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Transport of α- and β-D-Glucose by the Intact Human Red Cell†

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ABSTRACT: The kinetics of α- and β-D-glucose mutarotation and the transport of these anomers by intact human red cells were determined at 0.6 and 36.6 °C. The mutarotation coefficients for α- and β-D-glucose in cell-free tris(hydroxymethyl)aminomethane medium (pH 7.4) at 0.6 °C are (2.25 ± 0.2) and (1.73 ± 0.42) × 10^{-3} min^{-1}, respectively, and at 36.6 °C are (69 ± 12) and (75 ± 5) × 10^{-3} min^{-1}, respectively. These values are in good agreement with previous estimates. At 0.6 °C, the red cell contains no detectable mutarotase activity. Initial rates of sugar uptake were measured by using radiolabeled D-glucose and time courses of uptake by turbidimetry. The time courses of α- and β-D-glucose and an equilibrium mixture of α- and β-D-glucose infinite-cis entry are identical at 0.66 °C (n = 41) where negligible mutarotation is observed. The apparent K_i values for inhibition of radiolabeled D-glucose initial uptake by unlabeled α- or β-D-glucose at 0.6 °C are identical (1.6 mM). The calculated V_{max} parameters for uptake of the radiolabeled anomers at this temperature are also indistinguishable. The time courses of infinite-cis α- and β-D-glucose uptake at 36.66 °C are identical (n = 40). While D-glucose mutarotation is more rapid at this temperature, the anomers of D-glucose are not transported differently by the red cell hexose transfer system. These findings confirm the suitability of the use of the integrated rate analysis for infinite-cis entry kinetics and support rejection of the symmetric and asymmetric carrier models for red cell sugar transport [see Naftalin, R. J., & Holman, G. D. (1977) in *Membrane Transport in Red Cells* (Ellory, J. C., & Lew, V. C., Eds.) pp 257-300, Academic Press, New York].

Hexose transfer in intact human erythrocytes displays kinetic asymmetry (Widdas, 1980). K_m and V_m for D-glucose net exit are some 10-fold greater than K_m and V_m for influx (Geck, 1971; Regen & Tarpley, 1974; Baker & Widdas, 1973). Moreover, when the external hexose transfer sites are saturated with substrate, a second, kinetically distinct (high affinity, low K_m) transport site is detected at the inner surface of the plasma membrane both in infinite-cis entry experiments (Hankin et al., 1972; Ginsburg & Stein, 1975; Foster et al., 1979; Carruthers & Melchior, 1983a) and in infinite-trans exit determinations (Baker & Naftalin, 1979). These measurements were made either by analysis of initial rates of radiolabeled sugar entry and exit (Ginsburg & Stein, 1975; Baker & Naftalin, 1979) or by transformation of the time course of net sugar uptake according to the integrated form of the infinite-cis entry Michaelis–Menten equation (Hankin et al., 1972; Ginsburg & Stein, 1975; Foster et al., 1979; Carruthers & Melchior, 1983a) and thus indicate that the obtained results are independent of both the method of measurement and the method of kinetic analysis. These observations are incompatible with the asymmetric form of the simple mobile carrier model for red cell sugar transport (Widdas, 1952; Regen & Tarpley, 1974).

Gorga & Lienhard (1981) suggested a possible explanation for these findings—that α and β anomers of D-glucose are transported with different velocity constants (V_m) or with different affinities (K_m) by the red cell hexose transfer system. Under experimental conditions, the red cell may be exposed to a mixture of these anomers (the equilibrium ratio is 36.2:63.8 α:β; Pigman & Anet, 1972). Hence, analysis of hexose transfer must recognize both parallel uptake and competition for uptake between these anomers. This would mean that the steady-state analyses of hexose transfer (both initial *and* integrated Michaelis–Menten rate equations) employed in the above studies were not, in the most rigorous sense, valid. If correct, this suggestion has serious analytical implications. Nevertheless, this suggestion still fails to account for the two quite different transport sites detected at the interior of the cell under zero-trans and infinite-trans exit conditions. Moreover, it is not clear how differential transport of anomers can account for the loss of hexose transfer kinetic asymmetry upon removal of cellular contents (Carruthers & Melchior, 1983a).

The simplest test of the anomer hypothesis is to determine the kinetics of α- and β-D-glucose uptake by the red cell under

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conditions where the rate of mutarotation is negligible. The mutarotation of α-D-glucose is a first-order reversible reaction with a mutarotation coefficient of 0.00632 min^{-1} at 20 °C, pH 7, and an activation energy of 17.2 $kcal \cdot mol^{-1}$ (Pigman & Anet, 1972). The mutarotation coefficient for β-D-glucose under the same conditions is 0.00625 min^{-1}. It follows that at 0 °C, the half-times for mutarotation of α- and β-D-glucose are 683 and 690 min^{-1}, respectively. Our approach was to monitor both the kinetics of α- and β-D-glucose uptake at 0 °C and the mutarotation of the glucose anomers during the incubation period. The red cell was also assayed for endogenous mutarotase activity.

Materials and Methods

Solutions. Tris(hydroxymethyl)aminomethane (Tris)-buffered solution contained 150 mM KCl and 10 mM ethylenediaminetetraacetic acid (EDTA), pH 7.4 (adjusted by using 1 M Tris base at either 0 or 37 °C). This medium was employed to eliminate the possibility of net K exit-induced red cell volume changes (Carruthers & Melchior, 1983a).

Erythrocytes. Erythrocytes, obtained from freshly outdated blood bank whole blood, were washed 3 times in Tris medium and collected by centrifugation at 25000*g* for 15 min.

Sugar Transport Measurements. Sugar entry was determined under infinite-cis entry conditions by turbidimetry (Carruthers & Melchior, 1983a) and under zero-trans conditions by use of radiolabeled D-glucose.

Individual Procedures. (*A*) *Infinite-Cis Entry.* D-Glucose-free red cells (0.5 μL of packed cells) were injected into 400 μL of Tris medium containing 100 mM α- or β-D-glucose. Mixing and transport of the cell suspension to the light path of the thermojacketed photocell were complete within 100 ms. Upon exposure to D-glucose (100 mM), the cells first rapidly shrink due to osmotic water loss and then swell due to D-glucose entry. The turbidity of the suspension of cells reports the transport-induced volume changes (Carruthers & Melchior, 1983a) and may be used as a convenient means of monitoring the time course of sugar entry. The condition of infinite-cis entry requires that the external sugar transport sites are completely saturated with substrate. The external D-glucose concentration (100 mM) ensures that the external transport sites are 98.5% saturated (K_m = 1.6 mM, see below). The time course data may be plotted in the form

$$\ln (1 + S/P)/t \text{ vs. } S/T$$

and from the resulting linear transformation of the data, K_m is obtained from the slope, $(K_m + P + C_o)/[P(P + C_o)]$, and V_{max} from the y intercept, $-V_m K_m/[P(P + C_0)]$ (Baker & Naftalin, 1979; Carruthers & Melchior, 1983a). P is the osmolality of the sugar-free medium (332 mOsm), S the amount of sugar in the cells, and C_o the external sugar concentration (100 mM). It will be shown that both this transformation of the integrated infinite-cis Michaelis–Menten equation and the initial rate form of the equation provide accurate estimates of the Michaelis–Menten parameters for transport.

Turbidity measurements assume that the red cell behaves as a perfect osmometer and that the turbidity record is directly related to cell volume. Cell volume, V, is given by

$$V = \frac{PV_{rel} + S}{P + C_o}$$

where V_{rel} is the isotonic cell volume. Over the range of C_o values employed in this study, Rich et al. (1968) have shown that this relationship holds. Substituting D-glucose with sucrose or KCl (20–100 mOsm in excess of P), one observes for a given preparation of red cells that $E = b/V + c$ where b and c are experimentally variable constants and E is the apparent absorption of the red cell suspension. As osmotic water flow across the human red cell membrane is not subject to rectification (Viera et al., 1970) and red cell hydraulic conductivity is manyfold more rapid than hexose fluxes (Carruthers & Melchior, 1983b), these observations permit the rigorous application of the turbidity method to the determination of red cell hexose transfer rates.

(*B*) *Zero-Trans Entry.* D-Glucose-free red cells (125 μL of packed cells, maintained at 0.66 °C) were injected into 625 μL of ice-cold Tris medium (0.66 °C) consisting of D-[U-^{14}C]glucose containing α- or β-D-glucose (0, 0.066, 0.333, 0.666, 3.333, 6.666, or 16.666 mM) in a 1.5-mL Eppendorf tube. After 20 s, 0.5 mL of silicone oil (Dexter Hysol, XF-1792-B) was added, and the tube was centrifuged for 1 min in an Eppendorf bench centrifuge. Prior to centrifugation, the centrifuge rotor was maintained on ice. The cells are separated from the incubation medium within 10 s by this procedure and form a tight pellet isolated from the incubation medium by the oil. The carry-through of incubation medium into the pellet was estimated by simultaneous incubation in [^{3}H]inulin. The incubation medium was aspirated, the pellet was extracted in 40% perchloric acid and centrifuged, and the supernatant was counted. Each condition was assayed in triplicate. Estimates of initial rates of zero-trans D-glucose uptake at 0.66 °C obtained from 5-s incubation periods were not significantly different from those obtained with 20-s incubation periods (i.e., uptake was linear over 0–20 s).

Mutarotation Kinetics. A Zeiss polarimeter with a 2-dm thermojacketed polarimeter tube and a Na lamp were used to measure the specific rotations of D-glucose at 0.6 and 36.6 °C.

The ability of red cells to accelerate the rate of mutarotation of α-D-glucose was examined under three different experimental conditions. Red cells (30 mL of packed, washed cells) were incubated in 300 mM α-D-glucose (30 mL) for 1 h at 0.6 °C, the incubation medium was collected by centrifugation, and the specific optical rotation of the sugar in solution was determined. The cells were also collected, and the sugar within the cells was collected by two methods. Half of the cell pellet was immediately lysed in 7.5 mL of ice-cold hypotonic (KCl-free) Tris medium and the released sugar collected by centrifugation (to pellet the membranes). The remaining cells were placed in ice-cold sugar-free Tris medium (7.5 mL), and the sugar was collected by permitting sugar efflux to proceed for 0.5 h. Parallel measurements of the time course of sugar exit at this temperature by turbidimetry indicated that within this time intracellular glucose had equilibrated with the extracellular medium. Throughout the course of these experiments, 300 mM α-D-glucose medium was kept on ice to provide an estimate of the rate of mutarotation in the absence of red cells. Possible interference by hemoglobin was avoided by ultrafiltration of all samples at 0.6 °C using a 10000 molecular weight cutoff filter (Diaflo, YM10). Specific rotations (R) were calculated from observed rotations (R_o) according to the normal procedure:

$$R = \frac{100R_o}{cl}$$

where c is the concentration of sugar in grams per 100 mL and l the length of the polarimeter tube in decimeters.

Source of Materials. α and β anomers of D-glucose were purchased from Sigma Chemical Co. (St. Louis, MO). Purity was verified by measurement of specific optical rotations and by melting point analysis by differential scanning calorimetry

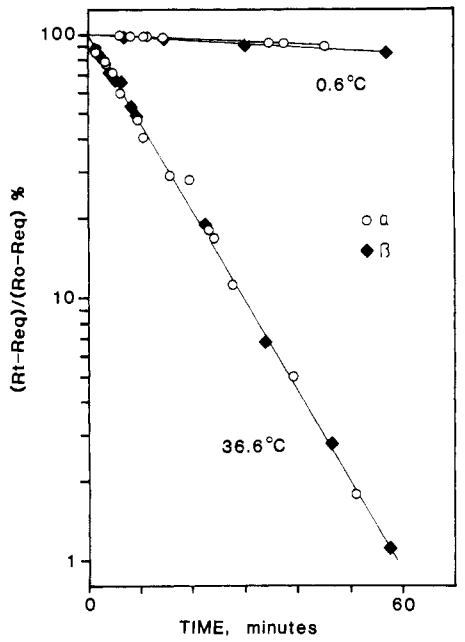

FIGURE 1: Time course of α- and β-D-glucose mutarotation at 0.6 and 36.6 °C. Ordinate: Observed specific optical rotation at time t (R_t) minus the specific optical rotation at equilibrium (R_{eq}) divided by the specific optical rotation at time 0 (R_0) minus R_{eq}. Abscissa: time in minutes. At time 0, 20 mL of fresh D-glucose anomer (20 g/100 mL) was added to the polarimeter tube, and optical rotations were measured at the indicated time points. For ease of presentation, the β-D-glucose data are plotted as $(R_{eq} - R_t)/(R_{eq} - R_0)$. α-D-Glucose data are represented by the circles (○) and β-D-glucose data by the diamonds (♦). The temperatures at which measurements were made are shown on the graph. The final specific optical rotation of all samples was 52.7°.

(Carruthers & Melchior, 1983b). The melting points of crystalline α- and β-D-glucose were 146.2 and 151.1 °C, respectively.

RESULTS

Specific Optical Rotations. (A) Time Courses. Figure 1 illustrates the time course of mutarotation of α- and β-D-glucose in Tris medium at 0.6 °C (pH 7.4) and 36.6 °C (pH 7.4). The mutarotation of α- and β-D-glucose follows first-order reaction kinetics, indicating that the main constituents of the equilibrium solution contain only α- and β-pyranose forms of the molecule with no substantial amounts of intermediate forms. The absence of significant amounts of forms of glucose other than the α- or β-pyranose anomers has been confirmed by chemical (Pigman & Anet, 1972) and NMR techniques (Angyal, 1972). The mutarotation coefficients for α- and β-D-glucose at 0.6 °C are (2.25 ± 0.192) and (1.73 ± 0.42) × 10^{-3} min^{-1}, respectively, and at 36.6 °C are (69.3 ± 11.5) and (75.2 ± 5.1) × 10^{-3} min^{-1}, respectively. These values are some 2-fold greater than those reported for mutarotation in water (pH 7; Pigman & Anet, 1972) and 65% of those reported for mutarotation in isotonic NaCl–phosphate buffer, pH 8 (Faust, 1960). These results are consistent with the observed acceleration of mutarotation at pH values greater than 7 (Pigman & Anet, 1972; Faust, 1960). Assuming that the mutarotation coefficients increase logarithmically with temperature, then the activation energies (E_a) for α- and β-D-glucose mutarotation are 16 and 17.6 kcal mol^{-1} K^{-1}, respectively. These values are not statistically significantly different from the reported value of 17.2 kcal mol^{-1} K^{-1} (Pigman & Anet, 1972).

(B) Red Cell Mutarotase Activity. Table I shows that the specific optical rotation of initially pure α-D-glucose was unaffected by exposure to the external surface of red cells, by exposure to the cells' interior, and by transport into or out of the cell at 0.6 °C. Concentrations of α-D-glucose were calculated by assuming an intracellular red cell water content of 70% and a mean estimate of equilibration of extracellular sugar with the cell water at this temperature during the period of incubation (44.5 ± 2.8%, obtained by turbidimetry). At 0.6 °C, metabolism of D-glucose is negligible (Baker & Naftalin, 1979). While the use of low temperature may not indicate whether the red cell has mutarotase activity at physiological temperature, the results are important for they indicate that at low temperature the transport assays are not compromised by accelerated mutarotation of the anomers.

Table I: Mutarotase Activity of the Red Cell[a]

condn	sp optical rotation	incubn time (min)	% mutarotation
300 mM α-D-glucose	111.9	0.5	0.17
incubation medium	105	70	12
lysate	105	80	12
effluent	104.2	110	13
control 300 mM α-D-glucose[b]	105	110	12
equilibrium	52.7		100

[a] Red cells were incubated in 300 mM fresh α-D-glucose for 60 min and then separated from the incubation medium by centrifugation. The cells were then either lysed to obtain intracellular sugar (lysate) or mixed with sugar-free medium for 30 min to obtain intracellular sugar by efflux (effluent). All experiments were performed at 0.6 °C. [b] This medium was not exposed to red cells.

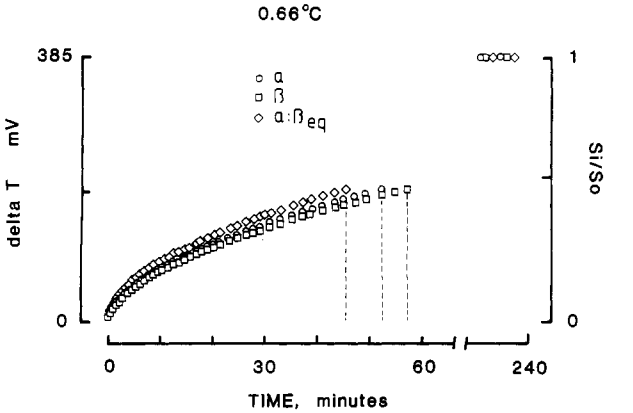

FIGURE 2: Time course of α- and β-D-glucose uptake and an equilibrium mixture of α- and β-D-glucose (36.2:63.8 molar ratio) uptake at 0.66 °C. Ordinates: change in turbidity of red cell suspension (spectrophotometer output) (in millivolts) (left ordinate) and the internal sugar concentration at time t (S_i) divided by the external sugar concentration (S_o, 100 mM) (right ordinate). Abscissa: time in minutes. The three curves shown [α-D-glucose (○); β-D-glucose (□); α-D-glucose–β-D-glucose (◊)] represent data that best approximate the mean behavior of each experimental group (see Table II). Kinetic analysis according to the integrated rate approach indicates the following: (○) K_m = 5.2 ± 2.7 mM, V_{max} = 6.4 ± 1.6 mmol L^{-1} min^{-1}; (□) K_m = 4.9 ± 2.6, V_{max} = 6.1 ± 1.5 mmol L^{-1} min^{-1}; (◊) K_m = 4.5 ± 2.3, V_{max} = 9.1 ± 1.4 mmol L^{-1} min^{-1}. The curves are discontinued at that time where S_i/S_o = 0.5. Final equilibrium levels are shown to the right of the curves. Within experimental error, the total turbidity changes for each group (for a given preparation of red cells) were identical. This final equilibrium is some 5 ± 3% lower than the turbidity of an equal volume of cells injected into isotonic medium, indicating that both α-D-glucose and β-D-glucose penetrate and equilibrate with the intracellular water to an equal extent.

Kinetics of α- and β-D-Glucose Transport. (A) Infinite-Cis Entry at 0.66 °C. Figure 2 illustrates the time course of infinite-cis α- and β-D-glucose uptake at 0.66 °C. Also shown is the time course of uptake of an equilibrium mixture (36.2:63.8 molar ratio) of α-D-glucose–β-D-glucose. Following the analysis of Faust (1960), the half-times for equilibration

with α-D-glucose, β-D-glucose, and the mixture of anomers are 51.6, 55.9, and 42.7 min, respectively. The differences between these numbers are somewhat misleading for in 41 experiments we found these variations to lie within the scatter of our measurements.

Table II summarizes the results of these studies. The standard deviations for mean half-times for equilibration are large (±27–61%). It may be concluded, therefore, that there is no significant difference in the uptake of the anomers with respect to the half-time for equilibration. Since there is no significant mutarotation during the first few minutes of uptake (as demonstrated by our polarimetry measurements), we can analyze these data by using the integrated form of the infinite-cis entry Michaelis–Menten equation (Hankin et al., 1972) to obtain values for V_{max} for entry and K_m for infinite-trans exit. Table II shows the results of these estimates together with calculations of the theoretical half-times for equilibration based on the obtained Michaelis–Menten parameters. These values can be expected to be accurate since both Michaelis–Menten parameters are obtained from the initial portion of the uptake curves where the rate of change of net flux is greatest, the intracellular D-glucose concentration is close to the K_m (4–5 mM), and the degree of mutarotation is negligible. These calculations confirm that there is no significant difference between red cell α- and β-D-glucose uptake under conditions where mutarotation is severely depressed.

(B) Flux Measurements at 0.66 °C with Radiolabeled D-Glucose. Flux measurements with radiolabeled D-glucose are complicated by the likely presence of both α and β forms of the radiolabeled sugar. The simplest test to perform is therefore to determine whether unlabeled α- or β-D-glucose inhibits radiolabeled sugar uptake with equal affinity. This test is straightforward for even in the most difficult analytical circumstances (e.g., K_m for α-D-glucose uptake $\neq$ K_m for β-D-glucose uptake and V_{max} for α-D-glucose uptake $\neq$ V_{max} for β-D-glucose uptake), K_i for inhibition of total radiolabel uptake by cold α- or β-D-glucose will provide an accurate estimate of K_m for transport of the D-glucose anomer (assuming competition between anomers for transport—noncompetitive inhibition and uncompetitive inhibition are ruled out by virtue of the ability of the red cell to transport both α- and β-D-glucose; see above).

Figure 3 illustrates the findings of our competition studies. Total radiolabel uptake is inhibited with equal affinity by α- and β-D-glucose (apparent K_i = 1.6 ± 0.38 mM). We, therefore, conclude that α-D-glucose and β-D-glucose are transported with equal affinity by the red cell at 0.66 °C. This conclusion is directly supported by our calculations of the K_m for infinite-trans exit of α- and β-D-glucose. These radiolabel studies may be further exploited to determine whether V_{max} for zero-trans entry of the anomers differs. Recalculation of the data to express (instead of inhibition of uptake) the total molar uptake of sugar during a 20-s incubation period results in identical estimates of the apparent V_{max} for uptake of α- and β-D-glucose (see Figure 2). Assuming true initial rates are monitored during this period, then total uptake of D-glucose may be expressed by

$$v = \frac{V_\alpha[\alpha]}{[\alpha] + K_\alpha(1 + [\beta]/K_\beta)} + \frac{V_\beta[\beta]}{[\beta] + K_\beta(1 + [\alpha]/K_\alpha)}$$

where $K_\alpha = K_\beta = K_i$ for inhibition of label uptake, V_α and V_β are V_{max} for uptake of α- and β-D-glucose, and [α] and [β] are concentrations of α- and β-D-glucose in solution. In the absence of cold α- or β-D-glucose, [α] + [β] = 30 μM, and we assume an equilibrium mixture of anomers exists. Over the range of cold α- or β-D-glucose concentrations employed (0.066–16.666 mM), uptake is dominated by the V_α or V_β terms. Indeed, as total sugar uptake is monitored from, for example, β-D-glucose Tris medium, the molar uptake of tracer α-D-glucose (which is minimal at 10 μM [α]) will fall with increasing [β] and hence will not compromise our determination of V_β. As the apparent V_α and the apparent V_β were not significantly different, we conclude that V_{max} values for zero-trans entry of the D-glucose anomers at 0.66 °C are identical. This is directly supported by our calculations of V_{max} for infinite-cis entry.

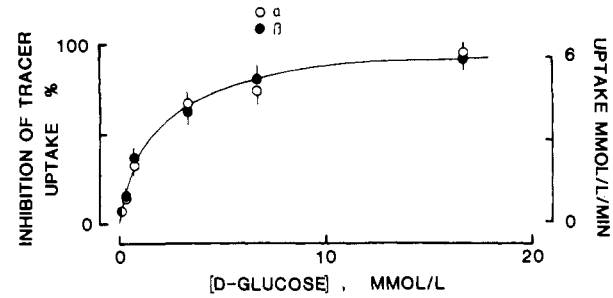

FIGURE 3: Effects of α- and β-D-glucose on uptake of D-[U-^{14}C]glucose (30 μM, specific activity = 2 μCi/mL) by intact red cells at 0.6 °C. Ordinates: percent inhibition of tracer uptake (left ordinate) and rate of total sugar uptake (in millimoles per liter of cell water per minute) (right ordinate). Abscissa: external α- or β-D-glucose concentration. Uptake rates were calculated on the assumption that tracer uptake was competitively inhibited by both D-glucose anomers. Figures 2 and 4 directly support this assumption by demonstration of α- and β-D-glucose uptake by red cells. The curve drawn through the points corresponds to a section of a single rectangular hyperbola with an apparent K_m and an apparent K_i of 1.6 ± 0.4 mM and maximum inhibition and V_{max} for uptake of 94.2 ± 5.2% and 6.45 ± 0.6 mmol (L of cell water)$^{-1}$ min^{-1}, respectively. Number of experiments per point, 3. Number of determinations per point per experiment, 3. The curve drawn through the points was calculated by nonlinear regression analysis assuming Michaelis–Menten kinetics according to the procedure of Duggleby (1981).

(C) Infinite-Cis Entry at 37 °C. Faust (1960) reported that the half-time for equilibration of D-glucose-free red cells with 300 mM β-D-glucose at 37 °C was some 3.5-fold lower than that for equilibration with α-D-glucose. We have repeated these experiments (using 100 mM D-glucose). Figure 4 shows one such experiment. Table III summarizes our findings. The mean half-time for equilibration of α- or β-D-glucose is not statistically different. In some 22 experiments with α-D-glucose, the standard deviation of the mean was ±89%, and in 18 experiments with β-D-glucose, the standard deviation of the mean was 87%. This variability cannot be accounted for by instrumental or analytical factors and most likely arises from differences in the various red cell preparations employed. These differences could include intrinsic membrane factors and residual cytosolic factors known to modulate sugar transport in the red cell (Carruthers & Melchior, 1983a). During the course of these experiments, significant mutarotation of D-glucose anomers occurs (see Figure 1). Nevertheless, K_m and V_{max} parameters are obtained from the first few seconds of the entry records (20–30 s) at which time less than 4% of the sugar has mutarotated. Once again, the calculated Michaelis–Menten parameters for infinite-cis α- and β-D-glucose uptake are indistinguishable.

Discussion

Intact human red cell hexose transfer shows an operational kinetic complexity that is not predicted by the intrinsic properties of either the symmetric or the asymmetric models of the simple mobile carrier hypothesis (Regen & Tarpley, 1974; Hankin et al., 1972; Baker & Naftalin, 1979; Widdas,

Table II: Kinetics of α- and β-D-Glucose Uptake at 0.6 °C[a]

	α						β						α-β					
					$t_{1/2}$ (min)						$t_{1/2}$ (min)						$t_{1/2}$ (min)	
expt	K_m (mM)	SD (mM)	V_{max}[b]	SD[b]	exptl	theor	K_m (mM)	SD (mM)	V_{max}[b]	SD[b]	exptl	theor	K_m (mM)	SD (mM)	V_{max}[b]	SD[b]	exptl	theor
1	7.70	4.14	5.76	1.46	42.70	42.04	6.80	3.55	10.62	2.61	24.80	25.20	1.80	0.90	19.20	4.51	42.70	45.42
	6.87	3.55	9.66	2.36	26.80	27.47	4.93	2.55	6.12	1.49	55.90	57.21	2.23	1.01	15.54	3.32	39.20	45.92
	3.07	1.57	16.26	3.93	31.60	32.72	4.76	2.54	6.48	1.63	56.05	55.69	1.36	0.70	10.14	2.45	108.40	112.23
2	6.46	3.49	6.60	1.68	43.10	42.28	1.22	0.63	9.66	2.35	127.30	130.73	1.10	0.54	30.00	6.95	43.10	46.51
	2.20	1.17	12.24	3.08	59.40	59.03	1.50	0.78	12.06	2.97	84.70	85.94	8.20	4.51	9.12	2.36	26.20	25.27
	2.95	1.58	11.82	3.00	47.30	46.67	1.64	0.86	10.26	2.53	91.40	92.81	8.50	4.73	9.12	2.39	25.80	24.57
3	1.70	0.90	12.06	3.01	76.20	76.32	1.46	0.77	12.48	3.09	84.30	85.21	4.50	2.31	11.42	2.76	32.10	33.16
	5.20	2.73	6.42	1.59	51.60	52.11	9.95	5.66	10.02	2.69	21.30	19.83	4.50	2.49	6.18	1.62	64.10	61.30
	5.90	3.07	6.06	1.49	48.80	49.64	9.94	4.83	10.02	2.30	18.20	19.85	3.72	1.41	6.24	1.12	51.30	71.76
4	3.10	1.66	10.02	2.53	53.10	52.63	9.99	5.06	9.66	2.31	19.60	20. 51	10.73	6.02	19.32	5.12	10.30	9.73
	2.30	1.23	10.92	2.76	64.20	63.49	4.21	2.19	9.96	2.45	39.60	40.31	5.61	3.05	11.28	2.89	28.50	27.82
	2.25	1.19	10.98	2.73	64.10	64.45	4.12	2.22	9.96	2.53	41.80	41.08	6.47	3.32	8.40	2.03	32.10	33.18
	2.27	1.21	10.98	2.77	64.50	63.92	3.92	2.01	9.48	2.29	43.60	45.09	2.00	1.04	12.00	2.94	64.50	65.82
													5.25	3.04	11.14	3.05	32.60	29.80
													5.07	2.57	11.24	2.69	29.10	30.42
mean ±	4.00 ±		9.98 ±		51.80 ±	51.75 ±	4.96 ±		9.75 ±		54.50 ±	55.34 ±	5.15 ±		11.97 ±		42.16 ±	43.96 ±
SD	2.11		3.06		13.97	13.76	3.29		1.78		33.53	34.27	2.85		6.32		25.23	27.38

[a] Experiments 1–4 represent paired experiments. Each set of kinetic parameters (K_m, V_{max}) represents the mean ± SD of three separate determinations. Experimental and theoretical half-times for equilibration ($t_{1/2}$) are shown for each data set. [b] V_{max} and SD in units of mmol L^{-1} min^{-1}.

Table III: Kinetics of α- and β-D-Glucose Uptake at 36.6 °C[a]

	α						β					
					$t_{1/2}$ (min)						$t_{1/2}$ (min)	
expt	K_m (mM)	SD (mM)	V_{max} (mmol L^{-1} min^{-1})	SD (mmol L^{-1} min^{-1})	exptl	theor	K_m (mM)	SD (mM)	V_{max} (mmol L^{-1} min^{-1})	SD (mmol L^{-1} min^{-1})	exptl	theor
1	1.60	0.79	102.60	25.40	9.41	9.50	3.50	1.74	78.00	19.37	6.02	6.06
	1.60	0.80	102.00	25.51	9.56	9.56	3.50	1.77	78.00	19.69	6.12	6.06
2	2.80	1.37	99.00	24.23	5.72	5.84	2.60	1.31	168.00	42.40	3.72	3.69
	2.90	1.45	117.60	29.44	4.77	4.76	3.10	1.56	84.00	21.11	6.31	6.28
	2.62	1.24	61.80	14.62	9.41	9.95	2.60	1.32	108.00	27.41	5.82	5.73
3	9.60	4.92	102.00	26.12	2.05	2.00	4.60	2.32	72.00	18.13	5.20	5.16
	10.40	5.24	60.60	15.28	3.20	3.17	11.30	5.85	264.00	68.37	0.71	0.69
4	3.60	1.82	234.00	59.09	1.99	1.97	3.90	1.98	72.00	18.26	6.05	5.96
	4.80	2.38	192.00	47.59	1.85	1.87	3.40	1.72	144.00	36.43	3.41	3.37
	3.20	1.58	246.00	60.82	2.06	2.08	4.30	2.10	204.00	49.89	1.89	1.93
5	7.20	3.70	162.00	41.60	1.62	1.58	9.20	4.38	228.00	54.24	0.88	0.92
	10.60	5.34	96.00	24.18	1.99	1.97	10.20	5.01	282.00	69.31	0.68	0.69
	10.40	5.24	60.60	15.28	3.20	3.17	9.00	4.74	342.00	90.01	0.66	0.63
6	6.90	3.30	324.00	77.41	0.78	0.82	6.90	3.38	324.00	79.39	0.80	0.82
	4.90	2.33	294.00	69.99	1.14	1.20	8.30	4.25	360.00	92.26	0.65	0.63
7	8.80	4.47	504.00	128.09	0.44	0.43	7.80	4.19	390.00	104.70	0.66	0.61
	14.60	7.16	450.00	110.38	0.33	0.34	11.30	5.85	264.00	68.37	0.71	0.69
8	3.20	1.56	510.00	124.37	0.98	1.00	6.10	3.21	474.00	124.79	0.65	0.62
9	2.30	1.17	792.00	201.30	0.89	0.88						
	1.10	0.55	270.00	67.80	5.19	5.17						
	2.00	0.99	96.00	23.86	8.18	8.23						
	2.01	1.01	63.60	15.98	12.42	12.36						
mean ±	4.97 ±		224.80 ±		4.27 ±	4.28 ±	5.95 ±		232.00 ±		2.82 ±	2.80 ±
SD	3.71		190.80		3.82	3.82	2.82		142.80		2.44	2.42

[a] Experiments 1–8 represent paired experiments. Each set of kinetic parameters (K_m, V_{max}) is the mean ± SD of three separate determinations. Experimental and theoretical half-times for equilibration ($t_{1/2}$) are shown for each data set.

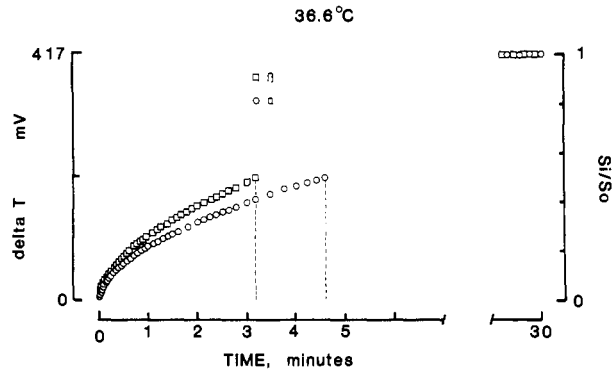

FIGURE 4: Time course of α- and β-D-glucose infinite-cis uptake at 36.6 °C. Ordinates: change in turbidity of red cell suspension (spectrophotometer output) (in millivolts) (left ordinate) and the internal sugar concentration at time t (S_i) divided by the external sugar concentration (S_o, 100 mM). Abscissa: time in minutes. Two sets of data are shown [(○) α-D-glucose and (□) β-D-glucose]. These data were chosen to best represent the statistical half-time averages of the two groups (complete data sets are given in Table III). The curves are discontinued when $S_i/S_o = 0.5$. Kinetic analysis indicates the following: (○) $K_m = 2.9 \pm 1.5$ mM, $V_{max} = 117 \pm 30$ mmol L^{-1} min^{-1}; (□) $K_m = 3.6 \pm 1.3$ mM, $V_{max} = 172 \pm 44$ mmol L^{-1} min^{-1}. Final equilibrium levels are shown to the right of the curves. Within experimental error, the total turbidity changes for each group (for a given preparation of red cells) were identical. As with turbidity measurements made at 0.6 °C, the final turbidity level was not significantly different from that of an equal volume of cells injected into isotonic medium.

1980; Carruthers & Melchior, 1983a). It has been suggested (Naftalin & Holman, 1977; Baker & Naftalin, 1979) that the observed complex operational kinetics of transfer arise from factors extrinsic to the transport system itself. The simplest model accounting for the observed properties of intact red cell hexose transfer proposes that the transport system is intrinsically kinetically symmetric and that the well-documented [see Naftalin & Holman (1977)], nonspecific adsorption of water and D-glucose by intracellular hemoglobin leads to an overestimation of K_m for zero-trans D-glucose exit and an underestimation of V_{max} for entry (Naftalin & Holman, 1977). This hypothesis was tested directly by removing intracellular hemoglobin from red blood cells together with other cytosolic factors by forming ghosts and then reexamining the kinetic properties of the system. D-Glucose transport kinetics in the nominal absence of cytosolic hemoglobin (and other cellular constituents) were observed to be symmetric (Carruthers & Melchior, 1983a). Further experiments demonstrated that the effector of operational kinetic asymmetry was not hemoglobin, but rather some cytosolic factor of molecular weight 10 000 or less (Holman, 1980; Carruthers & Melchior, 1983a). The general conclusion is, therefore, that the effectors of the operational complexity of transport are factors extrinsic to the transport system. Indeed, a number of recent studies indicate that ATP may modify human red cell hexose transfer kinetics (Jacquez, 1983) and that products of hexose metabolism may modify transport in rat and dog erythrocytes (Abumrad et al., 1984; A. Carruthers, D. L. Melchior, and H. M. Goodman, unpublished results).

While these considerations point to the extrinsic modulation of the transport system by cytosolic factors, the possibility still exists that systematic errors arising from assumptions in the analysis of transport could account for the complex operational kinetics of the transport system. Gorga & Lienhard (1981) have suggested that should α- and β-D-glucose be transported differently by the red cell, then the analytical assumptions of the above studies would be invalidated. Only one previous study has described the α- and β-D-glucose transport properties of the human red cell (Faust, 1960). This study, using turbidity measurements, reported that β-D-glucose (300 mM) penetrated the interior of the red cell some 3-fold faster than α-D-glucose. However, there are a number of reasons to believe that some systematic error may have been introduced into these determinations. (1) The reported complex pH dependence of D-glucose uptake (with maximum rates at pH 7) was not confirmed by later studies (Naftalin & Holman, 1977; Widdas, 1980). (2) The lack of dependence of half-times for equilibration at pH 7 upon D-glucose concentrations between 6 and 300 mM is inconsistent with both first- and second-order transport kinetics. (3) The reported experimental half-times for D-glucose and D-galactose (300 mM) equilibration at 29 and 37 °C are some 3-fold lower than would be expected from the known temperature dependence of transport of these sugars (Widdas, 1980).

Naftalin & Holman (1977) have interpreted the studies of Faust (1960) as being consistent with the view that β-D-glucose is transported with higher affinity (lower K_m) by the red cell than is α-D-glucose. Our studies demonstrate that this interpretation is incorrect. In fact, α-D-glucose and β-D-glucose share a common affinity (K_m) for the transfer system. The results of Faust (1960) would be more in keeping with the view that the V_{max} for β-D-glucose entry is greater than V_α or that the K_m for β-D-glucose infinite-trans exit is greater than K_α. As neither of these predictions has been confirmed experimentally, we believe that those systematic errors (possibly net K^+ exit-induced red cell volume changes) that influenced Faust's other findings may also have led to errors in the determination of half-times for α- and β-D-glucose entry.

Our studies demonstrate that K_m values for both zero-trans entry and infinite-trans exit of D-glucose anomers are indistinguishable under conditions where mutarotation of the anomers is negligible. Further, at temperatures where D-glucose mutarotation is accelerated but still slow with respect to the time points required for accurate determinations of V_{max} and K_m for infinite-cis entry or where the cells are presented with an equilibrium mixture of anomers, the time courses of sugar uptake are indistinguishable. Any mixture of α and β anomers of D-glucose therefore represents a homogeneous source of substrate for the transfer system. This means that the integrated rate analysis of infinite-cis entry is a rigorous analytical procedure for determination of velocity and Michaelis parameters for glucose transport [although see Baker & Naftalin (1979) and Foster et al. (1979) for limitations]. Moreover, the complex operational kinetic parameters of red cell hexose transfer remain inconsistent with the simple symmetric and asymmetric forms of the mobile carrier model for transport (Widdas, 1980; Gorga & Lienhard, 1981). Future studies describing both molecular and kinetic models for red cell hexose transport must account for these complex properties of the transport system.

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Boundary Centrifugation in Isovolumetric and Isokinetic Cesium Sulfate Density Gradients: Application to Cartilage Proteoglycans and Other Macromolecules†

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ABSTRACT: A boundary sedimentation methodology is described that avoids plateau dilution and simplifies the calculation of centrifugal parameters. The technique is designed for the preparative ultracentrifuge and uses a newly developed sectorial cell. It is based on previous developments of the transport method and depends on isokinetic or isovolumetric Cs_2SO_4 density and viscosity gradients. These gradients are prepared with a single-chamber mixing device, and the only two parameters required for their calculations are presented in a tabulated form for general use with most available rotors and cell sizes. Conditions are specified (1) to assure that the density and shape of the sedimenting molecules remain invariant through the selected electrolytic gradient, (2) to monitor the gradient profiles, and (3) to verify attainment of isokinetic or isovolumetric sedimentations. A set of equations is presented to calculate the average and transport sedimentation coefficients and the differential sedimentation coefficient distribution for both the isokinetic and isovolumetric centrifugal regimes. The method was applied to slowly diffusing polydisperse proteoglycan monomers, to a paucidisperse DNA from bacteriophage PM2, and to a diffusible monodisperse system (purified bovine serum albumin). In all cases, the expected results were obtained.

In earlier publications, we described the use of the transport method for the centrifugal characterization of cartilage proteoglycans and other biological macromolecules in the preparative ultracentrifuge (Pita & Muller, 1972, 1973a,b; Pita et al., 1978, 1979, 1983). The present work describes a velocity sedimentation technique that is based on the same principles and incorporates the use of specially designed exponential gradients in combination with a newly developed sectorial cell (Pita et al., 1983). The gradient can be made to avoid plateau dilution during centrifugation and to substantially simplify the mathematical analysis of the sedimenting boundaries. Special care will be taken in this work to select a gradient medium that will not erratically affect the sedimenting molecules during sedimentation. The present technique has been especially designed to study polydisperse and slowly diffusing molecules such as cartilage proteoglycans, DNA, hyaluronate, etc. The practical parameters given below in Table III, however, have been calculated with sufficient generality as to render them useful for a variety of biological molecules within a density range of 1.25–1.85 g/cm^3, and for almost all available centrifugation cells and swinging-bucket rotors.

Following a different approach, preparative rather than analytical, several researchers have used, previously, exponential gradients in connection with zonal velocity centrifugation. The latter technique has been used in the fractionation of complex cell particles and biological macromolecules in the presence of sucrose isokinetic or equivolumetric gradients (Schumaker, 1967; McCarty et al., 1968; Spragg et al., 1969; Noll, 1969; Pollack & Price, 1971; Vanduffel et al., 1975). Although there is no reason why zonal centrifugation could not be used as an analytical technique, the usual approach, however, for studying differential distribution functions of s

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