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LIPID-PROTEIN INTERACTIONS AT THE ERYTHROCYTE MEMBRANE

DIFFERENT INFLUENCE OF GLUCOSE AND SORBOSE ON MEMBRANE LIPID TRANSITION*

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SUMMARY

When observed over a temperature range, erythrocyte membrane lipids undergo a transition at 18–20 °C (Zimmer, G. and Schirmer, H. (1974) Biochim. Biophys. Acta 345, 314–320). This observation has prompted an investigation of the effects that substrate binding has on the transition of the red cell membrane. Glucose and sorbose were compared, since transport kinetics of these sugars still pose unresolved questions.

In membranes, preloaded with glucose, the break at the transition temperature was intensified, while it was abolished or reversed in membranes preloaded with sorbose.

These results were corroborated using different solubilization procedures (sonication, sodium dodecyl sulfate treatment) of the membranes, and also different techniques (viscosimetry, 90° light scattering, 1-anilino-naphthalene-8-sulfonate fluorescence).

In extracted membrane lipids, viscosimetry indicated a break at transition temperature after preloading with either glucose or sorbose.

Disc electrophoresis revealed a different binding pattern of the two sugars.

It is suggested, that the amplification of the discontinuity in red cell membranes by glucose and the abolition or reversal of the break by sorbose are mediated by membrane protein- and/or membrane lipid-protein interaction.

INTRODUCTION

Substrate binding to red cell membrane has been found to induce conformational changes within the membrane [1–3]. The implications of these changes

Abbreviation: ANS, 1-anilinonaphthalene-8-sulfonate.

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should be further analysed, in order to increase our understanding of the transport phenomenon.

Transport of substrate through the biological membrane should be dependent on lipid-protein interactions since, on the one hand, many substrates are bound specifically to membrane protein [4], while, on the other hand, the transition temperature of the membrane phospholipids plays an actual role in the transport event [5–7].

We use the binding of substrate to the red cell membrane in order to explore its influence on lipid transition. Different techniques are employed to observe substrate action on the solubilized membrane. Binding of glucose and sorbose to the red cell membrane was studied, because many discrepancies in transport kinetics of these two sugars have been reported in the past [8–13].

MATERIALS AND METHODS

Preparation of red cell membranes

Fresh human blood conserves or fresh erythrocyte concentrates were obtained from the blood bank. Red cell membranes were prepared after lysis of the concentrated erythrocytes with 10 volumes of 15 mM sodium phosphate buffer pH 7.5 over night, using the washing procedure of Dodge et al. [14] with 10 mM sodium phosphate buffer pH 7.5.

The membranes were washed until the supernatant became clear and special care was taken in removing all of the yellow-red precipitate (Fairbanks et al. [15]). The membranes were finally suspended in water, frozen overnight, centrifuged once more and lyophilized. The protein concentrations were estimated by the method of Lowry et al. [16] and varied from about 0.5–0.7 mg of protein per mg membrane.

Extraction of erythrocyte membrane lipids

The extraction procedure essentially followed the method described [17] which is based on the procedure of Dawson et al. [18]. After addition of 9 ml 0.9 % NaCl and separation into two phases at 5 °C the lower phase was concentrated to about 1/10 of the original volume with a rotatory vacuum dryer. Thereafter the lipids were dispersed with 10 ml of 0.9 % NaCl. Total lipid and cholesterol determinations were carried out using the methods of Zöllner and Kirsch [19] and Watson [20] as has been described previously [17].

Viscosity measurements

Viscosity measurements of red cell membranes [17] were carried out with Ostwald capillary viscosimeters with water flow speeds of 26.7 and 30.2 s. at 20 °C. For the extracted membrane lipids, viscosimeters with flow times of 48.2 s were used. The protein concentrations were varied from about 3 to 10 mg/ml.

50–100 mg of erythrocyte membrane suspension in 9 ml 0.9 % NaCl was sonicated for 15 s four times, using a Branson S-75 sonifier at maximal output (about 5.5 A). Sonication was performed in an ice bath, so that the temperature of the sample did not rise to more than 13 °C during each sonication period.

Alternatively, the membranes were suspended with 1 % sodium dodecyl

sulfate. This was performed by very gentle homogenization with a small Potter-Elvehjem homogenizer.

Subsequently the membrane suspensions, which looked clear or only slightly turbid, were filtered with paper (Schleicher and Schüll, Dassel, G.F.R., Nr. 595 1/2, pore width about 5 μm), in order to remove particles which might have disturbed the reproducibility of the measurements. Protein and lipid estimations before and after filtration indicated that there was no change of protein/lipid ratio during this step.

3 ml of the filtrates were then employed for the viscosity measurements. The accuracy of the repeated (five times at least at each temperature) measurements was good, an average standard deviation of ± 0.15 s being obtained. Calibration of the viscosimeters was carried out with water/glycerol standard mixtures of known densities. The temperature was held constant at about ± 0.2 °C with a Lauda ultrathermostat.

In viscosity measurements, we consider a discontinuity ('break') to be significant only if it has been found to occur repeatedly in different experiments and with different membrane preparations. We estimate such a break in the following manner: If there are two straight lines of different slope that intersect, we measure the angle between the lines. This method is, however, only practicable in cases where no shift of the lines occurs on the y-scale. It is not yet worked out how to deal with the problem of the estimation of a break in these cases.

All lines in the viscosity measurements were finally calculated by regression. Correlation coefficients ranging from 0.944–1.013 were found. Lines with correlation coefficients from 0.966–1.013 were drawn according to the regression. Lines with correlation coefficients from 0.944–0.956 were all above transition temperature, and also without exception exhibited an overestimation of the break, since experimental values just above the transition temperature (20–21 °C) and values at about 24–25 °C were averaged out. In these cases, lines were drawn by visual estimation according to the best fit of the experimental points.

Light scattering measurements

Light scattering measurements at 90° were carried out on the sonicated membrane suspensions. We used a Farrand fluorospectrophotometer (type Mark I) equipped with an amplifier of high sensitivity. The protein concentrations were in the range of 0.5 mg protein/ml. The sonicated membranes were suspended with 0.9 % NaCl. Peaks at 420 and at 600 nm were recorded at different temperatures. The constancy of the temperatures was held at ± 0.2 °C.

Fluorescence measurements

Fluorescence measurements were performed with the same Farrand fluorospectrophotometer that was used for the light scattering experiments. 20 mg of the membranes were solubilized with 9 ml 0.9 % NaCl by sonication. For sonication procedure, see under Viscosity Measurements. 0.4 ml of the membrane suspension after sonication was diluted with 4 ml of 0.9 % NaCl. 0.65 mg of 1-anilinonaphthalene-8-sulfonate (ANS) was dissolved with 1.0 ml of 0.9 % NaCl. 0.2 ml of this ANS solution was added to the diluted membrane suspension. The final protein concentrations were: 0.11 mg/ml. Excitation at 380, Emission at 462 nm. The temperature was held constant at about ± 0.2 °C.

Preincubation with the sugars

Preincubation with the sugars (D-(+)-glucose, L-glucose, L (−)-sorbose, sucrose and D (+)-galactose) was carried out at 0.3–1.5 % (w/v) concentrations in the viscosity measurements, and at 0.2–0.8 % (w/v) concentrations in the light scattering and fluorescence experiments. The preincubation time was always 30 min and the temperature 22 °C. After sonication or in sodium dodecyl sulfate the membrane suspensions were slowly stirred during preincubation.

Disc electrophoresis

Disc electrophoresis was performed as described previously (see ref. 31) according to the methods of Marchesi et al. [21] and Lenard [22]. After incubation of the red cell membranes with ^{14}C-labelled sugar at 6.6 mM concentration in the presence of 1.0 mg sodium dodecyl sulfate/mg protein for 30 min at 22 °C the membrane suspension was dialysed against H_2O/NH_3, pH 8, for at least 4 h. This time was sufficient to remove all of the unbound ^{14}C radioactivity. Aliquots of the dialysed material were applied to the disc columns.

Radioactivity measurements

For estimation of radioactivity a scintillation liquid consisting of 1000 ml of toluene, 5 g of permablend III (Packard) and 10 % (v/v) of soluene 350 (Packard) was used. A Packard liquid scintillation counter was employed. After dissection of the gels with a razor blade the material was allowed to stand in the scintillation cocktail for at least 12 h before counting.

D(+)-[^{14}C]Glucose (specific activity: 2.9 Ci/mol) and L(−)-[^{14}C]sorbose (specific activity: 3.0 Ci/mol) were purchased from the Radiochemical Centre, Amersham, England. D(+)-Glucose, D(+)-galactose and sucrose were obtained from Merck, Darmstadt, L(−)-sorbose was from Fluka, Buchs, Switzerland, L-glucose (Calbiochem, A-grade) was a gift from the laboratory of Dr Kinne, Max-Planck-Institut für Biophysik, Frankfurt. All other reagents were of the highest purity available.

RESULTS

Viscosimetry measurements are presented in Figs. 1–6 and 9, 10. Fig. 1 shows the effects of D(+)-glucose, L(−)-sorbose and L-glucose on the red cell membrane over the temperature range of 15–25 °C. It is clearly discernible, that the actions of D(+)-glucose and L(−)-sorbose are opposite: while a break at 19–20 °C, exhibited by the control, is enhanced by D(+)-glucose, it is abolished by L(−)-sorbose. L-Glucose exhibits a discontinuity which is comparable to that of the control.

In Fig. 2 and 3 the influences of glucose and sorbose at increasing concentrations are shown, demonstrating that the effects, observed in Fig. 1, are also obtained at about 0.75 % preloading concentrations.

When red cell membranes are dispersed in 0.9 % NaCl, the pH value of the dispersion is about 6.75. Adjusting the pH to the physiological value of 7.5 with 0.01 M sodium phosphate buffer essentially does not change the effects of glucose, sorbose and the control as may be seen in Fig. 4.

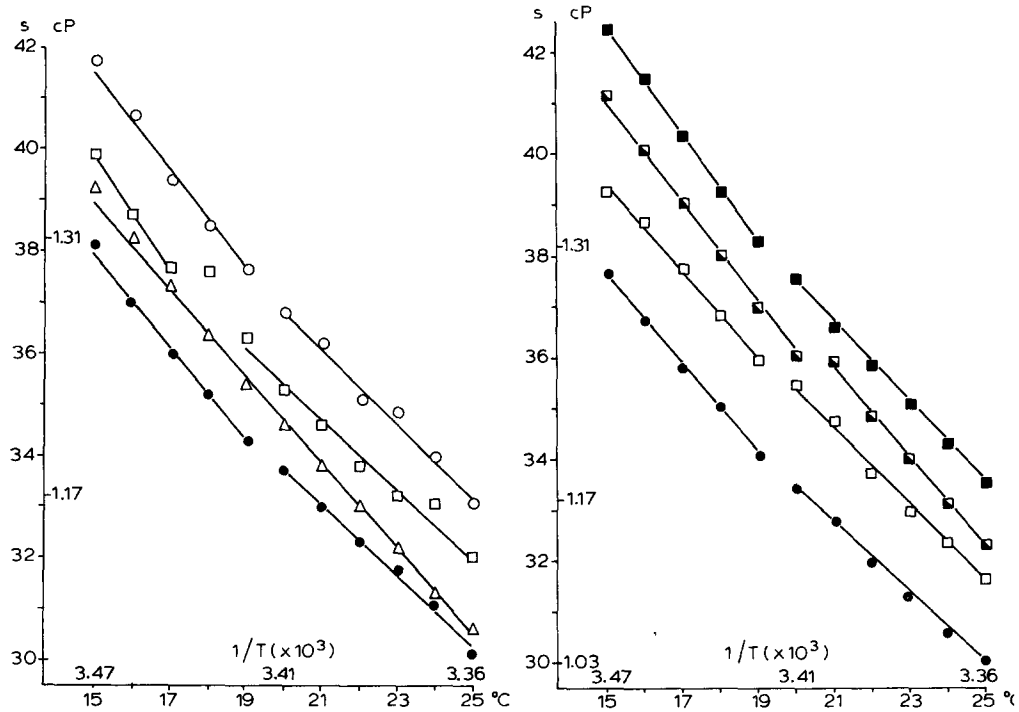

Fig. 1. Viscosity measurements of red cell membranes, sonicated in 0.9 % NaCl. The membranes were preloaded with 1.5 % of the sugars. Protein content: about 3–4 mg/ml. ●, control in the absence of added sugar; □, D(+)-glucose; ○, L-glucose; △, L(−)-sorbose. For better presentation L-glucose was displaced for +1 s and D(+)-glucose for 0.5 s on the y-scale. Ordinate: left flow time in s; right viscosity in cP.

Fig. 2. Viscosity measurements of red cell membranes, sonicated in 0.9 % NaCl. The membranes were preloaded with different D(+)-glucose concentrations. Protein content: about 3–4 mg/ml. ●, control in the absence of added sugar; □, 0.3 % glucose; ◧, 0.75 % glucose; ■, 1.5 % glucose. Ordinate: left flow time in s; right viscosity in cP.

Further viscosimetry measurements were carried out on red cell membranes dispersed in sodium dodecyl sulfate solution. It is known that intermediate concentrations of dodecyl sulfate do not inactivate certain membrane enzymes [23]. At concentrations of 0.55 mg dodecyl sulfate per mg protein, however, the discontinuities, normally observed, are effectively abolished. At a concentration of 0.25 mg dodecyl sulfate the behaviour, as observed in Figs. 1–4, reappears. (Figs. 5 and 6.) Sucrose preloading of the membranes does not show a difference when compared to the controls in the absence of added sugar.

The results, obtained by the viscosimetric procedure, were confirmed by 90° light scattering and ANS fluorescence. Fig. 7 shows the results of experiments, using the light scattering procedure. The light scattering intensities at 420 and 600 nm were recorded. We observe the already known behaviour of D(+)-glucose and L(−)-sorbose. Membranes preloaded with glucose show an enhancement of the break and also a shift to a lower temperature range, when compared to the control.

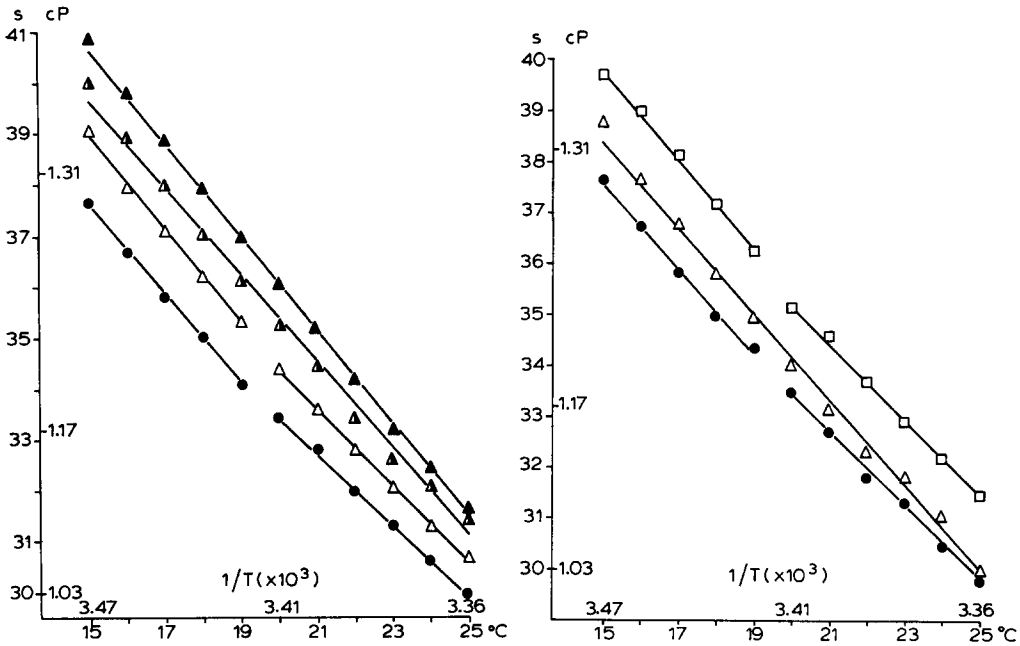

Fig. 3. Viscosity measurements of red cell membranes, sonicated in 0.9 % NaCl. The membranes were preloaded with different L(−)-sorbose concentrations. Protein content: about 3–4 mg/ml. ●, control in the absence of added sugar; △, 0.3 % sorbose; ▲, 0.75 % sorbose; ▲, 1.5 % sorbose. Ordinate: left flow time in s; right viscosity in cP.

Fig. 4. Viscosity measurements of sonicated red cell membranes. Solvent: 0.9 % NaCl; the pH was adjusted to 7.5 with 0.01 M sodium phosphate. Protein content: about 3–4 mg/ml. ●, control in the absence of added sugar; □, 0.75 % D(+)-glucose; △, 0.75 % L(−)-sorbose. For better presentation D(+)-glucose was displaced for +1 s in the y-scale. Ordinate: left flow time in s; right viscosity in cP.

No discontinuity can be found in the sorbose experiment. Differences are also clearly revealed in Table I, where the relative 90° light scattering intensity ratios of 420 to 600 nm are compared. The increase of the ratio in the control experiments between 16 and 23 °C is 25 %, while with glucose the ratio is about 38 %. No change in the ratios is apparent in the sorbose experiment.

ANS fluorescence experiments showed, as expected [24], an increase of the fluorescence intensity at transition temperature in the control and with D(+)-glucose. A decrease is observed with L(−)-sorbose. This reverse effect of sorbose as compared to glucose and the control may be interpreted to indicate a decrease in binding sites for the ANS molecule (Fig. 8).

Finally in this series of experiments, we have compared the competitive influence of glucose versus sorbose when given consecutively to the same membrane preparation at 0.75 % concentration each. Irrespective of the sequence of addition of the sugars, the break (re)-appears, demonstrating dominance of the glucose influence on the discontinuity. Galactose, added by the usual preloading procedure, exhibits a similar break compared to the control (Fig. 9).

The experiments shown in Fig. 1–9 were designed to clearly verify the differences of glucose versus sorbose action on the red cell membrane at transition

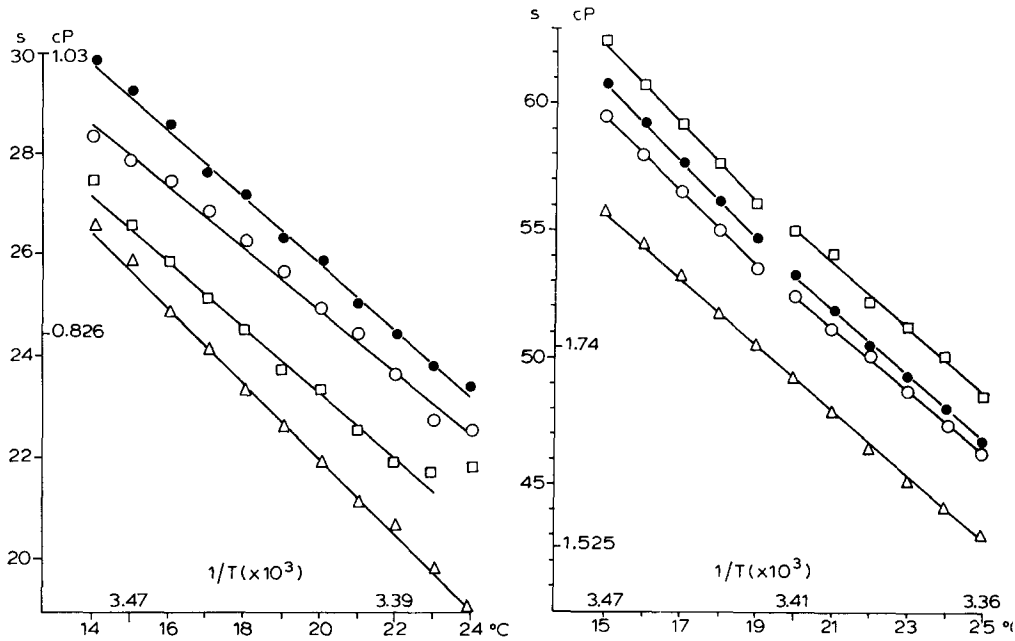

Fig. 5. Viscosity measurements of red cell membranes solubilized with 0.55 mg sodium dodecyl sulfate/mg protein. The membranes were preloaded with 1.5 % of the sugars. Protein content: about 10 mg/ml. ●, control in the absence of added sugar; □, D(+)-glucose; △, L(−)-sorbose; ○, sucrose. For better presentation the control curve was displaced for +1 s, the glucose curve for −1 s and the sorbose curve for −2 s in the y-scale. Ordinate: left flow time in s; right viscosity in cP.

Fig. 6. Viscosity measurements of red cell membranes solubilized with 0.25 mg sodium dodecyl sulfate/mg protein. The membranes were preloaded with 1.5 % of the sugars. Protein content: about 6 mg/ml. ●, control in the absence of added sugar; □, D(+)-glucose; △, L(−)-sorbose; ○, sucrose. For better presentation the glucose curve was displaced for +1 s in the y-scale. Ordinate: left flow time in s; right viscosity in cP.

temperature. Different effects, observed with extracted membrane lipids, would add to the specificity of the effects, seen at the red cell membrane.

Such different behaviour is clearly exhibited by Fig. 10. Sorbose as well as glucose show a break under these conditions of experiment. Similar observations were made with sonicated extracted membrane lipids.

At length, we have carried out disc electrophoretic experiments of red cell membrane in the presence of D(+)-[^{14}C]glucose and L(−)-[^{14}C]sorbose. The usually observed separation of protein bands stained with Coomassie blue is shown in Fig. 11. The radioactivity patterns of glucose and sorbose experiments are characteristically dissimilar.

While D(+)-glucose radioactivity is found to migrate with the main protein bands, L(−)-sorbose migrated with the dodecyl sulfate front of the gels, or stayed with the stacking gel. It has been found by numerous groups that lipids in dodecyl sulfate gel electrophoresis move with the dodecyl sulfate front of the gels [22, 25–27]. On the other hand, high molecular weight lipoproteins were suspected not to enter the separation gel, but to stay with the stacking gel, as has been found for some

TABLE I

90° LIGHT SCATTERING MEASUREMENTS: RATIOS OF 420/600 nm RELATIVE SCATTERING INTENSITIES

Temp. °C	14	15	16	17	18	19	20	21	22	23	24
Control	—	—	2.78	2.70	2.84	2.81	3.00	—	3.22	3.48	
Glucose	3.16	—	3.00	3.14	3.13	3.15	3.67	3.75	4.16	4.16	4.36
Sorbose	5.80	5.43	—	5.64	5.29	5.66	5.48	5.58	5.47	5.30	5.26

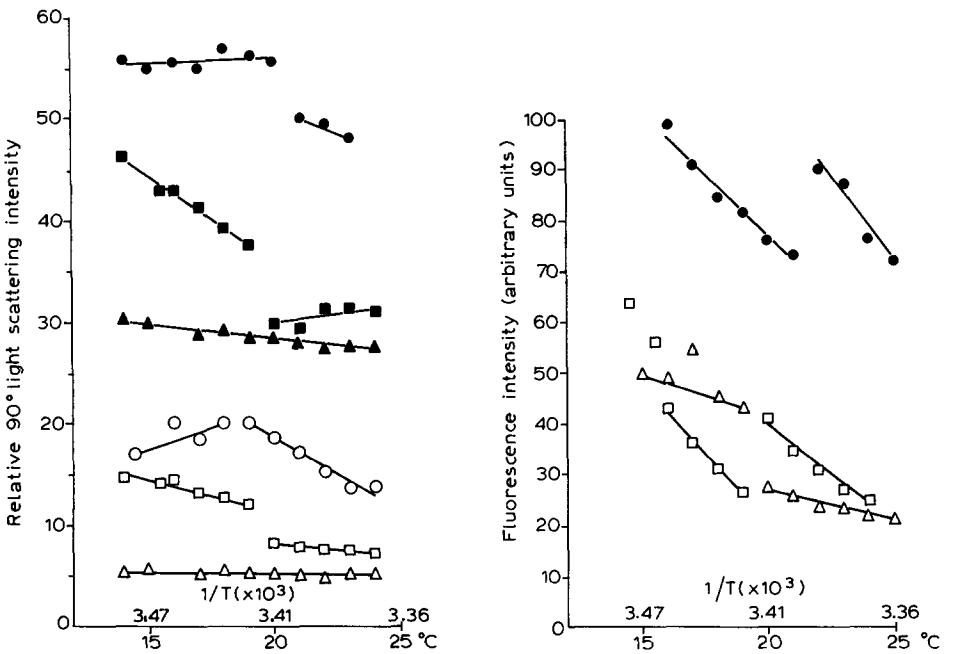

Fig. 7. 90° light scattering measurements of red cell membranes sonicated in 0.9 % NaCl. The membranes were preloaded with 0.8 % of the sugars. Protein content: about 0.5 mg/ml. ●, control curve observed at 420 nm; ■, D(+)-glucose observed at 420 nm; ▲, L(−)-sorbose observed at 420 nm; ○, control curve observed at 600 nm; □, D(+)-glucose observed at 600 nm; △, L(−)-sorbose observed at 600 nm.

Fig. 8. Fluorometric measurements of red cell membranes, sonicated in 0.9 % NaCl, with 1-anilinonaphthalene-8-sulfonate. The membranes were preloaded with 0.8 % of the sugars. Protein concentrations: about 0.11 mg/ml. ●, control; □, D(+)-glucose; △, L(−)-sorbose.

glycoproteins [28]. These points may potentially have some bearing on the results obtained with L(−)-sorbose.

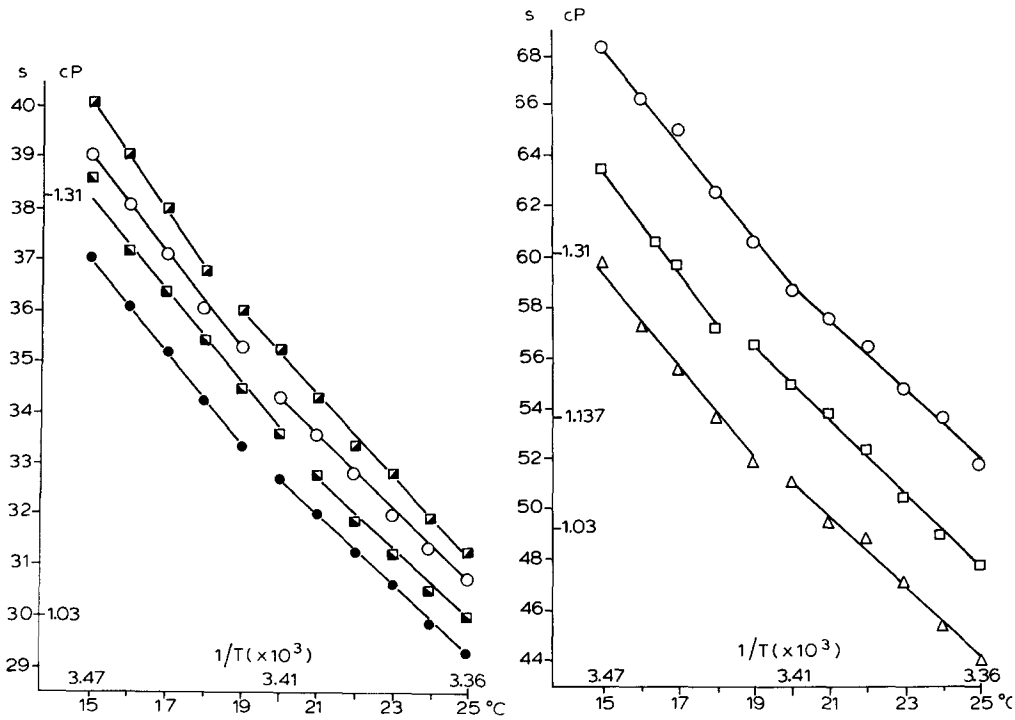

Fig. 9. Glucose-sorbose competition experiments. Viscosity measurements in sonicated red cell membranes similar to Figs 1–4. ●, control in the absence of added sugars; ○, 1.5 % D(+)-galactose; ◧, 0.75 % D(+)-glucose preincubated for 2 h, subsequently 0.75 % L(−)-sorbose added and incubated for 30 min; ◨, 0.75 % L(−)-sorbose preincubated for 2 h, subsequently 0.75 % D(+)-glucose added and incubated for 30 min. Ordinate: left flow time in s; right viscosity in cP.

Fig. 10. Viscosity measurements of the extracted lipids of red cell membranes, containing total lipid = 3.6 mg/ml, cholesterol = 1.06 mg/ml. Preloaded with 1.5 % of the sugars. ○, control in the absence of added sugar; □, D(+)-glucose; △, L(−)-sorbose. Ordinate: left flow time in s; right viscosity in cP.

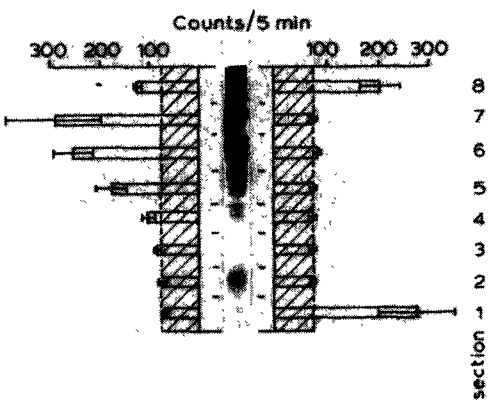

Fig. 11. ^{14}C radioactivity distribution in disc electrophoresis of erythrocyte membranes. The membranes were preincubated with 6.6 mM of D(+)-[^{14}C]glucose or L(−)-[^{14}C]sorbose. Counts/5 min ±S.D. was determined as described in Materials and Methods. Left, glucose (n = 5); right, sorbose (n = 5). Radioactivity in the front section 1 (n = 3), n = number of experiments, direction of electrophoresis: from top to bottom. Center: Electrophoresis pattern stained with Coomassie blue.

DISCUSSION

Transition temperature of membrane lipids has been used by some authors as a means for the investigation of structural features of membrane in the transport event [5–7].

It has been found that a correlation exists between transport activity and temperature in so far as below transition temperature the slope of log transport activity versus absolute temperature is different from the one observed above the transition temperature [5]. From this finding one may conclude that the structural changes occurring at the transition temperature play an essential role in substrate transport.

As there is a dependency of transition temperature on pH [29], ionic conditions and strength [30] as well may there be a dependency on the substrate itself. If there were such an influence, this may have some bearing on the competitive behaviour of substrates like glucose and sorbose for the sugar transport system of red cells.

Two different systems have been investigated to study the influence of glucose and sorbose at the membrane level: on the one hand we studied the red cell membrane itself, solubilized by different procedures, on the other hand, we studied the extracted membrane lipids.

The results obtained were characteristically different: In red cell membranes glucose preloading enhanced the break at transition temperature, while sorbose abolished or reversed it. In extracted membrane lipids both glucose and sorbose preloading revealed a similar break. Compared to the control, this break was not enhanced (Fig. 10).

It is thus clear that the enhancement of the break brought about by glucose and the abolition of the break by sorbose are due to membrane protein- or, alternatively, membrane lipid-protein interactions.

It should be noted at this point that interesting evidence is also supplied by experiments using infrared [31] and Raman spectroscopy [32]: The first method, concerning glucose and sorbose effects on the red cell membranes, the second about the transition of phosphatidylcholine and phosphatidylethanolamine.

We have previously observed, that glucose and sorbose loading of the red cell membranes caused different infrared spectra: While glucose caused a reduction of band intensity in the region of P=O, C–O–C at 1225 cm^{-1} wave number, sorbose caused an increase in this region's band intensity [31]. A change in the P=O or PO^- region was also found to be of importance at the transition temperature of the phospholipids investigated by Raman spectroscopy [32].

These findings correspond with one another. Since the region of phospholipid head groups is sensitive to changes at transition temperature [24, 32], the differences found in the infrared spectra may account for the different behaviour of glucose versus sorbose in the red cell membrane at transition temperature. However, in order to prove specificity of these observations, other sugars should also be investigated spectroscopically when bound to the red cell membrane.

Different binding sites of these sugars were moreover found with disc electrophoretic experiments. These findings correspond with earlier conclusions [31].

We refer to transport investigations in a very loose manner only. But we

wish to emphasize that the results obtained do not contradict transport data of the competition between glucose and sorbose in the red cell.

Competition seems not necessarily to be brought about by interaction at exactly the same binding site of the membrane [33]. One need not postulate such a common site [34]. Competition may as well result from binding to different sites with a certain overlap as can be deduced from work by Semenza and collaborators [35].

In conclusion, if there are different preferential binding sites, as we have shown, for glucose and sorbose at the red cell membrane, competition between the two sugars, nevertheless, may be attained by a reversible, opposite structural effect. The exact nature of this structural effect should be the goal of future investigation.

Recently, Gottlieb and Eanes [36] reported X-ray diffraction studies of erythrocyte membrane and membrane lipids, observing a transition from the rigid crystalline to the liquid crystalline state in the range from 2–(15)—20 °C [15], when phospholipids, containing 3 % cholesterol, were investigated. Similar broad transitions observed in X-ray diffraction studies as opposed to sharp transitions observed with other methods have been noted by Sullivan et al. [37]. Increasing the amount of cholesterol or experiments with red cell membranes did no longer yield evidence for a transition in this temperature range [36].

We also suggest that the different physical form of the materials [36] may have contributed to this discrepancy. The particles after sonication have a much larger surface in comparison to the bulk membrane material, investigated by Gottlieb and Eanes. A change in surface charge could eventually lead to reversible aggregation disaggregation phenomena, which would be detected by the viscosimetry, light scattering and ANS fluorescence measurements.

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