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TEMPERATURE DEPENDENCE OF D-GLUCOSE TRANSPORT IN RECONSTITUTED LIPOSOMES

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Sodium-dependent D-glucose uptake into proteoliposomes reconstituted from dimyristoylphosphatidylcholine (DMPC) and hog kidney brush border membrane extract is strongly affected by temperature and the physical state of the membranes. This dependence is defined by a nonlinear Arrhenius plot with a break point at 23°C, a temperature not significantly different from the phase transition temperature of the pure lipid (24°C). The transport process is characterized by different activation energies: 35.1 kcal/mol below and 5.5 kcal/mol above the transition temperature. The shift in the break point for the D-glucose transport activity from 15°C, in the brush border membranes, to 23°C in the reconstituted system leads us to conclude that the lipids surrounding the sodium/D-glucose cotransport system can exchange readily with the bulk lipid used for reconstitution. The results thus provide no evidence for the presence of an annulus of specific lipids surrounding the transport system.

Introduction

Temperature is one of the parameters that influences membrane-associated processes, such as membrane transport, enzymatic activities and membrane fusion [1–4]. The influence of the physical state of the membrane on the rate-limiting step of the transport process can be deduced from the appearance of break points on the Arrhenius plot and their correlation with thermotropic transitions of the system [1,2].

The dependence of D-glucose transport activity on temperature was studied in intact brush border membranes [5,6]. The Arrhenius plot for the transport activity shows a break point at about 15°C but no correlation has so far been established between this break point and the temperature-induced changes in membrane lipids.

A better understanding of the temperature effect on membrane-associated functions can be achieved when the temperature dependence studies are performed in parallel using both intact membranes and the corresponding reconstituted systems. A good example of this situation is provided by the studies with $(Na^+ + K^+)$-ATPase [7]. With the aim of clarifying the mechanism of temperature dependence and the interactions between the lipid and the D-glucose transport protein, studies on the temperature dependence of D-glucose uptake and phase transition were performed using the reconstituted system. Reconstitutions were performed using lipids with a phase transition temperature different from the temperature at

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Abbreviations: Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; DMPC, dimyristoylphosphatidylcholine.

which the break point in the Arrhenius plot occurred in natural membranes.

Part of the results has been published in abstract form at the IIIth Annual Meeting of the Sociedade Portuguesa de Fisiologia, 6 July, 1981, Lisbon (Portugal) and at the VIIth Congresso Nacional de Bioquímica, 29 November–2 December, 1981, Póvoa de Varzim (Portugal).

Methods and Materials

Isolation of brush border membranes. Fresh pig kidneys were obtained from a local slaughterhouse. Brush border membranes were isolated by calcium precipitation and differential centrifugation according to the method of Vannier et al. [8] as modified in our laboratory [9–11].

Preparation of membrane extracts. The brush border membranes were added under stirring to a detergent solution in buffer A (150 mM KCl, 20 mM Hepes, 0.2 mM dithiothreitol, 0.5 mM EDTA (K^+ salt), pH adjusted to 7.4 with Tris) until a final concentration of 1% octyl-β-D-glucoside was reached, the ratio of detergent to protein being kept constant at 4 : 1 (w/w) [10]. The suspension was incubated for 30 min and then centrifuged for 60 min at 100 000 × *g*. The protein in the supernatant was considered as solubilized and represented about 70 ± 3% of the total membrane protein. The lipid-to-protein ratio of the detergent membrane extracts was 0.3–0.4. All procedures were performed at 4°C.

Preparation of liposomes. Liposomes were prepared with DMPC. The lipid solubilized in chloroform was transferred into an evaporation flask and the solvent was evaporated in the presence of N_2. Then buffer A was added to the dried lipid and the lipid was suspended in the buffer by shaking gently. After the lipid was completely suspended in the buffer the mixture was sonicated under a stream of nitrogen for periods of 3 min with intervals of 1 min, the total sonication time being 9 min. Then centrifugation at 45 000 × *g* · 10 min followed, where aggregated lipids and liposomes were separated. The liposomes present in the supernatant were then further subdivided into large and small liposomes by centrifugation at 300 000 × *g* · 45 min. The larger liposomes assembled in the pellet were suspended in buffer A to a final concentration of 20–40 mg phospholipid per ml and were used for reconstitution experiments. Usually, about 35% of the total lipid was recovered as larger liposomes.

Incorporation method. The protein fractions were put into dialysis bags and were dialysed for about 4 h against 20% poly(ethylene glycol) (mol. wt. 40 000) dissolved in buffer A at 4°C [11]. When almost complete removal of the liquid inside the dialysis bag was achieved, fresh buffer was added and a new concentration step took place. This procedure was repeated two to three times (4–6 h). After each concentration step and addition of fresh buffer, the detergent concentration inside the dialysis bag was determined. After 3–4 h dialysis against poly(ethylene glycol) more than 90% of the detergent was removed, before incorporation.

Sonicated liposomes (20 mg/ml) were added to the concentrated protein. The ratio of lipid to protein used was 7–9 (w/w), higher than the minimal amount needed to reconstitute the transport activity (lipid/protein ratio of 2–3). The mixture of lipid, protein and the remaining detergent was dialysed against buffer A for 40–80 h. During this time, the dialysis buffer was changed six to ten times. In the last dialysis, Bio-Beads SM2 were added to the dialysis buffer to remove the last traces of detergent. The liposomes formed during the dialysis containing the incorporated protein were separated from the nonincorporated material by high-speed centrifugation. The pellet was suspended in buffer at a final protein concentration of about 1 mg/ml. Proteoliposomes were used for transport experiments directly.

Analysis of membrane properties and components. Protein measurements were carried out according to the method of Lowry et al. [12], with modifications described previously [11]. An additional centrifugation step was introduced in order to remove the lipids which interferred in the absorbance measurements.

The total phospholipid content was determined by estimating the inorganic phosphate, released after digestion with perchloric acid [13], by a technique modified from that of Fisk and SubbaRow [14].

Phase transition temperature determinations were carried out by turbidimetry according to Refs. 4 and 15. Liposomes suspended in buffer A

were added to a cuvette of a fluorescence spectrophotometer (Perkin-Elmer 650-40) connected to a thermostatically controlled bath. The temperature of the bath was changed by increments of 0.5 to 1°C, in the range between 10 and 30°C. After equilibration the temperature in the cuvette was monitored and the light scattering at 450 nm excitation and 450 nm emission was recorded. In the transition region an abrupt change of turbidity occurred which allowed the determination of the transition temperature.

For the transport experiments a rapid filtration technique was used [11]. The experiments were carried out under tracer-exchange conditions, i.e., the liposomes contained solutions identical with the incubation media: 20 mM Hepes-Tris, pH 7.4, 150 mM $NaNO_3$ or KNO_3 and 0.1 mM D-glucose. The media contained, in addition, radioactively labelled D-glucose. The transport experiments were performed at least in duplicate. The exact conditions for each experiment are given in the legends to the figures.

For temperature studies the media and the proteoliposomes were equilibrated, separately, at various temperatures from 13 to 26.5°C with intervals of 0.5–2°C. Then uptake experiments were initiated by incubation of proteoliposomes in medium of the same temperature.

Materials. All chemical reagents used were of the highest purity available. Radioactive isotopes were purchased from New England Nuclear (Boston, MA, U.S.A.).

Results

Transport activity

Fig. 1A illustrates the time course of D-glucose uptake by proteoliposomes, in the presence of

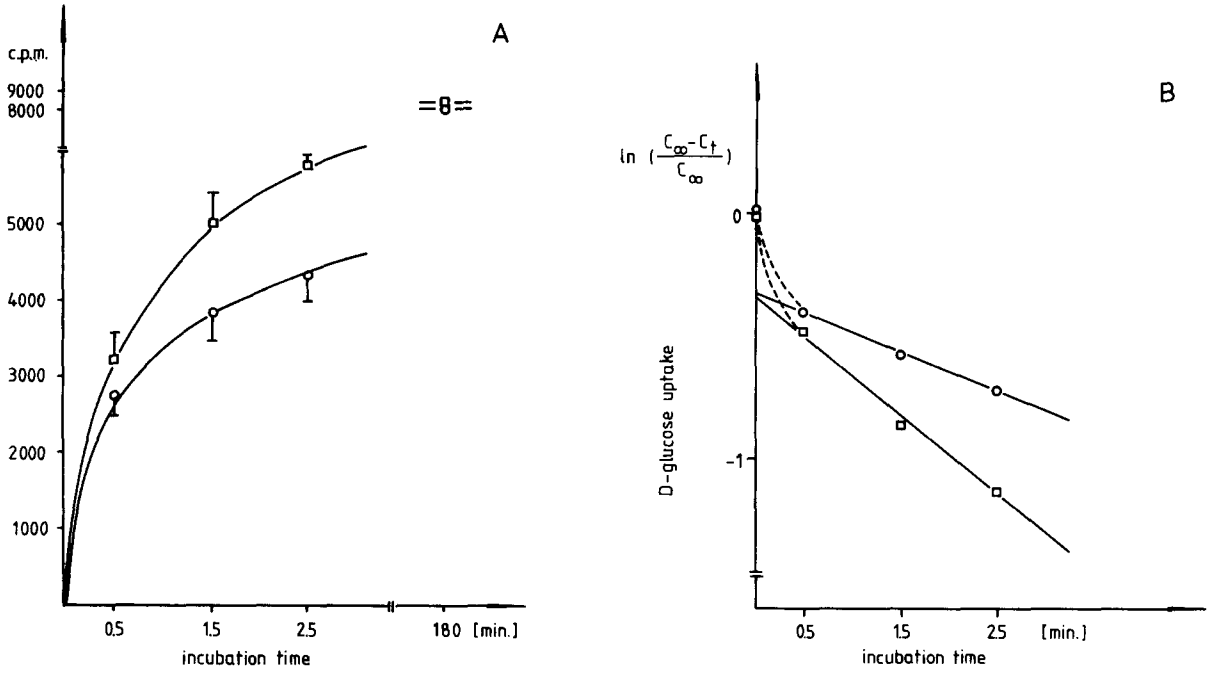

Fig. 1. (A) Time course of D-glucose uptake by proteoliposomes. Proteoliposomes were prepared from crude membrane extracts and DMPC with a lipid/protein ratio of 9. The proteoliposomes contained 50 mM KNO_3 and 20 mM Hepes-Tris. The uptake experiments were performed under tracer-exchange conditions at 30°C. The proteoliposomes were incubated, before the experiments at 30°C, with buffer until their internal final composition equalled that of the incubation media: 20 mM Hepes-Tris, 0.13 mM D-glucose and 150 mM KNO_3 (○) or 50 mM KNO_3 plus 100 mM $NaNO_3$ (□). The incubation media contained, in addition, 50 μCi D-[^{3}H]glucose. The experimental points represent averages of two to four experiments (B) Fractional D-glucose uptake by proteoliposomes. The ordinate units are ln $(\frac{C_\infty - C_t}{C_\infty})$; C_∞ refers the uptake at equilibrium and C_t the uptake at any given incubation time. The uptake values were derived from panel 1A.

$NaNO_3$ or KNO_3. The uptake of labelled D-glucose is faster in the presence of Na^+ than in the presence of K^+: 3099 cpm and 2713 cpm after 30 s, respectively. In the semilogarithmic representation of the results a curved and a linear part are seen (Fig. 1B). For times longer than 0.5 min the curve becomes a straight line. The deviation from linearity observed may be due to the heterogeneity of vesicle size and permeability. For a population of similar vesicles the rate constant k has two factors: the rate constant of the transport system itself and a factor dependent on the vesicle size and carrier density [16]. Small vesicles with a spherical shape will fill faster than larger ones of similar permeability. The curved part of the linearized fractional uptake may be due to a series of exponential terms which define the D-glucose uptake after short times of incubation [6,16]. To calculate rate constants the first time point was rejected. The rate constant of D-glucose exchange in the presence of Na^+ is 0.34 min^{-1}, more than 2-times higher than the rate constant in the presence of K^+, 0.15 min^{-1}. Alternatively, the uptake of D-glucose measured by the tracer-exchange procedure may be characterized by its half-time, experimentally calculated as the time for which C_t is $1/2$ of C_∞. The half-time values deduced from Fig. 1B are 65 and 150 s in the presence of Na^+ and K^+, respectively.

Phase transition of liposomes and proteoliposomes

The phase transition temperatures of plain DMPC liposomes and proteoliposomes after protein incorporation were determined. As shown in Fig. 2 an abrupt change in turbidity was observed between 23 and 25°C both for liposomes and proteoliposomes. This change of turbidity is attributed to the faster penetration of water between the layers of lipids, occurring in the thermotropic transition [4,15,17].

The mid temperature of the phase transition determined by the midpoint of the vertical projection between the inflexion points of the turbidimetric curves [4] is 24°C for liposomes as well as for proteoliposomes (Fig. 2A and A'). A more accurate estimation of the midphase transition temperature can be obtained by analysing the temperature of the maximum value of the first derivative of the absorbance in relation to the

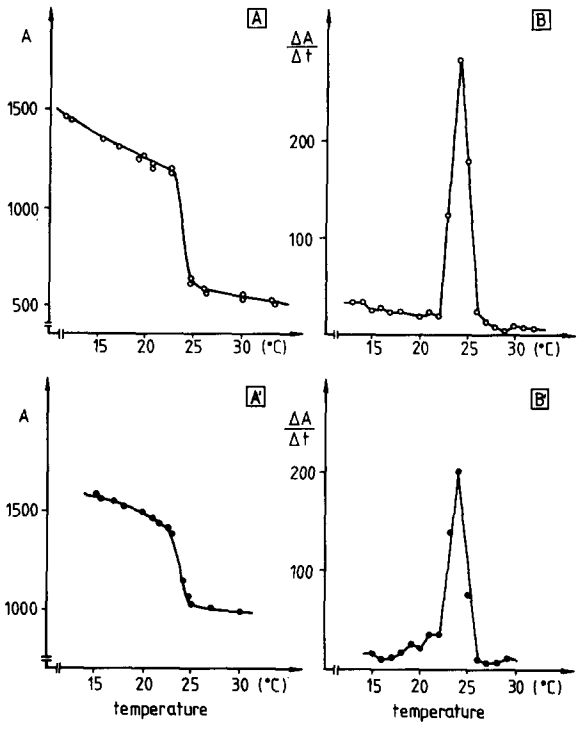

Fig. 2. Determination of phase transition temperature of liposomes and proteoliposomes. Turbidity curves (A,A') represent the change in absorbance of liposomes (open symbols) and proteoliposomes (closed symbols) with temperature, at 450 nm. The change in the first derivative of absorbance in relation to temperature with temperature is also represented (B,B'). Liposomes were prepared with DMPC. Proteoliposomes were prepared with DMPC and crude membrane extracts with a lipid/protein ratio of 7–9.

temperature. An approximation of the first derivative can be obtained by the ratio of the difference in absorbance at two different temperatures (ΔA) and the respective temperature range (Δt). The midpoint temperature of thermotropic transition derived by this method is again 24°C both for liposomes and proteoliposomes (Fig. 2 B and B'). The coincidence of the phase transition temperature in liposomes and proteoliposomes indicates that, at a lipid-to-protein ratio of 7–9, and probably also above, the incorporation of protein does not alter the behavior of the bulk lipids. Such a finding would be expected, since a calculation assuming an average molecular weight of protein of 70000 and that of phospholipids of 700 shows that the molar ratio between lipids in the lipo-

somal membrane and the protein incorporated is, under our experimental conditions, higher than 100. Thus, the disturbance of lipid by the incorporated protein should be minimal.

Temperature dependence of D-glucose uptake in proteoliposomes

Table I lists the rate constants of the D-glucose uptake as well as the apparent intravesicular space of proteoliposomes at different temperatures. The apparent intravesicular space was derived from the D-glucose uptake at equilibrium, experimentally determined for each temperature.

All parameters increased with temperature from 13 to 26.5°C. The apparent intravesicular space varies from 1.1 to 6 μl/mg protein at 13 and 26.5°C, respectively, showing a sharp increase between 20 and 24°C. For total and sodium-independent D-glucose uptake the highest temperature coefficients were observed between 22 and 25°C.

In Fig. 3 the change in D-glucose uptake with temperature is presented in the form of an

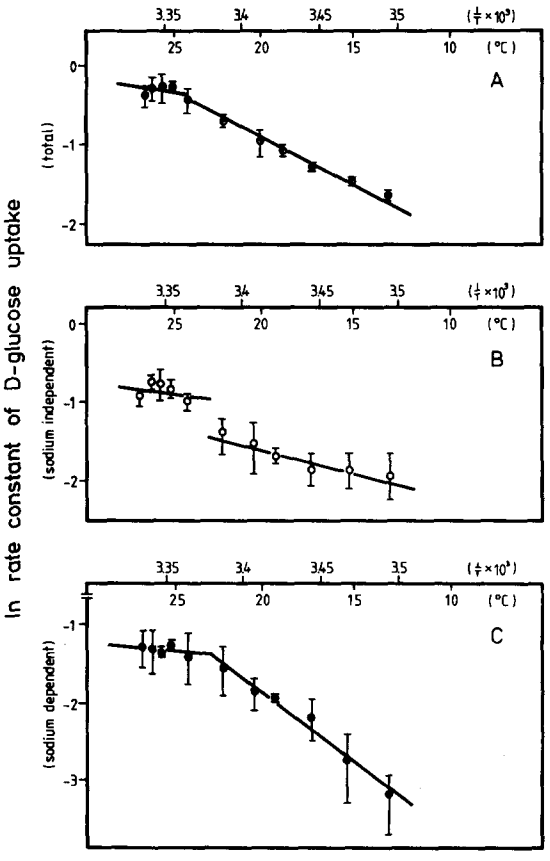

Fig. 3. Temperature dependence of D-glucose uptake in proteoliposomes: changes in rate constants of D-glucose uptake with temperature: total (A), sodium independent (B) and sodium dependent (C). The proteoliposomes were prepared with DMPC and crude membrane extracts at 28°C and with a lipid/protein ratio of 7–9. The transport experiments were carried out under exchange conditions, in the presence of 0.3 mM D-[^{3}H]glucose in buffered media and 100 mM $NaNO_3$ or KNO_3. The best fit for the experimental data was determined from regression lines by least-square analysis, using a programmable desk calculator.

TABLE I

RATE CONSTANT OF D-GLUCOSE UPTAKE BY PROTEOLIPOSOMES

The proteoliposomes were prepared with DMPC and crude membrane extracts, at 28°C with a lipid/protein ratio of 9. The data are mean values of the number of experiments indicated; standard deviations are given.

t (°C)	Rate constant of D-glucose uptake (min^{-1})			Apparent intravesicular space (μl/mg protein)	Number of determinations
	Total	Sodium independent	Sodium dependent		
13	0.18 ±0.004	0.14 ±0.004	0.04 ±0.01	1.1	3
15	0.213±0.006	0.15 ±0.035	0.063±0.027	0.6	3
17	0.26 ±0.045	0.15 ±0.035	0.11 ±0.034	1.9	5
19	0.323±0.02	0.178±0.02	0.143±0.01	2.6	3
20	0.36 ±0.09	0.21 ±0.07	0.15 ±0.025	4.0	3
22	0.46 ±0.03	0.245±0.07	0.215±0.07	6.0	4
24	0.615±0.16	0.365±0.03	0.25 ±0.06	7.25	4
25	0.76 ±0.038	0.465±0.03	0.290±0.07	7.6	6
25.5	0.73 ±0.05	0.48 ±0.06	0.25 ±0.01	8.0	5
26	0.75 ±0.12	0.485±0.04	0.265±0.07	9.5	2
26.5	0.66 ±0.11	0.38 ±0.04	0.28 ±0.07	6.0	2

Arrhenius plot. The Arrhenius plots were constructed using rate constants of the uptake at temperatures corresponding to well defined phases, above or below the transition region. Rate constants of the uptake at temperatures within the transition region were not considered. For total D-glucose uptake (A) a biphasic plot was found with a plateau above 24°C. The Arrhenius plot for the sodium-independent D-glucose uptake (B) shows a discontinuity between 22 and 24°C. For the sodium-dependent D-glucose uptake (C) a biphasic Arrhenius plot with a break point at about 23°C was obtained. The temperatures where break points and discontinuities were found in the Arrhenius plot are not statistically different from the phase transition temperature of DMPC proteoliposomes, indicating that changes in transport activity are related to changes in the physical state of the lipids surrounding the transport protein. The effect of those phase changes on membrane permeability and intravesicular space are evidenced by the higher increase observed for sodium-independent uptake and the uptake at equilibrium, near the transition region.

The following Arrhenius activation energies were derived from the slopes: for the total uptake 8.3 and 21.2 kcal/mol, above and below 24°C, respectively; for the sodium-independent D-glucose uptake, if we assume that the Arrhenius plot is composed of two straight lines, 9 and 12 kcal/mol, above and below the discontinuity and for the sodium-dependent D-glucose uptake 5.7 above and 35.1 kcal/mol below the break point, respectively.

Discussion

Effect of temperature on the characteristics of the proteoliposomes

Temperature has an effect on the permeability and the apparent internal space of reconstituted liposomes similar to that observed in nonreconstituted liposomes prepared with pure lipids.

Changes of 23–30% in the volume trapped inside the vesicles, increases of the order of 20% in the rate of water permeation and enhanced release of trapped solutes on passing the transition region were observed in liposomes prepared with several lecithins [17–19].

Reconstituted liposomes from crude membrane extracts and DMPC showed a continuous increase in apparent internal space and sodium-independent D-glucose uptake above and below the thermotropic transition region and sudden changes in those parameters within that region. If we correlate the sodium-independent D-glucose uptake with simple diffusion and also the volume trapped inside the vesicles with their internal space. we can conclude that the increase in those parameters on passing the transition region indicates that the permeability and the apparent internal space of the reconstituted system are strongly affected by the physical state of the membrane. The activation energies of the D-glucose uptake confirm this hypothesis, since smaller values were found for the uptake in the disordered phase, where higher lipid chain mobility is expected, than in the ordered phase [1]. This finding also agrees with the smaller activation energies observed above the transition temperature for water permeation in DMPC and dipalmitoylphosphatidylcholine liposomes [17].

In intact brush border membranes, where a sharp phase transition is not likely to occur due to the complexity of the lipid mixture, the diffusional D-glucose uptake increases continuously with temperature. Correspondingly, a single value of the activation energy is observed [5,6].

The effect of temperature on transport activity

In Table II studies on the temperature dependence of D-glucose transport in reconstituted proteoliposomes are compared with those in natural brush border membranes. Their respective activation energies are about 6–8-fold higher below the transition temperature than above. The high activation energy required for the transport process below the phase transition temperature can be interpreted, in DMPC proteoliposomes, to indicate that the transport system requires the lipids surrounding the protein to be in a mobile state in order to express its full activity. The appearance of a break point in the Arrhenius plot of the transport activity of brush border membranes could perhaps be related to phase separation and crystallization of the individual components of the lipid mixture or to changes in the mobility of the microenvironment of the protein [2]. The activation energy determined for the reconstituted trans-

TABLE II

TEMPERATURE DEPENDENCE OF D-GLUCOSE UPTAKE

The reconstitution and transport experiments were performed as described in Fig. 1. The activation energy was derived from the slopes of the Arrhenius plots for the corresponding uptake. The apparent activation energy of D-glucose uptake by brush border membranes is indicated in brackets (taken from Ref. 6 with kind permission of the publisher).

	Apparent activation energy (kcal/mol)		
	Total	Sodium independent	Sodium dependent
Above discontinuity or break point	8.2 –	9 (4.9)	5.7 (4.2)
Below discontinuity or break point	21.2 –	12 (4.9)	35.1 (32.6)
Temperature of apparent discontinuity or break point (°C)	24 –	22-24 (15)	23 –

port activity in the disordered phase is of the same range as that obtained in hog kidney brush border membranes above the break point, indicating that extraction of the transport protein from natural membranes and its reconstitution in lipid vesicles do not markedly change the characteristics or the energy requirements of the transport. This similarity also indicates that the transport process is not very much influenced by the composition of the lipids surrounding the transport protein provided that appropriate system fluidity is achieved.

Evidence that substrate translocation across the membrane is facilitated by the transport system is provided [20], in proteoliposomes, by the lower activation energy of the sodium-dependent D-glucose uptake as compared with total or diffusional uptake, above the break point.

Lipid-protein interactions

The possible existence of an annulus of lipids and the interactions of lipid with protein has not so far been elucidated concerning the sodium D-glucose cotransport system. Comparing our experiments on the temperature dependence of transport activity by proteoliposomes with those using brush border membranes (Table II), one is tempted to conclude that there is no strong interaction between the transport system and the original lipids surrounding it, including those remaining after detergent treatment, as they could be substituted easily by lipids from the new bulk phase. In fact, the break point observed at 15°C in the Arrhenius plot of brush border membranes shifted to 23°C when the transport system was reconstituted into a lipid with a phase transition temperature of 24°C. This conclusion is also supported by the similar activation energies found, above the transition temperature, for the transport process, using either the natural or reconstituted system.

We observed in our experiments a slight difference between the break point in the Arrhenius plot for D-glucose transport and the phase transition temperature of the proteoliposomes as determined by the turbidity measurements. This phenomenon has been considered to point to the existence of a shell of lipids which, due to the interaction with the hydrophobic membrane proteins, have physicochemical properties different from those of the bulk lipids, [1,2,23,24]. To determine the existence of such a shell and describe it in molecular terms, a purified D-glucose transport system and other methods, such as the use of lipid-soluble reporter molecules, have to be employed [1,2,23,24].

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