Effects of Bilayer Cholesterol on Human Erythrocyte Hexose Transport Protein Activity in Synthetic Lecithin Bilayers†

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ABSTRACT: In this study, we describe the effects of altered bilayer cholesterol content on reconstituted, protein-mediated sugar transport. The system used was the human erythrocyte sugar transporter (band 4.5) reconstituted into the bilayers of large unilamellar vesicles. Vesicle preparations were formed from synthetic lecithins whose bilayer cholesterol content ranged from 0 to 50 mol %. Transport was measured by microturbidimetric analysis over the temperature range of 0–65 °C while bilayer physical state was characterized by differential scanning calorimetry. Reconstituted transport activity was irreversibly lost between 62 and 65 °C. The K_m for reconstituted transport was found to increase only slightly with increasing temperature and was not systematically affected by bilayer cholesterol content. The most striking observation of this study is that over certain critical cholesterol concentrations, as little as a 2.5% change in bilayer cholesterol can result in as much as a 100-fold change in V_{max} per reconstituted protein. Our findings run counter to the view that increasing bilayer cholesterol content monotonically transforms a membrane into a state of "intermediate fluidity". Abrupt, cholesterol-induced bilayer reorganizations occurring at 15–20 and 30 mol % bilayer cholesterol are markedly reflected in altered sugar transport rates. Increasing the cholesterol content of crystalline distearoyllecithin bilayers *inhibits* the activity of the reconstituted transporter. It is apparent from these studies that bilayer "fluidity" is neither the sole nor a major determinant of the transport activity of the human erythrocyte hexose transport molecule. Indeed, we find the effect of cholesterol on transport activity is independent of its ability to fluidize membranes. This is in agreement with our previous study [Carruthers, A., & Melchior, D. L. (1984) *Biochemistry 23*, 6901–6911] in which bilayer lipid composition was shown to be a more important determinant of reconstituted transport rates than bilayer fluidity. As in our previous study, we find the activity of the sugar transporter is governed by the bulk properties of the membrane and not by features of a physically or compositionally distinct hypothetical boundary or "annular" lipid. In accord with other recent findings, these studies illustrate the potential for control of membrane transport protein activity by small changes in membrane lipid composition.

The plasma membrane is a primary determinant of the compositional asymmetry between a cell's cytosol and its extracellular environment. While the membrane lipid bilayer acts to limit the transmembrane movement of most hydrophilic solutes, integral membrane proteins selectively catalyze specific transbilayer fluxes. It is becoming increasingly apparent that for bilayer-spanning transport proteins, bilayer lipids serve more than to simply provide a structural matrix. Rather, certain features of the lipid bilayer (e.g., composition and fluidity) appear to control or modulate the activity of transport proteins situated within the membrane [for reviews, see Melchior & Steim (1976) and Overath & Thilo (1978)]. By analogy with water-soluble enzymes, it is suggested that these membrane translocases are sensitive to the "state" of their solvent, the lipid bilayer. As an example, a model offered for protein-mediated transport assumes that substrate-induced conformational changes within the transporter molecules are rate limited by the "fluidity" of the membrane bilayer (Kotyk & Janacek, 1969). Hence, as membrane fluidity increases, so translocase activity is accelerated.

The fluidity hypothesis has been questioned recently (East et al., 1984; Carruthers & Melchior, 1984b). While bilayer fluidity may be important, other lipid parameters such as hydrocarbon chain length and lipid composition seem to be the primary determinants of translocase activity. Here we report that the activity (turnover number) of the human erythrocyte hexose transport protein reconstituted into dipalmitoyllecithin bilayers is markedly affected by the cholesterol content of its resident bilayer. The observed effects of cholesterol on activity are not, however, consistent with the view that cholesterol monotonically transforms the bilayer state to one of intermediate fluidity. Using differential scanning calorimetry to determine the physical state (fluid/crystalline) of the reconstituted bilayers and kinetic analysis to determine reconstituted transporter activity, we show that sugar transport is remarkably sensitive to bilayer cholesterol concentrations of 15–20 and 30–40 mol %. Our data suggest that bilayer composition is a primary determinant of transporter activity while membrane fluidity is of secondary importance. A preliminary account of these findings has been reported elsewhere (Connolly et al., 1985).

Materials and Methods

Solutions. NaCl medium consisted of 25 mM NaCl, 5 mM tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl),[1]

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[1] Abbreviations: EDTA, ethylenediaminetetraacetic acid; LUVs, large unilamellar vesicles; DML, dimyristoyllecithin; DPL, dipalmitoyllecithin; DSL, distearoyllecithin; DAL, diarachidonoyllecithin; DOL, dioleoyllecithin; T_n, turnover number; T_m, onset temperature; T_f, completion temperature; ΔH, enthalpy; CCB, cytochalasin B; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; kDa, kilodalton(s); DSC, differential scanning calorimetry; FSD, full-scale deflection.

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and 2 mM EDTA, pH 7.4. Detergent medium consisted of NaCl medium plus 0.5% Triton X-100.

Hexose Transfer Protein Purification. Human erythrocyte hexose transfer protein (protein band 4.5, a 55-kDa glycoprotein) was purified as described previously (Carruthers & Melchior, 1984a,b). Briefly, integral membrane proteins are eluted from red cell ghosts by addition of 0.5% Triton X-100 (5 volumes of detergent medium to 1 volume of membranes; temperature, 1 °C; 30-min incubation). Solubilized integral membrane proteins are separated from the "Triton shells" (peripheral proteins) by centrifugation, and band 4.5 protein is obtained by application of the solubilized intrinsic proteins to a DEAE-cellulose column (Kasahara & Hinkle, 1977) equilibrated with detergent medium. Band 4.5 emerges from the column with the flow-through eluate. Protein bands 3, 4.1, 4.2, 5, and 6 are eluted with detergent medium containing 1 M NaCl. The band 4.5 preparation is contaminated by red cell membrane lipid (0.75 ± 0.05 nmol of protein/μmol of phospholipid) and by protein band 7 (6%). Protein band 4.5 runs as a diffuse band on 10% polyacrylamide gels and lacks the highly differentiated subbands seen in gels of intact red cell membranes. Following Triton X-100 removal by Biobeads (see below), the protein can be assayed for D-glucose-inhibitable cytochalasin B binding activity. The ratio of D-glucose displaceable cytochalasin B binding sites per milligram of band 4.5 preparation is 14 nmol/mg of protein. This represents a 13-fold purification of the cytochalasin B binding activity of the intact red cell membrane. Assuming an average molecular weight for band 4.5 of 55 000 (Baldwin et al., 1981) and correcting for contamination by band 7 protein, the ratio of binding sites to transport protein is 0.82:1. This is likely to be an underestimate due to protein denaturation (Baldwin et al., 1981).

Reconstitution of Band 4.5 Protein. Band 4.5 protein was reconstituted into bilayers of large unilamellar vesicles (LUVs) by the method of reverse-phase evaporation reconstitution (Carruthers & Melchior, 1983b, 1984a,b). Cholesterol was added to dipalmitoyllecithin (DPL) bilayers by initial cocrystallization of DPL and cholesterol from organic solvent prior to reconstitution. Organic solvents present at this step were removed by drying the lipids overnight under vacuum (100 μmHg). Cholesterol–lecithin bilayers formed by initial cocrystallization of lipids have been demonstrated to be homogeneous in cholesterol content (Melchior et al., 1980). Band 4.5 protein was added to the gel-phase lipids (40–120 μg of protein/40 mg of lipid) following removal of Triton X-100 from the solubilized protein fraction by overnight exposure to Biobeads SM-2 (Bio-Rad, Richmond, CA) at 4 °C (0.6 g of beads/2 mL of protein solution). Addition of protein in NaCl medium induces LUV formation. These LUVs may be collected readily by centrifugation at 30000*g* for 10 min and used immediately in transport assays.

Transport Determinations. V_{max} for infinite-cis D-glucose entry (saturated influx) was obtained from the time course of equilibration of glucose-free LUVs with 100 mM external D-glucose in NaCl medium using the integrated Michaelis–Menten infinite-cis entry rate equation (Hankin et al., 1972; Baker & Naftalin, 1979; Carruthers & Melchior, 1983a, 1984a,b). The obtained K_m (10–18 mM) shows little dependence upon temperature (Carruthers & Melchior, 1984b). Over the range of temperatures covered in this study (0–70 °C), the concentration of D-glucose ensured 85–91% saturation of external transport sites. While complete saturation is, by definition, required for this type of analysis, control experiments in which 200 mM external D-glucose was used resulted in no significant differences between the obtained V_{max} values. Further conditions for rigorous analysis required that the orientation of the reconstituted transporter is that of the native system or, if not, that the kinetics of transport are intrinsically symmetric. In addition, the major component of transport must be mediated by the saturable carrier. Native transporter orientation is lost upon reconstitution (Baldwin et al., 1981; Wheeler & Hinkle, 1981; Carruthers & Melchior, 1984b), but transport in the native and reconstituted systems is intrinsically symmetric [Traverna & Langdon, 1976; Carruthers & Melchior, 1983a, 1984b; Carruthers, 1984; however, see Wheeler & Hinkle (1981)]. Reconstituted hexose transport was, under all conditions studied here, more than 98% inhibited by a combination of 0.1 mM phloretin and 50 μM cytochalasin B—specific competitive inhibitors of native red cell, saturable sugar transport (Carruthers, 1984). Sugar fluxes were monitored by using the computer-controlled digital turbidimetric stop-flow apparatus described previously (Carruthers & Melchior, 1983b, 1984a,b). This system monitors glucose transport induced LUV volume changes. Data analysis requires that the LUVs behave as perfect osmometers both below and above the melting of their bilayers, that turbidity is related directly to LUV volume, and that osmotic water flux is not rate limiting for the accurate determination of hexose flux. These conditions have been previously shown to be satisfied (Carruthers & Melchior, 1983b, 1984a,b) and were confirmed in this study. Additional requirements applying to all reconstituted systems are that the LUVs be homogeneous in both size and transport protein content. Reconstitution by reverse-phase evaporation produces LUVs of very narrow size distribution as judged by photon correlation spectroscopy (Carruthers & Melchior, 1984b) and produces LUVs with uniform transporter content (Carruthers & Melchior, 1984b).

Reconstituted LUVs containing NaCl medium were first preincubated in D-glucose-free NaCl medium (±0.1 mM phloretin and 50 μM cytochalasin B) at the temperature of interest (controlled to ±0.2%) and then injected (0.5–3 μL of LUVs, 6×10^6 to 4×10^7 LUVs) into 400 μL of NaCl medium (at the same temperature) containing 100 mM D-glucose (±phloretin and cytochalasin B). Mixing was complete within 100 ms and the time course of entry monitored at sample intervals of 50–10 000 ms.

Cytochalasin B Binding Assays. The concentration dependence of ^{3}H-cytochalasin B binding to reconstituted LUVs was determined as described previously (Carruthers & Melchior, 1984a,b). Over the range of cytochalasin B concentrations employed (0.1–10 μM), binding consisted of two components: a saturable, D-glucose-displaceable component and a nonsaturable component. This latter component consisted of association with LUV lipid (1 molecule of cytochalasin B per 7000 molecules of lipid), equilibration with the intravesicular space (depending on LUV size, this component accounted for 9–40% of nonspecific "binding"), and activity present in the extravesicular space (4–6%).

Transport Determinations with Human Red Cells. V_{max} for zero-trans D-glucose exit was obtained by integration of the time course of zero-trans D-glucose exit from red cells loaded with 60 mM D-glucose. The details of these procedures were as described previously (Carruthers & Melchior, 1983a).

Analytical Procedures. Triton X-100 assays were performed according to the method of Garewal (1973) with modification by Lukacovic et al. (1981). Protein assays were as described by Lowry et al. (1951) with modification by Yu & Steck (1975). Lipids were extracted from LUVs by the method of Bligh & Dyer (1959). Phospholipid phosphorus assays were

Table I: Reconstituted LUV Size, Transporter Content, and Transport Activity

mol % cholesterol	LUV diameter (μm)	polydispersity factor	sites/LUV	sites μm^{-2} bilayer	V_{max}/n^a
0	1.9	0.09	45	8	$(1.2 \pm 0.2) \times 10^{-19}$
	3.5	0.1	474	85	$(1.4 \pm 0.3) \times 10^{-19}$
	4.9	0.2	720	4	$(1.0 \pm 0.2) \times 10^{-19}$
	1.8	0.06	93	5	$(1.2 \pm 0.3) \times 10^{-19}$
10	3.8	0.2	189	2	$(1.4 \pm 0.2) \times 10^{-19}$
	4.7	0.16	621	5	$(1.7 \pm 0.4) \times 10^{-19}$
	1.7	0.1	98	5	$(1.5 \pm 0.3) \times 10^{-19}$
12.5	0.8	0.06	18	20	$(2.1 \pm 0.4) \times 10^{-21}$
	0.9	0.14	35	7	$(1.8 \pm 0.3) \times 10^{-21}$
	0.38	0.09	21	23	$(2.2 \pm 0.3) \times 10^{-21}$
	1.5	0.2	32	3	$(1.9 \pm 0.2) \times 10^{-21}$
15	7.8	0.2	9941	26	$(2.0 \pm 0.5) \times 10^{-20}$
	6.5	0.16	16570	63	$(2.5 \pm 0.6) \times 10^{-20}$
	6.1	0.21	5501	24	$(2.2 \pm 0.4) \times 10^{-20}$
17.5	0.62	0.11	59	25	$(3.4 \pm 0.7) \times 10^{-21}$
	3.9	0.07	189	2	$(3.1 \pm 0.6) \times 10^{-21}$
	2.1	0.09	218	8	$(3.9 \pm 0.8) \times 10^{-21}$
	1.7	0.15	374	21	$(2.2 \pm 0.4) \times 10^{-21}$
20	5.0	0.05	1310	8	$(1.2 \pm 0.2) \times 10^{-19}$
	4.6	0.12	998	8	$(0.8 \pm 0.1) \times 10^{-19}$
	4.9	0.11	1761	12	$(1.6 \pm 0.1) \times 10^{-19}$
25	1.8	0.12			
30	8.5	0.18	7338	45	$(1.1 \pm 0.2) \times 10^{-19}$
	0.4	0.04	46	46	$(1.4 \pm 0.3) \times 10^{-19}$
	1.3	0.09	921	84	$(1.6 \pm 0.3) \times 10^{-19}$
35	5.3	0.14			
40	6.9	0.05	5983	20	$(2.0 \pm 0.4) \times 10^{-20}$
	4.2	0.18	4712	42	$(2.4 \pm 0.5) \times 10^{-20}$
	3.2	0.2	1780	28	$(1.7 \pm 0.5) \times 10^{-20}$
45	3.8	0.16			
50	8.7	0.1	8457	18	$(3.1 \pm 0.6) \times 10^{-20}$
	0.9	0.17	47	9	$(2.8 \pm 0.5) \times 10^{-20}$
	0.4	0.11	18	18	$(2.5 \pm 0.5) \times 10^{-20}$
	0.8	0.05	8	2	$(3.6 \pm 0.7) \times 10^{-20}$

[a] Turnover number of the reconstituted transporter (moles of hexose per second). Measured at 45 ± 2 °C. Number of measurements per reconstitution, 4 or more. Data are shown as mean ± 1 SE.

as described by Bartlett (1959). Fatty acid analysis was as reported previously (Carruthers & Melchior, 1983b). Sodium dodecyl sulfate–polyacrylamide gel electrophoresis was carried out on 10% gels as described by Laemmli (1970). Differential scanning calorimetry (DSC) was carried out by using the Du Pont Instruments 1090 thermal analyzer. DSC pans were loaded with LUVs (0.3–1.1 mg of lipid) and scanned at 10 °C/min. The thermal profiles obtained were not significantly altered by slowing the scan rate. Integration of thermograms, determination of onset temperatures, and curve decompositions were carried out by using the Du Pont general analysis utility program (version 1.0). LUVs were sized by using photon correlation spectroscopy (Coulter N4 submicron particle analyzer; Coulter Electronics, Hialiah, FL). Pearson V distribution function analysis of correlation coefficients resulted in polydispersity factors of 0.2 or less, indicating substantially monodispersed samples (McCally & Bargeron, 1977). The intensity of detected, scattered light was greater at low detection angles, indicating that the LUVs behaved as large, spherical particles. Kinetic constants for saturable cytochalasin B binding to reconstituted LUVs were obtained by curve fitting to the Michaelis–Menten formalism. This was achieved by weighted nonlinear regression employing "robust" methods for the modification of residuals (Duggleby, 1981).

Calculation of Sugar Fluxes. V_{max} obtained by the above procedures has units of moles per liter intravesicular water per unit time. These units were converted to moles per square centimeter of LUV surface area per unit time by calculating the surface area of that quantity of LUVs whose total intravesicular water content is 1 L. This was obtained from LUV size and water content analyses (Carruthers & Melchior, 1984a,b). Knowing the density of reconstituted transporters (sites per unit of LUV surface area), V_{max} (moles per square centimeter per second) was then converted to T_n (turnover number, V_{max} per site) with units of moles per site per second.

Results

Physical Properties of Reconstituted Systems. Using the Coulter N4 submicron particle sizer multiangle detection option, we detected maximum light scattering by reconstituted LUVs at small angles, indicating that the LUVs behaved as large spherical particles. With lecithin LUVs, vesicle size is dependent upon the fatty acyl component of the bilayer phospholipid (Carruthers & Melchior, 1984a). Cholesterol, however, had no systematic effect on the size of reconstituted LUVs formed from DPL and band 4.5 protein. For a given batch of LUVs, size analysis indicated population uniformity, but when repeated on a new batch of LUVs of identical DPL–cholesterol composition, LUV size could vary by up to 22-fold (see Table I). The reasons for size inconsistencies between these LUV preparations are not currently known.

Contamination of our reconstituted systems by carry-through of red cell phospholipid was directly proportional to the amount of band 4.5 protein reconstituted into LUV bilayers. This varied from 0.1 to 0.7 mol %. A potential effect of this contamination might have been altered bilayer phase behavior. This was tested by including similar amounts of red cell lipids [approximately 0.5–0.8 mol %, extracted by the technique of Bligh & Dyer (1959)] into the exogenous lipids and using the resulting mixture to form protein-free LUVs. The inclusion of endogenous red cell membrane lipid into the bilayers of protein-free LUVs was without significant effect on the resulting thermograms. This indicated that the carry-through of red cell lipid during reconstitution did not no-

Table II[a]

DPL LUV cholesterol content (mol %)	μg of protein added/mg of lipid	reconstituted CCB binding (pmol/mg of lipid)	V_{max} entry (pmol cm^{-2} s^{-1})
0	0.75 (1)	9 (1)	258 ± 52 (1)
0	1.7 (2.3)	20.3 (2.2)	577 ± 133 (2.3 ± 0.4)
0	3.5 (4.7)	42.5 (4.8)	1204 ± 289 (4.6 ± 1.1)
15	0.77 (1.03)	9.2 (1.02)	27 ± 5
15	1.3 (1.7)	15.1 (1.6)	44 ± 8
15	2.8 (3.7)	32.9 (3.6)	102 ± 24
20	1.2 (1.6)	13.9 (1.5)	387 ± 74
20	2.1 (2.8)	24.7 (2.7)	707 ± 163
30	0.66 (0.88)	7.9 (0.86)	216 ± 56
30	1.5 (2)	17.7 (1.9)	477 ± 105
50	1.1 (1.5)	13.2 (1.4)	37 ± 7
50	2.5 (3.33)	28.9 (3.2)	87 ± 23

[a] The numbers in parentheses indicate the increase in both protein added and cytochalasin B binding sites reconstituted relative to 0% cholesterol (0.75 μg of band 4.5 protein/mg of lipid). Each reconstituted LUV preparation was sized, and in agreement with Table I, no two preparations shared the same mean diameter. Transport determinations were made at 50 °C and are shown as mean ± 1 SD. The numbers in parentheses in the V_{max} column represent the increase in transport activity brought about by increasing the amount of reconstituted protein. As protein turnover number is markedly sensitive to bilayer cholesterol content (see Figure 3), it is only feasible to present these data for cholesterol-free bilayers while maintaining consistency with the other columns. As expected for unilamellar vesicles, reconstituted activity (flux per unit LUV surface area) is independent of LUV size and directly proportional to the amount of reconstituted transport protein.

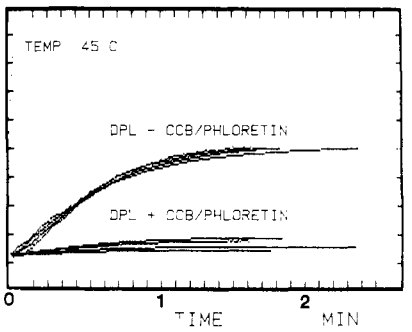

FIGURE 1: Effect of cytochalasin B (50 μM) and phloretin (100 μM) on infinite-cis D-glucose entry into reconstituted dipalmitoyllecithin (DPL) large unilamellar vesicles (LUVs). Ordinate: output of spectrophotometer in millivolts (FSD = 100 mV). Abscissa: time in minutes. Band 4.5 protein (40 μg) was reconstituted into LUVs formed from 40 mg of DPL. Mean LUV isotonic diameter, 2.1 ± 0.2 μm. Glucose-free LUVs were injected into NaCl medium containing 100 mM D-glucose ± 100 μM phloretin and 50 μM cytochalasin B. In the absence of transport inhibitors, the LUVs rapidly shrink due to osmotic water loss and then swell as glucose is transported into the intravesicular space. In the presence of inhibitors, the rate of transport-induced swelling is markedly reduced although the same final steady-state level of apparent absorption is achieved (this is a monoexponential process with a rate constant, k, of 0.009 min^{-1}; bilayer permeability coefficient for D-glucose flux = 9×10^{-9} cm·s^{-1}). Kinetic analysis of transport in the absence of inhibitors results in K_m = 14.2 ± 3.4 mM and V_{max} = 118 ± 15 mmol L^{-1} min^{-1} [i.e., permeability coefficient (V_{max}/K_m) = 1×10^{-5} cm·s^{-1}]. Hence, the presence of functional transport protein increases bilayer D-glucose permeability by 3 orders of magnitude. Temperature, 45 °C. Four digitized records are superimposed in each condition.

tably alter the bulk-phase behavior of the reconstituted LUVs. Another consequence of the presence of endogenous red cell lipids might also have been to "buffer" the transport protein from the bulk lipid of the LUV bilayers. This possibility was treated by enriching the purified protein preparation in dimyristoyllecithin [DML (C_{14}) bilayer melting temperature 22 °C; DML:red cell lipid molar ratio 4:1] prior to its reconstitution into higher melting distearoyllecithin [DSL (C_{18}) bilayer melting temperature 54 °C] bilayers (final DML molar content 2 mol %). Transport activity in these bilayers was indistinguishable from that observed in "pure" DSL bilayers. Further, we have previously shown that reconstituted activity is governed absolutely by the bulk lipid composition of the bilayer—activity increasing with increasing lecithin acyl chain length (Carruthers & Melchior, 1984b). These data indicate that the activity of the protein is governed by the bulk properties of the membrane and not by the properties of any physically and compositionally distinct hypothetical boundary or "annular" lipid. 99.8% of the Triton X-100 was removed from the solubilized band 4.5 preparation during exposure to Biobeads. The final Triton X-100:lipid molar ratio in the reconstituted systems ranged from 1:15000 to 1:12000. The number of reconstituted saturable cytochalasin B binding sites per LUV ranged from 8 to 17 000 (Table I). This wide distribution reflects the size ranges of the LUVs. When the saturable cytochalasin B binding sites per LUV are expressed as sites per square micrometer of LUV surface area, the range of reconstituted protein numbers is 2–85 sites/μm^2, representing a 40-fold variation in reconstituted protein numbers (Table I). The corresponding value for the human red cell is 2×10^3 sites/μm^2 (Carruthers, 1984). With LUVs of a given lipid composition, the number of reconstituted sites was directly proportional to the amount of protein added during reconstitution (Table II). Cholesterol appeared to have no systematic effect on the number of cytochalasin B binding proteins successfully reconstituted.

Band 4.5 protein mediated D-glucose flux is a saturable process inhibited competitively by low concentrations of cytochalasin B or phloretin (Kasahara & Hinkle, 1977; Wheeler & Hinkle, 1981; Carruthers & Melchior, 1984a,b). In this study, infinite-cis D-glucose entry (influx from medium containing saturating D-glucose levels) was almost completely inhibited (98–99%) by a combination of 100 μM phloretin and 50 μM cytochalasin B (Figure 1). This confirmed that the transmembrane hexose fluxes reported here were mediated by the saturable transfer process and not by passive leakage across the lipid phase of the bilayer or via the lipid/protein interface [e.g., see Carruthers & Melchior (1983b)].

Thermotropic Behavior of Reconstituted LUVs. Figure 2 summarizes the phase behavior of the various reconstituted LUV systems employed in this study. LUVs formed from DPL and band 4.5 preparation (0 mol % cholesterol) show a characteristic pretransition of low enthalpy (0.6 J/g of DPL) beginning at 28 °C and complete at 32.2 °C. This is followed by the main transition with an onset temperature (T_m) of 40.8 °C, a completion temperature (T_f) of 44 °C, and a heat of transition of 42 J/g of DPL. Increasing bilayer cholesterol to 10 or 12.5 mol % abolished the pretransition but was without significant effect on the T_m, T_f, and heat of the main transition. The narrow, main DPL transition was detected at all cholesterol levels studied except 15 and 20–35 mol % cholesterol where most likely it was obscured by the newly emergent cholesterotropic endotherms. At 50 mol % cholesterol, no thermal events were seen. At 15 mol % cholesterol, a broad endotherm, suggestive of a lipid complex distinct from pure DPL, was first detected as reduced T_m and increased T_f. This endotherm disappeared at 17.5 mol % cholesterol and was followed by a much broader endotherm appearing at cholesterol concentrations of 20–35 mol %. The total heat of transition was reduced at 15 and 17.5 mol % cholesterol. At 20–35 mol % cholesterol, the heat of the endotherm was indistinguishable from that of 0 mol % cholesterol. Thereafter, the heat fell markedly.

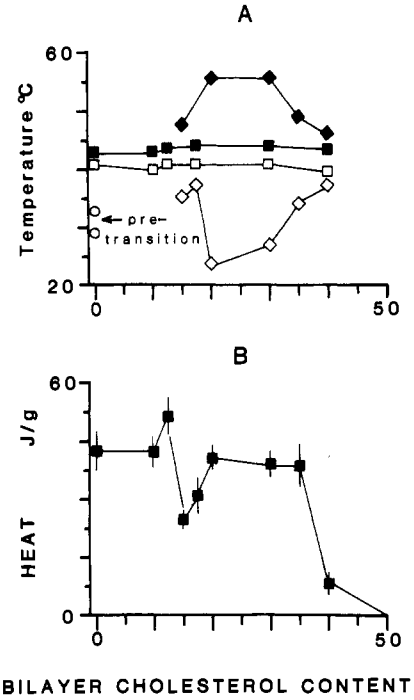

FIGURE 2: (A) Effects of cholesterol on the phase behavior of reconstituted DPL LUVs. Ordinate: temperature in degrees centigrade. Abscissa: bilayer cholesterol content (mole percent). The open squares and open diamonds indicate the onset melting temperatures (T_m) of the narrow and broad endotherms, respectively, detected by DSC. The closed squares and closed diamonds indicate the temperatures (T_f) at which these melts are complete. The open circles show T_m and T_f of the low-heat endotherm (pretransition) that typically precedes the main transition in DPL bilayers. A broad endotherm was first detected at 15 mol % cholesterol. No thermal events were detected at 50 mol % cholesterol. (B) Effects of increasing DPL LUV cholesterol content on the heat of bilayer transition. Ordinate: heat of transition in joules per gram of lipid. Abscissa: bilayer cholesterol content (mole percent). Integration and curve decompositions of DSC thermograms were carried out by using the Du Pont general analysis utility program version 1.0. The heats plotted are total heats.

D-Glucose Transport Properties of Reconstituted LUVs. Figure 3 summarizes the temperature dependence of reconstituted cytochalasin B binding protein D-glucose translocase activity in DPL LUVs containing 0–50 mol % cholesterol. This diagram illustrates how transporter turnover number, T_n, varied with temperature and bilayer cholesterol content. Here, turnover number has units of moles per second and represents the hexose flux catalyzed by a single transporter molecule under the experimental conditions of this study. T_n is given by V_{max}/n where n is the molar concentration of cytochalasin B binding sites per unit of LUV surface area and V_{max} is saturated transbilayer hexose flux expressed as molar flux per unit area of LUV (Lieb, 1982; Taverna & Langdon, 1976). These calculations require that the LUVs are spherical and that each cytochalasin B binding site corresponds to a single functional transport unit. We have shown above that LUVs behave as large, spherical particles, that reconstituted transport activity (V_{max}) is directly proportional to the number of cytochalasin B binding proteins reconstituted (Table II), and that the minimum estimate of the molar ratio of cytochalasin B binding sites per band 4.5 protein is 0.8 [see Materials and Methods, Carruthers & Melchior (1984b), and Baldwin et al. (1982)]. These findings directly support our calculations.

Where transport activity was measurable, T_n varied from 3.6×10^{-19} mol/s (in DPL bilayers at about 60 °C) to (3–6) $\times 10^{-22}$ mol/s (in DPL LUVs containing 12.5 mol % cholesterol at 19 °C). Over the same range of temperature, T_n in native membranes is 5.7×10^{-22} mol/s at 20 °C and 9.8×10^{-21} mol/s at 45 °C, assuming 3×10^5 cytochalasin B binding sites per human red cell (Jones & Nickson, 1981; see Figure 3). Assuming that the native transporter is not denatured at 60 °C and that the temperature dependence of activity is a monoexponential function over this range [activation energy E_a =18.5 kcal mol^{-1} K^{-1}; see also Hankin & Stein (1972)], T_n at 60 °C would be 3.7×10^{-20} mol/s. The available data fall into three groups classified by the cholesterol content of the reconstituted LUV bilayers. These groups are I (0, 10, and 20 mol % cholesterol), II (15, 40, and 50 mol % cholesterol) and III (12.5 and 17.5 mol % cholesterol). Reconstituted LUVs containing 30 mol % cholesterol are of interest for at low temperatures (<37 °C), T_n values are consistent with group II behavior and at higher temperatures are consistent with group I behavior. With all reconstituted LUVs, transport activity was irreversibly inhibited at temperatures between 60 and 70 °C.

Figure 4 shows the cholesterol dependence of V_{max} per cytochalasin B binding site at 20 and 45 °C. Included are red cell native transport data [the cholesterol content of human red cell membranes is 42 ± 3 mol % (Carruthers & Melchior, 1983b)]. At 20 °C, pure DPL LUV bilayers are in the "crystalline" state (Melchior & Steim, 1979) and support no detectable reconstituted transport activity. This lack of activity seems not to arise from inaccessibility of the substrate binding site to substrate, for D-glucose (50 mM) displaces cytochalasin B from band 4.5 protein reconstituted into these membranes with an apparent K_i of 10.5 ± 1.3 mM.[2] Activity in cholesterol-free DPL LUVs was first detected at 31 °C where the bilayer pretransition is almost complete. Thereafter, activity increased markedly with temperature until 33.6 °C. Beyond this temperature, activity showed a simple logarithmic dependence on temperature. This is surprising for between 41 and 45 °C, the LUV bilayers undergo a major transition from the liquid-crystalline to the fluid state. This marked sensitivity of reconstituted transport activity in DPL bilayers to the bilayer pretransition has been described previously distinguishes reconstituted DPL LUVs from all other reconstituted lecithin LUVs studied (Carruthers & Melchior, 1984b).

Effect of Cholesterol on Transport Activity Reconstituted into DSL Bilayers. The activity of the reconstituted transporter in distearoyllecithin (DSL; acyl chain carbon number, 18) LUVs differs strikingly from its behavior in DPL (acyl chain carbon number, 16) LUVs. Unlike reconstituted DPL LUVs, in DSL LUVs substantial activity exists at temperatures well below (more than 40 °C below) the liquid-crystalline to fluid phase transition temperature [Figure 5; see also

[2] The K_i for D-glucose displacement of cytochalasin B from reconstituted DPL LUVs was determined by analysis of cytochalasin B binding in the presence and absence of 50 mM intravesicular D-glucose in addition to the presence and absence of 1000 mM D-glucose (in both the extra- and intravesicular space). In the absence of the additional presence of 50 mM intravesicular D-glucose, the concentration dependence of that component of cytochalasin B binding displaced by 1 M D-glucose was well approximated by a section of a single rectangular hyperbola with an apparent K_d of 0.4 ± 0.1 μM and a B_{max} of 13.2 fmol/nmol of DPL. In the additional presence of 50 mM intravesicular D-glucose, the K_d for binding increased to 1.0 ± 0.1 μM with B_{max} unchanged. Assuming a random protein insertion pattern upon reconstitution (Carruthers, 1984) and that intravesicular D-glucose acts as a competitive inhibitor of binding to those sites facing the interior of the LUV alone (Yu & Steck, 1975), these data are consistent with two components of binding: site 1 (external site accessible only to cytochalasin B), K_d = 0.4 ± 0.1 μM and B_{max} = 6.6 fmol/nmol of DPL; site 2 (internal site accessible to both D-glucose and cytochalasin B), K_d' = 2.3 ± 0.3 μM and B_{max} = 6.6 fmol/nmol of DPL. The K_i for inhibition of binding by D-glucose is therefore given by K_i = [intravesicular D-glucose]/(K_d'/K_d − 1) = 10.5 ± 1.3 mM.

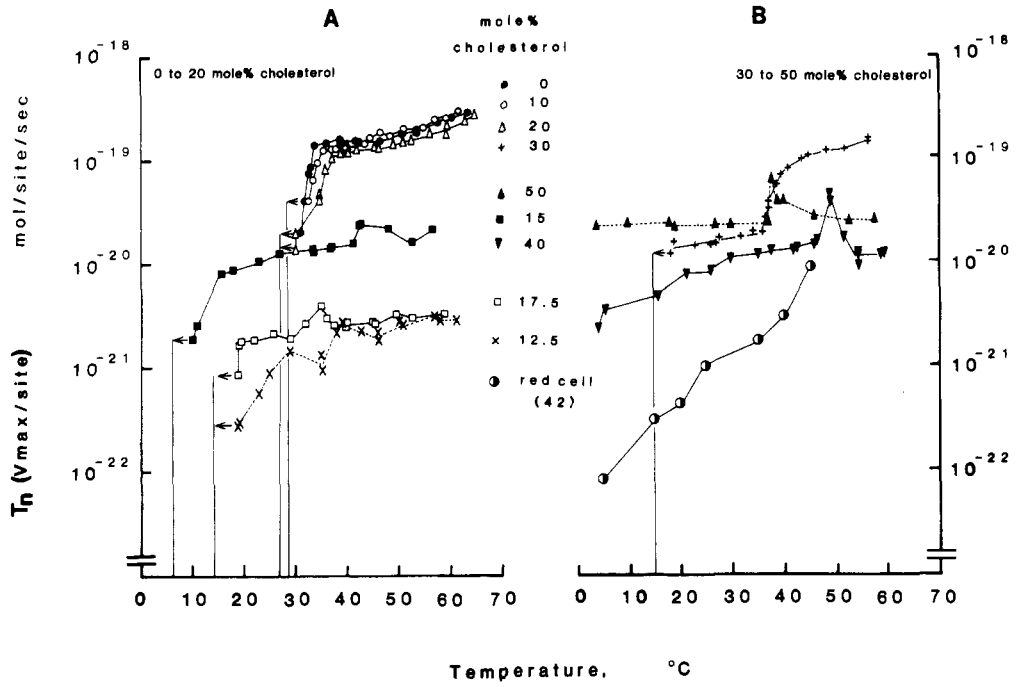

FIGURE 3: Effect of bilayer cholesterol content on the turnover number (T_n) of the transport protein reconstituted into DPL LUVs. Ordinate: T_n in moles of D-glucose per transporter per second. Abscissa: temperature in degrees centigrade. V_{max} (mol cm^{-2} s^{-1}) for infinite-cis D-glucose entry into the various LUVs was obtained as a function of temperature and the number of reconstituted cytochalasin B binding proteins, n (mol/cm^2 of bilayer), determined in parallel cytochalasin B binding assays ($T_n = V_{max}/n$). The data are divided into two graphs for clarity of presentation. Figure 3A shows data from DPL LUVs containing 0–20 mol % cholesterol. Figure 3B shows data from DPL LUVs containing 30–50 mol % cholesterol. The cholesterol content of the various bilayers is shown in the center of the graph. Number of reconstitutions per condition, 3 or more. Number of determinations per point per reconstitution, 5 or more. Variation between reconstitutions was in almost all cases less than 25% (see Table I). Human red cell data are also included for comparison (◑).

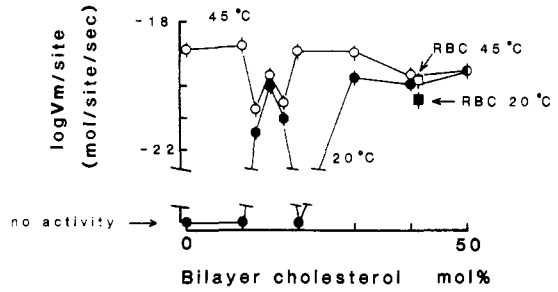

FIGURE 4: Effect of cholesterol on reconstituted transport activity in DPL bilayers. Ordinate: log V_{max} per reconstituted cytochalasin B binding protein in moles per site per second. Abscissa: bilayer cholesterol content (mol percent). The upper curve shows the cholesterol dependence of activity at 45 °C, where pure DPL bilayers are in the fully fluid state. The lower curve shows activity at 20 °C, where pure DPL bilayers are in the crystalline state. Human red cell data (bilayer cholesterol content, 42 mol %) are shown by the squares. Number of reconstitutions per condition, 3 or more. Number of determinations per point per reconstitution, 4 or more. Data are shown as mean ± 1 SE.

Carruthers & Melchior (1984b)]. As expected (Melchior & Czech, 1979), cholesterol (40 mol %) was found to allow sugar transport to proceed in crystalline DPL LUVs. It was of interest, therefore, to determine how transport in crystalline DSL bilayers (bilayers supporting high activity) would respond to 40 mol % bilayer cholesterol. Figure 5 shows that cholesterol (40 mol %) reduced the temperature dependence of transport in DSL LUVs (control activation energy E_a =16.2 ± 2.8 kcal mol^{-1} K^{-1}; E_a in the presence of 40 mol % cholesterol = 3.4 ± 1.6 kcal mol^{-1} K^{-1}). The effects of cholesterol on absolute activity in reconstituted LUVs were, however, more complex. At temperatures below 29 °C, cholesterol produced a marginal stimulation of reconstituted activity (V_{max}/site) of up to 2-fold. At higher temperatures (greater than 20 °C but less than 54 °C) where pure DSL bilayers are in the crystalline state, cholesterol inhibited reconstituted activity by up to 5-fold. As expected, activity in fluid DSL bilayers (temperature >54 °C) was inhibited by the introduction of cholesterol.

Effect of Cholesterol on K_m for Infinite-Cis Entry in DPL LUVs. Over the range of cholesterol levels employed, the obtained Michaelis constants for infinite-cis entry (i.e., K_m for infinite-trans exit) were within the range 9.8–17.6 mM. K_m was not systematically affected by LUV bilayer cholesterol content, and as previously reported for both native and reconstituted systems (Carruthers & Melchior, 1984b), K_m increased only slightly with increasing temperature (Figure 6).

Discussion

Our DSC and transport assays support recent biophysical studies indicating that cholesterol induces significant bilayer reorganization at concentrations of 15–20 and 30 mol % (Carruthers & Melchior, 1983b; Melchior et al., 1980; Mabrey et al., 1978; Estep et al., 1978; Rubenstein et al., 1979) and run counter to the view that increasing membrane cholesterol content monotonically transforms the bilayer to a state of intermediate fluidity (Yuli et al., 1981). Further, our findings demonstrate that cholesterol-induced bilayer reorganizations significantly modify the activity of the human erythrocyte hexose transport protein.

Current understanding of the influence of membrane lipid on integral membrane protein function largely derives from studies of biological membranes. In the case of protein-mediated membrane transport, the results of a number of studies have been integrated to form a working hypothesis of control of protein function by membrane fluidity [e.g., see Kotyk & Janacek (1969), Thilo et al. (1977), and Yuli et al. (1981)]. The term fluidity is an abstraction, summarizing interpretations of various spectroscopic studies on membranes. These

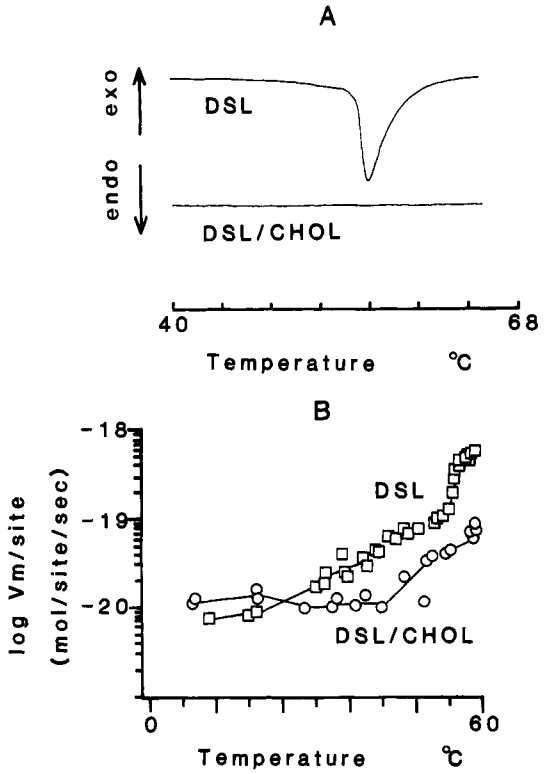

FIGURE 5: Effect of cholesterol on transport activity reconstituted into distearoyllecithin (DSL) LUVs. (A) DSC thermograms of DSL and DSL–cholesterol (60:40 molar ratio) reconstituted systems. Ordinate: heat flow. Abscissa: temperature in degrees centigrade. The upper curve indicates that DSL bilayers begin to melt at about 54 °C. This phase transition is abolished by the inclusion of 40 mol % cholesterol in DSL bilayers. (B) Ordinate: log V_{max} per reconstituted transport protein (moles per site per second). Abscissa: temperature in degrees centigrade. Reconstituted activity in DSL (□) and DSL–cholesterol (60:40 molar ratio, ○) is plotted as a function of temperature. The curvature in DSL data at high temperatures (onset = 54 °C) is the result of the bilayers undergoing the liquid-crystalline to fluid phase transition (see below). Number of determinations per point, 3–8.

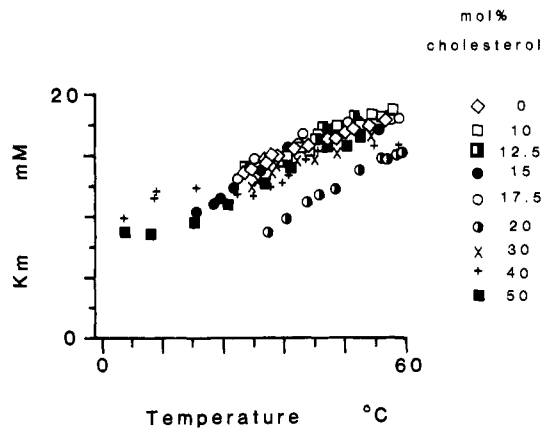

FIGURE 6: Effect of bilayer cholesterol content on K_m for infinite-cis D-glucose entry in reconstituted DPL LUVs. Ordinate: K_m in millimolar. Abscissa: temperature in degrees centigrade. Each point represents the mean of at least five separate determinations per reconstitution. The bilayer cholesterol content is indicated on the graph. K_m increases only slightly with temperature. van't Hoff plots of these data indicate that only 20 mol % cholesterol differs significantly in its effect on the enthalpy (ΔH) of transport (mean ΔH = 3 kcal mol^{-1} K^{-1}; 20 mol % cholesterol, ΔH = 5 cal mol^{-1} K^{-1}).

techniques sense the rate of motion of lipid molecules and their fatty acyl chains in the bilayer and the order of the bilayer (packing properties of the lipids; Stubbs, 1983). As the motion of the lipids increases and the bilayer expands, membrane fluidity is considered to increase. The fluidity hypothesis considers transport to be mediated by proteins undergoing significant conformational and positional change during transport—steps postulated to be directly dependent upon the fluidity of the bilayer. Agents that increase membrane fluidity are thereby assumed to stimulate transport.

This hypothesis is consistent with in vivo studies such as those of Thilo et al. (1977) which clearly demonstrated that the activity of the proton-coupled lactose permease of *Escherichia coli* increases as its resident membrane undergoes a liquid-crystalline to fluid phase transition. Studies such as these, however, cannot be simply interpreted as demonstrating the importance of membrane fluidity in governing a membrane enzyme's activity (as indeed they were not by Thilo et al.). The compositional heterogeneity and structural complexity of native membranes do not allow a molecular interpretation of existing data in terms of simply fluidity, specific lipid–protein interactions, or specific alterations of the bilayer in the immediate environment of a membrane enzyme. Indeed, as a membrane of heterogeneous lipid composition melts, its crystalline and fluid regions are continuously altered in their lipid composition. At the low-temperature end of the transition, fluid regions of the membrane are enriched in low melting point lipids while crystalline regions are enriched in high melting point lipids (Melchior & Steim, 1979). Membrane proteins have been shown to partition unequally between the fluid and crystalline areas of a membrane (van Herrikhuizen et al., 1975). Thus, during the course of a membrane melt, membrane proteins are surrounded by a lipid environment not only of changing fluidity but also of changing lipid composition. The problem of interpreting membrane enzyme activity and "membrane fluidity" data from heterogeneous membranes is illustrated experimentally by the dissonant findings of numerous studies using different bilayer fluidity reporter molecules on various membrane systems [for reviews, see Stubbs (1983) and Stubbs & Smith (1984)].

The importance of bilayer fluidity in controlling membrane enzyme activity has been recently rigorously questioned experimentally (East et al., 1984; Carruthers & Melchior, 1984b). Reconstituting the sarcoplasmic reticulum Ca^{2+}–Mg^{2+}-dependent ATPase into a variety of lipid bilayers, East et al. (1984) found no consistent relationship between bilayer fluidity and ATPase activity. Rather, they concluded that the activity of the enzyme may be directly related to bilayer lipid acyl chain length. Studies on the activity of the reconstituted human red cell sugar transport protein also came to the same conclusion (Carruthers & Melchior, 1984b). V_{max} per reconstituted hexose transport protein was found to be dependent upon the acyl chain carbon number of the bilayer lecithin. With short-chain disaturated lecithins (dimyristoyllecithin, C_{14}; DPL, C_{16}), activity was absent at temperatures where the bilayers were in the crystalline state but appeared in DML bilayers as the bilayers melted and in DPL bilayers during the pretransition (induced by increasing temperature). With the longer chain lecithins (DSL, C_{18}; DAL, C_{20}), activity was present in crystalline bilayers and, with DSL, increased during the bilayer phase transition. The most important observation of these studies, however, was that V_{max} per reconstituted transporter in crystalline DSL bilayers at 10 °C was indistinguishable from that supported by fluid DML bilayers and crystalline DAL bilayers at 60 °C. Moreover, the activity in crystalline DSL bilayers was greater at all temperatures than that supported by fully fluid dioleoyllecithin (DOL, C_{18}, a cis diunsaturate) membranes. Thus, while activity increases within a given bilayer during the course of its melt, absolute

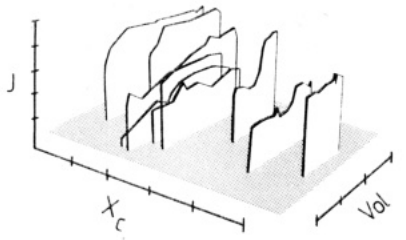

FIGURE 7: Relationship between bilayer cholesterol content, bilayer apparent partial specific volume, and V_{max} per site. This is a three-dimensional representation of these data. The axes are the following: x, bilayer cholesterol content in mole percent (X_c) (range = 0–50 mol %); z, V_{max} per site (J) [log (mol site^{-1} s^{-1}), range = −23 to −18]; y, bilayer apparent partial specific volume in milliliters per gram of lipid (range = 0.92–1.01). y axis data were obtained from the scanning dilatometry studies of DPL–cholesterol bilayers by Melchior et al. (1980).

activity is dependent upon the acyl chain length and the saturation–unsaturation of the bilayer lipid. This compositional dependence is apparently independent of the effects of lipid composition on membrane fluidity.

The results described in the present study confirm and extend our previous findings (Carruthers & Melchior, 1984b). The effects of cholesterol (40 mol %) activity reconstituted into DSL LUVs provide additional evidence against the view that bilayer fluidity is the sole or major determinant of transport protein activity. While cholesterol suppresses the liquid-crystalline to fluid phase transition of DSL LUVs [a finding consistent with cholesterol's ability to act as a membrane plasticizer (Melchior, 1982; Presti et al., 1982)] at temperatures where DSL bilayers are in the crystalline state, cholesterol *inhibits* the activity of the reconstituted transporter. Our findings, therefore, support the view that the effect of cholesterol on transporter activity in DSL bilayers is independent of its ability to fluidize crystalline bilayers.

If cholesterol influences transport activity reconstituted into DPL LUVs by altering bilayer order (lipid packing), then activity should be related to bilayer volume—a parameter dependent upon lipid packing. Figure 7 illustrates the dependence of V_{max} per site and bilayer apparent partial specific volume upon bilayer cholesterol content. The volume data are those determined by differential scanning dilatometry (Melchior et al., 1980). The salient feature of this analysis is the lack of any consistent relationship between bilayer volume and V_{max} per site. Rather, activity appears to be determined by bilayer cholesterol content. Assuming homogeneity in LUV cholesterol content, these findings rule out the simplest possibility—that protein activity is dependent upon a parameter related to bilayer packing per se. This assumption seems valid, for thermograms of LUVs differing in cholesterol content display the distinctive behavior observed with multilamellar vesicles of homogeneous cholesterol content (Melchior et al., 1980; Mabrey et al., 1978), except for the broader transitions and lower onset temperatures characteristic of LUVs (Carruthers & Melchior, 1983b; Duzgünes et al., 1983). It is quite significant, however, that those concentrations of cholesterol that induce altered bilayer molecular organization markedly alter transport protein activity.

It is not yet possible to interpret these transport findings in terms of specific molecular mechanisms. At a phenomenological level, however, we can conclude that both bilayer physical state and bulk bilayer composition influence the activity of the red cell sugar transport protein. Moreover, bulk bilayer composition appears to establish the basal properties of the transport molecule which may then be additionally modified by thermotropic or other types of physically induced alterations in bilayer state.

Registry No. DPL, 2644-64-6; DSL, 4539-70-2; D-glucose, 50-99-7; cholesterol, 57-88-5; cytochalasin B, 14930-96-2.

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Modulation of (Na^+,K^+)-ATPase Activity by the Lipid Bilayer Examined with Dansylated Phosphatidylserine†

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ABSTRACT: The fluorescent probe 8-(dimethylamino)naphthalene-1-sulfonylphosphatidylserine (Dns-PS) was incorporated into purified lamb kidney Na^+- and K^+-stimulated adenosinetriphosphatase (EC 3.6.1.3) [(Na^+,K^+)-ATPase] by using a purified phospholipid exchange protein. Phospholipase C was used to reduce phospholipid content. Up to 40% of the phospholipid could be hydrolyzed with only 10% inhibition of the (Na^+,K^+)-ATPase, but when 67% of the phospholipid was hydrolyzed, the enzyme was inhibited 53%. To examine the effect of protein on the phospholipid bilayer, the fluorescent parameters of the probe incorporated into the enzyme preparation were contrasted with the same parameters for the probe incorporated into the total lipid extract of the preparation. The polarization of fluorescence of the probe in the lipid extract was 0.118 while in the enzyme preparation it was 0.218. This reflected a decrease in fluidity of the glycerol region of the phospholipid bilayer which was mediated by the protein. This effect increased as the phospholipid content of the (Na^+,K^+)-ATPase preparation was reduced so that with maximal phospholipid reduction the polarization of fluorescence was 0.262. The protein caused a decrease in the transition temperature from gel to fluid states of the bilayer detected by polarization of the probe. The midpoint temperature transition of the enzyme preparation decreased from 33 °C when all phospholipids were present to 20 °C when 67% of the phospholipids were hydrolyzed. This decrease was not observed for the lipid extract of these samples. A direct correlation between the (Na^+,K^+)-ATPase specific activity and the polarization of fluorescence of Dns-PS was found. The reduction in phospholipid content did not affect the steady-state level of phosphorylation of the enzyme by ATP but did affect the rate of dephosphorylation which would require conformational changes of the enzymes. The data showed that the fluidity of the phospholipid bilayer can modulate the activity of the (Na^+,K^+)-ATPase.

The Na^+- and K^+-stimulated adenosinetriphosphatase, (Na^+,K^+)-ATPase[1] (ATP phosphohydrolase, EC 3.6.1.3), is an intrinsic membrane enzyme that couples the hydrolysis of ATP to the active transport of Na^+ and K^+ across biological membranes. This enzyme requires the presence of phospholipids for full activity since their removal will cause partial to complete loss of activity (Kimelberg & Papahadjopoulos, 1972; Jorgenson, 1982; Stahl, 1973a; Simpkin & Hokin, 1973). Partial recovery of activity has been achieved by reintroduction of phospholipids, but the results do not seem to clearly support a requirement for a specific phospholipid class (Jorgenson, 1982; Hokin & Hexum, 1972; Giraud et al., 1981; Ottolenghi, 1979).

Brotherus et al. (1980) suggested that negatively charged phospholipids preferentially associated with the (Na^+,K^+)-ATPase, on the basis of an electron spin resonance (ESR) study with spin-labeled phospholipids. In contrast, DePont et al. (1978), employing specific lipases, enzymatically converted all the phosphatidylserine in a (Na^+,K^+)-ATPase preparation to phosphatidylethanolamine with essentially no effect on (Na^+,K^+)-ATPase activity. These authors also enzymatically hydrolyzed all the phosphatidylinositol in the (Na^+,K^+)-ATPase sample, again without an effect on the enzyme activity. Hilden & Hokin (1976), using excess ex-

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[1] Abbreviations: (Na^+,K^+)-ATPase, Na^+- and K^+-stimulated adenosinetriphosphatase (EC 3.6.1.3); Dns-PS, 8-(dimethylamino)-naphthalene-1-sulfonylphosphatidylserine; PLEP, phospholipid exchange protein; PC, phosphatidylcholine; PS, phosphatidylserine; PI, phosphatidylinositol; PE, phosphatidylethanolamine; SM, sphingomyelin; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; Tris, tris(hydroxymethyl)aminomethane (Trizma base); SET, 250 mM sucrose, 50 mM Tris-HCl, and 1 mM ethylenediaminetetraacetic acid buffer, pH 7.4; BSA, bovine serum albumin; Me_2SO, dimethyl sulfoxide; T_m, midpoint temperature transition; PLP, phospholipid phosphorus; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; CDTA, *trans*-1,2-diaminocyclohexane-*N*,*N*,*N*′,*N*′-tetraacetic acid; ESR, electron spin resonance; Cl_3CCOOH, trichloroacetic acid.

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