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Preferential Uptake of D-Glucose by Isolated Human Erythrocyte Membranes*

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ABSTRACT: A sensitive method for the measurement of the stereospecific uptake of D-glucose by isolated human erythrocyte membranes has been developed. The method is based on the difference in uptake of L-[^{14}C]glucose and D-[^{3}H]glucose as measured by the retention of radioactivity by the membrane preparation. Detection of the preferential D-glucose uptake activity was found to be dependent on the presence of $(NH_4)_2SO_4$ and the uptake increased with increasing concentrations of this neutral salt. This effect of $(NH_4)_2SO_4$ could not be attributed to either ammonium or sulfate ions *per se* or to a restoration of D-glucose transport activity. Properties of the D-glucose uptake activity have been studied. The uptake was reversible and did not involve any chemical alteration of D-glucose. Changes of pH (5.7–8.8) and fragmentation of the erythrocyte membrane did not greatly affect the uptake; all D-glucose uptake activity was lost upon incubation at 50° or by boiling the membrane preparation for 5–10 min. The apparent dissociation constant for the D-glucose–membrane complex was 2.9×10^{-5} to 4.6×10^{-5} M at 0° and increased as the temperature was increased. From the temperature dependence, it was calculated that the apparent enthalpy of dissociation of the complex was 3.3 kcal/mol. In the presence of a finite concentration of substrate, the maximum capacity for D-glucose uptake varied from 394.5 to 519.1 pmoles per mg of membrane protein and was independent of temperature (0–37°). The D-glucose-uptake activity was shown to be closely associated with glucose transport in erythrocytes since it exhibited the following properties characteristic of this carrier-mediated transport system. (a) The uptake was specific for the D isomer of glucose. (b) Phloretin, a competitive inhibitor of erythrocyte monosaccharide transport, inhibited the uptake. (c) The uptake activity was inhibited by monosaccharides known to be competitive inhibitors of D-glucose transport in human erythrocytes; the degree of inhibition paralleled the apparent affinities of the monosaccharides for the glucose transport system. (d) Saturation of D-glucose uptake occurred with increasing concentrations of D-glucose. (e) The uptake activity was inhibited by sulfhydryl reagents previously reported to inhibit D-glucose transport. Evidence suggesting that the uptake activity represents the stereospecific binding of D-glucose to the erythrocyte membrane rather than the transport of the sugar into a vesicle lumen is discussed.

Of all the mammalian transport systems, that of monosaccharide transport in the erythrocyte has been studied the most extensively (LeFevre, 1954, 1961a; Wilbrandt and Rosenberg, 1961; Stein, 1967). The kinetic data accumulated on this process have generally been interpreted to indicate that the translocation of monosaccharides across the cell membrane does not occur by simple diffusion but involves an obligatory reversible association of the sugar with a membrane component during transport. However, the reactions essential to this carrier-mediated transport of monosaccharides have not been identified.

All models of carrier-mediated transport assume the first step to be the specific binding of the permeant to an active site on the extracellular surface of the cell membrane (Stein, 1967; Pardee, 1968). Within recent years several permeant-binding proteins associated with bacterial membrane transport systems have been isolated and characterized (Pardee, 1968; Heppel, 1967, 1969; Roseman, 1969). However, parallel studies in mammalian cells have not been reported. Several attempts to identify the glucose-binding component of erythrocyte membranes have been described. LeFevre *et al.* (1964) have demonstrated that phospholipids extracted from the

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erythrocyte membrane have the ability to form complexes with glucose. Furthermore, the passage of glucose through a chloroform layer separating two aqueous compartments was found to be accelerated markedly upon the addition of erythrocyte membrane phospholipids to the chloroform (Jung *et al.*, 1968; LeFevre *et al.*, 1968). However, the membrane phospholipids could not be cast in the role of the glucose-binding component involved in monosaccharide transport since they failed to demonstrate in their sugar associations the stereospecificity characteristic of the complete glucose-transport system (Jung *et al.*, 1968; LeFevre *et al.*, 1964, 1968).

The formation of a transient imine intermediate involving the aldehyde group of glucose and a lysyl side chain of the membrane protein of human erythrocytes has been postulated by Langdon and Sloan (1967) to be an essential reaction in glucose transport. This hypothesis has been seriously questioned by LeFevre (1967) upon a critical review of the data. In addition, Evans *et al.* (1967, 1969) reported there was no alteration of the kinetics of glucose transport when extensive amounts of the sugar had been irreversibly bound to the lysyl side chains of the membrane protein and Kahlenberg (1969) demonstrated that the reaction leading to the formation of the imine intermediate did not possess the same stereospecificity characteristic of glucose transport.

Bobinski and Stein (1966), Bonsall and Hunt (1966), and Levine and Stein (1967), using the methods of retardation chromatography and ultrafiltration, have reported on the preferential binding of D-glucose (as compared to the poorly transported analogs, L-sorbose or L-glucose) to human erythrocyte membranes or solubilized protein extracts. However considerable doubt has been cast upon the interpretation of these studies by the recent work of LeFevre and Masiak (1970) as well as our own (Urman, 1970) which provides convincing evidence that the apparent binding activity measured was due to the presence of residual carrier-mediated D-glucose transport activity in the membrane preparations employed.

The present communication describes a sensitive and reproducible method for the measurement of the uptake of D-glucose by the isolated human erythrocyte membrane. In contrast to previous reports on the binding of D-glucose to components of the erythrocyte membrane (Jung *et al.*, 1968; LeFevre *et al.*, 1964, 1968; Langdon and Sloan, 1967), the uptake activity reported in this paper was found to be specific for the D isomer of glucose. Many of the properties of the D-glucose-uptake activity that have been studied are identical with those of the overall glucose transport system in erythrocytes. With regard to the nature of the D-glucose uptake activity measured, it is unknown at present whether it represents the transport of D-glucose into a vesicle lumen or the binding of the sugar to a specific site on the erythrocyte membrane. Evidence in support of the latter possibility is discussed.

Experimental Section

Materials

The following radioactive monosaccharides were purchased from Amersham–Searle Corp.: L-[1-^{14}C]glucose (3 mCi/mmole), D-[U-^{14}C]glucose (309 mCi/mmole), D-[U-^{14}C]glucitol (7 mCi/mmole), D-[6-^{3}H]glucose (500 mCi/mmole), and D-[1-^{3}H]glucitol (200 mCi/mmole). New England Nuclear Corp. was the supplier of D-[2-^{3}H]glucose (310 mCi/mmole). ATP,[1] AMP, PEP, and *p*-chloromercuribenzenesulfonate were supplied by Sigma Chemical Co. L-Glucose was purchased from General Biochemicals. D-Fructose, D-galactose, D-mannose, D-xylose, 2-deoxy-D-glucose, hemin, bovine serum albumin, and phloretin were obtained from Nutritional Biochemicals Corp. Other monosaccharides were Pfanstiehl products. All other compounds were of reagent grade.

[1] The abbreviations used are: ATP, adenosine 5′-triphosphate; AMP, adenosine 5′-monophosphate; PEP, phosphoenolpyruvate.

Methods

Preparation of Hemoglobin-Free Human Erythrocyte Membranes. Erythrocyte membranes were prepared from recently out-dated whole human blood by the method of Dodge *et al.* (1963), using 20 mosm sodium phosphate buffer (pH 7.5) for lysis. The validity of using outdated blood in the present study is supported by the report of Miller (1968) that there are no significant differences in the kinetics of the erythrocyte–monosaccharide transport system of fresh *vs.* outdated blood-bank blood. Upon harvesting the membranes after the last centrifugation, only the clear white precipitate surrounding the small central buff-colored portion of the button was collected to yield a suspension with a protein concentration of 3–4 mg/ml. Protein was measured by the method of Lowry *et al.* (1951) using bovine serum albumin as a standard. The hemoglobin content of the erythrocyte membranes, assayed by the method of Rimington (1942) as modified by Dodge *et al.* (1963), constituted less than 0.3% of the total membrane protein. Examination of the membrane suspension by phase-contrast microscopy revealed characteristic erythrocyte "ghosts" (Dodge *et al.*, 1963) without any evidence of other contaminating cellular material. The membrane suspension, which was prepared fresh weekly, was stored at 2–5°.

Measurement of D-Glucose Uptake. Unless otherwise indicated, the preferential D-glucose-uptake activity of human erythrocyte membranes was measured in duplicate as follows. The incubation medium consisted of 0.4–0.6 mg of membrane protein, 60 μM D-[6-^{3}H]glucose (specific activity 16.7 mCi/mmole), and 60 μM L-[1-^{14}C]glucose (specific activity 3 miCi/mmole) in a final volume of 0.250 ml of 20 mosm sodium phosphate buffer (pH 7.3–7.4). After incubation in an ice–water bath for 30 min, 3 ml of ice-cold saturated $(NH_4)_2SO_4$ were added, bringing the final concentration of the sugars to 4.6 μM, and the reaction mixture was mixed. After incubation as described above, the sample was rapidly filtered with suction through a dry Millipore microfiber glass prefilter (AP25 022). The membrane protein, which is quantitatively retained by the prefilter, was rapidly washed with two 4-ml volumes of ice-cold saturated $(NH_4)_2SO_4$ to remove all unbound radioactivity from the sample. The prefilter was then transferred to the bottom of a liquid scintillation counting vial, mixed vigorously with 10 ml of counting solution, and counted as described below. Aliquots (25 μl) of the original incubation medium, lacking membrane protein, were analyzed for their ^{3}H and ^{14}C content and the ratio of D-glucose:L-glucose, *i.e.*, ^{3}H:^{14}C, was determined. This ratio in the erythrocyte membrane retained on the filter was four to five times higher than that of the original incubation medium.

The calculation of D-glucose uptake was similar to that recently described by Eichholz *et al.* (1969) for the binding of D-glucose to isolated intestinal brush borders. The distribution of D-[^{3}H]glucose (D_s) and L-[^{14}C]glucose (L_s) cpm in each sample and the ^{3}H to ^{14}C ratio for the original incubation medium (D_{im}/L_{im}) were determined. L-Glucose served as a measure of the nonspecific binding and/or entrapment of D-glucose (N) in each sample which was calculated as follows: $N = [(D_{im})/(L_{im})](L_s)$.

TABLE I: D-Glucose-Transport Activity of Different Preparations of Human Erythrocyte Membranes.[a]

Membrane Prepn	D-Glucose Transported (μmole/mg of Protein)
A	0.13
B	0.60
C	0.46
D	0

[a] Membrane preparations A, B, C, and D were isolated from the same sample of blood by the methods of Hoffman (1958), Garrahan and Glynn (1968), Lacko (1966), and Dodge *et al.* (1963), respectively. D-Glucose transport was measured as described under Methods.

TABLE II: Effect of Replacement of Ammonium Sulfate by Other Neutral Salts on the Detection of the Preferential D-Glucose-Uptake Activity of Human Erythrocyte Membranes.[a]

Salt Added	Concn (M)	D-Glucose Uptake (pmoles/mg of Protein)
None		−0.6
Ammonium sulfate	3.6	55.2
Ammonium citrate	2.5	2.8
Ammonium chloride	4.9	−0.6
Sodium sulfate	1.7	−2.3
Lithium sulfate	2.4	−0.7

[a] All salts were at a final concentration of 92.3% saturation at 0°. The D-glucose binding assay procedure described under Methods was modified by filtering the reaction mixtures through Millipore filters, 0.8 μ pore size (AAWP 025), surmounted by a microfiber glass prefilter. This modification was necessary in order to quantitatively filter the membrane protein in the absence of the added salts.

Subtracting this result from the sample's total D-glucose cpm (D_s), one obtained the counts per minute of D-glucose preferentially taken up by the membrane, which amounted to 80–90% of D_s. (D_s) − N = preferential D-glucose uptake (cpm).

From the specific activity of the D-[^{3}H]glucose in the incubation medium, expressed in counts per minute per picomole, the number of picomoles of D-glucose taken up was calculated.

Measurement of D-Glucose Transport. The assay was done in duplicate in the following manner. Membrane suspension (0.5 ml) was added with mixing to 1.0 ml of incubation medium consisting of 30 mM D-[6-^{3}H]glucose (specific activity 0.06 mCi/mmole) and 30 mM L-[1-^{14}C]glucose (specific activity 0.004 mCi/mmole) and incubated for 30 min at 37° with shaking. Glucose transport was interrupted and determined by the method of LeFevre and McGinness (1960) using 2 mM $HgCl_2$ in 310 mosm sodium phosphate buffer (pH 7.5) as the quenching mixture. L-Glucose, which is not a substrate for the erythrocyte–monosaccharide transport system (LeFevre, 1961a; Stein, 1967), was added to the assay medium to provide a measure of the amount of D-glucose entrapped in the extracellular fluid of the stroma.

Extraction of Radioactivity Associated with the Erythrocyte Membrane. In order to determine the identity of the radioactivity associated with the erythrocyte membrane, a large scale uptake experiment was performed. Extraction of the radioactivity from 30 replicate samples was performed as previously described (Kahlenberg *et al.*, 1968). The extract was dried by flash evaporation at 40°. The resulting residue, which contained the $(NH_4)_2SO_4$ associated with the radioactive samples from the assay procedure, was then washed with four 5-ml volumes of redistilled ethanol to extract the radioactivity from the salt residue. The ethanol extract was flash evaporated and reextracted with ethanol as described above. The final radioactive product obtained, which did not contain $(NH_4)_2SO_4$ as judged by the absence of any residue after flash evaporation, was dissolved in 2 ml of distilled water and used in the chromatography systems to be described (see Results).

Liquid Scintillation Counting. All samples were counted in 10 ml of a scintillation solution prepared as previously described (Kahlenberg, 1969). Double-isotope counting was done in a Packard Tri-Carb liquid scintillation spectrometer (Model 3320) using automatic external standardization to correct for the spillage, 18–20%, of ^{14}C into the ^{3}H channel. Counting efficiencies were of the order of 28 and 49% for ^{3}H and ^{14}C, respectively.

Results

D-Glucose Transport Activity of Hemoglobin-Free Human Erythrocyte Membranes. Erythrocyte membranes prepared by several different hemolytic procedures (Dodge *et al.*, 1963; Garrahan and Glynn, 1968; Hoffman, 1958; Lacko, 1966) were assayed for the presence of any residual D-glucose transport activity. As can be seen in Table I, membranes prepared by the method of Dodge *et al.* (1963), in contrast to the other preparations, lacked capacity for D-glucose transport. These results are in agreement with the report of Theodore and Robin (1965) who found that increasing penetration of erythrocyte stroma by high molecular weight solutes was associated with decreasing amounts of residual hemoglobin. Furthermore it was found (Urman, 1970) that the hemoglobin-free erythrocyte membranes prepared by the method of Dodge *et al.* (1963), in contrast to other membrane preparations retaining up to 25% of the original cell hemoglobin, did not yield a reconstituted D-glucose transport system when exposed to isotonic media of varying compositions (Hoffman, 1958; Garrahan and Glynn, 1968; Lacko, 1966; LeFevre, 1961b). Therefore in the D-glucose uptake experiments to be described, erythrocyte membranes prepared only by the method of Dodge *et al.* (1963) were used, thereby permitting an interpretation of the results obtained on the basis of an activity other than transport.

Measurement of Preferential D-Glucose-Uptake Activity. It has recently been reported that the noncovalent binding affinity between steroids and α_1-acid glycoprotein is increased many fold by neutral salts, such as Na_2SO_4, $(NH_4)_2SO_4$, or NaCl, which are salting-out reagents and which generally stabilize the conformational structure of globular proteins (Ganguly and Westphal, 1968). Consequently, since the binding of D-glucose to its transport site is likely to be noncovalent (Stein, 1967), the possibility that this activity would be detected in the presence of these neutral salts was investigated. As can

TABLE III: D-Glucose-Uptake Activity of the Human Erythrocyte Membrane Measured in the Presence of Different Mixtures of Radioactive Monosaccharides.[a]

Monosaccharides Added	D-Glucose Uptake (pmoles/mg of Protein)
D-[6-^{3}H]Glucose + L-[1-^{14}C]glucose	56.6
D-[2-^{3}H]Glucose + L-[1-^{14}C]glucose	52.1
D-[6-^{3}H]Glucose + D-[U-^{14}C]glucitol	56.4
D-[U-^{14}C]Glucose + D-[1-^{3}H]glucitol	56.0

[a] All monosaccharides were added at a final concentration of 4.6 μM. The assay procedure is described under Methods.

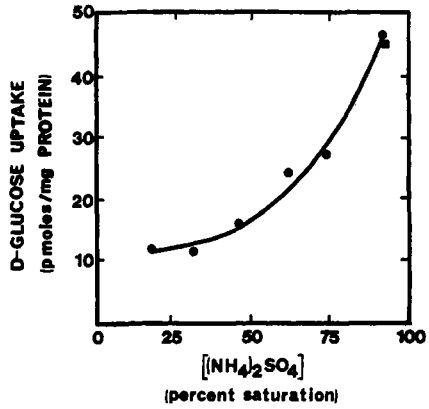

FIGURE 1: Uptake of D-glucose by erythrocyte membranes as a function of increasing concentrations of $(NH_4)_2SO_4$. The assay was performed as described under Methods except that the final concentration of $(NH_4)_2SO_4$ was varied as indicated. The washing step of the assay procedure was done with the corresponding salt concentration. The D-glucose-uptake activity obtained when $(NH_4)_2SO_4$ was added to the membrane suspension prior to the mixture of radioactive monosaccharides is indicated by the square.

be seen in Table II, preferential uptake of D-glucose by isolated erythrocyte membranes could be detected, but only in the presence of $(NH_4)_2SO_4$. This effect of $(NH_4)_2SO_4$ could not be attributed to either ammonium or sulfate ions *per se* (Table II). Furthermore, the uptake activity was found to increase with increasing concentrations of $(NH_4)_2SO_4$ (Figure 1). This uptake of D-glucose, detected in the presence of $(NH_4)_2SO_4$, was found to be completely reversible since all the radioactivity bound to the membrane could be removed by washing the sample with two 4-ml volumes of ice-cold 20 mosm sodium phosphate buffer (pH 7.5) (see Methods). In addition, in eight separate experiments, the same uptake activity was recorded when $(NH_4)_2SO_4$, at a final concentration of 92.3% saturation, was added to the membrane suspension prior to the mixture of radioactive sugars (Figure 1).

Similar D-glucose-uptake activity values were obtained when the usual mixture of radioactive monosaccharides added to the incubation medium was replaced by either D-[U-^{14}C]glucose and D-[1-^{3}H]glucitol or D-[2-^{3}H]glucose and L-[1-^{14}C]glucose and when D-[U-^{14}C]glucitol was substituted for L-[1-^{14}C]-glucose (Table III). Therefore, the D-glucose uptake measured in the present experiments cannot be due to an isotope effect.

Fragmentation of the hemoglobin-free erythrocyte membranes by repetitive freezing and thawing (Wheeler and Whittam, 1964; Rega *et al.*, 1968; LeFevre and Masiak, 1970) or by sonication (Rosenberg and McIntosh, 1968) did not destroy the uptake (Table IV). These results support our previous conclusion (see above) that the membrane preparation obtained by the method of Dodge *et al.* (1963) does not contain unhemolyzed cells or membrane vesicles possessing D-glucose transport activity.

Identity of Radioactivity Associated with Erythrocyte Membranes. The identity of the radioactivity taken up by the erythrocyte membrane was investigated. Erythrocyte membranes were incubated with D-[^{3}H]glucose and L-[^{14}C]glucose in the usual manner, but on a large scale (see Methods). The radioactivity associated with the membranes was extracted (see Methods) and applied to a Dowex 2-X8 formate column (1.5 × 17 cm). All of the radioactivity applied was recovered in the void volume of the column upon elution with distilled water, as would be expected for the uncharged glucose molecule.

The radioactivity recovered from the Dowex 2-X8 formate column was flash evaporated at 40° and dissolved in 1 ml of redistilled ethanol containing 2.5 mM each of D- and L-glucose. This material was then subjected to descending paper chromatography in duplicate on Whatman No. 1 and the chromatograms were counted as previously described (Yoda *et al.*, 1967). All of the radioactivity applied to chromatograms developed in 1-butanol–ethanol–water (2:1:1, v/v) or in methanol–ethanol–water (45:45:10, v/v) cochromatographed with D- and L-glucose which do not separate in these solvent systems. The locations of these two sugars on the chromatograms was determined by staining one of the duplicate chromatograms with 0.1 M ammoniacal silver nitrate.

It is therefore concluded that the stereospecific uptake of D-glucose by the erythrocyte membrane is a reversible process and does not involve a chemical change of the monosaccharide.

Properties of the Preferential D-Glucose-Uptake Activity of Human Erythrocyte Membranes. The D-glucose-uptake activity was shown to be directly proportional to the amount of membrane protein added to the incubation medium (Figure 2). In routine experiments, the incubation medium contained 0.4–0.6 mg of membrane protein. Higher amounts of membrane protein were found to clog the Millipore prefilter which resulted in a significant decrease in the rate of filtration of the

TABLE IV: Effect of Sonication and Repetitive Freezing and Thawing on D-Glucose Uptake.

Treatment	D-Glucose Uptake (% of Control)
None	100
Sonication for 1 min[a]	104 ± 13
2 min	105 ± 13
4 min	103 ± 12
6 min	97 ± 9
Repetitive freeze–thaw	73 ± 5

[a] Results for sonication and freezing and thawing treatments are the mean ± SEM from two and four experiments, respectively.

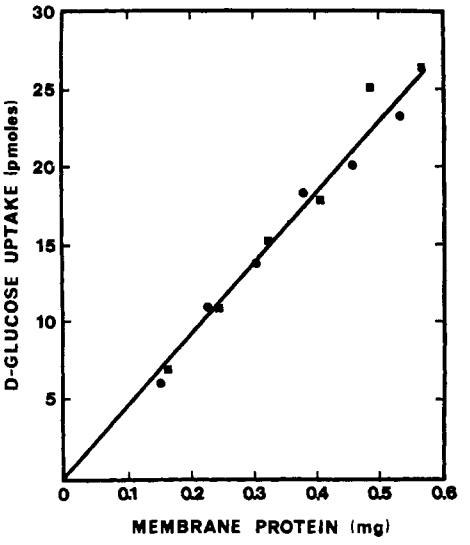

FIGURE 2: Uptake of D-glucose by erythrocyte membranes as a function of increasing amounts of membrane protein. The results obtained with two membrane preparations (circles and squares) are shown. The assay procedure is described under Methods.

reaction mixture (see Methods). This in turn prevented the washing out of all the unbound radioactivity from the filtered sample which is required to obtain reproducible D-glucose-uptake activity values.

The uptake activity was not affected by pH, over the range of 5.7–8.8, when the assay was carried out in the presence of 40 mM Tris-maleate buffer. This buffer had no effect on the uptake activity. Unless otherwise indicated, all uptake assays were performed at a pH of 7.3–7.4. All uptake activity was lost upon incubation at 50° or by boiling the membrane preparation for 5–10 min.

Effect of Sulfhydryl Reagents, Metal Ions, and Other Substances on Uptake Activity. The addition of Mg^{2+}, Na^{+}, or K^{+} to the incubation medium had no effect on the uptake activity (Table V). The presence of sulfhydryl reagents reported to inhibit D-glucose transport in erythrocytes (Stein, 1967) inhibited D-glucose uptake (Table V). Although 1-fluoro-2,4-dinitrobenzene is a powerful inhibitor of glucose transport, the level of this inhibitor's effect on D-glucose uptake (Table V) is in agreement with that reported by Bowyer and Widdas (1958) for glucose transport measured under similar conditions of incubation. The addition of ATP, AMP, and PEP, substrates known to participate in transport processes in bacteria (Pardee, 1968; Roseman, 1969) or to be closely coupled to the metabolism of glucose, had no effect on the uptake (Table V).

Specificity of Uptake. The inhibition by various monosaccharides of the preferential uptake of D-glucose by erythrocyte membranes is shown in Table VI. The monosaccharides used in this experiment are listed in a descending order of their apparent affinities for the human erythrocyte–monosaccharide transport system (Stein, 1967). The results indicate that D-glucose uptake was inhibited only by those monosaccharides which inhibit D-glucose transport (Stein, 1967; Lacko and Burger, 1961; LeFevre and Davis, 1951) and which belong to the homomorphous series having an L-arabino, D-xylo, and D-lyxo configuration. The degree of inhibition paralleled the apparent affinities of the monosaccharides for the transport system. D-Fructose and D-ribose, which are substrates for transport by human erythrocytes (LeFevre and Davis, 1951) but do not inhibit D-glucose transport (Lacko and Burger, 1961), did not inhibit D-glucose up-

TABLE V: Effect of Sulfhydryl Reagents, Metal Ions, and Other Substances on D-Glucose Uptake.[a]

Addition	D-Glucose Uptake (% of Control)
None	100
$HgCl_2$[b]	26
N-Ethylmaleimide[b]	73
Sodium iodoacetate	96
1-Fluoro-2,4-dinitrobenzene[b]	71
Sodium *p*-chloromercuribenzenesulfonate[b]	32
$MgCl_2$ + ATP	104
$MgCl_2$ + ADP	91
$MgCl_2$ + PEP	99
$MgCl_2$ (100 mM)	102
NaCl (100 mM)	91
KCl (100 mM)	99

[a] Unless indicated otherwise, all substances were added to the incubation medium at a final concentration of 1 mM. The assay procedure is described under Methods. [b] Sulfhydryl reagents that inhibit D-glucose transport in human erythrocytes (Stein, 1967).

TABLE VI: Inhibition by Monosaccharides of Preferential D-[^{3}H]Glucose-Uptake Activity of Human Erythrocyte Membranes.[a]

Conditions	D-Glucose Uptake (% of Control) [Monosaccharide] 0.46 mM	4.6 mM
Control	100	100
+ 2-Deoxy-D-glucose[b]	1	1
+ D-Glucose[b]	1	1
+ D-Mannose[b]	33	8
+ D-Galactose[b]	41	9
+ D-Xylose[b]	51	15
+ L-Arabinose[b]	83	54
+ D-Ribose	97	102
+ D-Fucose[b]	67	22
+ D-Lyxose[b]	84	67
+ D-Fructose	89	99
+ L-Glucose	91	107
+ D-Glucitol	98	101

[a] The monosaccharide concentrations quoted in this table were calculated on the basis of the volume of the reaction mixture after the addition of $(NH_4)_2SO_4$ (see Methods). The calculation of the D-[^{3}H]glucose uptake and the assay conditions are described under Methods. [b] Monosaccharides that competitively inhibit D-glucose transport in human erythrocytes (Stein, 1967; Lacko and Burger, 1961).

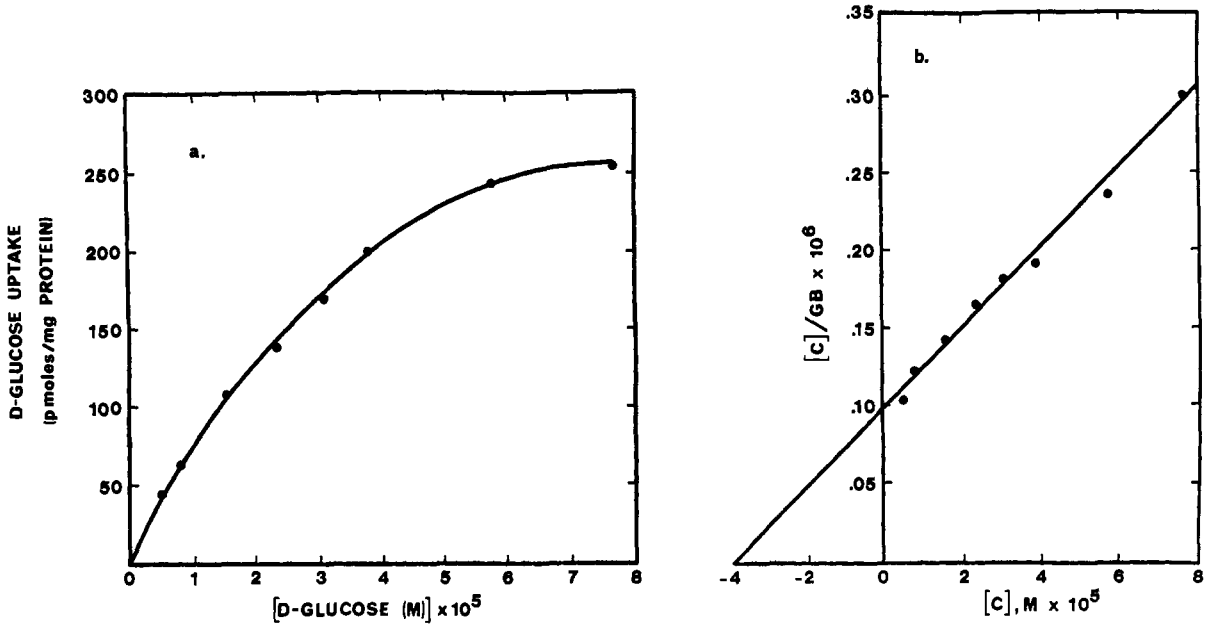

FIGURE 3: (a) Uptake of D-glucose by erythrocyte membranes as a function of increasing concentrations of D-glucose. The assay procedure is described under Methods. (b) The reciprocal plot of these data according to the method of Hanes (1932). The P value for the slope of the line obtained by calculation of the regression coefficient was <0.001. The coefficient of correlation was significant at the 1% level. The apparent dissociation constant and maximum capacity for D-glucose uptake calculated from these data are 3.9×10^{-5} M and 394.5 pmoles of D-glucose per mg of membrane protein, respectively.

take. The uptake of D-glucose was inhibited by D-lyxose but not by D-ribose, although according to LeFevre (1961a) the latter has a higher apparent affinity for the erythrocyte–monosaccharide transport system. These findings suggest, in agreement with the report of Lacko and Burger (1961), that the human erythrocyte possesses at least two separate carrier-mediated transport systems for monosaccharides, one of which is acessible only to D-glucose and monosaccharides belonging to the homomorphous series having an L-arabino, D-xylo, or D-lyxo configuration.

As expected, the addition of D-glucose to the assay medium decreased, through isotope dilution, the counts per minute of D-[^{3}H]glucose associated with the erythrocyte membrane (Table VI). The failure of D-glucitol or L-glucose to significantly inhibit the uptake of D-glucose is further indication of the high degree of stereospecificity of the uptake activity measured.

Phloretin in its ketonic form, which predominates at a pH of 6.12–6.95 (LeFevre and Marshall, 1959), is a competitive inhibitor of D-glucose transport in erythrocytes (Stein, 1967). Since the uptake of D-glucose was shown to be unaffected by changes in pH in the range of 5.5–8.5 (see above), we examined the effect of phloretin on D-glucose uptake when the assay medium was adjusted to pH 6.5. Phloretin, at a concentration of 10^{-5} and 10^{-6} M, was found to inhibit the preferential uptake of D-glucose by 84 and 34%, respectively. This result provides additional evidence that the D-glucose uptake reported in this communication is a reaction essential to the carrier-mediated monosaccharide transport system of the erythrocyte.

Apparent Dissociation Constant for D-Glucose Uptake and Maximum D-Glucose-Uptake Activity. D-Glucose-uptake activity was measured as a function of increasing concentrations of substrate. Since the same uptake activity was recorded when $(NH_4)_2SO_4$ was added to the membrane suspension prior to the mixture of radioactive monosaccharides (Figure 1), the D-glucose concentration used in these experiments was calculated on the basis of the volume of the reaction mixture after the addition of $(NH_4)_2SO_4$ and varied from 0.46 to 7.70×10^{-5} M. Figure 3a shows that the capacity of the erythrocyte membrane to take up D-glucose was saturated by a finite concentration of substrate. The apparent dissociation constant (K_D) for the D-glucose–membrane complex at 0°, determined in six separate experiments, varied from 2.9×10^{-5} to 4.6×10^{-5} M. It should be noted however, that due to the extensive hydration of the $(NH_4)_2SO_4$ present in the uptake assay medium, the effective concentration of glucose used in these experiments is considerably higher than that indicated in Figure 3. Therefore the K_D determined in the present study is an underestimation of the physiological K_D.

The slope of the line obtained from the reciprocal plot of the above data according to the method of Hanes (1932) corresponds to the pmoles^{-1} of D-glucose taken up per mg of membrane protein when the sugar is present at a saturating concentration (Figure 3b). In six separate experiments, this was found to vary from 394.5 to 519.1 pmoles per mg of membrane protein.

The K_D of the D-glucose–membrane complex was found to increase as the temperature was increased (Table VII). From a van't Hoff plot of these data the apparent enthalpy of the dissociation of this complex is 3.3 kcal/mole. The maximum capacity for D-glucose uptake did not change significantly as the temperature was varied from 0 to 37° (Table VII).

In the presence of a subsaturating level of substrate, the maximum uptake of D-glucose was reached within 10-min incubation (Figure 4). In contrast to this, however, when D-glucose was present at a saturating concentration, the maximum uptake activity was a time-independent value (Figure 4).

The rate of D-glucose uptake, assayed at a subsaturating level of substrate (Figure 4), was not altered in the presence of 0.46 mM D-galactose, an effective competitive inhibitor of D-glucose transport, but D-galactose did reduce the maximum

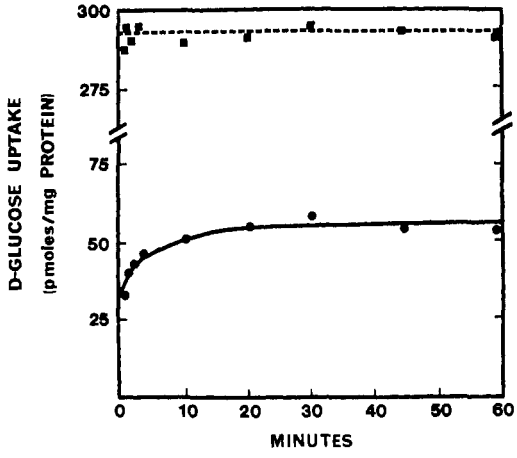

FIGURE 4: D-Glucose-uptake activity *vs.* time of incubation after the addition of $(NH_4)_2SO_4$. Uptake was measured as described under Methods, in the presence of subsaturating, 4.6×10^{-6} M (●), and saturating, 8.9×10^{-5} M (■), levels of substrate.

level of D-glucose uptake from 65.7 ± 1.0 (mean ± SEM) to 29.8 ± 0.8 pmoles of D-glucose per mg of membrane protein. Both the reduced and uninhibited levels of D-glucose uptake did not change significantly when assayed at ten intervals over a period of 120-min incubation after the addition of $(NH_4)_2SO_4$ (see Methods).

Discussion

Although considerable information is available on the overall properties of the carrier-mediated transport system for monosaccharides in the human erythrocyte, identification of the membrane-associated molecules involved in this process has not been made. The obvious difficulty in determining whether any membrane component participates in a transport system is that upon the lysis of the cells or the isolation of this component transport can no longer be measured. This paper represents an attempt to overcome this difficulty and describes a method for the measurement of the stereospecific uptake of the D isomer of glucose by isolated human erythrocyte membranes. The D-glucose-uptake activity was shown to be closely associated with the overall carrier-mediated-transport system for D-glucose since (a) the uptake activity exhibited the same stereospecificity characteristic of the D-glucose transport system in human erythrocytes. (b) Phloretin and sulfhydryl reagents which inhibit D-glucose transport in erythrocytes (LeFevre, 1961a; Stein, 1967) inhibited D-glucose uptake. (c) As is characteristic of carrier-mediated D-glucose transport (Stein, 1967), the uptake of D-glucose by the human erythrocyte membrane was not directly proportional to the concentration of D-glucose, but reached a limiting (saturation) value as the concentration of the monosaccharide was increased. (d) Both D-glucose transport (Sen and Widdas, 1962) and D-glucose uptake are independent of pH in the range of 5.7–8.8. The lack of any effect of pH on D-glucose uptake is in agreement with the findings reported for the binding activity of various monosaccharide-transport proteins isolated from bacteria (Heppel, 1969; Kaback, 1970) and suggests the absence of the involvement of any ionizable group on the membrane in the uptake mechanism.

TABLE VII: Effect of Temperature on the K_D and the Maximum D-Glucose-Uptake Activity.[a]

Temp (°C)	K_D (10^{-5} M)	D-Glucose Uptake (pmoles/mg of Protein)
0	2.9	403
17	6.6	397
25	10.9	416
37	15.0	393

[a] The dissociation constants (K_D) and the maximum uptake activity were determined and calculated as described in Figure 3b, except that the temperature of the complete assay was varied as indicated.

There are two possible explanations for the type of uptake recorded in the present experiments. It may represent uptake of D-glucose into the lumen of a vesicle, or it can represent the binding of the sugar to a specific site on the erythrocyte membrane. In view of the very small amount of D-glucose uptake detected in the presence of ammonium sulfate, only a very small percentage of the membrane preparation would have to be in the form of closed vesicles to account for the observed data. This small amount of transport activity could probably have escaped detection with the currently available transport assays. In addition, since the detection of D-glucose uptake was absolutely dependent on the presence of ammonium sulfate, the uptake activity measured could possibly be due to an ammonium sulfate induced formation of membrane vesicles possessing D-glucose transport activity However, that transport could account for the observed D-glucose uptake is weakened by the combined weight of the following observations, some of which are clearly inconsistent with transport and suggest that the uptake measured represents the binding of D-glucose to a specific site on the erythrocyte membrane. (a) The hemoglobin-free erythrocyte membrane preparation used in these studies was shown to lack capacity for detectable transport of D-glucose. This result is in agreement with the findings of Theodore and Robin (1965) that hemoglobin-free erythrocyte membranes are freely permeable to macromolecules, and do not selectively take up D-glucose. (b) Fragmentation of the membrane suspension by procedures reported to abolish all residual transport activity of nonhemoglobin-free membrane preparations (LeFevre and Masiak, 1970) did not destroy the uptake activity. (c) Exposure of the membrane preparation to various isotonic media or to saturated ammonium sulfate, followed by dialysis against isotonic buffer, did not result in the reconstitution of D-glucose transport activity (Urman, 1970). (d) In the presence of a saturating level of D-glucose, saturation of the uptake activity measured at 0°, the temperature at which net glucose transport is suppressed (Lacko and Burger, 1963), was independent of the time of incubation after the addition of ammonium sulfate. This is in direct contrast to the transport studies of LeFevre and McGinniss (1960) where the intracellular D-glucose concentration of erythrocytes incubated at 20° in the presence of a saturating concentration of sugar was shown to be maximal only after 100-min incubation. (e) In the present experiments, the same D-glucose-uptake activity was recorded whether ammonium sulfate was added to the membrane suspension before or after the addition of substrate (see Figure 1). However this would not have been the case if ammonium sulfate was inducing vesicle formation. The fluid entrapped in the vesicle lumen upon the addition of the salt to the incubation medium con-

taining the equimolar mixture of monosaccharides (see Methods) would have retained equal amounts of D- and L-glucose and therefore no preferential D-glucose uptake would have been detected. (f) Reduction in temperature normally increases the adsorption efficiency of any binding solute by lowering the energy of the bound molecules (Kipling, 1965). Thus as expected, but in contrast to the results of Levine and Stein (1966) and Miller (1968) who measured K_D indirectly by the glucose inhibition of sorbose efflux from erythrocytes, the K_D measured in the present study was found to increase as the temperature was increased. This finding supports the suggestion of Sen and Widdas (1962) that the decrease in the half-saturation constant for glucose transport in human erythrocytes at low temperatures is due to the dissociation of the glucose–membrane complex being an energy-dependent reaction. (g) Finally, it would be expected that if the D-glucose uptake measured here was due to transport, the rate of uptake but *not* the maximum uptake capacity would have been decreased in the presence of a competitive inhibitor of D-glucose transport (Stein, 1967). On the other hand, if the uptake measured represents the binding of D-glucose to a fixed number of sites on the erythrocyte membrane, then, as was shown to be the case in the present study, the maximum capacity for D-glucose uptake would be significantly reduced in the presence of the competitive inhibitor.

In summary, while the D-glucose-uptake activity reported in this paper exhibited properties characteristic of the overall erythrocyte–monosaccharide transport system, we believe we have provided evidence to characterize it as the initial step in transport; *i.e.*, the binding of D-glucose to a specific site on the erythrocyte membrane.

One human erythrocyte contains 0.8 pg of hemoglobin-free membrane protein (Ellroy and Keynes, 1968). If the D-glucose uptake measured by our assay system is due to binding, the number of specific D-glucose-binding sites, calculated from the maximum D-glucose-uptake activity (Figure 3b), varied in six experiments from 1.9×10^5 to 2.5×10^5 sites per erythrocyte. This estimate is compatible with that of LeFevre (1961c) who calculated that the number of D-glucose-binding sites involved in glucose transport was unlikely to be greater than 500,000/erythrocyte.

Recently Bosmann and Martin (1969) have demonstrated monosaccharide incorporation into glycoproteins of mitochondrial membranes in an *in vitro* system. Since in the present study all of the radioactivity retained by the erythrocyte membrane is released upon washing with buffer, it is unlikely that the sugar is covalently bound to the membrane.

Faust *et al.* (1968) and Eichholz *et al.* (1969) have presented evidence for the stereospecific binding of D-glucose to isolated hamster intestinal brush borders. However, the specificity exhibited by this binding activity was distinctly different from that of the intestinal active monosaccharide-transport system (Eichholz *et al.*, 1969). In fact, Parsons (1969) has subsequently demonstrated that the specificity profile of this binding activity was more closely related to the substrate specificity of intestinal brush border hexokinase than to that expected for the initial step of the active monosaccharide-transport system. In contrast to these findings, it is unlikely that the D-glucose-uptake activity reported in this paper represents the binding of the sugar to membrane-associated hexokinase. The specificity profile of the uptake activity paralleled that of the overall D-glucose-transport system in human erythrocytes which differs from that reported for human erythrocyte hexokinase (Bishop, 1964). Furthermore the isolated human erythrocyte membrane does not possess hexokinase activity (Schrier, 1963).

Finally, it is not known why the detection of the erythrocyte membrane's D-glucose-uptake activity is dependent on the presence of ammonium sulfate. In this connection, the recent report (Brush and Krawczyk, 1969) of the solubilization of a muscle membrane protein possessing phlorizin-inhibitable glucose-binding activity is of interest. The glucose-binding activity of that membrane protein was also detectable only in the presence of ammonium sulfate. However, additional description of the binding properties of the muscle membrane protein is required before it can be associated with the glucose-transport process in muscle.

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