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BICARBONATE EXCHANGE THROUGH THE HUMAN RED CELL MEMBRANE DETERMINED WITH [^{14}C] BICARBONATE

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SUMMARY

1. Bicarbonate transport across human red cell membranes was studied between 0 and 10 °C at alkaline pH values by determining the efflux of ^{14}C-labelled bicarbonate from resealed erythrocyte ghosts. Transfer of labelled CO_2 was eliminated as a source of error, when formation of intracellular $^{14}CO_2$ was inhibited with carbonic anhydrase inhibitors. The study showed that there are no fundamental differences between the characteristics of bicarbonate and of chloride self-exchange as has been inferred from previous studies of chloride–bicarbonate exchange.

2. Efflux of radioactivity could be reduced more than 99% by reversible and irreversible inhibitors of anion transport. Inhibition of both chloride and bicarbonate self-exchange was linearly related to the binding of 4,4′-diisothiocyanostilbene-2,2′-disulphonic acid (DIDS) to the membranes. Complete (i.e. > 99%) inhibition was obtained after binding of $1{\cdot}2 \times 10^6$ DIDS molecules per cell.

3. Bicarbonate self-exchange proved a saturable function of bicarbonate concentration, with a maximum at external and internal concentrations of ~100 mM, showing self-depression at higher bicarbonate concentrations, and half-maximum exchange flux at a concentration of 10 mM. The results were consistent with the hypothesis that the exchange mechanism has two anion binding sites, one mediating ion transport and the other causing transport inhibition.

4. Maximum exchange flux of bicarbonate was about 30% larger than that of chloride, and the affinity of bicarbonate for the transport site was about three times larger than that of chloride. The apparent activation energy of bicarbonate exchange was 28 kcal/mole, the same order of magnitude as found for other inorganic anions between 0 and 10 °C.

5. The ability of other inorganic anions to exchange with bicarbonate decreased in the sequence $Cl > NO_3 > F > Br \geqslant I$, corresponding to the sequence of the rate of self-exchange of halides.

6. Counter-transport of bicarbonate could be driven by a chloride gradient, when ghosts containing KCl were suspended in a medium containing traces of labelled bicarbonate in addition to a non-permeating anion. Concentration ratios (c_i/c_o) up to about 1000 could be obtained.

7. It is concluded that bicarbonate is transported by the inorganic anion exchange mechanism of the erythrocyte membrane. The slight differences between the exchange kinetics of chloride and bicarbonate were explained by differing affinities of the two anions for the two anion binding sites of the transport system.

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INTRODUCTION

This article describes a method for the measurement of [^{14}C]bicarbonate transport across the red cell membrane without interference from the transport of labelled CO_2.

The last decade has brought considerable insight into the kinetics of anion exchange. Moreover the identification of a putative anion transport protein in the red-cell membrane has joined transport physiologists and membrane biochemists in an endeavour to reveal the structural basis of the tightly coupled anion exchange, an exchange mechanism of the type first proposed by Ussing (1948). This development has recently been reviewed by Cabantchik, Knauf & Rothstein (1978). The necessity of characterizing anion kinetics, and the ease with which chloride transport can be studied by means of isotopes, has to some extent diverted the attention from the physiologically important phenomenon of bicarbonate transfer across the red-cell membrane. However, important observations in this area have been made through electrometric studies of the movements of H^+ ions stoichiometrically following the chloride–bicarbonate exchange, which can be induced by transfer of CO_2 and its subsequent hydration to bicarbonate and hydrogen ions (Chow, Crandall & Forster, 1976; Crandall, Obaid & Forster, 1978; Lambert & Lowe, 1978). The indirect studies of chloride–bicarbonate exchange, which have obviously physiological relevance, have raised a number of important questions regarding the implications of steady-state measurements of chloride exchange with regard to maximum transport capacity, transport saturability, and temperature dependence of the anion transport. It would be of obvious advantage, therefore, to supplement investigations of bicarbonate–chloride exchange with studies of steady-state bicarbonate exchange in order to reveal the causes of apparent discrepancies.

The present method for the determination of [^{14}C]bicarbonate transport across human erythrocyte membranes is based on the observation that the anion transport capacity is quantitatively retained, and may be studied at low temperatures in resealed ghosts even at very alkaline pH values (Funder & Wieth, 1976). The article presents a method for determination of bicarbonate transport and includes an elementary description of the kinetic characteristics of bicarbonate self-exchange.

METHODS

Preparation of resealed ghosts. A detailed description of the technique for preparing a uniform population of resealed ghosts with quantitative preservation of the anion transport capacity has been published previously (Funder & Wieth, 1976). The same reference contains information about labelling and packing of ghosts for ^{36}Cl-efflux experiments, determination of ghost volumes corrected for trapping of extracellular fluid, and calculation of unidirectional fluxes from the rate coefficients of tracers efflux. Fluxes were expressed in mol cm^{-2} sec^{-1}, assuming a membrane area of $1{\cdot}42 \times 10^{-6}$ cm^2 per cell (Funder & Wieth, 1976). To prepare ghosts for bicarbonate flux studies a few modifications were necessary. Catalysis of intracellular dehydration of labelled bicarbonate to $^{14}CO_2$, which permeates the membrane rapidly, was prevented by the use of enzyme inhibitors that do not interfere with anion transport in the concentrations used. When acetazolamide was sealed into the ghosts, it was added with the resealing electrolyte solution in an amount to yield a final concentration of 1 mM in the haemolysate, and, therefore, also in the resealed ghosts. Most of the ghost preparations were made as 'KCl-ghosts', but Cl^- exchanged readily with HCO_3^-, when ghosts made for studies of bicarbonate exchange were washed in the appropriate bicarbonate medium before use. As mentioned below, ghosts were also made from

red cells that had been treated with an irreversible inhibitor of anion transport. These ghosts were sealed in the presence of $KHCO_3$. The ghosts seal as completely, when $KHCO_3$ is used instead of KCl for reversal of tonicity in the haemolysate. Ghosts resealed with bicarbonate had a slightly smaller cell volume (e.g. 80 *vs.* 90 μm^3), most likely because the unsealed membrane has a slightly higher reflection coefficient to bicarbonate than to chloride (cf. Funder & Wieth, 1976). Ghosts were labelled with isotopes as described below and packed in nylon tubes to a cytocrit of 90–94 % for experiments. The packed ghosts were stored at 0 °C until they were used for experiments, usually on the day of preparation. In a few cases, as specified in the legends, experiments were carried out up to 24 hr after ghost preparation. It was found that maximum transport capacity decreased by 10–15 % when ghosts with an intracellular pH of 8·7 were stored at 0 °C for 24 hr. Although older ghosts were not used for experiments in the present work, it is worth noting that bicarbonate transport reached a stable plateau which was 60 % of the original transport capacity when ghosts were stored for 8–10 days. In brief, flux experiments were carried out by injecting 200 to 400 mg of packed ghosts into 40 ml. of a well-stirred electrolyte solution, thermostatted at the desired temperature. Efflux or – as in the experiment of Fig. 12 – influx of ^{14}C-labelled bicarbonate was followed by determinations of radioactivity in the cell-free extracellular fluid, which was isolated from the suspension by serial filtration as described by Dalmark & Wieth (1972). The vessel used for the experiment was covered with a Perspex lid. No other precautions were necessary to prevent the escape of labelled CO_2, because the loss of radioactivity under the most vigorous stirring employed was less than 0·1 % per min.

Inhibition of anion transport

Specific inhibition of anion transport (Fig. 1) was achieved by treating the red-cell membranes with 4,4′-diisothiocyanostilbene-2,2′-disulphonic acid (DIDS) as recently described by Funder, Tosteson & Wieth (1978). Attempts to treat red-cell membranes with DIDS in the presence of bicarbonate and acetazolamide were not successful. Therefore, fresh red cells washed in a 165 mM-KCl solution were exposed to the appropriate amount of DIDS for 45 min at 38 °C to bind a known number of DIDS molecules per membrane (Fig. 1). Ghosts were then prepared from the DIDS-treated red cells, and it was found that ^{36}Cl-exchange was inhibited over 99 %, when ghosts were prepared from red cells which had been treated with more than $1·2 \times 10^6$ DIDS molecules per cell.

Temperature dependence or bicarbonate transport. Apparent activation energies of bicarbonate transport were determined by linear regression analyses of the relation between the natural logarithm of the bicarbonate self-exchange flux and the reciprocal of the absolute temperature in uninhibited and in DIDS-treated ghosts (Figs. 7, 8). Standard deviations stated in the legends were calculated from the standard deviation of the slope of the regression line.

Electrolyte media. Unless otherwise stated, experiments were carried out in a 165 mM-$KHCO_3$ solution containing 1 mM-acetazolamide (Sigma Chem. Co., St Louis, Mo., U.S.A.). The pH of the medium was 8·7 at 0 °C. Ethoxzolamide was used as a rapidly permeating inhibitor of carbonic anhydrase in a few experiments (Fig. 6). It was a gift from The Upjohn Co., Mich., U.S.A. Bovine carbonic anhydrase (Sigma), 2800 Roughton-Boot units per mg was used in a concentration of 300 mg/l. to catalyse extracellular hydration of CO_2 (Figs. 3, 5). In the self-exchange experiments shown in Fig. 9, $KHCO_3$ concentrations were varied between 15 and 600 mM. Media used for determining the relative affinities of chloride and bicarbonate to the transport system (Fig. 10) were made up by mixing appropriate amounts of 165 mM-KCl containing 1 mM-acetazolamide with the $KHCO_3$ medium. The ability of bicarbonate to exchange with other inorganic anions in hetero-exchange experiments was determined with 165 mM-$KHCO_3$-ghosts in media containing 165 mM of the potassium salts of chloride, nitrate, fluoride, bromide or iodide (Fig. 11). Also these media contained 1 mM-acetazolamide and were titrated with KOH to pH 8·7 (0 °C).

Physical chemistry of the bicarbonate medium. Bicarbonate transport was studied at pH 8·7 to keep concentrations of CO_2 and of carbonate low compared to the bicarbonate concentration. The apparent pK′ values of bicarbonate, calculated from the data compiled by Siggaard-Andersen (1974), are: $pK_1(CO_2) = 6·4$, $pK'_2 = 10·3$ at 0 °C and an ionic strength of 0·16. Concentrations of CO_2, carbonate, and of bicarbonate are, therefore, 0·8, 4·1 and 157 mM in the so-called '165 mM-bicarbonate medium'. The P_{CO_2} is 8 mm Hg.

The rate of spontaneous dehydration of bicarbonate to CO_2 decreases with increasing pH.

According to the data of Magid & Turbeck (1968) the pseudo first-order rate coefficients of hydration (k'_h) and of dehydration (k'_d) are $3{\cdot}3 \times 10^{-3}$ sec^{-1} and $1{\cdot}65 \times 10^{-5}$ sec^{-1} at 0 °C, pH 8·7. The half-times of hydration and of dehydration at the constant pH are accordingly 3·5 and 700 min. It is the extremely low rate of spontaneous dehydration which makes it possible to determine transport of labelled bicarbonate without significant formation of labelled CO_2 from intracellular bicarbonate.

^{14}C-labelled bicarbonate was obtained from The Radiochemical Centre, Amersham, England, at a specific activity of 60 mCi/mmol. Ghosts were labelled, while suspended in the medium at a cytocrit of 30–50 % with about 2 μCi [^{14}C]HCO_3 per ml suspension. In experiments with an uninhibited transport system the ghosts were packed 1 min after the addition of radioactivity, as istotope equilibration is completed in a few seconds at room temperature. When ghosts were prepared from DIDS-treated red cells, the ghosts were heated to 38 °C for 3 min after addition of isotope to ensure tracer equilibration, which proceeds with a half-time of 20–30 s in inhibited cells at 38 °C.

Influx experiments. As shown in Fig. 12, counter-transport of [^{14}C]bicarbonate into ghosts driven by a chloride gradient can be followed at a low cytocrit, under conditions where the amount of permeating anions in the ghosts (S_1 mol) is significant compared to the amount of permeating anions in the extracellular phase (S_2 mol). The isotope can attain concentration ratios (a_i/a_o) as high as 1000. The experiment in Fig. 12 was carried out with ghosts loaded with 165 mM-KCl and 1 mM-acetazolamide. Preliminary experiments with gluconate and citrate media showed that gluconate is a weak competitive inhibitor of both chloride and of bicarbonate influx, when the extracellular concentration of the permeating anion is below 0·2 mM. The experiment was started by injecting the KCl ghosts into a medium containing 4·2 μM-labelled-bicarbonate, 25 mM-potassium citrate, 200 mM-sucrose, and 1 mM-acetazolamide (pH 8·7), being isosmotic with the 165 mM-KCl solution in the ghosts. It was checked that the ratio (a_o^∞/a_t) between the equilibrium concentration of radioactivity in the extracellular phase (a_o^∞) and the concentration of radioactivity in the total cell suspension (a_t) corresponded to the distribution of permeating anions: $S_2/(S_1+S_2)$. The equilibrium concentration of radioactivity in the extracellular phase was determined in samples of medium taken after 6–8 half-times of the isotope equilibration process. The rates of [^{14}C]bicarbonate and of $^{36}Cl^-$ influx from a 165 mM-potassium gluconate medium were only 50 % of the rates found in the citrate medium, but the rate of bicarbonate influx was approximately three times larger than that of $^{36}Cl^-$ influx in both media.

RESULTS

Table 1 compares the self-exchange fluxes of bicarbonate and chloride at extra- and intracellular anion concentrations of 165 mM. The ghosts used for the chloride and bicarbonate experiments were prepared from the same blood sample from each donor. The mean bicarbonate flux exceeded chloride exchange flux by ~20 %, a feature which is dealt with in the examination of the concentration dependence of bicarbonate exchange.

It was essential to demonstrate that the studies of bicarbonate transport were not hampered by an unperceived transfer of labelled CO_2 across the cell membranes. Useful information, therefore, could be obtained by comparing the inhibition of chloride and bicarbonate transport caused by irreversible binding of the amino-group reagent DIDS (Cabantchik & Rothstein, 1974). Fig. 1 shows that inhibition of chloride and bicarbonate transport was linearly related to the number of inhibitor molecules bound per cell membrane. The number causing 99 % inhibition of anion exchange ($1{\cdot}2 \times 10^6$ molecules per cell) was identical with previous findings (Lepke, Fasold, Pring & Passow, 1976; Ship, Shami, Breuer & Rothstein, 1977; Funder *et al.* 1978).

Although this result strongly suggested that shunting of labelled CO_2 is not a source of error, it was considered worthwhile to examine this point thoroughly.

Fig. 2 illustrates the experimental problem: resealed ghosts, labelled with radioactive bicarbonate, are suspended at a low cytocrit in an isotonic electrolyte medium. The extra- and intracellular pH is 8·7, where the ratio HCO_3^-/CO_2 is about 200 at 0 °C (cf. Methods section). The aim of the investigation is to examine the anion exchange by the process indicated as no. 1 in Fig. 2, without interference from transport of labelled CO_2 through the membrane by process no. 3. This is prevented

TABLE 1. Unidirectional self-exchange fluxes of chloride and of bicarbonate in resealed ghosts at 0 °C, pH 8·7. The experiments were carried out with ghost preparations from eight donors in 165 mM-KCl and $KHCO_3$ media containing 1 mM-acetazolamide. Rates of ^{36}Cl and of [^{14}C]-bicarbonate efflux were determined in duplicate within 24 hr after preparation of the ghosts. Unidirectional fluxes were calculated from the equation $J = k.(MCV/A.C_i$ mol cm^{-2} sec^{-1}), A being the surface area ($1{\cdot}42 \times 10^{-6}$ cm^2 per cell), MCV the mean cell volume, and C_i the intracellular anion concentration.

No.	Rate coefficient of ^{36}Cl exchange (sec^{-1})	MCV (μm)	Rate coefficient of $H^{14}CO_3^-$ exchange (sec^{-1})	MCV (μm^3)	Self-exchange flux (J) (10^{12} mol cm^{-2} sec^{-1}) Chloride	Bicarbonate	Relative flux $^JHCO_3/^JCl$
1	0·0269–0·0283	99·9	0·0391–0·0378	94·9	321	424	1·32
2	0·0255–0·0255	88·0	0·0329–0·0333	81·9	261	317	1·21
3	0·0250–0·0233	101·8	0·0329–0·0349	77·5	285	305	1·07
4	0·0281–0·0283	83·6	0·0342–0·0366	77·0	274	309	1·13
5	0·0236–0·0235	90·9	0·0346–0·0351	77·0	249	312	1·25
6	0·0222–0·0270	88·8	0·0354–0·0353	88·5	254	363	1·43
7	0·0233–0·0231	85·9	0·0351–0·0356	68·9	231	282	1·22
8	0·0207–0·0202	98·8	0·0327–0·0290	84·2	234	302	1·29
Mean	—	92·2	—	81·2	264	327	1·24
S.D.	—	7·0	—	8·0	30	45	0·11
S.E. of mean	—	2·6	—	3·0	11	17	0·04

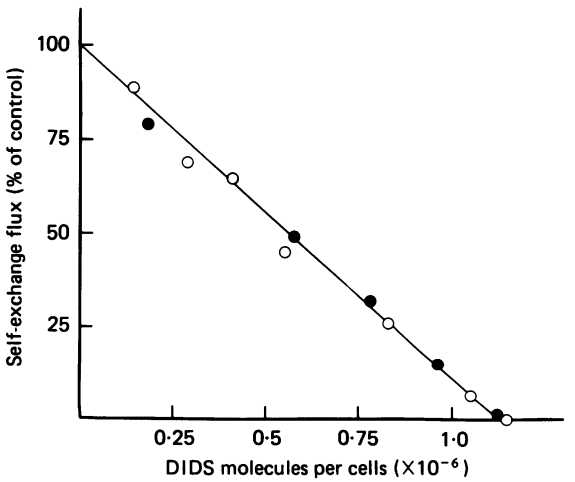

Fig. 1. Inhibition of chloride (○) and of bicarbonate (●) self-exchange fluxes in ghosts prepared from DIDS-treated human red cells as a function of the number of DIDS molecules bound per cell (cf. Methods). The ordinate represents the fractional self-exchange flux in percent of the value found in control samples of ghosts that had not been treated with DIDS.

by inhibiting the catalytic effect of residual intracellular carbonic anhydrase with a specific inhibitor, which does not interfere with the anion exchange mechanism. The spontaneous dehydration of HCO_3^- is so slow at the alkaline pH that the transport of labelled CO_2 will only contribute negligibly to the over-all release of ^{14}C from the ghosts. This was demonstrated experimentally. Fig. 3 shows the results from three experiments (*A*, *B*, and *C*). In *A*, anion exchange was inhibited by the presence of

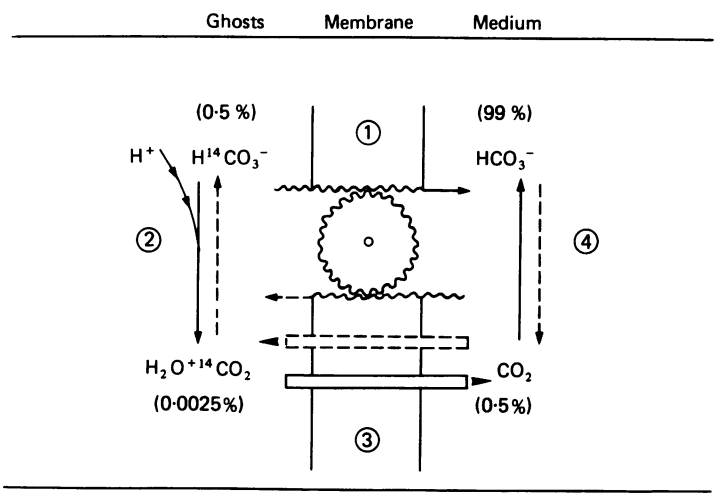

Fig. 2. Schematic representation of the pathways for release of ^{14}C from ghosts labelled with $H^{14}CO_3^-$. Initially 99·5 % of the ^{14}C is located in the intracellular bicarbonate when the cytocrit is 0·5 % at 0 °C, pH 8·7. The aim is to study the anion exchange mechanism, indicated as process no. 1. Therefore it is necessary to prevent catalysed dehydration of intracellular bicarbonate to CO_2 by process no. 2. If the formation of intracellular $^{14}CO_2$ is not inhibited, the tracer will permeate the membrane in the form of $^{14}CO_2$. Due to the high membrane permeability to CO_2, intra- and extracellular CO_2 will equilibrate rapidly. This will at a cytocrit of 0·5 % lead to a rapid loss of about 50 % of the tracer, because the amount of intracellular (bicarbonate plus CO_2) is equal to the pool of extracellular CO_2 (cf. the experimental results shown in Fig. 4). If carbonic anhydrase is added to the extracellular medium in the absence of intracellular carbonic anhydrase inhibition the release of ^{14}C will proceed rapidly by transport of $^{14}CO_2$, because the reaction can proceed rapidly until isotopic equilibrium is attained, when process no. 4., the extracellular hydration of CO_2 to bicarbonate, is catalysed (cf. the experimental results of Fig. 3).

phloretin, a potent inhibitor of anion transport. Virtually no radioactivity was lost from the ghosts during the first minute, indicating that the formation of CO_2 from labelled HCO_3 was prevented by the presence of 1 mM-acetazolamide. In contrast the tracer equilibrated monoexponentially with a half-time of about 20 s,when anion transfer was not blocked with phloretin (experiment *B*). In the third experiment, *C*, the intracellular radioactivity was allowed to be released through the CO_2 shunt indicated by the processes nos. 2–4 in Fig. 2. The rate of tracer movement was increased by the presence of uninhibited carbonic anhydrase on both sides of the cell membrane, catalysing the intracellular dehydration of bicarbonate and the extracellular hydration of CO_2. The concentration of extracellular carbonic anhydrase was sufficient to make the intracellular dehydration reaction rate limiting, and the

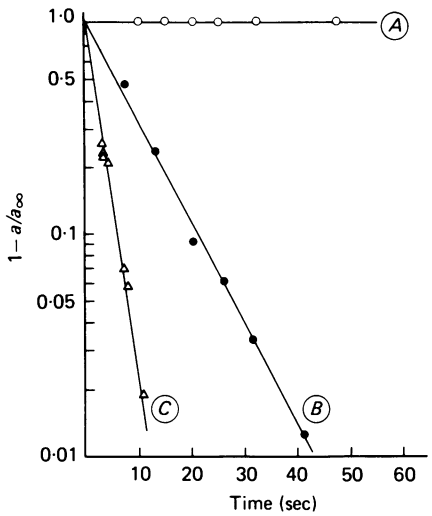

Fig. 3. Rate of tracer release from [^{14}C]bicarbonate labelled ghosts (0 °C, pH 8·7, cytocrit 0·5 %) illustrating the necessity of inhibiting residual intracellular carbonic anhydrase of the ghosts. The scale of the ordinate is logarithmic; a is the concentration of ^{14}C in the medium at the time of sampling, and a_∞ is the concentration after achievement of isotopic equilibrium. A small amount of trapped extracellular fluid is added to the medium with the packed ghosts, and the interception with the ordinate (0·92) corresponds well to the inulin space of 7·9 %, which was determined separately. Three experiments are shown: *A*, ○, anion exchange inhibited by phloretin (0·25 mM), carbonic anhydrase inhibited with acetazolamide; *B*, ●, intracellular carbonic anhydrase inhibited with acetazolamide; *C*, Δ, , carbonic anhydrase present in ghosts and medium (Bovine C.A. 300 mg/l. ~ $8{\cdot}4 \times 10^5$ Roughton-Booth units/l.).

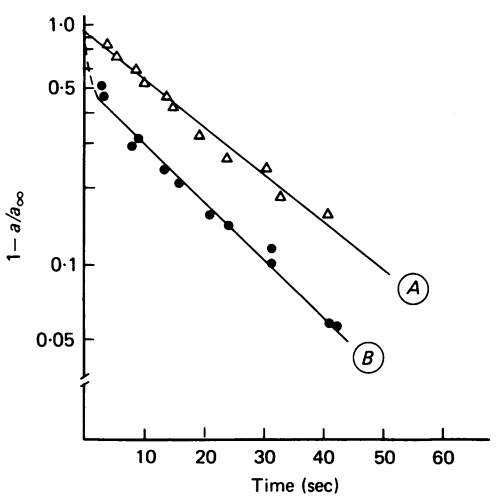

Fig. 4. Rate of tracer release from ^{14}C-bicarbonate-labelled ghosts (0 °C, pH 8·7, cytocrit 0·5 %), illustrating the rate limiting effect of the spontaneous extracellular hydration of $^{14}CO_2$ (cf. Fig. 2). Intracellular dehydration of intracellular bicarbonate was inhibited with acetazolamide in *A* (Δ) but was uninhibited in *B* (○), where there was a rapid initial loss of ^{14}C from the ghosts.

transfer of radioactivity took place both by the transport of labelled CO_2 (process 3 in Fig. 2), and via the anion exchange mechanism with a resultant half-time of about 2 sec. From comparison with experiment *B* it appears that about 90 % of the isotope transfer was carried by CO_2.

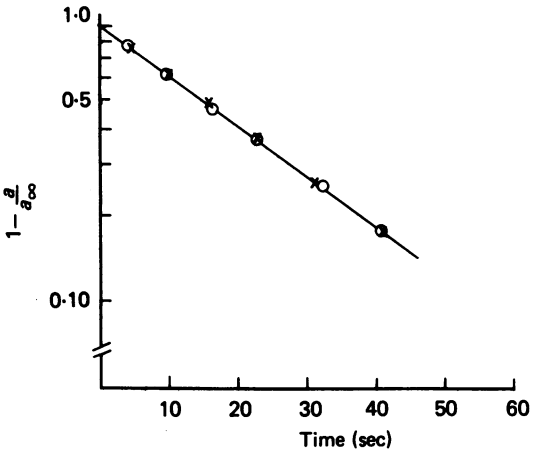

Fig. 5. Rate of tracer release from ^{14}C-bicarbonate-labelled ghosts containing acetazolamide (×; 0 °C, pH 8·7). Addition of bovine carbonic anhydrase to the medium (○) did not affect the rate of tracer efflux, showing that process no. 2 of Fig. 2 is efficiently inhibited by the intracellular carbonic anhydrase inhibition. ×, k = 0·040 sec^{-1} (S.D. 0·0004); ○, k = 0·039 sec^{-1} (S.D. 0·0004).

The necessity of inhibiting the intracellular carbonic anhydrase, also in the absence of an extracellular catalyst, is illustrated by Fig. 4, which shows the rate of release of intracellular ^{14}C in the presence (*A*) and in the absence (*B*) of intracellular carbonic anhydrase inhibitor. In the latter experiment there was an initial rapid release of about 50 % of the intracellular radioactive bicarbonate, whereafter the efflux progressed at a rate comparable to that found in *A*. The explanation of this time course is that the radioactivity is rapidly released as CO_2 until extra- and intracellular CO_2 attain isotopic equilibrium. This occurred after a loss of about 50 % of the cellular radioactivity, because the amount of intracellular (bicarbonate plus CO_2) at a cytocrit of 0·5 % is equal to the amount of extracellular CO_2 (cf. Fig. 2). Because hydration of extracellular CO_2 (by process no. 4 of Fig. 2) is relatively slow in the absence of carbonic anhydrase, (half-time 3·5 min according to the data stated in the Methods section) the main pathway of subsequent ^{14}C transfer is through the anion exchange mechanism (process no. 1 of Fig. 2). One might predict that the presence of extracellular carbonic anhydrase should not interfere with bicarbonate transport if the intracellular dehydration step is completely inhibited. This was confirmed by the result shown in Fig. 5. The exchange of bicarbonate was unaffected by extracellular carbonic anhydrase, when intracellular enzymatic activity was inhibited with azetazolamide, which has an extremely low membrane permeability at 0 °C (Holder & Hayes, 1965). For the same reason it is necessary to load the ghosts with acetazolamide before resealing. The red cell membrane is 10^4 times more

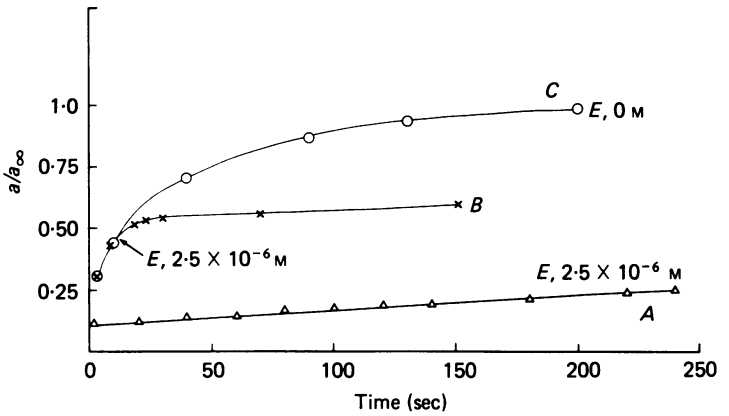

Fig. 6 The effect of a rapidly permeating carbonic anhydrase inhibitor (ethoxzolamide) on the rate of tracer release from ^{14}C-bicarbonate labelled ghosts prepared from red cells treated with 10^7 molecules DIDS per cell (0°, pH 8·7, cytocrit 1%). The ordinate has a linear scale, a is the concentration of radioactivity in the medium at the time of sampling, a_∞ is the concentration after achievement of isotopic equilibrium. There are three experiments: A, △, ethoxzolamide 2×10^{-6} M present in medium before injection of ghosts; B ×, ethoxzolamide 2×10^{-6} M added after 15 s; C, ○, no carbonic anhydrase inhibitor present. It is seen that the effect of ethoxzolamide on the rate of tracer release developed in a few seconds. The initial rapid tracer release in B and C was smaller than that shown in Fig. 4 because the amount of extracellular CO_2 is a smaller fraction of the amount of intracellular (bicarbonate plus CO_2) when the cytocrit is raised from 0·5 to 1%.

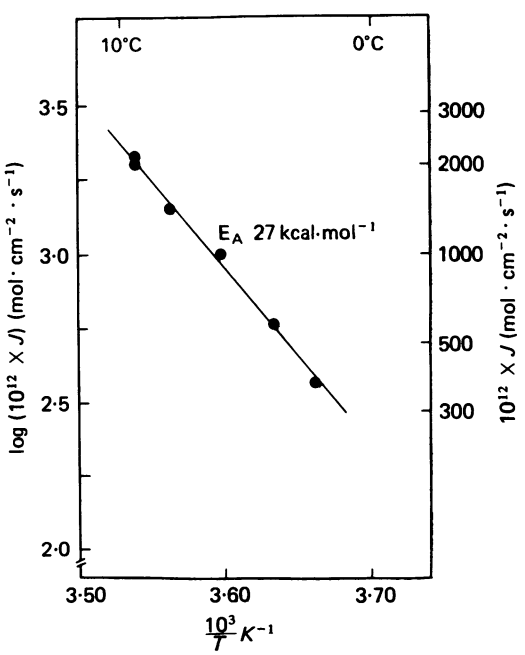

Fig. 7. Arrhenius diagram of the relation between the natural logarithm of the bicarbonate self-exchange flux in ghosts and the reciprocal absolute temperature (0–10 °C). The apparent activation energy calculated from the slope of the relation was 27 (± 0·7 S.D.) kcal/mol. The mean value of three experiments was 28·4 kcal/mol.

permeable to the potent carbonic anhydrase inhibitor ethoxzolamide than to acetazolamide (Holder & Hayes, 1965). Ethoxzolamide does not inhibit anion transport in concentrations up to 10^{-5} M. Even at 0 °C permeation of ethoxzolamide was sufficiently rapid to inhibit intracellular enzyme activity in a matter of seconds. This is shown in Fig. 6. The experiments were carried out with DIDS-treated cells, in order to be

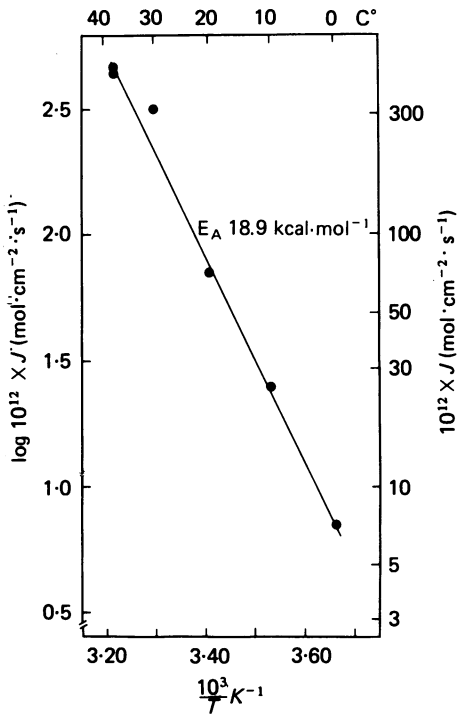

Fig. 8. Arrhenius diagram of the relation between the natural logarithm of the bicarbonate self-exchange flux in DIDS-treated ghosts and the reciprocal of the absolute temperature (0–38 °C). The ghosts were prepared from DIDS-treated red cells and the inhibition of anion exchange was ~ 99 % (cf. Fig. 7). It may be noted that the self-exchange flux of the inhibited ghosts at 38 °C is of the same magnitude as found in uninhibited ghosts at 0 °C. The apparent activation energy was 19 kcal/mol (± 1 S.D.). A similar decrease of the activation energy of chloride transport in DIDS-treated red cells has previously been shown by Brahm (1977).

able to monitor the formation of $^{14}CO_2$ from intracellular bicarbonate. There was a very slow release of tracer, when ghosts were injected into a medium containing $2{\cdot}5 \times 10^{-6}$ M-ethoxzolamide, and the much more rapid release of tracer taking place in the absence of inhibitor was stopped in a matter of seconds when ethoxzolamide was added to the suspension.

Fig. 7 is an Arrhenius diagram of the temperature dependence of bicarbonate self-exchange in ghosts from one donor. The mean value of apparent activation energy between 0 and 10 °C in three experiments was 28·4 kcal/mol (range 27·6–29·7) corresponding to a flux increase by a factor of six to seven between 0 and 10 °C. The apparent activation energy was also determined in DIDS-treated ghosts, in which the transport capacity was inhibited about 99 % at 0 °C (Fig. 8). The residual self-exchange flux increased from 7 to 440 pmol cm^{-2} sec^{-1} between 0 and 38 °C, and

the activation energy 18·8 kcal/mol was significantly lower than in the unmodified cells. The apparent activation energies are similar to those of the uninhibited and of the maximally DIDS-inhibited chloride exchange fluxes in human red cells (Brahm, 1977).

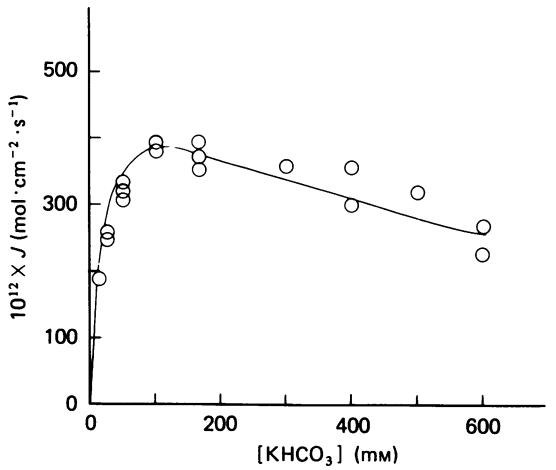

Fig. 9. Dependence of bicarbonate self-exchange flux of resealed ghosts on intra- and extracellular bicarbonate concentrations (pH 8·7 0 °C). Half-maximal flux was obtained at a bicarbonate concentration of ~ 10 mM, and the flux was depressed at bicarbonate concentrations of 4–600 mM. The qualitative shape of the concentration dependence is similar to that found for chloride by Funder & Wieth (1976), the quantitative differences are discussed in the text.

Fig. 9 shows a determination of the concentration dependence of bicarbonate self-exchange between 15 and 600 mM. As in the case of chloride exchange one finds that the self-exchange is a saturable function of anion concentration in ghosts and media. The transport is not a simple hyperbolic function of bicarbonate concentration. As in the case of chloride exchange in resealed ghosts (Funder & Wieth, 1976) the flux increased steeply at low anion concentrations, but 'self depression' of transport was apparent at higher concentrations. The graph in Fig. 9 showed the following quantitative differences from the concentration dependence of chloride exchange: the maximum flux of bicarbonate was 20–30 % higher than the maximal chloride flux found under similar conditions. The concentration, ($K_{\frac{1}{2}}$), causing half-maximum flux was about 10 mM in the bicarbonate experiments *vs.* 20–30 mM for chloride. Maximum bicarbonate flux was found at an anion concentration of ~100 mM, about 50 mM lower than the chloride concentration causing maximal chloride exchange. Self-inhibition was less pronounced for bicarbonate than for chloride flux. The flux was depressed by 35 % from its maximum at a bicarbonate concentration of 600 mM, to be compared with a 60 % reduction of chloride flux at the same chloride concentration. The curve in Fig. 9 was drawn according to the two site model of Dalmark (1976). This model assumes the existence of two anion binding sites in the transport system. One site is the transport site. Binding of the transported anion to this site is characterized by the dissociation constant, K_A. Binding of an anion to the second site, the so-called modifier site, is characterized by another dissociation constant (K_{AA}), and this binding leads to a non-competitive inhibition of anion transport,

hence the designation: self-inhibition. As dealt with in the Discussion, bicarbonate transport appeared to have a higher affinity to the transport site and a lower affinity to the modifier site than chloride. The affinity to the transport site at 0 °C appeared to be three times larger for bicarbonate than for chloride. A similar conclusion has been reached previously from studies of the competitive inhibitory effect of bicarbonate on chloride transport (Gunn, Falmark, Tosteson & Wieth, 1973; Dalmark, 1976).

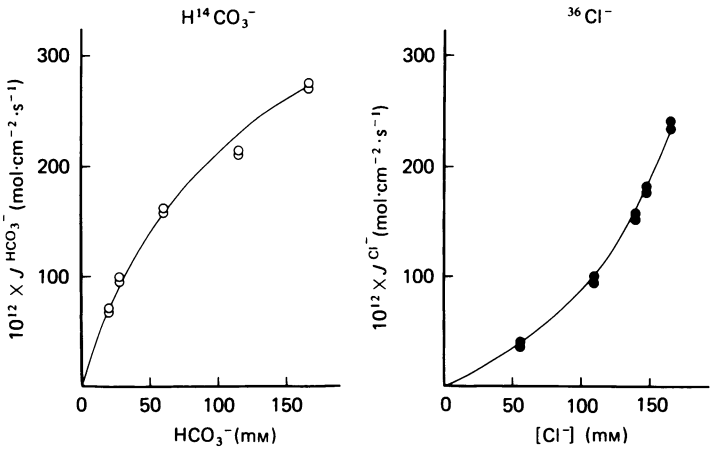

Fig. 10. Dependence of chloride and of bicarbonate self-exchange fluxes on anion concentrations in anion substitution experiments. The ion composition of extra- and intracellular water phases were identical in each experiment. The sum of (chloride + bicarbonate) was kept constant at 165 mM, but the two anions were substituted for each other to vary the concentration of both ions between 0 and 165 mM. All experiments were carried out at 0 °C, pH 8·7. The shapes of the graphs agree with the concept that the two anions compete for a common transport site, and that the bicarbonate affinity is three to four times larger than the chloride affinity (cf. Appendix of Gunn *et al.* 1973).

To fill the experimental pattern the inhibitory effect of chloride on bicarbonate exchange was examined in the present study (Fig. 10). The graph depicting bicarbonate transport as a function of bicarbonate concentration in anion substitution experiments, where the sum of ($Cl^- + HCO_3^-$) was kept constant (165 mM), was convex towards the ordinate, in contrast to the concavity of the graph representing chloride transport as a function of chloride concentration in similar substitution experiments. The graphs depicting anion transport *vs.* anion concentration (Fig. 10) would have been rectilinear if chloride and bicarbonate had identical affinities to the transport system. As stated in the legend of Fig. 10 the shapes of the graphs agree with the concept that the affinity of bicarbonate at 0 °C exceeds that of chloride by a factor of 3–4.

There are considerable differences in the ability of other inorganic anions to serve as exchange partners for bicarbonate. Fig. 11 presents the results of a series of hetero-exchange experiments, where ghosts with an intracellular bicarbonate concentration of 165 mM were injected into media containing 165 mM of the potassium salts of chloride, fluoride, nitrate, bromide or iodide. The initial rates of [^{14}C]bicarbonate efflux into these media are shown relative to the rate of tracer efflux into a 165 mM-bicarbonate medium. The efflux of bicarbonate took place with the same initial rate in the bicarbonate and in the chloride medium, but the rate decreased 5 times when

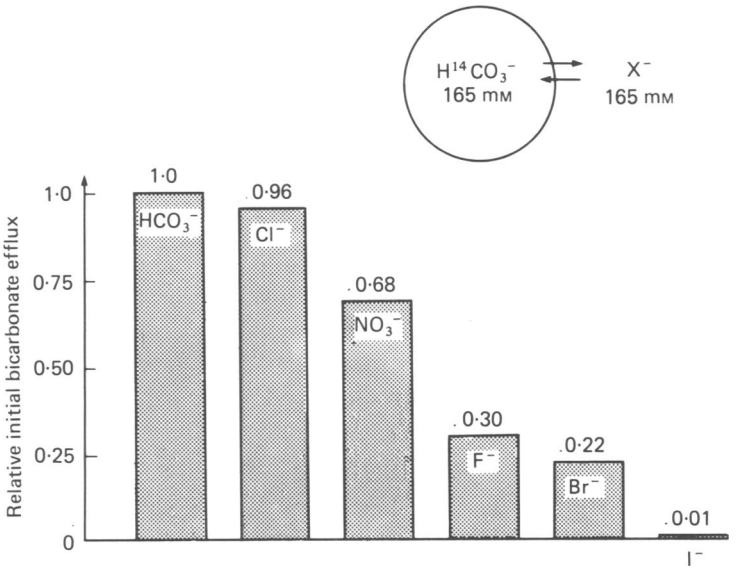

Fig. 11. The rate of exchange of [^{14}C]bicarbonate with monovalent anions (0 °C, pH 8·7). Bicarbonate loaded ghosts were injected into media containing 165 mM of the potassium salts indicated. The initial rates of bicarbonate efflux were determined and are expressed relative to the rate of bicarbonate self-exchange which in duplicate determinations had rate coefficients of 0·031 and 0·030 sec^{-1}. The ghosts used for these experiments were prepared 24 hr earlier.

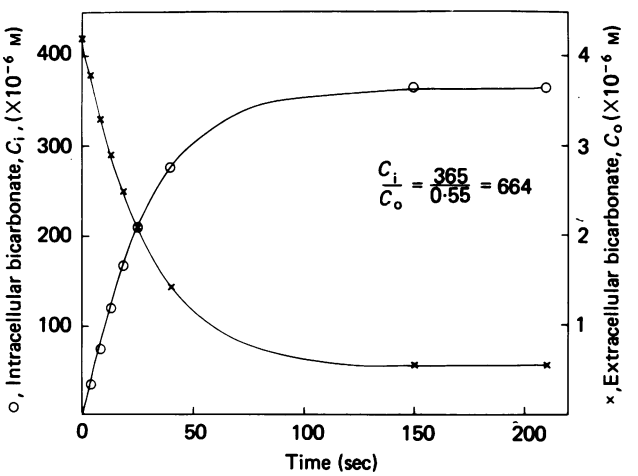

Fig. 12. Counter-transport of [^{14}C]bicarbonate into human erythrocyte ghosts driven by a chloride gradient. Ghosts containing 165 mM-KCl were injected into the citrate-sucrose medium described in the Methods (0 °C, pH 8·7, cytocrit 1 %). The medium contained initially 4·2 μM-$H^{14}CO_3^-$. Extracellular bicarbonate concentrations are indicated on the right-hand ordinate, intracellular concentrations on the left-hand ordinate; note difference of ordinate scales. The decrease of extracellular bicarbonate concentration, C_o(×), was followed by determinations of the extracellular radioactivity. The intracellular radioactivity, C_i(○), was calculated from the disappearance of [^{14}C]bicarbonate from the medium. It was checked that the radioactivity of the suspension remained constant during the experiment. The extracellular chloride concentration was 0·25 mM at the end of the experiment corresponding to a ratio C_i/C_o of 660.

the exchange partner was bromide, and was reduced a hundredfold in an iodide medium.

The sum of evidence presented so far indicates that bicarbonate and chloride ions are transferred by the same transport system: the anion exchange mechanism of the red-cell membrane. The transport is a tightly coupled electrically silent 1:1 exchange of anions as first quantified by Hunter (1971, 1977). Therefore, it should be possible to demonstrate counter-transport phenomena, e.g. an accumulation of bicarbonate ions in the intracellular phase driven by an oppositely directed chloride concentration gradient across the membrane. Experiments showed that concentration ratios of bicarbonate (C_i/C_o) up to one thousand could easily be achieved under appropriate conditions. An example is shown in Fig. 12. The extracellular medium was the isosmotic citrate-sucrose medium described in the Methods containing 4·2 μM-^{14}[C]bicarbonate. Citrate is an impermeant anion, and the experiment was started by injecting ghosts containing 165 mM-potassium chloride and 1 mM-acetazolamide into the medium to make a cytocrit of 1 %. Bicarbonate ions immediately began to accumulate in the intracellular phase, and the transport process continued until a steady-state ratio of internal to external bicarbonate of 660 (corresponding to the chloride distribution ratio) was achieved. The distribution was stable for several minutes. It must be noted that the ion transfer is not accompanied by any pH changes because carbonic anhydrase is inhibited. It was confirmed in other experiments that the accumulation of bicarbonate did not occur if anion transport was inhibited by phloretin or after DIDS-treatment of the ghosts. It was also found that the accumulation of labelled bicarbonate takes place about three times as fast as the accumulation of $^{36}Cl^-$ under these conditions, where the transport system is far from saturation. The experimental design (Fig. 12) resembles that of Lambert & Lowe (1978) except that in their experiments the presence of carbonic anhydrase on both sides of the membrane caused a recycling of CO_2 by a Jacobs–Stewart cycle (Jacobs & Stewart, 1942). This led to a net loss of anions from the cells and a concomitant transfer of OH^- from the extra- to the intracellular phase.

DISCUSSION

Transport of [^{14}C]bicarbonate

It has been demonstrated previously that the anion transport capacity of resealed ghost membranes is quantitatively retained at alkaline pH values (Funder & Wieth, 1976). Therefore, it was possible to determine the transport of bicarbonate ions at pH 8·7, where the spontaneous dehydration of bicarbonate with a half-time of 700 min is too slow to represent a significant source of error (cf. Methods). It must be assumed that labelled CO_2 present in the ghosts rapidly equilibrates with extracellular CO_2, but a subsequent shunting of $^{14}CO_2$ through the cell membranes (Fig. 2) is efficiently prevented by the use of carbonic anhydrase inhibitors (the slowly permeating acetazolamide, which does not interfere with anion transport (Cousin & Motais, 1976; Wieth, 1972), or the rapidly permeating ethoxzolamide, which only inhibits anion transport in concentrations over 10^{-5} M (J. O. Wieth, unpublished). It was necessary to use carbonic anhydrase inhibitors, because the preparation of pink ghosts removes only 97 % of the original cellular macromolecules, leaving about 6×10^{-6} M-carbonic

anhydrase in the resealed ghosts. The slowness of the conversion of $H^{14}CO_3^-$ to $^{14}CO_2$ explains why membrane transfer of ^{14}C in the presence of carbonic anhydrase inhibitors took place by a transport of bicarbonate ions, which could be efficiently inhibited (as shown in Fig. 1) by treating the membranes with a specific, irreversible inhibitor of anion transport (DIDS), or as shown in Fig. 3, by exposing the ghosts to phloretin, a potent reversible inhibitor of many facilitated transport systems, inhibiting anion transport with a K_I of 10^{-6} M (Wieth, Dalmark, Gunn & Tosteton, 1973). The necessity of preventing $^{14}CO_2$ formation from $H^{14}CO_3^-$ is evident from the finding of Gutknecht, Bisson & Tosteson (1977) that the permeability of a bimolecular lipid membrane to CO_2 is 4×10^{-1} cm sec^{-1}, five orders of magnitude larger than the apparent bicarbonate permeabilities dealt with in this article.

Maximum transport capacity and temperature dependence of transport

By direct comparison between chloride and bicarbonate transport (Table 1) it was demonstrated that unidirectional exchange fluxes of bicarbonate at an anion concentration of 165 mM exceeded those of chloride by 20–30 %. An explanation for this observation was provided by the study of the concentration dependence of bicarbonate self-exchange (Fig. 9). By comparison with earlier results on chloride exchange in ghosts (Funder & Wieth, 1976, fig. 3), Fig. 9 of the present work showed the following differences. Maximum bicarbonate exchange flux was found at a lower concentration (110 *vs.* 150–200 mM). The observed maximal bicarbonate flux was 400 pmol cm^{-2} sec^{-1}, about 33 % higher than that of chloride. The decrease of anion exchange with increasing anion concentration (self-inhibition) was considerably less pronounced in the case of bicarbonate (Fig. 9) than previously found for chloride (Cass & Dalmark, 1973; Dalmark, 1976; Funder & Wieth, 1976). The self-inhibition of transport at high anion concentrations has been interpreted by Dalmark (1976) to represent a non-competitive inhibitory effect of anion-binding to a so-called modifier site, which regulates the capacity of the transport system, because transport is inactivated when an anion is bound to the site. This phenomenon is analogous to the 'inhibition by substrate' in the kinetics of enzyme action (Laidler, 1958). Dalmark (1976) suggested that the concentration dependence of chloride self-exchange with an apparent half-saturation at a chloride concentration of 30 mM could be explained by a two-site reaction mechanism, with a transport site displaying a dissociation constant (K_A) for chloride of 67 mM, and a modifier site which is half-saturated with chloride at a concentration of 335 mM (K_{AA}). The corresponding values of bicarbonate concentration which were used for fitting the curve of Fig. 9, were 20 mM for the bicarbonate affinity to the transport site (K_A) and 600 mM for the half-saturation of the non-competitively inhibiting modifier site (K_{AA}). This agrees well with the conclusions made by Gunn *et al.* (1973) that the inhibitory effect of bicarbonate on chloride transport is predominantly competitive, a conclusion which was confirmed by Dalmark (1976), who estimated dissociation constants of 16 and 585 mM for K_A and K_{AA} of bicarbonate respectively. The concentration ($K_{\frac{1}{2}}$) at which the observed bicarbonate flux was half-maximal was about 10 mM (Fig. 9), in perfect agreement with evaluations based on the competitive effect of bicarbonate on chloride exchange (Gunn *et al.* 1973; Dalmark, 1976).

It was confirmed in the present study (Fig. 10) that the competition of bicarbonate

and chloride could be explained by assuming that the affinity of bicarbonate to the transport site at 0 °C was about three times higher than that of chloride. The same conclusion can be made from studies of the rates of [^{14}C]bicarbonate and of ^{36}Cl influx into red-cell ghosts injected into isotonic media containing only traces of chloride or bicarbonate (cf. Fig. 12). Under these conditions the rate of chloride influx was one third of the rate of bicarbonate influx. If it is assumed that the anion exchange mechanism follows Michaelis–Menten kinetics at low anion concentrations and has the same theoretical maximum flux (J_{max}^{theor}) for chloride and bicarbonate, relative transport rates at infinitely low 'substrate' concentrations approach the relative affinity of the two ions for the transport system because the apparent permeability (J/C) at low anion concentrations will approach the value J_{max}^{theor}/K_A. Relative affinities of chloride and bicarbonate, having the same, or nearly the same J_{max}^{theor}, can be measured, therefore, by comparing the rates of tracer influx in experiments of the type shown in Fig. 12, where extracellular anion concentration is small compared to the dissociation constants for anion binding to the transport site.

It has been reported repeatedly that the apparent activation energy of chloride–bicarbonate exchange, as measured by the rate of hydrogen ion transfer by the Jacobs–Stewart cycle (Jacobs & Stewart, 1942), is about 15–20 kcal/mol between 0 and 10 °C (Chow *et al.* 1976; Crandall *et al.* 1978; Lambert & Lowe, 1978), considerably lower than values of 28–37 kcal/mol found for the self-exchange of a considerable number of inorganic anions, which appear to be transported by the anion exchange mechanism (Dalmark & Wieth, 1972). The present study indicates that the unhibited bicarbonate self-exchange also has an apparent activation energy of about 30 kcal/mol (Fig. 7), comparable to that of other inorganic anions. Other apparent discrepancies are the findings by Lambert & Lowe (1978) of a half-saturated chloride–bicarbonate exchange at an extracellular bicarbonate concentration of 0·4 mM and of a significantly lower transport capacity than in the present study (Figs. 7 and 9). As noted in the Results, their experimental design resembled the one of Fig. 12, where bicarbonate concentration is kept low in the extracellular phase by substitution with a non-permeating anion. Work in progress has demonstrated that the apparent affinity for bicarbonate increases when only the extracellular bicarbonate concentration is varied (J. Brahm & J. O. Wieth, unpublished). Thus, our preliminary data show that the method presented in this paper will make it possible to sort out apparent differences between the kinetic properties of anion self-exchange and of chloride–bicarbonate hetero-exchange. At present it suffices to conclude that the fundamental characteristics of bicarbonate exchange between 0 and 10 °C do not differ from those of chloride transport.

Relative rates of exchange of bicarbonate with other inorganic anions

The ability of other inorganic anions to exchange with bicarbonate was examined by injecting 165 mM bicarbonate ghosts into media containing 165 mM of the potassium salts of chloride, fluoride, nitrate, bromide or iodide media. The rate of [^{14}C]-bicarbonate efflux was found to decrease through the above mentioned sequence (Fig. 11). It is worth noting that the exchange of bicarbonate with chloride took place at a rate which was insignificantly lower than the rate of bicarbonate self-exchange. The other inorganic anions caused a significant reduction of bicarbonate efflux.

Relative rates of self-exchange of chloride, bromide and iodide have previously been examined by Dalmark & Wieth (1972, Table 5). The rate of bromide self-exchange was 12 % and of iodide self-exchange 0·4 % of the rate of chloride self-exchange. In unpublished experiments the rate of fluoride self-exchange was 15 % of the rate of chloride exchange. It appears from a comparison of the relative rates of hetero-exchange shown in Fig. 11 that the ability of the anions to exchange with bicarbonate follows the sequence: Cl > F > Br ≫ I, similar to the sequence of magnitudes of halide self-exchange. The results of Fig. 11 showed that [^{14}C]bicarbonate exchanged at the same rates with chloride and with bicarbonate. In other experiments with chloride-loaded ghosts (not shown) it was found that $^{36}Cl^-$ also exchanged at identical initial rates with bicarbonate and chloride. The affinities of chloride and bicarbonate for the transport system differ appreciably, but the findings demonstrate that chloride and bicarbonate ions are transferred through the membrane with the same speed when the transport system is saturated at anion concentrations of 165 mM. In contrast, the rates of influx of fluoride, bromide, and iodide are all approximately doubled when the rates of hetero exchange are compared to rates of self-exchange. It has recently been reported that bromide exchanges considerably faster with intracellular chloride than with bromide (Gunn & Fröhlich, 1978), showing that membrane transfer of slowly transported anions can be 'trans-stimulated', when a more rapidly transported anion is available for the exchange process.

There is evidence that the red-cell membrane contains a transport pathway to organic oxyanions like lactate and pyruvate (Deuticke, 1977). This path is characterized by being relatively insensitive to inhibition with the classical disulphonic stilbene derivatives which block inorganic anion transport almost completely (i.e. by more than 99 %). It is worth considering whether this transport system transports bicarbonate ions, which may be said to take up an intermediate position between the organic oxyanions and the small inorganic anions. However, the results of Fig. 1 clearly show that bicarbonate transport was as sensitive to DIDS-binding to the membrane as was chloride transport. More evidence, indicating that the transport mechanism to inorganic anions is the quantitatively important pathway to bicarbonate transport, was found in unpublished experiments showing that the rate of bicarbonate–pyruvate hetero-exchange at an anion concentration of 165 mM was about 10 pmol cm^{-2} sec^{-1} (2 % of the bicarbonate self-exchange flux). This hetero-exchange flux was inhibited by a factor of four by DIDS-treatment of the membranes, suggesting that the major part of the small exchange flux of pyruvate occurred through the DIDS-sensitive inorganic anion transport mechanism, when pyruvate was forced to exchange with bicarbonate. Therefore, it appears that the inorganic anion exchange mechanism is the predominant pathway for the transfer of bicarbonate ions through the red-cell membrane.

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