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THE MECHANISM OF ANION TRANSLOCATION AND pH EQUILIBRATION IN ERYTHROCYTES

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SUMMARY

The process of anion translocation across the red cell membrane has been found to have the following properties:

1. The half-time for the electrogenic Cl^- translocation is of the order of 100 sec, about 500 times greater than that for the electroneutral Cl^- exchange.

2. The pH equilibration is the result of an exchange of Cl^- against HCO_3^- or of Cl^- against OH^-, and does not involve the translocation of H^+ as such.

3. The kinetics of Cl^- translocation show phenomena of competition and saturation.

The above properties are discussed from the point of view of the hypothesis that the translocation of anions across the red cell membrane is due to the operation of carriers.

INTRODUCTION

The high permeability of the red cell membrane to anions (for a review see ref. 1) has been explained by assuming a mechanism of simple diffusion through aqueous pores. TOSTESON[2], however, has noted that the exchange experiments do not permit the exclusion of an exchange diffusion mechanism.

More recently some observations have been reported which cast some doubt on the simple diffusion mechanism for anion translocation. HUNTER[3] found that the rates of change of the light-scattering during the anion translocation were much lower than the rates of anion exchanges measured by TOSTESON[2]. In further studies HUNTER[4] concluded that the rate of swelling of red cells due to the addition of NH_4Cl satisfies the requirements for carrier kinetics. SCARPA *et al.*[5], studying the NaCl penetration in gramicidin-treated red cells, found that Cl^- entered the erythrocytes at rates two or three orders of magnitude lower than the exchange rate.

In the present experiments attention has been focused on three questions: (a) the rate of electrogenic net Cl^- influx, (b) the mechanism of pH equilibration, and (c) the kinetics of Cl^- translocation. The results obtained will be discussed in relation to the hypothesis of anion carriers in the red cell membrane.

Abbreviation: FCCP, carbonylcyanide *p*-trifluoromethoxyphenylhydrazone.

METHODS

Red blood cells were usually prepared from guinea-pigs, but similar results were obtained with red cells from men and rats. The guinea-pig blood, drawn by cardiopuncture in isosmotic acid–citrate–dextrose, was centrifuged (about 1000 $\times$ *g*), and the supernatant fluid and buffy layer were removed by aspiration. The red cells were then washed thoroughly in isosmotic ice-cold choline chloride buffered at pH 7.4 with 10 mM Tris–HCl, suspended in a concentration of about 2 500 000/mm^3 in ice-cold isosmotic solution of sucrose or choline chloride as indicated in each experiment, and used immediately.

Absorbance was measured, at 546 nm in a stirred cuvette having a 1-cm light path, with an Eppendorf (Netheler and Hinz, Hamburg, Germany) photometer equipped with a recorder. Rates of swelling were calculated from the initial rates of absorbance change or from the half-time of the total changes of absorbance.

Changes in K^+ activity were monitored by a Beckman 39047 cationic electrode connected with a Radiometer Mod. PHM 26 pH meter (Radiometer, Copenhagen, Denmark) and a recorder. The calibration of the electrode was made in each experiment by adding, in a separate sample and under identical experimental conditions, amounts of a standard solution to obtain the same potential changes as observed in the experiments with the red cells.

External pH changes were monitored, sometimes simultaneously with K^+ activity, by a Schott and Gen 9259/8 combination electrode (Jena Glasswerk, Mainz, Germany) connected with a pH meter and recorder.

Internal pH changes of red cells were followed by making use of the fact that acid and alkaline methaemoglobin are spectroscopically different. According to the method of KEILIN AND MANN[6], red blood cells were treated with 0.156 M $NaNO_2$ which rapidly converted the intracellular haemoglobin into methaemoglobin. The cells were then washed twice in isosmotic choline chloride, and the conversion of alkali into acid methaemoglobin was followed with a dual wavelength (double beam) spectrophotometer in cuvettes having a 1-cm light path. The wavelengths used for this purpose were 600–615 nm, the latter being the isosbestic point and the former an absorbtion maximum for alkaline methaemoglobin. Qualitatively similar results were also obtained with a second wavelength pair of 540–520 nm.

Sometimes absorption differences at 600–615 nm (internal pH), changes in absorption at the isosbestic point at 615 nm (absorbance), and changes in the pH of the medium measured with a combination glass electrode, were followed simultaneously in the same cuvette equipped with a mechanical stirrer and recorded on a multichannel potentiometric recorder.

Gramicidin and *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulphonic acid (Hepes) were obtained from Calbiochem (Los Angeles, Calif., U.S.A.); glycylglycine was purchased from Schuchardt (Munich, Germany). Carbonylcyanide *p*-trifluoromethoxy phenylhydrazone (FCCP) was kindly given by Dr. P.G.Heytler.

All other reagents were of analytical grade.

RESULTS

Electrogenic influx of Cl^-

Two types of Cl^- flux can be studied in the red cells: (a) the electroneutral

exchange of Cl^- against other anions (Cl^-, HCO_3^- or OH^-); and (b) the electrogenic net translocation of Cl^-. The experiments of TOSTESON[2] were devised to measure the rate of electroneutral Cl^- exchange. The electrogenic translocation can be studied by using antibiotics that increase the permeability of the red cell membrane to the counter-ion translocated together with Cl^-. We have used gramicidin which increases the permeability of the membranes to Na^+, Li^+ K^+, Rb^+, Cs^+ and H^+ (refs. 8, 9).

Fig. 1 shows that the half-time for the swelling of gramicidin-treated red cells incubated in NaCl, $NaNO_3$, sodium tartrate or sodium succinate was dependent on both the pH and the anion species. With NaCl the rate of swelling increased considerably when the pH was lowered with a half-time decreasing from 150 at pH 8.5 to about 50 sec at pH 6.6. A similar sensitivity to pH was observed with sodium nitrate although the half-time was considerably greater. On the other hand the swelling occurring in the presence of tartrate and succinate was less sensitive to the pH of the medium.

Direct evidence was also obtained that the rates reported in Fig. 1 are expressions of the net translocation of NaCl into the cell. First, the swelling was dependent on the permeability of the membrane to both Na^+ and Cl^- (*cf.* ref. 5). When either of the two ions was replaced by a higher molecular weight ion, such as trihydroxymethylaminomethane (Tris) or ribonucleic ions, the swelling was abolished. Second, it was ascertained that the increase of cell water was accounted for by an increase of the Cl^- content of the cell (*cf.* Table I of ref. 5).

In the experiments of Fig. 1 the rate-limiting reaction was the translocation of the anions. This conclusion is supported by two lines of evidence. First, although the cation species was identical, the rate of swelling varied according to the anion

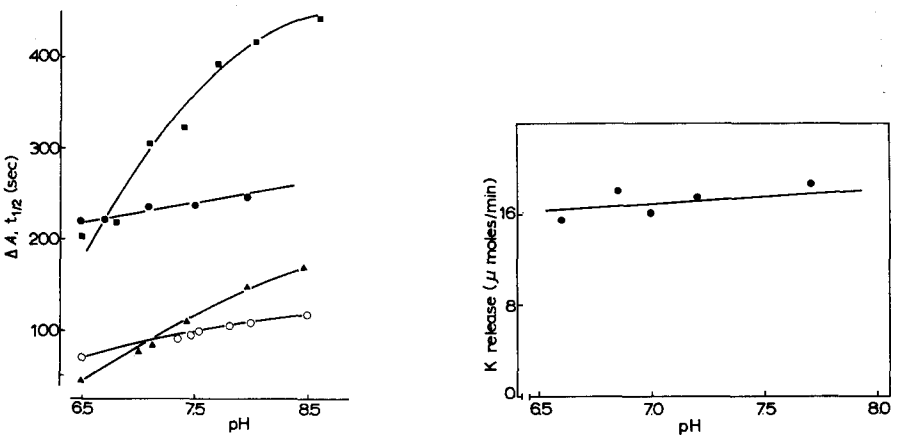

Fig. 1. Rate of swelling of erythrocytes in the presence of various anions and at various pH's. Packed, choline chloride-washed red cells (50 μl) were added to 1.95 ml of a medium containing the sodium salts indicated in the figure, at a concentration of 0.25 osM. The buffer, 5 mM Tris, was also made with the anions indicated in the figure. The values reported on the ordinate are the half time of the swelling phase measured as changes in absorbance induced by the addition of 10 μg gramicidin. Temperature 22°. ●—●, sodium tartrate; ■—■, sodium nitrate; ○—○, sodium succinate; ▲—▲, NaCl.

Fig. 2. Release of K^+ by addition of gramicidin at different pH's. The medium contained: 120 mM $LiNO_3$, 10 mM glycylglycine at the pH indicated in the figure, 1 mM KCl and 0.2 ml of red cells. The reaction was started by the addition of 2 μg gramicidin. Only the values of the initial rate of K^+ efflux are reported. Final volume 2 ml. Temperature 25°.

used. Second, the rate of cation exchange catalysed by gramicidin was far more rapid, and furthermore it was insensitive to the pH of the medium. This is shown in Fig. 2. Li^+ was used instead of Na^+ because the cation electrode is only slightly sensitive to Li^+, and gramicidin-treated red cells are highly permeable to Li^+ (ref. 7). Nitrate was used as anion because of the low rate of swelling of red cells in $NaNO_3$. Under these conditions all the K^+ efflux is coupled to the influx of Li^+. The $t_{1/2}$ of the K^+–Li^+ exchange, was of the order of 4 sec; furthermore it was insensitive to the pH of the medium.

From these experiments it appears that the half-time for the net Cl^- translocation is at least two orders of magnitude greater than the half-time for the cation exchange. The net Cl^- translocation shows the same pH sensitivity as the isotopic exchange of anions[1].

The mechanism of pH equilibration

To study the mechanism of pH equilibration use can be made of other ion carriers such as valinomycin and FCCP. Valinomycin has been shown to increase the permeability to K^+ but not to Na^+ or H^+ (ref. 9). In red cells, valinomycin does not cause K^+ release unless uncoupling agents are also added[9]. Since the uncoupling agents have been shown to act as H^+ conductors, it seems that to obtain a release of K^+ it is necessary to increase the permeability to H^+. These experiments therefore suggest that although the pH equilibration occurs across the membrane of red cells at a very high rate, the membrane is not permeable to H^+ as such.

An experiment relevant to the mechanism of pH equilibration is reported in Fig. 3. Amounts of HCl were added to a red cell suspension such as to give equivalent pH shifts. The pH trace after the acid pulse remained relatively constant at alkaline pH whereas at acidic pH it tended to return to the initial values, and this was faster the more acidic the pH. Since the process represents a pH equilibration across the red cell membrane it appears that this is faster at acidic and slower at alkaline pH. The slower rate of the pH equilibration at alkaline pH suggests that the process

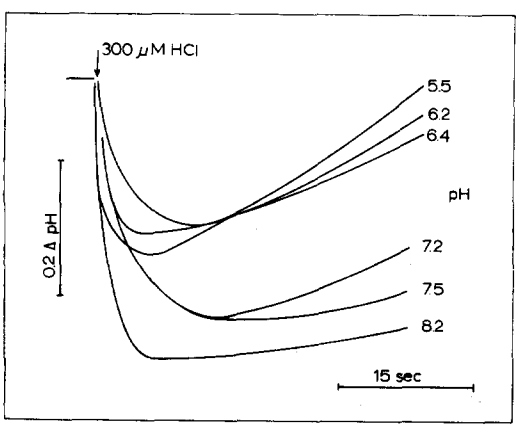

Fig. 3. Effect of pulses of HCl to erythrocytes suspended at different pH's. 0.2 ml of packed, sucrose-washed red cells was added to 1.8 ml of a medium containing 250 mM sucrose buffered at the pH indicated in the figure with glycylglycine. The amount of glycylglycine able to give the same buffer capacity at the various pH's was determined experimentally in parallel samples devoid of red cells. Temperature 25°.

involves an anion translocation. In fact similar pH dependence was observed when, instead of HCl pulses, NaCl pulses were added to red cells suspended in a sucrose medium. This observation suggests that the pH changes observed after the addition of either HCl or NaCl are expressions of the rate of anion exchange across the red cell membrane.

Fig. 4 compares the changes of the external and internal pH after the addition of various acids to red cells suspended in sucrose. Although the extent of external acidification was about the same, the internal acidification was much higher with Cl^- than with succinic or α-ketoglutaric acid. For comparison, Fig. 4 also shows the effect due to the addition of NaCl which also resulted in a decrease of the internal pH. The experiment of Fig. 4 thus indicates that the change of the internal pH is dependent on the type of anion which is translocated, and hence that the pH equilibration occurs through an anion exchange.

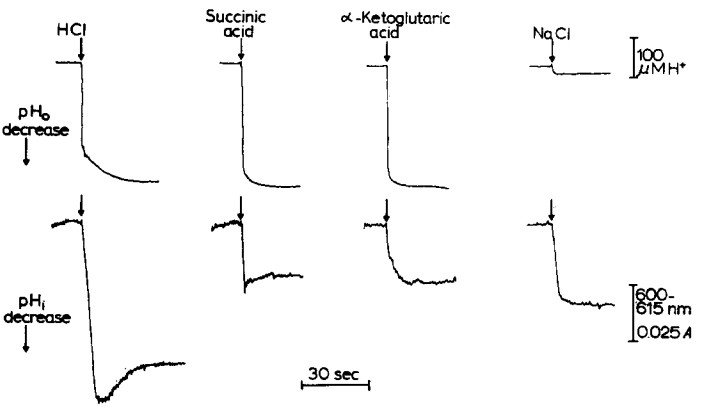

Fig. 4. The effect of various acid species on the external and internal pH. Experimental conditions as for Fig. 3. The reaction was started: with 0.5 mM HCl; with an amount of succinic or α-ketoglutaric acid able to produce the same decrease in the external pH as with HCl; with 1 mM NaCl. The external acidification produced by the addition of acids was in the range of 0.4 pH unit.

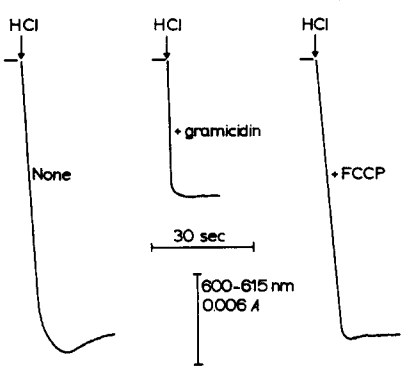

Fig. 5. The effect of gramicidin and FCCP on the internal acidification of red cells. 0.1 ml of red cells treated with nitrite and suspended in choline chloride was added to 1.9 ml of a medium containing 0.125 M choline chloride buffered at pH 7 with 5 mM Tris–HCl. When red cells were treated with gramicidin or FCCP the pH of the medium was brought back to 7 after equilibration. Gramicidin was 10 μg and FCCP 0.6 μM. Temperature 25°.

Fig. 5 shows the effects of gramicidin and FCCP on the decrease of the intracellular pH due to acid pulses. Neither the rate nor the extent of intracellular acidification was modified by the addition of FCCP. This experiment is open to two interpretations. First, that the permeability of the red cell membrane to H^+ is already so high that it cannot be further increased by H^+ conductors such as FCCP. However this interpretation is opposed by the observation that acidification of intracellular spaces occurs in the presence of valinomycin + FCCP, but not in the presence of valinomycin alone. The alternative explanation is that the intracellular pH is independent of the permeability to H^+ and changes only when an electroneutral anion exchange takes place. Fig. 5 also shows that a marked decease of the extent of the internal acidification was caused by gramicidin. With gramicidin, however, the permeability to univalent cations was also increased. Therefore gramicidin presumably causes a net movement of KCl due to the coupling of a H^+–K^+ exchange with the anion exchange.

Saturation and competition kinetics

Fig. 6 shows that the addition of a suspension of red cells to a sucrose medium resulted in a large acidification. Subsequent addition of 1 mM NaCl caused a large H^+ uptake. Treatment of the red cells with the inhibitor of carbonic anhydrase, acetazolamide (Diamox) resulted, as shown in Fig. 6B, in a strong inhibition of the rate of pH equilibration. Similar experiments were carried out by incubating the red cells in NaCl and varying the pH of the medium by HCl pulses. The rate of the pH equilibration was also markedly inhibited by the presence of acetazolamide.

These experiments lead to two conclusions: (a) the rate of anion exchange across the red cell membrane can be measured by recording the rates of pH change that accompany the anion exchange; and (b) the rapid rate of pH equilibration across the red cell membrane is primarily due to an exchange of Cl^- against HCO_3^- whereas the slower rate of equilibration in the presence of acetazolamide is due to an exchange of Cl^- against OH^-.

As shown above, the external alkalinization due to the addition of NaOH to

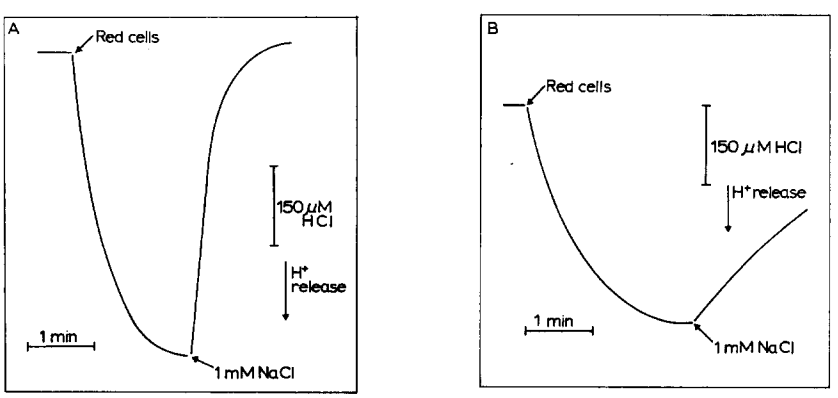

Fig. 6. (A) and (B) Acidification due to the addition of red cells to sucrose media and reversal by NaCl. The medium contained 250 mM sucrose and 2 mM glycylglycine (pH 7). In (B) 0.5 mM Diamox was also present. The reaction was started by the addition of 0.2 ml of packed, choline chloride-washed red cells. Final volume 2 ml. Temperature 25°.

the medium is followed by an increase of the internal pH. Fig. 7 shows that the rate and extent of internal alkalinization after the addition of NaOH were much greater in sucrose than in choline chloride media. Since the effect of a transmembrane Donnan potential, presumably larger in cells suspended in sucrose than in choline chloride, is negligible on the electroneutral HCO_3^-–Cl^- exchange, we attribute the lower rate of H^+ release in choline chloride as due to a competition between OH^- and Cl^- for the binding site of the anion translocator.

Fig. 8 shows the relation between the rate of alkalinization of the cell interior and the increase of the external Cl^- concentration. The alkalinization was obtained by adding NaOH pulses to red cells incubated in media at various Cl^- concentrations.

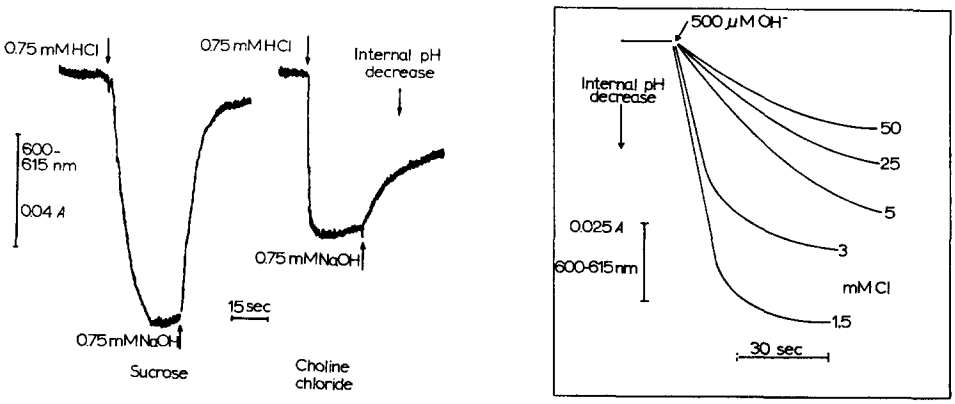

Fig. 7. Effect of pulses of HCl and NaOH on internal pH of erythrocytes incubated in media of sucrose and choline chloride. 0.1 ml of packed, choline chloride-washed, nitrite-treated, red cells, was added to a medium containing 250 mM sucrose or 125 mM choline chloride. Both media were buffered at pH 7.4 with 5 mM glycylglycine. Additions are reported in the figure. Final volume 2 ml. Temperature 22°.

Fig. 8. Effect of pulses of NaOH on internal pH of red cells incubated at various Cl^- concentrations. 0.1 ml of red cells, previously treated with nitrite and washed in sucrose, was added to 1.9 ml of a medium containing sucrose and different Cl^- concentrations up to a total osmolarity of 0.25. The Cl^- concentrations are indicated in the figure. Temperature 25°.

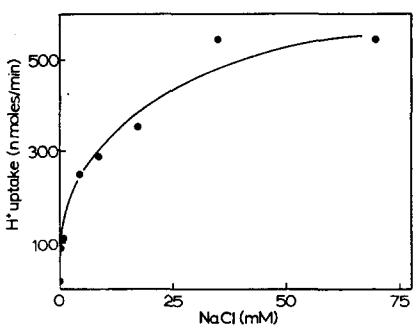

Fig. 9. Rate of pH equilibration in erythrocytes. 0.2 ml of packed, sucrose-washed red cells was added to a medium containing 0.25 M sucrose buffered at pH 7.4 with 10 mM glycylglycine. After equilibration of pH the amounts of NaCl reported in the abscissa were added in constant volume. The initial rates of the alkalinization of the medium following the addition of NaCl are reported in the ordinate. Final volume 2 ml. Temperature 25°.

In this experiment the inner pH of the red cells varied considerably. However the measurements were carried out in the region where the colour change of the pH indicator was proportional to the pH changes.

Fig. 9 shows the rate of alkalinization due to the addition of NaCl to red cells incubated in a sucrose medium. The rate of H^+ uptake increased with the increase of NaCl according to Michaelis–Menten kinetics. The apparent K_m was about 10 mM.

Figs. 10A–10C show a Dixon plot for the inhibitory effects of sulphate, succinate and nitrate on the rate of Cl^- translocation. The rate of Cl^- translocation was measured from the rate of pH equilibration after the addition of NaCl pulses to red cells incubated in sucrose at pH 6.5. The inhibition was of the competitive type with an apparent K_i of 90 mM, 125 mM and 300 mM for sulphate, succinate and nitrate respectively.

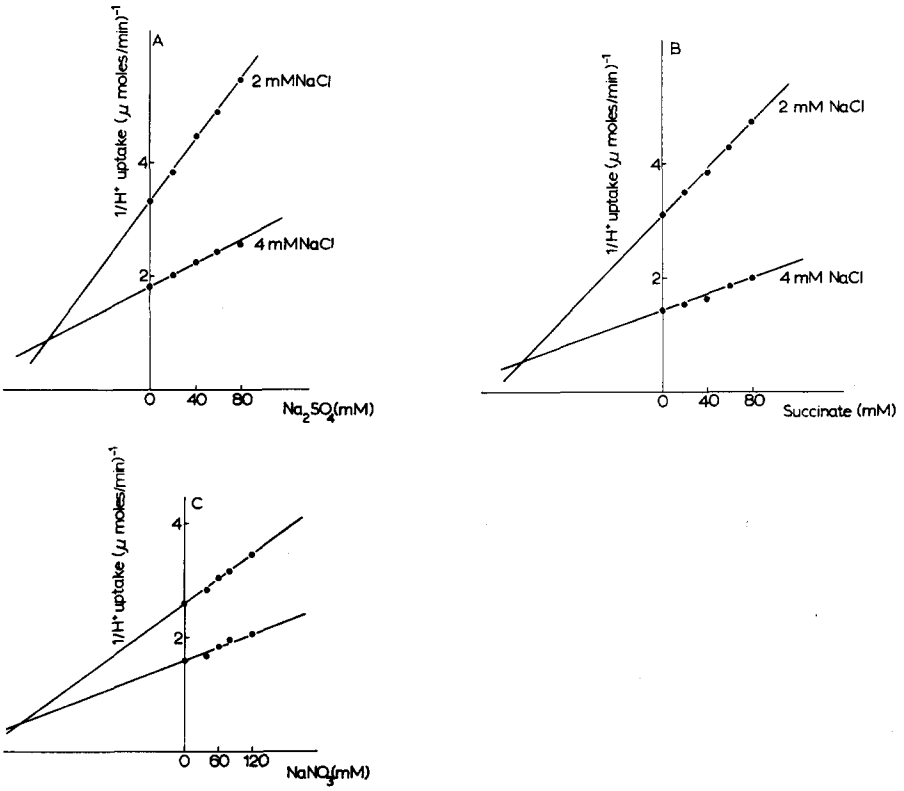

Fig. 10. Effect of SO_4^{2-} (A), succinate (B), and NO_3^- (C) on the pH equilibration due to the addition of Cl^-. The experimental conditions were as follows: 0.2 ml of packed, sucrose-washed red cells was added to 1.8 ml of sucrose medium containing 5 mM glycylglycine at pH 7, and concentrations of sucrose and anions as indicated on the figure up to a final osmolarity of 0.25. The reaction was started by the addition of 2 or 4 mM NaCl. The values reported in the ordinate represent the reciprocal of the initial rate of pH change.

DISCUSSION

The anions rapidly reach the Donnan equilibrium distribution across the red cell membrane. Two types of mechanism may account for the high reaction

rate, namely free diffusion *via* pores or translocation *via* carrier. Translocation *via* carrier is defined here as the occurrence of a catalysis factor for translocation[10,11]. This is obtained through binding of the transported species to a component of the membrane, whether fixed or movable. Such binding causes, during the kinetic analysis of the translocation, the appearance of phenomena of saturation and competition. The carrier hypothesis is therefore in essence an operational definition which is used to cope with a certain number of kinetic data. That such a hypothesis is feasible for anion translocation across the red cell membrane is supported by the present results.

The rate of electrogenic Cl^- diffusion was measured with gramicidin-treated red cells. The half-time for the Cl^- translocation, which is the rate-limiting reaction, was of the order of 100 sec as compared with the 0.1–0.5 sec found by TOSTESON[2] for the halide exchange. This difference in rate constants is hardly accounted for by a free diffusion of Cl^- through aqueous pores, since in this type of mechanism an electroneutral exchange reaction or an electrogenic translocation should occur at similar rates.

A membrane that allows OH^- to cross it only *via* a carrier requires a substantial impermeability to H^+ as such. A number of observations indicates that this is so for the red cell: (i) the lack of K^+ release by addition of valinomycin in the absence of uncouplers[9]; (ii) the dependence of the rate and the extent of pH equilibration on the type of anion present and not on the H^+ concentration; (iii) the lack of effect of the H^+ conductors on the rate of pH equilibration; and (iv) the pH dependence of the rate of pH equilibration which is similar to the pH dependence of the anion translocation.

The phenomena of competition and saturation represent the most direct indication that the translocation of anions across the red cell membrane involves the binding to a limited number of sites. Previous isotopic experiments with sulphate failed to demonstrate saturation kinetics. On the other hand a competition has been observed between SO_4^{2-} and Cl^- (ref. 1). The competition has been explained by assuming a common binding site constituted by fixed positive charges within the membrane. The competitive inhibition of Cl^- translocation by sulphate, succinate and nitrate is in agreement with the hypothesis of a limited number of common binding sites involved in the anion translocation mechanism. The inhibition of the pH equilibration observed at alkaline pH may also be an expression of competition of OH^- and Cl^- for the translocator.

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