# Kinetics of Passive Anion Transport across the Human Erythrocyte Membrane†

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ABSTRACT: An experiment is presented which allows detailed measurements to be made for the flux of an inorganic divalent anion across the human erythrocyte membrane. Dithionite ($S_2O_4^{2-}$) flux into resealed erythrocyte ghosts containing methemoglobin is measured. Methemoglobin serves to keep intracellular dithionite at a steady-state level during the initial portion of the reaction allowing measurement of the pseudo-zero-order transport velocity. The kinetics are measured at various constant initial concentrations of both cis and trans sulfate, varied independently and simultaneously. The effect of the nondiffusable anion, citrate, on the trans side of the membrane is studied, as are the effects of pH, temperature, and the anion transport inhibitor SITS (4-acetamido-4′-isothiocyanate-stilbene-2,2′-disulfonic acid). The results show several characteristic nonhyperbolic kinetic patterns which depend on the concentration of cis or trans sulfate. At pH 7, 25 °C, and low cis sulfate concentration, the kinetic pattern was characteristic of *activation by substrate followed by substrate inhibition*, with a maximum at about 25–35 mM and a self-inhibition constant estimated to be about 100 mM. Under these conditions, covalent reaction with SITS caused the velocities to decrease by a factor of about 5 and led to hyperbolic kinetic patterns, eliminating the substrate activation–inhibition behavior. While holding trans sulfate concentration constant through the use of nondiffusable cationic buffers, raising the concentration of cis sulfate also changed the pattern to one of substrate activation only and eliminated substrate inhibition. Increasing trans sulfate concentration by the same method *stimulated* the measured velocities, did not significantly affect substrate inhibition at high dithionite, but did cause the substrate activation pattern seen at lower dithionite concentrations to linearize on a double-reciprocal plot. Unexpectedly, increasing the trans citrate concentration also stimulated dithionite influx in a manner almost identical to trans sulfate, despite the fact that citrate is nondiffusable and cannot participate in a direct anion-exchange reaction. The nonhyperbolic nature of the kinetics, as well as the trans stimulation observed with a nondiffusable anion, would seem to rule out classical mobile carrier models for anion exchange. The results would seem more consistent with a multisite "allosteric" pore model.

Studies concerning the passive transport pathways of the erythrocyte membrane have as their general aim the assignment of a given transport process to certain membrane components, the kinetic characterization of transport, and the establishment of structure–function relationships (Deuticke, 1977). The kinetic characterization of inorganic anion transport has been almost exclusively performed with the equilibrium self-exchange method (Gardos et al., 1969) using radioactive tracers (Mond, 1927; Passow, 1964, 1969; Deuticke, 1970; Dalmark, 1976; Funder & Wieth, 1976; Schnell et al., 1977). In general, the exchange kinetics were dependent on pH, temperature, and the concentration of anions. The initial evidence from concentration-dependent measurements with only one anion present suggested that transport involves a simple facilitated diffusion mechanism, showing saturation kinetics which could be analyzed in terms of the Henri–Michaelis–Menten formalism (Gunn et al., 1973). However, recent work with both chloride and sulfate self-exchange suggests a more complex view (Dalmark, 1976; Funder & Wieth, 1976; Schnell et al., 1977). When exchange velocities were measured over a wide concentration range using both resealed ghosts and erythrocytes, substrate inhibition was observed. A self-inhibition constant of about 200 mM was found for sulfate at pH 6.5 (Schnell et al., 1977). This observation has been interpreted to indicate the presence of two anion binding sites: one involved in transport and the other an inhibitor site (Dalmark, 1976; Schnell et al., 1977).

Although equilibrium self-exchange measurements are important, they do not contain sufficient information to draw detailed mechanistic conclusions about transport (Lieb & Stein, 1974a,b; Eliam & Stein, 1974). For example, it is not possible to distinguish between a mobile carrier model vs. a pore model from self-exchange data alone. At least two zero-trans kinetic experiments are required in addition. If possible, several other types of kinetic experiments should be performed in order to completely characterize the functional properties of a transport process (Eliam & Stein, 1974). A zero-trans kinetic experiment involves measuring the unidirectional flux of a substrate across the membrane as a function of concentration while maintaining the concentration on the opposite (trans) side of the membrane at a very low, steady-state level through the use of some type of coupled intracellular reaction. Although this approach is acceptable when characterizing the transport of uncharged molecules, its use in the study of anion transport requires that both transmembrane chemical and electrical potential be considered.

It is possible to formulate an experiment for anion transport which would be analogous to the zero-trans experiment just described. Suppose, for example, that two divalent anions were initially present in the system, one anion on the outside of the membrane (A) and the other on the inside (B) with the concentration of A and B being equal. During the course of a given

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reaction, A would flux into the cell to form product P, and B would flux out, in exchange, to yield product Q. Ideally, one would study this A + B ↔ P + Q heteroexchange system in the *absence of products* by observing the initial rate of product formation using some type of coupled enzymatic trap for P and Q. A major difficulty arises if we view this heteroexchange experiment in a manner analogous to the two substrate problem of enzyme kinetics (Cleland, 1970; Segel, 1975). The experimental protocol for studying a two-substrate enzyme kinetic problem requires measuring the A → P reaction velocity at various constant concentrations of B and vice versa for the B → Q reaction. However, this would be done *at the expense* of maintaining constant transmembrane electrical potential. On the other hand, if the concentrations of A and B are always the same on either side of the membrane and varied together, we would not obtain meaningful results from the enzyme kinetic point of view. This apparent dilemma would be resolved if transmembrane electrical potential was not a significant driving force along the "kinetic pathway" for the transport process. Since there is no a priori way of knowing whether transmembrane electrical potential influences the *kinetics*, we elected to follow the procedures for a two-substrate enzyme kinetic problem and performed experiments with B anions which were diffusable and nondiffusable in order to test the influence of electrical potential.

The present communication shows results from a recently introduced[1] experiment, where dithionite ($S_2O_4^{2-}$) flux into resealed human erythrocyte ghosts containing methemoglobin is measured. The function of intracellular methemoglobin is to trap one product of the reaction, intracellular dithionite, thereby maintaining it at very low, steady-state levels during the initial portion of the reaction (see Results section for further details). Since we could not trap the other product of the reaction, extracellular sulfate, we did study its effect on the kinetics over a concentration range above and below the apparent $K_m$ determined from equilibrium self-exchange measurements. In addition, the kinetics were studied at various constant trans coanion and cis coanion concentrations, varied together and independently (see below). The effect of trans citrate, a nondiffusable anion (Garby, 1965), was also investigated at low cis and trans sulfate. Finally, the effects of temperature, pH, and the anion transport inhibitor SITS[2] were studied on entire saturation curves. A somewhat similar approach to the steady-state kinetics for transport of the organic anion pyruvate has also been introduced by Rice & Steck (1976, 1977) and by Halestrap (1976). An abstract based on the current results has been published (Salhany & Swanson, 1978).

## Materials and Methods

*Single-Beam Stopped-Flow Experiments on the Reduction of Methemoglobin by Dithionite in Solution.* Methemoglobin was prepared according to the method of Gibson et al. (1969). Single beam stopped-flow experiments were performed as described previously (Demma & Salhany, 1977), in 0.1 M phosphate buffer, 23 °C, under anaerobic conditions.

*Preparation of Resealed Ghosts Containing Methemoglobin.* Freshly outdated bank blood (or packed erythrocytes) from hematologically normal adult donors was used in this work. The blood was washed three times in cold (0–5 °C) PBS taking care to remove the "buffy coat." No blood sample was used past 7 days of its expiration date. Ice cold (0 °C) 5P (8) was prepared and all flasks were prechilled in an ice bath. The cold, washed, packed cells were lysed (1:5) and kept ice cold. The solution was then centrifuged at 15 000 rpm for 15 min at 0 °C in a Sorvall RC2B. The ghosts were collected and placed on ice. The hemolysate was saved. The hemolysate was used to make up the resealing solution which contained (before the mix with ghosts): 4 mM (heme) hemoglobin; 5P (8); 100 mM Tris; 100 mM Bistris; 40 mM sucrose and 280 mM NaCl, pH 9. This resealing solution was mixed 50:50 with the previous packed ghosts containing 4 mM (heme) hemoglobin plus 5P (8). The sample was then incubated at 37 °C for 1 h. Following incubation, the suspension was centrifuged at 10 000 rpm for 15 min at 0 °C in the Sorvall. The supernatant was removed and the oxyhemoglobin containing ghosts were suspended in the final wash solution used for the particular flux study but containing in addition 10 mM sodium nitrite. The ghosts were allowed to stand for 30 min at room temperature, open to air, during which time oxyhemoglobin was converted to methemoglobin within the cell. The ghosts were then washed once with the wash solution containing the required concentrations of sulfate, sucrose, Tris, and Bistris (minus sodium nitrite). They were then resuspended and incubated for 1 h at 37 °C in the wash solution after which they were washed two more times. Ghosts were used within 2 days after preparation. The ghosts were stored at 4 °C in the packed state in the last wash buffer. On the day of the flux studies, the ghosts were washed once again in the desired wash solution. The pH of the buffer solutions used to wash the ghosts was adjusted with sulfuric acid and the amount of added sodium sulfate adjusted accordingly as required.

*Control of Initial Sulfate Concentration on Either Side of the Membrane.* The basic principle we use to independently control the initial sulfate concentration on either side of the membrane is the Donnan equilibrium along with the fact that Tris and Bistris are nondiffusable buffers for the erythrocyte membrane (Battaglia et al., 1968). At pH 7, the concentration of sulfate inside and outside the cell is determined largely by the concentration of Tris on either side of the membrane. Using a Tris p$K$ of 8.1 and a Bistris p$K$ of 6.5 and assuming 2 equiv of Tris or Bistris per sulfate ion, a solution of 50 mM Bistris and 50 mM Tris base adjusted to pH 7 with sulfuric acid will contain 6 mM sulfate (Bistris contribution) plus 23 mM sulfate (Tris contribution) to equal 29 mM sulfate.

Variation in Intracellular Sulfate. Intracellular sulfate was varied by varying the amount of intracellular Tris incorporated at the resealing step described above. Intracellular sulfate was systematically varied at 6, 29, and 61 mM, corresponding to 50 mM Bistris plus 0, 50, and 119 mM intracellular Tris. Otherwise, the resealing solution described above was the same. *Intracellular sucrose was always incorporated to act as an osmotic buffer.*

Variation of Extracellular Sulfate. Extracellular sulfate concentrations were largely varied by changing the Tris concentration in the buffer wash solutions. There was one exception to this procedure. When 244 mM extracellular sulfate was desired at 29 mM intracellular sulfate, ghosts were prepared with 50 mM Bistris and 50 mM tris inside (i.e., 29 mM sulfate) and washed with the same buffer plus 200 mM sucrose, so that there was 29 mM sulfate on both sides. At the time of the experiment, they were mixed in the stopped-flow with the same

[1] The experimental approach presented in this report was introduced by J.M.S. to the Red Cell Club at the 61st Annual Meeting of the Federation of American Societies for Experimental Biology, Chicago, Illinois, April 3, 1977.

[2] Abbreviations used: SITS, 4-acetamido-4′-isothiocyanate-stilbene-2,2′-disulfonic acid; PBS, 5 mM sodium phosphate plus 150 mM NaCl, pH 8; 5P (8), 5 mM sodium phosphate, pH 8; Tris, tris(hydroxymethyl)aminomethane; Bistris, *N,N*-bis(2-hydroxyethyl)iminotris(hydroxymethyl)methane; 2,3-DPG, 2,3-diphosphoglycerate; ATP, adenosine triphosphate; IHP, inositol hexaphosphate.

buffer to which 430 mM sodium sulfate was added as well as the various dithionite concentrations, giving a final outside sulfate concentration of 244 mM, after the mix.

Variation of Extracellular and Intracellular Sulfate Simultaneously. In order to maintain the same intra- and extracellular sulfate, the Tris and Bistris concentrations on either side of the membrane were kept the same. Variation of Tris concentration on *both* sides of the membrane was used to control sulfate concentration up to 29 mM. Beyond that level, cis and trans sulfate concentrations were established by washing with sodium sulfate and bringing the system to equilibrium.

Effect of Intracellular Citrate. The procedures described at the beginning of this section were again employed in the preparation of ghosts to study the effect of intracellular citrate at 6 mM sulfate on both sides of the membrane. Besides the other necessary components, citrate was also present in the resealing solution so as to give a final intracellular concentration of 43 mM.

*Reaction of Ghosts with SITS.* Control ghosts with 6 mM sulfate on both sides (i.e., 50 mM Bistris), pH 7, were incubated with a stock solution containing 1 mM SITS in the same buffer (which also contained sucrose), pH 7, at 37 °C for 1 h at about a 10% hematocrit. The sample was then centrifuged at 0 °C and washed three to four times with the same buffer, free of SITS.

*Split-Beam Stopped-Flow Data Collection Procedures.* The split-beam attachment to the Gibson-Durrum stopped-flow is commercially available (Durrum Instrument Co., Palo Alto, Calif.). The basic design has been described in the literature (Millikan, 1933; Sirs & Roughton, 1963). Two narrow band pass filters (±3 nm, Ditric Optics, Marlboro, Mass.) were used, one at 560 nm (a reaction wavelength) and one at 600 nm (near a reaction isosbestic for met- vs. deoxyhemoglobin). The stopped-flow was interfaced to an On-Line-Instruments Systems (OLIS, Athens, Ga.) data acquisition system, described briefly elsewhere (Demma & Salhany, 1977). Programs were written at OLIS to acquire and analyze the output from the two photomultiplier tubes simultaneously. The program calculates $\Delta A$ at each increment of time for each wavelength and stores them separately. The difference in $\Delta A$ at the two wavelengths is then calculated for each point in time by the computer and this $\Delta\Delta A$ is normalized and initial time constants are calculated. The technique eliminates any artifacts due to mixing or volume changes which can occur during the course of the reaction. *It is our opinion that the split-beam stopped-flow system is essential for this type of work (see Results section).* The stopped-flow system was checked by studying the reaction of metmyoglobin with dithionite under heme limited conditions. The reactions were linear on a log scale over 95% of the time course. The cuvette path length used in all of this work was 2 mm. All mixing experiments were executed by hand pushing the drive syringes. Temperature was controlled by a Lauda K-2/RD circulating water bath. The ghosts suspension (~1.5 mL of packed ghosts suspended in 25 mL of buffer or about $1-3 \times 10^6$ cells/cm$^3$) was placed in a 50-mL Erylenmeyer flask capped with a rubber septum and thoroughly degassed with $N_2$ (Matheson, Inc., East Rutherford, N.J.). The buffer to which the dithionite was added was also thoroughly degassed with $N_2$. $N_2$ degassed buffer was also passed several times through the stopped-flow. The degassed ghosts and dithionite solutions were anaerobically transferred to the stopped-flow and reaction records acquired.

*Chemicals.* Sucrose, Tris base, Bistris, and sodium citrate were acquired from Sigma Chemical (St. Louis, Mo.). Sodium sulfate, sodium phosphate, sodium chloride, and sodium dithionite were acquired from J.T. Baker (Phillipsburg, N.J.). SITS was from ICN (Cleveland, Ohio).

## Results

### *Theory and Primary Characteristics of the Dithionite Transport System*

1. *Reduction of Methemoglobin by Dithionite.* Sodium dithionite is a reagent which is used to study the reduction kinetics of a wide variety of biological molecules, especially heme proteins (Lambeth & Palmer, 1973). It exists as an equilibrium mixture of dimeric and monomeric species, with the dimeric form being dominant ($S_2O_4^{2-} \leftrightarrow 2SO_2^-$; $K_{eq} = 1.4 \times 10^{-9}$ M). Either species can act as a reducing agent depending on the particular reaction being studied. The reaction of dithionite with metmyoglobin has been very thoroughly studied (Lambeth & Palmer, 1973; Olivas et al., 1977; Cox & Holloway, 1977). The evidence indicates that $SO_2^-$ is the reducing species for this reaction. The reaction of dithionite with methemoglobin has also been studied (MacQuarrie & Gibson, 1971), but, to our knowledge, there are no published data to indicate which of the two species is the effective reducing agent. As a necessary preliminary to the transport experiments, we studied the dependence of the reaction on pH and dithionite concentration. When about 56 $\mu$M (heme) of methemoglobin was reacted with 0.1% dithionite, two kinetic components were observed at all pH values studied and raising the pH from 6 to 8 caused both phases to decrease in rate by a factor of about 3.5, (MacQuarrie & Gibson, 1971). A log-log plot of the two observed rate constants vs. the concentration of $S_2O_4^{2-}$ at pH 6 gave straight lines with slopes of 0.5 ± 0.1 over a wide concentration range, indicating that the reducing species is $SO_2^-$. From these data it was possible to calculate an apparent second-order rate constant ($k_r$) for the reaction of $SO_2^-$ with each component according to the expression $k_{obsd} = k_r K_{eq}^{1/2} (S_2O_4^{2-})^{1/2}$ (Lambeth & Palmer, 1973). We have obtained values for $k_r$ of about $10^6$ M$^{-1}$ s$^{-1}$ for both phases, at pH 6. With these preliminary results, we are now able to discuss the application of this reaction to the study of anion transport across the human erythrocyte membrane.

2. *Theory of the Dithionite Transport System.* The theory of the assay system employed in this study is similar to that used for enzyme kinetic reactions where the reaction of interest, whose product cannot be measured directly, is coupled to a reaction whose product can be directly measured (McClure, 1969). The primary reactions to consider in the present system are: (A) the permeation of $S_2O_4^{2-}$ through the erythrocyte membrane; (B) the monomerization of $S_2O_4^{2-}$ to $SO_2^-$; and (C) the irreversible reduction of intracellular methemoglobin by dithionite.

(A) Permeation of Dithionite through the Membrane. It is necessary to assume that this process is pseudo-zero-order and irreversible. Furthermore, we have neglected extracellular $SO_2^-$ in our considerations owing to the very small monomerization equilibrium constant (Lambeth & Palmer, 1973). Over the concentration range we use, extracellular $SO_2^-$ never exceeds about 17 $\mu$M. Neglecting this very small concentration seems reasonable when one considers that most monovalent anions studied to date bind to the erythrocyte membrane with apparent $K_m$ values between 30 and 80 mM (Deuticke, 1977). The assumption of pseudo-zero-order kinetics also seems reasonable since there is virtually an infinite pool of extracellular dithionite from which very little is actually consumed during the reaction. Finally, the irreversibility of the permeation process is assumed since intracellular dithionite is continuously being removed during the observation period by re-

acting irreversibly with methemoglobin to form the gas sulfur dioxide.

(B and C) Dithionite Monomerization and Reduction of Intracellular Methemoglobin. As was shown in the first part of the Results section, the reaction of methemoglobin with dithionite can be represented as follows:

$$S_2O_4 \leftrightarrow 2SO_2^- \; k_1; \; k_{-1}; \; K_{eq} \qquad (1)$$

$$SO_2^- + F^{3+} \rightarrow F^{2+} + SO_2(\text{gas}) \; k_2 \qquad (2)$$

where $F^{3+}$ is a met heme and $F^{2+}$, a deoxy heme. It is not necessary to consider the differences in reduction rate between the $\alpha$ and $\beta$ chains (MacQuarrie & Gibson, 1971). Writing the differential equations and applying steady-state approximations yields:

$$2k_1(S_2O_4^{2-}) = (SO_2^-)(2k_{-1}(SO_2^-) + k_2(F^{3+})) \qquad (3)$$

The reduction of methemoglobin by dithionite within the cell occurs under dithionite limited conditions (i.e., $(S_2O_4^{2-}) \ll$ (heme)). During the initial portion of the permeation process, we would expect

$$k_2(F^{3+}) \gg 2k_{-1}(SO_2^-) \qquad (4)$$

Thus, the $2k_{-1}(SO_2^-)$ term of eq 3 can be neglected and we can solve the reduced form of eq 3 for $(SO_2^-)$ and substitute into the differential equation for the disappearance of met hemes (or the appearance of deoxy hemes) and obtain the following differential equation:

$$-d(F^{3+})/dt = d(F^{2+})/dt = 2k_1(S_2O_4^{2-}) \qquad (5)$$

The rate of this reaction is then first order with respect to intracellular $S_2O_4^{2-}$ and zero-order in heme concentration, with the dithionite monomerization rate being rate limiting ($k_1$ = 1.7 $s^{-1}$; Lambeth & Palmer, 1973). These intracellular conditions are exactly those required for our coupled assay system: *irreversible reduction of methemoglobin by dithionite with the reaction being first order with respect to intracellular* $S_2O_4^{2-}$. This condition will not hold when inequality 4 fails. Inequality 4 will not hold when the met hemes within the cell are nearly used up, at which time the reaction will become heme limited.

It is now possible to schematically represent $S_2O_4^{2-}$ permeation through the membrane, coupled to the reduction of intracellular methemoglobin as follows

$$(S_2O_4^{2-})_{out} \xrightarrow{k_T} (S_2O_4^{2-})_{in} \xrightarrow{k_1} 2(F^{2+})_{in} \qquad (6)$$

where $k_T$ is the pseudo-zero-order dithionite transport velocity (mol/(L·s)) and $k_1$ is the first-order monomerization rate ($s^{-1}$) of dithionite within the cell. The rate equation for this system is:

$$d(S_2O_4^{2-})_{in}/dt = k_T - k_1(S_2O_4^{2-})_{in} \qquad (7)$$

which integrates to

$$(S_2O_4^{2-})_{in} = (k_T/k_1)(1 - e^{-k_1 t}) \qquad (8)$$

Upon substitution of eq 8 into eq 5 and integration we have that

$$(F^{2+}) = 2k_T t + (2k_T/k_1)(e^{-k_1 t} - 1) \qquad (9)$$

Equation 9 shows that observation of the reduction of intracellular methemoglobin in the coupled transport system should show an initial lag period during which intracellular dithionite builds up to a steady-state concentration. When the steady-state concentration is reached, the time course will be linear with slope equal to $2k_T$. In the experiments reported below, the

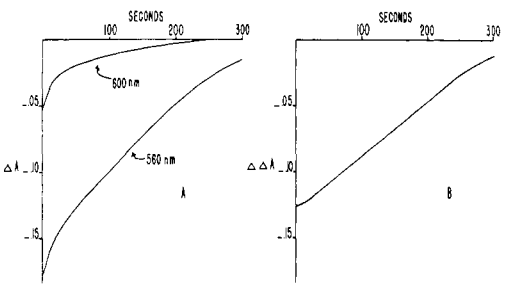


FIGURE 1: Split-beam stopped-flow data for the reaction of resealed erythrocyte ghosts containing methemoglobin with sodium dithionite. (A) Change in absorbance with time at the reaction wavelength (560 nm) and isosbestic (600 nm). (B) Computer calculated difference in $\Delta A$ at the two wavelengths ($\Delta\Delta A = \Delta A_{560} - \Delta A_{600}$). In the example shown, 600 data points were collected at each wavelength. The concentrations after the mix were: 5% ghost hematocrit; 3 mM sodium dithionite; 50 mM Tris; 50 mM Bistris; 20 mM sucrose; and 122 mM sulfate (pH 7.5, 25 °C).

*number* of met hemes within the cell is always the same and we observe the total conversion of intracellular hemoglobin from met to deoxy. Therefore, time constants calculated over the linear portion of normalized time courses will be directly comparable. Conversion of the measured time constants to velocities simply requires multiplication by 2 mM (i.e., (4 mM)/2).

It is of interest to know how long the induction period should last for our coupled reaction since transient phases for the anion transport system may occur and they need to be distinguished from the inherent induction period for the coupled assay system. It is possible to measure induction periods by extrapolating the linear portion of the time course to $t = 0$ with the line passing through a point where $(F^{2+}) = 0$ and intersecting the negative $(F^{2+})$ axis. The equation describing the extrapolated line is obtained from eq 9 by setting $e^{-k_1 t}$ equal to zero:

$$(F^{2+}) = 2k_T t - 2k_T/k_1 \qquad (10)$$

When $(F^{2+}) = 0$, we have that the lag time, $t'$, is

$$t' = 1/k_1 \qquad (11)$$

which should equal about 0.5 s at 25 °C since $k_1$ = 1.7 $s^{-1}$ (Lambeth & Palmer, 1973).

3. *Primary Characteristics for Dithionite Flux across the Erythrocyte Membrane.* Figures 1A and 1B show results from the on-line split-beam stopped-flow when resealed ghosts containing methemoglobin are mixed with 3 mM dithionite (after the mix) at pH 7.5, 25 °C, in the presence of 122 mM sulfate on both sides of the membrane. Figure 1A shows results ($\Delta A$ vs. time) from both photomultiplier tubes with band pass filters at 560 nm and 600 nm. Figure 1B shows the computer corrected data ($\Delta\Delta A = \Delta A_{560} - \Delta A_{600}$). The reaction shows an initial lag period followed by a substantial linear portion over which the initial velocity time constant can be calculated. Extrapolation of the linear portion of the time course to zero time, and extension of a horizontal line from the observed value of $\Delta\Delta A$ at time zero, shows an observed lag time of about 10 s. This is much longer than predicted from eq 11 and may suggest that part of the observed lag period represents a real pre-steady-state phenomenon for the transport of dithionite. We do not deal with pre-steady-state kinetics in this paper. All velocities presented here come from good linear time courses when plotted on a linear scale (see below).

The initial velocity time constant for intracellular methemoglobin reduction increased with incresing extracellular dithionite concentration (Figure 2). Figure 2A shows some time course data as a function of extracellular dithionite. The time

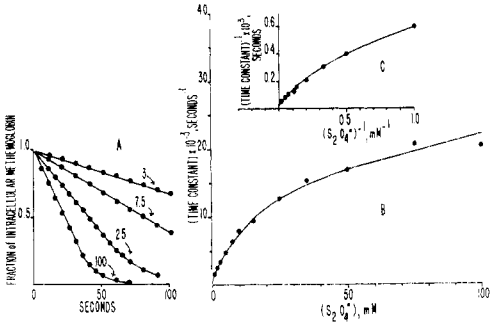

FIGURE 2: Intracellular methemoglobin reduction as a function of extracellular dithionite concentration. (A) Computer corrected, normalized time courses at 3, 7.5, 25, and 100 mM extracellular dithionite after the mix. (B) Plot of the time constant from the linear portion of curves in (A) vs. extracellular dithionite concentration (see text for method of calculation and presentation of the data). (C) Double-reciprocal or Lineweaver–Burk plot of the data in B. The lines are drawn through the data points and have no theoretical significance. Conditions are the same as those listed in the legend to Figure 1.

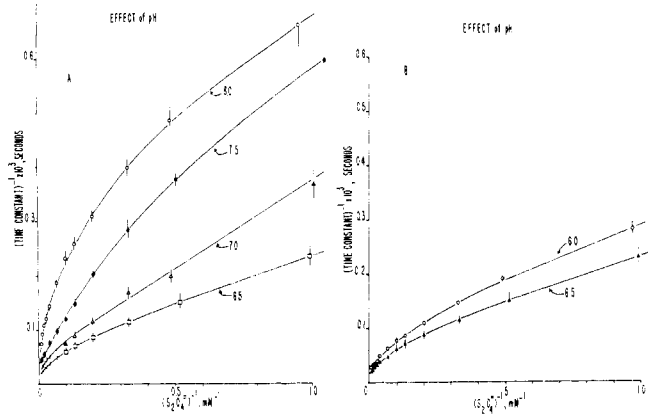

FIGURE 3: pH dependence of dithionite flux into resealed erythrocyte ghosts containing methemoglobin. Lineweaver–Burk plots for the effect of (A) raising the pH from 6.5 to 8 and (B) lowering the pH from 6.5 to 6.0. Temperature was 25 °C. Concentrations of sufate and other ingredients are as listed in the legend to Figure 1. pH was adjusted with $H_2SO_4$ and the amount of sodium sulfate added reduced so as to keep total sulfate constant. Error bars indicate the experimental range for two separate determinations on different days using different ghost preparations.

constant for the reaction was determined over the linear portion of each curve and plotted as a function of extracellular dithionite (Figure 2B). Under these conditions, the plot shows saturation-like behavior. An inspection of the double-reciprocal plot in Figure 2C shows that the kinetics are not hyperbolic. This was initially noticed in Salhany's original experiments with this new technique[1] and has since been observed by Halestrap (1976) for pyruvate flux, suggesting that the source of the nonlinearity in the double-reciprocal plots may be the transport mechanism and not an artifact. Rice & Steck (1976) did not report this nonlinearity since they restricted their data to a narrow pyruvate concentration range. In the next part of the Results section, we show that nonlinear Lineweaver–Burk plots are characteristic of the erythrocyte transport mechanism and that several identifiable kinetic patterns can be obtained depending on the concentration of coanion on either side of the membrane.

It is well known that pH and temperature affect the kinetics of anion exchange across the erythrocyte membrane (Deuticke, 1977; Schnell et al., 1977; Funder & Wieth, 1976; Gunn et al., 1973). Therefore, it is necessary to define the effects of these variables on the transport system being studied here. One interesting characteristic of the pH dependence for anion

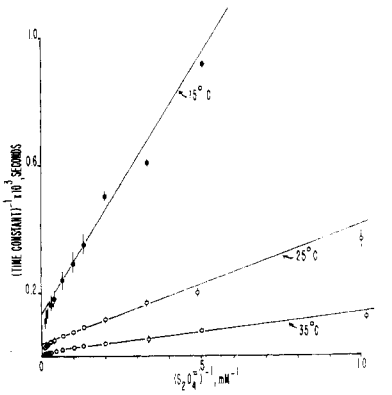

FIGURE 4: Effect of temperature on dithionite flux into resealed erythrocyte ghosts containing methemoglobin. The conditions are the same as in Figures 1–3 with respect to concentrations present. The pH was adjusted to 7.0 at each indicated temperature. Error bars mean the same as stated in the legend to Figure 3. Values of $K_m$ and $V_{max}$ were estimated using the weighted linear least-squares treatment of Wilkinson at dithionite concentrations between 2 and 15 mM. The lines drawn in the figure are the best fit of the data over this concentration range.

transport is that the exchange of *divalent* anions shows a *pH maximum* around 6.5, while monovalent anion exchange is characterized by the presence of a single titration with an apparent p$K$ between 6 and 6.5. In addition, the exchange process is characterized by a high Arrhenius activation energy (20 to 30 kcal/mol). We have investigated the effect of pH and temperature on dithionite flux over a wide concentration range. The results of Figure 3 show the pH dependence for transport. Although the double-reciprocal plots are nonlinear, it is clear that lowering the pH from 8 to 6.5 uniformly increases the velocities of dithionite flux (Figure 3A). As the pH continues to decrease, the initial velocities become slow (Figure 3B) consistent with a pH maximum near 6.5.

The effect of temperature is shown in Figure 4. Increasing the temperature has a marked effect on transport. Once again the plots are nonhyperbolic at the three temperatures studied. Therefore, calculation of Arrhenius activation energies using "$V_{max}$" cannot be considered entirely meaningful. Despite this obvious complication, it still seemed worthwhile to attempt to arrive at some number for an activation energy over a concentration range where hyperbolic behavior could be approximated. This was accomplished by writing a computer program to treat the kinetic data according to the method of Wilkinson (1961) (i.e., weighted linear least-squares fit). We used data between 2 and 15 mM to obtain values of $V_{max}$ and $k_m$. At least two complete sets of data were used at each temperature. A second least-squares fit of the log ($V_{max}$) vs. ($1/T$) values was made and was linear. The Arrhenius activation energy obtained was 20 kcal/mol. It may be worth noting that the "$K_m$" values determined from these calculations were *not* significantly different over the temperature range studied.

In summary, this new experiment does appear to provide a means for measuring the flux of dithionite ($S_2O_4^{2-}$) across the erythrocyte membrane under conditions where the membrane barrier is rate determining. The measurements show several characteristics of the erythrocyte anion transport system seen in sulfate self-exchange (Schnell et al., 1977) and certain kinetic experiments (Halestrap, 1976). It is clear that the next problem is to attempt to identify the meaning of the nonhyperbolic nature of the flux kinetics. In order to accomplish this we must turn to kinetic measurements as a function of coanion concentration on either side of the membrane.

*Effect of Sulfate Concentration on the Initial Velocity of Dithionite Flux.* In this section we present a systematic stud

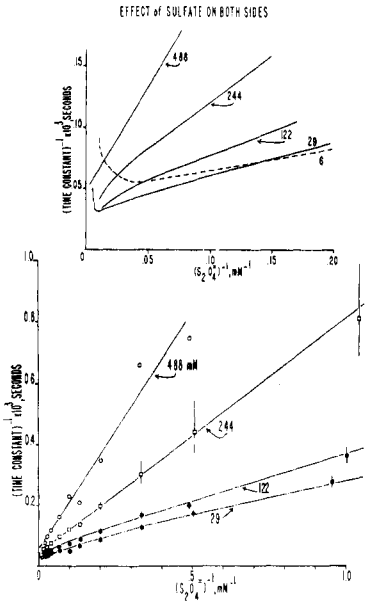


FIGURE 5: Effect of variation in sulfate concentration on *both* sides of the membrane, on dithionite flux. The lower portion of the figure shows the effect of increasing total sulfate from 29 mM to 488 mM on both sides. The insert for this figure shows an expanded view of the high dithionite concentration end of the curves and also includes data for 6 mM total sulfate. Data points have been omitted for clarity. The data were collected at pH 7, 25 °C. The reaction mixture for the experiments at 122, 244, and 488 mM sulfate contained (after the mix): 5% ghost hematocrit, 20 mM sucrose, 50 mM Tris base, 50 mM Bistris, adjusted to pH 7 with $H_2SO_4$ and sodium sulfate to give the total sulfate concentrations indicated (including buffer contribution). Ghosts were prepared as described in Materials and Methods and contained 50 mM Tris, 50 mM Bistris, 20 mM sucrose, and 4 mM heme. The conditions were the same for 6 and 29 mM total sulfate except that 200 mM and 120 mM extracellular sucrose was present. Sucrose concentration, per se, was shown not to affect the kinetics. The ghosts for the experiments with 6 mM total sulfate did not contain intracellular or extracellular Tris.

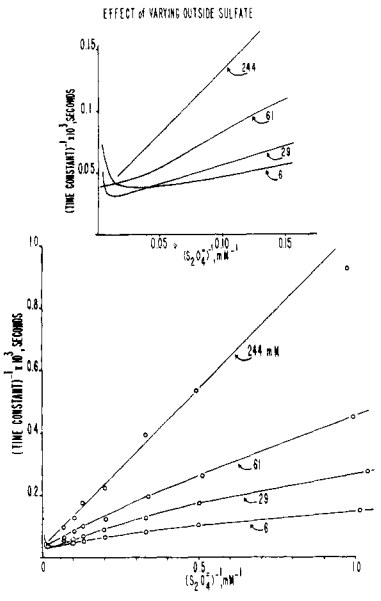


FIGURE 6: Effect of variation in extracellular sulfate at constant intracellular sulfate. The data show the effect of varying extracellular sulfate from 6 to 244 mM at constant (29 mM) intracellular sulfate, pH 7, 25 °C. The lower portion of the figure shows entire curves, with experimental points at concentrations greater than 35 mM omitted for clarity. The insert shows an expanded view of the high dithionite concentration end of the curves and again the data points are omitted for clarity. See Materials and Methods for further details concerning variation in extracellular sulfate at constant intracellular sulfate.

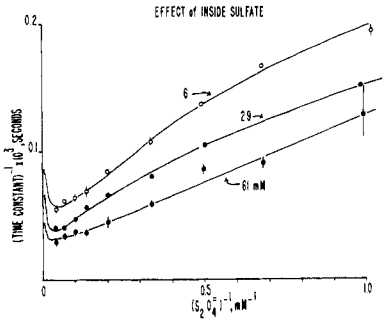


FIGURE 7: Effect of variation in intracellular (trans) sulfate at constant, *low*, extracellular (cis) sulfate. The data show the effect of varying *intracellular* sulfate from 6 to 61 mM at constant extracellular sulfate (6 mM) (see Materials and Methods section for details). At dithionite concentrations greater than 25 mM, points are omitted for clarity. The pH was 7.0 and temperature was 25 °C.

of the effect of variation in sulfate concentration: (a) on both sides of the membrane simultaneously; (b) on the outside (cis side with respect to dithionite) of the membrane at constant trans (inside) sulfate; and (c) on the trans side of the membrane at constant cis sulfate. The measurements are made at pH 7, 25 °C.

Figure 5 shows results from initial velocity measurements where the trans and cis sulfate concentrations are equal and increased together from 6 mM to 488 mM (see Materials and Methods for the techniques used to vary sulfate concentration). Several interesting features can be seen in these double-reciprocal plots. At 6 mM sulfate, the plots are quite nonlinear showing what can be characterized as *activation by substrate followed by substrate inhibition* (Segel, 1975). The double-reciprocal plot curves down toward the $1/(S_2O_4^{2-})$ axis initially and then curves up at higher dithionite (see insert to Figure 5). The minimum in this plot occurs around 25–35 mM. The substrate activation component is similar to that seen by Halestrap (1976) for pyruvate flux into red cells. Substrate inhibition has also been observed for sulfate in the equilibrium self-exchange measurements with erythrocytes or ghosts (Schnell et al., 1977).

In order to better understand how sulfate influences the observed kinetic pattern, we attempted to investigate the effect of independent variation in cis and trans sulfate concentration. The results of Figure 6 show the effect of variation in cis sulfate from 6 to 244 mM at constant trans sulfate (29 mM) (see Materials and Methods). At 6 mM cis sulfate the typical substrate activation–inhibition pattern is seen. As the concentration increases, substrate inhibition begins to disappear until only a weak substrate activation pattern remains at 244 mM cis sulfate (see insert to Figure 6). At this point it would appear that the substrate inhibition pattern seen at low coanion concentrations (Figure 5) is most strongly influenced by the concentration of cis sulfate.

The effect of varying the concentration of trans sulfate at low cis sulfate (6 mM; $K_m$ for sulfate 30 mM; Schnell et al., 1977) is shown in Figure 7. Increasing the fixed trans sulfate concentration from 6 mM to 61 mM caused the velocities to "uniformly" increase at all dithionite concentrations. This effect is quite unlike the effect of increasing the concentration of cis sulfate (Figure 6). Also dissimilar was the fact that the substrate inhibition pattern was not eliminated above 29 mM. The substrate activation behavior (concave down pattern at lower dithionite) was apparently less pronounced at higher trans sulfate.

In summary, we see that the cis and trans sulfate concen-

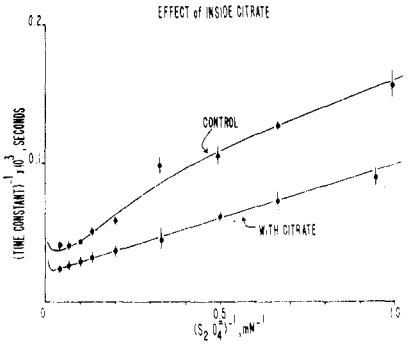


FIGURE 8: Effect of intracellular (trans) citrate on dithionite flux at low (6 mM) sulfate on both sides. Data from control ghosts (no citrate + 50 mM Bistris + 20 mM sucrose) and from experimental ghosts (43 mM citrate + 50 mM Bistris + 20 mM sucrose) are shown and consist of the average of two separate determinations on different days using different ghost preparations. The extracellular solution contained (after the mix): 200 mM sucrose, 50 mM Bistris (adjusted to pH 7 with $H_2SO_4$). The pH was adjusted to 7 with sulfuric acid (final $(SO_4^{2-})$ = 6 mM on both sides of the membrane) and the temperature was 25 °C. A weighted linear least-squares fit (Wilkinson, 1961) of the data for ghosts containing citrate, *before* the onset of substrate inhibition, gives $K_m$ = 3.4 ± 0.2 mM and $V_{max}$ = 92.4 ± 1.6 μM/s.

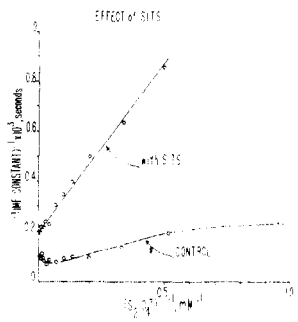


FIGURE 9: Effect of SITS on dithionite flux at low (6 mM) total sulfate. Note that covalent reaction of ghosts with SITS, under conditions stated in the text, gives essentially linear double-reciprocal plots, while control ghosts show the substrate activation–inhibition behavior usually seen at 6 mM sulfate, pH 7, 25 °C. A weighted linear least-squares fit (Wilkinson, 1961) of the data from SITS treated cells gives $K_m$ = 6.0 ± 0.8 mM and $V_{max}$ = 9.6 ± 0.4 μM/s.

trations have different effects on the kinetics. Increasing cis sulfate concentration shows competitive-like (product-like) inhibition and eliminates substrate inhibition at high dithionite concentrations. Trans sulfate does not significantly affect substrate inhibition over the concentration range studied but does diminish the substrate activation seen at lower dithionite concentrations. These separate effects of cis and trans sulfate seem to account for the observed behavior in the more conventional experiment where sulfate concentration was kept the same on both sides of the membrane and increased (i.e., activation–inhibition at low cis and trans sulfate and hyperbolic kinetics at very high cis and trans sulfate). The stimulation of dithionite flux by increasing trans sulfate concentration would be expected from a two substrate formulation. In the next section, we investigate the effect of a nondiffusable anion, citrate (Garby, 1965).

*Effect of Trans Citrate Concentration on the Initial Velocity of Dithionite Flux.* The effect of increasing the trans citrate concentration from 0 to 43 mM at low (6 mM) sulfate on both sides of the membrane is shown in Figure 8. The result is unexpected. Instead of decreasing the velocities, increasing trans citrate had an effect very similar to increasing trans sulfate concentration (a diffusable anion). The velocities significantly and reproducibly increased and the pattern became much more linear at lower dithionite concentrations. A weighted linear least-squares fit of the data before the onset of substrate inhibition gives a $K_m$ value of 3.4 ± 0.2 mM and a $V_{max}$ of 92.4 ± 1.6 μM/s. Substrate inhibition at higher dithionite concentration was apparently unaffected. The observation of *trans stimulation* by citrate (a nondiffusable anion) contrasts with the results of Rice & Steck (1976) for malate where no effect was observed. This could be due to a difference betwen the two anions in the strength of binding to sites on the trans side of the membrane. The observation of trans stimulation may also depend on the type and concentration of other anions in the system.

In summary, we see that the nondiffusable anion, citrate, trans stimulates dithionite flux across the membrane and eliminates substrate activation seen at low dithionite concentration when the trans concentration is increased. The results with trans citrate are the same as those found with the diffusable anion sulfate, when different initial sulfate concentration gradients were maintained with nondiffusable buffers.

*Effect of The Anion Transport Inhibitor, SITS.* SITS is a potent, nondiffusable, inhibitor of the anion transport system of the erythrocyte membrane (Maddy, 1964; Cabantchik & Rothstein, 1972). It binds both covalently and noncovalently. In order to study covalent inhibition, ghosts were first incubated in a buffer containing SITS. Free SITS was then washed away (see Materials and Methods). Figure 9 shows the results of covalent binding of SITS to the cis side of the membrane. The velocities are slower by about a factor of 5 and the kinetic pattern shows a loss of substrate activation and inhibition and is now linear. A weighted linear least-squares fit of the data gives a $K_m$ of 6.0 ± 0.8 mM and a $V_{max}$ of 9.6 ± 0.4 μM/s.

## Discussion

At equilibrium, anion exchange across the erythrocyte membrane involves an electrically silent, obligatory, one-for-one exchange (Tosteson et al., 1973; Hunter, 1968, 1971; Hoffman & Lassen, 1971; Lassen, 1972). The purpose of a kinetic experiment is to attempt to decide how this phenomenon occurs mechanistically. The approach to the kinetic problem was to organize the experiments within a two-substrate enzyme kinetic formalism. As stated in the introduction, this approach to the problem requires that transmembrane electrical potential not be a significant factor for the kinetics. Figures 7 and 8 suggest that this is the case. Increasing the intracellular concentration of Tris–sulfate stimulated dithionite influx (Figure 7). If transmembrane eletrical potential were important, increasing the intracellular concentration of sodium citrate (Figure 8) should have *decreased* dithionite influx since citrate is an impermeable anion while Tris is an impermeable cation. The fact that both Tris–sulfate and sodium citrate trans stimulated dithionite influx suggests that the chemical potential of the trans anion is the major influencing factor and favors the two-substrate enzyme kinetic interpretation. Within that formalism, two broad mechanistic categories should be considered: *sequential* (*ordered* or *random*) bireactant mechanisms and *ping-pong* bireactant mechanisms (Cleland, 1970). The first mechanism would require that *both* extracellular dithionite and intracellular sulfate combine with the transport system before transport can occur. Ping-pong mechanisms, on the other hand, require that one or more products be released *before* all substrates have added. Thus, ping-pong mechanisms are formally equivalent to mobile carrier models for anion exchange (Gunn, 1972, 1973).

There are two features of the kinetics which must be accounted for by any bireactant scheme: (a) nonhyperbolic patterns and (b) trans stimulation by an impermeable anion.

Rapid equilibrium sequential or ping-pong mechanisms will yield hyperbolic steady-state kinetics under all conditions (Segel, 1975). However, it is possible to account for substrate inhibition with either bireactant category by allowing substrate to combine with the wrong enzyme form leading to the formation of dead-end complexes. But this extra degree of freedom cannot explain both substrate activation and inhibition seen at low cis and trans sulfate (Figure 7). Activation–inhibition patterns could be explained with one of two types of more complex bireactant scheme: (a) *preferred pathway* bireactant kinetics; or (b) *allosteric* bireactant kinetics. Preferred pathway arguments require that: (1) rapid equilibrium *not* be attained prior to the formation of the ternary complex (i.e., breakdown of the ternary complex is *not* rate-limiting); (2) both pathways to the ternary complex *not* be equally favored kinetically; and (3) the two substrates *not* add in an obligatory fashion (Ferdinand, 1966; Fisher & Hoagland, 1968; Fisher, 1972; Dalziel, 1957). Bireactant allosteric systems have been discussed by several authors (Ainsworth, 1968; Kirtley & Koshland, 1967; Sumi & Ui, 1972; Pettigrew & Frieden, 1977). As is now well known, the fundamental idea of allosteric control is that various ligand binding sites interact *indirectly* (Wyman, 1972).

The evidence to discriminate between preferred pathway and allosteric bireactant schemes comes from the results which showed that citrate could trans stimulate dithionite influx at low coanion concentration and change the kinetic pattern from activation–inhibition to hyperbolic inhibition. Intracellular citrate may be regarded as a reversible inhibitor since it can not be transported across the membrane. Thus it should compete with intracellular sulfate for formation of a ternary complex. This should have led to a decrease in the influx velocity if a preferred pathway mechanism were operative. Thus, trans stimulation by citrate would seem to rule out preferred pathway mechanisms and favor an allosteric interpretation. Further support for an allosteric interpretation comes from the results with SITS (Figure 9). Reaction of a *covalent* inhibitor within a preferred pathway scheme where one ternary complex forms could account for the decreased velocities by decreasing the *number* of transport sites. However, this would not seem to explain the conversion of the kinetic pattern from activation–inhibition to hyperbolic (Figure 9). An allosteric scheme could explain both trans stimulation by citrate as well as the effect of SITS on the shape of the kinetic curve by allowing citrate to bind to a trans site and shift the "conformation" of the transporter toward some lower affinity, more rapid turnover state, with SITS covalently binding to a cis site and inhibiting such transitions.

One could phenomenologically account for substrate activation (or negative cooperativity) at low dithionite (Figure 7 or 8) by allowing dithionite to bind to a multisite transporter at one site and change the affinity or at least the velocity at the next site through some cooperative mechanism (e.g., shift to a lower affinity, faster flux T (trans) state at the next site). As dithionite concentration increases, binding to a cis effector site could occur and would shift the "conformation" back toward a higher affinity, slower flux C (cis) state. Raising the concentration of cis sulfate would also cause a transition to the C state and would be expected to diminish substrate inhibition. Direct competition between dithionite and sulfate for the transport site of the C state should also occur producing larger $K_m$ values than may be the case if that state were populated in the absence of competing anions. Covalent binding of SITS at low cis sulfate may "lock" the transporter in the C state, thus explaining the slower velocities and the hyperbolic nature of the curve (Figure 9). Trans stimulators, of which citrate is our only present example, would affect the system by progressively (with concentration) shifting the transporter toward the more rapid T state. This should be expected to *eliminate* substrate activation at low dithionite (Figures 7 and 8). Trans stimulators may also diminish substrate inhibition at high dithionite if binding were strong as compared with dithionite binding at cis sites.

The phenomenological picture just described is obviously *not* intended to be complete nor quantitative, but simply attempts a possible qualitative explanation of the current results. There are obviously many details which need to be known, especially the possibility that a functionally significant conformational change may occur in the membrane or one of its components with trans stimulation by citrate. In this regard, it has been suggested that band 3 (Steck, 1974) may be the transport protein and may undergo conformational changes (Rothstein et al., 1976). We are currently attempting to find better trans stimulators and better conditions under which to study trans stimulation (e.g., $Cl^-$ vs. $SO_4^{2-}$). Excellent trans stimulators, if they exist, could be expected to significantly increase the population of the hypothetical T state and may greatly increase dithionite influx. Some compounds currently being considered are 2,3-DPG, ATP, and IHP. The first organic phosphate is of obvious importance since its concentration undergoes large changes during oxygenation–deoxygenation because of its tight binding to deoxyhemoglobin and approximate equimolar concentration with hemoglobin tetramer within the cell (Benesch & Benesch, 1967; Chanutin & Curnish, 1967; Kilmartin & Rossi-Bernardi, 1973). Thus trans stimulation could be physiologically significant.

In conclusion, the results presented in this report offer the first detailed, systematic study of the kinetics for heteroanion exchange across the human erythrocyte membrane. The results suggest that obligatory exchange is not part of the *kinetic* mechanism. They also seem to rule out models where carriers are proposed to bind anions at either surface and *physically* traverse the membrane. The general view of anion transport, which seems most compatible with the results but is *not* proven by them, would be a multisite allosteric pore mechanism. The results of this paper should be helpful in organizing future structural and kinetic experiments in order to elucidate the mechanistic details.

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