Biochimica et Biophysica Acta 815 (1985) 75–84
Elsevier

BBA 72522

Asymmetric transport of a fluorescent glucose analogue by human erythrocytes

Louis Speizer [a], Richard Haugland [b] and Howard Kutchai [a]

[a] *Department of Physiology and Biophysics Program, University of Virginia, Charlottesville, VA 22908 and* [b] *Molecular Probes, Inc., 24750 Lawrence Road, Junction City, OR 97448 (U.S.A.)*

(Received August 8th, 1984)
(Revised manuscript received December 17th, 1984)

Key words: Hexose transport; Glucose analogue; Transport asymmetry; Fluorescence; (Erythrocyte membrane)

A fluorescent glucose analogue, 6-deoxy-*N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)-aminoglucose (NBDG), was synthesized and its interactions with the hexose transport system of the human red blood cell were investigated. NBDG entry is inhibited by increasing concentrations of D-glucose ($K_i = 2$ mM). However, NBDG exit is unaffected by D-glucose in red blood cells. Cytochalasin B was found to inhibit both NBDG entry and exit. NBDG accumulates in the red blood cell above the theoretical equilibrium concentration. Accumulation of NBDG is temperature-sensitive and is due to the binding of NBDG to some intracellular substance. The binding of NBDG to purified hemoglobin suggests that accumulation of NBDG by erythrocytes is due to the intracellular binding of NBDG to hemoglobin. NBDG does not accumulate in pink erythrocyte ghosts, while its rate of uptake is still inhibited by D-glucose and cytochalasin B. Although there was no apparent D-glucose inhibition of NBDG exit by intact red blood cells, D-glucose was able to inhibit NBDG exit by pink erythrocyte ghosts. The differing properties of NBDG influx and efflux support the interpretation that the hexose transport system of the human red blood cell appears asymmetric although it may be intrinsically symmetric.

Introduction

Current methods for monitoring sugar transport activity in erythrocytes depend primarily on either radioisotopically-labeled sugars or changes in light scattering caused by red cell volume changes induced by sugar transfer. Fluorescence spectroscopy offers a more sensitive method for monitoring transport. Prior to this report, only one other fluorescent glucose analogue had been reported; (2-dansylglucosamine), which was used as a competitive inhibitor of hexokinase [1]. NBDG is the first fluorescent hexose derivative synthesized to probe the nature of the glucose transport system.

D-Glucose and certain other monosaccharides enter the human red blood cell via the hexose transporter [2]. There are a number of inhibitors of glucose transport, such as cytochalasin B [3,4], ethylidene glucose [5,6], certain steroids [7], benzoic acid derivatives [8], sugar acetals [9] and 6-*O*-substituted hexoses [10,11]. Structure-activity relationships with various transport measurements have led to several theories on the mechanism of glucose transport, most of which were reviewed by Naftalin and Holman [12]. Additional reviews on the glucose transport system include those of Jung [13], LeFevre [14], Widdas [15] and Carruthers [16]. Current theory suggests that hexose transfer in human red blood cells is an asymmetric process

Abbreviation: NBDG, 6-deoxy-*N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)aminoglucose.

[12,15,17]. It has been suggested that the hexose transporter is intrinsically symmetric, but asymmetry develops due to some extrinsic feature, such as hemoglobin [12], or interaction of the transfer system with a low molecular weight cytosolic factor [17]. An apparent asymmetry in the glucose transport system has been demonstrated using ethylidene glucose [5,6,18,19], and 6-*O*-alkyl-substituted glucose analogues [10,11].

An ideal fluorescent analogue for the hexose transporter should be strongly fluorescent, and should have high affinity for the transporter. In designing a fluorescent analogue we chose the 7-nitrobenz-2-oxa-1,3-diazol-4-yl (NBD) group for its strong fluorescence and we chose the C-6 position of D-glucose as the site for covalent attachment of the fluorophore because it has been suggested that a hydrophobic region of the hexose transporter is in close proximity to this part of the glucose molecule [20,21] during some part of the transport cycle. In this paper, we describe the synthesis of NBDG and characterize its interaction with the glucose transport system of the human red blood cell.

Materials and Methods

Synthesis of NBDG. To 0.5 g 6-amino-6-deoxyglucose hydrochloride dissolved in 10 ml of 0.377 M Na_2CO_3 was added a solution of 0.5 g 4-chloro-7-nitrobenz-2-oxa-1,3-diazole (NBD chloride) in 20 ml acetonitrile. The reaction mixture was stirred at room temperature for 18 h during which time it became very dark. The reaction mixture was partially concentrated under vacuum to remove the acetonitrile. Dilution to 50 ml with water and adjusting to pH 2 with hydrochloric acid gave a dark solid which was filtered, washed with water and discarded. The filtrate was passed through a short column of DEAE-cellulose which retained additional dark impurities. The orange-colored eluate was evaporated to near dryness, then passed through a 22×350 mm Sephadex LH-20 column and eluted with H_2O. The fractions that contained the bright yellow fluorescent product and were free from the dull-yellow fluorescent impurity (4-hydroxy-7-nitrobenz-2-oxa-1,3-diazole) were combined, dissolved in a small volume of methanol and precipitated with ether to give a bright red solid. The yield of pure NBDG was 50–60 mg. On thin-layer chromatography on silica gel sheets (E. Merck) using butanol saturated with water, the product showed a single fluorescent spot with $R_F = 0.44$.

Preparation of erythrocytes. Human blood was used either immediately following venipuncture, or after storage (for no more than 1 week) in a citrate/phosphate/dextrose/anticoagulant storage solution (2.1 mM citric acid/10.8 mM sodium citrate/17.1 mM glucose/1.9 mM monobasic sodium phosphate). Prior to each experiment, the blood was washed four times in phosphate-buffered saline (5 mM Na_2HPO_4/150 mM NaCl/1 mM NaN_3 (pH 7.4)) and the buffy coat was removed.

Preparation of pink erythrocytes ghosts. Pink erythrocyte ghosts were made by hemolysis reversal, using the method of Jacquez [22] with the addition of 100 μM PMSF (phenylmethylsulfonyl fluoride). Washed red blood cells were suspended to a 50% hematocrit. 10 ml of red blood cells were added to 100 ml of hemolyzing solution at 4°C (hemolyzing solution: 4 mM $MgCl_2$/4.5 mM Tris-HCl/0.5 mM EGTA/1 mM KH_2PO_4/10 μM $CaCl_2$/100 μM PMSF (pH 7.1)) and hemolysis was allowed to proceed for 5 min. 15 ml of 1.254 M KCl was then added to restore tonicity to 308 mosmol. The cells were kept on ice for 5 additional min and then placed in a shaking waterbath at 37°C. After 60 min, the cells were centrifuged at $20\,000 \times g$ for 10 min. The pink ghosts were resuspended with phosphate-buffered saline and washed twice.

Measurement of intracellular solvent water. The intracellular water available for solvent exchange was determined by the method of Ponder [23]. Red blood cells or pink erythrocyte ghosts were placed in solutions of varying tonicities and the volume changes of the cells were measured by taking microhematocrits. The plot of the fractional change in volume versus tonicity yields a straight line with both slope and intercept equal to that fraction of cell water which participates in osmotic water movements.

Measurement of hemoglobin. The cyanmethemoglobin method was used to quantify the amount of hemoglobin present in red blood cells, lysates and erythrocyte ghosts [24].

Influx determinations. The fluorescence of

NBDG was used to estimate the amount of NBDG present in the red blood cells. Washed red blood cells were incubated at 37°C for 60 min with or without D-glucose, cytochalasin B or other chemical agent. The red blood cells (20% hematocrit) were then suspended in 108 μM NBDG (in phosphate-buffered saline), and placed in a shaking water-bath at 37°C. At various times, 0.2 ml aliquots of the red blood cell suspension were placed in a transport stopping solution [25] consisting of 1% NaCl/1 μM $HgCl_2$/1.25 mM KI/100 μM phloretin at 4°C. The red blood cells were sedimented in an Eppendorf Microfuge by spinning for 1 min at 15600 × *g* and washed two additional times in stopping solution. The red blood cell pellet was then hemolyzed in 2.5 ml Tris buffer (5 mM Tris-HCl/5 mM NaCl (pH 6.5)), and 0.38 ml of 20% trichloroacetic acid added to precipitate the hemoglobin. The fluorescence intensity of the supernatant was measured in a Perkin-Elmer 650-10S spectrofluorimeter. Excitation and emission wavelengths were 470 and 538 nm, respectively. The intracellular water available to NBDG which participates in osmotic water movements [23] was determined to be 0.62 ml water/ml red blood cell and 0.87 ml water/ml pink erythrocyte ghost. The intracellular concentration of NBDG was determined as follows:

V_i = intracellular solvent water = 0.2 ml × crit

× 0.62 (0.87 for pink ghosts)

$[NBDG]_{intracellular}$

$= [NBDG]_{measured\ spectrofluorimetrically}$

$\times (2.5 + 0.38 + V_i)/V_i$

Efflux determinations. Washed red blood cells (20% hematocrit) were incubated with 108 μM NBDG (in phosphate-buffered saline) at 37°C for 480 min. The NBDG-loaded red blood cells were then washed four times in phosphate-buffered saline at 4°C, and resuspended to a 2% hematocrit in either phosphate-buffered saline, or phosphate-buffered saline with either D-glucose or cytochalasin B. These cells were then incubated at 37°C in a shaking water-bath. At various times, aliquots of the red blood cells were washed three times in stopping solution and prepared for measurement of NBDG content as described above.

Pink erythrocyte ghosts were incubated with 200 μM NBDG (in phosphate-buffered saline) at 37°C for 130 min. The NBDG-loaded pink erythrocyte ghosts were then pelleted, the supernatant removed, and the ghosts resuspended to a 2% hematocrit in either phosphate-buffered saline or phosphate-buffered saline with either 32 mM D-glucose or 32 mM L-glucose. The ghosts were then incubated at 37°C in a shaking water-bath and at various times aliquots of the ghosts were washed three times in stopping solution and prepared for measurement of NBDG content as described above.

Equilibrium dialysis. The extent of NBDG binding to cellular constituents was estimated by equilibrium dialysis. Intact red blood cells were packed (93% hematocrit) and then lysed by repetitive freeze-thaw. In an equilibrium dialysis cell (Fisher Scientific), 0.2 ml of either cellular lysate, purified hemoglobin or intact red blood cells were placed on one side of a M_r 1000 cutoff dialysis membrane (Spectrapor), and 0.8 ml phosphate-buffered saline buffer containing NBDG was placed on the other side of the membrane. The dialysis cells were then placed at 37°C and the concentration of NBDG in both chambers of the dialysis cell was measured at equilibrium (1500 min later).

Data analysis. Non linear least-squares analysis of the data was performed on a PDP-11/23 minicomputer utilizing the program NONLIN [26].

Results

Spectra and sensitivity of NBDG

The structure of NBDG and its absorption and emission spectra are shown in Fig. 1A. In Tris buffer (pH 6.5), NBDG has an excitation maximum at 470 nm, and an emission maximum at 538 nm. NBDG in aqueous solution can be easily measured at concentrations as low as 50 nM (Fig. 1B). The fluorescence intensity of NBDG in ethanol is about 10-times greater than in H_2O.

D-Glucose inhibition of NBDG uptake

NBDG uptake by human red blood cells is inhibited by D-glucose at concentrations close to

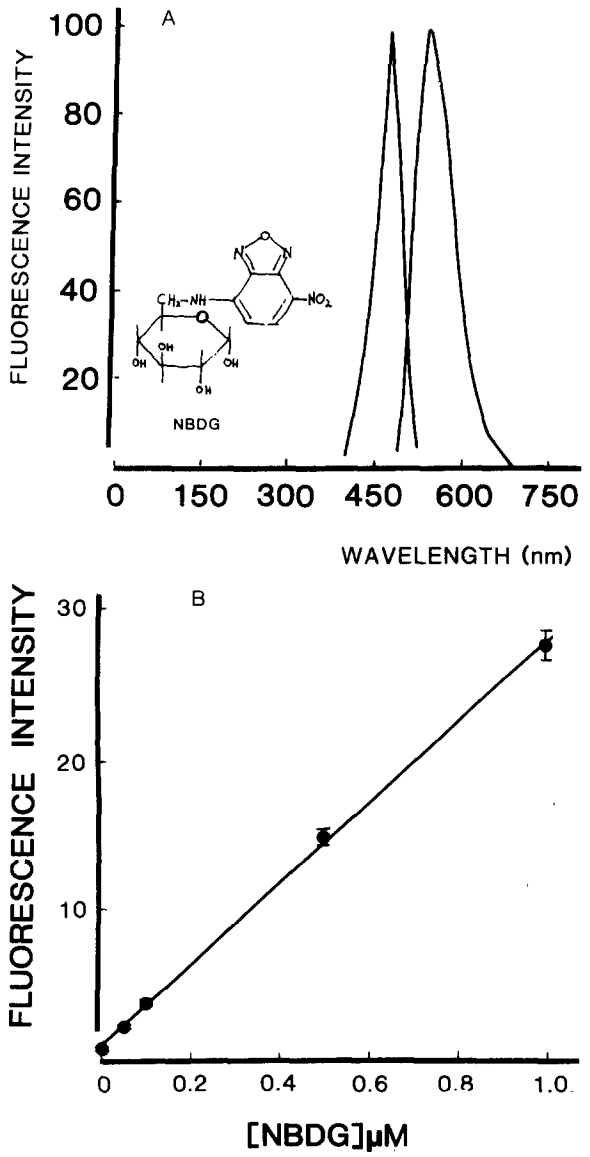

Fig. 1. (A) Excitation and emission spectra for NBDG in 5 mM Tris buffer (pH 6.5). (B) Fluorescence sensitivity of NBDG. 40 μl intact red blood cells were lysed with 2.5 ml 5 mM Tris buffer and 0.38 ml trichloroacetic acid (20%, w/v). Varying amounts of NBDG were added and fluorescence intensity of the supernatant was measured. Excitation and emission wavelengths were 470 and 538 nm, respectively. Displayed are the mean ± S.D. of seven determinations. The standard curve was fit with a straight line to: $RFI = 27.2 \times [NBDG]_{\mu M} + 0.852$ $r = 0.999$.

the K_m of D-glucose for hexose transport in the red blood cell (Fig. 2A). The K_m for glucose uptake by human red blood cells is about 2 mM [12].

Stereospecific inhibition of NBDG uptake

D-Glucose appears to inhibit the rate of NBDG uptake in a stereospecific manner. While D-glucose inhibits the rate of uptake of NBDG, L-glucose, a non-transported stereoisomer of D-glucose has no effect on NBDG uptake (Fig. 2B). 3-*O*-Methylglucose, a transported (non-metabolizable) analogue of D-glucose, also inhibits the rate of NBDG uptake into human red blood cells (Fig. 2C). Cytochalasin B has been shown to affect a number of cellular processes, including glucose transport [3,27]. Cytochalasin B also inhibits the rate of NBDG uptake in human red blood cells (Fig. 2C). Cytochalasin E competes with cytochalasin B for binding sites on actin; however, cytochalasin E does not inhibit glucose transport [4]. Cytochalasin E does not inhibit the uptake of NBDG (Fig. 2C). Thus, NBDG uptake is inhibited by compounds which enter the red blood cell by the hexose transporter (or compete for binding sites on the transporter), but the uptake of NBDG is not inhibited by closely related structural analogues which are not transported by the hexose transporter (or do not compete for binding sites on the transporter).

Lack of effect of D-glucose on NBDG exit

When red blood cells are preloaded with NBDG and then resuspended to a 2% hematocrit, the rate of exit of NBDG is unaffected by D-glucose concentrations as high as 32 mM (Fig. 3A). These results suggest that the exit of NBDG is not via the hexose transporter, but perhaps by diffusion.

Inhibition of NBDG exit by cytochalasin B

In contrast to the effects of D-glucose on NBDG exit, cytochalasin B does inhibit NBDG exit from the red blood cell (Fig. 3B). While D-glucose inhibits only NBDG influx by intact red blood cells, cytochalasin B inhibits both influx and efflux.

Accumulation of NBDG by red blood cells

NBDG accumulates inside the red blood cell above its theoretical equilibrium concentration. At equilibrium, if the intracellular and extracellular concentrations of NBDG are equal, the NBDG concentration should be 108 μM. However, the intracellular concentration of NBDG is more than twice the extracellular concentration of NBDG at equilibrium.

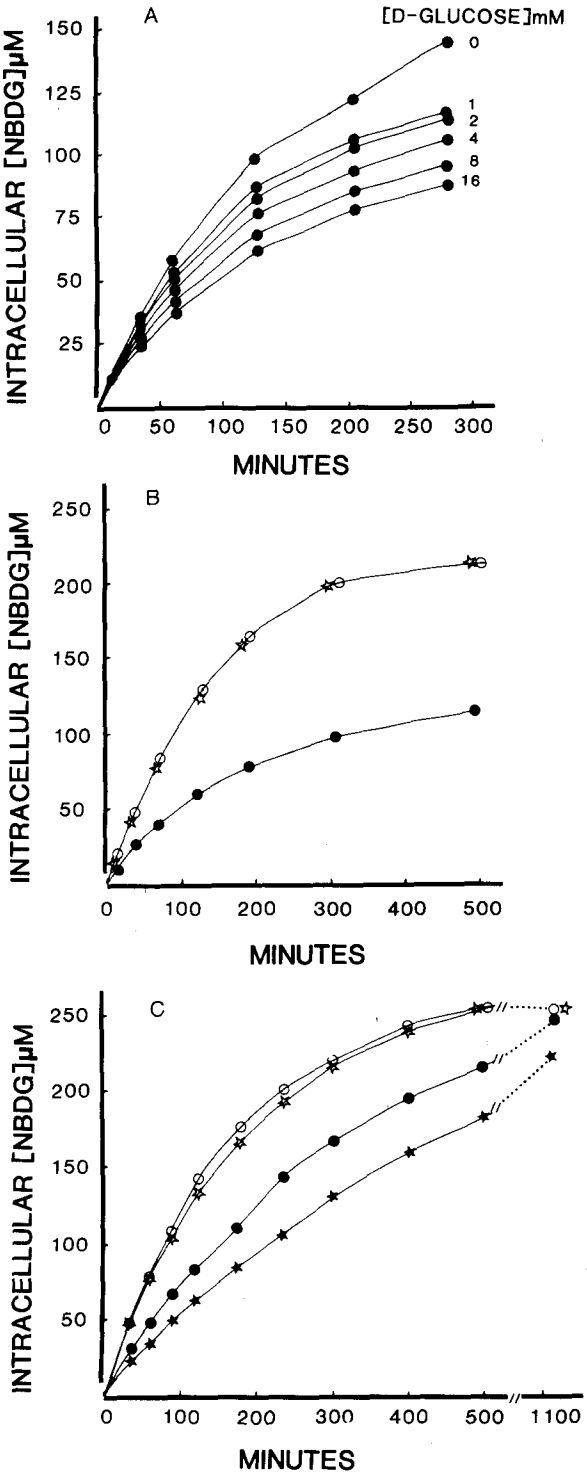

Fig. 2. (A) D-Glucose inhibition of NBDG uptake by human erythrocytes. (B) Stereospecific inhibition of NBDG uptake. NBDG uptake was determined in the absence (○) or presence (●) of 32 mM D-glucose or 32 mM L-glucose (☆). (C) NBDG uptake in the absence (○) or presence (●) of 32 mM 3-*O*-methylglucose, 10 μM cytochalasin B (★), or 10 μM cytochalasin E (☆).

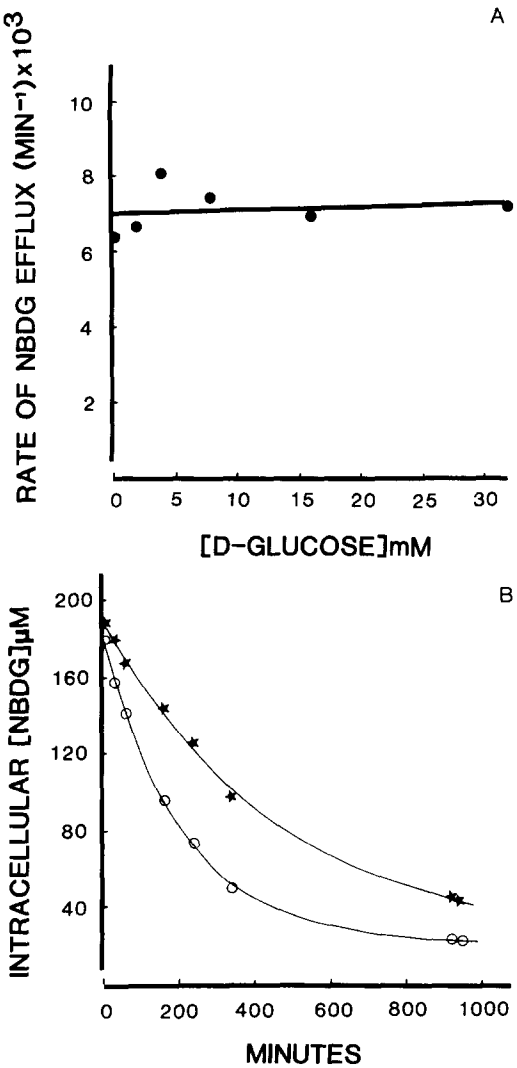

Fig. 3. (A) Lack of effect of D-glucose to inhibit the rate of NBDG exit from human erythrocytes. The efflux of NBDG from loaded red blood cells was first-order in time. Displayed are the first-order rate constants for NBDG exit plotted against D-glucose concentrations. The best-fit line through the data is also shown with a slope not significantly different from zero. (B) Inhibition of NBDG exit by cytochalasin B. NBDG-loaded red blood cells were resuspended in phosphate-buffered saline in the absence (○) or presence (★) of 10 μM cytochalasin B.

The accumulation of NBDG is temperature-sensitive (Fig. 4). Intact red blood cells accumulate NBDG much faster at 37°C than they do at 22°C.

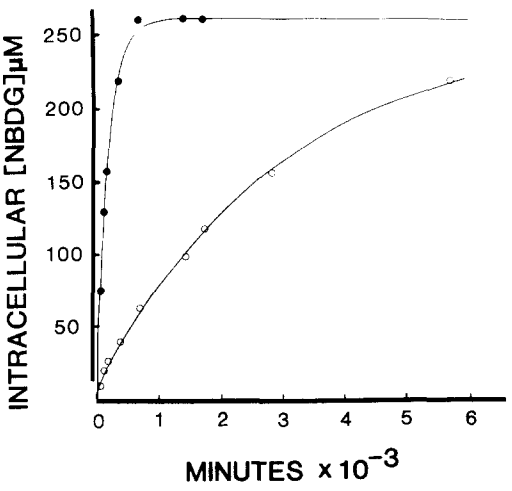

Fig. 4. Effect of temperature on NBDG uptake by human red blood cells at 22°C (○) or 37°C (●).

The rate of NBDG uptake at 37°C is over 10-times faster than it is at 22°C. The accumulation of NBDG in the red blood cell could come about by three processes: coupling of NBDG uptake to some other transport process (which would require metabolic energy), covalent modification of NBDG (e.g., metabolism), or intracellular binding of NBDG.

(1) Coupled transport (energy requiring). The coupling of NBDG uptake to some other transport system is unlikely, since iodoacetic acid has no effect on the accumulation of NBDG, nor does collapsing the Na^+ or K^+ gradients.

(2) Metabolism or covalent modification. Thin-layer chromatography of the intracellular lysate from red blood cells which had been incubated with NBDG resolved only one fluorescent spot whose location was identical with NBDG. This suggests that NBDG is not being modified intracellularly.

(3) Binding of NBDG to some intracellular substance.

NBDG binding to red blood cell lysate and intact red blood cells by equilibrium dialysis

Our results suggest that the accumulation of NBDG in the red blood cell is due to the binding of NBDG to some intracellular component (Table I). The concentration of NBDG in the lysate should be the same as that in the buffer at equilibrium if no accumulation were occuring.

NBDG was found to accumulate on the side of the dialysis cell containing the cellular lysate (Table I). Intact red blood cells placed in the dialysis cell also accumulated NBDG (as expected). There was no significant difference between the concentration of NBDG in the cellular lysate and the concentration of NBDG in an equivalent quantity of intact cells at 37°C. Neither D-glucose nor cytochalasin B prevented NBDG from binding to the cellular lysate, suggesting the inhibition of NBDG uptake by these agents is due to their interaction with the hexose transporter in the erythrocyte membrane.

There have been reports that hemoglobin [12], or perhaps some lower molecular weight cytosolic factor [17] can interact with the glucose transport system of the red blood cell. Dialyzing the intracellular lysate with an M_r of 50000 cut-off membrane had no effect on NBDG accumulation. This suggested that NBDG may be binding to a higher molecular weight component in the red blood cell; hemoglobin being the likely candidate. Purified hemoglobin A_0 was generously supplied by B.W. Turner (Johns Hopkins). NBDG was

TABLE I

NBDG BINDING TO RED BLOOD CELL LYSATE, INTACT RED BLOOD CELLS AND PURIFIED HEMOGLOBIN BY EQUILIBRIUM DIALYSIS

Condition	Chamber 1	Chamber 2	n	$\frac{[NBDG]_{chamber\ 1}}{[NBDG]_{chamber\ 2}}$	[Hb] (mM)
1	red blood cell lysate	phosphate-buffered saline	7	1.87 ± 0.14	3.04 ± 0.34
2	purified Hb	phosphate-buffered saline	3	1.89 ± 0.10	2.69 ± 0.23
3	intact red blood cells	phosphate-buffered saline	3	1.92 ± 0.15	3.90 ± 0.30
4	phosphate-buffered saline buffer	phosphate-buffered saline	2	0.97 ± 0.03	0.0

found to accumulate on the side of the dialysis cell containing the purified hemoglobin. There was no significant difference between NBDG binding to purified hemoglobin and its binding to cellular lysate.

NBDG uptake in pink erythrocyte ghosts

The uptake of NBDG by pink erythrocyte ghosts is shown in Fig. 5A. Plotted is the % equilibrium (where at 100% both intracellular and extracellular concentrations of NBDG are equal) versus time.

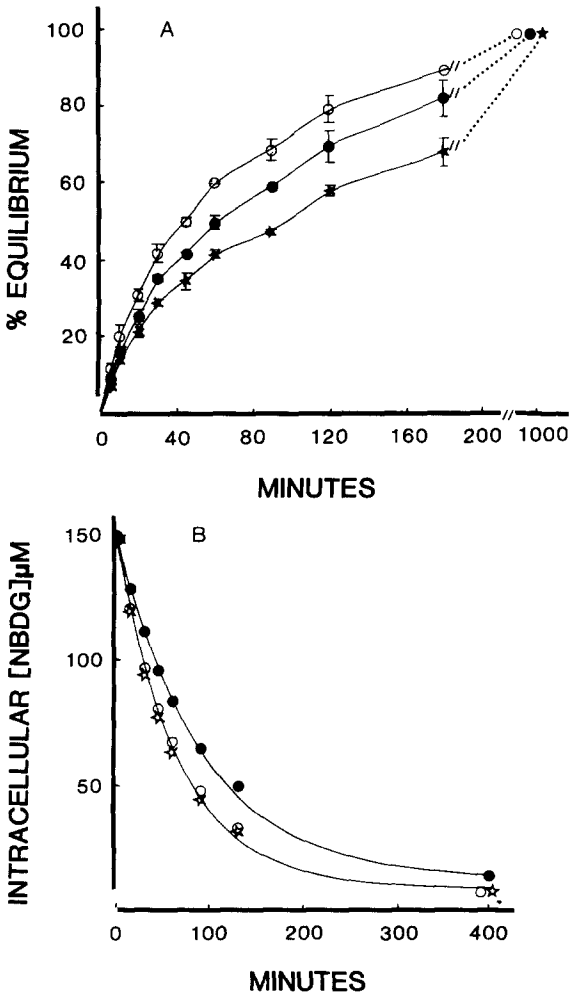

Fig. 5. (A) NBDG uptake by pink erythrocyte ghosts in the absence (○) or presence of 16 mM D-glucose (●) or 10 μM cytochalasin B (★). Displayed are the mean ± S.D. of two experiments. (B) NBDG efflux from pink erythrocyte ghosts in the absence (○) or presence of 32 mM D-glucose (●) or 32 mM L-glucose (☆).

NBDG does not accumulate in pink ghosts; however, the uptake of NBDG is still inhibited by D-glucose and cytochalasin B. The intracellular and extracellular concentrations of NBDG are equal at equilibrium. The inability of the ghost to accumulate NBDG suggests that some intracellular substance that binds NBDG has been largely removed. It also suggests that the accumulation is not due to the interaction of NBDG with the membrane.

NBDG efflux from pink erythrocyte ghosts

The efflux of NBDG by pink erythrocyte ghosts is shown in Fig. 5B. NBDG efflux from pink erythrocyte ghosts was inhibited by 32 mM D-glucose but not by 32 mM L-glucose. The presence of 32 mM D-glucose inhibited the rate of NBDG efflux from pink erythrocyte ghosts by 24%.

Discussion

NBDG appears to enter the human red cell via the hexose transport system (Fig. 2B). Concentrations of D-glucose near the K_m of D-glucose for the external transport site [12,28,29] markedly inhibit the rate of entry of NBDG. The K_i for D-glucose inhibition of NBDG uptake is close to the K_m of D-glucose for the external transport site, suggesting that NBDG enters the human red blood cell via the glucose transporter. Under our experimental conditions (108 μM NBDG, 37°C, pH 7.4), we estimate that the influx of NBDG is 40% via the hexose transporter and 60% by way of a glucose-insensitive pathway.

D-Glucose has no apparent effect on the efflux of NBDG from intact red blood cells. NBDG appears to exit the red blood cell by simple diffusion across the membrane, or by a facilitated transporter that has low affinity for D-glucose so that more than 32 mM D-glucose is required for inhibition. However, removal of a substantial fraction of the red blood cell cytosolic contents revealed a D-glucose-sensitive route of exit for NBDG in these pink erythrocyte ghosts.

Our efflux studies in pink erythrocyte ghosts suggest a D-glucose-sensitive route of exit for NBDG. It is conceivable that some cytosolic factor interacts with either the hexose transporter, or NBDG directly to induce this apparent asymmetry

of NBDG transport by human red blood cells. It has been suggested that although glucose transport in red blood cells appears asymmetric, this asymmetry may be induced by either hemoglobin or some lower molecular weight cytosolic factor [12,17]. Zero-*trans* influx and efflux experiments demonstrate significantly different K_m values of the external and internal sites for D-glucose [12,30]. Erythrocyte ghosts, however, which have had their cytosolic contents substantially depleted, transport glucose symmetrically, reflected by equal K_m values for the external and internal sites (measured by zero-*trans* entry and exit experiments) [17,31].

Barnett et al. [10,11] proposed a model of sugar transport whereby an unsubstituted group at the C-1 position of D-glucose is necessary for hexose entry, but hexose exit is dependent upon steric interactions with the C-6 position of D-glucose. The inability of D-glucose to inhibit NBDG efflux by intact red blood cells is consistent with the prediction of the Barnett hypothesis that the efflux of hexoses from human red blood cells via the hexose transporter is prevented by bulky substituents on the C-6 position of D-glucose. The Barnett hypothesis also predicts that glucose analogues with bulky groups on the C-6 position should bind to the transporter but not be transported, entering the human red blood cell via a glucose-insensitive route [10,11]. This part of the hypothesis was based on the failure of 6-*O*-propyl-D-galactose and bulkier related derivatives to be transported by the hexose transport system. Our finding that NBDG does enter via the hexose transporter suggests that other factors besides bulkiness of the C-6 substituent (perhaps its hydrophobicity) influences the ability of a compound to be transported by the hexose transfer system.

Based upon halogen and alkyl substitutions on positions C-1, C-2, C-3, C-4 and C-6 of D-glucose, it was determined that no single hydroxyl group is essential for binding to the hexose transporter [20]. These experiments also provided evidence for a hydrophobic site on the hexose transporter in close proximity to the C-6 position of D-glucose. Inhibition of glucose transport by hydrophobic compounds such as steroids [7,32], benzoic acid derivatives [8] and cytochalasins [3,4,27] further support a proposed hydrophobic region near the glucose transporter. Since NBDG has both hydrophilic (D-glucose) and hydrophobic (NBD) groupings, it is possible that the steric relationships between these sites and the transporter are such that recognition of the NBDG and its translocation by the transporter are possible.

The inhibition of glucose transport by cytochalasin B has been reported to be competitive [27]. The high affinity of cytochalasin B for the hexose transporter may be due to very similar spatial distributions of certain oxygen atoms found in the conformations of β-D-glucose and cytochalasin B [33,34]. Cytochalasin B has also been reported to bind asymmetrically to the hexose transporter, competing with glucose for the carrier on the inner surface of the cell membrane to a much greater extent than it competes for the outward-facing site of the carrier [35].

Our data demonstrate cytochalasin B inhibition for both influx and efflux of NBDG. If cytochalasin B partitions into the proposed hydrophobic area near the C-6 region of the hexose transporter, cytochalasin B could inhibit NBDG binding and subsequent translocation by the transporter. This may account for the inhibition of NBDG uptake by cytochalasin B we observe in human red blood cells.

The inhibition of NBDG exit from intact red blood cells by cytochalasin B is in contrast to the lack of apparent inhibition of NBDG exit by D-glucose. D-Glucose and cytochalasin B probably interact with the hexose transporter by different mechanisms. There is a marked contrast in the differential labeling of membrane proteins by fluorodinitrobenzene in the presence of cytochalasin B and D-glucose [36]. Gorga and Lienhard [37] have described changes in the intrinsic fluorescence of the purified hexose transporter (migrating in band 4.5). Both cytochalasin B and D-glucose induce a change in the intrinsic fluorescence of the transporter. The fluorescence intensity change induced with maximal glucose binding to the transporter is similar to, but smaller than, the change in fluorescence upon cytochalasin B binding. Studies using circular dichroism on a preparation of vesicles reconstituted with polypeptides from band 4.5 suggest that cytochalasin B induces a different conformation in the hexose transporter than does D-glucose [38]. Cytochalasin B might be binding to a site on the transporter inducing a conforma-

tional change in the transport protein which prevents the recognition and/or transport of NBDG. D-Glucose may not induce the same conformational change in the transporter as does cytochalasin B. Alternatively, cytochalasin B may be restricting the diffusion of NBDG in a manner not specific to glucose transport. Cytochalasin B has been shown to potently inhibit other transport processes, such as cyclic AMP transport in human erythrocyte ghosts [39], and cytosine transport in cultured Novikoff rat hepatoma cells [40].

Other compounds besides NBDG are known to accumulate in the red blood cell in a temperature-dependent manner. Catecholamines (norepinephrine and epinephrine) are accumulated in the human red blood cell reaching an equilibrium distribution ratio of 2 (intracellular/extracellular) at 37°C [41]. Various forms of vitamin B-6 also accumulate in the red blood cell in a temperature-dependent manner [42,43]. The accumulations of these substances by the red blood cell were assumed to be due to an active transport process. Since then investigators have demonstrated that certain catecholamines, pyridoxal (vitamin B-6) and pyridoxine accumulate in the red blood cell due to their binding to hemoglobin [44–46]. Hemoglobin is also thought to bind thyroid hormones [47] and phloretin [48]. The presence of aromatic structures is a shared feature of NBDG and the above compounds, which may be responsible for their accumulation in the red blood cell.

Hemoglobin A_{1c} is a glycosylated form of hemoglobin formed by the condensation of glucose with the N-terminal amino groups of the B-chains of hemoglobin A [49]. This nonenzymatic glycosylation was thought to take weeks to develop; however, recent studies suggest an interaction between glucose and hemoglobin occurs within minutes to hours [50,51]. Since the NBDG bound to purified hemoglobin in our experiments is released when the hemoglobin is precipitated by trichloroacetic acid, the binding of NBDG to hemoglobin we observed is apparently noncovalent. However, given enough time, NBDG might form a covalent bond with hemoglobin by a process similar to the nonenzymatic glycosylation of hemoglobin [49,51,52].

The entry rate for NBDG is much slower than that of other hexoses. This could be the result of NBDG having a low affinity for the transporter, or the V_{max} for NBDG uptake being much lower than that of D-glucose. Infinite-*cis* exit experiments via the method of Sen and Widdas [28,29] demonstrated a lack of inhibition by NBDG on 120 mM D-glucose efflux from human red blood cells using NBDG concentrations up to 500 μM (data not shown). A low-affinity external site for NBDG would be consistent with its slow rate of entry.

The present data demonstrate that 40% of NBDG uptake occurs via the hexose transporter (under our conditions). Our results with NBDG support the interpretation that the hexose transport system of human erythrocytes may appear asymmetric as a consequence of some interaction with a cytosolic factor; however, in the absence of this cytosolic factor, the hexose transport system may be intrinsically symmetric.

Interesting questions about the glucose transporter can be asked using fluorescent glucose analogues. Since glucose metabolism begins with phosphorylation occuring on the C-6 hydroxyl of D-glucose, the C-6-labeled glucose compounds are unmetabolizable and may be useful to other investigators due to their ability to be transported, but not metabolized.

Acknowledgements

Our research was supported by grants HL24460 and GM24168 from the National Institutes of Health. L.S. is a predoctoral trainee supported by NIH training grant HL07284. The laboratory of Dr. Adrian Gear (Biochemistry Department, University of Virginia) provided freshly-drawn blood samples. Dr. B.W. Turner (Biology Department, Johns Hopkins University) kindly provided purified human hemoglobin.

References

1 Lartey, P.A. and Derechin, M. (1979) Prep. Biochem. 9, 85–95
2 LeFevre, P.G. (1961) Pharmacol. Rev. 13, 39–70
3 Taverna, R.D. and Langdon, R.G. (1973) Biochim. Biophys. Acta 323, 207–219
4 Rampal, A.L., Pinkofsky, H.B. and Jung, C.Y. (1980) Biochemistry 19, 679–683

5 Baker, G.F. and Widdas, W.F. (1973) J. Physiol. 231, 129–142
6 Baker, G.F. and Widdas, W.F. (1973) J. Physiol. 231, 143–165
7 Lacko, L., Wittke, B. and Geck, P. (1975) J. Cell Physiol. 86, 673–680
8 Lacko, L., Wittke, B. and Zimmer, G. (1981) Biochem. Pharmacol. 30, 1425–1431
9 Novak, R.A. and LeFevre, P.G. (1974) J. Membrane Biol. 17, 383–390
10 Barnett, J.E.G., Holman, G.D. and Munday, K.A. (1973) Biochem. J. 135, 539–541
11 Barnett, J.E.G., Holman, G.D., Chalkley, R.A. and Munday, K.A. (1975) Biochem. J. 145, 417–429
12 Naftalin, R.J. and Holman, G.D. (1977) in Membrane Transport in Red Cells (Ellory, J.C. and Lew, V.L., eds.), pp. 257–300, Academic Press, London.
13 Jung, C.Y. (1975) in The Red Blood Cell, 2nd Edn., pp. 705–751, Academic Press, New York
14 LeFevre, P.G. (1975) in Current Topics in Membranes and Transport, Vol. 7, pp. 109–215, Academic Press, New York
15 Widdas, W.F. (1980) in Current Topics in Membranes and Transport, Vol. 14, pp. 165–223, Academic Press, New York
16 Carruthers, A. (1984) Prog. Biophys. Mol. Biol. 43, 33–69
17 Carruthers, A. and Melchior, D.L. (1983) Biochim. Biophys. Acta 728, 254–266
18 Baker, G.F. and Widdas, W.F. (1972) J. Physiol. 221, 10–11 P
19 Baker, G.F. and Widdas, W.F. (1972) J. Physiol. 226, 87–88 P
20 Barnett, J.E.G., Holman, G.D. and Munday, K.A. (1973) Biochem. J. 131, 211–221
21 Weber, T.M., Hussain, S. and Eichholz, A. (1983) Fed. Proc. 42, 607
22 Jacquez, J.A. (1983) Biochim. Biophys. Acta 727, 367–378
23 Ponder, E. (1948) in Hemolysis and Related Phenomenon, p. 101, Grune and Stratton, New York
24 Stadie, W.C. (1920) J. Biol. Chem. 41, 237
25 Karlish, S.J.D., Lieb, W.R., Ram, D. and Stein, W.D. (1972) Biochim. Biophys. Acta 255, 126–132
26 Johnson, M. (1983) Biophys. J. 44 (1), 101–106
27 Lin, S. and Spudich, J.A. (1974) J. Biol. Chem. 249, 5778–5783
28 Sen, A.K. and Widdas, W.F. (1962) J. Physiol. 160, 392–403
29 Sen, A.K. and Widdas, W.F. (1962) J. Physiol. 160, 404–416
30 Baker, G.F. and Naftalin, R.J. (1979) Biochim. Biophys. Acta 550, 474–484
31 Taverna, R.D. and Langdon, R.G. (1973) Biochim. Biophys. Acta 298, 412–421
32 Masiak, S.J. and LeFevre, P.G. (1974) Arch. Biochem. Biophys. 162, 442–447
33 Taylor, N.F. and Gagneja, G.L. (1975) Can. J. Biochem. 53, 1078–1084
34 Griffin, J.F., Rampal, A.L. and Jung, C.Y. (1982) Proc. Natl. Acad. Sci. U.S.A. 79, 3759–3763
35 Deves, R. and Krupka, R.M. (1978) Biochim. Biophys. Acta 510, 339–348
36 Shanahan, M.F. and Jacquez, J.A. (1978) Membrane Biochem. 1, 239–267
37 Gorga, F.F. and Lienhard, G.E. (1982) Biochemistry 21, 1905–1908
38 Jung, E., Pak, D., Chin, J. and Jung, C.Y. (1984) Fed. Proc. 43 (6), 1651
39 Holman, G.D. (1978) Biochim. Biophys. Ata 508, 174–183
40 Graff, J.C., Wolhueter, R.M. and Plagemann, P.G.W. (1977) J. Biol. Chem. 252 (12), 4185–4190
41 Danon, A. and Sapira, J.D. (1972) Clin. Pharm. Ther. 13, 916–922
42 Yamada, K. and Tsuji, M. (1968) J. Vitaminol. 14, 282–294
43 Yamada, K. and Tsuji, M. (1970) J. Vitaminol. 16, 237–242
44 Blakeley, A.G.H. and Nicol, C.J.M. (1978) J. Physiol. 277, 77–90
45 Mehansho, H. and Henderson, L.M. (1980) J.Biol. Chem. 255, 11901–11907
46 Ink, S.L., Mehansho, H. and Henderson, L.M. (1982) J. Biol. Chem. 257, 4753–4757
47 Davis, P.J., Yoshida, K., Schoenl, M. (1980) J. Lab. Clin. Med. 95, 714–724
48 Jennings, M.L. and Solomon, A.K. (1976) J. Gen. Physiol. 67, 381–397
49 Bunn, H.F., Haney, D.N., Kamin, S., Gabbay, K.H. and Gallop, P.M. (1976) J. Clin. Invest. 57, 1652–1659
50 Svendsen, P.A., Christiansen, J.S., Soegaard, U., Welinder, B.S. and Nerup, J. (1980) Diabetologia 19, 130–136
51 Goebel, F.D. and Fuessl, H. (1982 Br. Med. J. 284, 598–599
52 Bunn, H.F., Shapiro, R., McManus, M., Garrick, L., McDonald, M.J., Gallop, P.M. and Gabbay, K.H. (1979) J. Biol. Chem. 254 (10), 3892–3898