Effect of diethylpyrocarbonate on lactose/proton symport in *Escherichia coli* membrane vesicles

(Histidine/active transport/counterflow/facilitated diffusion/binding)

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ABSTRACT Exposure of *Escherichia coli* ML 308-225 membrane vesicles to the histidine-specific reagent diethylpyrocarbonate (DEPC) led to concentration- and time-dependent inactivation of active lactose transport, and the sensitivity of the system to inactivation was enhanced when an electrochemical proton gradient (ΔνH⁺, interior negative and alkaline) was generated across the vesicle membrane. Although β-D-galactopyranosyl 1-thio-β-D-galactopyranoside blocked DEPC inactivation, binding of p-nitrophenyl α-D-galactopyranoside was not significantly altered, indicating that DEPC does not react at the binding sites of the lac carrier protein. Strikingly, vesicles treated with DEPC exhibited an increased apparent Km for ΔνH⁺-driven lactose transport and counterflow but no change in the Vmax of these reactions and no change in the apparent Km or Vmax of facilitated diffusion. Moreover, DEPC treatment increased the apparent Km observed for ΔνH⁺-driven proline and D-lactate transport with no change in Vmax. Finally, the lactose counterflow activity of DEPC-treated vesicles was regenerated by subsequent exposure to hydroxylamine. It is suggested that a histidyl residue(s) in the lac carrier or another protein in the translocation complex is involved either in the binding and translocation of protons or in a conformational change that may occur upon protonation of the lac carrier protein.

The chemosmotic hypothesis of Mitchell (1–3) proposes that energy derived from respiration, photochemical reactions, or ATP hydrolysis can be transformed into a transmembrane electrochemical gradient of protons (ΔνH+) that is the immediate driving force for many active transport systems in bacterial cells. More specifically, the hypothesis states that substrate-specific membrane proteins (i.e., carriers, or permeases) mediate the coupling between ΔνH+ and substrate accumulation by catalyzing the translocation of substrate with protons, the substrate moving against and the protons with their respective electrochemical gradients (i.e., symport). Cytoplasmic membrane vesicles prepared from *Escherichia coli*, which retain the same polarity and configuration as the membrane in the intact cell (4–6) as well as the capacity to convert respiratory energy into a ΔνH+ (interior negative and alkaline) (7), catalyze the active transport of many substrates, including β-galactosides (8). Moreover, studies with intact cells (9) and this in vitro system (7, 10–14) provide virtually unequivocal evidence for the central obligatory role of chemosmotopic phenomena in active transport. Recent evidence with isolated membrane vesicles (15, 16) indicates that carrier-mediated lactose efflux down a concentration gradient is an ordered mechanism in which lactose is released first from the carrier, followed by loss of a proton, and that the unloaded carrier may be negatively charged. In addition, kinetic analyses of active transport and facilitated diffusion demonstrate that ΔνH+ reduces the apparent Km for lactose influx by a factor of about 100 (16). Although these observations provide insight into the translocation mechanisms catalyzed by the lac carrier, little information is available regarding the chemistry of the reactions, in particular, the functional groups that may be involved in binding and translocation of protons.

In this report, experiments documenting the effect of diethylpyrocarbonate (DEPC), a histidine-specific reagent (17), on lactose translocation in *E. coli* membrane vesicles are presented. The results demonstrate that this reagent increases the apparent Km of certain reactions catalyzed by the β-galactoside transport system without altering the Vmax of translocation or the ability of the system to bind substrate. It is suggested that a histidyl residue(s) in the lac carrier or another protein in the translocation complex (18) may play an important role in the response of the transport system to ΔνH+. Preliminary evidence indicates, in addition, that DEPC exerts a similar effect on other transport systems that are driven by ΔνH+.

MATERIALS AND METHODS

**Growth of Cells and Preparation of Membrane Vesicles**

*E. coli* ML 308-225 (lacI−, lacZ−, lacY+, lacA+) and ML 30 (lacI+, lacZ+, lacY+, lacA+) were grown on minimal medium A containing 1.0% disodium succinate (hexahydrate), and membrane vesicles were prepared as described (19, 20).

**Treatment with DEPC**

Vesicles suspended in 100 mM potassium phosphate (pH 6.6) were titrated to pH 6.0 with 100 mM monobasic potassium phosphate and adjusted to a final concentration of about 2.0 mg of protein per ml by adding appropriate volumes of 100 mM potassium phosphate (pH 6.0). Small portions of 2.0 M DEPC (freshly prepared in absolute ethanol) were added to the membrane suspensions such that the final concentration of ethanol was never more than 0.5% (at concentrations up to 1.0%, ethanol has no significant effect on any of the activities studied). Immediately after addition of DEPC, the suspensions were vigorously agitated to achieve complete mixing and incubated at room temperature on a magnetic stirrer for given periods of time. Reactions were terminated by 1:5 dilution with ice-cold 100 mM potassium phosphate (pH 7.0) and immediately centrifuged at 48,000 × g for 30 min. The supernatants were discarded and the pellets were resuspended in at least a 100-fold excess (vol/vol) of 100 mM potassium phosphate (pH 7.0). After centrifugation at 48,000 × g for 30 min, the supernatants were again discarded and the pellets were resuspended in 100 mM potassium phosphate (pH 7.0) to given concentrations. Where indicated, DEPC

Abbreviations: ΔνH+, electrochemical gradient of protons; DEPC, diethylpyrocarbonate; PMS, phenazine methosulfate; ΔΨ, membrane potential; TPP*, tetraphenylphosphonium; NphGal, p-nitrophényl α-D-galactopyranoside; Gal-S-Gal, β-D-galactopyranosylic 1-thio-β-D-galactopyranoside.

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treatment was carried out in the presence of potassium ascorbate and phenazine methosulfate (PMS) at final concentrations of 20 mM and 0.1 mM, respectively, under an atmosphere of water-saturated oxygen (21).

Transport Assays. Respiration-driven uptake of [1-14C]-lactose, [U-14C]-proline, and D-[U-14C]-lactate was assayed with reduced PMS as electron donor (21). Countercflow (15) and facilitated diffusion (16) were measured as described.

Measurement of Membrane Potential (ΔΨ, Interior Negative). The ΔΨ generated across the vesicle membrane as a result of reduced PMS oxidation was determined from the distribution of [3H]tetraphenylphosphonium (TPP+) by using flow dialysis (7, 10).

Binding of D-Nitrophenyl α-D-[6-3H]Galactopyranoside (Nph[3H]Gal). Binding of Nph[3H]Gal to membrane vesicles under energized conditions was assayed by flow dialysis (22). The upper chamber contained 100 mM potassium phosphate (pH 7.0) and 8.0 mg of membrane protein in a total volume of 0.2 ml. Potassium phosphate (100 mM, pH 7.0) was pumped from the lower chamber at a rate of 6.0 ml/min, and 1.7-ml fractions were collected. Assays were initiated by addition of given concentrations of Nph[3H]Gal to the vesicles in the upper chamber, and, after equilibrium was achieved, 1-thio-β-D-galactopyranosyl 1-thio-β-D-galactopyranoside (Gal-S-Gal) was added to the upper chamber to a final concentration of 2.5 mM. Specifically bound NphGal was quantitated from the increase in the dialyzable concentration of Nph[3H]Gal after addition of Gal-S-Gal.

Protein Determinations. Protein was measured as described by Lowry et al. (23) with crystalline bovine serum albumin as standard.

Materials. DEPC was obtained from Sigma, and [1-14C]-lactose, [U-14C]-proline, and D-[U-14C]-lactate were purchased from Amersham or New England Nuclear. [3H]TPP+ (bromide salt) was prepared by the Isotope Synthesis Group at Hoffmann-La Roche under the direction of Arnold Lieberman. Nph[3H]Gal was synthesized and purified as described (22). All other materials were reagent grade obtained from commercial sources.

RESULTS

Effect of DEPC Treatment on Δψp-Driven Lactose Transport. When membrane vesicles of E. coli ML 308-225 were treated with given concentrations of DEPC for 10 min, washed free of the reagent, and assayed for lactose accumulation in the presence of reduced PMS, half-maximal inactivation was observed at about 1.6 mM DEPC and approximately 90% inactivation at 5.0–6.0 mM DEPC (Fig. 1). Interestingly, when DEPC treatment was carried out in the presence of reduced PMS, the system was 2.5–3 times more sensitive to DEPC; half-maximal inactivation was observed at about 0.6 mM and 90% inactivation at 2.0–3.0 mM DEPC. This enhanced sensitivity was abolished by flushing the reaction mixtures with argon or, more importantly, by adding 1.0 mM dinitrophenol during treatment with the inactivator (data not shown). Thus, the effect of reduced PMS is due specifically to the generation of Δψp. The data shown in the Inset of Fig. 1 depict time courses of lactose uptake by control preparations and vesicles treated with 1.1 mM DEPC in the presence and absence of reduced PMS. It is apparent that DEPC depressed both the initial rate and steady-state level of accumulation. Although data are not shown, this statement applies generally to the time courses observed for vesicles treated with each of the DEPC concentrations given in the body of the figure.

At room temperature (ca. 20°C), inactivation of lactose transport proceeded slowly in the presence of 1.1 mM DEPC, and 70% of the control activity remained after treatment for 10 min (Fig. 2). On the other hand, when inactivation was carried out in the presence of reduced PMS, the rate was markedly enhanced: 50% inactivation was observed after 2 min and only 20% of the activity remained after 10 min. Inactivation kinetics similar to those observed for 1.1 mM DEPC in the presence of reduced PMS were observed for 0.7 mM and 0.1 mM DEPC (data not shown). These results demonstrate that DEPC treatment produces a dose-dependent inactivation of lactose transport with the rate of inactivation accelerated by reduced PMS.
presence of reduced PMS were obtained in the absence of the electron donor when the DEPC concentration was increased to 3.0 mM (data not shown).

Despite marked inactivation of lactose transport, it is apparent that vesicles treated with DEPC at concentrations up to 2.0 mM are not altered in their ability to generate $\Delta\mu_{H^+}$. Thus, when untreated vesicles and vesicles treated with DEPC over this concentration range were assayed for $[^3H]TPP$ accumulation at pH 7.0 in the presence of reduced PMS (7, 10), $\Delta\Psi$ values of about $-80$ mV were obtained in both preparations.

Gal-S-Gal Protection Against DEPC Inactivation. Gal-S-Gal protects the lac carrier protein from inactivation by certain sulfhydryl reagents, and this property of the system has allowed labeling, solubilization, and partial purification of the lacY gene product (24, 25). When ML 308-225 vesicles were treated with DEPC in the presence of Gal-S-Gal, the rate of inactivation of lactose transport was clearly diminished relative to samples treated with DEPC in the absence of Gal-S-Gal (Fig. 3). These findings and the demonstration that DEPC treatment did not alter the capacity of the vesicles to generate $\Delta\mu_{H^+}$ provide strong evidence for the argument that DEPC inactivates lactose transport at the level of the lac carrier. It should be noted, however, that evidence to be presented (cf. below) suggests that inactivation by DEPC is not due to a modification in the binding site of the lac carrier protein.

Kinetics of Lactose Translocation in DEPC-Treated Vesicles. The lac carrier protein catalyzes translocation in at least two different kinetic modes (15, 16). When lactose influx is driven by $\Delta\mu_{H^+}$ (i.e., active transport) or by $\Delta\mu_{\text{lac}}$ (i.e., counterflow), the system exhibits a low apparent $K_m$ (approximately 0.2 mM), whereas facilitated diffusion exhibits a high apparent $K_m$ (approximately 20 mM). As a result of these and other observations (15, 16), it was suggested that one of the primary effects of $\Delta\mu_{H^+}$ is to cause a marked decrease in the apparent $K_m$ for influx and that this phenomenon is related to the state of protonation of the lac carrier protein.

The effects of DEPC treatment on initial rates of reduced PMS-dependent lactose transport, counterflow, and facilitated diffusion are contrasted in Fig. 4, and the differences observed are striking. When the kinetics of active transport are examined

![Fig. 3. Effect of Gal-S-Gal on the rate of DEPC inactivation of lactose transport. E. coli ML 308-225 vesicles were incubated with 1.1 mM DEPC in the absence of reduced PMS for given times as described in Figs. 1 and 2 and in Materials and Methods (>). Where indicated (O), Gal-S-Gal was added to the reaction mixtures to a final concentration of 20 mM. Uptake of [1-14C]lactose was assayed as described for Fig. 1, and steady-state levels of accumulation (5-min incubations) are expressed as a percentage of appropriate control samples incubated in the presence and absence of Gal-S-Gal but without DEPC. In each case, the control values approximated 20 nmol/mg protein. The difference in inactivation rates observed here and in Fig. 2 (O) is due to the use of a different vesicle preparation.](image-url)

![Fig. 4. Effect of DEPC treatment on initial rates of ascorbate/ PMS-dependent lactose transport (A), counterflow (B), and facilitated diffusion (C). Data are presented in the form of double reciprocal plots. $v$, initial rate of transport in nmol/min per mg of protein; $S$, substrate concentration in mM. (A) Vesicles were incubated without DEPC (>) and with DEPC at final concentrations of 0.5 mM (△) and 1.1 mM (O) for 10 min in the presence of ascorbate and PMS, washed, and resuspended as described in Materials and Methods and in Figs. 1 and 2. Initial rates of lactose uptake were then measured in the presence of potassium ascorbate (20 mM, final concentration) and PMS (0.1 mM, final concentration) at times ranging from 2 to 15 sec at given concentrations of [1-14C]lactose. Over this time period, the initial rates were linear at each lactose concentration assayed. (B) Vesicles were incubated without DEPC (>) and with DEPC at a final concentration of 3.0 mM (O) for 10 min in the presence of ascorbate and PMS, washed, and resuspended to a concentration of 60 mg of protein per ml. The concentrated suspensions were reequilibrated with 10 mM lactose by incubation at room temperature for 4 hr (15). Initial rates of counterflow were then measured by diluting aliquots (2 μl) 1:200 into 100 mM potassium phosphate (pH 7.0) containing given concentrations of [1-14C]lactose (50 μCi/ml) and assaying samples at times ranging from 2 to 15 sec. Over this time period, the initial rates were linear at each lactose concentration studied. (C) Vesicles were incubated without DEPC (>) and with DEPC at a final concentration of 3.0 mM (O) for 10 min in the presence of ascorbate and PMS, washed, and resuspended to a concentration of 10 mg of protein per ml, and carbonyl cyanide m-chlorophenylhydrazone was added to a final concentration of 5 μM. Initial rates of facilitated diffusion were then measured at given concentrations of [1-14C]lactose (50 μCi/ml), using 100-μl aliquots and 47-mm cellulose nitrate filters as described (16). The data were corrected for passive diffusion by carrying out parallel experiments with vesicles prepared from uninduced E. coli ML 30.](image-url)
(Fig. 4A), it is apparent that treatment with 0.5 and 1.1 mM DEPC caused increases in the apparent $K_m$ from the control value of 0.2 mM to 0.5 mM and 1.25 mM, respectively, with no change in $V_{\text{max}}$ (20 nmol/min per mg protein in all three preparations). Similarly, after treatment with 3.0 mM DEPC, the vesicles exhibited an increase in the apparent $K_m$ for counterflow from approximately 0.25 mM in the control to about 1.5 mM in DEPC-treated vesicles with no change in $V_{\text{max}}$ (100 nmol/min per mg of protein) (Fig. 4B). Finally, and importantly, DEPC treatment caused no change in the kinetics of facilitated diffusion (Fig. 4C), and in this instance, both the control and DEPC-treated vesicles exhibited an apparent $K_m$ of about 20 mM and a $V_{\text{max}}$ of 50–60 nmol/min per mg of protein. It is clear from these results that a major effect of DEPC treatment is to modify the response of the $\text{lac}$ transport system to $\Delta\mu_{\text{H}^+}$ and $\Delta\mu_{\text{ac}}$. That is, the system no longer exhibits a low apparent $K_m$ when these forces are imposed across the membrane. On the other hand, neither the $V_{\text{max}}$ of translocation nor the apparent $K_m$ for lactose translocation down a chemical gradient appear to be altered.

**NphGal Binding.** NphGal is a potent competitive inhibitor of lactose transport in ML 308-225 vesicles that binds to the $\text{lac}$ carrier protein with a $K_d$ that corresponds to its $K_i$ (22). For the experiments presented in Fig. 5, binding of high specific activity Nph$[^3]$H$^+$Gal was studied under nonenergized conditions. In agreement with earlier observations (22), the $K_d$ in untreated vesicles (calculated from the slope of the function) was about 12 $\mu$M and the number of binding sites (the intercept with the abscissa) approximated 0.2 nmol/mg of protein. In DEPC-treated vesicles the $K_d$ was identical within experimental error, while the number of binding sites was slightly decreased to about 0.15 nmol/mg of protein, a difference that has borderline significance.

**Reversal of DEPC Inactivation.** Modification of certain soluble proteins by DEPC can be reversed by treatment with hydroxylamine at pH 7.0 (17). Under these conditions, the ethoxycarbonyl moiety is displaced from the imidazole nitrogen of histidine with regeneration of native structure and function. Respiration-driven active transport is inactivated by hydroxylamine, however, and in order to study the ability of the nucleophile to reactivate, counterflow was used to assay $\text{lac}$ carrier function. As shown in Fig. 6, DEPC treatment caused a marked reduction in counterflow (A), and well over 50% of the activity of the control samples was regenerated after treatment with hydroxylamine (B).

**Inactivation of Other Transport Systems by DEPC Treatment.** Because transport of many other solutes in addition to lactose is coupled to $\Delta\mu_{\text{H}^+}$, the effects of DEPC treatment on proline and d-lactate transport were also examined. Although not shown, both the concentration dependence and the time course of DEPC inactivation of these transport systems were similar to those observed for lactose (cf. Figs. 1 and 2). Importantly, moreover, as summarized in Table 1, DEPC treatment resulted in an increase in the apparent $K_m$ of each transport system with no change in $V_{\text{max}}$.

**DISCUSSION**

Treatment of *E. coli* membrane vesicles with the histidine reagent DEPC produces a unique effect on the $\beta$-galactoside transport system—an increase in the apparent $K_m$ for active transport and counterflow with no effect on the $V_{\text{max}}$ of these

![Fig. 5](image1.png)  
**FIG. 5.** Effect of DEPC treatment on NphGal binding. *E. coli* ML 308-225 vesicles were incubated without DEPC (○) or with DEPC (●) at a final concentration of 3.0 mM for 10 min in the presence of reduced PMS, washed, and resuspended to a concentration of 15 mg of protein per ml. Binding of Nph$[^6]$H$^+$Gal (2380 mCi/mmol) was assayed under nonenergized conditions at concentrations ranging from 1 to 25 $\mu$M as described (22) and in Materials and Methods. Data are plotted according to Scatchard (26).

![Fig. 6](image2.png)  
**FIG. 6.** Reactivation of lactose counterflow in DEPC-treated vesicles by hydroxylamine. (A) Vesicles were incubated without DEPC (○) and with DEPC (●) at a final concentration of 4.0 mM for 10 min in the presence of ascorbate and PMS, washed, and resuspended to a concentration of 60 mg of protein per ml. The suspensions were equilibrated with 10 mM lactose by incubation at room temperature for 4 hr and assayed for counterflow as described for Fig. 4B at an external lactose concentration of 0.4 mM. (B) Control vesicles (○) and vesicles treated with DEPC (●) were washed as described in A and resuspended in 100 mM potassium phosphate (pH 7.0) to a concentration of 2.0 mg of protein per ml. Hydroxylamine (titrated to pH 7.0 with potassium hydroxide) was added to a final concentration of 500 mM, and the vesicles were incubated at room temperature for 90 min, washed twice in 100 mM potassium phosphate (pH 7.0), and resuspended in 60 mg of protein per ml. The samples were then equilibrated with 10 mM lactose and assayed for counterflow as described for A.

**Table 1.** Effect of DEPC on the kinetics of $\Delta\mu_{\text{H}^+}$-driven proline and d-lactate transport

<table>
<thead>
<tr>
<th>Vesicles</th>
<th>Proline $K_m$, nmol/mg prot.</th>
<th>Proline $V_{\text{max}}$, nmol/mg prot./min</th>
<th>D-Lactate $K_m$, $\mu$M</th>
<th>D-Lactate $V_{\text{max}}$, nmol/mg prot./min</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>10.5</td>
<td>5.0</td>
<td>4.35</td>
<td>3.3</td>
</tr>
<tr>
<td>DEPC-treated</td>
<td>33.3</td>
<td>5.0</td>
<td>20.0</td>
<td>3.3</td>
</tr>
</tbody>
</table>

Membrane vesicles were incubated with 0.5 mM DEPC in the presence of reduced PMS for 10 min, washed, and resuspended. Initial rates of proline and d-lactate uptake were then measured in the presence of potassium ascorbate (20 mM, final concentration) and PMS (0.1 mM, final concentration) at times ranging from 2 to 20 sec, at the following concentration range of substrates: $[^1]$H$^+$proline (248 mCi/mmol), 0.5–16 $\mu$M; $[^1]$H$^+$d-$[^1]$C$^+$lactate (21 mCi/mmol), 2.0–80 $\mu$M. Over this time period, the initial rates were linear at each substrate concentration assayed. $K_m$ and $V_{\text{max}}$ were calculated from double reciprocal plots of initial rate versus substrate concentration.
reactions, no effect on facilitated diffusion, and no significant effect on NphGal binding. In other words, after treatment with DEPC, the lac transport system no longer responds to $\Delta \mu_{H^+}$ and $\Delta \mu_{\text{sys}}$ by exhibiting the low apparent $K_m$ characteristic of active transport and counterflow. Because it is unlikely that DEPC reacts at the binding site of the lac carrier protein, it is suggested that a histidyl residue(s) is involved either in the binding and translocation of protons or in a conformational transition that may occur upon protonation of the lac carrier (15).

Although Gal-S-Gal protects the lac transport system from DEPC, treatment with the reagent does not significantly alter the ability of the vesicles to bind NphGal, another high-affinity substrate of the lac carrier protein. In this context, it is interesting to contrast the effects of DEPC with those of sulphydryl reagents such as N-ethylmaleimide. It is well known that N-ethylmaleimide reacts at the binding site of the lac carrier protein (24, 25). Gal-S-Gal protects the lac transport system against sulphydryl inactivation (24, 25, 27), and treatment of vesicles with sulphydryl reagents blocks NphGal binding (22) and decreases the $V_{\text{max}}$ of lactose transport without altering the apparent $K_m$. Taken together, the observations suggest that DEPC does not modify the binding site of the lac carrier directly and that substrate binding may induce a conformational change resulting in decreased reactivity with DEPC, secondary perhaps to a change in the $pK_a$ of a critical histidyl residue(s). Lastly, the differential effects of DEPC on lactose transport and NphGal binding provide a strong indication that $K_m$ and $K_d$ may bear little relationship to each other, a situation that has been documented with certain soluble enzymes (28).

Two types of genetic lesions have been described that alter the coupling between $\beta$-galactoside transport and $\Delta \mu_{H^+}$. One type (29, 30) causes a specific defect in $\beta$-galactoside accumulation with no apparent defect in facilitated diffusion or counterflow. In addition, these mutants exhibit diminished $\beta$-galactoside/H$^+$ symport (31), and the site of the mutation is probably within the lacY gene (30, 32). Another type of mutant (18) is temperature sensitive and pleiotropically defective in a number of $\Delta \mu_{H^+}$-driven transport systems at the nonpermissive temperature despite a normal $\Delta \mu_{H^+}$. In this case, the mutation maps outside of the lac operon, and it was suggested that the gene (ecf) codes for a protein that may interact with various substrate-specific gene products, acting as the proton-translocating subunit of many transport systems. At the present time, it is not clear whether the chemical lesion produced by DEPC mimics either of these genetic lesions. In analogy to the ecf mutation, at least two other $\Delta \mu_{H^+}$-driven transport systems, in addition to the lac system, are altered by DEPC (Table 1), however, because these systems catalyze similar reactions, it is possible that the substrate-specific components react similarly with DEPC. In the same vein, DEPC-treated vesicles exhibit an increase in the apparent $K_m$ for active lactose transport, as well as counterflow, whereas the mutants isolated in Wilson's laboratory (29, 30, 32) exhibit normal or even enhanced counterflow activity.

Although it has not been demonstrated that the effects of DEPC are due to modification of membrane histidyl residues, a number of indirect lines of evidence support this contention: (1) DEPC treatment at pH 6.0 specifically modifies histidyl residues in a number of soluble proteins (17); (ii) hydroxylamine displaces the ethoxycarbonyl moiety from the imidazole ni-