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Membrane permeability of formate in human erythrocytes: NMR measurements

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Abstract The rate of the rapid exchange of formate mediated by band 3 in human erythrocytes, under equilibrium exchange conditions, was measured by using a T_1 relaxation method with ^{13}C-labelled formate and ^{13}C NMR, and a pulsed field-gradient spin-echo (PFGSE) method using ^{1}H NMR. The former analysis was based on large differences in T_1 between the inside and the outside of the cells brought about by added Mn^{2+}; the latter was based on large differences in the apparent diffusion coefficient inside and outside the cells. There was close agreement in the estimates of the membrane permeabilities made using both methods, suggesting a lack of interference of the exchange process by Mn^{2+}. Regression analysis yielded estimates (under the specified conditions, including 37°C) of V_{max} of $3.5\pm0.3\times10^{-9}$ and $3.8\pm0.4\times10^{-9}$ mol cm^{-2} s^{-1}, and K_m of 9.8±0.2 and 8.1±0.2 mM, for the T_1 and the PFGSE methods, respectively. These are new estimates made using methodology that has not previously been applied to measuring rapid (sub-second time scale) formate exchange in cells.

Key words Formate · Human erythrocyte · Band 3 · ^{13}C NMR relaxation analysis · Pulsed field-gradient NMR

Introduction

The permeability of red blood cells (RBC) to organic anions has been the subject of several studies (Aubert and Motais 1975; Motais 1977; Passow 1986). However, to our knowledge there has been only one study on the transport of formate into RBC (Aubert and Motais 1975) and only relatively recently has it become possible to make the measurements in a straightforward way by using NMR spectroscopy (Kuchel et al. 1992). Formate is normally only found in micromolar concentrations in human RBC and little is known about its physiological role in these cells. It is thought to be partly utilised by developing RBC in the biosynthesis of purine (Lowy et al. 1962; Wagner and Levitch 1973). It is known that formate is found in leukocytes in much higher concentrations than in RBC and may be involved in their apoptosis (Scott et al. 1966; Ahmed and Weidemann 1994).

A full appreciation of the mechanism of formate transport, as well as its physiological function, requires accurate estimates of parameters that characterize its transmembrane exchange kinetics. Beside its physiological functions, formate is an ideal compound on which to measure exchange in RBC by NMR methods, as it occurs as a singlet in the ^{1}H spectrum, its chemical shift is well separated from those of other cellular compounds, and it has partly resolved ^{13}C NMR spectral peaks from the extra- and intracellular populations (Kuchel et al. 1992).

The determination of formate membrane permeability parameters may be of importance for the characterization, by analogy, of bicarbonate transport since the bicarbonate and formate anions are structurally very similar and differ only in the substitution of the formate hydrogen atom by a hydroxyl group in the bicarbonate anion. Such comparisons as a route to insights into the mechanism of band 3 have already been elegantly employed for a wide range of anions (Galanter et al. 1993). Transport processes of bicarbonate across the membrane of RBC are of major physiological importance, especially in the transfer of carbon dioxide from peripheral tissues to the lungs (e.g., Dirken and Mook 1931; Lukner 1939). However, there have been major difficulties in the determination of the 'effective' membrane permeability of bicarbonate owing to its chemical equilibrium with carbon dioxide (Chapman et al. 1986; Kuchel et al. 1987). Thus it appeared to us that it would be useful to compare the membrane permeability coefficients of formate, determined by different NMR techniques, with those previously obtained for bicarbonate.

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Theory: NMR methods for measuring formate transport

T_1 relaxation times ('manganese doping' method)

Longitudinal relaxation times (T_1) can be used to study membrane exchange processes if the T_1 of a nucleus is much longer than the exchange time of the compound across the cell membrane, and if the external (or internal) signal of the compound is quenched in a time much smaller than the exchange time (e.g., Colon and Outhred 1972; Karan and Macey 1990). The quenching of the extracellular formate signal can be achieved by adding low concentrations of the impermeable paramagnetic ion Mn^{2+} to the buffer solution in which the RBC are suspended. The inversion recovery pulse sequence (Farrar and Becker 1971) is used to obtain the values of T_1. The principle of this NMR experiment is that the intra- and extracellular populations are magnetically labelled by a π pulse, but within a few milliseconds the external label disappears owing to rapid relaxation induced by the paramagnetic Mn^{2+}. The population leaving the RBC loses its 'label' upon contact with the external solution while the extracellular population entering the RBC is unlabelled. The decay of the label measured in a suspension of RBC is dominated by the membrane exchange, because of the much slower T_1 relaxation of the intracellular population. Therefore, the exchange time (T_{ex}) is calculated from

$$(T_{ex})^{-1}=(T_1^*)^{-1}-(T_1^{in})^{-1} \quad (1)$$

where T_1^* is the apparent T_1 in the RBC suspension containing extracellular Mn^{2+}, and T_1^{in} that for the intracellular population measured on an undoped packed cell suspension (Colon and Outhred 1972).

Pulsed field-gradient spin-echo NMR

A pulsed field-gradient spin-echo (PFGSE) NMR method for measuring membrane transport avoids the use of relaxation compounds or ions like Mn^{2+}, and it is unnecessary to have signals with different chemical shifts for the two compartments (see below; Waldeck et al. 1997).

The spin-echo signal attenuation for a solute in free solution in a PFGSE experiment, R, is given by (Stejskal and Tanner 1965):

$$R=\exp(-\gamma^2 g^2 D\delta^2(\Delta-\delta/3)) \quad (2)$$

where γ is the magnetogyric ratio, g the magnitude of the applied magnetic field gradient pulses, D the self-diffusion coefficient of the compound, δ the duration of the magnetic field gradient pulses, and Δ the time interval between the field gradient pulses. When the mean distance moved by solute molecules, in a specified time, is shorter than the distance moved by them in free solution, the molecules are said to undergo 'restricted' or, depending on the geometry of the system, 'obstructed' diffusion. This can arise in a suspension of cells where penetrable and impenetrable barriers (e.g., membranes) lead to a confining of the motion of some molecules (e.g., Stein 1986; Price et al. 1989; Kuchel et al. 1994). This means that the apparent diffusion coefficients in a cell suspension will differ from the diffusion coefficient in free solution.

The expression relating the attenuation of the NMR signal of a compound in a two-compartment system (e.g., a suspension of RBC in saline), undergoing exchange between the two regions, is described by (Kärger 1969, 1971, 1985; Kärger et al. 1988; and see Waldeck et al. 1997 for a recent derivation):

$$R=P_1\exp(-K D_1\Delta)+P_2\exp(-K D_2\Delta) \quad (3)$$

where D_1, D_2, P_1 and P_2 are the apparent self-diffusion coefficients and population fractions in the two compartments, respectively. These 'extrinisic' parameters are related to the 'intrinsic' parameters by the expressions:

$$D_1=(1/2)\{D_e+D_i+[K(\tau_e^{-1}+\tau_i^{-1})]^{-1} -[\{D_i-D_e+[K(\tau_e^{-1}+\tau_i^{-1})]^{-1}\}^2+4(K^2\,\tau_e\,\tau_i)^{-1}]^{1/2}\}$$

$$D_2=(1/2)\{D_e+D_i+[K(\tau_e^{-1}+\tau_i^{-1})]^{-1} +[\{D_i-D_e+[K(\tau_e^{-1}+\tau_i^{-1})]^{-1}\}^2+4(K^2\,\tau_e\,\tau_i)^{-1}]^{1/2}\}$$

$$P_1=1-P_2$$

$$P_2=(P_e D_e+P_i D_i-D_1)/(D_2-D_1)$$

$$K=\gamma^2 g^2\delta^2$$

where D_e and D_i are the diffusion coefficients of the molecules in the extra- and intracellular compartments, and P_e and P_i are the relative extracellular and intracellular population fractions, respectively. The mean residence lifetimes τ_e and τ_i are related by

$$\tau_e=P_e\,\tau_i/P_i$$

P_i and P_e were determined by ^{31}P NMR methods according to Raftos et al. (1988) and Kirk et al. (1988) (see below).

Equation (3) is based on the assumption that: (1) the intercompartmental exchange rate constants are much larger than the transverse relaxation rate constants of the nuclei under study, i.e., $k_{i,e} \gg (T_{2;i,e})^{-1}$; and (2) the magnetization-phase dispersion (due to diffusion) during δ is negligible compared with that occurring during Δ, i.e., $\Delta \gg \delta$.

Non-linear regression of Eq. (3) onto the data from the PFGSE experiments, in which g was varied, was employed to obtain an estimate for the intracellular lifetime of formate, τ_i (e.g., Waldeck et al. 1997).

Estimates of the apparent diffusion coefficients D_i and D_e were obtained from a PFGSE NMR experiment in which the RBC suspension in formate solution was incubated with 4,4′-dinitrostilbene-2,2′-disulfonate (DNDS). This inhibitor of the band 3 transporter also inhibits formate exchange. For slow exchange, Eq. (3) simplifies to

$$R=P_e\exp(-K D_e\Delta)+P_i\exp(-K D_i\Delta) \quad (4)$$

Non-linear regression of Eq. (4) onto the data of a PFGSE NMR experiment, with an RBC suspension incubated with DNDS, in which δ was varied, yielded estimates of D_e and D_i. These apparent diffusion coefficients for slow formate

exchange were used as starting values for the regression of Eq. (3) onto the data of the non-inhibited formate exchange.

Determination of the relative extra- and intracellular populations (P_e and P_i)

The relative extra- and intracellular mole fractions of formate depend on the volume of the intra- and extracellular compartments, i.e., the fraction of the cell volume available to water and solutes (α), and for a charged compound like formate it depends also on the membrane potential (V_m). Both α and V_m were determined by addition of small concentrations of dimethyl methylphosphonate (DMMP) and sodium hypophosphite (HPA) to the RBC suspension in the formate buffer solution, and the data were analysed according to Raftos et al. (1988) and Kirk et al. (1988). As the concentration of DMMP is the same for the intra- and extracellular compartments, the relative areas of the two well-separated DMMP peaks (I_i/I_e) can be used to calculate the fraction of cell volume (α) available to physicochemically similar solutes by using the expression

$$\alpha = I_i(1-H_c)/I_e H_c \quad (5)$$

where H_c denotes the haematocrit of the cell suspension. The hypophosphite ion is used as a probe of the membrane potential (Kirk et al. 1988). For cells in a metabolic and transport steady-state, the membrane potential is given by the Nernst equation:

$$V_m = (RT/F)\ln(a_i[\text{HPA}]/a_e[\text{HPA}]) \quad (6)$$

where R is the gas constant, T is the absolute temperature, F is the Faraday constant, the square brackets denote molar concentration, and the ratio of the intra- and extracellular activities of hypophosphite is given by:

$$a_i[\text{HPA}]/a_e[\text{HPA}] = I_i(1-H_c)\,(I_e\ \alpha\, H_c)^{-1} \quad (7)$$

It was assumed that the activity coefficients were closely approximated by 1, so the membrane potentials were able to be used to determine the ratio of the concentrations of formate in the RBC according to the Nernst equation:

$$[\text{formate}]_i/[\text{formate}]_e = \exp(V_m\, F/RT) \quad (8)$$

The relative population fractions of intra- and extracellular formate used in Eq. (3) were given by:

$$P_e = (1-H_c)\,([\text{formate}]_e/[\text{formate}]_i)/ [H_c\,\{\alpha-([\text{formate}]_e/[\text{formate}]_i)\}+([\text{formate}]_e/[\text{formate}]_i)] \quad (9)$$

$$P_i = \alpha\, H_c\ /\ [H_c\{\alpha-([\text{formate}]_e/[\text{formate}]_i)\} + ([\text{formate}]_e/[\text{formate}]_i) \quad (10)$$

Determination of the membrane permeability

The diffusional efflux membrane permeability for formate ($P_{formate}$) was calculated from the efflux rate constant (k_1) or the intracellular mean residence lifetimes (τ_i, T_{ex}), determined by the different NMR methods, as follows:

$$P_{formate} = (\tau_i)^{-1}(V^{cell}/A^{cell}) = k_1(\alpha \text{MCV}/A^{cell}) \quad (11)$$

where V^{cell}, A^{cell}, and MCV denote the volume of a single erythrocyte that is accessible to low molecular weight hydrophilic solutes and water, the membrane surface area of a single cell, and the mean cell volume, respectively. The concentration dependence of $P_{formate}$, for efflux from the cells, can be characterised by two transport parameters, V_{max}, the maximal transport rate, and K_m, the formate concentration required for half saturation of the transport:

$$P_{formate} = V_{max}\ /(K_m + [\text{formate}]_i) \quad (12)$$

Experimental

Erythrocyte preparations

Freshly drawn venous blood, from one donor (U.H.), was washed twice in ice-cold phosphate-buffered saline [PBS; 10 mM (Na_2HPO_4/NaH_2PO_4), 3 mM KCl, 1 mM KH_2PO_4, 0.6 mM $MgSO_4$, 0.6 mM $MgCl_2$, 140 mM NaCl]. Following centrifugation, the plasma, buffy coat and the uppermost RBC layers were removed by aspiration. Cells were stored before use for up to 24 h in PBS containing 5 mM glucose at 4°C. Just prior to an experiment, the RBC were washed in buffer containing 10 mM KH_2PO_4, 5 mM glucose, sodium formate and sucrose, and were then centrifuged (3000 *g*, 5 min, 4°C). This procedure was repeated five times, removing the supernatant by aspiration after the centrifugation step and allowing a 5 min period for equilibration of the chloride and formate ions between the intra- and extracellular compartments. The overall concentration of formate was varied between 5 to 140 mM. For experiments to measure the competitive inhibition of chloride on formate transport, solutions were used which contained the above compounds and NaCl of various concentrations (5–100 mM). Sucrose was used in all cases to adjust the osmolality of the solutions to 290 mOsmol kg^{-1}. The pH of the solutions was adjusted to ~7.4 prior to adjusting the osmolality.

Formate solutions containing a total of 1 mM $MnCl_2$ were used for the 'manganese doping' NMR experiments.

When required, an isotonic DNDS solution was added to the cells 15 min after their final wash in the formate solution. These cell suspensions were incubated at 37°C for another 15 min prior to the NMR experiments.

All of the cells were gassed with CO, to convert oxy- and deoxyhaemoglobin to carbonmonoxyhaemoglobin, which is diamagnetic and thereby ensures maximum spectral resolution (Fabry and San George 1983; Kirk and Kuchel 1988). The H_c were usually adjusted to 0.55–0.65.

The H_c of the RBC suspensions were determined using a haematocrit microcentrifuge (Clements, North Ryde, NSW, Australia). Haemoglobin concentrations and erythrocyte and leucocyte counts were obtained electronically

using a Sysmex Microcell Counter CL-130 and Autodilutor AD-241 (Toa Medical Electronics, Kobe, Japan). The osmolalities of the formate solutions was determined with a Wescor 5100C vapor pressure osmometer (Wescor, Logan, Utah, USA).

Chemicals

If not otherwise indicated, the chemicals were obtained from Sigma Chemical (St Louis, Mo., USA). Sucrose was obtained from May & Baker (Dagenham, UK), sodium formate and $MnCl_2$ were from British Drug Houses (Poole, UK) and ^{13}C-labelled sodium formate was from Isotec (Miamiburg, Ohio, USA).

A solution of dimethyl methylphosphonate (DMMP; Fluka, Buchs, Germany) and hypophosphite (HPA; Aldrich, Milwaukee, Wis., USA) in water was prepared for the determination of the fraction of the cell volume available to solutes (α) and the membrane potential (V_m) of the RBC in the various formate buffer solutions (Kirk et al. 1988; Raftos et al. 1988); the extracellular pH was adjusted to ~7.4. When added to suspensions of RBC, the solution was diluted in the respective formate solution so that the osmolality was ~290 mOsmol kg^{-1}. The final concentrations quoted for the two phosphorus compounds was that averaged over the whole cell suspension.

General NMR procedures

^1H, ^{13}C and ^{31}P NMR spectra were acquired on a Bruker AMX400 wb spectrometer at operating frequencies for ^1H, ^{13}C and ^{31}P of 400.13, 100.31 and 161.98 MHz, respectively. Some ^{13}C NMR spectra were acquired on a Bruker AMX600 spectrometer at an operating frequency of 150.92 MHz.

Typically, 16 transients were averaged into 8 k data points, with a spectral width of 4 kHz for ^1H NMR spectra; and 32 or 64 transients (depending on the concentration of the ^{13}C-labelled formate) were averaged into 16 k data points, with a spectral width of 8 kHz for the ^{13}C and ^{31}P spectra. Peak intensities were determined from the integrals of the NMR signals after an automatic baseline correction had been applied. Samples containing cells were not spun in order to avoid cell sedimentation in the NMR tube. The sample temperature was 37°C. The probe-thermostat setting was adjusted in some NMR experiments according to the method of Bubb et al. (1988) to compensate for decoupler-induced heating.

^{31}P NMR experiments were used to determine the MCV and the membrane potential of the RBC according to Raftos et al. (1988) and Kirk et al. (1988). From these data the relative extra- and intracellular compartments (P_e and P_i) were determined. The value of A^{cell} for a normal human RBC that is required for the calculation of $P_{formate}$ was taken to be 1.43×10^{-6} cm^2 (Brahm 1983). Control ^{31}P NMR time-course experiments were used to determine α and V_m values during an 8 h incubation in the NMR spectrometer.

Manganese doping experiments

The T_1 relaxation times of sodium formate and ^{13}C-labelled sodium formate in the ^1H and ^{13}C NMR spectra, respectively, were determined using the inversion-recovery pulse sequence (π_x-delay-$\pi/2$-acquire; Farrar and Becker 1971). Data analysis for the T_1 estimation used the standard Bruker software (*uxnmr*). Isotonic $MnCl_2$ solution was added to the formate-containing RBC suspension to a final concentration of 1 mM. Bovine serum albumin (BSA) was added to the buffer solution (2% w/v); it binds the Mn^{2+} and thus aids in trapping it in the extracellular medium (Pirkle et al. 1979; Kirk and Kuchel 1985). T_1^{in} was measured in packed RBC ($H_c > 0.94$) to which no Mn^{2+} had been added.

PFGSE NMR experiments

^1H PFGSE NMR experiments were performed with unlabelled sodium formate. Each sample of 0.5 ml was dispensed into flat-bottom NMR tubes and sealed with a Teflon vortex plug; the tubes were then placed in 10-mm NMR tubes filled with CCl_4. The vortex plug and the CCl_4 were necessary to minimise magnetic susceptibility differences at the boundaries of the sample within the region of the rf coils. The sample volumes were deliberately made small so that they resided in the linear region of the field-gradient generated by the NMR probe. A Hahn spin-echo pulse sequence was used, incorporating a field-gradient pulse in each τ period (Stejskal and Tanner 1965). A relaxation delay of 30 s (~5 T_1) was allowed between the transients. A low-intensity selective presaturation pulse was applied at the frequency of the ^1H_2O NMR signal during the relaxation delay. The PFG apparatus that was used for the generation of the magnetic field gradient pulses has been described previously (Kuchel and Chapman 1991). The parameters for the PFGSE experiments in which δ was varied ('little delta' experiments) were: δ =0.003–20 ms with increments of 0.3, 0.4 or 0.6 ms; Δ=50 or 100 ms; and g=0.5 T m^{-1}. In the experiments in which g was varied ('variable g' experiments) the parameters were identical to those in the 'little delta' experiments, except that δ=3, 4 or 5 ms and g=0.0002–0.5 T m^{-1} with increments of 0.015 T m^{-1}. 'Little delta' experiments were performed on RBC suspensions preincubated with DNDS while 'variable g' experiments were used for the non-inhibited RBC suspensions.

Numerical procedures

Data were processed using the program Origin 3.54 (Microcal Software, Northampton, Mass., USA) on an IBM 486 computer.

Results and discussion

Determination of α and V_m and tests of RBC integrity

The formate concentrations used in the experiments were unphysiologically high. Therefore the cell volume and the membrane potential of RBC in all buffer solutions were determined in control experiments in which small concentrations (5 mM) of DMMP and HPA were added to the respective solutions (Raftos et al. 1988; Kirk et al. 1988). The cell volume was also calculated from cell counts and haematocrit measurements (see Experimental). The cell volume varied between 86 and 90 fl and this is within the normal range for human RBC (Dacie and Lewis 1975). Values for α from 0.69 to 0.73 were obtained for the suspensions of RBC with a statistically insignificant increase with increasing formate concentrations from 5 to 140 mM. These values were in close agreement with the value of 0.717 obtained in previous studies of RBC suspended in isotonic saline (Gary-Bobo and Solomon 1968). The membrane potential was determined to be –7 to –10 mV for freshly prepared RBC suspensions in the formate-buffer solutions. No significant dependence of V_m on the formate concentration was found.

The experiments on formate exchange were performed over a period of up to 2 h. It was therefore of interest to determine if changes occurred in α and V_m or if haemolysis occurred in this time. ^{31}P NMR spectra of the RBC suspensions containing DMMP, HPA and formate (5, 50 and 140 mM) were recorded to monitor possible changes over a period of 8 h. Again, the ^{31}P NMR chemical-shift differences of the intra- and extracellular populations of DMMP (Raftos et al. 1988) were used to monitor changes in the cell volume and possible haemolysis. As the chemical-shift difference between the resonances of intra- and extracellular DMMP depends on the haemoglobin concentration, this peak separation would have been decreased by a mean volume increase of the RBC, or by the release of proteins by haemolysis. However, if the cell volume were to increase, the overall intensity of the intracellular DMMP resonance would have increased; if there were release of haemoglobin, the intensity of the extracellular DMMP resonance would have increased. Hence it was possible to distinguish between the phenomena. In addition, the extent of haemolysis was monitored by recording cell counts and haematocrits and also from inspecting the supernatant in the haematocrit capillaries, before and after the time courses.

No significant changes in α, V_m and the extent of haemolysis were observed for the first 3 h after the RBC had been suspended in the formate-buffer solution. After this time the membrane potential and the cell volume increased. The membrane potential was 0 mV and the cell volume was 90 fl for a formate concentration of 140 mM after 6 h of incubation at 37 °C; this represents the largest difference from the normal state, just after the dispersion of the cells in the formate solution. No changes in the extent of haemolysis (<5% for H_c=0.6) were found, compared to control experiments in which RBC were incubated in isotonic saline containing 5 mM glucose, 5 mM HPA and 5 mM DMMP.

The measurements on formate exchange were typically completed within 3 h of the preparation of the RBC suspensions; no RBC suspensions older than 3 h were used.

Apparent translational diffusion coefficients of formate

Figure 1A shows the attenuation of the PFGSE ^{1}H NMR signal that was detected using a typical 'little delta' experiment in an RBC suspension with 70 mM formate which was preincubated with DNDS (100 μM). It is known that DNDS inhibits the exchange of formate in RBC (Kuchel et al. 1992). Non-linear regression of Eq. (4) onto these data yielded the estimate of the apparent intra- and extracellular diffusion coefficients of formate, D_i and D_e, of 0.04±0.008 and 0.7±0.1 $\times 10^{-9}$ m^2 s^{-1}, respectively.

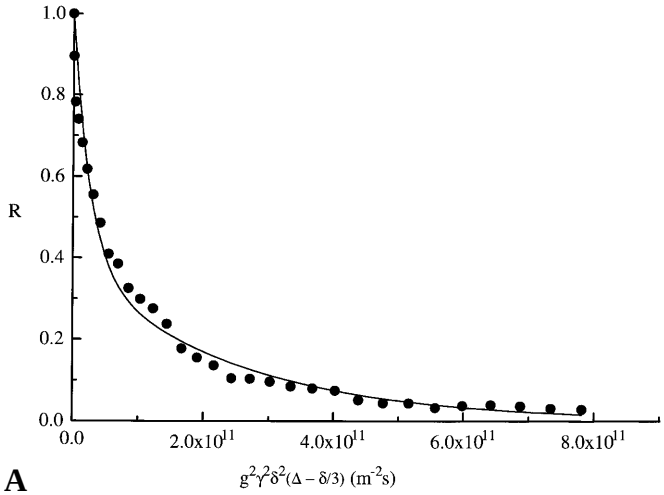
The self-diffusion coefficient of formate in free solution, D_{free}, was determined by non-linear regression of Eq. (2) onto the signal obtained from a 'little delta' PFGSE ^{1}H NMR experiment. Nilsson et al. (1983) and Jönsson et al. (1986) calculated obstruction corrections for the self-diffusion coefficient of small molecules in a suspension of particles of different shape in water solutions. An estimate of D_e of 1.1±0.1×10^{-9} m^2 s^{-1} for a RBC suspension with H_c=0.6 was calculated from D_{free} and an obstruction correction for particles with an oblate shape (1:5) of 0.71.

Estimates of the ratio D_e/D_i were calculated on the basis of a simulation of diffusion restricted to a sphere with the computer program 'Mathematica' (Lundberg and Kuchel 1997). A ratio D_e/D_i of 15 yielded D_i=7×10^{-11} m^2 s^{-1}.

Comparison of experimental and calculated values of D_i and D_e showed that they were in close agreement, respectively, and therefore the experimental values were good initial estimates for the diffusion coefficients, used in the non-linear regression of Eq. (3) onto the PFGSE NMR data obtained from RBC suspensions undergoing transmembrane formate exchange (Fig. 1B). However, it was found that if D_i and D_e were allowed to 'float' during the regression of Eq. (3) onto the data, the apparent diffusion coefficients were systematically 20–30% larger than those determined from the regression of Eq. (4) onto the data from DNDS-inhibited RBC.

Comparison of $P_{formate}$ determined by different NMR methods

The efflux permeability coefficient of formate was determined in suspensions of RBC in 70 mM sodium formate solution, by the three different NMR methods (see Theory section), namely ^{1}H and ^{13}C 'manganese doping' T_1 measurements and the diffusion method using PFGSE NMR. The efflux permeability coefficients were calculated to be (10^5×cm s^{-1}) 5.8±0.9, 6.2±0.4 and 6.8±0.5, respectively; the number of measurements was 5, 8 and 10, respectively. The standard deviation and systematic errors were used to



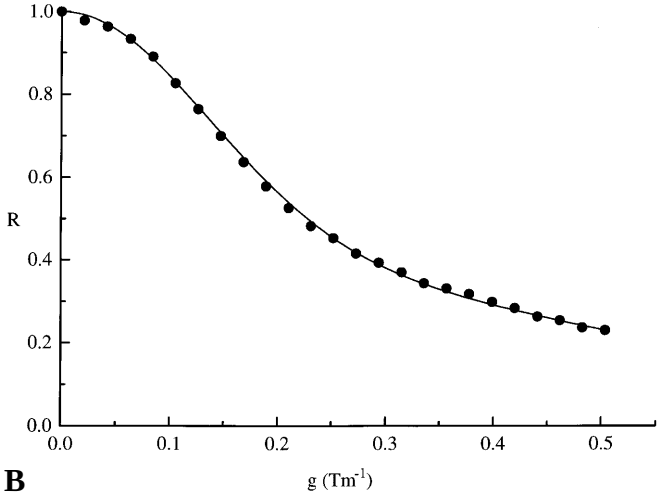


Fig. 1A, B Typical PFGSE ^{1}H NMR signal intensities of 70 mM sodium formate in a suspension of RBC of H_c=0.60, as a function of the Stejskal and Tanner parameter. A 'little delta' experiment was performed for RBC with formate exchange inhibited by incubation with DNDS **(A)**. Non-linear least-squares regression of Eq. (4) yielded the estimate of the apparent diffusion coefficients for the intra- and extracellular compartments of 0.04±0.008 and 0.7±0.1×10^{-9} m^2 s^{-1}. The NMR parameters for this experiment were: δ=0.003–20 ms with increments of 0.4 ms; Δ=50 ms; and g=0.5 T m^{-1}. **B** Results from a 'variable g' experiment in which the exchange of formate was not inhibited. Non-linear least-squares regression of Eq. (3) onto the data yielded the mean intracellular lifetime for formate, τ_i=0.85 s. The NMR parameters for this experiment were: g=0.0002–0.5 T m^{-1} with increments of 0.021 T m^{-1}; δ=3 ms; and Δ=50 ms. No error bars are shown as the dots are the original NMR signal intensities from the spectra in a single experiment

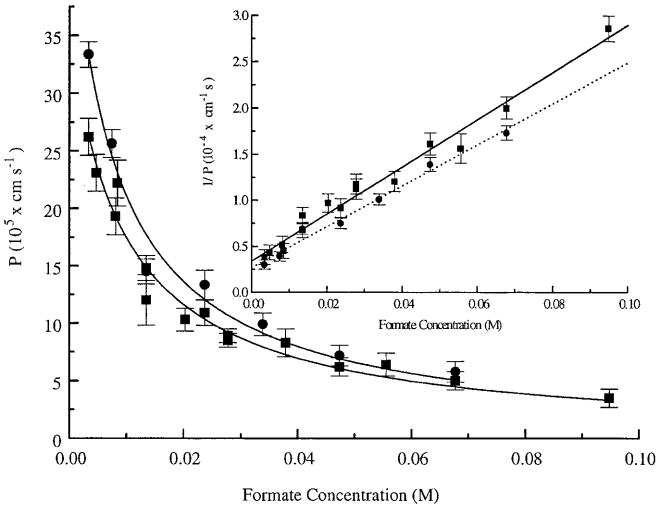


Fig. 2 Concentration dependence of the formate membrane permeability coefficient in a suspension of RBC. *Squares* denote $P_{formate}$ obtained from the ^{13}C-formate 'manganese doping' method, and *circles* denote results obtained from PFGSE NMR experiments. Non-linear least-squares regression of Eq. (12) onto the data yielded V_{max}=3.5±0.3×10^{-9} mol cm^{-2} s^{-1} ('manganese doping') and 3.8±0.4×10^{-9} mol cm^{-2} s^{-1} (PFGSE NMR), and K_m=9.8±0.2 mM ('manganese doping') and 8.1±0.2 mM (PFGSE NMR). The *inset* shows the respective Hanes-Woolf plots with lines regressed onto the data without any weighting. The *error bars* in the main graph and the *inset* denote ±1 SD

assess the 'quality' of these techniques for further studies. In a previous study by Kuchel et al. (1992), using saturation transfer, a permeability coefficient of 7.7±0.3×10^{-5} cm s^{-1} was estimated for formate efflux under the same conditions as used in the present work.

The ^{13}C NMR spectrum of ^{13}C-labelled formate displays two partially separated signals, representing the extra- and intracellular populations, as shown previously (Kuchel et al. 1992). The frequency difference of 28 Hz between the intra- and extracellular resonances, recorded at a spectrometer frequency of 161.98 MHz (magnetic field strength 14.1 T), and with an osmolality of 290 mOsmol kg^{-1} did not yield fully-resolved peaks. No *selective* saturation of one frequency was possible as partial saturation of the magnetisation giving rise to the other peak occurred. Therefore, a systematic over-estimate of the efflux rate constants, and $P_{formate}$, would have arisen; this outcome was confirmed by our results where the largest $P_{formate}$ estimate was obtained for the saturation transfer data. However, the differences between the $P_{formate}$ values was smaller than expected, indicating that the semiquantitative determination of the 'rf-power spillover' in the saturation transfer experiments yielded a reasonable correction of the data.

The membrane permeability for formate efflux, as a function of formate concentration, was determined using both the PFGSE diffusion method and the ^{13}C 'manganese doping' T_1 method. The ^{13}C T_1 of formate was longer than the ^{1}H T_1 for both packed RBC and RBC suspensions; values of 10.5±0.7 and 3±0.5 s were obtained from packed cells for the ^{13}C and ^{1}H T_1, respectively. As the relaxation and exchange rate constants are the reciprocals of the T_1 values [see Eq. (1)] the ^{13}C T_1 measurements gave more reliable estimates of the efflux rate constants than did the ^{1}H T_1 measurements, so they were used for all subsequent analysis.

Figure 2 shows $P_{formate}$ values determined with the ^{13}C T_1 'manganese doping' and the PFGSE NMR methods, as a function of intracellular formate concentration. The in-

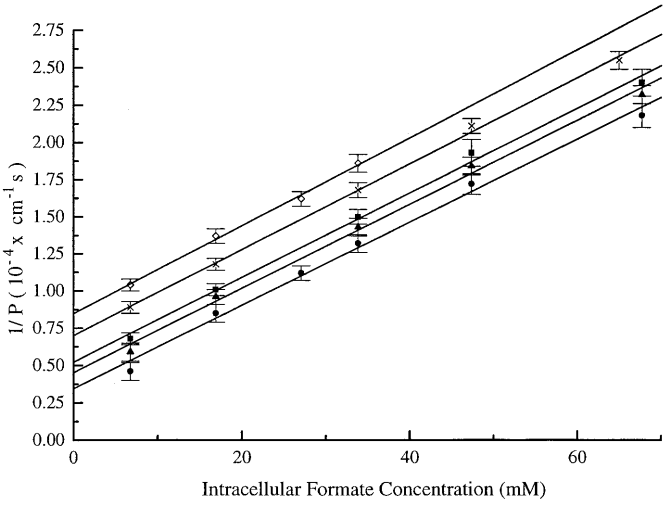


Fig. 3 Competitive inhibition of formate exchange by chloride. The symbols denote the following total NaCl concentrations in the RBC suspensions: *open diamond* 80 mM; *cross* 40 mM; *filled square* 20 mM; *filled triangle* 7 mM; and *filled circle* 0 mM. The *error bars* denote ±1 SD

Table 1 Influence of the total NaCl concentration in the RBC suspension on V_{max} and K_m of formate efflux from the RBC. The intracellular formate concentration was varied between 7 and 65 mM. The data were obtained from 13C-NMR 'manganese doping' experiments on RBC suspensions of H_c 0.55–0.65

NaCl concentration (mM)	K_m (mM)	V_{max} ($10^9 \times$mol cm^{-2} s^{-1})
0	9.8±0.2	3.51±0.3
7	15.9±1.5	3.53±0.5
20	18.3±1.4	3.51±0.4
40	24.2±0.9	3.46±0.5
80	28.7±1.0	3.38±0.5

set shows a linear dependence of $1/P_{formate}$ versus formate concentration (Hanes-Woolf plot). This behaviour is typical of saturable transport (Macey 1986). Similar values for $P_{formate}$ were found with both the ^{13}C NMR 'manganese doping' method and the PFGSE ^{1}H NMR diffusion measurements. Regression analysis yielded estimates of V_{max} of 3.5±0.3×10^{-9} and 3.8±0.4×10^{-9} mol cm^{-2} s^{-1} and K_m of 9.8±0.2 and 8.1±0.2 mM for the ^{13}C T_1 'manganese doping' and the PFGSE experiments, respectively, by regression of Eq. (12) onto the data in Fig. 2. These values are, to our knowledge, the first reported on formate exchange in RBC.

In another set of experiments the influence of DNDS, an inhibitor of the anion transporter band 3 (Cabantchik and Rothstein 1974), on the membrane permeability of formate was investigated. The efflux permeability of formate was reduced to approximately 10% of the value for RBC in the DNDS-free medium. The maximal inhibition of formate efflux was reached with concentrations larger 50 μM. The strong inhibition of formate exchange confirmed and quantified previous results that had suggested the involvement of the band 3 transporter in formate transport (Aubert and Motais 1975; Dalmark 1976; and Kuchel et al. 1992). The inhibitory effect (extent of inhibition) of DNDS on formate transport was similar to that on chloride transport (Gasbjerg et al. 1991; Brahm et al. 1992).

Previous workers have presented data on the initial penetration of formate determined by the rate of haemolysis of RBC in ammonium formate solutions (Aubert and Motais 1975). Therefore, no direct comparison of our data with other formate transport data was possible. Comparison of $P_{formate}$ with the membrane permeability of bicarbonate at 34 mM (Chapman et al. 1986) and at 113 mM (Kuchel et al. 1987) showed that $P_{formate}$ is ~0.26 and ~0.75 of that of bicarbonate at the respective concentrations. Also, the comparison of formate exchange rates with that of bicarbonate shows an expected similarity. We would expect similar membrane permeability coefficients for both ions as bicarbonate is an organic anion which uses the band 3 transport system (Dalmark 1976), and the penetration of anions depends on both size and configuration of the transported molecules (Galanter et al. 1993). Formate and bicarbonate are of approximately the same size with similar structures, but since formate is smaller than bicarbonate it might have been expected to be transported more rapidly. The fact that it is not suggests that hydrogen bonding of the additional oxygen atom of bicarbonate to residues in the band 3 transport protein is involved in the transport mechanism.

Measurements on the competitive inhibition of formate transport by chloride ions were obtained by varying the formate and chloride composition in the buffer solution in which the RBC were suspended. Figure 3 shows Hanes-Woolf plots of $1/P_{formate}$ versus formate concentration at several chloride concentrations. Table 1 shows the V_{max} and K_m at different chloride concentrations: these data provide a clear indication of competitive inhibition of formate transport by chloride ions. Regression analysis of the data yielded a value for the inhibition constant K_i of 38±10 mM.

In conclusion, relaxation methods, and methods relying on diffusion measurements carried out with NMR spectroscopy, provided a valuable means of studying the fast membrane transport processes involving formate that occurred on the sub-second time scale. Formate exchange under conditions of chemical and thermodynamic equilibrium was shown to be rapid and yet was amenable to the study of the concentration dependence of its rate. The structural similarity of formate to bicarbonate, and the similarity of the rates of exchange, make this anion a useful stable analogue of bicarbonate. Furthermore, the similarity of the exchange rate-constants measured using the two different NMR methods, one of which employed Mn^{2+} ions, suggests that the latter ion at the concentrations used do not affect the transport function of band 3.

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