Human Erythrocyte Hexose Transporter Activity Is Governed by Bilayer Lipid Composition in Reconstituted Vesicles[†]

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ABSTRACT: Purified membrane protein band 4.5 (a sugar transport protein) from human erythrocytes was reconstituted by reverse-phase evaporation into a variety of bilayers formed from the synthetic lecithins. The number of proteins reconstituted was estimated by determination of D-glucose-sensitive cytochalasin B binding sites. D-Glucose transport activity in reconstituted vesicles was assessed by monitoring cytochalasin B sensitive D-glucose fluxes using microturbidimetric analysis. The major points are as follows: (1) The sugar transport activity of the reconstituted system is directly proportional to the number of cytochalasin B binding sites reconstituted. The ratio of cytochalasin B binding sites per band 4.5 protein is 0.8. These data suggest that the functional protein unit is a monomer. (2) Inhibitor studies of reconstituted hexose transfer support the notion that the kinetics of reconstituted transport are intrinsically symmetric. (3) The turnover number for transport is not consistent with transport proceeding via water-filled pores. (4) The absolute activity of the reconstituted system (V_{max} per reconstituted cytochalasin B binding protein) is governed by the bulk lipid composition of the synthetic membrane. (5) At temperatures where bilayers formed from dimyristoyl- or dipalmitoyllecithin (DML and DPL, respectively) are "crystalline", hexose transport activity is not observed. Over the same temperature range, however, crystalline bilayers formed from the longer chain lecithins, distearoyl-, diarachidonoyl-, and dielaidoyllecithin (DSL, DAL, and DEL, respectively), support significant protein-mediated transport activity. (6) In a given synthetic membrane, the bilayer transition from the liquid-crystalline to the fluid state results in increased protein-mediated sugar transport activity. In the one synthetic membrane (DEL) in which the activation energy (E_a) for transport could be measured both above and below the bilayer phase transition, E_a was unaffected by the phase change. (7) E_a and the Arrhenius constant (A) for transport are dependent on lecithin acyl chain length and saturation. For both parameters, the order of increase is DML < DPL = dipalmitoleoyllecithin (DPOL) < DSL < DAL < DEL = dioleoyllecithin (DOL). This means that at 59–60 °C, the order of catalytic activity follows the lipid sequence DML < DPOL < DAL < DOL ≪ DPL = DEL < DSL. (8) Cholesterol (48 mol %) restores protein-mediated transport activity to crystalline DPL bilayers and reduces the activity supported by fluid DPL bilayers. This effect is not simply related to the effects of cholesterol on the bilayer partial specific volume. (9) The intuitive parameter "bilayer fluidity" appears to be a relatively unimportant determinant of protein-mediated hexose flux.

Among the integral membrane proteins, many serve as vectorial enzymes catalyzing the transbilayer flux of specific molecules. Kinetic analysis of protein-mediated transport indicates two general classes of transport systems. The pore or channel mechanisms describe substrate flux via presumed membrane-spanning hydrophilic channels. The carrier-type mechanisms describe flux via proteins that undergo conformational or positional change during transport (Lieb & Stein, 1974; Stein & Lieb, 1974). These kinetic schema, for the most part, employ conceptual changes in the protein state, not necessarily the actual physical transformations of the protein(s); hence, their utility in describing the molecular events underlying protein-mediated transport is limited. Such events might be studied more directly by employing physical techniques that address the molecular requirements of both the native and reconstituted transport systems.

Reconstituting transport systems into bilayers in which variables such as lipid composition and physical state are strictly controlled is an approach that should permit a more complete understanding of both the intrinsic and extrinsic molecular factors involved in protein-mediated transport. At the same time, this approach allows a direct investigation of how the different membrane lipids and their physical states influence transport processes. For most biomembranes, bilayer lipid may be considered a solvent of integral membrane proteins. By analogy with water-soluble enzymes, it would be expected that bilayer protein activity is governed in part by various parameters of its solvent (the bilayer lipid).

The membrane transport system chosen for study was the human erythrocyte hexose transporter. This system is particularly well suited to these studies for the following reasons: (1) The transport system is passive and thus is uncomplicated by additional reactions typical of active transport systems such as ATP hydrolysis or coupled cation transport. (2) The kinetics of the transport system are well-defined. (3) The lipid composition and organization of the red cell membrane have been extensively studied. (4) The transporters are abundant in the red cell membrane and may be readily purified without the additional complications of preexposure of tissue to enzymes or contamination by proteins from intracellular organelles. (5) The red blood cell is an ideal simple system for studying lipid effects on transport for there is no de novo synthesis of transporter nor any possibility of recruitment of transporter from cytosolic sites. Two red cell membrane proteins (band 4.5 and a component of band 3) appear to catalyze the saturable, selective transbilayer flux of D-glucose in synthetic membranes (Kasahara & Hinkle, 1977; Jones & Nickson, 1981; Shelton & Langdon, 1983; Carruthers & Melchior, 1984). Of these, band 4.5 proteins offer advantages of ease of purification and direct quantitation (Kasahara & Hinkle, 1977; Baldwin et al., 1980). Previous studies on the rat adipocyte hexose transport system reconstituted into syn-

thetic membranes indicated that hexose transport activity was most obvious in fluid bilayers and that the minimal activity observed in crystalline bilayers was enhanced by the presence of cholesterol (Melchior & Czech, 1979). This was a preliminary investigation, however, in which unfractionated membrane proteins were reconstituted with the kinetic analysis of transport restricted to flux determinations at a single sugar concentration.

Here we report the results of full kinetic studies with purified, reconstituted, human red cell band 4.5 protein. We show that reconstituted sugar transport activity (V_{max}) in lecithin bilayers is to a large extent determined by the bilayer, being systematically altered by bilayer physical state, lipid acyl chain length and unsaturation, and bilayer cholesterol content. Interestingly, the intuitive parameter "bilayer fluidity" appears to be a relatively unimportant determinant of protein-mediated hexose flux. Our findings suggest that the human red blood cell sugar transport protein may be of use in probing the microorganization of membranes.

Materials and Methods

Our approach was the following. The purified cytochalasin B binding component of the human erythrocyte hexose transport system was reconstituted into large unilamellar vesicles (LUVs)[1] by reverse-phase evaporation. The number of proteins reconstituted per nanomole of phospholipid phosphorus was determined by kinetic analysis of D-glucose-sensitive cytochalasin B binding to the LUVs. Contamination of the LUVs by carry through of endogenous (red cell) lipid was determined by fatty acid analysis of protein-free and protein-containing LUVs. The kinetic parameters for zero-trans D-glucose efflux from the reconstituted system were obtained by integration of the time course of sugar flux measured using microtubidimetric analysis. V_{max} for efflux was obtained over a range of temperatures for each system and the physical state of the LUV bilayers determined by differential scanning calorimetry (DSC).

Preparation of Red Cell Ghosts. Human erythrocyte ghosts were prepared from outdated blood bank whole blood as described previously (Carruthers & Melchior, 1983a).

Membrane Protein Solubilization and Purification. Red cell ghosts were incubated in 25 mM NaCl, 5 mM Tris-HCl, 0.2 mM EDTA, and 0.5% Triton X-100, pH 8, for 30 min at 4 °C (1 mL of ghosts per 5 mL of buffer). The resulting suspension was centrifuged at 45000g for 30 min and the supernatant collected and stored on ice. This initial purification procedure solubilizes red cell membrane protein bands 3, 4.5, and 6 and a small quantity of band 7 (50% of total membrane protein) and 95% of the lipid (Yu et al., 1973). The pellet formed from the remaining proteins (bands 1, 2, 4.1, 4.2, 5, and 7 and 25% of total band 3, 4.5, and 6 proteins) and lipid was washed once more with the same solution followed by centrifugation. The combined supernatants were concentrated by ultrafiltration (Diaflo YM10 membranes) and stored at -70 °C.

Band 4.5 proteins were separated from band 3 and 6 proteins by application of the membrane extract to a DEAE-cellulose column (20 × 1.8 cm) equilibrated with 0.5% Triton X-100

in 25 mM NaCl, 5 mM Tris-HCl, and 0.2 mM EDTA, pH 8. The flow-through fraction contains band 4.5 protein, residual hemoglobin, 7% band 7 protein, and lipid/Triton X-100 mixed micelles (Kasahara & Hinkle, 1977). Band 3 and 6 proteins were eluted by using 1 M NaCl, 5 mM Tris-HCl, 0.2 mM EDTA, pH 8, and 0.5% Triton X-100 (Kasahara & Hinkle, 1977). The band 4.5 fraction was further concentrated by ultrafiltration and stored at -70 °C. These procedures result in the recovery of 45% of total red cell band 4.5 protein. The cytochalasin B binding activity of the protein is (in the nominal absence of Triton X-100) 14 nmol/mg of protein. This represents a 13-fold purification over intact membranes (1.1 nmol/mg of protein) and, assuming a molecular weight of 55 000 for band 4.5, indicates a binding site:protein molar ratio of 0.8:1.

Reconstitution of Hexose Transfer Activity. Triton X-100 was removed from the protein fraction by addition of Biobeads SM-2 followed by gentle agitation at 4 °C for 4–14 h [0.6 g of beads/2 mL of protein solution (10–1000 µg of protein/mL) (Holloway, 1973)]. This produces a suspension of small unilamellar vesicles formed from residual lipid and protein. The Triton X-100 content of this suspension was reduced by more than 99.8%. The protein content of this nominally Triton X-100 free extract was reduced by 26%. This percentage appeared to be independent of the starting protein concentration. The Triton X-100 free protein suspension was used immediately for reconstitution into synthetic membranes.

Reconstitution was effected by using reverse-phase evaporation (Szoka & Papahadjopoulos, 1978) as modified by Carruthers & Melchior (1984). With this method, 25 mg of phospholipid in 0.25 mL of hexane was dried down to a thin film on the bottom of a 50-mL round-bottomed flask under N_2. The remaining organic solvent was removed in vacuo (100 µmHg) overnight. Diethyl ether (1.5 mL) was added to the lipids followed by 0.25 mL of buffer (25 mM NaCl, 5 mM Tris-HCl, and 0.2 mM EDTA, pH 7.5). This mixture was sonicated in a bath sonicator for 2 min, forming a visually homogeneous suspension. The ether was removed completely by rotary evaporation, resulting in a lipidic gel. Large unilamellar vesicles may then be formed by dispersion of the gel in buffer (Szoka & Papahadjopoulos, 1980; Carruthers & Melchior, 1984). Membrane protein reconstitution was effected by inclusion of the protein in the buffer (25 mM NaCl, 5 mM Tris-HCl, and 0.2 mM EDTA, pH 7.5) used to disperse the gel. The resulting vesicles were packed by centrifugation at 20000g for 15 min.

Hexose Transfer Determinations. D-Glucose efflux was measured by the turbidimetric method (Masaik & LeFevre, 1977; Carruthers & Melchior, 1983a,b, 1984) under zero-trans conditions (external sugar absent).

Glucose transport measurements made by using the turbidimetric method are based on the osmotic behavior of the vesicles. The vesicles have a water permeability some 1000–10000-fold greater than protein-mediated glucose permeability and behave as perfect osmometers both above and below the bilayer liquid-crystalline to fluid phase transition (Carruthers & Melchior, 1983b). Vesicles swell when D-glucose penetrates the intravesicular space and shrink when glucose is lost from the vesicles. As the vesicles act as osmometers, their size is related directly to the intravesicular glucose concentration. The time course of sugar transport induced vesicle size change may be monitored conveniently by measuring the turbidity of the suspension of vesicles as the turbidity is inversely proportional to the vesicle encapsulated volume (Carruthers & Melchior, 1983b). Turbidity was measured by using the computer-

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[1] Abbreviations: DML, dimyristoyllecithin; DPL, dipalmitoyllecithin; DSL, distearoyllecithin; DAL, diarachidonoyllecithin; DEL, dielaidoyllecithin; DOL, dioleoyllecithin; DPOL, dipalmitoleoyllecithin; DSC, differential scanning calorimetry; LUVs, large unilamellar vesicles; MLVs, multilamellar vesicles; CCB, cytochalasin B; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; EDTA, ethylenediaminetetraacetic acid.

controlled Digital microturbidimetric analysis stop-flow system described by Carruthers & Melchior (1983a,b). The reconstituted system (0.5-1 μL of packed vesicles; extravesicular water space, 50-70%), formed in NaCl buffer containing D-glucose, was injected into 400 μL of NaCl buffer and the time course of turbidity change monitored.

K_m and V_{max} for net zero-trans exit (initial D-glucose loading concentration 100 mM) were obtained by integration of the time course of flux (Hankin et al., 1972; Carruthers & Melchior, 1983a). Our previous studies have shown that the concentration dependence of the initial rate of radiolabeled D-glucose uptake in this reconstituted system shows saturation kinetics (Carruthers & Melchior, 1984). The kinetic parameters (V_{max} and K_m) obtained from these data agree closely with estimates made by using the integrated rate equation approach for analysis of time course data (Carruthers & Melchior, 1984). This is expected for both approaches are simply different mathematical expressions of the same steady-state kinetic analysis (Hankin et al., 1972).

Passive Permeability Properties of Vesicles. The passive fluxes of water and D-glucose across the liposomal membrane were estimated as described by Carruthers & Melchior (1983b). Estimates of encapsulated vesicle volume were made by determination of the total and extravesicular water contents of pelleted vesicles using [³H]inulin and wet and dry weight analyses (Carruthers & Melchior, 1983b).

Cytochalasin B Binding. The concentration dependence of cytochalasin B binding to reverse-phase LUVs was determined by incubation of pelleted LUVs (10 μL) in 100 μL of [³H]-cytochalasin B (0.1-10 μM concentration range) and NaCl buffer at 19-25 °C for 2 h. Following incubation, the LUVs were pelleted by centrifugation at 90000g for 20 min, the supernatant was aspirated, and the pellet was dissolved in 5% Triton X-100 and counted.

Contamination by extravesicular activity was 4.6%. Contamination by intravesicular activity depended upon the LUV diameter and ranged from 9% to 40% (DML and DAL, respectively). D-Glucose-sensitive [³H]cytochalasin B binding was determined by incubation ± 500 mM D-glucose. The apparent half-time for binding equilibrium at 19 °C was 12 min. Cytochalasin B inhibitable ¹⁴C-labeled D-glucose efflux from egg lecithin LUVs (loading concentration 0.5 mM) was measured as described previously (Carruthers & Melchior, 1984). Exit was consistent with a monoexponential process ($k = 0.8 \pm 0.1$ min⁻¹; temperature, 30 °C), indicating substantial population homogeneity in reconstituted protein content.

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 vesicles by the method of Bligh & Dyer (1959). Phospholipid phosphorus assays were as described by Bartlett (1959). Fatty acid analysis was as reported previously (Carruthers & Melchior, 1983b, 1984). Sodium dodecyl sulfate–polyacrylamide gel electrophoresis was carried out on 7% gels as described by Laemmli (1970). The "purified" band 4.5 fraction consists of band 4.5 (93%) and band 7 (7%) proteins. This fraction is referred to as band 4.5 protein in the text below.

Kinetic constants for cytochalasin B binding to the reconstituted system were obtained by subtraction of D-glucose-insensitive binding from total binding (Carruthers & Melchior, 1984) followed by curve fitting to the Michaelis–Menten formalism. This was achieved by weighted nonlinear regression
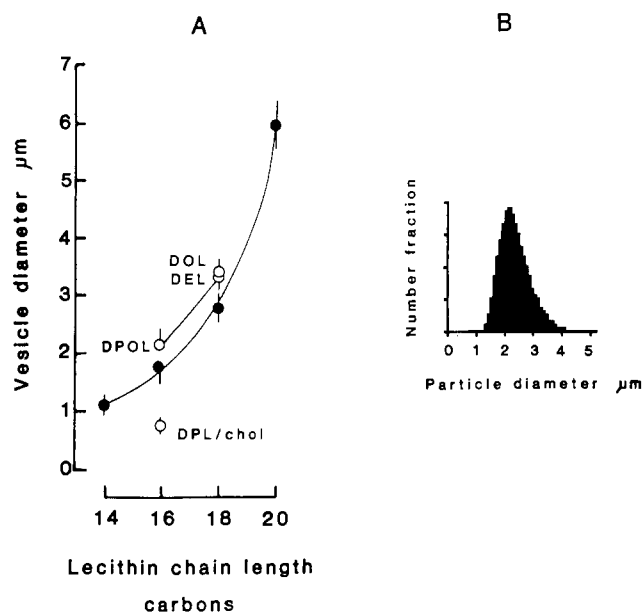

FIGURE 1: Dependence of vesicle diameter of the reconstituted system on phospholipid acyl chain length. The various lecithins used were dimyristoyllecithin (DML, C14), dipalmitoyllecithin (DPL, C16), dipalmitoleoyllecithin (DPOL, C16, a cis-9,10 diunsaturate), DPL/cholesterol (52:48 molar ratio), distearoyllecithin (DSL, C18), dielaidoyllecithin (DEL, C18, a trans-9,10 diunsaturate), dioleoyl-lecithin (DOL, C18, a cis-9,10 diunsaturate), and diarachidonoyl-lecithin (DAL, C20). Oridinate: vesicle diameter in micrometers. Abscissa: acyl chain carbon number for C14-C20. Each point represents the mean ± SD of at least four determinations. The saturates (DML, DPL, DSL, and DAL) are shown by the filled circles. (B) Size distribution of the DPOL reconstituted system. Temperature, 50 °C; polydispersity factor, 0.07; abscissa, diameter in micrometers.

employing "robust" methods for modification of residuals (Duggleby, 1981).

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 results in polydispersity factors of 0.1 or less, indicating substantially monodispersed samples (Ma-Cally & Bargeron, 1977). This homogeneity in size was confirmed by the recovery of only a single fraction of LUVs following application to a Sepharose 4B column. Mean LUV diameter was unaltered upon transit through the column.

Results

Characterization of the Reconstituted System. The size of LUVs formed during reconstitution of band 4.5 proteins is strikingly dependent on the phospholipid content of the bilayers. Figure 1 shows that LUV size increases with lecithin acyl chain length. These vesicles are large, ranging from 0.8 to 6 μm in diameter. LUV size was determined by using photon correlation spectroscopy. The limits of accuracy of this procedure are in the range 0.025-4 μm; hence, the estimate of the size of DAL LUVs (6 μm) should be interpreted cautiously. Nevertheless, the polydispersity factors obtained for each LUV type were, in most instances, significantly less than 0.1, indicating substantially monodisperse size populations. Figure 1 shows a typical size distribution analysis for the reconstituted system [in this case dipalmitoleoyllecithin (DP-OL) LUVs containing band 4.5 proteins]. Analysis of encapsulated volumes indicates that the LUVs are indeed unilamellar structures. The smallest LUVs formed by this procedure (from egg lecithin) have an average diameter of 0.12 ± 0.02 μm. Assuming these LUVs are spherical, unilamellar structures and that the average area of a lipid molecule is 50

$Å^2$ (Bangham et al., 1965), the calculated encapsulated volume is 3 μmol of lipid. The estimated value is 3.12 ± 0.06 μL/μmol of lipid. Similarly, with DEL LUVs (diameter, 3.36 ± 0.07 μm), the calculated encapsulated volume (84 ± 2 μL/μmol of lipid) corresponds closely with the estimated value of 79 ± 5 μL of lipid. In addition, examination of all LUVs by phase-contrast light microscopy confirmed that multilamellar vesicles were absent.

The number of copies of band 4.5 protein reconstituted per vesicle was estimated by analysis of D-glucose-sensitive cytochalasin B binding to the reconstituted system. Here, we have assumed that the binding site:protein molar ratio (0.8:1) corresponds to one site per protein. Knowing the ratio of cytochalasin B binding sites to moles of phospholipid, we may estimate the number of sites per LUV by assuming that the average lipid molecular area is 50 $Å^2$ and the LUVs are spherical. (This latter assumption was confirmed by multiple-angle analysis of the light-scattering properties of the LUVs using the particle sizer. In all cases, the LUVs scattered strongly at low angles, indicating that they behaved as large, spherical particles.) As Table I illustrates, there is little obvious correspondence between LUV lipid and number of sites reconstituted although our estimates indicate that the incorporation of protein is directly proportional to the amount of protein added during the reconstitution procedure. With DML bilayers, increasing the protein added during reconstitution 2.7-fold from 70 to 190 μg/40 mg of DML results in a corresponding increase in reconstituted D-glucose-sensitive cytochalasin B binding sites of (2.5 ± 0.3)-fold. With DOL, increasing the protein added during reconstitution by some 3.4-fold from 30 to 105 μg/40 mg of DOL results in a (3.2 ± 0.1)-fold increase in reconstituted cytochalasin B binding proteins. The density of the reconstituted transporter ranges between 4 and 100 sites/μm² (8–11 000 sites per LUV). This compares with the native density of 2 × 10³ sugar-sensitive cytochalasin B binding sites per μm² of human erythrocyte plasma membrane (Carruthers, 1984).

The magnitude of flux in a given reconstituted system is directly proportional to the number of cytochalasin B binding proteins reconstituted. In DOL bilayers at 40 °C, V_{max} increases (3.1 ± 0.1)-fold for a (3.2 ± 0.1)-fold increase in cytochalasin B binding sites reconstituted. In DML bilayers at 40 °C, a (2.3 ± 0.2)-fold increase in V_{max} for D-glucose exit is associated with a (2.5 ± 0.3)-fold increase in cytochalasin B binding protein reconstitution. As the molar ratio of cytochalasin B binding sites to band 4.5 protein is 0.8:1 (see Materials and Methods), these data suggest that unless multiple associations of proteins exclude all but one cytochalasin B binding site in the protein complex, the functional protein unit is a monomer.

Removal of Triton X-100 from the solubilized transport protein fraction results in the formation of a suspension of small (<500 Å) unilamellar vesicles consisting of red cell lipid and band 4.5 protein. Subsequent reconstitution of these proteins into synthetic bilayers is associated with the carry through of red cell lipid. The extent of this carry through was estimated by analysis of the fatty acid content of protein-free and protein-containing LUVs. In DML LUVs containing band 4.5 protein (molar ratio of protein to DML = 1.4 × 10⁻⁵), the carry through of red cell lipid amounts to 0.9 mol %. The Triton X-100 content of the reconstituted system is 0.08 mol % (1 detergent molecule per 1250 lipids).

Table I also includes estimates of the passive water and D-glucose permeabilities of the various reconstituted systems. D-Glucose penetrates the LUVs by two routes: a nonspecific,

Table I: Membrane System[a]

	DML	DPL	DPL/cholesterol	DPOL	DSL	DEL	DOL	DAL	egg lecithin	human red cell
unit diameter (μm)	1.11	1.76	0.77	2.16	2.79	3.36	3.41	5.99	0.12	7
K_d for CCB binding (μM)	0.2 ± 0.05	0.99 ± 0.21	0.45 ± 0.11	0.77 ± 0.25	0.49 ± 0.16	0.23 ± 0.05	0.97 ± 0.24	0.03 ± 0.01	0.1^b ± 0.05	0.05^c
phospholipids per CCB binding site	7 × 10⁴	4.2 × 10⁵	9.9 × 10⁵	3.7 × 10⁴	3.4 × 10⁵	2.7 × 10⁵	1.6 × 10⁵	4.1 × 10⁴		1.6 × 10³
sites per vesicle	223	93	8	1566	285	528	900	10890		3 × 10⁵
sites per μm²	58	10	4	107	12	15	25	97		1.9 × 10³
fatty acid carry-through (mol %)	0.9	0.2	0.4	0.9	0.6	0.5	0.7	1.1	0.08	
Triton X-100 contamination (mol %)	0.08	0.012	0.006	0.15	0.017	0.02	0.03	0.11		
P_f (cm·s⁻¹ × 10³) at 50 °C	11.8 ± 0.5	10.8 ± 1.5	7.8 ± 0.6	3.9 ± 0.2	9.9 ± 0.4	9.7 ± 0.7	8.9 ± 1.3	6.9 ± 0.5	11.4 ± 0.2	40
$P_{glucose}$ (cm·s⁻¹ × 10³) at 50 °C	2.5 ± 1.1	2.7 ± 0.7	2.3 ± 0.9	1.8 ± 0.3	1.9 ± 0.4	3.4 ± 0.7	3.4 ± 0.3	9.4 ± 0.6	2.5 ± 0.4	5 ± 0.7
$P_{sat(glucose)}$ (cm·s⁻¹) × 10⁶) at 50 °C	0.5 ± 0.1	6.2 ± 0.7	1.6 ± 0.5	15 ± 0.6	10 ± 0.2	24.2 ± 3.6	10.9 ± 1.3	9.8 ± 0.8	1.5 ± 0.1	178 ± 22
E_a (kcal/mol)	8.1 ± 2.7	12.5 ± 0.9	5.1 ± 1.7	11.8 ± 0.8	16.2 ± 2.8	23.2 ± 2.6	23.7 ± 1.1	22.3 ± 3.2	11.2 ± 0.9	18.4 ± 0.7
A (mol site⁻¹ s⁻¹)	2.5 × 10⁻¹⁶ ± 2.4 × 10⁻¹⁶	1.9 × 10⁻¹¹ ± 1.5 × 10⁻¹¹	3.7 × 10⁻¹⁷ ± 3.5 × 10⁻¹⁷	1 × 10⁻¹² ± 0.74 × 10⁻¹²	8.8 × 10⁻⁹ ± 8.7 × 10⁻⁹	5.2 × 10⁻⁴ ± 5.1 × 10⁻⁴	2 × 10⁻⁴ ± 0.8 × 10⁻⁴	8.9 × 10⁻⁶ ± 2.1 × 10⁻⁶		2.4 × 10⁻⁸ ± 0.1 × 10⁻⁸

[a] P_f is the osmotic water permeability coefficient; $P_{glucose}$ is the permeability coefficient for glucose flux in the absence of cytochalasin B (V_{max}/K_m). $P_{sat(glucose)}$ is the permeability coefficient for glucose flux in the presence of 10 μM cytochalasin B. [b] K_i for inhibition of zero-trans D-glucose exit. [c] Baldwin et al. (1980).

nonsaturable, low-permeability pathway and a saturable, selective, high-permeability route. This latter system is inhibited strongly by cytochalasin B (Carruthers & Melchior, 1983a,b, 1984). The permeability coefficient for nonspecific (cytochalasin B insensitive) flux is some 3–4 orders of magnitude lower than that for saturable D-glucose flux. Osmotic water flux proceeds some 100–1000-fold more rapidly than the saturable flux of hexose. These values are in close agreement with similar estimates made with both biological and synthetic membranes [see Carruthers & Melchior (1983b, 1984)] and attest to the structural integrity of the reconstituted membranes. This low nonspecific D-glucose permeability together with large vesicle size and narrow size range makes this reconstituted system particularly suited to kinetic analyses of protein-mediated hexose flux.

Characteristics of Reconstituted Hexose Transport. It is possible to use cytochalasin B binding data together with the effects of cytochalasin B on sugar flux to evaluate the directional orientation of the reconstituted transport proteins in the synthetic membranes. The human erythrocyte hexose transporter shows kinetic symmetry of transport in the absence of cellular solute (Taverna & Langdon, 1973; Carruthers & Melchior, 1983a) but biochemical asymmetry under the same conditions (Widdas, 1980). In inside-out red cell membrane vesicles in the absence of cytosolic solutes, K_m values for influx and efflux of D-glucose are identical. Nevertheless, cytochalasin B acts competitively with D-glucose at the interior of the intact cell and phloretin at the exterior (Krupka & Deves, 1981). In addition, trypsin inhibits glucose transport when applied to the interior of red cell ghosts but not to the exterior (Masaik & Le Fevre, 1977). This inhibition is associated with the loss of D-glucose-sensitive cytochalasin B binding sites in the plasma membrane (Baldwin et al., 1980). These sites are a component of band 4.5 protein and are exposed to the interior of the cell.

Assuming the directional orientation of the transport protein is random on reconstitution, this means that under the conditions of our experiments, 50% of the cytochalasin B binding sites will be facing sugar-poor medium (the extravesicular medium) and 50% sugar-rich medium (the intravesicular space). The kinetic consequence of this will be the overestimation of K_i for inhibition of sugar flux by cytochalasin B and K_d for binding of cytochalasin B due to competition between cytochalasin B and intravesicular glucose for the internal sites. V_{max} for exit will decrease by 50% due to the noncompetitive binding of cytochalasin B at the external sites, but the maximum number of measured binding sites will be unaffected. This was borne out with both zero-trans sugar exit experiments and cytochalasin B binding assays. With hexose flux determinations, the K_m for D-glucose exit in DML LUVs at 30 °C increases with increasing cytochalasin B concentration (control, $K_m = 11.9 \pm 1.3$ mM; +10 μM cytochalasin B, $K_m = 448 \pm 38$ mM), and the V_{max} falls to some 39 ± 5.1% of control values (cytochalasin B concentration 10 μM). This mixed inhibition resulting from the competitive and noncompetitive binding of cytochalasin B at both internally and externally oriented sites, respectively, indicates that orientation of the reconstituted cytochalasin B accessible site is essentially random (40:60 inside:outside). Cytochalasin B binding studies show that with DML LUVs assayed at 19 °C, where transport activity is negligible, intravesicular D-glucose increases the K_d from 0.2 ± 0.03 to 0.41 ± 0.04 μM cytochalasin B with maximum binding (B_{max}) unchanged at 12 fmol/nmol of DML. Assuming the above distribution of cytochalasin B binding sites (60:40 outside:inside) and a K_d for binding of cytochalasin B
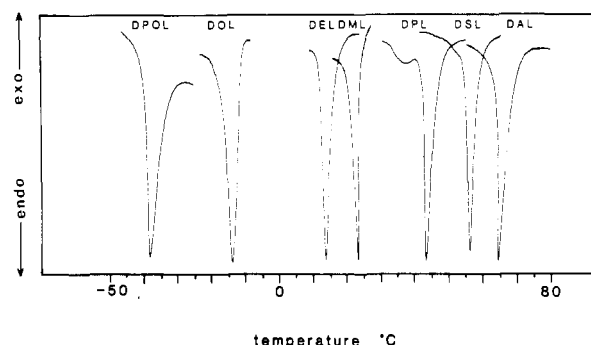

FIGURE 2: DSC thermograms of the various reconstituted systems. Ordinate: heat flow. Abscissa: temperature in degrees centigrade. The lipid composition of the LUVs is shown above the records. With DPOL and DOL, the sample pan was cooled to –50 °C by using liquid nitrogen and then scanned at 10 °C/min. Ethylene glycol was not added; hence, the water content of these samples was frozen. Each pan contained about 1.5 mg of lipid and 2 μg of band 4.5 protein.

of 0.2 μM, we can calculate the apparent K_d, K_d', for binding to the internal sites as

$$K_d' = (0.2 \ \mu M)(1 + [S]/K_m)$$

where [S] is the internal sugar concentration (100 mM) and K_m is the half-saturation constant for inhibition of cytochalasin B binding by D-glucose (10.1 ± 0.8 mM D-glucose; $n = 12$). Binding of cytochalasin B to the transporter is therefore described by a two-component parallel system as

$$b = \frac{0.6B_{max}[CCB]}{K_d + [CCB]} + \frac{0.4B_{max}[CCB]}{K_d' + [CCB]}$$

where b = moles of cytochalasin B (CCB) bound. Over the range of [CCB] used in our experiments (0.1–10 μM) and using the above values of K_d, K_d', and B_{max} and, in addition, assuming a homogeneous population of sites (as we do experimentally), kinetic analysis of binding will result in an apparent K_d of 0.39 ± 0.04 μM—a value close to the experimental K_d of 0.41 ± 0.04 μM.

Effects of Bilayer Crystalline to Fluid Transition on Reconstituted Transport Activity. Figure 2 shows thermograms of the various reconstituted systems obtained by differential scanning calorimetry (DSC). As with multilamellar vesicles formed from synthetic lecithins, LUVs formed from these same lipids show a characteristic pretransition and have broader main transitions than their multilamellar counterparts (Carruthers & Melchior, 1983b; Duzgunes et al., 1983). These pretransitions were absent in thermograms of DEL, DML, DSL, DPOL, and DAL LUVs containing the reconstituted transport protein. It is known that for multilamellar systems, the pretransitions are particularly sensitive to low concentrations of impurities in the bilayer (Melchior & Steim, 1977). Our analyses indicate that for the reconstituted systems reported here, the LUV bilayer contains 1 mol % endogenous (red cell) lipid, 0.08 mol % Triton X-100, and from 6 × 10⁻⁴ to 3 × 10⁻³ mol % cytochalasin B binding protein. DSC studies show that the pretransition of DML LUVs is unaffected by Triton X-100 (0.08 mol %) and its enthalpy approximately halved by 1 mol % red cell lipid. It is most likely that the absence of the premelt in these reconstituted systems results from the reconstituted proteins and the carry-through lipid acting as general impurities in the otherwise homogeneous phospholipid bilayers. The presence of a premelt in the DPL reconstituted system is consistent with a lower amount of reconstituted transporter (see Table I).

Figure 3 illustrates the temperature dependence of both V_{max} and K_m for zero-trans D-glucose exit in the reconstituted system
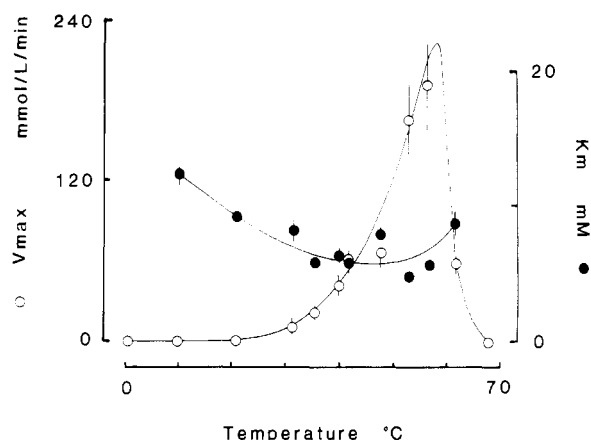
FIGURE 3: Temperature dependence of kinetic parameters for zero-trans D-glucose exit from DOL LUVs containing the reconstituted band 4.5 proteins. Ordinate: (O) V_{max} in millimoles per liter per minute; (●) K_m in millimoles per liter. Abscissa: temperature in degrees centigrade. Each point represents the mean ± SD of five separate determinations. The curve drawn through the V_{max} data between 10 and 57 °C corresponds to an activation energy (E_a) of 22.9 kcal mol^{-1} K^{-1}. All other curves are drawn by eye.
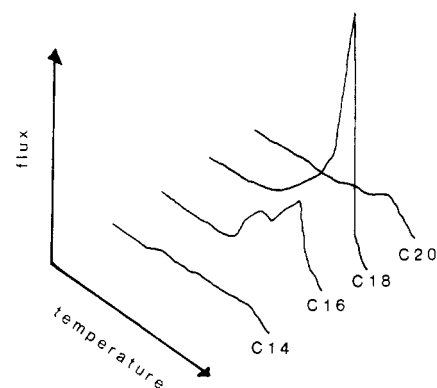

FIGURE 4: Dependence of reconstituted hexose transfer activity in disaturated lecithins on acyl chain length. This is a three-dimensional representation of the relative activity in DML, DPL, DSL, and DAL reconstituted systems. The x axis represents temperature between 10 and 70 °C. The y axis is activity (V_{max} per site) and ranges between 0 and 5 × 10^{-9} mol site^{-1} s^{-1}. The z axis shows the number of carbons per lecithin acyl chain (C14–C20). The points taken for activity are mean values of eight or more determinations in two separate reconstitutions. The variation between values from different runs is less than 25%.

formed from DOL and band 4.5 protein. In this system, the bilayers are fluid at all temperatures above –22 °C, the T_m of DOL bilayers. Arrhenius plots of these data indicate a simple logarithmic relationship between V_{max} and temperature (see Figure 6). The corresponding van't Hoff plot for K_m data indicates that as with transport in the erythrocyte membrane (Ginsburg & Yeroushalmy, 1978), K_m displays only a weak dependence on temperature. Between 17.5 and 20 °C, transport is absent. This lack of catalytic activity is a reversible phenomenon, activity being restored by lowering the temperature to 16 °C or raising the temperature to 22 °C. This behavior is observed only with DOL bilayers among the lecithins studied. The transport system denatures irreversibly at 60–62 °C.

Our primary interest here was to determine whether transport activity could be supported by both crystalline and fluid bilayers, and, if so, how their properties might differ. Reconstitution of band 4.5 protein into bilayers showing characteristic liquid-crystalline to fluid phase transitions above 0 °C results in hexose transfer activities rather different from those described for DOL (see Figure 4 for relative fluxes with the saturated lecithins). With DML, hexose transfer activity is not detectable at temperatures below the T_m of the bilayer (below 22 °C). As temperature is increased, however, and the bilayer begins to melt, transport activity is observed. As temperature is further increased beyond the melt of the bilayers, the V_{max} for efflux continues to increase in a fashion qualitatively similar to that observed with DOL bilayers. With DPL bilayers containing band 4.5 protein, similar behavior is observed. Transport activity is absent at low temperatures where the bilayers are in the crystalline state but is apparent at higher temperatures in fluid bilayers. The DPL experiments are particularly interesting in that they demonstrate that hexose transfer activity increases both during the premelt and also during the main phase transition. These data would seem to suggest that hexose transfer activity is absent in crystalline bilayers and that significant activity emerges only when the bilayers melt. However, when DSL and DAL bilayers which melt at 54 and 67 °C, respectively, are used, significant hexose transfer activity is seen to occur at temperatures as low as 10 °C. Activity increases with temperature in both DSL and DAL bilayers but shows an abrupt increase in DSL bilayers at about 55 °C–close to the T_m of the bilayer. With each of

these systems, hexose transfer is inhibited irreversibly at temperatures between 60 and 62 °C, presumably by heat denaturation of the proteins.

Effects of Cholesterol on Reconstituted Hexose Transfer Activity. Figure 5 shows the effects of 48 mol % cholesterol on the V_{max} for hexose transfer in DPL LUVs. At temperatures below 30 °C where DPL bilayers are crystalline and support no detectable protein-mediated hexose flux, cholesterol serves to restore transport activity. At higher temperatures where DPL bilayers are in the "fluid" state and transport activity is greatly increased, cholesterol reduces hexose transfer activity. There are some anomalous effects of cholesterol on sugar transport between 47 and 55 °C, where transport activity increases and then falls to lower levels. This particular effect could result from the melt of residual DPL not associated with cholesterol. The presence of a small but detectable endotherm in thermograms of these bilayers is consistent with this view. Fillipin, a polyene antibiotic, is believed to act on cholesterol-rich membranes by sequestering or precipitating cholesterol within the plane of the membrane (Severs & Robenek, 1983). We considered that if the transport activity of cholesterol-rich DPL bilayers reflects the ability of cholesterol to modulate bilayer fluidity, then the sequestration of cholesterol by fillipin might permit the transporter to function as in a pure DPL bilayer. With 75 μM fillipin, sugar transport was inhibited at all temperatures (5–60 °C) in cholesterol/DPL (48:52 molar ratio) while showing normal activity in the absence of fillipin.

Effects of Acyl Chain Length and Saturation on Reconstituted Hexose Transfer. It seemed important to determine in a bilayer of homogeneous lipid class (lecithin) how altered acyl chain composition affects reconstituted hexose transfer activity. The bilayers described above are formed from the disaturates DML, DPL, DSL, and DAL (C14, C16, C18, and C20) and the diunsaturate DOL (C18, a cis-9,10 unsaturate). We have also examined reconstituted hexose transfer activity in DEL (C18, a trans-9,10 unsaturate) and DPOL (C16, a cis-9,10 unsaturate) bilayers. Figure 6 shows the Arrhenius plots for V_{max} (per reconstituted cytochalasin B binding site) in these various bilayers. This analysis assumes that each of the cytochalasin B binding sites reconstituted is catalytically active. Although this assumption may not be valid, we show above that activity is a linear function in cytochalasin B binding
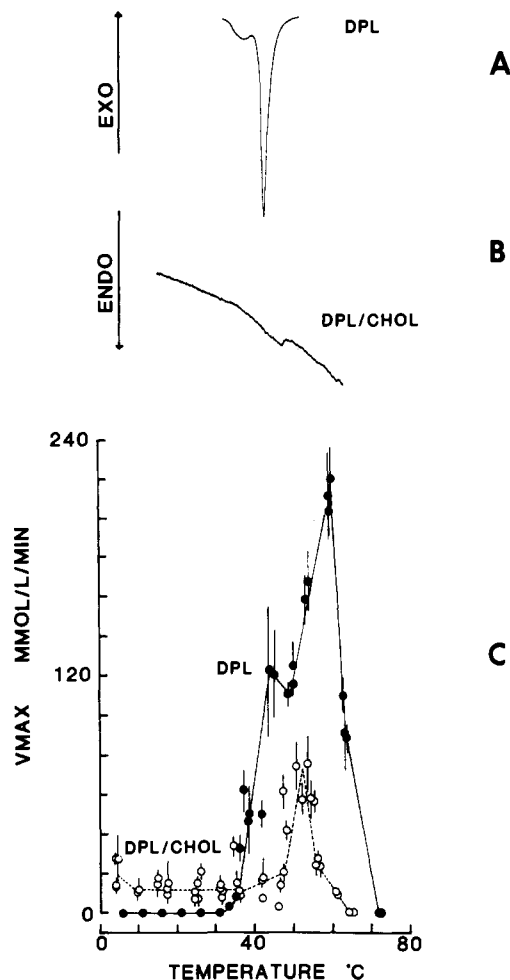
FIGURE 5: Effects of cholesterol on reconstituted hexose transfer activity in DPL bilayers. (A) DSC thermogram of the DPL reconstituted system. Ordinate: heat flow. Abscissa: temperature in degrees centigrade. (B) DSC thermogram of the DPL/cholesterol (52:48 mol %) reconstituted system. Axes as in (A) except that heat flow is shown at a much higher sensitivity than in (A). (C) Temperature dependence of reconstituted transport activity in DPL (●) and DPL/cholesterol (52:48, O) bilayers. Ordinate: V_{max} per liter per minute for zero-trans D-glucose exit (loading concentration 60 mM). Abscissa: as in (A) and (B). V_{max} for exit in DPL/cholesterol was incremented × 2.4 to adjust for the 2.4-fold lower density of reconstituted transporter in these vesicles (see Table I). Each point represents the mean ± SD of at least four separate determinations. The band 4.5 protein preparation was prepared from a single batch of red cells for these experiments.

protein numbers. As the activity measured is V_{max}, the Arrhenius equation takes the form

$$V_{max} = Ae^{-E_a/(RT)}$$

Figure 7 and Table I summarize the results of these analyses showing the relationship between acyl chain length and activation energy (E_a) and the preexponential Arrhenius constant (A). Both E_a and A increase linearly with acyl chain length in the saturates. With the unsaturates, E_a increases 2-fold over the range C16–C18 while DEL and DOL show no significant difference, and A increases by some 8 orders of magnitude between C16 and C18 [A(DEL) = 2A(DOL)]. In several experiments on DSL LUVs where the bilayer lipid used was made 2 mol % in DML, transport activity was identical with that for pure DSL. With egg lecithin (C16:C18:C20 molar ratio = 37.3:59.4:3.3; Arvidson, 1965) bilayers, E_a is 11.2 ± 0.9 kcal mol^{-1} K^{-1}, a value resembling that for flux in bilayers formed from disaturated or diunsaturated C16 lecithin.
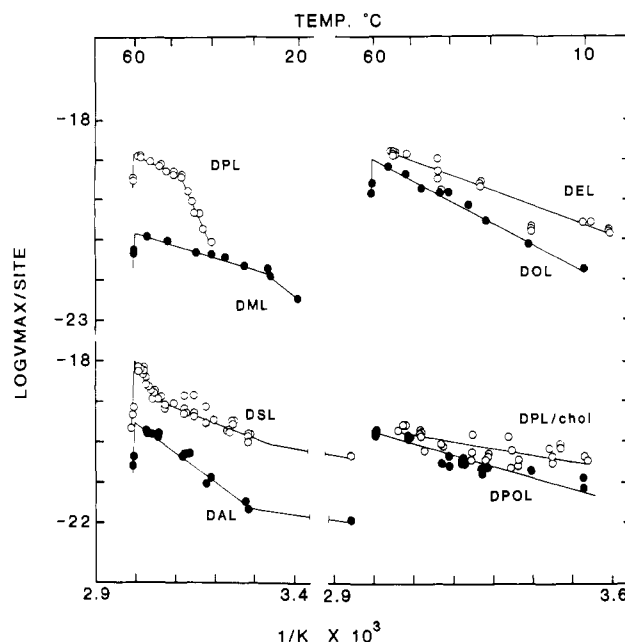

FIGURE 6: Arrhenius plots of V_{max} for D-glucose zero-trans exits in the various reconstituted systems. Ordinate: log (V_{max}/reconstituted site). Abscissa: 1/K. The lipid composition of the reconstituted systems is shown beside each curve. The lines drawn through the points are calculated by the method of least squares. With DSL and DAL, there is some deviation from the line of best fit at low temperature. Each point is the mean of at least four separate determinations. Loading D-glucose concentration, 60 mM.
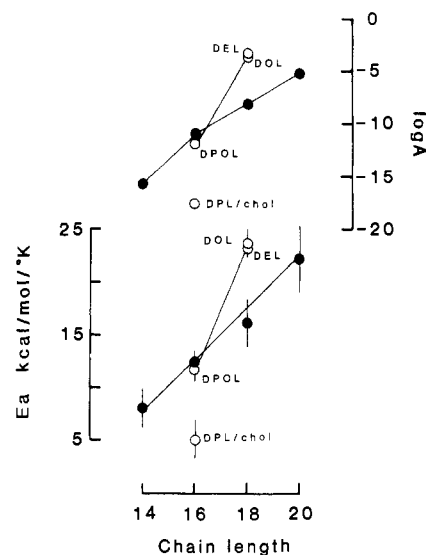

FIGURE 7: Dependence of E_a (activation energy) and A (Arrhenius constant) on lecithin acyl chain length. Ordinate: log A (in moles per site per second); E_a (in kilocalories per mole per degree kelvin). Abscissa: lecithin acyl chain carbon number. The saturates are indicated by the closed circles. These data were obtained by least-squares analysis of the data in Figure 6. E_a is obtained from the slope of the Arrhenius plot, and A is the y intercept of this plot. The values obtained for DML and DPL represent analysis of transport in the fluid bilayer. Those for DSL and DAL represent flux data from the crystalline bilayer.

Discussion

The reconstituted system reported here is well suited to transport studies. Individual unilamellar vesicles containing the reconstituted transport protein are large (0.8–6-μM diameter) and have a narrow size range—the mean diameter determined by the lipid composition of the bilayer. The great size of the vesicles is important for rapid fluxes are extremely

difficult to measure in small vesicles and difficult to interpret if the vesicle population size is overly heterogeneous. The number of transport proteins reconstituted per vesicle ranges from 8 in the smallest LUVs (DPL/cholesterol) to 1×10^4 in the largest (DAL LUVs). This represents a 20-fold variation in the density of reconstituted cytochalasin B binding proteins. The orientation of these proteins within the bilayer is essentially random. The maximum permeability coefficient [V_{max}/K_m; see Carruthers (1983)] for protein-mediated sugar flux was observed with DSL bilayers at 59 °C and is on the order of 10^{-5} cm/s—some 1–2 orders of magnitude less than that of native red cell membranes. This is consistent with the 100-fold greater concentration of cytochalasin B binding sites in the erythrocyte membrane (Jones & Nickson, 1981). However, as will be discussed, the absolute catalytic activity of the reconstituted systems is not simply related to carrier density but is also affected markedly by the lipid composition of the protein's resident bilayer. The direct comparison of permeability coefficients, turnover numbers, or specific activities in native and reconstituted systems is not, in light of our findings, strictly applicable. It is interesting to speculate that reported differences in the specific activities of reconstituted band 3 and band 4.5 proteins arise from the nature of the synthetic membrane rather than the proteins themselves (Shelton & Langdon, 1983; Carruthers & Melchior, 1984).

Our cytochalasin B binding studies demonstrate that D-glucose inhibits binding to band 4.5 protein in the nominal absence of transport activity in DML bilayers. This is interesting for it is often presumed that inhibition of cytochalasin B binding is the consequence of D-glucose binding at the transport site. According to this view, these data suggest that in the absence of transport activity, D-glucose may still bind to the acceptor or recognition site of the transporter; i.e., translocation is the rate-limiting step in transport. Indeed, measurements of the K_i(app) for inhibition of cytochalasin B binding by D-glucose and the K_m(app) for D-glucose transport in DSL bilayers at 20 °C (4.8 ± 0.9 mM, $n = 12$; 5.6 ± 0.4 mM, $n = 5$, respectively) indicate that K_i and K_m are indistinguishable. As the simple mobile carrier of Widdas (1952) predicts that K_m for transport derives from both binding and translocation constants [see Lieb & Stein (1974)], these data suggest that binding and K_m for transport are, in fact, governed by a single process and that the mobile carrier model must be rejected. However, it is important to recognize that both K_m and K_i may not reflect binding at a single recognition site but rather sequential binding at multiple distal sites as hexose moves through the transport system (Carruthers, 1984). Moreover, there is good reason to suspect that D-glucose inhibition of cytochalasin B binding to the transporter is an allosteric phenomenon rather than simple competition at a single site (Widdas, 1980). In view of these problems, mechanistic interpretation of the binding data is not currently justified.

Our findings are in agreement with the studies of Baldwin et al. (1980) and Wheeler & Hinkle (1981), indicating that the native directional orientation of the transport protein is lost upon reconstitution. Nevertheless, our conclusions concerning the kinetics of transport differ significantly from those of Wheeler & Hinkle (1981). Sugar transport in intact human erythrocytes shows an operational asymmetry in Michaelis and velocity constants for influx and efflux [K_m and V_{max} for zero-trans efflux are 10–16-fold greater than the corresponding values for influx (Widdas, 1980)]. In addition, two operational binding sites for hexose efflux are observed at the cytoplasmic surface of the plasma membrane [K_m for infinite trans exit

$\ll K_m$ for zero-trans exit (Baker & Naftalin, 1979)]. Wheeler & Hinkle (1981) used the trypsin sensitivity of the cytochalasin B sites of the transporter to inactivate the reconstituted transport proteins with reverse orientation, thus leaving the system with native orientation—the cytochalasin B binding sites exposed to the intravesicular medium. Under these conditions, V_{max} for D-glucose exit fell to some 70% of control values and V_{max} for entry to 21% of control influx. These data are consistent with a kinetic asymmetry in V_{max} of 3.2 ± 0.6 and support the view that the asymmetry of intact red cell transport is, in part (20%), preserved upon reconstitution. Our studies show that exposure of the reconstituted system to saturating (10 μM) cytochalasin B levels reduces V_{max} for zero-trans exit by 61 ± 5%. This results from the noncompetitive binding of cytochalasin B to cytochalasin B binding sites facing the external, sugar-free medium. The effect is, in essence, identical with trypsinization of the reconstituted system— the remaining functional transport is mediated by protein with a native orientation at which D-glucose and cytochalasin B compete for the binding. Had normal 10–16-fold asymmetry (Widdas, 1980) been preserved on reconstitution, V_{max} for exit would fall by 6–9%. Assuming the reported 3.2-fold asymmetry for reconstituted transport, exit would fall by 24%. We conclude that red cell hexose transport displays an experimental kinetic symmetry. This view is supported by the following two direct lines of evidence: (1) The kinetics of hexose transfer in inside-out vesicles from human erythrocytes are symmetric (Taverna & Langdon, 1973; Carruthers & Melchior, 1983a). Normal asymmetry in transport can be restored reversibly to inside-out vesicles by reexposure to red cell solute obtained upon cell lysis (Carruthers & Melchior, 1983a), indicating that operational transport asymmetry is conferred by cytosolic factors. (2) Measurements of infinite-cis entry kinetics estimate V_{max} for zero-trans entry and K_m for infinite-trans exit. Measurements with reconstituted band 4.5 indicate that K_m for infinite-trans exit is 7.5 mM and K_m for zero-trans entry is 8.4 mM (Carruthers & Melchior, 1984). Had intact cell transport asymmetry been preserved upon reconstitution, K_m for infinite-trans exit would have fallen to a value representing the mean of K_m for intact cell influx and for infinite-trans exit (1.6 mM; Hankin et al., 1972; Barker & Naftalin, 1979). The reasons for these rather different findings are not known but may derive in some way from the different reconstitution procedures employed [see, for example, Carruthers & Melchior (1984)] or from trypsinization of the protein.

Factors Influencing Reconstituted Transport Activity in Lecithin Bilayers. In a bilayer formed from a single lipid class, a number of factors might be expected to influence the catalytic activity of the reconstituted protein. These include lipid acyl chain length and saturation, bilayer physical state, and the addition of membrane-modulating lipids such as cholesterol (Melchior, 1982). Using lecithin bilayers, we have examined reconstituted transport activity in DML, DPL, DSL, and DAL bilayers (saturates with acyl chain lengths of C14, C16, C18, and C20, respectively). The unsaturates examined are DPOL, DOL, and DEL (C16, a cis-9,10 unsaturate; C18, a cis-9,10 unsaturate; and C18, a trans-9,10 unsaturate, respectively). In addition, we examined the effects of cholesterol on transport activity in DPL bilayers.

The most straightforward experimental manipulation is to melt the bilayer containing the transport protein. The practical working range of the reconstituted system lies between 0 and 60 °C. Beyond the higher temperature, significant reductions in activity are observed which presumably arise from protein
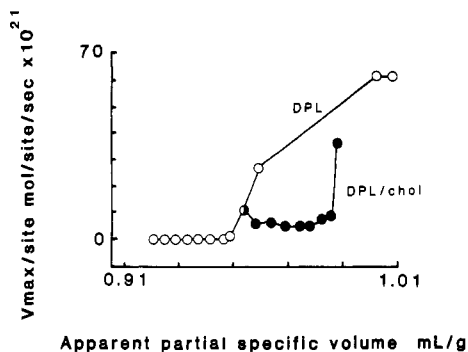
FIGURE 8: Dependence of reconstituted activity in DPL and DPL/cholesterol bilayers on bilayer apparent partial specific volume. Ordinate: V_{max} for zero-trans D-glucose exit (in millimoles per liter per minute); data taken from Figure 5. Abscissa: apparent partial specific volume (in milliliters per gram). The partial specific volumes were obtained for 0 and 50 mol % cholesterol in DPL bilayers from Figure 3 of Melchior et al. (1980). The curves through the pure DPL (O) and DPL/cholesterol (●) points were drawn by eye.

denaturation. This limited the synthetic membranes in which we could observe the effects of the bilayer phase transition on transport activity to DEL, DML, DPL, and DSL (T_m = 12, 22, 42, and 54 °C, respectively). DAL bilayers melt at 67 °C, and DPOL and DOL melt below 0 °C. With DML and DPL, transport activity is absent in fully crystalline bilayers but emerges when the bilayers undergo the liquid-crystalline to fluid phase transition. In DSL, DAL, and DEL, however, significant activity is observed at temperatures as low as 10 °C. On melting DSL bilayers, transport activity is seen to increase markedly. The inclusion of 2 mol % DML in DSL LUVs did not alter the thermal profile of sugar transport from what was observed in pure DSL bilayers. These findings indicate that although activity increases dramatically during the bilayer phase transition, the short-chain lecithins (C14 and C16) differ from their longer chain counterparts in their ability to support activity in the crystalline state. These results cannot be explained on the basis of simple protein/protein interaction. Such interactions would be expected to increase with protein concentration or membrane fluidity. At 60 °C, where all bilayers are fluid (except DAL and DPL/cholesterol), the order of reconstituted catalytic activity is DML < DPOL < DOL < DEL = DPL < DSL. The corresponding protein densities are DPL < DSL < DEL < DOL < DML < DPOL. Catalytic activity is also not reduced by protein/protein interaction for, in a given bilayer, activity is directly proportional to protein density. Cholesterol (48 mol %) restores transport activity to DPL bilayers at temperatures where cholesterol-free DPL would not support activity. At higher temperatures where DPL bilayers are fluid, cholesterol reduces transport activity. At first inspection, this appears to be consistent with the ability of cholesterol to plasticize crystalline DPL and condense fluid DPL bilayers (Melchior et al., 1980; Melchior, 1982). However, as Figure 8 illustrates, the relationships between bilayer partial specific volume [data taken from Melchior et al. (1980)] and hexose transfer activity (V_{max} per reconstituted site) in pure DPL and DPL/cholesterol bilayers are quite different. Assuming homogeneity in LUV cholesterol content, this rules out the simplest possibility that the transport protein responds to a parameter related simply to bilayer density per se and indicates that more subtle bilayer factors may influence the activity of the protein. This assumption seems valid for thermograms of LUVs differing in cholesterol content display the distinctive behavior observed with multilamellar vesicles (MLVs) (Mabrey et al., 1978; Melchior et al., 1980) except for the broader transitions and lower onset
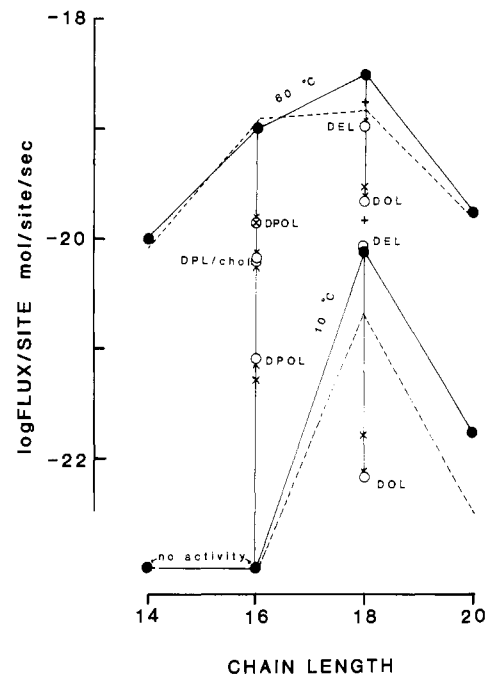

FIGURE 9: Dependence of reconstituted transport activity at 10 and 59–60 °C on lecithin acyl chain carbon number. Ordinate: log V_{max} per reconstituted cytochalasin B binding site (moles per site per second). Abscissa: acyl chain length (number of carbons). The upper continuous curve shows measured activity at 59–60 °C. The saturates (DML, DPL, DSL, and DAL) are shown by the closed circles. The lower continuous curve shows measured activities at 10 °C. Activity is absent (not measurable) in DML and DPL at 10 °C. The dashed lines indicate the calculated activities for the various systems at 10 and 60 °C. These values were obtained from the Arrhenius expression $V_{max}/site = Ae^{-E_a/(RT)}$. Calculated values for the cis and trans unsaturates (DPOL and DOL, and DEL, are indicated by (×) and (+), respectively.

temperatures characteristic of LUVs (Carruthers & Melchior, 1983b; Duzgunes et al., 1983). Initially, cocrystallizing lecithin with varying amounts of cholesterol has been demonstrated to give a population of bilayers (MLVs) homogeneous in cholesterol content (Melchior et al., 1980).

Figure 7 illustrates the relationship between bilayer lipid acyl chain length and activation energy (E_a) and the Arrhenius constant (A) for reconstituted transport. While these studies in no way discount the presence of or a role for hypothetical tightly bound lipid in protein-mediated hexose transport, they do indicate that the activity of the protein is governed by the bulk properties of the membrane. E_a increases with chain length (and in the case of C18 lecithins, with unsaturation). A increases by some 10 orders of magnitude over the range of saturates, C14–C20. With C18, unsaturation also increases A by some 5 orders of magnitude. As the rate-limiting step for transport appears to be translocation, it is reasonable to assume that E_a provides an estimate of the energy required for the formation of the transition-state conformer(s) of the transport protein/hexose complex committed to translocation. For water-soluble enzymes, A is related to the product of the probability of the reaction occurring when the reactants collide and the collision frequency of the reactants. It is not yet clear how this relates to the activity of an integral membrane transport protein.

Figure 9 illustrates the measured and calculated (from E_a and A) fluxes per reconstituted site for the various bilayers at 59–60 °C. The calculated value for DSL at 60 °C is somewhat low due to the values being calculated from Arrhenius plots of flux in crystalline DSL. This figure summarizes the effects of chain length and unsaturation on re-

constituted transport. These are the following: (a) increasing chain length increases the catalytic activity of the protein until an apparent optimum at C18; (b) unsaturation with C16 and C18 lecithins reduces the catalytic activity of the protein, the effect of the cis unsaturate being more marked than that of the trans unsaturate.

The behavior of DPOL and DOL reconstituted systems is, in some respects, surprising. These bilayers melt at -37 and -18 °C, respectively, and, over the range of temperatures examined in this study, might be considered to be more "fluid" than their saturated counterparts. Of some interest in view of the observations of others (Lee et al., 1974; Wunderlich et al., 1975; Baldassare et al., 1976) was our finding of loss of transport activity in fully fluid DOL bilayers at approximately 18 °C. Transport occurs both below and above this temperature. In an earlier study on water flux through LUVs, no change in water permeability was observed in DOL bilayers around 18 °C (Carruthers & Melchior, 1983b).

Many previous studies have characterized the effects of altered bilayer physical state on membrane transport activity in prokaryotes [for reviews, see Melchior & Steim (1979) and McElhaney (1982)]. Thilo et al. (1977) have provided strong evidence to show that E_a for proton-coupled β-galactoside transport in *Escherichia coli* is unchanged following the liquid-crystalline to fluid phase transition of the membrane, although the absolute activity of the permease is increased in the fluid membrane. Moreover, substrate binding to the lactose permease is unaffected in the crystalline biomembrane, indicating that translocation is the rate-limiting step for transport [Overath and co-workers; see McElhaney (1982)]. Our studies with the reconstituted transporter from cholesterol-rich human erythrocyte membranes are consistent with the findings of prokaryote studies, but significantly extend these findings to describe the effects of a variety of predetermined lipid environments on the activity of a eukaryotic transport protein. In addition, they indicate that the behavior of prokaryotic systems is unlikely to arise from secondary factors that influence transport. The most important conclusions differ, however, from those made from studies of prokaryotic systems (McElhaney, 1982), namely, that activity is not necessarily at an optimum in so-called fluid bilayers. Unsaturates may, in fact, reduce the apparent catalytic activity of the transport protein within the physiological range of temperature. Studies of reconstituted Na-dependent (active) D-glucose transport in DML bilayers have also shown that activity is increased in fluid DML (Da Cruz et al., 1983). Nevertheless, the apparent E_a for transport in crystalline DML was greater than that for transport in fluid DML. We refer to E_a measurements in this study as apparent E_a, for flux was measured only at subsaturating sugar concentrations. Changes in K_m or V_{max} for sugar transport or K_m for Na could greatly influence estimates of the apparent E_a. Our findings are in general accord with those of Da Cruz et al. (1983), showing that the *bulk* properties of the membrane greatly influence protein activity.

The molecular basis of protein-mediated hexose transport is unknown. The simplest transport model describing the basic (but not complete) kinetic properties of hexose transfer is the simple mobile carrier (Widdas, 1952; Krupka & Deves, 1981). This mobility is not implicit in the derivation of the kinetics although the model has often been interpreted in terms of components that "shuttle" between each hemileaflet of the bilayer. Indeed, the kinetics of a simple gated pore are identical with those of the mobile carrier (Lieb, 1982). The invariant topography of band 4.5 protein (C-terminus and carbohydrate exposed at the outer surface of the bilayer and N-terminus and cytochalasin B binding to the 20-kilodalton tryptic fragment at the interior of the cell; Baldwin et al., 1982; Deziel & Rothstein, 1984) argues against such a shuttle mechanism for transport. Nevertheless, the turnover time of the transporter (concentration of sites/V_{max}, $\simeq 1$ ms) is too rapid to be consistent with diffusion of hexose through a water-filled pore or channel of 5-nm length (Naftalin & Holman, 1977). Discussion of other possible mechanisms for transport is, at this stage, without experimental basis.

Two general types of model can be considered for the band 4.5 protein/lipid interaction. The "solubility model" requires that the protein adopts the most energetically favorable conformer in order to allow a minimum perturbation of the preferred packing of bilayer lipids. Here, the activity is a reflection of the protein conformer adopted. The "steric model" describes lipid/protein packing in which protein structure is relatively unaffected by the lipid but where activity depends upon steric interactions between lipid and protein. Our data do not permit distinction between these models. Studies of the substrate-induced modification of tryptophan fluorescence of band 4.5 protein (Gorga & Lienhard, 1982) may indicate whether possible conformational changes are prevented in these synthetic membranes or whether different conformers are adopted in different environments.

These studies remain to be extended to other classes of phospho- and glycolipids where lipid head-group interactions with the transport system can be examined and lipid preferences (if any) of the transport protein evaluated. Nevertheless, they raise the intriguing possibility that the characteristics of transport in native, eukaryotic membranes may be used as a probe of the lipid environment of the protein and consequently the microorganization of the membrane. Recognizing that lipid organization in the human erythrocyte plasma membrane is asymmetric (Op den Kamp, 1979) and, in addition, that the lecithins employed in the above studies are of limited class and species composition compared to those in the red cell membrane, some conclusions may be drawn from our studies. E_a and A for transport in the red cell ghost (see Table I) are similar to those for transport in synthetic membranes consisting of C16 and C18 saturated and unsaturated lecithins. In addition, the Arrhenius and E_a parameters are some 9 orders of magnitude and 4-fold greater, respectively, than the equivalent estimates made in DPL/cholesterol (52:48 mol %) bilayers. This might suggest that in native erythrocytes, hexose transport is mediated by transport proteins located in a substantially cholesterol-free membrane environment.

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