The phospholipid requirement for activity of the lactose carrier of Escherichia coli

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The transport activity of the lactose carrier of Escherichia coli has been reconstituted in proteoliposomes composed of different phospholipids. In previous studies, the passive channels or cation-driven transport proteins may influence the biological activity of membrane proteins. The lactose carrier reconstituted with amino phospholipids of increasing degrees of methylation (dipalmitylphosphatidylethanolamine, dioleoylmonomethylphosphatidylethanolamine, dioleoyldimethylphosphatidylethanolamine, and dioleoylphosphatidylethanolamine) revealed a progressive decrease in both counterflow and proton motive force-driven lactose uptake activities. Trinitrophenylation of phosphatidylethanolamine in the E. coli proteoliposomes resulted in a marked reduction in lactose carrier activity. Partial restitution of transport activity was obtained by detergent extraction of the carrier from these inactive proteoliposomes and reconstitution of the carrier into proteoliposomes containing normal E. coli lipid.

These results suggest that the amino group of the amino phospholipids (e.g. phosphatidylethanolamine and phosphatidylserine) is required for the full function of the lactose carrier from E. coli.

Studies in recent years have indicated that variations in the chemical structure of phospholipids of cell membranes may influence the biological activity of membrane proteins. Such protein-lipid interactions have been described for certain membrane-bound enzymes (1, 2) and for active transport proteins (3–8). On the other hand, much less is known concerning the role of phospholipids in the activity of passive channels or cation-driven transport proteins.

The lactose carrier of Escherichia coli is the best studied example of the cation-substrate cotransport systems. This membrane carrier couples the translocation of lactose and protons across the plasma membrane. In previous studies, the interactions between this carrier protein and the hydrocarbon chains of the membrane phospholipids were investigated (9–13). Alterations in the content of various unsaturated fatty acids in the membrane of fatty acid auxotrophs of E. coli resulted in profound changes in lactose transport. While activity of the carrier was normal in a fluid-lipid environment, the transport fell abruptly when the membrane lipid was below the transition temperature.

A possible role of the polar head groups of phospholipids in the lactose carrier activity has not been systematically investigated. A reduction in phosphatidylethanolamine and increase in phosphatidylserine found in a mutant of E. coli had no measurable effect on lactose transport (14). Although several other phospholipid mutants have been described, no data on lactose transport have been reported.

A new approach to this problem became possible after the successful reconstitution of the lactose carrier by Newman and Wilson (15). Extensive manipulation of the lipid environment can be readily accomplished without affecting passive permeability properties of the membrane. The experiments in this paper address the question of the role of the polar head groups of the phospholipids in activating the lactose carrier. Transport was assayed in reconstituted proteoliposomes prepared from mixtures of different phospholipids of defined composition. In addition, experiments are reported in which the phosphatidylethanolamine was modified chemically in the proteoliposome.

EXPERIMENTAL PROCEDURES

Bacteria—E. coli T206 (16), which carries the lac Y gene in a DNA plasmid, was kindly provided by Dr. Peter Overath of the Max-Planck-Institut für Biologie, Tübingen, West Germany.

Materials—The lipids were obtained from Avanti Polar-Lipids, Inc. (Birmingham, AL). Octyl glucoside and valinomycin were purchased from Calbiochem-Behring. 2,4,6-Trinitrobenzenesulfonic acid was from Eastman. p-Hydroxymercuribenzoate solution, Tris, isopropyl-β-D-thiogalactoside and dithioerythritol were from Sigma. MES was obtained from Boehringer Mannheim. 2-Mercaptoethanol was from Bio-Rad. [3H]Lactose and [14C]rubidium chloride were purchased from Amersham Corp. Radioactive lactose was purified by paper chromatography with propanol/H2O (3:1) as the solvent system. The phosphates were from Fisher.

Preparation of Membrane Vesicles—Cells were grown in Medium 63 (17) containing 1% tryptone. Isopropylthiogalactoside (0.5 mM) was added prior to the last two doublings. Membrane vesicles were prepared by passage through a French pressure cell. T206 membranes containing the lactose carrier labeled with [3H]NPG (10 Ci/mmol) were a generous gift from Dr. Ronald Kabeck (Roche Institute of Molecular Biology, Nutley, NJ).

Preparation of Stock Lipo acids—Lipids dissolved in organic solvent

1 The abbreviations used are: MES, 4-morpholinoethanesulfonic acid; MOPS, 4-morpholino propane sulfonic acid; PE, phosphatidylethanolamine; PC, phosphatidylcholine; PS, phosphatidylserine; CL, cardiolipin; DOPE, dioleoylphosphatidylethanolamine; DOPA, dioleoylphosphatidylarachidonic acid; DOPG, dioleoylphosphatidylglycerol; N-methyl-PE, dioleoylmethyethylphosphatidylethanolamine; N,N,N,N-dimethyl-PE, dioleoyldimethylphosphatidylethanolamine; DOPI, dioleoylphosphatidylcholine; TNBS, trinitrobenzenesulfonic acid; [3H]NPG, 4-nitro[2-3H]phenyl α-D-galactopyranoside.
Vortex dispersed. It was stored under Nz gas at methanol-extracted washed by a modification cations. The lactose carrier was solubilized by incubating (18). phospholipid was bath sonicated until clear. For most lipids, sonicated phospholipid a typical experiment, the reconstitution was performed by mixing (50) of the octyl glucoside extract with membranes plus about 100% of the octyl glucoside dilution method were very similar in the size, shape, and structure. The average size of both the proteoliposomes was approximately 100 nm in diameter, and all the structures examined were unilamellar liposomes.

**Preparation of the Proteoliposomes**—Proteoliposomes were prepared as described by Newman and Wilson (15) with certain modifications. The lactose carrier was solubilized by incubating T206 membrane vesicles (1 mg/ml) in 100 mM potassium phosphate buffer, pH 7.0, with E. coli proteoliposomes, 1.5% octyl glucoside, and excess phospholipid (3.7 mg/ml). When the effect of different lipids was investigated, the lipid being studied was added at this step (as well as the reconstitution step). Both 1.25 and 1.5% octyl glucoside concentrations have been used in preparing E. coli proteoliposomes, and no significant difference in lactose transport function was found (data not shown). The mixture was incubated on ice for 10 min and centrifuged at 175,000 × g, for 1 h at 4 °C. The pellet containing unextracted membranes plus about 80% of the exogenous lipid was discarded. In a typical experiment, the reconstitution was performed by mixing 788 μl of the octyl glucoside extract with 4 μl of the appropriate sonicated phospholipid (50 mg/ml), 78.3 μl of 15% octyl glucoside (final concentration, 1.25%). In experiments with phosphatidylinoline and other phospholipid mixtures, 22 μl of 15% octyl glucoside were added at this step (final concentration, 1.5%). The exogenous phospholipid was bath sonicated until clear. For most lipids, 15 min was adequate, but for DOPC, 20-30 min was required. After addition of this mixture (1 ml) on ice for 10 min, it was rapidly injected into 25 ml of 100 mM potassium phosphate buffer, pH 7.0, containing 1 ml dithiothreitol (dilution buffer) at 25 °C. The proteoliposomes formed during the detergent-dilution procedure were sedimented by centrifugation at 85,000 × g for 1 h at 4 °C. Approximately 50% of the liposome was found in the supernatant fluid and 50% in the proteoliposome pellet.

In the typical experiment, proteoliposomes were resuspended in 75 μl of the 100 mM potassium phosphate buffer, pH 7.0. The final concentrations of the protein in different preparations were 0.8–1.5 mg/ml; the phospholipid and detergent concentrations were 0.053–0.089 mg/ml. Protein was assayed by a modification (25) of the method of Schaffner and Weissmann (26). Phospholipid concentration in aqueous dispersion or proteoliposome suspension was determined by the method of Hellen (22). The proteoliposomes were treated with valinomycin by adding 2 μl of 1 mM valinomycin solution (in ethanol) to 50 μl of proteoliposome suspension and vortexed vigorously.

**Counterflow Assay**—The proteoliposomes were loaded with nonradioactive lactose by one of two different techniques. In most experiments, proteoliposomes were incubated in the presence of 20 mM lactose for 1 h at 25 °C to permit sugar equilibration. In a few experiments, 20 mM lactose was added to the dilution buffer during the proteoliposome reconstitution step. This method was found suitable since the passive leakage of phospholipid from the membrane was extremely slow and washing the liposomes with phospholipid-free solution was possible without loss from the internal compartment. All the proteoliposomes were prepared with 100 mM phosphate (unless otherwise indicated). Proteoliposomes (2 μl) were resuspended in 1 ml of 250 mM NaCl solution at 0 °C. After vortexing, the suspension was immediately filtered (using a 0.22-μm CMV Millipore filter) and washed with 1 ml of the ice-cold NaCl solution twice. The paper with the proteoliposome sample was transferred to a phosphate-free disposable glass tube. Sodium dodecyl sulfate (2 ml of a 10% solution) was added, the tube was vortexed to release the phosphate from the proteoliposome. The phosphate was estimated by the method of Dryer et al. (23).

**Trinitrophenylation of the E. coli Proteoliposomes**—E. coli proteoliposomes were trinitrophenylated with 2,4,6-trinitrobenzenesulfonic acid by a modification of the method of Rothman and Kennedy (24). In order to facilitate the trinitrophenylation reaction, the proteoliposomes of E. coli liposomes were formed in 100 mM potassium phosphate buffer at pH 8.0. Six microliters of 30 mM TNBS in 100 mM potassium phosphate buffer, pH 8.0, were added to 30 μl of resuspended proteoliposomes and incubated at room temperature for 30 min. Twelve microliters were diluted into 1.2 ml of 100 mM sodium phosphate buffer, pH 7.0, containing (1°C)lactose (0.018 mM) before the trinitrophenylation reactions started. As a control, 30 μl of TNBS-free 100 mM potassium phosphate buffer, pH 8.0, were added to an additional 30 μl of proteoliposomes.

The degree of the trinitrophenylation was determined by comparing the trinitrophenylated PE spot with the PE spot on the TLC plate. With the procedure given above, more than 60% of the PE of the proteoliposomes was converted to trinitrophenyl-PE.

**Measurement of the Valinomycin-induced Potassium Diffusion Potential**—The pellets of proteoliposomes were resuspended in 100 mM
potassium phosphate, pH 7.0, and valinomycin was added to give a final concentration of 40 μM. In measuring the potassium diffusion potential, the liposomes or proteoliposomes were diluted 50-fold into 100 mM potassium phosphate buffer, pH 7.0, with 20 μM [86Rb]rubidium chloride. Samples were filtered, washed, and counted at various times. The equilibrium level was measured by incubating the valinomycin-treated proteoliposomes with 100 mM potassium phosphate buffer, pH 7.0, with 20 μM [86Rb]rubidium chloride for 40 min to 1 h. Non-specific binding value of the [86Rb] onto the surface of the proteoliposomes and Millipore filter papers was estimated by using valinomycin-free proteoliposomes. This value was subtracted from all the points measured. All the samples were counted in a liquid scintillation counter.

RESULTS

Lactose Counterflow in Proteoliposomes Composed of E. coli Lipid or Egg Lecithin—The lactose carrier was extracted from membranes of induced cells of T206, and transport activity was reconstituted into proteoliposomes composed of E. coli lipid and egg lecithin. Phosphatidylycholine was chosen for study because of its well-known property of readily forming liposomes. In the first series of experiments, the assay for carrier activity was with the counterflow technique. Proteoliposomes were preloaded with 20 mM lactose and then exposed to a low concentration (0.4 mM) of [14C]lactose. Radioactive molecules enter via the carrier and accumulate within the proteoliposome due to competitive inhibition of exit by the high concentration of internal nonradioactive sugar. The accumulation is only temporary, however, since the exit of the preloaded lactose via the carrier leads to a progressive fall in its concentration and reduction of its inhibition of exit of radioactive sugar, leading ultimately to equilibration of radioactive and nonradioactive sugars across the membrane. Fig. 1 shows that the initial rate of lactose uptake for the counterflow assay in proteoliposomes prepared from egg lecithin was 80 nmol of lactose/mg of protein/min, about 12% of the initial rate of proteoliposomes prepared from E. coli lipid (650 nmol of lactose/mg of protein/min). The proteoliposomes containing E. coli lipid showed an earlier peak level of [14C]lactose and a faster fall in concentration compared with the lecithin-containing proteoliposomes. This observation is consistent with the view that the carrier is most active in the presence of E. coli lipid, and rapid exit of nonradioactive preloaded

FIG. 1. Lactose counterflow by reconstituted proteoliposomes of E. coli lipid and egg lecithin. Proteoliposomes were reconstituted as described under "Experimental Procedures" with 100 mM Tris/MES buffer, pH 6.0. The concentrated proteoliposome suspension was incubated with 20 mM lactose at 25 °C for 2 h to permit equilibration of internal and external lactose concentrations. The suspension was then diluted 50-fold into a solution containing 100 mM Tris/MES buffer, pH 7.0, plus 0.018 mM [14C]lactose (final concentration, 0.42 mM). Samples (100 μl) were filtered, washed, and counted. ● E. coli lipid; ○, egg lecithin.

FIG. 2. Lactose counterflow in proteoliposomes composed of different mixtures of E. coli lipid and egg lecithin. The two lipids in chloroform were mixed at the desired ratio, dried with N2 gas and lyophilized under vacuum for 2 h. The dried lipid was then resuspended in 2 mM mercaptoethanol at a lipid concentration of 50 mg/ml. Proteoliposomes were then formed as described under "Experimental Procedures" with 100 mM Tris/MES buffer, pH 6 (see Fig. 1). A, proteoliposomes were prepared from mixtures of differing percentage (w/w) of E. coli lipid and egg lecithin: 100% E. coli lipid (●), 60% E. coli lipid, 40% egg lecithin (○), 40% E. coli lipid, 60% egg lecithin (△), 20% E. coli lipid, 80% egg lecithin (○), and 100% egg lecithin (○). Lactose uptake was assayed at 20, 50, 90, and 120 s. B, initial rate of lactose uptake by proteoliposomes was plotted against the percentage of the E. coli lipid and egg lecithin. The initial uptake rate of the 100% E. coli lipid proteoliposome was taken as the control value of 100%.
lactose molecules allows exit of [14C]lactose. These transport assays were carried out at 22 °C which was well above the transition temperatures of the lipids used in the experiments.

In order to test the possibility that a minor component of the E. coli phospholipid was responsible for activating the lactose carrier, proteoliposomes were prepared from mixtures of E. coli lipid and egg lecithin. Counterflow activities of the carrier in proteoliposomes prepared from mixtures of these two lipids were compared. Fig. 2 shows that the initial rate of transport was increased in proportion to the content of the E. coli lipid in the proteoliposomes. The results provide no evidence that a trace amount of E. coli lipid could fully activate the lactose carrier or that a trace of lecithin was strongly inhibitory.

The fact that low concentrations of lecithin do not seriously interfere with the activity of E. coli lipid allowed the development of an experimental strategy to test different phospholipids. Lipids, which themselves fail to form liposomes (such as PE), were mixed with PC or DOPC, and the mixture was used to prepare stable proteoliposomes suitable for transport studies.

Counterflow in Proteoliposomes Prepared from Different Phospholipids—Proteoliposomes were prepared from a mixture of the three major phospholipids found in E. coli membranes, phosphatidylethanolamine, phosphatidylglycerol, and

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**Table I**

<table>
<thead>
<tr>
<th>Composition of proteoliposomes</th>
<th>Phosphate-trapping volume (μl/mg phospholipid)</th>
<th>[14C]Lactose equilibrium volume (μl/mg phospholipid)</th>
</tr>
</thead>
<tbody>
<tr>
<td>100% E. coli lipid</td>
<td>1.12 ± 0.18 (5)</td>
<td>1.40 ± 0.45 (3)</td>
</tr>
<tr>
<td>50% DOPC + 50% DOPE</td>
<td>1.04 ± 0.12 (4)</td>
<td>1.26 ± 0.32 (4)</td>
</tr>
<tr>
<td>50% DOPC + 50% DOPG</td>
<td>1.02 (2)</td>
<td>1.10 (2)</td>
</tr>
<tr>
<td>50% DOPC + 50% DOPA</td>
<td>1.08 (2)</td>
<td>1.20 (2)</td>
</tr>
<tr>
<td>50% DOPC + 50% cardiolipin</td>
<td>1.14 (2)</td>
<td>1.10 (2)</td>
</tr>
<tr>
<td>50% DOPC + 50% egg lecithin</td>
<td>0.96 (2)</td>
<td>0.85 (2)</td>
</tr>
<tr>
<td>100% DOPE</td>
<td>0.72 ± 0.26 (5)</td>
<td>0.72 ± 0.15 (6)</td>
</tr>
<tr>
<td>100% egg lecithin</td>
<td>1.16 (2)</td>
<td>1.08 (2)</td>
</tr>
<tr>
<td>100% DOPC + 50% N-methyl-PE</td>
<td>1.01 (2)</td>
<td></td>
</tr>
<tr>
<td>50% DOPC + 50% N,N-dimethyl-PE</td>
<td>0.98 (2)</td>
<td></td>
</tr>
</tbody>
</table>

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**Table II**

Incorporation of the lactose carrier in different proteoliposomes

T206 membrane vesicles were mixed in a 45:5 (protein/protein) ratio with T206 vesicles labeled with [3H]NPG (10 Ci/mmol). The specific activity of the mixture was 2 μCi/mg of membrane protein. In the experiment, this mixture was used to reconstitute the proteoliposomes of different phospholipid compositions with 100 mM potassium phosphate, pH 7.0. The lactose accumulation at 20 s was taken as the initial rate of uptake. The transport activity in proteoliposomes composed of E. coli lipid was taken as 100%.

Counterflow in proteoliposomes with different phospholipid compositions. The different phospholipid compositions are given on the figure. Proteoliposomes were reconstituted as described under "Experimental Procedures" with 100 mM potassium phosphate, pH 7.0. The lactose accumulation at 20 s was taken as the initial rate of uptake. The transport activity in proteoliposomes composed of E. coli lipid was taken as 100%.

![Graph showing initial rate of lactose uptake](image)
activity was the failure to form sealed proteoliposomes. The possibility, [3H]NPG-labeled lactose carrier protein (27) was DOPC and with the total protein/phospholipid ratios among different proteoliposomes of different lipid composition. To test this possibility in the incorporation of the lactose carrier protein into different types of proteoliposomes. The data given in Table I show that total protein/phospholipid ratios among different proteoliposomes are similar (from 9.9 to 12.0 µg/mg). The carrier/protein ratios vary from 1.6 to 2.7 nmol/mg.

A second possible explanation for differences in transport activity was the failure to form sealed proteoliposomes. The structure of the proteoliposomes was examined by electron microscopy with a few of the phospholipid combinations. Monolamellar vesicles of approximately 100 nm in diameter were observed for E. coli lipid, egg lecithin, and DOPC. There was somewhat more heterogeneity in size for E. coli proteoliposomes compared with the other two. In addition, the internal volume of the proteoliposomes with different phospholipid combinations was measured (Table II). The volume measured with the phosphate method was approximately 1 µl of water space/mg of phospholipid for each of the lipid mixtures except 100% DOPC which gave a value of 0.7. The volume calculated from the [14C]lactose equilibration experiments was of the same order of magnitude as the phosphate trapping volume. This calculation assumes equilibration between externally added [14C]lactose and the internal water space. It also assumes that each proteoliposome possesses at least one func-

### Table III

**Reactivation of the lactose carrier from DOPC proteoliposomes**

<table>
<thead>
<tr>
<th>Lactose uptake</th>
<th>Time (min)</th>
<th>100% E. coli lipid proteoliposomes (a)</th>
<th>100% DOPC proteoliposomes (b)</th>
<th>100% DOPC proteoliposomes reactivated with E. coli lipid (c)</th>
<th>100% DOPC proteoliposomes reactivated with DOPC lipid (d)</th>
</tr>
</thead>
<tbody>
<tr>
<td>100% E. coli lipid proteoliposomes</td>
<td>20</td>
<td>300</td>
<td>50</td>
<td>140</td>
<td>48</td>
</tr>
<tr>
<td>100% DOPC proteoliposomes</td>
<td>40</td>
<td>450</td>
<td>75</td>
<td>160</td>
<td>75</td>
</tr>
<tr>
<td>100% DOPC proteoliposomes reactivated with E. coli lipid</td>
<td>60</td>
<td>450</td>
<td>90</td>
<td>185</td>
<td>92</td>
</tr>
</tbody>
</table>

Cardiolipin (PE/PG/CL = 75/15/15% by weight). Proteoliposomes prepared from this combination were found to have the same activity as those from crude E. coli lipids (Fig. 3). From such an experiment, it seemed unlikely that a trace amount of a major component in E. coli lipid was essential for activity.

Certain experimental limitations to the preparation of mixtures suitable for liposome formation were encountered. For example, pure phosphatidylethanolamine will not form liposomes under the conditions of these experiments, while mixtures of PE with PC were quite satisfactory. Thus, egg PC or dioleoyl-PC was used extensively in these studies to facilitate preparation of liposomes. To simplify the interpretation of the results, most of the comparisons were made between different phospholipids with the same fatty acid moiety. In most cases, phospholipids used had the dioleoyl fatty acid moiety (cis-9-octade-cenoic acid) which provides all the phospholipids with phase-transition temperature much lower than the room temperature at which all the transport assays were carried out.

Proteoliposomes prepared from 90% DOPC, 10% DOPE (Fig. 3); 90% DOPC, 10% DOPG; and 90% DOPC, 10% cardiolipin (data not shown) showed no significant activation of the lactose carrier compared with DOPC alone (Fig. 3). This finding is consistent with the results given in Fig. 2, which showed that the lactose carrier required more than trace amount of the "factor" from E. coli lipids to significantly increase activity. Mixtures containing 50% DOPC plus 50% DOPE, DOPG, DOPA, or CL were compared with 100% DOPC and with E. coli lipid. Data in Fig. 3 show that proteoliposomes with a high PE content showed the highest transport activity. Proteoliposomes containing 50% DOPG, 50% DOPE gave 60% of the activity of E. coli lipid. One possible explanation for the differences observed in Fig. 3 was variability in the incorporation of the lactose carrier protein into proteoliposomes of different lipid composition. To test this possibility, [3H]NPG-labeled lactose carrier protein (27) was used to measure the incorporation of the lactose carrier into different types of proteoliposomes. The data given in Table I show that total protein/phospholipid ratios among different proteoliposomes are similar (from 9.9 to 12.0 µg/mg). The carrier/protein ratios vary from 1.6 to 2.7 nmol/mg.

**Fig. 4. Counterflow and membrane potential-driven lactose uptake in proteoliposomes of different phospholipid composition.** Proteoliposomes with different phospholipid composition, (100% E. coli lipid (a); 50% DOPC, 50% DOPG (b); 50% DOPC, 50% DOPG, 50% DOPE (c); 50% DOPC, 50% DOPG, 50% DOPA (d); 50% DOPC, 50% cardiolipin (e); and 100% DOPC (f)) were reconstituted as described under "Experimental Procedures." A, counterflow uptake. Valinomycin-treated proteoliposomes were first loaded with 20 mM cold lactose and then diluted 50-fold into the transport assay solution (100 mM potassium phosphate, pH 7.0, plus 0.018 mM [14C]lactose (final external concentration of lactose was 0.42 mM), B, membrane potential-driven uptake. Valinomycin-treated proteoliposomes loaded with 100 mM sodium phosphate buffer, pH 7.0, were diluted 100-fold into 100 mM sodium phosphate buffer, pH 7.0, with [14C]lactose (0.02 mM) at 25 °C for 45 min. Inset, the [85Rb]uptake was measured by diluting the valinomycin-treated proteoliposomes (preloaded with 100 mM potassium phosphate, pH 7.0) 10-fold into 100 mM sodium phosphate buffer, pH 7.0, plus 20 μM [85Rb]rubidium Cl. The equilibrium level was measured by diluting into 100 mM potassium phosphate buffer, pH 7.0, with 20 μM [85Rb]rubidium Cl. The membrane potential was calculated from the rubidium concentration ratio (In/Out) with the Nernst equation. All experiments shown in the figure were carried out on the same batch of proteoliposomes.
The Phospholipid Requirement for the Lactose Carrier

**Proteoliposomes with Different Phospholipid Compositions**

Proteoliposomes were reconstituted with 50 mM K2SO4, 50 mM MOPS, pH 7.2, as described under “Experimental Procedures.” A counterflow assay. Proteoliposomes were incubated in 20 mM lactose for 30 min at 22 °C. Such lactose-loaded proteoliposomes were diluted 50-fold into 50 mM K2SO4, 50 mM MOPS, pH 7.2, plus [14C]lactose (final concentration, 0.4 mM). E, proton motive force-driven uptake was carried out by diluting concentrated proteoliposomes (lactose-free) containing buffer at pH 7.2 50-fold into 50 mM Na2SO4, 50 mM MES, pH 6, with [14C]lactose (final concentration, 0.2 mM). Δ, 100% DOPC; ○, 100 E. coli lipid; □, 50% DOPC, 50% DOPE.

The average number of carrier molecules/proteoliposome was calculated from data on labeled sugar accumulated in the internal water space to a concentration that 16 times that in the incubation medium (Fig. 4B). A similar accumulation was observed with 50% DOPC, 50% DOPE. On the other hand, proteoliposomes containing 100% DOPC, 50% DOPC, 50% DOPE; or 50% DOPC, 50% CL accumulated only 3-4-fold under these conditions (Fig. 4B).

In order to estimate the membrane potential generated by the potassium diffusion potential, an aliquot of the proteoliposomes was exposed to a low concentration of 30m RB+ and uptake of the cation was measured. Since valinomycin readily transports rubidium, this cation distributes itself across the membrane in a manner determined by the membrane potential. The membrane potential was calculated from the Nernst equation with the measured rubidium ratio (in/out). The inset of Fig. 4B shows that the membrane potentials generated by the potassium diffusion potentials in the proteoliposomes of different composition were similar.

The same batch of proteoliposomes were tested for transport by the counterflow assay (Fig. 4A). Proteoliposomes composed of E. coli lipid or 50% DOPC, 50% DOPE showed good sugar accumulation, while those composed of 50% DOPC, 50% DOPE; 50% DOPC, 50% CL; or 100% DOPE showed much less activity.

**The Proton Motive Force-driven Lactose Uptake in Proteoliposomes with Different Phospholipid Composition**

To examine in more detail the role of the polar head group of the phospholipid for activating the lactose carrier, the ΔνH+ driven uptake activities of the carrier in different proteoliposomes were studied. In these experiments, a pH gradient (inside pH 7.5 and outside pH 6) as well as a membrane potential (inside negative) were established. The data shown in Fig. 5B indicate that E. coli lipid and 50% DOPC, 50% DOPE proteoliposomes accumulated 10-fold, while 100% DOPC showed no accumulation. Counterflow assays were performed on the same batch of proteoliposomes. Accumulation in E. coli and 50% DOPC, 50% DOPE reached approximately 15-fold, while the initial rate of accumulation in DOPC was much less (Fig. 5A).

**Trinitrophenylation of the E. coli Proteoliposomes and Lactose Carrier Activity**

An attempt was made to chemically alter the PE after reconstitution of the lactose carrier in proteoliposomes composed of E. coli lipid. The purpose of the experiment was to permit the carrier to become incorporated in its natural phospholipid environment and then modify one component, the other lipids being unaffected. TNBS is known to react with the amino group of PE without affecting the other lipids. The conditions of the reaction, pH 8, are sufficiently mild that the proteoliposomes would be quite stable. The lactose uptake function of the carrier was assayed with a proton motive force. The result shown in Fig. 6A indicates that TNBS-treated proteoliposomes have less than 30% of the activity of the untreated control. The inset in Fig. 6A showed no difference of the membrane potentials between TNBS-treated and untreated proteoliposomes.

An important control was to show that the lactose carrier protein was unaffected by TNBS. French press vesicles containing lactose carrier were exposed to TNBS under the same conditions as the previous experiment. The treated carrier was then extracted with octyl glucoside and reconstituted into proteoliposomes containing E. coli lipid. The TNBS-treated carrier showed full activity (Fig. 6B). In a second type of experiment to demonstrate that the carrier was unaffected by the reagent, the carrier was extracted from TNBS-treated proteoliposomes and “reactivated” by reconstituting it in normal E. coli lipid (Table IV).

An additional control was carried out to rule out the possibility that free TNBS affected the membrane potential by acting as a proton conductor or directly inhibiting transport.
Phospholipid Requirement for the Lactose Carrier

**FIG. 6. Lactose uptake activity in proteoliposomes composed of the E. coli lipid treated with TNBS.**

Proteoliposomes containing E. coli lipid were reconstituted with 100 mM potassium phosphate buffer, pH 7.0. The pellet was resuspended in 75 μl of 100 mM potassium phosphate buffer, pH 8.0, plus valinomycin (40 mM). Proteoliposomes were prepared by adding 6 μl of 30 mM TNBS solution (in 100 mM potassium phosphate buffer, pH 8.0), into 30 μl of the resuspended proteoliposomes and incubating at 25 °C for 30 min (A, open circles). Untreated proteoliposomes (A, solid circles) were incubated with 6 μl of 100 mM potassium phosphate buffer, pH 8.0. Treated and untreated proteoliposomes were diluted 50-fold into 100 mM sodium phosphate buffer, pH 6.0, containing 0.018 mM [14C]lactose. The equilibrium level, accumulation ratio, and membrane potential measurements (inset) were carried out as described in the legend to Fig. 4.

**TABLE IV**

Reactivation of the lactose carrier from trinitrophenylated E. coli lipid proteoliposomes

Proteoliposomes of E. coli lipid were reconstituted and trinitrophenylated as described under “Experimental Procedures.” The reactivation experiment was carried out by solubilizing 50 μl of TNBS-treated proteoliposomes (phospholipid concentration was 60 mg/ml) with 1.5% octyl glucoside and by adding 60 μl of additional sonicated E. coli lipid (50 mg/ml). The solution was well mixed and then diluted 25-fold into 100 mM potassium phosphate buffer, pH 7.0. Proteoliposomes formed were collected by centrifugation. Membrane potential-driven uptake of lactose was measured in proteoliposomes composed of E. coli lipid, TNBS-treated E. coli lipid, as well as reactivated proteoliposomes. The lactose concentration used in all these experiments was 0.018 mM.

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>Untreated proteoliposomes</th>
<th>TNBS-treated proteoliposomes</th>
<th>TNBS-treated proteoliposomes reactivated with E. coli lipid</th>
</tr>
</thead>
<tbody>
<tr>
<td>30</td>
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<td>13</td>
<td>23</td>
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<td>20</td>
<td>33</td>
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The experiment was repeated at pH 7, a condition which greatly reduces the reaction with amino groups. Although free TNBS was present in this experiment, no inhibition of lactose transport was observed.

**Effect of Methylation of PE on Lactose Transport**—It was of interest to investigate the transport activity of the lactose carrier in proteoliposomes containing phosphatidylethanolamine with various degrees of methylation on the amino group N-methyl-PE and N,N-dimethyl-PE were enzymatically synthesized with phospholipase D by Avanti. Counterflow and membrane potential-driven uptake were measured in proteoliposomes prepared from 100% E. coli lipid; 50% DOPC, 50% DOPE; 50% DOPC, 50% N-methyl-PE; 50% DOPC, 50% N,N-dimethyl-PE; and 100% DOPC. The results in Fig. 7 indicate that for membrane potential-driven transport by proteoliposomes, there was a graded activity among the different methylated species with PE giving 75% of the E. coli value, the N-methyl derivation giving 43%, the N,N-dimethyl giving 32%, and the PC giving 12%. The data for counterflow were approximately the same as for membrane potential-driven transport.

**DISCUSSION**

In order to carry out their biological functions, membrane proteins must interact in an appropriate manner with several aspects of an extremely complex environment. One portion of the molecule must recognize water-soluble ligands, while another region of the polypeptide must interact with phospholipids and other hydrophobic substances. This latter relationship between membrane proteins and phospholipids is
other lipids supported 20% of full activity. Ca²⁺ transport was stimulated by PE and galactosyl diglyceride (8). On the other hand, bacteriorhodopsin shows activity when reconstituted in phospholipids.

proteoliposomes composed of a wide variety of different phospholipid head groups. While lipids from this strain of thermophilic bacterium gave full activity, no activity was observed with soybean phospholipids or phosphatidylglycerol (35). The phospholipid requirement of the ATP-driven Ca⁺⁺ pump of sarcoplasmic reticulum has been studied in great detail (3–8). Bennett et al. (4) found that the ATP hydrolytic activity was maximal with PC and PE, although other lipids supported 20% of full activity. Ca⁺⁺ transport was stimulated by PE and galactosyl diglyceride (8). On the other hand, bacteriorhodopsin shows activity when reconstituted in proteoliposomes composed of a wide variety of different phospholipids.

Although there are several studies of the lactose carrier reconstituted into proteoliposomes (15, 19, 25, 36–40), this study reports the first systematic study of the effects of different phospholipid head groups on the biological activity of this membrane carrier. Previous reconstitution experiments on cation-substrate cotransport carriers have generally been carried out with complex lipids such as soybean or E. coli phospholipids. Studies with the reconstitution of lactose transport activity in proteoliposomes with purified phospholipids have permitted an evaluation of the role of lipids in carrier activity.

One question that must be considered is whether the lactose carrier is normally surrounded by an annulus of phospholipids to which it is rather firmly bound and the extraction and reconstitution process simply transfers the carrier plus annulus to a new environment. It seems likely that, in the presence of the nonionic detergent octyl glucoside and a large amount of added phospholipid, exchange between lipid associated with the carrier in the membrane and exogenous lipid would take place. The ratio of added phospholipid to endogenous lipid was 60:1. Thin-layer chromatography of the phospholipid in proteoliposomes confirmed that more than 98% of the phospholipid was derived from added lipid. Finally, the wide range of transport activities observed depending on the composition of added lipid argues against the notion that the lactose carrier retains the annulus of phospholipids from the natural membrane.

Two additional variables might be responsible for differences in the measured transport rate: variations in the incorporation of lactose carrier into different proteoliposomes and variations in size of proteoliposomes. The former possibility was tested experimentally by measuring the incorporation of labeled carrier molecules into different proteoliposomes. Table II shows that incorporation was similar in all types of lipid. The volume of different types of proteoliposomes was estimated morphologically and biochemically. Electron microscopy of proteoliposomes prepared from 100% E. coli lipid, 100% DOPC, or 100% egg lecithin indicated that all of the structures were sealed unilamellar liposomes with the average diameter of approximately 100 nm. In addition, the aqueous volume within the proteoliposomes was measured and was found to be approximately 1 µl of water/mg of phospholipid in most cases. DOPC proteoliposomes contained about 70% of the water volume measured for other lipids. It is therefore reasonable to assume that the alterations of transport activity by proteoliposomes of different compositions were due to differences in the lipid interaction with the lactose carrier.

We conclude that all of the results point to the importance of the amino group in the phospholipid for activation of the lactose carrier. Both PS and PE permit full activity of the transport system. Blockade of the amino group by either trinitrophenylation or methylation greatly reduce the function of the carrier. Such data are consistent with the hypothesis that hydrogen bonding may play an important role in the protein-lipid interaction.