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Topical Review

The *Lac* Carrier Protein in *Escherichia coli**

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Introduction

Although active transport (i.e., concentration of solute against a gradient at the expense of metabolic energy) has been a recognized phenomenon for many years, insight into the biochemistry of the reactions involved has begun to occur only recently. In the author's opinion, the reasons for the progression from the phenomenological to the molecular level are essentially threefold: (1) Formulation of the chemiosmotic hypothesis by Peter Mitchell (1961, 1963, 1966, 1968), which stipulates that the immediate driving force for many processes in energy-coupling membranes is an electrochemical gradient of hydrogen ion ($\Delta\bar{\mu}_{H^+}$)[1]. (2) Availability of isolated membrane vesicles and proteoliposomes reconstituted with purified components that can be manipulated biochemically and genetically so as to yield information on a molecular level. (3) Development of techniques that enable detection and quantitation of electrochemical ion gradients in systems too small for the introduction of microelectrodes.

This article is not intended as a general review. Rather, the purpose is to discuss the β-galactoside transport system in *Escherichia coli* as a representative example of current developments. Lest the reader be dissuaded from continuing, however, because the topic appears to be highly specialized, it is important to stress that an overall conceptual framework exists which allows generalization of such studies to a broad range of problems involving biological membranes. Thus, in much the same way that the genetic code has provided the backbone for molecular biology from bacteriophage to man, the chemiosmotic concept accounts for a wide range of bioenergetic phenomena from bacterial motility to the uptake and storage of neurotransmitters in presynaptic nerve termini. Unfortunately, however, generalities in the area are often obfuscated because the chemiosmotic hypothesis was formulated initially to explain oxidative and photophosphorylation, because biochemists in general are uncomfortable with ephemoral entities such as electrochemical ion gradients and finally because various disciplines use different terminologies to describe the same basic phenomena.

The Chemiosmotic Hypothesis and Active Transport

Although it is beyond the scope of this article to review the chemiosmotic hypothesis and its ramifications in detail, a few words are necessary in order to put the β-galactoside transport system into perspective. For more comprehensive discussions, the reader is referred to the "gray books" of Mitchell (1966, 1968), three particularly lucid reviews (Greville, 1969; Harold, 1972; Hinkle & McCarty, 1978) and a recent compendium (Skulachev & Hinkle, 1981) commemorating Mitchell's 60th birthday.

In its most general form (Fig. 1), the chemiosmotic concept postulates that the immediate driving force for many processes in energy-coupling

* This article is dedicated to the memory of a fine scientist and a good friend, Dr. Rudolf Weil, of Sandoz Forschungsinstitut, Vienna, Austria, who died January 27, 1983.

[1] Abbreviations: $\Delta\bar{\mu}_{H^+}$, the proton electrochemical gradient across the membrane; $\Delta\psi$, membrane potential; ΔpH, the pH gradient across the membrane; octylglucoside, octyl-β-D-glucopyranoside; NPG, *p*-nitrophenyl-α-D-galactopyranoside; SDS-PAGE, sodium dodecylsulfate polyacrylamide gel electrophoresis; CCCP, carbonylcyanide-*m*-chlorophenylhydrazone; Q_1H_2, ubiquinol-1; *p*-CMBS, *p*-chloromercuribenzene-sulfonate; DEPC, diethylpyrocarbonate; D-LDH, D-lactate dehydrogenase; HPLC, high performance liquid chromatography.

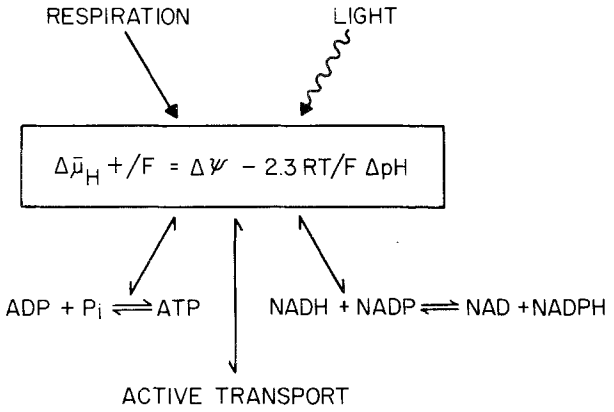

Fig. 1. Generalized chemiosmotic hypothesis. Respiration or absorption of light leads to the generation of a proton electrochemical gradient that provides the immediate driving force for a number of seemingly unrelated phenomena, which include oxidative and photophosphorylation, active transport, and transhydrogenation

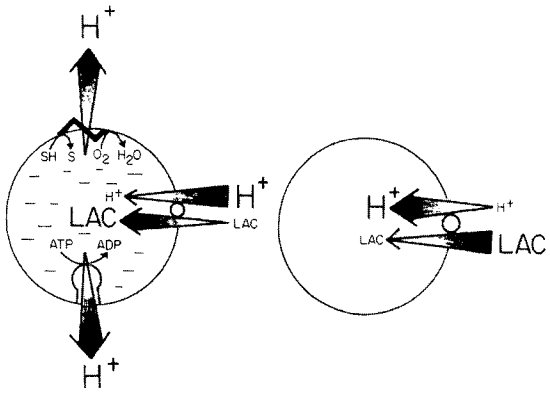

Fig. 2. The chemiosmotic hypothesis and lactose transport in *E. coli*. *Left:* uphill lactose transport in response to $\Delta\bar{\mu}_{H^+}$ (interior negative and alkaline) generated either by respiration or ATP hydrolysis. *Right:* uphill proton transport in response to an inwardly directed lactose gradient. ⌇, membrane bound respiratory with alternating proton and electron carriers; ♌, the proton ATPase; o, the *lac* carrier

membranes is a proton electrochemical gradient ($\Delta\bar{\mu}_{H^+}$) composed of electrical and chemical parameters according to the following relationship:

$$\Delta\bar{\mu}_{H^+}/F = \Delta\Psi - 2.3\,RT/F\ \ \Delta pH \qquad (1)$$

where $\Delta\Psi$ represents the electrical potential across the membrane and ΔpH is the chemical difference in proton concentration across the membrane (R is the gas constant, T is absolute temperature, F is the Faraday constant; $2.3RT/F$ is equal to 58.8 at room temperature).

Accordingly, the basic energy-yielding processes of the cell – respiration or absorption of light – generate $\Delta\mu_{H^+}$, and the energy stored therein is utilized to drive a number of seemingly unrelated phenomena such as the formation of ATP from ADP and inorganic phosphate, active transport, and transhydrogenation of NADP by NADH. More recently, it has become apparent that $\Delta\bar{\mu}_{H^+}$ or one of its components is involved in many other diverse phenomena such as bacterial motility (Doetsch & Sjoblad, 1980), nitrogen fixation (Laane et al., 1980), transfer of genetic information (Grinius & Berzinskiene, 1976; Kalasauskaite & Grinius, 1979; Labedan & Goldberg, 1979; Grinius, 1980; Wagner, Ponta & Schweiger, 1980; Santos & Kaback, 1981), sensitivity and resistance to certain antibiotics (McMurry, Petrucci & Levy, 1980; Mates et al., 1982), cellulose synthesis (Delmer, Benziman & Padan, 1982) and processing of secreted proteins (Date, Zwizniski, Ludmerer & Wickner, 1980; Daniels, Bole, Quay & Oxender, 1981; Enequist et al., 1981). Importantly, many of the processes driven by $\Delta\bar{\mu}_{H^+}$ are reversible. Thus, hydrolysis of ATP via the proton ATPase leads to the generation of $\Delta\bar{\mu}_{H^+}$. Similarly, transport of solutes down a concentration gradient (i.e. the reverse of active transport) can also lead to generation of $\Delta\bar{\mu}_{H^+}$ (Kaczorowski & Kaback, 1979). Clearly, therefore, the "common currency of energy exchange," particularly in the bacterial cell, is *not* ATP, but $\Delta\bar{\mu}_{H^+}$.

The salient features of the chemiosmotic hypothesis with respect to the β-galactoside transport system in *E. coli* are presented schematically in Fig. 2. As shown on the left, $\Delta\bar{\mu}_{H^+}$ can be generated either *via* the respiratory chain (aerobically or anaerobically with nitrate or fumarate as terminal electron acceptors) or through hydrolysis of ATP catalyzed by the proton ATPase. According to formal chemiosmotic doctrine, $\Delta\bar{\mu}_{H^+}$ acts to drive transport by different mechanisms (uniport, antiport or symport), depending on the nature of the solute (Mitchell, 1968; Kaback, 1977). Transport of β-galactosides, which are neutral solutes, occurs by symport. By this means, a solute-specific component in the membrane – in this case, the *lac* carrier protein or *lac* permease – is able to utilize the energy released by the downhill translocation of protons in response to $\Delta\bar{\mu}_{H^+}$, which is interior negative and alkaline, to drive the uphill transport of solute against a concentration gradient. Furthermore, since the flux of substrate is coupled to the flux of protons, downhill translocation of substrate along a concentration gradient results in uphill transport of protons and generation of $\Delta\bar{\mu}_{H^+}$ (Fig. 2, right). Although carrier-mediated downhill translocation is termed "facilitated diffusion" and the nomenclature has been applied traditionally to

describe β-galactoside transport under nonenergized conditions, this is not correct formally. That is, although one of the substrates, the β-galactoside, moves downhill, the other substrate, hydrogen ion, moves uphill against its electrochemical gradient, and it is important to realize that this is not a trivial point. As demonstrated by Konings and co-workers (Michels et al., 1979; Otto et al., 1980), for instance, this property of bacterial transport systems may have important consequences for the overall energetics of cell growth. Thus, carrier-mediated efflux of glycolytically-generated lactic acid can provide a significant increase in growth yield under certain conditions due to generation of $\Delta\bar{\mu}_{H^+}$.

The β-Galactoside Transport System

The β-galactoside or *lac* transport system in *E. coli* is the most intensively studied of bacterial transport systems. It was described originally in 1955 by Cohen and Rickenberg (1955; Rickenberg, Cohen, Buttin & Monod, 1956; Cohen & Monod, 1957; Kepes & Cohen, 1962; Kepes, 1971) and is encoded by the well-known *lac* operon which enables the organism to utilize the disaccharide lactose. In addition to its regulatory loci, the *lac* operon contains three structural genes: (i) the *z* gene encoding β-galactosidase, a cytosolic enzyme that cleaves lactose once it enters the cell; (ii) the *y* gene encoding the *lac* carrier protein or *lac* permease which catalyzes transport of lactose through the plasma membrane of the cell; and (iii) the *a* gene encoding thiogalactoside transacetylase, an enzyme that catalyzes the acetylation of thiogalactosides with acetyl-CoA as the acetyl donor and has no known physiological function.

In 1963, Mitchell (1963) postulated explicitly that lactose transport occurs in symport with protons (Fig. 2) and that $\Delta\bar{\mu}_{H^+}$ is the immediate driving force for accumulation against a concentration gradient. Subsequently, West (1970) and West and Mitchell (1972, 1973) demonstrated that addition of lactose to de-energized cells causes alkalinization of the external medium, thus providing the first evidence for lactose/proton symport in *E. coli*. During the ensuing five to six years, experiments with intact cells and membrane vesicles with the same and opposite polarities as the membrane in the intact cell provided virtually unequivocal evidence that *E. coli* generates a large $\Delta\bar{\mu}_{H^+}$ as the result of either electron transfer or ATP hydrolysis and that $\Delta\bar{\mu}_{H^+}$ or one of its components is the immediate driving force for the transport of a wide variety of solutes, particularly lactose and other β-galactosides (*cf.* Kaback, 1977; Harold, 1978; Skulachev & Hinkle, 1981, for reviews). Since the energetics of bacterial active transport are resolved to a great extent, the focus of the field is shifting to a more molecular, mechanistic approach, and the remainder of this article will deal with observations on this level.

Purification of Functional *lac* Carrier Protein

Although the kinetics (Kaczorowski & Kaback, 1979; Kaczorowski, Robertson & Kaback, 1979; Robertson, Kaczorowski, Garcia & Kaback, 1980; Ghazi & Shechter, 1981), substrate specificity (Sandermann, 1977) and genetics (Hobson, Gho & Müller-Hill, 1977) of the β-galactoside transport system were studied intensively, and the *lac y* gene product was shown to be a membrane protein (Kennedy, 1970), relatively little progress was made with respect to purification primarily because all attempts to solubilize the protein in a functional state were unsuccessful (*cf.* Padan, Schuldiner & Kaback, 1979*b*). In 1978 (Teather et al., 1978), however, the *lac y* gene was cloned into a recombinant plasmid, allowing the elucidation of its nucleotide sequence and the amino acid sequence of the *lac* carrier protein (Büchel, Gronenborn & Müller-Hill, 1980), as well as amplification of the carrier (Teather et al., 1980) and its synthesis *in vitro* (Ehring Beyreuther, Wright & Overath, 1980). Shortly thereafter, Newman and Wilson (1980) solubilized the carrier in octyl-β-D-glucopyranoside (octylglucoside) and successfully reconstituted lactose transport activity in proteoliposomes by using the octylglucoside dilution technique described by Racker et al. (1979). Accordingly, *E. coli* membranes containing the *lac* carrier were solubilized in octylglucoside in the presence of exogenous phospholipids, and proteoliposomes were formed by diluting the detergent under appropriate conditions (Fig. 3). After harvesting by high speed centrifugation, the proteoliposomes were found to catalyze counterflow as well as lactose accumulation in response to a valinomycin-mediated potassium diffusion potential (interior negative). Furthermore, similar preparations from a strain of *E. coli* mutated in *lac y* exhibited no activity. Almost simultaneously, it was demonstrated that *p*-nitrophenyl-α-D-galactopyranoside (NPG) is a highly specific photoaffinity label for the *lac* carrier protein (Kaczorowski, LeBlanc & Kaback, 1980). The use of this photoaffinity probe is based on the nucleophilic aromatic photosubstitution reactions of nitrophenyl ethers (Cornelisse, DeGunst

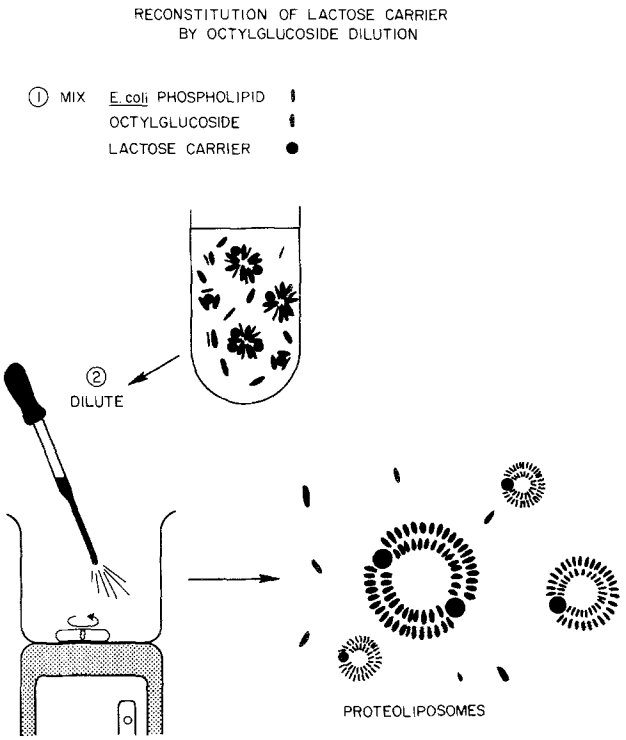

Fig. 3. Schematic representation of the octylglucoside dilution technique for reconstitution of the *lac* carrier protein into proteoliposomes

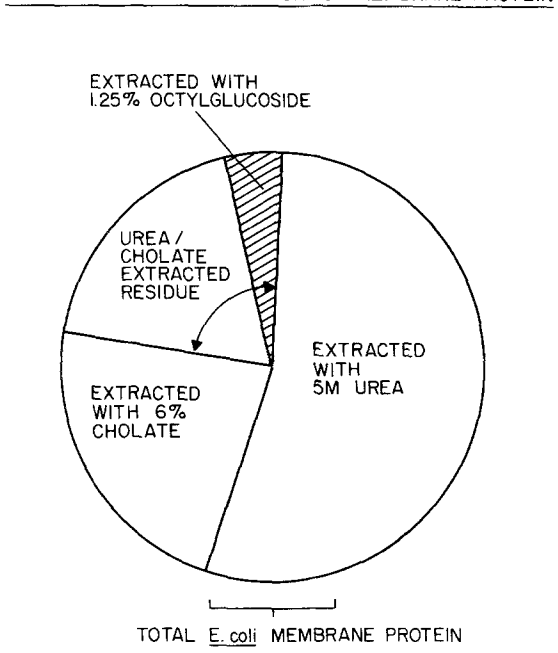

Fig. 5. Pie slice schematic showing fractionation resulting from differential solubilization of *E. coli* membrane proteins

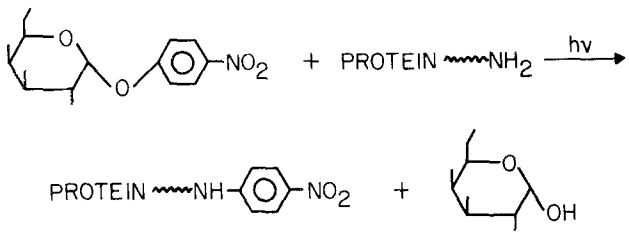

Fig. 4. Putative mechanism for photoaffinity labeling of the *lac* carrier protein with [^{3}H]NPG. (After Jelenc et al., 1978)

& Havinga, 1975; Cornelisse & Havinga, 1975; Jelenc, Contor & Simon, 1978). Thus, when NPG is irradiated with light above 300 nm, the phenolic carbon participating in the galactosidic bond is activated to a triplet state. Subsequently, if an appropriate nucleophile is in the vicinity, the activated carbon is attacked resulting in arylation of the nucleophile and the galactosyl portion of the molecule acts as a leaving group (Fig. 4). When *p*-nitro[2-^{3}H]phenyl-α-D-galactopyranoside ([^{3}H]NPG) is photolyzed in the presence of membrane vesicles under anaerobic reducing conditions, and the vesicles are subjected to sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE), essentially all of the radioactivity is associated with a band that migrates at an M_r of about 33 K (Kaczorowski et al., 1980). Furthermore, it is apparent from experiments with vesicles devoid of *lac* carrier, vesicles containing amplified levels of *lac* carrier, and the purified protein itself that this band represents the product of the *lac y* gene. By using a strain of *E. coli* with amplified levels of the *lac y* gene, [^{3}H]NPG to photolabel the carrier specifically and thus follow its distribution during purification and the transport activity of proteoliposomes reconstituted with the *lac* carrier, the product of the *lac y* gene was then purified to homogeneity in a functional state (Newman, Foster, Wilson & Kaback, 1981; Foster et al., 1982*b*).

The pie diagram presented in Fig. 5 summarizes a purification scheme in which *E. coli* membranes are first sequentially extracted with high concentrations of urea and cholate to effect about a threefold purification of the carrier *in situ*. Both of these operations are based upon earlier studies (Padan, Patel & Kaback, 1979*a*; Patel, Schuldiner & Kaback, 1975) demonstrating that treatment of right-side-out membrane vesicles with these reagents extracts considerable amounts of protein from the membrane with little or no effect on the *lac* carrier. Subsequent extraction with octylgluco-

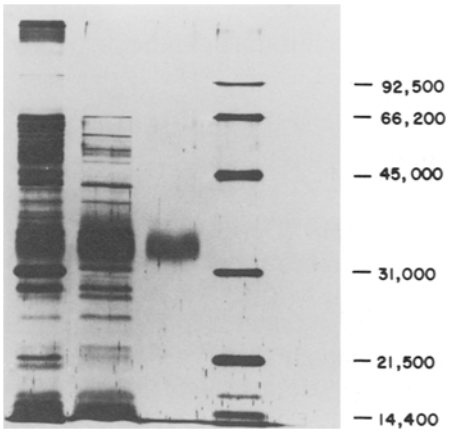

Fig. 6. SDS-PAGE of various fractions obtained during purification of the *lac* carrier. From the left, *Lane 1:* urea/cholate-extracted membrane, 4 μg; *lane 2:* octyglucoside extract, 4 μg; *lane 3:* pooled DEAE fractions, 0.8 μg; *lane 4:* molecular weight standards, 2 μg. Gels were silver stained. (From Newman et al., 1981)

side in the presence of *E. coli* phospholipids solubilizes most of the carrier, but only about 15% of the remaining protein, leading to an additional fourfold enrichment and 12-fold purification relative to the original membrane. The octylglucoside extract is then subjected to DEAE-Sepharose column chromatography under isocratic conditions at pH 6.0. Transport activity (*cf.* below) and most of the protein-associated photolabel is eluted in a symmetrical peak slightly behind the void volume of the column. Overall, the procedure results in a 35-fold purification relative to the crude membrane fraction and a yield of about 50%, based on the recovery of the photolabel.

Since photolabeling studies with [^{3}H]NPG indicate that 3% of the protein in the membrane of the amplified strain is *lac* carrier, a 35-fold enrichment of the photolabeled material suggests that a high degree of purification was achieved. This is confirmed by SDS-PAGE of the purified material (Fig. 6). Shown (from the left) are silver stained images obtained with urea/cholate-extracted membranes, the octylglucoside extract, pooled DEAE-sepharose fractions and molecular weight standards. Clearly, the pooled DEAE-sepharose fractions yield a single broad band[2] with an M_r of about 33K, which is in close agreement with published values for the molecular weight of the carrier as determined by SDS-PAGE (Teather et al., 1978; Jones & Kennedy, 1969). When membranes are prepared from cells that were not induced, the band corresponding to the purified carrier is only a minor constituent of the octylglucoside extract of urea/cholate-treated membranes, thus demonstrating that the purified protein exhibits an important property expected of the product of the *lac y* gene in the recombinant plasmid (Teather et al., 1980). Importantly, the amino acid composition of the purified protein closely matches the composition predicted from the DNA sequence of the *lac y* gene (Büchel et al., 1980). This result indicates that the functional *lac* carrier has a molecular weight similar to the value predicted (46,504) from the DNA sequence. Furthermore, N-terminal sequencing of the first 13 amino acids of the purified *lac* carrier yields results that are in complete agreement with the DNA sequence, providing additional evidence for the high degree of purity of the preparation.

It is not known why the carrier yields a spuriously low M_r on SDS-PAGE, although the high content of hydrophobic amino acids in the protein suggests that this phenomenon may be due to unusually high binding of SDS. Furthermore, it is not known why the carrier migrates as a broad band. However, it should be emphasized that when the protein is subjected to SDS-PAGE at increasing concentrations of polyacrylamide and the data are treated quantitatively (Neville, 1971; Banker & Cotman, 1972), an M_r of about 46K is obtained. A similar value is also obtained by gel permeation chromatography on Sephacryl S-300 in hexamethylphosphoric triamide (König & Sandermann, 1982).

Before progressing, the so-called "rapid assay" for transport activity (Newman et al., 1981; Foster et al., 1982*b*) should be discussed because it greatly facilitates measurement of transport activity during purification and is potentially applicable to other systems. During purification and particularly after chromatography on DEAE-Sepharose, many fractions are obtained in which the *lac* carrier is present at dilute concentrations. Although transport activity can be assayed in these fractions by reconstitution using the standard octylglucoside dilution technique, this requires centrifugation at high speeds or the use of another method to concentrate the proteoliposomes. An alternative method that is especially useful is shown schematically in Fig. 7. In this procedure, phospholipids dissolved in octylglucoside are added to an octylglucoside membrane extract or column fraction, and proteoliposomes are formed by dilution in the usual manner (in the presence of 20 mM lactose

[2] At higher protein concentrations, a less intense band is also observed at about M_r 60K. Since this band is observed after photoaffinity labeling with NPG (*cf.* Fig. 15) and reacts with antibody prepared against purified *lac* carrier (*cf.* Fig. 16), it is probably an aggregate of the *lac* carrier protein.

if counterflow activity is to be measured). The proteoliposomes which contain 20 mM lactose are then collected on a Millipore filter, and the vacuum is released. A solution containing radioactive lactose at a low concentration is then layered over the filter, and at a given time the vacuum is reapplied. By this or similar means, counterflow activity, as well as $\Delta\Psi$- and ΔpH-driven lactose accumulation, can be observed, and the need for time-consuming centrifugation procedures is obviated. It should be emphasized, however, that the activity observed in this manner is not quantitative (i.e., proteoliposomes in suspension exhibit much higher activity).

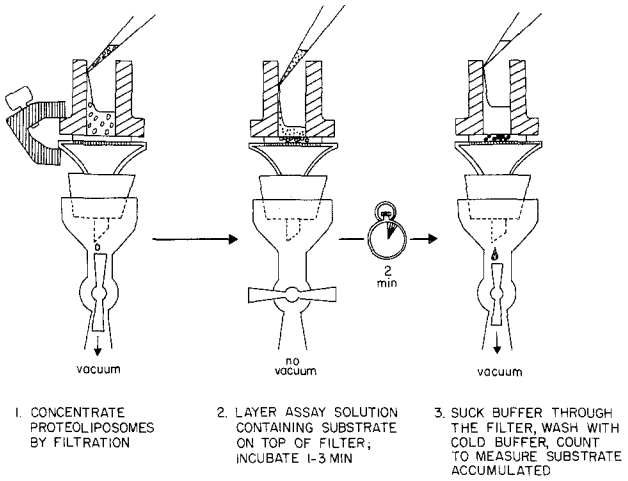

Fig. 7. Schematic representation of the "rapid filter assay" for transport in reconstituted proteoliposomes

Morphology and Ion Permeability of Proteoliposomes Reconstituted with *lac* Carrier Protein

The electron micrographs shown in Fig. 8 demonstrate that proteoliposomes prepared by octylglucoside dilution followed by freeze-thaw/sonication are unilamellar vesicles that exhibit no internal structure. Moreover, when the cross-sectional diameters of the vesicles are measured, about 80% of the proteoliposomes fall between 50 and 150 nm. In contrast, examination of preparations that were frozen and thawed but left unsonicated exhibit cross-sectional diameters that distribute over a considerably broader range (from 100 to 700 nm) (Garcia, Viitanen, Foster & Kaback, 1983).

Relatively low magnification electron microscopy of platinum/carbon replicas of freeze-fractured proteoliposomes containing purified *lac* carrier confirm the unilamellar nature of the preparation (Fig. 9*A*). Higher magnification reveals that both the convex and concave fracture surfaces exhibit a relatively uniform distribution of particles that are 85 to 90 Å in diameter (Fig. 9*B*). Since particles, but no pits, are observed on both surfaces

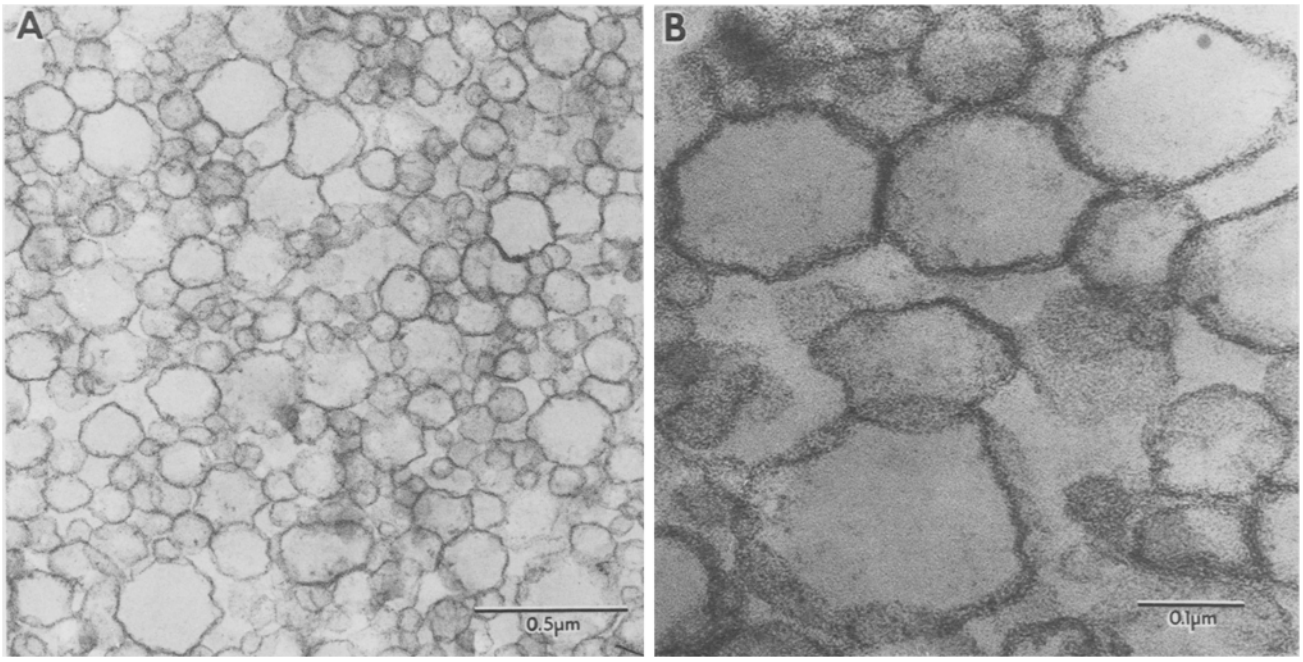

Fig. 8. Electron micrographs of reconstituted proteoliposomes. Proteoliposomes containing purified *lac* carrier were reconstituted by octylglucoside dilution followed by freeze-thaw/sonication. The preparation was fixed with 2% glutaraldehyde for 1 hr at 20 °C, postfixed with 1% OsO_4 in 50 mM cacodylate (pH 6.8) and embedded in Epon prior to sectioning. The study was performed by Miloslav Boublik and Frank Jenkins, Roche Institute of Molecular Biology

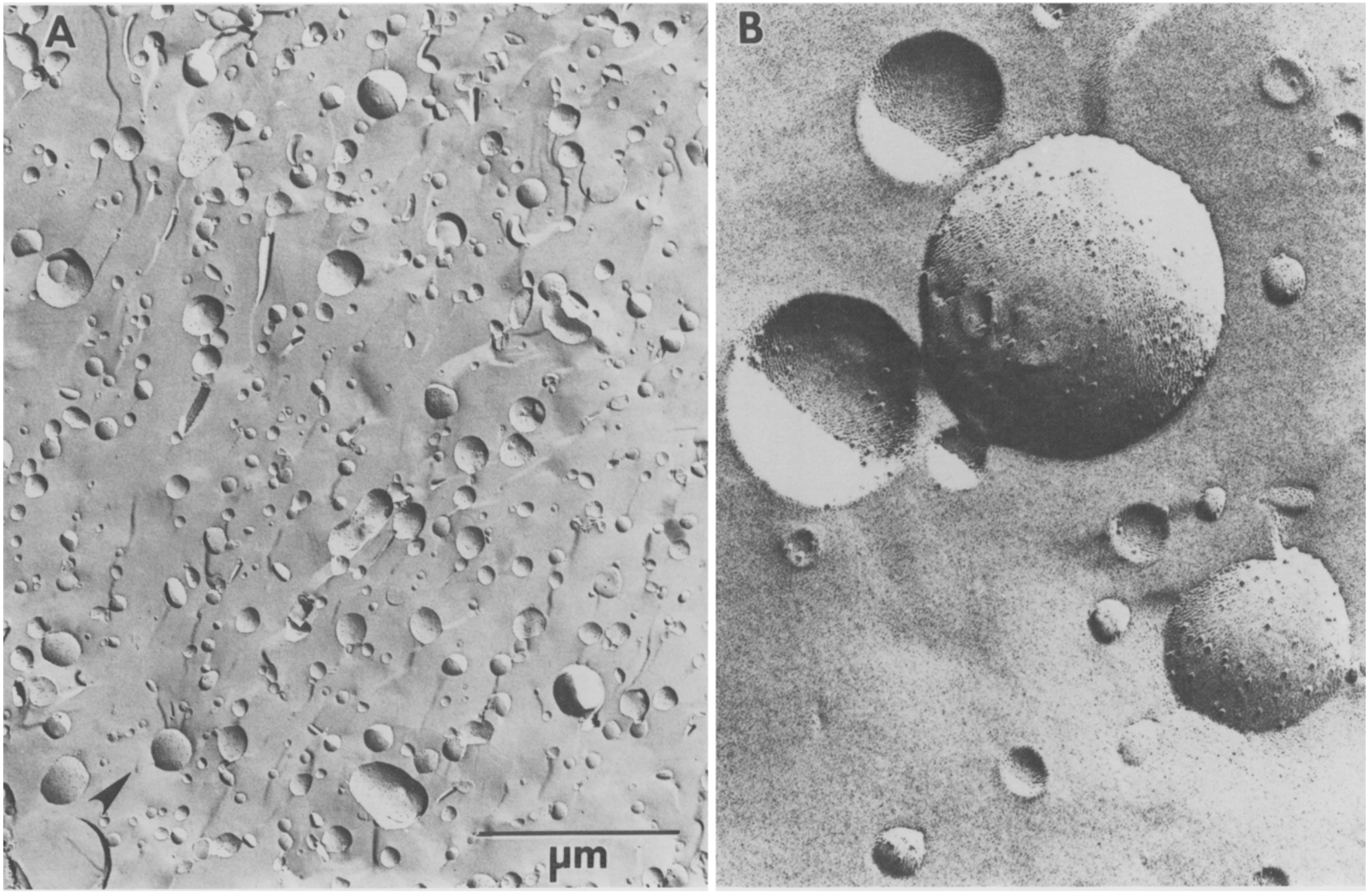

Fig. 9. Freeze fracture electron microscopy of proteoliposomes reconstituted with purified *lac* carrier. Platinum/carbon replicas of freeze-fractured proteoliposomes prepared by octyglucoside dilution followed by freeze-thaw/sonication are shown. The study was performed by Joseph Costello in the Department of Anatomy, Duke University, Medical Center

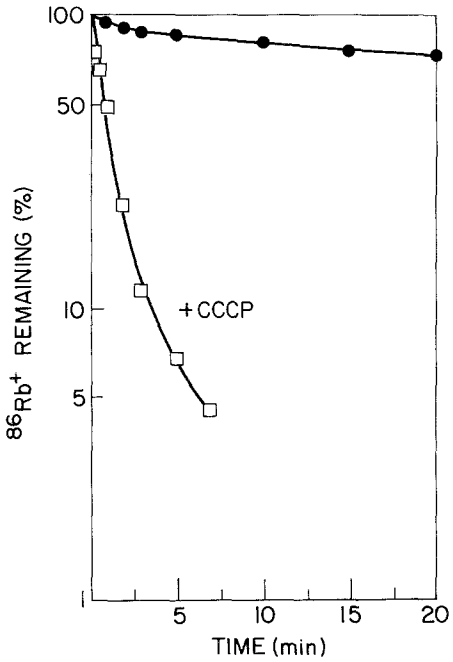

Fig. 10. $^{86}Rb^+$ efflux from proteoliposomes. Proteoliposomes containing purified *lac* carrier were treated with 20 μM valinomycin and equilibrated with $^{86}RbCl$. Aliquots were then diluted 200-fold into appropriate buffer at 25 °C. At the times indicated, samples were assayed by filtration. •, control; □, plus 20 μM CCCP. (*See* Garcia et al. (1983) for experimental details)

of the membranes, it seems likely that the *lac* carrier has equal affinity for the phospholipids in each leaflet of the bilayer. Given the mass of the *lac* carrier (46,504), a particle size of 85–90 Å suggests that the particles may contain one to three polypeptides, depending on the degree to which the shadowing increases the observed particle diameter.

When proteoliposomes containing purified *lac* carrier are equilibrated with $^{86}Rb^+$, treated with valinomycin, and diluted 200-fold into sodium phosphate, efflux of the cation occurs very slowly, and at 20 min the proteoliposomes still retain at least 80% of the label (Fig. 10). On addition of carbonylcyanide-*m*-chlorphenylhydrazone (CCCP), which increases permeability to protons specifically, a marked increase in the rate of Rb^+ efflux is evident. If the same experiments are performed in the absence of valinomycin, Rb^+ efflux is almost negligible, and addition of CCCP has no significant effect. The observations demonstrate, albeit indirectly, that the proteoliposomes are highly impermeable to the ions present in the reaction mixture (i.e., hydrogen ion, rubidium, sodium, chloride and phosphate). Thus, the slow rate of Rb^+ efflux observed in the presence of valinomycin is caused by the generation of $\Delta\Psi$ (interior negative) that is maintained for long periods of time because of the impermeability of the proteoliposomes to counterions. Addition of CCCP, on the other hand, provides a pathway for protons and results in dissipation of $\Delta\Psi$ with rapid downhill movement of Rb^+.

Clearly, these proteoliposomes are almost ideally suited for studies of proton/solute symport. Morphologically, the preparation consists of a population of unilamellar, closed, unit membrane-bound sacs that are relatively uniform in diameter and contain no internal structure, findings that correlate nicely with the pseudo-first-order efflux and exchange kinetics observed for Rb^+ and lactose (Garcia et al., 1983).Furthermore, it is apparent that the proteoliposomes are passively impermeable to many ions, a property that is highly advantageous. Thus, certain aspects of proton/lactose symport that were impossible to document with right-side-out vesicles (i.e., stimulation of efflux by ionophores; *cf.* below) are readily elucidated with the reconstituted system. Generally, proteoliposomes reconstituted with the *lac* carrier exhibit all of the phenomena described in right-side-out membrane vesicles, but the results are significantly more clear-cut and provide firmer support for certain ideas concerning reaction mechanisms.

A Single Polypeptide is Required for Lactose Transport

Although it is readily apparent that the *lac* carrier protein purified to apparent homogeneity catalyzes counterflow, proton influx and efflux in response to appropriately directed lactose concentration gradients and $\Delta\bar{\mu}_{H^+}$-driven lactose accumulation against a concentration gradient, evidence has been presented that was interpreted to indicate that active lactose transport may require more than a single polypeptide. Thus, it has been reported (Villarejo & Ping, 1978; Villarejo, 1980) that the *lac* carrier can be resolved into two distinct bands on SDS-PAGE ($M_r = 30K$ and $15K$) when solubilized at 100 °C in the presence of high concentrations of dithiothreitol, and it was proposed that both polypeptides are products of the *lac y* gene. Furthermore, mutants have been described (Hong, 1977; Plate & Suit, 1981) that map outside of the *lac y* gene and exhibit a pleiotropic loss of energy-coupled accumulation of solutes by several transport systems, including the *lac* system, despite the ability of the cells to maintain $\Delta\bar{\mu}_{H^+}$. Finally, Wright et al. (1982), using *lac* carrier partially purified and reconstituted by techniques different from those described (Newman et al., 1981; Foster

Table 1. Comparison of turnover numbers for the *lac* carrier protein: ML 308–225 membrane vesicles *versus* proteoliposomes reconstituted with purified carrier

Reaction[a]	Turnover numbers			
	Membrane vesicles[b]		Proteoliposomes	
	(sec^{-1})			
$\Delta\Psi$-driven influx ($\Delta\Psi$=100 mV)	16	(K_m=0.2 mM)	16–21	(K_m=0.5 mM)
Counterflow	16–39	(K_m=0.45 mM)	28	(K_m=0.6 mM)
Facilitated diffusion	8–15	($K_m \simeq$20 mM)	8–9	($K_m \simeq$3.1 mM)
Efflux	8	(K_m=2.1 mM)	6–9	(K_m=2.5 mM)

[a] All reactions were carried out at pH 7.5 and 25 °C.
[b] Determination of the amount of *lac* carrier protein in ML 308–225 membrane vesicles is based on photolabeling experiments with [^{3}H]NPG, which indicate that the carrier represents about 0.5% of the membrane protein.

et al., 1982*b*), were able to elicit counterflow activity, but unable to demonstrate $\Delta\Psi$- or ΔpH-driven lactose accumulation.

For these reasons, careful kinetic experiments were performed on proteoliposomes reconstituted with *lac* carrier protein purified to a single polypeptide species as judged by silver-stained SDS-PAGE (*cf.* Fig. 6). Turnover numbers were calculated for the carrier operating in various modes of translocation and compared to those calculated from published V_{max}*s* for right-side-out membrane vesicles (Table). As shown, both the turnover number of the *lac* carrier as well as its apparent K_m for lactose, are virtually identical in proteoliposomes and membrane vesicles with respect to $\Delta\Psi$-driven lactose accumulation, counterflow, facilitated diffusion (i.e., lactose influx under nonenergized conditions), and efflux.

In addition, Matsushita et al. (1983) have recently demonstrated that proteoliposomes simultaneously reconstituted with a purified *o*-type cytochrome oxidase and the *lac* carrier protein catalyze electron-transfer driven active lactose accumulation. The *o*-type cytochrome oxidase was purified from a mutant of *E. coli* defective in cytochrome *d* oxidase by extraction with octylglucoside after sequential treatment of membranes with urea and cholate. The oxidase was then purified to homogeneity by DEAE-Sepharose chromatography. The purified oxidase contains four polypeptides (M_r 66 K, 35K, 22K and 17K) and two *b*-type cytochromes (*b*558 and *b*563), and catalyzes the oxidation of ubiquinol-1 (Q_1H_2) and other electron donors with specific activities 20- to 30-fold higher

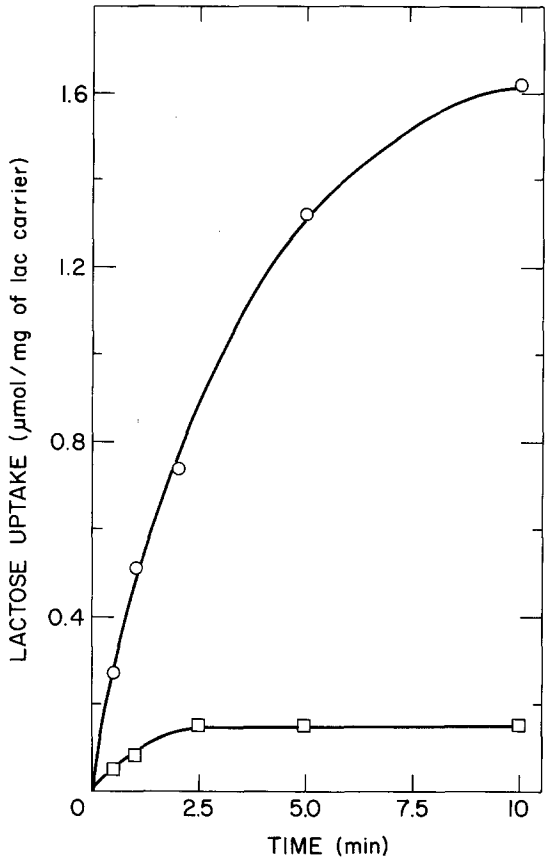

Fig. 11. Electron transfer-driven lactose accumulation by proteoliposomes simultaneously reconstituted with purified *o*-type cytochrome oxidase and purified *lac* carrier protein. Proteoliposomes containing purified cytochrome oxidase and *lac* carrier at a molar ratio of 1.3 to 1.0 were prepared by octylglucoside dilution followed by freeze-thaw/sonication. Lactose transport was assayed by filtration in the absence (□) and presence (○) of 16 μM Q_1H_2. In the presence of 20 μM valinomycin and 0.5 μM nigericin, values similar to those obtained in the absence of Q_1H_2 were observed (□)

than crude membranes. Proteoliposomes were reconstituted simultaneously with the purified oxidase and *lac* carrier protein by octylglucoside dilution followed by freeze-thaw/sonication. The reconstituted system generates a $\Delta\bar{\mu}_{H^+}$ (interior negative and alkaline) with Q_1H_2 as electron donor, and the magnitude of the $\Delta\bar{\mu}_{H^+}$ is dependent on the concentration of the oxidase in the proteoliposomes. As shown in Fig. 11, in the presence of Q_1H_2, the proteoliposomes accumulate lactose against a concentration gradient, and the phenomenon is completely abolished by addition of valinomycin and nigericin. Since uptake in the absence of Q_1H_2 or in the presence of valinomycin and nigericin represents equilibration with the medium, it is apparent that the steady-state level of lactose accumulation observed during oxidase turnover represents a concentration gradient of at least

10-fold. Moreover, by comparing lactose transport induced by Q_1H_2 to that induced by valinomycin-mediated potassium diffusion potentials ($K_{in} \rightarrow K_{out}$) and quantitating the magnitude of the $\Delta\Psi$s generated under each condition, it is apparent that the lactose transport activity observed is commensurate with the magnitude of $\Delta\bar{\mu}_{H^+}$.

Clearly, therefore, it is highly likely that a single polypeptide species, the product of the *lac y* gene, is necessary for each of the reactions catalyzed by the *lac* transport system in the *E. coli* membrane, including active transport energized by electron transfer. Furthermore, the double reconstitution experiment provides yet another strong line of evidence – this time on a molecular level – supporting the concept that active transport is driven by a transmembrane $\Delta\mu_{H^+}$.

Mechanistic Studies

Studies with right-side-out membrane vesicles (Kaczorowski & Kaback, 1979; Kaczorowski et al., 1979; Patel et al., 1982) demonstrate that carrier-mediated lactose efflux down a concentration gradient occurs in symport with protons and suggest that the translocation reaction is limited either by deprotonation of the carrier on the outer surface of the membrane or by a step corresponding to the return of the unloaded carrier to the inner surface of the membrane. In addition, the observations (Kaczorowski & Kaback, 1979; Kaczorowski et al., 1979) led to the following conclusions: (i) efflux occurs by an ordered mechanism in which lactose is released first from the carrier, followed by loss of the symported proton; (ii) the carrier recycles in the protonated form during exchange and counterflow; and (iii) reactions catalyzed by the unloaded carrier involve net movement of negative charge. Recent experiments with proteoliposomes reconstituted with purified *lac* carrier protein provide convincing support for many of these ideas (Garcia et al., 1983; Viitanen et al., 1983).

Transient accumulation of Rb^+ during lactose efflux in the presence of valinomycin argues strongly for the coupled translocation of a charged species with lactose which leads to the generation of $\Delta\Psi$ (interior negative). In addition to the phenomenon itself, the process is abolished by CCCP, and efflux-induced Rb^+ uptake is blocked by *p*-chloromercuribenzenesulfonate (*p*CMBS), a sulfhydryl reagent that completely inactivates the *lac* carrier protein. Furthermore, the rate of lactose efflux is enhanced by ionophores that collapse $\Delta\Psi$, and artificial imposition of $\Delta\Psi$ (interior negative) dramatically slows the rate of efflux with no significant change in apparent K_m.

The maximal rate of efflux is pH dependent, increasing more than 100-fold from pH 5.5 to pH 9.5 in a sigmoidal fashion with a midpoint at about pH 8.3. In contrast, experiments performed under identical conditions with equimolar lactose in the external medium (i.e., under exchange conditions) demonstrate that the exchange reaction is insensitive to pH and very fast relative to efflux, particularly at relatively acid pH values (below pH 7.5). Therefore, the rate-determining step for efflux must involve either deprotonation of the carrier on the external surface of the membrane or the reaction corresponding to return of the unloaded carrier to the inner surface of the membrane, as these are the only steps by which efflux and exchange differ (Fig. 12). Assuming that loss of lactose and protons from the carrier is necessary for reinitiation of an efflux cycle, external pH would influence the rate of turnover in either of two ways. First, deprotonation could be slow and thereby limit the overall rate of efflux in a pH-dependent manner. Although proton transfers between accessible amino acid residues and water in soluble enzymes are usually fast (Eigen, 1963), little is known about such reactions with hydrophobic membrane proteins. Alternatively, pH could alter the equilibrium between protonated and unprotonated forms of the *lac* carrier, favoring the unprotonated form at more alkaline pH. Since it is assumed that only the deprotonated form of the carrier can recycle, the rate of efflux would be at least partially controlled by external pH, and the rate-determining step might then involve "movement" of the unloaded carrier to the inner surface of the membrane. The observation that the rate of efflux increases with pH is consistent with either possibility. In contrast, if deprotonation of the carrier is not obligatory for exchange, protons might remain bound to the carrier during this mode of translocation, rendering exchange insensitive to pH. If efflux is an ordered mechanism in which the carrier releases lactose first, followed by loss of a proton (Fig. 12), deprotonation and/or return of the unloaded carrier could be slow and appear as the limiting step for efflux.

Counterflow experiments conducted at various pH values reveal that external lactose affects proton loss from the carrier and therefore provide strong support for the ordered efflux mechanism shown in Fig. 12. When external lactose is saturating, counterflow is unaffected by pH; moreover, transient formation of $\Delta\Psi$ observed during lactose efflux is abolished under these conditions. The re-

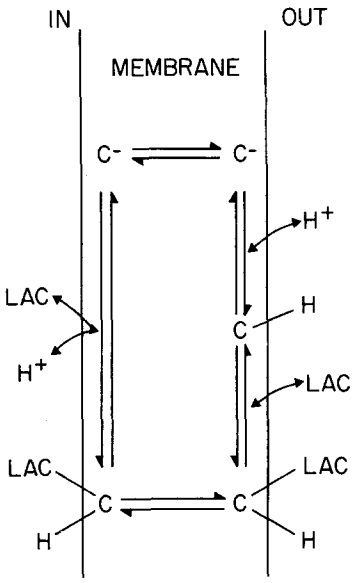

Fig. 12. Schematic representation of reactions involved in lactose efflux. *C* represents the *lac* carrier protein. The order of substrate binding at the inner surface of the membrane is not implied. Reprinted with permission from Kaczorowski and Kaback (1979). Copyright 1982 American Chemical Society.

sults can be interpreted in the following way. On initiation of efflux, lactose and protons bind to the carrier on the inner surface of the membrane (in an unspecified order) and are translocated to the outer surface. Lactose is released from the carrier, but in the presence of excess labeled substrate, rebinding and influx occur rapidly before deprotonation occurs. Under these conditions, therefore, proton release is infrequent and pH has no effect on the overall phenomenon. When external [^{14}C]lactose is limiting, however, rebinding of labeled substrate is less frequent, allowing deprotonation and return of the unloaded carrier. Moreover, as pH is increased, deprotonation and return of the unloaded carrier are enhanced, resulting in further diminution of counterflow. Inhibition of efflux-generated $\Delta\Psi$ formation by external lactose is also readily explained by this scheme. When lactose is present externally at saturating concentrations, release of lactose from the carrier and rebinding of substrate occur rapidly before deprotonation can occur, and the ability of the system to generate $\Delta\Psi$ is abolished.

Given the indication that proton loss may be a limiting step for efflux, it is apparent that one means by which to further investigate the suggested mechanism is to search for a solvent deuterium isotope effect (Kaczorowski et al., 1979; Viitanen et al., 1983). At equivalent pH and pD (i.e., pD = pH + 0.4) (Jencks, 1969), the rate of lactose facilitated diffusion (influx as well as efflux) is approximately 3–4 times slower in deuterated medium (with over 95% of the protium replaced with deuterium) relative to control conditions in protium, while the rate of exchange is identical in the presence of deuterium or protium. Furthermore, during counterflow with the external lactose concentration below the K_m of the carrier, the magnitude of the overshoot is 2–3 times *greater* in deuterium relative to protium. With respect to the kinetic model (*cf.* Fig. 12), high external lactose concentrations prevent deprotonation of the carrier (the C-H form), and it recycles across the membrane in the fully loaded state, catalyzing 1:1 exchange of internal unlabeled lactose with external [^{14}C]lactose. When the external lactose concentration is well below the K_m, however, D_2O increases the coupling efficiency for counterflow, particularly at higher pH (pD) values. Under these conditions, the C−H or C−D form of the carrier partitions between two pathways, one involving loss of protium or deuterium which results in net efflux and the other involving rebinding of lactose prior to loss of protium or deuterium which results in exchange (i.e., counterflow). The former pathway is favored at high pH (pD) values, but the C−D form of the carrier is deprotonated at a slower rate than the C−H form, favoring binding of external [^{14}C]lactose. Consequently, the frequency with which the carrier returns to the inner surface of the membrane in the loaded versus the unloaded form is enhanced in the presence of D_2O, and the effect is most pronounced at alkaline pH. At relatively acid pH (pD), where the coupling efficiency is already essentially 1:1, the D_2O effect is masked.

Remarkably, $\Delta\Psi$-driven lactose accumulation exhibits essentially no solvent deuterium isotope effect. Based on the observations as a whole, it is reasonable to suggest that under conditions where carrier turnover is driven by a lactose concentration gradient, the rate of translocation is determined by a step(s) involving protonation, deprotonation, or the subsequent step (i.e., a reaction corresponding to the return of the unloaded carrier). In contrast, when there is a driving force on the protons (i.e., in the presence of $\Delta\bar{\mu}_{H^+}$), this step(s) is no longer rate-determining. As a cautionary note, it should be emphasized that the solvent deuterium isotope effects described cannot be attributed definitively to a true kinetic isotope effect as opposed to a pK_a effect (i.e., deuterium increases the pK_a values of various functional groups from 0.4 to 0.7 pH units; Jencks, 1969) because the isotope effect on efflux disappears at pH 9.0 and above. On the other hand, at these alkaline

pHs, the rate of efflux approaches the rate of exchange, suggesting that the rate-determining step for efflux may change at high pH. In any event, it seems evident that different steps are limiting when carrier turnover is driven by $\Delta\bar{\mu}_{H^+}$ or by a solute concentration gradient.

A Secondary Structure Model for the *lac* Carrier Protein

Circular dichroic measurements on purified *lac* carrier indicate that $85 \pm 5\%$ of the amino acid residues are arranged in helical secondary structures whether the protein is solubilized in octylglucoside or reconstituted into proteoliposomes (Foster et al., 1982*a*). This finding led to a systematic examination of primary structure as determined from the DNA sequence of the *lac y* gene (Büchel et al., 1980). When the hydrophilicity and hydrophobicity (i.e., hydropathy) of the protein is evaluated along the amino acid sequence according to the method of Kyte and Doolittle (1982), it is apparent that the carrier contains a number of relatively long hydrophobic regions punctuated by shorter hydrophilic regions (Fig. 13). In light of the circular dichroism data, this finding suggests strongly that most, if not all, of these segments are α-helical. Furthermore, since the segments are markedly hydrophobic, it seems likely that they are embedded in the lipid layer. About 12 of the longest hydrophobic segments exhibit a mean length of 24 ± 4 amino acid residues, and they comprise approximately 70% of the length of the polypeptide. The mean length of these segments correlates remarkably well with the mean lengths calculated for similar domains found in four other integral membrane proteins involved in proton translocation (i.e., bacteriorhodopsin and the three subunits of the F_o portion of the proton ATPase). A 24-residue α-helical peptide would be expected to be a maximum of 36 Å in length, a distance that corresponds roughly to the thickness of the hydrophobic core of the membrane.

According to the secondary structure predictions of Chou and Fasman (1974), 18 regions of the *lac* carrier should contain reverse turns (180° reversals) (Fig. 13; *cf. arrows*). As shown, 15 (83%) of the putative turns fall within hydrophilic regions between the hydrophobic segments postulated to traverse the bilayer. The three exceptions are reverse turns that fall within two long hydrophobic segments included by amino acids 73–98 and 289–336.

Based on these considerations, the *lac* carrier is postulated to consist of 12 α-helical segments that traverse the membrane in a zig-zag fashion as suggested for bacteriorhodopsin (Henderson & Unwin, 1975; Engelman, Henderson, McLachlan & Wallace, 1980). It will become clear from the following discussion that some of the experimental tools necessary to test the model are already available. Thus, evidence regarding its validity or the lack thereof should be forthcoming.

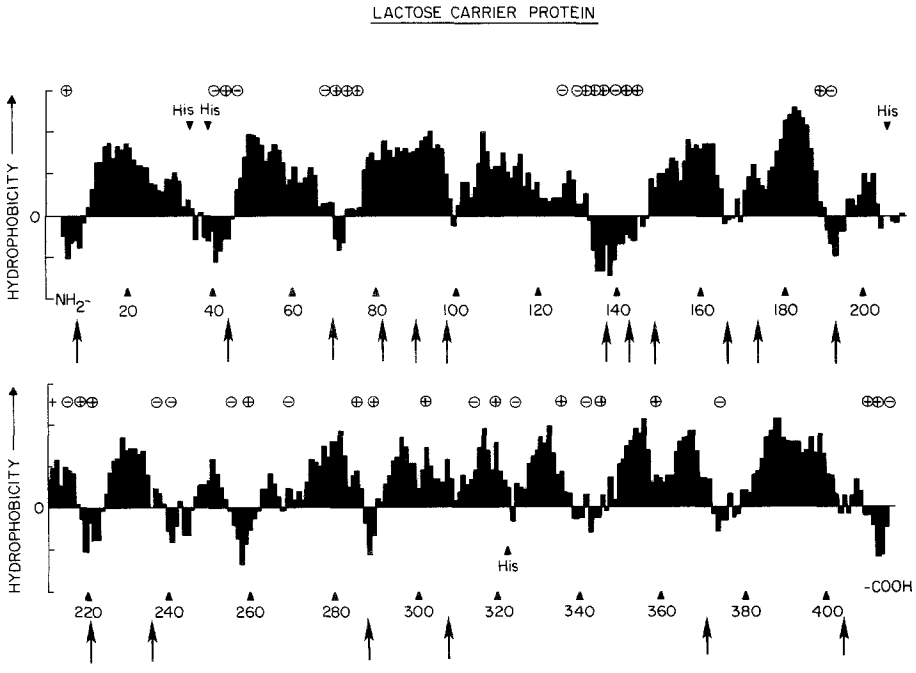

Fig. 13. Hydropathic profile of the *lac* carrier protein. (From Foster et al., 1982*a*)

Topology of the *lac* Carrier Protein in the Membrane

Proteolysis of right-side-out and inside-out membrane vesicles with chymotrypsin, trypsin, or papain inactivates *lac* carrier function in a symmetrical manner (Goldkorn, Kimon & Kaback, 1983). Concomitantly, the electrophoretic mobility of *lac* carrier protein photoaffinity labeled *in situ* with [^{3}H]NPG is altered, and the time course of proteolysis is almost identical in vesicles of opposite polarities (Fig. 14). Clearly, with-right-side-out and inside-out vesicles, the bulk of the radioactivity migrates at M_r 33K with a relatively minor density at a higher M_r (60 K). Since both bands are absent when photoaffinity labeling is carried out in the presence of excess β-D-galactopyranosyl 1-thio-β-D-galactopyranoside and the same bands are observed in similar proportions when the gels are assayed for binding of monoclonal antibody against purified *lac* carrier protein (*cf.* below), it is highly likely that the 60 K material represents an aggregate of the *lac* carrier. In any case, it is apparent that proteolytic digestion of either vesicle preparation leads to a decrease in the intensity of the bands at M_r 33 K and 60 K and the appearance of a new radioactive band at M_r 20 K (lanes *B–F*). A less intense band also appears at about 50 K which may be an aggregate of intact *lac* carrier with the 20 K fragments. Although it may not be readily apparent from the images presented, further addition of chymotrypsin after 3 hr incubation (*G* lanes) causes almost complete conversion of the 33 K band to the 20 K fragment when the films are quantitated by micro-densitometry. Also, similar results are obtained with papain and with trypsin. In contrast, solubilization of the vesicles in SDS, followed by proteolysis, causes fragmentation of the 33 K and 60 K bands into material that electrophoreses at the solvent front. Notably, proteolysis has no effect whatsoever on the ability of the *lac* carrier to bind substrate, as judged by photoaffinity labeling experiments. Furthermore, electrophoresis of samples proteolyzed prior to photoaffinity labeling exhibit the same patterns as those observed when the procedures are reversed. The results provide a clear and direct demonstration that the *lac* carrier protein spans the membrane and indicate that the binding site resides within a segment that is embedded in the bilayer.

At first glance, it is puzzling that proteolysis of the *lac* carrier in right-side-out and inside-out vesicles yields labeled fragments approximately 20 K in size, particularly in view of observations to be discussed which demonstrate that the protein is asymmetric in the membrane. However, consideration of the secondary structure model proposed for the *lac* carrier (Fig. 14) allows a tentative expla-

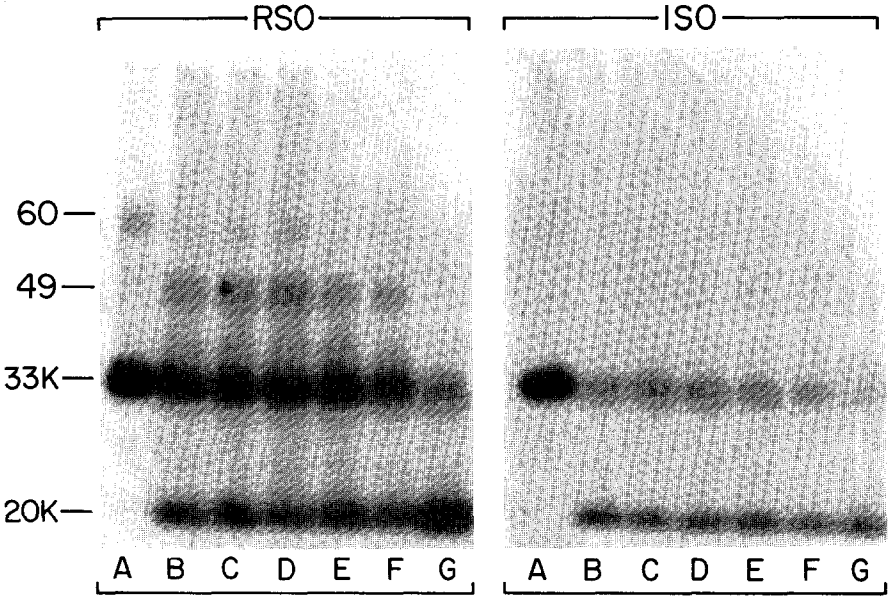

Fig. 14. Proteolysis of photoaffinity labeled *lac* carrier protein in right-side-out (RSO, left-hand panel) and inside-out (ISO, right-hand panel) membrane vesicles. Samples of RSO and ISO vesicles (100 μg protein) that were photoaffinity labeled with [^{3}H]NPG were incubated with 10 μg chymotrypsin for the following times: *A*, 0; *B*, 0.25 hr; *C*, 0.5 hr; *D*, 1 hr; *E*, 1.5 hr; *F*, 3 hr; *G*, 4 hr. Samples *G*, which were incubated with chymotrypsin for a total of 4 hr, were supplemented with an additional 40 μg protease after 3 hr incubation. The samples were then subjected to SDS-PAGE, and the gels were stained with Coomassie Briliant Blue G, destained, dried, and fluorographed. Similar results were obtained with trypsin or papain. (From Goldkorn et al., 1983)

nation of the findings. Assuming that the cysteine residue at position 148, which is protected from alkylation by substrate (Beyreuther, Bieselem, Ehring & Müller-Hill, 1981), is contained within the substrate binding site or in its vicinity, it seems a reasonable possibility that [^{3}H]NPG photolabeling occurs within the amino-terminal half of the molecule. Furthermore, it is suggested that proteolytic attack from either surface of the membrane generates labeled fragments that differ in lenth by only a single transmembrane α-helical segment of 16 to 20 amino acid residues. Thus, proteolysis on one surface between residues 188 and 222 and from the other surface between residues 237 and 260, for instance, would result in two labeled fragments that differ in size by as little as 1.5 K to 2.0 K, a difference that would not be resolved by the techniques utilized. Also implicit in the results is a marked difference in the susceptibility of the various nonhelical portions of the molecule to proteolytic attack, despite their apparent hydrophilic nature. Possibly, the differences arise from intramolecular interactions between the helices that prevent the connecting segments from fully interacting with the solvent at the surfaces of the membrane.

Structure/Function Considerations

Kinetic studies of lactose transport in right-side-out membrane vesicles in the absence and presence of $\Delta\bar{\mu}_{H^+}$ demonstrate that the primary effect of $\Delta\bar{\mu}_{H^+}$ is to cause a marked decrease in the apparent K_m of the system with little change in V_{max} (Kaczorowski et al., 1979; Robertson et al., 1980). Thus, in the absence of $\Delta\bar{\mu}_{H^+}$ transport exhibits an apparent K_m of about 20 mM for lactose, and when $\Delta\bar{\mu}_{H^+}$ is generated by means of an appropriate electron donor, the apparent K_m for lactose decreases about 100-fold to 0.2 mM. More detailed studies (Robertson et al., 1980) in which $\Delta\Psi$ or ΔpH were selectively dissipated show that either component of $\Delta\bar{\mu}_{H^+}$ causes the decrease in K_m, and quantitative measurements under various conditions demonstrate dramatically that the distribution of the transport reaction between the high and low K_m pathways varies as the *square* of $\Delta\Psi$ or ΔpH. Other studies demonstrate that inactivation of the *lac* transport system by various maleimides (Cohn, Kaczorowski & Kaback, 1981) is enhanced two- to threefold in the presence of $\Delta\bar{\mu}_{H^+}$, and a similar effect is observed with the histidine reagent diethylpyrocarbonate (DEPC) (Padan et al., 1979*a*). Thus, the shift between the two kinetic pathways may involve structural or conformational changes that alter the reactivity of certain function groups in the *lac* carrier. Furthermore, treatment of vesicles with DEPC or photooxidation in the presence of rose bengal, an operation that photooxidizes histidine residues, inactivates $\Delta\bar{\mu}_{H^+}$-driven lactose transport with no effect on the ability of the *lac* carrier to bind NPG or catalyze facilitated diffusion of lactose (Patel et al., 1982; Garcia, Patel, Padan & Kaback, 1982). In addition, treatment with DEPC prevents the carrier from responding to $\Delta\bar{\mu}_{H^+}$. That is, after treatment with DEPC, the *lac* carrier no longer exhibits a decrease in K_m in response to $\Delta\bar{\mu}_{H^+}$, and only the high K_m, facilitated diffusion pathway is observed[3]. In all likelihood therefore a histidine residue(s) in the *lac* carrier is (are) involved in the response of the protein to $\Delta\bar{\mu}_{H^+}$. Based on these observations, it was suggested very tentatively (Robertson et al., 1980) that the *lac* carrier might exist in two forms, monomer and dimer, that the monomer catalyzes facilitated diffusion (high K_m) and the dimer active transport (low K_m), and finally, that $\Delta\bar{\mu}_{H^+}$ causes a monomer-dimer transition in which histidine residues in the protein are somehow involved.

Application of electron inactivation analysis (Kempner & Schlegel, 1979) to the system provides support for some of these ideas. In these experiments, vesicles containing the *lac* carrier are frozen in liquid nitrogen before or after generation of $\Delta\bar{\mu}_{H^+}$ and then subjected to a high intensity electron beam for various periods of time. Since the vesicles become very permeable after short periods of irradiation, it is necessary to extract and reconstitute the *lac* carrier in order to assay activity. Thus, after irradiation, the samples are extracted with octylglucoside, reconstituted into proteoliposomes, and tested for counterflow activity (Goldkorn, Rimon, Kempner & Kaback, 1982). Under all conditions tested, the decrease in activity exhibits pseudo-first-order kinetics as a function of radiation dosage, allowing straightforward application of target theory for determination of functional molecular mass. When *lac* carrier activity solubilized from nonenergized vesicles is assayed under these conditions, the results obtained are consistent with a functional molecular weight of 45 K–50 K, a value similar to the molecular weight of the *lac* carrier protein as determined by other means (*cf.* above). Importantly, moreover, similar values are obtained when the octyglucoside extract is irradiat-

[3] It is noteworthy, however, that after the vesicles are inactivated with DEPC, proton influx in response to an inwardly-directed lactose gradient is no longer observed (Patel et al., 1982). Thus, modification of the carrier in this manner appears to uncouple lactose and proton movements at the level of the carrier.

ed, and target sizes observed for D-lactate dehydrogenase (D-LDH) and dicyclohexylcarbodiimide-sensitive ATPase activity in the same vesicle preparations are comparable to the known molecular weights of D-LDH and the F_1 portion of the proton ATPase. Strikingly, when the same procedures are carried out with vesicles that were energized with appropriate electron donors (D-lactate or reduced phenazine methosulfate) prior to freezing and irradiation, a functional molecular weight of 85 K–90 K is obtained for the *lac* carrier with no change in the target volume of D-LDH. In contrast, when the vesicles are energized in the presence of the protonophore CCCP, the target mass of the *lac* carrier returns to 45 K–50 K.

In addition to these observations, genetic studies demonstrating that certain *lac y* mutations are dominant (Mieschendahl, Büchel, Bocklage & Müller-Hill, 1981) also lend credence to the idea that oligomeric structure may be important for *lac* carrier function.

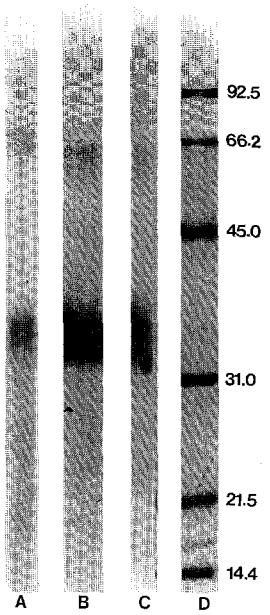

Fig. 15. Specificity of 4B1 hybridoma supernatant for the *lac* carrier protein. Purified *lac* carrier protein and right-side-out T206 membrane vesicles extracted with 5 M urea were subjected to SDS-PAGE, and the protein bands were transferred to nitrocellulose. Individual strips were incubated with 4B1 hybridoma supernatant, washed, incubated with ^{125}I-labeled protein A, and autoradiographed. Lanes: *A*, purified *lac* carrier protein after SDS-PAGE and staining with Coomassie Brilliant Blue G; *B* and *C*, autoradiograms of immunoblotted *lac* carrier protein and T206 membrane vesicles, respectively; *D*, M_r standards after SDS-PAGE and staining with Coomassie Brilliant Blue G. (From Carrasco et al., 1982)

Monoclonal Antibodies as Structure/Function Probes for the *lac* Carrier

Recently, Carrasco et al. (1982) described the preparation, characterization, and properties of monoclonal antibodies directed against the *lac* carrier. The antibodies were prepared by somatic cell fusion of mouse P3X63Ag8.653 myeloma cells with splenocytes from a mouse immunized with purified *lac* carrier protein. A number of clones were shown to produce antibodies that react with the purified protein as demonstrated by solid-phase radioimmunoassay (SP-RIA), and all of the hybridomas chosen for expansion react with the major polypeptide in the purified *lac* carrier preparation that migrates at M_r 33 K (Fig. 15). In addition, a protein of higher apparent M_r (60 K) reacts with the supernatants. Because the same bands are observed with purified *lac* carrier photoaffinity labeled with [^{3}H]NPG and with photoaffinity labeled vesicles containing *lac* carrier (*cf.* Fig. 14), it is likely that the material at 60 K is an aggregate of the *lac* carrier protein. In any event, essentially identical results are obtained with membrane vesicles containing the *lac* carrier (Fig. 15), providing even stronger evidence for the high degree of specificity of the antibodies for the *lac* carrier.

Five hybridomas (3G12, 4A10R, 4B1, 4B11 and 5F7) were subcloned by limiting dilution in two stages (Nowinski et al., 1979). Cloned cells were expanded in culture, injected into mice for ascites fluid production, and the antibodies purified by protein A-Sepharose chromatography. Analyses of the purified antibodies for IgG subclass show that 4B1, 4B11 and 5F7 are IgG2a immunoglobulins, whereas 4A10R and 3G12 are in subclass IgG3. Competition studies (Carrasco, Herzlinger & Kaback, 1983) with [^{125}I]labeled 4B1, 5F7 and 4A10R demonstrate that each of these antibodies is directed against an independent epitope in the *lac* carrier. Furthermore, binding studies performed with right-side-out and inside-out membrane vesicles show that the epitopes for 4B1 and 5F7 are present virtually exclusively on the exterior surface of the membrane, while the epitope(s) for 4A10R appears to be present on the interior surface. Importantly, antibody 4B1 binds essentially stoichiometrically to proteoliposomes reconstituted with purified *lac* carrier protein, indicating that the orientation of the protein in the reconstituted system is similar to that in the native membrane.

In order to correlate immunochemical proper-

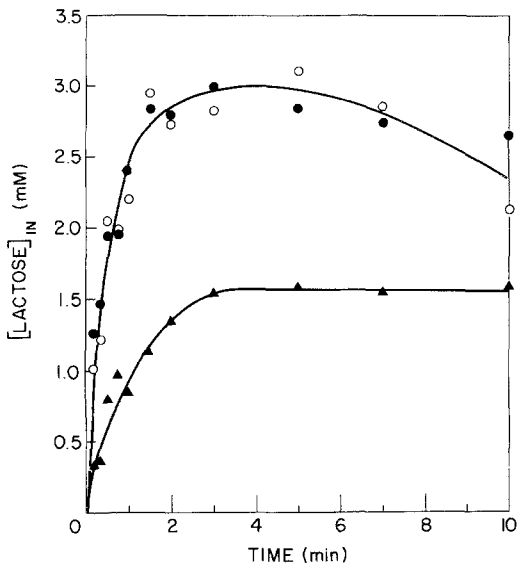

Fig. 16. Effect of antibody 4B1 on $\Delta\Psi$-driven lactose transport in proteoliposomes reconstituted with purified *lac* carrier protein. ▲, Proteoliposomes treated with antibody 4B1; ○, untreated proteoliposomes; ●, proteoliposomes treated with either monoclonal antibody against D-LDH or IgG2a from UPC10 cells. (*See* Carrasco et al. (1982) for experimental details).

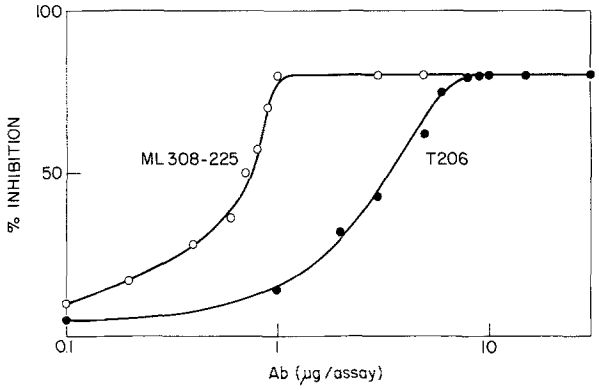

Fig. 17. Inhibitory efect of antibody 4B1 on initial rates of respiration-driven lactose transport in membrane vesicles from ML 308-225 (○) and T206 (●). Right-side-out membrane vesicles from ML 308-225 and T206, as indicated, were incubated with given amounts of antibody 4B1 for 10 min. Subsequently, initial rates of lactose transport were measured at 5 sec in the presence of reduced phenazine methosulfate. Results are presented as percentage inhibition relative to control samples incubated in the absence of antibody. (From Carrasco et al., 1982)

ties with function, supernatants from the hybridomas were screened initially for effects on lactose transport in right-side-out membrane vesicles, and inhibition ranging from 20 to 90% was observed, depending on the hybridoma supernatant tested. Antibody 4B1 exhibited the most pronounced effect. Subsequently, antibodies were purified from the five hybridoma clones and retested for inhibitory activity, and 4B1 was the only antibody observed to give potent inhibition. Further studies demonstrate that antibody secreted by hybridoma 4B1 is the only one of over 50 tested that inhibits lactose transport. Thus, 4B1 was selected for intensive study.

Antibody 4B1 inhibits active transport of lactose in proteoliposomes reconstituted with purified *lac* carrier protein (Fig. 16) and in right-side-out membrane vesicles. Furthermore, the amount of antibody needed for a given degree of inhibition is proportional to the amount of *lac* carrier in the membrane. Thus, with T206 vesicles, which contain five to six times the amount of *lac* carrier as ML 308–225 vesicles, about five times more 4B1 is required to achieve 50% inhibition of the initial rate of lactose transport (Fig. 17). Notably, antibody-induced inhibition occurs within seconds (Carrasco et al., 1982), an observation that is consistent with the external localization of the 4B1 epitope (*cf.* above), and Fab fragments prepared from 4B1 inhibit transport as effectively as intact antibody.

Although 4B1 markedly inhibits $\Delta\bar{\mu}_{H^+}$-driven lactose accumulation, the antibody has no effect on the exchange reaction and no effect on NPG binding. Given these observations, and evidence suggesting that $\Delta\bar{\mu}_{H^+}$ may induce a major conformational alteration in the *lac* carrier (e.g., dimerization), it is tempting to speculate that the antibody might inhibit active transport by preventing this conformational transition.

In any event, initial efforts to localize and identify some of the epitopes within the *lac* carrier protein are promising. When the purified protein is fragmented with cyanogen bromide, 4B1 epitope activity is retained, and a number of cyanogen bromide fragments can be resolved and identified by high performance liquid chromatography (HPLC). In addition, preliminary use of monoclonal affinity columns prepared with antibodies 4B1 and 4A10R appears to be encouraging with regard to epitope isolation and identification. Clearly, these early experiments suggest that further characterization of antibody 4B1 and some of the other monoclonal antibodies combined with detailed structure/function studies may provide important insight into the structure and mechanism of action of the *lac* carrier protein.

References

Banker, G.A., Cotman, C.W. 1972. *J. Biol. Chem.* **247**:5856–5861

Beyreuther, K., Bieselem, B., Ehring, R., Müller-Hill, B. 1981. Methods in Protein Sequence Analysis. M. Elzina, editor. p. 139. Humana Press, Clifton, N.J.
Büchel, D.E., Groneborn, B., Müller-Hill, B. 1980. *Nature (London)* **283**:541–545
Carrasco, N., Tahara, S.M., Patel, L., Goldkorn, T., Kaback, H.R. 1982. *Proc. Natl. Acad. Sci. USA* **79**:6894–6898
Carrasco, N., Herzlinger, D., Kaback, H.R. 1983. *Fed. Proc. (abstr.) (in press)*
Chou, P.Y., Fasman, G.D. 1974. *Biochemistry* **13**:222–245
Cohen, G.N., Monod, J. 1957. *Bacteriol. Rev.* **21**:169–194
Cohen, G.N., Rickenberg, H.V. 1955. *Compt. Rend.* **240**:466–8
Cohn, D., Kaczorowski, G.J., Kaback, H.R. 1981. *Biochemistry* **20**:3308–3313
Cornelisse, J., DeGunst, G.P., Havinga, E. 1975. *Adv. Phys. Org. Chem.* **11**:225
Cornelisse, J., Havinga, E. 1975. *Chem. Rev.* **75**:353–388
Daniels, C.J., Bole, D.G., Quay, S.C., Oxender, D.L. 1981. *Proc. Natl. Acad. Sci. USA* **78**:5396–5400
Date, T., Zwizniski, C., Ludmerer, S., Wickner, W. 1980. *Proc. Natl. Acad. Sci. USA* **77**:827–831
Delmer, D.P., Benziman, M., Padan, E. 1982. *Proc. Natl. Acad. Sci. USA* **79**:5282–5286
Doetsch, R.N., Sjoblad, R.D. 1980. *Ann. Rev. Microbiol.* **34**:69–108
Ehring, R., Beyreuther, K., Wright, J.K., Overath, P. 1980. *Nature (London* **283**:537–540
Eigen, M. 1963. *Angew. Chem. Int. Ed. Engl.* **3**:1–19
Enequist, H.G., Hirsh, T.R., Havayama, S., Hardy, S.J.S., Randall, L.L. 1981. *Eur. J. Biochem.* **116**:227–233
Engelman, D.M., Henderson, R., McLachlan, A.D., Wallace, B.A. 1980. *Proc. Natl. Acad. Sci. USA* **77**:2023–2027
Foster, D.L., Boublik, M., Kaback, H.R. 1982*a*. *J. Biol. Chem.* **258**:31–34
Foster, D.L., Garcia, M.L., Newman, M.J., Patel, L., Kaback, H.R. 1982*b*. *Biochemistry* **21**:5634–5638
Garcia, M.L., Patel, L., Padan, E., Kaback, H.R. 1982. *Biochemistry* **21**:5800–5805
Garcia, M.L., Viitanen, P., Foster, D.L., Kaback, H.R. 1983. *Biochemistry (in press)*
Ghazi, A., Shechter, E. 1981. *Biochim. Biophys. Acta* **645**:305–315
Goldkorn, T., Rimon, G., Kaback, H.R. 1983. *Proc. Natl. Acad. Sci. USA (in press)*
Goldkorn, T., Rimon, G., Kempner, E.S., Kaback, H.R. 1982. *Fed. Proc.* **41**:1415
Greville, G.D. 1969. *Curr. Topics in Bioenerg.* **3**:1
Grinius, L. 1980. *FEBS Lett.* **113**:1–10
Grinius, L., Berzinskiene, J. 1976. *FEBS Lett.* **72**:151–154
Harold, F.M. 1972. *Bacteriol. Rev.* **36**:172–230
Harold, F.M. 1978. *In:* The Bacteria. I.C. Gunsalus, L.N. Ornston, and T.R. Socatch, editors. Vol. 6, pp. 463–521 Academic Press, New York
Henderson, R., Unwin, P.N.J. 1975. *Nature (London)* **257**:28–32
Hinkle, P.C., McCarty, R.E. 1978. *Sci. Am.* **238**(3):104–123
Hobson, A.C., Gho, D., Müller-Hill, B. 1977. *J. Bacteriol.* **131**:830–838
Hong, J.-S. 1977. *J. Biol. Chem.* **252**:8582–8588
Jelenc, P.C., Cantor, C.R., Simon, S.R. 1978. *Proc. Natl. Acad. Sci. USA* **75**:3564–3568
Jencks, W.P. 1969. *In:* Catalysis in Chemistry and Enzymology. McGraw-Hill, New York
Jones, T.H.D., Kennedy, E.P. 1969. *J. Biol. Chem.* **244**:5981–5987
Kaback, H.R. 1977. *J. Cell. Physiol.* **89**:575–593
Kaczorowski, G.J., Kaback, H.R. 1979. *Biochemistry* **18**:3691–3697
Kaczorowski, G.J., LeBlanc, G., Kaback, H.R. 1980. *Proc. Natl. Acad. Sci. USA* **77**:6319–6323
Kaczorowski, G.J., Robertson, D.E., Kaback, H.R. 1979. *Biochemistry* **18**:3697–3704
Kalasauskaite, E., Grinius, L. 1979. *FEBS Lett.* **99**:287–291
Kempner, E.S., Schlegel, W. 1979. *Anal. Biochem.* **92**:2–10
Kennedy, E.P. 197 . *In:* The Lactose Operon. J.R. Beckwith and D. Zipser, editors. p. 49. Cold Spring Harbor Laboratory, New York
Kepes, A. 1971. *J. Membrane Biol.* **4**:87–112
Kepes, A., Cohen, G.N. 1962. *In:* The Bacteria. I.C. Gunsalus and R. Stanier, editors. Vol. 4, p. 179–221. Academic Press, New York
König, B., Sandermann, H., Jr. 1982. *FEBS Lett.* **147**:31
Kyte, J., Doolittle, R.F. 1982. *J. Mol. Biol.* **157**:105–132
Laane, C., Krone, W., Konings, W., Haaker, H., Veeger, C. 1980. *Eur. J. Biochem.* **103**:39–46
Labedan, B., Goldberg, E.B. 1979. *Proc. Natl. Acad. Sci. USA* **76**:4669–4673
Mates, S., Eisenberg, E.S., Mandel, L.J., Patel, L., Kaback, H.R., Miller, M.H. 1982. *Proc. Natl. Acad. Sci. USA* **79**:6693–6697
Matsushita, K., Patel, L., Gennis, R.B., Kaback, H.R. 1983. *Fed. Proc. (abstr.) (in press)*
McMurry, L., Petrucci, R.E., Levy, S.B. 1980. *Proc. Natl. Acad. Sci. USA* **77**:3974–3977
Michels, P.A.M., Michels, J.P.J., Boonstra, J., Konings, W.N. 1979. *FEMS Microbiol. Lett.* **5**:357–364
Mieschendahl, M., Büchel, D., Bocklage, H., Müller-Hill, B. 1981. *Proc. Natl. Acad. Sci. USA* **78**:7652–7656
Mitchell, P. 1961. *Nature (London)* **191**:144–148
Mitchell, P. 1963. *Biochem. Soc. Symp.* **22**:142–169
Mitchell, P. 1966. Chemiosmotic Coupling in Oxidative and Photophosphorylation. Glynn Research, Bodmin, England
Mitchell, P. 1968. Chemiosmotic Coupling and Energy Transduction. Glynn Research, Bodmin, England
Neville, D.M. 1971. *J. Biol. Chem.* **246**:6328–6334
Newman, M.J., Foster, D., Wilson, T.H., Kaback, H.R. 1981. *J. Biol. Chem.* **256**:11804–11808
Newman, M.J., Wilson, T.H. 1980. *J. Biol. Chem.* **255**:10583–10586
Nowinski, R.C., Lostrom, M.E., Tam, M.R., Stone, M.R., Burnette, W.N. 1979. *Virology* **93**:111–126
Otto, R., Sonenberg, A.S.M., Veldkamp, H., Konings, W.N. 1980. *Proc. Natl. Acad. Sci. USA* **77**:5502–5506
Padan, E., Patel, L., Kaback, H.R. 1979*a*. *Proc. Natl. Acad. Sci. USA* **76**:6221–6225
Padan, E., Schuldiner, S., Kaback, H.R. 1979*b*. *Biochem. Biophys. Res. Commun.* **91**:854–861
Patel, L., Garcia, M.L., Kaback, H.R. 1982. *Biochemistry* **21**:5805–5810
Patel, L., Schuldiner, S., Kaback, H.R. 1975. *Proc. Natl. Acad. Sci. USA* **77**:3387–3391
Plate, C.A., Suit, J.L. 1981. *J. Biol. Chem.* **256**:12974–12980
Racker, E., Violand, B., O'Neal, S., Alfonzo, M., Telford, J. 1979. *Arch. Biochem. Biophys.* **198**:470–477
Rickenberg, H.V., Cohen, G.N., Buttin, G., Monod, J. 1956. *Ann. Inst. Pasteur* **91**:829–857
Robertson, D.E., Kaczorowski, G.J., Garcia, M.L., Kaback, H.R. 1980. *Biochemistry* **19**:5692–5702
Sandermann, H., Jr. 1977. *Eur. J. Biochem.* **80**:507–515
Santos, E., Kaback, H.R. 1981. *Biochem. Biophys. Res. Commun.* **99**:1153–1160
Skulachev, V.P., Hinkle, P.C. (editors). 1981. Chemiosmotic

Proton Circuits in Biological Membranes. Addison-Wesley, Reading, Mass
Teather, R.M., Müller-Hill, B., Abrutsch, V., Aichele, G., Overath, P. 1978. *Mol. Gen. Genet.* **159:**239–248
Teather, R.M., Bramhall, J., Riede, I., Wright, J.K., Furst, M., Aichele, G., Wilhelm, V., Overath, P. 1980. *Eur. J. Biochem.* **108:**223–231
Viitanen, P., Garcia, M.L., Foster, D.L., Kaczorowski, G.J., Kaback, H.R. 1983. *Biochemistry (in press)*
Villarejo, M. 1980. *Biochem. Biophys. Res. Commun.* **93:**16–23
Villarejo, M., Ping, C. 1978. *Biochem. Biophys. Res. Commun.* **82:**935–942
Wagner, E.F., Ponta, H., Schweiger, M. 1980. *J. Biol. Chem.* **255:**534–539
West, I.C. 1970. *Biochem. Biophys. Res. Commun.* **41:**655–661
West, I.C., Mitchell, P. 1972. *J. Bioenerg.* **3:**445–462
West, I.C., Mitchell, P. 1973. *Biochem. J.* **132:**587–592
Wright, J.K., Schwarz, H., Straub, E., Overath, P., Bieseler, B., Beyreuther, K. 1982. *Eur. J. Biochem.* **124:**545–552

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