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Exchange of fluorinated glucose across the red-cell membrane measured by ^{19}F-n.m.r. magnetization transfer

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The ^{19}F n.m.r. spectrum of 3-fluoro-3-deoxy-D-glucose (3FG) in a red-cell suspension was observed to contain separate resonances from the intra- and extra-cellular populations of both the α- and β-anomers. This phenomenon was used with an n.m.r. spin-transfer procedure to measure the rate of exchange of the anomers across the human red-cell membrane under equilibrium-exchange conditions at 37 °C. The β-anomer crossed the membrane significantly more quickly than the α-anomer. At a total 3FG concentration of 9.3 mM, the first-order rate constants for the efflux of the α- and β-anomers were 0.41 ± 0.15 and $0.88 \pm 0.20\ s^{-1}$ respectively. The measurable 3FG exchange was inhibited by 75 and 100 % respectively by the glucose-transport inhibitors cytochalasin B and phloretin. Glucose inhibited the exchange of 3FG, and the results were consistent with glucose and 3FG binding to the hexose-transport protein with similar affinity.

INTRODUCTION

The facilitated transport of sugars across human red-cell membranes has been extensively documented and studied [1,2], and yet the mechanism of transport is still not fully understood [3]. Under physiological conditions, glucose transport is remarkably fast [1], which complicates determinations of initial rates of transport [3]. Therefore many studies of the kinetics of glucose transport have been carried out at well below physiological temperatures [4–6] or in cells from animal species with lower rates of glucose transport, such as rat erythrocytes [3].

3-Fluoro-3-deoxy-D-glucose (3FG) has been used as a probe compound in studies of the 'binding requirements' for sugar transport in human red blood cells [7]. Riley & Taylor [8] found that glucose and 3FG cross the red-cell membrane via the same transporter and that the binding of the two sugars to this transport system is 'equivalent'. Thus 3FG is a useful compound for studying the glucose-transport protein; however, the difficulties of precisely measuring rapid transport at physiological temperatures remain.

We observed, in the present work, that the ^{19}F n.m.r. spectrum of 3FG in a red-cell suspension contained four resonances; the two peaks (from the α- and β-anomers) that are present in a spectrum of 3FG in water are each split into peaks corresponding to the intra- and extracellular populations of the compound. This phenomenon can be exploited to measure separately the transmembrane exchange of the anomers of 3FG by an n.m.r. spin-transfer technique. These techniques have been used previously for studying fast-chemical-exchange events at chemical equilibrium, such as enzyme-catalysed reactions [9,10] and membrane transport [11–13]. In the present work we used ^{19}F-n.m.r. saturation transfer to measure the equilibrium exchange influx of 3FG into human red cells at 37 °C. We also measured competition with this exchange by glucose, and observed the effects of the glucose-transport inhibitors cytochalasin B and phloretin on 3FG exchange.

MATERIALS AND METHODS

Materials

3FG, cytochalasin B and phloretin were from Sigma, St. Louis, MO, U.S.A. ^2H_2O (99.75 atom %) was from the Australian Institute of Nuclear Science and Engineering, Lucas Heights, N.S.W., Australia. All other reagents were of A.R. grade.

Erythrocytes

Human blood was obtained from the Red Cross Blood Transfusion Service (N.S.W.), and it was used within 5 days of collection. Before each experiment the cells were washed three times (2200 *g*, 5 min, 5 °C) in 3 vol. of iso-osmotic saline; they were then resuspended in phosphate-buffered saline (PBS; 141 mM-NaCl/20 mM-Na_2HPO_4/NaH_2PO_4 pH 7.4, 5 °C), bubbled with CO (5 min), centrifuged (2200 *g*, 5 min, 5 °C), and washed once more with PBS (prebubbled with CO). Bubbling the red-cell suspensions with CO ensures that all haemoglobin is in the fully diamagnetic state, thus minimizing magnetic-field gradients in the n.m.r. samples. The cells were stored at 4 °C until required for sample preparation.

Unless otherwise stated, samples (0.8 ml) were prepared by mixing cells and PBS to give a haematocrit (Ht) of ~ 0.4, and 3FG (0.2 M in PBS) was added to give a final concentration of ~ 9.3 mM with respect to the total solute-accessible volume. An aliquot (0.5 ml) of this sample was placed in a 5 mm (outer diameter) n.m.r. tube for the transport measurements. The remainder of the sample was kept on ice for later determinations of Ht, and the number of cells/ml, which were measured with an Ht centrifuge and a Coulter particle counter (model ZF; Coulter Electronics, Dunstable, Beds., U.K.). Lysates were produced from the n.m.r. samples by

Abbreviations used: 3FG, 3-fluoro-3-deoxy-D-glucose; PBS, phosphate-buffered saline (for composition and pH, see the text); Ht, haematocrit.

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freeze–thawing three times in liquid N_2. Supernatants were isolated by centrifugation (2000 *g*, 5 min, 20 °C).

N.m.r.

Field/frequency locking was achieved by using a coaxial capillary filled with ^2H_2O. Broad-band proton-decoupled ^{19}F-n.m.r. spectra were acquired at 282.35 MHz in the Fourier-transform mode using a Bruker CXP-300 spectrometer. Selective irradiation was achieved using a DANTE pulse-train [14]. A 0.3 μs pulse was repeated every 0.333 ms for a duration of 12 s ($> 5 \times T_1$ where T_1 is the longitudinal relaxation time). The sample temperature in the magnet was estimated by using an ethylene glycol capillary [15]. The samples were not spun in order to minimize cell sedimentation. T_1^s (the T_1 of 3FG while saturation is applied at a selected frequency) was estimated using a standard inversion-recovery pulse sequence. N.m.r. parameters are provided in the Figure legends. In all spectra the chemical shift of the extracellular α-3FG resonance was arbitrarily set to 0 p.p.m. The spectra shown were processed with a 3 Hz line-broadening factor; however, peak areas were measured on spectra with a 1 Hz line-broadening factor.

Calculation of rate constants and permeability coefficients

We used ^{19}F-n.m.r. saturation transfer to measure the first-order rate constants of 3FG exchange across human red-cell membranes. The n.m.r. protocol was similar to that described previously for ^{31}P n.m.r. [13]. The first-order rate constant for 3FG influx (k_1) was calculated directly from the areas of the extracellular resonance in the saturation transfer and control experiments by using the following expression:

$$k_1 = \frac{\Delta M/M}{(1-\Delta M/M)T_1^s} \quad (1)$$

where $\Delta M/M$ is the relative diminution of the extracellular resonance in the presence of the saturation radio-frequency field and in the presence of exchange [12]. Thus $\Delta M/M$ was calculated from the area of the extracellular resonance in two experiments in which (*a*) the intracellular resonance in a cell suspension was selectively saturated and (*b*) selective irradiation was applied at the same distance (in Hz) from the extracellular resonance, but in a lysate of a sample of the same or similar composition. Thus, for example, in determining the effect of glucose concentration on the rate of 3FG exchange, the control experiments were performed on two lysates (of differing glucose concentration) and the mean results used for all the samples. T_1^s is the T_1 of the extracellular 3FG resonance in the absence of exchange, measured while irradiating at the position (in Hz) of the intracellular resonance. T_1^s values were measured on the day of the experiment in the supernatant that was separated from a cell suspension of Ht $\sim$ 0.4 and with a 3FG concentration of $\sim$ 9.3 mM. It was assumed that a change in the glucose concentration would not affect the T_1^s of 3FG and that its main determinant would be the proximity of the frequency of selective irradiation to the 3FG resonance frequency.

Much of the current literature quotes the efflux rate constants for glucose. The efflux rate constant for 3FG exchange, k_{-1}, can be calculated from the following equation:

$$k_{-1} = \frac{k_1 V^{OUT}}{V^{INT}} \quad (2)$$

where V^{OUT} is the extracellular volume/ml of suspension, which can be calculated from the Ht ($V^{OUT} = 1 - Ht$) and V^{INT} is the solute-accessible intracellular volume/ml of suspension ($V^{INT} = Ht \times 0.717$) [16]. The permeability coefficient, *P*, which is required in the analysis of glucose inhibition of 3FG exchange, can be calculated by using the following expression:

$$P = \frac{k_1 V^{OUT}}{A^{TOTAL}} \quad (3)$$

where A^{TOTAL} is the red-cell membrane area/ml of suspension; this can be calculated from the known area of the membrane and the number of cells/ml.

RESULTS

The ^{19}F-n.m.r. spectrum of 3FG contains two resonances that have been assigned previously [17]; the higher-frequency resonance is from the β-anomer and the other is from the α-anomer. The ratio of the areas of the α- and β-peaks (measured in a lysate sample in the present work) was $43 \pm 5\%/57 \pm 5\%$, a value which is consistent with that obtained in previous work [7,18] in which ratios of $\sim 50/\sim 50$ and 45/55 respectively were observed. For the purposes of calculating the concentrations of β-3FG and α-3FG the anomeric ratio was approximated to 1.

Fig. 1(*a*) shows the ^{19}F-n.m.r. spectrum of 3FG in a high-Ht (0.65) cell suspension. The α- and β-resonances are split into peaks corresponding to the intra- and extracellular populations of 3FG. Such a phenomenon has been reported recently for other fluorinated compounds [19]. For both anomers, decreasing the Ht of the cell suspension (Fig. 1*b*) diminishes the intensity of the higher-frequency component.

We calculated first-order rate constants, for the efflux of 3FG, of $0.41 \pm 0.15\,s^{-1}$ and $0.88 \pm 0.20\,s^{-1}$ for the α- and β-anomers respectively (the error is the S.D. of the results of four experiments). The corresponding permeability coefficients (see eqn. 3) were $1.70 \pm 0.61\,cm \cdot s^{-1}$ and $3.60 \pm 0.80\,cm \cdot s^{-1}$. Incubating the cells with cytochalasin B (0.15 mM, 120 min) decreased the rate of measurable β-3FG exchange by 75%, and incubation with phloretin (0.9 mM, 150 min) totally inhibited the exchange. Fig. 2 shows the decrease in the extent of saturation transfer in the phloretin-inhibited Fig. 2(*b*) cell suspension compared with the extent of saturation transfer in a normal (Fig. 2*a*) cell suspension.

The inhibition by glucose of 3FG exchange was studied by measuring k_1 (influx rate constant) in five samples with constant 3FG concentration and varying glucose concentrations (Fig. 3). The results were analysed by non-linear least-squares regression of the following expression [20] on to flux versus inhibitor-concentration data [20]:

$$v = \frac{V_{max}[S]}{K_s(1 + \frac{[I]}{K_i}) + [S]} \quad (4)$$

In this expression v is the flux which, under equilibrium-exchange conditions, is given by $v = P \times [S]$, where [S] is the substrate concentration and *P* is the permeability

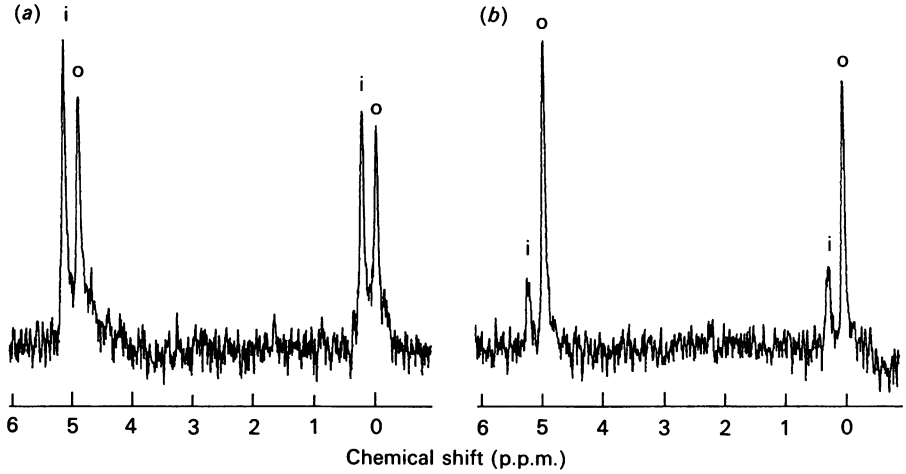

Fig. 1. ^{19}F-n.m.r. spectra of 3FG in red-cell suspensions of Ht 0.65 (*a*) and 0.35 (*b*)

The 3FG concentration in both samples was 9.3 mM. The higher-frequency pair of resonances are from the β-anomer. The intra- and extra-cellular resonances are indicated by 'i' and 'o' respectively. N.m.r. parameters: spectral width, 6667 Hz; 8000 data points per transient; 32 transients; line-broadening factor, 3 Hz; and inter-transient delay, 2 s.

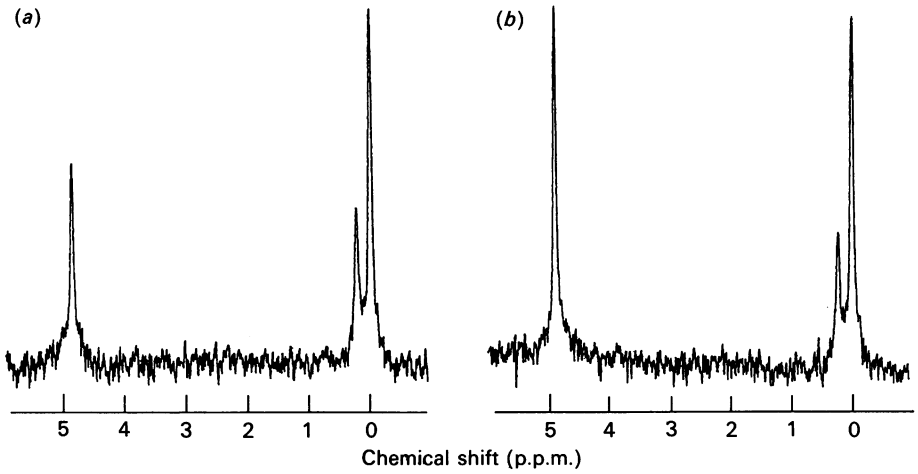

Fig. 2. ^{19}F-n.m.r. saturation-transfer spectra showing the effect of inhibition of 3FG exchange by phloretin

In both spectra selective irradiation was applied at the frequency of the intracellular resonance of β-3FG (see Fig. 1). The extent of saturation transfer observed was smaller in the presence of phloretin (*b*) than that observed in normal cells (*a*). The preparation of the samples was as described in the Materials and methods section. N.m.r. parameters: spectral width, 6667 Hz; 8000 data points per transient; 32 transients per spectrum; line-broadening factor, 3 Hz; duration of selective irradiation, 12 s; DANTE delay, 0.33 ms; pulse width, 0.3 μs; $\pi/2$ acquisition pulse, 45 μs. In the cell suspension shown in (*a*) the linewidth at half-height of the α-3FG resonance was 20 Hz, and in the lysate used for the control experiment (spectrum not shown) this linewidth was 18 Hz.

coefficient. V_{max} is the maximal flux, and K_s is the Michaelis–Menten constant for 3FG exchange [21]. K_i is the inhibition constant for the inhibition by glucose and α-3FG of β-3FG exchange. Thus we assume that the K_i of α-3FG for the transport of β-3FG is the same as the K_i of glucose for the transport of β-3FG. Barnett *et al.* [7] found that 3FG and glucose bind to the glucose transporter with similar affinity. Also, 3FG and glucose are transported at similar rates and with the same $V_{max.}$ [8]. Thus, for the present work, we considered it to be valid to assume that, in eqn. (4), $K_s = K_i$. Thus eqn. (4) was simplified to:

$$P = \frac{V_{max.}}{K_s + [I] + [S]} \quad (5)$$

In the present experiments, only the transport of the β-anomer was measured, thus [S] = [β-3FG] and [I] = [α-3FG] + [glucose]. The non-linear least-squares analysis with [S] = 4.7 mM yielded a $V_{max.}$ of 25 ± 1.2 mmol·s^{-1}·litre^{-1} and a K_s of 20 ± 1.7 mM (using a red-cell membrane area of 1.43×10^{-6} cm^{-2}). These values are comparable with the $V_{max.}$ and K_s of 30 mmol·s^{-1}·litre^{-1} and $\sim$ 16 mM respectively, determined for glucose influx under equilibrium-exchange conditions at 37 °C and using a non-n.m.r. method [2].

DISCUSSION

In the present work it was observed that, in a red-cell suspension, the ^{19}F-n.m.r. resonance of the intracellular

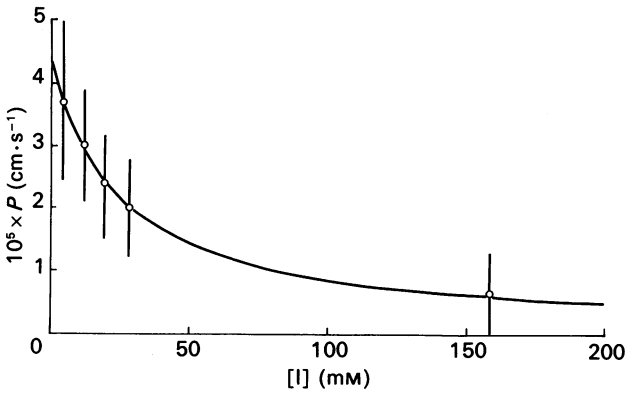

Fig. 3. Inhibition of 3FG exchange by glucose

[I] is the concentration of the inhibiting species (glucose and α-3FG) and P is the corresponding permeability coefficient measured for β-3FG transport across the red-cell membranes. Details of the parameter values are given in the text. The error bar on the first point indicates the mean error from two experiments, whereas the error bars on the other points indicate the result of an estimated 10% uncertainty in measuring the peak areas. The line was fitted by a non-linear least-squares regression of eqn. (5) on to the data.

3FG population is shifted to high frequency with respect to that of the extracellular population. This is consistent with recent work [19] in which separate intra- and extracellular resonances were observed for trifluoroacetamide and trifluoroacetate in human red-cell suspensions, and with our own observations on difluorophosphate, monofluorophosphate and hexafluorophosphate (results not shown). For each of these compounds the intracellular resonance is to high frequency of the extracellular resonance.

From the present work we concluded that the β-anomer of 3FG is transported across the red-cell membrane significantly more quickly than the α-anomer. This is consistent with the results of Faust [22]; he found that the β-anomer of glucose was transported approx. 3 times more quickly than the α-anomer. Carruthers & Melchior [23] were unable to detect a difference in the values of $V_{max.}$ and K_s for the two anomers, but the ranges of their measured values were so large as to conceal possibly a difference [2].

Riley & Taylor [8] found that 3FG crosses the human red-cell membrane via the glucose-transport protein. The present results supported this finding by showing that phloretin and cytochalasin B, both potent glucose-transport inhibitors [24,25], inhibited 3FG exchange.

Our results are also consistent with 3FG and glucose being transported across the human red-cell membrane with similar values of K_s and $V_{max.}$ (Fig. 3), thus confirming the suitability of this compound as a probe of the properties of the red-cell glucose-transport protein.

In conclusion, from the present as well as previous results [7,8] it can be seen that 3FG is a useful compound for the study of the human red-cell glucose transporter. Since it is transported as rapidly as glucose, the difficulties of measuring its exchange across the cell membranes remain. However, our particular adaptation of an n.m.r. spin-transfer technique is a convenient means of measuring the fast transmembrane exchange process. Although we previously developed a method for measuring glucose transport in red cells using ^{13}C n.m.r. [11], the present technique is more sensitive and direct and thus may be useful for measuring changes in glucose transport under various experimental conditions. As with all 'tracer-exchange' techniques, the present one is relatively insensitive at high substrate concentrations; but this problem can be partly overcome by using cell suspension of high Ht, and thereby increasing the level of saturation transfer observed for a given rate of exchange.

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