J. Membrane Biol. 12, 177 – 188 (1973)

A Study of the Dependence of the Human Erythrocyte Glucose Transport System on Membrane Sulfhydryl Groups

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Received 14 August 1972

Summary. A brief review of the data relating the glucose transport system and other membrane functions of red cells to surface sulfhydryl groups is presented. The effect of a variety of sulfhydryl reagents on glucose efflux rates from loaded red cells was studied. Neither iodoacetate nor iodoacetamide at 5 mM inhibited efflux. Several maleimide derivatives and disulfides inhibited efflux in 0.7 to 2.0 mM concentrations. Organomercury compounds, on the other hand, were active in the 0.07 to 0.1 mM range. These data suggest that, if sulfhydryl groups are important in the glucose efflux process, they are not equally accessible to the above reagents; and that the primary effect of these reagents may be on structural elements near membrane sulfhydryl groups.

The inhibition of glucose transfer in erythrocytes by organic mercurials was studied in considerable detail by LeFevre (1948). The dependence of the glucose transport system of human erythrocytes upon membrane-associated sulfhydryl groups and the possible location of essential sulfhydryls in the membrane were suggested by Van Steveninck, Weed and Rothstein (1965) from their studies of the reaction of various organic mercury compounds with human erythrocytes.

Uptake experiments have shown that a small amount of *p*-chloromercuri-benzenesulfonate (PCMBS) reacted very rapidly with the cell, binding to 0.8×10^6 sites per cell (Sutherland, Rothstein & Weed, 1967). With time there was additional slower uptake of the agent. At 0.5 mM

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PCMBS, maximum uptake occurred after 5 hr, corresponding to 6.5×10^6 molecules per cell. These authors suggested that the initial reaction was with groups on the outer surface of the membrane, and that these were responsible for the inhibition of glucose efflux from red cells. Experiments (Scott, Knight, Settlemire & Brierley, 1970) on the differential effects of mercury reagents on membrane thiols of mitochondria indicated that the polarity of PCMBS was a major feature governing its initial reaction with and lack of penetration of membranes.

Other cell membrane functions, such as cation permeability (Passow, 1964) and Rh antigen activity (Green, 1967) were also altered when red cells were treated with organo-mercurials. In these cases it was assumed that they acted to block an essential sulfhydryl needed for the maintenance of these functions.

The inhibition of the red cell glucose transport by N-ethyl-maleimide (NEM) was studied by Dawson and Widdas (1963). NEM was known to penetrate the red cell membrane and readily react with intracellular thiols (Jacob & Jandl, 1962; Beutler, Duron & Kelly, 1963). The initial uptake of NEM by cells, however, was not associated with blocking glucose transport but longer reaction times of NEM with the cell resulted in inhibition, as seen in a later study (Forsling, Remfry & Widdas, 1968).

Green (1967) studied the effect of sulfhydryl agents on the Rh antigen activity of the red blood cell membranes, noting that there was an initial rapid uptake of NEM by the membrane with no loss of antigen activity. Further reaction with the membrane, during 2 hr, resulted in complete loss of antigen activity paralleling the increased uptake of NEM. These results would indicate that the most reactive sulfhydryls (SH's) were not those involved with antigenic activity, whereas some less reactive groups were.

Although organic mercury compounds and NEM, which are widely used as sulfhydryl reagents, block erythrocyte glucose transport, a classic sulfhydryl reagent, iodoacetate, has been reported as having no effect on glucose entrance into cells (LeFevre, 1948). The ineffectiveness of iodoacetate in altering certain cell membrane functions while NEM and organic mercurials do affect them is not unique to glucose transport processes. Passow (1964) reported that iodoacetate produced no effect on passive cation permeability, whereas mercurials and NEM had a marked effect. To our knowledge, iodoacetamide, a sulfhydryl reagent with no ionic charge at pH 7, has not been studied in these systems. Green's (1967) work with erythrocyte membrane Rh antigen activity is an example where iodo-

acetamide was ineffective while mercurials and NEM altered a cell membrane property. Work done with various sulfhydryl reagents led Shapiro, Kollman and Martin (1970) to suggest that classes of thiol groups of the red blood cell membrane show differential reactivity towards various agents.

The differences in effectiveness of sulfhydryl agents poses the question of whether more than one type of sulfhydryl group may function to maintain the glucose transport system.

Another possibility remains: that inhibition of glucose transport by these reagents is not caused by reaction with sulfhydryl groups, but with some other membrane-associated groups. Organo-mercurials and NEM have been shown to be reactive towards, in addition to sulfhydryls, amino and imidazole groups, under certain conditions (Horowitz & Klotz, 1956; Smyth, Nagamatsu & Fruton, 1960; Smyth, Blumenfeld & Konigsberg, 1964). The present study was an attempt using a variety of thiol reagents, with differing specificities toward sulfhydryls, to determine if the glucose transport system of the human erythrocyte was dependent upon membrane-associated sulfhydryl groups.

Materials and Methods

Sources of Materials

5,5′-Dithiobis (2-nitrobenzoic acid) (DTNB) and 5,5′-dithio dipyridine (DTP) were obtained from the Aldrich Chemical Company; N-ethylmaleimide (NEM), iodoacetamide, and ethylene-(dinitrilo)-tetraacetic acid (EDTA) from Eastman Organic Chemicals; N-butyl, N-cyclohexyl, and N-phenylmaleimides from Nutritional Biochemicals; *p*-chloromercuribenzoic acid (PCMB) from Mann Research Laboratories; PCMBS from Sigma Chemical Company; and glucose oxidase and *o*-dianisidine from Worthington Biochemicals Corporation. Other compounds were reagent grade or better.

Solutions

Phosphate-buffered saline (PBS) used was 0.9% NaCl, 0.01 M NaH_2PO_4, adjusted to pH 7.4.

Anhydrous glucose was dissolved in PBS to give 2.5% or 3.0% glucose. The "exiting" solution into which glucose-loaded cells were placed was, in a few early experiments, 1.7% NaCl, 0.02 M NaH_2PO_4, adjusted to pH 7.4, chosen to be approximately isoosmotic with the cell contents after equilibration with 5.0% glucose (van Steveninck *et al.*, 1965). In the majority of the experiments, however, efflux was into a solution of 1.4% NaCl, 0.01 M NaH_2PO_4, adjusted to pH 7.4.

Solutions of sulfhydryl reagents were prepared on the day of the experiment to insure that a minimum of breakdown would occur. DTNB solutions, however, were kept

under refrigeration for several days. Use of solutions differing from these will be noted in the results of individual experiments.

Red Cells

Human blood was collected from staff and students of this Medical Center utilizing "Vacutainers" containing disodium ethylenediamine-tetraacetic acid (EDTA) as anticoagulant. After centrifugation, the plasma and white cells were removed by suction and the red cells washed twice with PBS at room temperature.

Glucose Loading

Following the method of van Steveninck *et al.* (1965), these red cells were suspended in an equal volume of PBS containing 3% glucose. The suspension was incubated at 37 °C for 1 hr, mixing at about 15-min intervals to insure an even distribution of cells. In some experiments the cells were loaded in the presence of inhibitor.

Efflux Rate Measurements

After centrifugation of the loaded cells and removal of the supernatant, the cells were cooled on ice and mixed with 10 to 15 volumes of cold exiting solution of known inhibitor concentration. The temperature of the cell suspension was kept between 4 and 6 °C. Glucose concentration was evaluated in the supernatant after centrifuging 1 to 3-ml aliquots of the suspension. The glucose oxidase method (Washko & Rice, 1961) was used to assay the supernatant.

Calculations

The % Inhibition (I) was computed as follows:

$$I = 100\left[1 - \frac{\Delta G_i}{\Delta G_c}\right]$$

where

ΔG_i = the difference in the glucose concentration of the supernatant of the inhibitor-treated sample at given time t and at zero time.

ΔG_c = the difference in the glucose concentration of the supernatant of the control sample (i.e., run without inhibitor) at the final time (routinely 90 min) and at zero time.

The analytical methods used resulted in a range of 10 to 15% of the mean values obtained for I in any one experiment.

Results

Organic Mercury Compounds

At a PCMB concentration of 0.1 mM (Fig. 1), a 70 to 80% inhibition was observed whether the organo-mercury compounds were present during the loading and exiting phases or only during the exiting stages (Table 1).

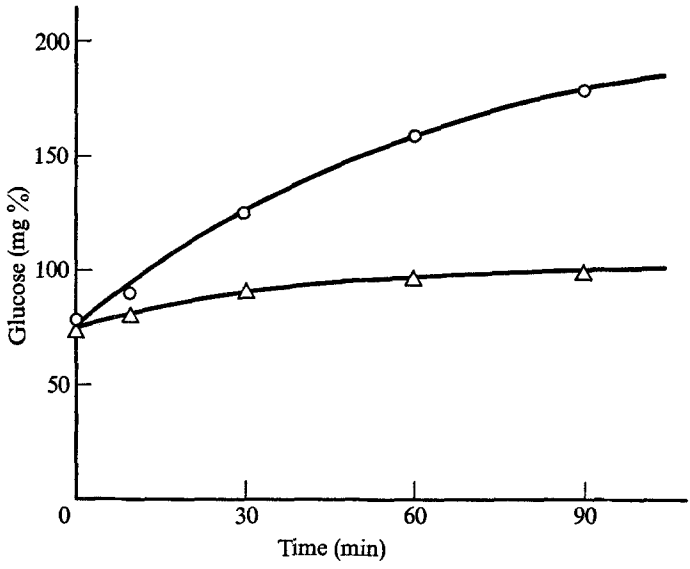

Fig. 1. Glucose levels, as a function of time, in the supernatants of samples of glucose-loaded red cells treated with 0.1 mM PCMB (Δ) or no inhibitor (0)

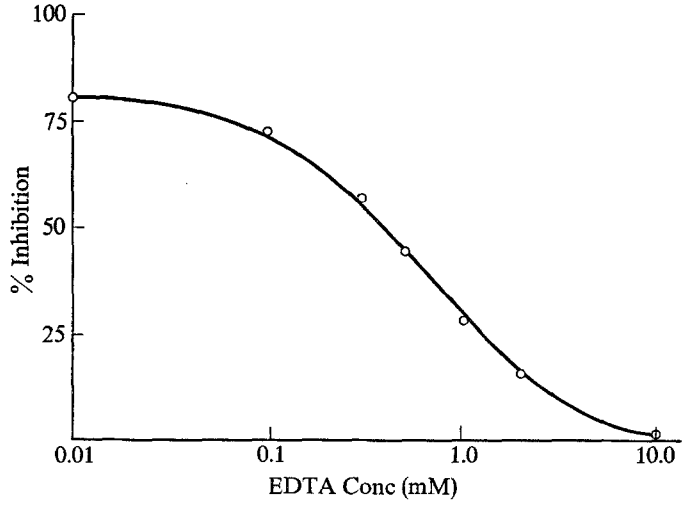

Fig. 2. The effect of varying concentrations of EDTA on the inhibition of glucose efflux from glucose-loaded red cells caused by PCMBS (0.1 mM for 90 min)

Table 1. Effect of sulfhydryl reagents on glucose efflux

Reagents	Concentration (mM)	Preincubation time (min)	Inhibition (%)
Organic mercury compounds:			
PCMB	0.1	0	75
	0.1	30	73, 77
PCMBS	0.1	0	80, 78, 77
	0.1	30	85
Maleimides:			
Ethyl	0.1	60	0
	1.0	30	0
	3.3	60	30
	4.0	0	15
	5.0	0	20, 30
		60	30
	8.0	0	35, 40
		60	50, 50
	16.0	0	50, 60
		60	80
Butyl	0.9	0	35, 40
	2.0	0	60, 65
Phenyl	0.4	0	12
	0.6	0	25
	0.9	0	40, 45, 50
Cyclohexyl	0.3	0	15
	0.4	0	25
	0.6	0	45
Disulfide:			
Dithiodipyridine	1.0	0	20
	1.5	0	30
	2.5	0	70, 65

Horowitz and Klotz (1956) suggested that glycine-chelated mercury ion would have a greater specificity for reaction with sulfhydryl groups than the unchelated ion. For our experiments, 50 mM glycine in the exiting solution had negligible effects either at pH 7.4 or at pH 8.5. However, EDTA, which is a more efficient chelator of mercury ions than is glycine (Bjerrum, Schwarzenbach & Sillen, 1957), did have an effect on the inhibition caused by PCMBS. Fig. 2 shows the effect of varying concentration

of EDTA upon the inhibition of the glucose transport system caused by 0.1 mM PCMBS. The chelating action of EDTA would also be expected to increase the specificity of organic mercurials towards SH groups.

Maleimide Derivatives

Initial experiments using N-ethylmaleimide (NEM) were done using preincubations of 0 and 30 min and a concentration of 1 mM. Under these conditions no effect on the rate of efflux of glucose from treated cells was observed. When the concentration was raised to 3.3 mM, inhibition was noted. Although preincubation of the cells with NEM was not necessary at this concentration to cause inhibition, it appeared that preincubation at a given concentration of NEM caused greater inhibition than the same concentration with no preincubation (Table 1).

The N-butyl, N-cyclohexyl, and N-phenyl derivatives of maleimide were investigated for their abilities to inhibit efflux from glucose-loaded RBC's. Fig. 3 is a plot of the per cent inhibition given by varying concentrations of the different derivatives. It was noted that the efficiency of derivatives increased as the water solubility decreased (Table 2).

Entry of N-ethylmaleimide into red cells was assessed by measuring the glutathione content of the cells after treatment with NEM. In 1 hr, the level was reduced to less than 10% of the normal values (Table 3).

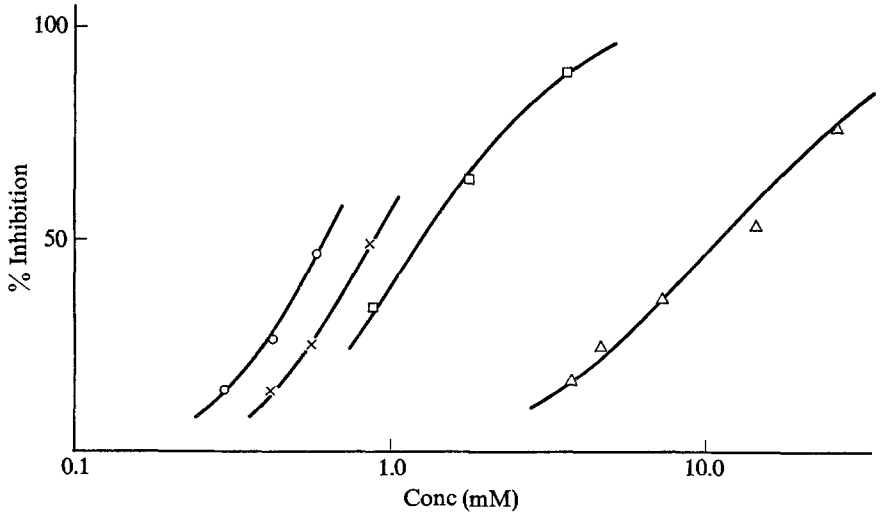

Fig. 3. The inhibition of glucose efflux as a function of concentration of various maleimide derivatives: N-ethyl (Δ), N-butyl (□), N-phenyl (X), and N-cyclohexyl (0). Each point represents the % inhibition after 90 min of incubation of glucose-loaded red cells at the indicated maleimide concentration

Table 2. Effectiveness of maleimide derivatives in inhibiting glucose efflux

Reagent	ED 50 (mM)	Solubility (mM)
NEM	14	306
NBM	1.5	33
NPM	1.0	2.9
NCM	0.7	1.1

The concentrations required to produce 50% inhibition of glucose efflux from glucose-loaded cells (ED 50) as taken from Fig. 3 are presented. Also shown are the water solubilities of the various maleimide derivatives.

Table 3. Entry of reagents into red cells; glutathione content

Reagent	mmoles/liter RBC
None	2.08
NEM	0.15
DTNB	1.95
Iodoacetate	0.31
Iodoacetamide	0.06

Red cells were washed in phosphate-buffered saline. They were then resuspended in an equal volume of this medium with or without the reagent listed (5 mM final concentration) and were incubated at room temperature for 1 hr. The treated cells were washed in PBS and the glutathione content determined (Beutler *et al.*, 1963).

Aromatic Disulfides

4,4′-Dithiodipyridine (DTP) has been used by Grassetti and Murray (1967*b*) for the determination of sulfhydryl groups. DTP was tested for its effect on glucose efflex. In the concentration range 1.0 to 2.5 mM, DTP caused an inhibition of efflux from glucose-loaded red cells (Table 1).

5,5′-Dithiobis-(2-nitrobenzoic acid) (DTNB), a reagent which reacts with protein sulfhydryls by way of disulfide exchange (Ellman, 1959), was shown to react with whole erythrocyte sulfhydryl groups by the production of the characteristic yellow anion. DTNB, however, did not appreciably inhibit glucose efflux from loaded cells. Concentrations of 0.01 to 10 mM and preincubation times up to 60 min were used with no effect. DTNB, unlike NEM or iodoacetate or its amide, showed limited ability to enter red cells in the period of incubation (Table 3).

Iodoacetate and its Amide

Neither compound inhibited glucose efflux at 5 mM, whether a pre-incubation period of 30 or 120 min was used.

Discussion

The data obtained with organic mercury compounds essentially confirm the findings of LeFevre (1948) and of van Steveninck *et al.* (1965); that is, that PCMB or PCMBS in concentrations of 0.1 mM inhibits glucose efflux from preloaded cells. The inhibition is relatively rapid, and occurs whether the net glucose flux is positive or zero. Thus, it appears that glucose does not "protect" the transport site from interacting with organo-mercury compounds.

The experiments using glycine or EDTA in an attempt to increase the specificity of the mercury compounds for reaction with sulfhydryl groups can be interpreted in terms of their chelation constants. Horowitz and Klotz (1956) concluded that organo-mercury compounds are completely selective for and react stoichiometrically with protein thiols when used in glycine buffer above pH 7.0. They attributed the selectivity to the fact that the amino group of glycine forms a strong competing ligand for the mercury ion against all competitors except thiols. Glycine at 500 times the concentration of PCMBS would be expected to prevent the reaction of PCMB or PCMBS with membrane functional groups other than sulfhydryl groups. Since glycine showed no effect in this system, it was assumed that it was the reaction of PCMB and PCMBS with membrane thiols that was responsible for the inhibition of glucose efflux. EDTA has a chelation constant 10^{10} times greater for mercury than does glycine (Bjerrum *et al.*, 1957). Its ability to block inhibition due to PCMBS can be explained in terms of its increased chelating ability (effectively lowering the PCMBS concentration). The results obtained indicate that the inhibitory action of organo-mercury compounds may be due to their reaction to thiols, although ones which have reduced reactivity.

The data from N-ethylmaleimide experiments suggest that surface thiol groups may not be involved in its effect on the glucose efflux mechanism. Our data (*see also* Forsling *et al.*, 1968) demonstrate that concentrations of 3 to 5 mM were necessary to observe appreciable inhibition of glucose efflux. It is of some interest that the total sulfhydryl content of red cells is of the order of 4 to 6 mM. Thus, the depression of efflux by a highly specific reagent which is known to penetrate the cells rapidly requires that a substantial portion (perhaps over 90%) of the total cellular sulfhydryl be blocked

before the efflux mechanism is appreciably affected. The NEM may block efflux as a result of reacting with some internal SH, possibly on the inner membrane.

The reactions of the N-butyl, N-cyclohexyl and N-phenyl derivatives of maleimide, however, suggest an alternate explanation. Their effectiveness at lower concentrations than NEM and their decreased water solubility (Table 2) suggest that the ethyl derivative may not enter a lipidlike region as efficiently or to the same extent as the butyl, cyclohexyl or phenyl derivatives. Thus, their reactivity with thiol groups in lipid portions of the membrane may well be enhanced by their increased lipid solubility. Assuming that the specificity and reactivity of all the maleimides with sulfhydryl groups is the same, one then would suppose that the sulfhydryl group of interest may well be within a lipidlike phase of the cell. Furthermore, the effect of the size of the organic moiety on the maleimide nitrogen needs to be considered; certainly, the larger the group, the more disorientation of the lipidlike region might be expected. This idea would fit our data, also, as the phenyl and cyclohexyl derivatives are more inhibitory than the butyl derivative.

The reaction of the two disulfides DTNB and DTP is also in line with this interpretation. The charged groups on DTNB appear to keep the molecule out of the cell (Table 3), although it is able to react with surface sulfhydryl groups. DTP, on the other hand, probably penetrates cell membranes (Grassetti & Murray, 1967*a*) and could thereby react more readily with sulfhydryl groups within the lipid regions of the cell membrane.

The effects of iodoacetate and iodacetamide on glucose efflux rates were negligible. These molecules, like N-ethylmaleimide, probably penetrate the cell membrane and react rapidly with the high internal content of sulfhydryl groups before reacting with any membrane-lipid "protected" thiols.

Recent studies of sulfhydryl groups have suggested that they tend to orient hydrocarbonlike phases around them and may be a major structural determinant for simple peptides or more complex protein structures (Nementhy & Scheraga, 1962). These studies suggest to us that the thiol groups responsible for the reaction with DTP or the maleimides may be somewhat inaccessible to the more water-soluble and ionic reagents and that their interaction with the membrane sulfhydryls produces a modified membrane structure whose ability to transport glucose is thereby impaired.

The response of the glucose transport system to the variety of sulfhydryl agents tested indicates that blockage of thiols in different membrane locations results in alteration of glucose transport. This suggests that the thiol groups

that are reactive may not be central to the transport system as reactive sites, but may participate by a mechanism concerned with maintaining the structural features of this system.

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