydroxyl ion concentrations (at least 500 times); that is, so many chloride ions are readily available for movement across the membrane (relative to the number of hydroxyl ions) that chloride ion mobility could not become a limiting factor. Furthermore in our experiments hydroxyl ion movement is driven by a large concentration gradient, while we arranged conditions so that there was approximately zero chloride ion activity gradient initially. Thus, the chloride ion movement can be thought of as resulting solely from the membrane potential changes secondary to the OH^- gradients.

Plateau pH Values

The extracellular pH values in the mixture measured at time zero after mixing are theoretically calculable on the basis of dilution ratios and buffers present in the extracellular fluid. However, the buffering observed could not be explained by the action of water and the measured extracellular hemoglobin. After considerable investigation, we concluded that the extra buffering action could be fully explained by the additional lactate and phosphate that were leaking from the cells into the extracellular fluid. The concentrations of lactate and phosphate in the supernatant from suspension A were measured and found to increase with time of contact with the red cells. Three extra washings reduced these concentrations by only one-third or less, indicating that lactate and phosphate did not reach diffusion equilibrium during the washings, which is not surprising in the light of their low permeability values (Giebel and Passow, 1960; LaCelle and Passow, 1966). There were even significant concentrations of lactate and phosphate in the supernatant from suspension A obtained by spinning suspension A immediately after it was prepared, showing that a considerable volume of extracellular fluid was trapped in the packed cells.

Inclusion of all these buffers theoretically in the computations would not

have been practical, so an empirical buffering curve, as described above, was used. It is perhaps worth special mention that for the special requirements of these experiments, the standard washing procedure does not suffice to prevent the appearance of all diffusible buffer substances in the cell suspension.

Experiments at Extreme Acid Conditions

Several experiments were performed with solution B at both extremes of pH, at 0.9, 2.1, and 11.7. The results when the pH of B was 11.7 are not reliable,

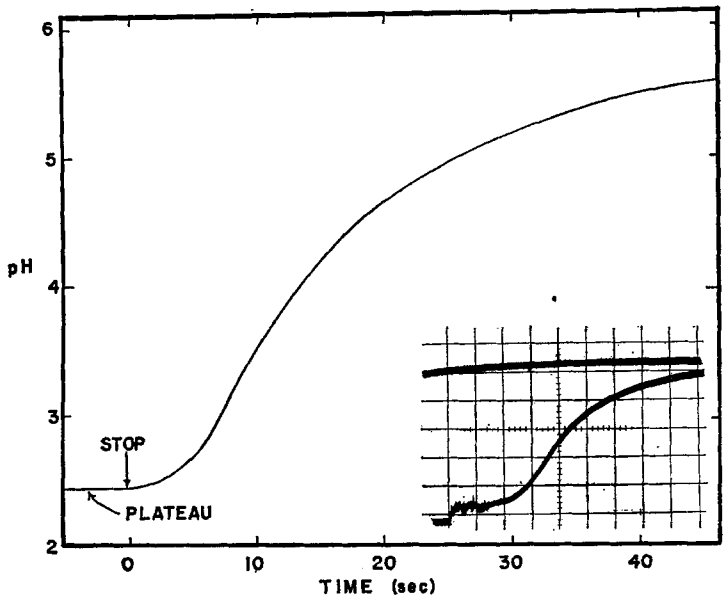

FIGURE 7. Time course of pH_o after mixing equal volumes of suspension A and solution B at pH 2.12. Donor, R. F. Included in the figure is the oscilloscope tracing from which the curve was drawn (in which one horizontal division = 5 sec, and one vertical division = 20 mv).

since the alkaline error of the electrode is quite large above pH 10.5. When pH_B was 0.9, the cells lysed too rapidly after mixing for anything to be said about their permeability behavior.

The results when the pH of B was 2.1, however, exhibit some interesting phenomena. While the cells did eventually lyse about 1–2 min after mixing, enough of a time course of extracellular pH was obtained as to be worth reporting, although the conclusions to be drawn from the results are extremely tentative.

A typical time course is shown in Fig. 7, where pH_B is 2.12. As can be seen, the curve has a sigmoid shape, not exponential as in Figs. 4 and 5 (or indeed, as in all curves obtained when pH_B was between 3.1 and 10.3). Suspension A

for Fig. 7 was the same as that for which the data are given in Table I. The plateau pH is 2.44, and the final pH (at pseudoequilibrium) is about 5.8.

In Fig. 8, the P_{OH^-} values calculated from experiments in which pH_B was 2.12 are plotted against pH_o along with the regression line for the data for $pH_B > 3$ (equation 8). When the pH of the mixture exceeded 4.0, the permeability values, while higher than those obtained at corresponding pH in experiments with pH_B greater than 3.0, were decreasing with increasing pH. The permeability values when the mixture was less than 4.0 were, however,

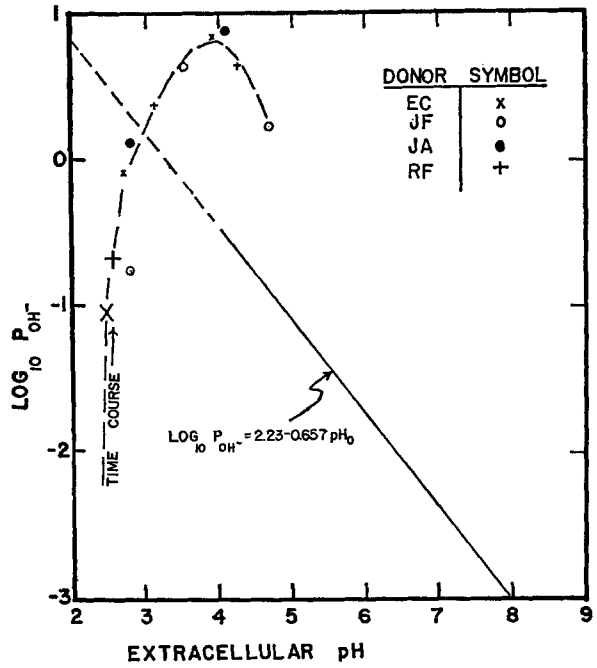

FIGURE 8. $Log_{10} P_{OH^-}$ as a function of extracellular pH, from experiments in which $pH_B = 2.12$. The line is the linear regression line for all points from experiments in which $3.1 < pH_B < 10.3$ (equation 8).

lower than these latter values and moving in the opposite direction with pH.

The reasons for this behavior are not apparent, but suggest that the cell membrane is altered during exposure to an extremely acid environment, so that its permeability to hydroxyl ion is greatly decreased (though not nil) at first (possibly involving cell stiffening) and considerably increased later. Eventually the cells lyse, perhaps due to permanent damage to the membrane (due to extreme external acid conditions).

Changes in internal acidity, and resultant water movements, may also play an important role in cell lysis, because in those experiments in which lysis occurred ($pH_B = 2.12$), internal pH must have decreased significantly. We

calculate that in the experiment shown the internal pH fell about 1.1 units, or to about 5.9 at the pseudoequilibrium, as compared to a change of less than 0.13 unit in all the other cases. Unfortunately the relative importance of swelling vs. membrane alterations cannot be assessed from these few experiments.

We wish to thank Miss Dietrun Kamp for performing many of the measurements reported here. We are also indebted to Mr. Joseph Pili, machinist extraordinaire, for construction of major parts of the equipment.

This work was supported in part by Grant No. 4108 from the National Heart and Lung Institute. Dr. Crandall was the recipient of US Public Health Service Special Fellowship No. 2-F03-GM-38,982-02, and Dr. Klocke was supported by Training Grant No. 5430 from the National Institutes of Health.

The availability to us of the University of Pennsylvania Medical School Computer Facility, under National Institute of Health Grant No. RR-15, is gratefully acknowledged.

Received for publication 16 October 1970.

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