

Anion Inhibitor-Sensitive Unidirectional Sodium Movements in the Human Erythrocyte

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ABSTRACT The increased unidirectional sodium influx found when human erythrocytes are suspended in isotonic salt solutions containing bicarbonate ions as a replacement for chloride ions was examined. The increased sodium movement appears to have the transport characteristics of anion movement. Inhibitors of anion transport such as furosemide, fluorodinitrobenzene (FDNB), and 4-acetamido-4'-isothiocyano-stilbene-2-2'-disulfonic acid (SITS) drastically inhibit these augmented sodium movements. An ion-pair mechanism appears to phenomenologically describe much of the data. A possible role for carbamino groups is considered. Such a model, however, requires additional assumptions to explain the selectivity and the anion inhibitor effects.

INTRODUCTION

Passive ion movements of anions and cations across the erythrocyte membrane, although usually considered separately, exhibit some common properties in certain experimental situations. Wieth and Funder (1965) and Wieth (1969), as well as Gunn and Tosteson (1971), showed that anions themselves, both organic and inorganic, could also alter cation movement.

In a later study Wieth (1970*a*) postulated that in some special cases, namely when erythrocytes are suspended in bicarbonate media, the observed increase in cation (Na^+ and Li^+) movement may be due not to an effect on the membrane but rather to ion pairing between the cation and the carbonate ion.

This intriguing mechanism was based on similarities of flux movements for Li and Na in erythrocytes suspended in bicarbonate media. The Na^+ influx rate constant of erythrocytes suspended in both NaCl and LiCl media, estimated from tracer ^{22}Na uptake, was dramatically increased (2.5–3 times) when the chloride in these solutions was replaced by bicarbonate.

The similarity of ion movements with Na^+ and Li^+ in the bicarbonate media and the selective increase in the movements of these cations contrasted with other monovalent cation movements suggested that a complex of Li and Na with the divalent carbonate ion could be involved. The pH dependence of earlier reported Na influx measurements in erythrocytes suspended in 120 mM bicarbonate media (X^-) appeared also to parallel the estimated carbonate concentrations (Wieth and Funder, 1965). A direct effect of P_{CO_2} which involves

the presence of membrane-bound carbaminos, however, cannot as yet be dismissed (Callahan, 1972; Callahan and Goldstein, 1972).

Chemical modifiers of membrane transport, such as specific inhibitors of anion or cation pathways, provide a means to test the ion-pair hypothesis. Chemical probes used to study the ion permeation mechanism vary with respect to reaction specificity, size, charge distribution, and accessibility to functional control sites.

Some agents, such as sulfhydryls, increase cation permeability but appear to have no direct effect on anion movement (Knauf, 1970; Knauf and Rothstein, 1971). Other agents, such as 4-acetamido-4'-isothiocyano-stilbene-2-2'-disulfonic acid (SITS) and 4,4'-diisothiocyano-2-2'-disulfonic-stilbene (DIDS), appear specific for anion movement under normal physiological conditions (Knauf and Rothstein, 1971*a*, Cabantchik and Rothstein, 1972). Still other agents, especially some amino reactive agents, fluorodinitrobenzene (FDNB) and 2-methoxy-5-nitropropone (MNT), can affect both anion and cation flux. The relative specificity for anions and cations can be altered by varying concentration of the agent or by addition of ethanol (Passow and Schnell, 1969; Poensgen and Passow, 1971).

Because of the occurrence of the biologically important and omnipresent $\text{CO}_2/\text{HCO}_3^-$ system and because ion pairing may represent an important transport mechanism, it was of interest to seek additional evidence bearing on the importance of the mechanism in the erythrocyte experiments.

METHODS

On the day of the experiments fresh human erythrocytes were drawn by venipuncture and defibrinated by stirring. The cells were centrifuged at 1,500 *g* for 10 min, and the white cells and plasma removed by suction pipette. The cells were then washed with the electrolyte medium of the experiment, spun for 10 min at 1,500 *g* and the supernate removed. This procedure was repeated for three wash cycles. The cells were then suspended in fresh medium to a predetermined volume, the hematocrit taken in duplicate, and the cell suspension allowed to incubate for about an hour at pH 7.4 and 38°C. The pH was controlled by a modified Vestergaard-Bogind (1962) gasometric pH-stat which utilized CO_2 gas mixtures.

Electrolyte Media

The buffer media composition for the cation flux experiments, unless otherwise noted, was as follows: Na^+ 142 mM, K^+ 3.7 mM, Ca^{++} 1.5 mM, Mg^{++} 1.0 mM, HCO_3^- 22.0 mM, Cl^- 6.5 mM (derived from the addition of CaCl_2 , MgCl_2 , and KCl), PO_4^- 1.1 mM as K_2HPO_4 , and X^- 120 mM (X^- represents either Cl^- or HCO_3^-). The media also contained 0.1 mM adenosine and 7.5 mM dextrose.

Sodium Influx

Unidirectional sodium movements were monitored by following the time-course of radioactive ^{24}Na movement. Unless otherwise noted, ouabain (ICN Nutritional Biochemicals Div., International Chemical & Nuclear Corp., Cleveland, Ohio) at a final concentration of 3×10^{-4} M was added to the cell suspensions in the sodium uptake experiments. Initial measurements of sodium movement were made 40–60 min after the addition of ouabain.

Radioactive ^{24}Na was added in a high specific activity sodium chloride or sodium bicarbonate carrier medium. 1-ml samples of supernate were taken immediately after adding the isotope and at the end of the experiment. The time-course of radioactive distribution was followed by withdrawing duplicate 0.2-ml samples of cell suspension at time intervals on the order of 15–20 min throughout the course of the experiment. These cell samples were washed three times with isotonic choline chloride and centrifuged at 5°C . Net sodium and potassium movements were measured by taking samples of the supernate and cells at various time intervals and analyzing for sodium and potassium concentration on a flame photometer (Instrumentation Laboratory, Inc., Lexington, Mass.) with an internal lithium standard.

All samples were taken in duplicate. Hematocrits of the cell suspensions ranged from 0.2–0.4 and were measured throughout the experiment.

Sodium influx constants expressed as K_{sc} (centimeters per minute) were estimated as follows. The change in the amount of radioactivity in the cells in 1 ml suspension is equal to the difference of the amounts of radioactivity moving from supernate to cell and from cell to supernate.

$$\frac{dP_c}{dt} = \frac{dP_{sc}}{dt} - \frac{dP_{cs}}{dt}, \quad (1)$$

where P_c is the amount of radioactivity in the cells (counts per minute), dP_{sc} and dP_{cs} are the amount of radioactivity that penetrates from the supernate to the cell and the cell to the supernate, respectively, in the same period of time (t).

Inasmuch as a number of variables, such as the number and volume of cells as well as concentration, vary as a function of time, the descriptive equations were left in the differential form. The rate of change in the amount of radioactivity in the cells (cpm) per milliliter of suspension can be written as:

$$\frac{dP_c}{dt} = NV_c \frac{A_c}{V_c} \left(K_{sc} \frac{X_s^*}{V_s} - K_{cs} \frac{X_c^*}{V_i} \right) \quad (2)$$

and the volume change as:

$$\frac{dV_c}{dt} = \frac{\lambda - \beta V_c}{N}, \quad (3)$$

where N is the number of cells per milliliter of suspension, V_c is the cell volume (water plus solid), A_c is the surface area of an erythrocyte, V_i is the cell water content, V_s is the volume of supernate, X_s^* and X_c^* are the amount of radioactivity in the supernate and cells, respectively, per milliliter of suspension, and K_{sc} and K_{cs} are constants describing the influx and efflux rate of sodium through the erythrocyte membrane. The parameters λ and β are the time rates of change of the hematocrit (dH/dt) and the cell number (dN/dt) evaluated from the experimental data. The parameters K_{sc} and K_{cs} were estimated by the numerical solution of the two differential Eqs. 2 and 3. The equations were solved using the fourth-order Runge-Kutta method (Ralston and Wilf, 1967), and the best estimates of K_{sc} and K_{cs} were obtained by fitting the experimental data to the above equations by a nonlinear least-squares fit program. All the above calculations were performed on an IBM 360 model 44 digital computer (IBM Corp., White Plains, N.Y.). The approach is designed as a general analysis of flux measurement. The second rate constant (K_{cs}) can be evaluated from the nonlinearity in an uptake (^{24}Na) vs. time curve. In the experiments reported herein, the duration was short enough so the uptake of tracer sodium was linear with time. A single rate constant, K_{sc} , was therefore obtained.

RESULTS

The flux measurements used to evaluate the ion-pair hypothesis are of the type presented in Fig. 1. This typical ^{24}Na uptake into erythrocytes illustrates the magnitude and linearity of the phenomenon. The rate constant K_{sc} (cm/min) is based on the flow through a unit membrane area of the erythrocyte (surface area taken to be 1.65×10^{-6} cm²/cell [Ponder, 1948]). The use of an influx constant in these units is considered to reflect more of an intrinsic membrane property than one in reciprocal time. The unidirectional rate constant of ^{24}Na

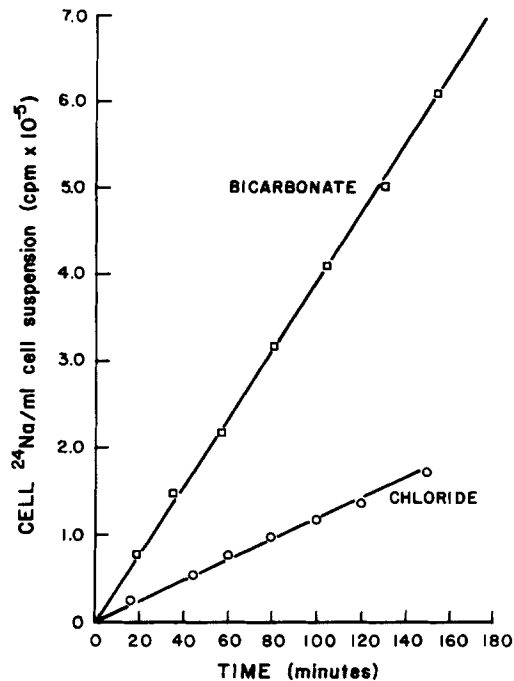


FIGURE 1. Time-course of radioactive ^{24}Na uptake into human erythrocytes suspended in electrolyte media of various anionic composition. Two typical experiments are illustrated. Electrolyte compositions are described in Methods (chloride, X^- 120 mM Cl; bicarbonate, X^- is 120 mM HCO_3^-). Cell suspensions, maintained at pH 7.4 and 38°C, contained 3×10^{-4} M ouabain. Cell counts corrected for small differences in the external suspension counts by multiplication with a constant to equalize external cpm, for illustration.

movement into erythrocytes suspended in the chloride medium, K_{sc} , is 1.53×10^{-8} (SE 0.12) cm/min, whereas the uptake in the bicarbonate medium is 4.17×10^{-8} (SE 0.28) cm/min.

There are two types of evidence to consider in the evaluation of ion-pair formation. One type is physicochemical in nature, the other is phenomenological.

Phenomenological Evidence

If a complex of Na^+ and CO_3^- is formed, NaCO_3^- , one might expect it to

possess the transport characteristics of an anion. As pointed out by Wieth (1970*a*), the ion concentrations are in the right direction for the transport of sodium into the cell and then for the complex to dissociate. The intracellular concentrations of hydrogen ions and of Na^+ are respectively higher and lower than the corresponding extracellular concentrations.

The observed phenomenon, a unidirectional Na influx increase in HCO_3^- media, should then be sensitive to inhibitors of anion movement rather than cation flux inhibitors. Many agents that cause a decrease in anion movement cause a corresponding increase in cation movement. The changes elicited by such an agent would be difficult to resolve because the ion-pair anionic movement down its electrochemical gradient is superimposed on the normal Na^+ cationic movement down its electrochemical gradient. The problem is, therefore, to find inhibitors of anion movement that are selective only for anions so as to simplify the interpretation of the results. We have used three inhibitors in our experiments.

Furosemide

The experimental results with this inhibitor are shown in Table I. Previous work established furosemide as an inhibitor of anion transport in human erythrocytes. Deuticke (1970) reported that furosemide inhibited phosphate transport, and Brazy and Gunn reported (1976) that furosemide inhibited chloride exchange. The diuretic furosemide was added at a concentration of 10^{-3} M to cells suspended in the bicarbonate medium or the chloride medium, and the Na influx rate constant was evaluated. Passive cation transport, in contrast, is not altered by furosemide at these same concentrations (Deuticke, 1970). We can see that in HCO_3^- medium the furosemide reduces the influx rate constant (K_{sc}) from 4.17 to 1.96, whereas in the chloride medium there is essentially no change. The bicarbonate-induced sodium movement, taken as the difference in the influx rate constant between the high bicarbonate and the chloride medium in the absence of inhibitors, is inhibited 84% by 10^{-3} M furosemide. This value is similar to the 90% reduction for the chloride self-exchange flux reported by Brazy and Gunn (1976) at 10^{-3} M furosemide. This result is consistent with an anion-dependent Na movement in the HCO_3^- medium.

FDNB

In 1971 Poensgen and Passow reported that FDNB could affect both cation and anion movements depending upon concentration, time, and temperature. FDNB at a concentration of 0.5 mM inhibited sulfate flux 75–80%, whereas net K efflux was not significantly increased. Only above a threshold concentration of 0.5–1.0 mM does K^+ efflux increase drastically. No threshold seems to exist for inhibition of anion permeability. If FDNB is added at a concentration of 0.5 mM, allowed to react for 10–15 min at room temperature, and the cells are washed twice with suspension media (a condition in which only anion fluxes should be affected), we see a dramatic decrease in the HCO_3^- -mediated Na^+ influx rate constant (Table I). The rate constant in the bicarbonate medium drops from a value of 4.17×10^{-8} (SE 0.28) to 1.99×10^{-8} (SE 0.30) cm/min in the presence of 0.5 mM FDNB, a reduction of 82%. The small increase in the

rate constant found in the chloride medium, with and without 0.5 mM FDMB, may represent a slight cation effect. The results of experiments in which cells were treated as described above but with 5.3 mM FDNB (a condition that should affect cation as well as anion movements) are also summarized in Table I. A greater than sevenfold increase in the Na^+ influx constant is observed, $K_{sc} = 30.4 \times 10^{-8}$ (SE 3.64) cm/min. This large increase is interpreted to be a general cation permeability increase similar to what Passow observed with K efflux at high FDNB levels. This increase overshadows the more subtle bicarbonate-dependent sodium influx increase.

TABLE I
SODIUM INFLUX RATE CONSTANT IN PRESENCE OF INHIBITORS K_{sc} *

	Medium, X^- concentration, mM	
	Bicarbonate, 120 mM	Chloride, 120 mM
Control	4.17 (0.28)‡ <i>n</i> = 11§	1.53 (0.12) <i>n</i> = 6
+ Furosemide	1.96 (0.16) <i>n</i> = 2	1.26 (0.11) <i>n</i> = 2
+ FDNB (0.5 mM)	1.99 (0.30) <i>n</i> = 2	2.06 (0.31) <i>n</i> = 3
+ FDNB (5.3 mM)	30.37 (3.64) <i>n</i> = 2	
+ SITS	1.97 (0.29) <i>n</i> = 4	1.42 (0.13) <i>n</i> = 3
SITS (+ albumin wash)	2.92 (0.21) <i>n</i> = 3	

Sodium influx rate constant in chloride and bicarbonate media in the presence and absence of the inhibitors furosemide (10^{-3} M), FDNB (0.5 mM for 15 min or 5.3 mM for 15 min), and SITS (0.3 mg/ml packed cell volume for 10 min at 38°C). Media composition described in Methods. Cell suspensions were maintained at pH 7.4 and 38°C and contained 3×10^{-4} M ouabain.

* $\left(\frac{\text{cm}}{\text{min}} \times 10^8\right)$.

‡ Standard error.

§ Number of separate experiments.

SITS

A third agent, perhaps better for testing this hypothesis because of its reported specificity, is the stilbene-disulfonic acid, SITS. This compound was shown by Knauf and Rothstein (1971) to decrease the anion permeability of human erythrocytes, as measured by equilibrium sulfate exchange effluxes. It has no effect on cation movements in solutions of normal ionic strength. Table I illustrates the results of adding 0.3 mg/ml packed cell volume of SITS to erythrocytes suspended in various ionic media. The experiments were performed with cells exposed to SITS for 10 min at 38°C and a hematocrit of 20% and washed twice with the respective SITS-free medium.

SITS has an almost identical effect as does 0.5 mM FDNB. The value of K_{sc} is reduced from 4.17×10^{-8} (SE 0.28) to 1.97×10^{-8} (SE 0.29) cm/min.

In line with our studies characterizing the total CO_2 effect, and in light of Cabantchik and Rothstein's report in 1972 that part of the SITS-dependent anion flux could be removed by albumin washing, an additional experiment was performed. The cells were reacted with 0.3 mg/ml SITS for 15 min at 37°C , washed once with buffer, twice with 1 g/100 ml albumin solution (allowing the cells to sit for 10 min in the albumin solution before centrifuging), followed by a final wash in buffer before resuspension and flux measurement.

As was observed with the sulfate equilibrium-exchange fluxes by Cabantchik, there is a decrease in the effect of SITS after washing with albumin. Even after the albumin washes, however, SITS retains 50% of its effect. Cells in 120 mM HCO_3^- medium without SITS additions and washed with albumin have a Na^+ influx constant of 3.98×10^{-8} cm/min. SITS-treated albumin washed cells show a decrease to 2.92×10^{-8} (SE 0.21) cm/min.

A consistent pattern in all the above experiments is that the increased Na^+ influx observed in erythrocytes suspended in high bicarbonate media is dramatically reduced in the presence of inhibitors of anion movement. These data are entirely consistent with the ion-pair hypothesis. The concentration dependence of the SITS effect in the bicarbonate medium is illustrated in Fig. 2. The difference between the sodium influx rate constant, K_{sc} , in the high bicarbonate medium in the absence of SITS and the K_{sc} in the chloride medium is considered a measure of the bicarbonate-induced sodium influx. The percent inhibition of this enhanced sodium influx by SITS is measured at various SITS concentrations. The dose-response curve for SITS inhibition of sodium influx is similar to that obtained by Cabantchik and Rothstein (1972) for inhibition of sulfate exchange. In the sodium influx studies, the cells were exposed to SITS for 10 min at 38°C and washed twice with the bicarbonate medium. The sulfate fluxes were measured after exposing the cells to SITS for 30 min at 5°C , and washed three times with 5 vol of buffer (Cabantchik and Rothstein, 1972). It is felt that the results are comparable because both conditions (10 min at 30°C and 30 min at 0°C) represent maximal binding conditions. In both cases no further significant increase in binding or inhibition of flux is seen as a function of time, and both methods yield a similar 80–85% reduction in sulfate-exchange flux at 10^{-4} SITS concentration (Knauf and Rothstein, 1971; Cabantchik and Rothstein, 1972). These results support the concept that the bicarbonate-induced sodium influx responds as an anion.

Physicochemical Evidence

In 1961 Garrels et al. reported the ability of Na^+ ions and CO_3^{2-} to form a complex, and calculated the equilibrium constant for its dissociation. This topic is dealt with briefly in the Discussion. It should be possible to correlate ion-pair formation of the type NaCO_3^- with flux changes. As the HCO_3^- concentration is increased from 22 to 142 mM, the number of NaCO_3^- ion pairs, calculated according to Garrels et al. (1961) from mass equilibrium data and the carbonic acid dissociation constants of $\text{pK}^1 = 6.10$, $\text{pK}^2 = 9.8$ (Siggaard-Andersen, 1974), increases linearly from 0.4 to 2.9×10^{-4} M.

The influx constant for Na^+ reported as K_{sc} in $\text{cm}/\text{min} \times 10^8$ appears also to be a linear function of the HCO_3^- concentration in the medium (Fig. 3). These data confirm similar observations by Funder and Wieth (1967) that the increased influx of sodium at a fixed pH is a linear function of bicarbonate concentration.

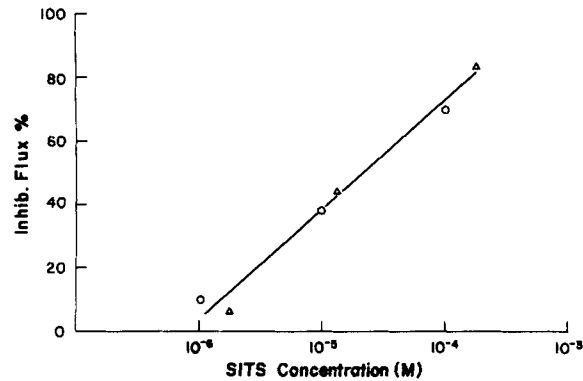


FIGURE 2. The effect of SITS concentration on bicarbonate-induced sodium influx rate constant ($\Delta-\Delta$) and sulfate exchange ($O-O$). SITS reacted for 10 min, 38°C . High bicarbonate (X^- 120 mM) medium composition described in Methods. Flux measured at pH 7.4, 38°C . Dose response of SITS on sulfate exchange data from Cabantchik and Rothstein (1972).

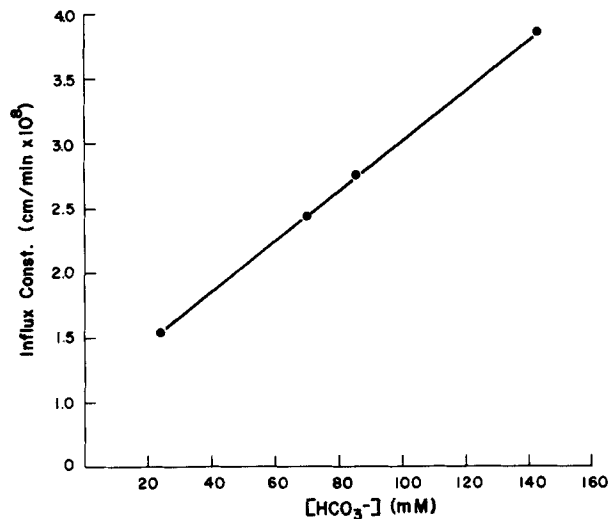


FIGURE 3. Sodium influx rate constant as a function of bicarbonate concentration in the medium. The bicarbonate concentration varies from control level of 22 mM (X^- 120 mM Cl^-) to 142 mM HCO_3^- (X^- 120 mM HCO_3^-). Cell suspensions maintained at pH 7.4, 38°C , and contained 3×10^{-4} M ouabain.

The various parameters of the bicarbonate system need to be measured under these experimental conditions, however, to further test the ion-pair hypothesis.

DISCUSSION

The increased Na influx movements in ouabain-treated human erythrocytes

suspended in HCO_3^- media certainly appear to possess properties usually ascribed to anion movements. The separation of causative factors in HCO_3^- -based solutions in a multiphase system like erythrocyte suspensions is always difficult. Selective ion pairing is known to exist for Na^+ and Li^+ but not K^+ , Cs^+ , Rb^+ in hydroxide solutions (Robinson and Stokes, 1959). Ion-pair formation between Na^+ and CO_3^{2-} had been suggested previously by Siggaard-Andersen in 1962 in an attempt to explain the pH dependence of the pK of carbonic acid. However, the existence of such complexes for carbonates had not been noted in the biomedical literature. If, however, the ion-pair complex constants of Garrels et al. (1961) can be used, and the data of the sodium flux enhancement in the presence of increasing HCO_3^- concentrations are interpreted with these in mind, there is a correlation of NaCO_3^- ion-pair formation with increased Na flux movements. The absence of evidence in their study of a complex between K^+ and CO_3^{2-} fits the observations by Wieth (1970a) that the sodium influx rate constant, but not the potassium rate constant, is increased in erythrocytes suspended in HCO_3^- solutions.

There are other permeation mechanisms which suggest the necessity to consider multiple forms of species present. One example of this is the salicylate movements in erythrocytes (Dalmark and Wieth, 1972). Salicylate is thought to penetrate as an undissociated acid, a form of membrane penetration that is well recognized and is somewhat different from the sodium movements described here in that H^+ is involved rather than Na^+ .

Quantitation of ion pairing in the bicarbonate medium must take into account ion-complex formation and ion activities within the membrane phase. The use of the relationship of Garrels et al. (1961) for activity coefficients and association constants considers only the bathing solutions, not the cell compartment, and assumes that ion-pair complexes found at 25°C are present at 37°C although the actual magnitudes will change slightly with temperature.

The activity coefficients of HCO_3^- and CO_3^{2-} were determined in aqueous solutions of NaCl , MgCl_2 and NaCl-MgCl_2 mixtures. Garrels et al. (1961) felt the approximate equivalence of the activity coefficient of carbonate, $\gamma_{\text{CO}_3^{2-}}$, as determined theoretically from Debye-Huckel theory and experimentally in K_2CO_3 solution, indicated that in solutions containing only potassium salts there is little ion association, and the total or apparent activity coefficient $\gamma_{\text{CO}_3^{2-}(\text{T})}$ of K_2CO_3 equals the actual activity coefficient, $\gamma_{\text{CO}_3^{2-}}$ (the ratio of activity of a dissolved species to the concentration of "uncomplexed" species). On the other hand, the marked decrease of the total or apparent activity coefficient $\gamma_{\text{CO}_3^{2-}(\text{T})}$ in NaCl solutions compared to that in KCl solutions is strong evidence for the presence of complex NaCO_3^- ions.

The question of whether there are enough ion pairs to account for the increased Na^+ movement as NaCO_3^- , of course, depends upon the mechanism of anion movement. The ratio of $P_{\text{Cl}}/P_{\text{Na}}$ currently is estimated at 10^2 to 10^6 depending on whether one considers values for P_{Cl} as estimated from what is considered a current carrying conductance pathway or from steady-state isotope "exchange" measurements (Hunter, 1971; Gunn, 1973; Tosteson et al., 1973).

To describe the observed flux changes, a selectivity of 10^6 (corresponding to the permeability calculated from anion exchange), requires $<0.1\%$ of the

carbonate to exist as ion pairs. In fact in sodium salt solutions of similar ionic strength, Garrels et al. (1961) reported 15% of the carbonate existed as the ion-pair complex. Therefore, there are enough ion pairs present at 25°C to account for such a mechanism if the selectivity $P_{(\text{ion pair})}/P_{\text{cation}}$ were as low as 2×10^3 .

It would appear that if ion pairs are responsible for the Na influx, they must utilize the anion exchange mechanism because they would penetrate too slowly by the net anion transport system. Even though HCO_3^- appears to be an effective "exchange" partner for chloride, it is possible that NaCO_3^- might be less efficient because there is a spread of several orders of magnitude in the rate coefficients of exchange of various monovalent inorganic ions (Wieth, 1972).

What does appear to be consistent throughout the experiments with increasing HCO_3^- concentration is that the increased Na movements are sensitive to what ordinarily are considered anion flux inhibitors. It is possible that the functional specificity of these anion probes is lost in the HCO_3^- media. Certain probes like SITS are reported to lose their specificity in altered experimental conditions like low ionic strength (Knauf, 1970).

The quantitative amounts of anion inhibitors used are the same as those reported in the literature to cause similar anion inhibition. FDNB and SITS are thought to have some molecular specificity for the observed anion inhibition, especially in the case of SITS. Reports by Cabantchik and Rothstein (1972) indicated that there was a reversible and irreversible binding component to SITS. The degree of inhibition does not seem to depend on the nature of the binding but rather on the total amount bound, whether reversible or irreversible. Over 50% of the present SITS effect is retained even after washing twice with 1 g/100 ml albumin. The molecular mechanism of furosemide action is, at present, unknown.

It is evident that replacement of 120 mM chloride (X^-) ion with the bicarbonate ion involves changing more than one variable even if the pH is maintained at 7.4. This is a fundamental problem associated with bicarbonate systems. Although it appears that ion-pair formation is a plausible mechanism for the increased sodium influx in high bicarbonate solutions, a direct action on the membrane should also be considered. A substitution for chloride by anions of the lyotropic series was shown to increase the sodium flux according to the sequence: ($\text{Cl}^- = \text{Br}^- < \text{I}^- < \text{NO}_3^- < \text{SCN}^-$) (Weith, 1970*b*). Binding of the lyotropic anions to fixed cationic charges in the membrane, decreasing the positive charge, was considered a likely explanation of the permeability changes.

One view of the passive ion movement in the erythrocyte is the concept of a pore structure (Passow and Schnell, 1969; Gary-Bobo and Solomon, 1971) associated with positive-charged, dissociable groups which can control permeability (Passow, 1965). An alternate, more recent view of anion movement is by a titratable carrier (Gunn, 1973). In the first hypothesis a decrease in positive charge is thought to increase cation permeability and decrease anion permeability; for an increase in positive charges the converse is true (Passow, 1965).

In the "carbamino hypothesis" when erythrocytes are placed in increased concentrations of bicarbonate medium, maintaining the pH with increased P_{CO_2} , the direct combination of CO_2 with the un-ionized form of amino groups would result in carbamino group formation. The reaction is a highly reversible

one. At the existing pH values in our experiments, the carbamino group would most likely exist in its dissociated form. The net result of the reaction is for CO_2 to react with the un-ionized amino groups, thus pulling the reaction $\text{NH}_3^+ \leftrightarrow \text{NH}_2 + \text{H}^+$ to the right and converting the NH_2 groups to COO^- . The overall result could convert positively charged membrane permeability barriers to negatively charged carbaminos. Such a reaction would be expected to increase the cation flux and decrease the anion flux.

The extent of the reaction in a system as complicated as the erythrocyte depends on a number of parameters and interdependent relationships. The type of amino group involved, location in the membrane, and local pH and pK values all affect the extent of the carbamino reactions.

The extent of carbamino formation can be estimated under conditions similar to those found in our experimental sodium influx determination. The role of pH, P_{CO_2} , and composition of the medium on the fraction of amino groups in the carbamino form can be calculated with the aid of some simplifying assumptions (see Appendix). In the absence of the actual membrane carbamino measurements and because of the availability of existing data, hemoglobin is taken as the model for illustration. Table II shows the calculated fraction of

TABLE II
CALCULATED FRACTION OF HEMOGLOBIN AMINO GROUPS PRESENT AS
CARBAMINO COMPOUNDS FROM THEORETICAL CONSIDERATIONS*

Electrolyte medium	pH	P_{CO_2}	Z‡
Chloride (X^- 25 mM HCO_3^-)	6.8	146	0.17
	7.4	37	0.30
	7.7	18	0.33
Bicarbonate (X^- 120 mM HCO_3^-)	6.8	9.44	0.56
	7.4	237	0.73
	7.7	119	0.77

* See Appendix.

‡ Fraction of amino groups present as carbaminos.

amino groups in the carbamino form for both a low and high bicarbonate medium at three pH values.

There are a number of points which deserve further mention. It can be seen that the fraction of amino groups existing in the carbamino form can be substantially increased in the high bicarbonate medium relative to the low, at all pH values examined. Coincidentally, the increase in carbamino fraction from the low to high bicarbonate medium at pH 7.4 is similar in magnitude to the flux change noticed in human erythrocytes suspended in low vs. high bicarbonate media at the same pH. The fraction of carbaminos formed in the respective media increased with pH.

Previous investigators (Funder and Weith, 1967) thought it unlikely that P_{CO_2} is involved in the increased sodium influx measurements in human erythrocytes suspended in high bicarbonate media. This was based on their observation of further increase in sodium influx in the presence of a lowered P_{CO_2} value. However, the pH was simultaneously increased. A comparison of the last two columns, P_{CO_2} and Z, in Table II, illustrates that one cannot, a priori, rule out a

role for carbamino formation in sodium transport. Carbamino formation is a function of both pH and P_{CO_2} and in the hemoglobin system examined here the fraction of aminos in the carbamino form is seen to increase at the higher pH values in spite of a lower P_{CO_2} .

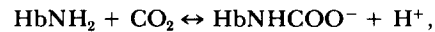
With a fixed charge model one could see how carbamino formation could enhance cation movement, but it is difficult to explain the selectivity for sodium. Ion-pair formation appears to best describe the existing data.

APPENDIX

Hemoglobin-Carbamino Estimations

The extent of carbamino formation under conditions similar to those found in our experimental sodium influx determinations can be estimated in hemoglobin solutions. The role of pH, P_{CO_2} , and composition of the medium on the fraction of amino groups in the carbamino form can be calculated with the aid of some simplifying assumptions and existing data in the literature.

If we consider the following relationships to be descriptive of the major amino reactions in a hemoglobin solution,



and assume the system has reached equilibrium and is thus describable by the following relationships

$$K_z \text{ (ionization constant)} = \frac{[\text{HbNH}_2][\text{H}^+]}{[\text{HbNH}_3^+]}$$

$$K_c \text{ (equilibrium constant)} = \frac{[\text{HbNHCOO}^-][\text{H}^+]}{[\text{HbNH}_2][\text{CO}_2]},$$

the Law of Mass Action can be applied to the above relations to estimate the proportion of amino groups combined as carbamino (Rossi-Bernardi and Roughton, 1967).

Fraction amino groups

$$\text{as carbamino} = Z = \frac{[\text{HbNHCOO}^-]}{[\text{HbNH}_2] + [\text{HbNH}_3^+] + [\text{HbNHCOO}^-]}.$$

Substitution of the above equations into this latter relationship yields

$$Z = \frac{1}{\frac{[\text{H}^+]}{[\text{CO}_2]K_c} + \frac{[\text{H}^+]^2}{[\text{CO}_2]K_zK_c} + 1}.$$

Estimates of K_c and K_z have been published by Rossi-Bernardi and Roughton (1967) from the data of Ferguson (1936) on human hemoglobin. There are tentative estimates and not true thermodynamic constants. CO_2 is also assumed to follow Henry's Law, and the proportionality constant, α , relating the partial pressure of CO_2 and the concentration of CO_2 in solution, is taken as 0.03 mmol/liter, mm Hg, P_{CO_2} . The pH and CO_2 values are related through the Henderson-Hasselbalch equation, using the value of pK, 6.1, as the apparent first dissociation constant of carbonic acid according to Siggaard-Andersen (1974): $\text{p}K_c = 4.62$, $\text{p}K_z = 7.18$, $\alpha = 0.03$ mmol/liter, mm Hg, P_{CO_2} at 38°C, $\text{pH} = 6.1 + \log \frac{[\text{HCO}_3^-]}{\alpha P_{\text{CO}_2}}$.

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