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Urea exchange across the human erythrocyte membrane measured using ^{13}C NMR lineshape analysis

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Abstract. The ^{13}C NMR spectrum of ^{13}C-urea in a suspension of human red cells of reduced mean cell volume was observed to contain partially resolved resonances arising from the intra- and extracellular populations of the compound. It was shown that at 25 °C and a magnetic field strength of 9.4 T, the rate of exchange of urea between the intra- and extracellular populations was such that the NMR lineshape was sensitive to a change in the rate of ^{13}C-urea exchange, induced either by the addition of the urea transport inhibitor phloretin, or by the addition of ^{12}C-urea. Total lineshape analysis of ^{13}C NMR spectra of ^{13}C-urea in red cell suspensions containing different concentrations of ^{12}C-urea resulted in a weighted mean estimate for the K_m and V_{max} for urea equilibrium exchange from three experiments of 44 ± 18 mM and $3.1 \pm 0.6 \times 10^{-8}$ mol cm^{-2} s^{-1}, respectively (the errors denote the weighted mean standard deviations). These estimates of K_m and V_{max} were significantly lower than previous values reported in the literature and determined using other techniques.

Key words: Erythrocyte urea exchange – NMR lineshape analysis

Introduction

The human erythrocyte membrane possesses a very high permeability to urea (Brahm 1983; Mayrand and Levitt 1983) which, it has been suggested, is required for the erythrocytes to maintain osmotic stability in their passage through the renal medulla where they are exposed to high urea concentrations (Macey 1984; Macey and Yousef 1988). Urea transport across the human erythrocyte membrane exhibits several of the characteristics that are associated with facilitated exchange. The transport has been shown to be saturable (Hunter 1970; Brahm 1983; Mayrand and Levitt 1983), inhibited by phloretin (Macey and Farmer 1970; Brahm 1983) and by the sulphydryl reagent pCMBS (Macey and Farmer 1970; Naccache and Sha'afi 1974; Brahm 1983; Toon and Solomon 1986). Urea exchange has also been shown to be competitively inhibited by a number of analogues of urea including thiourea and dimethylurea (Mayrand and Levitt 1983). Despite this evidence that urea is transported across the membrane *via* a specific protein-mediated pathway, the identity of the urea transport protein is as yet unknown.

It has been suggested that the human erythrocyte anion transport protein, Band 3, may be involved in the exchange of urea across the red cell membrane (Toon and Solomon 1985; Ojcius and Solomon 1988). However, the observation that red cells with the phenotype Jk(a-b-) appear to lack a facilitated urea transport mechanism and yet which appear to exhibit normal anion exchange (Fröhlich et al. 1991) would indicate that urea transport is separate from the transport of anions.

The studies that have been carried out to determine the kinetic parameters of urea equilibrium exchange across the human erythrocyte membrane have indicated that the exchange obeys Michaelis-Menten kinetics (Brahm 1983) but they have also resulted in a wide range of estimates of the Michaelis constant (K_m) and the maximal velocity (V^S_{max}). This is probably due, at least in part, to the difficulties associated with measuring such rapid exchange. However, the unequivocal determination of the kinetic parameters of urea exchange across the red cell membrane should result in a better understanding of the mechanism of exchange and how it is related to its important proposed physiological function. In the present work we show that ^{13}C NMR lineshape analysis can be used to obtain estimates of the K_m and V^S_{max} for urea exchange across the human erythrocyte membrane, under equilibrium exchange conditions and at 25 °C. This study resulted in an estimate of the permeability coefficient (extrapolated to zero substrate concentration) that was reasonably consistent with those reported in the literature. However, the values of both V^S_{max} and K_m were significantly lower than the values measured previously using other techniques.

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Experimental

^{13}C-urea (90% isotopic purity) was from CEA Labelled Compounds, Cedex, France, and phloretin was from Sigma, St. Louis, MO, USA. All other reagents were of A.R. grade. $^{2}H_2O$ (99.75 atom%) was from the Australian Institute of Nuclear Science and Engineering, Lucas Heights, NSW, Australia.

Unless otherwise stated, cell suspensions were prepared as described below. Human erythrocytes were obtained fresh by venipuncture from the same healthy donor (JRP) in our laboratory. Cells were either prepared for the NMR experiment immediately or washed twice in isotonic saline and stored overnight at 4 °C in 5 mM glucose. Prior to the NMR bandshape experiments, cells were prepared in the following manner: cells were centrifugally washed (4×) in three volumes of ice-cold isotonic saline; cells were then resuspended in phosphate-buffered saline (PBS; pH 7.40, 10 mM glucose, 20% $^{2}H_2O$ (v/v)) prior to being bubbled with carbon-monoxide (CO) for 5 min at 4 °C. Cells were then washed in PBS. For the transport experiments, cell suspensions of haematocrit (Ht) ~0.40 with the appropriate concentrations of ^{12}C-urea and ^{13}C-urea were prepared by combining the packed cells with solutions containing saline (50% $^{2}H_2O$ (v/v)), urea/saline (50% $^{2}H_2O$ (v/v); pH 7.2±0.1) and ^{13}C-urea (1.5 M). The mean cell volume of the suspensions was reduced to ~60–70 fl by the addition of aliquots of a NaCl (4 M) solution to the cell suspensions. The samples were incubated for 15 min at 37 °C to allow for full equilibration of the urea across the membrane and they were then stored at 4 °C. Prior to the NMR experiment the samples were incubated at 25 °C for 10 min. Following the NMR experiment the Ht of each cell suspension was determined in quadruplicate and the number of cells per ml of suspension was measured using a Coulter particle counter (model ZF), Coulter Electronics, Dunstable, UK.

^{13}C NMR spectra were principally acquired at 100.62 MHz in the Fourier transform mode on a Varian 400 MHz VXR/XL NMR spectrometer. Later experiments employed a Bruker AMX400 wb NMR spectrometer. $^{2}H_2O$ was routinely incorporated into the samples to facilitate field/frequency locking. The temperature in the samples was measured using an ethylene glycol capillary (Bubb et al. 1988). Typical spectral acquisition parameters were as follows: fully relaxed spectra were acquired by allowing a 200 s (>5 T_1 values) delay between transients, the acquisition time was 2 s, the spectral width was 2 000 Hz and the number of data points collected was 8 000. In the transport experiments the free-induction decay was Fourier transformed using an exponential apodisation function that resulted in 1 Hz of line-broadening in the resultant spectrum.

After phase-correction, the required region of each digitised NMR spectrum was transferred to an HP 220 computer using a portable 'spooler'; a frequency axis was then assigned to the digitised intensities. The assigned frequency axis had the opposite direction to the axis in the original NMR spectrum, but this was easily accounted for in the analysis. The final form of the spectra for fitting of the bandshape equations was weighted in favour of the region of the spectrum containing the two resonances by using a program that selected only every sixth point in the regions of the spectrum with low signal intensity to high and low frequency of the ^{13}C-urea resonances, and selected every point in the region of the two resonances. The selection of these two regions was carried out by eye. The spectra were then transferred to a Silicon Graphics IRIS 4D/20 workstation for nonlinear least-squares fitting (Osborne 1976) of the bandshape equation to the spectra and for the determination of the K_m and V^S_{max} for urea exchange. For clarity, the procedure described above has been summarised in a flow chart (Fig. 1).

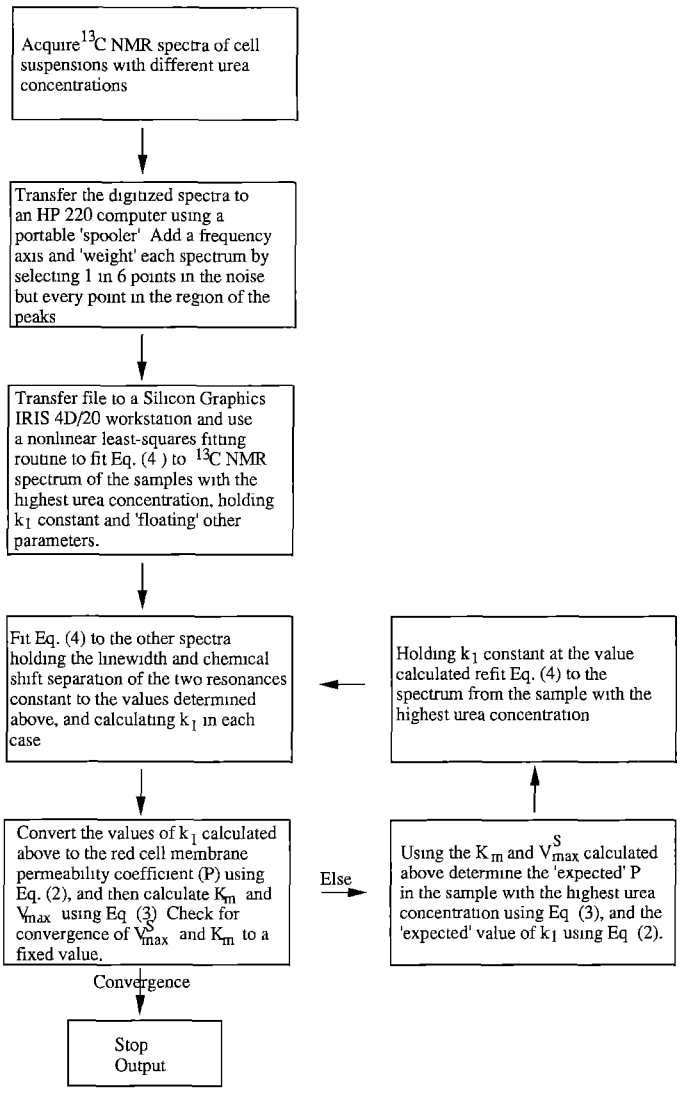

Fig. 1. Flow chart illustrating the computer-based procedure for the determination of the K_m and V^S_{max} of urea exchange in red cells, from ^{13}C NMR spectra

Theory

When two populations of nuclear spins of the same nuclide exist in sufficiently different chemical environments

they give rise to resonances in the NMR spectrum with distinct chemical shifts. If these two populations are in chemical exchange that is slow on the NMR timescale, then two resonances are visible in the NMR spectrum, and the shape of each line is not significantly altered as a result of the exchange. Alternatively, if exchange between the two populations of spins is rapid on the NMR timescale, then the spectrometer can only distinguish an average chemical environment for the nuclei and only a single resonance will be observed in the NMR spectrum (Akitt 1983). In the 'intermediate' region of exchange (i.e. between these two extremes), an increase in the rate of exchange between the populations results in a broadening of the two NMR lines; this is due to the presence of spins in each population, of spins with the characteristics of that population, plus those spins that have just entered the population and have retained the characteristics of their original population. There also exist those spins with characteristics intermediate between these two states (Dwek 1973). For a given rate of exchange, whether two populations of spins are in slow, intermediate or fast exchange on the NMR timescale, will depend on the chemical shift separation of the resonances in the absence of exchange, and thus also on the magnetic field strength at which the spectrum is acquired.

As mentioned above, when two populations are in intermediate exchange on the NMR timescale, a change in the rate of exchange results in an alteration in the lineshape of the resonances. Analysis of NMR lineshapes to measure the rates of exchange between two populations of spins is known as dynamic NMR (DNMR) spectroscopy. The advent of high speed computers has resulted in total lineshape analysis becoming more practicable. However, estimates of the rates of exchange between two populations of nuclei have also been obtained using approximation methods in which, for example, changes in the linewidth or chemical shift separation of the resonances were used (Sandström, 1982; Oki 1985). In the past, DNMR was primarily used to determine the activation energies of various chemical exchange processes. More recently, Alger and Prestegard (1979) studied the temperature dependence of the permeation of acetic acid through large unilamellar vesicles, by observing the change in the proton NMR spectrum of acetic acid in a suspension of vesicles where an impermeable paramagnetic shift reagent had been added to the external compartment. Hoffman and Henkens (1987) used ^{13}C NMR lineshape analysis to measure CO_2 transport across red cell membranes, however this experiment relied on the exchange between CO_2 and a second ($H^{13}CO_3^-$) species rather than simply exchange between the intra- and extracellular populations of CO_2.

In the present work, we observed that under certain experimental conditions (i.e. at 25 °C, at a magnetic field strength of 9.4 T and a mean cell volume of 60–70 fl) partially-resolved ^{13}C NMR resonances were observed for the intra- and extracellular populations of ^{13}C-urea in a red cell suspension. Under the same conditions, urea exchange across the human erythrocyte membrane was 'intermediate' in the NMR timescale with the lineshape of the ^{13}C NMR spectrum of urea being sensitive to the rate of exchange between the intra- and extracellular populations. Also, in the present work we observed that the lineshape of the ^{13}C NMR spectrum of ^{13}C-urea in a red cell suspension was dependent on the total concentration of urea that was added to the cells. Therefore, we used total lineshape analysis to determine the first-order influx rate constant for ^{13}C-urea in suspensions to which varying concentrations of ^{12}C-urea had been added and thus calculated a K_m and V^S_{max} for urea exchange across the red cell membrane under equilibrium exchange conditions.

We treated the ^{13}C-urea/^{12}C-urea system as being one where the exchange of ^{13}C-urea (S) was measured in the presence of increasing concentrations of a competitive inhibitor of the exchange reaction i.e. ^{12}C-urea (I in (1)). Brahm (1983) showed that human erythrocyte urea transport exhibits saturation kinetics of the Michaelis-Menten type; thus, the equation describing the first-order flux (v) of ^{13}C-urea across the membrane in the presence of ^{12}C-urea is given by,

$$v = k_1 [S_0] = \frac{V^S_{max} [S_0]}{K_m + \frac{K_m}{K_I}[I_0] + [S_0]}, \quad (1)$$

where K_m and K_I are the Michaelis constants for the exchange of ^{13}C-urea and ^{12}C-urea, respectively (we assume that there is no isotope effect on the binding and kinetics and therefore $K_m = K_I$), across the human erythrocyte membrane. V^S_{max} is the maximal velocity for the exchange of ^{13}C-urea across the red cell membrane and k_1 is the apparent first-order influx rate constant for the exchange of ^{13}C-urea across the red cell membrane. $[S_0]$ and $[I_0]$ are the extracellular concentrations of ^{13}C- and ^{12}C-urea, respectively. As the first-order influx rate constant is dependent on the Ht of the cell suspension (i.e. on the volume of the extracellular compartment), in order to avoid the need to correct for small differences in Ht between NMR samples, we calculated an Ht-independent permeability coefficient using:

$$P = k_1 \frac{V^{out}}{A^{total}}, \quad (2)$$

where A^{total} is the area of the red cell membrane per ml of cell suspension; it was calculated from the number of cells per ml of suspension and the known area of the human erythrocyte membrane ($1.43 \pm 0.08 \times 10^{-6}$ cm^2; e.g. Kirk and Kuchel 1986). V^{out} is the extracellular volume per ml of cell suspension and was calculated from the Ht of the cel suspension ($V^{out} = 1 - Ht$). These studies were carried out at equilibrium and as urea is distributed passively across the membrane, $[S_0] = [S_i] = [S]$ and $[I_0] = [I_i] = [I]$, where $[S_i]$ and $[I_i]$ are the intracellular concentrations of ^{13}C- and ^{12}C-urea, respectively. Also, if V^S_{max} is calculated with the units 'mol cm^{-2} s^{-1}' then (1) becomes;

$$P_S = \frac{V^S_{max}}{K_m + [X]}, \quad (3)$$

where $[X]$ is the total concentration of urea in the cell suspension. In calculating the total urea concentrations in this work we assumed first, that all of the intracellular

water, which has been calculated to have a volume that is 0.717 of the total cell volume (Savitz et al. 1964; Kirk et al. 1988), acts as a solvent for urea, and secondly that the absolute volume of each cell that is inaccessible to urea does not change significantly as the cell volume is reduced from its normal volume of 85 fl (Dacie and Lewis 1975) to 60–70 fl (Savitz et al. 1964).

The NMR bandshape (v) of two exchange-broadened Lorentzian resonances is given by the following equation (Rogers and Woodbrey 1962; Sandström 1982):

$$v = -C_0 \frac{[S(1+V)+QR]}{S^2+R^2} + b_c \qquad (4)$$

and

$$\Delta\nu = \nu_A - \nu_B$$

$$B = 0.5(2\nu_A - \Delta\nu) - \nu$$

$$A = \pi^2 (\Delta\nu^A{}_{1/2})(\Delta\nu^B_{1/2}) - 4\pi^2 B^2$$

$$S = \frac{p_B}{k_1}[A + \pi^2(\Delta\nu)^2] + \pi[(1-p_B)(\Delta\nu^A_{1/2}) + p_B(\Delta\nu^B_{1/2})]$$

$$Q = \frac{\pi p_B}{k_1}[2B - \Delta\nu(1-2p_B)]$$

$$T = \left(1 + \frac{p_B}{k_1}\pi(\Delta\nu^A_{1/2} + \Delta\nu^B_{1/2})\right)$$

$$R = 2\pi BT + \pi^2 \frac{p_B}{k_1}\Delta\nu(\Delta\nu^B_{1/2} - \Delta\nu^A_{1/2}) + \pi\Delta\nu(1-2p_B)$$

$$V = \pi\frac{p_B}{k_1}[p_B(\Delta\nu^B_{1/2}) + (1-p_B)(\Delta\nu^A_{1/2})]$$

$$\pi\Delta\nu^A_{1/2} = \frac{1}{T_{2,A}} \quad \text{and} \quad \pi\Delta\nu^B_{1/2} = \frac{1}{T_{2,B}}.$$

The above equations are essentially identical to those of Sandström (1982) except for the substitution of the final two expressions above for the $1/T_2$ terms. In the above expressions, ν_A and ν_B are the resonance frequencies of the extra- and intracellular populations of ^{13}C-urea, respectively, in the absence of exchange and ν is the spectral 'offset' frequency. p_A and p_B are equivalent to the relative accessible volumes of the extra- and intracellular compartments, respectively, and in the present work they have been normalised such that $p_A + p_B = 1$. $\Delta\nu^A_{1/2}$ and $\Delta\nu^B_{1/2}$ are the linewidths-at-half-height of the extra- and intracellular resonances, respectively, in the absence of exchange. As has been shown, they are related to the spin-spin relaxation rate constants $1/T_{2,A}$ and $1/T_{2,B}$, respectively. The first-order influx rate constant for urea exchange is k_1. The parameter b_c was included to account for any baseline variation in the spectra, but in most cases, adequate fits were obtained when this parameter was held constant at zero. C_0 is proportional to the concentration of ^{13}C-urea.

Results

Observation of separate resonances for the intra- and extracellular populations of ^{13}C-urea in a suspension of human erythrocytes

Figure 2A shows the ^{13}C NMR spectrum of ^{13}C-urea (~13 mM with respect to the total sample volume) in a red cell suspension of Ht ~ 0.5; there are clearly two partially resolved resonances instead of the single resonance that might have been expected. Figure 2B shows a ^{13}C NMR spectrum of the same sample but after lysing the red cells by freeze/thawing the sample (3 ×) in liquid N_2. The two resonances in Fig. 2A collapsed to a single resonance, suggesting that they arose from the intra- and extracellular populations of urea. Figure 2D shows the effect of the addition of Mn^{2+} (0.4 mM, calculated with respect to the total extracellular volume) to the ^{13}C NMR spectrum of ^{13}C-urea in a red cell suspension of Ht ~ 0.33 (Fig. 2C). The buffer contained 2% (w/v) bovine serum albumin; the protein will bind to the Mn^{2+} and thus assist in trapping it in the extracellular medium (Pirkle et al. 1979; Kirk and Kuchel 1985). The presence of paramagnetic Mn^{2+} results in broadening of the resonances of the extracellular species, thus we assigned the higher frequency resonance to the extracellular population of ^{13}C-urea. This assignment was confirmed by comparing Fig. 2A and C. In the spectrum of the higher Ht cell suspension (Fig. 2A), as expected, the proposed intracellular resonance is of greater intensity with respect to the proposed extracellular resonance than in the lower Ht cell suspension (Fig. 2C). The mean cell volume was reduced to ~62 fl and 66 fl in the experiments shown in Fig. 2A and C, respectively. The reduction in cell volume resulted in an increase in the chemical shift separation of the intra- and extracellular resonances (results not shown) thus improving the resolution between these resonances.

Figures 3 and 4 show the effect of a change in the rate of exchange of ^{13}C-urea across the red cell membrane on the appearance of the NMR spectrum of ^{13}C-urea in a red cell suspension. In Fig. 3 the NMR spectrum of ^{13}C-urea (~25 mM) in a red cell suspension of Ht ~ 0.45 (Fig. 3A) was compared with the spectrum of the sample but after addition of phloretin (1 mM with respect to the total sample volume; Fig. 3B). Phloretin has been shown previously to be an effective inhibitor of urea exchange (Macey and Farmer 1970; Brahm 1983). In Fig. 4 the effect of the addition of ^{12}C-urea to the cell suspension on the appearance of the spectrum of ^{13}C-urea is shown. ^{12}C-urea is not NMR-visible (apart from the 1.1% of natural abundance ^{13}C-urea contained in the sample), however as urea transport has been shown to be saturable, the rate of ^{13}C-urea exchange across the membrane will be reduced as the ^{12}C-urea concentration is increased. In both Figs. 3 and 4 a reduction in the rate of ^{13}C-urea exchange across the red cell membrane resulted in a reduction in the linewidths of the two resonances and thus an increase in resolution between the intra- and extracellular resonances. Thus, under these conditions, exchange of urea across the red cell membrane was clearly intermediate on the NMR timescale (according to the

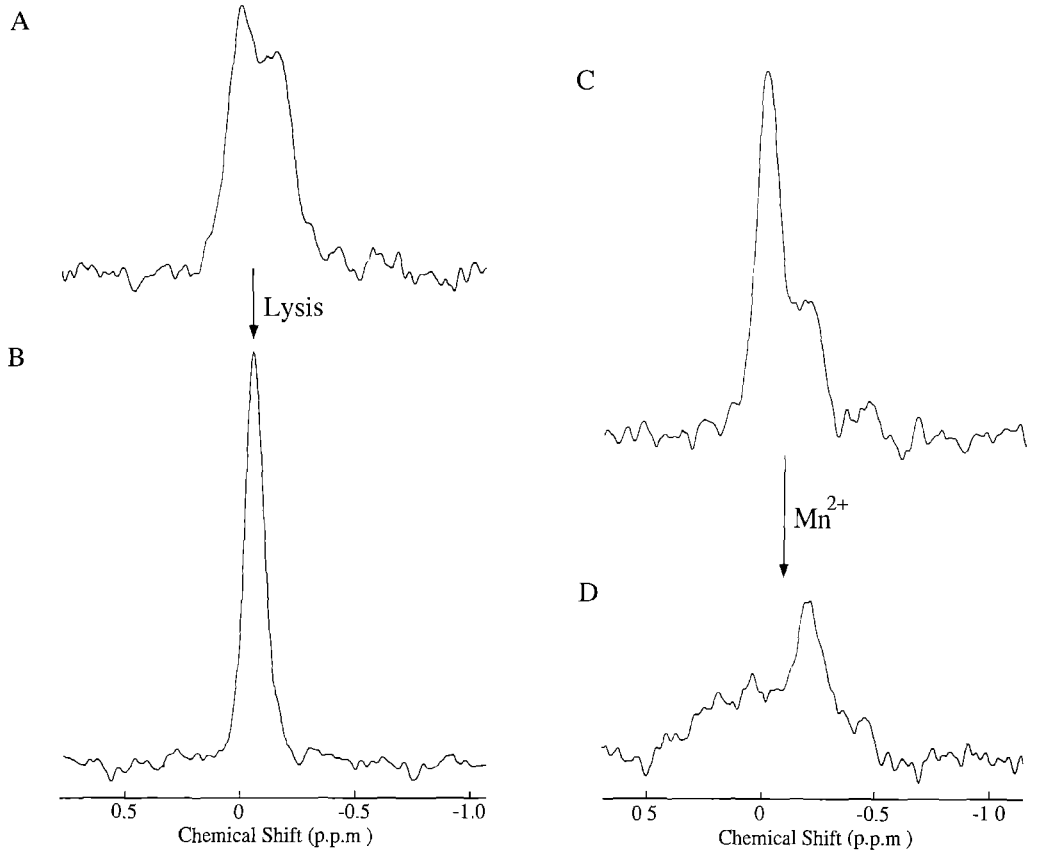

Fig. 2. **A** The ^{13}C NMR spectrum of ^{13}C-urea (13 mM with respect to the *total* sample volume) in a cell suspension of Ht ~ 0.5. Cell volume was reduced by the addition of NaCl such that the mean cell volume in the cell suspension was 62 fl. **B** The ^{13}C NMR spectrum of the sample in **A** but acquired after the erythrocytes had been lysed by freeze/thawing the sample (3 ×) in liquid N_2. **C** The ^{13}C NMR spectrum of ^{13}C-urea (13 mM with respect to the total sample volume) in a cell suspension of Ht ~ 0.33. The mean cell volume (reduced by the addition of NaCl to the cell suspension) was ~ 66 fl. **D** The result of addition of Mn^{2+} (0.4 mM) to the cell suspension. BSA was present at approximately 2% w/v in the extracellular medium. For the four spectra the NMR acquisition parameters were as follows; inter-transient delay 300 s, spectral width 2 000 Hz, 8 transients digitized into 8 000 data points with an acquisition time of 2 s, and $\pi/2$ pulse was 29 μs. The free-induction decay was Fourier transformed using an exponential apodisation function that resulted in 3 Hz of line-broadening in the resultant spectrum. Cell preparation was as described in Methods

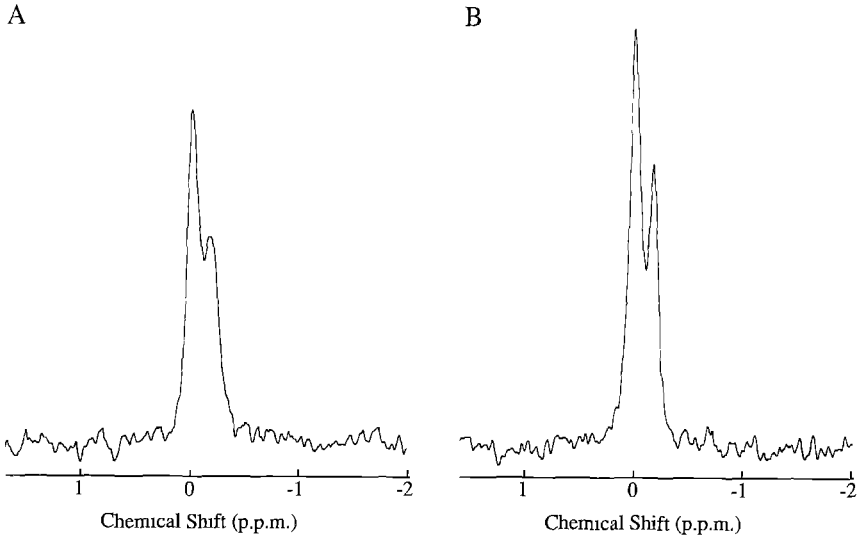

Fig. 3. **A** The ^{13}C NMR spectrum of ^{13}C-urea (~ 27 mM with respect to the total accessible volume) in a red cell suspension of Ht ~ 0.45 and a mean cell volume (reduced by addition of NaCl to the cell suspension) of ~ 66 fl. **B** The ^{13}C NMR spectrum of the same sample but after addition of phloretin (and incubation for 10 min at 25 °C). NMR acquisition parameters: $\pi/2$ pulse, 26 μs; intertransient delay, 200 s; other parameters were as for Fig. 2

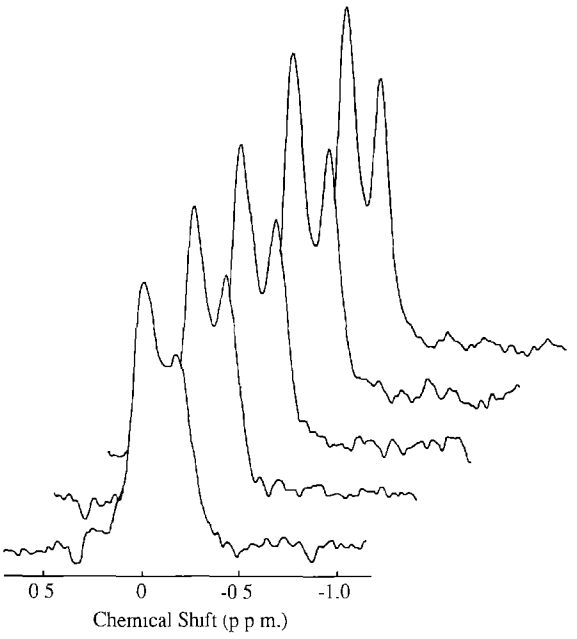

Fig. 4. ^{13}C NMR spectra of ^{13}C-urea (~26 mM) in cell suspensions to which had been added different concentrations of ^{12}C-urea. The concentrations were calculated with respect the total accessible volume for urea (see Methods). The total concentrations were (from the front to the back spectrum) 0.029 M, 0.081 M, 0.14 M, 0.29 M, and 0.87 M. NMR acquisition parameters: $\pi/2$ pulse, 30 μs; inter-transient delay, 200 s. The free-induction decay was Fourier transformed using an exponential apodisation function that resulted in 1 Hz of line-broadening in the resultant spectrum. The other parameters were as for Fig. 2

definition described in Theory), with the intra- and extracellular ^{13}C-urea NMR resonances being exchange-broadened.

Determination of the V^S_{max} and K_m for urea exchange

NMR lineshape analysis was used to calculate the rate constants for ^{13}C-urea influx under equilibrium exchange conditions in samples to which different concentrations of ^{12}C-urea had been added (as in Fig. 4). Fully relaxed NMR spectra were acquired of each sample at 25 °C. Equation (4) was fitted to the spectrum with the highest urea concentration (~870–940 mM). Holding the rate constant at a starting value (initial estimate obtained from K_m and V^S_{max} values quoted in the literature; Brahm 1983; Mayrand and Levitt 1983), the values for the other parameters that describe the lineshape were estimated by using nonlinear least-squares regression of (4) onto the spectrum. The linewidths and chemical shift separation of the resonances were then held constant at their calculated values in subsequent regression of (4) onto the spectra with lower urea concentrations. The rate constants calculated were converted to permeability coefficients (2) and the K_m and V^S_{max} for urea exchange were determined using a nonlinear least-squares regression of (3) onto a graph of the permeability coefficient as a function of the total urea concentration. In this calculation of K_m and V^S_{max} the permeability coefficient measured in the sample with the highest urea concentration was not included, as this spectrum was used to generate initial estimates for the next reiteration in the analysis. The calculated V^S_{max} and K_m were then used to calculate the expected rate constant at the highest urea concentration and then the initial non-linear least-squares regression of (4) onto this spectrum was repeated, holding the value of k_1 constant at this new value. This iterative procedure was fully automated in the computer program; iteration continued until a V^S_{max} and K_m of 'best fit' were determined. We performed this analysis on three occasions using blood from the same donor and obtained the results shown in Table 1.

Table 1. Values of K_m and V^S_{max} for urea exchange across the human erythrocyte membrane, measured under equilibrium exchange conditions and at 25 °C using ^{13}C NMR lineshape analysis. The concentration range of urea in each analysis was 0.029 M to 0.87 M, 0.029 M to 0.94 M, and 0.030 M to 0.90 M, respectively, down the table

n*	V^S_{max} ($10^8 \times$ mol cm^{-2} s^{-1})	K_m (mM)
8	3.1±0.5	42±14
9	2.8±0.7	35±21
6	3.7±0.8	58±24

* n refers to the number of samples used in the determination of the K_m and V^S_{max}

Figure 5 compares the *calculated* lineshapes for samples with (A) the lowest and (B) the highest urea concentrations with the acquired ^{13}C NMR spectra of these samples. Figure 6 is a plot of permeability coefficient as a function of the total urea concentration. The point for the sample with the highest urea concentration was included in the figure, though it was not used in the final calculation of K_m and V^S_{max}. The curves were drawn using (a) the K_m and V^S_{max} determined by Mayrand and Levitt (1983), (b) the K_m and V^S_{max} reported by Brahm (1983), and (c) the K_m and V^S_{max} calculated using the data shown and determined by the procedure described above.

The analysis was tested for its sensitivity to the chosen *starting* value of k_1, and we found that in each set of experiments, a change in the starting value of k_1 from ~0.5 s^{-1} to ~4 s^{-1} resulted in no significant difference in the values of K_m and V^S_{max} calculated.

To test the program we simulated NMR spectra similar to the experimental spectra using (4). A program was written which allowed similar weighting of the spectrum and using assigned values for all parameters in (4) (except for the first-order influx rate constant) to calculate the lineshape according to the rate constant that was predicted from (3) using assigned values of K_m and V^S_{max} to calculate the k_1 at a particular urea concentration. These simulated data were then analysed in the same manner as described above for the experimental data. We confirmed that the values of K_m and V^S_{max} calculated were the same as had been used in the generation of the simulated data.

Karan and Macey (1990) measured the $K_{1/2}$ and V^S_{max} for urea exchange across human erythrocyte membranes under equilibrium exchange conditions using an alterna-

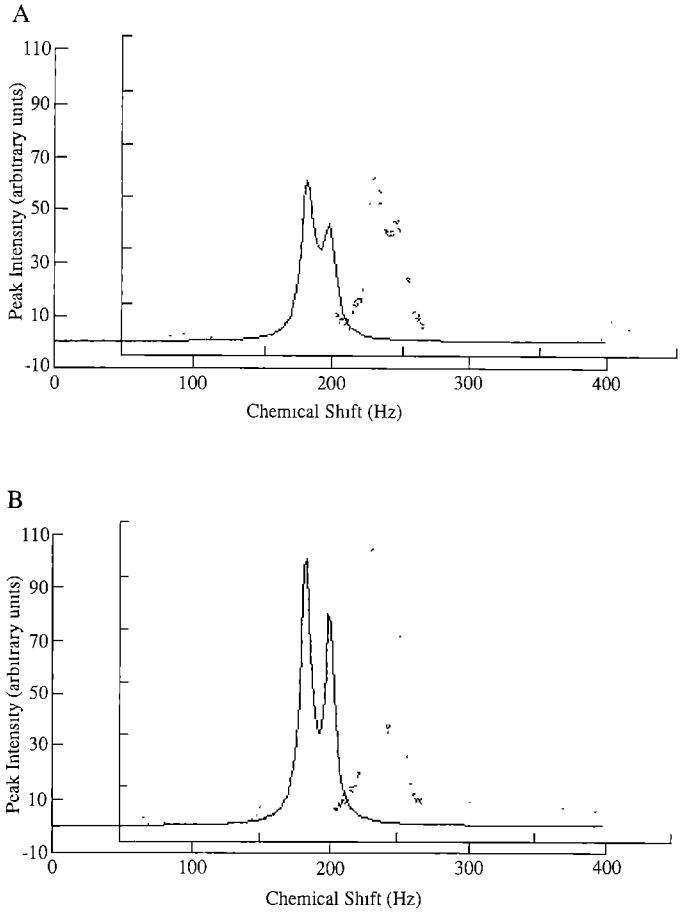

Fig. 5A, B. Comparison of acquired spectra with the lineshape of best fit, determined using nonlinear least-squares regression (Osborne 1976) of (4) onto the spectra. Spectra and lineshapes for the **A** lowest and **B** highest concentrations of urea used (see Fig. 4) are shown

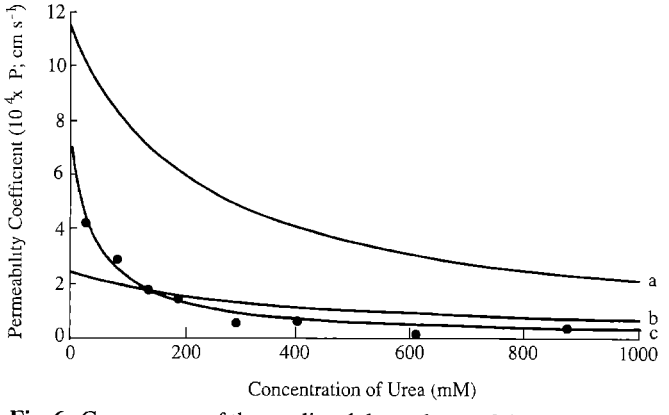

Fig. 6. Comparison of the predicted dependence of the permeability coefficient for urea exchange on the concentration of urea in the red cell suspension. The lines were drawn using (3) using the values of K_m and V^S_{max} determined (a) by Mayrand and Levitt (1983) of 218 mM and 25×10^{-8} mol cm^{-2} s^{-1}, respectively, (b) by Brahm (1983) of 334 mM and 8.2×10^{-8} mol cm^{-2} s^{-1}, respectively and (c) in the present study, using the data shown, of 42 mM and 3.09×10^{-8} mol cm^{-2} s^{-1}, respectively

tive ^{13}C NMR technique and obtained estimates of these parameters that are significantly higher than those measured in the present work. In this experiment, Mn^{2+} is added to the extracellular medium to greatly reduce the value of the longitudinal relaxation time (T_1) of the extracellular urea, such that the value of the T_1 for intracellular urea, can be used to calculate the rate of exchange of urea across the red cell membrane. In order to compare the results of the two experiments, in the present work the K_m and V^S_{max} for urea exchange was measured using the two different techniques but using blood from the same donor and under similar experimental conditions. Cells were prepared similarly to those described in Methods, however the cells were washed in isotonic saline rather than in PBS, and the ^2H_2O concentration in the solutions was 20%. ^{13}C NMR spectra were acquired at 100.62 MHz in the Fourier transform mode on a Bruker AMX400 NMR spectrometer. Fully-relaxed spectra were acquired by allowing >5 T_1 values between transients. The spectral acquisition time was 0.8 s, the spectral width was 5 000 Hz with the free-induction decay digitised into 8 K ('zero-filled' to 16 K) data points and processed using, in most cases, an apodisation function that resulted in 1 Hz of linebroadening. In some of the T_1 experiments a linebroadening factor of 3 Hz was used.

In the Mn^{2+}-doping experiment, four cell suspensions of Ht ~ 0.3 were prepared containing urea concentrations in the range 21.5 mM to 839 mM and with a mean cell volume of 70.4 ± 0.9 fl where this value is the mean $\pm$ standard deviation of the mean cell volume in each of the samples. Mn^{2+} was added to a concentration of 10 mM just prior to the NMR experiment. The T_1 of the intracellular urea was measured in each sample using a standard inversion-recovery experiment (Farrar and Becker 1971). The T_1 of intracellular urea was also measured in a sample of high (0.9) Ht. The efflux rate constant (k_{-1}) and subsequently the permeability coefficient were calculated as described by Karan and Macey (1990). The K_m and V^S_{max} for urea exchange of 560 ± 90 mM and $(1.03 \pm 0.14) \times 10^{-7}$ mol cm^{-2} s^{-1}, respectively, were determined from the dependence of the permeability coefficient on the total urea concentration using nonlinear least-squares regression of (3) onto the data.

In the lineshape analysis experiment, nine samples were prepared with total urea concentrations in the range 28.9 mM to 862 mM. The mean cell volume was 69.8 ± 0.7 fl (where this is the mean $\pm$ standard deviation of the mean cell volume in the nine samples). After phase correction, the digitised spectra were transferred to the Silicon Graphics IRIS 4D/20 workstation (Methods) *via* a network. K_m and V^S_{max} for urea exchange of 14 ± 14 mM and $(1.4 \pm 0.4) \times 10^{-8}$ mol cm^{-2} s^{-1}, respectively, were calculated similarly to the procedure summarised in Fig. 1. Using the erythrocytes prepared for the lineshape experiment, the permeability coefficient for urea exchange at a single (123 mM) urea concentration was measured using the Mn^{2+}-doping technique. At this concentration of urea the measured permeability coefficient of $(1.4 \pm 0.4) \times 10^{-4}$ cm s^{-1} was not significantly different from that predicted from the above values of K_m and V^S_{max} of $(1.0 \pm 0.3 \times 10^{-4}$ cm s$^{-1})$.

Discussion

Observation of separate resonances for the intra- and extracellular populations of ^{13}C-urea

We showed that the ^{13}C NMR spectrum of ^{13}C-urea in an erythrocyte suspension of reduced mean cell volume, contained two partially resolved resonances that arose from the intra- and extracellular populations of the compound. The chemical shift separation of the two resonances, and the rate of exchange of urea across the membrane under the conditions used, were such that the exchange was 'intermediate' on the NMR timescale. This enabled the determination of the K_m and V^S_{max} for the equilibrium exchange of urea across the human red cell membrane at 25 °C.

There are two likely contributors to the separation of the intra- and extracellular ^{13}C-urea resonances observed in a suspension of human erythrocytes. First, part of the observed separation could be accounted for by the difference in the magnetic susceptibility between the intra- and extracellular compartments. Prior to the NMR experiments, the cell suspensions were bubbled with CO. Both carboxy- and oxy-haemoglobin are diamagnetic (Philo et al. 1984) but CO binds more tightly to haemoglobin than oxygen (Lehman and Huntsman 1966) and thus by bubbling the cells with CO, the rate of formation of paramagnetic deoxyhaemoglobin (Fabry and San George 1983) is slowed. The effect of the lower bulk magnetic susceptibility inside the erythrocyte (due to carboxyhaemoglobin) will result in a shift of the intracellular resonance to low frequency (Fabry and San George 1983) with respect to the chemical shift of the extracellular resonance.

Kirk and Kuchel (1988a) found that a number phosphoryl compounds, when placed in a suspension of human erythrocytes, had well-resolved ^{31}P NMR resonances arising from the intra- and extracellular populations; the intracellular resonance having the lower frequency. In an extensive study of this phenomenon, it was concluded that for trimethyl phosphate (TMP) the separation between the resonances could be accounted for on the basis of the difference in magnetic susceptibility between the intra- and extracellular compartments, while for other compounds the chemical shift difference was too large to be accounted for on the basis of magnetic susceptibility effects alone.

Second, it has been suggested by Maciel and Natterstad (1965) that one explanation for the observed sensitivity to environment of the chemical shift of some carbonyl compounds, when they were placed in protic solvents, may lie in the tendency of these solvents to form hydrogen bonds to the carbonyl group. It has been proposed (Kirk and Kuchel 1988b) that for a number of phosphoryl compounds (*vide supra*) the separation between the intra- and extracellular resonances observed in ^{31}P NMR spectra of these compounds in suspensions of red cells, may arise in part from the disruption, by intracellular haemoglobin, of hydrogen bonds between the phosphoryl oxygen and the solvent water. Recently we pointed out that this phenomenon is also probably the explanation for the chemical shift separation of the intra- and extracellular ^{19}F NMR resonances of some fluorinated compounds in red cell suspensions (Xu et al. 1991). Thus, this proposed disruption of hydrogen bonding by intracellular haemoglobin may also contribute to the observed separation of the intra- and extracellular resonances in the ^{13}C NMR spectrum of ^{13}C-urea in a red cell suspension.

Regardless of the basis of the separation between the intra- and extracellular ^{13}C-urea resonances, we have shown that this phenomenon of separate resonances can be used to measure the K_m and V^S_{max} of urea exchange across the red cell membrane under equilibrium exchange conditions.

Comparison with previous results

Mayrand and Levitt (1983) used a 'rapid-flow' technique to measure urea equilibrium exchange at 23 °C. They measured a K_m of 218 mM and extrapolated their analysis to a value for the permeability coefficient at zero substrate concentration (P_0) of 1.16×10^{-3} cm s^{-1}. Applying simple Michaelis-Menten kinetics, these values can be used to calculate a V^S_{max} of 25×10^{-8} mol cm^{-2} s^{-1} at 25 °C. Brahm (1983) measured urea exchange under similar conditions but using a continuous flow technique obtained a K_m of 334 mM and a V^S_{max} of 8.2×10^{-8} mol cm^{-2} s^{-1}. Using thes values we calculated P_0 to be 2.5×10^{-4} cm s^{-1}. Karan and Macey (1990) used a ^{13}C NMR technique in which the effect of exchange with the Mn^{2+}-doped extracellular compartment on the longitudinal relaxation time of the intracellular population was analysed. They measured a K_m for urea exchange at 25 °C of 840 mM, a value for V^S_{max} of 15×10^{-8} mol cm^{-2} s^{-1} and a corresponding value for P_0 of 1.8×10^{-4} cm s^{-1}. Yousef and Macey (1989) used the 'perturbation method', in which they measured small changes in red cell volume in response to a specific alteration in the salt tonicity and/or the substrate concentration in the cell suspension, to study the exchange of urea across the human erythrocyte membrane. They measured a K_m for urea exchange at room temperature of 685 ± 41 mM at pH 7.8 and a V^S_{max} that ranged from $18-41 \times 10^{-8}$ mol cm^{-2} s^{-1} in the cells from four individuals. In the present work we used the calculated values of K_m and V^S_{max} to obtain a weighted mean P_0 of $7 \pm 3 \times 10^{-4}$ cm s^{-1}, a value which lies within the range of values quoted above. The results of the calculation of K_m and V^S_{max} are shown in Table 1, the weighted mean values (weight = 1/variance) over the three experiments were $3.1 \pm 0.6 \times 10^{-8}$ mol cm^{-2} s^{-1} and 44 ± 18 mM (the errors denote the weighted standard deviation). Thus we note that the K_m and V^S_{max} values estimated in the present work are significantly lower than all of the previously determined values.

There has clearly been a wide variation in the values of K_m and V^S_{max} measured using different techniques. This is probably an indication of the difficulties involved in measuring such rapid transmembrane exchange. Another example of widely varying estimates of parameters for this exchange system is the temperature dependence of the K_m for urea exchange. Karan and Macey (1990) measured an

increase in $K_{1/2}$, from 1.01 M to 3.53 M, on increasing the temperature from 25 °C to 38 °C. In contrast Brahm (1983) reported that at 25 °C the K_m for urea exchange was 334 mM while at 38 °C it was 396 mM.

In the present work we also measured the K_m and V^S_{max} for urea exchange using the Mn^{2+}-doping method described by Karan and Macey (1990). We obtained estimates of the standard deviations of the parameters by using a nonlinear least-squares regression of (3) onto the data rather than using the graphical procedures (Hanes-Woolf plot) used by Karan and Macey (1990). While the estimates of K_m and V^S_{max} were lower (560 ± 90 mM, and $1.03 \pm 0.14 \times 10^{-7}$ mol cm^{-2} s^{-1}) than those measured by Karan and Macey (1990; 840 mM, and 1.5×10^{-7} mol cm^{-2} s^{-1}) at the same temperature, the differences were not sufficient to account for the far lower values obtained using the ^{13}C NMR lineshape analysis technique. This result indicates that neither physiological variation between donors, nor the reduced mean cell volume used in the lineshape analysis experiments, accounts for the difference in the results obtained using the two techniques. Also when the Mn^{2+}-doping experiment was repeated at a single urea concentration of 123 mM, there was no significant difference between the permeability coefficient measured using the Mn^{2+}-doping experiment and the permeability coefficient predicted using the K_m and V^S_{max} measured using lineshape analysis under similar experimental conditions and on the same day.

The lineshape analysis technique described here resulted in estimates of the K_m for urea transmembrane exchange that were significantly lower than previous estimates obtained using other techniques, including an alternative ^{13}C NMR technique. This may indicate that these other techniques are not sufficiently sensitive at low substrate concentrations to measure the steep dependence of the permeability coefficient on the substrate concentration in this concentration range that was apparent when the ^{13}C NMR lineshape analysis method was employed.

Conclusions

In the present work we applied an established NMR technique in a novel context, namely the measurement of the kinetic parameters for the exchange of urea across the human erythrocyte membrane at 25 °C. While the ratio of V^S_{max}/K_m (i.e. P_0) calculated was reasonably consistent with the results of previous studies, the absolute values of these two parameters were consistently lower than earlier estimates obtained using other techniques.

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