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BICARBONATE EXCHANGE KINETICS AT EQUILIBRIUM ACROSS THE ERYTHROCYTE MEMBRANE BY ^{13}C NMR

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The rate of exchange of ^{13}C-labelled bicarbonate across the membranes of human erythrocytes in suspension, at thermal and chemical equilibrium, was measured using ^{13}C NMR spectroscopy: the permeability coefficient (3.34×10^{-4} cm s^{-1}) agrees well with previous values obtained with other methods. Data analysis was complicated by the need to consider the Donnan ratio of the charged species inside and outside the cells. This work appears to be the first, involving the present NMR procedure, for studying fast membrane transport of a molecule other than water.

The kinetics of bicarbonate-CO_2 exchange across the erythrocyte membrane has been the subject of many studies due to its physiological importance in the transport of carbon dioxide out of body tissues. As examples, Chow *et al.* (1) determined the kinetics of bicarbonate-chloride exchange in the erythrocyte from the time course of extracellular pH in a stopped-flow apparatus during transfer of H^+ into the cell via the CO_2 hydration-dehydration cycle. Itada and Forster (2) followed the exchange of ^{18}O in $C^{18}O^{16}O$ with ^{16}O in water, by mass spectroscopy, as a function of intracellular carbonic anhydrase activity and permeability of the cell membrane to bicarbonate. The methods are experimentally complex and give an indirect measure of bicarbonate permeability after considerable analysis and simplifying assumptions.

A relatively simple Nuclear Magnetic Resonance (NMR) method has been successfully used by a number of authors (3,4,5) to study the diffusional permeability of erythrocytes to water. Addition of a paramagnetic species, such as manganous ions, to the extracellular compartment results in a dramatic fall in the transverse relaxation time of extracellular water compared with intracellular water. Exchange of water between compartments results in a moderation of these relaxation times. A line fitting analysis (6,7) of the transverse magnetisation decay, after labelling the water

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spins with a radio-frequency pulse, yields the rate constants for diffusion of water across the membrane.

For this communication we used this NMR method to measure the permeability of the erythrocyte membrane to bicarbonate ions.

MATERIALS AND METHODS

N-2-Hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES), 5,5-dimethyl-2,4-oxazolidinedione (DMO) and 4,4'-Diisothiocyano-2,2'-disulfonic acid stilbene (DIDS) were obtained from Sigma Chemical Co., St. Louis, MO, USA. [^{14}C]DMO was obtained from Amersham International, Amersham, Buckinghamshire, UK. Methazolamide was obtained from American Cyanamid Co., Pearl River, NY, USA and Na[^{13}C]HCO_3 from Stohler Isotope Chemicals, Waltham, MA, USA. All other reagents were of Analytical grade.

Freshly drawn venous blood, from the one donor (PWK), was washed twice in cold isotonic saline and twice in the following buffer: HEPES 15 mM, NaCl 90 mM, Na[^{13}C]HCO_3 50 mM and 1% bovine serum albumin. The buffer was made isotonic (290 mOm) by addition of a small amount of NaCl, the pH adjusted to 7.4 and 5 mM glucose was added. The carbonic anhydrase inhibitor, methazolamide (2 mg/ml) was added to the final wash. Samples used for NMR studies (2 ml) were; (1) supernatant from the final wash, (2) packed cells and (3) a cell suspension of haematocrit 0.70. Where appropriate, $MnCl_2$ to a final extracellular fluid (ECF) concentration of 25 μM was added. For certain experiments where the band 3 anion transporter was inhibited, DIDS (0.5 mg/ml cells) was also added. Samples were placed in 10 mm o.d., NMR tubes and sealed with a 10 mm small volume sample bulb (Wilmad Catalog No. 529E-10) which served as a vortex plug. Then a 2 cm layer of paraffin oil was placed on top of the bulb to provide an air-tight seal.

^{13}C NMR spectra were measured at 37°C, and at 100.62 MHz in the Fourier mode using a Bruker WM-400 spectrometer. The Carr-Purcell-Meiboom-Gill (CPMG) pulse sequence (8) $\pi/2\text{-}(\tau\text{-}\pi\text{-}\tau)_n$ with $\tau = 300$ μs was used to measure the transverse magnetization of the sample as a function of total-pulse-sequence time, $t = 2n\tau$. At the midpoint and, end of each experiment a spectrum was obtained using the initial (shortest) value of t. This was used as a check for loss of bicarbonate as CO_2. Typically 90-95% of the original bicarbonate was present at the end of the experiment. The intensity of the peaks was adjusted to account for this small loss, prior to statistical analysis. A total of 8 or 16 transients were averaged using a spectral width of 4000 Hz and 16384 data points. Spectra were acquired with the spectrometer unlocked, the magnetic field homogeneity having been previously adjusted using a D_2O sample of the same volume. The chemical shifts are quoted relative to external tetramethylsilane at 0.000 ppm.

The transmembrane pH difference and thus the membrane potential was determined by the distribution of radioactive DMO across the erythrocyte using a standard method (9,10).

RESULTS AND DISCUSSION

The CO_2-HCO_3^- exchange reaction catalysed by carbonic anhydrase results in a dramatic broadening and a consequent low signal-to-noise ratio of the

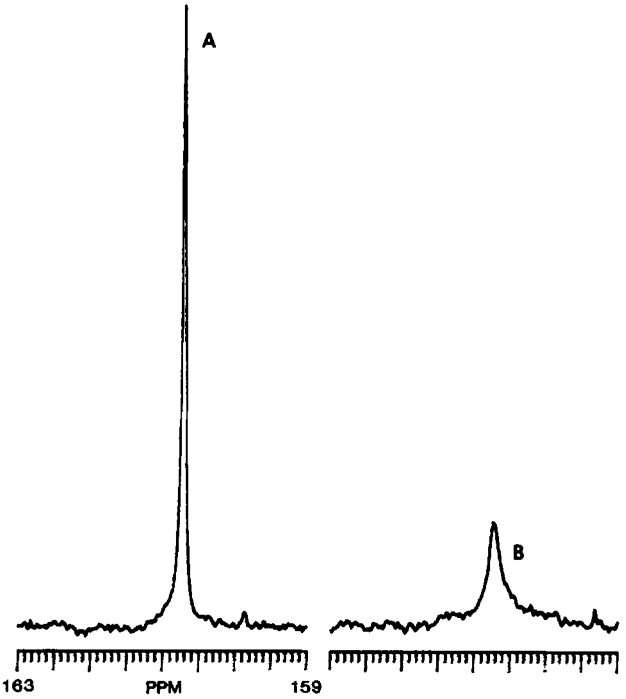

Fig. 1. ^{13}C NMR spectra of 50 mM bicarbonate in packed erythrocytes with (A) and without (B) methazolamide.

^{13}C NMR bicarbonate peak. This exchange is effectively inhibited by methazolamide (11). Fig. 1 shows spectra of bicarbonate in packed cells with and without methazolamide: all samples used for transverse relaxation time (T_2) measurements contained this compound.

The decay of the bicarbonate transverse magnetization in the various samples was measured using the CPMG sequence as described in Materials and Methods. The magnetization in supernatant and packed cells decayed as a single exponential characterized by T_2's of 27 and 580 ms, respectively. Fig. 2 shows spectra of the bicarbonate peak as a function of t (the CPMG sequence time) for cell suspensions of hematocrit 0.70 with and without the band 3 anion transport inhibitor DIDS. In the presence of DIDS the signal has an initial rapid fall (spectrum at 9.6 ms), due to decay of extracellular magnetization relaxed by the manganous ions, followed by a slower rate of decay of the trapped, intracellular, bicarbonate. In the absence of DIDS the decay rate is intermediate between the two rates discussed above due to the exchange of bicarbonate between the intra- and extracellular compartments. The magnetization decay in the absence of DIDS is graphed in Fig. 3.

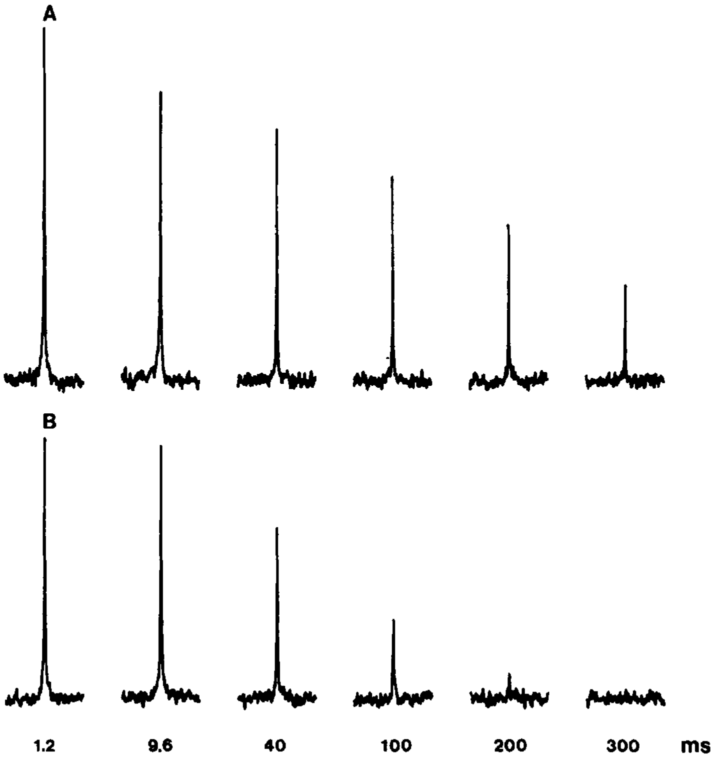

Fig. 2. ^{13}C NMR spectra of 50 mM bicarbonate in Hc-0.7 cell suspensions as a function of the CPMG pulse sequence time. (A) with DIDS, (B) without DIDS. 25 μM of Mn^{2+} was present in the extracellular compartment of both samples.

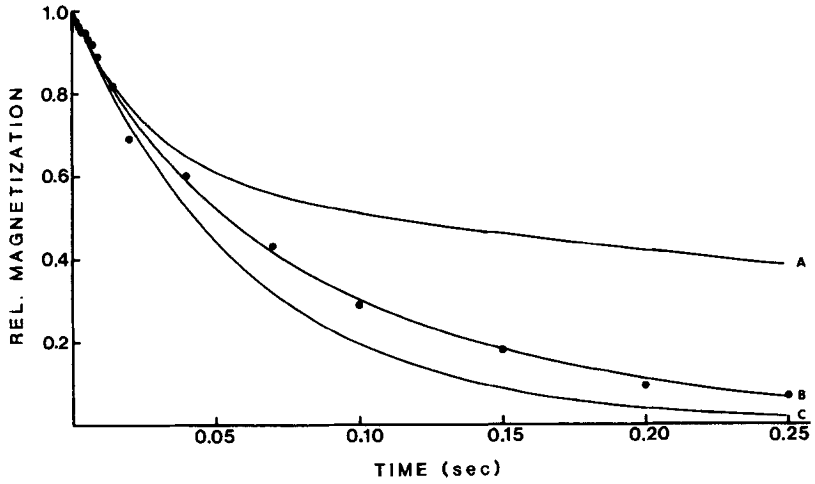

Fig. 3. Decay with time of bicarbonate magnetization in the absence of DIDS. Data from Fig. 2B. Solid lines calculated for three values of k_2 (A) 0.0 s^{-1} (B) 7.58 s^{-1} and (C) 20.0 s^{-1}

The following equation, derived from the 2-site spin-exchange differential equations (4), was used to fit the magnetization decay in Fig. 3.

$$M\ (t) = (\phi_1 \exp\ (\lambda_1 t) + \phi_2 \exp\ (\lambda_2 t))/(M_o + M_i)$$

where

$$\lambda_1 = -\ [A + (A^2 - 4B)^{1/2}]/2$$
$$\lambda_2 = -\ [A - (A^2 - 4B)^{1/2}]/2$$
$$A = 1/T_2{}^o + 1/T_2{}^i + k_1 + k_2$$
$$B = 1/(T_2{}^o T_2{}^i) + k_1/T_2{}^o + k_2/T_2{}^i$$

$$\phi_1 = [C + D - \lambda_1 (M_o + M_i)]/(\lambda_2 - \lambda_1)$$
$$\phi_2 = -[C + D - \lambda_2 (M_o + M_i)]/(\lambda_2 - \lambda_1)$$
$$C = M_o/T_2{}^i + (M_o + M_i) k_2$$
$$D = M_i/T_2 + (M_o + M_i) k_1$$
$$M_o = M_i\ k_2/k_1$$

$T_2{}^o$ and $T_2{}^i$ are the transverse relaxation rates of bicarbonate outside and inside the cells respectively; k_1 and k_2 are the out-to-in and in-to-out transport rate constants. M_o and M_i are the initial magnetizations, directly proportional to the amounts S_o and S_i, of bicarbonate outside and inside the cells. M(t) is the reduced observable magnetization. M_i was arbitrarily set to 100 as the fitting treated the magnetization in a scaled form.

Attempts to fit the data by floating both rate constants, k_1 and k_2 were unsuccessful, yielding degenerate solutions depending on the initial trial values of k_1 and k_2. This arose because the fitting method determined the amounts of intra- and extracellular bicarbonate from the rate-constant ratio. By effectively varying both magnetizations and rate constants a number of solutions is possible.

For a neutral species the intra- and extracellular concentrations may be determined from the volume ratio of both compartments, as in the water-exchange-rate measurements (3,4,5). However, bicarbonate being a charged species distributes between the intra- and extracellular compartments according to the Donnan equilibrium. If DIDS completely inhibited the exchange reaction then the amounts in each compartment could be determined by an exponential peel of the DIDS-inhibited-cell data which would be the sum of two simple exponentials. As this was not the case (see below) the membrane potential of the cells in this buffer system was determined by the distribution of radioactive DMO across the membrane. Values of -10.3 and -10.7 mV were obtained in two separate experiments.

From the membrane potential the distribution of intra- and extracellular bicarbonate may be determined using the relationship

$$\exp(\psi zF/RT)=[S_o]/[S_i]=S_oV_i/S_iV_o$$

where ψ is the membrane potential, z the bicarbonate charge, F the Faraday constant, R the gas constant, T the temperature and V_i and V_o the intra and extra-cellular volumes. V_i was set to 0.49 (0.7 times the haematocrit (7)) and V_o to 0.3. The first order rate constant, k_1, for entry of bicarbonate into the cell is also fixed with respect to k_2, the exit rate constant, by $k_1=S_ik_2/S_o$. Thus the analysis of the data is reduced to a single parameter fit of k_2. Fig. 3 shows theoretical lines for three values of k_2.

The best fit of the data was obtained with $k_2=7.58\pm.02\ s^{-1}$. This gives a permeability, $P = k_2\ V/A$, of the erythrocyte membrane to bicarbonate of 3.34×10^{-4} cm s^{-1} if V is the intracellular solvent volume 6.3×10^{-11} cm^3, and A the area of an erythrocyte, 1.43×10^{-6} cm^2.

The transverse magnetization decay curve for DIDS-inhibited cells showed some residual bicarbonate transport. Analysis of the data using the above method gave a best fit with $k_2=0.48\pm0.02$ s^{-1}, equivalent to a bicarbonate permeability of 0.21×10^{-4} cm s^{-1}. This exchange could be due to incomplete inhibition of band 3 anion transporter; however, in view of the DIDS concentration used this is unlikely (12). Although the carbonic anhydrase catalysed CO_2-HCO_3^- exchange reaction is inhibited by methazolamide the chemical exchange-reaction still takes place at a lower rate in both the intra- and extracellular compartments. As the diffusion of CO_2 across the erythrocyte membrane is extremely rapid (13) this would provide a mechanism for an apparent transport of bicarbonate ions across the erythrocyte membrane.

The value of the human erythrocyte bicarbonate permeability coefficient in this study (3.34×10^{-4} cm s^{-1}) compares favorably with literature values: Itada and Forster (2) obtained values between 2.21 - 4.13 x 10^{-4} cm s^{-1} for five different subjects. While our present NMR work involved the use of additional, non-NMR, experiments to determine the Donnan ratio, and thus overcome a redundancy problem in data-fitting, it is possible to determine the Donnan ratio from the ΔpH across the red cell membrane; this can be measured using NMR but as yet reliable pH-probe molecules and analysis exist only for ^{31}P (e.g., 12).

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