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Biosynthesis of Sulfated Saccharides *N*-Glycosidically Linked to the Protein via Glucose

PURIFICATION AND IDENTIFICATION OF SULFATED DOLICHYL MONOPHOSPHORYL TETRASACCHARIDES FROM HALOBACTERIA*

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A novel type of *N*-glycosidic linkage, asparaginyl glucose, occurs in the cell surface glycoprotein of halobacteria (Wieland, F., Heitzer, R., and Schaefer, W. (1983) *Proc. Natl. Acad. Sci. U. S. A.* 80, 5470-5474). Sulfated oligosaccharides containing glucuronic acids are attached to the polypeptide chain via this linkage.

Here we describe the isolation and chemical characterization of lipid-linked precursors of these saccharides, and these have the following new features. 1) Rather than the bacterial undecaprenol, a C_{60}-dolichol is the carrier lipid. 2) The oligosaccharide is bound to this lipid via a monophosphate, rather than a pyrophosphate bridge. 3) Sulfation of the saccharides is completed while they are linked to lipid and does not occur after transfer of the saccharides to protein.

The most common linkage between carbohydrates and proteins involves *N*-acetylglucosamine attached to the amido nitrogen of asparagine. For a long time, this has been the only *N*-glycosidic linkage known (2), and its biosynthesis has been elucidated during the last decade; a unique carbohydrate core is established by sequential addition of nucleotide- or lipid monophosphate-activated sugars to dolichyl pyrophosphoryl *N*-acetylglucosamine.

After completion, this saccharide core is transferred "en bloc" from the lipid pyrophosphate to the protein. Only after this transfer does processing of this unique core occur, resulting in the different types of *N*-linked saccharides. Chemical modification of glycoconjugates, *e.g.* sulfation or phosphorylation, has been reported to occur only at the protein-bound level (3, 4).

Recently, glycopeptides from the cell surface glycoprotein of the archaebacterium *Halobacterium halobium* have been described, which contain glucose, glucuronic acid, and sulfate (1). The linkage unit to the protein of these saccharides is asparaginyl glucose and not asparaginyl GlcNAc. The question arose as to how this novel *N*-glycosidic linkage is synthesized, and the aim of this work was to isolate and characterize precursors of these sulfated saccharides.

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EXPERIMENTAL PROCEDURES

Analytical Methods

Carbohydrate Analyses—Neutral sugars were analyzed by GLC[1] as their alditol acetates (5) on a Durabond 1701 capillary column (30 m, ICT Laboratories, Frankfurt). Samples were hydrolyzed in 2 M trifluoroacetic acid for various times at 100 °C. Colorimetric estimations were done with the untreated samples using the anthrone method (6) for neutral sugars, with glucose as standard. Uronic acids were analyzed by either of the following methods: quantitatively by the colorimetric assay described in Ref. 7, with glucuronic acid as standard; qualitatively by methanolysis (0.5 N HCl in methanol at 80 °C for 20 h) and subsequent GLC as their pentafluoropropionyl derivatives on a Durabond capillary column (30 m, carrier gas He, 1 ml/min, starting at 120 °C with a temperature gradient of 2 °C/min). Pentafluoropropionylation was performed as described for trifluoroacetylation in Ref. 8. The use of pentafluoropropionic anhydride rather than trifluoroacetic anhydride for derivatization has the advantage of allowing the separation, by GLC, of the main peaks resulting from Glc and GlcUA. This cannot be accomplished on a Durabond 1701 capillary with the respective trifluoroacetylmethylglycosides. Alternatively, uronic acids were analyzed by GLC of their peracetylated neutral sugar alditol derivatives. For this purpose, after hydrolysis (2 N trifluoroacetic acid, 100 °C, 4 h) the samples were repeatedly lactonized and reduced with sodium borohydride according to Ref. 9.

Determination of reducing sugars was performed by reduction with sodium [^{3}H]borohydride hydrolysis and subsequent high-voltage thin-layer electrophoresis in sodium borate, mainly according to the procedure described in Ref. 10.

Amino sugars were analyzed using an automated amino acid analyzer (LC 5000, Biotronic, FRG) after hydrolysis of the samples in 4 N trifluoroacetic acid for various times at 100 °C.

Quantification of Sulfate and Phosphate—Sulfate was determined with the colorimetric assay described in Ref. 11 after hydrolysis of the samples for 2 h at 100 °C in 6 N HCl. Phosphate was determined after heating of the samples in 250 μl of 7.2 N sulfuric acid for 30 min at 300 °C. After cooling to room temperature, 20 μl of 30% H_2O_2 (Perhydrol, Merck, Darmstadt) were added, and the samples were heated again to 300 °C for 15 min. Then 2.75 ml of water and 250 μl of a solution of 3% (w/v) ammonium molybdate in 0.12% Triton X-100 (Serva, Heidelberg) were added. The samples were mixed, and after exactly 20 min at room temperature, the turbidity was measured at 510 nm against a blank. This procedure is adapted from Ref. 12 and is described in detail in the data sheet of a commercially available phosphate test (Serva, Heidelberg).

Thin-layer Chromatography—Thin-layer chromatography was performed on aluminum sheets (Silica Gel 60, 0.2 mm, Merck, Darmstadt) using one of the following solvents: A, propan-1-ol/H_2O (65:35); B, $CHCl_3$/CH_3OH/H_2O (60:25:4); C, $CHCl_3$/CH_3OH/HCOOH/H_2O (70:18.5:7:0.5).

Spots were visualized by spraying with one of the following reagents: 1) 50% H_2SO_4, 100 °C, 10 min for organic material; 2) orcinol reagent (13) for saccharides; 3) aniline reagent (14) for reducing

[1] The abbreviations used are: GLC, gas-liquid chromatography; SP, sulfated precursor; HPLC, high-performance liquid chromatography; GlcUA, glucuronic acid.

sugars; 4) anisaldehyde reagent (15) for unsaturated lipids; 5) spray for phosphate-containing lipids according to Ref. 16. Radioactive spots were detected by autoradiography.

Purification of Glycopeptides from the H. halobium Cell Surface Glycoprotein

Pronase digestion and purification of acidic glycopeptide fractions containing sulfate is described in Ref. 1. The fractions corresponding to bands III and IV in Fig. 2 of Ref. 1 were further purified by chromatography on "Mono Q" (Pharmacia, Sweden) under the following conditions. Binding to the column and subsequent washing for 10 min was performed using 0.1 M *N*-methylmorpholine acetate buffer, pH 7.0. Then a linear gradient was applied from 0 to 50% of 1 M LiCl in the above buffer over the course of 50 min. The flow rate was 1 ml/min. Saccharides were detected by spotting onto silica gel and spraying with orcinol reagent. Peak fractions of carbohydrate were pooled, and after evaporation the dry residues were extracted twice with 3–5 ml of absolute ethanol to eliminate buffer salts as well as LiCl. Desulfation, permethylation, and ^2H reduction of the samples was performed as described under "Experimental Procedures."

Preparation and Characterization of Sulfated Lipid-linked Oligosaccharides

Halobacterial Growth and $^{35}SO_4^{2-}$ Labeling—H. halobium strain R_1M_1 was grown in 50-liter cultures in complex medium as described in Ref. 17. For $^{35}SO_4^{2-}$ labeling, cells were grown in 40-ml cultures of sulfate-depleted synthetic medium (18) in 100-ml Erlenmeyer flasks with shaking (100 rpm, 37 °C). At early stationary phase, the cells were concentrated 20-fold in sulfate-free basal salts and illuminated as in Ref. 17. After 60 min, 125 μCi of sodium [^{35}S]sulfate (carrier free, Amersham Corp.) was added per 1 ml, and the cell suspension was further illuminated for 6 h at 37 °C with gentle stirring. Thereafter the cells were harvested by centrifugation (10,000 × *g* for 10 min).

Isolation of Sulfated Lipid-linked Oligosaccharides SP—Cells were lysed by hypoosmosis. The pellet from a 50-liter culture was mixed with 2 liters of deionized water containing 5 mg of DNase I (Boehringer Mannheim) and homogenized for 4 h with vigorous stirring. After centrifugation (38,000 × *g* for 14 h), the combined pellets were washed with 1 liter of water and centrifuged again (38,000 × *g* for 4 h). The final pellet was resuspended in water to give a total volume of 54 ml. The homogenized membrane fraction was extracted with 1140 ml of $CHCl_3/CH_3OH$ (3:2) and centrifuged (23,000 × *g* for 10 min). The pellet was resuspended in 900 ml of $CHCl_3/CH_3OH$ (3:2) with mechanical stirring and sonification and centrifuged (23,000 × *g* for 10 min) again. This step was repeated once. The pellet was extracted twice with 300 ml of $CHCl_3/CH_3OH/H_2O$ (10:10:3) with mechanical stirring, sonification, and centrifugation as above. The combined supernatants after extractions with $CHCl_3/CH_3OH/H_2O$ (10:10:3) were mixed with $^{35}SO_4^{2-}$-labeled material, which had been isolated in the same way, and evaporated to give a final suspension of about 15 ml. The following steps were monitored by counting the added radioactivity to indicate the presence of SP. Propan-1-ol was added to the suspension to give a final concentration of 90%. A precipitate formed and was removed by centrifugation (23,000 × *g* for 10 min). The pellet was extracted twice with 20 ml of propan-1-ol/H_2O (1:1), with mechanical stirring, but omitting sonification. After centrifugation (23,000 × *g* for 10 min), the combined supernatants were brought to 0.05 M ammonium acetate and submitted to chromatography on DEAE-cellulose (1.5 × 5 cm, DEAE-Sephacel, Pharmacia, Sweden) in the above buffer.

After washing with 4 bed volumes of starting buffer, the column was eluted with 1 M ammonium acetate in propan-1-ol/H_2O (1:1). Fractions containing radioactivity were pooled and brought to propanol-1/H_2O = 9:1. The precipitate was removed by centrifugation (35,000 × *g* for 10 min) and extracted twice with 4 ml of $CHCl_3/CH_3OH/H_2O$ (10:10:3) with sonification and subsequent centrifugation as above. The $CHCl_3/CH_3OH/H_2O$ (10:10:3) extracts were combined and submitted to HPLC on silica gel (25 × 1 cm, LiChrosorb, Si60, 7 μm, Merck, Darmstadt) according to Ref. 19 in solvent A ($CHCl_3/CH_3OH$/25% NH_3, 65:29:6). After washing with 120 ml of solvent A, a first gradient from 0–30% solvent B ($CHCl_3/CH_3OH$/25% NH_3/H_2O, 40:42:6:10) was run for a period of 20 min with a flow rate of 4 ml/min. Subsequently, a second gradient, ranging from 30–47% solvent B was applied over the course of 80 min and at a flow rate as above.

Fractions containing radioactivity were pooled as indicated, concentrated, and desalted by passage through a Bio-Gel P 2 column (6 × 1 cm, 200–400 mesh, Bio-Rad) in propan-1-ol/H_2O (1:1).

All steps except HPLC and desalting were performed at 4 °C.

Acid Hydrolyses—For mild acid hydrolysis the samples were incubated in 10 mM HCl with a drop of propan-1-ol for 5 min at 100 °C. These conditions are even more gentle than those described in Ref. 20 for the hydrolysis of the bond between the reducing end of dolichyl-linked oligosaccharides and the lipid-pyrophosphate residue.

Strong acid hydrolysis implies conditions under which a dolichyl pyrophospho-oligosaccharide will be cleaved into the corresponding lipid phosphate, inorganic phosphate, and the oligosaccharide. Two different conditions were used: 1 N HCl in 50% CH_3OH for 5 min at 100 °C, and 0.15 N HCl in 1-propanol for 5 min at 100 °C (21, 22).

Desulfation—The sample was subjected to mild acid hydrolysis, and after partition between chloroform and water, the carbohydrate (water phase) was reduced first with sodium [^{3}H]borohydride and then with unlabeled sodium borohydride. Subsequently desulfation was achieved by mild methanolysis; the sample was desalted by passage through a column of Dowex 50W-H^+ ion-exchange resin and repeated evaporation with methanol and, after drying, redissolved in 50 mM HCl in methanol and incubated for 2 h at 37 °C. This procedure is adapted from Ref. 23. The degree of desulfation was checked by gel filtration on Bio-Gel P-2; all of the $^{35}SO_4^{2-}$ radioactivity was eluted in the included volume of the column.

Permethylation and ^2H Reduction of Uronic Acids—The samples were desulfated as above, incubated at room temperature for 30 min in 5% NH_3/water (w/v) to hydrolyze uronic acid methyl esters, dried, and permethylated once according to Ref. 24 with the modifications in Ref. 25. The uronic methyl esters obtained after permethylation and purification on a Sep-Pack cartridge ((RP-18, Waters) 1. wash: 8 ml of H_2O; 2. wash: 6 ml of acetonitrile/H_2O (3:14); 3. wash: 2 ml of acetonitrile/H_2O (1:3), elution: 100% acetonitrile) were reduced with sodium [^{2}H]borohydride in tetrahydrofuran/ethanol as described in Ref. 26. After hydrolysis (4 N trifluoroacetic acid for 4 h at 100 °C), reduction with sodium borohydride, and peracetylation, the alditol derivatives were analyzed by GLC-mass spectroscopy on a Hewlett-Packard 5995 GLC/MS system equipped with a Durabond 1701 capillary column (30 m).

RESULTS

Structure of a Sulfated Asparaginyl Glucose-linked Saccharide—The cell surface glycoprotein from *H. halobium* contains sulfated saccharides consisting of Glc and GlcUA (1). Some of these saccharides were further characterized by permethylation. For that purpose, the samples (bands III and IV, Fig. 2 in Ref. 1) were subjected to ion-exchange chromatography as described under "Experimental Procedures." Two different resulting glycopeptides, containing Asp/Asn, Glc, and GlcUA in a ratio of 1:0.9:2.6 and 1:1.7:2.2, respectively, were desulfated, permethylated, and reduced with sodium [^{2}H]borohydride in order to label the uronic acids (for details see "Experimental Procedures"). After hydrolysis the monosaccharides were analyzed as their peracetylated methyl alditol derivatives by GLC-mass spectroscopy (*e.g.* Fig. 1). The compounds identified are listed in Table I. These data allow the following structures to be assigned to the tetrasaccharides: GlcUA(1→4)GlcUA(1→4)GlcUA(1→4)-Glc and Glc(1→4)GlcUA(1→4)GlcUA(1→4)Glc.

Detection of Sulfated Precursors—Long-term labeling of *H. halobium* with $^{35}SO_4^{2-}$ yields a single radioactive polypeptide, the cell surface glycoprotein (27). All of the radioactivity incorporated is recovered as sulfate after acid hydrolysis. Thus, pulse labeling with $^{35}SO_4^{2-}$ is the method of choice to study the biosynthesis of this glycoprotein. *In vivo* pulse labeling with $^{35}SO_4^{2-}$ for 30 min (corresponding to 5% of the generation time) according to Ref. 17 and subsequent analysis of cell lysates by sodium dodecyl sulfate-polyacrylamide gel electrophoresis yields the fluorograph shown in Fig. 2, *lane B*. After a chase with unlabeled sulfate for the periods indicated, the patterns in *lanes C* to *G* were obtained. Clearly, a low-

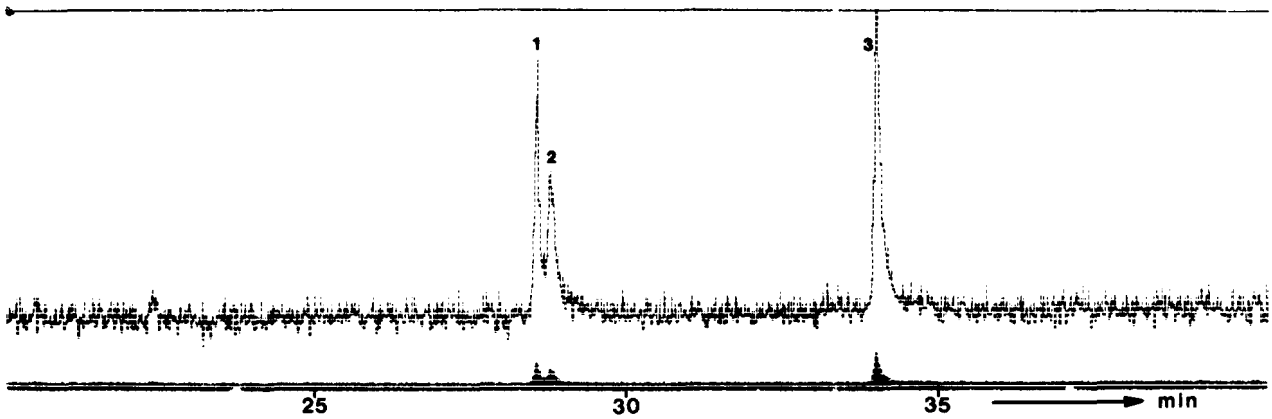


FIG. 1. **GLC-mass spectroscopic analysis of an asparaginyl saccharide after permethylation, reduction of the resulting uronic methyl esters with Na[^{2}H]BH_4, hydrolysis to monosaccharides, reduction with $NaBH_4$, and peracetylation; total ion profile.** For details see "Experimental Procedures." *1*, 1,4,5-tri-*O*-acetyl-2,3,6-tri-*O*-methylhexitol; *2*, 1,5,6-D_2-tri-*O*-acetyl-2,3,4-tri-*O*-methylhexitol; *3*, 1,4,5,6-D_2-tetra-*O*-acetyl-2,3-di-*O*-methylhexitol.

TABLE I

Partially methylated peralditol acetates as identified by GLC/mass spectroscopy after methylation

A, asparaginyl saccharides from the cell surface glycoprotein; B, fraction 2; and C, fraction 3 after gel filtration (*cf.* Fig. 5) of the saccharide moiety of SP-II, respectively. For details see "Experimental Procedures."

A	B	C	Compound originating from:
1,4,5-Tri-*O*-acetyl-2,3,6-tri-*O*-methylhexitol			R-$_4$Glc$_1$-Asn
1,5,6-D_2-Tri-*O*-acetyl-2,3,4-tri-*O*-methylhexitol		1,5,6-D_2-Tri-*O*-acetyl-2,3,4-tri-*O*-methylhexitol	GlcUA$_1$-R
1,5-Di-*O*-acetyl-2,3,4,6-tetra-*O*-methylhexitol	1,5-Di-*O*-acetyl-2,3,4,6-tetra-*O*-methylhexitol		Glc$_1$-R
	4-*O*-Acetyl-1,2,3,5,6-penta-*O*-methylhexitol	4-*O*-Acetyl-1,2,3,5,6-penta-*O*-methylhexitol	R-$_4$Glc$_1$-ol
1,4,5,6-D_2-Tetra-*O*-acetyl-2,3-di-*O*-methylhexitol	1,4,5,6-D_2-Tetra-*O*-acetyl-2,3-di-*O*-methylhexitol	1,4,5,6-D_2-Tetra-*O*-acetyl-2,3-di-*O*-methylhexitol	R-$_4$GlcUA$_1$-R

molecular-weight component (*lower arrow* in Fig. 2) exhibits a high rate of turnover. All of the radioactivity of this component is finally incorporated into the cell surface glycoprotein, as found by quantitative evaluation of a long-term chase experiment. This sulfated low-molecular-weight component is consequently a likely candidate for a biosynthetic precursor. The material will, therefore, be termed SP and is the subject of this study. A set of additional labeled components of intermediate molecular weight also seems to be involved in the biosynthesis of the glycoprotein but will not be dealt with in this paper.

Isolation of the SP—The sulfated precursor behaves like a lipid with strong amphiphilic character. It is soluble in $CHCl_3$/CH_3OH/H_2O, 10:10:3, like lipid-linked oligosaccharides but is soluble in 50% aqueous propan-1-ol as well. On the other hand, it can be precipitated by increasing the concentration of propan-1-ol to 90% (v/v). In Scheme 1, the individual steps in the isolation of the sulfated precursor are listed. After adsorption to DEAE-cellulose, elution with 1 M ammonium acetate, precipitation from 90% aqueous propan-1-ol, and desalting by gel filtration on Bio-Gel P-2, the radioactive fraction was further purified by HPLC on silica gel. Fig. 3*A* shows the radioactivity profile obtained by applying a gradient of the solvents composed as described under "Experimental Procedures." Three main peaks were observed and will be referred to hereafter as SP-I, -II, and -III. Clearly, these pools do not represent homogeneous substances. As will be shown in the following section for SP-II, this pool consists of three compounds differing in their saccharide moieties. However, each of the pools on sodium dodecyl sulfate-polyacrylamide gel electrophoresis behaves like SP (see *arrow* in Fig. 2). Silica gel thin-layer chromatography showed that each pool contained substantial amounts of organic material co-migrating with the added tracer radioactivity, as revealed by comparison of the patterns obtained by fluorography and detection with sulfuric acid (not shown).

The observed heterogeneity possibly reflects different stages in the biosynthesis of the saccharide moiety. To check this suggestion, an *in vivo* $^{35}SO_4^{2-}$ pulse was performed in the presence of ethidium bromide (at a concentration which leads to a drastic decrease of incorporation of ^{35}S-labeled methionine into *H. halobium* proteins). Under such conditions of suppressed protein synthesis one should expect an arrest of SP synthesis at a defined stage leading to a more homogeneous product. This experiment is shown in Fig. 3*B*; peaks I and II decrease and peak III increases drastically. Therefore, at least part of the observed heterogeneity (Fig. 3*A*) reflects different stages during biosynthesis of the sulfated precursor.

The Purified SPs Contain Carbohydrate, Lipid, and Phosphate—Each fraction obtained after HPLC on silica gel contains neutral sugars and uronic acids as well as phosphate. Sulfate is found in amounts equivalent to uronic acid. This

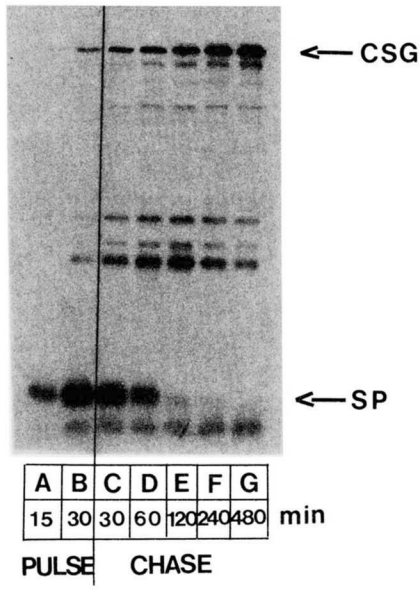
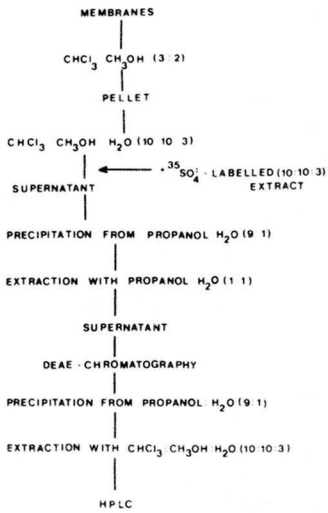


FIG. 2. **$^{35}SO_4^{2-}$ pulse-chase labeling of halobacteria.** *H. halobium* strain R_1M_1 was grown in a sulfate-depleted medium, concentrated in sulfate-free basal salts, and illuminated (17). After 5 min $^{35}SO_4^{2-}$ sulfate (100 μCi, carrier free, Amersham, Frankfurt) was added per ml. At the times indicated aliquots were taken and immediately lysed by hypoosmosis. The chase was performed after 30 min by addition of 1 volume of complex medium (which contains 80 mM $MgSO_4$). The lysed samples were submitted to sodium dodecyl sulfate-gel electrophoresis on a 12% polyacrylamide gel as described in Ref. 17. The figure shows a fluorograph of the gel. *CSG*, cell surface glycoprotein.



SCHEME 1. **Individual steps during isolation of sulfated precursors.** Details are given under "Experimental Procedures."

composition is compatible with that of the glycopeptides mentioned above. Repeated purifications yielded different amounts of SP-I, -II, and -III. We have collected the material corresponding to SP-II, which predominated in most of the preparations, and this material was further investigated. To obtain a first insight into the structure, the material was incubated under conditions known to hydrolyze lipid-pyrophosphate oligosaccharides (10 mM HCl, 5 min at 100 °C (20)). After partition between chloroform and water the products in both phases were analyzed by silica gel thin-layer chromatography (see Fig. 4). Spots on the chromatograms were visualized by different reagents with specificities for organic material (Fig. 4, *lane A*), carbohydrate (*lane B*), reducing sugar (*lane C*), unsaturated lipid (*lane D*), lipid phosphate ester (*lane E*), and $^{35}SO_4^{2-}$ radioactivity was visualized by autoradiography (*lane F*). From the patterns obtained, one must conclude that mild acid hydrolysis splits the material into a lipid phosphate ester (chloroform phase) and a sulfated carbohydrate (water phase).

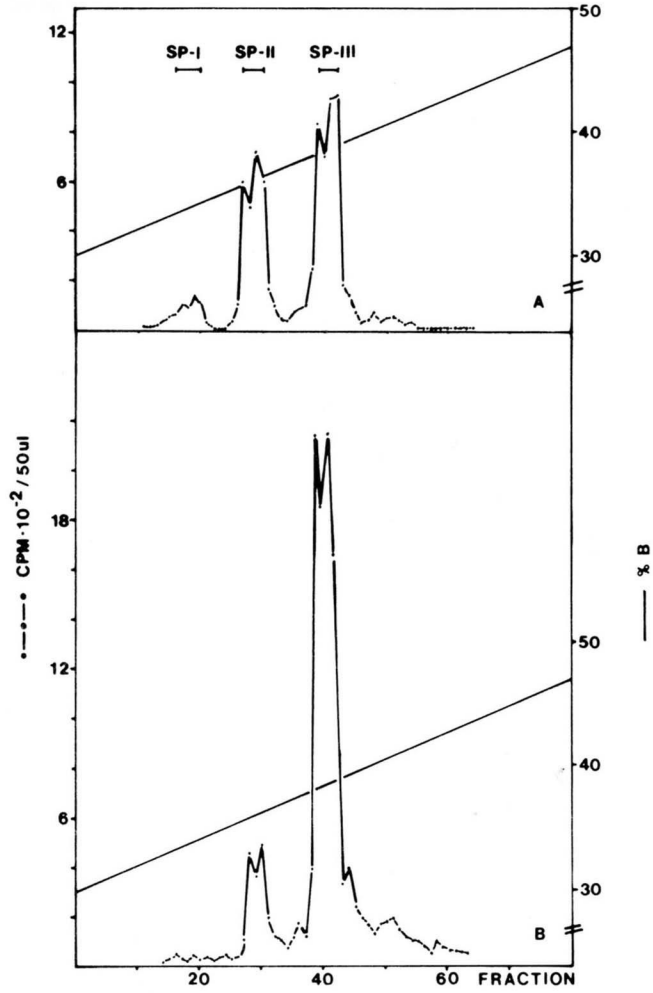


FIG. 3. **Separation of sulfated precursors by HPLC.** The radioactive sample obtained after ion-exchange chromatography and precipitation from propan-1-ol/H_2O (9:1) and extraction with chloroform methanol/water (10:10:3) was chromatographed on a silica gel column as described under "Experimental Procedures." •–•–•, radioactivity profile; ——, proportion of solvent B. Fractions indicated by *bars* were pooled. *A*, SP from cells labeled with $^{35}SO_4^{2-}$ without inhibition of protein synthesis (for details see "Experimental Procedures"). *B*, SP from cells labeled in the presence of ethidium bromide (40 μg/ml) to inhibit protein synthesis.

The saccharide moiety of SP-II, after mild acid hydrolysis and reduction, was desulfated and subjected to gel filtration as described under "Experimental Procedures." The sugar composition in each of the three resulting fractions (see Fig. 5) was determined quantitatively by colorimetric assays and in the amino acid analyzer. Only neutral sugars and uronic acids were detected. The nature of these sugars was determined by GLC after methanolysis and derivatization with pentafluoropropionic anhydride or as their peralditol acetates. The following compositions were found: fraction 1, 2 GlcUA:1 Glc and an additional unidentified compound; fraction 2, 1 GlcUA:1 Glc; and fraction 3, 2–3 GlcUA:1 Glc.

As we have not yet identified the unknown compound in fraction 1, fractions 2 and 3 were further investigated.

The Carbohydrate Is Linked to the Lipid via Glucose—To

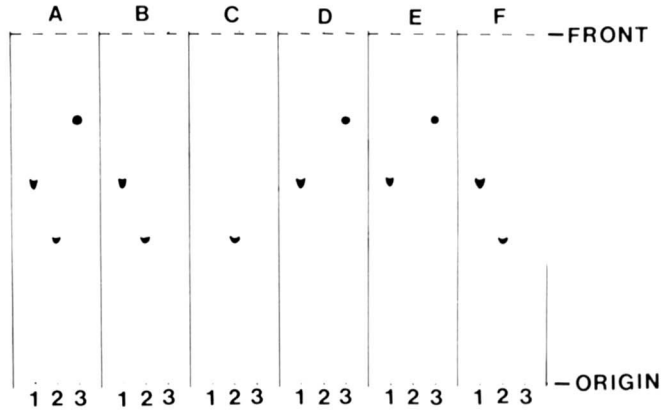


FIG. 4. **Thin-layer chromatographic analysis on silica gel of SP-II before and after mild acid hydrolysis, followed by partitioning between chloroform and water.** The figure is a drawing of chromatograms after staining with different sprays for: *A*, organic material; *B*, saccharides; *C*, reducing sugar; *D*, unsaturated lipids; *E*, lipid phosphates. *F* is an autoradiograph to detect $^{35}SO_4^{2-}$ radioactivity. *Lane 1*, untreated SP-II; *Lane 2*, H_2O phase after acid treatment; *Lane 3*, chloroform phase after acid treatment. Conditions are described under "Experimental Procedures."

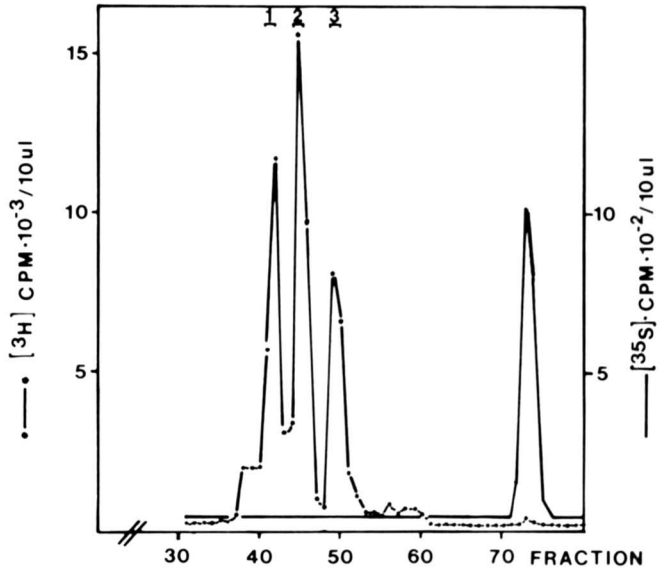


FIG. 5. **Gel filtration of SP-II after mild acid hydrolysis, desulfation, and subsequent ^3H reduction.** The sample (H_2O phase after partitioning between H_2O and chloroform) was ^3H reduced and desulfated as described under "Experimental Procedures" and chromatographed on a column (160 × 0.8 cm) of Bio-Gel P-2 (minus 400 mesh) in pyridine acetate (0.1 M, pH 5.5). The fractions indicated by *bars* were pooled. •–•–•, ^3H radioactivity; ——, $^{35}SO_4^{2-}$ radioactivity.

analyze the sugar linked to the lipid phosphate, the ^3H-reduced fractions 2 and 3 in Fig. 5 were hydrolyzed (2 N trifluoroacetic acid for 4 h at 100 °C) and subjected to high-voltage thin-layer electrophoresis in the presence of borate. As shown in Fig. 6, the only radioactive compound resulting from fraction 3 is glucitol (*lane B*). Analysis of fraction 2 gave the same result. Consequently, glucose is the link sugar.

Permethylation Analysis of the Carbohydrate Moiety—After permethylation of the reduced and desulfated material of fractions 2 and 3 and reduction of the resulting glucuronyl methyl esters with sodium [^2H]borohydride, the samples were hydrolyzed and analyzed by GLC-mass spectroscopy as their peracetylmethylalditol derivatives. The substances observed are listed in Table I. The occurrence of a pentamethylhexitol

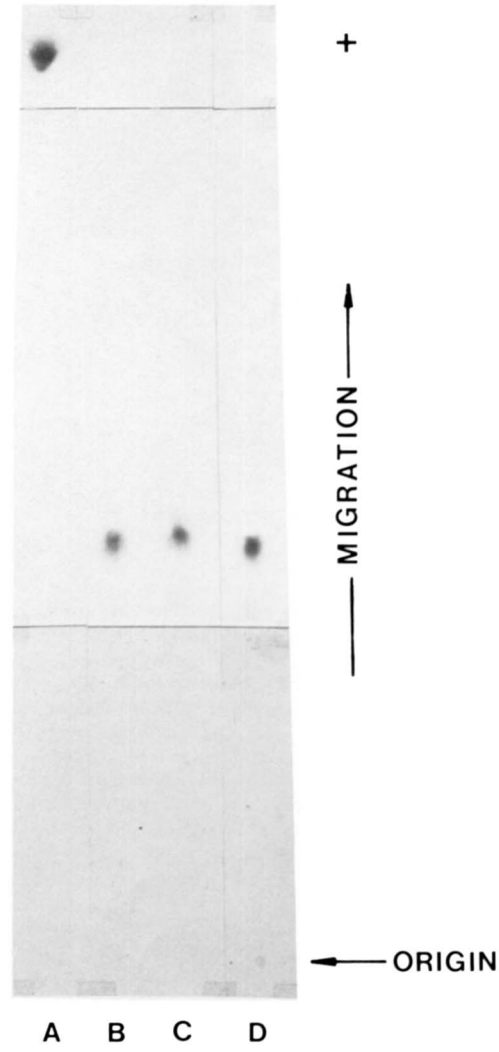


FIG. 6. **Determination of the reducing end.** The sample (pool 3 in Fig. 5) was submitted to high-voltage thin-layer electrophoresis as described under "Experimental Procedures" and the resulting electropherogram was fluorographed. *A*, untreated sample; *B*, after hydrolysis (2 M trifluoroacetic acid for 4 h at 100 °C); *C*, [^3H]glucitol; *D*, mixture of *B* and *C*.

TABLE II

Quantification of phosphate in SP-II and after hydrolysis of SP-II and partition between H_2O and chloroform. For details see "Experimental Procedures."

Experiment	PO_4^{3-}		
	SP-II	Chloroform phase	H_2O phase
	nmol		
1	24.0	23.5	0.25
2	41.0	39.2	0.5

confirms a glucose residue to be the linkage sugar. The C_6-dideuterated dimethylhexitol tetraacetates must be derived from 1,4-linked glucuronic acids, whereas the tetramethylhexitoldiacetate derives from a peripherally bound glucose (peak 2) and the trimethyl C_6-dideuterated hexitoltriacetate (peak 3) reflects a peripherally bound glucuronic acid. The sequences of the saccharides are, therefore: peak 2, Glc(1→4)GlcUA(1→4)GlcUA(1→4)Glc-ol; and peak 3, GlcUA(1→4)(GlcUA(1→4))$_{1-2}$Glc-ol.

The structures of fractions 2 and 3 are in agreement with

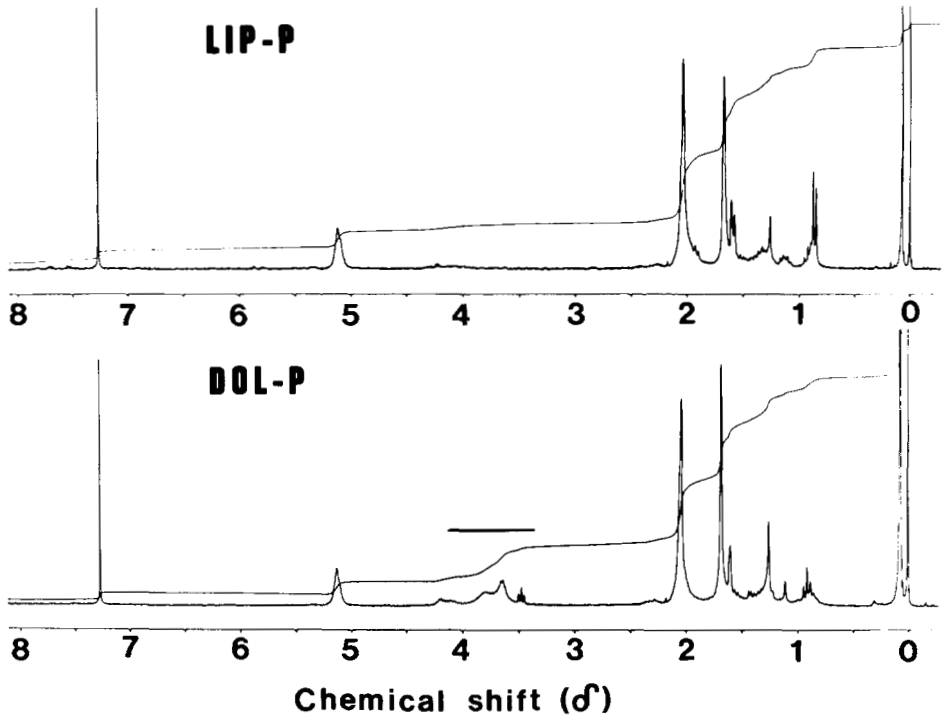


FIG. 7. **Proton NMR spectroscopy.** *Lip-P*, the chloroform phase after partial acid hydrolysis of SP-II and partitioning between chloroform and water. Samples were dissolved in $[^2H]CHCl_3$. *Dol-P*, commercially available dolichyl phosphate with a chain length of 80 to 105 carbon atoms. The *bar* indicates contaminating material in commercially available dolichyl phosphate. (C_{80-105}). The spectra were taken at 250 MHz using the Fourier transform method.

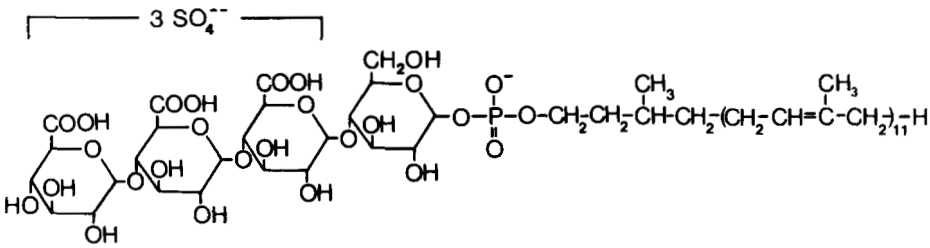


FIG. 8. **Molecular structure of one sulfated precursor as deduced from the data given under "Results."**

those of the asparaginyl saccharides described above.

One Mol of SP Contains Only One Mol of Phosphate—All lipid-linked oligosaccharide precursors known so far are bound to their lipid residue via pyrophosphate. To investigate further the structure of SP, phosphate was quantitatively determined. After strong acid hydrolysis (conditions that split a dolichyl pyrophospho-oligosaccharide into phosphate, dolichyl phosphate, and saccharide) and partitioning between chloroform and water, all the phosphate of SP-II is exclusively recovered in the organic phase (Table II). This excludes the existence of a pyrophosphate bridge in SP-II.

This conclusion is further supported by the ratio of uronic acids to phosphate, determined to be 2:1 in SP-II.

The Lipid Is of the Eucaryotic Dolichol Type—Thin-layer chromatography of the chloroform phase after mild acid hydrolysis of SP-II (Fig. 4, *lanes A, D,* and *E*) had indicated homogeneity of the lipid moiety. Therefore, this fraction was analyzed by proton NMR spectroscopy. Fig. 7 shows the spectrum of isolated lipid phosphate and commercially available (Sigma) dolichyl phosphate. The signals with a bar are due to contamination of the authentic dolichyl phosphate. Comparison of the spectra shows that the lipid phosphate isolated in the chloroform phase after acid hydrolysis of SP-II is a polyprenyl compound. This was further substantiated by mass spectroscopy. With the electron impact method, an authentic lipid phosphate as well as our fraction yielded mass spectra typical of polyprenyl compounds, showing a series with mass differences of 68. Most probably, the phosphate residue is eliminated thermally by this method, leaving a volatile lipid.

The highest mass peak found with the lipid phosphate was 818. This is in accord with a C_{60}-dolichyl phosphate (α-saturated) after elimination of the phosphate residue.

Further evidence for a chain length of 12 isoprene units comes from silica thin-layer chromatography of the lipid phosphate. The sample co-migrated with authentic C_{60}-dolichyl phosphate in two different solvent systems (B and C, described under "Experimental Procedures").

All efforts to hydrolyze the phosphate from the compound failed. It was resistant to 3 M HCl in methanol (12 h, 100 °C), to 60% aqueous hydrogen fluoride (overnight at room temperature), to 6 M HCl (overnight at 100 °C), and to condensed hydrogen fluoride (3 h at room temperature). As α-desaturated undecaprenyl phosphate is known to be cleaved by milder acidic conditions (1 N HCl in 50% (v/v) CH_3OH/H_2O, 100 °C, 5′ (21)), these negative results clearly reveal that the lipid must contain a saturated isoprenyl residue in the α position.

Taken together the above data show that the lipid moiety of SP-II is an α-saturated dodecaprenol.

DISCUSSION

From the above data the structure of a lipid-linked sulfated saccharide precursor was established and is shown in Fig. 8. The carbohydrate moiety is structurally identical to a saccharide *N*-glycosidically linked to the *H. halobium* cell surface glycoprotein via the novel Asn-Glc linkage. The sulfate residues are most probably linked to the 2′- or 3′-position of the uronic acids since (i) during all steps in the preparation of the glycopeptides, glucuronic acid and sulfate copurified in a

1:1 stoichiometry, and (ii) glucuronic acids were resistant to periodate oxidation before desulfation and could be destroyed by periodate after desulfation.

A first amino acid sequence analysis of one isolated tryptic glycopeptide demonstrated an attachment site matching the established acceptor sequence Asn-*X*-Thr(Ser) (28) for classical *N*-glycosidic linkages (data not shown). On the other hand, the precursor exhibits the following unusual features. The saccharide is built up on and transferred from a dolichyl monophosphate and not a pyrophosphate. In addition, the carbohydrate is fully sulfated before its transfer to the polypeptide rather than becoming sulfated at the polypeptide level (3). The lipid carrier in *H. halobium* turned out to be of the eucaryotic type rather than a procaryotic undecaprenol. This is another indication of the eucaryotic properties of Archaebacteria. A main experimental difficulty during this work was caused by heterogeneity of the sulfated precursor fraction. In view of the elucidated structures, a major cause of this diversity most probably was the differing degree of sulfation and variation of chain lengths of the carbohydrates, reflecting biosynthetic intermediates. Furthermore, a lipid-linked sulfated saccharide occurs which contains an additional compound of sugar nature that we have not yet identified. Since no equivalent compound has been found in the glycoprotein, this residue might indicate processing of the saccharides.

Lipid-linked monosaccharides have tentatively been characterized in previous studies of Halobacteria (29). One of them has been described as containing a GlcNAc residue. This compound might be a precursor of the glycosaminoglycan-like sulfated repeating unit saccharide we have characterized recently in the cell surface glycoprotein (18).

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