Mechanism of Lactose Transport in *Escherichia coli* Membrane Vesicles: Evidence for the Involvement of Histidine Residue(s) in the Response of the *lac* Carrier to the Proton Electrochemical Gradient†

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ABSTRACT: Exposure of *Escherichia coli* ML 308-225 membrane vesicles to diethyl pyrocarbonate or to light in the presence of rose bengal and air causes inactivation of active lactose transport and/or counterflow in a manner that is blocked by substrates of the *lac* carrier protein. The effect of pH on inactivation indicates that loss of activity is due to modification of an amino acid residue with a pK_a between 6.0 and 6.5, and the pH profiles for inactivation are very similar to those observed for the reaction of histidine. Furthermore, titration experiments with diethyl pyrocarbonate imply that acylation of a single site in the β-galactoside transport system is sufficient for inactivation. In contrast, neither reagent inhibits the ability of the carrier to bind *p*-nitrophenyl α-D-galactopyranoside nor its ability to catalyze facilitated diffusion of lactose [Padan, E., Patel, L., & Kaback, H. R. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 6221]. Experiments carried out over an extended range of lactose concentrations demonstrate that treatment with diethyl pyrocarbonate causes the *lac* transport system to exhibit biphasic kinetics. One component of the overall process exhibits kinetic parameters typical of active transport (i.e., low apparent K_m), and the other has the characteristics of facilitated diffusion (i.e., high apparent K_m). Since partial dissipation of the proton electrochemical gradient leads to similar biphasic kinetics [Robertson, D. E., Kaczorowski, G. J., Garcia, M.-L., & Kaback, H. R. (1980) *Biochemistry 19*, 5692] and treatment of the vesicles with diethyl pyrocarbonate induces a similar alteration with no reduction in the proton electrochemical gradient, it is suggested that a histidine residue(s) in the *lac* carrier is (are) involved in the response of the protein to the proton gradient. In the following paper [Patel, L., Garcia, M. L., & Kaback, H. R. (1982) *Biochemistry* (following paper in this issue)], the effects of diethyl pyrocarbonate on lactose-induced proton movements in isolated membrane vesicles are examined.

Cytoplasmic membrane vesicles prepared from *Escherichia coli* by osmotic lysis retain the same polarity as the membrane in the intact cell (Kaback, 1971, 1974a; Short et al., 1975; Owen & Kaback, 1978, 1979a,b) and catalyze the active accumulation of many substrates by mechanisms in which chemiosmotic phenomena (Mitchell, 1961, 1968, 1973, 1979) play a central, obligatory role (Ramos et al., 1976; Ramos & Kaback, 1977a–c; Konings & Boonstra, 1977; Reenstra et al., 1980; LeBlanc et al., 1980; Hugenholtz et al., 1981). Despite widespread agreement on the chemiosmotic nature of active transport, however, little is known about the mechanism of the phenomenon and the chemical reactions and/or functional groups involved and whether or not they are affected by the proton electrochemical gradient ($\Delta\bar{\mu}_{H^+}$).[1]

Kinetic studies of lactose transport in the vesicle system have provided some insight into the effects of $\Delta\bar{\mu}_{H^+}$ on the overall translocation reactions. Comparison of active transport and facilitated diffusion of lactose shows that either a membrane potential ($\Delta\Psi$, interior negative) or a pH gradient (ΔpH, interior alkaline) causes a dramatic decrease in the apparent K_m (Kaczorowski et al., 1979; Robertson et al., 1980). More recently, it was demonstrated (Robertson et al., 1980) that the β-galactoside transport system catalyzes two distinctly different reactions, one exhibiting a high apparent K_m (in the absence of $\Delta\bar{\mu}_{H^+}$) and the other a much lower apparent K_m (in the presence of $\Delta\bar{\mu}_{H^+}$), and that the interconversion of the high and low K_m pathways is dependent on the magnitude of $\Delta\Psi$ or ΔpH to the second power. In order to explain the observations, it was tentatively suggested that $\Delta\bar{\mu}_{H^+}$ causes a structural change in the *lac* carrier protein; however, it was emphasized that a change in the rate-limiting step for transport might account for the findings without a structural alteration in the *lac* carrier.

With regard to the role of functional groups, several porters in *E. coli* membrane vesicles, the *lac* carrier in particular, are inactivated by sulfhydryl reagents (Kepes, 1960; Fox & Kennedy, 1965; Barnes & Kaback, 1971; Kaback & Barnes, 1971; Kaback & Patel, 1978), and recent experiments (Cohn et al., 1981) indicate that $\Delta\bar{\mu}_{H^+}$ (interior negative and alkaline) brings about an increase in the reactivity of sensitive sulfhydryl groups in these transport systems. In addition, Padan et al. (1979) reported that $\Delta\bar{\mu}_{H^+}$-driven lactose transport and counterflow (i.e., $\Delta\mu_{lac}$-driven lactose transport), but not facilitated diffusion, are inhibited by diethyl pyrocarbonate (DEPC). Moreover, inhibition by the reagent is accelerated by $\Delta\bar{\mu}_{H^+}$, partially blocked by substrate, and reversed by treatment with hydroxylamine. These findings and the observation that photooxidation of the vesicles in the presence of rose bengal (Westhead, 1965), an operation known to destroy histidine residues, causes similar effects led to the preliminary conclusion that a histidyl residue(s) in the *lac* carrier may play an important role in the response of the β-galactoside transport system to $\Delta\bar{\mu}_{H^+}$.

The results presented here provide further evidence for an essential role of a histidine residue(s) in the mechanism of action of the *lac* carrier protein. In the following paper (Patel et al., 1982), the effect of DEPC on lactose-induced proton movements in isolated membrane vesicles is examined.

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[1] Abbreviations: $\Delta\bar{\mu}_{H^+}$, proton electrochemical gradient; $\Delta\Psi$, membrane potential; ΔpH, pH gradient; DEPC, diethyl pyrocarbonate; PMS, phenazine methosulfate; NPG, *p*-nitrophenyl α-D-galactopyranoside; TDG, β-D-galactopyranosyl 1-thio-β-D-galactopyranoside.

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Experimental Procedures

Materials. Rose bengal was obtained from Fisher Scientific Co. and purified by passage through a column of Dowex 50 in the sodium form (Westhead, 1965). DEPC was purchased from Sigma and [1-^{14}C]lactose (57.7 mCi/mmol) from Amersham/Searle. All other materials were reagent grade and obtained from commercial sources.

Growth of Cells and Preparation of Membrane Vesicles. *E. coli* ML 308-225 ($i^-z^-y^+a^+$) was grown on minimal medium A (Davis & Mingioli, 1959), containing 1.0% disodium succinate (hexahydrate), and membrane vesicles were prepared as described previously (Kaback, 1971; Short et al., 1975).

For experiments at various pHs, vesicles prepared in 0.1 M potassium phosphate (pH 6.6) and stored in liquid nitrogen were thawed and resuspended in a 50-fold excess of a given buffer at the desired pH. After incubation at room temperature for 30 min, the vesicles were collected by centrifugation (45000*g* for 30 min), washed once with the same buffer, and resuspended at an appropriate protein concentration.

Rose Bengal Catalyzed Photooxidation. Treatment of vesicles with rose bengal was carried out essentially as described by Westhead (1965) in a water-jacketed tube of 1.0-cm internal diameter. The water jacket, containing a 1.0 M solution of sodium nitrate to filter out wavelengths below 400 nm, was maintained at 25 °C by means of a circulating pump. The sample (up to 5.0 mL) at a final concentration of 2.0 mg of protein/mL was stirred with a magnetic stirrer and irradiated with a 500-W slide projector. The lens of the projector was placed 10 cm from the reaction vessel. Aliquots were withdrawn at appropriate intervals and diluted with a 40-fold excess of 0.1 M potassium phosphate (pH 7.0) containing 0.01 M lactose, and the vesicles were collected by centrifugation (45000*g* for 30 min), washed once with the same medium, and resuspended in a minimal volume to give as concentrated a suspension as possible (ca. 40 mg of protein/mL). Control samples were treated identically, but without rose bengal, with rose bengal in the dark or under argon.

Treatment with DEPC. Vesicles were treated with DEPC as described (Padan et al., 1979). After addition of the reagent, aliquots were withdrawn at given times, diluted with a 40-fold excess of either 0.1 M potassium phosphate (pH 7.0) containing 0.01 M lactose (counterflow experiments) or 0.1 M potassium phosphate (pH 6.5) ($\Delta\bar{\mu}_{H^+}$-driven uptake). The vesicles were collected by centrifugation (45000*g* for 30 min), washed once in the same buffer, and resuspended to an appropriate protein concentration.

Transport Assays. Uptake of [1-^{14}C]lactose at given concentrations was determined in the presence and absence of reduced phenazine methosulfate (PMS) as described (Kaback, 1974b; Robertson et al., 1980). Lactose counterflow was measured as described by Kaczorowski & Kaback (1979).

Protein. Protein was measured according to Lowry et al. (1951) with bovine serum albumin as standard.

Results

Effect of Rose Bengal Photooxidation and DEPC Treatment on lac Carrier Function. Unlike DEPC, which inactivates $\Delta\bar{\mu}_{H^+}$-driven lactose transport with no effect on the generation of $\Delta\bar{\mu}_{H^+}$ (Padan et al., 1979), rose bengal catalyzed photooxidation markedly impairs the ability of the vesicles to generate $\Delta\bar{\mu}_{H^+}$ in the presence of reduced PMS (data not shown). Thus, counterflow or another activity of the carrier that is independent of respiration must be used to monitor *lac* carrier function. When *E. coli* ML 308-225 vesicles are subjected to photooxidation in the presence of rose bengal, it is apparent that both the initial rate of counterflow and the magnitude of the overshoot are diminished and that the degree of inhibition increases with the concentration of rose bengal (Figure 1). Furthermore, illumination of the vesicles in the absence of dye or oxygen (i.e., illumination was carried out under argon) and incubation with rose bengal in the dark have no effect whatsoever on lactose counterflow activity. The time course of photoinactivation with rose bengal at a concentration of 0.06 μM is pseudo first order with a half-time of inactivation (i.e., $t_{1/2}$) of about 15 min at this concentration of dye (data not shown).

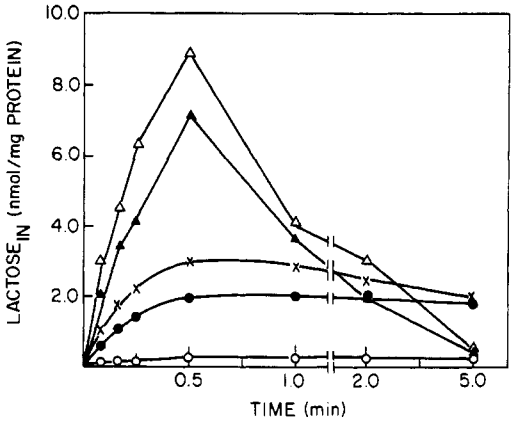

FIGURE 1: Effect of illumination in the presence of rose bengal and air on lactose counterflow. *E. coli* ML 308-225 membrane vesicles were suspended in 0.1 M potassium phosphate (pH 6.0) at a final concentration of 2.0 mg of protein/mL and placed in a jacketed tube that was maintained at 25 °C by means of a circulating water pump. Samples were stirred with a magnetic stirrer, rose bengal was added to concentrations given below, and illumination was carried out with a 500-W slide projector for 30 min as described under Experimental Procedures. The vesicles were then washed and resuspended in 0.1 M potassium phosphate (pH 7.0) to a final concentration of 40 mg of protein/mL. The concentrated suspensions were equilibrated with 0.01 M lactose by incubation at room temperature for 4 h. Counterflow was measured by diluting aliquots (2 μL) into 400 μL of 0.1 M potassium phosphate (pH 7.0) containing 0.4 mM [1-^{14}C]lactose (19 mCi/mmol) and assaying samples at times ranging from 2 s to 5 min. Experimental controls were carried out by treating membranes under identical conditions with rose bengal in the dark or by illumination in the absence of the dye or under argon. (△) Control vesicles treated with rose bengal in the dark or illuminated without rose bengal or oxygen; (▲) vesicles illuminated in the presence of 0.014 μM rose bengal; (×) vesicles illuminated in the presence of 0.06 μM rose bengal; (●) vesicles illuminated in the presence of 0.28 μM rose bengal; (○) vesicles illuminated in the presence of 1.4 μM rose bengal.

Treatment of vesicles with DEPC also inactivates counterflow with pseudo-first-order kinetics (not shown), and a plot of the inactivation rates (i.e., $t_{1/2}^{-1}$) vs. DEPC concentration yields a linear function with a slope of 1 (Figure 2). The findings imply that acylation of a single site in the transport system is sufficient to inactivate counterflow.

Previous experiments (Padan et al., 1979) demonstrate that inactivation of the *lac* carrier by DEPC is blocked by substrate although treatment of vesicles with the reagent does not inhibit binding of *p*-nitrophenyl α-D-galactopyranoside (NPG). Similarly, rose bengal catalyzed photoinactivation of lactose counterflow is almost completely blocked by β-D-galactopyranosyl 1-thio-β-D-galactopyranoside (TDG; Figure 3). Furthermore, although data will not be presented, the K_d for NPG in vesicles photooxidized with rose bengal is identical with that observed in control preparations (i.e., about 12 μM; Rudnick et al., 1976), while the number of binding sites is only marginally decreased, if at all (0.15–0.2 nmol/mg of membrane protein).

pH Dependence of Inactivation. Since the rate of acylation of imidazole by DEPC, as well as its photooxidation by rose

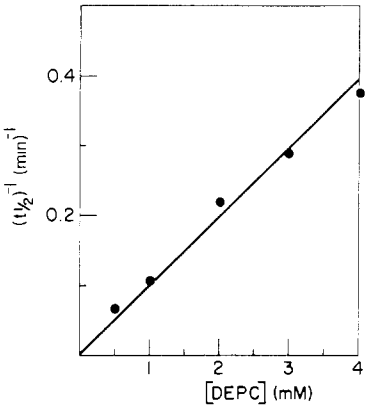

FIGURE 2: Effect of DEPC concentration on the rate of inactivation of lactose counterflow. ML 308-225 membrane vesicles were suspended in 0.1 M potassium phosphate (pH 6.0) to a concentration of 2.0 mg of protein/mL and treated with DEPC at concentrations ranging from 0.5 to 4.0 mM. Aliquots were withdrawn at appropriate time intervals, washed free of the reagent, and resuspended in 0.1 M potassium phosphate (pH 7.0) containing 0.01 M lactose to a final concentration of about 40 mg of protein/mL. Initial rates of lactose counterflow were measured as described in Figure 1, and at each DEPC concentration tested, the rate of inactivation was pseudo first order (data not shown). Half-times of inactivation ($t_{1/2}$) were calculated from semilogarithmic plots. Inactivation rates (i.e., $t_{1/2}^{-1}$) are plotted as a function of DEPC concentration.

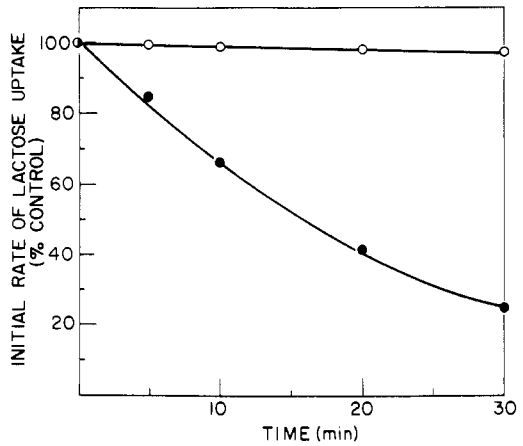

FIGURE 3: Effect of TDG on rose bengal photoinactivation of lactose counterflow. *E. coli* ML 308-225 membrane vesicles were illuminated in the presence of 0.06 μM rose bengal and air as described under Experimental Procedures (●). Where indicated (○), TDG was added to the inactivation mixtures to a final concentration of 20 mM. At given times, samples were washed and assayed for initial rates of lactose counterflow as described in Figure 1. Initial rates of counterflow are expressed as percentages of appropriate control samples incubated in the absence or presence of TDG but without rose bengal. In each case, the control values approximated 60 nmol min^{-1} (mg of membrane protein)$^{-1}$.

bengal, is inversely related to the protonation state of imidazole, the pH dependence of inactivation is a useful strategem for approximating the nature of the amino acid residue(s) involved. When vesicles are treated with 2.0 mM DEPC over a range of pH values from pH 5.0 to 8.0, the time course of inactivation of lactose counterflow is pseudo first order at each pH tested (data not shown), and the relationship between inactivation rate (i.e., $t_{1/2}^{-1}$) and pH is shown in Figure 4. The rate of inactivation is almost negligible at pH 5.0 and increases with pH, exhibiting an inflection at approximately pH 6.5. Although the reason for the increased rates of inactivation at pH values above 7.0 is unknown, DEPC will react with functional groups other than histidine that become nucleophilic at alkaline pH (Miles, 1977). In any case, the profile observed is similar to that observed for histidine in aqueous solution

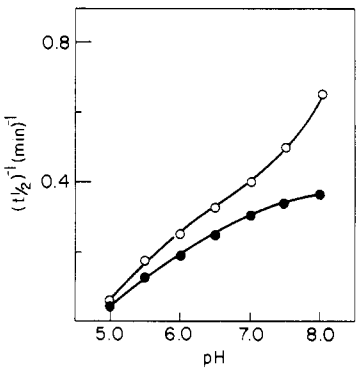

FIGURE 4: pH dependence of DEPC inactivation and rose bengal photoinactivation of lactose counterflow. *E. coli* ML 308-225 vesicles were resuspended in 0.1 M potassium phosphate at a given pH to a final protein concentration of 2.0 mg/mL and treated with 2.0 mM DEPC (○) or illuminated in the presence of 0.06 μM rose bengal and air (●) as described previously (Padan et al., 1979) and under Experimental Procedures. Aliquots were withdrawn at appropriate intervals, washed free of reagent, and resuspended in 0.1 M potassium phosphate (pH 7.0) to a final concentration of 40 mg of protein/mL. The concentrated suspensions were equilibrated with 0.01 M lactose by incubation at room temperature for 4 h. Initial rates of lactose counterflow were then measured as described in Figure 1. At each pH, the rate of inactivation was pseudo first order, and half-times of inactivation ($t_{1/2}$) were obtained from semilogarithmic plots. Inactivation rates (i.e., $t_{1/2}^{-1}$) are plotted as a function of pH.

Table I: Effect of Reduced PMS on DEPC Inactivation of Lactose Counterflow as a Function of pH[a]

pH	$t_{1/2}$ of inhibition (min)	
	−PMS	+PMS
5.0	20	10
6.0	4	2.2
7.0	2.5	1.2
8.0	1.2	0.5

[a] *E. coli* ML 308-225 membrane vesicles were resuspended in 0.1 M potassium phosphate at a given pH to a concentration of 2 mg of protein/mL and treated with 2.0 mM DEPC in the absence or presence of reduced PMS for different periods of time at 25 °C (Padan et al., 1979). The vesicles were washed free of excess reagent and resuspended in 0.1 M potassium phosphate (pH 7.0) containing 0.01 M lactose. Initial rates of lactose counterflow were assayed as described in Figures 1 and 2. The $t_{1/2}$ of inhibition was determined from semilogarithmic plots which exhibited pseudo-first-order kinetics at each pH listed (data not shown).

(Holbrook & Ingram, 1973; data not shown). As shown previously (Padan et al., 1979), DEPC inactivation of lactose transport is enhanced in the presence of $\Delta\bar{\mu}_{H^+}$. When vesicles are treated with DEPC at various pHs in the absence and presence of reduced PMS, the $t_{1/2}$ of inactivation is diminished about 2-fold from pH 5.0 to 8.0 (Table I). On the other hand, the inflection remains at pH 6.0–6.5.

In a parallel series of experiments, vesicles were subjected to photooxidation in the presence of rose bengal at pHs ranging from 5.0 to 8.0, washed free of reagent, and assayed for lactose counterflow. Again, at each pH tested, the rate of inactivation is pseudo first order (data not shown). Moreover, when $t_{1/2}^{-1}$ at each pH is plotted as a function of pH, it is apparent that the inactivation rate increases almost linearly from pH 5.0 to 7.0 and exhibits saturation behavior at higher pH values (Figure 4). The profile, with the midpoint between pH 6.0 and 6.5, corresponds very closely to that obtained for rose bengal catalyzed photooxidation of histidine (Westhead, 1965).

These and the previous results (Padan et al., 1979), particularly those demonstrating that DEPC inactivation of lactose counterflow is reversed by hydroxylamine, suggest that a

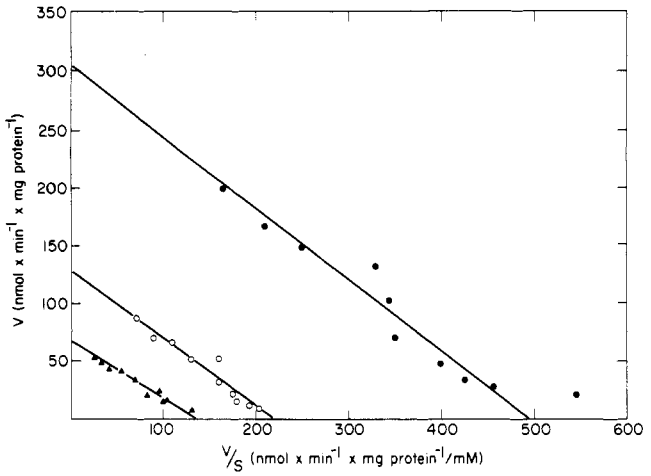

FIGURE 5: Effect of DEPC and rose bengal on the kinetics of lactose counterflow. *E. coli* ML 308-225 membrane vesicles were resuspended in 0.1 M potassium phosphate (pH 6.0) to a final concentration of 2.0 mg of protein/mL and treated with DEPC at a final concentration of 1.0 mM for 10 min at 25 °C (○). Alternatively, a sample was placed in a jacketed tube which was maintained at 25 °C by means of a circulating water pump, rose bengal was added to a final concentration of 0.1 μM, and the sample was illuminated for 30 min (▲). The preparations were then washed and resuspended in 0.1 M potassium phosphate (pH 7.0) containing 0.01 M lactose to a final concentration of 40 mg of protein/mL and incubated at room temperature for 4 h. Initial rates of counterflow were measured by diluting aliquots (2 μL) 200-fold into 0.1 M potassium phosphate (pH 7.0) containing various concentrations of [1-^{14}C]lactose and assaying samples at times ranging from 2 to 15 s. Over this time period, initial rates of lactose uptake were linear at each lactose concentration tested. Control samples (●) were treated identically, except that they were incubated in the absence of DEPC for 10 min or illuminated in the absence of rose bengal for 30 min. No significant difference was observed in the control samples.

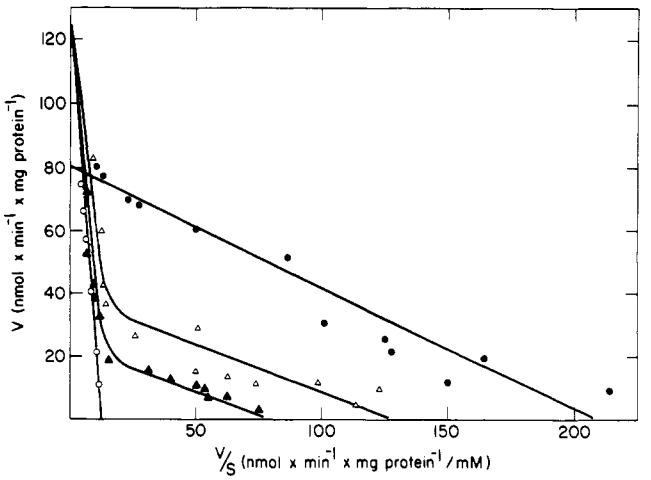

FIGURE 6: Effect of DEPC on the kinetics of $\Delta\bar{\mu}_{H^+}$-driven lactose transport. *E. coli* ML 308-225 membrane vesicles were incubated without DEPC (●, ○) or with DEPC at final concentration of 0.5 (△) or 1.0 mM (▲) for 10 min at 25 °C, washed free of the reagent, and resuspended in 0.1 M potassium phosphate (pH 6.5) to a final concentration of 4.0 mg of protein/mL. Aliquots (0.5 mL) were diluted to a final volume of 1.0 mL in 16 × 125 mm test tubes containing, in final concentrations, 50 mM potassium phosphate (pH 6.5) and 10 mM magnesium sulfate (Robertson et al., 1980). Where indicated (○), carbonyl cyanide *m*-chlorophenylhydrazone was added to a final concentration of 10 μM. The samples were incubated for 30 s to 1 min at 25 °C under an atmosphere of water-saturated oxygen (Kaback, 1974b). Unless indicated otherwise (○), potassium ascorbate and PMS were added to final concentrations of 20 and 0.1 mM, respectively, and the incubations were continued at 25 °C under oxygen for 30 s. At this time, [1-^{14}C]lactose (2.0 mCi/mmol) was added at concentrations ranging from 0.04 to 15 mM. Uptake was allowed to proceed for 10 s, when the reactions were terminated by addition of 5.0 mL of 0.1 M potassium phosphate (pH 5.5) containing 0.1 M lithium chloride. The samples were immediately and rapidly filtered through 47-mm Amicon microporous filters (0.45-μm pore size), and the filters were washed with an additional 5.0 mL of quench buffer and immediately removed from the filtration apparatus. All data were corrected for passive permeability by performing identical experiments with vesicles that were pretreated with 0.1 mM *N*-naphthylmaleimide for 30 min at 25 °C (Robertson et al., 1980). Although not shown, samples assayed in the presence of carbonyl cyanide *m*-chlorophenylhydrazone and the absence of reduced PMS (○) were unaffected by treatment with DEPC (i.e., the kinetics of facilitated diffusion are not affected by treatment with the acylating agent).

histidyl residue(s) in the *lac* carrier is (are) involved in the translocation mechanism.

Kinetics of Lactose Transport in Vesicles Modified with DEPC or Rose Bengal. In an effort to study the mechanistic consequences of histidine modification, vesicles were treated with DEPC or rose bengal under appropriate conditions, and initial rates of counterflow were measured at given lactose concentrations. The data were then plotted according to Hofstee (1954), where the slope of the function represents the apparent K_m and the y intercept is V_{max} (Figure 5). Untreated control vesicles exhibit an apparent K_m of approximately 0.45 mM and a V_{max} of 250–300 nmol min^{-1} (mg of membrane protein)$^{-1}$, while vesicles treated with DEPC or subjected to rose bengal photooxidation manifest a marked decrease in V_{max} and no significant change in the apparent K_m.

Given these and previous results (Padan et al., 1979; Kaczorowski et al., 1979; Robertson et al., 1980), the effects of DEPC treatment on the kinetics of $\Delta\bar{\mu}_{H^+}$-driven lactose transport were examined (Figure 6). Initial rates of lactose transport were determined at pH 6.5 over an extended range of lactose concentrations such that $\Delta\bar{\mu}_{H^+}$-driven transport and facilitated diffusion could be assayed under identical conditions (Robertson et al., 1980). With control samples in the presence of reduced PMS, the initial rate of lactose transport increases markedly at external lactose concentrations ranging from 0.04 to about 0.4 mM, and the V vs. V/S plot yields an apparent K_m of about 0.4 mM and a V_{max} of 80 nmol min^{-1} (mg of membrane protein)$^{-1}$. In the absence of $\Delta\bar{\mu}_{H^+}$, initial rates of uptake are very low below 1.0 mM lactose and increase markedly between 1.0 and 8.0 mM, and the V vs. V/S plot yields an apparent K_m of about 18 mM and a V_{max} of about 120 nmol min^{-1} (mg of membrane protein)$^{-1}$. In both instances, the values are in reasonable agreement with those reported previously (Kaczorowski et al., 1979; Robertson et al., 1980). A third and fourth set of samples were treated with 0.5 and 1.0 mM DEPC, respectively, and in these samples, it is readily apparent that the kinetics become biphasic. That is, after DEPC treatment, a component of the total uptake is mediated by a process that exhibits a low apparent K_m, while a second component exhibits an apparent K_m typical of facilitated diffusion. Furthermore, although the data are not presented here, it is important that the kinetics of facilitated diffusion are not altered by treating the vesicles with DEPC (Padan et al., 1979; Patel et al., 1982) nor is the ability of the vesicles to generate $\Delta\bar{\mu}_{H^+}$ in the presence of reduced PMS (Padan et al., 1979).

Discussion

With a single exception to be discussed below, the findings presented here confirm and extend the previous results of Padan et al. (1979) which suggest that a histidyl residue(s) in the *lac* carrier play(s) an important role in the mechanism of proton/lactose symport. Acylation of vesicles with DEPC or photooxidation in the presence of rose bengal, operations known to modify histidine residues in various soluble proteins relatively specifically, leads to inactivation of *lac* carrier

function. Loss of activity occurs when function is monitored by substrate counterflow (DEPC or rose bengal) or by $\Delta\bar{\mu}_{H^+}$-driven active transport (DEPC), and experiments with DEPC (Figure 2) indicate that acylation of a single site in the carrier is sufficient for inactivation. Importantly, however, neither reagent interferes with the ability of the carrier to bind NPG nor with its ability to catalyze facilitated diffusion (Padan et al., 1979; Patel et al., 1982).

Although it has not been demonstrated here that the effects of DEPC and rose bengal are due specifically to modification of histidyl residues in the *lac* carrier, a number of lines of evidence support this argument. First, both DEPC and rose bengal have been shown to specifically modify histidyl residues in a number of soluble enzymes (Westhead, 1965; Hoffee et al., 1967; Brand et al., 1969; Holbrook & Ingram, 1973; Miles, 1977; Saluja & McFadden, 1980, 1982; Meyer & Cromartie, 1980). In addition, both reagents have been used to modify histidyl residues involved in the binding of EF-Tu to aminoacyl-tRNA and/or ribosomes (Jonak & Rychlik, 1980). Second, hydroxylamine displaces the ethoxycarbonyl moiety from the imidazole nitrogen of histidine (Heinrikson & Kramer, 1974) and regenerates counterflow activity in DEPC-treated vesicles (Padan et al., 1979). Third, pH titrations of both DEPC inactivation and rose bengal catalyzed photooxidation suggest that loss of activity is due to modification of an amino acid residue with a pK_a between pH 6.0 and 6.5, and in both instances, the pH profiles are similar to those observed for histidine. Finally, experiments with purified *lac* carrier (Newman et al., 1981) demonstrate directly that rose bengal photooxidation specifically modifies histidyl residues in the protein. In these studies, photooxidation of the carrier in the presence of 1.0 μM rose bengal results in the disappearance of two of the four histidine residues in the protein (Büchel et al., 1980; Newman et al., 1981) with no significant alteration in any other amino acid residue (M. L. Garcia, D. L. Foster, L. Patel, and H. R. Kaback, unpublished experiments).

Padan et al. (1979) presented kinetic data indicating that DEPC treatment of ML 308-225 vesicles leads to an increase in the apparent K_m for $\Delta\bar{\mu}_{H^+}$- and $\Delta\mu_{lac}$-driven lactose transport with no significant effect on V_{max} and no effect on the kinetics of facilitated diffusion. Subsequently, Robertson et al. (1980), by studying the effects of varying $\Delta\Psi$ and ΔpH on the kinetics of lactose transport over an extended range of lactose concentrations, were able to demonstrate that in addition to acting as the driving force for active accumulation, $\Delta\bar{\mu}_{H^+}$ alters the distribution of the *lac* carrier between two different kinetic modes, one with a high apparent K_m (facilitated diffusion) and one with a low apparent K_m ($\Delta\bar{\mu}_{H^+}$-driven active transport). In contradistinction to the results of Padan et al. (1979), the kinetic experiments presented here, which were performed as described by Robertson et al. (1980), demonstrate clearly that DEPC treatment or rose bengal catalyzed photooxidation causes a decrease in the V_{max} of the low apparent K_m component with no effect on its apparent K_m. The findings are important with regard to mechanistic considerations, in addition to highlighting the importance of performing transport kinetics over a sufficiently wide range of substrate concentrations. Since partial dissipation of $\Delta\bar{\mu}_{H^+}$ leads to biphasic kinetics (Robertson et al., 1980) and treatment of the vesicles with DEPC causes the same kinetic effect with no reduction in $\Delta\bar{\mu}_{H^+}$ (Padan et al., 1979; data not shown), it seems reasonable to conclude that a histidyl residue(s) in the *lac* carrier is (are) involved in the response of the carrier to $\Delta\bar{\mu}_{H^+}$. Furthermore, observations demonstrating that $\Delta\bar{\mu}_{H^+}$ enhances the rates of inactivation of the *lac* carrier by various maleimides (Cohn et al., 1981) and by DEPC (Table I) support the suggestion that the shift between the two kinetic forms of the carrier may involve structural or conformational changes that alter the reactivity of certain essential functional groups in the protein. In any event, regardless of the interpretation of the phenomena, experiments performed with purified *lac* carrier demonstrate that DEPC and rose bengal produce effects similar to those reported here with isolated membrane vesicles (M. L. Garcia, D. L. Foster, L. Patel, and H. R. Kaback, unpublished experiments). That is, treatment of the carrier with either reagent causes a decrease in V_{max} for counterflow with no effect on the apparent K_m, and the effect is blocked by substrate.

In the following paper (Patel et al., 1982), lactose-induced proton movements are described in isolated membrane vesicles for the first time, and the effect of DEPC treatment on this reaction is described.

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Direct Measurement of Lactose/Proton Symport in *Escherichia coli* Membrane Vesicles: Further Evidence for the Involvement of Histidine Residue(s)†

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ABSTRACT: Addition of lactose to *Escherichia coli* ML 308-225 membrane vesicles under nonenergized conditions induces transient alkalinization of the medium, and the initial rate of proton influx is stimulated by valinomycin and abolished by nigericin or carbonyl cyanide *m*-chlorophenylhydrazone. A functional *lac y* gene product is absolutely required as the effect is not observed in ML 308-225 vesicles treated with *N*-ethylmaleimide nor with vesicles from uninduced *Escherichia coli* ML 30. Furthermore, the magnitude of the phenomenon is enhanced about 3-fold in vesicles from *Escherichia coli* T206, which contain amplified levels of the *lac* carrier protein. Kinetic parameters for lactose-induced proton influx are the same as those determined for lactose-facilitated diffusion, and quantitative comparison of the initial rates of the two fluxes indicates that the stoichiometry between protons and lactose is 1:1. Treatment of ML 308-225 vesicles with diethyl pyrocarbonate causes inactivation of lactose-induced proton influx. Remarkably, however, treatment with the histidine reagent enhances the rate of lactose-facilitated diffusion in a manner suggesting that the altered *lac* carrier catalyzes lactose influx without the symport of protons. The results are consistent with the hypothesis that acylation of a histidyl residue(s) in the *lac* carrier protein dissociates lactose influx from proton influx and indicate that this residue(s) play(s) an important role in the pathway of proton translocation.

According to the chemiosmotic hypothesis (Mitchell, 1961, 1966, 1968, 1973), a proton electrochemical gradient ($\Delta\bar{\mu}_{H^+}$,[1] interior negative and alkaline) is the immediate driving force for accumulation of β-galactosides in *Escherichia coli* (Mitchell, 1963). Thus, transport is postulated to be catalyzed by a β-galactoside-specific membrane protein (the product of the *lac y* gene) that translocates β-galactosides with protons in a symport reaction, the protons moving down their electrochemical gradient and driving the uphill translocation of sugar. Over the past decade, virtually unequivocal experimental support for this concept has been presented.

Several laboratories have investigated proton/β-galactoside symport in intact *E. coli* by studying substrate-induced proton fluxes. West (1970) and West & Mitchell (1972, 1973) demonstrated that addition of lactose to cells under energy-limited conditions causes alkalinization of the medium. Other workers have demonstrated accumulation of the lactose analogue methyl 1-thio-β-D-galactopyranoside (TMG) in response to an artificially imposed membrane potential ($\Delta\psi$) or pH gradient (ΔpH) (Flagg & Wilson, 1977). Similarly, efflux of TMG from preloaded cells drives accumulation of other solutes because coupled proton movements lead to the generation of $\Delta\bar{\mu}_{H^+}$ (Flagg & Wilson, 1978).

Proton/β-galactoside symport has also been studied intensively in cytoplasmic membrane vesicles from *E. coli*. Right-side-out (Kaback, 1971, 1974a; Short et al., 1975; Owen & Kaback, 1978, 1979a,b) and inverted (Hertzberg & Hinkle, 1974; Rosen & McClees, 1974) vesicles generate a $\Delta\bar{\mu}_{H^+}$ of similar magnitude but opposite polarity (Ramos et al., 1976; Ramos & Kaback 1977a; Reenstra et al., 1980), and in both instances, β-galactoside transport is coupled to $\Delta\bar{\mu}_{H^+}$ (Ramos & Kaback, 1977b,c; Lancaster & Hinkle, 1977a,b). Lactose accumulation in right-side-out vesicles leads to partial collapse of $\Delta\psi$ (Schuldiner & Kaback, 1975) and ΔpH (Ramos &

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[1] Abbreviations: $\Delta\bar{\mu}_{H^+}$, transmembrane proton electrochemical gradient; $\Delta\psi$, membrane potential; ΔpH, pH gradient; TMG, methyl 1-thio-β-D-galactopyranoside; NNM, *N*-(2-naphthyl)maleimide; DEPC, diethyl pyrocarbonate; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone.

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