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Effect of Different Phospholipids on the Reconstitution of Two Functions of the Lactose Carrier of *Escherichia coli*

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Summary. The lactose carrier was extracted from membranes of *Escherichia coli* and transport activity reconstituted in proteoliposomes containing different phospholipids. Two different assays for carrier activity were utilized: counterflow and membrane potential-driven uptake. Proteoliposomes composed of *E. coli* lipid or of 50% phosphatidylethanolamine—50% phosphatidylcholine showed very high transport activity with both assays. On the other hand, proteoliposomes containing asolectin, phosphatidylcholine or 25% cholesterol/75% phosphatidylcholine showed good counterflow activity but poor membrane potential-driven uptake. The discrepancy between the two types of transport activity in the latter group of three lipids is not due to leakiness to protons, size of proteoliposomes, or carrier protein content per proteoliposome. Apparently one function of the carrier molecule shows a broad tolerance for various phospholipids, while a second facet of the membrane protein activity requires very restricted lipid environment.

Key Words proteoliposomes · counterflow · lactose carrier · phospholipid requirement · *Escherichia coli* · reconstitution

Introduction

The lactose transport system of *E. coli* is capable of the transfer of galactosides across the plasma membrane under the influence of two different types of driving forces. The first is the movement of substrate from a high external concentration to low internal concentration, commonly designated facilitated diffusion (Danielli, 1954). For this process no external source of energy is needed. A special case of facilitated diffusion is illustrated by the counterflow phenomenon in which the entry of substrate A into the cell (preloaded with a high concentration of substrate B) results in intracellular accumulation of substrate A (Widdas, 1952; Park et al., 1956; Rosenberg & Wilbrandt, 1957; Winkler & Wilson, 1966; Wong & Wilson, 1970; Wilbrandt, 1972; Kaczorowski & Kaback, 1979). Substrate B competitively blocks the exit of A leading to high intracellular concentrations of A. With time the concentration of B progressively declines due to its exit on the carrier and its inhibiting effect on A falls correspondingly, leading to the ultimate equilibration of both substrates across the membrane. Counterflow on the lactose carrier does not require the presence of ion gradients as it occurs in the presence of metabolic inhibitors such as azide (Winkler & Wilson, 1966; Kaczorowski & Kaback, 1979) and the facilitated entry of *o*-nitrophenylgalactoside is insensitive to proton ionophores such as dinitrophenol (Cohen & Monod, 1957).

The second form of membrane translocation is the ion gradient-driven accumulation of galactosides. Since the mechanism of lactose transport involves the obligatory coupling between H^+ movement and lactose transport on the carrier (Mitchell, 1963), a protonmotive force (membrane potential plus pH gradient of the appropriate orientation) will drive galactosides across the membrane against considerable concentration gradients.

When these two types of carrier activity, counterflow and ion gradient-driven accumulation, were first tested in reconstituted systems both types of phenomena were observed (Newman & Wilson, 1980; Foster et al., 1982; Viitanen et al., 1983; Garcia, Viitanen, Foster & Kaback, 1983; Wright & Overath, 1984). However, during a study of the effect of different phospholipids on lactose transport a very unexpected dissociation between the two types of transport was observed in the case of certain phospholipids. Reconstitution of the lactose carrier with asolectin, phosphatidylcholine, or phosphatidylcholine (75%) plus cholesterol (25%) showed counterflow but extremely weak ion-gradient-driven uptake. Evidence is presented that the failure to accumulate in response to protonmotive force with these phospholipids is not due to "leakiness" of the membranes for protons but to some other phenomenon.

Materials and Methods

Bacterial Strains and Growth

E. coli strains used in this study were X71/F'W3747 (*lac* I Z^+Y^+ *pro* C^- str^R *trp*$^-$ B_1 /F' *lac* $I^+Z^+Y^+$ *pro* C^+) and T206 (Teather et al., 1980) which carries the lac *Y* gene on a plasmid (*lac* I^+Z Y / F' *lac* $I^QZ^-Y^-$/pl *lac* Y^+). Strain X71/F'W3747 was grown to midlog-phase in minimal Medium 63 (Cohen & Rickenberg, 1956) with 0.4% glycerol as the carbon source and supplemented with 20 μg/ml tryptophan, 0.5 μg/ml thiamine and 200 μg/ml streptomycin. Stock cells in midlog-phase were diluted into minimal Medium 63 containing glycerol supplement and 1 mM isopropyl-β-D-thiogalactopyranoaide (IPTG). T206 was grown in Medium 63 with 1% tryptone (Difco) as the carbon source. IPTG (0.1 mM) was added two doublings before harvest. The cells were grown to late logarithmic phase.

Preparation of Membrane Vesicles

The cells were harvested, washed once with Medium 63, and resuspended at a concentration of 5 ml/g wet weight of cells in a buffer containing 50 mM potassium phosphate, pH 7.5, 1 mM dithiothreitol (DTT), 20 mM lactose, 5 mM magnesium sulphate, and 1 mM phenylmethylsulfonylfluoride (PMSF). DNAase (10 μg/ml) was added to the suspension, and the cells were disrupted by passing through an Aminco French pressure cell at 20,000 psi. The unbroken cells were removed by centrifugation at 11,700 × *g* for 10 min. The supernatant was centrifuged at 145,000 × *g* for 1 hr. The pelleted membrane vesicles were washed once with a buffer consisting of 50 mM potassium phosphate, pH 7.5, 1 mM DTT, 20 mM lactose and 1 mM phenylmethylsulfonylfluoride and then centrifuged at 145,000 × *g* for 1 hr. The washed membrane vesicles were resuspended in 50 mM potassium phosphate, pH 7.5, 0.5 mM DTT and 10 mM lactose. For storage, the membrane vesicles were divided into small aliquots, frozen in liquid N_2 and stored at −80°C.

Reconstitution of Lactose Transport

The reconstitution method was that of Newman and Wilson (1980) with the modifications indicated. The membrane vesicles were extracted at 0°C with a buffer containing 100 mM potassium phosphate, 25 mM 4-morpholine-ethanesulfonic acid (MES), pH 6, 1.3 mM DTT, 3.9 mg *E. coli* lipid/ml and 1.3% octylglucoside. The protein concentration of the suspension was approximately 1 mg/ml. The suspension was incubated on ice for 10 min and then centrifuged at 180,000 × *g* for 1 hr at 4°C. Reconstitution was carried out by adding 650 μl of the supernatant to 165 μl of bath sonicated *E. coli* liposomes plus 15 μl of 15% octylglucoside. The final concentration of octylglucoside was 1.29%. The suspension was diluted into 25 ml of buffer (at 22°C) to form proteoliposomes. In most experiments the buffer consisted of 100 mM potassium phosphate, 25 mM MES (for the pH *see* figure legends) and 1 mM DTT with or without 20 mM lactose. In one experiment (Table 3) proteoliposomes were preloaded with 50 mM K_2SO_4 plus 50 mM MOPS pH 7.2. The proteoliposomes were collected by centrifugation at 145,000 × *g* for 1 hr at 4°C.

In experiments with phospholipids other than *E. coli* lipids the procedure was similar to that described above except that the octylglucoside concentration was 1.5% in both the extraction and reconstitution steps. In the case of asolectin the amount of lipid used in the reconstitution step was two times higher than for the *E. coli* lipid experiments.

Counterflow Assay

The proteoliposomes were resuspended in 75 μl of a buffer containing 100 mM potassium phosphate, 25 mM MES, pH 6, 20 mM lactose and 1 mM DTT. Proteoliposomes were diluted 50-fold into an assay buffer at 22°C. The assay medium contained 100 mM potassium phosphate, 25 mM MES, pH 6, and 0.5 μCi/ml [^{14}C]-lactose (0.01 mM). The final concentration of lactose in the reaction mixture was 0.41 mM. Samples (100 μl) were removed at various time intervals and placed onto the center of a 0.22 μm Millipore filter (type GSTF) without a chimney. After filtration the proteoliposomes were washed with 5 ml cold buffer containing 100 mM potassium phosphate and 25 mM MES, pH 6. In one experiment (Fig. 6) the buffer in the assay medium was 100 mM potassium phosphate (pH 7.5) and in another (Table 2) the buffer was 50 mM K_2SO_4/50 mM MOPS.

Ion Gradient-Driven Transport Assay

In most experiments the proteoliposomes were resuspended in 100 mM potassium phosphate, 25 mM MES, pH 7.5, and 1 mM DTT. Valinomycin was added to the resuspended proteoliposomes to give a concentration of 19 μM. In two experiments (Fig. 6 and Table 2) the valinomycin concentration was raised to 40 μM in the stock proteoliposomes. Proteoliposomes (12 μl) were added to 1.2 ml of assay buffer (at 22°C) containing 100 mM potassium or sodium phosphate, 25 mM MES (for pH *see* figure legends) and 1 μCi/ml [^{14}C]-lactose (0.2 mM). Samples (220 μl) were removed at the various time intervals, filtered, and washed as described above. The composition of the wash buffer was the same as the incubation medium without lactose.

Measurement of Internal Water Space of Proteoliposomes and Liposomes

Sugar Trapping Method

Liposomes (without added protein) were prepared in a manner designed to trap radioactive sugars in the internal water space. *E. coli* lipid or asolectin was added to octyglucoside (in the absence of membrane vesicles) and centrifuged at 4°C. The supernatant was mixed with sonicated lipid plus octylglucoside as described in the "Reconstitution of Lactose Transport" section. This lipid-detergent suspension (50 μl for *E. coli* lipid and 61 μl for asolectin) was added to 1.65 ml buffer at 22°C consisting of 100 mM potassium phosphate, 25 mM MES, pH 6, 1 mM DTT and radioactive sugars. The radioactive sugars were: 3.6 μCi/ml [^{3}H]-raffinose (0.9 mM) and 0.9 μCi/ml [^{14}C]-lactose (0.9 mM). The liposomes were collected by centrifugation at 180,000 × *g* for 1 hr at 4°C. The liposomes were resuspended with 1.5 ml buffer containing 100 mM potassium phosphate, 25 mM MES, pH 6 and 1 mM DTT. The resuspended liposomes (1 ml) were filtered onto a 0.22 μM Millipore filter (type GSTF) without a chimney. The liposomes were washed with 10 ml cold buffer containing 100 mM potassium phosphate plus 25 mM MES, pH 6. As a control non-preloaded liposomes were exposed to the radioactive sugars and

then centrifuged. Samples were filtered and washed as above. This "blank" was subtracted from the experimental values.

Phosphate Trapping Method

The internal water space of the proteoliposomes was also measured by determining the inorganic phosphate trapped inside during the process of formation. This method was found suitable since the passive leakage of phosphate across the membrane was extremely slow and washing of the liposomes with phosphate-free solution was possible without loss from the internal compartment. All the proteoliposomes were prepared with 100 mM phosphate. Proteoliposomes (2 μl) were added to 1 ml of 250 mM NaCl solution at 0°C. After vortexing, the suspension was immediately filtered through the 0.22 μM GSTF Millipore filter paper, washed with 5 ml of the ice-cold NaCl solution twice. The paper with the proteoliposome sample was transferred to a phosphate-free disposable glass tube. Sodium dodecylsulfate (2 ml of a 10% solution) was added and the tube vortexed to release the phosphate from the proteoliposome. The phosphate was estimated by the method of Dryer Tammes and Routh (1957).

Measurement of Membrane Potential ($\Delta\psi$) by Accumulation of ^{86}Rb

Proteoliposomes or liposomes were preloaded in 100 mM potassium phosphate, 25 mM MES, pH 7.5, and 1 mM DTT. Valinomycin (an ionophore for Rb^+ as well as K^+) was added to the resuspended proteoliposomes or liposomes to give a concentration of 19 μM. Proteoliposomes or liposomes (12 μl) were added to 1.2 ml of assay buffer (at 22°C) containing 100 mM potassium or sodium phosphate, 25 mM MES, pH 7.5, and 1 μCi/ml ^{86}Rb sulfate (0.2 mM). Samples (220 μl) were removed at various time intervals, filtered, and washed with 5 ml cold buffer. The Millipore filters were soaked with 0.2 mM $RbSO_4$ and washed with 2 ml buffer before the samples were filtered. The wash buffer was the same as the incubation medium without Rb. All the values were corrected for a blank which was obtained by filtering 0.22 ml of [^{86}Rb]-containing buffer without liposomes and washed as above. The filter "blank" when washed with Na^+ buffer was somewhat higher than the "blank" washed with K^+ buffer.

The membrane potential was calculated from the rubidium concentration ratio using the Nernst Equation. The ^{86}Rb uptake by liposomes exposed to sodium phosphate ($\Delta\psi$ present) divided by the uptake by liposomes exposed to potassium phosphate (no $\Delta\psi$ present) was used for this calculation.

Direct Measurement of H^+ Entry into Proteoliposomes or Liposomes

E. coli and asolectin proteoliposomes or liposomes were preloaded with 100 mM potassium phosphate, 25 mM MES, pH 7.5, and 1 mM DTT. Proteoliposomes or liposomes (25 μl) were diluted into a 2.5 ml medium containing 100 mM sodium sulfate plus 25 MES, pH 7.5. The external potassium phosphate concentration was 1 mM. The extracellular pH change was monitored with a combination glass electrode and a pH meter was connected to a recorder. The change of extracellular pH was initiated by addition of valinomycin (final concentration was 0.5 μM). At the end of the experiment *p*-chlorocarbonylcyanide phenylhydrazine (CCCP) was added as a control to show that protons entered liposomes when a proton ionophore was present.

Protein Determination

Protein was determined by a modification of the methods of Schaffner and Weissmann, (1973) and Newman, Foster, Wilson and Kaback (1981). The final concentrations of sodium dodecylsulfate and trichloroacetic acid were increased to 1 and 20%, respectively.

Phospholipid Determination

Phospholipid was determined by the method of Hallen (1980). Bath sonicated lipid of the appropriate type was used as a standard to determine the phospholipid content (e.g., a DOPC standard was used in determining DOPC).

Preparation of Stock Lipids

Lipids dissolved in organic solvent (usually chloroform) were mixed together in appropriate proportions and dried under a stream of N_2 gas. Traces of solvent were then removed under vacuum for 3 hr. The dried lipid was suspended in 2 mM mercaptoethanol at a concentration of 50 mg lipid/ml and vortex-dispersed. It was stored under N_2 gas at −80°C. Chloroform/methanol extracted *E. coli* lipid (from Avanti) and asolectin were each acetone/ether washed by a modification (Newman & Wilson, 1980) of the method of Kagawa and Racker (1971).

Chemicals

[^{14}C]-Lactose was obtained from Amersham (Arlington Heights, IL) and was purified by descending paper chromatography in 1-propanol/H_2O (3 : 1) before use. [^3H]-Raffinose was from New England Nuclear (Boston, MA). ^{86}Rb was from Amersham. Octylglucoside and valinomycin were obtained from Calbiochem (San Diego, CA). Lactose and isopropylthiogalactoside were from Sigma (Saint Louis, MO). Dioleoylphosphatidylcholine (DOPC), dioleoylphosphatidylethanolamine (DOPE) and crude *E. coli* lipid were purchased from Avanti Polar-Lipids (Birmingham, AL). Asolectin was purchased from Associated Concentrates (Woodside, NY). Cholesterol was from Eastman (Rochester, NY). [^3H]NPG-labeled membranes were kindly provided by Dr. Ronald Kaback (Roche Institute, Nutley, NJ).

Results

In the first experiment a comparison was made between the counterflow activities of the lactose carrier in proteoliposomes whose phospholipids were derived from *E. coli* compared with carrier activity of proteoliposomes from soybean (asolectin). In this transport assay accumulation of a low concentration of [^{14}C]-lactose (0.4 mM) results from the inhibition of exit of the radioactive molecules by the high concentration (20 mM) of preloaded nonradio-

active lactose. The lactose accumulation in proteoliposomes composed of *E. coli* lipid reached 20-fold in 5 min and fell to 15-fold in 20 min (Fig. 1). The pattern of accumulation with asolectin was somewhat different. The initial rate of uptake was slower than that of *E. coli*, but the level of accumulation at 20 min was about the same. The transport inhibitor α-*p*-nitrophenylgalactoside (αpNPG) blocked uptake in the two lipids.

The same proteoliposomes were assayed for transport activity with a membrane potential (inside negative) as the source of energy (Fig. 2). In each experiment proteoliposomes were prepared in the same potassium phosphate buffer but in the absence of 20 mM lactose. Valinomycin was added to the concentrated proteoliposomes and the mixture diluted 100-fold into sodium phosphate plus [^{14}C]-lactose. The final K^+ in the medium was 1 mM. The exit of K^+ on the ionophore gave rise to a membrane potential inside negative. This electrical potential difference provided the driving force for the inward movement of protons with lactose on the lactose carrier. The mean value for the [^{14}C]-lactose accumulation in four experiments was sevenfold at 5 min and sixfold at 15 min (Fig. 2). In contrast, the accumulation in asolectin proteoliposomes was only twofold at 5 min and 1.5-fold at 15 min.

An even more striking difference between the accumulation in proteoliposomes of the two lipids was observed when both a membrane potential and a pH gradient (inside alkaline) were imposed (Fig. 3). At 5 min lactose accumulated within the *E. coli* proteoliposomes to a concentration 24 times higher than the external medium, while the concentration in the asolectin proteoliposomes was only twice as high as the external medium.

One possible explanation for the failure of asolectin-containing proteoliposomes to support $\Delta\psi$-driven uptake was a high permeability of these membranes to protons. Such a leak of protons would cause an inward diffusion of H^+ and fall in the $\Delta\psi$. Evidence against this possibility was provided by several types of experiments. One such experiment was to measure the membrane potential by following the accumulation of a low concentration of added $^{86}Rb^+$ in the presence of valinomycin. Since this ionophore transfers Rb^+ (as well as K^+) across the membrane the final equilibrium concentration ratio of the ion may be used to calculate the membrane potential with the use of the Nernst equation. Table 1 shows that the calculated membrane potential in the asolectin liposomes or proteoliposomes was very similar to that for *E. coli*. These data indicate that membranes prepared from these two types of lipid are relatively impermeable to H^+.

In a second type of experiment, designed to test

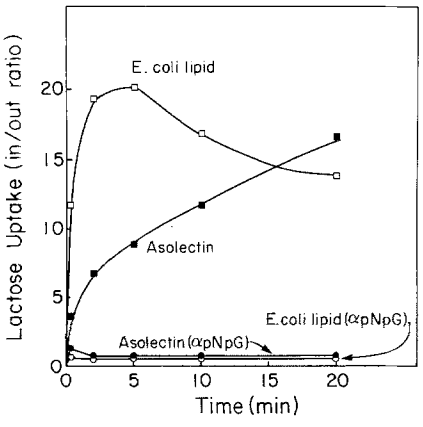

Fig. 1. Lactose counterflow in proteoliposomes prepared with *E. coli* lipid or asolectin. Proteoliposomes prepared with an extract of X71/F'W3747 membranes were preloaded with 20 mM lactose and diluted 50-fold into an assay medium containing 100 mM potassium phosphate, 25 mM MES, pH 6, 0.5 μCi/ml [^{14}C]-lactose with or without 10 mM αpNPG. The final external concentration of [^{14}C]-lactose was 0.41 mM. The protein concentrations in all the experiments were approximately 20 μg/ml. Open symbols represent the *E. coli* proteoliposomes and closed symbols represent the asolectin proteoliposomes. The mean values of four experiments are given

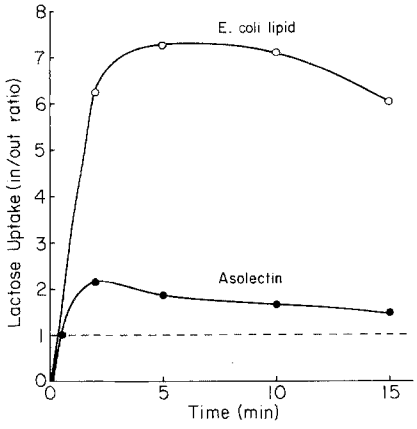

Fig. 2. $\Delta\psi$-driven lactose uptake in *E. coli* and asolectin proteoliposomes. Proteoliposomes prepared with an extract of X71/F'W3747 membranes were preloaded with 100 mM potassium phosphate, 25 mM MES, pH 7.5, and 1 mM DTT. Valinomycin was added to the concentrated proteoliposomes to give a concentration of 19 μM. The proteoliposomes were diluted 100-fold into an assay medium containing 100 mM sodium phosphate, 25 mM MES, pH 7.5, and 1 μCi/ml [^{14}C]-lactose (0.2 mM). The protein concentrations in all the experiments were approximately 10 μg/ml. The open symbols represent the *E. coli* proteoliposomes, and the closed symbols represent the asolectin proteoliposomes. The mean values of six experiments are given. The counts obtained with *E. coli* liposomes and asolectin liposomes were subtracted from the values obtained with *E. coli* and asolectin proteoliposomes, respectively

proton permeability, pH measurements were made with liposomes with a $\Delta\psi$ (inside negative). Valinomycin was added to liposomes preloaded with high K^+ and suspended in low K^+. This K^+ diffusion potential provided a strong driving force for the inward movement of H^+. Such liposomes were suspended in an unbuffered solution, and the pH of the medium was monitored continuously. A relatively small pH change was observed in 2 min for either liposome, although there was a slightly greater H^+ entry with asolectin (Fig. 4*A* and *B*). The proton ionophore CCCP allowed rapid H^+ entry in both liposomes.

The same experiment was carried out with proteoliposomes composed of the two different lipids (Fig. 5*A* and *B*). The proton movement resulting from valinomycin addition to *E. coli* or asolectin proteoliposomes was similar in the two cases. Thus it appeared that membranes composed of either *E. coli* lipid or asolectin showed an equally low permeability to protons.

In additional experiments liposomes composed of *E. coli* lipid or asolectin were found to show an equal ability to maintain a 2-pH unit difference across the membrane as assayed by the 9-aminoacridine technique (data not shown). This indicates that a pH gradient (as well as $\Delta\psi$) can be effectively maintained by membranes containing these two phospholipids.

Lactose transport in proteoliposomes consisting of 75% DOPC/25% cholesterol was next investigated. Counterflow with this mixture of lipids was similar to that seen with *E. coli* lipid; asolectin gave a slower rate of uptake (Fig. 6*A*). When $\Delta\psi$ was the driving force proteoliposomes with DOPC/cholesterol or with asolectin failed to show significant accumulation (Fig. 6*B*). Proton leakage was not a factor in this experiment as the membrane potentials were similar for all these lipids (Fig. 6*B*, insert).

In the final experiment three artificial phospholipids were tested in comparison with *E. coli* lipid for

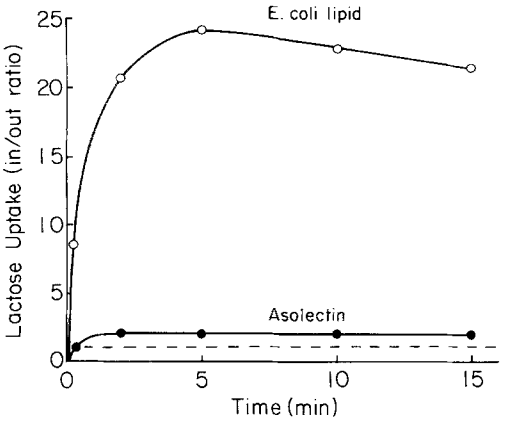

Fig. 3. ΔpH and $\Delta\psi$-driven lactose uptake in *E. coli* and asolectin proteoliposomes. The preparation of proteoliposomes and the assay conditions were the same as in Fig. 2 except that the pH of the assay buffer was 6 instead of 7.5. The mean values of four experiments are given

Table 1. Effect of lipid composition on membrane potential

Lipids	Liposomes (mV)	Proteoliposomes (mV)
E. coli	103 ± 4.3	106 ± 1.3
Asolectin	95 ± 10.7	101 ± 1.2

The [^{86}Rb] uptake was measured in liposomes and proteoliposomes as described in Materials and Methods. The calculated membrane potential ($\Delta\psi$) at 3 min is given. The $\Delta\psi$ at either 10 or 15 min was similar (data not shown). The mean values from three experiments with liposomes and four experiments with proteoliposomes are given.

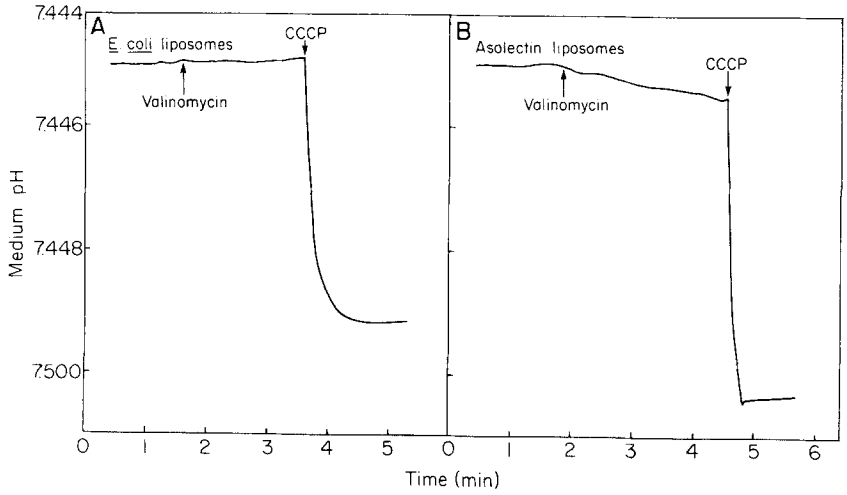

Fig. 4. Direct measurement of H^+ entry into liposomes. *E. coli* (*A*) and asolectin (*B*) liposomes were preloaded with 100 mM potassium phosphate, 25 mM MES, pH 7.5, and 1 mM DTT. Liposomes (25 μl) were diluted into 2.5 ml medium containing 100 mM sodium sulfate plus 25 mM MES, pH 7.5. The final external concentration of potassium phosphate was 1 mM. The change of the medium pH was initiated by addition of valinomycin. At the termination of the experiment the proton ionophore CCCP was added. The final concentration of valinomycin or CCCP was 0.5 μM and ethanol was 2%

protonmotive force-driven lactose accumulation (Table 2). Proteoliposomes containing *E. coli* lipid or DOPC/DOPE showed good lactose carrier activity with either type of transport assay. On the other hand proteoliposomes composed of DOPC/cholesterol or DOPC alone showed counterflow but very weak protonmotive force-driven uptake. The proton-impermeability of DOPC and DOPC/DOPE have previously been reported for experiments of this type (Chen & Wilson, 1984).

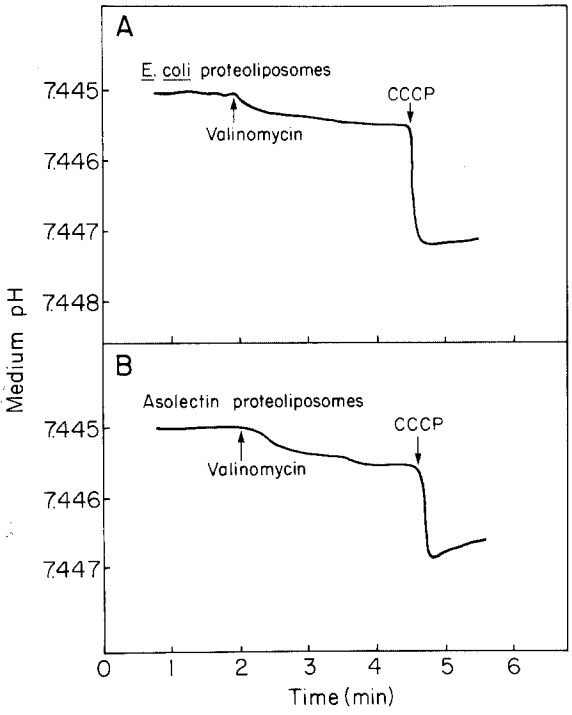

Fig. 5. H^+ entry into proteoliposomes. Lactose carrier (X71/F'W3747) was reconstituted into proteoliposomes of *E. coli* lipid (*A*) and asolectin (*B*) preloaded with 100 mM potassium phosphate (*see* Fig. 4). The pH of the suspension was measured as in Fig. 4

The possibility that membranes with different lipid compositions incorporated variable amounts of lactose carrier protein was considered. The quantity of lactose carrier present in the proteoliposomes was estimated by mixing photoaffinity-labeled carrier with normal T206 membranes and subsequently determining the amount of label in the final proteoliposomes. The data in Table 3 show that there is no direct relationship between the carrier content and the ability to carry out $\Delta\bar{\mu}_{H^+}$-driven uptake. Although the *E. coli* lipid proteoliposomes took up the maximum amount of carrier, DOPC/DOPE (which shows excellent $\Delta\bar{\mu}_{H^+}$-driven uptake) shows about the same carrier incorporation as asolectin, DOPC or DOPC/cholesterol.

The final variable considered as a possible contributing factor to some of the variations in behavior of different lipids was the volume of liposomes or proteoliposomes. The internal water space of proteoliposomes prepared from different lipids was measured by determining the sugar or inorganic phosphate trapped inside the vesicles during the process of formation. The volume of each of the four different types of proteoliposomes was of the same order of magnitude although asolectin vesicles were slightly smaller than the other three (Table 4).

Discussion

This paper described the unexpected finding that two types of lactose carrier activity can be dissociated by reconstituting the carrier in different types of lipid. In previous studies these two types of ac-

Table 2. Counterflow and $\Delta\bar{\mu}_{H^+}$-driven lactose uptake by proteoliposomes composed of different phospholipids

	Counterflow (In/Out ratio)			$\Delta\bar{\mu}_{H^+}$-driven uptake (In/Out ratio)		
	1 min	5 min	10 min	1 min	5 min	10 min
E. coli lipid	16	15	10	7	11	12
DOPC (50%) + (DOPE (50%)	10	14	12	7	9	10
DOPC (75%) + cholesterol (25%)	16	14	9	1.5	2	1.5
DOPC	3	6	8	1.5	2	1.5

Proteoliposomes were reconstituted with an extract of T206 membranes in the presence of 50 mM K_2SO_4 plus 50 mM MOPS, pH 7.2, and treated with 40 mM valinomycin. Prior to the counterflow assay, proteoliposomes were incubated in 20 mM lactose for 30 min at 22°C. Such lactose-loaded proteoliposomes were then diluted 50-fold into 50 mM K_2SO_4/50 mM MOPS, pH 7.2, plus [^{14}C]-lactose (final concentration = 0.4 mM). Protonmotive force-driven uptake was carried out by diluting concentrated proteoliposomes (preloaded with buffer at pH 7.2 but no sugar) 100-fold into 50 mM Na_2SO_4 plus 50 mM MES, pH 6, with [^{14}C]-lactose (final concentration 0.2 mM; 1 μCi/ml).

Table 3. Incorporation of the lactose carrier in different proteoliposomes

Proteoliposome	Protein/phospholipid (μg/mg)	Carrier number/protein (pmol/μg)
100% *E. coli* lipid	12.0	2.3
50% DOPC + 50% DOPE	9.9	1.6
75% DOPC + 25% cholesterol	10.0	1.2
100% DOPC	11.0	1.7
Asolectin	11.0	1.4

T206 membrane vesicles were mixed in 90 : 10 (protein/protein) ratio with T206 vesicles labeled with [^{3}H]NPG (4-nitrophenyl-α-D-galactopyranoside) (10 Ci/mmol). The specific activity of the mixture was 270 μCi/mg of membrane protein. This mixture was used to reconstitute the proteoliposomes of different phospholipid compositions in the presence of 100 mM potassium phosphate, pH 7. The number of the lactose carriers incorporated was estimated from the radioactivity of the labeled carrier and dilution factor. Protein and phospholipid measurements were carried out on each group of proteoliposomes.

Table 4. Internal water space of proteoliposomes and liposomes

	Water space (μl water/mg lipid)	
	Proteoliposomes[a]	Liposomes[b]
E. coli	1.12 ± 0.18 (n = 5)	1.25 ± 0.04 (n = 8)
Asolectin	0.86 ± 0.23 (n = 3)	0.77 ± 0.05 (n = 8)
DOPC 50% + DOPE 50%	1.02 (n = 2)	
DOPC 75% + cholesterol 25%	1.27 ± 0.29 (n = 4)	

[a] Phosphate trapping technique (*see* Materials and Methods).
[b] Sugar trapping technique (*see* Materials and Methods).

tivity, (i) counterflow and (ii) $\Delta\bar{\mu}_{H^+}$-driven accumulation, have always been found together in studies of intact cells, isolated cell membranes, and reconstituted systems.

Both activities increase in parallel during the process of induction (Kepes, 1960; Koch, 1964). Both are blocked by lactose analog (Kepes, 1960; Koch, 1964) and by SH-reagents (Kepes, 1960; Fox & Kennedy, 1965). Proton ionophores block accumulation because of their effects in reducing protonmotive force but have much less effect on facilitated diffusion, presumably because the sugar gradient is sufficient to permit entry even with an unfavorable $\Delta\bar{\mu}_{H^+}$ (Cohen & Monod, 1957; Koch, 1964). One interesting inhibitor is diethylpyrocarbonate, which inhibits accumulation but not facilitated diffusion without affecting the protonmotive force (Padan, Patel & Kaback, 1979; Garcia, Patel, Padan & Kaback, 1982; Patel, Garcia & Kaback, 1982).

While most mutants of the *lac* Y gene result in concomitant changes in both types of activity there is one class of mutants in which the carrier shows normal facilitated diffusion but severely defective $\Delta\bar{\mu}_{H^+}$-driven uptake (Wilson, Kush & Kashket, 1970; Wong, Kashket & Wilson, 1970; Fried, 1977).

In the present experiments there is a marked "discrepancy" between the two types of activity in proteoliposomes composed of asolectin, DOPC or DOPC-cholesterol. When an artificial protonmotive force ($\Delta\psi$ plus ΔpH) is imposed across the membrane of proteoliposomes with these two phospholipids, little or no accumulation is observed although counterflow is present. The most striking example is DOPC/cholesterol, which shows counterflow activity equal to that of *E. coli* lipid (more than 10-fold accumulation) but $\Delta\bar{\mu}_{H^+}$-driven uptake is virtually absent. The "discrepancy" is apparently not due to leakiness of protons (Table 1, Figs. 4-6), nor is it correlated with size (Table 4) or carrier content (Table 3) of proteoliposomes. Perhaps one may consider the two activities of the carrier to represent sugar recognition and translocation (counterflow), on the one hand, and cation-coupling ($\Delta\bar{\mu}_{H^+}$-driven uptake), on the other. According to this view, the sugar recognition and translocation aspect of the carrier is supported by a lipid environment with many different types of phospholipids while the proton-coupling aspect of the carrier can function only with a high concentration of phosphatidylethanolamine.

The dissociation between two different activities of other membrane transport systems has been studied by the reconstitution technique. The ATPase hydrolysis activity of the Ca^{2+}−ATPase of sarcoplasmic reticulum can be supported by reconstitution in many lipid mixtures (Hidalgo, Ikimoto & Gergely, 1976; Navarro, Toivio-Kunnucan & Racker, 1984), while the ATP-driven Ca^{2+} translocation requires PE (Navarro et al., 1984). When the Na^+-channel of rat brain was reconstituted in proteoliposomes, Na^+ translocation and tetrodotoxin binding show good activity when the phospholipid was phosphatidylchilone or a mixture of phosphatidylcholine and rat brain lipids (Tamkun, Talvenheimo & Catterall, 1984). However, the binding of scorpion toxin was found only with a mixture containing brain lipids; no such binding activity was found with reconstitution in PC.

These examples emphasize the complexity of the protein-lipid interactions in biological membranes. One portion of the transport molecule (or at least one biological function) shows a broad tolerance for

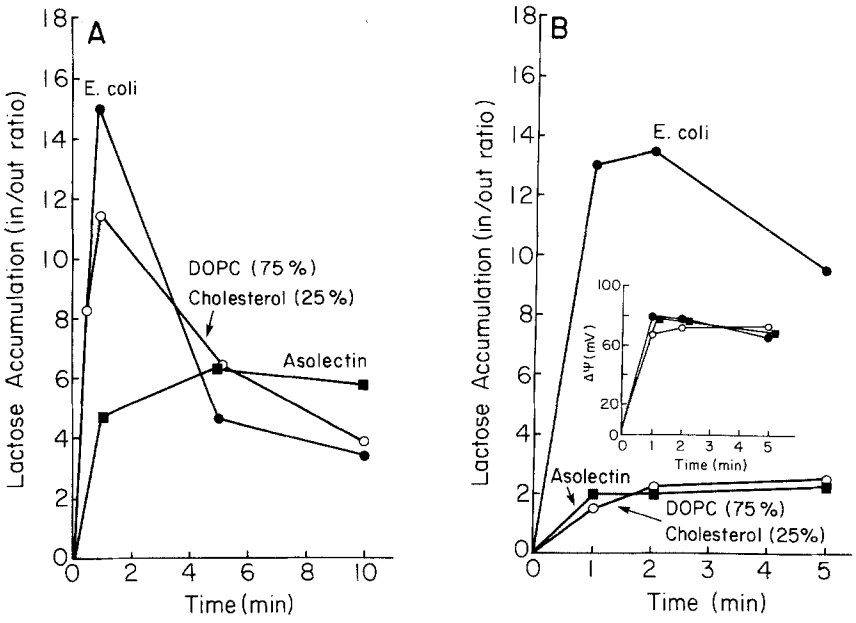

Fig. 6. Counterflow and $\Delta\psi$-driven lactose uptake in proteoliposomes of different composition. Proteoliposomes prepared with extracts of T206 membranes were preloaded with 100 mM potassium phosphate, pH 7.5, centrifuged and resuspended in 75 μl of 100 mM potassium phosphate, pH 7.5. Valinomycin was added to this concentrated suspension to give a final concentration of 40 μM. (*A*) For counterflow experiments valinomycin-treated proteoliposomes were equilibrated with 20 mM lactose solution at 22°C for 1 hr. Twelve μl of the proteoliposomes containing 20 mM lactose were diluted into 600 μl potassium phosphate buffer pH 7.5 containing 0.018 mM [^{14}C]-lactose (1 μCi/ml). The final concentration of lactose in the external medium was 0.42 mM. Samples were removed at various times, filtered, washed, and counted. (*B*) For membrane potential-driven lactose uptake experiments, 12 μl of the valinomycin treated proteoliposomes (not incubated with lactose) were diluted into 1.2 ml of 100 mM sodium phosphate buffer at pH 7.5 containing 0.018 mM [^{14}C]-lactose. Samples (0.2 ml) were removed at various times, filtered, washed, and counted. The equilibrium level of lactose was determined by diluting the proteoliposomes into 100 mM potassium phosphate buffer containing 0.018 mM [^{14}C]-lactose and incubated at 22°C for 2 hr. Nonspecific binding (and/or inadequate washing) was measured by diluting the proteoliposomes into ice-cold 100 mM potassium phosphate buffer containing 2 mM PCMB and sampled immediately after mixing. This value was subtracted from all experimental points. The membrane potential was measured with ^{86}Rb as described in Materials and Methods

various phospholipids, while another facet of the membrane protein activity requires very restricted lipid environment. It is difficult to speculate on the molecular significance of these observations without more detailed knowledge of the three-dimensional structure of membrane proteins and interactions of various domains with the lipid environment.

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References

Chen, C.C., Wilson, T.H. 1984. The phospholipid requirement for activity of the lactose carrier of *Escherichia coli*. *J. Biol. Chem.* **259**:10150–10158

Cohen, G.N., Monod, J. 1957. Bacterial permeases. *Bacteriol. Rev.* **21**:169–194

Cohen, G.N., Rickenberg, H.V. 1956. Concentration specifique reversible des amino acids chez *Escherichia coli*. *Ann. Inst. Pasteur Paris* **91**:693–720

Danielli, J.F. 1954. The present position in the field of facilitated diffusion and selective active transport. *Proc. Symp. Colston Res. Soc.* **7**:1–4

Dryer, R.L., Tammes, A.R., Routh, J.I. 1957. The determination of phosphorus and phosphatase with N-phenyl-*p*-phenylenediamine. *J. Biol. Chem.* **225**:177–183

Foster, D.L., Garcia, M.L., Newman, M.J., Patel, L., Kaback, H.R. 1982. Lactose-proton symport by purified *lac* carrier protein. *Biochemistry* **21**:5634–5638

Fox, C.F., Kennedy, E.P. 1965. Specific labeling and partial purification of the M protein, a component of the β-galactoside transport system of *Escherichia coli*. *Proc. Natl. Acad. Sci. USA* **54**:891–899

Fried, V.A. 1977. A novel mutant of the *lac* transport system of *E. coli*. *J. Mol. Biol.* **114**:477–490

Garcia, M.L., Patel, L., Padan, E., Kaback, H.R 1982. Mechanism of lactose transport in *Escherichia coli* membrane vesicles: Evidence for the involvement of histidine residue(s) in the response to the *lac* carrier to the proton electrochemical gradient. *Biochemistry* **21**:5800–5805

Garcia, M.L., Viitanen, P., Foster, D.L., Kaback, H.R. 1983. Mechanism of lactose translocation in proteoliposomes reconstituted with *lac* carrier protein purified from *Escherichia coli*. I. Effect of pH and imposed membrane potential on

efflux, exchange, and counterflow. *Biochemistry* **22**:2524–2531

Hallen, R.M. 1980. Colorimetric estimation of phospholipids in aqueous dispersions. *J. Biochem. Biophys. Methods* **2**:251–255

Hidalgo, C., Ikemoto, N., Gergely, J. 1976. Role of phospholipids in calcium-dependent ATPase of the sarcoplasmic reticulum. Enzymatic and ESR studies with phospholipid-replacement membranes. *J. Biol. Chem.* **251**:4224–4232

Kaczorowski, G.J., Kaback, H.R. 1979. Mechanism of lactose translocation in membrane vesicles from *Escherichia coli*. 1. Effect of pH on efflux, exchange, and counterflow. *Biochemistry* **18**:3691–3704

Kagawa, Y., Racker, E. 1971. Partial resolution of the enzymes catalyzing oxidative phosphorylation. XXV. Reconstitution of vesicles catalyzing $^{32}P_i$-adenosine triphosphate exchange. *J. Biol. Chem.* **246**:5477–5487

Kepes, A. 1960. Etudes cinétiques sur la galactoside-perméase d'*Escherichia coli*. *Biochim. Biophys. Acta* **40**:70–84

Koch, A.L. 1964. The role of permease in transport. *Biochim. Biophys. Acta* **79**:177–200

Mitchell, P. 1963. Molecule, group and electron translocation through natural membranes. *Biochem. Soc. Symp.* **22**:142–168

Navarro, J., Toivio-Kunnucan, M., Racker, E. 1984. Effect of lipid composition on the calcium/adenosine 5′-triphosphate coupling ratio of the Ca^{2+}-ATPase of sarcoplasmic reticulum. *Biochemistry* **23**:130–135

Newman, M.J., Foster, D.L., Wilson, T.H., Kaback, H.R. 1981. Purification and reconstitution of functional lactose carrier from *Escherichia coli*. *J. Biol. Chem.* **256**:11804–11808

Newman, M.J., Wilson, T.H. 1980. Solubilization and reconstitution of the lactose transport system from *Escherichia coli*. *J. Biol. Chem.* **255**:10583–10586

Padan, E., Patel, L., Kaback, H.R. 1979. Effect of diethylpyrocarbonate on lactose/proton symport in *Escherichia coli* membrane vesicles. *Proc. Natl. Acad. Sci. USA* **76**:6221–6225

Park, C.R., Post, R.L., Kalman, C.F., Wright, J.H., Jr., Johnson, L.H., Morgan, H.E. 1956. The transport of glucose and other sugars across cell membranes and the effect of insulin. *Ciba Found. Colloq. Endocrinol.* **9**:240–260

Patel, L., Garcia, M.L., Kaback, H.R. 1982. Direct measurement of lactose/proton symport in *Escherichia coli* membrane vesicles: Further evidence for the involvement of histidine residue(s). *Biochemistry* **21**:5805–5810

Rosenberg, T., Wilbrandt, W. 1957. Uphill transport induced by counterflow *J. Gen. Physiol.* **41**:289–296

Schaffner, W., Weissman, C. 1973. A rapid, sensitive, and specific method for the determination of protein in dilute solution. *Anal. Biochem.* **56**:502–514

Tamkun, M.M., Talvenheimo, J.A., Catterall, W.A. 1984. The sodium channel from rat brain. Reconstitution of neurotoxin-activated flux and scorpion toxin from purified components. *J. Biol. Chem.* **259**:1676–1688

Teather, R.M., Bramhall, J., Riede, I., Wright, J.K., Furst, M., Aichele, G., Wilhelm, U., Overath, P. 1980. Lactose carrier protein of *Escherichia coli*: Structure and expression of plasmids carrying the *Y* gene of the *lac* operon. *Eur. J. Biochem.* **108**:223–231

Viitanen, P., Garcia, M.L., Foster, D.L., Kaczorowski, G.J., Kaback, H.R. 1983. Mechanism of lactose translocation in proteoliposomes reconstituted with *lac* carrier protein purified from *Escherichia coli*. II. Deuterium solvent isotope effects. *Biochemistry* **22**:2531–2536

Widdas, W.F. 1952. Inability of diffusion of account for placental glucose transfer in the sheep and consideration of the kinetics of a possible carrier transfer. *J. Physiol. (London)* **118**:23–29

Wilbrandt, W. 1972. Carrier diffusion in biomembranes. *In*: Passive Permeability of Cell Membranes. F. Kreuzer and J.F.G. Slegers, editors. Vol. 3, pp. 79–99. Plenum, New York

Wilson, T.H., Kusch, M., Kashket, E.R. 1970. A mutant in *Escherichia coli* energy-uncoupled for lactose transport: A defect in the lactose-operon. *Biochem. Biophys. Res. Commun.* **40**:1409–1414

Winkler, H.H., Wilson, T.H. 1966. The role of energy coupling in the transport of β-galactosides by *Escherichia coli*. *J. Biol. Chem.* **241**:2200–2211

Wong, P.T.S., Kashket, E.R., Wilson, T.H. 1970. Energy coupling in the lactose transport system of *Escherichia coli*. *Proc. Natl. Acad. Sci. USA* **65**:63–69

Wong, P.T.S., Wilson, T.H. 1970. Counterflow of galactosides in *Escherichia coli*. *Biochim. Biophys. Acta* **196**:336–350

Wright, J.K., Overath, P. 1984. Purification of the lactose: H^+ carrier of *Escherichia coli* and characterization of galactoside binding and transport. *Eur. J. Biochem.* **138**:497–508

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