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Different Binding Sites for Glucose and Sorbose at the Erythrocyte Membrane, Studied by Gel Filtration and Infrared Spectroscopy

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Summary. Human red blood cell membranes were solubilized with sodium dodecylsulfate and incubated with various concentrations of ^{14}C-glucose and ^{14}C-sorbose. After gel filtration on Sephadex G-100, which separated lipoproteins of differing lipid content, it was observed that the radioactivity of the bound glucose coincided with the protein peak. Radioactivity of bound sorbose was found mainly before and after the protein peak. This distribution of bound sugars was confirmed by double labeling experiments in which ^{3}H-glucose and ^{14}C-sorbose were applied simultaneously. Infrared spectroscopy revealed differences between the membranes loaded with sorbose and glucose. Particularly, the band in the C–O–C and P=O region at 1,225 cm^{-1} was intensified in the sorbose-loaded membranes. Compared to serum albumin, the erythrocyte membranes were found to bind 4 times as much ^{14}C-glucose per mg of protein. It is concluded from the results obtained by gel filtration that glucose and sorbose preferentially bind at different sites of the erythrocyte membrane. The results obtained by infrared spectroscopy correspond with this conclusion.

Much of our knowledge of sugar transport in the red blood cell is derived from the extensive kinetic studies which have been performed with this system (Le Fevre, 1954; Wilbrandt, 1960; Sen & Widdas, 1962; Levine & Stein, 1966). However, despite the large amount of information obtained by this approach, interpretation of certain results presents some difficulty (Lacko & Burger, 1961; Levine, Oxender & Stein, 1965; Lacko, 1967; Stein, 1969; Lieb & Stein, 1970).

In the present work, therefore, we have adopted a different approach in studying the binding of sugars to components of red cell membranes. Erythrocyte membranes, solubilized with sodium dodecylsulfate, were in-

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cubated with the labeled sugars (glucose and sorbose), gel filtered on Sephadex G-100 columns, and individual fractions were analyzed for lipid and protein content and for radioactivity. Infrared spectroscopic studies were also performed on glucose- or sorbose-loaded membranes.

Materials and Methods

Human erythrocytes were obtained from a blood bank either as an erythrocyte concentrate or as fresh blood conserve. The erythrocytes were then lysed with 10 vol of H_2O containing a few drops of NH_4OH at a pH of about 8.0. They were then washed with 10 mM phosphate buffer pH 7.5 at 5 °C by the method of Dodge, Mitchell and Hanahan (1963). This washing procedure results in a loss of water-soluble membrane proteins (Hogeveen, Juliano, Coleman & Rothstein, 1970). The washed membranes were subsequently lyophilized.

Incubation of Erythrocyte Membranes with Radioactively Labeled Sugars

A portion of 20 mg of the lyophilized membrane preparation (containing about 40% protein) was incubated at 22 °C with 0.5 ml of 1% sodium dodecylsulfate (this corresponds to about 0.6 mg of sodium dodecylsulfate per mg of membrane protein) for about 20 min. Then 2.5 ml of 0.15 M NaCl containing the radioactively labeled sugars were added, and the incubation was continued as indicated in the tables and figures. Immediately after incubation, the dissolved membranes were gel filtered.

Gel Filtration

Columns of Sephadex G-100 (450 × 12 mm) were equilibrated with 0.5 M NaCl containing 0.01 M K_2HPO_4 at pH 8.4, except for the experiment summarized in Table 3, where sodium dodecylsulfate was used for equilibration and elution. The dissolved membranes were layered onto the column and gel filtration was carried out at 22 °C. The transmission at 280 nm (T_{280}) was recorded continuously using an LKB uvichord. Fractions of about 1.5 ml were collected. The elution rate was 8 to 10 ml/hr.

Estimation of Protein

Protein determinations of the effluent were carried out by the Lowry method (1951) using 0.05- to 1.0-ml aliquots of the fractions.

Estimation of Lipid

The lipid contents of 0.1-ml aliquots of the fractions were determined by the method of Zöllner and Kirsch (1962) using the Merckotest (E. Merck, Darmstadt, Germany).

Determination of Radioactivity

Radioactivity was determined on 0.2-ml aliquots of the fractions, using a liquid scintillation counter.

Infrared Spectroscopy

A sample of 20 mg of erythrocyte membranes was suspended by stirring in 2 ml of 0.9% NaCl for 1 hr at 22 °C. The membranes were then centrifuged in a Sorvall RC2b centrifuge for 10 min at 15,000 × *g* and at 5 °C. The membrane pellets were resuspended in 1 ml of a medium containing 0.5% sodium dodecylsulfate, 3.15 mM glucose or sorbose, and 0.45% NaCl, and incubated for 30 min at 22 °C. Controls were incubated in a similar medium containing no glucose or sorbose. After the incubation period, gel filtration on Sephadex G-100 was performed. The peaks at 280 nm were collected and dialyzed against H_2O containing a few drops of NH_4OH and at a pH of about 8.0 for 6 hr at 5 °C. The material was then lyophilized. About 0.1 to 1.0 mg of the lyophilizate was dissolved in 0.5 ml of 40% KBr (for spectroscopy, Merck A. G.). The samples were dried overnight. IR-spectra were obtained by recording microdiscs of 5 × 1 mm size with about 30 to 50 µg substance (20 to 30 mg KBr) using a mirror condenser in the Beckman IR-10 spectrophotometer.

^{14}C-D (+) glucose (specific activity: 2.9 mC/mM), ^{14}C-L (−) sorbose (specific activity: 3.0 mC/mM) and ^{3}H-glucose (specific activity: 500 mC/mM) were purchased from the Radio-chemical Centre, Amersham, England.

Results

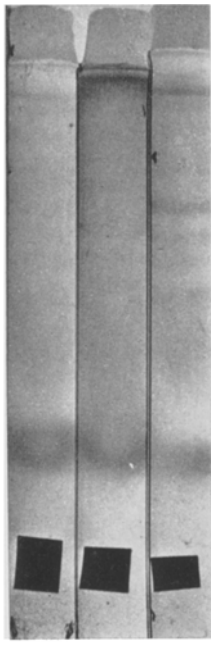
A. Separation of Larger and Smaller Membrane Lipoproteins

The partial separation of larger lipoproteins from smaller ones with a lower lipid content was achieved on Sephadex G-100 as shown in Table 1.

Table 1. Lipid and protein contents of fractions after gel filtration[a]

Fraction no.[b]	Lipid (% from total)	Protein (% from total)	Ratio (Lipid/ Protein)
1	1.5±0.7	1.7±0.1	(0.9)
2	8.5±1.3	5.3±0.1	1.6
3	20.7±1.7	14.9±1.6	1.39
4	25.0±1.6	18.1±1.5	1.38
5	20.5±6.1	18.8±2.2	1.09
6	10.6±2.7	14.2±1.2	0.74
7	4.6±1.5	9.3±1.1	0.49
8	2.4±0.2	6.3±1.2	0.38
9	1.6±0.2	3.8±1.3	0.42

[a] Means of 3 experiments ± SD.
[b] The fractions comprise those within the peak at 280 nm (lipoprotein peak).



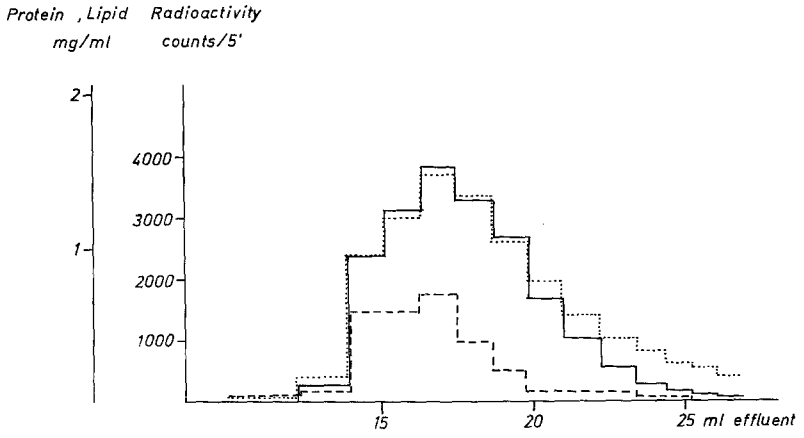
a b c

Fig. 1. Disc electrophoretic pattern from different fractions after gel filtration. A portion of 20 mg of erythrocyte membranes was dissolved in 0.5 ml 1 % sodium dodecylsulfate to which was added 2.5 ml 0.9 % NaCl, and the solution was stirred for 45 min at 22 °C. Gel filtration was performed as described in Materials and Methods. Consecutive fractions from the lipoprotein peak at 280 nm were pooled and dialyzed (6 hr at 5 °C) as follows: (*a*) Fractions from the beginning to the top of the peak; (*b*) Fractions from the top to the upper half of the descending portion of the peak; (*c*) Fractions from the lower half of the descending portion of the peak. Disc electrophoresis was carried out according to the methods of Marchesi, Steers, Marchesi and Tillack (1970) and by Lenard (1970) after pretreatment of the membranes with 2.4 % of sodium dodecylsulfate and 0.14 M beta-mercaptoethanol in 0.1 M phosphate buffer pH 7.8 and heating to 100 °C. The separation gels were made with 7 % polyacrylamide and 1 % sodium dodecylsulfate. 0.1 M Na-phosphate buffer pH 7.1 was 1 % with sodium dodecylsulfate

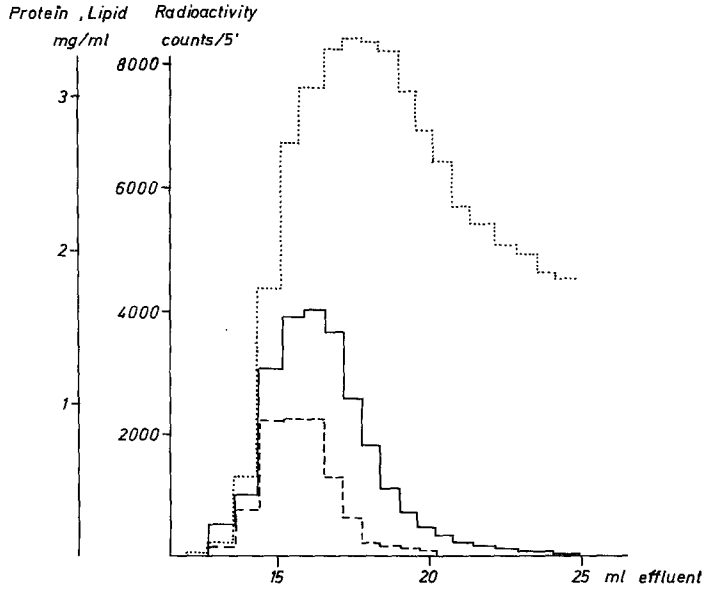
The lipid/protein ratio changes from above 1 to about 0.4 as the gel filtration proceeds, thus indicating that the lipoproteins with the highest lipid content are eluted first. Disc electrophoresis of the peak at 280 nm obtained by gel filtration allows a better separation of membrane proteins of the descending portion (Fig. 1*c*; *compare* to Fig. 1*a* and 1*b*). This may, possibly, be due to the smaller lipid content of these particular lipoproteins. Partial separation of membrane lipoproteins by gel filtration provides a tool for differentiating binding sites at the erythrocyte membrane, as will be shown.

B. Binding of ^{14}C-Glucose and ^{14}C-Sorbose

Fig. 2 shows the differences in binding of ^{14}C-glucose and ^{14}C-sorbose at a concentration of 5 mM. There was a parallel distribution of ^{14}C-glucose and protein, suggesting that glucose specifically binds to protein rather than to lipid (Fig. 2*a*). This holds always for high concentrations of ^{14}C-glucose (2 to 5 mM). However, there was no parallel distribution of ^{14}C-sorbose and protein (Fig. 2*b*). This pattern was also observed with low (0.2 mM) concentrations of ^{14}C-sorbose, for example, as shown in Fig. 3*b*, where the



a



b

ig. 2. Binding of ^{14}C-glucose or ^{14}C-sorbose to different membrane fractions. A portion ' 20 mg of erythrocyte membranes was incubated for 60 min with 5 mM ^{14}C-glucose · ^{14}C-sorbose in the presence of dodecylsulfate and NaCl (*see* Materials and Methods). fter gel filtration, ^{14}C radioactivity, protein and lipid concentrations were determined the fractions. (*a*) Incubated with glucose; (*b*) incubated with sorbose; ····· radiotivity (Radioactivity of the free sugars appears far to the right, past the final effluent volume shown.); ——— protein; ------ lipid

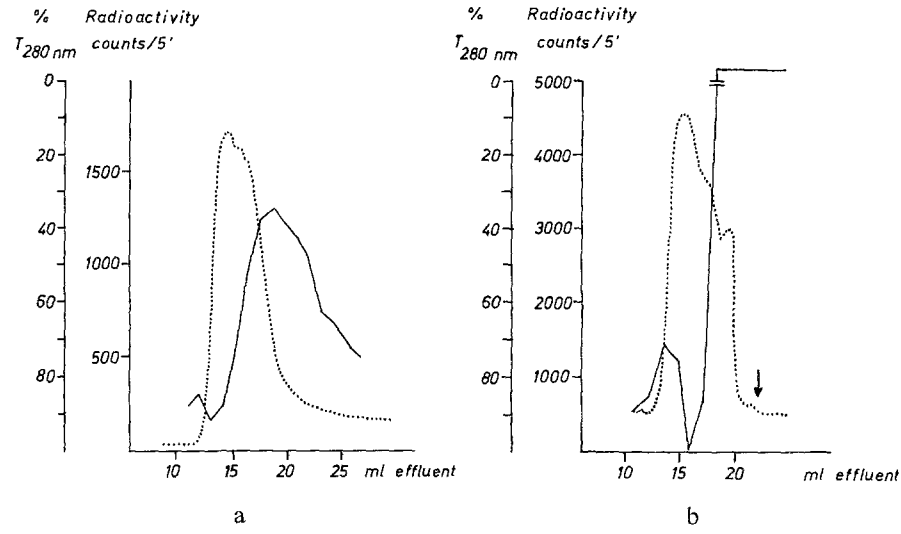


Fig. 3. Binding of ^{14}C-glucose or ^{14}C-sorbose to different membrane fractions. A portion of 20 mg or erythrocyte membranes was incubated for 30 min with 0.21 mM of ^{14}C-glucose or ^{14}C-sorbose in the presence of dodecylsulfate and NaCl (*see* Materials and Methods). The T_{280} was recorded continuously by an LKB uvichord during gel filtration. Afterwards, ^{14}C radioactivity was determined in the fractions. (*a*) Incubated with glucose; (*b*) incubated with sorbose; ····· T_{280} nm; ——— radioactivity (Radioactivity of the free sugars appears far to the right, past the final effluent volume shown. The background count in each fraction (0.2 aliquot) was about 250 counts/5 min and was not subtracted from the values obtained.); ↓ maximum of radioactivity

maximum of radioactivity was found at 22 ml effluent (*see* arrow Fig. 3*b*) and the T_{280} peak had reached the basal level. For comparison of the results of a simultaneous determination of protein content and T_{280} *see* Table 3.

Using 0.2 mM ^{14}C-sorbose and membranes from fresh blood conserves, we frequently observed two peaks of radioactivity. One peak of radioactivity was found at the ascending portion of the T_{280} peak the other at the descending portion or behind it (Fig. 3*b*). When the separation of lipoproteins was not adequate, or if high concentrations of ^{14}C-sorbose were used, these two peaks merged into one broad peak of radioactivity (Fig. 2*b*). There was no significant ^{14}C-glucose peak at the ascending portion of the T_{280} peak even with low (0.2 mM) concentrations of glucose, the radioactivity being always found at the descending portion of the T_{280} peak (Fig. 3*a*). The maximum of radioactivity corresponded to the protein maximum at concentrations of 2 to 5 mM ^{14}C-glucose (Fig. 2*a* and Table 3).

Table 2. Double-labeling experiments [a]

Fraction no.[b]	$^3H/^{14}C$ ratio	Relative molar ratios of bound sugars (glucose/sorbose)
1	1.6 ± 0.8	1:17.0
3	2.4 ± 0.4	1:11.3
5	3.4 ± 0.1	1:8.0
6	3.7 ± 0.17	1:7.3
3	3.4 ± 0.4	1:8.0
10	2.4 ± 0.5	1:11.3

[a] Ratios of $^3H/^{14}C$ radioactivities (cpm) in fractions obtained by gel filtration; means of 3 experiments $\pm$ SD. A portion of 20 mg of erythrocyte membranes was incubated with 0.5 μmoles of ^3H-glucose and 0.35 μmoles of ^{14}C-sorbose in the presence of 0.5 ml of 1% sodium dodecylsulfate and 1.5 ml of 0.9% NaCl with stirring for 30 min at 22 °C. After gel filtration, the fractions were analyzed for ^3H and ^{14}C radioactivity.
[b] The fractions comprise those within the peak at 280 nm.

C. Simultaneous Binding of ^3H-Glucose and ^{14}C-Sorbose

The results presented in Table 2 show that ^3H-glucose and ^{14}C-sorbose preferentially bind to different fractions when the two sugars are applied simultaneously. The ratios of ^3H-glucose/^{14}C-sorbose vary from about 1.6 to 3.7. Relatively more sorbose than glucose is bound at the ascending portion (fraction 1) and at the last fractions of the descending portion (fraction 10) of the T_{280} peak. There is, however, much more ^{14}C-sorbose than ^3H-glucose bound on a molar basis, when the differences of specific activities are considered (*see* Materials and Methods; *see*, for comparison, also Figs. 2 and 3). Similar results were obtained when the concentrations of ^3H-glucose and ^{14}C-sorbose were varied.

D. Infrared Spectroscopy of Membranes Incubated with Glucose and Sorbose

The experimental results presented in sections B and C suggest that glucose and sorbose do not bind preferentially to the same sites of the membrane components. Infrared spectroscopy was used to determine whether there would be significant differences between the spectra of membranes loaded with glucose and sorbose. The spectra of the membranes which had been incubated with glucose and sorbose were, in fact, distinctly different from each other and from the controls (Fig. 4). In the presence of

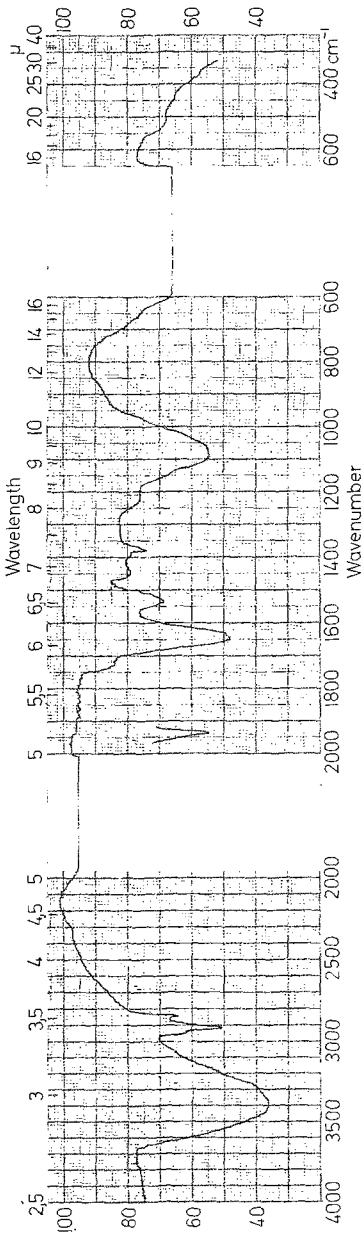

Wavelength
Wavenumber
μ
400 cm⁻¹

Fig. 4a

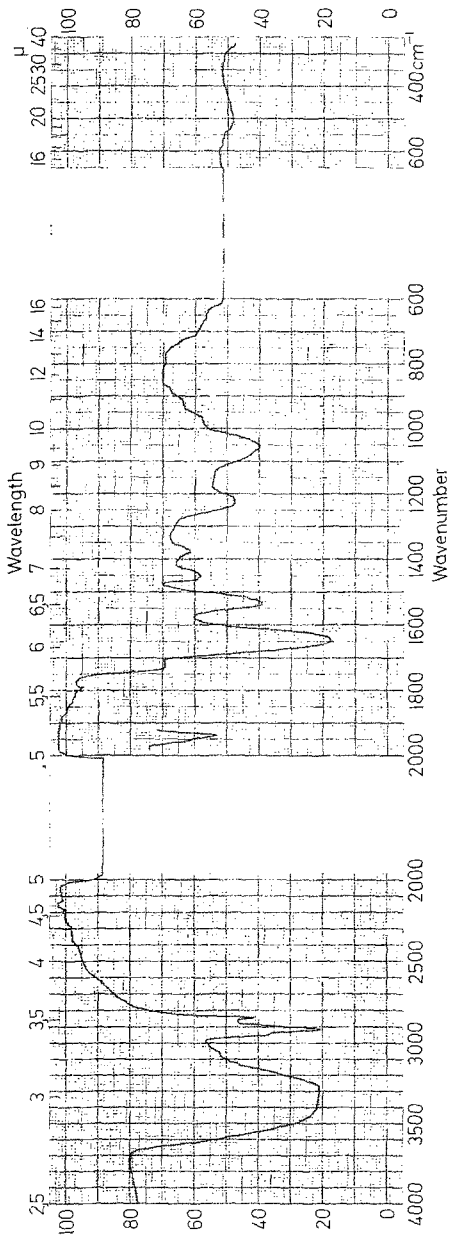

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Fig. 4b

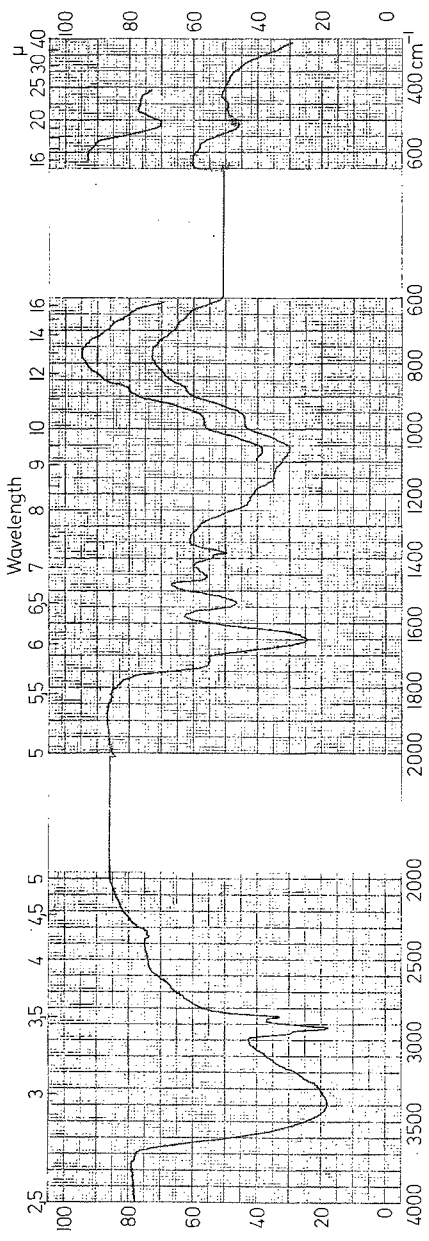

Fig. 4c

Fig. 4. Infrared spectroscopy of membranes incubated with glucose or sorbose. According to Tipson and Isbell (1962), and to personal experience, glucose and sorbose do not contribute to any absorption band in the area of 1,630 to 1,650 cm^{-1} of the spectra. For experimental details *see* Materials and Methods. (*a*) Incubated with glucose; (*b*) incubated with sorbose; (*c*) controls in the absence of added glucose or sorbose. The bands, recorded at the 90 % line, are obtained by weakening the reference beam so that the intensity of the light of the sample beam is increased. This affords a more sensitive recording of the bands

glucose the P-O-C band at 1,050 to 1,060 cm^{-1} was broader and shifted to higher wavenumbers, and the P=O and C-O-C band at 1,220 to 1,240 cm^{-1} was strongly reduced. In the presence of sorbose, the 1,220 to 1,240 cm^{-1} band was intensified. Spectra which were obtained from other membrane preparations gave similar results under the same conditions. A slight broadening and splitting of the amide I band to 1,630 cm^{-1} as indicated in Fig. 4*a* possibly reflects an increase of beta-conformation of the proteins (Miyazawa & Blout, 1961; Wallach & Zahler, 1966; Chapman, Kamat & Levene, 1968).

E. Comparison of ^{14}C-Glucose Binding to Erythrocyte Membranes and to Serum Albumin

To determine the extent to which glucose binds to proteins in general, we compared the binding of glucose to serum albumin (as a representative

Table 3. Comparison of ^{14}C-glucose binding to erythrocyte membranes and to bovine serum albumin [a]

Fraction no.	% T_{280}	mg prot/fract	cpm/mg prot	cpm/fract
Erythrocyte membranes:				
1	95	<0.2	–	240
2	85	<0.2	–	352
3	70	1.3	761	989
4	80	1.4	733	1,026
5	90	1.1	499	549
6	95	0.5	720	360
		4.3		3,516
Bovine serum albumin:				
1	95	<0.2	–	133
2	80	<0.2	–	126
3	65	1.0	189	189
4	50	2.1	123	259
5	75	2.6	129	336
6	90	1.8	163	294
7	95	0.8	218	175
8	95	0.3	420	126
		8.6		1,638

[a] A sample of 10 mg of erythrocyte membranes (or 10 mg of serum albumin) was suspended with 0.2 ml of 0.9 % NaCl and 0.8 ml of 1 % sodium dodecylsulfate in the presence of 2.1 mM ^{14}C-glucose for 30 min at 22 °C. Gel filtration on Sephadex G-100 columns, equilibrated with 1 % sodium dodecylsulfate (elution fluid: 1 % sodium dodecylsulfate), was then carried out.

protein) and to erythrocyte membranes. As shown in Table 3, the erythrocyte membranes bound about 4 times more of the labeled glucose than did bovine serum albumin when the results are expressed on a per mg protein basis.

Discussion

During recent years, a number of different procedures have been used for solubilizing erythrocyte membranes (Maddy, 1966; Bakerman & Wasemiller, 1967; Blumenfeld, 1968; Marchesi & Steers, 1968; Rosenberg & Guidotti, 1969; Lenard, 1970). The retention of biological activity in the solubilized state depends upon the concentration and type of solubilizing agent. For example, enzymatic activities were found to be preserved to an appreciable degree in Ehrlich ascites tumor cell membranes in the presence of low concentrations of sodium dodecylsulfate (Wallach, 1969). We used a low concentration of sodium dodecylsulfate as the dissolving agent for the erythrocyte membrane, since we have previously shown that the binding specificity for glucose is retained after this treatment (Zimmer & Lacko, 1971).

To separate the solubilized membrane components from the unbound sugars we use gel filtration. Zonal separation techniques have been used for some time in studying the binding of small molecules to larger ones; for example, to proteins (Wood & Cooper, 1970). Moreover, we sought an approach which would allow a differentiation of membrane components of the erythrocyte. Therefore, we chose a gel (Sephadex G-100) which does not completely exclude the membrane constituents after solubilization, but instead provides a partial separation of lipoproteins of different lipid content. [The method of Hummel and Dreyer (1962), which allows quantitative binding studies, is not suitable for this purpose because it requires a gel that completely excludes the larger of the interacting molecules].

Under the conditions of our experiments, glucose (5 mM) binds to the protein moiety of the erythrocyte membrane, as is evident from the parallel course of radioactivity and protein concentrations in the fractions of Fig. 2*a*. In contrast, the radioactivity from ^{14}C-sorbose (5 mM) reached a maximum either after the protein maximum (Fig. 2*b*) or, in the case of low concentrations of ^{14}C-sorbose, before and after the protein maximum (Fig. 3*b*). This different behavior of glucose and sorbose was confirmed by a double-labeling procedure.

There was, furthermore, a marked difference between the amounts of glucose and sorbose which were bound (Figs. 2 and 3). This could likewise

be attributed to different binding sites for the two sugars and suggests that sorbose does not dissociate as easily from its binding site as glucose does. Another possibility would be that sorbose is bound to a greater extent than glucose. In any case, the binding is not irreversible and a dissociation does occur. This is discernible from the marked tailing of ^{14}C-sorbose after the peak at 280 nm (or the lipoprotein peak) has reached the basal level (Fig. 2*b*).

The binding of glucose and sorbose to the membrane results in different changes in the IR-spectra particularly in the P=O and C-O-C group frequency at 1,225 cm^{-1}. These results are compatible with the presence of different binding sites, indicated by the results of the gel filtration studies.

The binding sites which are discussed in this paper do not necessarily belong to the hypothetical carriers for the sugars. However, the binding capacity for glucose, which we have found in the erythrocyte membrane, does not seem to be a general property of proteins.

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