Energy-dependent Binding of Dansylgalactosides to the β-Galactoside Carrier Protein*

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SUMMARY

Fluorescent β-galactosides (1-(N-dansyl)amino-β-D-galactopyranoside (DG₁), 2'-(N-dansyl)aminoethyl-β-D-thiogalactopyranoside (DG₂), 2''-(N-dansyl)aminoethyl-β-D-galactopyranoside (oxy-DG₂), and 6''-(N-dansyl)aminohexyl-β-D-thiogalactopyranoside (DG₆)) competitively inhibit lactose transport by membrane vesicles from Escherichia coli ML 308-225, but are not actively transported. An increase in the fluorescence of these dansylgalactosides is observed upon addition of D-lactate, imposition of a membrane diffusion potential (positive outside), or dilution-induced, carrier-mediated lactose efflux. The increase is not observed with 2''-(N-dansyl)aminoethyl-β-D-thiogalactopyranoside nor with membrane vesicles lacking the β-galactoside transport system. Moreover, the D-lactate-induced fluorescence increase is blocked or rapidly reversed by addition of β-galactosides, sulfhydryl reagents, inhibitors of D-lactate oxidation, or uncoupling agents. The fluorescence increase exhibits an emission maximum at 500 nm and excitation maxima at 345 nm and at 292 nm. The latter excitation maximum is absent unless D-lactate is added, indicating that the bound dansylgalactoside molecules are excited by energy transfer from the membrane proteins. Titration of vesicles with dansylgalactosides in the presence of D-lactate demonstrates that the lac carrier protein constitutes 3 to 4% of the total membrane protein, and that the affinity of the carrier for substrate is directly related to the length of the alkyl chain between the galactosidic and the dansyl moieties of the dansylgalactosides. In addition, there is excellent agreement between the affinity constants of the various dansylgalactosides as determined by fluorimetric titration and their apparent Kₐ's for lactose transport (Kₐ's and/or apparent Kₐ's are approximately 550, 30, 40, and 5 µM for DG₁, DG₂, oxy-DG₂, and DG₆, respectively). Polarization of fluorescence measurements with DG₂ and DG₆ demonstrate a dramatic increase in polarization on addition of D-lactate which is reversed by addition of lactose or anaerobiosis. These findings provide strong evidence for the contention that the fluorescence changes observed on "energization" of the membrane are due to binding of the dansylgalactosides per se, rather than binding followed by transfer into the hydrophobic interior of the membrane.

The results are consistent with the suggestion that the lac carrier protein is inaccessible to the external medium unless energy is provided, and that energy is coupled to one of the initial steps in transport. It is proposed furthermore that at least one aspect of "energization" may be the generation of a membrane potential (positive outside) resulting in increased accessibility of a negatively charged lac carrier protein to the external solvent.

It is not known how energy released from D-lactate oxidation is coupled to active β-galactoside transport in Escherichia coli membrane vesicles. Several contending theories attempt to provide an answer to this question, but experimental evidence does not allow a clear choice to be made between them (2).

One model proposed by Kaback and Barnes (3) depicted the carriers as electron transfer intermediates in which a change from the oxidized to the reduced state results in translocation of the carrier-substrate complex to the inner surface of the membrane and a concomitant decrease in the affinity of the carrier for substrate. A very different hypothesis, one that emphasizes the positioning of respiratory components within the matrix of the membrane, was proposed by Mitchell (4). As visualized by this chemiosmotic hypothesis, oxidation of electron donors is accompanied by extrusion of protons into the external medium, leading to a pH gradient or electrical potential across the membrane or both. This "proton motive force" is postulated to be the driving force for β-galactoside uptake via a carrier which catalyzes β-galactoside-proton symport (4). Recent studies by Reeves et al. (3) using a fluorescent β-galactoside, 2'-(N-dansyl)aminoethyl-β-D-galactopyranoside

1 The abbreviations used are: dansyl, 5-dimethylaminonaphthalene-1-sulfonic acid; DG₁, 1-(N-dansyl)amino-β-D-galactopyranoside; DG₂, 2'-(N-dansyl)aminoethyl-β-D-thiogalactopyranoside; oxy-DG₂, 2''-(N-dansyl)aminoethyl-β-D-galactopyranoside; DG₆, 6''-(N-dansyl)aminohexyl-β-D-thiogalactopyranoside; methylumbelliferyl-galactoside, methylumbelliferyl-β-D-galactopyranoside.

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that binding of DGs to the lac carrier protein is dependent upon n-lactate oxidation. These studies suggest that energy is coupled to one of the initial steps in transport and that facilitated diffusion, therefore, cannot be the rate-limiting step for active transport of β-galactosides. Koch (6) has reached a similar conclusion as a result of studies with starved intact cells, and transport of P-galactosides. Koch (6) has reached a similar conclusion as a result of studies with starved intact cells, and transport of P-galactosides.

A variety of dansylgalactoside analogues are shown to be competitive inhibitors of lactose transport, but are not transported themselves. Fluorescence studies with these analogues indicate that the lac carrier protein is not accessible to the external medium unless the membrane is "energized" by n-lactate oxidation, imposition of a membrane potential or charge separation within the membrane. Polarization of fluorescence measurements support the proposal that the fluorescence changes observed upon energization of the membrane are due specifically to binding of substrate to the lac carrier protein. It is proposed that the lac carrier protein is negatively charged, and can be "moved" to the external surface of the membrane by creation of either a diffusion potential or charge separation within the membrane.

**Experimental Procedure**

**Growth of Bacteria and Preparation of Membrane Vesicles**

*Escherichia coli* ML 306-225 (i′z′y′-a′), ML 3 (i′z′y′-a′), and ML 30 (i′z′y′-a′) were grown in Minimal Medium A containing 1% sodium succinate (hexahydrate) as sole source of carbon, and membrane vesicles were prepared as described previously (8).

**Transport Assays**

[1-14C]Lactose (20 nCi per mmol) uptake by membrane vesicles was assayed as described previously (8).

Uptake of DGs, [1H]DGs, oxy-DGs, DGs, and methylumbelliferonyl-β-galactopyranoside was assayed as follows: membrane vesicles (2 mg per ml of protein) suspended in 50 mM potassium phosphate (pH 6.6) and 10 mM magnesium sulfate (0.5 ml, final volume, in 10-ml reaction vessels) were incubated at 25° in the presence of 20 mM lithium n-lactate, DGs, [1H]DGs, oxy-DGs, methylumbelliferyl-galactoside, or [1-14C]lactose. The samples were gassed with oxygen for 5 min preceding the addition of substrate; TDG, P-n-galactosyl-I-thio-b-D-galactopyranoside; TMG, methyl-1-thio-p-D-galactopyranoside.

**Fluorescence Measurements**

Fluorescence was measured at an angle of 90° with an Amino-Bowman spectrofluorometer using cuvettes (1 X 1 cm) as described previously (10). The excitation light for the polarization experiments was defined by a blocking filter combination of a Corning 3-75 filter, a sodium nitrite filter, and an analyzer made of a glass-enclosed quartz polarizer. The sample chamber was maintained at 25° by circulating water bath. Excitation spectra were measured in the ratio mode without further correction. Light band-pass for excitation and emission spectra was 6 nm. Additions to the cuvette were made with Hamilton microsyringes and mixing was accomplished within 3 to 5 s using a small plastic stirrer.

**Synthesis of Dansylgalactosides**

The structures of the compounds to be described are shown in Fig. 1.

**Preparation of 1-(N-Dansyl)amino-β-β-galactopyranoside (DGs)**

**Step 1**: Preparation of 2,3,4,6-Tetra-O-acetyl-1-(N-dansyl)-amino-β-β-galactopyranose (I) – 2,3,4,6-Tetra-O-acetyl-1-amino-β-β-galactopyranose; TMG, methyl-1-thio-β-β-galactopyranoside.

in 0.1 M potassium phosphate buffer, pH 6.6, were rapidly diluted 3.7-fold into aqueous solutions of DGs, oxy-DGs, or DGs at concentrations of 6.9 mM. After 5 min, the osmolarity was adjusted by addition of 5 volumes of 0.1 M potassium phosphate buffer (pH 6.6), and the suspensions were centrifuged at 40,000 X g for 15 min. The supernatants were carefully aspirated and discarded, and the pellets were resuspended in a small volume of 50 mM potassium phosphate (pH 6.6) containing the dansylgalactoside to a final protein concentration of 40 mg per ml. Counterflow was initiated by diluting 2-µl aliquots of this membrane suspension into 100 µl of 50 mM potassium phosphate (pH 6.6) containing 10 mM magnesium sulfate and 0.4 mM [1-14C]lactose (200 Ci per mmol). Uptake was assayed as a function of time in the same manner as that described for n-lactate or azocarbene-phenazinc methosulfate-driven lactose uptake (8). Positive controls for these experiments were performed with vesicles loaded with 20 mM lactose rather than dansylgalactosides.

**Synthesis of Dansylgalactosides**

The structures of the compounds to be described are shown in Fig. 1.

**General**—Melt points were determined on a Reichert hot stage. Thin layer chromatography was carried out on plates coated with Merck Silica Gel G. Spots were located by fluorescence at 254 nm. The analyser filter was adjusted to transmit light polarized horizontally (i.e. perpendicular to the vector of the incident beam) or vertically (i.e. parallel to the vector of the incident beam). The calculated polarization parameters was (I0 – I1)/(I1 + I0). Under these conditions, the polarization parameter for a 3 µm solution of rhodamine B was 0.57 with excitation at 547 nm.

**Preparation of 1-(N-Dansyl)amino-β-β-galactopyranoside (DGs)**

**Step 1**: Preparation of 2,3,4,6-Tetra-O-acetyl-1-(N-dansyl)-amino-β-β-galactopyranose (I) – 2,3,4,6-Tetra-O-acetyl-1-amino-β-β-galactopyranose.
β-D-galactose (11) (1.4 g, 4 mmol) was dissolved in dry pyridine (80 ml) and a solution of dansyl chloride (1.2 g, 4.4 mmol) in dry pyridine (5 ml) was added. After standing overnight at 4°, the mixture was poured into ice and water (400 ml). The syrup product that formed was dissolved in chloroform (100 ml), washed successively with 1 N HCl (4 x 50 ml), saturated sodium hydrogen carbonate (2 x 50 ml), and water (2 x 50 ml), dried with CaCl₂, and evaporated to dryness. The residue was chromatographed on a silica gel column (5 x 60 cm) with benzene-methanol (9:1, v/v). Fractions positive for both carbohydrate and fluorescence were combined and concentrated. The residue was crystallized from 2-propanol to afford yellow crystals of Compound I. The yield was 0.51 g (22%). An analytical sample (m.p. 155-156°) was obtained by two additional crystallizations from ethanol.

**Step 2: Preparation of L-(N-Dansyl)aminoethyl-1-thio-β-D-galactopyranoside (IV)**—Compound III (0.1 g, 0.5 mmol) was dissolved in methanol (100 ml) presaturated with ammonia at 0°, and the solution was kept at 0° for 12 hours. The solvent was evaporated, the residue was dissolved in methanol and chromatographed on a Merck gel OR-PA 500 column (5 x 90 cm) (Merek, Darmstadt) in methanol. The insoluble material was removed from appropriate fractions, and the residue was triturated in hot 2-propanol. The yield was 0.24 g (82%), m.p. 205° (decomposition).

**Preparation of 2'-{(N-Dansyl)aminoethyl}-1-thio-β-D-galactopyranoside (IV)**—A solution of 2',3',4',6'-tetra-O-acetyl-2'-{N-dansyl}uridine-1-thio-β-D-galactopyranoside (13) (8.8 g, 4.4 mmol) in dry benzene (10 ml) was added, and stirring was continued for 2 hours until the solution was clear. Evaporation of the methanolic solution yielded 0.24 g (16%) of Compound V as a glassy mass which was dissolved in water and freeze-dried.

**Preparation of 2'-{(N-Dansyl)aminoethyl}-β-D-galactoside (oxy-DG₁)**

Step 1: 2',3',4',6'-Tetra-O-acetyl-2'-{(N-dansyl)aminoethyl}-β-D-galactopyranoside (IV)—To a solution of L-dansylaminomethane (14) (1.6 g, 4 mmol) in dry benzene (20 ml) was added silver carbonate (1.65 g, 4 mmol) and Drierite (3 g). After stirring for 2 hours, the solution was filtered and washed with ether. The residue was crystallized from ethanol to obtain 0.6 g (12%) of a yellow amorphous material. Compound VIII was dansylated and the reaction product was isolated as described for Compound IV. Efforts to crystallize the compound after chromatography on Sephadex LH-20 failed. Therefore, an additional chromatographic purification step on silica gel with benzene-methanol (1:1) as the eluant was performed giving homogeneous Compound IX as determined by thin layer chromatography and nuclear magnetic resonance. The purity of Compound VIII was confirmed by thin layer chromatography in Solvents C and D and by nuclear magnetic resonance.

**Preparation of 2'-{(N-Dansyl)aminoethyl}-β-D-glucoside (oxy-DG₂)**

Step 1: 2',3',4',6'-Tetra-O-acetyl-2'-{(N-dansyl)aminoethyl}-β-D-galactoside (oxy-DG₁)—The compound was synthesized by following the procedure described for the analogous galactose derivative. 1'-Thio-β-D-glucose sodium salt (hydrate) (16) (4.8 g, 0.019 mol) was added, yielding 2.9 g (67%) of Compound VIII, m.p. 19°.

**Preparation of 6'-{(N-Dansyl)aminoethyl}-1-thio-β-D-galactopyranoside (DG₃) (V)**

A mixture of 6'-aminoethyl-1-thio-β-D-galactopyranoside (13) (0.8 g, 2.7 mmol) and dansyl chloride (0.8 g, 3 mmol) in 50% acetonitrile-water (30 ml) was kept at room temperature for 2 hours, and the pH was maintained at 9.5 by autotitration with 0.5 N NaOH. The reaction mixture was neutralized by addition of 0.5 N HCl and evaporated. The resulting crude syrup was placed on the top of a column (60 x 100 cm) of silica gel packed in benzene. The column was washed with benzene until the eluate was free of fluorescent by-products. Elution was then performed with benzene-methanol-acetic acid (7:2:1, v/v/v). Appropriate fractions were combined and evaporated. The residue was rechromatographed on Sephadex LH-20 essentially as described for Compound IV. Thin layer chromatography of the fractions containing both carbohydrate and fluorescence revealed only one spot in several solvent systems. The radioactive material was isolated by thin layer chromatography. A mixture of 6'-aminoethyl-1-thio-β-D-galactopyranoside, isolated from one of the fractions, was found to have the same properties as the authentic compound.

**Materials**

Methylumbelliferyl-β-D-galactopyranoside was generously provided by Dr. W. Breugel of the University of Konstanz, Konstanz, W. Germany. [1-14C]Lactose was purchased from American/Searle. [U-14C]Dansyl chloride was obtained from American (England). All other chemicals were of reagent grade obtained from commercial sources.

**RESULTS**

Effects of Dansylgalactosides on Lactose Transport by Escherichia coli MIL 208-285 Membrane Vesicles—DG₁, DG₂, oxy-DG₃, and DG₄ are competitive inhibitors of lactose transport in membrane vesicles of Escherichia coli MIL 208-285.
vesicles prepared from *E. coli* ML 308-225 (Fig. 2). The apparent inhibition constants ($K_i$), given by the negative inhibitor concentration at the point of intersection, are 550 $\mu$M for DG6, 32 $\mu$M for DG3, 42 $\mu$M for oxy-DG3, and 6.5 $\mu$M for DG4. Over the range of concentrations tested, none of these dansylgalactosides has an effect on either the rate or extent of proline uptake by membrane vesicles. Moreover, 2-(N-dansyl)-desobutyl-DG3 does not inhibit lactose transport (data not shown). These results suggest that each of these dansylgalactoside analogues interacts specifically with the binding site of the $\beta$-galactoside carrier protein. It is also apparent from these studies that an increase in the length of the alkyl chain between the galactosidic terminals, membrane vesicles were incubated for 10 min in the presence of the appropriate $\beta$-galactoside with and without $\beta$-lactate. The samples were then centrifuged, and the pellets were assayed for fluorescence or radioactivity as described under “Experimental Methods.” In the absence of $\beta$-lactate, less than 1 nmol of each compound is bound per mg of membrane protein. On addition of $\beta$-lactate, 1.5 to 2.5 nmol of DG4,oxy-DG3, or DG4 are bound per mg of membrane protein, a quantity which approximates the amount of lac carrier protein as determined by fluorescent titration (see below). When [H]DG4 is used so that the determinations can be made radioactively rather than fluorometrically, essentially identical results are obtained. In contrast to the results obtained with dansylgalactosides, methylumbelliferyl $\beta$-$\beta$-galactopyranoside is actively accumulated by the vesicles in the presence of $\beta$-lactate (5). As shown in Table I, uptake of this fluorescent glycoside is essentially the same as that of lactose.

Another indication that the dansylgalactosides described above are not transported is that no counterflow of lactose can be detected in vesicles loaded with high concentrations of DG3, oxy-DG3, or DG4 as described under “Experimental Procedure” (data not shown). It should be stressed that vesicles loaded with lactose, on the other hand, exhibit typical counterflow phenomena.

**Fluorescence Properties of Dansylgalactosides**—As shown previously for DG2 (5), addition of ML 308-225 membrane vesicles to an aqueous solution of this dansylgalactoside has no effect on either the excitation or emission spectrum in the absence of $\beta$-lactate. Although similar results have been obtained with DG3, oxy-DG3, and DG4, only those obtained with DG4 are presented in detail (Fig. 3). The excitation spectrum of the $\beta$-lactate-induced increase in DG4 fluorescence (Fig. 3A, ———) exhibits a small shift to longer wavelengths, and in addition, there is a maximum at 292 nm which is absent from the excitation spectrum of DG4 in the absence of $\beta$-lactate (————). The maximum at 292 nm coincides with the excitation maximum for membrane-protein fluorescence, and indicates that the DG4 molecules responsible for the fluorescence increase can be excited by energy transfer from the tryptophanyl residues of the membrane proteins. As demonstrated previously with DG2 (5), this suggestion is confirmed by the finding that the fluorescence of the membrane proteins, measured at 335 nm, decreases when $\beta$-lactate is added to the membranes in the presence of dansylgalactosides. No such decrease is observed in the absence of dansylgalactoside.

Although not shown in Fig. 3B, a small shift (approximately 5 nm) to the blue region of the emission spectrum of DG4 is observed when membranes are added to the cuvette in the presence of lactose.

**Uptake of Various Galactosides by Escherichia coli ML 308-225 Membrane Vesicles in Presence of $\beta$-lactate**

<table>
<thead>
<tr>
<th>Compound</th>
<th>$\beta$-Lactate-dependent transport (nmol/mg membrane protein/10 min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DG2</td>
<td>1.8</td>
</tr>
<tr>
<td>[H]DG2</td>
<td>1.5</td>
</tr>
<tr>
<td>oxy-DG3</td>
<td>2.4</td>
</tr>
<tr>
<td>DG4</td>
<td>2.0</td>
</tr>
<tr>
<td>Umbelliferyl-galactoside</td>
<td>41.3</td>
</tr>
<tr>
<td>[1-14C]Lactose</td>
<td>44.1</td>
</tr>
</tbody>
</table>
absence of n-lactate. This effect is due to nonspecific binding since it is also observed with membrane vesicles devoid of the lac transport system (i.e. vesicles prepared from uninduced E. coli ML 30 or E. coli ML 3, a y^- mutant). Moreover, this shift is not affected by the addition of 5 mM lactose. When n-lactate is added to the cuvette, however, a large increase in fluorescence occurs. This increase is not observed with membrane vesicles prepared from either uninduced E. coli ML 30 or ML 3. Furthermore, the changes can be completely prevented or reversed by addition of lactose (Fig. 5). The difference in the emission spectrum with and without n-lactate shows a maximum at 500 nm, a region well below the emission maximum of DG2 in aqueous solution (approximately 540 nm).

**Fluorescence Polarization Studies**—The fluorescence parameters measured above do not distinguish between binding per se as opposed to binding followed by translocation of dansylgalactosides into the hydrophobic environment of the membrane. Polarization of fluorescence, on the other hand, can be used to assay binding specifically since changes in this parameter reflect alterations in the rotation of molecules in solution.

The data presented in Table II exhibit a small degree of polarization of the emission of DG2 in the presence of ML 308-225 vesicles. However, when n-lactate is added to the cuvette, a marked increase in polarization from 0.015 to 0.041 is observed and then abolished when the medium becomes anaerobic or when 5 mM lactose is added. With vesicles prepared from uninduced ML 30 or ML 3, polarization values of 0.012 and 0.013 are observed, respectively, and these values do not change upon addition of n-lactate. Moreover, the low polarization values observed in ML 308-225 vesicles in the absence of n-lactate or in ML 30 and ML 3 vesicles are not diminished by addition of excess lactose. Although not shown, similar data were obtained with DG6. These observations provide strong evidence in support of the hypothesis that the increase in dansylgalactoside fluorescence observed on addition of n-lactate is due specifically to binding to the lac carrier protein.

**Fluorimetric Titration Studies**—As demonstrated previously (5), the quantity of DG2 bound to the vesicles in the presence of n-lactate can be estimated from the increase in fluorescence at 500 nm. By measuring binding at various concentrations, the affinity constant of the lac carrier protein for DG2 was determined. The data presented in Fig. 4 represent an analogous study with DG6. As shown, the increase in DG6 binding induced by n-lactate is a saturable function of DG6 concentration. The apparent K_d, calculated from the Scatchard plot (18) shown in the inset, is approximately 4.7 μM, a value which is very similar to the apparent K_d for DG2 inhibition of lactose uptake (cf. Fig. 2 and Table III). In addition, the amount of DG6 bound at saturation is approximately 2.0 nmol per mg of membrane protein. Although titration curves for the other dansylgalactosides used in these studies will not be presented, the data derived from these studies are tabulated in Table III along with the apparent K_d obtained for inhibition of lactose transport.

**Table II**

<table>
<thead>
<tr>
<th>Membrane vesicles</th>
<th>Experimental μ (θ = 1/(1 + θ))</th>
</tr>
</thead>
<tbody>
<tr>
<td>(-) n-Lactate</td>
<td>(+) n-Lactate (20 μM)</td>
</tr>
<tr>
<td></td>
<td>(-) O_2</td>
</tr>
<tr>
<td>ML 308-225</td>
<td>0.015</td>
</tr>
<tr>
<td>ML 3</td>
<td>0.013</td>
</tr>
<tr>
<td>ML 30</td>
<td>0.011</td>
</tr>
</tbody>
</table>

![Fig. 3. Excitation (A) and emission (B) spectra of DG2 in the presence of membrane vesicles. DG1 (5 μM) was added to a cuvette containing 0.05 M potassium phosphate (pH 6.8) plus 0.01 M magnesium sulfate and Escherichia coli ML 308-225 membrane vesicles (0.4 mg of protein per ml, final concentration) in a total volume of 1.5 ml. Spectra were recorded before (---) and 1 min after (----) addition of 20 mM lithium n-lactate. The difference between the spectra obtained with and without n-lactate is given by the dotted lines (-----). Difference spectra were obtained by calculation, and plotted on a magnified scale relative to the other spectra shown. For emission spectra, the excitation wavelength was 340 nm; for excitation spectra fluorescence was monitored at 520 nm. The light band-pass was 6 nm.](http://www.jbc.org/)

![Fig. 4. Effect of DG6 concentration on the n-lactate-induced fluorescence increase. The percentage increase in DG6 fluorescence upon addition of n-lactate was determined at each DG6 concentration (excitation, 340 nm; fluorescence, 500 nm). Experimental conditions were the same as those described in Fig. 3, except that the concentration of DG6 was varied as indicated. The number of bound DG6 molecules at each concentration was calculated assuming that the fluorescence of the bound molecules in creased by a factor of 25. Inset, data plotted according to the method of Scatchard (18).](http://www.jbc.org/)
In each case, where the appropriate comparisons were made, the $K_D$ for binding as determined by fluorimetric titration compares favorably with the apparent $K_d$ determined kinetically. These studies provide direct support therefore for the suggestion that the affinity of the lac carrier protein ill-\textit{titration} compares favorably with the apparent $K_D$ for binding as determined by fluorimetric titration. Which analogue is used for the titration.

Properties of $\nu$-Lactate induced Dansylgalactoside Binding—

The time course of DG$_3$ binding induced by $\nu$-lactate is shown in Fig. 5. At $5 \mu M$ DG$_3$, addition of $20 \mu M$ $\nu$-lactate produces about an 80% increase in fluorescence measured at 500 nm; the fluorescence changes are half complete in 10 to 13 s at 23°C.

**Table III**

$K_D$, $K_{DP}$, and number of binding sites for various dansylgalactosides in ML 308-225 membrane vesicles

<table>
<thead>
<tr>
<th>Dansylgalactoside</th>
<th>$K_i$</th>
<th>$K_D$</th>
<th>Number of sites</th>
</tr>
</thead>
<tbody>
<tr>
<td>DG$_3$</td>
<td>550</td>
<td>31</td>
<td>9.2 mm bound/mg membrane protein</td>
</tr>
<tr>
<td>DG$_2$</td>
<td>32</td>
<td>31</td>
<td>1.4</td>
</tr>
<tr>
<td>Oxy-DG$_2$</td>
<td>42</td>
<td>40.4</td>
<td>1.35</td>
</tr>
<tr>
<td>DG$_4$</td>
<td>6.5</td>
<td>4.7</td>
<td>1.0</td>
</tr>
</tbody>
</table>

Once the steady state has been attained, the fluorescence remains stable until the cuvette becomes anaerobic. At this point, there is an abrupt increase in fluorescence due to reduction of a membrane component which may be menaquinone (19), followed by a decay of DG$_3$ fluorescence to its original level. Membranes prepared from uninduced \textit{E. coli} ML 30 or ML 3 exhibit no change in DG$_3$ fluorescence when $\nu$-lactate is added. Upon anaerobiosis, the fluorescence of these membranes increases and thereafter remains steady. Similar data were obtained with oxy-DG$_3$ and reported previously with DG$_2$ (5), except that the changes observed with DG$_3$ occur at a much lower concentration (i.e. 5 $\mu M$ for DG$_3$, as opposed to 30 or 40 $\mu M$ for DG$_2$ and oxy-DG$_2$, respectively). In marked contrast, no change in the fluorescence of 2'-N-dansylaminoethyl-$\beta$-n-thioglucopyranoside is observed with ML 308-225 vesicles when $\nu$-lactate is added (data not shown).

Addition of lactose completely and rapidly reverses DG$_3$ binding (Fig. 5). Moreover, addition of lactose before $\nu$-lactate practically abolishes the fluorescence changes induced by $\nu$-lactate (data not shown). Similar results were obtained with TDG and TMG. The fluorescence changes observed with oxy-DG$_2$ also exhibit similar properties.

Since the relative effects of various electron donors and different metabolic inhibitors on $\nu$-lactate-induced DG$_3$ and oxy-DG$_2$ binding are virtually identical with those previously reported for DG$_2$ (5), these data are not presented in detail. $\nu$-Lactate is clearly the most effective energy source for stimulating both the rate and extent of binding. $\nu$-Lactate, $\alpha$-hydroxybutyrate, and succinate induce much less dramatic changes in fluorescence, and neither addition of ATP nor sudden acidification of the medium ($\Delta pH = 1$ to 2) results in binding of these dansylgalactosides. $\nu$-Lactate-induced binding of DG$_3$ and oxy-DG$_3$ is also blocked and rapidly reversed by the electron transfer inhibitors 2-heptyl-4-hydroxyquinoline $N$-oxide ($40 \mu M$), amytal ($10 \mu M$), and potassium cyanide ($10 \mu M$), and by the uncoupler carbonyl cyanide $m$-chlorophenylhydrazone ($5 \mu M$). Potassium oxalate ($20 \mu M$) and potassium oxamate ($20 \mu M$), potent competitive inhibitors of $\nu$-lactate dehydrogenase (3), reduce both the rate and extent of $\nu$-lactate-dependent DG$_3$ and oxy-DG$_2$ binding when added before $\nu$-lactate. However, when added after $\nu$-lactate, these compounds cause only minimal reduction in DG$_3$ and oxy-DG$_2$ fluorescence. $N$-Ethylmaleimide also inhibits DG$_3$ and oxy-DG$_2$ binding in the presence of $\nu$-lactate. However, when added to the cuvette after $\nu$-lactate, $N$-ethylmaleimide causes a gradual decay of DG$_3$ and oxy-DG$_2$ fluorescence back to their original levels. As discussed previously (5), this finding represents a striking difference between the properties of the dansylgalactoside fluorescence changes and lactose transport activity. Sulfhydryl reagents block lactose uptake in membrane vesicles, but do not cause efflux. The failure to observe efflux is due to reaction of these reagents with the lac carrier protein itself. The difference between the effects of $N$-ethylmaleimide on $\beta$-galactoside transport and the fluorescence behavior of the dansylgalactosides indicates that binding changes observed do not reflect events that occur subsequent to transport of the dansylgalactosides into the vesicles.

**Dansylgalactoside Binding Induced by Imposition of Membrane Diffusion Potential (Positive Outside)—**As described above, binding of dansylgalactosides to the Lac carrier protein is induced by $\nu$-lactate oxidation. The experiment presented in Fig. 6A demonstrates that binding can also be induced by valinomycin-induced potassium efflux (i.e. under conditions in which a membrane potential (positive outside) is presumably imposed across...
the membrane). In the experiment shown, membrane vesicles prepared in 0.1 M potassium phosphate were diluted into a medium devoid of potassium containing 5 μM DG₄. On addition of valinomycin, a rapid increase in fluorescence is observed which reaches a maximum in less than 10 s, and then slowly decreases to the initial level if the experiment is carried out for a longer period of time (i.e. 10 to 15 min). When dilution is made into potassium-containing buffer, no change in fluorescence is observed on addition of valinomycin. Although not shown, addition of lactose before valinomycin practically abolishes the fluorescence change induced by valinomycin. Similarly, the uncoupler carbonyl cyanide m-chlorophenylhydrazone (5 μM) blocks the valinomycin effect. On the other hand, addition of 20 mM potassium cyanide causes only partial inhibition of DG₄ binding (approximately 15%) under these conditions. It should be stressed that this effect is not specific for valinomycin, since analogous results were obtained with monactin (in these experiments, potassium-loaded vesicles were diluted into choline phosphate).

Similar results are obtained by addition of sodium thiocyanate to the external medium (Fig. 6B). Since the thiocyanate anion is more permeable than the sodium cation, these conditions presumably induce a transient diffusion potential across the membrane (negative inside). As described in Fig. 6B, membrane vesicles prepared in 0.1 M sodium phosphate (pH 6.6) were diluted into medium containing 0.1 M sodium phosphate (pH 6.6) and 5 μM DG₄. On addition of 10 mM sodium thiocyanate, a rapid increase in fluorescence is observed which reaches a maximum in less than 5 s and then rapidly decreases to the initial level. As shown, the fluorescence increase is not observed when carbonyl cyanide m-chlorophenylhydrazone is added prior to sodium thiocyanate.

As opposed to the experiments described above, DG₄ binding is not caused by monactin-induced sodium efflux (vesicles prepared in 0.1 M sodium phosphate, pH 6.6, were diluted into choline phosphate, pH 6.6, and 1 to 10 μM monactin was added to initiate sodium efflux) nor by carbonyl cyanide m-chlorophenylhydrazide-induced proton efflux (vesicles prepared in 0.1 M potassium phosphate, pH 5.5, were diluted into potassium phosphate, pH 8.0, and 5 μM carbonyl cyanide m-chlorophenylhydrazide was added to initiate proton efflux).

The amount of DG₄ or DG₄ bound in response to potassium efflux depends upon the magnitude of the potassium gradient across the membrane. Thus, from the Scatchard plot shown in Fig. 7, it is apparent that the number of DG₄ binding sites exposed by a potassium gradient of about 150 (inside/outside) is approximately 0.28 nmol per mg of membrane protein. When the gradient is decreased by a factor of 10, the number of binding sites decreases to approximately 0.16 nmol per mg of membrane protein (Fig. 7), while increasing the potassium gradient to 600-fold increases the number of binding sites by approximately 2-fold (data not shown). It is also apparent from the data in Fig. 7 that the Kᵦ for DG₄ binding is independent of the magnitude of the potassium gradient, and is very similar to the Kᵦ observed with α-lactate-induced binding (i.e. 28 μM versus 31 M, respectively). On the other hand, the number of DG₄ binding sites generated by a 600-fold potassium gradient is not as great as that observed with α-lactate (i.e. 0.5 molecules versus 1.25 nmol per mg of membrane protein, respectively).

Dansylgalactoside Binding Induced by Lactose Efflux—Dansyl-galactoside binding can also be induced by carrier-mediated, passive lactose efflux (Fig. 8). In this experiment, ML 308-225 vesicles were loaded passively with 20 mM lactose, and subsequently diluted 100-fold into a cuvette containing DG₄. As shown, a transient increase in DG₄ fluorescence is observed, and the increase is absent when dilution is made into a medium...
n-Lactate. Scatchard (18). The results obtained with o-lactate (in the presence or absence of DNP or KCN) were obtained as described in Fig. 4. Results for valinomycin-induced binding were obtained as described in Fig. 7.

The apparent $K_D$ is 5 $\mu$M and the number of binding sites at infinite DG₄ concentration is 1.2 nmol per mg of membrane protein, values which are almost identical with those observed with d-lactate (Table III).

**DISCUSSION**

Fluorescent compounds that exhibit polarity-dependent fluorescence properties have been used as probes to study the structure of biological membranes (20). Two such compounds, 1-anilino-8-naphthalene sulfonate (21) and dansylphosphatidylethanolamine (22) have been used previously to study changes associated with transport in membrane vesicles of *E. coli*. However, the non-specificity of those probes limits the type of information that can be obtained. In this paper, fluorescent dansylgalactosides are used as specific probes for the $\beta$-galactoside transport system in membrane vesicles from *E. coli*.

Although the results obtained with the different analogues are qualitatively similar, quantitative differences are apparent. Strikingly, there appears to be a direct relationship between the length of the alkyl chain between the galactosidic and dansyl moieties of the molecules and their affinity for the lac carrier protein. Efforts are currently in progress to synthesize dansylgalactosides with increasingly longer alkyl chains between the galactosidic and dansyl moieties of the molecules. Presumably, at a particular length, the compound should behave as a competitive inhibitor of the $\beta$-galactoside transport system, but fail to exhibit the polarity shift in the emission spectrum or the energy transfer band in the excitation spectrum or both. This length should be related to the distance between the binding site in the carrier and the external solvent.

Although the affinity of the dansylgalactosides for the lac carrier protein increases with increasing length of the alkyl chain between the dansyl and galactosidic moieties of the molecules, none of the analogues tested is apparently transported. This observation is surprising and is not understood at the present time. Varying the chemical species mediating the galactosidic linkage (i.e. DG₂ versus oxy-DG₂) also does not make a discernable difference in this respect. In contrast to DG₂, oxy-DG₂, and DG₆, methylumbelliferyl-galactoside, a galactoside coupled to another heterocyclic system, is transported as well as lactose. Studies with other dansylated galactosides such as 2'- (N-dansyl)-aminoethyl-o-lactoside, the synthesis of which is currently in progress, may help to resolve this problem.

As shown by fluorescence titration (cf. Fig. 4, Table III, and Ref. 5), *E. coli* ML 308-225 membrane vesicles bind 1 to 2 nmol of each dansylgalactoside tested per mg of membrane protein in the presence of d-lactate, a value similar to that obtained from centrifugation experiments in which binding is assayed directly (Table I). Assuming that 1 dansylgalactoside molecule is bound per molecule of lac carrier protein and that the molecular weight of the lac carrier protein is approximately 30,000 (23), 1 to 2 nmol per mg of membrane protein is equivalent to 8 to 6% of the membrane protein. This number is if the same order of magnitude as that reported by Jones and Kennedy (23), as discussed previously (5). In this regard, a recent paper by Kennedy et al. (24) demonstrates binding of [3H]TDG in sonicated preparations from *E. coli* K-12. This binding is not in Fig. 8. The data were plotted as double reciprocal plots (i.e. 1/DG₄ bound versus 1/DG₄ added at each external lactose concentration), and the apparent $K_D$ was estimated from the point of intersection with the x-axis. This treatment was necessary because external lactose added during loading and dilution competes with DG₄ for binding sites.

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**TABLE IV**

*Binding constants and number of DG₄ binding sites under various conditions in ML 308-225 membrane vesicles*

<table>
<thead>
<tr>
<th>Condition</th>
<th>$K_D$ (pM)</th>
<th>Number of sites</th>
</tr>
</thead>
<tbody>
<tr>
<td>n-Lactate</td>
<td>31</td>
<td>1.25 nmol/mg membrane protein</td>
</tr>
<tr>
<td>K⁺ gradient ($K_{in}/K_{out} = 120$)</td>
<td>28</td>
<td>0.28</td>
</tr>
<tr>
<td>K⁺ gradient ($K_{in}/K_{out} = 15$)</td>
<td>26</td>
<td>0.16</td>
</tr>
<tr>
<td>n-Lactate + DNP, 67 μM</td>
<td>29</td>
<td>0.51</td>
</tr>
<tr>
<td>n-Lactate + KCN, 2 mM</td>
<td>21</td>
<td>0.48</td>
</tr>
</tbody>
</table>

* DNP, 2,4-dinitrophenol; KCN, potassium cyanide.
dependent apparently upon addition of an energy source. As pointed out by the authors, however, the binding obtained represents a small percentage of the total amount of lac carrier protein present in their preparations. Moreover, the preparations were not assayed for endogenous respiration.

There are at least three possible mechanisms by which energy might lead to dansylgalactoside binding to the lac carrier protein. These mechanisms are shown schematically in Fig. 9. In the first scheme (I), the carrier is accessible to the external medium, and binding occurs spontaneously. In this case, energy coupling results in partial translocation of the bound ligand, resulting in its exposure to the hydrophobic interior of the membrane, and thus to the fluorescence changes observed. This scheme appears to be ruled out by the polarization of fluorescence studies presented in Table II which provide a strong indication that binding to the lac carrier protein does not occur in the absence of energy coupling. The second scheme (II) suggests that the carrier is accessible to the external medium in the absence of energy coupling, but that its affinity is increased when energy is supplied. In the third scheme (III), the carrier is inaccessible to the external medium, and energy coupling causes the carrier to “move” to the external surface of the membrane where it can bind solute in the external medium. Although the latter two schemes are not mutually exclusive, the observation that carrier-mediated, passive lactose efflux results in binding of dansylgalactosides tends to favor the third scheme, as it is difficult to envisage how this process could increase the affinity of lac carrier protein on the external surface of the membrane (Scheme III).

With regard to induction of dansylgalactoside binding by ionophore-mediated potassium efflux or addition of sodium thiocyanate, a number of points should be discussed. Certainly the most simple explanation of these phenomena is that the conditions described result in the generation of a membrane potential (positive outside), and that the potential increases the accessibility of dansylgalactoside binding sites on the external surface of the vesicle membrane. If it is postulated moreover that the lac carrier protein has a negative charge, the appearance of binding sites on the exterior surface of the membrane could be easily explained. In Scheme III, for instance, if the carrier were negatively charged, imposition of a membrane potential (positive outside) would cause “movement” of the carrier to the external surface of the membrane and binding of dansylgalactoside. It should be emphasized, however, that if this is the case, the generation of the membrane potential by means of ion gradients should not be specific for potassium or thiocyanate. As demonstrated by Murer and Hopfer (25), for example, transport of glucose by membrane vesicles prepared from intestinal brush border can be induced by a membrane potential generated with proton gradients in addition to potassium or thiocyanate gradients. As discussed above, dansylgalactoside binding has been demonstrated with potassium and thiocyanate gradients only. An analogous situation exists with respect to lactose transport in this system. Although transport of lactose and other solutes can be induced by potassium efflux (26-29), transport is not observed with sodium or proton gradients in either direction.

Recent experiments have indicated that β-lactate oxidation by E. coli membrane vesicles also generates a membrane potential (positive outside) (26-28), and some of these observations have been corroborated and extended in this laboratory (2, 29). Thus, during β-lactate oxidation, lipophilic cations such as dimethylbenzylammonium (in the presence of tetraphenylboron) (26, 28, 30), safranine, and triphenylmethylphosphonium are accumulated. Moreover, there is a quantitative correlation between the uptake of rubidium in the presence of valinomycin and triphenylmethylphosphonium.

Although the interpretation of some of these data can be questioned (29), it is tempting to speculate that the basic mechanism of respiratory-dependent active transport, like potassium efflux-induced uptake, involves the generation of a membrane potential (positive outside) by means of proton extrusion or charge separation within the membrane or both. Either mechanism would then result in the “movement” of negatively charged carriers to the external surface of the membrane.

Insofar as the translocation and accumulation of lactose is concerned, the data presented here have little bearing on this aspect of the problem. Nonetheless, in view of the previous discussion, in order to translocate a negatively charged carrier back to the inner surface of the membrane in the presence of a membrane potential (positive outside), the charge on the carrier would have to be neutralized. This could be accomplished by the binding of protons as postulated by Mitchell (4) or other cations, or by shielding of the charge secondary to conformational changes induced by binding of ligand.

In the following paper (32), evidence is presented which is consistent with the proposal that energy is coupled to one of the initial steps in β-galactoside transport. These studies demonstrate that specific inactivation of the lactose transport system in E. coli membrane vesicles by 2-nitro-4-azidophenyl-b-D-thiogalactopyranoside, a photoaffinity label, is also dependent upon energy coupling.

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