

## In Vitro Synthesis of a Lipid-Linked Trisaccharide Involved in Synthesis of Enterobacterial Common Antigen

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The heteropolysaccharide chains of enterobacterial common antigen (ECA) are made up of linear trisaccharide repeat units with the structure  $\rightarrow 3$ - $\alpha$ -D-Fuc4NAc-(1  $\rightarrow$  4)- $\beta$ -D-ManNAcA-(1  $\rightarrow$  4)- $\alpha$ -D-GlcNAc-(1  $\rightarrow$ ), where Fuc4NAc is 4-acetamido-4,6-dideoxy-D-galactose, ManNAcA is N-acetyl-D-mannosaminuronic acid, and GlcNAc is N-acetyl-D-glucosamine. The assembly of these chains involves lipid-linked intermediates, and both GlcNAc-pyrophosphorylundecaprenol (lipid I) and ManNAcA-GlcNAc-pyrophosphorylundecaprenol (lipid II) are intermediates in ECA biosynthesis. In this study we demonstrated that lipid II serves as the acceptor of Fuc4NAc residues in the assembly of the trisaccharide repeat unit of ECA chains. Incubation of *Escherichia coli* membranes with UDP-GlcNAc, UDP-[<sup>14</sup>C]ManNAcA, and TDP-[<sup>3</sup>H]Fuc4NAc resulted in the synthesis of a radioactive glycolipid (lipid III) that contained both [<sup>14</sup>C]ManNAcA and [<sup>3</sup>H]Fuc4NAc. The oligosaccharide moiety of lipid III was identified as a trisaccharide by gel-permeation chromatography, and the in vitro synthesis of lipid III was dependent on prior synthesis of lipids I and II. Accordingly, the incorporation of [<sup>3</sup>H]Fuc4NAc into lipid III from the donor TDP-[<sup>3</sup>H]Fuc4NAc was dependent on the presence of both UDP-GlcNAc and UDP-ManNAcA in the reaction mixtures. In addition, the in vitro synthesis of lipid III was abolished by tunicamycin. Direct conversion of lipid II to lipid III was demonstrated in two-stage reactions in which membranes were initially incubated with UDP-GlcNAc and UDP-[<sup>14</sup>C]ManNAcA to allow the synthesis of radioactive lipid II. Subsequent addition of TDP-Fuc4NAc to the washed membranes resulted in almost complete conversion of radioactive lipid II to lipid III. The in vitro synthesis of lipid III was also accompanied by the apparent utilization of this lipid intermediate for the assembly of ECA heteropolysaccharide chains. Incubation of membranes with UDP-[<sup>3</sup>H]GlcNAc, UDP-ManNAcA, and TDP-Fuc4NAc resulted in the apparent incorporation of isotope into ECA polymers, as determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and fluorography. In addition, the in vitro incorporation of [<sup>3</sup>H]Fuc4NAc into ECA heteropolysaccharide chains was demonstrated with ether-treated cells that were prepared from  $\Delta rfbA$  mutants of *Salmonella typhimurium*. These mutants are defective in the synthesis of TDP-Fuc4NAc; as a consequence, they are also defective in the synthesis of lipid III and they accumulate lipid II. Accordingly, incubation of ether-permeabilized cells of  $\Delta rfbA$  mutants with TDP-[<sup>3</sup>H]Fuc4NAc resulted in the incorporation of isotope into both lipid III and ECA heteropolysaccharide chains.

Enterobacterial common antigen (ECA) is a cell surface glycolipid that is possessed by all members of the family *Enterobacteriaceae* (12, 15, 18). The carbohydrate portion of ECA consists of linear heteropolysaccharide chains that are made up of N-acetyl-D-glucosamine (GlcNAc), N-acetyl-D-mannosaminuronic acid (ManNAcA), and 4-acetamido-4,6-dideoxy-D-galactose (Fuc4NAc) (10). These amino sugars are linked, forming trisaccharide repeat units with the structure  $\rightarrow 3$ - $\alpha$ -D-Fuc4NAc-(1  $\rightarrow$  4)- $\beta$ -D-ManNAcA-(1  $\rightarrow$  4)- $\alpha$ -D-GlcNAc-(1  $\rightarrow$ ) (10). The heteropolysaccharide chains are linked to a phospholipid aglycone which presumably serves to anchor the polymer in the outer membrane. In addition to phospholipid-linked heteropolysaccharide chains, certain rough mutants also possess ECA heteropolysaccharide chains that are covalently linked to the core region of lipopolysaccharide (15).

Previous studies demonstrated that GlcNAc-pyrophosphorylundecaprenol (lipid I) and ManNAcA-GlcNAc-pyrophosphorylundecaprenol (lipid II) are intermediates in ECA synthesis, and the in vitro synthesis of these intermediates has been studied in detail (1, 22). In this study we demonstrate that membranes from *Escherichia coli* possess transferase activity that catalyzes the transfer of Fuc4NAc from TDP-Fuc4NAc to lipid II to yield Fuc4NAc-ManNAcA-

GlcNAc-pyrophosphorylundecaprenol (lipid III). Data are also presented which indicate that lipid III is an intermediate in the process involved in the assembly of ECA heteropolysaccharide chains.

### MATERIALS AND METHODS

**Bacterial strains and growth conditions.** Experiments were done with *E. coli* F1312 O8:K27<sup>-</sup> *his pro pyr met rha* Str<sup>r</sup> (27), *Salmonella typhimurium* PR122 *hisF1009 trpB2 metA22 xyl-1 rpsL galE nagB F<sup>-</sup>* (23), *S. typhimurium* SH5150 *rff4720 ivl-1190 his-809* (extended deletion in the *his-rfb* region) (13, 22), and *S. typhimurium* SH5150-2 (*ilv<sup>+</sup> rff<sup>+</sup>* transconjugant from a cross between HfrK1-2 and SH5150) (22). *Pseudomonas aeruginosa* 7700 was obtained from the American Type Culture Collection (Rockville, Md.). Cultures of *E. coli* and *S. typhimurium* were grown with vigorous aeration in Proteose Peptone (Difco Laboratories, Detroit, Mich.)-beef extract medium (25) containing 0.2% glucose or L-medium containing 0.2% glucose (2), as indicated. Cultures of *P. aeruginosa* were grown in 3% glycerol plus salts medium (3).

**Radiochemicals, chemicals, and reagents.** UDP-N-acetyl-D-[6-<sup>3</sup>H]glucosamine (UDP-[<sup>3</sup>H]GlcNAc; 20.4 Ci/mmol), UDP-N-acetyl-D-[U-<sup>14</sup>C]glucosamine (UDP-[<sup>14</sup>C]GlcNAc; 283 mCi/mmol), D-[1-<sup>3</sup>H]glucose ([<sup>3</sup>H]glucose; 15.5 Ci/mmol), and En<sup>3</sup>Hance were purchased from Dupont, NEN

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Research Products (Boston, Mass.). *N*-Acetyl-D-[<sup>3</sup>H]glucosamine ([<sup>3</sup>H]GlcNAc; 1.7 Ci/mmol) was purchased from Amersham Corp. (Arlington Heights, Ill.). UDP-*N*-acetyl-D-[<sup>14</sup>C]mannosaminuronic acid (UDP-[<sup>14</sup>C]ManNAcA) and UDP-ManNAcA were synthesized enzymatically from UDP-[<sup>14</sup>C]GlcNAc and UDP-GlcNAc, respectively, as described previously (1). D-[<sup>3</sup>H]glucose-6-phosphate was synthesized from [<sup>3</sup>H]glucose by using yeast hexokinase. TDP-D-[<sup>3</sup>H]glucose (TDP-[<sup>3</sup>H]glucose) was synthesized enzymatically from [<sup>3</sup>H]glucose-6-phosphate by using partially purified TDP-glucose pyrophosphorylase prepared from extracts of *P. aeruginosa* 7700, as described by Kornfeld and Glaser (6). TDP-4-acetamido-4,6-dideoxy-D-[<sup>3</sup>H]galactose (TDP-[<sup>3</sup>H]Fuc4NAc) and TDP-Fuc4NAc were synthesized from TDP-[<sup>3</sup>H]glucose and TDP-glucose, respectively, as described by Matsushashi and Strominger (14). Tunicamycin (mixture of isomers A, B, C, and D) was purchased from Sigma Chemical Co. (St. Louis, Mo.). Stock solutions of tunicamycin were prepared by dissolving the drug in 25 mM NaOH to give a final concentration of 0.4 mg/ml. Bio-Solv BBS-3 and Liquifluor were purchased from Beckman Instruments, Inc. (Fullerton, Calif.). All other chemicals and reagents were purchased from standard commercial sources.

**Reaction mixtures and assay procedures.** Cell envelopes were prepared as described previously (1). Reaction mixtures for the synthesis of lipid III included the following in a final volume of 55  $\mu$ l: 50 mM Tris hydrochloride (pH 8.2), 30 mM MgCl<sub>2</sub>, 5 mM 2-mercaptoethanol, 0.16 U of bacterial alkaline phosphatase, 5  $\mu$ M UDP-GlcNAc, 5  $\mu$ M UDP-ManNAcA, 5  $\mu$ M TDP-Fuc4NAc, and cell envelope membranes (200 to 250  $\mu$ g of protein). TDP-[<sup>3</sup>H]Fuc4NAc (2.56  $\times$  10<sup>4</sup> dpm), UDP-[<sup>14</sup>C]ManNAcA (8.5  $\times$  10<sup>3</sup> dpm), or both were added to reaction mixtures, where indicated, in place of the corresponding unlabeled nucleotide sugars. In addition, tunicamycin was added to reaction mixtures, where indicated, to give a final concentration of 10  $\mu$ g/ml. Reactions were incubated at 37°C for 30 min and then terminated by the addition of 1.0 ml of chloroform-methanol (3:2, by volume). The radioactive products were extracted from reaction mixtures as described previously (1), and they were either analyzed directly by ascending paper chromatography with SG-81 filter paper (Whatman, Inc., Clifton, N.J.) or partially purified by DEAE-cellulose chromatography as described below. Alternatively, the extracted products were treated directly with mild acid, and the radioactive oligosaccharides released from the glycolipids were analyzed as described below.

Two-stage reactions for the conversion of [<sup>14</sup>C]ManNAcA-containing lipid II to lipid III were carried out as follows. Cell envelope membranes (1.5 to 1.8 mg of protein) were initially incubated for 30 min at 37°C with 50 mM Tris hydrochloride (pH 8.2)–30 mM MgCl<sub>2</sub>–5 mM 2-mercaptoethanol–0.96 U of bacterial alkaline phosphatase–5  $\mu$ M UDP-GlcNAc–UDP-[<sup>14</sup>C]ManNAcA (8.5  $\times$  10<sup>3</sup> dpm) in a final volume of 312  $\mu$ l. Following the incubation, 0.2 ml of cold 15 mM Tris hydrochloride (pH 8.0) was added, and the membranes were pelleted by centrifugation (45 min, 160,000  $\times$  g, 4°C). The pellet was washed with the same buffer and suspended in 50 mM Tris hydrochloride (pH 8.2)–30 mM MgCl<sub>2</sub>–5 mM 2-mercaptoethanol–0.96 U of bacterial alkaline phosphatase in a final volume of 282  $\mu$ l. The second stage of the reaction was initiated by the addition of TDP-Fuc4NAc to the membrane suspension to give a final concentration of 6  $\mu$ M, and the reaction mixture was incubated at 37°C. Portions (50  $\mu$ l) were removed just prior to the addition of

TDP-Fuc4NAc and at various times following the addition of TDP-Fuc4NAc, and they were added to 1 ml of chloroform-methanol (3:2, by volume). The radioactive lipid-linked products were extracted as described above, and the amount of radioactivity in lipid II and lipid III oligosaccharides was determined following their release with mild acid (see below).

Reaction mixtures for the *in vitro* synthesis of [<sup>3</sup>H]GlcNAc-containing ECA polymers were the same as those described above for the synthesis of lipid III, with the exception of UDP-[<sup>3</sup>H]GlcNAc (2  $\times$  10<sup>5</sup> dpm) was added together with UDP-ManNAcA and TDP-Fuc4NAc. Incubations were carried out at 37°C for 2 h, and the reaction mixtures were subsequently diluted by adding 0.1 ml of cold 15 mM Tris hydrochloride (pH 8.0). Five reaction mixtures were pooled, and the membranes were pelleted by centrifugation (2 h, 160,000  $\times$  g, 4°C). The pellet was solubilized by boiling it for 5 min in 150  $\mu$ l of sample buffer consisting of 0.5 M Tris hydrochloride (pH 6.8), 10% glycerol, 2% sodium dodecyl sulfate (SDS), and 5 mM EDTA. Insoluble material was removed by centrifugation, and bromophenol blue was added to the supernatant to give a final concentration of 0.004%. The samples were analyzed by SDS-polyacrylamide gel electrophoresis (PAGE) in 12% gels by the method of Laemmli (8) and analyzed by fluorography with En<sup>3</sup>Hance (20).

**Ether treatment.** Cultures of *S. typhimurium* SH5150-2 and SH5150 were grown with vigorous aeration in L-medium (200 ml) containing 0.2% glucose at 37°C to an A<sub>600</sub> of 0.5 to 0.6. The cells were then permeabilized with ether by previously described methods (16, 30). Briefly, the cells were diluted immediately with 80 ml of cold basic medium (40 mM Tris hydrochloride [pH 7.4], 80 mM KCl, 7 mM MgCl<sub>2</sub>, 2 mM EGTA [ethylene glycol-bis( $\beta$ -aminoethyl ether)-*N*, *N*, *N'*, *N'*-tetraacetic acid], 0.4 mM spermidine hydrochloride 0.5 M sucrose) and harvested by centrifugation (7,000  $\times$  g, 15 min, 4°C). The pellet was suspended in 2 ml of basic medium, to which was added 2 ml of diethyl ether. The cells were gently agitated, and the phases were allowed to separate at room temperature. The ether phase was discarded and the treated cells were harvested by centrifugation (8,500  $\times$  g, 10 min, 4°C), washed once with basic medium, and suspended in basic medium to give a protein concentration of 10 mg/ml. The suspensions were stored until use at –20°C.

**Release of oligosaccharides by treatment with mild acid.** Radioactive glycolipids were incubated with 0.1 N HCl at 100°C for 5 min. The hydrolysates were neutralized with 0.5 N NaOH and subsequently incubated at room temperature with snake venom phosphodiesterase (0.4 U) and bacterial alkaline phosphatase (0.4 U) for 2 h. The samples were then spotted onto Whatman no. 3 filter paper and analyzed by paper electrophoresis as described below.

**Analysis of lipid III oligosaccharide by gel-permeation chromatography.** [<sup>3</sup>H]Fuc4NAc-containing lipid III was synthesized as described above, and the radioactive glycolipid was partially purified by DEAE-cellulose chromatography. The partially purified lipid III was incubated with 0.1 N HCl at 100°C for 15 min, and the hydrolysate was dried under vacuum over NaOH pellets and P<sub>2</sub>O<sub>5</sub>. The water-soluble fraction (2  $\times$  10<sup>4</sup> dpm) was analyzed by gel-permeation chromatography by using Bio-Gel P2, as described below.

**Chromatographic and electrophoretic procedures.** Paper chromatography was carried out with SG-81 filter paper (Whatman), which was prepared as described previously (2). Samples were spotted onto the filter paper, and the chro-

matogram was developed with chloroform-methanol-water-concentrated ammonium hydroxide (88:48:10:1, by volume). Lanes of the dried chromatogram were then cut into 1-cm-wide sections, and the sections were soaked in 0.5 ml of 1.25% SDS for 6 to 8 h at 42°C. The samples were then analyzed for radioactivity by liquid scintillation counting.

Paper electrophoresis was carried out on Whatman no. 3 filter paper by using pyridine-acetic acid-water (1:10:289, by volume; pH 3.5). Electrophoresis was conducted at 50 V/cm with cooling by using a flat-plate electrophoresis apparatus (Savant).

DEAE cellulose (acetate form; DE-52; Whatman) was prepared as described by Rouser et al. (26). The poured columns were washed thoroughly with chloroform-methanol-water (2:3:1, by volume) and were then washed with methanol prior to sample application. Following sample application, columns were washed with one bed volume each of methanol and chloroform-methanol-water (2:3:1, by volume), respectively. Columns were eluted with a linear gradient of ammonium acetate (0 to 0.15 M) in chloroform-methanol-water (2:3:1, by volume). The fractions composing the major peak of radioactive material eluting with approximately 85 mM ammonium acetate were pooled, desalted by extraction with water (0.2 volume), and dried by rotary evaporation at room temperature.

Gel-permeation chromatography was carried out by using Bio-Gel P2 (Bio-Rad Laboratories, Richmond, Calif.). Columns (1.5 by 90 cm) were preequilibrated with 25 mM acetic acid containing 1 mM EDTA, and they were developed with the same solvent.

**Counting procedures.** Counting procedures were performed as described previously (21).

## RESULTS

**In vitro synthesis of lipid III.** Incubation of membranes from strain F1312 with UDP-GlcNAc, UDP-[<sup>14</sup>C]ManNAcA, and TDP-[<sup>3</sup>H]Fuc4NAc resulted in the incorporation of [<sup>14</sup>C]ManNAcA and [<sup>3</sup>H]Fuc4NAc into lipid-linked material that migrated as a single component when analyzed by both DEAE-cellulose chromatography and ascending chromatography on silica gel-impregnated paper (data not shown). The identification of the [<sup>14</sup>C]ManNAcA- and [<sup>3</sup>H]Fuc4NAc-labeled lipid as an apparent single component was also indicated when reaction mixtures were analyzed by thin-layer chromatography, which was performed with a variety of solvent systems (data not shown). However, interpretation of these data was hampered by the fact that the radioactive product migrated with the same chromatographic mobility as did authentic lipid II regardless of the chromatographic system used. Accordingly, analysis of the intact lipid by these methods precluded differentiation between [<sup>14</sup>C]ManNAcA-labeled lipid II and the presumed next intermediate in the pathway, lipid III, resulting from the transfer of [<sup>3</sup>H]Fuc4NAc to [<sup>14</sup>C]ManNAcA-labeled lipid II. Thus, the lipid-linked products of the reaction were subjected to mild acid hydrolysis, and the oligosaccharides released into the water-soluble fraction were analyzed by paper electrophoresis and gel permeation chromatography.

Treatment of the [<sup>3</sup>H]Fuc4NAc- and [<sup>14</sup>C]ManNAcA-containing lipid fraction with mild acid released two radioactive oligosaccharides, as determined by paper electrophoresis at pH 3.5 (Fig. 1). The minor radioactive component appeared to be labeled with only [<sup>14</sup>C]ManNAcA and was identified as the lipid II disaccharide ManNAcA-GlcNAc based on a comparison of its electrophoretic mobility with

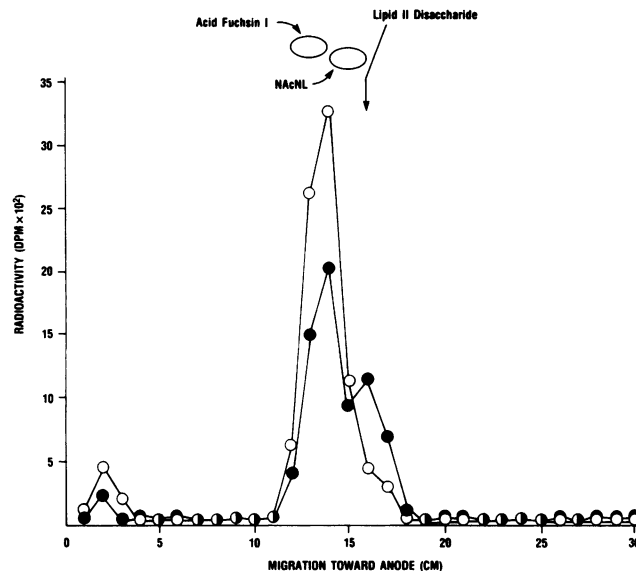


FIG. 1. Paper electrophoresis of the [<sup>14</sup>C]ManNAcA- and [<sup>3</sup>H]Fuc4NAc-containing oligosaccharide components of glycolipids synthesized in vitro. Cell envelope membranes were incubated with UDP-GlcNAc, UDP-[<sup>14</sup>C]ManNAcA, and TDP-[<sup>3</sup>H]Fuc4NAc. Radioactive products that were extracted into chloroform-methanol (3:2, by volume) were analyzed by DEAE-cellulose chromatography. Material that eluted with 85 mM ammonium acetate as a single peak of radioactivity containing both <sup>14</sup>C and <sup>3</sup>H was incubated with 0.1 N HCl for 5 min at 100°C. The water-soluble radioactive products were analyzed by paper electrophoresis at pH 3.5 along with standards of acid fuchsin I, N-acetyl-neuramin-lactose (NAcNL), and authentic lipid II disaccharide. Additional details are provided in the text. The mobility of acid fuchsin I denotes that of the acid fuchsin component with the least electrophoretic mobility under the conditions that were used. Symbols: ●, <sup>14</sup>C; ○, <sup>3</sup>H.

that of an authentic ManNAcA-GlcNAc standard. In contrast, the major radioactive component contained both [<sup>14</sup>C]ManNAcA and [<sup>3</sup>H]Fuc4NAc, and its electrophoretic mobility was decreased relative to that of the lipid II disaccharide, as was expected for a trisaccharide composed of Fuc4NAc, ManNAcA, and GlcNAc. In addition, treatment of [<sup>3</sup>H]Fuc4NAc-labeled lipid with mild acid followed by Bio-Gel P2 gel permeation chromatography of the water-soluble radioactive component revealed that essentially all of the radioactivity coeluted with the trisaccharide chitotriose (Fig. 2). Additional minor components eluted in the region of chitobiose and GlcNAc; these most likely represented degradation products that were generated from the [<sup>3</sup>H]Fuc4NAc-labeled oligosaccharide during acid hydrolysis. Accordingly, the Fuc4NAc-containing oligosaccharide was tentatively identified as Fuc4NAc-ManNAcA-GlcNAc.

**Precursor-product relationship between lipid II and lipid III.** In previous studies (2) it has been demonstrated that incubation of *E. coli* membranes with UDP-[<sup>14</sup>C]ManNAcA and UDP-GlcNAc results in the synthesis of [<sup>14</sup>C]ManNAcA-labeled lipid II as the only radioactive product. The data presented above suggest that the addition of TDP-Fuc4NAc to reaction mixtures containing UDP-ManNAcA and UDP-GlcNAc results in the transfer of Fuc4NAc to the nonreducing terminal ManNAcA residue of lipid II to yield lipid III. Evidence in support of this conclusion was obtained by demonstrating that optimal synthesis of [<sup>3</sup>H]Fuc4NAc-labeled lipid III was dependent on the presence of both UDP-GlcNAc and UDP-ManNAcA in reaction mixtures

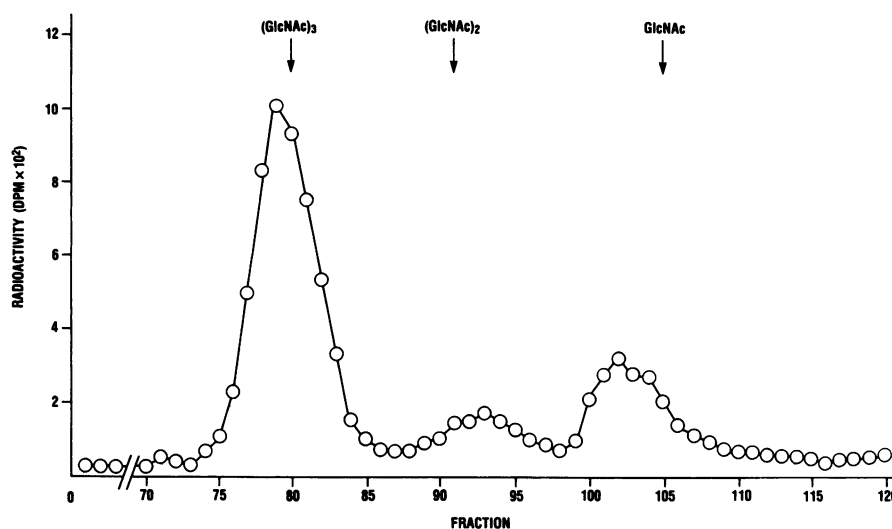


FIG. 2. Bio-Gel P2 chromatography of the [ $^3\text{H}$ ]Fuc4NAc-containing oligosaccharide released from lipid III by treatment with mild acid. [ $^3\text{H}$ ]Fuc4NAc-containing lipid III was treated with 0.1 N HCl at 100°C for 15 min. A portion of the water-soluble fraction (20,000 dpm), together with unlabeled standards of GlcNAc, chitobiose [(GlcNAc) $_2$ ], and chitotriose [(GlcNAc) $_3$ ] was applied to the bed of a Bio-Gel P2 column and eluted with 25 mM acetic acid containing 1 mM EDTA. Fractions of 1 ml were collected, and the radioactivity in 0.5 ml of each fraction was determined. The location of standards was determined by assaying 0.2 ml of each fraction by the ferricyanide method of Park and Johnson (17). Additional details are provided in the text.

(Table 1). In addition, lipid III synthesis was abolished by the addition of tunicamycin to reaction mixtures. Tunicamycin specifically inhibits the enzymatically catalyzed transfer of GlcNAc-1-phosphate from UDP-GlcNAc to polyprenyl monophosphate acceptors (4, 11, 28, 29). Thus, lipid III synthesis was precluded because of the inhibition of lipid I synthesis by the drug (1, 19). A low level of lipid III synthesis was observed even when UDP-GlcNAc, UDP-ManNAcA, or both were not added to reaction mixtures. It seems likely that the residual synthesis of lipid III observed under these conditions was caused by the presence of small amounts of endogenous UDP-GlcNAc and UDP-ManNAcA in membrane preparations.

Additional evidence for a precursor-product relationship between lipid II and lipid III was established by using two-stage reactions to demonstrate the conversion of lipid II to lipid III. Membranes were initially incubated with UDP-

GlcNAc and UDP-[ $^{14}\text{C}$ ]ManNAcA to allow the synthesis of [ $^{14}\text{C}$ ]ManNAcA-labeled lipid II. The membranes were then washed free of nucleotide-sugar substrates and subsequently incubated with TDP-Fuc4NAc. The amount of radioactivity in lipid II and lipid III was then determined at various times following the addition of TDP-Fuc4NAc by determining the amount of radioactivity in lipid II disaccharide and lipid III trisaccharide, respectively, following treatment of the lipid intermediate with mild acid. The addition of TDP-Fuc4NAc to reaction mixtures resulted in the conversion of lipid II to lipid III (Fig. 3). Accordingly, the amount of [ $^{14}\text{C}$ ]ManNAcA-labeled lipid II disaccharide decreased 80%, and the increase in radioactive lipid III trisaccharide (700 dpm) was in good agreement with the loss of radioactivity from the

TABLE 1. In vitro synthesis of [ $^3\text{H}$ ]Fuc4NAc-containing lipid III<sup>a</sup>

Addition	Radioactivity (dpm) incorporated into lipid III <sup>b</sup>
UDP-GlcNAc, UDP-ManNAcA .....	3,325
UDP-GlcNAc, UDP-ManNAcA, tunicamycin ....	88
UDP-GlcNAc .....	686
UDP-ManNAcA .....	405
None.....	214
	188

<sup>a</sup> Cell envelope membranes were incubated with TDP-[ $^3\text{H}$ ]Fuc4NAc in the presence or absence of UDP-GlcNAc, UDP-ManNAcA, and tunicamycin, as indicated. The amount of radioactivity incorporated into lipid III was subsequently determined by ascending chromatography with SG-81 filter paper. Experimental details are provided in the text.

<sup>b</sup> Values are the results obtained from two assays.

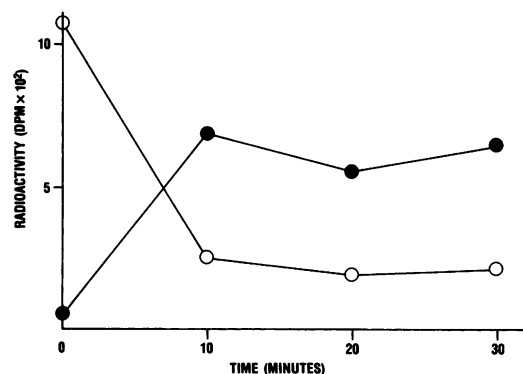


FIG. 3. Conversion of [ $^{14}\text{C}$ ]ManNAcA-containing lipid II to lipid III. Cell envelope membranes were incubated with UDP-GlcNAc and UDP-[ $^{14}\text{C}$ ]ManNAcA to allow synthesis of [ $^{14}\text{C}$ ]ManNAcA-containing lipid II. The membranes were then washed to remove nucleotide sugar substrates and subsequently incubated with TDP-Fuc4NAc. Portions of the reaction mixture were removed at the indicated times, and the amount of isotope in lipid II and lipid III oligosaccharides were determined following their release with mild acid. Additional details are provided in the text. Symbols: ●, lipid III trisaccharide; ○, lipid II disaccharide.



FIG. 4. In vitro incorporation of [ $^3\text{H}$ ]GlcNAc into ECA heteropolysaccharide chains. Cell envelope membranes were incubated with UDP-[ $^3\text{H}$ ]GlcNAc, UDP-ManNAcA, and TDP-Fuc4NAc (complete system). Membranes were also incubated in the complete system that lacked either UDP-ManNAcA or TDP-Fuc4NAc or that contained tunicamycin (10  $\mu\text{g/ml}$ ), as indicated below. The incorporation of radioactivity into ECA heteropolysaccharide chains was then determined by SDS-PAGE and fluorography, as described in the text. In addition, cultures of *S. typhimurium* PR122 were incubated with [ $^3\text{H}$ ]GlcNAc in the presence or absence of tunicamycin (10  $\mu\text{g/ml}$ ), and the incorporation of radioactivity into ECA was determined by SDS-PAGE and fluorography as described previously (19, 22). Lanes: 1, PR122; 2, PR122 plus tunicamycin; 3, complete system; 4, complete system minus UDP-ManNAcA; 5, complete system minus TDP-Fuc4NAc; 6, complete system plus tunicamycin.

lipid II disaccharide (825 dpm). In addition, the loss of radioactivity from lipid II disaccharide and the appearance of isotope in lipid III trisaccharide occurred with the same kinetics; maximal conversion of lipid II to lipid III was complete within 10 min after the addition of TDP-Fuc4NAc.

**In vitro synthesis of ECA heteropolysaccharide chains.** The data described above suggest that lipid III is involved in the biosynthesis of ECA. Thus, experiments were conducted in order to demonstrate that lipid III is an intermediate in the assembly of ECA heteropolysaccharide chains. Membranes were incubated with UDP-[ $^3\text{H}$ ]GlcNAc, UDP-ManNAcA, and TDP-Fuc4NAc; the radioactive material that was associated with the membranes was subsequently analyzed by SDS-PAGE and fluorography in an attempt to detect isotopically labeled ECA polymers. Similar experiments were also carried out in which membranes were incubated with UDP-[ $^3\text{H}$ ]GlcNAc without added UDP-ManNAcA, TDP-Fuc4NAc, or both.

Radioactivity from [ $^3\text{H}$ ]GlcNAc was incorporated into two major polymeric species as well as into faster-moving material that migrated closer to the bottom of the gel (Fig. 4). Although the identity of the faster-moving radioactive components has not been established, the major radioactive species are most likely the UDP-[ $^3\text{H}$ ]GlcNAc that was not completely removed from the membranes and a previously noted unidentified [ $^3\text{H}$ ]GlcNAc-containing component(s) that was unrelated to ECA (1). Separate experiments also revealed that lipid III migrated to the bottom of the gel when

the same conditions for analysis by SDS-PAGE were used (data not shown). The electrophoretic mobilities of the [ $^3\text{H}$ ]GlcNAc-containing ECA polymers indicated that their apparent degrees of polymerization approximated those of the major species of ECA chains synthesized *in vivo*. The *in vitro* synthesis of the polymers was dependent on the addition of both TDP-Fuc4NAc and UDP-ManNAcA to the reaction mixtures. In addition, the *in vitro* incorporation of [ $^3\text{H}$ ]GlcNAc into the polymers was abolished by tunicamycin, in agreement with both the demonstrated ability of the drug to preclude synthesis of lipid III (Table 1) and the previously established effect of tunicamycin on the *in vivo* synthesis of ECA (19, 22).

Additional support for the involvement of lipid III in the assembly of ECA chains was provided by *in vitro* experiments which revealed that the conversion of lipid II to lipid III is accompanied by synthesis of ECA heteropolysaccharide chains. Mutants of *S. typhimurium* that lack the structural gene for TDP-glucose pyrophosphorylase (*rfbA*) are severely impaired in their ability to synthesize TDP-glucose, an obligatory precursor of TDP-Fuc4NAc (9). Accordingly,  $\Delta rfbA$  mutants of *S. typhimurium* are defective in lipid III synthesis, and they accumulate lipid II (22). Incubation of ether-permeabilized cells of *S. typhimurium* SH5150-2 ( $\Delta rfbA$ ) with TDP-[ $^3\text{H}$ ]Fuc4NAc resulted in the pronounced incorporation of isotope into lipid III (data not shown). In addition, the synthesis of [ $^3\text{H}$ ]Fuc4NAc-labeled lipid III was accompanied by the incorporation of radioactivity into ECA heteropolysaccharide chains, as demonstrated by SDS-PAGE and fluorography (Fig. 5, lane 1). No incorporation of isotope into either lipid III or ECA chains was observed when similar experiments were conducted with the parental strain *S. typhimurium* SH5150 ( $\Delta rfbA rff$ ), which was unable to synthesize lipid II because of the *rff* lesion (Fig. 5, lane 2). The data presented above strongly support the conclusion that lipid III is an intermediate in the assembly of ECA polymers.

## DISCUSSION

Recent studies in our laboratory have demonstrated the accumulation of ManNAcA-GlcNAc-pyrophosphorylundecaprenol (lipid II) in mutants of *S. typhimurium* that are defective in the synthesis of TDP-Fuc4NAc (22). This observation suggests that lipid II serves as the acceptor of Fuc4NAc residues in the synthesis of the ECA trisaccharide repeat unit. The data presented here support this conclusion, and they provide evidence for the utilization of Fuc4NAc-ManNAcA-GlcNAc-pyrophosphorylundecaprenol (lipid III) in the process that is involved in the assembly of ECA polymers.

Incubation of *E. coli* membranes with UDP-GlcNAc, UDP-[ $^{14}\text{C}$ ]ManNAcA, and TDP-[ $^3\text{H}$ ]Fuc4NAc resulted in the synthesis of a lipid-linked oligosaccharide that contained both [ $^{14}\text{C}$ ]ManNAcA and [ $^3\text{H}$ ]Fuc4NAc. The availability of only small quantities of the glycolipid product precluded the complete structural analysis of the molecule; however, the product was identified as lipid III based on several observations. Incubation of the glycolipid with mild acid released the oligosaccharide portion of the molecule, which was characterized as a trisaccharide by gel-permeation chromatography. The electrophoretic mobility of the oligosaccharide at pH 3.5 was also in agreement with its identification as an acidic trisaccharide containing a single carboxyl group. The *in vitro* synthesis of the glycolipid was dependent on the prior synthesis of lipids I and II. Accordingly, the synthesis

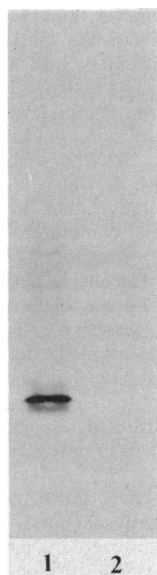


FIG. 5. Addition of TDP- $^3\text{H}$ Fuc4NAc to *S. typhimurium* mutants permeabilized with ether. Cells from strains SH5150-2 and SH5150 were treated with ether as described in the text. Portions (1 mg of protein) were incubated at 37°C for 30 min with 50 mM Tris hydrochloride (pH 8.2)–30 mM  $\text{MgCl}_2$ –5 mM 2-mercaptoethanol–0.32 U of alkaline phosphatase–TDP- $^3\text{H}$ Fuc4NAc ( $1.02 \times 10^5$  dpm) in a final volume of 0.2 ml. The particulate fraction was then washed twice with cold water and suspended in 150  $\mu\text{l}$  of sample buffer, and the incorporation of radioactivity into ECA polymers was determined by SDS-PAGE and fluorography as described in the text. Lane 1, SH5150-2; lane 2, SH5150.

of the glycolipid was abolished by tunicamycin, which specifically inhibits the synthesis of lipid I (1, 19). The direct conversion of lipid II to the glycolipid was also demonstrated; the addition of TDP-Fuc4NAc to reaction mixtures containing [ $^{14}\text{C}$ ]ManNAcA-labeled lipid II resulted in the almost complete conversion of radiolabeled lipid II to the glycolipid. These observations strongly support the identification of the glycolipid as Fuc4NAc-ManNAcA-GlcNAc-acylphosphorylundecaprenol-lipid (lipid III).

The *in vitro* synthesis of lipid III was also accompanied by the apparent synthesis of ECA heteropolysaccharide chains. Accordingly, incubation of membranes with UDP- $^3\text{H}$ GlcNAc, UDP-ManNAcA, and TDP-Fuc4NAc resulted in the incorporation of radioactivity into polymeric species with electrophoretic mobilities similar to those of ECA polymers synthesized *in vivo*. The incorporation of [ $^3\text{H}$ ]GlcNAc into these polymers was dependent on the presence of both UDP-ManNAcA and TDP-Fuc4NAc in reaction mixtures, and synthesis of the radiolabeled polymers was abolished when tunicamycin was included in the reaction mixtures. Further evidence for a precursor-product relationship between lipid III and ECA polymers was obtained by using  $\Delta\text{rfbA}$  mutants of *S. typhimurium* that accumulate lipid II because of their inability to synthesize TDP-Fuc4NAc. Mutant cells permeabilized by treatment with ether incorporated [ $^3\text{H}$ ]Fuc4NAc into both lipid III and ECA heteropolysaccharide chains when they were incubated with TDP- $^3\text{H}$ Fuc4NAc. In addition, the radiolabeled polymers appeared to be identical in size to ECA polymers that were synthesized *in vivo*, as determined by SDS-PAGE.

In contrast, no radioactive ECA polymers were detected following the incubation of TDP- $^3\text{H}$ Fuc4NAc with ether-treated cells of a mutant that was unable to synthesize lipid II because of a lesion in the *rff* locus. Although direct utilization of lipid III for heteropolysaccharide synthesis has not yet been demonstrated, the data presented here provide good indirect evidence in support of the conclusion that lipid III is involved in the assembly of ECA heteropolysaccharide chains.

In contrast to the homologous series of ECA polymers that were synthesized *in vivo* and by ether-treated cells, the *in vitro* incorporation of [ $^3\text{H}$ ]GlcNAc into only two polymeric species was observed when cell envelope membranes were incubated with UDP- $^3\text{H}$ GlcNAc, UDP-ManNAcA, and TDP-Fuc4NAc. However, the apparent degrees of polymerization of these polymers were equivalent to those of the major species of ECA synthesized *in vivo*. Nothing is yet known concerning the factors that govern the degree of polymerization of ECA polymers and other polysaccharides such as lipopolysaccharide O side chains during biosynthesis. However, it does not seem unreasonable to assume that this process might be disrupted as a result of the sonication procedures used in this study for the preparation of membranes for *in vitro* analyses. In addition, it is not yet known whether the heteropolysaccharide chains synthesized *in vitro* are still linked to the undecaprenol carrier lipid or if they are linked to phospholipid, as is the case for the haptenic form of ECA (7, 15).

The mechanism involved in the assembly of ECA heteropolysaccharide chains remains to be established. It is possible that the synthesis of ECA chains involves the polymerization of lipid-linked trisaccharide repeat units by a mechanism similar to that described for O-antigen synthesis in *Salmonella newington* (24). Alternatively, chain elongation may occur by the successive transfer of individual sugars from the appropriate nucleotide-sugar donor to the nonreducing terminal sugar of the growing polysaccharide chain. The latter mechanism has been reported for the assembly of lipopolysaccharide O side chains in *E. coli* O8 and *E. coli* O9 (5). Further studies are necessary in order to define the mechanism of ECA heteropolysaccharide chain elongation and to more closely establish the role of lipid III in this process.

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