JOURNAL OF BACTERIOLOGY, Jan. 1977, p. 225–236
Vol. 129, No. 1
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Role of a Sugar-Lipid Intermediate in Colanic Acid Synthesis by *Escherichia coli*

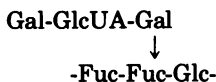
JAMES G. JOHNSON AND DAVID B. WILSON*

Division of Biological Sciences, Cornell University, Ithaca, New York 14853

Received for publication 1 July 1976

Membrane fractions from a *lon* strain of *Escherichia coli* but not a wild-type strain catalyze the incorporation of fucose from guanosine 5'-diphosphate-fucose into a lipid and into polymeric material. Both incorporation reactions specifically require only uridine 5'-diphosphate (UDP)-glucose. The sugar lipid was shown to be an intermediate in the synthesis of the polymer which was related to colanic acid. The sugar lipid had the structure (fucose$_3$, glucose$_2$)-glucose P-P-lipid. Its behavior on column and thin-layer chromatography, the rates of its hydrolysis in acid and base, and the response of its synthesis to inhibitors are all identical to the other sugar-lipid intermediates which have been shown to contain sugars attached to the C_{55}-polyisoprenol, undecaprenol, by a pyrophosphate linkage. The membrane fractions from both the *lon* strain and the wild-type strain also catalyzed the incorporation of either glucose from UDP-glucose or galactose from UDP-galactose into a lipid fraction which was shown to contain the free sugar attached by a monophosphate linkage to an undecaprenol-like lipid. This lipid was isolated and its nuclear magnetic resonance spectra was identical to undecaprenol. The membrane fractions from both strains also incorporated glucose from UDP-glucose into glycogen and into a polymer that behaved like *Escherichia coli* lipopolysaccharide. Conditions were found where the incorporation of glucose could be directed specifically into each compound by adding the appropriate inhibitors.

Mucoid (*lon* or *capR*) mutants of *Escherichia coli* produce an extracellular polysaccharide called colanic acid (9), whose repeat unit structure is (35):



The same polymer, also known as the M-antigen, is produced by strains of *Salmonella* and *Aerobacter* (13).

The *lon* gene is of particular interest because its mutation causes many effects, including mucoidy, radiation sensitivity, inhibition of protein degradation (3), and enzyme induction (19). The induced enzymes are scattered around the genetic map and include those of the *gal* operon as well as all other operons whose gene products catalyze the synthesis of precursors of the polymer. Biochemically the *lon* gene behaves as though its product were a repressor protein which normally prevents colanic acid synthesis (20). This means that the *gal* operon is subject to regulation by two repressors (12, 18). Knowledge of the biosynthetic path to colanic acid would allow genetic studies to be extended to include the as yet unidentified sugar transferase enzymes producing the polymer, some of which probably increase dramatically after mutation of the *lon* gene to account for the more than 100-fold increase in colanic acid synthesis resulting from mutation in the *lon* gene.

Studies of the biosynthesis of several complex bacterial heteropolysaccharides have shown that one common feature of these reactions is the preassembly of repeating sugar oligosaccharides on a carrier lipid coenzyme, undecaprenol phosphate (2, 24, 30, 37).

This paper provides evidence that colanic acid is also made via a recycling lipid carrier. A hexasaccharide P-P-lipid intermediate probably related to an intermediate in colanic acid biosynthesis was characterized. In addition, a glucose-P-lipid which is apparently unrelated to the colanic acid system was also identified. This sugar lipid closely resembles that identified as being an intermediate in *Salmonella* O-antigen modification (21, 41). Both of the lipids identified in this work resemble undecaprenol phosphate. In all, five incorporation reactions were characterized:

GDP-fucose + UDPG → mucoid lipid → mucoid polymer (1 and 2)

UDPG → glucose lipid → glucose polymer (3 and 4)

UDPG → glucan polymer (5)

where UDPG is uridine diphosphate glucose.

A preliminary report of this paper has appeared (J. G. Johnson, Fed. Proc. 32:523).

MATERIALS AND METHODS

Reagents. Enzymes and unlabeled nucleotides were purchased from Sigma Chemical Co., except for guanosine 5'-diphosphate (GDP)-fucose, which was made by the procedure of Ginsburg (8). Uridine 5'-diphosphate (UDP)-[U-^{14}C]glucose, UDP-[U-^{14}C]galactose, UDP-[U-^{14}C]glucuronic acid, and GDP-[^{14}C]fucose were obtained from Amersham/Searle Corp. [^{14}C]mannosyl-1-phosphoryl-undecaprenol from the micrococcal mannan system (30) was a generous gift of C. J. Waechter of Johns Hopkins University.

Bacterial strains. *E. coli* K-12, strain 3300 (nonmucoid parent) and strain M6 (mucoid mutant carrying *capR6*) were obtained from A. Markovitz (20). Strain M6-2 used in this work was a highly mucoid, single colony isolated from strain M6. Mutants of M6-2 lacking UDP-galactose-4-epimerase were selected from nonmutagenized cultures by picking colonies resistant to 2-deoxygalactose. The procedure used was a modification of the procedure of K. Floyd and was as follows. Strain M6-2 was grown in minimal glycerol medium containing 10^{-3} M D-fucose to abut 5×10^8 cells/ml. Cells (0.1 ml) were spread on a minimal agar plate containing 0.2% 2-deoxygalactose, 10^{-3} M D-fucose, and 0.4% glycerol. The plate was incubated at 37°C for 48 h, and the colonies which grew were tested on eosin methylene blue (EMB)-galactose plates. The colonies which were Gal$^-$ were picked from the original plate, streaked for single colonies on a minimal glucose plate, and then grown and assayed for UDP-galactose-4-epimerase activity. Cultures were grown on rotary shakers at 28°C in a minimal medium (18) supplemented with 10 μg of thiamine per ml and 0.2% D-glucose.

Particulate preparation. Cells were collected and washed twice with cold 0.14 M NaCl and then suspended in 0.14 M NaCl, 30 mM tris(hydroxymethyl)aminomethane(Tris), and 1 mM dithiotheritol (DTT), pH 7.5, at a concentration of 1 g (wet weight) of cells per 40 ml of buffer. The suspension was sonicated in 70-ml batches at full power in the chamber of a Raytheon 10-kc, 250-W, model DF101 sonic oscillator for 7.5 min in 30-s bursts. The sonic extract was centrifuged at $3,000 \times g$ for 20 min. The pellet was suspended in buffer A (1 mM DTT, 30 mM Tris, pH 7.5, 30 mM NaCl, 10% glycerol) to a concentration of 25 mg of protein per ml and quick frozen at −80°C, at which temperature it was stable for weeks.

Incorporation reactions. Incubation mixtures contained 40 μM GDP-fucose, 150 μM UDP-glucose, 80 mM Tris, pH 7.5, 11 mM $MgCl_2$, 5 mM ethylenediaminetetraacetate (EDTA), and about 16 mg of particulate protein per ml in a total volume of 0.1 ml. ^{14}C-labeled sugar nucleotides were used at a specific activity of 2,000 to 7,000 cpm per nmol. Incorporations of sugar nucleotides into colanic acid-related polymer or lipid were carried out at 28°C and into glucose-containing polymers at 37°C. Incorporation into the lipid fraction and into the polymer fraction was assayed by the procedures of Troy et al. (37).

Preparation of colanic acid-related oligosaccharides. Sugar lipid was extracted by the procedure of Troy et al. (37) from reactions mixtures, scaled up 40-fold from the standard assay, which were incubated for 30 min at 12°C. The organic extract was dried under nitrogen, dissolved in a few drops of $CHCl_3$, and diluted with 5 ml of methanol. The copious white precipitate which formed was removed by centrifugation. The above steps were repeated until no precipitate appeared after storage in methanol overnight at −20°C. Little phosphate or radioactivity was lost in this procedure. The sugar lipid in the supernatant was then chromatographed on a Sephadex LH-20 column.

Salt was removed from the column fractions by extraction with water, and the sugar lipid was dried, taken up in 0.22 ml of methanol, and diluted with 0.22 ml of water. A 50-μl amount of 1 N KOH was added, the tube was left overnight at 30°C, and 20 μl of 1 M Tris, pH 7.5, 3.5 ml of water, and 2 ml of $CHCl_3$ were added. The mixture was separated and the organic phase was washed with water. The aqueous extracts were evaporated to remove methanol and lyophilized. Raffinose was added to serve as a marker and as a carrier, the solution was made up to 0.2 ml with 0.1 M glycine-0.01 M $MgCl_2$ (pH 8.5), 3 U of alkaline phosphatase was added, and the mixture was incubated for 3 h at 37°C. The mixture was then applied to a Bio-Gel P-2 column to separate the released oligosaccharides.

Sugar hydrolysis. Samples were dissolved in 1 ml of 1 N H_2SO_4, sealed in air, and hydrolyzed at 105°C for 3 h. They were then diluted with water and passed through a 2-ml Dowex-1 (formate) column.

Reducing-terminus determination. The oligosaccharide was reduced in 0.2 ml of 4 mM KBH_4 for 3 h at 20°C and then hydrolyzed directly as above. The hydrolysis products were separated by paper electrophoresis in buffer B (39).

Chromatography and electrophoresis. Solvents for paper and silica gel chromatography were: (i) pyridine-ethyl acetate-acetic acid-water (5:5:1:3); (ii) 95% ethanol-*n*-butanol-water (10:1:2); (iii) $CHCl_3$-methanol-water (65:25:4); (iv) $CHCl_3$-methanol-water (20:12:3); (v) di-isobutyl ketone-acetic acid-water (64:25:6). High-voltage paper electrophoresis buffers were: (i) 45 ml of pyridine per liter adjusted to pH 6.2 with about 2.6 ml of acetic acid and (ii) 86 mM $Na_2MoO_4 \cdot 2H_2O$ adjusted to pH 5.0 with concentrated H_2SO_4. Electrophoresis was carried out at 35 V/cm on a Shandon model L25 instrument.

Sugars were visualized with aniline phthaline (25) or periodate-permanganate (15).

Column chromatography. Sephadex LH-20 was swollen in 99% methanol for 24 h and then packed to

form a column (55 by 1.1 cm). The column was equilibrated and run at a flow rate of 8 ml/h with 0.1 M ammonium acetate dissolved in 99% methanol. A diethylaminoethyl (DEAE)-cellulose column was prepared and run exactly as described by Rouser et al. (27). The Bio-Gel P_2 column (87 by 1.5 cm) was equilibrated and run at a flow rate of 16 ml/h with 0.1 M ammonium formate. The Bio-Gel A-150 column (59 by 1.6 cm) was equilibrated and run at a flow rate of 3 ml/h with 0.03 M Tris-0.03 M NaCl, pH 7.5. The Sephadex G-25 superfine column (84 by 1.5 cm) was equilibrated and run at a flow rate of 12 ml/h with 0.1 M ammonium formate.

Colorimetric assays. These substrates were determined by the following assays: phosphate by the ascorbic acid-molybate procedure (1); protein by the Lowry method (17); total carbohydrate by the phenol-H_2SO_4 procedure (7); fucose by the cysteine-H_2SO_4 procedure with readings at 396 and 427 nm (6); aldohexase by the cysteine-H_2SO_4 procedure with a 3-min incubation and readings at 414 and 380 nm; glucuronic acid by the carbazole-H_2SO_4 method (5); 2-keto-3-deoxyoctonic acid by the periodate-thiobarbituric acid procedure as described by Osborn (23).

RESULTS

Polymer production in vivo. Colanic acid was released into the culture medium and did not adhere to the cell as a capsule. Boiling the cultures for 15 min before centrifugation produced no more polymer than did direct centrifugation. Cells washed with saline at room temperature and then placed back into the growth medium began growth and polymer production immediately at the same rates they were exhibiting before washing. Cell pellets of strain M6-2 grown at 30°C were always covered by a viscous, loose layer of slime, even after centrifugation at 30,000 × *g*. The slime layer could be partially separated from the tighter pellet by repeatedly pouring it off from the pellet, resuspending it in saline, and centrifuging again. Other than in appearance, the resulting slime and pellet were indistinguishable, both containing about half the total protein and half the viable cells. The slime contained no fucose or glucuronic acid, so its properties were not due to the colanic acid polymer itself. But the slime was related to polymer production, for mutants of M6-2 lacking UDP-galactose-4-epimerase (which produce no polymer when grown on glucose) gave cell pellets with no slime, as did the nonmucoid parent strain 3300. The slime, characteristic of mucoid M6-2, seems to consist of altered but intact cells and is not a result of disruption and leakage during washing. The slime disappeared during sonication, after which there was no problem in sedimenting particulate material.

The maximal rate of polymer production during late exponential growth was 150 μg of polymer per h per ml per 10^9 cells, and the maximal total polymer production was 510 μg of polymer per ml of culture. This represented 25% of the total substrate glucose originally present. Higher cell and polymer yields occurred at higher glucose concentrations.

Mucoid polymer was purified by both the long (9) and short (28) procedures of Goebel. Both products and the crude undialyzed culture supernatant gave identical sugar compositions in colorimetric and paper chromatography assays. The ratio of glucuronic acid-fucose-(glucose plus galactose) was 1.0:1.9:2.7, compared to 1:2:3 for the theoretical hexamer (35). Titration of the purified polymer using cetylpyridinium bromide by Scott's procedure (31) gave a value of 2.2 anionic groups per hexamer or an equivalent weight of 490. This value is in good agreement with the calculated equivalent weight of 533. Final evidence that the polymer we isolated was cholanic acid was the presence in hydrolysates of the polymer of an acid-resistant disaccharide behaving like aldobiuronic acid, which is characteristic of colanic acid (26).

The M6-2 mucoid polymer thus matches colanic acid as isolated and studied by Goebel (9), Markovitz (19), and Sutherland (35). It was the only fucose- or glucuronic acid-containing polymeric material produced by strain M6-2. This is shown by the fact that the culture supernatant and the purified polymer had identical compositions. In addition, the crude supernatant eluted as a single broad peak during chromatography on Bio-Gel A-150 (Fig. 1) with a constant ratio of fucose-glucuronic acid-total carbohydrate across the peak. This material is fairly homogeneous in size, with no visible small chains. The washed slime layer of cells (see above) contained no fucose or glucuronic acid. Therefore, fucose was used as a specific marker for colanic acid and its intermediates in the rest of this work.

Abnormal polymer produced in vivo. Polymer produced by early log cells had the same composition as the purified polymer reported above. When cells harvested at midlog phase were washed in saline at room temperature, resuspended to the original concentration in fresh growth medium, and allowed to continue exponential growth for one doubling time, they produced polymer that lacked a quarter of the normal side-chain terminal galactose and glucuronic acid. Perhaps older cells produce incomplete polymer, or the washing selectively harmed the transferase responsible for adding glucuronic acid. Sutherland has suggested that colanic acid generally lacks some glucuronic acid (36).

In vitro incorporation of fucose. Particulate

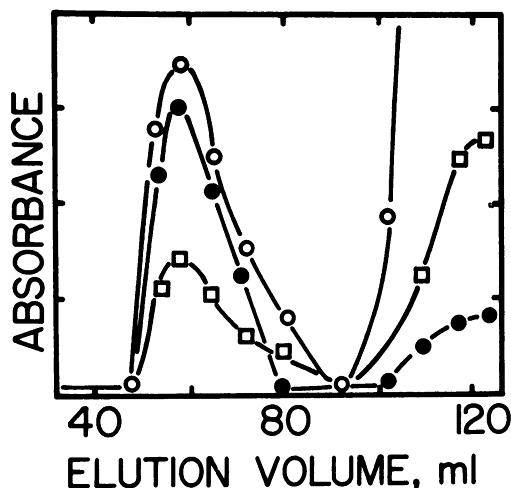


FIG. 1. *Agarose chromatography of in vivo-synthesized mucoid polymer. A sample of the culture supernatant from strain M6-2 grown in minimal salts medium was applied directly to a Bio-Gel A-150 column (1.6 by 59 cm) equilibrated with 0.03 M Tris-0.03 M NaCl, pH 7.5. The column was run at a flow rate of 3 ml/h, and portions were assayed for total carbohydrate (○), fucose (●), and glucuronic acid (□) by the procedures described in the text.*

preparations from strain M6-2 incorporated [^{14}C]fucose from GDP-[^{14}C]fucose into both the lipid fraction and the polymer fraction (Table 1 and Fig. 2). There was no incorporation of fucose into either lipid or polymer when the particulate preparation from strain 3300 was used. This in vitro incorporation of fucose had the same temperature optimum (28°C) as the in vivo synthesis of colanic acid (data not shown). The maximum rate of fucose incorporation into polymer is 0.45 μg of fucose per h per mg of protein. Although this rate is low compared with the in vivo rate of colanic acid synthesis, it is similar to the in vitro rate of fucose incorporation by particulate preparations from *Aerobacter* (37).

The only requirement for fucose incorporation was the specific presence of UDP-glucose (Table 1). The particulate preparation contained 0.5% of the cell's UDP-galactose-4-epimerase (epimerase) so UDP-galactose could normally replace UDP-glucose. Epimerase could be removed by washing the particulate material with cold buffer A. This reduced the fucose incorporation activity to 30% of normal, but the residual activity specifically required UDP-glucose. Mutants lacking epimerase also required UDP-glucose for fucose incorporation. As described below, direct analysis of the sugar lipid showed glucose alone to be present, even though UDP-galactose was present in the incubation mixture due to epimerase action.

The addition of UDP-glucuronic acid and phosphoenolpyruvate, the other presumed precursors of colanic acid, to the reaction mixture did not stimulate fucose incorporation into either lipid or polymer. Furthermore, there was no incorporation of glucuronic acid from UDP-

TABLE 1. *Incorporation of labeled sugars into the mucoid lipid and mucoid polymer fractions*

Strain	Substrates	Incorporation[a] (nmol/mg of protein)	
		Lipid	Polymer
M6-2	GDP-[^{14}C]fucose	0.08	0.04
M6-2	GDP-[^{14}C]fucose + UDP-glucose	0.4	0.7
3300	GDP-[^{14}C]fucose + UDP-glucose	0.04	0.03
M6-s[b]	UDP-[^{14}C]glucuronic acid + GDP-fucose + UDP-glucose	0.01	0.02

[a] Incorporation of the indicated substrate was carried out as described in the text. The incorporation into the lipid and polymer fractions was determined by the procedure used in reference 37.

[b] UDP-galactose was present in all normal reactions which contained UDP-glucose due to the presence of the enzyme UDP-galactose-4-epimerase.

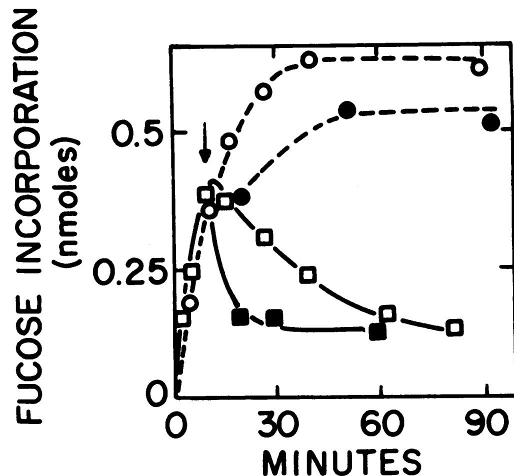


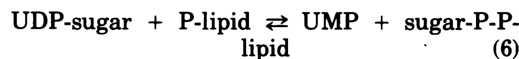
FIG. 2. *Effect of EDTA on the incorporation of fucose into the lipid and polymer fractions. The standard reaction mixture containing 0.049 mM GDP-[^{14}C]fucose (7,480 cpm/nmol) and 0.168 mM UDP-glucose was incubated at 28°C. At the time indicated by the arrow the sample was divided into two equal parts and EDTA was added to one to give a concentration of 30 mM. Samples were taken at the indicated times, and the amount of incorporation of fucose into lipid and polymer was determined as described in reference 37. When EDTA was present from the start of the reaction, the lipid and polymer incorporations were 0.027 nmol in 35 min and 0.064 nmol in 160 min, respectively. Symbols: (□) control lipid, (■) inhibited lipid, (○) control polymer, (●) inhibited polymer.*

[^{14}C]glucuronic acid into either lipid or polymer even in the presence of all of the presumed precursors of colanic acid (Table 1). All incorporations reported below contained only UDP-glucose and GDP-fucose.

Inhibitors of incorporation. When present from the beginning of the incorporation reaction, inhibitors affected the lipid and polymer to about the same extent (Table 2). Fucose did not compete with GDP-fucose. UDP, GMP, and GDP were not inhibitors. The optimal $MgCl_2$ concentration was 10 mM, with higher concentrations causing inhibition (80% inhibition at 100 mM). EDTA (30 mM) totally inhibited incorporation in the presence of 10 mM $MgCl_2$. Triton X-100 inhibited both lipid and polymer incorporation by 50%. This shows that Triton must affect the formation of sugar lipid, and not just the formation of polymer from sugar lipid as it does in the mannan system (29).

The inhibition of fucose incorporation into both the polymer and lipid fractions by uridine 5′-monophosphate (UMP) is shown in Fig. 3. When UMP was added after incorporation had occurred, fucose was lost from the lipid fraction and further polymer production ceased. The production of sugar lipid from GDP-fucose and UDP-glucose appeared to be a reversible reaction, as the level of sugar lipid was nearly the same whether UMP was present from the start or was added during the reaction (Table 3, line 3). When UMP was added at a later time (line 4) there was reversal, but it was not as complete. This could result from the inactivation of the system which seems to occur during the incubation reaction.

Reversal by UMP, and not UDP, of sugar-lipid formation is characteristic of the translocase reaction:



UDP-sugar + P-lipid ⇄ UMP + sugar-P-P-lipid (6)

as shown in peptidoglycan, O-antigen, and capsular polysaccharide synthesis where the acceptor lipids are C_{55}-polyisoprenol phosphates (2, 24, 37). That UMP, rather than guanosine 5′-monophosphate (GMP), reverses the reaction shows that glucose rather than fucose is the first sugar attached to the lipid. However, this reversal by UMP differs from the above examples because the labeled sugar being followed is fucose, not glucose. The fucose would be attached via a second or later reaction of the type:

GDP-fucose + Glc-P-P-lipid ⇄ GDP + Fuc-Glc-P-P-lipid (7)

if the incorporation reaction being studied follows the pathway seen for the synthesis of other

TABLE 2. *Inhibition of fucose incorporation into the lipid and polymer fractions*

Compound	Concn	% Inhibition[a]	
		Lipid	Polymer
Fucose	2.5 mM	3	5
UDP	2.4 mM	0	7
GMP	2.5 mM	2	3
GDP	2.5 mM	4	0
ADP	2.9 mM	3	0
Bacitracin and then EDTA	0.7 mg/ml	25	55
EDTA and then bacitracin	1.0 mg/ml	19	43
EDTA then bacitracin	1.0 mg/ml	0	0
Trition X-100	0.6 mg/ml	50	50
UMP	0.14 mM	50	37
UMP	0.3 mM	82	75

[a] The incorporation of fucose into lipid and polymer was measured as described in the text except that the indicated concentration of each inhibitor was present during the incubation. In the experiments with bacitracin, a reaction mixture with all components except UDP-glucose and GDP-fucose was incubated for 5 min and then the reaction was started by adding the UDP-glucose and GDP-fucose. In the experiment in line 7, bacitracin was present during the preincubation and 13 mM EDTA was added along with UDP-glucose and GDP-fucose, whereas in line 8, 13 mM EDTA was present during the preincubation and bacitracin was added along with UDP-glucose and GDP-fucose.

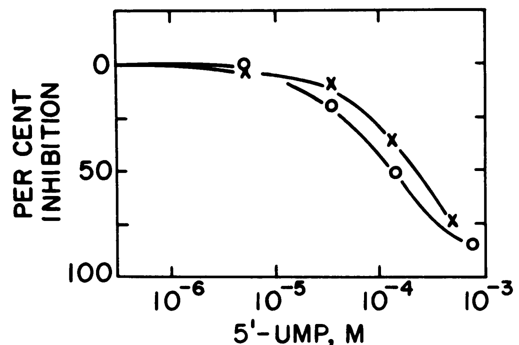


FIG. 3. *Inhibition of fucose incorporation by UMP. Standard reaction mixtures of 0.070 ml containing 0.054 mM GDP [^{14}C]fucose (7,750 cpm/nmol), 0.070 mM UDP-glucose, 0.7 mg of protein, and the indicated concentration of UMP were incubated at 28°C. Lipid was assayed after 40 min and polymer was assayed after 90 min by the methods in reference 37. In the absence of UMP the incorporation into lipid was 0.19 nmol/mg and into polymer was 1.4 nmol/mg. Symbols: (○) lipid, (×) polymer.*

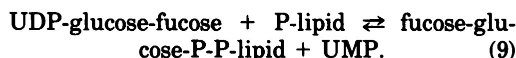
bacterial heteropolysaccharides (2, 24, 30, 34). This should be an irreversible reaction, unaffected by GDP (as was found), and should "lock" the terminal glucose onto the lipid and make the glucose-to-lipid bond insensitive to UMP (37). Another possibility would be the following set of reactions:

UDP − glucose + GDP − fucose ⇄ UDP-glucose − fucose + GDP (8)

TABLE 3. *Reversibility of the reaction between UMP and sugar lipid*

Reaction[a]	Time of UMP addition	Fucose in lipid (nmol)	
		Before UMP	At 45 min
1	None added		0.19
2	0		0.043
3	15	0.10	0.053
4	30	0.13	0.081

[a] The reactions were run at 12°C and the reaction mixture was as described in the text except that it contained 0.043 mM GDP-[^{14}C]fucose and 0.153 mM UDP-glucose. UMP (2.7 mM) was added at the indicated time. Incorporation into lipid was assayed at the indicated times as described in the text.



UDP-glucose-fucose + P-lipid ⇄ fucose-glucose-P-P-lipid + UMP. (9)

The last reaction would be inhibited by UMP as we observed. Further work will be required to determine the exact mechanism of the incorporations observed in this system.

Effect of bacitracin. Bacitracin at 700 μg/ml inhibited polymer production by 55% and sugar lipid by 25% (Table 2). Bacitracin specifically complexes with C_{55}-polyisoprenol pyrophosphate, preventing it from being monodephosphorylated and recycling through reaction 5. Its effect is highly indicative of the involvement of such a lipid carrier (2, 24, 37). The inhibitory complex forms only if free divalent cation is present. Once the complex has formed, the divalent cation cannot be extracted from it by a chelator like EDTA (33). Thus, EDTA will prevent bacitracin inhibition unless the inhibitory complex is formed before EDTA is added. The data in Table 2 shows that the bacitracin inhibition depended upon bacitracin being added before the EDTA (which was in excess of the divalent cation Mg^{2+}). This result implicates a C_{55}-polyisoprenol pyrophosphate as a component of the colanic acid biosynthetic system, even though the inhibition is not as great as seen in other systems.

Sugar lipid as precursor of polymer. The sugar lipid and polymer are circumstantially related by their specific incorporation of fucose, the time course of incorporation (Fig. 2), and their parallel response to inhibitors. As has been shown in the micrococcal mannan system (30), EDTA blocked sugar-lipid synthesis without affecting polymer production from preexisting sugar lipid. This property was exploited to demonstrate directly that the sugar lipid was a quantitative, obligatory precursor to the polymer.

EDTA was added to a reaction mixture at various times during an incorporation (Fig. 2). The amounts of polymer made, and of lipid lost, between the time of addition and the end of reaction are plotted in Fig. 4. The amount of polymer made is directly proportional and nearly equal (average slope is 85%) to the lipid lost. When present from the start of the reaction, EDTA inhibited over 90% of both lipid and polymer production.

Incorporation of glucose. Although glucose was incorporated into both the mucoid lipid and mucoid polymer, there were other incorporation reactions that interfered with the study of glucose incorporation into the mucoid lipid and mucoid polymer. One of these was the incorporation of glucose into another lipid molecule and some of the properties of this reaction are shown in Table 4. This reaction was catalyzed by particulate fractions from both strains M6 and 3300. The temperature optimum for this reaction was 37°C and both glucose from UDP-glucose and galactose from UDP-galactose were incorporated into this lipid. This incorporation was not inhibited by glucose, adenosine diphosphoglucose (ADP-glucose), ADP, or UMP. The reaction was inhibited 60% by 100 μg of bacitracin per ml, but the order of addition of EDTA, $MgCl_2$, and bacitracin did not affect the inhibition. This is in contrast to the incorporation of GDP-fucose into the mucoid lipid. UDP was a reversible inhibitor of the incorporation of

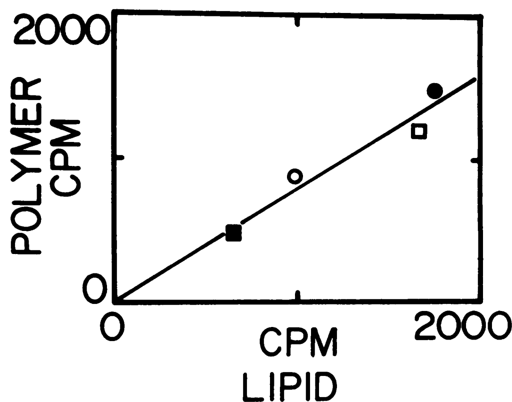


FIG. 4. *Mucoid sugar lipid is a precursor to the mucoid polymer. Samples of the reaction mix described in the legend to Fig. 2 were added to EDTA at 4, 10, 15, and 40 min from the start of the reaction, and the reaction was allowed to proceed for an additional 60 min. The amount of [^{14}C]fucose in lipid and polymer was also determined just before the addition of EDTA to each sample. The increase in polymer incorporation is plotted against the decrease in lipid incorporation. Symbols: (○) 4 min, (●) 10 min, (□) 15 min, (■) 40 min.*

TABLE 4. *Incorporation of glucose into nonmucoid lipid and polymeric material*

Strain	Inhibitors	Incorporation[a] (nmol/mg of protein)	
		Lipid	Polymer
3300	None	0.51	1.2
3300	10 mM UDP	0.02	0.96
3300	13 mM ADP		0.80
3300	10 mM UDP + 13 mM ADP	0.06	0.06
M6	None	0.72	1.7
M6	2 mM UDP	0.03	1.8
M6	13 mM ADP	0.68	1.55
M6	2 mM UDP + 13 mM ADP		0.18
M6	3.8 mM AMP	0.72	0.80
M6	0.37 mM ADP-glucose	0.72	1.38

[a] The reactions were run as described in the text using 0.05 mM UDP-[^{14}C]glucose.

sugar into the nonmucoid lipid (Fig. 5). The data in Fig. 5 can be used to calculate the equilibrium constant for the reaction: UDP-hexose + lipid ⇄ UDP + hexose-lipid; the value found was 7 ± 1. This data can also be used to calculate the pool of acceptor lipid (34) in the cell, which was found to be 550 pmol/mg of particulate protein.

Another interfering reaction was the incorporation of glucose from UDP-glucose into a glucan polymer. This reaction also was catalyzed by particulate fractions from both strains M6-2 and 3300. The polymer produced was hydrolyzed by both α-amylase and amyloglucosidase, which is a property of glycogen. Galactose from UDP-galactose was not incorporated into this polymer. The incorporation reaction was inhibited by AMP, ADP, and ADP-glucose (Table 4) but not by glucose UMP, UDP, bacitracin, EDTA, or Triton X-100.

The final interfering reaction also resulted in the synthesis of a polymeric material. This reaction was only observed when the incorporation into the glucose polymer was inhibited by ADP. This reaction was inhibited by UDP and EDTA just as was the incorporation of glucose into the nonmucoid lipid. This polymer was resistant to α-amylase and behaved like lipopolysaccharide on extraction (40). The fact that the incorporation of glucose into the nonmucoid lipid and into the lipopolysaccharide polymer showed identical responses to UDP inhibition (Fig. 5) suggests that the nonmucoid lipid may be a precursor of the lipopolysaccharide, although this was not shown directly.

Kinetic constants of the incorporation reaction. Approximate K_m and V_{max} values were determined for six of the incorporation reactions discussed above and the values found are given in Table 5. The conditions of the assay

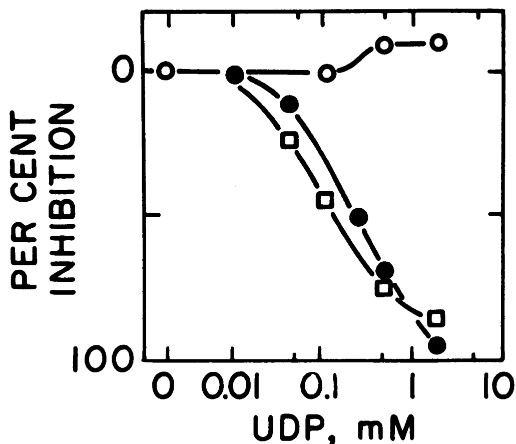


FIG. 5. *Inhibition by UDP of sugar incorporation into lipid and polymer. Standard assay mixtures (see text) containing 0.051 mM UDP-[^{14}C]galactose (11,600 cpm/nmol), 0.49 mg of protein and the indicated concentration of UDP were incubated at 28°C for 30 min for lipid assay and 90 min for polymer assay. The 100% incorporation values were 1.2 nmol/mg for polymer with no addition, 0.99 nmol/mg for polymer with 13 mM ADP, and 0.40 nmol/mg for lipid with 13 mM ADP. Symbols: (○) polymer with no addition, (□) polymer plus 13 mM ADP, (●) lipid plus 13 mM ADP.*

TABLE 5. *Kinetic constants of the incorporation reactions*

Reaction	K_m[a] (mM)	V_{max}[b] (nmol/h per mg)
UDP-glucose → mucoid lipid	0.03	0.7
GDP-fucose → mucoid lipid	0.01	0.7
UDP-glucose → mucoid polymer	0.03	3.3
GDP-fucose → mucoid polymer	0.2	3.3
UDP-glucose → glycogen	0.5	1.3
UDP-galactose → galactose lipid	0.02	1.4
UDP-galactose → lipopolysaccharide	0.4	0.7

[a] The K_m and V_{max} for the incorporations of UDP-glucose and GDP-fucose into the mucoid lipid and polymer were determined by the procedures described in the text except that the appropriate substrate was varied and the reactions contained 5 mM UDP and 13 mM ADP to inhibit the nonmucoid incorporation reactions. The K_m and V_{max} values for the incorporation of UDP-glucose into the glucose polymer were determined by the procedures described in the text, except that the assay reaction mix contained 10 mM UDP to inhibit the incorporation of UDP-glucose into the other glucose polymer. The K_m and V_{max} for the incorporation of UDP-galactose into the galactose lipid and into polymer were determined by the procedures described in the text except the incubations were run at 38°C and contained 13 mM ADP.

[b] These values do not reflect initial rates as the samples were taken at times (20 min for lipid and 90 min for polymer) at which the reactions were no longer linear.

are such that these are values for total incorporation, rather than for the initial rates of incorporation.

Isolation and characterization of the carbohydrate attached to the mucoid sugar lipid. The mucoid sugar lipid was isolated from an in vitro reaction mixture as described in Materials and Methods and was hydrolyzed in 50% methanol to yield intact oligosaccharides. Half-times of hydrolysis (from linear semilog plots) were 7 min (0.05 N KOH, 37°C), 0.5 min (0.1 N HCl, 100°C), and 140 min (0.01 N HCL, 37°C). These times are similar to those of known pyrophosphate and phosphodiester-linked sugar lipids (4, 42). The products of base hydrolysis were chromatographed on Bio-Gel P-2 columns (Fig. 6). The three major peaks had essentially identical fucose-glucose ratios of 1.15 (Table 6), and when chromatographed in high salt on G-25 Sephadex to minimize charge effects, peaks B and D eluted in the same position. This position indicated a molecular weight of 900 (Table 7). Peak B was the major product of mild-base hydrolysis and it was converted quantitatively to peak A by further base hydrolysis. Peak A was converted to peak D by treatment with alkaline phosphatase. These results indicate that the mucoid lipid contains a single oligosaccharide which appears to be a hexasaccharide containing three fucose and three glucose residues (calculated 942 molecular weight). There was no galactose present in the oligosaccharide as none was found when the oligosaccharide was hydrolyzed, and the products were chromatographed using solvents 1 and 2 on either paper or thin-layer plates. Mild-base hydrolysis released this oligosaccharide in a form that behaved as a cyclic phosphate (peak B) which was converted to an open phosphate (peak A) by further hydrolysis. The open phosphate was converted to a neutral oligosaccharide by treatment with alkaline phosphatase. The results of paper electrophoresis using buffer B were consistent with the proposed structures for the peaks, as peak D did not migrate on electrophoresis, whereas peaks B and A migrate at 0.42 and 0.52 times the rate of glucuronic acid, respectively.

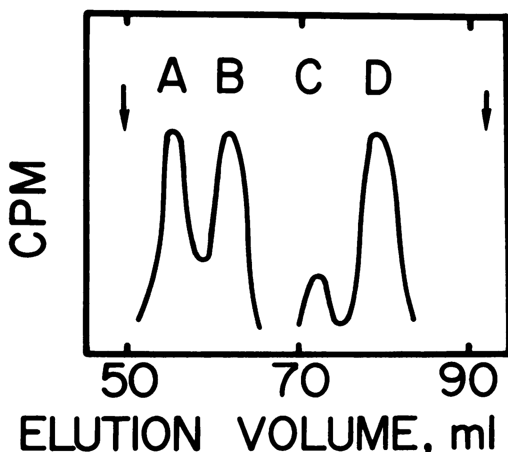


FIG. 6. *Bio-Gel P_2 chromatography of the hydrolysis products of the mucoid lipid. Mucoid lipid was isolated and hydrolyzed, and the hydrolysis products were chromatographed on Bio-Gel P_2, as described in the text.*

The reducing terminal sugar of the oligosaccharide was determined by borohydride reduction and found to be glucose. This is the result one would expect from the fact that UMP but not GMP inhibited the incorporation into the mucoid lipid.

The nonmucoid sugar lipid was isolated from incubations containing UDP-[^{14}C]glucose and hydrolyzed in 50% methanol containing 0.01 M HCl at 37°C (half-time = 160 min). The products were identified as the monosaccharides glucose and galactose by chromatography in solvents 1 and 2 and chromatography on Bio-Gel 2. The galactose was presumably formed by the action of epimerase on the UDP-glucose. The glucose lipid was somewhat resistant to base hydrolysis in 50% methanol (half-times of 3 and 35 min at 100°C in 0.5 and 0.05 N KOH, respectively). It was not hydrolyzed under deacylating conditions (0.11 N NaOH, 37°C, 10 min).

Mucoid polymer product in vitro. Soluble labeled mucoid polymeric material could be recovered from a reaction mixture by extracting it with water, 0.2 N NaOH, or cold 7% trichloroacetic acid or by washing with saline after heating it at 100°C for 10 min. These treatments suggest that the product was truly polysaccharide. The yields were 30 to 70% of the amounts seen in the paper chromatography assay for polymer. Polymer could also be recovered from the aqueous phase of the Folch wash used for lipid assay.

The polymeric product was entirely excluded from Sepharose-6B run in 0.05 M ammonium acetate. It was insensitive to α-amylase and amyloglucosidase.

It was difficult to determine accurately the fucose-glucose ratio because glucose was incorporated in large amounts in the other two polymeric forms described above. The ratio was determined by using inhibitors (ADP and UDP) which blocked these other incorporations without affecting mucoid incorporation. The result in Table 7, fucose-glucose = 1.0, shows that the in vitro polymer product has a composition similar to that of its putative precursor, the sugar

TABLE 6. *Characterization of the oligosaccharides from the mucoid lipid*

P_2 peak[a]	Substance	Mol wt		Fucose-glucose ratio
		Bio-Gel P_2	Sephadex G-25	
Starting material	Sugar lipid			1.16
A	Oligomer-phosphate	2,500	1,700	1.12
B	Oligomer-cyclic phosphate	1,700	900	1.15
C	Neutral oligomer	1,180	930	1.10
D	Neutral oligomer	900	900	1.11
	Glucose-6-P	700	430	

[a] The peaks A, B, C, and D were prepared by acid hydrolysis of isolated sugar lipid as described in the methods section. Peaks C and D were also obtained by base hydrolysis followed by alkaline phosphatase digestion as described in the text. The P_2 column was run as described, whereas the Sephadex G-25 column (1.5 by 85 cm, superfine) was run in 0.5 M NaCl, 0.1 M ammonium formate at 10 ml/h. The fucose-to-glucose ratio was determined for each peak as described.

TABLE 7. *Determination of the fucose-glucose ratio of the mucoid polymer synthesized in vitro*

Incubation	Substrate[a]	Incorporation (nmol/h per mg)
1	GDP-[^{14}C]fucose	0.067
2	GDP-[^{14}C]fucose + UDP-glucose	0.148
3	UDP-[^{14}C]glucose	0.023
4	UDP-[^{14}C]glucose + GDP-fucose	0.105
	Ratio of fucose to glucose is no. 2 − no. 1/no. 4 − no. 3 = 1.0	

[a] Each reaction was run as described in the text except that 12.1 mM ADP and 9.3 mM UDP were present to inhibit nonmucoid polymer synthesis and 0.44 mg of membrane was used. The reactions were run for 90 min at 28°C. The concentration of GDP-fucose was 0.44 mM and the concentration of UDP-glucose was 0.059 mM.

lipid, whose ratio was 1.15. The ratio was also determined to be 1.07 by destroying most of the other polymeric materials with α-amylase and amyloglucosidase, leaving the mucoid polymer relatively untouched.

Characterization of the mucoid sugar lipid. Sugar lipid was extracted from a scaled-up standard reaction mixture by the Folch procedure and purified on Sephadex LH-20 (Fig. 7, left) and on DEAE-cellulose (Fig. 7, right) columns. The column profiles closely match those of Rha-Gal-P-P-undercaprenol (4). The elution position from the DEAE column shows the sugar lipid to be a doubly charged anion, as expected of a pyrophosphate. Both columns were reproducible and quantitative, accounting for all the label and phosphate applied. Subsequent silica-gel thin-layer chromatography (TLC) was necessary for more complete purification of the sugar lipid (R_f in solvent 3 to 0.06).

A large-scale isolation of sugar-lipid yielded 80 pmol/mg of particulate protein, corresponding to 0.1% of the cell's total phospholipid, about the same as found for the peptidoglycan (38) and capsule (37) sugar-lipid intermediates.

An attempt was made to isolate the active

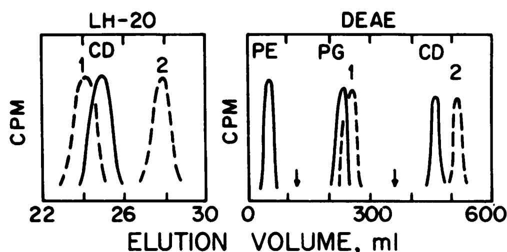


FIG. 7. *Column chromatography of sugar lipids. The Sephadex LH-20 column was 55 by 1.1 cm and was run in 0.1 M ammonium acetate-0.13 M acetic acid in 99% methanol (4). The DEAE-cellulose column, 40-ml total volume, was prepared as described by Dankert et al. (4). After sample application, the column was eluted first with 99% methanol, then with a linear gradient from 0 to 15 mM ammonium acetate in 99% methanol (first arrow), and finally with a second linear gradient from 15 to 150 mM ammonium acetate in 99% methanol (second arrow). Peak 1 is [^{14}C]mannose-P-undecaprenol, and peak 2 is the mucoid sugar lipid. Abbreviations: PE, Phosphatidylethanolamine; PG, phosphatidyl glycerol; and CD, cardiolipin. Phosphate (——); ^{14}C (-----). Peak 1 from the LH-20 column also contains PE and PG.*

acceptor lipid itself from total cell lipids, using as an assay the regeneration of incorporating activity in a delipidated particulate preparation upon the addition of lipid fractions. The procedure described by Troy et al. (37) was followed. Very weak stimulatory activity was found in the lipid fractions eluting just beyond cardiolipin on the DEAE column (Fig. 7, right). No activity was found elsewhere, particularly in the region near phosphatidylglycerol where P-lipid might be expected (14). The stimulatory lipid is therefore doubly charged and might be sugar(s)-P-P-lipid itself. Perhaps the delipidated particulate system is so damaged that it requires lipid already carrying some sugar, rather than the expected P-lipid. The stimula-

tory activity was extremely weak and its isolation was not pursued further.

Characterization of the glucose lipid. The glucose lipid was isolated from scaled-up reaction mixtures by the procedure described.

It was inseparable from authentic mannose-P-undercaprenol on column and TLC (Fig. 7; R_f = 0.16, 0.44, and 0.03 on silica gel TLC in solvents 3, 4, and 5, respectively). A large-scale preparation from M6-2 yielded 400 pmol/mg of particulate protein, about 0.5% of total cell phospholipid. Figure 8 shows the nuclear magnetic resonance spectrum of the glucose lipid, which closely resembles published spectra of undercaprenol lipid carriers (11). The ratios of *cis*-to-*trans* (—CH = $\underset{|}{\overset{CH_3}{C}}H_3$ CH$_2$—) groups, i.e., τ = 8.34/τ = 8.40, is about 3:1.

DISCUSSION

The mucoid strain M6-2 appears to make a single extracellular polysaccharide containing fucose and glucuronic acid, identical with colanic acid. In vitro, a single fucose containing sugar lipid and its product polymer are produced from nucleotide sugars. The nonmucoid parent strain 3300 produces no polysaccharide fucose in vivo and incorporates no fucose in vitro. On the basis of the results of this work colanic acid is concluded to be synthesized by the scheme nucleotide sugars → sugar-lipid intermediate(s) → polymer.

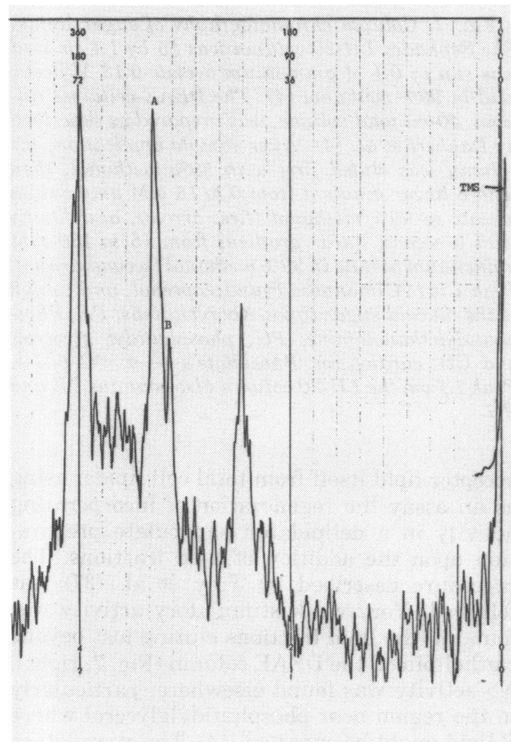


FIG. 8. *Nuclear magnetic resonance spectrum of the glucose lipid. Purified glucose lipid (340 nmol in 0.4 ml of $CDCl_3$ + 1% internal TMS) was scanned at 90 M Hz on a Bruker HX-90 nuclear magnetic resonance spectrometer at 30°C.*

The single sugar-lipid intermediate seen in this work has the structure (fucose$_3$, glucose$_2$)-glucose-P-P-lipid. The pyrophosphate was shown by the UMP reversal, the bacitracin inhibition, and the elution position on DEAE-cellulose. Other properties, such as the rate of hydrolysis by acid and base and its mobility on silica gel TLC, were consistent with this structure. Bacitracin inhibition suggests that the lipid may act as a cycling coenzyme carrier.

The sugar lipid and the polymer made in vitro possess only fucose and glucose in equal amounts rather than the six sugars composing the repeat unit of colanic acid (35). The system is probably damaged during preparation so that it produces in vitro an aberrant version of the colanic acid precursor. The hexasaccharide containing equal amounts of fucose and glucose which we find attached to the lipid is difficult to relate to colanic acid. One possibility would be that the system synthesizes a dimer of the repeat unit which has side-chain glucose rather than the galactose found in colanic acid. Lack of specificity in in vitro reactions is a common hazard, particularly when the system is dependent on organized membrane structures. A relevant example is that *Salmonella* in vivo utilizes only the full O-antigen tetrasaccharide sugar lipid

for polymerization into O-chains (43), whereas, in vitro, O-chains of the same length are made from the trisaccharide sugar lipid lacking side-chain abequose (24). The fact that the polymer made in vitro also has a ratio of glucose-to-fucose of one is consistent with the other evidence that the fucose-containing sugar lipid is an intermediate of the fucose-containing polymer. Since these compounds are only made by strain M6 and colanic acid appears to be the only fucose-containing polymer made in strain M6, it seems highly probable that the reactions we are studying are related to those involved in colanic acid biosynthesis.

This work was complicated greatly by the presence of several incorporation systems acting simultaneously (reactions 3, 4, and 5). The

various reactions could be separated by using appropriate substrate levels (see K_m values in Table 6) or, most efficiently, by selectively inhibiting them. At the substrate concentrations normally used, 5 mM UDP completely blocked mucoid incorporations, 5 mM UDP blocked the glucose lipid and glucose polymer, and 13 mM ADP blocked glucan production.

It would be interesting to know whether the lipid portion of both the mucoid sugar lipid and the glucose lipid is the same and whether it is the same C_{55}-polyisoprenol found in other membrane polymer synthesis systems (16). Chromatographically, both sugar lipids behaved as known pyrophosphate- or phosphodiester-undecaprenols. The glucose lipid was inseparable from authentic mannose-P-undecaprenol and had a similar nuclear magnetic resonance spectrum. The bacitracin inhibition of the mucoid system indicates a C_{55}-isoprenyl pyrophosphate. Bacitracin inhibited the glucose lipid, but not in a way so precisely characteristic of C_{55}-isoprenol pyrophosphate, and, in view of the strong evidence for the glucose lipid being a phosphodiester, this inhibition appears artifactual. Both lipids appear similar to the classic undecaprenol carrier lipids.

ACKNOWLEDGMENTS

This investigation was supported by Public Health Service grant 501 AM 11923 from the National Institute of Arthritis, Metabolism and Digestive Diseases.

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