Lactose Carrier Protein of *Escherichia coli*: Interaction with Galactosides and Protons†

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ABSTRACT: The binding of galactosides to the lactose carrier of cytoplasmic membrane vesicles derived from *Escherichia coli* strains T185 or T206 which harbor plasmids bearing the *lac*Y gene is measured and compared to the active transport of galactosides in vesicles or cells of the haploid strain ML308-225 in terms of the apparent affinity. Lactose and other galactosides bind only to a single site on the carrier protein. The affinity of lactose for this site is low (K_D = 14 ± 5 mM), while the half-saturation constant for active transport, K_T, is 0.085 ± 0.007 mM, approximately 160-fold smaller. Other galactosides exhibit smaller differences between K_D and K_T, the ratio of K_D to K_T ranging from 48 to 1. The turnover number of the carrier for lactose transport is 2.9 s^{-1} in membrane vesicles and 48 s^{-1} in EDTA-treated cells. Galactoside binding and transport parameters are independent of the bulk pH in the range 5.5–8.0. The binding of β-D-galactosyl 1-thio-β-D-galactoside to the lactose carrier elicits the binding of less than 0.1 proton per galactoside. The fatty acid composition of the plasmid-harboring unsaturated fatty acid auxotroph strain T200E1a is manipulated by supplementation with elaidate, palmitelaidate, or oleate. The midpoint of the ordered ↔ fluid transition of the membrane phospholipids lies at 32, 27, or 14 °C, respectively. Arrhenius plots of the rate of *o*-nitrophenyl β-D-galactoside transport (in vivo) exhibit corresponding downward changes in slope at 34, 26, or 15 °C, respectively. In contrast to the effect upon transport, the lipid phase transition does not affect substrate binding to the carrier. Over the same temperature interval (40–14 °C), the dissociation constant, K_D, for *p*-nitrophenyl α-D-galactopyranoside decreases from 50 to about 10 μM, and a plot of log K_D vs. $1/T$ is linear throughout the temperature range of the phase transition. The number of binding sites remains constant at about 0.5 nmol/mg of crude membrane protein. The experimental results are discussed in terms of a galactoside–proton symport model which has the following features. There is a single galactoside binding site on the carrier protein. Galactoside and proton binding occur in a random order independently of one another. Galactoside and proton binding represent rapid preequilibria to the relatively slow translocation steps. Galactoside–proton symport arises from the coupling of fluxes of the cosubstrates and not from the coupling of their binding. Because binding, in contrast to transport, is not affected by the lipid phase transition, a carrier translocation step is most likely adversely influenced by the transition. This finding is in agreement with assertions that galactoside binding is more rapid than the transport cycle. The apparent increase in affinity from the equilibrium dissociation constant, K_D, to the half-saturation constant for active transport, K_T, observed for lactose and certain other substrates is discussed in terms of either a change in the affinity of binding or an increase in the mobility of the loaded carrier in the presence of a transmembrane electrochemical potential difference. The physiological importance of this effect is that kinetic saturation of the carrier in the presence of an electrochemical potential difference, $\Delta\tilde{\mu}_{H^+}$, is attained at substrate concentrations far below the equilibrium binding constant measured in the absence of $\Delta\tilde{\mu}_{H^+}$.

Although β-galactoside transport in *Escherichia coli* has been under continued study ever since its first description in 1956 (Rickenberg et al., 1956), the detailed mechanism of substrate translocation by the lactose carrier (lactose permease, "M-protein", product of the Y gene of the *lac* operon) across the cytoplasmic membrane remains unknown. The demonstration by West & Mitchell (1972, 1973) that the carrier catalyzes the concomitant translocation of a sugar molecule and a proton has led to a clarification of the energetic aspects of active uptake. At low sugar concentrations, galactoside accumulation tends to come into equilibrium with the transmembrane electrochemical potential difference ($\Delta\tilde{\mu}_{H^+}$)[1] in a fashion suggesting a 1:1 proton:galactoside symport (West & Mitchell, 1972, 1973; Ramos & Kaback, 1977; Padan et al., 1976; Zilberstein et al., 1979; Booth et al., 1979). Carrier-mediated galactoside movement across the membrane responds to both components of $\Delta\tilde{\mu}_{H^+}$, the electrical potential difference, $\Delta\psi$, and the pH difference, ΔpH (West, 1970; West & Mitchell, 1972; Hirata et al., 1973, 1974; Altendorf et al., 1974; Schuldiner & Kaback, 1975; Flagg & Wilson, 1976, 1977, 1978; Kaczorowski et al., 1979). At high substrate concentrations, the level of solute accumulation departs from this ideal behavior because the intracellular concentration attains a maximal value (Rickenberg et al., 1956; Kepes, 1960, 1971). Therefore, substrate accumulation is kinetically modulated by a passive or carrier-mediated leak or both which opposes the driving force, $\Delta\tilde{\mu}_{H^+}$. Consistent with the partic-

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[1] Abbreviations used: lactose, 4-*O*-β-D-galactosyl-α-D-glucose; MeSGal, methyl 1-thio-β-D-galactoside; PrSGal, isopropyl 1-thio-β-D-galactoside; NpβGal, *o*-nitrophenyl β-D-galactoside; NpαGal, *p*-nitrophenyl α-galactoside; DnsLac, 2′-*N*-dansylaminoethyl β-lactoside; GalSGal, β-D-galactosyl 1-thio-β-D-galactoside; DnsEtOGal, 2′-*N*-dansylaminoethyl 1-*O*-β-D-galactoside; DnsEtSGal, 2′-*N*-dansylaminoethyl 1-thio-β-D-galactoside; melibiose, 6-*O*-α-D-galactosyl-α-D-glucose; raffinose, *O*-α-D-galactosyl-(1→6)-α-D-glucosyl β-D-fructoside; stachyose, α-D-galactosyl-α-D-galactosyl-α-D-glucosyl-β-D-fructose; $ClHgBzSO_3$, *p*-(chloromercuri)benzenesulfonate, sodium salt; $ClPhzC(CN)_2$, carbonyl cyanide *m*-chlorophenylhydrazone; $\Delta\tilde{\mu}_{H^+}$, the transmembrane gradient of the proton electrochemical potential composed of the pH gradient, ΔpH, and the electrical potential gradient, $\Delta\psi$; β-galactosidase, EC 3.2.1.23; elaidate, *trans*-9-octadecenoate; palmitelaidate, *trans*-9-hexadecenoate; oleate, *cis*-9-octadecenoate; 12:0, dodecanoate; 14:0, tetradecanoate; 16:0, hexadecanoate; 16:1, hexadecenoate; 18:0, octadecanoate; 18:1, octadecenoate; T_h, T_m, and T_l, temperatures at the onset, midpoint, and completion of the fluid → ordered lipid phase transition.

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ipation of the carrier in this leak pathway is the observation that the level of substrate accumulation depends on the number of carriers per cell (Maloney & Wilson, 1973).

The initial steps in the transport cycle likely consist of the binding of galactoside and proton to the carrier protein. Binding of β-D-galactosyl 1-thio-β-D-galactoside (GalSGal) to the carrier present in crude membrane fractions of *E. coli* was first detected by Fox, Kennedy, and co-workers and led to the initial characterization of the *lac*Y gene product (Fox & Kennedy, 1965; Carter et al., 1968; Jones & Kennedy, 1969; Kennedy et al., 1974). More recently, studies of galactoside binding to the carrier have been conducted by employing membranes with elevated carrier levels derived from strains bearing *lac*Y-containing hybrid plasmids. Galactoside binding is a spontaneous reaction, and the stoichiometry of galactoside–carrier binding is close to 1 (Teather et al., 1978; Overath et al., 1979; Teather et al., 1980).

First, an analysis of the sugar–carrier interaction is presented. The carrier translocates a large variety of α- and β-galactosides (Kepes & Cohen, 1962; Kennedy, 1970; Schuldiner & Kaback, 1977; Sandermann, 1977). A puzzling observation made in Kennedy's laboratory was that several substrates including the physiological substrate lactose did not inhibit the binding of the high-affinity substrates GalSGal and *p*-nitrophenyl α-D-galactoside (NpαGal) (Kennedy et al., 1974). These authors concluded that the carrier contains two different sugar binding sites. As discussed in preliminary reports (Wright et al., 1979; Wright & Overath, 1980; Overath & Wright, 1980) and documented in detail in this paper, the transport protein possesses, in fact, only a single type of binding site which recognizes all transported substrates.

Second, the relationship between galactoside and proton binding is investigated. The binding of GalSGal to the carrier does not induce proton binding, suggesting that substrate and proton binding are independent and represent rapid pre-equilibria to the translocation steps.

Finally, galactoside binding to the carrier protein in membranes which have an altered acyl chain composition of the membrane phospholipids is measured. These membranes, therefore, undergo an ordered ↔ fluid phase transition in the physiological temperature range. Binding is not influenced by the phase transition, suggesting that the extensively documented effect of the physical state of the membrane on the transport rate [Schairer & Overath, 1969; Wilson et al., 1970; see Overath & Thilo (1978) for a review] is exerted on the level of translocation rather than binding.

Materials and Methods

Strains, Growth of Bacteria, and Isolation of Membranes. The plasmid-harboring *Escherichia coli* strains T185 [*lac* $I^+O^+Z^-Y^-(A^+)$/F′ *lac* $I^qO^+Z^{U118}(Y^+A^+)$/pTE18 *lac* Δ-(I)O^+Δ(Z)Y^+Δ(A)] and T206 [*lac* $I^+O^+Z^-Y^-(A^+)$/F′ *lac* $I^qO^+Z^{U118}(Y^+A^+)$/pGM21 *lac* Δ(I)O^+Δ(Z)Y^+Δ(A); see Teather et al. (1978, 1980) for exact genotypes of all strains] were grown in minimal medium in the presence of pyrrolidinomethyltetracyclin (Hoechst, Frankfurt am Main, FRG). Cytoplasmic membrane vesicles were isolated by a variation of the procedure of Osborn et al. (1972) as described by Teather et al. (1980). Crude membrane preparations were obtained by disruption of cells chilled in an ice bath by a Branson B-12 sonicator operating at 80–100 W. After removal of cells by centrifuging 20 min at 1500*g*, the membranes were isolated by centrifugation for 90 min at 150000*g*. The preparation was washed an additional 2 times. Strain ML308-225 (*lac* $I^-Z^-Y^+A^+$) was grown in minimal medium containing 0.4% sodium succinate (Devor et al., 1976). Active transport competent vesicles were isolated as described by Short et al. (1975) and Kabak (1971). Strain ML308 (*lac* $I^-Z^+Y^+A^+$) used for the measurement of NpβGal hydrolysis in vivo was grown identically.

The fatty acid auxotroph and plasmid-harboring strain T200E1a was constructed as follows. A *fab*$^-$ derivative of strain T27RT [*lac* $I^+O^+Z^+Y^-(A^+)$] was isolated after *N*-methyl-*N*-nitrosoguanidine mutagenesis which only grew in the presence of oleate. From strain AM 41, an F′ factor [*lac* $I^qO^+Z^+Y^-(A^+)$] was introduced by conjugation. This strain was further transformed with the plasmid pGM21 carrying the genes for the lactose carrier and tetracyclin resistance, yielding strain T200 which has the same *lac* genotype as strain T206. The derivative T200E1a was isolated by two passages on minimal medium agar plates containing methionine, threonine, and elaidate. T200E1a was grown in the same medium as T206 containing 0.5% Brij 35 and 0.01% elaidic, palmitelaidic, or oleic acid additionally as noted in the text. Cells used for the measurement of NpβGal hydrolysis were harvested from a culture in which lactose carrier synthesis had been induced by 0.1 mM PrSGal and washed twice in minimal salts medium. Crude membrane vesicles were prepared where indicated by sonication as described above. For certain measurements, cells of strain ML308-225 and ML308 were subjected to the EDTA treatment of Leive (1965).

Sugars. DnsEtOGal and DnsEtSGal were synthesized according to the method of Schuldiner et al. (1975). DnsLac was the gift of Dr. R. Weil of Sandoz, Vienna, Austria. All other sugars were of the best available grades. [6-^{3}H]Lactose, [6-^{3}H]NpαGal, and [6,6′-$^3H_2$]GalSGal were prepared as described by Kennedy et al. (1974). Some experiments were performed with [6,6′-$^3H_2$]GalSGal which was the gift of Dr. J.-P. Belaich, Marseille, France. [*methyl*-^{14}C]MeSGal was purchased from CEA, Gif-sur-Yvette, France.

Galactoside Binding Studies. The binding of radioactively labeled galactoside was measured by flow dialysis as previously described (Teather et al., 1980). The protein concentration was between 7 and 12 mg/mL for cytoplasmic membrane vesicles (T strains) and at least twice this value for crude membrane preparations. Samples contained 4–8 kBq [6,6′-$^3H_2$]GalSGal or 20–30 kBq [6-^{3}H]NpαGal. The flow dialysis cell was equipped with a thermostated collar in the upper compartment for temperature control. Binding curves were obtained by titrating a sample with a concentrated solution of the unlabeled ligand (total binding) or by measuring the amount of ligand released after the addition of $ClHgBzSO_3$ (0.4 mM) to different samples (sulfhydryl-dependent binding). The binding of lactose and other unlabeled galactosides were measured by inhibiting the binding of a labeled substrate. Two methods were used. The concentration of the unlabeled ligand was constant, and the concentration of the labeled ligand was varied. This experiment was repeated at several concentrations of the unlabeled ligand. The apparent dissociation constant ($K_{D,app}$) of the labeled ligand in the presence of the unlabeled one was evaluated, and the dissociation constant of the unlabeled ligand was determined from a replot of the apparent K_D against the concentration of unlabeled ligand, and the dissociation constants were determined according to

$$K_{D,app} = K_D\left(1 + \frac{[G]}{K_G}\right)$$

where K_D is the true dissociation constant of the labeled ligand and K_G is the dissociation constant of the unlabeled galactoside (G). Alternatively, the concentration of the labeled ligand was held constant, and the concentration of the unlabeled ligand

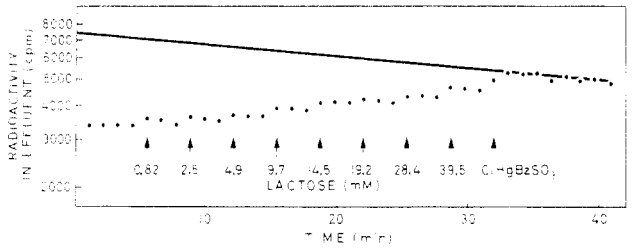

FIGURE 1: Lactose inhibition of NpαGal binding measured by flow dialysis. The sample in the upper compartment contained cytoplasmic membrane vesicles from strain T206 (8.3 mg of protein/mL) and 9.7 μM NpαGal (activity 2.7 TBq/mol). The logarithm of the radioactivity in the effluent from the lower chamber is plotted against time. Addition of lactose to final concentrations of 0.82, 2.5, 4.9, 9.7, 14.5, 19.2, 28.4, and 39.5 mM (first eight arrows) increases the fraction of NpαGal not bound to the carrier which is reflected by an increase in the radioactivity passing into the lower chamber. The addition of the inhibitor $ClHgBzSO_3$ (0.4 mM) to the membrane suspension releases all galactosides from the carrier (last arrow). The subsequent points are used to construct a line corresponding to the absence of NpαGal binding to the carrier. Specific binding to the carrier is indicated by the difference between this line and the measured radioactivity. The addition of lactose causes the rapid attainment of a new equilibrium between bound and unbound NpαGal as demonstrated by the stepwise increase of radioactivity in the effluent. Because of the high carrier level (3.1 nmol/mg of protein) and the small size of the sonicated vesicles, equilibration of galactoside across the membrane should be rapid.

was varied. The data were evaluated from the slopes of $\bar{\nu}^{-1}$ against the concentration of the unlabeled ligand, where $\bar{\nu}$ is the occupancy of the binding sites by labeled ligand (e.g., NpαGal, GalSGal) for different concentrations of labeled galactoside. The dissociation constant of the unlabeled galactoside (K_G) was obtained from

$$K_G = R\left(1 + \frac{[S]}{K_S}\right)^{-1}$$

where R is the ratio of intercept to slope in the preceding plot and S is the concentration of free, labeled galactoside whose dissociation constant is K_S. Under the conditions of the flow dialysis experiment, S is constant to within 6% during the study. Experiments were conducted at 18–20 °C in 50 mM potassium hydrogen phosphate buffer, and the pH was 6.6 unless otherwise indicated. The indirect determination of the K_D for lactose by flow dialysis is presented in Figure 1. The binding of dansylated galactosides was determined fluorometrically (Overath et al., 1979). The binding of some galactosides was measured by their ability to inhibit the binding of fluorescent galactoside. Dissociation constants were evaluated from replots of the data as described by the first equation above.

Transport in Vesicles and Cells. Vesicles from strain ML308-225 were diluted in 50 mM potassium hydrogen phosphate and 1 mM $MgSO_4$ of the appropriate pH, centrifuged for 10 min at 12000g, and resuspended in the same buffer. The suspension (2.5 mg/mL, 300–450 μg/sample) was thermostated at 25 °C and aerated under a stream of oxygen. Lithium D-lactate (Paesel KG, Frankfurt, FRG) was added to a final concentration of 20 mM, and the sample was further incubated for 60 s, whereupon labeled substrate (0.3–2 kBq) was added. Transport was halted after 3, 5, and 10 s by the addition of 5 mL of phosphate buffer containing 2 mM $HgCl_2$. The inclusion of $HgCl_2$ in the stop and wash buffers ensured that only *accumulated* galactoside was measured, as $HgCl_2$ inhibits galactoside binding. $HgCl_2$ also appears to render the vesicles very impermeable to sugars. Vesicles were collected on 6VG glass fiber filters (Schleicher & Schuell, Dassel, FRG) and washed 3 times under suction with 5 mL of the above solution. The wash process lasts approximately 6 s. When the accumulation of NpαGal 2 min after addition of substrate was measured, the filters were washed only once with 4 mL of buffer. The substrate uptake was linear up to 10 s.

EDTA-treated cells of strain ML308-225 and ML308 were suspended in 50 mM potassium hydrogen phosphate and 2 mM $MgSO_4$, pH 6.0, containing 20 mM sodium succinate at a concentration of 0.6–0.9 mg of protein/mL. The suspensions were thermostated at 25 °C and aerated for 60 s. Substrate was added, and transport was halted after 5, 10, or 15 s by the addition of 50 mM phosphate buffer, pH 6.0, containing 2 mM $HgCl_2$. The cells were collected on glass fiber filters and washed thrice with 5 mL of the above solution. Filters were transferred to vials with 5 mL of Quickszint 212 (Zinsser, Frankfurt/M., FRG), gently shaken, and counted. Initial rates were linear only for 5–10 s. The steady-state level of substrate accumulation was also determined by flow dialysis. A solution of 800 μL of 50 mM potassium hydrogen phosphate and 2 mM $MgSO_4$, pH 6.0, containing 20 μM radioactive substrate (2–7 kBq) was present in the upper chamber. After the collection of eight effluent fractions, cells of strain ML308-225 were added so that the ratio of intracellular to extracellular volume was 1:81 [using a value of 5 μL/mg of cell protein (Zilberstein et al., 1979)]. Accumulated substrate was released by the addition of a freshly prepared ethanolic solution of ClPhzC-$(CN)_2$ to a final concentration of 20 μM. The transport of fluorescent substrates in vesicles was performed as previously described in O_2-saturated buffer (Overath et al., 1979) with lithium D-lactate (20 mM) as the substrate of the respiratory chain. The protein concentration was 0.35 mg/mL. The inhibition of the transport of fluorescent galactosides by certain galactosides was used to determine kinetic inhibition constants which are also identified with K_T, the half-saturation constant for active transport. NpαGal hydrolysis in vivo was measured in minimal salts medium, pH 7.0. A millimolar absorptivity of 3.0 mM^{-1} cm^{-1} for *o*-nitrophenol was employed. The pH dependence of cellular NpβGal hydrolysis was measured by a discontinuous transport assay. To 1.0 mL of minimal salts medium, pH 5.5, 6.6, or 7.8, were added 5–200 μL of 25 mM NpβGal, 25 μL of H_2O or 0.2 M GalSGal, and 50 μL of cells, 2 mg/mL. After 0.5, 1.0, or 2.0 min, 2 mL of 1 M Na_2CO_3 was added, and the cells were pelleted by centrifugation. The absorbance of the samples was measured at 420 nm. A millimolar absorptivity for *o*-nitrophenol of 4.7 mM^{-1} cm^{-1} was determined under these conditions.

pH Measurements for H^+ Binding Studies. A suspension of T206 cytoplasmic membrane vesicles (1 mL) was magnetically stirred in a glass vessel thermostated at 25 °C. The contents of glass syringes containing 10.1 mM HCl, 10.1 mM NaOH, or 20 mM GalSGal were introduced into the sample via Teflon tubing. The pH of the suspension was monitored by an Ingold 405-M5 combined electrode. The signal was transferred to a recorder by using the offset mode of a Knick 645 potentiometer. The response of the system was calibrated by the addition of standard acid and base solutions to the suspension. The electrode and sample vessel were enclosed in a Faraday cage to achieve additional stability.

Results

Binding Constants for Sugar–Carrier Interaction. Binding constants, K_D, provide basic information on the initial interaction of sugars with the transport protein. Therefore, K_D values for a series of substrates which, with the possible exception of glucose and stachyose, are known to be transported via the lactose carrier have been determined. Binding was

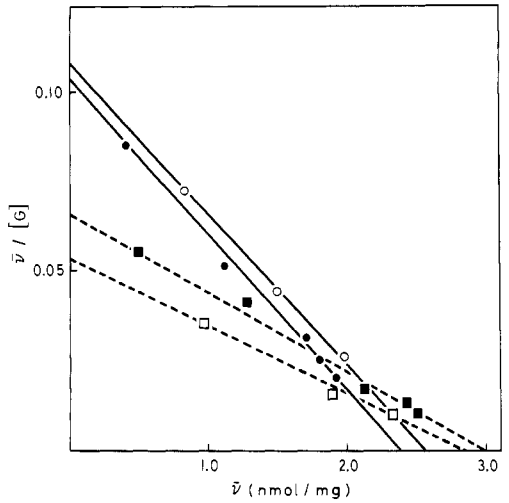

FIGURE 2: Binding of galactosides to cytoplasmic membrane vesicles derived from strain T206. The data are presented as a plot of $\bar{\nu}/[G]$ vs. $\bar{\nu}$, where $\bar{\nu}$ is the mean binding site occupancy (nanomoles of galactoside per milligram of vesicle protein) and [G] is the concentration of unbound galactoside (micromolar). Both the total (●) and $ClHgBzSO_3$-sensitive (○) binding of NpαGal to the carrier are indistinguishable in terms of affinity (K_D = 23 vs. 24 μM) and stoichiometry (n = 2.4 vs. 2.6 nmol of galactoside/mg of protein). Similarly for GalSGal, total (■) and $ClHgBzSO_3$-sensitive (□) binding are identical (K_D = 45 and 52 μM; n = 3.0 and 2.8 nmol of galactoside/mg of protein, respectively). In all cases, the number of binding sites is nearly identical.

measured by the flow dialysis method of Colowick & Womack (1965) by using sonicated cytoplasmic membranes from strains T185 or T206 which harbor the *lac*Y gene on multicopy plasmid vectors (Teather et al., 1980). Within the time scale of flow dialysis titration experiments, transport via the carrier rapidly establishes a new equilibrium concentration of substrate across the vesicular membrane (see Figure 1).

Equilibrium binding constants and total substrate binding can be determined either by the stepwise displacement of bound labeled substrate by an excess of unlabeled ligand or by the release of bound substrates through the addition of $ClHgBzSO_3$, a sulfhydryl reagent and a potent inhibitor of substrate binding and translocation. Binding curves obtained by these two methods (Figure 2) for NpαGal are essentially identical in terms of substrate affinity (K_D = 23 vs. 24 μM) and binding stoichiometry (n = 2.4 vs. 2.6 nmol/mg of protein). A comparison of GalSGal binding to the same membrane preparation confirms this observation (K_D = 45 vs. 52 μM; n = 3.0 vs. 2.8 nmol/mg of protein). In both cases, only a single class of sites with respect to affinity and $ClHgBzSO_3$ sensitivity is evident in the concentration ranges examined. The number of binding sites for both ligands appears identical. Cytoplasmic membrane vesicles derived from strains T206 or T185 not induced for the *lac* carrier bind less than 0.1 nmol/mg of protein of either substrate within the same concentration ranges. Galactoside binding to the carrier is spontaneous and is not inhibited by NaN_3, gramicidin J or D, $ClPhzC(CN)_2$, valinomycin, or nigericin (Overath et al., 1979; Teather et al., 1980).

Lactose binding to carrier-rich cytoplasmic membrane vesicles cannot be measured up to a lactose concentration of 1 mM, although the half-saturation constant for active transport, K_T, is about 0.1 mM (see below). Lactose inhibits NpαGal binding markedly only at higher concentrations (10–40 mM, Figure 3). Analysis of the concentration dependence of this competitive inhibition is consistent with an equilibrium inhibition constant (for simplicity, also designated K_D) of 14 ± 2 mM for lactose. The lactose binding sites detected by the inhibition of NpαGal binding are homogeneous with respect to affinity, as the double logarithmic plot of a NpαGal saturation function vs. the lactose concentration has a slope of unity (range 0.8–1.2, Figure 3B). The inhibition of GalSGal binding by lactose follows an analogous pattern: lactose inhibition is competitive and of low affinity (Table I, line 1; Wright & Overath, 1980), K_D = 18 mM. The average value of K_D for lactose is 14 ± 5 mM.

The K_D values for other sugars were determined similarly (Table I). Methyl 1-thio-β-D-galactoside (MeSGal) possesses a dissociation constant of 42 mM, which is only slightly smaller than that for D-galactose (Table I, lines 2 and 10). A potent inducer of the *lac* operon, isopropyl 1-thio-β-D-galactoside (PrSGal), exhibits a K_D of 6 mM. A galactoside employed in the determination of carrier activity in *lac* Z^+ (β-galactosidase positive) strains, *o*-nitrophenyl β-D-galactoside

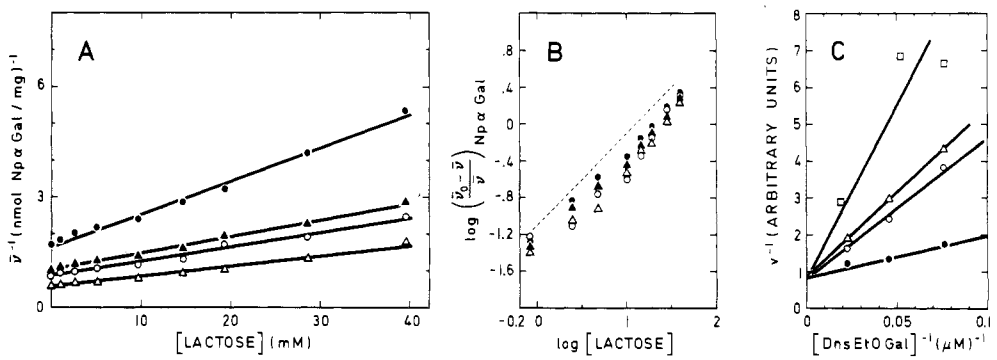

FIGURE 3: Interaction of lactose with the carrier. (A) Lactose binding to the carrier in cytoplasmic membrane vesicles derived from strain T206 is quantified by analysis of the inhibition of NpαGal binding at pH 6.6. The amount of NpαGal bound ($\bar{\nu}$, nanomoles of NpαGal per milligram of vesicle protein) to the carrier at total NpαGal concentrations of 9.7 (●), 19.8 (▲), 32.8 (○), and 40.4 (△) μM is determined as a function of the lactose concentration (0.82–39.5 mM). Lactose competitively inhibits NpαGal binding, and an inhibition constant, K_D = 14 ± 2 mM, can be computed from the relationship $K_D = R(1 + [\text{NpαGal}]/K_D')^{-1}$, where R is the ratio of intercept to slope and K_D' is the dissociation constant for NpαGal. (B) Lactose binds to a single class of noninteracting sites. The double logarithmic plot of $(\bar{\nu}_0 - \bar{\nu})/\bar{\nu}$, where $\bar{\nu}$ is the amount of NpαGal bound (see above) and the subscript indicates this value in the absence of lactose, against the lactose concentration is linear with a slope of unity (the dashed line is a reference with unit slope). The slope varies from 0.8 at low lactose concentrations to 1.2 at the highest concentration. Both the lactose and NpαGal concentrations cover a large section of the binding curve so that conditions of low and high binding site occupancy are examined. The presence of lactose binding sites with different affinities would be reflected in a deviation of the replot of the inhibition data from the appearance of the reference line. (C) Lactose inhibits the active transport of DnsEtOGal supported by D-lactate oxidation by transport-competent vesicles derived from strain ML308-225. The initial velocity of DnsEtOGal uptake (v, in arbitrary units) is measured at pH 6.6 in the presence of 0 (●), 87 (○), 177 (△), and 443 μM (□) lactose. The inhibition is competitive with K_T = 94 μM.

Table I: Equilibrium and Kinetic Constants for the Interaction of Sugars with the Lactose Carrier

	binding[a]		active transport[b]					
			vesicles			cells		
ligand	K_D (mM)	$\Delta\bar{G}_0$ (kJ/mol)	K_T (mM)	k_{cat} (s^{-1})	K_D/K_T[c]	K_T (mM)	k_{cat} (s^{-1})	accumulation[d]
(1) lactose	18 (7)	−10	0.073	2.9	160	0.27	48	709
	14 (5)		0.094 (8)					
	9 (8)		0.082 (9)					
	10 (9)		0.091 (7)					
	15 (5)							
(2) MeSGal	42 (5)	−8			48	0.87	37	341
(3) PrSGal	6 (8)	−12	0.15 (8)		40			
(4) NpβGal	15 (5)	−10			16	0.91	140	
(5) NpαGal	0.022	−26	0.008	0.69	3	0.024	7, 75[e]	99
(6) DnsLac	0.068	−23	0.029		2			
(7) GalSGal	0.053	−24	0.043	0.82	1	0.044	14	903
(8) DnsEtOGal	0.017	−27	0.019	0.4	1			
(9) DnsEtSGal	0.032	−25	0.034	0.4	1			
(10) galactose	50 (5)	−7			4	13 (4)		
(11) glucose	~300 (5)	−3			1	~400 (4)		
(12) melibiose	0.51 (5)	−18			1	0.50 (4)		
(13) raffinose	0.98 (5)	−17			1	0.89 (4)		
(14) stachyose	1.05 (5)	−17			2	0.58 (4)		

[a] Binding to cytoplasmic membrane vesicles derived from strains T206 and T185 with the exception of the last value for lactose which was determined with a crude membrane preparation. The half-saturation constant for binding for certain ligands was determined indirectly by monitoring the binding of a second ligand identified by the number in parentheses next to the value of K_D. The standard free energy of binding was computed from the relation $\Delta\bar{G}_0 = -RT \ln K_D$. The pH was 6.6, and the temperature was about 20 °C. [b] Active transport was measured in vesicles prepared from strain ML308-225 and in EDTA-treated cells of strain ML308-225 or in EDTA-treated cells of strain ML308 when NpβGal (4) was employed. The half-saturation constant for active transport for certain ligands was determined by measuring the inhibition of the transport of a second ligand identified by the number in parentheses next to the value of K_T. The maximal velocity is expressed as the turnover number k_{cat} by assuming 0.2 nmol of lactose carrier/mg of vesicle protein and that vesicles contain 15% of the original cell protein (Kaback, 1971). The temperature was 25 °C and the pH 6.0 except for measurements of NpβGal hydrolysis in vivo which was measured at 28 °C and pH 7.0. [c] The ratio of binding and active transport half-saturation constants. Where K_T values from vesicles were lacking, values from cells were used. [d] Substrate accumulation in washed cells of strain ML308-225 with succinate as substrate. All galactosides were present at an initial concentration of 20 μM. The ratio of the extracellular to intracellular spaces was 81 for an intracellular water content of 5 μL/mg of protein (Zilberstein et al., 1979). Uptake was measured in 50 mM potassium hydrogen phosphate, 2 mM $MgSO_4$, and 10 mM sodium succinate, pH 6.0. [e] Hydrolysis of NpαGal in strain DS-338-2 (Overath et al., 1979).

(NpβGal), binds with a K_D value of 15 mM as judged from the inhibition of NpαGal binding. Both D-galactose and D-glucose are weak inhibitors of NpαGal binding (K_D = 50 and ca. 300 mM; Table I, lines 10 and 11). The sum of the free energy of binding, ΔG_0, for these two sugars approximates the free energy of lactose (galactosylglucose) binding (−10 kJ/mol; Table I, lines 1, 10, and 11). While in the case of lactose, the galactose–carrier interaction provides the greater portion of the binding energy (−7 kJ/mol; Table I, line 10), aromatic aglycon moieties contribute the more substantial part for other galactosides (Table I, lines 5, 6, 8, and 9; see, however, GalSGal, line 7). Melibiose, raffinose, and stachyose have binding energies 7–8 kJ/mol more negative than that of lactose (Table I, lines 12, 13, 14, and 1).

Half-Saturation Constant, K_T, and the $K_D \rightarrow K_T$ Shift. In contrast to K_D, the half-saturation constant, K_T, is a kinetic parameter obtained from the concentration dependence of the initial rate of the active uptake of galactoside. Although half-saturation constants and maximal velocities for active transport have been determined by many authors [see, for example, Kepes & Cohen (1962), Kennedy (1970), Schuldiner & Kaback (1977), and Sandermann (1977)], these data are difficult to compare because of differences in strains, growth, and assay conditions used. The data summarized in Table I are comparable because they have all been determined for EDTA-treated cells of haploid ML strains (ML308 and ML308-225) in the presence of succinate or for membrane vesicles of strain ML308-225 with D-lactate as an energy source (Kaback, 1971).

The initial rate of NpαGal uptake in membrane vesicles (Figure 4, upper curve) and the accumulation of NpαGal in the steady state [Figure 4, lower curve; cf. Rudnick et al. (1976)] vary with the extravesicular concentration of galactoside hyperbolically as evidenced by the linear appearance of double reciprocal plots of these data. The kinetic constant derived from initial rate measurements, K_T = 8 μM, is reproducibly smaller than the equilibrium constant, K_D = 22 μM (Table I, line 5). The saturation of the steady-state substrate accumulation yields a half-saturation constant of 6.4 μM, a value similar to that of K_T. This phenomenon is a nonideality of this system with respect to the chemiosmotic hypothesis (Mitchell, 1966), as the substrate accumulation approaches a theoretical maximum (determined by K_T, V_{max}, and passive efflux) only at the limit of zero substrate concentration (Rickenberg et al., 1956; Kepes, 1960). While for NpαGal K_D differs from K_T only by a factor of three, this discrepancy is most pronounced for the physiological substrate lactose (K_D/K_T = 160; cf. Table I, line 1). Whether measured directly or by the inhibition of uptake of other galactosides (see Figure 3C and Table I), values for K_T agree closely (range 0.07–0.09 mM, average 0.085 ± 0.007 mM) while the average K_D is 14 mM.

The K_D/K_T ratio for various substrates ranges from the extreme value for lactose (K_D/K_T = 160) through intermediate values for MeSGal (K_D/K_T = 48), PrSGal (K_D/K_T = 40), and NpβGal (K_D/K_T = 16) to values equal or close to 1 for the substrates listed in lines 5–14 of Table I. For some substrates, K_T has been determined for both vesicles and EDTA-treated cells. In the case of lactose and NpαGal, K_T for cells appears to be 3 times larger than that for vesicles (Table I, lines 1, 5, and 7).

Accumulation Ratio and Maximum Rate of Active Transport. Galactosides with K_D/K_T = 1 are actively transported into cells of strain ML308-225 as are those with

Table II: Dependence of Galactoside Binding and Transport upon the Bulk External pH

	binding			transport			
		NpαGal		lactose[c]		NpβGal[d]	
pH	lactose[a] K_D (mM)	K_D (mM)	n[b] (nmol/mg of protein)	K_T (mM)	k_{cat} (s^{-1})	K_T (mM)	k_{cat} (s^{-1})
4.0		>0.10	~1				
5.0	21	0.032	2.9				
5.5	11	0.022	1.9[e]	0.20	2.0	0.94	252
				0.3	1.8[f]		
6.0	13	0.024	3.3				
6.6	10	0.023	2.2[e]	0.05	2.7	0.86	283
7.0	15	0.021	2.9				
7.6	16	0.019	1.8[e]	0.17	2.4		
7.8						0.96	231
8.0	~25	0.046	3.1				
9.0	~50	~0.08	~1.5				

[a] Lactose binding was measured indirectly through the inhibition of NpαGal binding to cytoplasmic membrane vesicles. Unless otherwise indicated, vesicles were derived from strain T206. K_D is the equilibrium dissociation constant in units of millimolar. [b] NpαGal binding sites expressed as nanomoles per milligram of vesicle protein. [c] Lactose transport was measured in vesicles derived from strain ML308-225. The respiratory chain substrate was D-lactate (10 mM). The maximal velocity is expressed as a turnover number k_{cat}, and the half-saturation constant for active transport, K_T, is given in units of millimolar. [d] NpβGal hydrolysis in cells of strain ML308 was measured in a discontinuous assay. The absorbance of *o*-nitrophenol was determined after the addition of Na_2CO_3. NpβGal hydrolysis was completely inhibitable by 5 mM GalSGal at all substrate concentrations and pH values. [e] Cytoplasmic membrane vesicles from strain T185. [f] The substrate of the respiratory chain was sodium succinate (20 mM) instead of lactate. Under these conditions, no transmembrane pH gradient is formed in vesicles (Ramos et al., 1976).

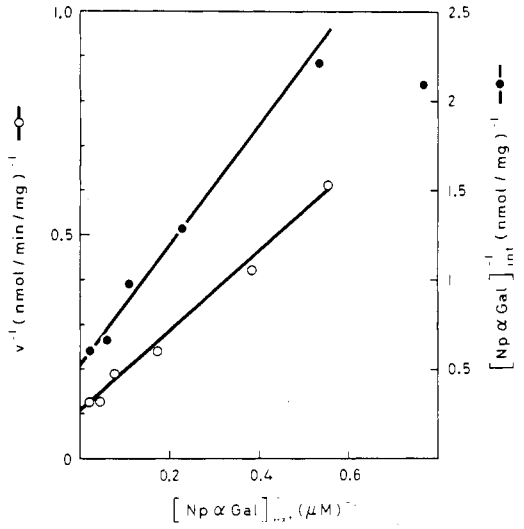

FIGURE 4: NpαGal uptake in vesicles derived from strain ML308-225. A double reciprocal plot of the initial velocity [v, nmol min^{-1} (mg of vesicle protein)$^{-1}$] against the extravesicular concentration of galactoside ([NpαGal]$_{ext}$, μM) yields values of 9.1 μM for the half-saturation constant for active transport, K_T, and 8.3 nmol min^{-1} (mg of protein)$^{-1}$ for the maximal velocity (○, left ordinate). This latter value can be expressed as a turnover number k_{cat} by assuming a carrier level of 0.2 nmol/mg of vesicle protein and corresponds to 0.69 transport cycles s^{-1} at saturation. Analysis of the accumulation of NpαGal ([NpαGal]$_{int}$, nmol/mg of protein) in a double reciprocal plot against the extravesicular concentration of NpαGal in the steady state (●, right ordinate) demonstrates that the intravesicular concentration of galactoside attains a maximum, 1.9 nmol/mg of protein. This amount of galactoside represents only transported substrate, because the vesicles are collected by filtration in the presence of 2 mM $HgCl_2$. The half-saturation constant for this phenomenon is 6.4 μM, nearly identical with the value of K_T. A simple model for galactoside accumulation would require that the accumulation ratio ([NpαGal]$_{int}$/[NpαGal]$_{ext}$) be independent of [NpαGal]$_{ext}$; i.e., the data should lie on a line passing through the origin.

$K_D/K_T > 1$ (Table I, lines 1, 2, 5, and 7). The accumulation ratio ([galactoside]$_{in}$/[galactoside]$_{out}$) varies over an order of magnitude, from 903 for GalSGal to 99 for NpαGal, although the initial substrate concentrations, the ratio of intra- to extracellular volume, and the physiological state of the cells are identical in every case (Table I, lines 1, 2, 5, and 7, and Materials and Methods). Thus, the accumulation of galactoside is not solely dependent upon the magnitude of $\Delta\tilde{\mu}_{H^+}$ (see Discussion).

In vesicles from strain ML308-225, lactose is transported with a greater maximal velocity (expressed here as the turnover number k_{cat}) than that of any of four other galactosides, 2.9 vs. 0.4–0.8 s^{-1} (Table I, line 1 vs. line 5, 7, 8, and 9). At kinetic saturation, the carrier catalyzes 2.9 transport cycles/s with lactose, whereas the turnover number for GalSGal is 3-fold lower. This may be related to the large K_D/K_T ratio of lactose. The expression of maximal velocity in molecular terms emphasizes the relative sluggishness of galactoside transport under these conditions and also facilitates comparison to data from cells. Both the active transport turnover number k_{cat} and the transport constant K_T are larger in EDTA-treated cells than in vesicles (Table I). Lactose and MeSGal (K_D/K_T = 160 and 48; Table I, lines 1 and 2) exhibit larger turnover numbers than NpαGal and GalSGal (K_D/K_T = 3 and 1; Table I, lines 5 and 7), viz., 48 and 37 vs. 7 and 14 s^{-1}, respectively. This is a repetition of the pattern in vesicles where substrates with a large $K_D \rightarrow K_T$ shift are transported more rapidly. These values of k_{cat} are about 10 times larger than the comparable values for vesicles, e.g., 0.82 vs. 14 s^{-1} for GalSGal (Table I, line 7). In the case in which transport is monitored continuously by the hydrolysis of substrate in vivo (NpβGal and NpαGal; Table I, lines 4 and 5), even larger maximal velocities are observed, 75–140 s^{-1}.

Independence of Proton and Galactoside Binding: pH Dependence of Galactoside Binding and Transport. Within the framework of the galactoside–proton symport model for the lactose carrier, the ternary complex of the transport protein with its two cosubstrates is assumed to reorient across the membrane. The coupling of cosubstrate transport can be exerted either on the level of binding or on the level of translocation. If coupling occurs on the level of binding, the order of galactoside and H^+ binding can dictate an obligatory pH dependence of the apparent galactoside dissociation constant [see Discussion and Van den Broek & van Stevenick (1980)]. Both the number of NpαGal binding sites and the affinity of this galactoside for the carrier in cytoplasmic

Table III: Independence of GalSGal and Proton Binding to Lactose Carrier

initial pH	[GalSGal] (mM)	proton uptake by carrier (nmol) obsd[a]	proton uptake by carrier (nmol) theor[b]	remarks
6.11	0.5	2	27	cytoplasmic membranes from induced strain T206, 4.1 nmol of carrier/mg of vesicle protein, 7.4 mg of protein/mL
	1.0	3	29	
6.19	0.5	1	27	membrane preparation as above + 20 μM $ClPhzC(CN)_2$
	1.0	1	29	
6.21	0.5	3	0	membrane preparation as above, pretreated with $ClHgBzSO_3$[c]
	1.0	4	0	
6.17	0.5	1	27	membrane preparation as above + 13 nmol gramicidin J/mg of protein
	1.0	2	29	
6.52	0.5	1	27	membrane preparation as above + 20 μM $ClPhzC(CN)_2$
	1.0	2	29	
6.30	0.1	1	10	membrane preparation as above, 4 mg of protein/mL
	0.2	2	13	
	0.3	4	14	
	0.4	5	14	
6.15	0.5	3	0	membranes from uninduced strain T206, 4 mg of protein/mL
6.18	0.5	2	0	membranes from uninduced strain T206, 7.8 mg of protein/mL
	1.0	5	0	

[a] Numbers correspond to uptake of H^+ (or release of OH^-), i.e., alkalinization of the suspension. [b] Values computed by assuming a 1:1 binding of H^+ and GalSGal to the carrier and by using a value of $K_D = 50$ μM for GalSGal. [c] After $ClHgBzSO_3$ treatment, the vesicles were washed to remove free mercurial ion. No NpαGal binding could be observed.

membrane vesicles from strains T185 and T206 manifest broad maxima between pH 5.5 and 8.0 (Table II). The NpαGal dissociation constant K_D increases sharply as the pH approaches 4.0. This decrease in affinity is coincident with the gross flocculation of the sample and is probably ascribable to adverse structural changes in the carrier. The affinity is lower at pH 9.0 than near neutrality, probably for similar reasons. The profile of the lactose affinity for the carrier over the same pH range is congruent to that for NpαGal. Significantly, the lactose binding constant K_D does not approach the K_T value as the proton concentration is raised.

Kinetic constants for lactose active transport at various pH values are presented in Table II for vesicles from strain ML308-225 with either D-lactate or succinate as an energy source. Neither the maximal velocity expressed as the turnover number k_{cat} nor the half-saturation constant for active transport K_T varies appreciably between extravesicular pH values of 5.5 and 7.6. Ramos et al. (1976) have demonstrated that the intravesicular pH remains constant at 7.6 in this range during oxidation of D-lactate, whereas the internal pH is not regulated by succinate oxidation in vesicles. Thus, variation of the intravesicular pH from 5.5 to 7.6 at an external pH of 5.5 through the choice of the substrate of the respiratory chain is without great impact on the transport parameters (Table II). As will be described in a forthcoming publication [see also Wright & Overath (1980)], facilitated diffusion of lactose into vesicles in the presence of gramicidin J and at pH values from 5.5 to 7.6 on both faces of the membrane shows closely similar values for K_T ($K_T \simeq 25$ mM) and k_{cat} ($k_{cat} \simeq 3\ s^{-1}$). Finally, the transport of NpβGal in EDTA-treated cells of strain ML308 exhibits virtually no pH dependence of k_{cat} or of K_T (Table II) and is completely inhibitable by 5 mM GalSGal between pH 5.5 and 7.8.

Substrate Binding to the Carrier Does Not Induce Proton Binding. In a fashion strictly analogous to the case for galactoside binding, the apparent affinity of the carrier for the symported proton may depend upon the presence of the galactoside cosubstrate. The binding of galactoside could, therefore, induce H^+ binding or a thermodynamically equivalent process. The pH of a suspension of cytoplasmic membrane vesicles from strain T206 with a specific binding activity of 4.1 nmol of GalSGal/mg of protein was monitored. The initial pH was between 6.1 and 6.5. A sufficient quantity of GalSGal was introduced, so that >90% of the binding sites were occupied. If a proton (hydroxide ion) binds, the bulk pH rises (falls). However, no pH change was observed accompanying the addition of GalSGal (Figure 5, Table III) beyond a small alkalinization of the suspension, which was observed also in cytoplasmic membranes from strain T206 not induced for the lactose carrier and in vesicles where the carrier was inhibited by pretreatment with $ClHgBzSO_3$. Calibrations with HCl and NaOH showed that 10% of the expected pH change based upon a 1:1 galactoside–H^+ (or OH^-) binding stoichiometry should have been detectable (Figure 5). The vesicles can be made highly permeable to H^+ by the addition of gramicidin J or $ClPhzC(CN)_2$. No pH change attributable to the binding of GalSGal to the lactose carrier was observed under these circumstances (Table III, experiments 2, 4, and 5). Thus, an intravesicular pH change cannot elude detection, because, in the absence of protonophores, H^+ might not equilibrate across the membrane. The vesicles were sonicated so that the sample should contain vesicles of a normal as well as inverted topology with respect to the cell. In summary, the experiments demonstrate that GalSGal induces the binding of less than 0.1 mol of H^+/mol of carrier binding site.

Galactoside Binding to the Carrier Is Not Affected by the Phospholipid Phase Transition. The well-established effect of the ordered ↔ fluid lipid phase transition upon the transport rate of the lactose carrier [see Overath & Thilo (1978) for a review] could be exerted on the level of substrate binding. NpαGal binding to a crude membrane fraction of strain T200E1a, a *lac*Y plasmid-harboring fatty acid auxotroph supplemented with elaidate, palmitelaidate, or oleate (cf. Figure 6B,E, data for oleate-supplemented cells not shown), has been measured as a function of temperature. For comparison, the temperature dependence of NpβGal hydrolysis in vivo and the temperature range of the ordered ↔ fluid phase transition are presented in panels A and D and in panels C and F, respectively, of Figure 6.

Neither the dissociation constant, K_D, nor the number of total binding sites for NpαGal are affected by the phase transition. A plot of log K_D vs. $1/T$ is linear throughout the region of the phase transition, and the number of binding sites decreases marginally with decreasing temperature. The values of the van't Hoff enthalpy of binding computed from the temperature dependence of K_D are independent of the fatty

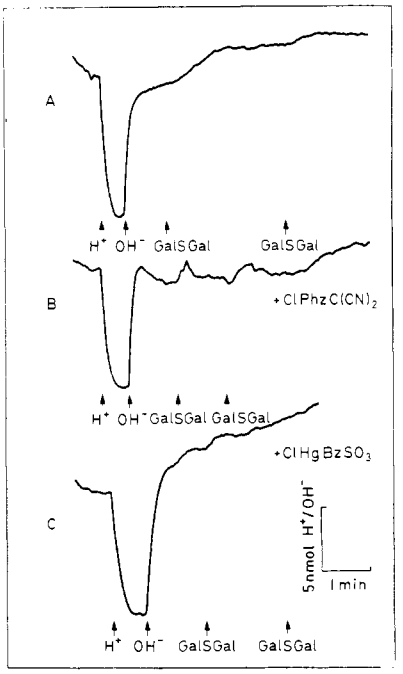

FIGURE 5: GalSGal binding does not induce proton binding. The pH of a suspension of vesicles derived from strain T206 was monitored. The samples contained 7.4 mg of vesicle protein/mL with a specific binding activity of 4.1 nmol of GalSGal/mg of vesicle protein (i.e., 30.3 μM lactose carrier) in 50 mM KCl. A downward deflection corresponds to acidification of the medium. The initial decrease and increase in curves A, B, and C correspond to the addition of 10.1 nmol of HCl and NaOH, respectively, as indicated by the arrows. (A) The initial pH was 6.11. After calibration of the pH meter response, GalSGal was added in succession to the suspension to final concentrations of 0.5 and 1.0 mM (cf. arrows). The small alteration of the bulk pH corresponds to the uptake of 2 and 3 nmol of proton after the first and second addition of GalSGal, respectively. For comparison, assuming a 1:1 stoichiometry of binding of proton and galactoside, a total of 27 and 29 protons would have been expected to be bound after the first and second addition of GalSGal, respectively. (B) Conditions were the same as those for (A) except that 20 μM $ClPhzC(CN)_2$ was present and the initial pH was 6.19 (see Table III, line 2). Addition of GalSGal to final concentrations of 0.5 (first arrow) and 1.0 mM (second arrow) caused a marginal pH change corresponding to the uptake of about 1 nmol of proton after each addition. (C) Conditions were the same as those in (A) except that the initial pH was 6.21 and the lactose carrier was inactive because of pretreatment with $ClHgBzSO_3$. Addition of GalSGal to the membranes washed free of $ClHgBzSO_3$ to final concentrations of 0.5 (first arrow) and 1.0 mM (second arrow) induced the uptake of 3 and 4 nmol of protons, respectively. Similar results were obtained when vesicles from cells uninduced for the synthesis of the carrier were employed (Table III).

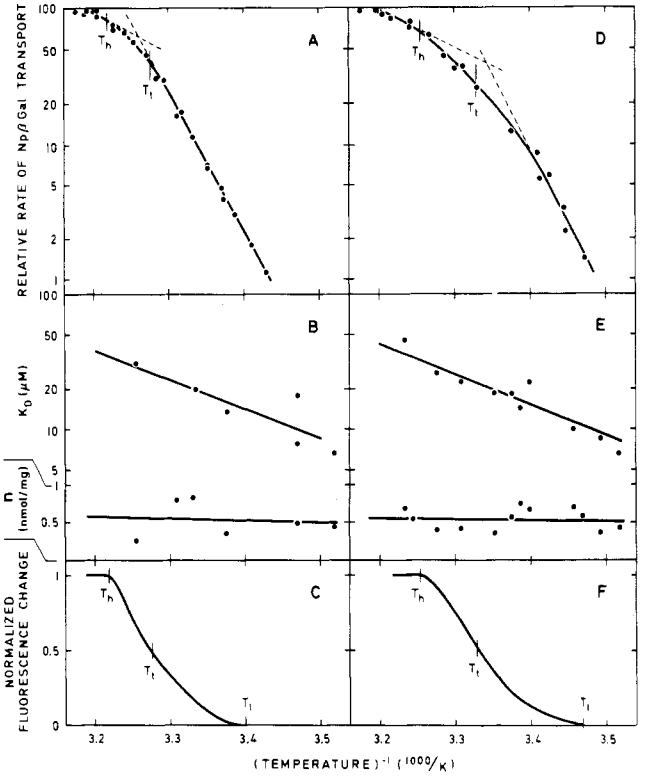

FIGURE 6: Temperature dependence of NpαGal binding and NpβGal transport. (A and D) Temperature dependence of the normalized rate of in vivo NpβGal hydrolysis for cells of strain T200E1a supplemented with either elaidate (A) or palmitelaidate (D). The absolute rate was 1.8 μmol min^{-1} (mg of protein)$^{-1}$ at 40 °C for elaidate- and 1.6 μmol min^{-1} (mg of protein)$^{-1}$ at 40 °C for palmitelaidate-supplemented cells. (B and E) Dissociation constant, K_D, and total number of binding sites, n (nanomoles per milligram of protein), for crude membranes from strain T200E1a using NpαGal as substrate. Binding curves were obtained by stepwise displacement of radioactive NpαGal with unlabeled NpαGal at various temperatures as described for Figure 1. Measurements were performed in 50 mM potassium phosphate–10 mM $MgSO_4$, pH 6.6, in the presence of 10 mM NaN_3. Protein concentration was 10 mg/mL. (C and F) Normalized ordered ↔ fluid phase transition measured in cells or crude membranes in the presence of *N*-phenyl-1-naphthylamine (rising-temperature scans). T_l and T_h refer to the low- and high-temperature ends of the transition, respectively, and T_t refers to the midpoint of the phase transition. The fatty acid compositions of membrane phospholipids (mole percent) were the following: elaidate-supplemented cells, 12:0, 0.4; 14:0, 2.2; 16:0, 38.5; 16:1, 12.5; 18:0, 1.3; 18:1, 46.3; palmitelaidate-supplemented cells, 12:0, 0.8; 14:0, 1.0; 16:0, 43.7; 16:1, 47.4; 18:0, 1.4; 18:1, 5.8. Cis and trans unsaturated fatty acids cannot be differentiated under the conditions of gas chromatography used. The low content of 18:1 in palmitelaidate-supplemented cells most likely represents *cis*-Δ^{11}-18:1 formed by residual endogenous synthesis of unsaturated fatty acids. By analogy, part of 18:1 in elaidate-supplemented cells may be *cis*-Δ^{11}-18:1 rather than medium-derived *trans*-Δ^{9}-18:1.

acid supplement: −41 kJ/mol (elaidate), −43 kJ/mol (palmitelaidate), and −38 kJ/mol (oleate). As the standard free energy of binding for NpαGal is −26 kJ/mol (Table I), the binding of this substrate is enthalpy driven. Belaich et al. (1976) have demonstrated microcalorimetrically that the binding of GalSGal is also enthalpy driven, $\Delta H_0 = -88$ kJ/mol.

The fatty acid composition of strain T200E1a (cf. legend to Figure 6) is much more heterogeneous than that for the previously used strain T105 (Thilo et al., 1977). Therefore, the phase transition is broader for strain T200E1a than for T105, i.e., $\Delta T = 16.5$ °C and $\Delta T = 10$ °C, respectively, for elaidate-supplemented cells [compare Figure 6C with Figure 3 in Thilo et al. (1977)]. The midpoint of the transition, T_t, lies at 32 °C for elaidate-supplemented cells, 27 °C for palmitelaidate-supplemented cells, and 14 °C for oleate-supplemented cells. The temperature dependence of in vivo NpβGal hydrolysis exhibits downward changes in slope at extrapolated temperatures of 34, 26, and 15 °C, respectively, i.e., close to T_t as observed previously. The broadness of the transition is reflected in the relative flatness of the curves. In contrast to the earlier studied strain T105, a second upward change in slope at the low-temperature end of the transition could not be observed. As the lower change in slope formed the basis for a mechanistic interpretation of these Arrhenius plots (Thilo et al., 1977), this discrepancy must be clarified by further studies, preferentially in a reconstituted system with defined phospholipids. The low-temperature change in slope may escape detection in a membrane with a broad phase transition having an ill-defined lower end.

Discussion

A general model of galactoside–proton symport is depicted in Figure 7. The carrier (C) possesses one binding site for galactoside (G) and one for the symported proton (H^+). The

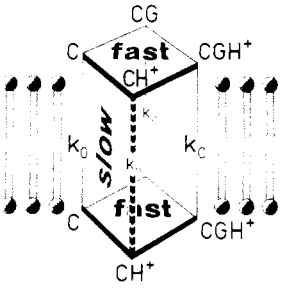

FIGURE 7: Model of galactoside–proton symport. Symbols are the following: G, galactoside; CG, carrier–galactoside complex; CH^+, carrier–proton complex; CGH^+, ternary complex; k_0, rate constant for translocation of free carrier; k_G and k_H, rate constants for the binary complexes; k_c, rate constant for the ternary complex. The magnitude of rate and equilibrium constants may depend upon internal and external pH and upon $\Delta\psi$.

binding of the cosubstrates gives rise to the formation of binary complexes (CG and CH^+) and a ternary complex (CGH^+). All carrier species may translocate across the membrane. The identity of internal and external dissociation constants and the identity of forward and reverse rate constants are not assumed. Any of these constants potentially depends upon $\Delta\tilde{\mu}_{H^+}$ or one of its components. With reference to this model, the following points are discussed: (i) cosubstrate binding, (ii) translocation, (iii) the $K_D \rightarrow K_T$ shift, and (iv) the effect of the lipid phase transition.

Cosubstrate Binding. Only one class of galactoside binding site exists on the carrier. The identity of NpαGal and GalSGal binding stoichiometries, the identity of total and sulfhydryl-dependent binding, and competitive nature of the inhibition of substrate binding by lactose and other galactosides, and the ability to describe binding or inhibition by a single equilibrium constant, K_D (Figures 1–3, Table I) are consistent with the homogeneous nature of substrate binding detectable in the concentration ranges studied. Included in this statement is the assertion that the carrier is functionally symmetrical with respect to galactoside binding in the absence of $\Delta\tilde{\mu}_{H^+}$. There are several demonstrations that the carrier is also functionally symmetrical with respect to active transport (Teather et al., 1977; Lancaster & Hinkle, 1977a,b). Moreover, Overath et al. (1979) and Teather et al. (1980) have shown that the lactose carrier binds approximately one substrate molecule per polypeptide chain. Although the distribution of the binding site orientation with respect to the two faces of sonicated membranes is not known, this site appears to be accessible from both sides of the membrane. Within the time scale of flow dialysis titration experiments, transport via the carrier rapidly establishes a new equilibrium concentration of substrate across the vesicular membrane.

The unique galactoside binding site on the carrier apparently consists of three subsites. The galactosyl moiety binds in one and serves as an anchor for the weakly binding substrates, e.g., lactose or NpβGal. Binding of a galactosyl moiety to this subsite protects the essential SH group. The binding energy provided by this site amounts to about −7 kJ/mol ($\Delta\tilde{\mu}_{H^+}$ = 0, cf. Table I, line 10). Glucose and other carbohydrate residues (e.g., galactose in GalSGal) bind at a second site which provides binding energy between −3 (glucose) and −14 kJ/mol (GalSGal, Table I). The variability of the interaction at this second site is discussed in the context of the lactose $K_D \rightarrow K_T$ shift below. The third subsite appears to be a hydrophobic pocket which in the case of DnsEtOGal delivers −20 kJ/mol (Table I, line 8). The binding of amphipathic molecules at this putative subsite may explain the sensitivity of substrate binding to detergents (Kennedy et al., 1974; Wright et al., 1979; J. K. Wright, unpublished experiments).

The variable discrepancy between the equilibrium parameter, K_D, and the kinetic parameter, K_T, resolves the issue of two classes of galactoside binding sites as postulated by Kennedy and co-workers (Fox & Kennedy, 1965; Carter et al., 1968; Kennedy et al., 1974). Substrates with a large K_D/K_T ratio, i.e., lactose, MeSGal, or PrSGal, are those substrates originally designated type I on the basis of their apparent inability to protect an essential SH group or to displace carrier-bound GalSGal while type II substrates, i.e., NpαGal or GalSGal, with a K_D/K_T ratio close to 1 are those that did protect the SH group and competed for the binding site. Because K_T is a gross overestimate of the affinity of lactose for the carrier (K_D = 14 mM), at least in the absence of $\Delta\tilde{\mu}_{H^+}$, the concentration of lactose present during these earlier experiments (5 mM) was subsaturating. Lactose protects this thiol group; lactose binding as that of GalSGal (Kennedy et al., 1974) is sensitive to Triton X-100, and lactose inhibits GalSGal binding [Wright et al., 1979; Overath & Wright, 1980; Wright & Overath, 1980; see also Kepes (1971)]. Therefore, all observations can be explained by the assumption of a single binding site.

Galactoside and proton binding are independent processes in the absence of $\Delta\tilde{\mu}_{H^+}$. Neither the binding of NpαGal (K_D/K_T = 3) nor the binding of lactose (K_D/K_T = 160) is markedly influenced by the bulk pH in the range 5–8 (Table II). Kennedy et al. (1974) demonstrated that GalSGal (K_D/K_T = 1) binding is also not strongly pH dependent. The binding of a galactoside, regardless of whether a $K_D \rightarrow K_T$ shift is evident, is not influenced by the protonation of any group on the carrier in this pH region. If lactose and proton binding were coupled, then the apparent lactose binding constant must be a function of pH. The lack of an effect of pH on the apparent binding constant is in accordance with the observation that nearly complete saturation of the galactoside binding site of the carrier by GalSGal occasions the binding of less than 0.1 proton per binding site (Figure 5, Table III). In contrast, the binding of proline to its carrier, a proton symporter in *E. coli*, is pH dependent (Amanuma et al., 1977; Motojima et al., 1979). However, no evidence (mutation, chemical modification) demonstrates that the proton whose binding increases the affinity of the carrier for the substrate and the symported proton are one and the same.

The present analysis of proton binding assumes, however, that alteration of the bulk pH results in a change in the local pH in the environment of the carrier and that the occupation of the proton binding site changes between pH 5.5 and 7.6, i.e., $5.5 < pK < 7.6$. Kell (1979) has proposed that a significant portion of the protons generated by respiration do not enter the bulk phase, implying that these two compartments are not in equilibrium with one another. The tendency of galactoside uptake to come into equilibrium with the bulk value of $\Delta\tilde{\mu}_{H^+}$ would tend to justify the assumption.

In terms of the galactoside–proton symport model, the main conclusion from these experiments is that proton and galactoside binding proceed in a random order with noninteracting proton and galactoside sites. These features are illustrated in Figure 7 by the two rhomboids on either face of the membrane. Furthermore, arguments will be presented below that the binding of each cosubstrate constitutes a rapid preequilibrium to the translocation steps.

Translocation. The maximal velocity of active transport in membrane vesicles expressed as the turnover number k_{cat} is 2.9 s^{-1} for lactose and only 0.82 s^{-1} for GalSGal (Table I). The turnover numbers of the lactose carrier in EDTA-treated

cells are about 10-fold higher, $k_{cat} = 48\ s^{-1}$ for lactose and $k_{cat} = 14\ s^{-1}$ for GalSGal [Table I, see also Kepes (1960)]. The rates of hydrolysis of NpαGal and NpβGal in cells are even more rapid (75–250 s^{-1}, cf. Tables I and II). Differences among the values of k_{cat} for different substrates in vesicles or in cells could arise from different values for the rate constant for the translocation of the ternary complex, k_c^+. Alternatively, the initial rate determinations for the uptake of lactose or GalSGal could be underestimates due to failure to correct for substrate efflux, or the rates of translocation of NpαGal and NpβGal may be overestimates due to failure to correct for the accelerating effect of counterflux of hydrolysis products [cf. Huber et al. (1980)]. Similar tendencies are apparent in the work of other authors. Expressing the maximal velocities as k_{cat}, they are as follows: 190 s^{-1} (NpβGal hydrolysis in cells; Winkler & Wilson, 1966); 328 (NpβGal) and 197 s^{-1} (lactose hydrolysis; Maloney & Wilson, 1978); 87 s^{-1} (lactose hydrolysis; Huber et al., 1980); 30–60 s^{-1} (lactose-induced proton flux in cells; Page & West, 1980); and 4 s^{-1} (lactose transport in vesicles; Kaczorowski et al., 1979). The reasons for these differences are at present unknown.

The equilibrium binding constants of the magnitude observed for the carrier are normally characterized by association-dissociation rate constants which are much larger than the turnover numbers of transport. This implies that translocation steps rather than binding are rate determining in the transport cycle. In fact, the turnover numbers lie within the same time scale as that for protein isomerizations. Therefore, substrate binding represents a rapid preequilibrium to slower translocation steps. This view is supported by the fact that the K_T and K_D values for GalSGal are the same [cf. Heinz (1978) and Devês & Krupa (1979)], for which the existence of rapid preequilibria is a necessary but not sufficient condition (cf. eq 2). In the case of substrates with $K_T \neq K_D$, a rapid preequilibrium is also expected as the values of K_T are larger than that for GalSGal (i.e., dissociation of galactoside from the carrier is probably faster) and k_{cat} for these substrates is similar to that for GalSGal. As noted below, the maximal velocities of lactose active transport and facilitated diffusion (influx) are of similar magnitude. In the latter case, $K_T = K_D$, and thus the designation of lactose binding as a rapid preequilibrium appears warranted in both instances.

The active transport parameters do not vary significantly between bulk intra- and extravesicular pH values of 5.5 and 7.6 (Table II). Oxidation of D-lactate by vesicles from strain ML308-225 imposes a nearly pH-independent transmembrane potential difference ($\Delta\psi$ = 60–70 mV) and maintains the intravesicular pH at 7.5 (Ramos et al., 1976). Oxidation of succinate by vesicles produces no transmembrane pH gradient, ΔpH, so that at an external pH of 5.5 the internal pH is also 5.5 ($\Delta\psi$ = 64 mV; Ramos et al., 1976). Thus, the portions of the carrier in the inner and outer aspects of the cytoplasmic membrane are not functionally affected by variation of the pH between 5.5 and 7.5 in the presence of $\Delta\tilde{\mu}_{H^+}$. These data permit the extension of the criterion of independent binding to the case of active transport and help to clarify the mechanism of cotransport. Efficient coupling of galactoside and proton fluxes occurs when the fluxes of the ternary complex (CGH^+) and unloaded carrier (C), $k_c[CGH^+]$ and $k_0[C]$, respectively, are larger than the fluxes of the binary complexes, $k_H[CH^+]$ and $k_G[CG]$ (see Figure 7). Coupling is strongest when these latter fluxes are zero, which occurs in general if $[CH^+] = [CG] = 0$ (equilibrium control) or if $k_H = k_G = 0$ (kinetic control). Independent binding of the cosubstrates implies coupling is maintained by kinetic control, since binary complexes are always present.

$K_D \rightarrow K_T$ Shift. Recent experiments have shown that the half-saturation constant for influx of lactose in the absence of $\Delta\tilde{\mu}_{H^+}$ is similar to the equilibrium binding constant, K_D. Kaczorowski et al. (1979) have determined a value of 19 mM for vesicles of strain ML308-225 in the presence of ClPhzC-$(CN)_2$; Wright & Overath (1980) found a value of 24 mM in the presence of gramicidin J. Moreover, Page & West (1980) demonstrated that in starved, anaerobic, and NaN_3-poisoned cells lactose-induced proton uptake occurs with a K_T value for lactose of 8 mM. Therefore, the $K_D \rightarrow K_T$ shift is dependent upon $\Delta\tilde{\mu}_{H^+}$ or one of its components. However, the maximum rate of influx ($\Delta\tilde{\mu}_{H^+} = 0$) in the presence of gramicidin J and active transport are similar [cf. Wright & Overath (1980) and Table I; see also Kaczorowski et al. (1979)].

Three features of the transport model suggest an explanation of the $K_D \rightarrow K_T$ shift. Two explanations may be grouped under the heading of "affinity effects". If the affinity of the carrier for lactose increases by a factor of R in the presence of $\Delta\tilde{\mu}_{H^+}$ or one of its components, then the half-saturation constant for active transport is related to the reference equilibrium constant in the absence of $\Delta\tilde{\mu}_{H^+}$ as follows:

$$K_T = \frac{K_D}{R} \tag{1}$$

The change in affinity may be ascribable to an alteration in the conformation of the carrier or to the appearance of a proton–galactoside interaction via the carrier (i.e., coupled binding). This latter explanation appears less plausible, because neither does k_{cat} increase nor does K_T decrease strongly with decreasing pH. There appear to be sufficient carrier–galactoside interactions possible, i.e., for the structurally similar GalSGal, to warrant the interpretation of the small K_T of lactose as a K_D value in the presence of $\Delta\tilde{\mu}_{H^+}$ or one of its components. Certain galactosides by virtue of their structure would be unresponsive to this putative conformational change ($K_D = K_T$).

The second explanation of the $K_D \rightarrow K_T$ shift is a "mobility effect". Under the assumption of a rapid preequilibrium for substrate binding, transport parameters are expressed in terms of fundamental rate constants as follows:

$$K_T = \frac{k_0^-}{k_c^+ + k_0^-} K_D \tag{2}$$

and

$$k_{cat} = \frac{k_0^- k_c^+}{k_0^- + k_c^+} \tag{3}$$

where plus and minus superscripts indicate forward and reverse rate constants (cf. Figure 7). Clearly, the term before K_D would be unity in the case of GalSGal and $^1/_{160}$ in the case of lactose. Since k_c^+ is the only substrate-dependent rate constant in this model, this constant must be much larger for lactose than for GalSGal. While a large value of the rate constant for the translocation of the tertiary lactose–proton–carrier complex affects the K_D/K_T ratio dramatically in comparison to that for GalSGal, the value of k_{cat} for lactose would only be increased by a relatively small factor (ca. 3) in comparison with k_{cat} for GalSGal, because the reorientation of the unloaded carrier eventually becomes rate determining ($k_{cat} \leq k_0^-$).

The change from K_D to K_T is not a fundamental aspect of active transport, since a number of galactosides are actively transported which do not exhibit this effect. Its physiological

significance is that kinetic saturation of the carrier in the presence of $\Delta\tilde{\mu}_{H^+}$ is attained at substrate concentrations far below the equilibrium binding constant measured in the absence of $\Delta\tilde{\mu}_{H^+}$. The demonstration of the change from K_T to K_D for lactose or other substrates, e.g., NpβGal, may provide a most sensitive test for the complete deenergization ($\Delta\tilde{\mu}_{H^+} = 0$) of cells.

Effect of the Lipid Phase Transition. The affinity of the carrier and the number of substrate binding sites are not affected by the ordered ↔ fluid phase transition. This result provides a further means to differentiate between binding and transport [see discussion of the results of Thêrisod et al. (1977) in Overath et al. (1979)]. Moreover, this finding is in accord with the view of galactoside binding as a rapid preequilibrium to the slower translocation events.

The effect of the lipid phase transition on transport has previously been discussed with the explicit assumption that it adversely affects the carrier itself rather than the electrochemical driving force (Thilo et al., 1977). Although proof for this assumption eventually rests on the demonstration of this effect in a reconstituted system with a purified carrier, there is reasonable circumstantial evidence to support this notion. First, the phase transition affects the carrier-mediated efflux of MeSGal, a non-energy-requiring process (Overath et al., 1970). Second, in vesicles of strain ML308-225, the electrochemical potential is not affected by the phase transition; i.e., the driving force remains constant under conditions where active transport of 6-*N*-dansylaminohexyl 1-thio-β-D-galactoside is affected (Thêrisod et al., 1978). Third, the phase transition does not induce the $K_T \rightarrow K_D$ shift for NpβGal transport; i.e., the K_T remains essentially unaffected (Thilo et al., 1977). In view of the results presented in this and a previous paper (Wright & Overath, 1980), this implies that at least part of the electrochemical gradient is maintained throughout the transition. The available data suggest that the ordered ↔ fluid transition affects translocation, the most interesting step in the transport cycle.

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Transbilayer Distribution in Small Unilamellar Phosphatidylglycerol–Phosphatidylcholine Vesicles†

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ABSTRACT: The transbilayer distribution of the phospholipids in small unilamellar vesicles comprised of egg phosphatidylglycerol (PG) and egg phosphatidylcholine (PC) was ascertained by ^{31}P NMR. These vesicles, containing 10–75 mol % PG, were formed by sonication (pH 7.6) and fractionated by centrifugation. Data from spectra accumulated in the presence and absence of a paramagnetic shift reagent, Mn^{2+}, indicated that the phospholipids are randomly arranged across the bilayer. The absence of compositional asymmetry, which contradicts earlier reports, is also exhibited by small unilamellar vesicles (50 mol % PG) prepared by the rapid ethanol injection method. Control experiments showed that Mn^{2+} did not induce fusion, permeate the vesicles, or cause the phospholipids to migrate across the bilayer. It has been proposed that the transbilayer distribution of charged phospholipids in membranes is a consequence of the different surface charge densities on the opposing sides of the membrane. Our results suggest that it is the difference in the effective polar headgroup volumes of the components rather than the net charge of one component that determines the packing constraints for mixtures of phospholipids with the same acyl chains, at least in highly curved bilayers.

It seems reasonable to expect membrane constituents to be asymmetrically distributed in biological membranes since the different functions performed on opposing sides of the membrane probably require different structural components. In fact, there is much evidence to support the notion that constituents of biological membranes are vectorially arranged within the bilayer [see review by Op den Kamp (1979)]. Generally, the proteins are distributed with an absolute asymmetry while the various classes of phospholipids are present in both monolayers, albeit in unequal amounts.

Several investigators have attempted to understand the intermolecular interactions that might induce the spontaneous formation of asymmetric phospholipid bilayers by studying binary mixtures of phospholipids in small unilamellar vesicles. Elegant studies by Litman (1973, 1974) indicate that egg phosphatidylethanolamine in small unilamellar vesicles (SUV)[1] comprised of this lipid and egg phosphatidylcholine (PC)[1] is preferentially localized in the outer monolayer at low phosphatidylethanolamine concentrations (<10 mol %) and in the inner monolayer at higher phosphatidylethanolamine concentrations. Phosphatidylserine in small vesicles containing phosphatidylcholine also prefers the inner monolayer (Berden et al., 1975), at least at low phosphatidylserine concentrations (Barsukov et al., 1980). For other mixtures, there has not always been complete agreement. Phosphatidic acid in sonicated phosphatidic acid–phosphatidylcholine vesicles was reported by Berden et al. (1975) to be preferentially localized in the inner monolayer while more recent work (Koter et al.,

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[1] Abbreviations used: PC, phosphatidylcholine; PG, phosphatidylglycerol; SUV, small unilamellar vesicles; EDTA, ethylenediaminetetraacetic acid.

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