STUDIES ON ERYTHROCYTE SUGAR TRANSPORT

A Dissertation

Presented to

The Faculty of the Department of Biology

University of Houston

In Partial Fulfillment

Of The Requirements For The Degree

DOCTOR OF PHILOSOPHY

By

Roger Allan Novak

May, 1972

620851

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ABSTRACT

Sugar transport was studied in bovine and human erythrocytes. It was concluded that the bovine RBC possesses a facilitated diffusion system for the transport of various sugars, based on the demonstration of saturation kinetics and competitive inhibition. However, sugar transport in the bovine RBC, as compared to the human RBC, is characterized by (1) a slow rate of transport, (2) lack of substrate alteration of carrier mobility, and (3) insensitivity to protein sulfhydryl reagents.

Membrane components of the human RBC sugar transport system were identified by dual isotopic, differential labeling with 2-fluoro-2,4-dinitrobenzene (DNFB). The labeling technique was based on the ability of high affinity substrates to induce conformational changes in the transport mechanism, thereby, increasing its incorporation of DNFB. The results indicate that, under these conditions, the site of DNFB incorporation is the membrane-phospholipids, rather than membrane protein(s). Thus it appears that membrane phospholipids are an integral part of the sugar transport system.

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INTRODUCTION

The limiting cell membrane of all cells contains specific permeability systems, i.e., transport systems, to control selectively the movement of many metabolites into and out of the cell. Kinetic analysis of the various systems has led to the generally accepted hypothesis that translocation requires the binding of the permeant molecule to a specific membrane component, presumably a membrane protein (Stein, 1967). While several permeant-bonding proteins associated with bacterial membrane transport systems have been isolated and characterized (Pardee, 1968; Heppel, 1969; Roseman, 1969), their isolation from cell membranes of higher organisms has not been achieved.

The most extensively studied mammalian transport system, from the standpoint of kinetics and molecular mechanisms, has been the monosaccharide transport system of human erythrocytes (LeFevre, 1961a; Stein, 1967). Several attempts have been made to elucidate the nature of the membrane, glucose-binding component. Phospholipids extracted from the erythrocyte membrane were shown to form complexes with substrates of the transport system and to accelerate the passage of sugar between two aqueous compartments separated by a hydrophobic layer; however, the system did not distinguish between optical enantiomorphs as does the <u>in situ</u> transport system (LeFevre et al., 1964; Jung et al., 1968; LeFevre, 1968).

Bobinski and Stein (1966), Bonsall and Hunt (1966), and Levine and Stein (1967), using the methods of retardation chromatography and ultrafiltration, have reported the preferential binding of D-glucose to human erythrocyte membranes or solubilized protein extracts; however, it was shown that the measured binding activity was due to the presence of residual, carrier-mediated, D-glucose transport activity of membrane vescicles in their preparations (LeFevre and Masiak, 1970).

Studies on the sugar transport mechanism in other mammalian tissues have resulted in the demonstration of stereospecific binding of D-glucose to fractions of the isolated brush border of hamster intestine. However, the binding activity was not associated with the membrane fraction and did not show the specificity profile of the in situ transport system (Eichholz et al., 1969). Instead, binding resembled that of intestinal brush border hexokinase (Parsons, 1969). The isolation of a muscle membrane protein possessing phlorizin-inhibitable, glucose-binding activity in the presence of ammonium sulfate has been reported (Brush and Krawczyk, 1969). However this protein has yet to be associated with glucose transport in muscle.

All previous attempts to identify a membrane, permeant-binding component or "carrier molecule" have been based on the standard biochemical methodology of extract and purify.

This procedure necessitates the destruction of membrane integrity prior to the assay, i.e., binding of permeant by a subfraction of the membrane. However, it appears that present techniques for disassembly of the membrane result in the loss of permeant binding capability (LeFevre, 1969). The solution to this problem is to develop techniques whereby an identifying label can be attached to the permeant binding site in situ so that its isolation can be monitored during fractionation.

A presumed in situ labeling technique was developed by Langdon and Sloan (1967), who demonstrated that D-glucose was incorporated into erythrocyte membrane proteins by the formation of a Schiff's base with lysyl residues. The formation of this intermediate was proposed to be the primary reaction between glucose and the permeant binding site. This hypothesis has been rejected because the reaction does not inhibit the transport of D-glucose and lacks the necessary stereospecificity (Evans et al., 1969; Kahlenberg, 1969).

The permeant binding site of a transport system is analogous to the active center of an enzyme. Thus, techniques used to identify enzyme active centers could be used to attach an identifying label to the carrier molecule in situ. One such technique is differential labeling.

In differential labeling, the enzyme is treated with an

irreversible inhibitor in the presence of excess substrate. The amino acid residues within the active center are protected from reaction with the inhibitor by the bound substrate while all unprotected residues outside of the active center react. Consequently, substrate will decrease the rate of inactivation of enzymatic activity. The substrate is then removed and a radioactive form of the inhibitor is allowed to react with those residues which had previously been protected (Vallee and Riordan, 1969). This technique has been used to identify the lactose carrier molecule of E. coli (Fox and Kennedy, 1967; Jones and Kennedy, 1969). Thus, this technique, or a modification thereof, seems suitable for the identification of the erythrocyte membrane components involved in monosaccharide transport.

STATEMENT OF THE PROBLEM

The basic assumption for all models of membrane transport is that the permeant molecule undergoes an obligatory, reversible interaction with a specific membrane component, presumably a protein. All previous attempts to identify a mammalian transport protein have been unsuccessful due to inherent difficulties with the techniques employed.

The basis of this thesis is the development of a technique, whereby an identifying label can be incorporated into those components of the erythrocyte membrane involved in monosaccharide transport to allow their subsequent identification upon fractionation of the membrane.

Characteristics of sugar transport were studied in both bovine and human red blood cells, which are markedly different with respect to their rates of sugar transport, in order to choose the cell more suitable for isolation of the protein.

MATERIALS AND METHODS

Materials

Bovine blood was collected by exsanguination into ACD medium (.08 M sodium citrate; .04 M citric acid; .05 M D-glucose; 200 ml added to 800 ml whole blood) and stored up to one week at 4° C. Human blood was obtained from Hermann Hospital blood bank and used within six weeks of withdrawal. There are no significant differences in the kinetics of sugar transport in fresh *vs* outdated human blood (Miller, 1968).

Prior to all experimental procedures, erythrocytes were washed at least three times with a four-fold volume of buffered saline (.13 M NaCl; .03 M sucrose; .01 M sodium phosphate; pH 7.2). The plasma and top layer of cells were removed in the course of these washings.

^{3}H-inulin, D-(^{14}C)-glucose, L-(^{14}C)-glucose, 3-O-($^{14}CH_3$)-D-glucose(3MG), and N-(^{14}C)-ethylmaleimide(NEM) were obtained from New England Nuclear Corp. (^{3}H) and (^{14}C)-1-fluoro-2,4-dinitrobenzene(DNFB) were obtained from Amersham-Searle Corp.

Alloxan, L-glucose, N-ethylmaleimide(NEM), and p-chloromercuribenzenesulfonate(PCMBS) were obtained from Sigma Chemical Co. DNFB and alpha-bromo-acetophenone (a-BRAC) were purchased from Aldrich Chemical Co. N-cyclo-hexylmaleimide(NCM) was obtained from Nutritional Biochemicals. All chemicals used were of reagent grade.

Methods

Measurement of Sugar Transport. Two methods were employed to measure sugar transport across bovine and human erythrocyte membranes.

Method I.

Bovine erythrocytes were incubated with an equal volume of buffered saline containing tracer amounts of (^{3}H)-inulin as an extracellular space marker and various concentrations of (^{14}C)-3MG. At appropriate times, 1 ml aliquots of the suspension were placed in polyethylene tubes (.45 x 9 cm) containing 0.2 ml of dibutyl phthalate and centrifuged at 10,000 x g for 10 min at 0° C. During centrifugation the dibutyl phthalate layered between the packed cells and supernatant, preventing cross contamination and effectively stopping transport. Both packed cells and supernatant (0.2 ml) were extracted with 15% (w/v) trichloroacetic acid and aliquots of the extract were counted in a Beckman LS - 150 liquid scintillation spectrometer. The scintillation cocktail contained toluene (900 ml/L), Beckman Biosolve BBS-3 (100 ml/L), and Liquifluor (42 ml/L). The degree of quenching was found, by use of the external standard, to be constant.

Method II.

Entrance studies. One volume of packed bovine or human erythrocytes were suspended in a six-fold volume of buffered

saline containing the radioactive test sugar. Uptake was halted by the rapid addition of a ten-fold volume of ice-cold stopping fluid (2% NaCl; 2 mM $HgCl_2$; 1.25 mM KI) which stops all glucose transport (Levine and Stein, 1965). The cells were then centrifuged for 5 min at 10,000 x g at 22° C. The supernatant was removed by aspiration and the cell pellet was analyzed as in Method I. The amount of sugar trapped in the extracellular space of the cell pellet was estimated by mixing the cells with the stopping fluid prior to addition of radioactive sugar.

Exit studies. Bovine erythrocytes were incubated with either 5 mM (^{14}C)-3MG or 5 mM (^{14}C)-D-glucose for 3 hours at 37° C. 10 mM NEM was present during D-glucose incubation to inhibit metabolism (Webb, 1966). Human erythrocytes were incubated with the same sugars for 30 min at 22° C. Following sugar loading, the cells were packed by centrifugation at 10,000 x g for 15 min at 22° C. 0.5 ml of the packed cells was resuspended in 5 ml of the test solution and exit was halted by the addition of 30 ml of ice-cold stopping fluid. The resulting suspension was analyzed as previously described. For determination of the initial cell sugar content, the stopping fluid was added directly to the cells followed by the test solution.

Inhibitor studies. One volume of packed erythrocytes were suspended in six volumes of buffered saline containing

the test compounds (inhibitors and/or sugars) for a given time and temperature. Ethanol was included in the incubation media at a percentage equal to the mMolar concentration of inhibitor when water-insoluble inhibitors (a-BRAC, NEM, DNFB) were used. The cells then were washed once with a tenfold volume of buffered saline containing 5 mM 2-mercaptoethanol to remove any remaining sulfhydryl-reagent and at least twice with buffered saline. In cases where the cells were incubated with an inhibitor in the presence of a sugar, the cells were suspended in the wash media for 15 min prior to each centrifugation to ensure removal of intracellular sugar. Sugar transport was assayed by method II; the only modification being that for exit studies the cells were loaded to an internal concentration of ca. 75 mMolar. Controls were carried through the same experimental procedure without the addition of inhibitor.

Differential labeling of the transport system with DNFB. Human erythrocytes were exposed to radioactive DNFB under the optimal inhibitory conditions as determined by methods described above. These conditions were: pretreatment of the erythrocytes with 10 mM NEM at a 50% Hct. for 30 min at 37° C, followed by exposure to 2 mM (^{3}H) or (^{14}C)-DNFB in the presence or absence of 100 mM test sugars, respectively. The cells were extensively washed to remove unbound inhibitor and membranes were obtained by

hypotonic lysis in 10 mM sodium phosphate buffer (pH 7.4) at a cell to medium ratio of 10:1 (Dodge et al., 1963).

The membranes were prepared for co-electrophoresis by mixing 0.8 ml of (^{14}C)-membranes, 0.2 ml of (^{3}H)-membranes and 0.2 ml of a sodium dodecylsulfate (SDS) stock solution. The SDS stock solution contained 50% glycerol (v/v); 13% SDS (w/v); 0.1 M Tris-PO_4, pH 6.8; 10 mM EDTA; 200 mM dithiothreitol (DTT); 50 ug/ml bromphenol blue. The resulting solution was heated to 56° C for 30 min to promote reduction of disulfide bonds by DTT (Fairbanks et al., 1971).

Protein was measured by the method of Lowry et al., (1951), using bovine serum albumin as a standard, prior to mixing of the membranes. The final protein concentration of the prepared sample was calculated to be 6.5 mg/ml.

Electrophoresis was preformed on 5% acrylamide gels (1.3 x 18 cm) containing 0.1% SDS and 8 M urea (Swank and Munkres, 1971). The buffer system was 0.1% SDS, 0.1 M H_3PO_4 adjusted to pH 6.8 with powdered Tris. The protein load was 1.3 mg/gel and the gels were run at 10 mA/gel for 24 hours. Following electrophoresis the gels were stained with 0.01% coomassie brilliant blue in 15% methanol, 7.5% acetic acid (v/v, respectively) for 10 to 24 hours; destained by diffusion with 15% methanol, 7.5% acetic cid for at least 48 hours and then scanned at 560 nm in a Joyce-Loeb densitometer. The gels were then sliced into

2.8 mm segments and the individual slices were incubated in scintillation vials with 2 ml of Protosol (New England Nuclear) at 65° C for 24 hours. Aquasol (15 ml, New England Nuclear) containing 2% glacial acetic acid (v/v) was added and the vials were dark aged for at least 48 hours and then counted to a 3% error for each isotope. In some cases, following counting, the vials were scanned at 560 nm in a Beckman B spectrophotometer to measure the dye eluted from the slices.

Distribution of the radioactive DNFB between the membrane lipids and protein was determined by extracting the membranes with chloroform:methanol (2:1;v/v). An aliquot of 0.050 ml of the labeled membranes was added to 0.2 ml of unlabeled membranes followed by addition of methanol (3 ml) and of chloroform (6 ml). The mixture was allowed to stand for 15 min at 22° C and then centrifuged at 6000 x g for 15 min. An aliquot of the supernatant (lipids) was taken for counting and the precipitated proteins were washed once with methanol and dissolved in Protosol and counted.

RESULTS

Sugar transport in bovine erythrocytes.

Uptake of 3-O-methyl-D-glucose and D-glucose by bovine erythrocytes suggested that a two component system for uptake was present (Fig. 1). At relatively low concentrations velocity vs substrate curves suggested a saturable component but D-glucose uptake was linear at high concentrations (1.0 - 10 mM) suggesting that either diffusion was occuring or that there was a second transport system with a high K_m. The slope (1.2 nmoles/mM) or the linear portion or the velocity vs substrate curve was used to correct D-glucose uptake for diffusion. Lineweaver-Burke plots yielded an apparent K_m and V_{max} of 0.97 mM and 62 umoles L^{-1} RBC min^{-1} for 3MG uptake and 54 uM and 6 umoles L^{-1} RBC min^{-1} for D-glucose uptake (Fig. 2).

Competition for entry between 3MG and D-glucose was measured by incubating the erythrocytes with buffered saline containing a fixed concentration of (^{14}C)-3MG (5 mM) and various concentrations of (^{3}H)-D-glucose (1.25-10.0 mM). Entry of 3MG was progressively inhibited as the concentration of D-glucose was raised. 3MG (5 mM) inhibited D-glucose entry only at the lowest D-glucose concentration tested (1.25 mM) (Table 1).

Bovine erythrocytes failed to exhibit the phenomena of "accelerative exchange diffusion." Erythrocytes loaded

FIGURE 1. Uptake of 3-O-methyl-D-glucose and D-glucose by bovine erythrocytes. Erythrocytes were incubated at various substrate concentrations for 5 minutes at 37°C. 3-O-methyl-D-glucose uptake was assayed by METHOD I; D-glucose uptake was assayed by METHOD II. The amount of sugar which entered the cells (VELOCITY) is plotted against the concentration of sugar in the external media. The vertical lines represent the SEM of four values.

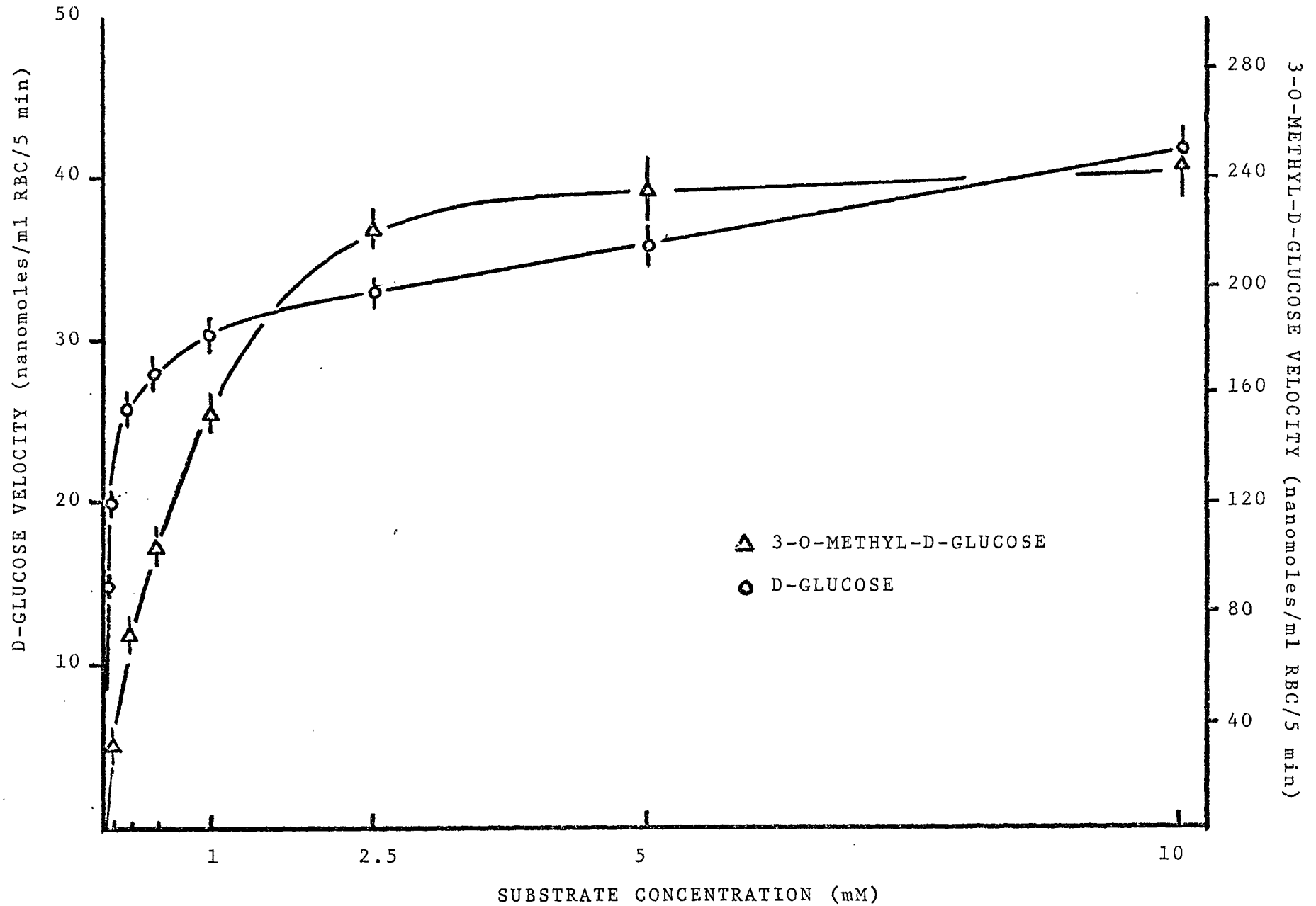
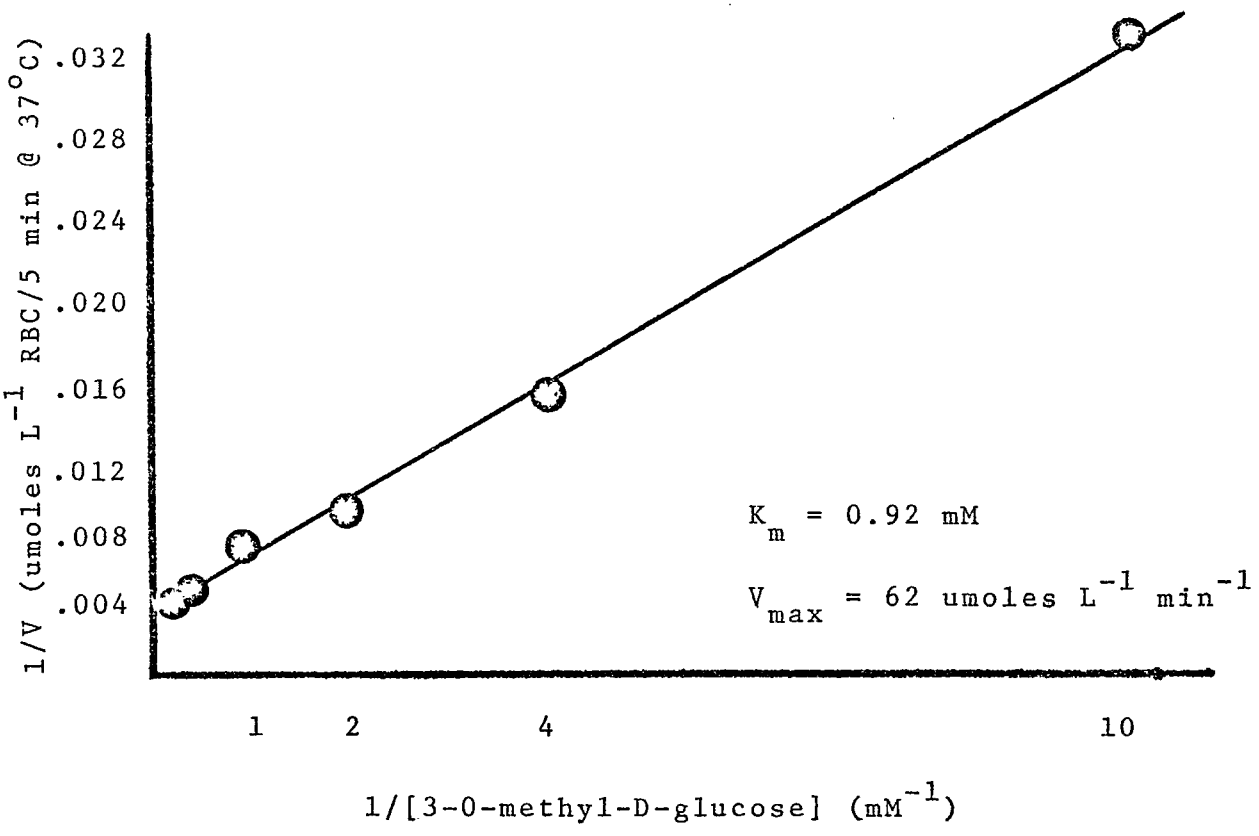

50
40
30
20
10
D-GLUCOSE VELOCITY (nanomoles/ml RBC/5 min)
280
240
200
160
120
80
40
3-O-METHYL-D-GLUCOSE VELOCITY (nanomoles/ml RBC/5 min)
3-O-METHYL-D-GLUCOSE
D-GLUCOSE
1
2.5
5
10
SUBSTRATE CONCENTRATION (mM)

FIGURE 2. LINEWEAVER-BURKE PLOTS OF 3-O-METHYL-D-GLUCOSE AND D-GLUCOSE ENTRY INTO BOVINE ERYTHROCYTES. DATA ARE TAKEN FROM FIGURE 1. THE LINES WERE FIT BY LEAST SQUARES REGRESSION ANALYSIS.


1/V (umoles L^{-1} RBC/5 min @ 37°C)
.032
.028
.024
.020
.016
.012
.008
.004
1
2
4
10
1/[3-0-methyl-D-glucose] (mM^{-1})
K_m = 0.92 mM
V_{max} = 62 umoles L^{-1} min^{-1}

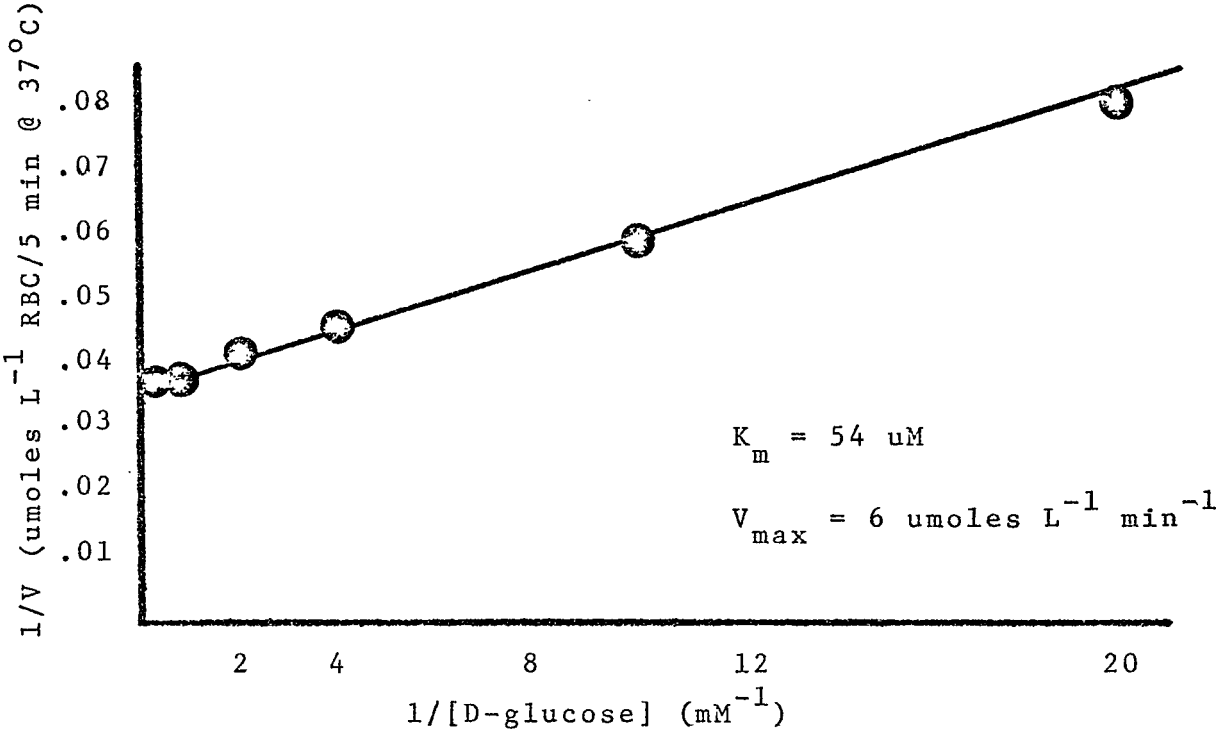

1/V (umoles L^{-1} RBC/5 min @ 37°C)
.08
.07
.06
.05
.04
.03
.02
.01
2
4
8
12
20
1/[D-glucose] (mM^{-1})
K_m = 54 uM
V_{max} = 6 umoles L^{-1} min^{-1}

TABLE 1

COMPETITION BETWEEN 3-O-METHYL-D-GLUCOSE AND D-GLUCOSE FOR ENTRY INTO BOVINE ERYTHROCYTES

SUGAR(S) IN THE EXTERNAL MEDIA		UPTAKE (nanomoles/ml RBC ± SEM)	
3MG and	D-glucose	3MG	D-glucose
5 mM	0	214 ± 18	0
5 mM	1.25 mM	132 ± 4	24 ± 2*
5 mM	2.5 mM	100 ± 4	32 ± 1
5 mM	5.0 mM	66 ± 4	39 ± 2
5 mM	10.0 mM	44 ± 2	50 ± 2
D-glucose			
1.25 mM			28 ± 1
2.5 mM			33 ± 6
5.0 mM			37 ± 5
10.0 mM			46 ± 8

Bovine erythrocytes were incubated with either (14C)-3MG and (3H)-D-glucose or (3H)-D-glucose for 5 minutes at 37°C and uptake was assayed by METHOD II.

D-glucose uptake in the presence and absence of 3MG was compared by t test (n=3).

* indicates a significant difference at $p < .05$.

with either labeled 3MG or D-glucose were placed in buffered saline containing various concentrations of unlabeled 3MG or D-glucose and exit of the label from the intracellular compartment was measured. When the same sugar was present both inside and outside of the cell exit of the label from the intracellular compartment was progressively inhibited as the external concentration increased (Fig. 3, Fig. 4).

Human erythrocytes exhibited "accelerative exchange diffusion" when the same sugar (D-glucose) was in both compartments (Fig. 5). A 36% increase in the efflux rate occured at 30 mM external D-glucose, however, at low external D-glucose (0.1 mM) efflux was 28% inhibited. This phenomena has not previously been reported in the literature.

The bovine sugar transport system was not inhibited by N-ethylmaleimide (NEM) or 1-fluoro-2,4-dinitrobenzene (DNFB); these agents are sulfhydryl reagents known to be potent inhibitors of sugar transport in human erythrocytes. However, the system was sensitive to heavy metals (Table 2).

An enhancement of sugar uptake in bovine erythrocytes was observed when erythrocytes were exposed to the two inhibitors sequentially. The enhancement was due to the inclusion of ethanol in the media for solubilization of DNFB (Novak, unpublished data).

FIGURE 3. EFFLUX OF 3-O-METHYL-D-GLUCOSE (3MG) FROM BOVINE ERYTHROCYTES INTO 3MG. ERYTHROCYTES WERE LOADED WITH (14C)-3MG TO AN INTERNAL CONCENTRATION OF 1.3 mM AND RESUSPENDED IN SALINE CONTAINING VARIOUS CONCENTRATIONS OF UNLABELED 3MG. THE AMOUNT OF (14C)-3MG LOST FROM THE RBC IN FIVE MINUTES AT 37°C IS PLOTTED AGAINST THE EXTERNAL CONCENTRATION OF UNLABELED 3MG. EACH POINT IS THE MEAN OF FOUR REPLICATES; THE VERTICAL LINES REPRESENT THE SEM.

THE FORMULA FROM THE LEAST SQUARES REGRESSION LINE IS $Y = -.00226X + 0.206$.

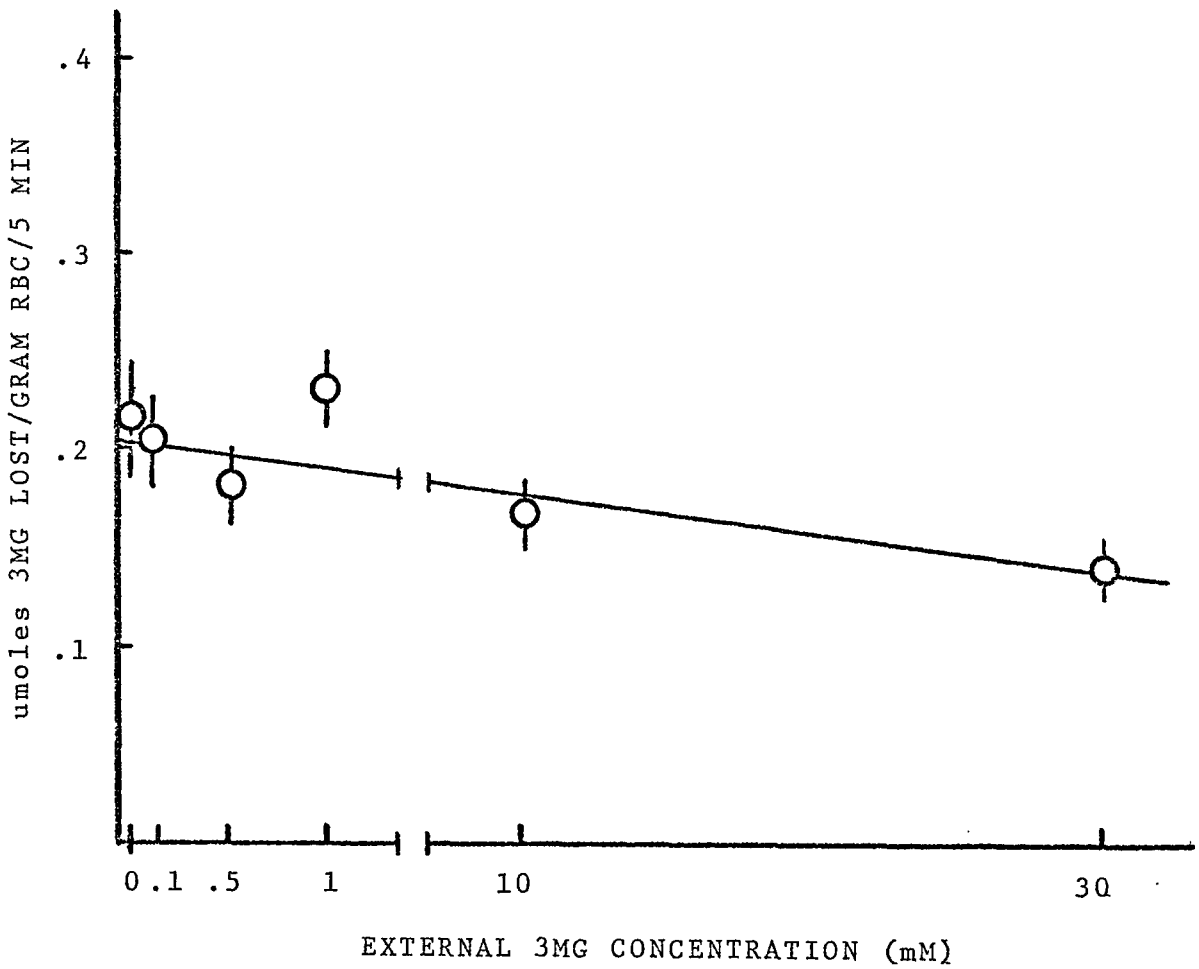

.4
.3
.2
.1
umoles 3MG LOST/GRAM RBC/5 MIN
0
.1
.5
1
10
30
EXTERNAL 3MG CONCENTRATION (mM)

FIGURE 4. EFFLUX OF D-GLUCOSE FROM BOVINE ERYTHROCYTES INTO D-GLUCOSE. ERYTHROCYTES WERE LOADED WITH (3H)-D-GLUCOSE TO AN INTERNAL CONCENTRATION OF 0.34 mM AND RESUSPENDED IN SALINE CONTAINING VARIOUS CONCENTRATIONS OF UNLABELED D-GLUCOSE. THE AMOUNT OF (3H)-D-GLUCOSE LOST FROM THE RBC IN FIVE MINUTES AT 37°C IS PLOTTED AGAINST THE EXTERNAL CONCENTRATION OF UNLABELED D-GLUCOSE. EACH POINT IS THE MEAN OF THREE REPLICATES; THE VERTICAL LINES REPRESENT THE SEM.

THE FORMULA FOR THE LEAST SQUARES REGRESSION IS
$Y = -.00003X + .01883$.

NOTE THAT THE ABSCISSA IS NOT TO SCALE.

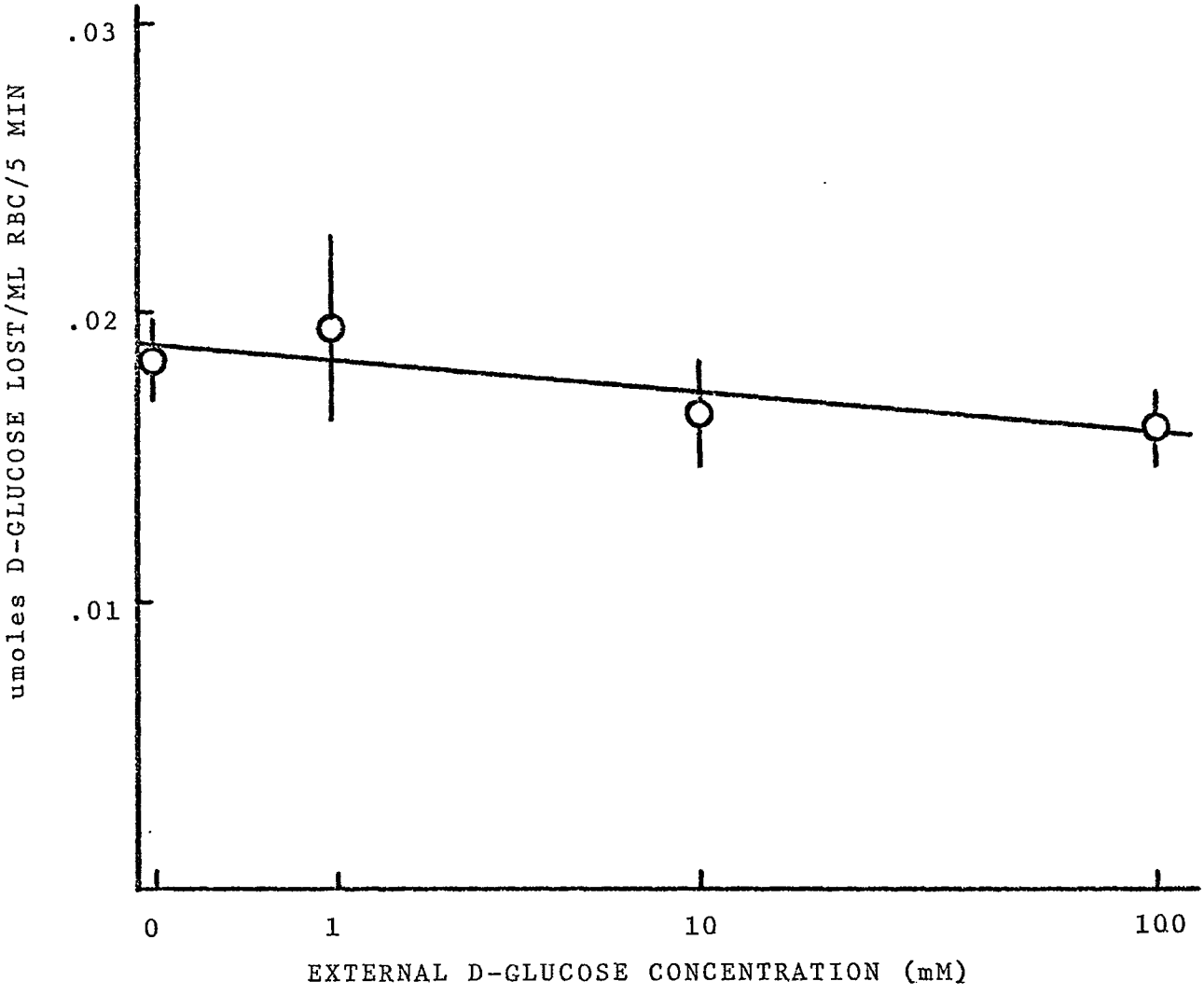

.03
.02
.01
umoles D-GLUCOSE LOST/ML RBC/5 MIN
0
1
10
100
EXTERNAL D-GLUCOSE CONCENTRATION (mM)

FIGURE 5. EFFLUX OF D-GLUCOSE FROM HUMAN ERYTHROCYTES INTO D-GLUCOSE. ERYTHROCYTES WERE LOADED WITH (14C)-D-GLUCOSE TO AN INTERNAL CONCENTRATION OF 1.6 mM AND RESUSPENDED IN SALINE CONTAINING VARIOUS CONCENTRATIONS OF UNLABELED D-GLUCOSE. THE AMOUNT OF (14C)-D-GLUCOSE LOST FROM THE ERYTHROCYTES IN 10 SECONDS AT 3°C IS PLOTTED AGAINST THE EXTERNAL CONCENTRATION OF UNLABELED D-GLUCOSE. EACH POINT IS THE MEAN OF FOUR REPLICATES; THE VERTICAL LINES REPRESENT THE SEM.

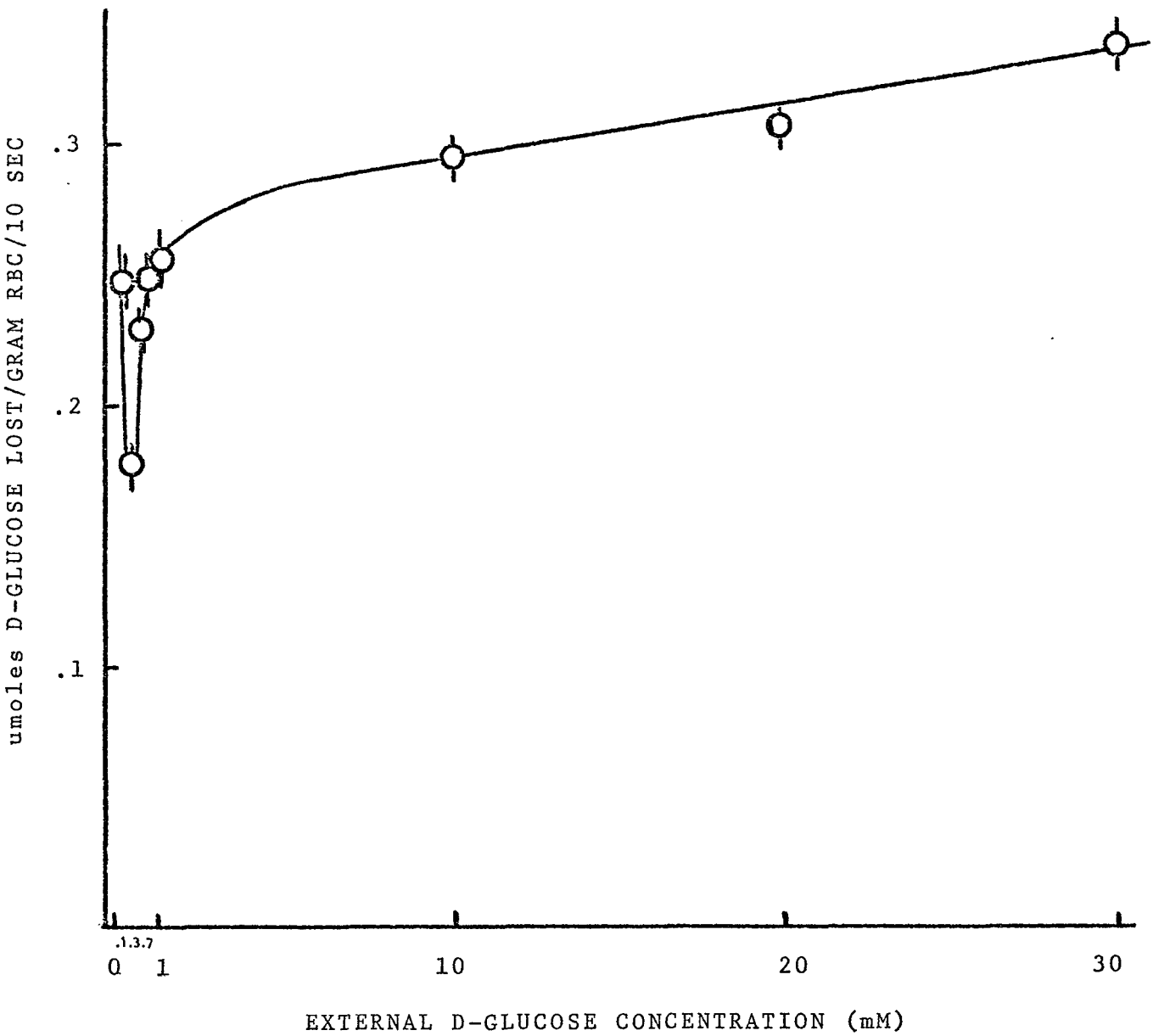

umoles D-GLUCOSE LOST/GRAM RBC/10 SEC
.3
.2
.1
.1.3.7
0
1
10
20
30
EXTERNAL D-GLUCOSE CONCENTRATION (mM)

TABLE 2

EFFECTS OF SULFHYDRYL REAGENTS ON SUGAR TRANSPORT IN BOVINE AND HUMAN ERYTHROCYTES

CELL TYPE	SH-REAGENT	TIME OF EXPOSURE	%INHIBITION
Human	NEM (30 mM)	30 min @ 37°	49.5, 48.9
Human	DNFB (5 mM)	15 min @ 37°	96.4, 96.6
Bovine	NEM (50 mM)	60 min @ 37°	1.1, 7.3
Bovine	NEM (20 mM)	60 min @ 37°	
	plus DNFB (2 mM)	60 min @ 37°	-15, -21
Bovine	stopping fluid	present during uptake @ 37°	93, 94

Sugar transport in human erythrocytes was measured as 10 second D-glucose entry at 22°C. Bovine erythrocyte sugar transport was measured as 5 minute 3MG entry at 37°C. All determinations were preformed in duplicate and all resulting values are presented.

A negative % inhibition signifies sugar uptake following treatment was greater than the corresponding controls.

Inhibition of sugar transport in human erythrocytes by sulfhydryl reagents.

Human erythrocytes were exposed to a variety of proven sulfhydryl reagents in the presence or absence of D-glucose to determine if D-glucose could influence the reaction of the reagents with the transport system. The presence of D-glucose enhanced the irreversible inactivation of the transport system by N-ethylmaleimide (NEM), alloxan, a-bromoacetophenone (a-BRAC) but not by N-cyclohexylmaleimide (NCM).

Erythrocytes were exposed to a combination of a reversible sulfhydryl reagent, p-chloromercuribenzenesulfonate (PCMBS), and an irreversible reagent, DNFB. (The PCMBS sensitive sulfhydryl groups of the erythrocyte membrane have been reported to be the essential sulfhydryl groups of the glucose transport system (van Stevenick et al., 1965); therefore PCMBS should protect the transport system from irreversible inactivation by DNFB.) After washing to remove PCMBS, the percent inhibition resulting from the treatments was: PCMBS 1-2%, DNFB + PCMBS 89.6 ± 1.6%, DNFB alone 95.5 ± .71% ($p < .05$) (Table 4). PCMBS did not affect the rate of DNFB inactivation but served to protect a fraction (6%) of the transport system from reaction with DNFB (Fig. 6).

TABLE 3

EFFECT OF D-GLUCOSE ON THE INTERACTION OF VARIOUS SULFHYDRYL REAGENTS WITH THE HUMAN ERYTHROCYTE GLUCOSE TRANSPORT SYSTEM

REAGENT	TIME OF INCUBATION AT 37°C (min)	D-GLUCOSE CONC. (mM)	% INHIBITION
N-ethylmaleimide (30 mM)	10 min	0.0	54.1, 56.2
		100.0	67.4, 64.2
N-cyclohexyl-maleimide (5 mM)	10 min	0.0	82.7
		100.0	83.2
	20 min	0.0	91.0
		100.0	91.9
Alloxan (10 mM)	10 min	0.0	0.2, 0.8
		100.0	9.1, 19.9
a-bromoaceto-phenone (2 mM)	15 min	0.0	10.6, 19.7
		100.0	29.8, 35.1

% inhibition is the irreversible inhibition resulting from exposure of the erythrocytes to the various reagents.

Sugar transport was measured as 10 second D-glucose entry at 22°C. Determinations were preformed either singly or in duplicate. All resulting values are presented.

TABLE 4

EFFECT OF p-CHLOROMERCURIBENZENESULFONATE (PCMBS) ON THE DEVELOPMENT OF IRREVERSIBLE DNFB INHIBITION OF GLUCOSE TRANSPORT IN HUMAN ERYTHROCYTES

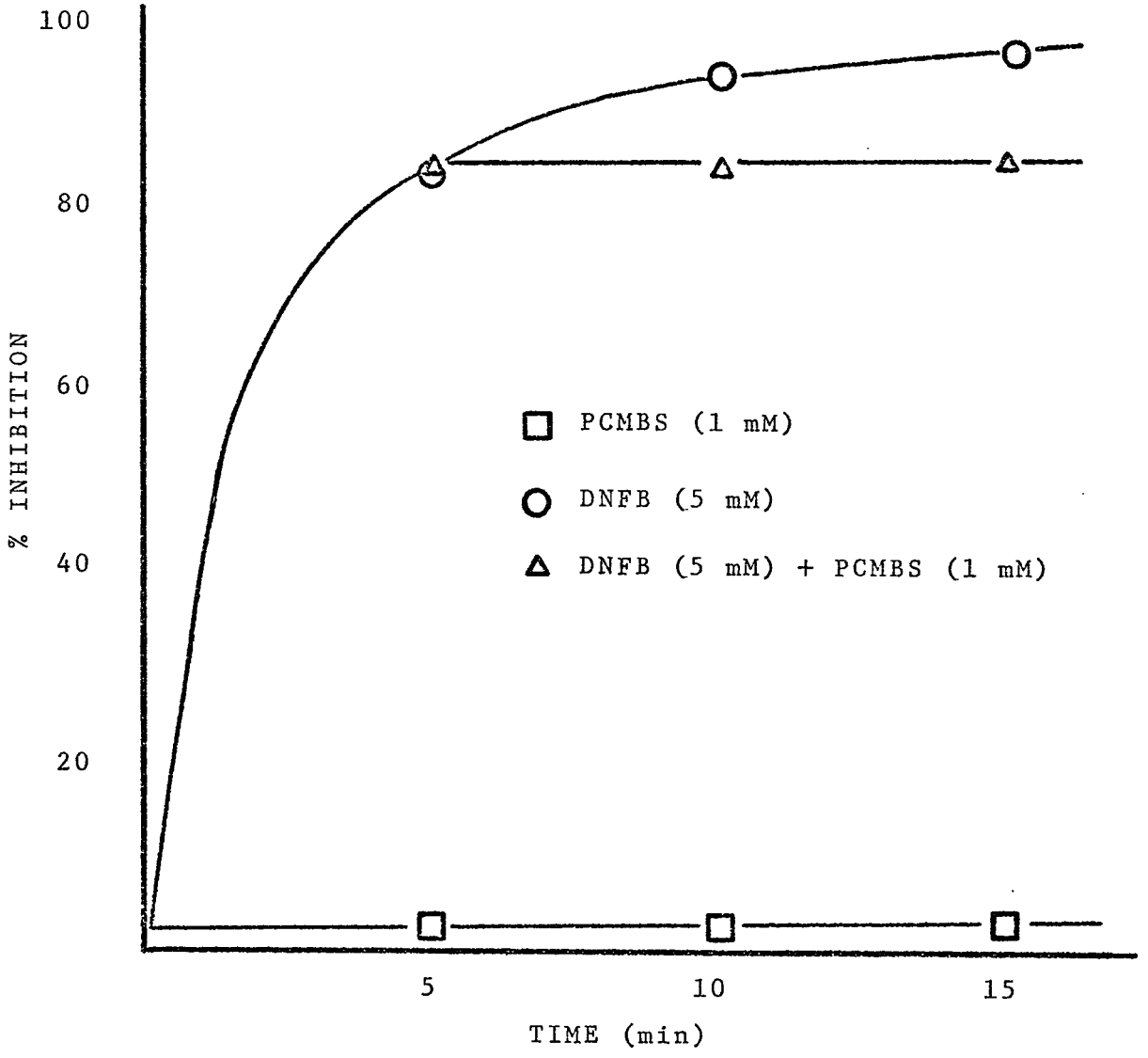
	% INHIBITION		
TRIAL	PCMBS	PCMBS + DNFB	DNFB
1	2.0	89.0	97.0
2	1.0	95.2	96.2
3	-	85.5	93.3
4	-	93.1	96.6
5	-	89.7	93.2
6	-	85.5	94.0
MEAN ± SEM		89.6 ± 1.6*	95.5 ± .71*

Each trial represents blood from a different donor.

Erythrocytes were incubated with or without 10^{-4}M PCMBS at a 16% Hct. for 15 minutes at 37°C. DNFB was then added to the appropriate samples to a final concentration of 5 mM and the incubation was continued for an additional 15 minutes. The cells were then washed with 5 mM mercaptoethanol in saline to remove the inhibitors and assayed for glucose entry at 22°C. Controls were manipulated in a similar fashion without addition of the inhibitors.

* indicates the means were significantly different by t test at $p < .05$.

FIGURE 6. PCMBS PROTECTION OF THE GLUCOSE TRANSPORT SYSTEM AGAINST IRREVERSIBLE DNFB INHIBITION. HUMAN ERYTHROCYTES WERE INCUBATED FOR VARIOUS PERIODS OF TIME AT 37°C WITH EITHER DNFB, PCMBS OR DNFB + PCMBS. THE REAGENTS WERE REMOVED BY WASHING THE CELLS WITH 5 mM MERCAPTOETHANOL-SALINE AND THE CELLS ASSAYED FOR GLUCOSE ENTRY (10 SEC) AT 22°C. EACH POINT REPRESENTS A SINGLE DETERMINATION.


100
80
60
40
20
% INHIBITION
PCMBS (1 mM)
DNFB (5 mM)
DNFB (5 mM) + PCMBS (1 mM)
5
10
15
TIME (min)

Differential labeling of the human erythrocyte glucose transport system with DNFB.

Treatment of erythrocytes with DNFB in conjunction with 2-deoxy-glucose resulted in an inhibition of glucose exit which was 2 to 4 times greater than that seen in erythrocytes treated only with DNFB (Fig. 7). Similar results have also been reported (Bowyer and Widdas, 1958; Krupka, 1971) and were interpreted as meaning that binding of a substrate induces a conformational change in the carrier so that a chemical group attacked by DNFB becomes more exposed or more reactive than in the free carrier (Krupka, 1971). This phenomena allowed for the design of an experiment to identify the carrier. An outline of the experimental procedure is shown in Figure 8.

Erythrocytes were treated with a sub-inhibitory concentration of NEM to block sulfhydryl groups not essential to transport and then exposed to either (^{3}H)-DNFB + 2-deoxy-glucose (2dG), (^{3}H)-DNFB + L-glucose, or only (^{14}C)-DNFB. (The specific activities of DNFB were 16.8, 17.2, and 1.12 uc/uM respectively.) The intact cells were extensively washed to remove inhibitor and substrate and the membranes were isolated by hypotonic hemolysis.

The effect of the treatments on sugar transport was determined by treating a parallel sample of erythrocytes with unlabeled DNFB under the same conditions. Glucose

FIGURE 7. ENHANCEMENT OF DNFB INHIBITION OF GLUCOSE EXIT BY 2-DEOXY-GLUCOSE. HUMAN ERYTHROCYTES WERE TREATED WITH DNFB IN THE PRESENCE OR ABSENCE OF 2-DEOXY-GLUCOSE (2dG) AT A 14% HCT. FOR VARYING PERIODS OF TIME AT 22°C. THE INHIBITOR AND SUBSTRATE WERE REMOVED BY WASHING AND THE CELLS WERE LOADED TO 75 mM WITH (3H)-D-GLUCOSE. GLUCOSE EXIT WAS MEASURED AS THE AMOUNT OF GLUCOSE LOST FROM THE RBC IN 10 SECONDS AT 22°C.

ERYTHROCYTES WERE PRETREATED WITH 10 mM N-ETHYLMALEIMIDE PRIOR TO EXPOSURE TO 2 mM DNFB.

EACH POINT IS THE AVERAGE OF DUPLICATE DETERMINATIONS.

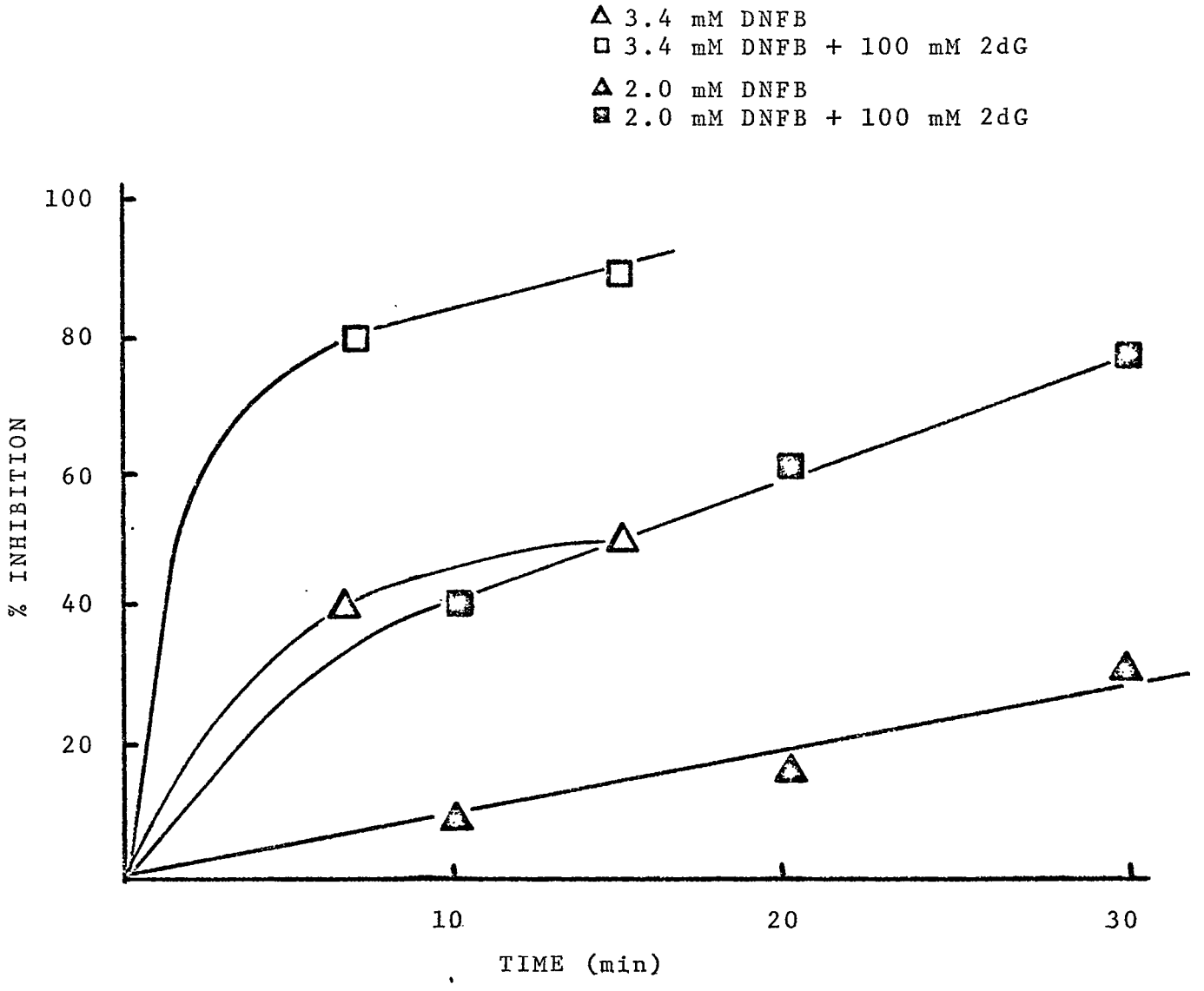
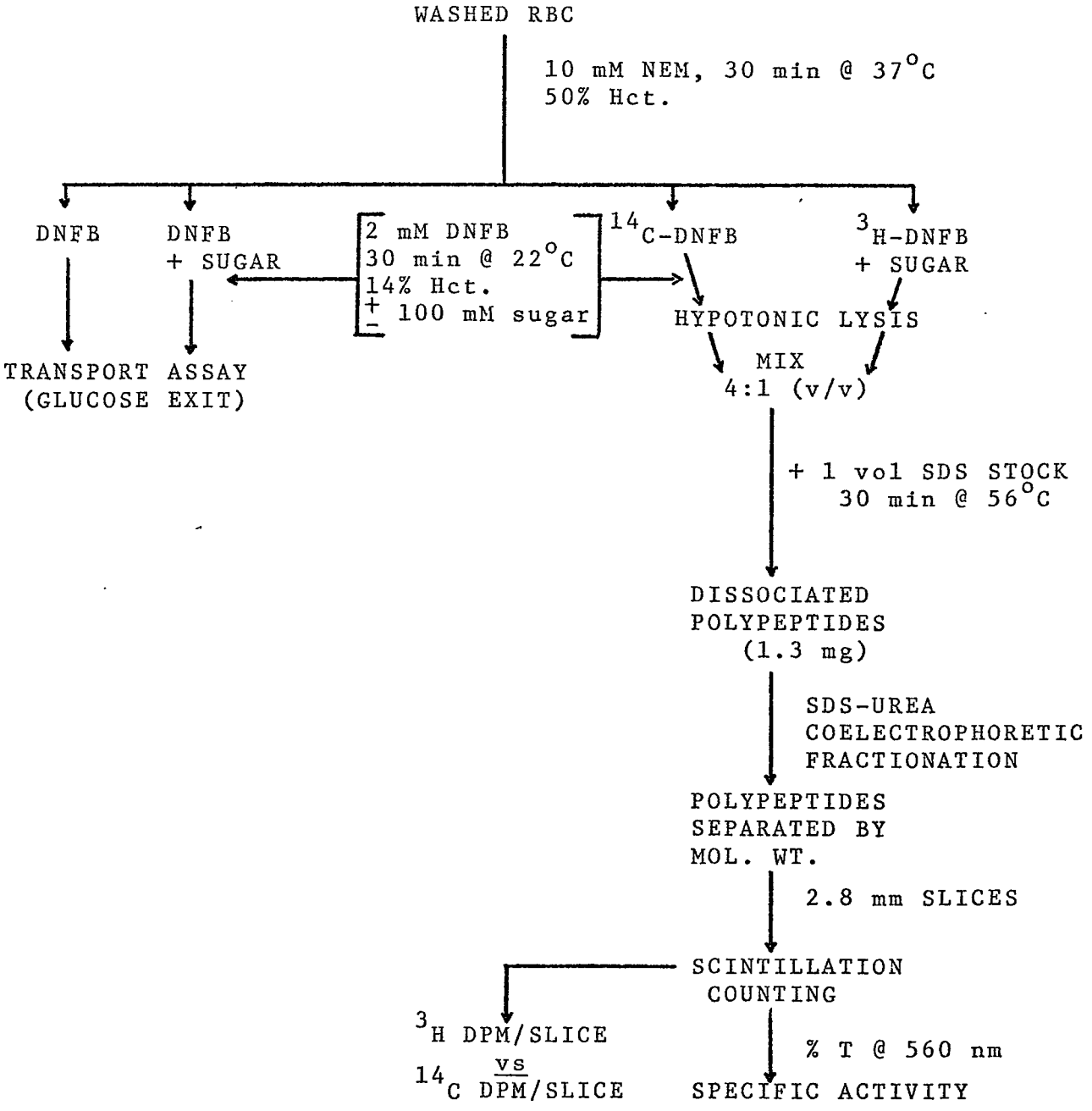

△ 3.4 mM DNFB
□ 3.4 mM DNFB + 100 mM 2dG
▲ 2.0 mM DNFB
■ 2.0 mM DNFB + 100 mM 2dG
100
80
60
40
20
% INHIBITION
10
20
30
TIME (min)

FIGURE 8. Experimental procedure for dual-isotopic differential labeling of membrane proteins of the glucose transport system of human erythrocytes.


WASHED RBC
10 mM NEM, 30 min @ 37°C
50% Hct.
DNFB
DNFB
+ SUGAR
2 mM DNFB
30 min @ 22°C
14% Hct.
+ 100 mM sugar
14C-DNFB
3H-DNFB
+ SUGAR
TRANSPORT ASSAY
(GLUCOSE EXIT)
HYPOTONIC LYSIS
MIX
4:1 (v/v)
+ 1 vol SDS STOCK
30 min @ 56°C
DISSOCIATED
POLYPEPTIDES
(1.3 mg)
SDS-UREA
COELECTROPHORETIC
FRACTIONATION
POLYPEPTIDES
SEPARATED BY
MOL. WT.
2.8 mm SLICES
SCINTILLATION
COUNTING
3H DPM/SLICE
vs
14C DPM/SLICE
% T @ 560 nm
SPECIFIC ACTIVITY

exit was inhibited 22% by the DNFB treatment and 80% by the DNFB + 2-deoxy-glucose treatment (Table 5).

The membranes derived from the above treatments were extracted with chloroform:methanol to determine if the presence of sugar altered the distribution of DNFB between membrane lipids and protein (Table 5). The sugars (L-glucose, 2-deoxy-glucose) caused an increased incorporation of DNFB into membrane lipids while protein-bound DNFB was unaltered. L-glucose caused a 10% and 2-deoxy-glucose a 24% increase in lipid-bound DNFB. Only the 2-deoxy-glucose incorporation was significantly different from the control incorporation (absence of sugar; $p < .05$). The average protein-bound DNFB (7.39 nmoles/mg) corresponded to 3.5×10^6 DNFB molecules/cell. The increase in lipid-bound DNFB by 2-deoxy-glucose (3.03 nmoles) corresponded to 1.45×10^6 DNFB molecules/cell.

Co-electrophoretic fractionation of the membranes was performed to determine if the presence of sugars altered the incorporation of (^{3}H)-DNFB into the various membrane proteins relative to the (^{14}C)-DNFB incorporation. However, the procedure required the use of whole membranes dissociated with SDS so that both membrane lipids and proteins were applied to the acrylamide gel. Since a significant amount of DNFB was bound to membrane lipids it was essential to determine the behavior of the lipids on the electro-

TABLE 5

DISTRIBUTION OF DNFB BETWEEN RBC MEMBRANE LIPIDS AND PROTEIN

TREATMENT	% INHIBITION OF GLUCOSE EXIT	N	PROTEIN DNFB (10^{-9} moles/mg protein)	LIPID DNFB	% TOTAL DNFB IN LIPID
^{14}C-DNFB	22%	4	7.67 ± .51	11.30 ± 1.04	59.6 ± .83
^{3}H-DNFB + L-glucose	(not tested)	5	7.11 ± .26	12.51 ± 0.78	63.6 ± 1.89
^{3}H-DNFB + 2-deoxy-glucose	80%	5	7.38 ± .31	14.41 ± 0.72*	66.1 ± 1.10*

% inhibition of glucose exit is the average of duplicate determinations using unlabeled DNFB.

Labeled DNFB values are the mean (± SEM) of N replicate chloroform:methanol extractions. Protein DNFB is the amount of DNFB in the precipitated proteins and lipid DNFB is that found in the supernatant.

Means were compared by t test.
* indicates a value which was significantly different from the control value (^{14}C-DNFB) at $p < .05$.

phoretic columns. To this end, the electrophoretic procedure was performed using a sample of unlabeled membranes containing (^{14}C)-phosphatidylethanolamine. One gel was sliced and counted while a duplicate gel was stained for protein (Fig. 9). The results indicated that the membrane lipids had a R_f of 1.66 relative to the leading protein band. Therefore, the electrophoretic separation of membrane proteins was carried out for a period of time sufficient to elute the lipids from the gel (24 hours).

Following liquid scintillation counting of the co-electrophoretic gels the ^{3}H and ^{14}C dpm of each slice was converted to a percentage of the total ^{3}H or ^{14}C dpm on the gel. The results from corresponding slices of five gels for each treatment (2-deoxy-glucose or L-glucose) were then pooled and analyzed by two-way analysis of variance with replication. No significant difference was observed between the two isotopes in either treatment.

Distribution of (^{14}C)-DNFB and the specific activity of the membrane proteins from a representitive gel is presented in Figure 10. The major peak of activity and highest specific activity was found in a major component of the erythrocyte membrane (component III). Component III is the only membrane protein found at both faces of the membrane; all other membrane proteins being located at the interior face of the membrane (Carraway et al., 1971; Bretscher, 1971;

FIGURE 9. MIGRATION OF PHOSPHATIDYLETHANOLAMINE ON SDS-UREA ACRYLAMIDE GELS. A SAMPLE OF HUMAN RBC MEMBRANES CONTAINING (14C)-PHOSPHATIDYLETHANOLAMINE WAS ELECTROPHORIESED ON 0.1% SDS, 8 M UREA, 5% ACRYLAMIDE GELS. (■) REPRESENTS THE 14C CPM/SLICE. (——) REPRESENTS A DENSITOMETER TRACING OF A DUPLICATE GEL STAINED FROM PROTEIN. THE ARROWS INDICATE THE POSITION OF THE TRACKER DYE FOLLOWING ELECTROPHORIESIS. B = BROMPHENOL BLUE; P = PYRONIN Y.

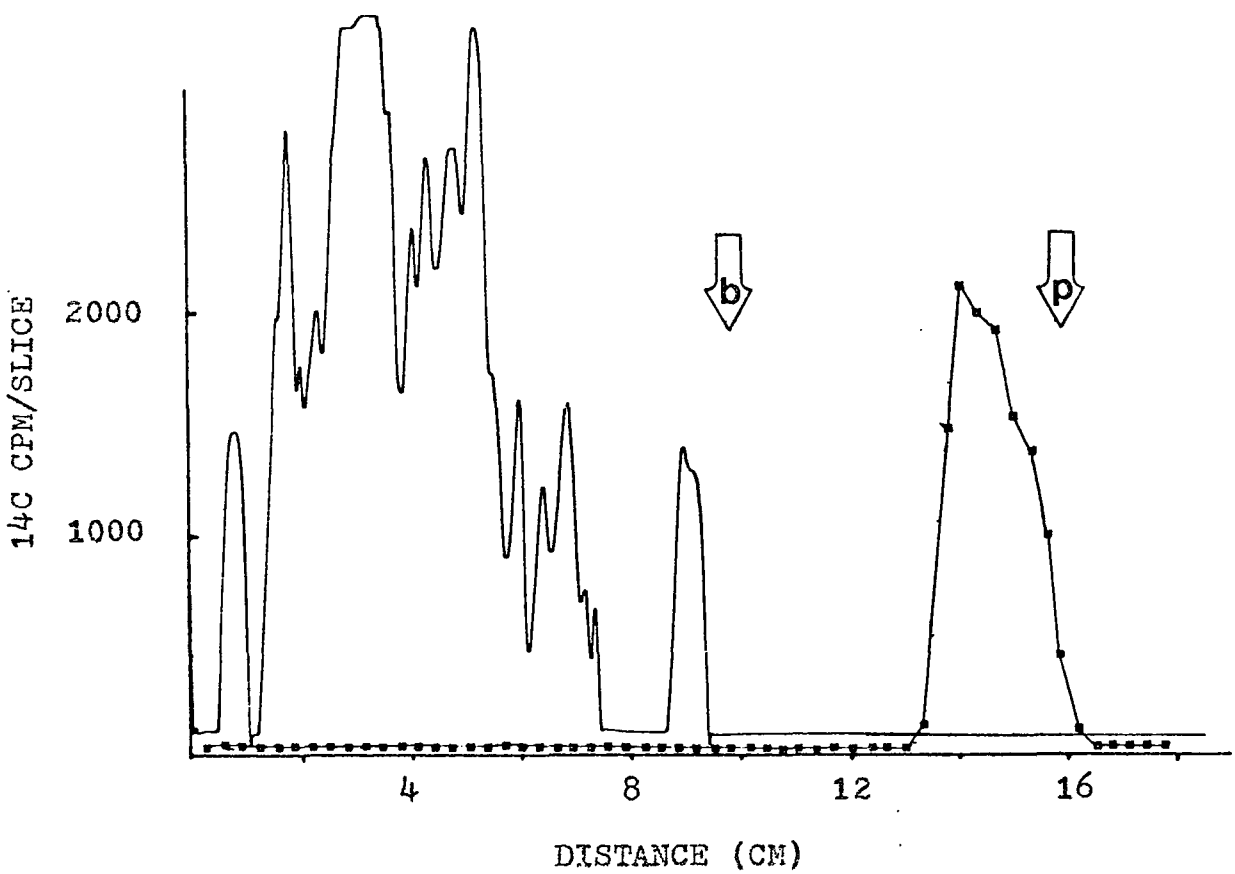
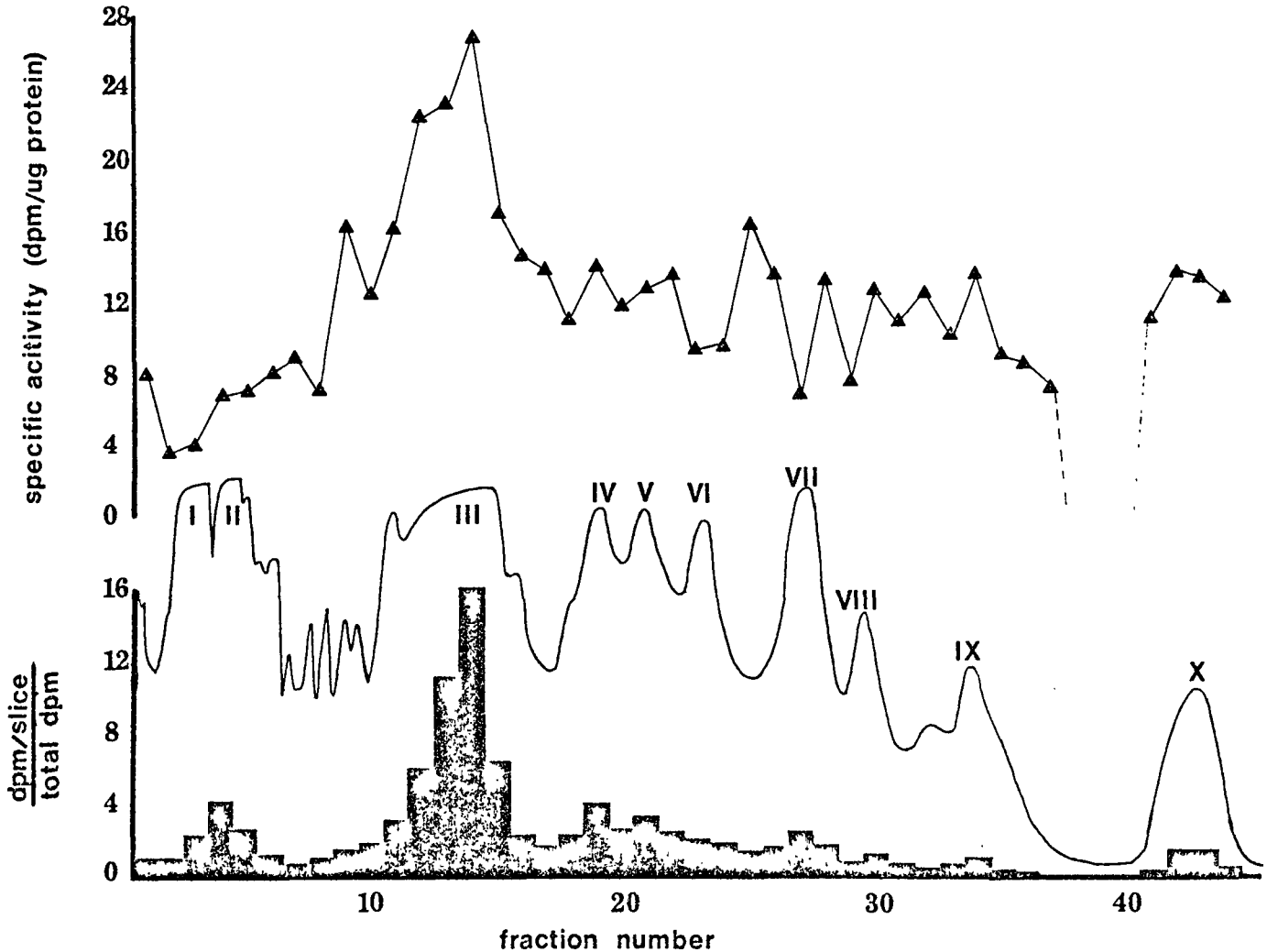

14C CPM/SLICE
2000
1000
b
p
4
8
12
16
DISTANCE (CM)

FIGURE 10. ^{14}C-DNFB INCORPORATION INTO ELECTROPHORETICALLY SEPARATED ERYTHROCYTE MEMBRANE PROTEINS. THE GRAPH REPRESENTS THE SPECIFIC ACTIVITY (▲), DENSITOMETER TRACING (——), AND THE % OF TOTAL ACTIVITY/GEL SLICE (▣) FROM AN L-GLUCOSE COELECTROPHORESIS GEL. THE MAJOR PROTEIN COMPONENTS ARE LABELED (I-X). THE GEL COMPOSITION WAS 5% ACRYLAMIDE, 0.1% SDS, 8 M UREA, pH 6.8.


specific acitivity (dpm/ug protein)
28
24
20
16
12
8
4
0
I
II
III
IV
V
VI
VII
VIII
IX
X
dpm/slice / total dpm
16
12
8
4
0
10
20
30
40
fraction number

Bender et al., 1971; Phillips and Morrison, 1971).

The distribution of radioactivity in component III was compared between treatments (2-deoxy-glucose vs L-glucose). The % total ^{3}H in each slice was divided by the % total ^{14}C in the same slice to yield a normalized ratio of ^{3}H to ^{14}C. (Component III extended over four slices, therefore, n=20 for each treatment.) The ratio of ^{3}H to ^{14}C in component III was 1.035 ± 0.129 (mean ± SEM) and 0.985 ± 0.0767 for L-glucose and 2-deoxy-glucose, respectively. These ratios were significantly different ($p < .05$) as determined by t test. The ratios corresponded to the binding of + 6400 and - 5000 (^{3}H)-DNFB molecules/cell for L-glucose and 2-deoxy-glucose, respectively.

DISCUSSION

Kinetic analysis of sugar transport in human RBC has led to the mobile carrier hypothesis which can be summarized as follows (Stein, 1967):

(1) The carriers are present in cell membranes in limited amounts.

(2) Carriers combine with permeants by undefined bonds but to an extent which depends on the nature of the carrier and permeant.

(3) The carrier-permeant complex can cross the membrane and must by physically movable in order to be available to permeant first from one side of the membrane and then from the other.

(4) The rates of transit of free carrier and carrier-permeant complex through the membrane can differ.

To date there has been no definitive evidence as to the biochemical nature of the hypothetical carrier in mammalian cells, although it is generally assumed to be a cell membrane protein (Stein, 1967; LeFevre, 1971). Since the various investigators have concentrated exclusively on the human erythrocyte, it was possible that the sugar transport system in non-human RBC might prove amendable to carrier isolation techniques. The bovine RBC appeared suitable because of its availability and investigations were undertaken to compare sugar transport in bovine vs human RBCs. The results are tabulated as follows:

	BOVINE RBC		HUMAN RBC
	3MG	D-glucose	D-glucose
K_m	0.97 mM	54 uM	4 mM*
V_{max} (L^{-1} min^{-1})	62 umoles	6 umoles	130 mmole*
AED**	absent	absent	present
Competitive Inhibition	yes	?	yes*
Chemical Inhibition (NEM, DNFB)	no	not tested	yes

* data from Stein, 1967
** Accelerative exchange diffusion

Sugar transport in bovine RBCs occurs via a facilitated diffusion system. This conclusion is based on the demonstration of saturation kinetics and competitive inhibition. However, the bovine system differs markedly from the human system in its lack of accelerative exchange diffusion, insensitivity to protein alkylating reagents and the slow rate of transport.

The phenomena of accelerative exchange diffusion (AED) is a direct consequence of the mobile carrier hypothesis and can be demonstrated as follows:

> Erythrocytes loaded with radioactive glucose to a low level internally are transferred to a large volume of external medium containing saline, together with glucose at a series of increasing concentrations. The efflux of labeled glucose in a fixed time period is plotted against the level of external glucose. Since the Km for this system is known, the experimental conditions are so chosen that with no glucose

> present externally the amount of glucose transferred in the extracellular fluid of the packed erythrocytes, together with that leaving the cell, produce a concentration of external glucose far below Km. A true unidirectional efflux of labeled glucose is therefore measured (Stein, 1967).

The results from such an experiment indicate that there is a direct acceleration of efflux to some limiting value as the external glucose concentration is raised (Levine et al., 1965). This is interpreted as meaning that the carrier-permeant complex has a greater mobility in the membrane than the free carrier; hence, the increased rate of return of carriers from the external face (as the carrier-permeant complex) to the internal face of the membrane will cause an increased rate of efflux. The ratio of the mobility of the loaded to unloaded carrier is 4 in human RBC (Levine et al., 1965; Levine and Stein, 1966).

In our investigations of this phenomena in human RBCs the external glucose concentration was varied from 0.1 mM to 30 mM as opposed to 1.0 mM to 30 mM previously reported (Levine et al., 1965). These data indicate that the AED phenomena is actually biphasic, with inhibition of glucose efflux occuring at low external glucose concentrations (0.1 - 1.0 mM) and acceleration at higher levels ($>$1.0 mM; see Fig. 5).

The biphasic nature of glucose efflux can be reconciled with the mobile carrier hypothesis if it is assumed that the sugar flux is mediated by a carrier having two sites of

differing affinities for glucose. When only the high affinity site is occupied, the carrier mobility is less than the mobility of free carrier and when both sites are occupied the carrier mobility is greated than that of free carrier (ie., glucose*carrier*glucose > carrier > carrier*glucose). A system of this nature has been hypothesized for sodium transport in human RBC (Gardner et al., 1972).

Sugar transport in bovine RBC does not exhibit AED, leading to the conclusion that in these cells carrier mobility is unaltered by bound substrate. While the presence of AED is strong evidence for a mobile carrier transport system, the converse is not true (Stein, 1967).

The data also suggest that the bovine RBC has two sugar transport systems since (1) the V_{max} for 3MG and D-glucose differ by a factor of 10 and (2) D-glucose inhibits 3MG uptake but 3MG did not appear to inhibit D-glucose transport; however, there are insufficient data to draw any firm conclusion.

Further studies on bovine RBC sugar transport were discontinued when it became apparent that the system was not inhibited by NEM or DNFB. The lack of inhibition by NEM could not be ascribed to a failure of the reagent to react with the membrane proteins because (^{14}C)-NEM was incorporated into membrane proteins (Novak, unpublished data).

The glucose carrier of human RBCs is thought to be of a proteinaceous nature. This concept is based on the observations that various reagents which react with protein sulfhydryl groups will inhibit transport (for a review see Stein, 1967). The sulfhydryl groups of the RBC membrane can be divided into various subpopulations based on their relative reactivity (Shapiro et al., 1970). Therefore, if one can manipulate the reactivity of the sulfhydryl groups essential for sugar transport and therefore, their incorporation of a radiolabeled inhibitor, it should be possible to identify the membrane protein(s) involved in the transport process.

One procedure for carrier identification is based on the observation that reaction of 0.6% of the membrane sulfhydryl groups (700,000/cell) with the organic mercurial p-CMBS will inhibit glucose transport by 80% (van Stevenick et al., 1965; LeFevre, 1971). p-CMBS is a reversible thiol reagent and protects the membrane sulfhydryl to which it is bound from irreversible reaction with DNFB (Knauf and Rothstein, 1971). Therefore, p-CMBS should protect 80% of the transport activity from irreversible inhibition by DNFB. Regeneration of the p-CMBS sensitive sulfhydryls followed by exposure to radiolabeled DNFB will then result in the selective incorporation of radiolabeled DNFB into the moiety containing sulfhydryl groups essential for

transport. However, the observed protection from DNFB by p-CMBS was 5.38 ± 1.27% (mean ± SEM). This implies that p-CMBS inhibition of glucose transport is not necessarily related to the binding of p-CMBS to carrier sulfhydryl groups. If the 700,000/cell p-CMBS sensitive sulfhydryls are responsible for 5.38% of the transport activity, then the total number of transport sulfhydryl groups must be on the order of 1.05 - 1.7 X 10^7/cell. This is two orders of magnitude greater than the predicted density of carrier sites (LeFevre, 1961b), but it is of the same order as the number of bound N-ethylmaleimide molecules required to cause 95% inhibition of transport (4 - 5 X 10^7/cell; LeFevre, 1971).

The carrier or substrate binding site of the transport system is analogous to an enzyme active center for which, various identification techniques are available (Vallee and Riordan, 1969). The technique most applicable to the identification of the RBC carrier moiety is differential labeling. The basis of this technique is the altered reactivity of protein groups following ligand or substrate binding. Substrate can either protect amino acid residues by shielding them from the attacking reagent or enhance the reactivity of certain groups via a conformational change in protein structure following binding (Koshland et al, 1962).

Glucose transport shows increased reactivity to a

variety of sulfhydryl reagents, particularly DNFB, when glucose is present (Fig. 9; Table 3; Bowyer and Widdas, 1958; Krupka, 1971). For DNFB the increase in reactivity is related to the affinity of the substrate for the transport system and is the result of the formation of a 1:1 complex between sugar and carrier (Krupka, 1971). These observations indicate that the carrier undergoes a conformational change upon binding of substrate so that a protein group becomes more reactive to attack by DNFB. Therefore, if one exposes RBC to (^{3}H)-DNFB plus 2-deoxy-glucose (a high affinity substrate) or only to (^{14}C)-DNFB there should be a selective incorporation of ^{3}H over ^{14}C into the carrier moiety.

DNFB is a highly reactive protein reagent, reacting primarily with cysteinyl (sulfhydryl) residues and to a lesser extent with lysyl, tyrosyl, and histidyl residues of RBC membrane proteins (Forsling and Widdas, 1967; Stein, 1968). In addition, DNFB reacts with RBC membrane phospho-lipids, being incorporated into the amino group of phospha-tidylethanolamine but not phosphatidylserine (Forsling and Widdas, 1967, Passow and Schnell, 1969).

Our data indicate that the amount of DNFB bound to membrane proteins is approximately 3.6 X 10^{6} molecules/cell. This is 40-fold less than the amount of N-ethylmaleimide binding required for 95% inhibition of transport (LeFevre, 1971).

The upper limit of the number of carrier sites is put at 500,000/cell (LeFevre, 1961b). In addition, measurement of glucose binding to RBC membranes indicates 250,000 binding sites/cell (Kahlenberg et al., 1971). This would seem to indicate that approximately 10% of the protein-bound DNFB is incorporated into the transport system.

The reaction of DNFB with the transport system shows first order kinetics; therefore, one DNFB reacts with one carrier to yield one inactive carrier (Krupka, 1971). Assuming (1) that this reaction is enhanced by a transported sugar, (2) the number of carrier sites/cell is 250,000, and (3) in the presence and absence of 2-deoxy-glucose 80% and 20% of the carrier sites react with DNFB, respectively; the calculated binding of DNFB to membrane proteins in the presence and absence of 2-deoxy-glucose should be on the order of 6.69 and 7.12 X 10^{-9}moles/mg membrane protein, respectively. However, the overall incorporation of DNFB into membrane proteins is unaltered (see Table 5) by the presence of a transported sugar (2-deoxy-glucose) or a non-transported sugar (L-glucose; LeFevre and Marshall, 1958). Even though the small calculated difference between treatments could be obscured by the variability of the extraction procedure, one would expect to see a trend towards greater DNFB incorporation in the presence of 2-deoxy-glucose.

In light of the assumptions mentioned above, it is

possible to calculate the ^{3}H enrichemnt of the carrier under the conditions of our experiments for the following cases:

> CASE 1) The carrier is a singly polypeptide chain, present in the membrane in fixed amounts. The carrier (3H/14C) enrichment ratio (dpm:dpm) would then equal 58:1, while a non-carrier protein (3H/14C) ratio would be 14.4:1. This represents a carrier enrichment of 400%.
>
> CASE 2) Special Site Hypothesis. The carrier can bind a certain amount of DNFB with little or no effect on transport. However, the carrier also contains a sugar-sensitive group, such that incorporation of DNFB into this group will block transport. It follows that the (3H/14C) ratio of the carrier will be variable, depending on the amount of non-inhibitory DNFB binding. Assuming that the non-specific binding occurs rapidly, the carrier (3H/14C) ratio will be enhanced over the non-carrier (3H/14C) ratio by 46%, 26% and 18% for the non-specific binding of 1, 2, or 3 DNFB molecules, respectively.

Coelectrophoretic analysis of the ^{3}H and ^{14}C content of the individual polypeptide chains failed to demonstrate a peak of ^{3}H enrichment approaching that predicted by CASE 1 or CASE 2. In fact, <u>all</u> membrane polypeptides incorporate both isotopes in equal amounts, ie., there is no significant difference between the ^{3}H and ^{14}C content of the various gel fractions. Therefore, the hypothesis that the carrier is a simple membrane protein is not tenable.

Taking into account the dilution factors involved in preparing the membranes for electrophoresis and the counting background, the lower limit of carriers which could be detected is ca. 5×10^{3}/cell. This value assumes that the

carrier has a molecular weight that distinguishes it from the rest of the membrane proteins. The coelectrophoretic fractionation is based on molecular weight; however, homogeneity of molecular weight does not imply homogeneity of physiological function. If the carrier is present in small amounts and has a molecular weight identical to that of a major membrane component, the expected ^{3}H enrichment would be obscured by the larger amount of non-specific ^{3}H binding. The grand mean of the normalized (3H/14C) ratios is 0.9989; the 95% confidence limits are 0.9510 and 1.0468. Using the confidence limits as an estimate of the variability, it appears that the ^{3}H content of a slice would have to differ from the ^{14}C content by ca. 10% to be significantly different.

Comparison between the two sugar treatments (L-glucose vs 2-deoxy-glucose) reveals that the sugars have a significant effect on the incorporation of DNFB into component III (C-III) of the membrane polypeptides (see Figure 10). L-glucose enhances the binding of DNFB (+ 6400 molecules/cell) while 2-deoxy-glucose inhibits DNFB binding (- 5000 molecules/cell) to this component.

C-III appears to be a mixture of polypeptide chains of almost identical molecular weight (100,000 daltons) and is poorly resolved by electrophoresis. C-III comprises ca. 20% of the membrane protein and binds 28% of the total

incorporated DNFB. In the intact RBC, C-III is the only membrane protein which is exposed to the environment at both the inner and outer faces of the membrane (Bender et al., 1971; Carraway et al, 1971; Bretscher, 1971; Phillips and Morrison, 1971). This unique property of C-III and the face that sugars can alter its ability to incorporate DNFB suggests that it has some role in the sugar transport process. However, the pattern of DNFB incorporation is just the opposite of that predicted from the transport data. 2-deoxy-glucose should enhance DNFB incorporation, but protects C-III from DNFB attack. L-glucose, with no measurable affinity for the transport system (LeFevre and Marshall, 1958) should not affect DNFB binding by C-III, but enhances binding. These observations can be reconciled; sugars, in general can bring about changes in protein structure (Laskowski, 1966). Therefore, the effect of L-glucose could be due to a non-specific interaction with C-III. The 2-deoxy-glucose effect is consistent with protection of a binding site involved in transport, however, 2-deoxy-glucose enhances DNFB inhibition of transport. Therefore, 2-deoxy-glucose must direct DNFB incorporation into a translocation-specific site.

The data indicate that the translocation-specific site is located in the membrane lipid fraction because 2-deoxy-glucose increases the incorporation of DNFB into

this fraction (see Table 5).

It is generally assumed that inhibition of sugar transport by protein reagents is the result of their interaction with a specific membrane protein (Stein, 1967). However, for the most part, the reaction of inhibitors with membrane lipids has been neglected. Only two inhibitors have been shown to bond covalently to membrane phospholipids: N-ethylmaleimide (Forsling, 1967) and DNFB (Table 5; Forsling 1967; Forsling and Widdas, 1967; Passow and Schnell, 1969).

Phospholipids form phospholipid-sugar complexes which will carry sugars into highly nonpolar solvents and sugar solubilization is inhibited if the phospholipids are first treated with DNFB (LeFevre et al., 1964). Furthermore, the transport system is sensitive to hydrolysis of membrane phospholipids by various phospholipases (Jung, 1971). The above findings suggest that phospholipids are intimately involved in the transport process. However, phospholipids do not exhibit the selectivity toward sugar stereoisomers that is characteristic of the in vivo transport system. Therefore, the carrier could be a membrane proteolipid or lipoprotein, such that binding of sugar is a function of the protein moiety while translocation of the sugar-carrier complex is associated with the phospholipid molecules (LeFevre, 1968).

This hypothesis is the one which is most consistent

with our data, ie., binding of 2-deoxy-glucose to C-III causes (1) a protection of the binding site from DNFB and (2) a conformational change such that DNFB-reactive phospholipids are exposed to DNFB attack; the end result being a 2-deoxy-glucose enhancement of DNFB inhibition of transport. The true nature of the DNFB-binding material in the lipid extract is not known. The extraction procedure will remove proteolipids from RBC (Folch et al., 1959; LeFevre, 1968) and thus it is possible that the intact carrier is removed with cell lipids. The DNFB-binding material migrates with the solvent front on thin layer chromatograms using the common phospholipid solvent systems, while the membrane protein remains at the origin (Novak, unpublished data). Proteolipids behave in a similar fashion, ie., the lipids migrate while the protein remains at the origin (Folch et al., 1959). Thus it appears that the DNFB-binding material is a membrane phospholipid, probably phosphatidylethanolamine, which has been rendered more nonpolar by the incorporation of DNFB.

The assays for inhibition of transport are based on the movement of substrate between two compartments. Thus, the observed inhibition is the result of inhibition of translocation and/or the inhibition of formation of the sugar-carrier complex, ie., binding. At present there is no way to distinguish between the two possibilities. This

greatly confounds any attempt to estimate the number of transport sites/cell from our data. Sugar transport in the control cells (^{14}C-DNFB) is inhibited by 20% but there are no data to indicate the percentage contributed by translocational and/or binding inhibition. However, it appears that a certain proportion of the 20% inhibition of sugar transport is due to inhibition of binding because 2-deoxy-glucose protects C-III while enhancing inhibition by 60%. The 5000 sites/cell protection could represent only a very small percentage of the total number of binding sites. For example, assuming (1) that DNFB inhibition in the absence of 2-deoxy-glucose is due only to the destruction of 20% of the total binding sites and (2) DNFB inhibition in the presence of 2-deoxy-glucose is the sum of a 19% reduction in the number of binding sites and a 61% decrease in translocation, the 5000 sites/cell would only represent 1% of the total number of sites. If it is assumed that the 60% difference in inhibition between the two treatments (with and without 2-deoxy-glucose) is due entirely to incorporation of DNFB into membrane lipids, then the total number of lipid DNFB-sensitive sites is 2.4 x 10^6/cell. This can be considered an overestimate since the actual transport-specific lipid sites could very possibly be a subpopulation of the total number of lipid sites.

L-glucose causes a small, nonsignificant increase

in the incorporation of DNFB into the membrane lipid fraction (9.04×10^{-19} mole/cell). Because L-glucose does not interact with the transport system (LeFevre and Marshall, 1958), the increased DNFB incorporation is probably the result of a non-specific perturbation of membrane structure by L-glucose. Using the L-glucose data as an estimate of non-specific DNFB binding, the total number of lipid DNFB-sensitive sites can be corrected for non-specific sugar effects to yield 1.53×10^{6}/cell. This value is ca. five-fold greater than the accepted number of transport sites (Stein, 1967). However, the accepted values are derived from indirect measurements, ie., inhibition, etc.; there is no direct evidence for the selective binding of glucose by RBC membranes under physiological conditions. Since there is no basis for comparison, the 1.53×10^{6} lipid DNFB-sensitive sites may or may not represent the actual number of transport sites.

SUMMARY

The distinguishing features of bovine as opposed to human RBC sugar transport are (1) a slow rate of transport, (2) lack of substrate alteration of carrier mobility, and (3) insensitivity to reagents capable of reacting with membrane proteins and lipids.

In human RBCs the sugar carrier appears to be a membrane-bound lipoprotein which undergoes a conformational change upon substrate binding. The conformational change alters the reactivity of membrane phospholipids associated with the transport system resulting in their greater incorporation of DNFB; the end result is that substrate enhances the inhibition of the system by DNFB.

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