Membrane-derived oligosaccharides, found in the *Escherichia coli* periplasmic space (Schulman, H., and Kennedy, E. P. (1979) *J. Bacteriol.* 137, 686-688), are composed of 8-10 units of glucose, the sole sugar, in \( \beta_{1-2} \) and \( \beta_{1-6} \) linkages (Schneider, J. E., Reinhold, V., Rumley, M. K., and Kennedy, E. P. (1979) *J. Biol. Chem.* 254, 10135-10138). Oligosaccharides in this family are variously substituted with succinyl ester residues, as well as with sn-1-phosphoglycerol and phosphoethanolamine, both derived from membrane phospholipids. These negatively charged oligosaccharides may function in cellular osmoregulation since their synthesis is under osmotic control (Kennedy, E. P. (1979) *Proc. Natl. Acad. Sci. U. S. A.* 79, 1092-1095).

We now report initial characterization of an enzyme catalyzing transfer of phosphoglycerol residues from phosphatidylglycerol to membrane-derived oligosaccharides or to synthetic \( \beta \)-glucoside acceptors. The products are sn-1,2-diglyceride and \( \beta \)-glucoside-6-phosphoglycerol. Localized in the inner membrane, the transferase has a requirement for divalent cations, of which manganese is most effective, and a pH optimum of 8.9 in vitro.

**MDO** are a family of compounds found in the periplasmic space of *Escherichia coli* and other Gram-negative bacteria (1). Glucose, the sole sugar, forms a branched backbone of 8-10 units joined by \( \beta_{1-2} \) and \( \beta_{1-6} \) linkages (2). The glucose backbones are substituted to varying degrees with lipid-derived sn-1-phosphoglycerol and phosphoethanolamine in phosphodiester linkage (3, 4), as well as with succinic acid in ester linkage (5).

The synthesis of MDO is regulated by the osmolarity of the medium (6). When osmolarity is low, MDO may constitute 7% of the cells' dry weight. Two proposed functions of MDO are maintenance of the high periplasmic osmolarity and, with succinyl ester residues, as acceptors for \( \beta \)-glucoside-6-phosphoglycerol residues transferred from phosphatidylglycerol by the membrane-bound transferase whose discovery is described here. A second, periplasmic phosphoglycerol transferase has been partially purified and characterized (8). It has been proposed that the periplasmic enzyme, which does not utilize phosphatidylglycerol as a source of phosphoglycerol residues, functions in the formation of multiply substituted soluble forms of MDO in the periplasmic compartment (8).

**MATERIALS AND METHODS**

**Bacterial Strains**—*E. coli* strains DP214 (zwf pgi) (10), LIN205 (glpA glpD glpR phoA) (11), and ABI (12) were grown at 37 °C with vigorous aeration on a rotary shaker.

**Preparation of Enzyme**—DP214 was grown in low osmolality medium (6) to mid-log phase. Subsequent steps were carried out at 0-4 °C. Cells harvested through centrifugation were washed once, resuspended in 0.05 M Tris-chloride buffer, pH 7.8, and disrupted at 1,100 atm in an Amino French press. After unbroken cells were removed by centrifugation at 3,200 \( \times \) g, membranes were pelleted at 100,000 \( \times \) g for 1 h. They were resuspended using a Dounce homogenizer in 0.05 M Tris-chloride buffer, pH 7.8, and frozen at -20 °C in several portions. Enzyme thawed more than twice was not used.

**Phosphoglycerol Transferase Assay**—The incubation mixture in a final volume of 0.2 ml contained 0.05 M Tris-chloride buffer, pH 8.8, 5.0 mM \( \beta \)-glucose, 1.0 mM 2-mercaptoethanol, 0.25 mM MnCl\(_2\), 1 mg/ml of sodium taurodeoxycholate, and 40 nmol of racemic phosphatidylglycerol (Sigma) plus *E. coli* sn-[2-\( ^3 \)H]glycerol-labeled phosphatidylglycerol to yield a specific activity of 2000-5000 cpm/nmol of racemic phosphatidylglycerol. The Sigma product is produced by transphosphatidylation of egg phosphatidyicholine, the principal fatty acids of which are 16:0, 18:0, 18:1, and 18:2. After addition of the membrane enzyme (usually about 0.1 mg of protein), the mixture was incubated for 2 h at 37 °C. The reaction was stopped by addition of 0.5 ml of NaOH (0.1 N). After 10 min at room temperature, 0.1 ml of bovine serum albumin (60 mg/ml) was added, immediately followed by 0.5 ml of trichloroacetic acid (0.8 M). After 10 min on ice, precipitates were removed in an Eppendorf centrifuge. A portion (1.0 ml) of each supernatant was shaken with 0.5 ml of a suspension of Norit A charcoal (60 mg/ml) for 15 min on an Eberbach reciprocating shaker. The charcoal was washed twice by centrifugation with 1.0-ml portions of water to remove unadsorbed radioactive substances. MDO and the synthetic \( \beta \)-glucosides employed in this work were strongly adsorbed onto charcoal under the conditions employed. The radioactive product formed by the enzymic transfer of phosphoglycerol from sn-[2-\( ^3 \)H]glycerol-labeled phosphatidylglycerol to the glucoside acceptors was then eluted by shaking the charcoal with 1.2 ml of benzyl alcohol-saturated water for 1 h or with 1.2 ml of 15% (v/v) aqueous pyridine. After centrifugation, radioactivity in a portion of the eluate was determined with a scintillation counter. For calculations, equal specific activities of the phosphatidylglycerol backbone and head group were assumed. The total amount of added racemic phosphatidylglycerol was used as the basis for the calculation of specific activity, although it is possible that only the naturally occurring phosphatidyl-sn-1-glycerol is the enzymically active stereosomer.

Recoveries measured for the radioactive 6-phosphoglycerol derivatives of various glucosides were as follows after elution with aqueous pyridine: o-nitrophenyl-\( \beta \)-glucoside, 79%; octyl-\( \beta \)-glucoside, 73%; amygdalin, 79%; and arbutin, 45%. With o-nitrophenyl-\( \beta \)-glucoside as the acceptor for tritiated phosphatidylglycerol, the specific activity of the acceptor was found to be 2000 cpm/nmol. The specific activity of phenyl-\( \beta \)-glucoside was found to be 580 cpm/nmol; 4-amino-\( \beta \)-glucoside, 1800 cpm/nmol; guaiaconic acid, 1400 cpm/nmol; and saligenin, 1100 cpm/nmol.
**A Membrane-bound Phosphoglycerol Transferase**

**Working model for biosynthesis of MDO.** From Goldberg et al. (8) with permission of the publishers. 

**PHOSPHATIDYLGLYCEROL**

**MEMBRANE TRANSFERASE**

**DIGLYCERIDE**

**PERIPLASMIC TRANSFERASE**

**HYDROLASE**

**WORKING MODEL FOR BIOSYNTHESIS OF MDO**

**Table I**

<table>
<thead>
<tr>
<th>MDO acceptor added</th>
<th>Total count recovered in MDO peak</th>
<th>mM</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>0.1</td>
<td>276</td>
<td></td>
</tr>
<tr>
<td>1.0</td>
<td>2338</td>
<td></td>
</tr>
<tr>
<td>2.0</td>
<td>4000</td>
<td></td>
</tr>
<tr>
<td>1.0 + 1 mM gentiobiose</td>
<td>1681</td>
<td></td>
</tr>
<tr>
<td>1.0 + 5 mM gentiobiose</td>
<td>828</td>
<td></td>
</tr>
</tbody>
</table>

**FIG. 1. Working model for biosynthesis of MDO.** From Goldberg et al. (8) with permission of the publishers. OGL, glyceral.

acceptor in the standard assay, replicate assays agreed within 5%. The reaction was linear with time from 20 min to at least 2.5 h, and linear with added membrane protein from 0-0.13 mg.

**Preparation of sn-[2-3H]Glycerol-labeled Phosphatidylglycerol—** A 10-ml mid-log culture of L12295 was labeled for 20 min with 1 ml of [2-3H]glycerol (New England Nuclear) 2.0 mCi/ml, 5-10 Ci/mmole. Total lipids were extracted (13) and chromatographed on a Brinkmann Silica Gel G plate in chloroform/methanol/acetic acid (65:25:8, v/v/v). The region of the plate expected to contain phosphatidylglycerol was scraped and extracted (13). Upon re-chromatography, the sn-[2-3H]glycerol-labeled phosphatidylglycerol mixed with carrier phosphatidylglycerol standard (Sigma) migrated as a radioactive iodine-stainable spot with $R_f = 0.57$.

**Synthesis of α-Nitrophenyl β-D-glucoside-6-sn-[3H]phosphoglycerol—** The synthesis of α-nitrophenyl β-D-glucoside-6-sn-[3H]phosphoglycerol from α-nitrophenyl β-D-glucoside and racemic sn-1(3),2-isopropylidenglycerophosphate was carried out by condensation in the presence of dicyclohexylcarbodiimide on thin layer plates. The principal product was characterized as the α-nitrophenyl β-D-glucoside-6-phosphoglycerol derivative by methods developed by Schneider and Kennedy (4) for the characterization of glucose-6-phosphoglycerol. Briefly, the glycerol moiety was removed from the product with the specific phosphodiesterase II from Aspergillus niger, followed by partial acid hydrolysis for the removal of α-nitrophenol. This procedure led to the formation of in good yield of glucose-6-phosphate, quantitatively determined with glucose-6-phosphate dehydrogenase.

Further details of the synthesis and characterization will be published elsewhere.

**Membrane Fractionation—** Separation of inner and outer membranes from AB1133 was carried out according to the procedure of Osborn and Munson (15). Phospholipids had been labeled by growth on [2-3H]glycerol. Phosphoglycerol transferase was assayed as described in the text. Phosphatidylether decarboxylase (16) was assayed as an inner membrane marker; phospholipase A (17) was assayed as an outer membrane marker. Density was determined with a Bausch and Lomb refractometer.

**RESULTS**

**Evidence for a Membrane-bound Phosphoglycerol Transferase—** Cells of strain DF214, labeled by growth on [2-3H]glycerol, were used as a source of labeled spheroplast membranes. The isolated membranes, containing endogenously labeled phosphatidylglycerol, were incubated with soluble MDO of the pre-A fraction, which contains a maximum of one phosphatidylglycerol residue/mol. The reaction was stopped by addition of chloroform/methanol (1:1, v/v), and a portion of the aqueous phase was chromatographed on G-25 to assess production of labeled MDO (1, 8). The results (Table I) revealed a transfer of label, presumably from endogenous phosphatidylglycerol, to MDO in a reaction that was almost linearly dependent upon the concentration of acceptor MDO. This low affinity for soluble MDO is consistent with the postulate that carrier-linked MDO (Fig. 1) is the physiological substrate for the membrane transferase.

Since β-gentiobiose and other relatively simple β-glucosides can be used as model substrates for the periplasmic phosphoglycerol transferase (8), the effect of added β-gentiobiose on the transfer of radioactivity to MDO from endogenously labeled membranes was tested; it was found to act as an effective competitor (Table I).

**Synthetic β-D-Glucosides as Model Substrates—** The experiment of Table I indicates that simple β-glucosides may serve as model substrates for the membrane-bound phosphoglycerol transferase. A number of glucosides were tested (Table II) in the assay described under “Materials and Methods” in which unlabeled membranes were used as the enzyme source with purified E. coli sn-[2-3H]glycerol-labeled phosphatidylglycerol as labeled substrate.

β-glucosides with aromatic aglycones proved very effective substrates (Table II). The enzyme is specific for β-glucosides as shown by comparison of α-nitrophenyl-α- and β-D-glucosides. MDO contain exclusively β-linkages.

α-Nitrophenyl-β-D-glucoside was employed as substrate in other experiments here because the synthetic 6-phosphoglycerol derivative was available.

**Optimum pH—** The pH dependence of phosphoglycerol transferase activity with 5 mM α-nitrophenyl-β-D-glucoside as acceptor was determined in buffers containing 50 mM Tris-glycine of variable pH. The actual pH of the incubation...
mixture was determined with a glass electrode. The pH optimum was 8.9, with 50% of optimal activity at pH 7.8 or 9.7. This highly alkaline optimum presumably reflects assay conditions rather than the pH at which the enzyme functions in vivo.

Effects of EDTA and Divalent Cations—If the membrane fraction was not treated with chelating agents, full activity was observed in the absence of added divalent cations. When the enzyme was treated with 0.1 mM EDTA for 1 h at 0 °C, activity in the subsequent assay was reduced to 40% of the control value. Activity of the EDTA-treated enzyme was fully restored by addition of 0.5 mM MnCl₂ to the assay system. The activity with 0.5 mM MgCl₂, the optimal concentration for that cation, was 67% that observed with the same concentration of MnCl₂. In earlier experiments employing [2-3H]glycerol-labeled membranes and omitting taurodeoxycholate, treatment of enzyme with a final concentration of 2.7 mM EDTA prior to assay abolished 90% of activity, relative to control. In terms of restoring activity, 0.25 mM divalent cation was effective in descending order: Mn²⁺, Mg²⁺, Ca²⁺.

Identification of sn-1,2-Diglyceride as a Product of the Reaction—The transfer of phosphoglycerol residues from phosphatidylglycerol should lead to the formation of sn-1,2-diglyceride as a lipid product. In the experiment shown in Fig. 2, a chloroform extract was prepared from the total lipids of a 0.4 ml incubation mixture. Authentic 1,2-dipalmitin, 1,3-diglyceride, dipalmitin, and phosphatidylglycerol (all from Sigma) were added as carriers. To remove most of the labeled phosphatidylglycerol, the chloroform extract was passed over a small column of silica gel. The neutral lipid fraction, obtained by elution with chloroform, was concentrated and analyzed by thin layer chromatography. Some residual polar lipid remained at the origin. A peak of radioactivity was recovered in the region containing sn-1,2-diglyceride, visualized in an adjacent track by ultraviolet light after spraying with 0.1% 2,7'-dichlorofluorescein in ethanol. A smaller amount of radioactivity (about 17% of total diglyceride count) was recovered from the region containing sn-1,3-diglyceride; The appearance of labeled diglyceride was almost completely dependent on the presence of added acceptor (Fig. 2, bottom). The small amount of diglyceride produced in the absence of added acceptor may result from activity of some unrelated enzyme or may reflect a limited hydrolytic activity of the membrane-bound phosphoglycerol transferase in the absence of acceptor.

Since the equilibrium constant for interconversion of diglyceride acyl isomers is 1 (18), the 1,3-isomer cannot be the primary product. Rather, a small amount of 1,3-diglyceride is generated by acyl migration, which occurs readily during chromatography (18). We conclude that sn-1,2-diglyceride is a product of the enzymic reaction of phosphatidylglycerol and o-nitrophenyl-β-D-glucoside.

Identification of the 6-Phosphoglycerol Derivative of o-Nitrophenyl-β-D-glucoside as a Product of the Reaction—The water-soluble radioactive product of the assay system, derived from sn-[2-3H]glycerol-labeled phosphatidylglycerol and o-nitrophenyl-β-D-glucoside, was eluted from charcoal with aqueous 20% pyridine following preparation of the substituted glucoside in the standard assay system. A total of 14,000 cpm so obtained was mixed with 9 μmol of synthetic o-nitrophenyl-β-D-glucoside-6-phosphoglycerol and chromatographed on a column (1 × 20 cm) of DEAE to which a gradient of 0–0.04 M LiCl in 25% 1-propanol was applied. All of the radioactivity emerged in a single sharp peak at 0.02 M LiCl, exactly coincident with the peak of synthetic material (determined as o-nitrophenol after alkaline hydrolysis).

DEAE-chromatography under the conditions described...
does not effectively separate the 6-phosphoglycerol from the 3- and 4-phosphoglycerol derivatives of o-nitrophenyl-β-D-glu-
coside, but these can be readily resolved by thin layer chromatography. Another sample of labeled product was therefore
analyzed as shown in Fig. 3. The radioactivity migrated as a single spot with an RF value of 0.16, indistinguishable from the
synthetic 6-phosphoglycerol derivative of o-nitrophenyl-β-D-glu-
coside (detected with ultraviolet light). In this solvent system, synthetic 3- and 4-phosphoglycerol derivatives migrate distinctly more rapidly, with RF values between 0.3 and 0.4. The radioactive product also migrates with authentic o-
nitrophenyl-β-D-glucoside-6-phosphoglycerol (RF = 0.11) on Silica Gel 60 F254 plates (EM Reagents) developed in 1-propanol, 1 N ammonium formate (9:2, v/v). Glucosides were identified as dark spots under ultraviolet light. Lanes were then cut and counted to localize the product of the enzymic reaction.

**Discussion**

Considerable evidence indicates that the membrane-bound phosphoglycerol transferase described here catalyzes an important step in the biosynthesis of MDO. Phosphatidylglycerol is the donor of sn-1-phosphoglycerol residues to MDO in vivo (9) and in vivo (19) and in the reaction described here. Phospho-
glycerol residues are attached to the 6-position of glucose units in MDO (4); the membrane-bound transferase similarly
transfers phosphoglycerol to the 6-position of glucose units in model substrates. MDO are composed of glucose units in β-
linkage (2), and the membrane-bound transferase greatly prefers β- to α-glucosides (Table II). The transferase is active
with soluble species of MDO (Table I) but the affinity for soluble MDO is low. This suggests that carrier-linked MDO
are the physiological substrates, although direct evidence on this point is lacking.

The membrane-bound transferase may be sharply distinguished from the soluble periplasmic transferase previously studied by Goldberg et al. (8). The periplasmic transferase exists in a different cellular compartment and cannot utilize phosphatidylglycerol as a phosphoglycerol donor. Readily interchanging phosphoglycerol residues between species of MDO, the periplasmic enzyme is thought to catalyze the multiple substitution of soluble MDO with phosphoglycerol residues from carrier-linked MDO, proposed substrate for the membrane-bound enzyme (Fig. 1).

The following evidence indicates that the active site of the transferase is oriented on the outer aspect of the inner mem-
brane. Table II indicates this enzyme readily transfers phos-
phosphoglycerol to the β-glucoside arbutin. Intact cells of strain CA 198 (galU) growing in medium supplemented with arbutin also transfer phosphoglycerol residues to the exogenous β-glucoside, which is not taken up by K12 cells in the absence of a specific mutation (20). This galU strain cannot make UDP-glucose, and hence cannot make MDO (9). Because the strain lacks MDO, phosphoglycerol residues on MDO cannot be the source of the phosphoglycerol residues transferred to arbutin. The membrane transferase, and not the periplasmic enzyme, is therefore presumed to transfer phosphoglycerol from phosphatidylglycerol to arbutin in vivo. Since the membrane enzyme is localized on the inner cytoplasmic membrane, its working site must be oriented on the outer aspect of the inner membrane, where the exogenous β-glucoside is available.

The production of sn-1,2-diglyceride catalyzed by the transferase studied here sheds light on the observations of Raetz and Newman (19) who studied the accumulation of diglyceride in a mutant with defective diglyceride kinase. Accumulation of diglyceride was maximal and growth of the mutant was slowest in medium of low osmolarity. It has since been shown that MDO synthesis, and therefore production of diglyceride from phosphatidylglycerol catalyzed by the membrane transferase, occurs at highest rates in medium of lowest osmolarity (6).

Acknowledgments—We thank Marilyn K. Rumley for the data in Table I and Dr. Jean-Pierre Bohin for permission to cite unpublished data. We also thank Phillip Temples for typing the manuscript.

Jean-Pierre Bohin, unpublished results.
The biosynthesis of membrane-derived oligosaccharides. A membrane-bound phosphoglycerol transferase.
B J Jackson and E P Kennedy