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VISCOSITY CHANGES OF ERYTHROCYTE MEMBRANE AND MEMBRANE LIPIDS AT TRANSITION TEMPERATURE

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SUMMARY

A non-linearity in the changes of viscosity with temperature was found in sonicated human erythrocyte membranes at 18–19 °C. At the same temperature, a break was observed in the viscosity of the extracted membrane lipids, the cholesterol content of which was varied by means of Sephadex LH 20 column chromatography. It is inferred that the break observed in the membranes corresponds to the transition temperature of the erythrocyte membrane lipids. The applied method of direct viscosimetry is relatively simple and cheap in comparison to the well known methods of ESR spectroscopy or differential scanning calorimetry, which have been hitherto widely used in determining thermal transition points in different systems.

Viscosity measurements may be compared to light scattering or fluorescence measurements, introduced recently for the determination of phase transitions (Träuble, H. (1971) Naturwissenschaften 58, 277–284, and Lussan, C. and Faucon, J. F. (1971) FEBS Lett. 19, 186–188).

INTRODUCTION

Raison et al. [1] have demonstrated an interrelationship of lipid transition temperature in mitochondria to O_2 uptake and to some ESR parameters. The role played by membrane lipids in transport mechanisms is also going to be established [2–5]. Thus it was disclosed that the transition temperature of membrane lipids is dependent on fatty acid supplementation to the growth medium of *Escherichia coli* k 12, and that glucoside and galactoside transport rates revealed different slopes below and above this temperature [4]. Similarly, a break in glucose transport rates was found to occur in erythrocytes at about 19 °C [6].

In the present paper, we use an additional physical parameter, namely, direct viscosity measurements, which proved suitable to follow changes in membrane and lipid structure. Viscosity measurements may be compared to light-scattering or fluorescence measurements, which were recently introduced for the determination of phase transitions [7, 8].

MATERIALS AND METHODS

Preparation of erythrocyte membranes

Human erythrocytes were obtained from the blood bank as fresh blood conserves. They were lysed with 15 mM sodium phosphate buffer for about 17 h and washed with 15 mM and subsequently 10 mM phosphate buffer, pH 7.5, as described by Dodge et al. [9]. After completion of the washing procedure the suspensions were frozen overnight, centrifuged, and afterwards resuspended with water and lyophilized.

Extraction of erythrocyte membrane lipids

The extraction of lipids followed essentially the method of Dawson et al. [10] in chloroform–methanol (2 : 1, v/v). 200 mg of freshly prepared erythrocyte membranes (protein content: about 0.5 mg/mg lyophilized membrane preparation) were suspended with 2 ml of 0.9% NaCl. The suspension was homogenized in a Potter–Elvehjem homogenizer and 14 ml of methanol were added. Afterwards the suspension was stirred with the addition of 28 ml of chloroform. The resulting fine suspension was filtered with paper (No. 595 1/2, porewidth about 5 μm) Schleicher and Schüll, Dassel, West-Germany) after standing for 15 min. To the filtrate, 9 ml of 0.9% NaCl was added, shaken, and the charge was then allowed to separate into two phases at 5 °C. After 2 h no lipid could be detected in the upper phase, which was subsequently withdrawn by suction. The lower phase was filtered and concentrated by a rotatory vacuum dryer to about 1/5 of the original volume. The final volume was 3–5 ml.

Sephadex LH 20 column chromatography

About 2–3 ml of lipid extract, containing about 40 mg of total lipids were layered onto a column of Sephadex LH 20 (200 mm $\times$ 19 mm) which was equilibrated with chloroform–methanol (1 : 1, v/v). Chromatography was carried out in this system with an elution speed of 3 ml/h at 21 °C. Fractions of 0.5–1.0 ml were collected.

Lipid and lipid phosphorus determinations

Lipid content of extracted phospholipids was determined as previously described [11] using the method of Zöllner and Kirsch [12]; 0.05 ml of the fractions after column chromatography was employed. Lipid phosphorus was determined by the method of Bartlett [13].

Determination of cholesterol

For the determination of cholesterol the method of Watson [14] was applied. A Biochemica test combination from Boehringer (Mannheim, Germany) was employed. 0.1 ml of the fractions after column chromatography was used.

Protein determinations

Protein was estimated by the method of Lowry et al. [15], using crystalline serum albumin as a standard.

Viscosity measurements

Viscosity measurements were carried out in Ostwald capillary viscosimeters. One of those with a water flow speed of 26.7 s at 20 °C was employed in experiments with erythrocyte and mitochondrial membranes. 50 mg of erythrocyte membrane suspension in 9 ml 0.9% NaCl was sonicated four times for 15 s using a Branson S-75 sonifier at maximal output (5–6 A). Red cell membranes were suspended with 0.9% NaCl because many workers on sugar transport kinetics also made use of this medium. Sonication was carried out in a 10-ml beaker which was put into crushed ice. Temperature controls before and immediately after sonications revealed that the range of changes was in between 5 and 13 °C. The pH remained stable at around 6.75 before and after sonication. Subsequently the membrane suspension was filtered with paper (No. 595 1/2, Schleicher and Schüll). Filtration was performed in order to remove particles larger than the porewidth of the filter paper (5 μm), which might have interfered with the measurements. Protein and lipid estimations before and after filtration revealed that no loss of membrane protein or lipid did occur during this step. 3 ml of the filtrate were then used for measurement of viscosity. Another viscosimeter with a water flow speed of 60 s at 20 °C was used for measurements on extracted and chromatographed lipids. 3 ml were employed for the viscosity measurements. Calibration of the viscosimeters was performed with water–glycerol standard mixtures of known densities. The temperature was held constant at $\pm$0.2 °C with a Lauda ultrathermostat. An average accuracy of 0.15 s S.D. was obtained with the viscosity measurements, which were repeated five times at each temperature.

Preparation of rat liver mitochondrial membranes

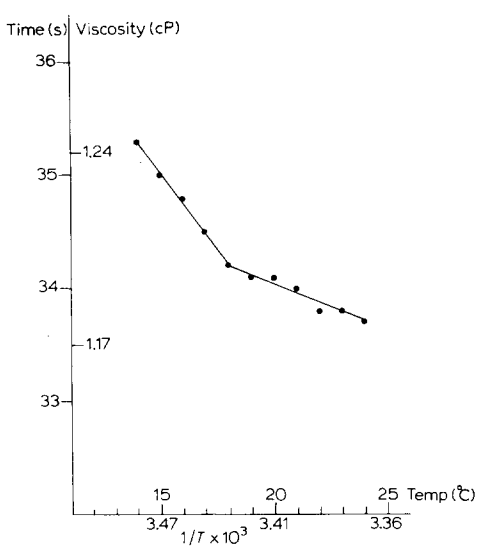
Liver mitochondria from 4 rats (Sprague–Dawley, 200 g) were prepared in 0.25 M sucrose by the conventional method described previously [16]. After the last centrifugation the mitochondrial pellet was suspended with 5 ml of 0.25 M sucrose and pipetted into 10 vol. of ice-cold water. Sonication of the ice cold suspension was performed with a Branson sonifier S-75 four times for 15 s and at 5–6 A. After sonication the suspension was centrifuged for 15 min at 20 000 $\times g$ in a Sorvall RC2b centrifuge and subsequently for 60 min at 100 000 $\times g$ in a Beckman ultracentrifuge. The membrane pellets were suspended with 5 ml water and frozen overnight. After filtration 3 ml (protein content 25 mg/ml) were analysed for viscosity changes with temperature.

RESULTS

We have found a non-linearity in viscosity changes of sonicated erythrocyte membranes with temperature at about 18–19 °C. The slope of viscosity changes becomes flatter after this temperature is reached (Fig. 1).

In order, to prove a wider applicability of the method, viscosity measurements were also carried out on sonicated mitochondrial membranes. The known transition temperatures at 22–23 °C [1, 17] and at 35–36 °C [17] were confirmed (Fig. 2). Further work was executed on the erythrocyte membrane system.

Following the changes that were observed in the extracted erythrocyte membrane lipids, a break at the same temperature (18–19 °C) occurred. Sephadex LH



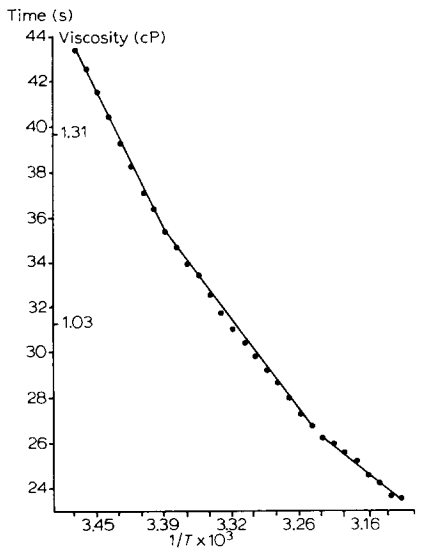


Fig. 1. Viscosity measurements of erythrocyte membranes, sonicated in 0.9 % NaCl. Protein content in final volume (3 ml): 8.8 mg. Ordinate: left, flow time in s; right, viscosity in cP.
Fig. 2. Viscosity measurements of mitochondrial membranes sonicated in 0.025 M sucrose–water. Protein content in final volume (3 ml): 75 mg. Ordinate: left, flow time in s; right, viscosity in cP.

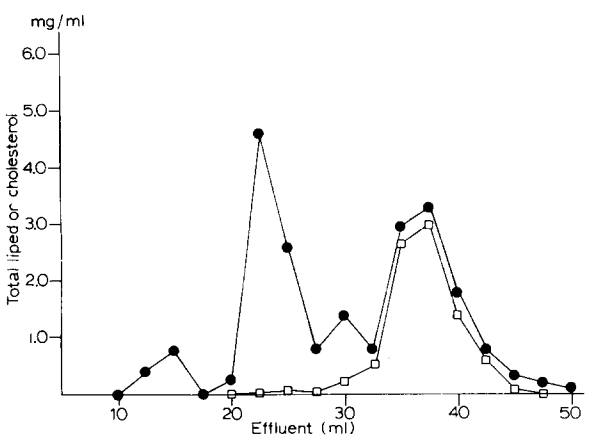


Fig. 3. Sephadex LH 20 column chromatography of extracted erythrocyte membrane lipids. Solvent: chloroform–methanol (1:1, v/v). ●, total lipid; □, cholesterol.

20 column chromatography of the extracted membrane lipids afforded a good separation of phospholipid and cholesterol (Fig. 3). Phosphorus determinations of the fractions after column chromatography revealed a run parallel to the differences of total lipid and cholesterol. It was, therefore, possible to vary the phospholipid/cholesterol ratios by means of pooling different fractions after chromatography. Vis-

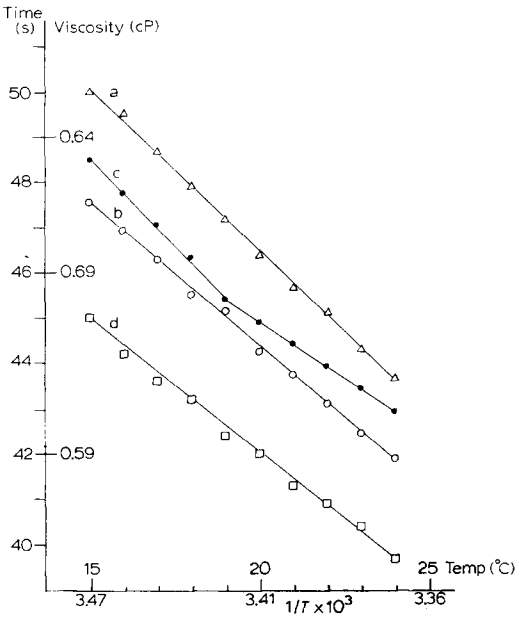


Fig. 4. Viscosity measurements of chromatographed lipids. Solvent: chloroform–methanol (1/1, v/v). Fractions were pooled after total lipid and cholesterol determinations, in order to obtain different cholesterol contents. Cholesterol contents: (a) no cholesterol; (b) 5 % cholesterol; (c) 10 % cholesterol; (d) 75 % cholesterol. Total lipid content in the final volume (3 ml); 10–20 mg. Ordinate: left flow time in s; right viscosity in cP.

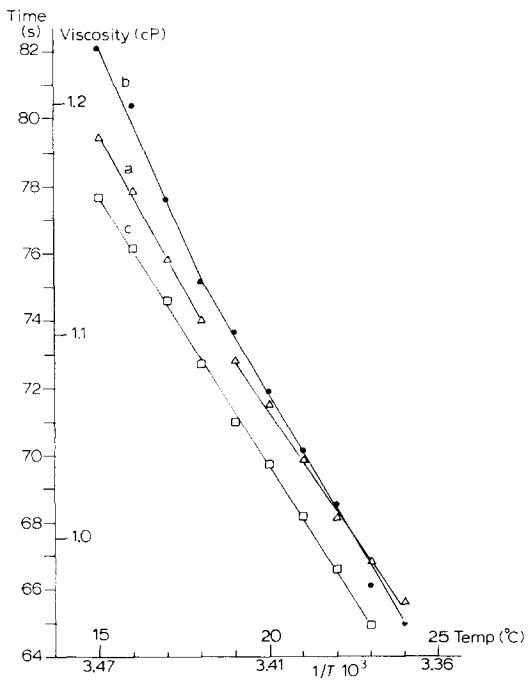


Fig. 5. Viscosity measurements of chromatographed lipids. Solvent: 0.9 % NaCl. Fractions were pooled after total lipid and cholesterol determinations, in order to obtain different cholesterol contents. Cholesterol contents: (a) no cholesterol; (b) 10 % cholesterol; (c) 50 % cholesterol. Total lipid content in the final volume (3 ml): 5–15 mg. Ordinate: left, flow time in s; right, viscosity in cP.

cosity changes of the chromatographed lipids, which were measured in chloroform–methanol (1 : 1, v/v), were then compared to those, obtained after evaporation of organic solvent and dispersion of the lipids in 0.9% NaCl. A comparison of Figs 4 and 5 reveals that, on the one hand, membrane phospholipid free of cholesterol shows a discontinuity in aequous medium (Fig. 5a), whereas this is not found in organic solvent (Fig. 4a). On the other hand, the break in viscosity changes of phospholipid containing 10% cholesterol is similar in both environments (Figs 4c, 5b). This may be interpreted in a such way, that a small content of cholesterol helps in changing the solubility properties of phospholipids, or in building and/or maintaining phospholipid micelles. Possibly, micelles may not exist in organic solvent, when phospholipid, free of cholesterol is measured. A larger amount of cholesterol, however, will prevent the cooperative effect of the phase transition of the phospholipids (Figs 4d, 5c). In this respect, a 25–50% (w/w) content of cholesterol may roughly correspond to an equimolar ratio of phospholipid/cholesterol. Therefore, this observation is in accordance with the findings of Ladbrooke et al. [18], using a lecithin–cholesterol–water system.

DISCUSSION

It is well known, that viscosity and the 'relative free volume' of liquids change inversely when the temperature is raised (Hildebrand [19]). Basing on investigations of Chapman et al. [20] and using dilatometric measurements it was found by Träuble and Haynes [21] that the phase transition in lipid bilayer lamellae was accompanied by an abrupt volume increase. Thus the observed change in slope of viscosity of erythrocyte membranes and membrane lipids with temperature may be attributed to volume and structural alterations that have been described [21]. In other words these changes may preclude a similar decline of viscosity below and above transition temperature. Differences in electrical charge (on the particle surface) may also contribute. As a result, the slope of viscosity changes will become flatter above the transition temperature, as is shown in the figures. This effect is, however, concentration dependent. At higher viscosities sometimes a steeper decrease was observed. The temperature range of the transition was, however, constant. Similar results were obtained with light scattering measurements at 420–600 nm (Zimmer, G. and Schirmer, H., unpublished).

So far the physiological implications of these observations of a phase transition in erythrocyte membrane lipids at 18–19 °C are concerned, the work of Hankin and Stein [22] on glucose transport reveals a discontinuity in a similar temperature range in the equilibrium exchange experiments. This point was, however, not taken into consideration by the authors. Also similar deviations may be found in the data of Sen and Widdas [23], so far the half saturation constant of glucose transport is concerned. We propose the observed phase transition to be the rationale serving to explain the discontinuities in glucose transport kinetics at 18–20 °C [6, 22, 23]. These interrelations are, therefore, suited to stress the importance of investigations on lipid–protein interactions in the sugar-transport system of human erythrocytes. Our experiments suggest that direct viscosity measurements may provide a relatively simple and interesting tool to investigate membrane structure and function.

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