BICARBONATE-CHLORIDE EXCHANGE IN ERYTHROCYTE SUSPENSIONS

STOPPED-FLOW pH ELECTRODE MEASUREMENTS

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ABSTRACT A pH-sensitive glass electrode was used in a temperature-controlled stopped-flow rapid reaction apparatus to determine rates of pH equilibration in red cell suspensions. The apparatus requires less than 2 ml of reactants. The electrode is insensitive to pressure and flow variations, and has a response time of <5 ms. A 20% suspension of washed fresh human erythrocytes in saline at pH 7.7 containing $NaHCO_3$ and extracellular carbonic anhydrase is mixed with an equal volume of 30 mM phosphate buffer at pH 6.7. Within a few milliseconds after mixing, extracellular HCO_3^- reacts with H^+ to form CO_2, which enters the red cells and rehydrates to form HCO_3^-, producing an electrochemical potential gradient for HCO_3^- from inside to outside the cells. HCO_3^- then leaves the cells in exchange for Cl^-, and extracellular pH increases as the HCO_3^- flowing out of the cells reacts with H^+. Flux of HCO_3^- is calculated from the dpH/dt during HCO_3^--Cl^- exchange, and a velocity constant is computed from the flux and the calculated intracellular and extracellular $[HCO_3^-]$. The activation energy for the exchange process is 18.6 kcal/mol between 5°C and 17°C (transition temperature), and 11.4 kcal/mol from 17°C to 40°C. The activation energies and transition temperature are not significantly altered in the presence of a potent anion exchange inhibitor (SITS), although the fluxes are markedly decreased. These findings suggest that the rate-limiting step in red cell anion exchange changes at 17°C, either because of an alteration in the nature of the transport site or because of a transition in the physical state of membrane lipids affecting protein-lipid interactions.

INTRODUCTION

Anion exchanges across the erythrocyte membrane are of particular physiological significance because of the importance of rapid HCO_3^--Cl^- exchange for CO_2 transport in lung and tissue capillaries (1). Recent studies (2, 3) have suggested that a specialized transport system facilitates Cl^--Cl^- self-exchange across the membrane, based on the observation of saturation kinetics, competitive inhibition, high activation energy at low temperature, and the behavior of exchange flux as a function of pH. The exchange may be obligatory and electrically neutral, since membrane conductance appears to be much lower than the calculated exchange permeabilities would suggest (4). This has led to speculation that both exchange and net translocation pathways for anions exist in the red cell membrane, and that both pathways may share common sites (5, 6).

These data have been made more convincing by the demonstration that specific sites in the red cell membrane are likely to be involved in anion exchange processes (7). Much of the kinetic data has been obtained at low temperatures, but Brahm (3) has recently shown that similar phenomena occur at 37°C. Gunn (6) has proposed a model for anion exchanges that may explain the experimental data for divalent and monovalent anions, and includes net translocation as a probabilistic event occurring through the exchange pathway.

Red cell membrane HCO_3^--Cl^- exchange, because of its critical role in gas exchange, has been studied by a number of workers over the last 50 years (8–11). Since the half-time of this process may be of the same order of magnitude as the transit time of red cells through capillary beds during exercise, the exchange might limit the amount of CO_2 that can be transported between blood and tissue or between blood and alveolar gas. Recently, a new approach was developed (12) to study HCO_3^--Cl^- exchange in red cells (or any closed permeable vesicle) over a wide range of physiological conditions. The method is based on observing the rate of pH change in the extracellular fluid of a suspension under conditions where the HCO_3^--Cl^- exchange is rate-limiting. A large quantity of H^+ is added to a red cell suspension that contains some HCO_3^- and a high concentration of carbonic anhydrase in its extracellular fluid. As a result, some H^+ combines with HCO_3^- in the extracellular fluid rapidly to form CO_2 (Fig. 1). This dissolved CO_2 quickly enters the red cells and rehydrates to form H^+ and HCO_3^-, setting up an electrochemical potential gradient for HCO_3^- from inside to outside the cells. As a result of these rapid readjustments in intra- and extracellular [HCO_3^-], HCO_3^- flows out of the cells in exchange for Cl^-. As HCO_3^- enters the extracellu-

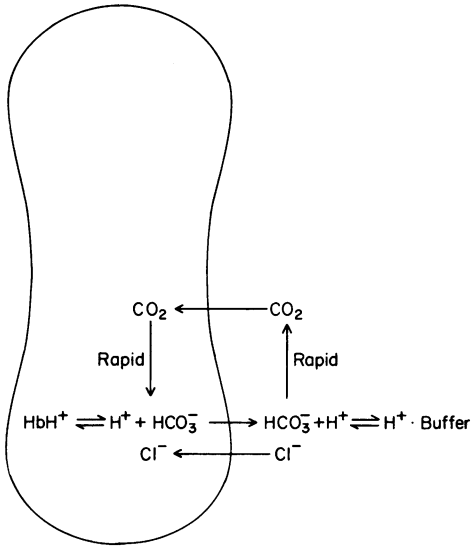

FIGURE 1 Schematic diagram of the Jacobs-Stewart cycle in the presence of extracellular carbonic anhydrase.

lar fluid, however, it rapidly combines with H^+ there to form CO_2 and raise extracellular pH. The CO_2 re-enters the cell and rehydrates to form HCO_3^- and H^+. The net result of a complete cycle of one bicarbonate ion leaving the cell and CO_2 entering to reform HCO_3^- is the transfer of one H^+ and one Cl^- (with the necessary osmotic flow of water) from outside to inside the cell (Fig. 1). This process has been called the Jacobs-Stewart cycle (13,14). dpH/dt in the extracellular fluid after the initial rapid readjustments of intra- and extracellular $[HCO_3^-]$ is a measure of the rate of exchange of HCO_3^- for Cl^- across the red cell membrane.

In the present study, we have used the stopped-flow rapid reaction apparatus to measure pH changes in human erythrocyte suspensions after pulsing the extracellular fluid with acid at temperatures ranging from 5° to 40°C. The flux of HCO_3^- in the Jacobs-Stewart cycle was calculated from the measured dpH/dt, along with a velocity constant based on computed intracellular and extracellular $[HCO_3^-]$. We have shown that there is a transition at 17°C from an activation energy of 18.6 kcal/mol below to 11.4 kcal/mol above 17°C. SITS, a potent anion exchange inhibitor (4-acetamide-4′-isothiocyanostilbene-2,2′-disulfonic acid), does not change the activation energies or the transition temperature, although the fluxes are markedly decreased. The data agree with recently reported measurements of Cl^--Cl^- self-exchange (3), and suggest different rate-determining steps for anion exchange across the red cell membrane above and below the transition temperature. The change in activation energy with temperature may be mediated by a change in the nature of the transport site of the membrane, or by alterations in protein-lipid interactions due to changes in the physical state of lipids in the cell membrane.

METHODS AND MATERIALS

Apparatus

The stopped-flow rapid reaction apparatus used in these experiments has been described previously (15) and is shown schematically in Fig. 2. In the apparatus, equal volumes of a red cell suspension A and phosphate-buffered saline solution B are forced through a four-jet mixer (0.004 ml) into a 0.1-ml measuring chamber. A pH-sensitive glass electrode (117145 Leeds & Northrop Co., North Wales, Pa.) is used to follow the pH of the mixture as a function of time, both before and after flow stops. The reference electrode liquid junction is a KCl-saturated cotton wick bridging a snug-fitting Teflon plug, and is pressure- and flow-insensitive (16). The voltage across the electrodes is amplified (Transidyne General Corp., Ann Arbor, Mich., MPA-6 with its own power source MPS-15) and monitored on a storage oscilloscope screen (5103N, Tektronix, Inc., Beaverton, Ore.). A measure of flow velocity is simultaneously monitored on the oscilloscope screen by recording voltage output from a magnet-in-coil device mounted on the stopped-flow apparatus drive block. The entire apparatus is water-jacketed, and the experiments reported here were carried out between 5°C and 40°C.

The response time of the electrode system has been estimated to be <5 ms, using a ramp change in pH due to carbonic acid dehydration as a test reaction. The lag time of the apparatus (elapsed time between mixing and reaching the glass electrode) is less than 20 ms at the linear flow rates used in these experiments (25–50 cm/s). Further details of the characteristics of the rapid-reaction apparatus are available in the literature (15–17).

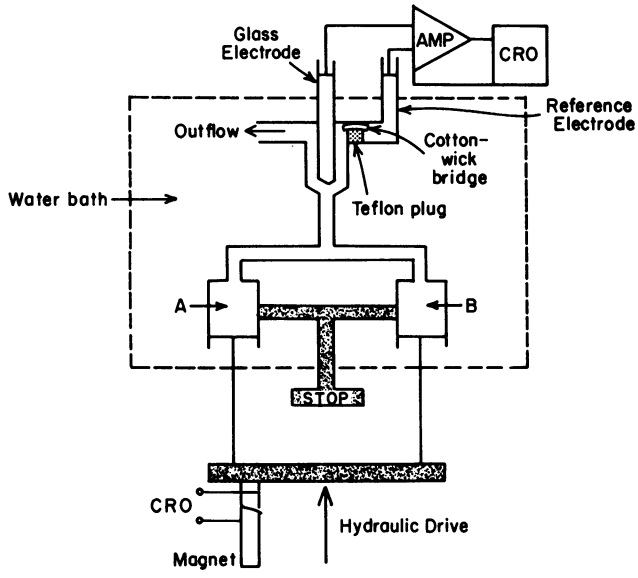

FIGURE 2 Schematic diagram of the stopped-flow rapid reaction apparatus with the pH-sensitive glass electrode as the measuring device.

Solutions and Procedure

Fresh heparinized human blood was centrifuged for 10 min at 3,000 *g*. The cells were resuspended in 10 times their volume of 146.5 mM NaCl, 3.5 mM KCl, and recentrifuged, this procedure being repeated three times. The washed cells were then resuspended in 146.5 mM NaCl, 3.5 mM KCl to about 20% hematocrit to form suspension A. NaOH was added to the suspending medium to reach a final pH_A of 7.7. Carbonic anhydrase (bovine carbonate hydrolase, Sigma Chemical Co., St. Louis, Mo.) was added to the suspension to a concentration of 80,000 Wilbur-Anderson U/100 ml of suspension. Freshly-prepared $NaHCO_3$ was then added to a final concentration of 4.4 mM and the suspension maintained in a closed tonometer thereafter. The phosphate-buffered solution B consisted of 112.5 mM NaCl, 15 mM Na_2HPO_4, and 15 mM KH_2PO_4 with pH_B of 6.7. All suspensions and solutions were prepared at room temperature.

Hematocrit (Hct) of suspension A was measured in standard Wintrobe tubes. pH was determined anaerobically in a Radiometer BMS3 Mk 2 blood gas machine (Radiometer Co., Copenhagen, Denmark). Supernatant hemoglobin concentration in suspension A was measured spectrophotometrically at 541 nm in a Perkin-Elmer Coleman 124 spectrophotometer (Perkin-Elmer Corp., Norwalk, Conn.). Intracellular pH was determined from red cell lysates produced by freezing and thawing packed cells separated by centrifugation. A mixture of equal volumes of suspension A and solution B was collected in a syringe after the mixture had passed through the rapid reaction apparatus. After centrifugation, the supernatant from the mixture was titrated anaerobically with a microburette (S-1100A, Roger Gilmont Instruments, Inc., Great Neck, N.Y.) and a water-jacketed titration chamber. The buffer capacity β of the extracellular fluid of the mixture as a function of pH (around 6.7) was obtained by differentiation of the titration curve.

For the experiments in which cells were treated with an anion exchange inhibitor, the red cells were washed three times as described above and resuspended to 10% hematocrit in a solution containing 146.5 mM NaCl, 20 mM Tris buffer (pH 7.4), 0.2% ethanol and 0.11 mM

SITS (PolyScience Corp., Niles, Ill.). This suspension was incubated at 37°C for 10 min with constant agitation. The cells were then rewashed three times with 146.5 mM NaCl, 3.5 mM KCl, resuspended to 20% hematocrit, and treated further as described above.

Computations

The flux ϕ of HCO_3^- out of the red cells per unit of membrane surface area was determined from the initial dpH/dt observed in the mixture after stopping flow in the rapid reaction apparatus: $\phi = \beta(dpH/dt)(1 - Hct)/(Hct \times A/V)$, where V = volume per cell and A = surface area per cell. The intracellular and extracellular $[HCO_3^-]$ at the time of stopping flow were computed from the measured extracellular pH at that time ("plateau" pH) with the equilibrium constant for carbonic acid and the assumptions that $[CO_2]$ is the same intra- and extracellularly and that total CO_2 content remains constant. The equations, given previously (12), were solved on a PDP-10 digital computer (Digital Equipment Corp., Marlboro, Mass.) using an iterative procedure. A velocity constant for HCO_3^--Cl^- exchange was then calculated: $k = \phi/([HCO_3^-]_i - [HCO_3^-]_0)$.

RESULTS

A typical experimental record is shown in Fig. 3. The upper tracing represents the pH of the fluid in the measuring chamber as a function of time. The lower trace indicates where flow of reactants starts and stops. Each trace was swept across the screen several times. Before flow starts, solution B (pH 6.75) is in the measuring chamber. During flow, the "plateau" pH is that of the mixture during flow, about 20 ms after mixing (pH 6.77). This is the extracellular pH after the rapid movement of HCO_3^- into the cells (described above), but before significant HCO_3^--Cl^- exchange has had time to occur (see Fig. 1). After flow stops, the pH of the mixture in the measuring chamber rises towards its final equilibrated value (pH 7.14), as the Jacobs-Stewart cycle effects the transfer of H^+-equivalents from outside to inside the erythrocytes.

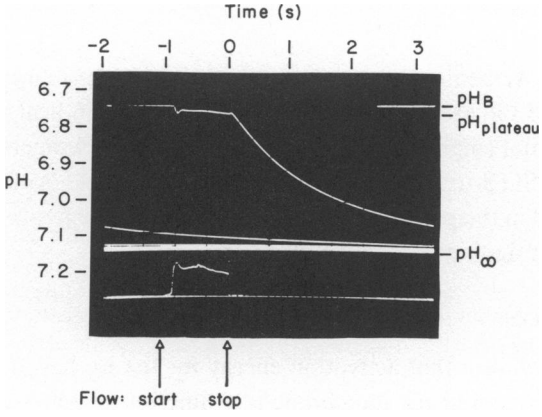

FIGURE 3 Oscilloscope tracing of the change of pH with time in a mixture of equal volumes of solution B and suspension A. In this experiment, suspension A was at pH 7.7 with hematocrit of 16.3%, total CO_2 content of 4.4 mM, and carbonic anhydrase concentration of 800 Wilbur-Anderson U/ml. The times of starting and stopping flow in the rapid reaction apparatus are indicated. Further details of this record, solution B, and suspension A are given in the text.

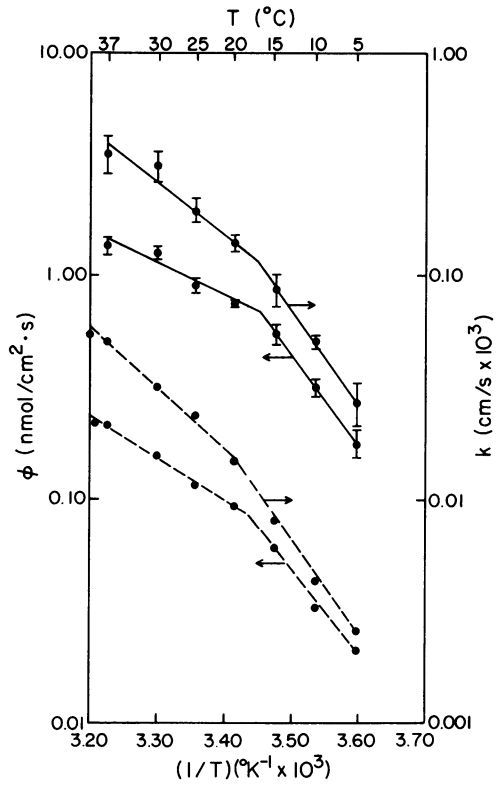

FIGURE 4 Arrhenius plot of the logarithm of flux ϕ and velocity constant k vs. $1/T$ for bicarbonate-chloride exchange. Data for untreated cells are represented by the points on the solid lines (means ± SE, $3 \leq n \leq 8$), and those for cells exposed to SITS are given by the points on the broken lines (means, $n = 2$). The activation energies quoted in the text are based on the velocity constant data.

Fig. 4 shows the Arrhenius plot of the logarithm of both ϕ and k versus $1/T$. The activation energy of the exchange process changes from 18.6 kcal/mol (low temperature) to 11.4 kcal/mol (high temperature) at the transition temperature of 15°C. The Arrhenius plot for SITS-treated cells is also shown in Fig. 4. Although the fluxes are markedly decreased in the presence of SITS, the activation energies and the transition temperature are not significantly changed.

DISCUSSION

Although the observation that activation energy for the exchange of bicarbonate for chloride ions across the red cell membrane is a function of temperature has been reported earlier (12), the present data show that this relationship can be characterized by just two values for activation energy, with the change occurring at about 17°C. Similar behavior has been measured for red cell glucose transport (18) and for viscosity of suspensions of red cell membrane fragments (19), both showing transition temperatures

in the vicinity of 17°C. It was suggested (19) that the change in activation energy is due to an alteration in the physical state of membrane lipids.

Previous studies on Cl^--Cl^- and other anion exchange processes across the red cell membrane had failed to show a change in activation energy with temperature (20), primarily due to the inability to follow rapid fluxes at higher temperatures by isotope techniques. The rapid reaction method reported here, capable of determining fluxes over a wide range of temperature, revealed the transition phenomenon. Very recently, Brahm (3) used a modification of a continuous flow rapid reaction apparatus to study Cl^--Cl^- self-exchange across the red cell membrane. It was found that the activation energy is a function of temperature, being 30 kcal/mol below and 20 kcal/mol above 15°C. Given that we are studying HCO_3^--Cl^- exchange rather than Cl^--Cl^- self-exchange, and allowing for the differences in the experimental conditions, the similarities in the data are notable.

A change in activation energy for anion exchanges at a transition temperature implies that different processes are limiting the rate of the exchanges below and above this temperature. One possible explanation is that the membrane transport mechanism is not the same in the cold as it is at higher temperatures. For example, a carrier-mediated mechanism might limit the rate of exchange at low temperature, while a diffusion process determines the rate at higher temperature (12). This possibility was made less likely by the demonstration (3) that at both low and high temperature, a saturation phenomenon and a maximum flux at $7<pH<8$ characterize Cl^--Cl^- exchange. It is more likely that the change in activation energy is mediated by alterations involving the membrane proteins that may participate in anion exchanges (7). These alterations at the transition temperature could include modifications within a given pathway, a switch of transport from one saturable pathway to another, or changes in the physical state of membrane lipids. The latter possibility, however, would not explain the transition temperature of 25°C for Br^--Br^- self-exchange (3) compared to that for Cl^--Cl^- or HCO_3^--Cl^- exchanges. Brahm (3) has suggested a maximum turnover number at a specific site in the membrane as the factor that determines the rate-limiting step for anion exchanges.

SITS-treated cells, although flux was uniformly reduced by 90%, showed transition temperature and activation energies not significantly different from those for untreated cells. This agrees with data reported for cells exposed to another anion exchange inhibitor, DIDS (4,4′-diisothiocyano-2,2′-stilbene disulfonic acid) (3). These data are interpreted as being consistent with unchanged transport pathway and kinetics at a given temperature, but a diminished number of sites available for the exchange. Further investigation is needed to elucidate the specific mechanism underlying the change in activation energy for transport processes across the erythrocyte membrane.

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REFERENCES

1. CRANDALL, E. D., and R. E. FORSTER. 1973. Rapid ion exchanges across the red cell membrane. *Adv. Chem. Ser.* **118**:65–87.
2. GUNN, R. B., M. DALMARK, D. C. TOSTESON, and J. O. WIETH. 1973. Characteristics of chloride transport in human red blood cells. *J. Gen. Physiol.* **70**:185–206.
3. BRAHM, J. 1977. Temperature-dependent changes of chloride transport kinetics in human red cells. *J. Gen. Physiol.* **70**:283–306.
4. LASSEN, U. V., L. PAPE, B. VESTERGAARD-BOGIND, and O. BENGTSON. 1974. Calcium-related hyperpolarization of the *Amphiuma* red cell membrane following micropuncture. *J. Membr. Biol.* **18**:125–144.
5. KNAUF, P. A., G. F. FUHRMANN, S. ROTHSTEIN, and A. ROTHSTEIN. 1977. The relationship between anion exchange and net anion flow across the human red blood cell membrane. *J. Gen. Physiol.* **69**: 363–386.
6. GUNN, R. B. 1977. A titratable lock-carrier model for anion transport in red blood cells. *Proc. Int. Union Physiol. Sci.* **12**:122.
7. ROTHSTEIN, A., Z. I. CABANTCHICK, and P. KNAUF. 1976. Mechanism of anion transport in red blood cells: role of membrane proteins. *Fed. Proc.* **35**:3–10.
8. DIRKEN, M. N. J., and H. W. MOOK. 1931. The rate of gas exchange between blood cells and serum. *J. Physiol. (Lond.).* **73**:349–360.
9. Luckner, H. 1948. Die temperaturabhängigkeit des anionen-austausches roter blutkörperchen. *Pflügers Arch. Eur. J. Physiol.* **250**:303–311.
10. HEMINGWAY, A., C. J. HEMINGWAY, and F. J. W. ROUGHTON. 1970. The rate of chloride shift of respiration studied with a rapid filtration method. *Respir. Physiol.* **10**:1–9.
11. KLOCKE, R. A. 1976. Rate of bicarbonate-chloride exchange in human red cells at 37°C. *J. Appl. Physiol.* **40**:707–714.
12. CHOW, E. I., E. D. CRANDALL, and R. E. FORSTER. 1976. Kinetics of bicarbonate-chloride exchange across the human red blood cell membrane. *J. Gen. Physiol.* **68**:633–652.
13. JACOBS, M. H., and D. R. STEWART. 1942. The role of carbonic anhydrase in certain ionic exchanges involving the erythrocyte. *J. Gen. Physiol.* **25**:539–552.
14. FORSTER, R. E., and E. D. CRANDALL. 1975. Time course of exchanges between red cells and extracellular fluid during CO_2 uptake. *J. Appl. Physiol.* **38**:710–718.
15. CRANDALL, E. D., R. A. KLOCKE, and R. E. FORSTER. 1971. Hydroxyl ion movement across the human erythrocyte membrane. *J. Gen. Physiol.* **57**:664–683.
16. CRANDALL, E. D., and J. DELONG. 1976. A pressure- and flow-insensitive reference electrode liquid junction. *J. Appl. Physiol.* **41**:125–128.
17. GROS, G., R. E. FORSTER, and L. LIN. 1976. The carbamate reaction of glycylglycine, plasma and tissue extracts evaluated by a pH stopped flow apparatus. *J. Biol. Chem.* **251**:4398–4407.
18. LACKO, L., B. WITTE, and P. GECK. 1973. The temperature dependence of the exchange transport of glucose in human erythrocytes. *J. Cell. Physiol.* **82**:213–218.
19. ZIMMER, G., and H. SCHIRMER. 1974. Viscosity changes of erythrocyte membrane and membrane lipids at transition temperature. *Biochim. Biophys. Acta.* **345**:314–320.
20. DALMARK, M., and J. O. WIETH. 1972. Temperature dependence of chloride, bromide, iodide, thiocyanate and salicylate transport in human red cells. *J. Physiol. (Lond.).* **224**:583–610.

DISCUSSION

BECKER: Have you measured the phase transition temperature and activation energies in red cells having different cholesterol content?

CRANDALL: Not yet. That is one of the things we have on the drawing board. We have a way to make the cholesterol-enriched and cholesterol-depleted cells, and we are about to do that. That is one of the variations we have not done yet, and it should be interesting.