Acidic residues which are found on transmembrane segments within the lactose permease may play an important role in $H^+$ and/or sugar recognition. To examine the functional roles of Glu-269 and Glu-325, we have constructed a variety of amino acid substitutions (e.g. aspartate, glycine, alanine, serine, or glutamine) via site-directed mutagenesis. At position 269, all mutations appear to have a detrimental effect on sugar affinity, downhill transport, and counterflow. The Asp-269 mutant was able to accumulate lactose against a concentration gradient, whereas all of the nonionizable substitutions at position 269 were completely defective. Nevertheless, in spite of their inability to actively accumulate sugars, Gly-269, Ala-269, and Gln-269 mutants were observed to transport $H^+$ upon the addition of galactosides. Mutations at position 325 had a markedly different phenotype. For example, the Asp-325, Gly-325, and Gln-325 mutants exhibited an apparent $K_m$ for lactose transport (e.g. 0.21, 0.47, and 0.50 mM, respectively), which was actually lower than that of the wild-type strain (1.44 mM). In counterflow assays, all position 325 mutants also appear to catalyze lactose exchange. Similar to the results obtained at position 269, the Asp-325 mutant exhibited moderate levels of accumulation, whereas none of the nonionizable mutations at position 325 were able to accumulate galactosides against a concentration gradient. However, unlike the position 269 mutants, no $H^+$ transport was observed in the Gly-325, Ala-325, Ser-325, or Gln-325 strains upon the addition of lactose, $S$-$\beta$-$\beta$-galactopyranosyl-$\beta$-$\beta$-thiogalactopyranoside, 1-O-methyl-$\beta$-$\beta$-galactopyranoside, or melibiose. Furthermore, in these mutants, the efflux of lactose during counterflow assays became insensitive to $\Delta$$\nu$H. Overall, these results are consistent with the notion that an acidic residue at position 325 is required for $H^+$ transport via the lactose permease. Alternative hypotheses are also discussed.

The lactose permease is found within the inner membrane of *Escherichia coli* and cotransports lactose and $H^+$ into the bacterial cytoplasm (1, 2). The inwardly directed $H^+$ electrochemical gradient provides the driving force for the active accumulation of lactose (3, 4). The lactose permease has been well characterized in whole cells and membrane vesicles (5, 6). It has been purified to homogeneity and reconstituted into functional proteoliposomes (7, 8). The gene encoding the lactose permease, *lacY*, has been cloned on multicopy vectors and sequenced (9, 10). The DNA sequence revealed an open reading frame coding for a protein of 417 amino acid residues. Several topological studies are consistent with a secondary structural model in which the lactose permease contains twelve transmembrane segments in an $\alpha$-helical conformation (11-13).

In a variety of different types of transport proteins, acidic amino acids have been implicated to be involved with cation binding and transport. In the case of the bacteriorhodopsin, Asp-96 is involved with $H^+$ binding from the cytoplasm and Asp-85 releases $H^+$ into the extracellular medium (14). In the F-type ion motive ATPases, Asp-61 within subunit $c$ of the $F_0$ sector of the *E. coli* protein has been identified as the DCCD-sensitive site (15). This residue has also been shown to be critical for $H^+$ transport (16). In P-type ATPases, acidic residues also appear to be important for cation recognition. For example, in the Ca$^{2+}$-ATPase of the sarcoplasmic reticulum, Glu-309, Glu-771, Asp-800, and Glu-908 appear to be important for Ca$^{2+}$ binding and transport (17). Overall, a common theme is that aspartate and glutamate residues are functionally important among transport proteins which have a binding site for cations. Moreover, these acidic residues are found on transmembrane segments within their respective proteins.

In the case of the lactose permease, 4 acidic residues (Asp-237, Asp-240, Glu-269, and Glu-325) are found on putative transmembrane segments. Asp-237 and Asp-240 have been implicated to form salt bridges with other charged residues within the permease (Lys-358 and Lys-319, respectively; Refs. 13, 18, and 19). An acidic residue at position 237 appears to be relatively unimportant, since double mutants containing nonionizable side chains at position 237 and 358 are functionally active. In general, Asp-240 replacements do have significant effects on transport, although double mutants containing neutral residues at positions 240 and 319 do exhibit significant uphill transport (19). While the preceding results indicate that these 2 aspartic residues are not required for cation binding by the permease, other studies have indicated that Glu-269 and Glu-325 may be essential residues for active lactose transport. As far as Glu-269 is concerned, it has been reported that a valine substitution results in a white phenotype on lactose MacConkey plates (20) and that alanine or cysteine substitutions abolish active lactose transport (19). Very recently, it has been suggested that Glu-269 may interact with Lys-319 in the lactose permease (21). A Glu-269 mutant was defective in the uphill accumulation of galactosides and showed little or no $H^+$ transport upon the addition of lactose or melibiose. The authors concluded that Glu-269 plays a critical role in proton translocation and its coupling with sugar transport. Similarly, strong evidence for the importance of an acidic residue within the lactose permease concerns Glu-325. In previous work, it has been shown that an acidic residue at position 325 is required for the uphill accumulation of lactose and $H^+$ transport, but not for exchange (22, 23). The authors speculated that this acidic
residue might play a critical role in the deprotonation step of the reaction mechanism. In the current study, we have generated a series of site-directed mutations at positions 269 and 325. The effects of these mutations on sugar recognition, transport, and H⁺ transport have been examined in detail.

MATERIALS AND METHODS

Reagents—Lactose (O-β-D-galactopyranosyl-(1,4)-α-D-glucopyranose), melibiose (O-α-D-galactopyranosyl-(1,6)-D-glucopyranose), TDG1 (S-β-D-galactopyranosyl-(1,4)-β-D-glucopyranoside), and TMG (1-O-methyl-β-D-galactopyranoside) were purchased from Sigma. [¹⁴C]Lactose was purchased from Amer sham Corp., and [¹⁴C]TMG was from DuPont NEN. The remaining reagents were analytical grade.

Methods—Plasmid DNA was isolated by the NaOH-sodium dodecyl sulfate method (24) and introduced into the appropriate bacterial strain by the CaCl₂ transformation procedure of Mandel and Higa (25).

Stock cultures of cells were grown in YT medium (26) supplemented with tetracycline (0.01 mg/ml). For transport assays, cells were grown to midlog phase in YT medium containing tetracycline (0.005 mg/ml) and 0.25 mM isopropylthiogalactoside to induce the synthesis of the lactose permease.

Site Transport Assays—For the experiments of Figs. 1 and 2, midlog cells were washed in phosphate buffer, pH 7.0, containing 60 mM K₂HPO₄ and 40 mM KH₂PO₄ and resuspended in the same buffer to a density of approximately 0.5 mg of protein/ml. Cells were then equilibrated at 30 °C and radioactive sugar (final concentration = 0.1 μM) was added. At the appropriate time intervals, 0.25-ml aliquots were withdrawn and filtered over a membrane filter (pore size = 0.45 μm). The external medium was then washed away with 5–10 ml of phosphate buffer, pH 7.0, by rapid filtration. For the uptake experiments, 10 μM HgCl₂ was included in the wash buffer to rapidly inhibit the lactose permease and thereby minimize sugar efflux during the removal of the extracellular medium. As a control, the lacY− strain, HS4006TP [lacY−/pACYC184 (no lacY insert), was also assayed for radiolabeled sugar uptake in order to obtain an accurate value for nonspecific sugar uptake. The control sample was then subtracted from all experimental samples to provide a value for the amount of lactose permease-mediated uptake.

For the counterflow experiments of Figs. 4 and 6, midlog cells were washed in phosphate buffer, pH 7.0, and resuspended to a density of approximately 0.5 mg of protein/ml. An aliquot of cells was then added to a phosphate buffer solution, pH 7.0, containing 30 mM sodium azide and 20 mM lactose (nonradiolabeled). The cells were then incubated at room temperature for 30–60 min to allow the lactose to equilibrate within the cells. The cells were then spun for 10 min at 6,000 rpm in a clinical centrifuge. The supernatant was discarded, and the tube was carefully wiped to remove any remaining liquid. The cell pellet was then resuspended in a 15°C buffer solution containing 30 mM sodium azide and 0.5 μM [¹⁴C]lactose (0.5 μCi/ml) in 100 mM phosphate buffer at pH 5.9, 6.7, or 7.5. At the designated time intervals, 0.15-ml aliquots were withdrawn and filtered over a membrane filter (pore size = 0.45 μm). The external medium was then washed away with 5–10 ml of phosphate buffer, pH 7.0, by rapid filtration. The amount of [¹⁴C]lactose retained within the cells was determined by liquid scintillation counting of the membrane filters.

H⁺ Transport—For the H⁺ transport experiments of Fig. 3, cells were grown to midlog phase and washed twice with 120 mM KCl. The cells were then suspended in 120 mM KCl, 15 mM potassium thiocyanate to a density of approximately 5 mg of protein/ml. 2.5 ml of cells were placed in a closed vessel with a lid containing tight-fitting openings for the insertion of a pH electrode, the introduction of argon, and the insertion of (gas-impermeable) Hamilton syringes. Cells were made anaerobic by a continuous stream of argon for at least 30 min. To initiate sugar-induced H⁺-transport, an anaerobic solution containing lactose, melibiose, TMG, or TDG was added to the designated final concentration of 2 or 10 mM. The change in external pH was measured with a Radiometer pH meter (PHM82) and electrode (GR2401C). Changes in pH were continuously recorded on a Radiometer chart recorder which had been modified to expand the scale of pH changes to a range where a 0.1-unit pH change caused a 10-cm deflection in the chart recording.

Site-directed Mutagenesis—The plasmid, pET28 (Ref. 27), was digested with EcoRI to yield a 2,300-base pair fragment containing the entire lacY gene. This fragment was ligated to the vector M13mp18 (Ref. 28) in such a way that the antisense strand of the lacY gene was colinear with the viral DNA. Site-directed mutagenesis was then performed by the method of Zoller and Smith (29) as modified by Kunkel et al. (30) using oligonucleotide primers producing the desired base change. Clones containing the appropriate mutation were identified by DNA sequencing (see below). The double-stranded RF DNA was then isolated and digested with EcoRI to produce the 2,300-base pair fragment containing the lacY gene. This fragment was then ligated into the EcoRI site of pACYC184 (Ref. 31). Following transformation of E. coli strain T184, cells harboring hybrid plasmids with a lacY insert were identified by their loss of chloramphenicol resistance. The hybrid clones were restriction mapped to verify the presence of the mutation and to be certain that no other secondary mutations had occurred. At least two independent clones for each mutant type were saved for further study.

In order to construct the double Asp-269/Asp-325 mutant, plasmids containing the respective single aspartic mutations were digested with NdeI and SalI. NdeI cleaves at a single site within the lacY coding sequence between position 269 and 325, whereas SalI has a unique site within the pACYC184 vector sequence. Fragments containing the first portion of the lacY (with an aspartic 269 mutation) and the second portion of the lacY (containing the aspartic 325 mutation) were ligated together. The plasmid DNA was transformed into T184 and plated onto rich plates containing tetracycline. Single colonies were picked, and plasmid DNA was isolated and sequenced to verify the presence of both mutations.

DNA Sequencing—Single-stranded viral DNA was sequenced by the Sanger dideoxy method (32) using oligonucleotide primers which anneal within the lacY gene. Double-stranded plasmid DNA was isolated and sequenced as described by Kraf t et al. (33).

Immunoblot—As described by Lolkema et al. (34), the amount of lactose permease protein was determined using a polyclonal antibody preparation directed against the carboxyl-terminal decapeptide of the lactose permease.

RESULTS

Isolation of Site-directed Mutants—In the current study, we have utilized the technique of site-directed mutagenesis to construct a group of lactose permease mutants which contain a variety of substitutions at position 269 and/or 325. To determine whether these mutations affect the level of lactose permease protein within the cell, the amount of protein was measured by using an antibody preparation directed against the carboxyl-terminal tail of the permease. These results are given in Table I. Although some differences are noted, the results of Table I indicate that cells with position 269 and 325 mutations...
whereas a white phenotype indicates a lack of significant fermentation. As expected, the wild-type strain showed the highest colony phenotype. As shown in Table I, the wild-type and mu-

ein. The only exception was the Ala-269 mutant, which was white on a rather high sugar concentration within the plate (i.e., 10 mM). Extracellular lactose concentration remains higher than the position 269 mutant had a much higher $K_m$ (i.e., 10.1 mM), whereas the Asp-325, Gly-325, and Gln-325 mutants had even lower $K_m$ values than the wild-type strain (0.21, 0.47, and 0.50 mM, respectively). At both positions, aspartic mutations had a moderately detrimental effect on the $V_{max}$ value and nonionizable substitutions had an even greater effect. It is possible to reconcile the data in Fig. 3 and the phenotype on MacConkey plates (Table II) by proposing that the nonionizable substitutions affect the kinetic parameters for transport in different ways. It was observed that nonionizable substitutions at position 325 enhance the apparent affinity for lactose but greatly decrease the velocity. Unfortunately, it wasn't possible to measure kinetic parameters in the Gly-269, Ala-269, and Gln-269 mutants. Nevertheless, as is the case with the Asp-269 mutant, it may be that nonionizable substitutions at position 269 decrease the affinity for lactose but only moderately decrease the $V_{max}$ value. This would explain their red phenotype on MacConkey plates which contain a relatively high sugar concentration (i.e., 10 mM).

Another important aspect of $H^+/\text{lactose}$ cotransport is the ability to accumulate sugars against a concentration gradient. In order to accomplish secondary active transport, the uptake of sugar must be coupled to the uptake of $H^+$ ions so that the proton electrochemical gradient can provide the driving force for the accumulation of sugar (3, 4). In Fig. 2, the wild-type and mutant strains were assayed for their ability to accumulate lactose (left panel) or TMG (right panel). As expected, the wild-type strain was able to accumulate these sugars to high intracellular levels. The Asp-325 mutant was also able to accumulate both of the sugars to moderate levels, whereas the Asp-269 mutant only showed significant accumulation of lactose but not TMG. In contrast, none of the other mutations at position 269 or 325 exhibited any active accumulation of either sugar.

### Sugar-induced $H^+$ Transport

The results of Fig. 2 indicate that nonionizable substitutions at positions 269 and 325 cause an inability to accumulate galactosides against a concentration gradient. One possible explanation would be that these glutamates are essential for $H^+$ binding and/or transport. To explore this possibility, a pH electrode was used to measure the ability of the wild-type and mutant strains to cotransport $H^+$ and sugar. $H^+$ transport induced by the addition of melibiose is shown in Fig. 3. A striking dichotomy was observed between the position 269 and 325 mutants. For position 269 mutants, melibiose-induced $H^+$ transport was readily observed. Indeed, as shown in Table IV, position 269 mutants showed substantial $H^+$ transport in the presence of most of the sugars tested. In a few cases, however, no detectable $H^+$ transport was observed. For example, the Gln-269 and Ala-269 mutants showed no $H^+$ transport upon the addition of lactose. Based upon the low levels of downhill transport, it seems likely that this result is due to the fact that these two mutants have extremely low levels of lactose uptake (see Fig. 1). In any case, the results of Fig. 3 and Table IV indicate that an ionizable residue at position 269 is not required for $H^+$ transport via the lactose permease. In sharp contrast, none of the nonionizable substitutions at position 325 showed any detectable $H^+$ transport with lactose, TMG, TDG, or melibiose (see Fig. 3 and Table IV). Two possible explanations could account for this observation. First, it may be that the position 325 mutants are incapable of $H^+/sugar$ cotransport but that the rate of unidirectional sugar influx is too low to induce a detectable level of alkalization. A second explanation would be that sugar influx is uncoupled. The ability to distinguish between these two possibilities is problematic. On the one hand, significant lactose-induced $H^+$ transport was ob-

<table>
<thead>
<tr>
<th>Strain</th>
<th>10 mM lactose</th>
<th>10 mM melibiose</th>
</tr>
</thead>
<tbody>
<tr>
<td>pACYC-LacY</td>
<td>Red</td>
<td>Red</td>
</tr>
<tr>
<td>pE269D</td>
<td>Red</td>
<td>Red</td>
</tr>
<tr>
<td>pE269G</td>
<td>Red</td>
<td>Red</td>
</tr>
<tr>
<td>pE269A</td>
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<td>Red</td>
</tr>
<tr>
<td>pE269Q</td>
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<td>Red</td>
</tr>
<tr>
<td>pE325D</td>
<td>Red</td>
<td>Red</td>
</tr>
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<td>White</td>
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</tr>
<tr>
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</tr>
<tr>
<td>pE259D/E325D</td>
<td>Red</td>
<td>Red</td>
</tr>
</tbody>
</table>

**Functional Roles of Glu-269 and Glu-325 within the lac Permease** 7381
Functional Roles of Glu-269 and Glu-325 within the lac Permease

**FIG. 1.** Downhill lactose transport by parental and mutant strains. The uptake of [14C]lactose was measured at 30 °C as described under "Materials and Methods." The strain HS4068F2·2·Y- (Ref. 45) containing the plasmids pACYC-LacY (○); pE269D (●); pE269G, pE269A, pE269Q, and pE269D/pE325D (+); pE325D (●); pE325G (△); pE325A (■); pE325S (○); and pE325Q ( ● ) was assayed at an external lactose concentration of 0.1 mM.

**TABLE III**

<table>
<thead>
<tr>
<th>Strain</th>
<th>$K_m$ (mM)</th>
<th>$V_{max}$ (nmol lactose/min/mg protein)</th>
</tr>
</thead>
<tbody>
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<td>pACYC-LacY</td>
<td>1.44</td>
<td>756</td>
</tr>
<tr>
<td>pE269D</td>
<td>10.1</td>
<td>46.1</td>
</tr>
<tr>
<td>pE325D</td>
<td>0.21</td>
<td>93.0</td>
</tr>
<tr>
<td>pE325G</td>
<td>0.47</td>
<td>11.2</td>
</tr>
<tr>
<td>pE325S</td>
<td>0.50</td>
<td>9.1</td>
</tr>
</tbody>
</table>

served in the Asp-269 and Gly-269 mutants, even though their downhill lactose transport at an external concentration of 0.1 mM was substantially lower than most of the position 325 mutants (see Fig. 1). From this observation, one might argue that the inability to detect lactose-induced H+ transport in the position 325 mutants would be due to a true “uncoupling.” On the other hand, it was also observed that the Gly-325, Gln-325, Ala-325, and Ser-325 were white on 10 mM MacConkey plates, whereas the position 269 mutants were usually red. From these results, one might argue that the levels of unidirectional sugar uptake are very low and may be too low to induce an alkalization.

**Lactose Counterflow**—The transport experiments described thus far have been aimed at measuring the unidirectional transport of lactose or H+ via the lactose permease. However, the lactose permease is able to catalyze an exchange reaction as well. One way to measure this is via a counterflow assay. In the experiment of Fig. 4, cells were poisoned and preloaded with a high concentration of nonlabeled lactose. The cells were then diluted into a medium containing a low concentration of radiolabeled lactose. During the early time points, radiolabeled lactose is exchanged for nonlabeled lactose on the inside. This reaction is rapid and leads to an apparent transient accumulation of radiolabeled lactose on the inside of the cell. However, a slow rate of unidirectional lactose efflux ultimately leads to a decrease in the accumulation value. As seen in Fig. 4, this type of biphasic curve is observed for the wild-type strain. In contrast, the mutant strains behave quite differently. The position 269 mutants appear to be defective in exchange, since the uphill accumulation of radiolabeled lactose was not observed. However, the position 325 mutants are able to catalyze rapid exchange and thereby accumulate radiolabeled lactose to high
Functional Roles of Glu-269 and Glu-325 within the lac Permease

**[14C]-Lactose Accumulation**

<table>
<thead>
<tr>
<th>Time (min)</th>
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<th>0.5</th>
<th>1.0</th>
<th>1.5</th>
<th>2.0</th>
<th>2.5</th>
<th>3.0</th>
<th>3.5</th>
</tr>
</thead>
<tbody>
<tr>
<td>[IN]/[OUT]</td>
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<td>45</td>
<td>40</td>
<td>35</td>
<td>30</td>
<td>25</td>
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<td>15</td>
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</table>

**[14C]-TMG Accumulation**

<table>
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<th>Time (min)</th>
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<th>0.5</th>
<th>1.0</th>
<th>1.5</th>
<th>2.0</th>
<th>2.5</th>
<th>3.0</th>
<th>3.5</th>
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<tr>
<td>[IN]/[OUT]</td>
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<td>35</td>
<td>30</td>
<td>25</td>
<td>20</td>
<td>15</td>
</tr>
</tbody>
</table>

**FIG. 2. Uphill accumulation of lactose or TMG.** In the left panel, the T184 strain (Ref. 27) containing the plasmids pACYC-LacY (○); pE269D (●); pE325D (△); and pE269G, pE269A, pE269Q, pE325G, pE325A, pE325S, pE325Q, and pE269D/pE325D (+) was assayed for lactose accumulation as described under "Materials and Methods." In the right panel, the HS4006/F'14Z-Y- strain containing the plasmid pACYC-LacY (○); pE269D (●); pE325D (△); and pE269G, pE269A, pE269Q, pE325G, pE325A, pE325S, pE325Q, and pE269D/pE325D (+) were assayed for TMG accumulation.

**FIG. 3. H+ transport upon the addition of 2 mM melibiose.** H+ transport was measured in the strain T184 containing the designated plasmids as described under "Materials and Methods."

---

Table IV

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Lactose</th>
<th>Melibiose</th>
<th>TDG</th>
<th>TMG</th>
</tr>
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<tbody>
<tr>
<td>pACYC-Lac+</td>
<td>+++*</td>
<td>++</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td>pE269D</td>
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<td>−</td>
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<tr>
<td>pE325D</td>
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<td>++</td>
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<td>++</td>
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<tr>
<td>pE325G</td>
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<td>pE325S</td>
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<td>pE325Q</td>
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</tr>
<tr>
<td>pE269D/E325D</td>
<td>++</td>
<td>+++</td>
<td>−</td>
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</tbody>
</table>

*The level of H+ transport was divided into different categories based upon the relative extent of alkalization: +++, high alkalization; ++, moderate alkalization; +, low alkalization; and −, no detectable alkalization. See Fig. 3 for a quantitative comparison of these values. The strain T184, which can metabolize melibiose, but not lactose, was transformed with the designated plasmids.

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levels. An interesting difference between the wild-type and position 325 mutants is that the efflux phase of the counterflow assay appears to be much slower in the mutants.

The results up to this point indicate that an acidic residue at position 325 is not required for sugar translocation (i.e., exchange) but is required for uphill sugar accumulation and sugar-induced H+ transport. These results are consistent with the notion that Glu-325 in the wild-type permease is essential for cation binding. If this is the case, mutants with a nonionizable side chain would be expected to behave as if they could only catalyze simple facilitated diffusion. Therefore, unlike the wild-type permease which is influenced by a pH gradient across the membrane, the position 325 mutants would be expected to have lost this characteristic. To explore this possibility, counterflow experiments were conducted in which the external pH was varied. As previously seen in Fig. 4, the data in a counterflow experiment exhibit two phases. During the first phase, the dominant reaction is the exchange of nonlabeled lactose on the inside with radiolabeled lactose on the outside. This exchange reaction is at least 10 times more rapid than unidirectional...
lactose efflux and therefore leads to a plateau in which the radiolabeled lactose is accumulated against a gradient (36). Furthermore, at saturating external lactose concentrations (i.e. 0.43 mM), this exchange reaction appears to be insensitive to changes in ΔpH and Δψ (35, 36). From this observation, it has been hypothesized by others that the exchange does not require protonation and concomitant deprotonation (36, 37). A kinetic mechanism which is consistent with observation is depicted in Fig. 5. The exchange reaction occurs via the ternary complex but does not require deprotonation. In other words, H⁺ does not have to be released in order for another lactose molecule to bind and be translocated in the opposite direction. The second phase of the counterflow experiment involves a gradual drop in the accumulation level of radiolabeled sugar on the inside. This kinetically slower process is due to the unidirectional efflux of lactose (which is initially at a higher chemical concentration on the inside of the cell). Eventually, since the cells are poisoned, this efflux phase will ultimately lead to a 1:1 equilibration of both nonlabeled and radiolabeled sugar across the membrane.

Provided that the above assumptions are correct, an alteration in the transmembrane pH gradient should have a significant effect on counterflow results. In particular, an increase in the external pH should enhance the rate of unidirectional efflux since it will favor the deprotonation of the carrier on the outside of the membrane (see Fig. 5). This would be expected to cause the plateau level of accumulation (due to exchange) to

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**Lactose Counterflow at Variable pH**

![Lactose Counterflow at Variable pH](image_url)

**Fig. 4. Lactose counterflow in wild-type and mutant strains.** The T184 strain containing the plasmids pACYC-LacY (□); pE269D (○); pE269G, pE269A, pE269Q, and pE269D/pE325D (●); pE325D (●); pE325G (△); pE325A (■); pE325S (▲); and pE325Q (●) were assayed for lactose counterflow as described under "Materials and Methods."

**Fig. 5. Mechanism of lactose transport.** This model depicts the binding and translocation of H⁺ and lactose via the lactose permease. C₁ and C₂ indicate different conformations of the carrier, which are accessible to substrates from the inside or outside, respectively. Even though the binding of both substrates to the carrier may not be strictly ordered, the model is drawn in this fashion to emphasize the fact that under certain conditions the exchange of lactose via the ternary complex is rapid and does not appear to require protonation and concomitant deprotonation.

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**Fig. 6. Lactose counterflow at different external pH values.** Lactose counterflow flow was carried out in the T184 strain containing the designated plasmids at an external pH of 5.9 (△) pH 6.7 (□), or pH 7.5 (○).
last a shorter period of time and to decrease at a faster rate. As shown in Fig. 6, left panel, these predicted results were obtained with the wild-type permease. At an external pH of 5.9, a high plateau value was rapidly achieved which gradually decreased over a longer time course of several minutes. As the external pH was increased to pH 6.7 and 7.5, this efflux phase was observed to increase dramatically. Indeed, at pH 7.5, the efflux phase became so rapid that a maximal plateau was not achieved. Similar results were also obtained with the Asp-325 mutant, although the time course was significantly different (see Fig. 6, right panel). Strikingly, however, the Gln-325 and Gly-325 mutants behaved as if they had become completely pH insensitive. At all three pH values tested, these mutants were observed to have similar maximal plateau values and similar rates of efflux. Taken together, these results are consistent with the notion that a nonionizable residue at position 325 converts the lactose permease into a carrier which is relatively insensitive to ΔpH. Alternatively, it may be that the rates of efflux in the Gln-325 and Gly-325 are so low that it is not possible to detect any effect of a pH gradient.

**DISCUSSION**

The results of the current study describe the kinetic characterization of mutations in which Glu-269 and Glu-325 have been changed to aspartates or nonionizable residues. At position 269, mutations appear to have a detrimental affect on the affinity for sugar and on the maximal velocity for transport. In addition, nonionizable substitutions at position 269 were unable to accumulate sugars against a concentration gradient. Nevertheless, it was observed that these mutants were still able to couple the translocation of sugar and H+.

Therefore, an acidic residue at position 269 is not essential for H+/lactose cotransport via the lactose permease. 

The reason for the inability of position 269 mutants to accumulate remains unclear. One explanation would be related to their apparent low rate of sugar influx (see Fig. 1). Since the influx of sugar via other pathways (i.e. passive diffusion) is significant, the observed levels of sugar accumulation reflect a competition between the rate of uptake via the permease-mediated pathway and decreases brought about by the leak pathways. Therefore, mutations which substantially diminish the rate of permease-mediated sugar influx will also affect the overall accumulation levels, since a smaller difference will exist between the rates of the permease-mediated and leak pathways.

As far as H+ recognition is concerned, the results of this study and previous studies are consistent with the notion that an acidic residue at position 325 is essential for H+ transport via the lactose permease (22, 23). Due to its negative charge, it is tempting to speculate that this residue provides a binding site for the H+ ion which is cotransported with lactose. Moreover, based upon the effects on sugar recognition seen in Table III as well as its proximity to other residues which have been identified in sugar specificity studies (e.g. Lys-319 and His-322, Refs. 38 and 39), one could also speculate that this site is involved with sugar recognition as well. Such a physical "coupling" between cation binding and sugar binding is an attractive way to explain the functional coupling via symporters. Along these same lines, it is also interesting to comment on the possible roles of other ionizable residues within the permease such as Arg-302, Lys-319, and His-322. Although these residues are not essential for H+ transport via the permease, mutations at these sites do have marked effects on sugar specificity and on the active accumulation of sugar (38–43). In the wild-type permease, these residues could be involved with sugar recognition and/or influence the pKₐ of an essential residue (i.e. Glu-325) for H+ binding. It is interesting to note that in bacteriorhodopsin, for example, the proximity of Arg-82 to Asp-85 has been suggested to lower the pKₐ for the aspartic acid at position 85 (Ref 14).

Although the above speculations are compelling, it is also important to consider that other explanations are consistent with the available data. For example, it has been suggested that H+ translocating proteins may actually transport hydroxion ions rather than protons (44). If this is the case with the lactose permease, it may be that an acidic residue at position 325 is essential for the proper formation of a more complex hydroxion ion binding site within the protein. And finally, it still remains a reasonable possibility that a requirement for an acidic residue at position 325 is not due to an effect on cation binding per se. For example, the available data would be consistent with a model in which an acidic residue at position 325 is necessary for the unloaded carrier to isomerize at an appreciable rate. Unfortunately, the types of kinetic experiments described in this paper cannot distinguish among these various possibilities. In future work, it will be necessary to employ techniques which can determine whether residues such as Glu-325 become protonated/deprotonated during transport.

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**REFERENCES**

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Functional Roles of Glu-269 and Glu-325 within the lac Permease