Sequence of the Halobacterial Glycosaminoglycan*

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The cell-surface glycoprotein of halobacterium contains a sulfated repeating unit saccharide chain, similar to the mammalian glycosaminoglycans. The composition of a presumptive repeating pentasaccharide unit of this glycosaminoglycan is 1 GlcNAc, 1 GalNAc, 1 Gal, 1 GalA (where GalA represents galacturonic acid), 1 3-O-methyl-GalA, and 2 SO$_4^{2-}$. Linkage to protein of this glycoconjugate involves the hitherto unique unit Asn-GalNAc, with the N-linked asparagine residue being the second NH$_2$-terminal amino acid and part of the common N-linked glycosyl acceptor sequence Asn-X-Thr(Ser). Transfer of the completed, sulfated glycosaminoglycan from its lipid precursor to the protein occurs at the cell surface, and the presence of this sulfated saccharide chain in the cell-surface glycoprotein seems to be required to maintain the structural integrity of the rod-shaped halobacteria.

In this paper, we report the complete saccharide structure of this N-linked glycosaminoglycan. This structure is deduced from chemical analyses of fragments that were isolated after hydrazinolysis and subsequent nitrous acid deamination or after mild acidic hydrolysis of purified Pronase-derived glycosaminoglycan-peptides. The halobacterial glycosaminoglycan consists, on the average, of 10 repeating pentasaccharide units of the following structure.

\[
\begin{align*}
\text{GalA-3-O-CH$_3$} & \\
\text{GalNAc} & \\
\text{3-GalNAc} & \\
\text{fGal} & \\
\end{align*}
\]

The reducing end N-acetylgalactosamine residue is linked directly to the asparagine, without a special saccharide linker region.

Archaeobacteria are the only bacteria presently known to contain true glycoproteins. The main constituent of the halobacterial cell membrane is a cell-surface glycoprotein (1), which contains two different types of sulfated saccharides (2). These saccharides are linked to the protein via two unique types of N-glycosidic linkages: 1) several small sulfated oligosaccharides are linked to the β-amino nitrogen of asparagine residues via glucose (3), and 2) one long glycosaminoglycan-like saccharide chain constructed of a repeating saccharide unit is linked via N-acetylgalactosamine to the β-amino nitrogen of an asparagine residue that represents the second NH$_2$-terminal amino acid residue of the core protein (4, 5). The structure and biosynthesis of the small sulfated oligosaccharides and the biosynthesis of the glycosaminoglycan-like saccharide chain have previously been described (4, 6, 7). Transfer of both types of sulfated saccharides to the protein occurs at the surface of the halobacterial cell. Both types of N-glycosidic linkage units involve asparagine residues that are constituents of the common N-glycosidic acceptor sequence Asn-X-Thr(Ser) found in eucaryotes. One N-linked glycosaminoglycan chain occurs in each molecule of cell-surface glycoprotein. This unusual glycoconjugate is composed of GlcNAc, GalNAc, Gal, GalA, and 3-O-methyl-GalA. The latter methylylated uronic acid is a rare constituent of natural carbohydrates; but, unlike stated in our previous publication (6), it has actually been described before, namely as a constituent of the methylated polysaccharide associated with the coccoliths of the alga *Emiliania huxleyi* (Lohman) Kamptner (8).

The cell-surface glycoprotein covers the halobacterial cell in a hexagonal, two-dimensional crystalline layer, and the presence of the glycosaminoglycan chain in this glycoprotein seems to be required to maintain the structural integrity of the rod-shaped cells (2, 9).

To obtain more insight into the arrangement (and interaction) of these highly glycosylated cell-surface protein molecules, we are interested in the molecular structure of their glycoconjugates. The structure of the N-linked small sulfated oligosaccharides has been described (3, 6); and here, we report the complete structure of its glycosaminoglycan chain. This structure has been obtained by chemical characterization of fragments isolated from Pronase-derived glycosaminoglycan-peptides.

**EXPERIMENTAL PROCEDURES**

Growth of *Halobacterium halobium* mutant R1M1 as well as conditions for radioactive labeling with $^{35}$S$^\text{O}_4^-$ have been described (2). Isolation and characterization of tryptic and Pronase-derived glycosaminoglycan-peptides are described in Ref 5.

**Analytical Methods**—For total hydrolysis of saccharides, samples were treated with 40% (v/v) trifluoroacetic acid for 4–6 h at 100 °C in a vacuum-sealed ampule. They were then dried either in a Speed Vac (Savant Instruments, Inc.) concentrator centrifuge or by rotary evaporation. Total hydrolysis of permethylated saccharides (after reduction of uronic acid carboxyl groups) was mainly as described (10). Samples were dissolved in 200 µl of 0.5 N H$_2$SO$_4$ in 95% acetic acid. After 14 h at 80 °C, 200 µl of water was added and the sample was incubated for a further 5 h at 80 °C. Sulfuric acid was removed by passing the samples over a small column of Dowex AG 1-X2 ion-exchange resin (acetate form). The partially methylated monosaccharide of a presumptive repeating pentasaccharide was obtained by passing the column and evaporation.

The abbreviations used are: GalA, galacturonic acid; GLC/MS, combined gas liquid chromatography/mass spectrometry; FPLC, fast protein liquid chromatography; GalNAc-ol, N-acetylgalactosaminol.
riders eluting with H₂O were taken to dryness and analyzed as described below.

Methodology was performed essentially according to Ref. 6 for 20 h at 80°C. Thereafter, the samples were dried under a stream of nitrogen. The methylglycosides obtained either were analyzed by reductive aminopropionylation and GLC (6) or were hydrolyzed (i.e., with HCl as acid at 121°C for 2 h) and Sep-Pak RC cartridges for further analysis with the alditol peracetate method.

Hydrazinolysis and Deamination—Hydrazinolysis and deamination were performed essentially according to Ref. 13. The saccharide sample was dried thoroughly over P₂O₅ in vacuo and then suspended in anhydrous hydrazine (J. T. Baker Chemical Co.) that contained 10 mg of hydrazine sulfate/mL. The saccharide concentration was kept in the range of 1-3 mg/mL, and the glass tubes were sealed under vacuum. After 18 h at 100°C, hydrazine was removed by repeated rotary evaporation with the repeated addition of tolune. For deamination, 250 μl of a solution of 50 mg of NaNO₂ in 1 ml of 0.5 M sodium acetate, pH 3.5, was added to the dry sample. After 30 min at room temperature, another 250 μl of the HNO₂ solution was added. The HNO₂ solution was freshly prepared for each addition. Excess HNO₂ was destroyed by addition of solid ammonium sulfamate, and the solution was washed with methanol.

Reduction of Fragments—The mixture of fragments obtained after hydrazinolysis and nitric acid deamination was subject to reduction of the reducing ends generated. To this end, samples were dissolved in a minimal volume of 0.1 M NaOH (20-80 μl). After 5 min at room temperature and washing of the carrier-free NaBH₄ (Amersham Corp.) in dimethyl formamide (25 μL/mL), the mixture was added, and the mixture was kept at 37°C for 15 min. 100 μl of a 1 M solution of NaBH₄, (or NaBH₄, where indicated) in 0.1 M NaOH was added to complete the reduction of the saccharide. After a further 30 min at 37°C, the excess of NaBH₄ was destroyed by careful dropwise addition of 2 M acetic acid (performed under a ventilated hood), and cations were removed by chromatography on Dowex AG 50X-H⁺ in water. The eluent was dried by rotary evaporation, and residual boric acid was removed by rotary evaporation of its trimethyl ester after addition of methanol.

Isolation of Fragments—The fragments obtained after hydrazinolysis, deamination, and reduction were chromatographed on a Sephadex G-25 column (1 x 140 cm) in 0.1 M pyridine buffer, pH 5.5. Pooled fractions were dried by rotary evaporation, redissolved in 0.01 M ammonium formate buffer, pH 3.5, and applied to a Mono-Q FPLC column (Pharmacia P-L Biochemicals). After washing for 10 min, a 0.01–0.4 M gradient of the above buffer was used in 30 min. Flow was 1 ml/min. Pooled fractions were concentrated by rotary evaporation and desalted by passage through a small column of Dowex AG 50W-H⁺ in water, and the eluent was lyophilized.

Methylated Acid Hydrolyzate of Glycopeptide—The methylated acid hydrolyzate was dissolved at a concentration of ~10 mg/ml in 0.25 M HCl and incubated for 15 min at 100°C. Hydrolysis was stopped by chilling on ice and immediate neutralization with NaOH. The fragments obtained were either reduced with NaBH₄/NaBH₄ (or NaBH₄) as described above and separated according to their m/z by chromatography on Sep-Pak P-10 (Bio-Rad) (2.5 x 50 cm) in 0.1 M pyridine acetate, pH 5.5, or, if 25SO⁻ labeled glycosaminoglycan (2) was used, to the gel filtration without prior labeling with reduction with NaBH₄.

Desulfation of Saccharides—In order to desulfate carbohydrate samples under mild conditions, essentially the solvolysis method described in Ref. 12 was used. The dry samples were dissolved in methanol containing ~50 mM HCl (1 ml of dry methanol plus 3.5 μl of acetyl chloride) and incubated at 37°C for 2 h. Thereafter, they were brought to dryness with a stream of nitrogen.

Permethylation Analysis—Permethylation of saccharides was performed according to Ref. 13 with the modifications given in Refs. 10 and 14. Reduced saccharide (0.02-2 mg) was dried in vacuo over P₂O₅ overnight. After addition of 250 μl of dry dimethyl sulfoxide, the sealed sample was stirred for 4 h at room temperature. 60 μl of sodium dimyel reagent (15) was added, and the mixture was stirred for 4 h at room temperature. At this point, the presence of excess sodium dimyel in the sample was assayed with triphenylmethane; and, if required, another 60-μl aliquot of reagent was added. In this case, stirring was for another 2 h. Finally, 35 μl of methyl iodide was added, and the mixture was stirred overnight at room temperature. After addition of 300 μl of methylylated NaBH₄, peracetates were purified by a Sep-Pak RP₈ cartridge (Waters Associates). The cartridge was pre-washed with 40 ml of ethanol, 4 ml of acetonitrile, and 6 ml of water. After application of the sample, it was washed with 10 ml of H₂O and eluted stepwise with 5 ml of acetonitrile/H₂O (3:14), 2 ml of acetonitrile/H₂O (1:3), and 3 ml of acetonitrile. Carbohydrate in the fractions was assayed either by the orcinol method (17) or by determining H radioactivity, where applicable. The samples were dried; and, if they contained uronic acids, their carboxyl methyl esters were reduced with NaBH₄ in tetrahydrofuran as described (14). The samples were then hydrolyzed as described above. The resulting partially methylated monosaccharides were reduced with NaBH₄ peracetyleated, and submitted to GLC and GLC/MS on a Hewlett-Packard system 5995 with a Durabond 1701 capillary column (30 m) and helium as carrier gas (1 ml/min). The temperature of the interface was 280°C, and the entrance voltage of the mass detector was 70 kV. All the GLC analyses were performed with a temperature gradient of 2°C/min (starting at injection). The start temperature for analysis of partially methylated alditol peracetates was 140°C and of alditol peracetates was 200°C.

RESULTS

Isolation of Pronase-derived glycosaminoglycan-peptides from the halobacterial cell-surface glycoprotein has been described previously (5). Glycodipeptides are obtained which contain 1 asparagine and 1 alanine residue and which differ in the length of their repeating unit saccharide chain. Lengths between about 7 and 13 pentasaccharide units are typically obtained, with an average chain length of 10 units. The repeating pentasaccharide unit consists of 1 GlcNAc, 1 GalNAc, 1 GalA, 1 Gal, 1 3-O-methyl-GalA, and 2 SO₂⁻ residues. Two methods proved to be useful in fragmentation of the chain, leading to fragments of different compositions: 1) graded acid hydrolysis leads to tetrasaccharides as the smallest fragments, and 2) hydrazinolysis with subsequent nitrous acid deamination leads to a pentasaccharide, a triasaccharide, and a disaccharide as the smallest fragments. Our strategy to elucidate the complete structure of the glycosaminoglycan was to isolate and characterize these fragments chemically and to compare their patterns obtained after permethylation analysis with those patterns obtained after permethylation analysis of larger repeating unit saccharide fragments, before and after desulfation.

Isolation of Fragments after Hydrazinolysis and Nitrous Acid Deamination—Hydrazinolysis normally desylates N-acetylamino sugars, yielding the free amino group and acetic acid hydrazide. When the liberated amino group then is deaminated by treatment with nitric acid, the skeletons of the amino sugars rearrange to yield 2,5-anhydrosugars, a process during which their glycosidic linkages are cleaved. Therefore, O-glycosidic linkages involving the reducing end of an amino sugar are generally cleaved by the combination of hydrazinolysis and nitrous acid deamination. However, if the N-acetylamino sugar is substituted at its carbon 3, the N-acetyl group is much more slowly deacylated by hydrazinolysis under standard conditions (16); and therefore, glycosidic linkages of amino sugars that are substituted in position 3 are cleaved to a lesser extent by the subsequent treatment with nitric acid. Isolated glycosaminoglycan-peptides were hydrazinolized and treated with nitric acid under the standard conditions described under "Experimental Procedures." Thereafter, the aldehydes representing carbon 1 of the resulting anhydro sugars residues were reduced with a low concentration of sodium borotritide, and then reduction was completed with sodium borodeuteride. The mixture of the 3H-containing saccharide fragments was subject to gel permeation chromatography on Sephadex G-25. A resulting 3H radioactivity elution profile can be seen in Fig. 1. The amounts of radioactivity found in the two peaks marked with arrows (fractions 39-43 (peak I) and 48-51 (peak II)) correlated with carbohydrate mass as analyzed by the orcinol method (17) (the high radioactivity peak (fractions 61-65) contained tritiated water). Capillary GLC/MS analysis with the alditol peracetate method of sam-
Glycosaminoglycan-peptide was fragmented and 3H-labeled as described under "Experimental Procedures." Thereafter, the sample was chromatographed on a Sephadex G-25 column (1.0 × 140 cm) in 0.1 M pyridine acetic, pH 5.5. 1.3-ml fractions were collected, and 20 μl of each was used to determine 3H radioactivity. Peak fractions (arrows) were pooled as indicated in text.

Table I
Qualitative composition analysis of the materials in peaks I and II of Fig. 1

| Saccharides                           | Peak I | Peak II |
|---------------------------------------|--------|---------|
| Anhydromannose (product of deamination of glucosamine) | +      | +       |
| Anhydrotalose (product of deamination of galactosamine) | -      | +       |
| 3-O-Methylgalacturonic acid           | +      | +       |
| Galacturonic acid                     | +      | +       |
| Galactose                             | -      | -       |
| N-Acetylgalactosamine                 | -      | +       |
| N-Acetylgalactosamine                 | +      | -       |

Purification on a Sep-Pack cartridge
Reduction of uronic acid methyl esters with sodium borodeuteride
Hydrolysis
Reduction of liberated monosaccharides with sodium borohydride
Permethylation
Peracetylation
Analysis by combined capillary GLC/MS

Scheme I. Individual steps in the permethylation analysis were performed according to Ref. 14.

Fig. 1. Separation by gel filtration of saccharides obtained after hydrazinolysis and nitrous acid deamination. Isolated glycosaminoglycan-peptide was fragmented and 3H-labeled as described under "Experimental Procedures." Thereafter, the sample was chromatographed on a Sephadex G-25 column (1.0 × 140 cm) in 0.1 M pyridine acetic, pH 5.5. 1.3-ml fractions were collected, and 20 μl of each was used to determine 3H radioactivity. Peak fractions (arrows) were pooled as indicated in text.

Fig. 2. Separation of saccharides in peak II of Fig. 1 by ion-exchange chromatography. Lyophilized pooled fractions of peak II in Fig. 1 were dissolved in 0.01 M ammonium formate buffer, pH 3.5, applied to a Mono-Q FPLC column (Pharmacia P-L Biochemicals) equilibrated with the same buffer, and washed for 10 min. A gradient of ammonium formate, pH 3.5, was applied from 0.01 to 0.4 M in 30 min. Flow was 1 ml/min throughout. 20 μl of each fraction (1 ml) was used to determine the 3H radioactivity.

Table II
Qualitative composition analysis of the materials in peaks IIA and IIB in Fig. 2

| Saccharides                           | Peak IIA | Peak IIB |
|---------------------------------------|----------|----------|
| Anhydromannose                       | -        | -        |
| Anhydrotralose (fragment IIB)         | -        | +        |
| 3-O-Methylgalacturonic acid           | +        | -        |
| Galacturonic acid                     | -        | +        |
| Galactose                             | +        | -        |
| C-1-D-reduced saccharide              |          |          |
| Permethylation                        |          |          |
| Purification on a Sep-Pack cartridge  |          |          |
| Reduction of uronic acid methyl esters with sodium borodeuteride |          |          |
| Hydrolysis                            |          |          |
| Reduction of liberated monosaccharides with sodium borohydride |          |          |
| Permethylation                        |          |          |
| Peracetylation                        |          |          |
| Analysis by combined capillary GLC/MS |          |          |

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| 3-O-Methylgalacturonic acid           | +        | -        |
| Galacturonic acid                     | -        | +        |
| Galactose                             | +        | -        |
| C-1-D-reduced saccharide              |          |          |
| Permethylation                        |          |          |
| Purification on a Sep-Pack cartridge  |          |          |
| Reduction of uronic acid methyl esters with sodium borodeuteride |          |          |
| Hydrolysis                            |          |          |
| Reduction of liberated monosaccharides with sodium borohydride |          |          |
| Permethylation                        |          |          |
| Peracetylation                        |          |          |
| Analysis by combined capillary GLC/MS |          |          |
Combined with the composition analyses of the isolated fragments, these results reveal the following structures: fragment IIA, 3-O-methyl-GalA-6-anhydromannitol; and fragment IIB, GalA-1–3(or 4)-anhydrotalitol. The second substituent in position 3 or 4 is a sulfate residue (see below).

Structure of the Pentasaccharide Fragment I—The isolated pentasaccharide fragment I, which contained anhydromannitol as well as N-acetylgalactosamine, but no anhydrotalitol, was permethylated before and after desulfation according to Scheme I. Desulfation is achieved by mild methanolysis, as described under “Experimental Procedures,” a co-association that quantitatively cleaves the bond of the labile galactose residue. Therefore, after desulfation of the pentasaccharide, a tetrasaccharide is obtained that lacks galactose. The free, unreduced galactose residue is destroyed by the permethylation procedure. The ion profiles obtained by GC/MS after permethylation of the untreated and desulfated fragment I are shown in Fig. 6 (A and B), and the partially methylated alditol peracetates corresponding to the mass spectra of peaks B–F are listed in Table III. According to these compounds, the following structural elements are present in fragment I: a furanosidically linked unsubstituted hexose (peak B), an unsubstituted hexuronic acid (peak C), a monosubstituted hexuronic acid (peak D), and a disubstituted N-acetylamino hexose (peak E); whereas in the desulfated fragment I, unsubstituted hexuronic acid was found (peak C), together with a monosubstituted N-acetylamino hexose (peak E). In addition, both samples contained a substance giving rise to peak A, the spectrum of which is shown in Fig. 6C. This mass spectrum may be derived from an anhydrohexitol that was disubstituted either at its 3- and 6-positions or at its 4- and 6-positions. Typical fragments are indicated in Fig. 5 (C and D). By combining these structural elements of the pentasaccharide and the tetrasaccharide (desulfated) with the structures of

**Table III**

| Compound assigned                  | Mass spectrum of peak |
|------------------------------------|-----------------------|
| B 1,4-Di-O-acetyl-2,3,5,6-tetra-O-methylhexitol | 15 23 30 35 40 45 |
| C 6-D$_3$1,5,6-Tri-O-acetyl-2,3,4-tri-O-methylhexitol | 15 23 30 35 40 45 |
| D 6-D$_3$1,3,5,6-Tetra-O-acetyl-2,4-di-O-methylhexitol | 15 23 30 35 40 45 |
| E 1,3,5-Tri-O-acetyl-4,6-di-O-methyl-2-deoxy-2-methylaminohexitol | 15 23 30 35 40 45 |
| F 1,3,4,5-Tetra-O-acetyl-6-O-methyl-2-deoxy-2-methylaminohexitol | 15 23 30 35 40 45 |
fragments IIA (3-O-methyl-GalA-6-anhydromannitol) and IIB (GalA-4(or 3)-anhydrotalitol), Structure I is established for the pentasaccharide obtained after hydrazinolysis and nitrous acid deamination. The disaccharide fragment IIB, obtained in smaller amounts after hydrazinolysis and nitrous acid deamination, contained GalA in 1-3(or 4)-linkage to anhydrotalitol, the derivative of GalNAc. Therefore, GalA is linked to 3- or 4-position of GalNAc; and, in addition, this amino sugar contains a sulfate residue in its 3- or 4-position. From the composition of fragment IIA, we know that 3-O-methyl-GalA is linked to the 6-position of anhydromannitol, the derivative of GlcNAc. The existence of a 3,6- or 4,6-disubstituted anhydromannitol and the occurrence of the minor fragment IIB containing anhydrotalitol show that fragment IIB is linked to fragment IIA via GalNAc to the 3- or 4-position of GlcNAc. Before desulfation and the concomitant degalactosylation of the pentasaccharide, a 3-substituted and a peripherally hexuronic acid was found (peaks C and D in Fig. 6A); whereas after desulfation and degalactosylation, only peripheral hexuronic acid was found (accounting for both, the 3-O-methyl-GalA and GalA, because a peripheral GalA and a methylated GalA cannot be distinguished by permethylation analysis) (peak C in Fig. 6B). As the sulfated residue is linked to the GalNAc residue (see fragment IIB), the Gal residue must have been linked to the 3-position of the GalA residue. The GlcNAc residue quantitatively converted to anhydromannose by hydrazinolysis and nitrous acid deamination is most likely substituted at its 4-position rather than its 3-position within the glycosaminoglycan chain. The peripheral Gal residue is linked furanosidically to the GalA residue. This explains its extreme lability toward acid.

Partial Acid Hydrolysis of the Glycosaminoglycan-peptides—To establish how the pentasaccharides are linked together in the saccharide chain and to determine whether the GalNAc residue is linked to the 3- or 4-position of GlcNAc, we isolated fragments produced by cleavage at sites different from those cleaved by nitrous deamination. This was achieved by graded acid hydrolysis as described in Refs. 4 and 18 and under “Experimental Procedures.” This method led to a quantitative liberation of the fucosidic Gal residues as well as to partial loss of SO$_3$$. By gel filtration a tetra-, an octa-, and a dodécasaccharide fraction could be separated (18), all of which had the composition 1 GalA, 1 3-O-methyl-GalA, 1 GalA, and 1 GlcNAc. Reduction of the partial hydrolysate with NaB$_2$H$_4$ before gel filtration and quantitative evaluation of mass spectra revealed that the tetrasaccharide fraction was a 10:1 mixture of tetrasaccharides with GalNAc (10 parts) and with GalA (1 part) at the $^3$$^H$-reduced end if the glycosaminoglycan was desulfated by methanolation prior to graded acid hydrolysis. If untreated saccharide was partially hydrolyzed, a 1:1 mixture was obtained. This finding suggests a neighbor catalytic function of a sulfate residue during graded acid hydrolysis of the saccharide chain.

Structure of a Tetrasaccharide Fraction—As we were interested in the linkage between the pentasaccharide units (fragments I) and in the substitution pattern of the GlcNAc residue, we decided to isolate the tetrasaccharide fraction after graded hydrolysis of the desulfated saccharide (with a ratio of 10 tetrasaccharides with 1-D-GalNAc-ol to 1 tetrasaccharide with 1-D-GalA-ol) for permethylation analysis. The tetrasaccharide fraction was isolated by gel filtration on Bio-Gel P-10 as described (18) and was submitted to permethylation analysis. The total ion profile resulting from a capillary GLC/MS analysis is shown in Fig. 7. The compounds assigned to the mass spectra of peaks A–E are listed in Table IV. These partially methylated alditol per acetates are derived from a peripherally linked hexuronic acid (peak A), a 3-mono substituted reducing end aminohe xo (peak B), a monosubstituted hexuronic acid (peak C), and a disubstituted amino sugar (peak E). The substance of peak D is a result of incomplete methylation of the 1-position of the reducing end aminohe xo. The occurrence of a 4-substituted hexuronic acid reveals that the linkage of fragments I within the chain is GlcNAc-4$^\&$GalA. The amino sugar with the incorporated deuterium must be the GalNAc residue, as the reducing end sugar of this tetrasaccharide has been shown to be GalNAc (see above). Furthermore, the substitution at position 6 of the GalNAc by a peripheral 3-O-methyl-GalA as well as the 3-substitution of GalNAc (peak B in Fig. 7) are confirmed. By combination of these results with the structural data on fragments I, IIA, and IIB, we obtain the following structure of the major tetrasaccharide, generated by graded acid hydrolysis: 3-O-$^\&$CH$_3$-GalA-1-6-GlcNAc-1-4-GalA-3-GalNAc-ol.

Structure of an Octasaccharide Fraction—At this point, it remained to be established whether the GalNAc is linked to the 3- or 4-position of the GlcNAc residue within the intact glycosaminoglycan chain. To answer this question, an octasaccharide fraction was isolated as described (18) and submitted to permethylation, as outlined for the other fragments. The corresponding GLC/MS analyses showed, in addition to the peaks found in Fig. 7, a signal corresponding to a 3-substituted amino sugar, thus confirming the disubstituted GalNAc residue (peak A in Fig. 8A), and a signal corresponding to a 4,6-disubstituted amino sugar (peak B in Fig. 8A). The mass spectrum of the latter is depicted in Fig. 8B.

Thus, the pattern of glycosidic linkages found in fragments after hydrazinolysis and nitrous deamination is confirmed by permethylation analyses of two fragments obtained after
graded acid hydrolysis of the glycosaminoglycan chain. In addition, the elucidation of the repeating unit structure is completed by the finding of a 4-6-branch in the N-acetylgalactosamine residue. This 4-substitution instead of 3-substitution of the amino sugar was expected, as a 3-substituted N-tetrasaccharide in the legend to Fig. 4 obtained after graded acid hydrolysis of desulfated glycosaminoglycan-peptide. The sample was prepared as described for the tetrasaccharide in the legend to Fig. 7. A, total ion profile; B, mass spectrum of peak B in A.

Furthermore, in the fragments after graded acid hydrolysis, a 4-substituted GalA residue was found. As we know that the peripheral furanosidic Gal residue is linked to the 3-position of GalA and is lost after graded acid hydrolysis, the pentasaccharide units (fragments I) in the glycosaminoglycan chain must be linked together via R-GlcNAc-4GalAl-3GalNAc-01-GlcNAc units (fragments I) in the glycosaminoglycan chain. This pentasaccharide unit must contain another sulfate residue. Unfortunately, so far we have not been able to localize this residue in one of the fragments investigated presumably because it is cleaved by the methods we have employed to fragment the chain. This pentasaccharide unit is repeated 10 times on the average, linked together via GalNAc-4-GlcNAc to yield a glycosaminoglycan chain of about 10-12 kDa. Hydrolysis of the carbohydrate chain at its GalNAc-GlcNAc bonds as well as at its GalA-GalNAc bonds under conditions as mild as 0.25 N HCl at 100 °C for 15 min might be explained by a possible neighbor

**TABLE V**

| Mass spectrum of peak | Compound assigned |
|-----------------------|-------------------|
| A                     | 6-D2-1,5,6-Tri-O-acetyl-2,3,4-tri-O-methyl-hexitol |
| B                     | 6-D2-1,4,5,6-Tetra-O-acetyl-2,3-di-O-methyl-hexitol |
| C                     | 1,3,5-Tri-O-acetyl-4,6-di-O-methyl-2-deoxy-2-methylaminohexitol |
| D                     | 1,5,6-Tri-O-acetyl-3,4-di-O-methyl-2-deoxy-2-methylaminohexitol |

**FIG. 9.** GLC/MS analysis of a permethylated tryptic tetrasaccharide peptide derived by graded acid hydrolysis of a tryptic halobacterial glycosaminoglycan peptide. A total ion profile is shown.

**FIG. 10.** Structure of the halobacterial glycosaminoglycan chain.

(from the GlcNAc residue with its 6-linked 3-O-methylgalacturonic acid) (peak D).

**DISCUSSION**

Using several methods to cleave the halobacterial glycosaminoglycan and elucidating the structures of fragments obtained, we have determined the sequence and the linkage pattern of this sulfated, repeating unit saccharide. The structure obtained is shown in Fig. 10. The repeating unit consists of a pentasaccharide, with 1 sulfate residue esterified to the 4-position of the N-acetylgalactosamine residue. According to previous analyses, the pentasaccharide unit must contain another sulfate residue. Unfortunately, so far we have not been able to localize this residue in one of the fragments investigated presumably because it is cleaved by the methods that we have employed to fragment the chain. This pentasaccharide unit is repeated 10 times on the average, linked together via GalNAcI-4-GlcNAc to yield a glycosaminoglycan chain of about 10-12 kDa. Hydrolysis of the carbohydrate chain at its GalNAc-GlcNAc bonds as well as at its GalA-GalNAc bonds under conditions as mild as 0.25 N HCl at 100 °C for 15 min might be explained by a possible neighbor

**FIG. 8.** GLC/MS analysis of a permethylated octasaccharide obtained after graded acid hydrolysis of desulfated glycosaminoglycan-peptide. The sample was prepared as described for the tetrasaccharide in the legend to Fig. 7. A, total ion profile; B, mass spectrum of peak B in A.

**TABLE II**

**Structure II**

**Structure of a Tryptic Linkage Unit Glycopeptide.—** The glycosaminoglycan is linked to the polypeptide chain of the halobacterial cell-surface glycoprotein via asparaginyl-N-acetylglactosamine (5). To elucidate the saccharide structure at this linkage region, we isolated a tryptic glycosaminoglycan-peptide as described (5) and submitted it to desulfation and graded acid hydrolysis as shown above. The resulting fragments were separated according to size by gel filtration on Bio-Gel P-10 and purified further by reversed-phase high pressure liquid chromatography under the conditions described (5) for the purification of the complete tryptic glycosaminoglycan-peptide. We obtained a tetrasaccharide peptide with 1 3-O-methyl-GalA, 1 GalA, 1 GlcNAc, and 1 GalNAc. Permethylation of this tetrasaccharide peptide gave rise to the total ion profile after GLC/MS analysis as shown in Fig. 9. The partially methylated alditol peracetates of peaks A–D are listed in Table V, and these derivatives are in agreement with the linkage unit tetrasaccharide peptide having a tetrasaccharide (dimeric repetitive unit lacking 2 peripheral Gal residues) as that shown in Structure II.

GlcNAc-4GalAl-3GalNAc-14GalA-5GalNAc-01

1 3-O-CH3-GalA 3-O-CH3-GalA

**FIG. 10.** Structure of the halobacterial glycosaminoglycan chain.
Sequence of the Halobacterial Glicosaminoglycan

Halobacteria grown in the presence of this drug no longer maintain their rod shape, but are converted to spherical cells (9). Thus, the glycosaminoglycan chain presented in this paper might be involved in maintaining the structural integrity of the organism. To investigate the possible role of carbohydrate-carbohydrate or carbohydrate-protein interaction maintaining cell structure, we are interested in the three-dimensional structure of this saccharide chain. A first insight into its three-dimensional structure might be obtained by x-ray fiber diffraction analysis.

With the structure mentioned in this paper, the characterization at the molecular level of the glycoconjugates linked to the cell-surface glycoprotein of halobacteria is nearly completed. A schematic diagram of this complex procaryotic glycoprotein is given in Fig. 11.

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Fig. 11. Structure of the cell-surface glycoprotein of halobacteria. GAG, glycosaminoglycan; IdA, iduronic acid.

Group catalytic effect exerted by sulfate residues. However, after desulfation, the chain is degraded under the same conditions by preferential hydrolysis of its GalNAc-GlcNAc bonds. To explain this, one could speculate that after desulfation, uronic acid carboxyl groups might exert neighbor group catalysis as well.

To date, protein-linked sulfated saccharide chains made up of repeating units have been found exclusively in eucaryotes. These glycosaminoglycans generally contain sulfated disaccharides as repeating units. Two different saccharide-protein linkage units have been described: O-glycosidic linkage to serine residues via a linker region Ser-O-Xyl-Gal-Gal (where Xyl represents xylose) typically present in the chondroitin sulfates (19) and an N-glycosidic linkage that contains a typical N-linked GlcNAc-Man core. The two branches of this core are elongated by the repeating sulfated disaccharide unit in corneal keratan sulfate. The biosynthesis of this unusual large glycoconjugate has been shown to involve a lipid-linked precursor that is completed (including sulfation) before transfer to the protein occurs. The transfer takes place at the surface of the halobacterial cell. So far, it is unknown how the complicated and highly sulfated saccharide chain is translocated through the membrane to the cell surface. Either this might be achieved en bloc by a specific transport system or the long carbohydrate chain might be established stepwise after translocation through the membrane of smaller precursor molecules, similar to the biosynthesis of the bacterial lipopolysaccharide O side chain. Incorporation of the glycosaminoglycan into the polypeptide chain is inhibited by the antibiotic bacitracin (2).