GLYCOCONJUGATE BIOCHEMISTRY: STRUCTURE-FUNCTION RELATIONSHIP

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A thesis submitted for the degree of Doctor of Science

University of Edinburgh

1996

STATEMENT TO REGULATION 1.1.4.

(a) The research work presented in this thesis has not been submitted for the award of any other degree or diploma.

(b) This is further certified that 70-85% of the published and unpublished research work included in this thesis has been performed by the undersigned or was conducted under his supervision and guidance.

Nasir-ud-Din

Dedicated to Amina, my daughter.

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Unpublished Work

ABSTRACT

Glycoconjugates are ubiquitously distributed in nature and have been implicated in distinctly significant biological functions including energy resource, protection, lubrication, structural support, cell adhesion, molecular and cellular recognition, receptors for hormones and viruses. In this thesis studies on the following glycoconjugates are presented: bacterial, Micrococcus lysodeikticus, cell wall, simian cervical mucin, human pulmonary mucin, bovine gallbladder mucin, sperm surface glycoproteins and glycoconjugates of malarial parasite, Plasmodium falciparum. The information obtained on the structure of these complex carbohydrates utilising chemical, enzymatic, immunological and physical methods provided insight into the understanding, in particular, of structure-function relationship, degradation and biosynthesis of these macromolecules. Studies on model compounds and analytical methods, all of which are vitally important tools in the study of glycoconjugates, are also described.

The carbohydrate prosthetic group of Micrococcus lysodeikticus cell wall was shown to consist of a glycan moiety linked to the protein and an antigenic polysaccharide attached to the glycan moiety of the peptidoglycan through a phosphodiester group. A variety of model compounds were synthesised to establish the structure of the carbohydrate moiety as well as to study the kinetics of the acid hydrolysis of the phosphodiester group linked to muramic acid and to the reducing terminus of glucose. The study was performed on Micrococcus lysodeikticus cell wall polymer resistant to lysozyme, elaborating the structural requirement for stability to the enzyme. Furthermore, a water soluble polymer from the Micrococcus lysodeikticus cell wall was isolated and characterised, a novel observation. The study on this polymer suggested the possible deficiencies in the biosynthesis or possible autolysis of the cell wall polymer.

A large number of model compounds were chemically synthesised to identify the structural features of the cell wall peptidoglycans and those of the antigenic polysaccharide. In addition, several compounds were chemically synthesised to obtain the model compounds necessary to conduct kinetic studies to define the type of linkage, i.e., differentiate between the monophosphate or pyrophosphate, between the cell wall polysaccharide and peptidoglycan, more precisely the linkage between muramic acid 6-phosphate of the peptidoglycan and the reducing terminal residue, glucose, of the polysaccharide.

Comprehensive studies on simian cervical mucus glycoproteins were performed to relate the morphological and biophysical changes during the ovarian cycle to biochemical changes in the biochemical structure of the mucus. The biophysical and biochemical changes in the mucus are of vital significance in human reproductive physiology. The bonnet monkey produces larger amounts of mucus and its ovarian / menstrual cycle is similar to that of human. The extensive structural investigations on cervical glycoproteins of different phases of the ovarian cycle led to the elucidation of several interesting and novel structural features, such as the linkage of sialic acid to ultimate N-acetylgalactosoamine residues linked to serine or threonine of the protein core as well as to the galactose residues further away from the protein core. These two differentially linked sialic acid residues have been postulated to contribute to the function of mucus glycoproteins as well as to biophysical properties. Similarly, sulfate groups in the glycoprotein safeguard the integrity of the macromolecule. Also, a novel type of α-galactosyl-linkage has been shown by us in the midcycle cervical mucus. In addition, it was also discovered by us that α-linked N-acetylgalactosoamine residues were present in the midcycle cervical glycoproteins. The functional role of these α-linked residues is not clear. It is believed that this type of galactose residues are uncommon in man and old world monkey. The structural studies on luteal phase glycoproteins demonstrated significant differences in the structure of oligosaccharides. Similarities in the oligosaccharides structures were also observed. Polyclonal antibodies to midcycle bonnet monkey glycoproteins clearly demonstrated the functional role of the cervical mucus. It was discovered by us that the glycoproteins that line the cervical mucus channels are responsible for sperm migration. Our study, first of its nature, distinctly defined the functional role of the cervical glycoproteins and the significant contributions of the sugar residues. It was further shown that sperm penetration was inhibited by the antibody-mucus complex. The antibody against the midcycle glycoprotein cross-reacted with the midcycle mucus and weakly with the luteal phase mucus. Similarly the antibody against the luteal phase glycoprotein cross-reacted with the luteal phase as well as, though weakly, with the midcycle mucus. These observations clearly suggested some common epitopes amongst glycoproteins of different phases of the ovarian cycle, interalia some common structural features in the glycoproteins of the different phases. The goal of this study, i.e., to relate the structure function relationship of the cervical mucus glycoproteins, was achieved. Initial work on sperm surface glycoproteins and gall bladder glycoprotein have been performed. Currently pursued research indicates that sperm surface glycoproteins may provide useful means to regulate fertility.

Over a period of several years, extensive and detailed biosynthetic, biochemical and immunological studies were pursued to elaborate the role of the carbohydrate moiety of the variant glycoprotein antigens of the malarial parasite, Plasmodium falciparum. For many years it was known that sugars,

such as N-acetylglucosamine, mannose and galactose were incorporated in several antigens including the vaccine candidates, i.e., MSP-1, 195 kDa glycoprotein, and MSP-2, 43-52 kDa glycoprotein. Labelled sugars were metabolically incorporated in MSP-1 and MSP-2. The labelled glycoproteins from the asexual erythrocytic stages of Plasmodium falciparum were purified to homogeneity by SDS-PAGE and the labelled sugars were identified. It was further observed that N-acetylglucosamine was incorporated in different glycoproteins in much more significant amounts than mannose, and mannose was in abundance compared to galactose. From these observations it was clear that N-acetylglucosamine was incorporated in glycoproteins in abundance in regions other than anchor as well. Our researches, for the first time, showed that N-acetylglucosamine was the sugar residue that provided the O-glycosyl type linkage to serine or threonine. Thus, the linkage point between the sugar moiety and the protein-core was defined. The studies on the carbohydrate moiety of these glycoproteins have become significantly important as these may provide clues to the immune evasion properties of the variant glycoprotein antigens.

Reviews on secretory glycoproteins have been published. A review by us on malarial glycoprotein is in press. More recently a research article on the integration of glycans utilising glycophosphatidylinositol into plasma membranes and their possible role and interaction with the targeted cells has been discussed.

Reprinted from

Carbohydrate Research, 28 (1973) 243–251

SYNTHESIS OF THE 3- AND 4-METHYL, 3,4-DIMETHYL, AND 3,4,6-TRIMETHYL ETHERS OF METHYL 2-ACETAMIDO-2-DEOXY-α-D-MANNOPYRANOSIDE*†

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(Received December 7th, 1972; accepted for publication, December 13th, 1972)

ABSTRACT

The methyl ethers of 2-amino-2-deoxy-D-mannose are reference compounds in studies, by the methylation procedure, of the chemical structure of polysaccharides containing 2-amino-2-deoxy-D-mannose and 2-amino-2-deoxy-D-mannuronic acid residues. Methylation of methyl 2-acetamido-2-deoxy-α-D-mannopyranoside (**1**) gave the 3,4,6-trimethyl ether. Methylation of the 6-trityl ether of **1**, followed by detritylation, gave the 3,4-dimethyl ether of **1**. Methylation of the 4,6-*O*-benzylidene derivative (**6**) of **1**, followed by removal of the benzylidene group, gave the 3-methyl ether of **1**. Benzoylation of **6**, followed by removal of the benzylidene group and monobenzoylation, gave the 3,6-dibenzoate of **1**, which was methylated, and the product saponified, to give the 4-methyl ether of **1**; the latter compound was also obtained by a similar route *via* the 3-*O*-acetyl-6-*O*-benzoyl derivative.

INTRODUCTION

The polysaccharide linked to the peptidoglycan of the cell wall of *Micrococcus lysodeikticus* contains residues of both D-glucose and 2-amino-2-deoxy-D-mannuronic acid[1]. The chemical structure of this polysaccharide was established by the methylation procedure, the polysaccharide being methylated, the ether reduced, and the product methanolyzed[2]. In order to identify the resulting fragments, the 3-methyl, 4-methyl, and 3,4-dimethyl ethers of methyl 2-acetamido-2-deoxy-α-D-mannopyranoside were synthesized, because the methanolysis gives a mixture of the anomeric forms in which the α-D-pyranoside form preponderates. In addition, the synthesis

*Dedicated to Dr. Louis Long, Jr., in honor of his 70th birthday.

†Amino Sugars LXXXIII. This is publication No. 603 of the Robert W. Lovett Memorial Group for the Study of Diseases Causing Deformities, Harvard Medical School at the Massachusetts General Hospital, Boston, Massachusetts. This investigation was supported by a research grant (AI-06692) from the National Institute of Allergy and Infectious Diseases, National Institutes of Health, U. S. Public Health Service.

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of the 3,4,6-trimethyl ether is reported. These methyl ethers are also of interest for the elucidation of the structure of polysaccharides containing 2-amino-2-deoxy-D-mannose residues; such carbohydrates have been isolated from Salmonella groups J and T, *Escherichia coli* O_{31}, Arizona 15 (Ref. 3), and from a capsular polysaccharide[4] of Pneumococcus Type IV.

RESULTS AND DISCUSSION

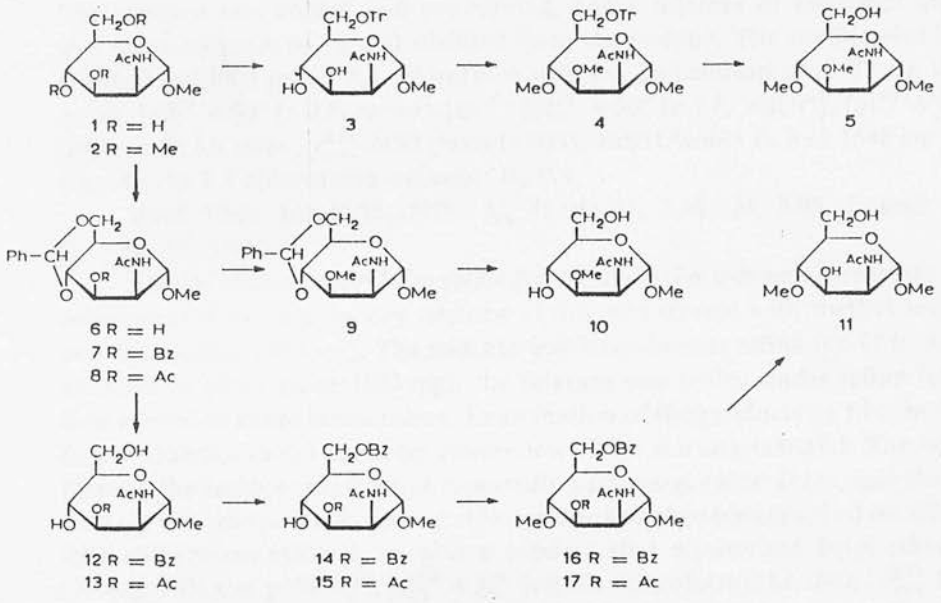
(*a*) Methyl 2-acetamido-2-deoxy-α-D-mannopyranoside (**1**) was obtained in 9% yield by glycosidation of 2-acetamido-2-deoxy-D-mannose, followed by separation of the α- and β-D furanosides and pyranosides by means of Dowex-1 resin and preparative paper-chromatography[5]; this method, the second step of which required 55 sheets of Whatman No. 1 paper for the preparation of 218 mg of **1**, is not practical for the preparation of larger amounts of starting material. (*b*) The separation of the glycosides by using only a Dowex-1 column[6] probably gave a mixture of the two furanosides and two pyranosides. (*c*) Preparation of the 3,4,6-tri-*O*-acetyl derivative of **1** *via* the 2-methyl-4,5-(3,4,6-tri-*O*-acetyl-2-deoxy-D-mannopyrano)-2-oxazoline gave a very low yield[7]. (*d*) Application of the improved synthesis of the oxazoline according to Khorlin *et al.*[8] did not, in our hands, give the high yields (82%) reported. (*e*) However, removal of the benzylidene group of methyl 2-acetamido-4,6-*O*-benzylidene-2-deoxy-α-D-mannopyranoside (**6**), which had been obtained from methyl 4,6-*O*-benzylidene-α-D-glucopyranoside by the methods of Foster *et al.*[9], Buss *et al.*[10], and Sinaÿ *et al.*[11], gave **1** in syrupy form and 89% yield. This compound showed an optical rotation identical with that of the compound previously described[5].

In order to avoid *N*-methylation, the classical method of Purdie and Irvine[12] was applied to **1**, and the crystalline 3,4,6-trimethyl ether (**2**) was obtained in 70% yield.

Treatment of **1** with chlorotriphenylmethane gave a crystalline 6-trityl ether (**3**), which was methylated by the Purdie reagents to give the crystalline 3,4-dimethyl ether (**4**); removal of the trityl group gave the crystalline 3,4-dimethyl ether (**5**) of **1** in an overall yield of 65% (calculated on **1**).

Methylation of the benzylidene derivative **6**, followed by removal of the benzylidene group from the crystalline intermediate **9**, gave the amorphous 3-methyl ether (**10**) of **1** in an overall yield of 62%.

Synthesis of the 4-methyl ether (**11**) of **1** was achieved *via* two different routes. In the first, the 3-hydroxyl group of the benzylidene derivative **6** was protected with a benzoyl group, to give the crystalline monobenzoate **7**. Removal of the benzylidene group thereof was followed by monobenzoylation at O-6 of the crystalline intermediate **12**, to give the crystalline 3,6-dibenzoate (**14**). Methylation of **14** afforded the crystalline 4-methyl ether **11** in an overall yield of 27% (based on **6**). A similar sequence of reactions that used an acetyl group at O-3 for protection, and proceeded *via* the known[11] crystalline 3-*O*-acetyl-4,6-*O*-benzylidene (**8**), amorphous 3-*O*-acetyl (**13**), crystalline 3-*O*-acetyl-6-*O*-benzoyl (**15**), and syrupy 3-*O*-acetyl-6-*O*-



benzoyl-4-*O*-methyl (**17**) derivatives, gave the 4-methyl ether **11** in an overall yield of 25% (based on **6**); methylation of **15** was performed in the presence of boron trifluoride etherate by the method of Gros *et al.*[13], which has been shown not to cause migration of the *O*-acetyl groups. The location of the 4-*O*-methyl group was ascertained by the preparation of a 6-trityl ether (which was not further characterized).

EXPERIMENTAL

General. — Melting points were determined with a Mettler FP-2 apparatus, and correspond to "corrected melting points". Optical rotations were measured, in semimicrotubes, with a Perkin–Elmer Model 141 polarimeter. I.r. spectra were recorded, for potassium bromide discs, with a Perkin–Elmer Model 237 spectrophotometer. The chloroform used was analytical-reagent grade, and contained 0.75% of ethanol. Column chromatography was performed on Silica Gel Merck (70–325 mesh; E. Merck, Darmstadt, Germany), used without pretreatment. The ratio of weight of substance to weight of adsorbent was 1:70 to 1:100. The volume of the fractions eluted was 4–5 ml per gram of the substance to be chromatographed. T.l.c. was performed on precoated silica gel plates (without fluorescence indicator; layer thickness 0.25 mm; E. Merck, Darmstadt, Germany); all compounds showed only one spot. The R_{TMG} values refer to the mobilities on plates of silica gel, relative to that of methyl 2-acetamido-2-deoxy-3,4,6-tri-*O*-methyl-α-D-glucopyranoside. Evaporations were conducted *in vacuo*, with the bath temperature kept below 40°. Microanalyses were performed by Dr. W. Manser, Zürich, Switzerland.

Methyl 2-acetamido-2-deoxy-α-D-mannopyranoside (**1**). — Compound **6** (80 mg) was heated with 60% acetic acid (3.5 ml) for 1 h on a water bath at 80°.

The solution was cooled and evaporated, and a mixture of methanol and toluene was repeatedly added to and distilled from the residue. The residue was chromatographed on silica gel in 7:3 chloroform–ethanol, and elution gave 52 mg (89%) of a syrup, $[\alpha]_D^{20}$ +50° (*c* 0.9, water) {lit.[5] : $[\alpha]_D^{24}$ +50° (*c* 1.6, water)}, $[\alpha]_D^{20}$ +47° (*c* 0.8, methanol); i.r. data : ν_{max}^{KBr} 3450 (broad; OH), 1650 (Amide I), and 1548 cm^{-1} (Amide II); t.l.c. in 7:3 chloroform–ethanol: R_F 0.4.

Anal. Calc. for $C_9H_{17}NO_6$: C, 45.95; H, 7.28; N, 5.95. Found: C, 45.83; H, 7.26; N, 5.93.

*Methyl 2-acetamido-2-deoxy-3,4,6-tri-*O*-methyl-α-*D*-mannopyranoside* (**2**). — A solution of **1** (45 mg) in dry acetone (1 ml) was treated with methyl iodide (3 ml) and silver oxide (200 mg). The mixture was boiled under reflux for 12 h. After a new addition of silver oxide (100 mg), the mixture was boiled under reflux for 6 h, and then cooled to room temperature. Examination of the products by t.l.c. in 9:1 chloroform–ethanol showed the total conversion of the starting material. The mixture was filtered, the residue was washed repeatedly with warm chloroform, and the combined filtrates were evaporated *in vacuo*. The residue was chromatographed on silica gel with 19:1 chloroform–ethanol, to give a product that crystallized from ether in plates (37 mg, 70%), m.p. 98–99°, $[\alpha]_D^{20}$ +39° (*c* 0.33, chloroform); i.r. data : ν_{max}^{KBr} 3250 (NH), 1630 (Amide I), and 1550 cm^{-1} (Amide II); t.l.c. in 19:1 chloroform–ethanol : R_F 0.5; R_{TMG} 1.0. The product contained one molecule of water per molecule.

Anal. Calc. for $C_{12}H_{23}NO_6 \cdot H_2O$: C, 48.80; H, 8.53; N, 4.74. Found: C, 49.00; H, 8.25; N, 4.88.

After being melted *in vacuo* and cooled, the compound showed the following analytical values.

Anal. Calc. for $C_{12}H_{23}NO_6$: C, 51.97; H, 8.36; N, 5.05; O, 34.62; OMe, 44.72. Found: C, 51.93; H, 8.34; N, 4.89; O, 34.82; OMe, 44.26.

*Methyl 2-acetamido-2-deoxy-6-*O*-trityl-α-*D*-mannopyranoside* (**3**). — A solution of **1** (65 mg) in dry pyridine (3 ml) was treated with chlorotriphenylmethane (88 mg) for 46 h at room temperature, and then the mixture was poured onto crushed ice. The precipitate was dissolved in chloroform (15 ml), and the solution was washed with cold water (4 × 4 ml), dried (sodium sulfate), and evaporated; repeated addition and distillation of toluene gave a residue that, on examination by t.l.c. in 4:1 chloroform–ethanol, showed two components, one corresponding to triphenylmethanol. The mixture was chromatographed on silica gel, with 9:1 chloroform–ethanol, to give a compound that crystallized from ethanol–ether as refringent plates (117 mg, 89%), m.p. 122–123°, $[\alpha]_D^{20}$ +6° (*c* 0.61, methanol); i.r. data : ν_{max}^{KBr} 3460 (OH), 3300 (NH), 1650 (Amide I), 1560 (Amide II), 1490 (Ar), 1450 (Ar), and 670 cm^{-1} (Ph); t.l.c. in 9:1 chloroform–ethanol: R_F 0.5.

Anal. Calc. for $C_{28}H_{31}NO_6$: C, 70.42; H, 6.54; N, 2.93; O, 20.10. Found: C, 70.41; H, 6.60; N, 2.82; O, 20.36.

*Methyl 2-acetamido-2-deoxy-3,4-di-*O*-methyl-6-*O*-trityl-α-*D*-mannopyranoside* (**4**). — A solution of **3** (115 mg) in methyl iodide (3 ml) was boiled under reflux with silver oxide (250 mg) for 12 h, and then for 6 h after a further addition of silver

oxide (100 mg). Examination of the mixture by t.l.c. in 19:1 chloroform–ethanol showed total conversion of the starting material. After filtration, the solids were washed with warm chloroform, and the combined filtrates were dried (sodium sulfate) and evaporated. The residue was chromatographed on silica gel, with 19:1 chloroform–ethanol, to give 99 mg (81%) of a compound that crystallized from ethanol as plates, m.p. 117–119°, $[\alpha]_D^{20}$ +7° (*c* 0.51, methanol); i.r. data : ν_{max}^{KBr} 3425–3430 (NH), 1675 (Amide I), 1515 (Amide II), 1600, 1490, and 1450 cm^{-1} (Ar); t.l.c. in 9:1 chloroform–ethanol: R_F 0.6.

Anal. Calc. for $C_{30}H_{35}NO_6$: C, 71.27; H, 6.98; N, 2.77; OMe, 18.41. Found: C, 71.17; H, 6.93; N, 2.73; OMe, 18.75.

*Methyl 2-acetamido-2-deoxy-3,4-di-*O*-methyl-α-*D*-mannopyranoside* (**5**). — A solution of **4** (90 mg) in glacial acetic acid (3 ml) was heated on a water bath (80°); then, water (2 ml) was added dropwise, heating was continued for 45 min, the mixture was cooled to room temperature, and water (8 ml) was added. The precipitated triphenylmethanol was filtered off, and the filtrate was diluted to 100 ml with water, and freeze-dried to give a syrup. Chromatography of this syrup on silica gel, with 19:1 chloroform–ethanol, gave 42 mg (90%) of material that crystallized from methanol–ether as needles, m.p. 175–176°, $[\alpha]_D^{20}$ +52° (*c* 0.44, ethanol); i.r. data : ν_{max}^{KBr} 3545 (OH), 3250 (NH), 1635 (Amide I), and 1555 cm^{-1} (Amide II); t.l.c. in 19:1 chloroform–ethanol: R_F 0.2; R_{TMG} 0.7.

Anal. Calc. for $C_{11}H_{21}NO_6$: C, 50.18; H, 8.04; N, 5.32; OMe, 35.36. Found: C, 50.11; H, 8.01; N, 5.26; OMe, 34.97.

*Methyl 2-acetamido-4,6-*O*-benzylidene-2-deoxy-3-*O*-methyl-α-*D*-mannopyranoside* (**9**). — A mixture of compound[11] **6** (100 mg), methyl iodide (8.5 ml), and silver oxide (300 mg) in dry tetrahydrofuran (1.5 ml) was boiled under reflux for 8 h, and heating was continued for 12 h after a further addition of silver oxide (150 mg). After filtration, the residue was successively washed with three 50-ml portions of warm chloroform and two 5-ml portions of methanol, and the combined filtrates were evaporated. The residue was chromatographed on silica gel with 9:1 chloroform–ethanol. A pure fraction was obtained that crystallized from methanol to give 84 mg (80%) as plates, m.p. 81–84°, $[\alpha]_D^{20}$ +15° (*c* 0.54, chloroform); i.r. data : ν_{max}^{KBr} 3300 (NH), 1650 (Amide I), 1540 (Amide II), 1450 (Ar), and 640 cm^{-1} (Ph); t.l.c. in 19:1 chloroform–ethanol: R_F 0.5.

Anal. Calc. for $C_{17}H_{23}NO_6$: C, 60.52; H, 6.87; N, 4.15; O, 28.45; OMe, 18.40. Found: C, 60.38; H, 6.95; N, 4.00; O, 28.57; OMe, 18.34.

*Methyl 2-acetamido-2-deoxy-3-*O*-methyl-α-*D*-mannopyranoside* (**10**). — A solution of **9** (80 mg) in 60 % acetic acid (8 ml) was heated for 1 h on a water bath (80°), and evaporated; the residue was dried by repeated addition and distillation of toluene, dissolved in water (100 ml), and freeze-dried to give a syrup. The syrup was chromatographed on silica gel with 4:1 chloroform–ethanol to give 46 mg (78%) of amorphous **10**, $[\alpha]_D^{20}$ +20° (*c* 0.69, methanol); i.r. data : ν_{max}^{film} 3500 (broad, OH), 1650 (Amide I), and 1545 cm^{-1} (Amide II); t.l.c. in 4:1 chloroform–ethanol: R_F 0.5; R_{TMG} 0.4.

Anal. Calc. for $C_{10}H_{19}NO_6$: C, 48.19; H, 7.68; N, 5.62; OMe, 24.50. Found: C, 48.12; H, 7.60; N, 5.66; OMe, 24.46.

Methyl 2-acetamido-3-O-benzoyl-4,6-O-benzylidene-2-deoxy-α-D-mannopyranoside (7). — A solution of **6** (250 mg) in dry pyridine (4 ml) was cooled to −60°, treated with benzoyl chloride (89 µl) for 4 h at −20° and 12 h at −5°, and then diluted with chloroform (10 ml). The clear solution was successively washed with ice-cold, saturated solutions of sodium hydrogen sulfate and sodium hydrogen carbonate, and with ice-cold water, dried (sodium sulfate), and evaporated to give a syrup which was chromatographed on silica gel with 19:1 chloroform–ethanol. The product (220 mg, 75%) crystallized from chloroform–ethanol as small needles, m.p. 236–237°, $[\alpha]_D^{20}$ −22° (*c* 0.59, methanol); i.r. data: ν_{max}^{KBr} 3405 (NH), 1700 (ester), 1670 (Amide I), 1550 (Amide II), 715, and 690 cm^{-1} (Ph); t.l.c. in 19:1 chloroform–ethanol: R_F 0.6.

Anal. Calc. for $C_{23}H_{25}NO_7$: C, 64.63; H, 5.90; N, 3.28; O, 26.20. Found: C, 64.40; H, 5.86; N, 3.20; O, 26.48.

Methyl 2-acetamido-3-O-benzoyl-2-deoxy-α-D-mannopyranoside (**12**). — A solution of **7** (225 mg) in glacial acetic acid (4 ml) was heated on a water bath (80°); the hot solution was diluted with water (2 ml), and heating was continued for 1 h. The solution was cooled and evaporated, the residue was dissolved in water (150 ml), and the solution was freeze-dried to give a syrup (154 mg). Chromatography of this syrup on silica gel in 7:3 chloroform–ethanol gave 142 mg (80%) of material that crystallized from ethanol–chloroform to give plates containing one molecule of chloroform per molecule, which was not removed by drying the melted product in high vacuum; m.p. 115–116°, $[\alpha]_D^{20}$ +35° (*c* 0.75, methanol); i.r. data: ν_{max}^{KBr} 3330 (OH), 3200 (NH), 1710 (ester), 1650 (Amide I), 1550 (Amide II), 775 (C–Cl), and 710 cm^{-1} (Ph); t.l.c. in 4:1 chloroform–ethanol: R_F 0.4.

Anal. Calc. for $C_{16}H_{21}NO_7 \cdot CHCl_3$: C, 44.51; H, 4.83; Cl, 23.18; N, 3.05; O, 24.41. Found: C, 45.06; H, 4.87; Cl, 23.08; N, 3.21; O, 24.81.

Crystallization of **12** from methanol–ether gave prisms that contained 0.5 molecule of ether per molecule, m.p. 107–109°.

Anal. Calc. for $C_{16}H_{21}NO_7 \cdot 0.5\ C_2H_5OC_2H_5$: C, 57.43; H, 6.96; N, 3.72. Found: C, 57.56; H, 7.08; N, 3.84.

Methyl 2-acetamido-3,6-di-O-benzoyl-2-deoxy-α-D-mannopyranoside (**14**). — A solution of **12** (90 mg) in dry pyridine (3 ml) was cooled to −70° and benzoyl chloride (33 µl) was slowly added. The mixture was kept for 2 h at −20° and 12 h at −5°, and then processed as described for compound **7**. The product was chromatographed on silica gel, with 9:1 chloroform–ethanol, and a syrupy material (82 mg, 70%) was obtained that crystallized from chloroform–ether; m.p. 208–209°, $[\alpha]_D^{20}$ +89° (*c* 0.32, chloroform); i.r. data: ν_{max}^{KBr} 3530 (OH), 3280 (NH), 1710 and 1690 (ester), 1660 (Amide I), 1600 (Ar), and 1530 cm^{-1} (Amide II); t.l.c. in 9:1 chloroform–ethanol: R_F 0.7.

Anal. Calc. for $C_{23}H_{25}NO_8$: C, 62.30; H, 5.68; N, 3.16; O, 28.86. Found: C, 62.04; H, 5.63; N, 3.23; O, 29.14.

Methyl 2-acetamido-3,6-di-O-benzoyl-2-deoxy-4-O-methyl-α-D-mannopyranoside (**16**). — Compound **14** (45 mg) in dry acetone (1 ml) was treated with methyl iodide (2 ml) and silver oxide (150 mg) for 32 h at room temperature, with vigorous stirring of the reaction mixture. A further 100 mg of silver oxide was added, and stirring was continued for 12 h. The solids were filtered off, and washed with warm chloroform, and the combined filtrate and washings were evaporated. The residue was dissolved in methyl iodide (3 ml) and re-treated with silver oxide (200 mg) for 32 h. After processing the mixture as described for **2**, the residue was chromatographed on silica gel. Elution with 29:1 chloroform–ethanol gave 36 mg (78%) of material that, on crystallization from ether, gave stout needles, m.p. 151–152°, $[\alpha]_D^{20}$ +59° (*c* 0.37, chloroform); i.r. data: ν_{max}^{KBr} 3200 (NH), 1720 (ester), 1655 (Amide I), 1585 (Ar), and 1550 cm^{-1} (Amide II); t.l.c. in 19:1 chloroform–ethanol: R_F 0.8.

Anal. Calc. for $C_{24}H_{27}NO_8$: C, 63.01; H, 5.95; N, 3.06; OMe, 13.57. Found: C, 63.00; H, 5.86; N, 3.07; OMe, 13.43.

Methyl 2-acetamido-2-deoxy-4-O-methyl-α-D-mannopyranoside (**11**). — *A. From* **16**. A solution of **16** (28 mg) in dry methanol (2 ml) was treated with 0.1M methanolic sodium methoxide (0.1 ml) for 18 h at 4°, and then diluted with methanol (2 ml), and de-ionized with Rexyn 300 (H^+, OH^-) ion-exchange resin (1 ml), and evaporated. The residue was chromatographed on silica gel with 4:1 chloroform–ethanol to give 12.6 mg (83%) of material that crystallized from methanol–ether as plates, m.p. 121–123°, $[\alpha]_D^{20}$ +43° (*c* 0.53, methanol); i.r. data: ν_{max}^{KBr} 3370 (OH), 3260 (NH), 1650 (Amide I), 1550 (Amide II), and 1130–1090 cm^{-1} (CH–O–CH_2–); t.l.c. in 4:1 chloroform–ethanol: R_F 0.5; R_{TMG} 0.3.

Anal. Calc. for $C_{10}H_{19}NO_6$: C, 48.19; H, 7.68; N, 5.62. Found: C, 47.97; H, 7.54; N, 5.74.

B. From **17**. A solution of **17** (26 mg) in methanol (2 ml) was treated with 0.1M methanolic sodium methoxide (0.1 ml) for 2 h at 0° and for 8 h at room temperature. The solution was treated with Rexyn 300 (H^+,OH^-) ion-exchange resin (1 ml) and evaporated. The residue crystallized, as plates, from methanol–ether (12.9 mg, 80%), m.p. 120–122°, $[\alpha]_D^{20}$ +43° (*c* 0.42, methanol); t.l.c. in 4:1 chloroform–ethanol: R_{TMG} 0.3, a value identical with that for the product obtained from **16**.

A solution of **11** (1.5 mg) in dry pyridine (1 ml) was treated with chlorotriphenylmethane (1.2 mg) for 48 h at room temperature. The solution was evaporated, and the residue was fractionated by t.l.c. in 9:1 chloroform–ethanol to give triphenylmethanol and the trityl ether of **11**. This compound was treated with 60% acetic acid (1 ml) for 1 h at 80°; t.l.c. in 9:1 chloroform–ethanol then indicated the presence of **11** and triphenylmethanol.

Methyl 2-acetamido-3-O-acetyl-4,6-O-benzylidene-2-deoxy-α-D-mannopyranoside (**8**). — Acetic anhydride (2 ml) was added to a solution of **6** (150 mg) in dry pyridine (2 ml), cooled to 0°, and the solution was kept for 30 h at room temperature, diludet with chloroform (10 ml), and evaporated; repeated addition and distillation of toluene gave a crystalline residue. Recrystallization from methanol–ether gave 145 mg (85%) of needles, m.p. 210–212°, $[\alpha]_D^{20}$ +34° (*c* 0.62, methanol); $[\alpha]_D^{20}$ +31° (*c* 0.46,

chloroform); i.r. data: ν_{max}^{KBr} 3405 (NH), 1700 (ester), 1670 (Amide I), and 1530 cm^{-1} (Amide II); t.l.c. in 19:1 chloroform–ethanol: R_F 0.55. Compound **8** has been reported[11] to exist in a crystalline form containing 0.5 molecule of water per molecule, m.p. 205–208°, $[\alpha]_D^{25}$ −11° (*c* 1.0, pyridine); $[\alpha]_D^{25}$ +32° (*c* 0.6, chloroform).

Anal. Calc. for $C_{18}H_{23}NO_7$: C, 59.17; H, 6.34; N, 3.83; O, 30.65. Found: C, 59.13; H, 6.37; N, 3.78; O, 30.75.

*Methyl 2-acetamido-3-*O*-acetyl-2-deoxy-*α*-*D*-mannopyranoside* (**13**). — Compound **8** (130 mg) was heated with 60% acetic acid (5 ml) for 1 h on a water bath (80°). The solution was cooled and concentrated, and toluene was repeatedly added and distilled off. The residue was chromatographed on silica gel, and elution with 7:3 chloroform–ethanol gave 89 mg (90%) of amorphous **13**; $[\alpha]_D^{20}$ +42° (*c* 1.72, methanol); i.r. data: ν_{max}^{film} 3540 (OH), 3290 (NH), 1750 (OAc), 1665 (Amide I), and 1555 cm^{-1} (Amide II); t.l.c. in 4:1 chloroform–ethanol: R_F 0.3.

Anal. Calc. for $C_{11}H_{19}NO_7$: C, 47.65; H, 6.91; N, 5.05; O, 40.39. Found: C, 47.64; H, 6.91; N, 4.92; O, 40.56.

*Methyl 2-acetamido-3-*O*-acetyl-6-*O*-benzoyl-2-deoxy-*α*-*D*-mannopyranoside* (**15**). — A solution of **13** (80 mg) in dry pyridine (4 ml) was cooled to −60°. Benzoyl chloride (34 µl) was slowly added, and the mixture was kept for 1 h at −20° and for 12 h at −5°, and then processed as described for **7**. Chromatography of the resulting syrup (95 mg) on silica gel with 19:1 chloroform–ethanol gave **15**, which crystallized from ether–benzene as needles (65 mg, 60%), m.p. 96–100°, $[\alpha]_D^{20}$ +44° (*c* 0.73, methanol); i.r. data: ν_{max}^{KBr} 3500 (OH), 3300 (NH), 1720 (ester), 1695 (Amide I), and 1560 cm^{-1} (Amide II); t.l.c. in 9:1 chloroform–ethanol: R_F 0.5.

Anal. Calc. for $C_{18}H_{23}NO_8$: C, 56.69; H, 6.08; N, 3.67; O, 33.56. Found: C, 56.58; H, 6.04; N, 3.60; O, 33.56.

*Methyl 2-acetamido-3-*O*-acetyl-6-*O*-benzoyl-2-deoxy-4-*O*-methyl-*α*-*D*-mannopyranoside* (**17**). — A solution of **15** (46 mg) in anhydrous ether (4 ml) was cooled to −10°, boron trifluoride etherate (0.05 ml of a 3.3% solution in ether) was added, and the mixture was treated with diazomethane in ether until the yellow color of diazomethane persisted. T.l.c. in 19:1 benzene–methanol showed incomplete conversion of **15**. The solution was filtered through a sintered funnel (to remove polymeric material), the filtrate was evaporated, and the residue was dried under high vacuum. The methylation was repeated, and the resulting syrup was chromatographed on silica gel with 14:1 benzene–chloroform, to give amorphous **17** (33 mg, 69%), $[\alpha]_D^{20}$ +54° (*c* 0.5, methanol); i.r. data: ν_{max}^{KBr} 3300 (NH), 1775 and 1730 (ester), 1650 (Amide I) and 1540 cm^{-1} (Amide II); t.l.c. in 19:1 chloroform–ethanol: R_F 0.6.

Anal. Calc. for $C_{19}H_{25}NO_8$: C, 57.71; H, 6.37; N, 3.54; OMe, 15.69. Found: C, 57.64; H, 6.44; N, 3.57; OMe, 15.40.

ACKNOWLEDGMENT

The authors thank Dr. P. H. Gross for a gift of methyl 2-acetamido-4,6-*O*-benzylidene-2-deoxy-α-D-mannopyranoside.

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Reprinted from

Carbohydrate Research, 38 (1974) 205–216

SYNTHESIS OF THE 6-METHYL AND 3,6- AND 4,6-DIMETHYL ETHERS OF METHYL 2-ACETAMIDO-2-DEOXY-α-D-MANNOPYRANOSIDE*

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(Received August 10th, 1974; accepted September 6th, 1974)

ABSTRACT

The 6-mono- (**6**) and 4,6- (**16**) and 3,6-di-methyl (**25**) ethers of methyl 2-acetamido-2-deoxy-α-D-mannopyranoside have been synthesized from 6-*O*-trityl, 4,6-*O*-benzylidene, and 3-*O*-methyl derivatives, respectively, by way of *O*-benzoyl and of *O*-allyl derivatives. The yields were respectively 37 and 43% for **6**, 34 and 50% for **16**, and 14 and 25% for **25**. These ethers are used as standard compounds for the structure elucidation, by methylation, of polymers containing 2-amino-2-deoxy-D-mannose.

INTRODUCTION

2-Amino-2-deoxy-D-mannose[1] and 2-amino-2-deoxy-D-mannuronic acid[2] are components of the bacterial cell-wall. For the structural investigation of the complex polymers containing these two sugars, methyl ethers of 2-amino-2-deoxy-D-mannose are needed, as the uronic acid derivatives can be reduced to the corresponding hexose derivatives. We have previously described[3] the synthesis of the 3- and 4-mono-, 3,4-di-, and 3,4,6-tri-methyl ethers of 2-amino-2-deoxy-D-mannose. We now describe the synthesis of the remaining methyl ethers susceptible to being formed by methylation of 2-amino-2-deoxy-D-mannopyranose, namely, the 6-mono- (6) and 4,6- (**16**) and 3,6-di-methyl (**25**) ethers. In addition to the benzoyl group conventionally used, the allyl group[4] was examined for the protection of the hydroxyl groups on C-3 and C-4; it was found to be very convenient, as etherification therewith readily proceeds to completion, the derivatives formed are isolated in good yield, and the removal of

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*Amino Sugars XCII. This is publication No. 646 of the Robert W. Lovett Memorial Group for the Study of Diseases Causing Deformities, Harvard Medical School and the Massachusetts General Hospital, Boston, Massachusetts. This work was supported by research grants from the National Institute of Allergy and Infectious Diseases (AI-06692) and from the National Institute of Arthritis, Metabolism, and Digestive Diseases (AM-03564), National Institutes of Health, U. S. Public Health Service. A preliminary communication has been presented [*Abstr. Papers Amer. Chem. Soc. Meeting*, 168 (1974) CARB-16].

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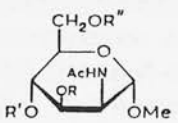
the protecting groups is conveniently performed with tris(triphenyl)phosphine-rhodium chloride and 1,4-diazabicyclo[2.2.2]octane (the Corey reagents[5]), followed by treatment with Dowex 50 (H^+) ion-exchange resin.

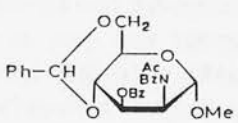
RESULTS AND DISCUSSION

Benzoylation of methyl 2-acetamido-2-deoxy-6-*O*-trityl-α-D-mannopyranoside (**1**) with 2 molar equiv. of benzoyl chloride in the presence of pyridine at −60° (in order to avoid *N*-benzoylation[3,6]) gave the 3,4-dibenzoate (**2**) in 72% yield. A more polar compound, having properties corresponding to those of a monobenzoic ester, was isolated in 10% yield; it was probably[7] the 3-benzoate (**3**). Removal of the trityl group gave crystalline methyl 2-acetamido-3,4-di-*O*-benzoyl-2-deoxy-α-D-manno-pyranoside (**4**), which was methylated with methyl iodide and silver oxide (the Purdie reagents[8]) to give the crystalline 6-methyl ether (**5**), subsequently hydrolyzed under alkaline conditions to afford crystalline methyl 2-acetamido-2-deoxy-6-*O*-methyl-α-D-mannopyranoside (**6**) in an overall yield (from **1**) of ~37%.

Compound **6** was also synthesized from **1** through the crystalline 3,4-di-*O*-allyl-6-*O*-trityl derivative (**7**), obtained in 85% yield. In order to minimize *N*-substitution during the allylation of the hydroxyl groups[9], sodium hydride was replaced by sodium hydroxide, a milder reagent. Compound **7** was detritylated in 85% yield to give the syrupy 3,4-di-*O*-allyl derivative (**8**), which was methylated with the Purdie reagents. The resulting, crystalline 6-methyl ether (**9**), obtained in 82% yield, was treated with the Corey reagents[5], a procedure less cumbersome than the isomerization of allyl into 1-propenyl groups with dimethyl sulfoxide[4]; although, in this case, the isomerization was not complete, repetition of the procedure raised the yield to over 70%. The 1-propenyl derivative obtained was directly hydrolyzed, without purification, with a sulfonic resin to give **6** in an overall yield of 43% from **1**.

Comparison of the yields of **6** respectively obtained *via* the benzoate and the allyl derivatives shows that little migration of the benzoate groups occurred during methylation with the Purdie reagents. The acid- and alkali-stable allyl derivatives





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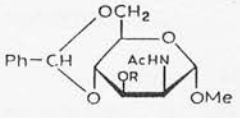
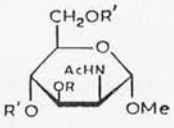
1 R = R' = H, R'' = Tr
2 R = R' = Bz, R'' = Tr
3 R = Bz, R' = H, R'' = Tr
4 R = R' = Bz, R'' = H
5 R = R' = Bz, R'' = Me
6 R = R' = H, R'' = Me
7 R = R' = $-CH_2-CH=CH_2$, R'' = Tr
8 R = R' = $-CH_2-CH=CH_2$, R'' = H
9 R = R' = $-CH_2-CH=CH_2$, R'' = Me

were found to be convenient intermediates, and the removal of the allyl groups under mildly acidic conditions suggests their use in the synthesis of alkali-labile, oligosaccharide-containing compounds.

Re-investigation of the benzoylation of methyl 2-acetamido-4,6-*O*-benzylidene-2-deoxy-α-D-mannopyranoside (**11**) showed that treatment with only one molar equiv. of benzoyl chloride at −5° results in *N*-benzoylation, with formation of the *N*-benzoyl-3-*O*-benzoyl derivative (**10**) in 47% yield, and that a mixing temperature of −60°, followed by −20°, was needed in order to avoid *N*-benzoylation and obtain **12**, as previously reported[3]. Purdie methylation of the previously described[3] methyl 2-acetamido-3-*O*-benzoyl-2-deoxy-α-D-mannopyranoside (**14**), and saponification of the resulting crystalline 4,6-dimethyl ether (**15**) gave, in an overall yield of 34% from **11**, amorphous methyl 2-acetamido-2-deoxy-4,6-di-*O*-methyl-α-D-mannopyranoside (**16**). In a similar sequence of reactions, amorphous **16** was obtained, in an overall yield of 50%, by way of the crystalline 3-*O*-allyl-4,6-*O*-benzylidene (**13**), 3-*O*-allyl (**17**), and 3-*O*-allyl-4,6-di-*O*-methyl (**18**) derivatives.





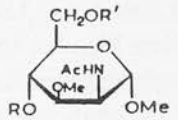
11 R = H
12 R = Bz
13 R = −CH$_2$−CH=CH$_2$

14 R = Bz, R' = H
15 R = Bz, R' = Me
16 R = H, R' = Me
17 R = −CH$_2$−CH=CH$_2$, R' = H
18 R = −CH$_2$−CH=CH$_2$, R' = Me

Treatment of methyl 2-acetamido-2-deoxy-3-*O*-methyl-α-D-mannopyranoside[3] (**19**) with chlorotriphenylmethane under vigorous conditions gave the 6-trityl ether (**20**) in only 61% yield. For an evaluation of the steric and electronic interactions of the hydroxyl groups on C-3 and C-4, it is of interest to compare the yield with that of the tritylation of methyl 2-acetamido-2-deoxy-α-D-glucopyranoside[10] (82%) and its 3-methyl ether[10] (70%), and of methyl 2-acetamido-2-deoxy-α-D-galactopyranoside[11] (88%) and its 3-methyl[12] (86%) and 3,4-dimethyl[13] (43%) ethers.

Benzoylation of **20** at very low temperature afforded the crystalline 4-*O*-benzoyl-3-*O*-methyl-6-*O*-trityl derivative (**21**), which was detritylated to give the crystalline 4-*O*-benzoyl-3-*O*-methyl derivative (**22**). Methylation with the Purdie reagents in tetrahydrofuran resulted in the formation of methyl 2-acetamido-4-*O*-benzoyl-2-deoxy-3,6-di-*O*-methyl-α-D-mannopyranoside (**23**), contaminated with some of the 6-*O*-benzoyl-3,4-di-*O*-methyl derivative (**24**). A similar migration of the benzoyl group from O-4 to O-6 under the mild alkaline conditions of the Purdie methylation, probably through the formation of an intermediate orthobenzoate, has previously been observed in the *gluco*[14] and *galacto*[12] series. Removal of the 4-*O*-benzoyl group gave amorphous methyl 2-acetamido-2-deoxy-3,6-di-*O*-methyl-α-D-mannopyranoside (**25**) in an overall yield of 14%.

In a similar sequence of reactions, **25** was obtained from **20** (in an overall yield of 25%) by way of the crystalline 4-*O*-allyl-3-*O*-methyl-6-*O*-trityl (**26**), amorphous 4-*O*-allyl-3-*O*-methyl (**27**), and crystalline 4-*O*-allyl-3,6-di-*O*-methyl (**28**) derivatives.



19 R = R' = H
20 R = H, R' = Tr
21 R = Bz, R' = Tr
22 R = Bz, R' = H
23 R = Bz, R' = Me
24 R = Me, R' = Bz
25 R = H, R' = Me
26 R = $-CH_2-CH=CH_2$, R' = Tr
27 R = $-CH_2-CH=CH_2$, R' = H
28 R = $-CH_2-CH=CH_2$, R' = Me

EXPERIMENTAL

General. — Melting points were determined with a Mettler FP-2 apparatus and correspond to "corrected melting points". The i.r. spectra were recorded with a Perkin–Elmer Model 237 spectrophotometer. Optical rotations were measured in 1-dm semimicrotubes with a Perkin–Elmer Model 141 polarimeter. Gas–liquid chromatography (g.l.c.) of the per(trimethylsilyl)[15] ethers was performed with a Perkin–Elmer Model 900 gas chromatograph equipped with a flame-ionization detector and a stainless-steel column (1.5 m) of 3% of OV-225 on Gas Chrom Q (100–200 mesh) programmed for a rise of 10°.min^{-1} from 80 to 250°, with nitrogen as the carrier gas. The chloroform used was analytical-reagent grade, and contained 0.75% of ethanol. Column chromatography was performed on Silica Gel Merck (70–325 mesh; E. Merck, Darmstadt, Germany), used without pretreatment. The proportion of weight of substance to weight of silica gel was 1:70 to 1:90. The volume of the fractions eluted was 3–4 ml per g of the substance to be chromatographed. Thin-layer chromatography (t.l.c.) was performed on plates of silica gel (without fluorescence indicator; layer thickness 0.25 mm; E. Merck). The compounds were detected by spraying the plates with (*A*) 1:1:18 (v/v) anisaldehyde–conc. sulfuric acid–ethanol, or (*B*) 1:10 (v/v) conc. sulfuric acid–ethanol. The R_{TMG} values refer to the mobilities on plates of silica gel, relative to that of methyl 2-acetamido-2-deoxy-3,4,6-tri-*O*-methyl-α-D-glucopyranoside as unity. Evaporations were conducted *in vacuo*, with the bath temperature kept below 40°. G.l.c.–mass spectrometry (g.l.c.–m.s.) was performed with an analytical system consisting of an IBM 1800 computer fed raw data generated by a single-focussing, mass spectrometer (Perkin–Elmer–Hitachi RMU-6). In all cases, the analyses were conducted on the per(trimethylsilyl) ethers of the sugars. Microanalyses were performed by Dr. W. Manser, Zurich, Switzerland.

*Methyl 2-acetamido-3,4-di-*O*-benzoyl-2-deoxy-6-*O*-trityl-α-*D*-mannopyranoside* (**2**). — A solution of compound **1** (110 mg) in dry pyridine (10 ml) was treated with benzoyl chloride (39 μl) at −60°. The mixture was kept for 8 h at −20° and for 20 h at −10°, and then diluted with chloroform (10 ml) and successively washed with ice-cold, saturated solutions of sodium hydrogensulfate, sodium hydrogencarbonate, and ice-cold water, dried (sodium sulfate), and evaporated to give a syrup which was chromatographed on silica gel with 19:1 (v/v) dichloromethane–ethanol. The major, syrupy product was crystallized from ether to give small needles (111 mg, 72%), m.p. 129–131° (with softening at 113°), $[\alpha]_D^{20}$ −22° (*c* 0.65, chloroform); i.r. data: ν_{max}^{KBr} 3290 (NH), 1770 (ester), 1655 (Amide I), and 1545 cm^{-1} (Amide II); t.l.c. in 19:1 (v/v) chloroform–ethanol: R_F 0.63.

Anal. Calc. for $C_{41}H_{39}NO_8$: C, 73.09; H, 5.83; N, 2.08; O, 18.99. Found: C, 72.99; H, 5.78; N, 2.13; O, 18.86.

*Methyl 2-acetamido-3-*O*-benzoyl-2-deoxy-6-*O*-trityl-α-*D*-mannopyranoside* (**3**). — The later fractions eluted from the silica gel column, described in the preceding paragraph, gave a compound [R_F 0.3 in 19:1 (v/v) chloroform–ethanol] which crystallized from benzene–hexane to give needles (15 mg), m.p. 223–225°, $[\alpha]_D^{20}$ +50° (*c* 0.59, chloroform); i.r. data: ν_{max}^{KBr} 3355 (NH), 1740 and 1680 (ester), 1655 (Amide I), and 1560 cm^{-1} (Amide II); t.l.c. in 19:1 (v/v) chloroform–ethanol: R_F 0.31.

Anal. Calc. for $C_{34}H_{35}NO_7$: C, 71.69; H, 6.19; N, 2.46; O, 19.66. Found: C, 72.04; H, 6.29; N, 2.40; O, 19.17.

A solution of **3** (3 mg) in dry methanol (1 ml) was treated with 0.1M methanolic sodium methoxide (0.01 ml) for 12 h at 4°, diluted with methanol (2 ml), de-ionized with Rexyn 300 (H^+, OH^-) ion-exchange resin (1 ml), and evaporated. T.l.c. of the residue in 9:1 (v/v) chloroform–methanol indicated the presence of **1**.

*Methyl 2-acetamido-3,4-di-*O*-benzoyl-2-deoxy-α-*D*-mannopyranoside* (**4**). — A suspension of **2** (90 mg) in acetic acid (5 ml, 60%) was heated on a water bath for 1 h at 80°, cooled to room temperature, diluted with water to 250 ml, and lyophilized. The syrupy residue was chromatographed on a column of silica gel with 14:1 (v/v) chloroform–ethanol. Triphenylmethanol was eluted first, and then **4**. Crystallization from ether–heptane gave 51 mg (86%) of prisms, m.p. 104–105°, $[\alpha]_D^{22}$ −26° (*c* 0.22, chloroform); i.r. data: ν_{max}^{KBr} 3430 (broad, OH and NH), 1730 (ester), 1655 (Amide I), and 1545 cm^{-1} (Amide II); t.l.c. in 14:1 (v/v) chloroform–ethanol: R_F 0.35.

Anal. Calc. for $C_{23}H_{25}NO_8$: C, 62.30; H, 5.68; N, 3.16. Found: C, 62.41; H, 6.06; N, 2.81.

*Methyl 2-acetamido-3,4-di-*O*-benzoyl-2-deoxy-6-*O*-methyl-α-*D*-mannopyranoside* (**5**). — Compound **4** (40 mg) was dissolved in methyl iodide (4 ml), and silver oxide (100 mg) was added. The mixture was vigorously stirred for 12 h at room temperature, and then boiled under reflux for 1 h. The mixture was cooled, diluted with chloroform, and filtered, and the solids were washed with warm chloroform. The filtrate and washings were combined and evaporated, and the residue was crystallized from ether–hexane to give a chromatographically pure material (31 mg, 75%). Recrystallization from ether–hexane gave prismatic needles, m.p. 168–169°, $[\alpha]_D^{20}$ −9° (*c* 0.70,

chloroform); i.r. data: ν_{max}^{KBr} 3320 (NH), 1710 (ester), 1670 (Amide I), and 1520 cm^{-1} (Amide II); t.l.c. in 19:1 (v/v) dichloromethane–ethanol: R_F 0.40.

Anal. Calc. for $C_{24}H_{27}NO_8$: C, 63.01; H, 5.95; N, 3.06; O, 27.98; OMe, 13.55. Found: C, 62.90; H, 5.98; N, 3.09; O, 28.04; OMe, 13.68.

Methyl 2-acetamido-2-deoxy-6-O-methyl-α-D-mannopyranoside (**6**). — *Method A. From* **5**. A solution of **5** (21 mg) in dry methanol (3 ml) was treated with 0.1M methanolic sodium methoxide (0.1 ml) for 12 h at 4°, and then for 8 h at room temperature. The solution was de-ionized with Rexyn 300 (H^+, OH^-) ion-exchange resin (2 ml), and evaporated. The residue was dissolved in methanol, the suspension filtered through charcoal–Celite, and the filtrate evaporated. The syrupy residue was crystallized from ethanol–ether to give needles (9 mg, 78%), m.p. 169–171°; $[\alpha]_D^{24}$ +38° (*c* 0.31, methanol); i.r. data: ν_{max}^{KBr} 3450–3250 (broad, OH and NH), 1655 (Amide I), and 1545 cm^{-1} (Amide II); t.l.c. in 9:1 (v/v) chloroform–ethanol: R_F 0.16; R_{TMG} 0.31.

Anal. Calc. for $C_{10}H_{19}NO_6$: C, 48.19; H, 7.68; N, 5.62; OMe, 24.50. Found: C, 48.00; H, 7.64; N, 5.60; OMe, 25.07.

Method B. From **9**. To a solution of **9** (25 mg) in ethanol (3 ml, 90%) was added tris(triphenyl)phosphinerhodium chloride (12 mg, 13 μmoles) and 1,4-diazabicyclo-[2.2.2]octane (5 mg). The mixture was boiled under reflux for 3 h, and the solution cooled and evaporated, the residue dispersed in water, and the suspension extracted with ether. The extract was dried (magnesium sulfate) and evaporated. A solution of the residue in methanol (3 ml) was treated with Dowex-50 X-8 (H^+) (100–200 mesh, 2 ml) ion-exchange resin for 12 h at 37°, the suspension filtered, and the filtrate evaporated. The residue was chromatographed on silica gel with 9:1 (v/v) chloroform–ethanol, to give a compound that crystallized as platelets (9 mg), m.p. 170–172°; $[\alpha]_D^{24}$ +37° (*c* 0.42, methanol); t.l.c. in 4:1 (v/v) chloroform–ethanol: R_F 0.16, R_{TMG} 0.31. The first fractions eluted from the silica gel column gave unreacted **9**, which was treated as just described to give additional **6** (total yield: 15 mg, 73%).

Methyl 2-acetamido-3,4-di-O-allyl-2-deoxy-6-O-trityl-α-D-mannopyranoside (**7**). — A solution of **1** (150 mg) in dry acetone (1 ml) and benzene (10 ml) was mixed with allyl bromide (54 μl) and powdered sodium hydroxide (500 mg), and the mixture boiled under reflux for 4 h, and cooled; sodium hydroxide (150 mg) was added, and the mixture was stirred for 12 h at room temperature, and diluted with benzene (5 ml). The solid was filtered off and washed with 7:3 (v/v) benzene–acetone (5 ml), the filtrate and washings were combined and evaporated, and the residue was dissolved in chloroform. The solution was washed with water (3 × 5 ml), dried (sodium sulfate), and evaporated. The syrupy residue was chromatographed on silica gel with 19:1 (v/v) chloroform–ethanol. A syrupy material was obtained that crystallized from 2-isopropoxypropane–heptane to give needles (145 mg, 85%), m.p. 110–112°, $[\alpha]_D^{24}$ +16° (*c* 0.82, chloroform); i.r. data: ν_{max}^{KBr} 3295 (NH), 1665–1635 (–CH_2–CH–CH_2, Amide I), and 1560 cm^{-1} (Amide II); t.l.c. in 19:1 (v/v) chloroform–ethanol: R_F 0.63.

Anal. Calc. for $C_{34}H_{39}NO_6$: C, 73.23; H, 7.05; N, 2.51; O, 17.21. Found: C, 73.20; H, 7.15; N, 2.37; O, 17.10.

Methyl 2-acetamido-3,4-di-O-allyl-2-deoxy-α-D-mannopyranoside (**8**). — A solution of **7** (125 mg) in glacial acetic acid (3 ml) was heated on a water bath (80°). The hot solution was diluted with water (1.5 ml), and heating was continued for 45 min. After dilution with cold water (150 ml), the solution was lyophilized, and the residue was chromatographed on silica gel with 14:1 (v/v) dichloromethane–ethanol to give 60 mg (85%) of a syrup, $[\alpha]_D^{24}$ +50° (*c* 0.60, methanol); i.r. data: ν_{max}^{KBr} 3440 (OH), 3250 (NH), 1660 ($-CH_2-CH=CH_2$), 1645 (Amide I), and 1545 cm^{-1} (Amide II); t.l.c. in 14:1 (v/v) chloroform–ethanol: R_F 0.31.

Anal. Calc. for $C_{15}H_{25}NO_6$: C, 57.13; H, 7.99; N, 4.44; O, 30.44. Found: C, 57.05; H, 8.01; N, 4.39; O, 30.44.

Methyl 2-acetamido-3,4-di-O-allyl-2-deoxy-6-O-methyl-α-D-mannopyranoside (**9**). — Compound **8** (50 mg) in dry tetrahydrofuran (2 ml) was treated with methyl iodide (3 ml) and silver oxide (250 mg) for 6 h under reflux, with vigorous stirring. A further 100 mg of silver oxide was added, and stirring was continued for 12 h. The solids were filtered off and washed with hot chloroform, and the combined filtrate and washings were evaporated. The residue was chromatographed on silica gel. Elution with 19:1 (v/v) chloroform–ethanol gave a material that crystallized from chloroform–heptane in plates (43 mg, 82%), m.p. 73–75°, $[\alpha]_D^{24}$ +44° (*c* 1.00, chloroform); i.r. data: ν_{max}^{KBr} 3290 (NH), 1670 ($-CH_2CH=CH_2$), 1640 (Amide I), and 1550 cm^{-1} (Amide II); t.l.c. in 19:1 (v/v) chloroform–ethanol: R_F 0.32.

Anal. Calc. for $C_{16}H_{27}NO_6$: C, 58.34; H, 8.26; N, 4.25; O, 29.14. Found: C, 58.27; H, 8.21; N, 4.33; O, 29.11.

Methyl 2-(N-acetylbenzamido)-3-O-benzoyl-4,6-O-benzylidene-2-deoxy-α-D-mannopyranoside (**10**). — Methyl 2-acetamido-4,6-*O*-benzylidene-2-deoxy-α-D-mannopyranoside[3] (**11**, 100 mg) in dry pyridine (2 ml) was treated with benzoyl chloride (54 ml) for 20 h at −5°. The mixture was diluted with chloroform (6 ml), and then processed as described for **2**. The chloroform extract was dried (sodium sulfate), filtered, and evaporated. The residue was chromatographed on silica gel with 19:1 (v/v) chloroform–ethanol to give a syrup (90 mg) that crystallized from methanol as needles (78 mg, 47%), m.p. 161–163°, $[\alpha]_D^{26}$ −70° (*c* 0.51, chloroform); i.r. data: ν_{max}^{KBr} 1715 (ester), 1680 (*N*-Ac and *N*-Bz), 1590 and 1580 (Ar), 1450 (CO), and 740 cm^{-1} (Ar); no Amide II band was observed; t.l.c. in 19:1 (v/v) chloroform–methanol: R_F 0.76.

Anal. Calc. for $C_{30}H_{29}NO_8$: C, 67.79; H, 5.50; N, 2.63; O, 24.08. Found: C, 68.24; H, 5.48; N, 2.73; O, 23.88.

Methyl 2-acetamido-3-O-benzoyl-2-deoxy-4,6-di-O-methyl-α-D-mannopyranoside (**15**). — Methyl 2-acetamido-3-*O*-benzoyl-2-deoxy-α-D-mannopyranoside[3] (**14**) (25 mg) in dry tetrahydrofuran (4 ml) was treated with methyl iodide (1.5 ml) and silver oxide (100 mg) for 16 h at room temperature, followed by boiling under reflux for 4 h. The solids were filtered off and washed with warm chloroform, the combined filtrate and washings were evaporated, and the residue was chromatographed on silica gel. Elution with 19:1 (v/v) chloroform–ethanol gave 18 mg (65%) of material that, on crystallization from chloroform–ether–pentane, gave platelets containing

0.5 molecule of water per molecule, m.p. 121–123°; $[\alpha]_D^{22}$ +35° (*c* 0.41, chloroform); i.r. data: ν_{max}^{KBr} 3500 (OH), 3275 (NH), 1740 (ester), 1655 (Amide I), 1550 (Amide II), and 1455 cm^{-1} (Ar); t.l.c. in 19:1 (v/v) chloroform–ethanol: R_F 0.48.

Anal. for $C_{18}H_{25}NO_7 \cdot 0.5H_2O$: C, 57.43; H, 6.96; N, 3.72. Found: C, 57.59; H, 6.96; N, 4.12.

*Methyl 2-acetamido-2-deoxy-4,6-di-*O*-methyl-α-*D*-mannopyranoside* (**16**). — *Method A. From* **15**. Compound **15** (13 mg) in dry methanol (2 ml) was treated with 0.1M methanolic sodium methoxide (50 ml) for 18 h at 4°, and then the solution was diluted with methanol, de-ionized with Rexyn 300 (H^+, OH^-) ion-exchange resin (1 ml), and evaporated. The residue was chromatographed on silica gel with 9:1 (v/v) chloroform–ethanol to give 8 mg (86%) of material that crystallized from cold chloroform–ether–hexane as plates; at room temperature, the crystalline material turned partly amorphous; $[\alpha]_D^{20}$ +49° (*c* 0.36, ethanol); t.l.c. in 9:1 (v/v) chloroform–ethanol: R_F 0.4, R_{TMG} 0.67.

Anal. Calc. for $C_{11}H_{21}NO_6$: C, 50.18; H, 8.04; N, 5.32; OMe, 35.36. Found: C, 50.05; H, 8.13; N, 5.21; OMe, 35.05.

Method B. From **18**. A solution of **18** (30 mg) in 90% ethanol (4 ml) was treated with tris(triphenyl)phosphinerhodium chloride (8 mg) and 1,4-diazabicyclo[2.2.2]-octane (2 mg). The mixture was processed as described for the preparation of **6** from **9**, and the resulting product was chromatographed on silica gel with 9:1 (v/v) chloroform–ethanol to give an amorphous substance (14 mg) and unconverted **18**. A second treatment gave additional pure **16** (7 mg; total yield 81%), $[\alpha]_D^{20}$ +48° (*c* 0.42, ethanol); t.l.c. in 9:1 (v/v) chloroform–ethanol: R_F 0.4, R_{TMG} 0.67.

*Methyl 2-acetamido-3-*O*-allyl-4,6-*O*-benzylidene-2-deoxy-α-*D*-mannopyranoside* (**13**). — A solution of **11** (170 mg) in dry acetone (2 ml) and benzene (15 ml) was treated with allyl bromide (46 ml) and sodium hydroxide (400 mg). The mixture was boiled under reflux for 2 h, and then cooled, and sodium hydroxide (150 mg) was added. After being vigorously stirred for 12 h at room temperature, the mixture was filtered, and the insoluble material washed with warm, 1:1 (v/v) benzene–acetone (10 ml). The combined filtrate and washings were evaporated, the residue was dissolved in chloroform, and the solution was washed with water, dried (sodium sulfate), and evaporated. The residue was chromatographed on silica gel with 19:1 (v/v) dichloromethane–methanol to give a material that crystallized from 2-isopropoxypropane in prismatic needles (163 mg, 85%), m.p. 93–94°, $[\alpha]_D^{20}$ +9° (*c* 0.52, chloroform); i.r. data: ν_{max}^{KBr} 3275 (NH), 1670 (CH_2–CH=CH_2), 1635 (Amide I), and 1550 cm^{-1} (Amide II); t.l.c. in 19:1 (v/v) dichloromethane–methanol: R_F 0.4.

Anal. Calc. for $C_{19}H_{25}NO_6$: C, 62.80; H, 6.93; N, 3.85; O, 26.42. Found: C, 62.79; H, 6.89; N, 3.84; O, 26.49.

*Methyl 2-acetamido-3-*O*-allyl-2-deoxy-α-*D*-mannopyranoside* (**17**). — Compound **13** (140 mg) in 60% acetic acid (5 ml) was treated for 1 h on a water bath (80°). The solution was cooled and concentrated, and toluene was repeatedly added and distilled off. The residue was chromatographed on silica gel; elution with 7:3 (v/v) chloroform–ethanol gave 94 mg (89%) of **17** that tenaciously retained chloroform after being

dried *in vacuo*, as indicated by variable values for its chlorine content; $[\alpha]_D^{22}$ +33° (*c* 0.30, methanol); i.r. data: ν_{max}^{film} 3400–3250 (broad, OH, NH), 1660–1645 ($CH_2CH=CH_2$, Amide I), 1575 (Amide II), and 750 cm^{-1} (C–Cl); t.l.c. in 4:1 (v/v) chloroform–ethanol: R_F 0.60.

Anal. Calc. for $C_{12}H_{21}NO_6 \cdot 0.1CHCl_3$: C, 50.59; H, 7.40; N, 4.88. Found: C, 50.32; H, 7.49; N, 4.64.

Methyl 2-acetamido-3-O-allyl-2-deoxy-4,6-di-O-methyl-α-D-mannopyranoside (**18**). — A solution of **17** (65 mg) in dry tetrahydrofuran (2 ml) was mixed with methyl iodide (3 ml) and silver oxide (250 mg), and the mixture was boiled under reflux for 12 h. After a new addition of silver oxide (150 mg), the mixture was boiled under reflux for 8 h, and then cooled to room temperature and filtered. The residue was washed with warm chloroform, and the combined filtrate and washings were evaporated. The residue was chromatographed on silica gel with 19:1 (v/v) chloroform–ethanol to give a product that crystallized from chloroform–heptane as needles (59 mg, 82%), m.p. 86–88°, $[\alpha]_D^{24}$ +36° (*c* 0.50, chloroform); i.r. data: ν_{max}^{KBr} 3275 (NH), 1670 (CH_2–CH=CH_2), 1635 (Amide I), and 1550 cm^{-1} (Amide II); t.l.c. in 19:1 (v/v) chloroform–ethanol: R_F 0.25.

Anal. Calc. for $C_{14}H_{25}NO_6$: C, 55.43; H, 8.31; N, 4.62; O, 31.64. Found: C, 55.34; H, 8.29; N, 4.68; O, 31.58.

Methyl 2-acetamido-2-deoxy-3-O-methyl-6-O-trityl-α-D-mannopyranoside (**20**). — A solution of methyl 2-acetamido-2-deoxy-3-*O*-methyl-α-D-mannopyranoside[3] (**19**, 400 mg) in dry pyridine (40 ml) was treated with chlorotriphenylmethane (530 mg) for 6 h at 100°. The mixture was cooled and evaporated; repeated addition and distillation of toluene gave a residue that, on examination by t.l.c. in 14:1 (v/v) chloroform–ethanol, showed three components, corresponding to triphenylmethanol, unreacted **19**, and **20**. The mixture was chromatographed on silica gel with 14:1 (v/v) chloroform–ethanol to give a material that crystallized from ether as microcrystals (480 mg, 61%), m.p. 182–184°, $[\alpha]_D^{20}$ +3° (*c* 0.70, chloroform); i.r. data: ν_{max}^{KBr} 3290 (NH), 1645 (Amide I), 1545 (Amide II), 1490, and 1450 cm^{-1} (Ar); t.l.c. in 14:1 (v/v) chloroform–ethanol: R_F 0.31.

Anal. Calc. for $C_{29}H_{33}NO_6$: C, 70.86; H, 6.77; N, 2.85; O, 19.53. Found: C, 70.74; H, 6.89; N, 2.75; O, 19.78.

From the later fractions from the silica gel column, **19** was recovered; it was converted into **20** to give an additional 180 mg of the latter.

Methyl 2-acetamido-4-O-benzoyl-2-deoxy-3-O-methyl-6-O-trityl-α-D-mannopyranoside (**21**). — A solution of **20** (270 mg) in dry pyridine (10 ml) was cooled to −60°, treated with benzoyl chloride (65 μl) for 6 h at −20° and 12 h at −10°, diluted with chloroform (10 ml), and processed as described for **2**. Chromatography of the resulting syrup on silica gel with 24:1 (v/v) chloroform–ethanol gave a material that crystallized from ether–pentane as prisms (240 mg, 76%), m.p. 198–199° (softening at 145°), $[\alpha]_D^{20}$ +12° (*c* 0.54, chloroform); i.r. data: ν_{max}^{KBr} 3300 (NH), 1720 (ester), 1650 (Amide I), 1550 (Amide II), 1490, 1450 (Ar), and 700 cm^{-1} (Ar); t.l.c. in 19:1 (v/v) dichloromethane–ethanol: R_F 0.50.

Anal. Calc. for $C_{36}H_{37}NO_7$: C, 72.59; H, 6.26; N, 2.35; O, 18.80. Found: C, 72.54; H, 6.36; N, 2.39; O, 18.73.

Methyl 2-acetamido-4-O-benzoyl-2-deoxy-3-O-methyl-α-D-mannopyranoside (**22**). — Compound **21** (240 mg) was heated with 60% acetic acid (12 ml) for 1 h on a water bath (80°). The solution was cooled, diluted with water (150 ml), and lyophilized. The residue was chromatographed on silica gel with 14:1 (v/v) dichloromethane–ethanol to give a material that crystallized from chloroform–ether–pentane as plates (100 mg, 70%) containing 0.5 molecule of pentane per molecule, m.p. 76–77°, $[\alpha]_D^{20}$ −3° (*c* 1.32, chloroform); i.r. data: ν_{max}^{KBr} 3440–3280 (broad, OH, NH), 1740 (ester), 1655 (Amide I), and 1540 cm^{-1} (Amide II); t.l.c. in 14:1 (v/v) dichloromethane–ethanol: R_F 0.22.

Anal. Calc. for $C_{17}H_{23}NO_7 \cdot 0.5C_5H_{10}$: C, 60.29; H, 7.26; N, 3.60. Found: C, 60.24; H, 6.83; H, 4.06.

Methyl 2-acetamido-4-O-benzoyl-2-deoxy-3,6-di-O-methyl- (**23**) *and methyl 2-acetamido-6-O-benzoyl-2-deoxy-3,4-di-O-methyl-α-D-mannopyranoside* (**24**). — Compound **22** (80 mg) in dry tetrahydrofuran (2 ml) was treated with methyl iodide (4 ml) and silver oxide (300 mg) for 24 h at room temperature, with vigorous stirring. A further 150 mg of silver oxide was added, and stirring was continued for 16 h. T.l.c. in 19:1 (v/v) chloroform–ethanol showed maximum conversion of **22** into two components moving very closely. After processing the mixture as described for **15**, the residue was chromatographed on silica gel with 29,1 (v/v) chloroform–ethanol. Early fractions gave an amorphous solid (50 mg, 60%) having 0.5 molecule of hexane per molecule, $[\alpha]_D^{20}$ +19° (*c* 0.24, chloroform); i.r. data: ν_{max}^{film} 3270 (NH), 1745 (ester), 1655 (Amide I), and 1550 cm^{-1} (Amide II); t.l.c. in 19:1 (v/v) chloroform–ethanol, R_f 0.23.

Anal. Calc. for $C_{18}H_{25}NO_7 \cdot 0.5C_6H_{12}$: C, 61.60; H, 7.63. Found: C, 61.52; H, 8.07.

T.l.c. of the later fractions eluted from the column indicated the presence of two components (**23** and **24**); these were not separable by use of 29:1 (v/v) chloroform–ethanol, 29:1 (v/v) dichloromethane–ethanol, 19:1 or 14:1 (v/v) benzene–methanol, or 14:1 (v/v) ether–acetone. The mixture of **23** and **24** (22 mg) in methanol (2 ml) was treated for 16 h at 4° with 0.1M methanolic sodium methoxide (50 ml). The solution was de-ionized with Rexyn 300 (H^+, OH^-) ion-exchange resin, and examination of the product by t.l.c. in 19:1 (v/v) chloroform–ethanol showed the presence of two components. Examination of the mixture by g.l.c. after preparation of the per(trimethylsilyl) ethers indicated the presence of two components, one corresponding to methyl 2-acetamido-2-deoxy-3,4-di-*O*-methyl-6-*O*-(trimethylsilyl)-α-D-mannopyranoside. The mixture (16 mg) was chromatographed on silica gel in 19:1 (v/v) chloroform–ethanol. G.l.c.–m.s. of the isolated components as the per(trimethylsilyl) ethers showed the products to be methyl 2-acetamido-2-deoxy-3,6-di-*O*-methyl-α-D-mannopyranoside and methyl 2-acetamido-2-deoxy-3,4-di-*O*-methyl-α-D-mannopyranoside.

Methyl 2-acetamido-2-deoxy-3,6-di-O-methyl-α-D-mannopyranoside (**25**). — *Method A. From* **23**. A solution in dry methanol (4 ml) of the early fractions of the preparation of **23** (40 mg) was treated with 0.1M methanolic sodium methoxide

(100 ml) for 20 h at 4°, and the solution was processed as described for **16**. The residue was chromatographed on silica gel with 19:1 (v/v) chloroform–ethanol to give a material that crystallized from chloroform–hexane in the cold, and turned amorphous (21 mg, 76%) at room temperature, $[\alpha]_D^{24}$ +27° (*c* 1.13, methanol); i.r. data: ν_{max}^{film} 3450–3250 (OH, NH), 1655 (Amide I), and 1540 cm^{-1} (Amide II); t.l.c. in 19:1 (v/v) chloroform–ethanol: R_F 0.23, R_{TMG} 0.72.

Anal. Calc. for $C_{11}H_{21}NO_6$: C, 50.18; H, 8.04; N, 5.32. Found: C, 50.00; H, 7.98; N, 5.06.

Method B. From **28**. A solution of **28** (32 mg) in 90% ethanol (5 ml) was treated with tris(triphenyl)phosphinerhodium chloride (8 mg) and 1,4-diazabicyclo[2.2.2]-octane (2.5 mg). The mixture was processed as described for compound **6**, and the residue was chromatographed on silica gel with 14:1 (v/v) dichloromethane–ethanol to give an amorphous solid (14 mg). The earlier fractions eluted from the column contained **28**, which was again treated as just described, to give an additional amount of **25** (7 mg; total yield 76%), $[\alpha]_D^{24}$ +28° (*c* 0.42, methanol); t.l.c. in 19:1 (v/v) dichloromethane–ethanol: R_F 0.23, R_{TMG} 0.72; g.l.c.–m.s. of the per(trimethylsilyl) ether showed the product to be identical to that obtained from **23**.

*Methyl 2-acetamido-4-*O*-allyl-3-*O*-methyl-6-*O*-trityl-*α*-*D*-mannopyranoside* (**26**). — A solution of **20** (200 mg) in dry benzene (20 ml) was treated with allyl bromide (36 ml) and powdered sodium hydroxide (600 mg). The mixture was boiled under reflux for 3 h with vigorous stirring. A further 200 mg of sodium hydroxide was added, and stirring was continued for 12 h at room temperature. The mixture was processed as described for **7**, and chromatography of the resulting syrup with 19:1 (v/v) dichloromethane–ethanol gave a material that crystallized from chloroform–ether as needles (173 mg, 80%), m.p. 138–140°, $[\alpha]_D^{25}$ +18° (*c* 0.71, chloroform); i.r. data: ν_{max}^{KBr} 3300 (NH), 1660 (CH_2–CH–CH_2), 1645 (Amide I), and 1540 cm^{-1} (Amide II); t.l.c. in 19:1 (v/v) dichloromethane–ethanol: R_F 0.56.

Anal. Calc. for $C_{32}H_{37}NO_6$: C, 72.30; H, 7.01; N, 2.63; O, 18.06. Found: C, 72.21; H, 7.00; N, 2.58; O, 18.08.

*Methyl 2-acetamido-4-*O*-allyl-3-*O*-methyl-*α*-*D*-mannopyranoside* (**27**). — Compound **26** (150 mg) was treated with 60% acetic acid (6 ml) for 1 h on a water bath (80°). The solution was cooled and concentrated, and toluene was repeatedly added and distilled off. Chromatography of the residue on silica gel with 9:1 (v/v) chloroform–ethanol gave 70 mg (85%) of amorphous **27**, $[\alpha]_D^{24}$ −38° (*c* 0.67, chloroform); t.l.c. in 19:1 (v/v) chloroform–ethanol: R_F 0.47.

Anal. Calc. for $C_{13}H_{23}NO_6$: C, 53.97; H, 8.01; N, 4.84; O, 33.18. Found: C, 53.89; H, 7.93; N, 4.84; O, 33.29.

*Methyl 2-acetamido-4-*O*-allyl-3,6-di-*O*-methyl-*α*-*D*-mannopyranoside* (**28**). — A solution of **27** (55 mg) in dry acetone (2 ml) mixed with methyl iodide (3 ml) and silver oxide (200 mg) was boiled for 12 h under reflux. A further 100 mg of silver oxide was added, and heating was continued for 8 h. After the mixture had been processed as described for **15**, the residue was chromatographed on silica gel. Elution with 19:1 (v/v) dichloromethane–ethanol gave a material that crystallized from chloroform–

ether as prisms (45 mg, 78%), m.p. 116–118°, $[\alpha]_D^{20}$ +48° (*c* 0.95, chloroform); i.r. data: ν_{max}^{KBr} 3300 (NH), 1660–1640 (CH_2–CH=CH_2, Amide I), and 1540 cm^{-1} (Amide II); t.l.c. in 19:1 (v/v) dichloromethane–ethanol: R_F 0.29.

Anal. Calc. for $C_{14}H_{25}NO_6$: C, 55.43; H, 8.31; N, 4.62; O, 31.64. Found: C, 55.35; H, 8.25; N, 4.65; O, 31.72.

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Reprinted from
Carbohydrate Research, 47 (1976) 245–260

THE CHEMICAL STRUCTURE OF A FRAGMENT OF *Micrococcus lysodeikticus* CELL-WALL*

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(Received January 4th, 1976; accepted for publication, January 14th, 1976)

ABSTRACT

A fragment of *Micrococcus lysodeikticus* cell-wall obtained by cetylpyridinium precipitation from the nondialyzable portion of the degradation products of egg-white lysozyme was studied by the periodate oxidation and methylation procedures. The fragment consists of a polysaccharide chain composed of about 40 repeating (1→4)-*O*-(2-acetamido-2-deoxy-β-D-mannopyranosyluronic acid)-(1→6)-*O*-(α-D-glucopyranosyl) residues with D-glucopyranosyl residues at both ends. The α-D-glucopyranose residue at the reducing end is linked to a phosphate group that is also linked to C-6 of a 2-acetamido-3-*O*-(D-1-carboxyethyl)-2-deoxy-β-D-glucopyranosyl residue of a peptidoglycan chain composed of four repeating (1→4)-*O*-(2-acetamido-2-deoxy-β-D-glucopyranosyl)-(1→4)-*O*-[2-acetamido-3-*O*-(D-1-carboxyethyl)-2-deoxy-β-D-glucopyranosyl] residues. The peptidoglycan chain has, as nonreducing group, a 2-acetamido-2-deoxy-β-D-glucopyranosyl group, and, as reducing residue, a 2-acetamido-3-*O*-(D-1-carboxyethyl)-2-deoxy-β-D-glucose residue.

INTRODUCTION

Perkins[6] has described the isolation in low yield of the external polysaccharide chains of *Micrococcus lysodeikticus* cell-wall by trichloroacetic acid treatment, and the chemical structure of these chains has been partially elucidated by Hase *et al.*[7]. A fragment representing 26% of the total cell-wall, 10% of the peptidoglycan, and 50% of the external polysaccharide chains was isolated from *Micrococcus lysodeikticus* cell-wall[8]. This fragment, obtained by degradation with egg-white lysozyme, precipitation with cetylpyridinium chloride, and Bio-gel chromatography, was shown[8] to

*Amino Sugars CII and *Micrococcus lysodeikticus* cell-wall V (for preceding papers in this series, see Refs. 1–4). This is publication No. 694 of the Robert W. Lovett Memorial Group for the Study of Diseases Causing Deformities, Harvard Medical School and Massachusetts General Hospital. This investigation was supported by a research grant from the National Institute of Allergy and Infectious Diseases (Grant AI-06692). A preliminary communication has been presented[5].

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consist of a peptidoglycan moiety composed of four alternating, (1→4)-linked 2-acetamido-2-deoxy-β-D-glucopyranosyl and 2-acetamido-3-*O*-(D-1-carboxyethyl)-2-deoxy-β-D-glucopyranosyl (*N*-acetylmuramic acid) residues to which is attached an external, polysaccharide chain composed of ~40 *O*-(β-D-mannopyranosyluronic acid)-*O*-(α-D-glucopyranosyl) repeating units linked through a phosphate group to C-6 of one of the muramic acid residues of the peptidoglycan chain; in addition, all the carboxyl groups of the muramic acid residues are substituted by peptide chains. The present paper describes the elucidation of the chemical structure of the external polysaccharide chain and of the glycan part of the peptidoglycan moiety by the periodate and methylation procedures.

RESULTS AND DISCUSSION

Extensive, periodate oxidation at 4° in the dark, which has been shown to cause no overoxidation of 2-acetamido-2-deoxy sugar residues[9], gave an amount of formic acid (0.9 mol/D-glucose residue) corresponding to the oxidation of all of the D-glucose residues, suggesting that these residues are linked at C-6 (see Table I). The

TABLE I

SEQUENTIAL PERIODATE OXIDATION, SODIUM BOROHYDRIDE REDUCTION, AND HYDROLYSIS OF FRACTION CPC_{A-2} [a]

Components[b], *reagent, and compounds formed*	*Fraction* CPC_{A-2}	*Sequential degradation*			
		First		*Second*	
		Dl[c]	*Ndl*[c]	*Dl*[c]	*Ndl*[c]
D-Glucose[d]	28		1		0
2-Amino-2-deoxy-D-mannuronic acid	37[e]		12[f]		0
2-Amino-2-deoxy-D-glucose[g]	7		9		13
Muramic acid[g]	9		14		20
Muramic acid 6-phosphate[g]	1.4		2.2		
Glycerol		3.3[h]	7	0	0
Alanine[i]	4		10		14
Glutamic acid[i]	3		8		6
Glycine[i]	2		4		6
Lysine[i]	3		5		8
Periodate consumed[j] (μmol/mg)		3.65			
Formic acid released[k] (μmol/mg)		1.3			
Formaldehyde released[l] (nmol/mg)		65			

[a]For conditions, see Experimental section. [b]Component contents expressed in %. [c]Abbreviations: Dl, dialyzable fraction; Ndl, nondialyzable fraction. [d]Determined by the anthrone colorimetric method[20]. [e]Determined by g.l.c. as 2-amino-2-deoxy-D-mannose, relative to the D-glucose content. [f]Determined by g.l.c. as 2-amino-2-deoxy-D-mannose. [g]Determined by a modified Elson–Morgan, colorimetric method[21]. [h]Expressed in %, relative to fraction CPC_{A-2}. [i]Determined with an amino acid analyzer (see Experimental section). [j]Determined by u.v. absorption at[26] 223 nm. [k]Determined by titration with sodium hydroxide. [l]Determined by the chromotropic acid, colorimetric method[27].

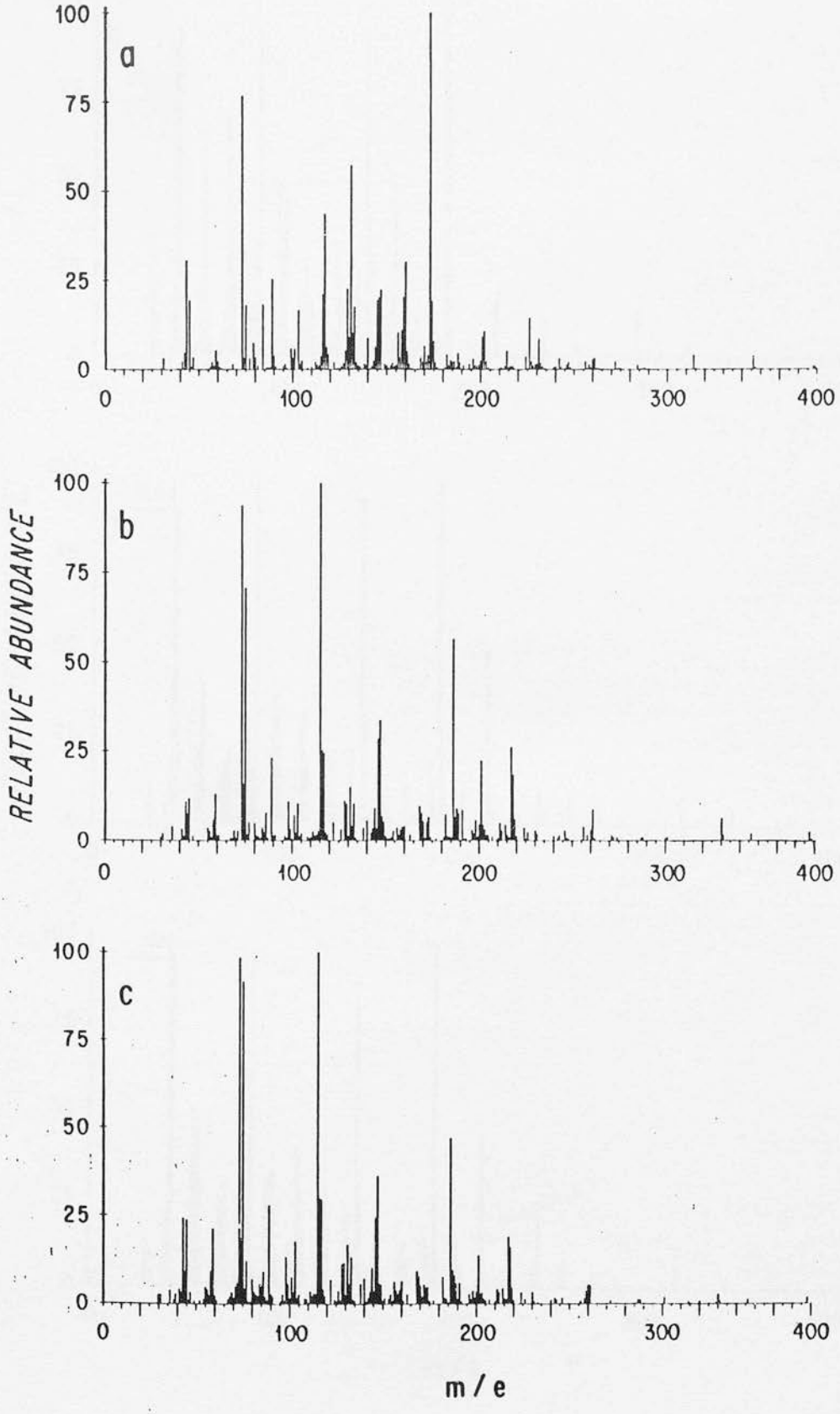
release of formaldehyde (65 nmol/mg) corresponds to the oxidation of the reducing, terminal, muramic acid residue of the peptidoglycan fragment. The oxidized fragment was reduced with sodium borohydride, the resulting polyalcohol hydrolyzed by mild hydrolysis with acid, and the hydrolyzate dialyzed. The dialyzable fraction contained only one quarter of the expected amount of glycerol, and the remainder consisted of 2-amino-2-deoxy-D-mannuronic acid. This compound, after treatment by methanolic hydrogen chloride and reduction, was identified by g.l.c. as methyl 2-acetamido-2-deoxy-α,β-D-mannopyranoside, and, after hydrolysis and *N*-acetylation, as crystalline 2-acetamido-2-deoxy-D-mannose.

The presence of about 1% of unoxidized D-glucose and 7% of glycerol in the oxidized, hydrolyzed, and nondialyzable material indicates clearly a limitation of the periodate–sodium borohydride–mild acid hydrolysis method for structure determination of complex carbohydrate molecules. The resistance of the D-glucose residues to periodate oxidation may suggest a (1→3)-linkage, but this observation was not confirmed by the methylation procedure, and it is more probable that steric effects were responsible for this inhibition of the periodate oxidation. The presence of glycerol shows that the conditions of hydrolysis were not sufficient to hydrolyze the oxidized, reduced, external chains completely, but stronger conditions would have hydrolyzed the phosphate bonds and, possibly, the 2-acetamido-2-deoxy-D-glycopyranosyl linkages. The total amount of glycerol found in the dialyzable and nondialyzable fractions corresponds to about half of the value expected, the loss probably being due to hydrolysis during purification of the reduced fractions. No erythrose was observed, thus confirming that all of the D-glucose residues are linked at C-6. A second periodate oxidation of the oxidized, reduced, nondialyzable fraction degraded the remaining D-glucose residues.

Methylation was performed by a modified Haworth procedure[10], in order to avoid degradation of alkali-sensitive bonds. Methanolysis, followed by acetylation and hydrolysis, gave a mixture that was fractionated on a column of Dowex 50 ion-exchange resin. The total recovery from the column was ~70%, from which 86% was further fractionated by paper chromatography.

The D-glucose component was recovered in 52% yield as 2,3-di-, 2,3,4-tri-, and 2,3,4,6-tetra-methyl ethers. In addition to identification by g.l.c. and paper chromatography, the first two compounds were identified, after reduction, as the crystalline *p*-phenylazobenzoyl derivatives, and the last-mentioned compound was obtained in crystalline form. Direct methanolysis and g.l.c. indicated that the 2,3-dimethyl ether was the product of hydrolysis of the 2,3,4-trimethyl ether. The ratio of 2,3,4-tri- to 2,3,4,6-tetra-methyl ether suggested a chain-length of ~50 repeating units, in good agreement with the value of ~40 found by determination of the reducing end linked to the phosphate group[8].

No methyl ethers of the 2-acetamido-2-deoxy-D-mannuronic acid residues were recovered as such, probably because of extensive decarboxylation during the hydrolysis. On reduction of the product of methanolysis, the 3-methyl ether of the corresponding 2-amino-2-deoxy-D-mannose residue was obtained in good yield by g.l.c.,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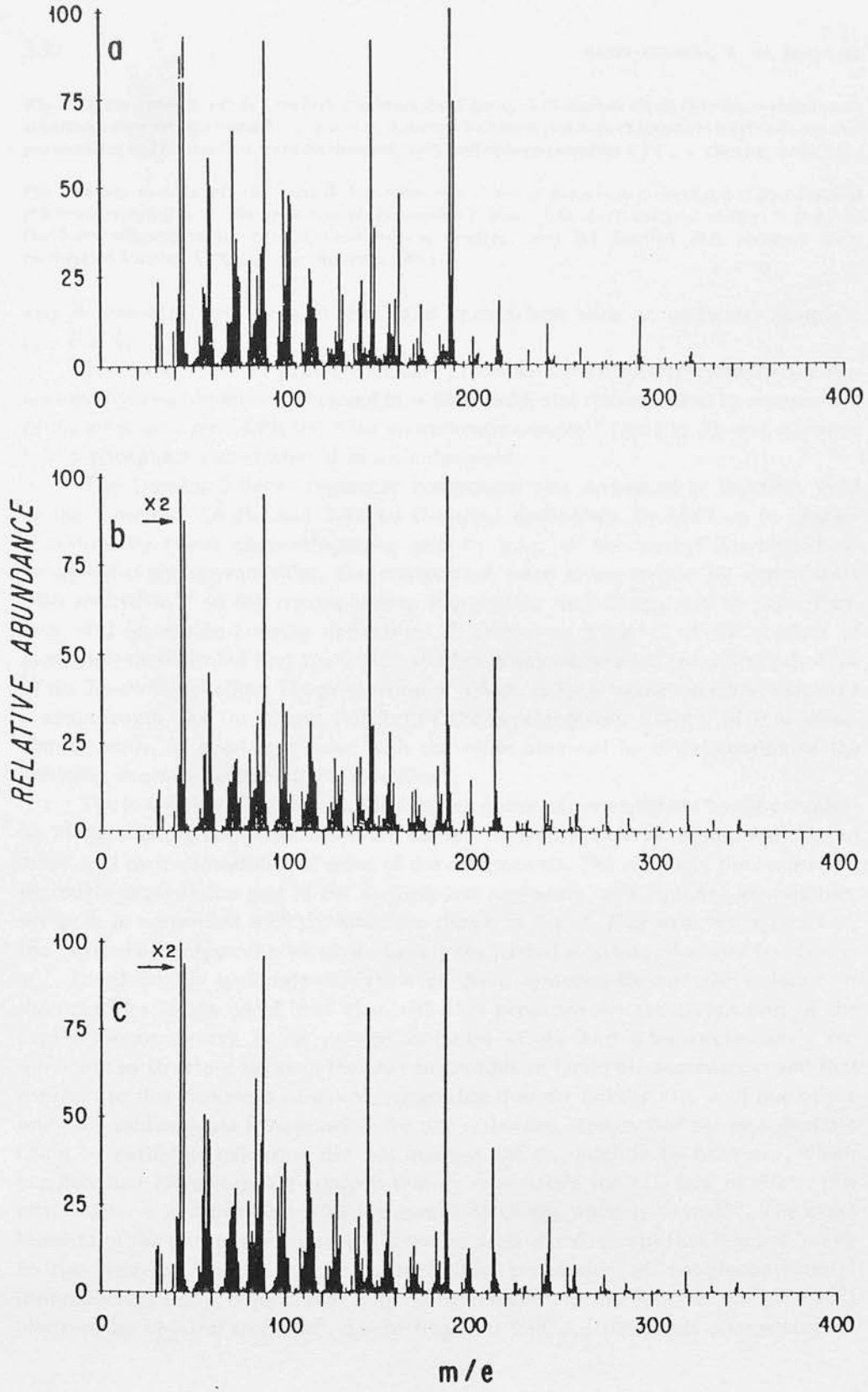
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RELATIVE ABUNDANCE
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Fig. 1. Mass spectra of: (a) methyl 2-acetamido-2-deoxy-4-*O*-methyl-3,6-di-*O*-(trimethylsilyl)-α-D-mannopyranoside; (b) methyl 2-acetamido-2-deoxy-3-*O*-methyl-4,6-di-*O*-(trimethylsilyl)-α-D-mannopyranoside; and (c) fraction from methylated, carboxyl-reduced fraction $CPC_{A\,2}$. (See fig. page 248.)

Fig. 2. Mass spectra of: (a) methyl 2-acetamido-6-*O*-acetyl-2-deoxy-4-*O*-methyl-3-*O*-[D-1-(methyl ethylcarboxylate)]-α-D-glucopyranoside; (b) methyl 2-acetamido-4-*O*-acetyl-2-deoxy-3-*O*-[D-1-(methoxycarbonyl)ethyl]-6-*O*-methyl-α-D-glucopyranoside; and (c) fraction d(*i*) obtained from methylated fraction CPC_{A-2}. (See fig. page 249.)

and it was identified by g.l.c.–m.s. and comparison with an authentic sample[11] (see Fig. 1).

The 6-methyl ether of muramic acid [2-amino-3-*O*-(D-1-carboxyethyl)-2-deoxy-6-*O*-methyl-D-glucose] was obtained in ~50% yield, and characterized by comparison of the g.l.c.–m.s. data with those for an authentic sample[12] (see Fig. 2), and muramic acid 6-phosphate was recovered in a similar yield.

The 2-amino-2-deoxy-D-glucose component was recovered in excellent yield as the 3-mono-, 3,6-di-, and 3,4,6-tri-*O*-methyl derivatives. In addition to characterization by paper chromatography and by g.l.c. of the methyl 2-acetamido-2-deoxy-α,β-D-glucopyranosides, the compounds were characterized by degradation with ninhydrin[13] to the corresponding D-arabinose derivatives, and by crystallization of 2-acetamido-2-deoxy derivatives. Examination by g.l.c. of the product of methanolysis indicated that the 3-methyl ether obtained resulted from the hydrolysis of the 3,6-dimethyl ether. The proportion of 3,6-di- to 3,4,6-tri-methyl ethers indicated a chain-length, for the glycan portion of the peptidoglycan moiety, of four disaccharide units, in good agreement with the value obtained by determination of the reducing, muramic acid end-group residue[8].

The low yields of methylated derivatives obtained are explained by the complexity of the mixture composed of seven different *O*-methyl derivatives and four amino acids, and by the instability of some of the components. The results of the sequential, periodate degradation and of the methylation procedure, although not quantitative, are both in agreement with the structure shown in Fig. 3. This structure agrees with the "external" polysaccharide chain having the partial structure proposed by Hase *et al.*[7], based on the periodate oxidation of the polysaccharide and the isolation of disaccharides in low yield, and also with that proposed for the glycan part of the peptidoglycan moiety based on the isolation of di- and tetra-saccharides[1]. No difference in structure between the part susceptible to lysozyme degradation and that resistant to this enzyme is observed, suggesting that the linkage at C-6 of one of the muramic acid residues is responsible for this resistance. Removal of the carbohydrate chain by periodate oxidation did not increase the degradation by lysozyme, which suggests that the phosphate group is mainly responsible for this lack of effect; this observation is in agreement with the specificity of egg-white lysozyme[14]. The exact location of the phosphate group could not be determined, except that it is not linked to the reducing muramic acid residue[8]. The proportion of α-D-glucopyranosyl (nonreducing) end-groups is also in good agreement with the mol. wt. ($22–27 \times 10^3$) observed by physical methods[8]. As the fragment CPC_{A-2} represents a large propor-

Fig. 3. Tentative structure of fraction CPC_{A-2} of the *Micrococcus lysodeikticus* cell-wall. [The chain composed of ManANAc*p* and Glc*p* residues can be linked to any of the three MurNAc*p* residues marked by an asterisk. Abbreviations: Glc*p*, glucopyranosyl; ManANAc*p*, 2-acetamido-2-deoxy-D-mannopyranosyluronic acid; GlcNAc*p*, 2-acetamido-2-deoxy-D-glycopyranosyl; MurNAc*p*, 2-acetamido-3-*O*-(D-1-carboxyethyl)-2-deoxy-D-glucopyranosyl.]

tion of the cell wall, it may be assumed that most of the lysozyme-resistant part of the cell wall of *M. lysodeikticus* has the chemical structure depicted in Fig. 3.

EXPERIMENTAL

General methods. — Evaporations were performed under diminished pressure, and the temperature of the bath was maintained at or below 40°. Optical rotations were determined with a Perkin–Elmer Model 141 polarimeter. Melting points were recorded with a Mettler FP-2 hot-stage equipped with a microscope, and correspond to corrected melting points. The microanalyses were performed by Dr. W. Manser, Zurich, Switzerland.

General analytical methods. — Paper chromatography on Whatman Nos. 1 and 3MM papers, and thin-layer chromatography on plates of cellulose F (E. Merck A. G., Darmstadt) were performed with the following solvent-systems (v/v): (*A*) 10:4:3 ethyl acetate–pyridine–water; (*B*) 7:7:6 pentanol–pyridine–water; (*C*) 4:1:5 (upper layer) butanol–ethanol–water; (*D*) 4:1:5 (upper layer) butanol–acetic acid–water; and (*E*) 6:4:3 butanol–pyridine–water. R_{Me_4Glc}, R_{Me_3GlcN}, and R_{GlcN} refer to the mobilities relative to 2,3,4,6-tetra-*O*-methyl-D-glucose, 2-amino-2-deoxy-3,4,6-tri-*O*-methyl-D-glucose, and 2-amino-2-deoxy-D-glucose, respectively. Amino sugars and amino acids were detected with ninhydrin[15], reducing sugars with the aniline phthalate reagent[16], reducing and nonreducing sugars with the alkaline silver nitrate[17] and periodate–benzidine reagents[18], lactones and esters with the hydroxylamine–ferric chloride reagent[19], and uronic acids with Bromothymol Blue indicator. Hexoses were quantitatively determined by the anthrone colorimetric method[20], and phosphate groups by the procedure of Chen *et al.*[21]. Mixtures of muramic acid 6-phosphate, muramic acid, and 2-amino-2-deoxy-D-glucose were separated on Dowex 50 (H^+) ion-exchange resin, and quantitatively estimated by use of a modified Elson–Morgan reaction[22]. Amino acids in acid hydrolyzates were quantitatively determined with a Beckman Model 116 amino acid analyzer.

Gas–liquid chromatography and mass spectrometry. — G.l.c. of reducing and nonreducing sugars was performed according to the procedure of Reinhold[23]. The fragments resulting from sequential periodate oxidation–sodium borohydride reduction–mild acid hydrolysis (Smith degradation)[24] were separated on columns of OV-1 and OV-11, and methyl glycosides of methyl ethers of hexoses and hexosamines on columns of OV-1, OV-11, OV-17, OV-25, and HI-EFF-8BP, with a Perkin–Elmer gas chromatograph equipped with dual ionization detector and integrator. G.l.c.–m.s. was performed with an analytical system consisting of an IBM 1800 computer fed raw data generated by a single-focusing, Hitachi–Perkin–Elmer RMU-6 mass spectrometer interfaced with a gas chromatograph (Perkin–Elmer Model 990). In all cases except those involving fully methylated sugars and muramic acid derivatives, analyses were performed on the trimethylsilyl derivatives of the sugars.

N-Acetylation. — *N*-Acetylation of small samples of amino sugars was performed by dissolution in methanol and addition of a 3-molar excess of acetic anhydride.

After 4 h at room temperature, the mixture was evaporated under a stream of nitrogen.

Hydrolysis. — Small-scale hydrolyses were performed on 1–2 mg of material with 4M hydrochloric acid for 16 h at 100°. The acid was removed by evaporation under a stream of nitrogen, followed by repeated addition and evaporation of ethanol and toluene.

Methanolysis. — Small-scale methanolysis was performed by treating the dried material (1–5 mg) with 0.5–1M methanolic hydrogen chloride (0.5–1.5 ml) for 8–12 h at 100°. The methanolic hydrogen chloride was removed by evaporation under a stream of nitrogen, followed by repeated addition and evaporation of methanol and toluene.

Reduction with sodium borohydride. — Ester and aldehyde groups were reduced by treating the cooled, aqueous solution of the substance (2–4 mg in 100–200 µl of water) with a 3–4M sodium borohydride solution (100–200 µl). The excess of borohydride was decomposed by addition of acetic acid or of Dowex 50 (H^+) ion-exchange resin. The solution was de-ionized with Dowex 50 ion-exchange resin (when necessary), and the borate ions were removed as methyl borate by repeated addition and evaporation of methanol.

Demethylation. — Methylated sugars were demethylated with boron tribromide or boron trichloride according to the procedure of Bourne and assoc.[25].

Ninhydrin degradation of hexosamines. — This degradation was performed according to Stoffyn and Jeanloz[13].

Materials. — The CPC_{A-2} fraction was obtained from *Micrococcus lysodeikticus* cell-walls, prepared according to Sharon and Jeanloz[2], by lysozyme degradation and cetylpyridinium fractionation, as described by Nasir-ud-Din *et al.*[8].

Sequential degradation by periodate oxidation–sodium borohydride reduction of fraction CPC_{A-2}. — *First treatment.* Fraction CPC_{A-2} (0.69 g in 100 ml of water, pH 3.8) was treated with sodium metaperiodate (1.925 g) for 24 h at 4° in the dark. The consumption of periodate, measured spectrophotometrically[26] at 223 nm, was constant after 8 h (2.3 mol/D-glucose residue). The formic acid released (0.9 mol/D-glucose residue) was estimated by titration with 20µM sodium hydroxide, and the formaldehyde released (1.35 mg), with the chromotropic acid reagent[27] (2-acetamido-2-deoxy-D-glucitol as the control). The excess of periodate was decomposed with 1,2-ethanediol, and the solution was dialyzed for three days at 4° against distilled water, to give a nondialyzable fraction (0.546 g after lyophilization). To a portion of this material (0.525 g) in water (25 ml) at 4° was added sodium borohydride (0.2 g) in four portions. An additional quantity of sodium borohydride (0.03 g) was added, and the solution was kept for 24 h in the cold. The excess of borohydride was decomposed with M acetic acid, and the solution was dialyzed for two days against distilled water. The nondialyzable portion was concentrated, and borate ions were removed as methyl borate by repeated addition and evaporation of methanol (6 × 50 ml). The residue was dissolved in water, the solution lyophilized (0.52 g), and the product treated with 0.5M sulfuric acid (15 ml) for 2 h at room temperature; the solution was then dialyzed against distilled water for three days. The nondialyzable fraction

was lyophilized, to give a periodate-resistant fraction (0.256 g), and kept for further treatment with periodate. The composition of this fraction is reported in Table I.

The dialyzable fraction was concentrated, and the solution passed through a column (1.8 × 16 cm) of Dowex 1 X-8 (AcO^-) ion-exchange resin (200–400 mesh), which was eluted with water (80 ml) and 10mM acetic acid (80 ml). The eluate was concentrated to 40 ml, and lyophilized (0.23 g). Thin-layer chromatography on cellulose, and paper chromatography in Solvents *B*, *C*, and *D*, with detection with alkaline silver nitrate, Bromothymol Blue, and the hydroxylamine–ferric chloride reagent, indicated the presence of glycerol, 2-acetamido-2-deoxy-D-mannuronic acid, and 2-acetamido-2-deoxy-D-mannuronolactone. G.l.c. on columns of OV-1 and OV-17 of the derived methyl glycosides showed the presence of glycerol (3.3%), in addition to unidentified compounds.

Hydrolysis of a portion of the dialyzable fraction with M hydrochloric acid for 16 h at 100°, and examination by paper chromatography in Solvent *D*, indicated the presence of glycerol and 2-amino-2-deoxy sugars. Another portion of the dialyzable fraction was treated with 0.5M methanolic hydrogen chloride for 6 h at 100°. The acid-free residue was *N*-acetylated, and the product treated in aqueous methanol (1:1) with sodium borohydride in the usual way. The reduced material was hydrolyzed with M hydrochloric acid for 4 h at 100°, and the product *N*-acetylated. Examination by paper chromatography in Solvent *D* (detection with alkaline silver nitrate), and in Solvent *E* on borate-treated paper[28] (Whatman No. 3MM) with detection with 0.5M sodium hydroxide in ethanol (u.v. light)[28], showed the presence of 2-acetamido-2-deoxy-D-mannose and glycerol. This result was confirmed by g.l.c. of the methyl glycosides.

The dialyzable fraction remaining was separated into two fractions on Whatman paper No. 3MM in Solvent *C*. The first fraction (5 mg) (R_{GlcN} 0.26) of the chromatogram contained glycerol, as indicated by t.l.c. The second fraction (38 mg) was composed of two compounds, respectively having $R_{D\text{-}Glucuronolactone}$ 1.0 and 1.21, positive to Bromothymol Blue and to the hydroxylamine–ferric chloride reagent. It was eluted from the paper, glycosidated and esterified with 0.5M methanolic hydrogen chloride (5 ml) for 6 h at 100°, and the product acetylated with pyridine and acetic anhydride. The product was reduced with sodium borohydride (25 mg) in the usual way, and the product hydrolyzed with M hydrochloric acid (1.5 ml) for 6 h at 100°. The hydrolyzate was adsorbed on a column (1 × 8 cm) of Dowex 1 X-8 (AcO^-) ion-exchange resin, and the column was eluted with aqueous methanol. The eluate was evaporated, the residue *N*-acetylated, and the product crystallized from aqueous ethanol (1:1, v/v) and acetone. Recrystallization from a mixture of aqueous ethanol and acetone gave platelets having m.p. 102–103°, and mixed m.p. with authentic 2-acetamido-2-deoxy-D-mannose, 102–105°; $[\alpha]_D^{20}$ −32° [after 5–10 min; *c* 0.42, 1:1 (v/v) methanol–water]; lit.[29] m.p. 105–108°, $[\alpha]_D$ −21 → +10° (*c* 1.0, water).

Second treatment. A small portion of the nondialyzable material resulting from the first treatment (12.0 mg) was dissolved in 0.1M sodium metaperiodate (5 ml), and the solution kept for 24 h at 4°. The consumption of periodate was constant

after 4 h, and then corresponded to 26 mmol/D-glucose residue. The solution was processed as described for the first treatment, except that the duration of the acid hydrolysis with 0.5M sulfuric acid was extended to 4 h. The nondialyzable material was lyophilized (11.0 mg). Examination of the dialyzate by paper chromatography showed the absence of reducing and nonreducing sugars.

Lysozyme degradation of periodate-degraded CPC_{A-2} *fraction.* — Twice periodate-treated fraction CPC_{A-2} (10 mg) in 10mM ammonium acetate (4 ml) was treated with lysozyme (50 mg; General Biochemicals, Division of the Mogul Corporation, Laboratory Park, Chagrin Falls, Ohio 44022) for 24 h at 37° in the presence of a drop of toluene. The resulting solution was dialyzed to give a nondialyzable fraction (9 mg). Examination of the dialyzable fraction by paper chromatography in Solvent *D* showed the absence of reducing and nonreducing sugars.

Application of the methylation procedure to fraction CPC_{A-2}. — *Methylation.* A cooled solution of fraction CPC_{A-2} (0.45 g) in water (8 ml) was treated at 4° with M sodium hydroxide (2 ml) and dimethyl sulfate (2 ml), added in small portions, while the mixture was vigorously stirred under an atmosphere of nitrogen. Sodium hydroxide (6 ml, 30%) and dimethyl sulfate (6 ml) were further added dropwise at 4°, and the mixture was stirred for 16 h. After seven additions of 6 ml of 30% sodium hydroxide and 6 ml of dimethyl sulfate, the mixture was dialyzed against distilled water, and the nondialyzable material was further methylated by three repetitions of the whole procedure just described, to give 0.4 g, $[\alpha]_D^{20}$ +9° [*c* 0.32, 1:1 (v/v) methanol–water].

Anal. Calc. for fully methylated polymer: OCH_3, 20.0. Found: OCH_3, 20.7.

Attempts to raise the methoxyl content further by methylation with silver oxide and methyl iodide[30], or with barium oxide–barium hydroxide–methyl iodide in *N*,*N*-dimethylformamide[31], showed no increase in methoxyl content, but excessive degradation took place. The i.r. spectrum indicated complete methylation.

Hydrolysis. A portion (0.325 g) of the methylated material just described was dissolved in M methanolic hydrogen chloride (15 ml), and the mixture was boiled under reflux for 16 h, and evaporated. The residue was freed of hydrochloric acid by repeated addition and evaporation of methanol, and treated with anhydrous pyridine (4 ml) and acetic anhydride (3 ml) at 4° and then for 2 h at room temperature. The solution was evaporated, the residue was hydrolyzed with M hydrochloric acid (6 ml) for 6 h at 100°, and the solution was diluted with water (to 10 ml) and applied to a column (0.9 × 20 cm) of Dowex 1 X-8 (AcO^-) ion-exchange resin. Elution with water (25 ml) and then with 0.1M acetic acid (45 ml) gave, after evaporation, a residue (0.30 g) that was dried by several additions and evaporations of water, methanol, and toluene.

Identification of methyl ethers of monosaccharides by isolation. A solution of the residue in water was applied to a column (1.8 × 38 cm) of Dowex 50 X-8 (H^+) ion-exchange resin. The carbohydrate components were eluted with water (500 ml) and then with a gradient of 0.05–2.5M hydrochloric acid (1 liter); 8-ml fractions were

collected, examined by t.l.c. and paper chromatography, and combined into seven fractions (*a–g*).

(*a*). This fraction (11 mg) was separated into two compounds (2.0 and 5.0 mg, respectively) on Whatman 3MM paper in Solvent *D*.

(i) The first compound had R_{Me_4Glc} 0.11, identical with that of muramic acid 6-phosphate. Treatment of 0.5 mg with acid phosphatase (wheat-germ, Sigma Chemical Co., St. Louis, Mo. 63178) in 50mM sodium acetate–acetic acid buffer (400 μg/ml; pH 5.0) for 4 h at 37°, followed by heating for 1 min, treatment with Dowex 50 X-8 (H^+) ion-exchange resin, and elution of the resin with 10mM hydrochloric acid, gave a residue showing in t.l.c. (Solvent *D*) the same rate of migration as that of muramic acid (R_{GlcN} 2.02).

(ii) A portion of the second compound (R_{Me_4Glc} 0.73) was demethylated to give a compound that showed in t.l.c. (Solvent *A*) R_{Glc} 1.0, identical with that of D-glucose. A second portion was methanolyzed; g.l.c. of the methanolyzate on a column of OV-11 showed R_T 8.7 and 10.9, identical with the R_T values of methyl 2,3-di-*O*-methyl-α,β-D-glucopyranoside. Reduction of a third portion with sodium borohydride, and treatment of the product with *p*-phenylazobenzoyl chloride in pyridine[32] gave 2,3-di-*O*-methyl-1,4,5,6-tetra-*O*-(*p*-phenylazobenzoyl)-D-glucitol, m.p. 181–184° and mixed m.p. 183–185°; lit.[32] m.p. 181°.

Anal. Calc. for $C_{60}H_{50}N_8O_{10}$: C, 69.06; H, 4.83; N, 10.77. Found: C, 69.06; H, 4.91; N, 10.80.

(*b*). The fraction (34 mg) was purified on Whatman 3MM paper (Solvent *C*) to give a compound (26 mg) showing $[\alpha]_D^{20}$ +68° (*c* 0.41, water) and R_{Me_4Glc} 0.92. A portion of this compound was demethylated to give a product showing in t.l.c. (Solvent *C*) the same migration as that of D-glucose. The compound was identified by g.l.c. on a column of OV-11 (R_T 9.4 and 10.7) as methyl 2,3,4-tri-*O*-methyl-α,β-D-glucopyranoside; this was confirmed by reduction with sodium borohydride, and treatment of the product with *p*-phenylazobenzoyl chloride in pyridine[32] to give 2,3,4-tri-*O*-methyl-1,5,6-tri-*O*-(*p*-phenylazobenzoyl)-D-glucitol[32], m.p. 88–90° and mixed m.p. 88–91°; lit.[32] m.p. 85°.

Anal. Calc. for $C_{48}H_{44}N_6O_4$: C, 67.91; H, 5.22; N, 9.90. Found: C, 67.88; H, 5.26; N, 9.88.

(*c*). The fraction (32 mg) was separated on Whatman 3MM paper (Solvent *C*) into two compounds (10 mg and 4 mg, respectively).

(i) The first compound (R_{Me_4Glc} 0.92) was methanolyzed, and the product identified by g.l.c. on an OV-11 column (R_T 9.4, 10.7) as methyl 2,3,4-tri-*O*-methyl-α,β-D-glucopyranoside.

(ii) A portion of the second compound (R_{Me_4Glc} 1.0) was demethylated to give a compound showing in t.l.c. (Solvent *A*) R_{Glc} 1.0, identical with that of D-glucose. Methanolysis of a second portion, and g.l.c. of the product on an OV-11 column showed R_T values of 8.9 and 9.2, identical with those of authentic methyl 2,3,4,6-tetra-*O*-methyl-α,β-D-glucopyranoside. The compound crystallized, and was recrys-

tallized from warm hexane to give 2,3,4,6-tetra-*O*-methyl-D-glucose, m.p. 86–88°; lit.[33] m.p. 96°.

(*d*). This fraction (40 mg) was separated on Whatman 3MM paper (Solvent *D*) into two components (R_{Me_3GlcN} 0.95 and 0.14).

(i) The first compound (14 mg) showed, in t.l.c. (Solvent *D*), R_{Glc} 2.5 identical with that of 2-amino-3-*O*-(D-1-carboxyethyl)-6-*O*-methyl-D-glucose (6-*O*-methylmuramic acid)[12]. Demethylation of this compound gave a compound showing, in t.l.c. (Solvent *D*), R_{GlcN} 1.0, identical with that of 2-amino-2-deoxy-D-glucose. A portion of the compound was *N*-acetylated, the product methanolyzed, and the product acetylated with acetic anhydride–pyridine. G.l.c.–m.s. showed an R_T 19.0 and mass spectrum corresponding to those of methyl 2-acetamido-4-*O*-acetyl-2-deoxy-3-*O*-[D-1-(methoxycarbonyl)ethyl]-6-*O*-methyl-α,β-D-glucopyranoside, but different from those of the 4-*O*-methyl analogue (see Fig. 2). On periodate oxidation of the *N*-acetylated, sodium borohydride-reduced compound, 1.0 mol of oxidant/mol of 2-acetamido-3-*O*-(D-1-carboxyethyl)-6-*O*-methyl-D-glucitol was used up, and no formaldehyde was detected by the chromotropic acid method[27].

(ii) The second compound (8 mg) showed, in t.l.c. (Solvent *D*), R_{Me_3GlcN} 0.14. It was reducing, and showed the presence of an amino group and the absence of a carboxyl group. A portion of this compound was demethylated, and paper chromatography (Solvent *D*) of the product showed the presence of 3 compounds, having R_{GlcN} 0.8, 1.15, and 2.0, respectively. This compound was not further investigated.

(*e*). This fraction (48 mg) was separated on Whatman 3MM paper (Solvent *D*) into 3 compounds.

(i) The first compound (4 mg) showed an R_{GlcN} value of 1.7 (Solvent *D*), identical with that of 2-amino-2-deoxy-3-*O*-methyl-D-glucose. A portion of this compound was demethylated to give a compound showing, in t.l.c. (Solvent *D*), an R_{GlcN} value of 1.0, identical with that of 2-amino-2-deoxy-D-glucose. Another portion was degraded with ninhydrin to give a compound showing, in t.l.c. (Solvent *D*), an R_F value identical with that of authentic 2-*O*-methyl-D-arabinose. Another fraction was *N*-acetylated; the crystalline compound resulting was recrystallized from methanol–acetone, m.p. 190–192°, m.p. on admixture with 2-acetamido-2-deoxy-3-*O*-methyl-D-glucose[34], m.p. 191–193°; lit.[34] m.p. 195–198°.

(ii) The second compound (8 mg; R_{GlcN} 2.9, Solvent *D*) showed an R_{GlcN} value identical with that of 2-amino-2-deoxy-3,6-di-*O*-methyl-D-glucose[35]. A portion of the compound was demethylated to give a compound showing, in t.l.c. (Solvent *D*), an R_{GlcN} value of 1.0, identical with that of 2-amino-2-deoxy-D-glucose. G.l.c. of the product obtained after treatment with methanolic hydrogen chloride showed an R_T value of 9.1, identical with that of methyl 2-acetamido-2-deoxy-3,6-di-*O*-methyl-α,β-D-glucopyranoside[35,36].

(iii) A mixture of compounds (14 mg; R_{GlcN} <1.68) was purified by chromatography on Whatman 3MM paper, first in Solvent *B* and then in Solvent *C*, to give a homogeneous product (5 mg) showing R_{GlcN} 1.7 (Solvent *C*), the presence of reducing and amino groups, and the absence of carboxyl groups. A portion of

the compound was *N*-acetylated, and the product treated with 0.1M sodium metaperiodate for 4 h at 4°; t.l.c. (Solvent *C*) of the resulting compound showed unchanged starting-material. A second portion of the compound (1.5 mg) was *N*-acetylated, the product reduced with sodium borohydride, and the product treated with 10mM sodium metaperiodate (3 ml) for 4 h at 4°; ~ 1 mol of periodate (relative to 1 mol of hexose) was consumed, and t.l.c. (Solvent *C*) showed a reducing spot having R_{Me_3GlcN} 0.62, and a trace of a nonreducing compound having R_{Me_3GlcN} 0.4. A third portion of the compound was treated with ninhydrin[13]; t.l.c. (Solvent *C*) showed an R_{Glc} value of 3.1, different from that of 3-*O*-methyl-D-arabinose (obtained by ninhydrin degradation of 2-amino-2-deoxy-4-*O*-methyl-D-glucose). The compound showed properties different from those of known methyl ethers of 2-amino-2-deoxy-D-glucose, suggesting that it was a 2-amino-2-deoxy-3-*O*-methylpentose, possibly arising from the decarboxylation of 2-amino-2-deoxy-D-mannuronic acid.

(*f*). This compound (24 mg) was purified further by chromatography on Whatman 3MM paper (Solvent *D*). It showed an R_{GlcN} value of 2.9, identical with that of 2-amino-2-deoxy-3,6-di-*O*-methyl-D-glucose[35]. A portion of the compound was treated with ninhydrin, and t.l.c. (Solvent *D*) showed an $R_{2,5\ Di\ O\text{-}methylarabinose}$ value of 1.0, identical with that of 2,5-di-*O*-methylarabinose and different from the values for 2,3- (0.85), 3,4- (0.70), and 3,5-di-*O*-methylarabinose (1.10). The compound was *N*-acetylated, and the product was crystallized from ethanol–ether–pentane to give 2-acetamido-2-deoxy-3,6-di-*O*-methyl-D-glucose, m.p. 214–216°, mixed m.p. 212–214°; lit.[35] m.p. 232–233° (α anomer).

(*g*). This compound (18 mg) was purified by chromatography on Whatman 3MM paper (Solvent *D*), to give a compound (8 mg) that showed an R_{Me_3GlcN} value of 1.0, identical with that of 2-amino-2-deoxy-3,4,6-tri-*O*-methyl-D-glucose. A portion of the compound was demethylated, and t.l.c. (Solvent *D*) of the resulting compound (R_{GlcN} 1.0) indicated that it was identical with 2-amino-2-deoxy-D-glucose. G.l.c. of the *N*-acetylated methyl glycosides on an OV-25 column showed R_T 6.4, identical with that of methyl 2-acetamido-2-deoxy-3,4,6-tri-*O*-methyl-α,β-D-glucopyranoside[36]. The compound was *N*-acetylated, and the product crystallized from ethanol, to give 2-acetamido-2-deoxy-3,4,6-tri-*O*-methyl-D-glucose as needles, m.p. 231–233°, mixed m.p. 230–233°; lit.[37] m.p. 234°.

*Identification of methyl ethers of monosaccharides by gas–liquid chromatography. — Methyl ethers of 2-amino-2-deoxy-*D*-mannuronic acid.* These methyl ethers were identified as methyl ethers of 2-amino-2-deoxy-D-mannose after reduction as follows. A solution of fraction CPC_{A-2} (55 mg) in 10mM methanolic hydrogen chloride (5 ml) was kept for 14 h at 22°, and then evaporated. The residue was treated by several additions and evaporations of toluene, and then dissolved in 3:2 (v/v)water–ethanol (5 ml). Potassium borohydride (10 mg) was added, and the solution was kept for 4 h at 4°, and then for 2 h at 22°; it was processed in the usual way, to give carboxyl-reduced fraction CPC_{A-2} (42 mg).

A portion of this fraction (20 mg) was methanolyzed with M methanolic hydrogen chloride (4 ml) for 16 h at 100°, and the solution evaporated. A solution

of the residue in water (0.5 ml) was applied to a column (0.8 × 12 cm) of Dowex 50 X-8 (H^+) ion-exchange resin, which was eluted with water (50 ml) and then with M hydrochloric acid (40 ml). The hydrochloric acid eluate was concentrated to 15 ml, and the solution applied to a column (0.8 × 7 cm) of Dowex 1 X-8 (AcO^-) ion-exchange resin. The column was successively eluted with water and 0.1M acetic acid (15 ml), the eluates were combined and lyophilized. and the residue was *N*-acetylated. G.l.c. of the trimethylsilyl ethers of the *N*-acetylated methyl glycosides on a column of OV-17 showed a component having R_T 10, identical with that of methyl 2-acetamido-2-deoxy-3-*O*-methyl-α-D-mannopyranoside[11], and different from that of methyl 2-acetamido-2-deoxy-3-*O*-methyl-α,β-D-glucopyranoside. The mass spectrum of the component having R_T 10 was identical with that of authentic methyl 2-acetamido-2-deoxy-3-*O*-methyl-α-D-mannopyranoside[11], and different from that of the 4-*O*-methyl analogue[11].

A second portion (3 mg) of the methylated, reduced, and methanolyzed fraction $CPC_{A-2}$was freed of hydrogen chloride by repeated addition and evaporation of toluene (1 ml) and methanol (1 ml), and then *N*-acetylated. Quantitative g.l.c. of the trimethylsilyl ether on an OV-1 column showed the presence of 32% (w/w of methylated, carboxyl-reduced fraction CPC_{A-2}) of methyl 2-acetamido-2-deoxy-3-*O*-methyl-α-D-mannopyranoside.

Methyl ethers of D-*glucose and 2-acetamido-2-deoxy*-D-*glucose.* A sample of methylated fraction CPC_{A-2} (6 mg) was methanolyzed. A solution of the acid-free methanolyzate in the minimal volume of water was adsorbed on a column (0.8 × 8 cm) of Dowex 50 X-8 (H^+) ion-exchange resin. Elution was successively conducted with water (35 ml), 50mM acetic acid, and 2.5M hydrochloric acid. The water and acetic acid eluates were combined and evaporated. G.l.c. of the residue on a column of OV-11 showed the presence of methyl 2,3,4,6-tetra- and 2,3,4-tri-*O*-methyl-α,β-D-glucopyranoside in the ratio of 1:49, and the absence of methyl 2,3-di-*O*-methyl-α,β-D-glucopyranoside. The hydrochloric acid eluate was evaporated to dryness, and the acid removed as previously described. The residue was *N*-acetylated, and g.l.c. on a column of OV-25 showed the presence of methyl 2-acetamido-2-deoxy-3,4,6-tri- and 3,6-di-*O*-methyl-α,β-D-glucopyranoside in the ratio of 1:3, and the absence of methyl 2-acetamido-2-deoxy-3-*O*-methyl-α,β-D-glucopyranoside.

ACKNOWLEDGMENTS

The authors thank Dr. V. N. Reinhold for recording the mass spectra, and Drs. G.O. Aspinall and R. Begbie for a gift of methyl ethers of D-arabinose.

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THE JOURNAL OF IMMUNOLOGY
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Vol. 119, No. 5, November 1977
Printed in U.S.A.

FURTHER PURIFICATION AND CHARACTERIZATION OF A CIRCULATING ANTIGEN IN SCHISTOSOMIASIS

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Previous studies showed that an antigen found in the circulation of animals heavily infected with *Schistosoma mansoni* was extracted in a trichloroacetic acid soluble-chloroform insoluble fraction (TCA-S-C) of adult worms. Antigenic activity was destroyed by periodate treatment but remained unaltered after treatment with proteolytic enzymes, DNase, RNase, and lyophilization. In the present study, chromatography of TCA-S-C on a DEAE cellulose column revealed six substances, one of which was antigenic. After electrophoresis in agarose antigenic activity corresponded to a slower moving, toluidine blue-staining material. A faster moving, toluidine blue-staining substance seems to be responsible for the large 260 nm. absorbing peak. Analysis of a fraction containing only antigen revealed a large amount of carbohydrate, primarily *N*-acetylglucosamine and D-glucuronic acid but also galactase, glucose, *N*-acetylglucosamine, and trace amounts of other sugars. Amino acids accounted for about 11% of the weight of the antigen. The antigen appears to be a proteoglycan.

Schistosomiasis is a major parasitic infection of the tropical world. Although there is much research interest in schistosomiasis, few antigens have been characterized. Unlike most trematodes, the adult schistosomes reside in the small venules of the gastrointestinal or genitourinary tract. Their excretory and secretory products are therefore discarded into the bloodstream of the host. In animals heavily infected with *Schistosoma mansoni* or *S. japonicum*, an antigen derived from the adult worms appears in the circulation (1–3). Previous studies suggested that this antigen is a large m.w., negatively charged polysaccharide that originates from the gut of the schistosome (1, 4, 5). It was extracted from the adult schistosomes in a trichloroacetic acid soluble-chloroform insoluble (TCA-S-C)[1] (1, 6) fraction which was stable to boiling and treatment with pronase, trypsin, DNase, RNase, and alkaline phosphatase but was destroyed by periodate oxidation (2). Since almost all the carbohydrate found was glucose, this did not adequately explain the presence of negative charges or the 260 nm-absorbing peak (1).

Received for publication June 3, 1977.
Accepted for publication August 2, 1977.
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[1] Abbreviations used in this paper: TCA-S-C, trichloroacetic acid soluble-chloroform insoluble; PAS, periodate-Schiff staining; g.l.c., gas liquid chromatography.

In the present study the TCA-S-C fraction of *S. mansoni* was found to contain at least six substances, only one of which was shown to be antigenic. The antigen was composed mostly of carbohydrate with a small proportion of amino acids. *N*-acetylgalactosamine and D-glucuronic acid were the most common sugars, accounting for 42 and 39%, respectively, of the weight of the known carbohydrate components. Lesser amounts of *N*-acetylglucosamine, galactose, and glucose were found with trace amounts of fucose, mannose, and xylose.

MATERIALS AND METHODS

Extraction of antigen. Adult *S. mansoni* were collected as previously described, lyophilized, and weighed (1). One batch of worms, donated by Dr. Darwin Murrel, of the Naval Medical Research Institute, Bethesda, Maryland was collected in Hanks' balanced salt solution and frozen. A TCA-S-C extract was prepared, as previously described, except homogenization was performed in water instead of dilute NaOH. The maximum amount of TCA-S-C obtained from about 8.7 g of lyopholized worms was 50 mg.

Fractionation of extract. A series of DEAE cellulose columns containing Whatman DE 52 preswollen, microgranular anion-exhange cellulose (Whatman Biochemical Ltd., Springfield Mill, Maidstone, Kent, England) were prepared. The resin was washed with 0.5 M HCl, and then with 0.5 M NaOH, and equilibrated with 0.05 M sodium phosphate, pH 7.2, before use. The initial columns were made from 10-ml glass pipets, with a bed volume of about 8 ml. With larger amounts of material, a 40 ml Econo-Column (Biorad, Richmond, Calif.) was used. After loading the TCA-S-C, the column was extensively washed with 0.05 M sodium phosphate buffer, pH 7.2. Then, the column was eluted with a linear gradient of starting buffer and 1.0 M NaCl in starting buffer. In later experiments, the column was further eluted with 0.05 M HCl (see Fig. 1). Analyses were performed on individual tubes or pooled fractions as indicated. Pooled samples were de-ionized by extensive dialysis against water or on a P-4 column (Bio-Rad, Richmond, Calif.). The dialysis tubing was boiled extensively in a saturated solution of $NaHCO_3$ before use. Antigenicity was determined by the presence of a precipitan line when the substances in tubes or fractions were reacted against rabbit anti-antigen as described below. Fractions of the larger DEAE cellulose column were pooled for analyses as follows: fractions 2W-5W consisted of the nonabsorbed material; fractions 10–16, the first peak reacting to the phenol-sulfuric acid reagent; fractions 17–23, the first half of the second peak reacting to the phenol-sulfuric acid reagent; fractions 24–31, last half of the second peak reacting to the phenol sulfuric acid reagent; fractions 32–39, an uncontaminated second antigenic peak; and fractions 40–50, remaining antigenic activity and last peak

reacting to the phenol-sulfuric reagent (Fig. 1). Fractions were extensively dialyzed against water, lyophilized, and weighed to the nearest 50 μg. Samples were then diluted with water to contain about 2 mg/ml.

Electrophoresis. Countercurrent electrophoresis was performed as previously described, except a 0.05 M barbital buffer, pH 8.6, was used (1). Immunoelectrophoresis and electrophoresis were done in agarose (B grade, Lot. 001874, Calbiochem, Los Angeles, Calif.), with a 0.05 M barbital buffer, pH 8.2. Cordis II plates (Cordis, Miami, Fla.) were employed for immunodiffusion studies. Electrophoresis of samples was done in either Ionagar (Colab Laboratories, Inc., Gleenwood, Ill.) for periodate-Schiff staining (PAS) or in agarose for PAS, amino black, and toluidine blue staining. Either 10 or 20 μl of samples containing 10 to 23 μg of material was electrophoresed on 8- x 10-cm glass plates layered with 10 ml of 0.9% agarose or Ionagar in 0.05 M barbital buffer, pH 8.2. Electrophoresis was carried out at 90 to 120 volts with bromphenol blue as a migration marker in two separated wells. Sodium heparin (10,000 units/ml., Abbott Laboratories, North Chicago, Ill.) and purified chondroitin 6-sulfate (purified by Dr. A. Hallén) were used as positive controls. When the indicator dye had migrated two-thirds of the length of the plates, electrophoresis was terminated and the plates were stained. For toluidine blue staining the plates were placed in a solution of 0.1% of toludine blue in 1% acetic acid for 15 min (7). The plate was then rinsed briefly in water and dried with a nonheated air stream while covered with filter paper. Positive staining was clearly seen as a dark blue area against a lighter blue background. For PAS staining, the plates were exposed to 0.2% periodic acid in 7.5% acetic acid for 15 to 20 min, and then to the Schiff's reagent for 45 min, followed by rinsing in 7.5% acetic acid. Protein staining was accomplished by fixing the plate in 7.5% acetic acid and staining with amido black, as previously described (1). Additionally, plates were fixed in 95% ethanol, and then stained with PAS, amido black, or toluidine blue.

For immunoelectrophoresis previously prepared antisera to the circulating antigen was used (1). Antigen titers were determined by countercurrent electrophoresis. A solution of antisera of standard dilution was electrophoresed against serial 2-fold dilutions of antigen in normal saline. The highest dilution giving a precipitin line was taken as the titer.

Acrylamide electrophoresis was performed following the method of Holden *et al.* (8); 90 μg of sample was used.

Pevikon (Mercer Chemical Company, N. Y.) electrophoresis was performed as described by Müller-Eberhard (9) and Osterland (10) in a 0.5 M barbital buffer, pH 8.6, in a lucite block, 10.3 cm x 25.5 cm. Bromophenol blue was used as a migration marker. One-centimeter strips were cut out and eluted with two separate 2-ml washes of water on a fine sintered glass funnel.

Analysis. Absorption spectra were determined with a Zeiss spectrophotometer. Change of 260-nm absorption after addition of NaOH was measured by the procedure of Hotchkiss (11). Samples for amino acid analyses were hydrolyzed with 6 M HCl under a nitrogen atmosphere at 100°C for 15 to 24 hr. Hydrogen chloride was evaporated under a stream of nitrogen after addition of a mixture of ethanol and toluene. Amino acids were analyzed by the procedure of Miller and Piez (12).

Carbohydrate determinations were performed with the phenol-sulfuric acid method on a 0.2-ml sample containing 0.1 ml of 5% solution of phenol in water, and 0.5 ml of concentrated H_2SO_4 (13). Sulfate content was measured on 100 μg by the procedure of Spencer (14). Gas liquid chromatography (g.l.c.) was performed with a Perkin Elmer 900 gas chromatograph equipped with columns containing glass beads coated with OV-17, OV-1, or SE-30. The method of Reinhold (15) was followed, except that 1.0 M methanolic HCl was used for methanolysis for 12 to 15 hr at 100°C. Acetylation was done at room temperature for 4 hr and O-deacetylation was done in some samples with methanolic ammonia for 1 hr at 65°C.

The unknown peaks on g.l.c. were identified by mass spectroscopy as derivatives of a hexuronic acid. The uronic acid was identified as D-glucuronic acid by reduction with sodium borodeuteride as follows: the sample (0.25 mg) was treated with 1 M H_2SO_4 (1 ml) for 16 hr at 100°C. The cooled solution was diluted with water (1 ml) and applied to a column (8 x 0.8 cm) of AG 1 X-8 (OAc-, 100-200 mesh) ion-exchange resin. The column was eluted with water (25 ml), followed by 50 mM CH_3COOH (25 ml). The combined eluates were evaporated to dryness, the residue was dissolved in methanol (3 ml), and toluene (3 ml), and the solvents were evaporated. This process was repeated three times. The residue was dissolved in pyridine (0.5 ml); acetic anhydride (250 μl) was added, and the solution was kept for 2 hr. After evaporation of the solvents, the residue was treated with 1 M methanolic hydrogen chloride for 21 hr at 100°C. The solution was evaporated and the residue was acetylated with pyridine (0.5 ml) and acetic anhydride (0.25 ml). The acetylated mixture was evaporated, and the residue in 20% methanol (1 ml) was treated with sodium borodeuteride (10 mg) at room temperature for 4 hr. Enough AG 50 X-8 (H^+, 20-50 mesh) ion-exchange resin (Bio-Rad) was added to decrease the pH of the solution to 4. After filtration, the solution was evaporated and then repeatedly treated with methanol to remove the borate ions as methyl borate. The sample was examined by g.l.c.–mass spectroscopy. Quantitative determinations of D-glucuronic acid were done by g.l.c. by comparison with authentic D-glucuronic acid.

Gas-liquid chromatography–mass spectrometry was performed with an analytical system consisting of an IBM 1800 computer fed raw data generated by a single focusing, Hitachi-Perkin-Elmer RMU-6 mass spectrometer interfaced with a gas chromatograph (Perkin-Elmer 900).

RESULTS

The elution pattern of TCA-S-C from a large DEAE cellulose column is shown in Figure 1. Most of the carbohydrate determined by the phenol-sulfuric acid method was found in the nonabsorbed (wash) fraction. Another PSA-positive peak was eluted in tubes 10–16, followed by a smaller PSA-positive peak. After addition of 0.05 M HCl (tube 30), another broad PSA-positive peak appeared.

The antigenic activity was eluted in two peaks. The first antigen active peak began at tube 17, declined in titer, and then increased again after the addition of 0.05 M HCl to reach the maximum titer obtained. The location of the PSA-positive material did not correspond to the antigenic activity (Fig. 1). As shown in Figure 2, the antigen present in various tubes was identical on examination by immunodiffusion. A single line of identity formed between the antigen in tubes 17, 28, 31, and 34, and the serum obtained from mice heavily infected with *S. mansoni*. On another plate (Fig. 3), a single line of identity was seen between material from tubes 17, 28, 40, 43, and a water-soluble extract of *S. mansoni*. Furthermore, the antigen in all fractions showed the same migration pattern on immunoelectrophoresis. As seen from Figure 1, the antigenic activity did not coincide with 260-nm absorption. In fact, the

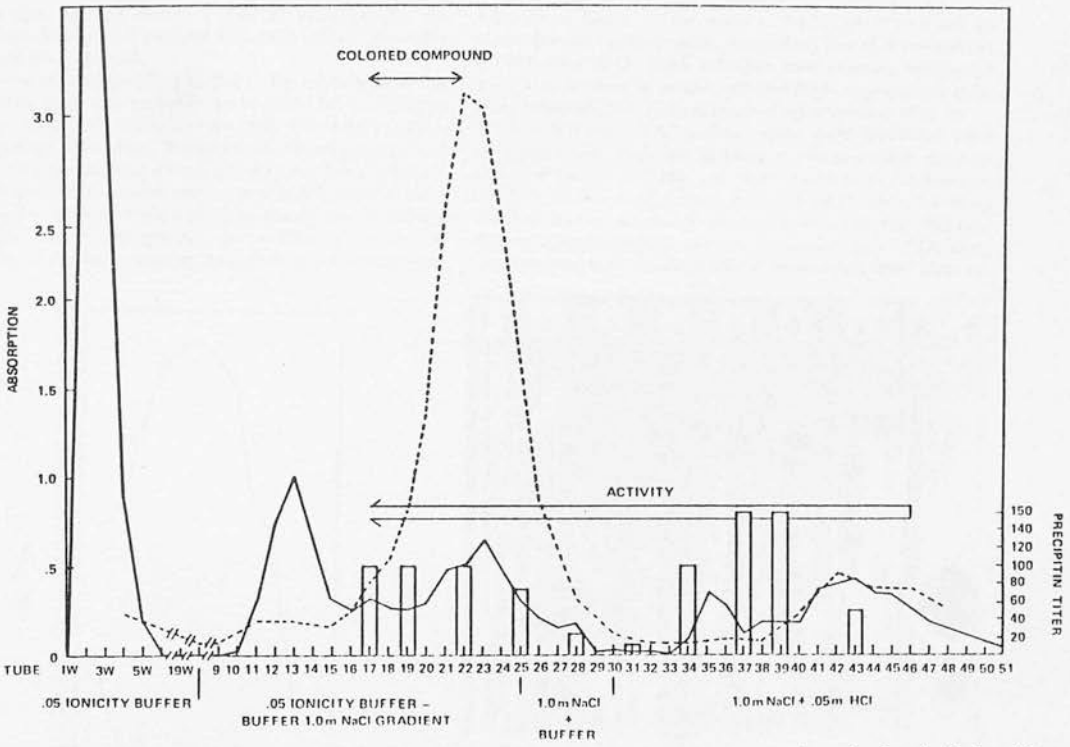

Figure 1. Elution pattern of TCA-S-C from a DEAE cellulose column. The solid line represents reactivity to the phenol-sulfuric acid test; the dotted line represents the 260-nm absorption. The vertical bars represent the precipitin titers of antigen in selected tubes by countercurrent electrophoresis. Antigenic activity was found in all tubes from 17 to 46; the limits of detectable antigenic activity are seen by the horizontal double line. The limits of a visible, dark brown eluate are noted by the single horizontal line. The same absorption scale is used for both the phenol-sulfuric acid test and 260-nm absorption.

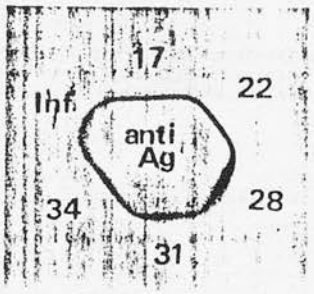

Figure 2. Immunodiffusion plate containing eluted fractions from the DEAE column shown in Figure 1. Rabbit anti-antigen is in the center well. The peripheral wells contain samples from individual tubes from the eluate of the DEAE cellulose column shown in Figure 1, and sera obtained from mice heavily infected with *S. mansoni* (Inf.). A line of identity is seen between infected sera and antigen containing tubes of both the earlier and the later antigen peaks.

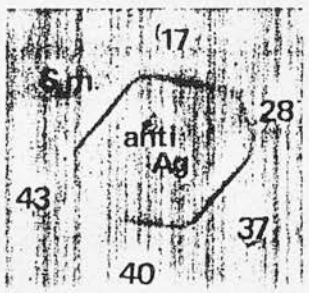

Figure 3. Immunodiffusion plate containing eluted fractions from the DEAE column shown in Figure 1 with rabbit anti-antigen in the center well. The peripheral wells contain samples from individual antigen containing tubes and a water soluble extract of adult *S. mansoni* (S.m.). A line of identity is shown between *S. mansoni* extract and the antigen containing tubes from both the earlier and the later antigen peaks.

absorption spectra of tubes 17 and 40 failed to show a 260-nm absorbing peak, although they did contain antigen (Fig. 4).

The characteristics of the pooled fractions on electrophoresis in agar or agarose, are shown in Table I. Of the two fractions reacting with toluidine blue substances the slower moving one was detected in the fractions containing antigenic activity (Fig. 5). The precipitin line observed after immunoelectrophoresis corresponds exactly to the margin of the slower moving

toluidine blue staining material (Fig. 6). Additionally, the material was faintly PAS positive and could be fixed with 95% ethanol and then stained.

The material corresponding to the faster moving toluidine blue-reacting spot was probably responsible for the 260-nm absorping peak. This material was only found in fractions showing 260-nm absorption. Furthermore, electrophoresis and staining of dilute solutions of material obtained from tubes 17, 19, and 22 revealed the faster moving material in tube 22 only. Therefore, the tube that showed peak absorption at 260-nm appeared to contain the greatest concentration of material responsible for the faster moving, toluidine blue-reacting spot. Addition of NaOH to the material from tube 22 did not increase the 260-nm absorption, suggesting that this was neither a DNA or a RNA. Both toluidine blue-reacting substances were also present in crude, saline-soluble supernatant solutions obtained from homogenates of schistosomes (Fig. 5).

Three different, PAS-positive spots were identified after electrophoresis in agarose or Ionagar; one present in the non-absorbed (wash) fraction, one in fractions 10–16, and one in fractions 40–50. A pigmented material was seen in fractions 17–23; it had no maximum absorption between 220–1000 nm. After electrophoresis in agarose or Ionagar none of the fractions stained with amido black. A fraction obtained from an-

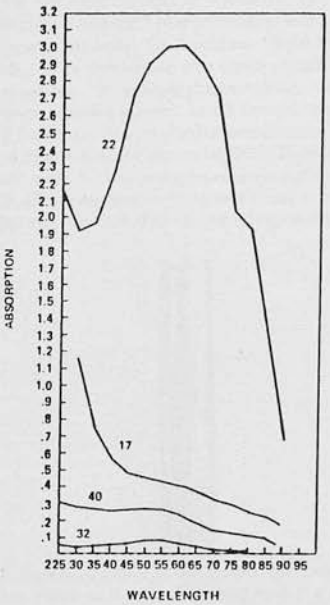

Figure 4. Absorption spectra of individual tubes from the eluate of the DEAE cellulose column shown in Figure 1. Fractions 17 and 40, although containing antigen, fail to show a significant 260-nm absorption.

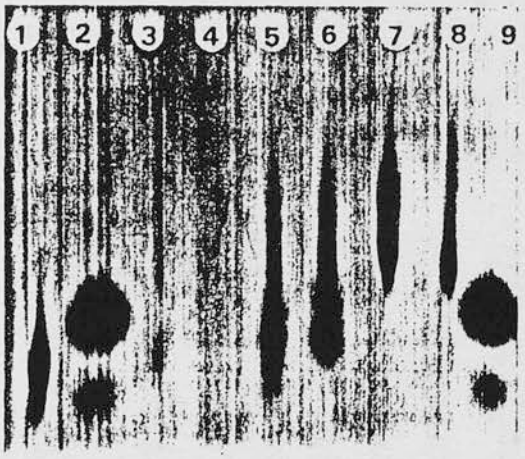

Figure 5. Electrophoresis in agarose of pooled fractions stained with toluidine blue, from the eluate of the DEAE cellulose column shown in Figure 1. Well 1 is chondroitin 6-sulfate; wells 2 and 9, bromophenol blue marker dye; wells 4, 5, 6, 7, and 8 contain pooled fractions 10–16, 17–23, 24–31, 32–39, and 40–50, respectively. Well 3 contains a concentrated saline extract of *S. mansoni* adults. Antigen-containing fractions (wells 5, 6, 7, 8) show a slower moving, anodic oblong spot, but only the fractions encompassing the 260-nm absorbing peak also show a faster moving, toluidine blue-positive spot. *S. mansoni* extract shows both toluidine blue-positive spots. Fractions 10–16 (well 4) did not demonstrate significant toluidine blue staining.

TABLE I

Properties of pooled fractions

Pooled Fractions	Antigenic Activity	Toluidine Blue Staining after Electrophoresis	260 Absorption	PAS after Electrophoresis	Other
Wash	Not present	Not present	Not present	Cathodic in Ionagar, no migration in agarose	
10–16	Not present	Not present	Not present	Barely anodic in Ionagar	
17–23	Present	Faster and slower toluidine blue spots	Present	Only antigen	Pigment
24–31	Present	Faster and slower toluidine blue spots	Present	Only antigen	
32–39	Present highest titer	Slower toluidine blue spot only	Not Present	Only antigen	
40–50	Present	Slower toluidine blue spot only	Slight	Antigen peak and another barely anodic spot	

other DEAE cellulose column, equivalent to tubes 17–40, did not stain with amido black after electrophoresis in acrylamide gel.

The proportion of the pooled fraction to the total material eluted from the DEAE cellulose column is shown in Table II. The nonabsorbed (wash) fractions represented 57%, fractions 10–16, 15.0%, and fractions 17–23, 17.6% of the total eluate. The remaining substances were less than 10% of the total. Although an accurate weight of the TCA-S-C put on this column was not obtained, data from previous columns showed that total recovery from the column ranged from 81 to 106% before elution with 0.05 M HCl.

Analyses of the pooled fractions for carbohydrates and amino acids are summarized in Table II. The nonabsorbed (wash) fractions contained predominately carbohydrate components, mostly glucose. In fractions 10–16 the amount of amino acids and carbohydrate was about equal. Galactose, *N*-acetylglucosamine, *N*-acetylgalactosamine, and mannose were the preponderant sugars. In all the subsequent antigen containing fractions, *N*-acetylgalactosamine was the sugar in the highest proportion. As shown in Table II, D-glucuronic acid was present only in the antigen-containing fractions. Fractions 32–39, which on electrophoresis did not contain any other identifiable material other than the antigen, showed a carbohydrate content of 41% and an amino acid content of 5%. D-glucuronic acid and *N*-acetylgalactosamine were present in almost equal proportions and together accounted for 81% of the carbohydrate present.

Figure 6. Superimposition of the immunoelectrophoresis pattern formed when rabbit anti-antigen reacted with the antigen in fractions 32–39 and the colored spot obtained with toluidine blue-reacting material in fractions 32–39. The immunoelectrophoresis pattern corresponds closely to the edge of the slower moving, toluidine blue-staining material.

The amino acid content of the various fractions is presented in Table III. Fractions 32–39, the only fractions containing antigen without other contaminating substances had glycine as the most common amino acid (18.8 μmol/100 μmole). Notably, there was a very small proportion of tyrosine and phenylalanine. Their absence explains the lack of absorption at 280 nm.

Pevikon electrophoresis of a fraction comparable to fractions 17–23 did not separate the antigenic acitvity from the 260-nm absorbing material. Analysis of the strips containing the antigen revealed amino acid and carbohydrate compositions that approach those of fraction 24–31. Although glucose was present in the antigenic fraction, it was also present in the dialysis control in roughly equal amounts. Sulfate represented almost 2.6% of the material by weight.

Sulfate determinations of pooled fractions of an earlier experiment showed an insignificant sulfate content in TCA-S-C

TABLE III

μmole amino acid/100 μmole of amino acids

Amino Acid	Fraction TCA-S-C[a]	Wash[b]	10–16[b]	17–23	24–31	32–39	40–50
$MetSO_4$	0.6	0	0	0	0	0	0
Asp	8.5	5.1	15.7	22.8	16.4	8.8	10.9
Thr	6.7	17.8	18.3	4.1	5.7	7.4	5.3
Ser	14.5	19.2	15.2	12.8	11.7	14.9	19.7
Glu	5.0	5.4	16.1	16.5	11.8	8.9	13.4
Pro	3.5	9.1	3.9	3.4	3.0	4.6	Unknown
Gly	15.9	9.6	6.7	19.2	27.9	18.8	24.1
Ala	12.6	6.6	3.1	3.2	6.5	6.3	6.9
Val	4.0	3.7	5.4	2.4	3.5	3.2	3.5
Ile	3.6	7.6	3.7	4.3	1.9	2.3	2.3
Leu	5.1	8.6	5.5	1.9	4.4	4.1	2.1
Tyr	Small	2.4	0.4	0.8	0.4	0.6	1.2
Phe	Small	2.2	0.6	0.3	Small	1.1	Small
Lys	14.9	2.7	3.1	4.7	3.2	12.0	6.7
His	3.4	Small	1.7	2.1	1.7	5.6	3.9
Arg	1.7	Small	0.6	1.5	1.9	1.3	0

[a] Separate batch of TCA-S-C.

[b] Same batch TCA-S-C but different DEAE column. Small refers to present but not quantifiable.

TABLE II

Composition of the pooled fractions[a]

Fraction	% of Peaks by Weight	Weight Peak	% CBH[b]	% Amino Acid	% by Weight CHB + Amino Acid	% Glu	% Gal	% GlcNac	% GalNac	% Fuc	% Man	% GlcUA	% Xyl
		mg											
TCA-S-C[c]			70	6	75	58	2.7	4.2	2.4	1.1	1.3		n.q.
Wash[d]	57.2	19.850	84	4	88	62	7.3	7.8	2.6	4.7	n.q.	0	n.q.
10–16[e]	15.0	5.200	27	28	55	1.0	9.0	5.0	7.3	1.3	3.5	0	n.q.
17–23[e]	17.6	6.105	15	21	36	1.9	1.5	1.2	6.5	0.4	0.5	2.9	0.3
24–31[f]	2.5	0.875	33	8	41	1.3		1.7	18.8	1.1	n.q.	10.3	0.2
32–39	5.8	2.005	41	5	46	3.3	2.0	1.9	17.2	n.q.	0.4	16.1	n.q.
40–50	1.9	0.675	23	8	31	4.8	1.0	1.7	11.7	n.q.	n.q.	4.0	n.q.

[a] Per cent sugars calculated as per cent of total sample analyzed.

[b] Abbreviations: CBH = carbohydrate; GlcNac = *N*-acetylglucosamine; GalNac = *N*-acetylgalactosamine; Glc = glucose; Gal = galactose; Man = mannose; Fuc = fucose; GlcUA = glucuronic acid; Xyl = xylose; n.q. = not quantifiable.

[c] Results with a TCA-S-C batch different from one separated on the DEAE cellulose column. Glucuronic acid not determined. Per cent amino acid of different TCA-S-C batches ranged from 2.6 to 6.0%.

[d] Carbohydrate and amino acid analysis of a different DEAE cellulose column but the same batch of TCA-S-C.

[e] Small amounts of *N*-acetylneuraminic acid found in fractions 10–16 and 17–23.

[f] Galactose not quantified. Per cent calculated without galactose, therefore underestimated.

and nonabsorbed (wash) fractions, 2.7% in the material comprising the first peak reacting to the phenol-sulfuric acid reagent, and 4.0% in the antigenic material eluted with 1.0 M NaCl alone.

DISCUSSION

Unlike other trematodes, adult schistosomes are intravascular parasites that reside in the small venules of the gastrointestinal or genitourinary tract of the host. Schistosomes derive nutrients from the blood of their host and all metabolic products are extruded into the intravascular space. Since schistosomes do not have an anus, digestive products are regurgitated into the blood stream.

Eggs are also released into the small venules where they incite an inflammatory response. They may pass into the lumen of the bowel, bladder, or ureter. Other eggs remain in the tissues or embolize to other organs, primarily the liver.

Although many persons are infected with schistosomes, few develop clinically apparent disease. Except for certain special circumstances, higher worm burden correlates with the presence of disease or risk of developing disease; the higher worm burden is reflected in a higher tissue concentration of eggs and a greater excretion of eggs in the urine or feces. Therefore, the number of eggs per unit of feces or urine is a measure of the risk of disease. Unfortunately, there are many variables associated with correlating egg excretion to disease, such as changes in egg excretion with duration of infection and the day to day variation in number of eggs excreted. Therefore, the significance of a single or even several egg counts is difficult to interpret in an individual patient.

Measurement of secretion or excretion products offers another means of quantifying infection in schistosomiasis. Previous studies in heavily infected mice and hamsters correlated the amount of antigen in the serum with the worm burden of the host (2, 3). Failure to detect the antigen in human sera may have been due to insensitivity of the assay system or possibly to binding of the antigen by antibody. Previous studies suggested that the circulating antigen was polysaccharide in nature (1). The results presented here have confirmed the previous findings and further define the composition of the antigen.

Chromatography of TCA-S-C on DEAE cellulose separated the crude TCA-S-C into a number of components. Although antigenic activity occurred in the last four pooled fractions, only fractions 32-39 contained a single component. Analysis of this fraction revealed primarily carbohydrate residues with a lesser proportion of amino acids. The large content of *N*-acetylgalactosamine and glucuronic acid suggests that the main component of the antigenic fraction is a proteoglycan, a polymer consisting of hexosamine and hexuronic acid residues generally sulfated and linked to a protein core. Although the sulfate concentration increased in the antigen-containing fractions, these fractions were impure and the presence of sulfate was not definitely established. Like the fractions observed here, the proteoglycans are also stained with toluidine blue because of their high negative-charge density (16, 17).

Known carbohydrate and amino acids components accounted for 30 to 42% of the total weight of the material analyzed. Material resistant to hydrolysis or incompletely derivatized, salts, and water may account for the remaining weight.

Previous studies (1, 2) had suggested that the circulating antigen had a 260-nm absorbing peak. In the present work this property was separated from the antigenic acitvity and it appears to be associated with the faster moving, toluidine blue-staining material. Although the nature of this material is unknown, numerous attempts have failed to show that this absorption was due to DNA or RNA.

In addition to the circulating antigen, five other substances were identified. Although no attempt was made to purify the fractions containing these substances, analysis of the fractions revealed carbohydrate-containing materials with varying amounts of amino acids. They undoubtedly have a role in the metabolism and structural integrity of the organisms.

Previous studies have shown that the antigen is localized in the epithelial cells of the schistosome gut and at the interface between these cells and the lumen of the gut (4, 5). Since the antigen appears in the circulation during infection and is found in the lumen of the gut, the antigen is probably secreted into the lumen, and then regurgitated into the bloodstream. The role that this antigen plays in the physiology of the schistosome is speculative; perhaps it protects the schistosome from digestive enzymes or host materials. Once in the circulation of the host this highly charged compound could interfere with the normal surface interactions of the host cells, possibly altering immune responses or clotting, for instance.

Little is known about glycoproteins and proteoglycans of schistosomes, although in other organisms these compounds are particularly important as structural material and surface constituents. Definition of their composition and structure can lead to a clearer understanding of the relationship between parasite and host.

Acknowledgment. The authors thank Dr. Vernon Reinhold for performing the mass spectroscopy and Mr. Michael Bynes for performing amino acid analyses. The helpful criticisms of Dr. Allen Cheever is greatly appreciated.

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Reprinted from

Carbohydrate Research, 57 (1977) C1–C3

Preliminary communication

Amino-sugar phosphates from the cell wall of *Micrococcus lysodeikticus**

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(Received February 22nd, 1977; accepted for publication, February 28th, 1977)

The presence of 2-amino-3-*O*-(D-1-carboxyethyl)-2-deoxy-D-glucose 6-phosphate (muramic acid 6-phosphate) in the cell walls of *Micrococcus lysodeikticus*[1–3] and several other bacteria has been reported[4–13]. The occurrence, in *Micrococcus lysodeikticus*, of another amino-sugar phosphate, with an electrophoretic mobility similar to that of 2-amino-2-deoxy-D-glucose 6-phosphate (glucosamine 6-phosphate), has also been reported[14]. On removal of the phosphate group, this sugar liberated an amino sugar that did not correspond to any known amino sugar on paper chromatography[14]. We now report the isolation and characterization of glucosamine 6-phosphate, as well as of muramic acid 6-phosphate, from the lysozyme-resistant cell-walls of *Micrococcus lysodeikticus*.

The lysozyme-resistant material obtained from cell walls, prepared according to the procedure of Sharon and Jeanloz[15], was treated with 4M hydrochloric acid for 10 h at 80°. After evaporation, the acid-free residue in water was adsorbed on a column of Dowex 50 X-8 (H^+, 200–400 mesh) ion-exchange resin, and the column was eluted with water. Three fractions were obtained. The first mainly contained D-glucose. The second fraction showed a single component on thin-layer chromatography (t.l.c.) and reacted positively with ninhydrin, the Hanes–Isherwood[16] and Park–Johnson[17] reagents, and with the modified Elson–Morgan reagent[18] to give an absorption maximum at 510 nm, indicating the presence of a reducing sugar having an amino and a phosphate group. Finally, treatment of the sugar phosphate with alkaline phosphatase (calf mucosa, Sigma Chemical Company, St. Louis, MO) liberated muramic acid.

In t.l.c., the third fraction showed a single component that gave a positive stain

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*Dedicated to the memory of Sir Edmund Hirst, C.B.E., F.R.S. Amino Sugars III and *Micrococcus lysodeikticus* cell-wall VI (for preceding paper, see Ref. 1). This is publication No. 731 of the Robert W. Lovett Memorial Group for the Study of Diseases Causing Deformities, Harvard Medical School and Massachusetts General Hospital. This investigation was supported by a research grant from the National Institute of Allergy and Infectious Diseases (Grant AI-06692).

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TABLE I

HYDROCHLORIC ACID TREATMENT OF SYNTHETIC MURAMIC ACID 6-PHOSPHATE

Hydrochloric acid (molarity)	*Time (h)*	*Temp. (°)*	*Compounds formed*[a]		
			Muramic acid 6-phosphate	*Muramic acid*	*Glucosamine 6-phosphate*
2	16	100	++++	+	–
4	16	100	++	+++	trace
4	10	80	+++++	–	–
6	1	100	++++	+	–
6	2	110	++	+++	trace

[a]The products of hydrolysis were examined by t.l.c. in 4:1:5 (v/v, upper layer) 1-butanol–acetic acid–water, and 1:3:1 (v/v) methanol–chloroform–water.

with ninhydrin and the Hanes–Isherwood reagent[16]. The sugar was reducing[17], and gave a positive Elson–Morgan reaction with an absorption maximum at 530 nm. Treatment of the sugar phosphate with alkaline phosphatase released a sugar, identical with glucosamine (t.l.c.), which gave arabinose (t.l.c.) on degradation with ninhydrin[19]. Periodate treatment of *N*-acetylglucosamine phosphate degraded the sugar, and no release of formaldehyde was detected. The results strongly suggest the presence of a phosphate group at C-6 of glucosamine.

In order to further establish that glucosamine 6-phosphate is an original sugar component of the cell walls and does not arise as the de-etherification product of muramic acid 6-phosphate during acid hydrolysis, synthetic muramic acid 6-phosphate[3] was treated with various concentrations of hydrochloric acid for several time-intervals (see Table I). The products of hydrolysis clearly indicate that the ether bond in muramic acid 6-phosphate is stable to the acid conditions used and that these treatments removed only the phosphate group. During acid hydrolysis, D-glucosamine 6-phosphate might arise from D-glucosamine 4-phosphate[20]; however, this seems unlikely, as the D-glucosamine residues in cell walls are linked at C-4, and nonreducing terminal 2-acetamido-2-deoxy-D-glucose residues were shown[1], by methylation studies, to be free of substituents.

As no inorganic phosphate was released by treatment of the nondialyzable cell-wall with alkaline phosphatase[21], it is probable that the D-glucosamine 6-phosphate residues serve, like the muramic acid 6-phosphate residues, as a link between the antigenic polysaccharide chains and the peptidoglycan chain.

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Pakistan J. Sci. Ind. Res., Vol. 21, Nos. 5-6, October - December 1978

STUDIES OF A CARBOHYDRATE-CONTAINING POLYMER FROM *CORDIA MYXA*

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(Received March 7, 1978; revised October 15, 1978)

A carbohydrate-containing polymer has been isolated from the mucilage of *Cordia myxa* and purified by gel filtration and ion exchange chromatography. The polymer has been shown to contain xylose, glucose, galactose, mannose and N-acetylglucosamine in addition to small amounts of amino acids. Hemagglutination inhibition studies and compositional analysis of this material suggest that the polymer is of the glycoprotein type.

INTRODUCTION

Cordia myxa (Vern. -Lasuri, Eng. - Sebestan plum), belongs to the plant family Boraginaceae. It is distributed throughout Pakistan and India. The fruits of the plant find use in the local materia medica as a remedy against coughs, chest infections and irritation of the urinary tract [1].

The chemical nature of the mucilage has been recently elucidated in two independent studies employing paper chromatographic techniques. Whereas Kassem *et al.* [2] have shown that the mucilage is composed of glucose, fructose and galacturonic acid, Ifzal and Qureshi [3] have reported the mucilage to consist of glucose, galacturonic acid, arabinose and xylose.

The present communication describes the isolation, purification and some structural features of the carbohydrate-containing ploymer obtained from *Cordia myxa* and presents results indicating a significantly different composition of the mucilage.

EXPERIMENTAL

The ripe *Cordia myxa* fruits were collected in the month of July. The fruits were macerated in water and the aqueous solution of the pulp was filtered through muslin cloth. The filtrate was acidified with HCl (0.5%) and the polysaccharide was precipitated by adding 95% ethanol. The precipitate was filtered through a sintered glass funnel, washed with ethanol and dried under low pressure at 40°. The dried powder was used for subsequent investigations.

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The powder (0.5 g) was dispersed in 10 *mM* NaOH (100 ml) and the mixture was stirred for 16 hr at 4° under nitrogen. The insoluble material was removed by centrifugation (10,000 rev/min) and the solution was dialyzed against three exchanges of distilled water at 4°. The non-diffusible material was freeze-dried to give a residue (100 mg).

The residue (70 mg) in 5 *mM* tris-HCl (pH 7.3; 3 ml) was applied to a column (2 x 40 cm) of Bio-gel P-100. The carbohydrate-containing fractions were combined and freeze dried. A portion of the residue (40 mg) in 50 *mM* sodium phosphate (pH 7.0, 2 ml) was applied to a column (2.2 x 50 cm) of DEAE-cellulose. The column was eluted with a gradient of 50 *mM*-1 *M* sodium phosphate (pH 7.0; 250 ml) followed by 0.05-1 *M* LiCl. The carbohydrate-containing fractions were combined and freeze-dried to give the polymer (25 mg). The eluates from both the columns were examined for carbohydrates by the phenol–sulphuric acid procedure [4] and the presence of amino acids was detected by absorption at 280 nm. The sugar residues were identified after methanolysis as trimethylsilyl derivatives according to the procedure of Reinhold [5].

Canavalia ensiformis hemagglutinin [6], *Ricinus communis* hemagglutinin [7] and *Triticum vulgaris* hemagglutinin [8] were purified by affinity chromatography. *Glycine max* hemagglutinin was used as a crude extract. The titration and inhibition assays were performed with human erythrocytes using the method of Matsumoto and Osawa [9]. The cells used for inhibition assays on concanavalin and glycine max hemagglutinin were trypsin treated [9].

RESULTS AND DISCUSSION

The material examined in these studies was dark brown

Table 1. Composition of the extract obtained.

Sugar	Retention time*		Molar ratio†
D - Xylose	0.66	0.71	1
D - Glucose	0.91	0.93	6
D - Galactose.	0.85	0.87	22
D - Mannose	0.79	0.82	2
2 - Acetamido-2-deoxy-D-glucose	1.15	1.18	2
Unidentified	0.60	0.61	
Unidentified	1.00	1.05	+++++
Unidentified	1.09	1.10	++

*Retention time relative to inositol

†Molar ratio relative to D – xylose

and mainly contained carbohydrates (Table 1). Column chromatography on Bio-gel P-100 showed the presence of a single macromolecular component containing carbohydrate and a small amount of protein. Ion exchange chromatography on DEAE cellulose also showed the presence of a homogenous material consisting mainly of sugar residues, though the presence of amino acids was also indicated. The proportion of sugars to amino acids in the macromolecule was approximately 9:1 and the two components together accounted for only 35% of the total weight. It is possible that the dark brown substance, present in the extract and eluted with the carbohydrate-containing material from the Bio-gel P-100 and DEAE cellulose columns, contributed to the remainder of the weight. The nature of this residue will be reported subsequently.

Gas liquid chromatography of the sugar residues showed the presence of a pentose, a mixture of hexoses, a 2-acetamido-2-deoxy-hexose and unidentified sugars (R_{ins} 0.60, 0.61, 1.00 1.05, 1.09 and 1.10 Table 1).

The inhibition studies on this polymer indicated weak inhibitory activity against *Glycine max* hemagglutinin, concanavalin A, *Triticum vulgaris* hemagglutinin and *Solanum tuberosum* hemagglutinin, but no activity was observed in the inhibition of *Ricinus communis* hemagglutinin. The known specificities of the above hemagglutinins [10] suggest that the polymer contains an α-D-glucopyranosyl and/or α-D-mannopyranosyl residue, a 2-acetamido-2-deoxy-β-D-glucopyranosyl residue and a non-reducing terminal 2-acetamido-2-deoxy-β-D-galactopyranosly residue, but it does not have a β-D-galactopyranosyl residue as a terminal sugar.

These results are consistent with the compositional data (Table 1) except in that no detectable amount of 2-acetamide-2-deoxy-D-galactose was identified by GLC. Inhibitory hemagglutination activity with concanavalin A is in agreement with the fair proportion of glucose and mannose present. The inhibition specificities based upon mono- and disaccharides do not always correspond to the polymeric structure [11]. The results, however, suggest certain structural similarities. Insignificant activity against *Ricinus communis* hemagglutinin despite the large proportion of galactose in the polymer suggest two possible structural features of this sugar residue.

It is either located in a terminal position in an inactive α-configuration or is attached in an inactive sequence. Both the Cal→GlcNAc and Cal→Glc linked 1,4 would be expected to be active. The composition of this polymer is unusual and is unlike those of the known polysaccharides of plant origin, it is possible that this may constitute a plant glycoprotein.

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Phytochemistry, 1978, Vol. 17, pp. 2059–2060. © Pergamon Press Ltd. Printed in England. 0031-9422/78/1201-2059 $02.00/0

GLYCOPROTEINS FROM *USTILAGO TRITICI*

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(*Revised received* 21 *April* 1978)

Key Word Index—*Ustilago tritici*; fungus; wheat smut; glycoproteins; structural determination; antigens.

Abstract—A complex mixture of glycoproteins has been isolated from *Ustilago tritici* cells obtained from diseased *Triticum vulgaris* plants. Composition analysis and hemagglutination inhibition assay of the purified material shows the presence of an unusual glycoprotein in the fungal spore.

INTRODUCTION

Ustilago tritici causes a commonly known infection of the seeds of *Triticum vulgaris* which is observed as soon as wheat begins to ear. The disease is characterized by a black powdery mass of spores. Antisera against the whole cell preparation and the supernatant cell fractions of *Ustilago maydis*, corn smut, has been reported [1]. The present study was aimed at isolating and identifying an antigenic material, and we report the isolation, composition and nature of a complex mixture of glycoproteins in the water-soluble portion of the cells.

RESULTS AND DISCUSSION

The water extract was dark brown in color and contained carbohydrates and amino acids. Gel filtration of the extract on Sepharose 6B showed the presence of at least two high MW materials containing carbohydrates and proteins. The two components (A and B) contained sugars and amino acids (Table 1) and were present in the ratio of 3:7. Polyacrylamide gel electrophoresis revealed a complex mixture of polymers which gave a positive reaction with periodate-Schiff reagent and Coomassie blue suggesting the presence of glycoproteins. Fraction B, a major fraction from Sepharose 6B, was further fractionated on a column of Sepharose 2B into two fractions, B_1 and B_2. The composition of the two fractions is reported in Table 2. The proportion of carbohydrate to amino acids in the glycoprotein was *ca* 4:1, and the two components together accounted for only 40% of the total wt. It is likely that the brown material, which was present in the H_2O extract and eluted with the glycoproteins on gel filtration from Sepharose 6B and Sepharose 2B, contributed to the remainder of the wt.

Fraction B_2 was eluted as a single component from the DEAE cellulose column with phosphate buffer. The inhibition studies on Fraction B_2 indicated weak but significant inhibitory activity against *Glycine max* hemagglutinin, concanavalin A, *Triticum vulgaris* hemagglutinin and *Solanum tuberosum* hemagglutinin [2], but no significant activity was observed in the inhibition of agglutination by *Ricinus communis* hemagglutinin. Although hemagglutination inhibition specifities based upon mono- and disaccharides do not always apply to macromolecular structure [3], the results suggest certain structural features. From the known specifities of these hemagglutinins [2] it is possible that this glycoprotein may contain an α-D-glucopyranosyl and/or an α-D-mannopyranosyl residue, a 2-acetamido-2-deoxy-β-D-glucopyranosyl and a non-reducing terminal 2-acetamido-2-deoxy-β-D-galactopyranosyl residue in the

Table 1. Composition of glycoproteins separated on a column of Sepharose 6B

Components	Fraction A	Fraction B
*Amino acids**		
Asp	97	140
Thr	67	95
Ser	119	147
Glu	121	149
Pro	45	65
Gly	155	158
Ala	89	82
Cys 1/2	16	
Val	59	38
Ile	41	20
Leu	70	31
Tyr	11	9
Phe	32	16
Lys	37	25
His	22	9
Arg	19	16
Carbohydrates†		
L-Fucose	1.0	1.0
D-Galactose	13.0	24.5
D-Glucose	4.5	7.0
D-Mannose	1.5	5.0
N-Acetylglucosamine	1.5	2.5
N-Acetylgalactosamine	0.2	0.2

* Residues per 1000 residues.
† Molar ratio relative to L-fucose.

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Table 2. Composition of glycoproteins separated on a column of Sepharose 2B

Components	Fraction B_1	Fraction B_2
*Amino acids**		
Asp	60	123
Thr	66	77
Ser	191	193
Glu	73	91
Pro	56	61
Gly	265	173
Ala	101	91
Val	22	41
Ile	43	35
Leu	115	40
Tyr	2	7
Phe	—	12
Lys	—	12
His	2	22
Arg	4	22
Carbohydrates†		
L-Fucose	1.0	1.0
D-Galactose	13.0	22.0
D-Glucose	5.0	6.0
D-Mannose	2.0	4.0
N-Acetylglucosamine	1.0	1.8
N-Acetylgalactosamine	0.1	0.2

* Residues per 1000 residues.
† Molar ratio relative to L-fucose.

structure, but that it does not have a β-D-galactopyranosyl residue as a terminal sugar.

These results are consistent with the compositional data (Table 2). Inhibitory activity against hemagglutination with concanavalin A is in agreement with the large proportion of mannose and glucose present. The weak inhibition activity against *R. communis* hemagglutinin, despite the larger proportion of galactose in B_2 raises two possibilities. Since galactose represents over 65% of the total carbohydrate residues (Table 2), a high proportion of this component must reside in a terminal position in an active α-configuration or is attached β in an inactive sequence, possibly Gal→Man, which to our knowledge has not been investigated. Both Gal→GlcNAc and Gal→Glc linked β 1,4 would be expected to be active.

The composition of this glycoprotein is unlike those of other glycoproteins of fungal origin, both in secretion and in the cell envelope [4], as it not only contains L-fucose and *N*-acetylhexosamines but also an unusual proportion of D-glucose, D-galactose and D-mannose. In addition, a very small amount of a sugar was detected by GLC with R_t similar to that of L-rhamnose. The function of this glycoprotein in *Ustilago tritici* cells is unclear although it may have some immunogenic significance.

EXPERIMENTAL

Ustilago tritici was collected from diseased plants (*Triticum vulgaris*) and the glycoproteins extracted by dispersing the spores (1 g; previously extracted with Et_2O and EtOH) in H_2O (100 ml) at 4° for 24 hr. The dispersion was centrifuged and the supernatant was lyophilized (10 mg). Gel chromatography was carried out on columns of Sepharose 6B and 2B using 5 mM Tris·HCl (pH 7.3) containing 50 mM NaCl and 50 mM NaPi (pH 7), respectively. Ion-exchange chromatography on DEAE-cellulose was performed with a gradient of 0.1–1 M NaPi (pH 6.8), followed by elution with 0.2 M LiCl. The eluates were examined for carbohydrates using the PhOH–H_2SO_4 reagent [5] and the presence of protein was detected by A at 278 nm. Gel electrophoresis was carried out by the modified procedure of ref. [6]. The gels were prepared by mixing polyacrylamide (2%) and agarose (0.75%). The sample soln, running gel and electrode buffer contained 0.1% SDS. Carbohydrate-containing polymers were detected with the periodate-Schiff reagent and proteins were stained with Coomassie blue. Sugar residues were identified and estimated by GLC [7]. Amino acid analyses were performed on glycoprotein samples, after hydrolysis with 6 M HCl for 20 hr at 105° on an amino acid analyser. *Canavalia ensiformis* hemagglutinin [8], *Ricinus communis* hemagglutinin [9], *Triticum vulgaris* hemagglutinin [10] and *Solanum tuberosum* hemagglutinin were purified by affinity chromatography. *Glycine max* hemagglutinin was used as a crude extract. The titration and inhibition assays were performed with human erythrocytes according to the method of ref. [3]. The cells used for inhibition assays on Concanavalin A and *Glycine max* hemagglutinin were trypsin-treated [11].

Acknowledgements—The authors wish to thank the plant pathologist of the Ayub Agricultural Research Institute for a gift of *Ustilago tritici* spores and Dr. John F. Codington for helpful discussions.

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Carbohydrate Research, 64 (1978) 43–56

SYNTHESIS OF METHYL (OR PROPYL) 2-ACETAMIDO-2-DEOXY-α-D-GLUCOPYRANOSIDE 6-(α-D-GLUCOPYRANOSYL PHOSPHATE) AND DERIVATIVES FOR THE STUDY OF THE PHOSPHORIC ESTER LINKAGE IN THE *Micrococcus lysodeikticus* CELL-WALL*

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(Received August 22nd, 1977; accepted for publication, October 27th, 1977)

ABSTRACT

Methyl 2-acetamido-3-*O*-allyl-2-deoxy-4-*O*-methyl-α-D-glucopyranoside, methyl 2-acetamido-2-deoxy-4-*O*-methyl-α-D-glucopyranoside, and methyl 2-acetamido-3,4-di-*O*-allyl-2-deoxy-α-D-glucopyranoside, prepared from methyl 2-acetamido-2-deoxy-α-D-glucopyranoside, were coupled with 2,3,4,6-tetra-*O*-acetyl-α-D-glucopyranosyl phosphate (**13**), to give the phosphoric esters methyl 2-acetamido-3-*O*-allyl-2-deoxy-4-*O*-methyl-α-D-glucopyranoside 6-(2,3,4,6-tetra-*O*-acetyl-α-D-glucopyranosyl phosphate) (**16**), methyl 2-acetamido-2-deoxy-4-*O*-methyl-α-D-glucopyranoside 6-(2,3,4,6-tetra-*O*-acetyl-α-D-glucopyranosyl phosphate) (**23**), and methyl 2-acetamido-3,4-di-*O*-allyl-2-deoxy-α-D-glucopyranoside 6-(2,3,4,6-tetra-*O*-acetyl-α-D-glucopyranosyl phosphate) (**17**). Compound **13** was prepared from penta-*O*-acetyl-β-D-glucopyranose by the phosphoric acid procedure, or by acetylation of α-D-glucopyranosyl phosphate. Removal of the allyl groups from **16** and **17** gave **23** and methyl 2-acetamido-2-deoxy-α-D-glucopyranoside 6-(2,3,4,6-tetra-*O*-acetyl-α-D-glucopyranosyl phosphate) (**19**), respectively. *O*-Deacetylation of **23** gave methyl 2-acetamido-2-deoxy-4-*O*-methyl-α-D-glucopyranoside 6-(α-D-glucopyranosyl phosphate) (**26**) and *O*-deacetylation of **19** gave methyl 2-acetamido-2-deoxy-α-D-glucopyranoside 6-(α-D-glucopyranosyl phosphate) (**24**). Propyl 2-acetamido-2-deoxy-α-D-glucopyranoside 6-(α-D-glucopyranosyl phosphate) (**25**) was prepared by coupling **13** with allyl 2-acetamido-3,4-di-*O*-benzyl-2-deoxy-α-D-glucopyranoside, followed by catalytic hydrogenation of the product to give the propyl glycoside, which was then *O*-deacetylated. Compounds **24**, **25**, and **26** are being employed in structural studies of the *Micrococcus lysodeikticus* cell-wall.

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*Amino Sugars CXVI. This is publication No. 753 of the Robert W. Lovett Memorial Group for the Study of Diseases Causing Deformities, Harvard Medical School and Massachusetts General Hospital. This investigation was supported by research grants from the National Institute of Allergy and Immunology (AI-06692), National Institutes of Health, U.S. Public Health Service. A preliminary communication has been presented (see ref. 1).

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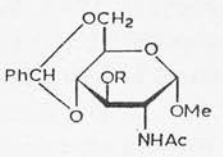

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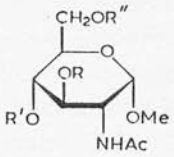
2 R = All
All = $CH_2CH{=}CH_2$



3 R = All, R′ = R″ = H
4 R = All, R′ = H, R″ = Bz
5 R = All, R′ = Me, R″ = Bz
6 R = All, R′ = Me, R″ = H
7 R = R″ = H, R′ = Me
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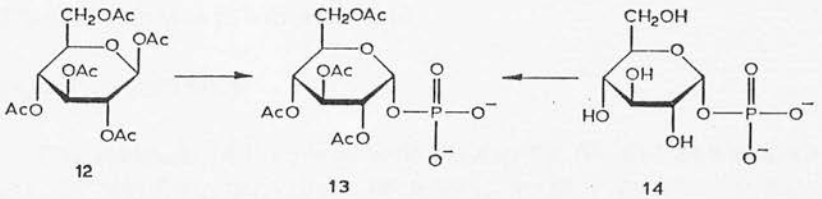

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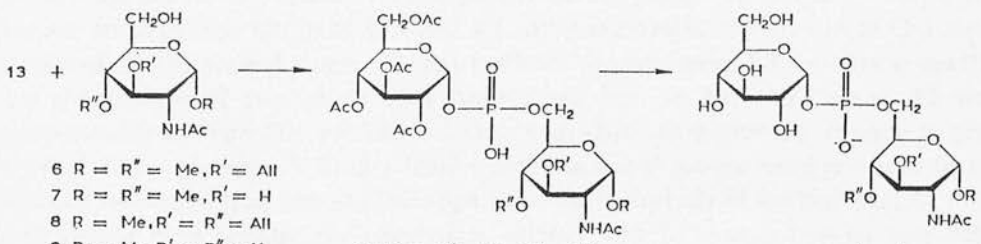

6 R = R″ = Me, R′ = All
7 R = R″ = Me, R′ = H
8 R = Me, R′ = R″ = All
9 R = Me, R′ = R″ = H
15 R′ = All, R′ = R″ = Bzl

Pr = $CH_2CH_2CH_3$
Pre = $CH{=}CH{-}CH_3$

16 R = R″ = Me, R′ = All
17 R = Me, R′ = R″ = All
18 R = Me, R′ = R″ = Pre
19 R = Me, R′ = R″ = H
20 R = R″ = Me, R′ = Pre
21 R = All, R′ = R″ = Bzl
22 R = Pr, R′ = R″ = H
23 R = R″ = Me, R′ = H

24 R = Me, R′ = R″ = H
25 R = Pr, R′ = R″ = H
26 R = R″ = Me, R′ = H

INTRODUCTION

Structural studies of the *Micrococcus lysodeikticus* cell-wall indicate the presence of 2-acetamido-2-deoxy-D-glucose 6-phosphate residues[1,2], and suggest that these residues perform a function similar to that of the *N*-acetylmuramic acid 6-phosphate residues generally present in bacterial cell-walls, including those of *Micrococcus lysodeikticus*, namely, that of linking the external, antigenic polysaccharide to the peptidoglycan chains[3]. In the *Micrococcus lysodeikticus* cell-wall, the antigenic polysaccharide consists of polysaccharide chains of D-glucose and 2-acetamido-2-deoxy-D-mannuronic acid residues and it is likely that the terminal D-glucose residue of this chain is linked to a muramic acid 6-phosphate or 2-acetamido-2-deoxy-D-glucose 6-phosphate residue *via* a phosphoric ester group[4]. Evidence for this type of structure

arose mainly from studies on acid- and alkali-catalyzed hydrolysis. In order to rationalize the results of those studies, it was necessary to investigate the behavior of model compounds. This paper reports the synthesis of three suitable compounds, propyl 2-acetamido-2-deoxy-α-D-glucopyranoside 6-(α-D-glucopyranosyl phosphate) (**25**), methyl 2-acetamido-2-deoxy-α-D-glucopyranoside 6-(α-D-glucopyranosyl phosphate) (**24**), and methyl 2-acetamido-2-deoxy-4-*O*-methyl-α-D-glucopyranoside 6-(α-D-glucopyranosyl phosphate) (**26**). Compounds **24** and **25** are models for a phosphoric ester linkage between C-1 of a D-glucose residue and C-6 of a 2-acetamido-2-deoxy-D-glucose residue linked in the peptidoglycan chain at C-1 only, whereas **26** is a model for the analogous situation in which the 2-acetamido-2-deoxy-D-glucose residue is linked at both C-1 and C-4. In related work[1], other compounds have been prepared that are models for a phosphoric ester linkage between a D-glucose residue and C-6 of a residue of muramic acid.

RESULTS AND DISCUSSION

The synthesis of the phosphoric diesters **24**, **25**, and **26** was achieved in three stages. In the first, derivatives of methyl or allyl 2-acetamido-2-deoxy-D-glucopyranoside (*a*) having readily removable protecting groups at O-3 and O-4, or (*b*) having an *O*-methyl group at O-4 and an easily removable substituent at O-3 were prepared. In the second stage, efficient methods for preparing 2,3,4,6-tetra-*O*-acetyl-α-D-glucopyranosyl phosphate (**13**) were developed. In the third stage, **13** was coupled with the partially protected 2-acetamido-2-deoxy-D-glucopyranoside to give a per-*O*-acetyl phosphoric diester from which the acetyl groups were removed by an alkaline treatment that was mild enough to avoid hydrolysis of the product. At first, allyl groups were selected for protection, as they may be removed under very mild conditions[5,6] unlikely to hydrolyze a phosphoric diester linkage. Methyl 2-acetamido-4,6-*O*-benzylidene-2-deoxy-α-D-glucopyranoside[7] (**1**) was allylated at O-3 to give **2**, from which the benzylidene group was removed by mild, acid hydrolysis to give methyl 2-acetamido-3-*O*-allyl-2-deoxy-α-D-glucopyranoside (**3**) in good yield. Conversion of **3** into methyl 2-acetamido-3-*O*-allyl-2-deoxy-4-*O*-methyl-α-D-glucopyranoside (**6**) was achieved by selective benzoylation at O-6 to give **4**, methylation at O-4 with methyl iodide and silver oxide, and *O*-debenzoylation of the resulting **5**. Compound **6** was employed (*a*) as starting material for the synthesis of the phosphoric diester **16** and (*b*) for conversion, by removal of the allyl substituent, into methyl 2-acetamido-2-deoxy-4-*O*-methyl-α-D-glucopyranoside[8] (**7**), in order to determine whether or not such a compound as **7**, having free hydroxyl groups at C-3 and C-6, could be used for phosphoric diester synthesis, with the expectation that the reaction would take place almost exclusively at C-6. The allyl group in **6** was removed very readily in the conventional way, by isomerization to the 1-propenyl derivative with tris(triphenylphosphine)rhodium chloride[5]; this result contrasts with the poor yields and side reactions unexpectedly encountered in similar treatment of the phosphoric diesters, especially when two vicinal allyl groups were involved, as with **17** (see later).

For the synthesis of methyl 2-acetamido-3,4-di-*O*-allyl-2-deoxy-α-D-glucopyranoside (**8**), methyl 2-acetamido-2-deoxy-α-D-glucopyranoside[9] (**9**) was converted into the known 6-trityl ether[10] **10**, which was allylated with allyl bromide and sodium hydroxide to give **11**. Finally, *O*-detritylation by mild, acid treatment gave **8**.

2,3,4,6-Tetra-*O*-acetyl-α-D-glucopyranosyl phosphate (**13**) was prepared by two different methods. The first is a modification of the phosphoric acid procedure[11], similar in some respects to that previously developed for the corresponding derivative of D-galactose[12]. Fusion under vacuum of a mixture of 1,2,3,4,6-penta-*O*-acetyl-β-D-glucopyranose[13] with crystalline phosphoric acid, followed by cautious neutralization with ammonium hydroxide, gave a syrup that contained **13**. Unlike 2,3,4,6-tetra-*O*-acetyl-α-D-galactopyranosyl phosphate[12], compound **13** did not crystallize and had to be purified by preparative layer chromatography (p.l.c.) before it was useful for phosphoric diester synthesis. In the alternative approach, α-D-glucopyranosyl phosphate (**14**) was acetylated with acetic anhydride and, as the base, either tetraethylammonium acetate[14] or pyridine. In the latter case, it is known[14] that a cyclic 1,2-phosphate will be formed in a competing reaction, but because the hydroxyl groups of **14** were acetylated very quickly, it was found possible to stop the reaction before this cyclic by-product was formed to any great extent. P.l.c. gave **13** in good yield.

For the phosphoric diester synthesis, equal amounts of **13**, in the pyridinium form, and one of the derivatives of 2-acetamido-2-deoxy-D-glucose having OH-6 unprotected (**6** or **8**) was thoroughly dried and then treated with a solution of 2,4,6-triisopropylbenzenesulfonyl chloride[15] in anhydrous pyridine. Total exclusion of moisture was critical for the success of the coupling reaction. Earlier, this was achieved by repeated additions and evaporations of dry pyridine[15] or toluene[16], prior to the addition of the coupling reagent. However, in this study, drying the starting compounds over phosphorus pentaoxide in a vacuum desiccator was found to be sufficient. The coupling reaction usually took place during two days at room temperature, after which time the product was isolated by p.l.c., **6** and **8** giving **16** and **17**, respectively. These fully protected phosphoric diesters were solids that showed no crystalline form under a microscope, but did have definite melting points, and were characterized by optical rotation, i.r. spectrum, and elementary analysis.

In the removal of protecting groups, *O*-deallylation was performed conventionally by isomerization of the allyl to a 1-propenyl group, followed by hydrolysis. It was not possible to use potassium *tert*-butoxide[17] for this isomerization, owing to the presence of ester groups, and so tris(triphenylphosphine)rhodium chloride[5] was the reagent of choice. This had worked well in the preparation of **7** from **6** (see earlier) but, with **17**, the reaction was very sluggish, requiring multiple additions of catalyst, and a prolonged reaction-time. When the isomerized material containing **18** was subjected to hydrolysis with mercuric chloride[6], t.l.c. showed the presence, in addition to **19**, of two other compounds lacking unsaturated groups. This result arose through partial hydrogenation of the allyl or 1-propenyl groups to give propyl groups (resistant to hydrolysis), a side reaction previously reported[18]. When

16, which has only one allyl group, was similarly treated, the isomerization to **20** and subsequent hydrolysis, were more facile. Apparently, the difficulty in the isomerization of the allyl groups in **17** is associated with both the presence of a phosphoric ester and the presence of vicinal substituents that are involved in the reaction.

In an attempt to overcome this problem, direct coupling of methyl 2-acetamido-2-deoxy-α-D-glucopyranoside (**9**) with **13** was tried, in the hope that reaction would occur preferentially at O-6. Unfortunately, analysis of the product indicated the presence of three phosphoric diesters, showing that reaction at O-3 and O-4 had also occurred. Therefore, *O*-deacetylation was performed by brief treatment with a dilute solution of sodium methoxide in methanol, after which, separation of **24** was readily achieved by p.l.c.

In another approach to the synthesis of a phosphoric diester having unprotected hydroxyl groups at C-3 and C-4, allyl 2-acetamido-3,4-di-*O*-benzyl-2-deoxy-α-D-glucopyranoside[18] (**15**) was coupled with **13**. The resulting compound was hydrogenated to remove the benzyl groups, and the allyl group was concomitantly converted into a propyl group. The resulting compound (**22**) was not pure (t.l.c.) and *O*-deacetylation was performed without prior purification of **22**. The product (**25**) was shown to be pure by both elementary analysis and chromatographic methods.

As mentioned earlier, the 4-methyl ether **16** was more readily *O*-deallylated than **17**; it gave **23**, which was *O*-deacetylated by the same mild method used for **19** and **22**, to yield the deprotected phosphoric diester **26**. In order to determine whether **23** (and hence **26**) could be obtained by a direct coupling-reaction, not employing an intermediate having a protecting group at O-3, methyl 2-acetamido-2-deoxy-4-*O*-methyl-α-D-glucopyranoside (**7**) was coupled with **13**, and the product was shown to contain only one phosphoric diester; it was obtained in good yield and corresponded on chromatograms to **23**. Thus, the 3-OH group in **7**, unlike that in **9**, is sterically hindered enough to prevent reaction with the glycosyl phosphate **13** under the conditions employed in this study.

The synthetic phosphoric diesters **24** and **26** were examined by field-desorption mass spectrometry[19]. In each case, the spectrum showed a molecular ion, together with fragmentation ions arising from D-glucose and 2-acetamido-2-deoxy-D-glucose 6-phosphate residues (from **24**) or the 4-methyl ether of the latter (from **26**), thus providing unequivocal proof of the structures assigned to **24** and **26**. These structures were also confirmed by treatment of the compounds with cation-exchange resin[20] (H^+ form) at 65°, the products identified being D-glucose and 2-acetamido-2-deoxy-D-glucose 6-phosphate (from **24**), and 2-acetamido-2-deoxy-4-*O*-methyl-D-glucose 6-phosphate (from **26**).

EXPERIMENTAL

General methods. — Melting points were determined with a Mettler FP-2 apparatus and correspond to "corrected melting points". Optical rotations were determined for solutions in 1-dm semimicro tubes with a Perkin–Elmer model 141

polarimeter. I.r. spectra were recorded with a Perkin–Elmer model 237 spectrophotometer. The cation-exchange resin used was AG-50W X8 (200–400 mesh) (BioRad Lab., Richmond, CA, 94804, unless stated otherwise) and in all instances the amount of resin used was in at least a two-fold excess over the quantity necessary to effect complete ion-exchange. All proportions of solvents are v/v. Evaporations were conducted *in vacuo*, with the bath temperature kept below 30°. Microanalyses were performed by Dr. W. Manser, CH-8704 Herrliberg (Switzerland).

Chromatographic separations. — T.l.c. was performed on precoated plates of Silica gel G (E. Merck A.-G., Darmstadt, Germany). The plates supplied (20 × 20 cm) were cut to a length of 6 cm and used without pretreatment. Preparative-layer chromatography was performed on precoated plates (2-mm or 0.5-mm thickness) of Silica Gel G (Merck). Unless otherwise stated, the spray reagent was 1:1:18 (v/v) anisaldehyde–sulfuric acid–ethanol[21], and the plates were heated to 125°. Unsaturation was detected with a 1% aqueous solution of potassium permanganate in 2% sodium carbonate. The spray reagent of Dittmer and Lester[22] was used to detect phosphate groups. Solvent systems used for chromatography were: *A*, 60:25:4 chloroform–methanol–water; *B*, 60:35:6 chloroform–methanol–water; and *C*, 10:10:3 chloroform–methanol–water. When plates were eluted more than once with the same solvent, they were dried in a stream of air for at least 30 min between each elution. The R_F values were calculated from measurement of the distance from the origin of the chromatogram to the point of maximum intensity of the spot after development. Column chromatography was performed on silica gel (70–325 mesh; Merck), used without pretreatment. The proportion of weight of substance to weight of silica gel was 1:60 to 1:90.

Methyl 2-acetamido-3-O-allyl-4,6-O-benzylidene-2-deoxy-α-D-glucopyranoside (**2**). — A suspension of methyl 2-acetamido-4,6-*O*-benzylidene-α-D-glucopyranoside[7] (**1**, 300 mg) in 1:1 benzene–tetrahydrofuran (100 ml) was treated with powdered sodium hydroxide (1.5 g) and allyl bromide (85 µl) for 3 h at boiling temperature under reflux, and for 12 h at room temperature with vigorous stirring. The mixture was filtered, and the insoluble material washed with hot, 1:1 benzene–chloroform (30 ml). The combined filtrate and washings were evaporated, the residue was dissolved in chloroform, and the solution was washed with water, dried (sodium sulfate), and evaporated. The residue was chromatographed on a column of silica gel with 19:1 chloroform–ethanol to give a syrup that crystallized from chloroform–ether as needles (267 mg, 79%), changing into long needles at 242–247°, m.p. 284–285° (dec.), $[\alpha]_D^{20}$ +30° (*c* 0.6, chloroform); ν_{max}^{KBr} 3295 (NH), 1645 (allyl), 1630 (Amide I), and 1550 cm^{-1} (Amide II); t.l.c. (19:1 chloroform–ethanol) R_F 0.37.

Anal. Calc. for $C_{19}H_{25}NO_6$: C, 62.80; H, 6.93; N, 3.85; O, 26.42. Found: C, 62.67; H, 6.91; N, 3.75; O, 26.37.

Methyl 2-acetamido-3-O-allyl-2-deoxy-α-D-glucopyranoside (**3**). — A suspension of **2** (240 mg) in 60% acetic acid (15 ml) was kept for 1 h at 80°. The resultant solution was cooled, concentrated, and toluene was repeatedly added to it and distilled off. The residue was chromatographed on a column of silica gel with 4:1 chloroform–

ethanol to give a syrup (162 mg, 89%) that crystallized as needles from chloroform (147 mg, 81%), m.p. 178–179°, $[\alpha]_D^{20}$ +117° (*c* 0.82, methanol); ν_{max}^{KBr} 3400–3275 (broad, OH, NH), 1655–1625 (allyl, Amide I), and 1575 cm^{-1} (Amide II); t.l.c. (9:1 chloroform–ethanol) R_F 0.12.

Anal. Calc. for $C_{12}H_{21}NO_6$: C, 52.35; H, 7.69; N, 5.09; O, 34.87. Found: C, 52.32; H, 7.64; N, 5.00; O, 34.81.

Methyl 2-acetamido-3-O-allyl-6-O-benzoyl-2-deoxy-α-D-glucopyranoside (**4**). — A solution of **3** (127 mg) in dry pyridine (15 ml) was cooled to −60° and treated with freshly distilled benzoyl chloride (54 µl) for 6 h at −20°, and for 16 h at −10°. The solution was diluted with dichloromethane (20 ml), and successively washed with ice-cold, saturated solutions of sodium hydrogensulfate, sodium hydrogencarbonate, and ice-cold water, dried (sodium sulfate), and evaporated to give a syrup that was chromatographed on a column of silica gel with 19:1 chloroform–ethanol. The syrupy product crystallized from chloroform–heptane as microneedles (141 mg, 80%), m.p. 216–218°, $[\alpha]_D^{20}$ +100° (*c* 0.49, chloroform); ν_{max}^{KBr} 3275 (NH), 1665 (allyl), 1645 (Amide I), and 1540 cm^{-1} (Amide II); t.l.c. (14:1 chloroform–ethanol) R_F 0.35.

Anal. Calc. for $C_{19}H_{25}NO_7$: C, 60.15; H, 6.64; N, 3.69; O, 29.52. Found: C, 60.07; H, 6.73; N, 3.81; O, 29.61.

Methyl 2-acetamido-3-O-allyl-6-O-benzoyl-2-deoxy-4-O-methyl-α-D-glucopyranoside (**5**). — A solution of **4** (120 mg) in dry tetrahydrofuran (6 ml) was mixed with iodomethane (3 ml) and silver oxide (200 mg), and the mixture was boiled for 2 h under reflux. After a further addition of silver oxide (100 mg), the mixture was boiled for 2 h under reflux, and then stirred for 6 h at 22°. The mixture was filtered, the residue was washed with warm chloroform, and the combined filtrate and washings were evaporated. The residue was chromatographed on a column of silica gel with 24:1 dichloromethane–ethanol, to give a product that crystallized from ether–pentane as prisms (82 mg, 66%), m.p. 189–192°, $[\alpha]_D^{20}$ +95° (*c* 0.76, chloroform); ν_{max}^{KBr} 3290 (NH), 1670 (allyl), 1645 (Amide I), 1555 (Amide II), 1490, and 1455 cm^{-1} (Ar); t.l.c. (24:1 chloroform–ethanol) R_F 0.44.

Anal. Calc. for $C_{20}H_{27}NO_7$: C, 61.06; H, 6.92; N, 3.56; O, 28.47. Found: C, 60.98; H, 6.96; N, 3.52; O, 28.29.

Methyl 2-acetamido-3-O-allyl-2-deoxy-4-O-methyl-α-D-glucopyranoside (**6**). — A solution of **5** (70 mg) in dry methanol (10 ml) was treated with 0.1M methanolic sodium methoxide (0.2 ml) for 20 h at 4°, and then diluted with methanol (5 ml), and de-ionized with Rexyn 300 (H^+, OH^-) ion-exchange resin (3 ml, Fisher Scientific Co., Fair Lawn, N.J. 07410). The solution was evaporated, and the residue was chromatographed on a column of silica gel with 14:1 chloroform–ethanol to give 46 mg (89%) of material that crystallized from ethanol–ether as clusters of needles, m.p. 198–199°, $[\alpha]_D^{20}$ +25° (*c* 0.16, chloroform); ν_{max}^{KBr} 3290 (NH), 1650 (Amide I), 1640 (allyl), and 1555 cm^{-1} (Amide II); t.l.c. (9:1 chloroform–ethanol) R_F 0.27.

Anal. Calc. for $C_{13}H_{23}NO_6$: C, 53.97; H, 8.01; N, 4.84. Found: C, 53.63; H, 8.08; N, 4.56.

Methyl 2-acetamido-2-deoxy-4-O-methyl-α-D-glucopyranoside (**7**). — A solution

of **6** (20 mg) in 90% ethanol (4 ml) was treated with tris(triphenylphosphine)rhodium chloride (5 mg, Ventron, Danvers, MA 01923) and 1,4-diazabicyclo[2.2.2]octane (1.6 mg). The mixture was boiled for 3 h under reflux. The resulting solution was cooled and evaporated, and the residue was dispersed in water and extracted with ether. The extract was dried (magnesium sulfate) and evaporated. A solution of the residue in methanol (6 ml) was treated with cation-exchange resin (H^+, 100–200 mesh; 2 ml) for 12 h at 37°. The suspension was filtered and the filtrate evaporated. The residue was chromatographed on a column of silica gel with 4:1 chloroform–ethanol to give a material (14 mg, 81%) that crystallized from ethanol–ether as needles, m.p. 228–229°, mixed m.p. 225–226° (lit.[8] m.p. 232–233°).

*Methyl 2-acetamido-3,4-di-*O*-allyl-6-*O*-trityl-α-*D*-glucopyranoside* (**11**). — A solution of methyl 2-acetamido-6-*O*-trityl-α-D-glucopyranoside[10] (**10**,145 mg) in dry tetrahydrofuran (4 ml) and benzene (10 ml) was treated with powdered sodium hydroxide (1 g) and allyl bromide (54 µl), and the mixture was boiled for 4 h under reflux. To the cooled mixture, sodium hydroxide (0.40 g) was added. The mixture was vigorously stirred for 12 h at room temperature, and then diluted with benzene (10 ml). The solid was filtered off and washed with warm 9:1 chloroform–ethanol (10 ml). The filtrate and washings were combined and evaporated. The residue was dissolved in chloroform, and the solution was washed with water (3 × 6 ml), dried (sodium sulfate), and evaporated. The syrupy residue was chromatographed on a column of silica gel, and elution with 24:1 dichloromethane–ethanol gave a material that crystallized from 2-isopropoxypropane–hexane to give microcrystals (130 mg, 74%), m.p. 167–169°, $[\alpha]_D^{20}$ + 77° (*c* 0.39, chloroform); ν_{max}^{KBr} 3290 (NH), 1670–1635 (allyl, Amide I), 1580 (Ar), 1540 (Amide II), and 1455 cm^{-1} (Ar); t.l.c. (19:1 chloroform–ethanol) R_F 0.75.

Anal. Calc. for $C_{34}H_{39}NO_6$: C, 73.23; H, 7.05; N, 2.51; O, 17.21. Found: C, 73.26; H, 6.97; N, 2.40; O, 17.23.

*Methyl 2-acetamido-3,4-di-*O*-allyl-2-deoxy-α-*D*-glucopyranoside* (**8**). — A solution of **11** (110 mg) in acetic acid (4 ml) was heated on a water bath at 80°. The hot solution was diluted with water (2 ml), and heating was continued for 1 h. The solution was diluted with cold water (200 ml) and lyophilized. The residue was chromatographed on a column of silica gel in 14:1 dichloromethane–ethanol to give a syrup that crystallized from 2-isopropoxypropane, affording **8** (58 mg, 80%) as needles, m.p. 186–187°, $[\alpha]_D^{20}$ +105° (*c* 0.98, methanol); ν_{max}^{KBr} 3290 (NH), 1665–1630 (allyl, Amide I), and 1555 cm^{-1} (Amide II); t.l.c. (9:1 chloroform–ethanol) R_F 0.28.

Anal. Calc. for $C_{15}H_{25}NO_6$: C, 57.13; H, 7.99; N, 4.44; O, 30.44. Found: C, 57.06; H, 7.90; N, 4.35; O, 30.55.

*2,3,4,6-Tetra-*O*-acetyl-α-*D*-glucopyranosyl phosphate* (**13**). — (*a*) *From 1,2,3,-4,6-penta-*O*-acetyl-β-*D*-glucopyranose* (**12**). To crystalline phosphoric acid (1.2 g, Tridom Chemical Inc., Hauppauge, N.Y. 11787), dried *in vacuo* over magnesium perchlorate for 48 h at room temperature and then fused by heating at 65° (oil bath), was added **12** (ref. 13) (1.0 g). The mixture was stirred *in vacuo* (oil pump equipped with a carbon dioxide–acetone trap) for 2 h at 65°. A solution of the product in dry

tetrahydrofuran was cooled to $-10°$, and treated with 15M ammonium hydroxide (~2 ml) to bring the pH to ~6. The precipitated salts were filtered off (Celite). Evaporation of the filtrate gave crude **13** (1.0 g, ammonium$^+$), which was purified by p.l.c. The product, in methanol (2 ml), was applied to ten 20 × 20-cm plates (2 mm thick) which, after drying, were eluted twice with solvent *B*. After detection of **13** with the phosphate-specific spray reagent (it was the major phosphate-containing band), the appropriate zones of silica gel were removed from each plate, combined and ground to a powder, and then stirred overnight with solvent *C*. Filtration (Celite) and evaporation gave a residue that was extracted with 2:1 chloroform–methanol. The resulting solution was filtered and evaporated to give **13** (0.37 g, 27%, ammonium$^+$) showing a single spot in t.l.c. (solvent *B*).

For synthetic purposes, **13** (0.37 g) was converted into the pyridinium$^+$ salt by dissolution in water and slow passage through a column of cation-exchange resin (pyridinium$^+$). The column was washed with 3 vol. of water and the combined eluates were evaporated to dryness. Portions of toluene (2 ml) were added and evaporated off (three times), and then the amorphous **13** (0.37 g, pyridinium$^+$) was dissolved in dry dichloromethane (3.7 ml). Aliquots of this solution were employed for synthesis of phosphoric diesters.

For characterization, **13** (0.37 g, ammonium$^+$) was dissolved in water (5 ml) and converted into the potassium form by stirring with a large excess of cation-exchange resin (K^+) for 24 h at room temperature. The resin was filtered off and washed with water (10 ml), and the combined filtrates were evaporated to a solid. Crystallization from ether–methanol gave the dipotassium salt of **13** (0.21 g, 50%), m.p. 136–137°, $[\alpha]_D^{20}$ +110° (*c* 0.70, 1:1 methanol–water).

Anal. Calc. for $C_{14}H_{19}K_2O_{13}P$: C, 33.33; H, 3.80. Found: C, 33.34; H, 4.09.

(*b*) *From α-D-glucopyranosyl dipotassium phosphate.* Compound **14** (1.0 g, Sigma Chemical Company, St. Louis, MO 63118) was converted into the pyridinium$^+$ form by dissolution in water (10 ml) and passage through a column of cation-exchange resin (pyridinium$^+$), as just described for **13**. After washing of the column, evaporation of the combined eluates, and repeated addition and evaporation of toluene, α-D-glucopyranosyl pyridinium phosphate (**14**) was obtained as a syrup.

(*i*). Acetylation was performed by treatment with tetraethylammonium acetate tetrahydrate (10 g, Aldrich Chemical Co., Inc., Milwaukee, WI 53233). Water was removed by repeated additions and evaporations of pyridine (10 ml), and then of toluene (10 ml), and the resulting gum was treated with acetic anhydride (20 ml). The mixture was kept overnight at room temperature, treated with pyridine (10 ml) and water (10 ml), and kept for a further 2 h at room temperature. Evaporation, followed by two additions and evaporations of toluene (10 ml), gave a residue containing inorganic material. This was removed by dissolution of the residue in chloroform and treatment with ether to the point of turbidity. After a few min, the crystalline solid (inorganic) was filtered off, and evaporation of the filtrate gave **13** (0.6 g, 61%, pyridinium$^+$), showing one major spot in t.l.c. (solvent *B*), corresponding to the product obtained by method (*a*).

(*ii*). Alternatively, acetylation was performed by treatment with acetic anhydride (2.5 ml) and pyridine (5 ml). The mixture was kept for 3 h at room temperature, whereupon t.l.c. (solvent *B*) showed a major product (R_F 0.38) that corresponded to 2,3,4,6-tetra-*O*-acetyl-α-D-glucopyranosyl phosphate (**13**) as prepared by method (*a*), plus several other compounds having higher R_F values (presumably including an acetylated, cyclic 1,2-phosphate). Water was added (until no more heat was evolved), and after evaporation, followed by two additions and evaporations of toluene (5 ml each), the product was purified by p.l.c. as described in (*a*) to give **13** (0.63 g, 64%, pyridinium$^+$), $[\alpha]_D^{20}$ +114° (*c* 2.2, dichloromethane).

Phosphoric diester synthesis. — A mixture of equal quantities (20–50 mg) of **13** (pyridinium$^+$) and the substituted derivative of methyl (or allyl) 2-acetamido-2-deoxy-α-D-glucopyranoside was kept *in vacuo* over phosphorus pentaoxide for 24–48 h at room temperature, and then treated with the appropriate volume of a solution of 2,4,6-triisopropylbenzenesulfonyl chloride (TPS, Aldrich) in dry pyridine, with rigorous exclusion of moisture. The reaction tube was stoppered tightly, and the reactants were thoroughly mixed with a Vortex mixer to give a clear solution, which was kept for 48 h at room temperature. The mixture was treated with methanol (1 ml) and kept overnight at room temperature, and then the solvents were evaporated (nitrogen) and residual pyridine was removed by two additions and subsequent evaporations of toluene. The residue was dissolved in methanol and examined (*a*) by t.l.c. with solvent *A* (detection with the anisaldehyde, potassium permanganate, and phosphate-specific spray reagents), and (*b*) by withdrawing a very small portion and treating the sample with an excess of 3% sodium methoxide in dry methanol to effect *O*-deacetylation, followed by t.l.c. When these steps showed that a good yield of the required compound had been formed in the condensation, the compound was isolated by p.l.c. of the crude product, solvent *A* or *B* being used for elution of the plates, and the potassium permanganate and phosphate-specific spray reagents for detection (for compounds containing allyl groups); otherwise, a 1-cm strip was cut from the plate and sprayed with the anisaldehyde reagent. The silica gel was removed from the plate, ground to a fine powder, and stirred overnight at room temperature with solvent *C*. After filtration (Celite), the resulting solution was evaporated to dryness, and the residue triturated with 2:1 chloroform–methanol to give a suspension that was filtered through sintered glass and evaporated (nitrogen) to yield the required phosphoric diester as an amorphous solid. For compounds **24–26**, methanol was employed for the second extraction, instead of 2:1 chloroform–methanol. As the amorphous compounds **24–26** were rather unstable, it was generally not possible to remove the solvents completely and obtain acceptable elementary analyses, and homogeneity was demonstrated only by t.l.c.

*Methyl 2-acetamido-3-*O*-allyl-2-deoxy-4-*O*-methyl-α-D-glucopyranoside 6-(2,3,4,6-tetra-*O*-acetyl-α-D-glucopyranosyl phosphate)* (**16**). — Compounds **13** (30 mg) and **6** (30 mg) were mixed and treated with TPS (36 mg) in pyridine (0.7 ml) by the general method just described. P.l.c. was performed as described for the preparations of **17** and **21** (see later paragraph), but t.l.c. of the product showed that it contained

a contaminant (not a carbohydrate) migrating just ahead of **16** (R_F 0.65, solvent *A*). Therefore, the chromatography was repeated on 2 thin-layer plates (0.25 mm thick), after which, processing, as described in the general methods, gave pure **16** (30 mg, 41 % based on **6**), m.p. 141–144°, $[\alpha]_D^{20}$ +70° (*c* 1.27, 1:1 chloroform–methanol); ν_{max}^{KBr} 3400 (OH, v. broad), 2970, 1750, 1665 (Amide I), 1560 (Amide II), 1375, 1230 (broad), 1095, 1050, 950, 875, and 675 cm^{-1}.

Anal. Calc. for $C_{27}H_{42}NO_{18}P$: C, 46.34; H, 6.05; N, 2.00. Found: C, 46.43; H, 6.14; N, 1.89.

Methyl 2-acetamido-2-deoxy-4-O-methyl-α-D-glucopyranoside 6-(2,3,4,6-tetra-O-acetyl-α-D-glucopyranosyl phosphate) (**23**). — (*a*) *From* **16**. A solution of **16** (25 mg) and 1,4-diazabicyclo[2.2.2]octane (5 mg) in 9:1 ethanol–water (2 ml) was stirred at 77° and treated with tris(triphenylphosphine)rhodium chloride (10 mg). The mixture was stirred for 2 h at 77°, at which time t.l.c. (solvent *A*) showed nearly complete conversion of **16** into the 1-propenyl derivative **20**. After the addition of more rhodium derivative (10 mg), the mixture was stirred a further 1 h at 77°, when t.l.c. showed no residual allyl group (anisaldehyde). The solvents were evaporated off, and the residue, consisting of unpurified **20**, was dissolved in 5:1 acetone–water (2 ml), and the resulting solution treated with mercuric chloride (25 mg). After 15 min at room temperature, t.l.c. (solvent *A*) showed the formation of **23** (R_F 0.40), and of a by-product (presumably a propyl derivative) that resisted hydrolysis, had the same R_F value as **16** and **20**, but gave a negative test for unsaturation (potassium permanganate reagent). After evaporation of the solvents, the residue was dissolved in 2:1 chloroform–methanol and **23** was purified by p.l.c. on one plate (0.5 mm thick, 20 × 20 cm) with solvent *B* and was detected with the anisaldehyde reagent. Processing by the general method gave **23** as the mercuric salt (11 mg, 40.5 %), m.p. 151–155°, $[\alpha]_D^{20}$ +97° (*c* 1.1, 1:1 chloroform–methanol); ν_{max}^{KBr} 3380 (OH,NH), 2955, 1750, 1655 (Amide I), 1545 (Amide II), 1375, 1230 (broad), 1125, 1040, and 960 cm^{-1}; t.l.c. (solvent *B*) R_F 0.50.

Anal. Calc. for $C_{24}H_{38}NO_{18}P \cdot 5Hg^{2+}$: C, 37.95; H, 5.04; N, 1.84. Found: C, 38.07; H, 4.96; N, 2.20.

(*b*) *From* **13** *and* **7**. Compound **13** (25 mg) and **7** (25 mg) were mixed and treated with TPS (30 mg) in dry pyridine (0.62 ml) by the general method. P.l.c. on one plate (2 mm thick, 20 × 20 cm), with solvent *B* for elution and the anisaldehyde reagent for detection, gave **23** (27 mg, 41 % based on **7**), pure according to t.l.c. (solvents *A* and *B*) and cochromatographing with the product obtained by method *a*.

Methyl 2-acetamido-2-deoxy-α-D-glucopyranoside 6-(2,3,4,6-tetra-O-acetyl-α-D-glucopyranosyl phosphate) (**19**). — A solution of **17** (50 mg) and 1,4-diazabicyclo-[2.2.2]octane (10 mg) in 9:1 ethanol–water (2 ml) was stirred at 78° and treated with tris(triphenylphosphine)rhodium chloride (10 mg). The mixture was stirred for 2 h at 78°, whereupon t.l.c. (solvent *A*) showed partial conversion of **17** into the 1-propenyl derivative **18**, having the same R_F value as **17** but lacking the color reaction with the anisaldehyde reagent that is characteristic of an allyl group[18]. In order to complete the conversion, it was necessary to perform four additional treatments with

tris(triphenylphosphine)rhodium chloride (10 mg), at 2-h intervals, while stirring at 78°. After evaporation of solvents, partial purification of **18** was achieved by p.l.c. on two plates, (0.5 mm thick, 20 × 20 cm), with solvent *B* for elution and the potassium permanganate spray for detection. Processing as described under "phosphoric diester synthesis" gave **18** (35 mg, 70%), t.l.c. (solvent *A*) R_F 0.62, showing the presence of some triphenylphosphine oxide as a pale-blue spot having a lower R_F value.

The 1-propenyl groups of **18** (35 mg) were hydrolyzed by dissolution in 5:1 acetone–water (3 ml), and treatment with mercuric chloride (50 mg). After 15 min at room temperature, t.l.c. (solvent *A*) showed formation of **19** (R_F 0.25) and of two by-products (R_F 0.62 and 0.50) that gave a negative test for unsaturation (potassium permanganate), and which presumably contained one or two propyl ether groups. Compound **19** was purified by p.l.c. on one plate (0.5 mm thick, 20 × 20 cm) with solvent *B* for elution and the anisaldehyde spray for detection. Processing, as described under "phosphoric diester synthesis", gave **19** as the mercuric salt (7 mg, 14%), m.p. 165–167°, $[\alpha]_D^{20}$ +69° (*c* 0.7, 1:1 chloroform–methanol); ν_{max}^{KBr} 3390 (OH, NH), 2950, 1750, 1660 (Amide I), 1545 (Amide II), 1375, 1230, 1145, 1110, 1045, and 955 cm^{-1}.

Anal. Calc. for $C_{23}H_{36}NO_{18}P \cdot 0.5Hg^{2+}$: C, 37.04; H, 4.86; N, 1.88. Found: C, 37.48; H, 5.15; N, 2.26.

Methyl 2-acetamido-2-deoxy-α-D-glucopyranoside 6-(α-D-glucopyranosyl phosphate) (**24**). — (*a*) *From* **19**. Compound **19** (1 mg) was treated with 1.5% sodium methoxide in dry methanol, and the mixture kept for 30 min at room temperature, after which time t.l.c. showed disappearance of **19** and formation of **24** (R_F 0.35, solvent *C*). The excess of base was removed by addition of cation-exchange resin (pyridinium$^+$), and the resin was removed by filtration and washed with 1:1 methanol–water. Evaporation gave amorphous **24**, which migrated more slowly in t.l.c. than the corresponding propyl glycoside **25** (R_F 0.52, solvent *C*). The sample of **24** prepared by this route was employed for chromatographic purposes only (see *b*).

(*b*) *From* **13** *and* **9**. A mixture of **13** (50 mg) and **9** (ref. 9) (50 mg) was treated with TPS (60 mg) as described in the general method for phosphoric diester synthesis. The product was isolated by p.l.c. on two plates, (2 mm thick, 20 × 20 cm) with solvent *B* and detection with the anisaldehyde reagent, to give an amorphous solid (35 mg), but t.l.c. (solvent *B*) showed that this was not homogenous. Therefore, *O*-deacetylation was performed (as described in *a*), after which, t.l.c. (solvent *C*) showed the formation of **24** (R_F 0.35, identified by comparison with the product from *a*) plus two other compounds, having a higher and a lower R_F value, respectively (presumably phosphoric diesters linked to O-3 and O-4 of the 2-acetamido-2-deoxy-D-glucose residue). Purification of **24** was achieved by p.l.c. on two plates (0.25 mm thick, 20 × 20 cm), with solvent *C* (two elutions) and detection with the anisaldehyde reagent. Processing by the general method previously described gave **24** (16 mg, 12% based on **9**), as an amorphous solid having no definite m.p. and containing residual solvent, $[\alpha]_D^{20}$ +87° (*c* 0.95, 1:1 methanol–water), $[\alpha]_D^{20}$ +121° (after correction

for solvent content); ν_{max}^{KBr} 3380 (OH, NH), 2940, 1650 (Amide I), 1550 (Amide II), 1375, 1230, 1145, 1090, 1035, 940, and 875 cm^{-1}.

Anal. Calc. for $C_{15}H_{28}NO_{14}P \cdot CHCl_3 \cdot 4H_2O$: C, 30.53; H, 5.57; N, 2.09. Found: C, 30.32; H, 5.45; N, 2.24.

Allyl 2-acetamido-3,4-di-O-benzyl-2-deoxy-α-D-glucopyranoside 6-(2,3,4,6-tetra-O-acetyl-α-D-glucopyranosyl phosphate) (**21**). — Compound **13** (50 mg) and **15** (ref. 18, 50 mg) were treated with TPS (60 mg) in pyridine (1.25 ml), and **21** was isolated as described in the general method for phosphoric diester synthesis, by p.l.c. on two plates (0.5-mm thick, 20 × 20 cm); yield 57 mg (55% based on **15**), m.p. 163–166°, $[\alpha]_D^{20}$ +88° (*c* 2.0, 2:1 chloroform–methanol), $[\alpha]_D^{20}$ +94° (after correction for residual solvent); ν_{max}^{KBr} 3400 (NH), 2950, (broad) 1750, 1650 (Amide I), 1550 (Amide II), 1500 (Ar), 1455, 1375, 1230 (broad), 1125, 1075, 1045, 950, 740, and 685 cm^{-1}; t.l.c. (solvent *A*) R_F 0.71.

Anal. Calc. for $C_{39}H_{50}NO_{18}P \cdot 0.5CHCl_3$: C, 52.06; H, 5.58; N, 1.54. Found: C, 51.98; H, 5.72; N, 1.71.

Propyl 2-acetamido-2-deoxy-α-D-glucopyranoside 6-(α-D-glucopyranosyl phosphate) (**25**). — Compound **21** (51 mg) was dissolved in methanol (4 ml) and hydrogenated at 1.5 atm over 10% palladium-on-charcoal (25 mg, Tridom). After 3 h, t.l.c. showed formation of a major product **22** (R_F 0.48, solvent *B*) together with a minor product having a slightly lower R_F value. The catalyst was filtered off and washed with methanol, and a small portion of the filtrate was treated with fresh catalyst and hydrogenated again for 3 h. As t.l.c. showed no change, a small portion of the original filtrate was *O*-deacetylated by treatment with an excess of sodium methoxide for 30 min at room temperature. T.l.c. now showed formation of pure **25** (R_F 0.52, solvent *C*), suggesting that the contaminant in **22** had resulted from partial *O*-deacetylation during hydrogenation. Therefore, the methanolic solution containing **22** was evaporated, and **22**, without purification, was *O*-deacetylated by treatment with 1.5% sodium methoxide in methanol for 30 min at room temperature. The mixture was treated with cation-exchange resin (pyridinium$^+$), the resin was filtered off (sintered glass) and washed with methanol, and the combined filtrates were evaporated to give **25** (27 mg, 95.5%) as an amorphous solid having no definite m.p., $[\alpha]_D^{20}$ +108° (*c* 1.2, 1:1 water–methanol); ν_{max}^{KBr} 3365 (OH, NH), 2940, 1650 (Amide I), 1550 (Amide II), 1490, 1375, 1225, 1090, 1030, 935, 875, 745, and 670 cm^{-1}; t.l.c. (solvent *C*) R_F 0.52.

Anal. Calc. for $C_{17}H_{32}NO_{14}P$: C, 40.40; H, 6.38; N, 2.77. Found: C, 40.42; H, 6.40; N, 2.71.

Methyl 2-acetamido-3,4-di-O-allyl-2-deoxy-α-D-glucopyranoside 6-(2,3,4,6-tetra-O-acetyl-α-D-glucopyranosyl phosphate) (**17**). — A mixture of **13** (50 mg) and **8** (50 mg) was treated with TPS (60 mg) in pyridine (1.25 ml), and the product **17** (58 mg, 50% based on **8**), isolated as described for **21**, had m.p. 117–120°, $[\alpha]_D^{20}$ +74° (*c* 1.5, 2:1 chloroform–methanol); ν_{max}^{KBr} 3425 (NH), 2960, 2875, 1750, 1650 (Amide I), 1550 (broad, Amide II), 1375, 1230 (broad), 1085, 1050, 1020, 950, 875, and 675 cm^{-1}; t.l.c. (solvent *A*) R_F 0.62.

Anal. Calc. for $C_{29}H_{44}NO_{18}P$: C, 47.96; H, 6.11; N, 1.93. Found: C, 48.06; H, 6.11; N, 1.74.

Methyl 2-acetamido-4-O-methyl-2-deoxy-α-D-glucopyranoside 6-(α-D-glucopyranosyl phosphate) (**26**). — Compound **23** (20 mg, obtained by method *b*) was *O*-deacetylated by treatment with 1.5% sodium methoxide in methanol, as described for the preparation (*a*) of **24**. Compound **26** (pyridinium$^+$ form) was obtained pure according to t.l.c. (solvent *C*), as an amorphous solid (17 mg, 77%) having no definite melting point and containing residual solvent; $[\alpha]_D^{20}$ +80° (*c* 1.7, 1:1 methanol–water), $[\alpha]_D^{20}$ +102° (after correction for residual solvent); ν_{max}^{KBr} 3350 (OH, NH), 2940, 1650 (Amide I), 1550 (Amide II), 1495, 1380, 1230, 1120, 1080, 1040, 945, 875, 750, and 675 cm^{-1}.

Anal. Calc. for $C_{16}H_{30}NO_{14}P \cdot C_5H_5N \cdot CHCl_3 \cdot 2H_2O$: C, 36.40; H, 5.55; N, 3.86. Found: C, 36.71; H, 6.32; N, 3.84.

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GLYCOCONJUGATE RESEARCH / Volume I
Proceedings of the Fourth International Symposium on Glycoconjugates

The Chemical Structure of a Glycoprotein from the Cervical Mucus (Premenstrual Phase) of *Macaca radiata*

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Cervical mucus is a hydrophilic, gel-like, dynamic epithelial secretion, which performs a key role in mammalian reproductive process. The mucus displays distinct differences in biophysical and physiological properties during the ovulatory cycle, and these changes are accompanied by alterations in carbohydrate composition (1,2). The bonnet monkey, whose menstrual cycle is very similar to that of the human cycle and which produces large amounts of mucus was used for this study.

The mucus was purified (3), fractionated (4), and the oligosaccharides from the major glycoprotein were prepared by reductive β-elimination as described by Iyer and Carlson (5). The oligosaccharides were separated on a column of Bio-Gel P-4 and the main oligosaccharide component (Table I) was investigated by methylation studies.

The results of the methylation studies showed the presence of terminal fucose; terminal and 2-linked, and 6- and 3-linked galactose; 4-linked *N*-acetylglucosamine; and 6-linked, and 3- and 6-linked *N*-acetylgalactosamine residues.

Acid cleavage of sialic acid residues from a glycoprotein is generally accomplished by treatment with 50 mM sulfuric acid at 80° for 60 min. Mild acid treatment of the methylated oligosaccharide under these conditions resulted in the removal of sialic acid residues, as demonstrated by subsequent methylation and introduction of a trideuteromethoxyl group at C-3 of D-galactose chain residues. The introduction of a trideuteromethoxyl group (~10%) at C-3 of terminal D-galactose residues was also observed. This incorporation of a trideuteromethoxyl group at terminal residues arising from partial removal of L-fucose from D-galactose residues is unlikely, as the ratio of L-fucose to terminal D-galactose, before and after acid treatment, was very similar. Furthermore, the incorporation of the trideutero-

ISBN 0-12-301301-1

methoxyl group at C-3 of the D-galactose residue only shows that the cleavage of the sialic acid residues was selective.

Table I. Composition of the Major Glycoprotein Separated on Sepharose 2B(I) and of Alkali-borohydride-treated Oligosaccharides (II)

Components[a]	I		II	
	%	Molar ratio[b]	%	Molar ratio[b]
L-Fucose	6.0	0.90	8.5	0.76
D-Galactose	16.0	2.10	25.0	2.04
N-Acetyl-galactosamine	14.0	1.50	11.0	0.73
N-Acetyl-glucosamine	9.2	1.00	15.0	1.00
N-Acetyl-neuraminic acid	10.0	0.77	11.2	0.53
N-Acetyl-galactosaminitol			14.5	0.96

[a] Determined by gas-liquid chromatography
[b] Molar ratio relative to N-acetylglucosamine

Structural studies of the oligosaccharides obtained from the peri-ovulatory glycoprotein have shown that D-galactose residues are linked at C-3, C-2, and C-6 (5,6). Similarly, *N*-acetyl-galactosamine residues were shown to be linked at C-3 and C-6, and the sialic acid residues linked to the C-6 of *N*-acetyl-galactosamine residues (6).

From the structural studies of the peri-ovulatory phase and methylation studies of the pre-menstrual-phase mucus glycoprotein, it is concluded that the linkages of D-galactose and *N*-acetylgalactosamine, and the point of attachment of sialic acid residues are different in these two glycoproteins (see Table II).

Table II. Linkages of Carbohydrate Components of the Main Glycoproteins of Peri-ovulatory and Premenstrual Phases of Monkey Cervical Mucus

Components	Linkages in glycoproteins	
	Peri-ovulatory phase	Premenstrual phase
D-Galactose	L-Fucp-(1→2)-D-Galp D-Galp-(1→3)-D-Galp D-GlcNAcp-(1→4)-D-Galp	L-Fucp-(1→2)-D-Galp D-Galp-(1→6)-D-Galp NeuAcp-(2→3)-D-Galp
N-Acetylglucosamine	D-Galp-(1→4)-D-GlcNAcp	D-Galp-(1→4)-D-GlcNAcp
N-Acetylgalactosamine	NeuAcp-(2→6)-D-GalNAcp D-Galp-(1→3)-D-GalNAcp D-GlcNAcp-(1→3)-D-GalNAcp	D-Galp- or D-GlcNAcp-(1→3,6)-D-GalNAcp

Variations in the points of attachment of sugar residues in glycoproteins of secretions, particularly in the blood-group active glycoproteins (7), are well known. The role played by these changes in the glycoprotein structures during the ovulatory cycle is not known, nor whether changes are restricted to the carbohydrate component of the glycoprotein.

The pre-menstrual glycoprotein was reduced with dithiothreitol and alkylated with iodoacetic acid. This procedure was repeated four times, and the alkylated glycoprotein was chromatographed on DEAE-cellulose; two components containing different proportions of cysteine and *S*-carboxymethylcysteine were obtained, indicating incomplete reduction and *S*-carboxymethylation, and clearly suggesting that cysteine is a component of the glycoprotein.

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GLYCOCONJUGATE RESEARCH /Volume II
Proceedings of the Fourth International Symposium on Glycoconjugates

Role of Synthetic Phosphate Diesters in Study of Bacterial Cell Wall

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The antigenic polysaccharide of *Micrococcus lysodeikticus* consists of alternating residues of D-glucose and *N*-acetylmannosaminuronic acid (1). Chemical and enzymic investigation of a fraction of the *Micrococcus lysodeikticus* cell wall containing both the peptidoglycan and antigenic polysaccharide (2) moieties, indicated that these macromolecules may be linked by a phosphate group between D-glucose (of the antigenic polysaccharide) and C-6 of *N*-acetylmuramic acid or *N*-acetylglucosamine (of the peptidoglycan). In order to confirm this, model compounds were prepared, consisting of phosphate diesters in which D-glucose was linked by the phosphate group to C-6 of (a) methyl 2-acetamido-3-*O*-(D-1-carboxyethyl)-2-deoxy-α-D-glucopyranoside (*N*-acetylmuramic acid methyl α-glucoside), or (b) methyl 2-acetamido-2-deoxy-α-D-glucopyranoside, either unsubstituted, or having a methyl group at O-3 or O-4.

The compounds were synthesized by coupling 2,3,4,6-tetra-*O*-acetyl-α-D-glucopyranosyl phosphate with a suitably protected derivative of *N*-acetylmuramic acid or 2-acetamido-2-deoxy-D-glucose in the presence of triisopropylbenzenesulfonyl chloride (TPS) and dry pyridine, chromatographic purification of the product, removal of protecting groups (usually allyl ethers), and careful *O*-deacetylation. 2,3,4,6-Tetra-*O*-acetyl-α-D-glucopyranosyl phosphate was prepared by a modified MacDonald (3) method, consisting of fusion of crystalline phosphoric acid with 1,2,3,4,6-penta-*O*-acetyl-β-D-glucopyranose at 60° for 2 h, followed by neutralization with 15 M ammonium hydroxide. After the coupling reaction to form a phosphate diester, allyl protecting groups were removed by isomerization to 1-propenyl groups with chlorotris(triphenylphosphine)rhodium (4), followed by hydrolysis with mercuric chloride.

ISBN 0-12-301302-X

The synthetic phosphate diesters were characterized by their i.r. spectra, optical rotation, and elemental analysis. For unequivocal confirmation of structure, field desorption mass spectrometry was performed. The resulting spectra showed in each case a molecular ion, together with fragmentation ions derived from the D-glucose and 2-acetamido-2-deoxy-D-glucose 6-phosphate residues. When the synthetic compounds were treated with mild alkali (2 M ammonium hydroxide for 20 min at 37°C), they were recovered unchanged, whether or not O-3 and O-4 of the 2-acetamido-2-deoxy-D-glucose residue were unsubstituted, and thus, available for possible cyclic phosphate formation. In contrast, a similar treatment of UDP-glucose caused complete hydrolysis to glucose phosphates.

When the model compounds were treated with Dowex 50 H^+ ion-exchange resin at 65°C, the glucosyl phosphate bond was cleaved in each case, yielding D-glucose and a 6-phosphate derivative of *N*-acetylmuramic acid or *N*-acetylglucosamine. When the fraction from the *M. lysodeikticus* cell wall, and synthetic α-D-glucopyranosyl [methyl 2-acetamido-3-*O*-(D-1-carboxyethyl)-2-deoxy-α-D-glucopyranoside]-6-yl phosphate (Glc-P-Mur) were treated together, the hydrolytic behavior of each was very similar, giving further support to the concept of a phosphate diester linkage between antigenic polysaccharide and peptidoglycan.

The probable occurrence of a phosphate diester linkage raises the possibility that a lipid intermediate may be involved in its formation. Similar intermediates could also participate in the biosynthesis of the D-glucose-containing antigenic polysaccharide. Because the search for lipid intermediates is helped by the availability of authentic compounds, both anomers of ficaprenyl D-glucopyranosyl phosphate have been synthesized, ficaprenol being employed as a close structural analog of the C_{55} polyprenol active in bacterial cell wall biosynthesis (5).

2,3,4,6-Tetra-*O*-acetyl-β-D-glucopyranosyl phosphate was prepared by a modified MacDonald fusion reaction, employing a very short reaction time (1 min). The per-*O*-acetyl α- or β-D-glucopyranosyl phosphate was coupled with ficaprenol in the presence of TPS. In each case, the resulting per-*O*-acetyl phosphate diester was partially purified, *O*-deacetylated, and the product purified by preparative t.l.c. For comparison, the α- and β-linked dolichyl glucosyl phosphates were similarly prepared.

The properties of the synthetic "lipid intermediates" were examined to establish methods for distinguishing (a) between ficaprenyl (allylic) and dolichyl (non-allylic) compounds, (dolichol having a saturated terminal isoprene residue), and (b) between α and β anomers. When briefly treated with hot, dilute acid, the dolichyl derivatives yielded D-glucose and dolichyl phosphate, whereas the ficaprenyl derivatives both gave a D-glucosyl phosphate. This cleavage of the allylic ester bond also occurred when the ficaprenyl compounds were subjected to catalytic hydrogenation: with the dolichyl compounds, the only reaction observed was saturation of the polyprenol residue. The α and β anomers of the glucosyl polyprenyl phosphates behaved very differently when treated with 0.1 M sodium hydroxide at 65°C. Whereas the α anomers were almost completely stable, both β anomers underwent a rapid conversion into ficaprenyl (or dolichyl) phosphate, together with a derivative of D-glucose that migrated differently from the 1,6-anhydro derivative on t.l.c. and paper chromatograms. As this compound was also produced by similar treatment of *p*-nitrophenyl β-D-glucopyranoside, it was tentatively identified as a 1,2-anhydro derivative (6). The outcome of these experiments is the finding that D-glucose-containing "lipid intermediates" can be readily identified, with regard to nature of polyprenyl moiety and anomeric configuration, by these straightforward chemical methods.

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FERTILITY AND STERILITY
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Vol. 32, No. 2, August 1979
Printed in U.S.A.

IMMUNOLOGICALLY INDUCED ALTERATION IN THE MORPHOLOGY OF THE CERVICAL MUCUS OF MACACA RADIATA*†

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Cervical mucus possesses a number of biophysical properties, such as viscosity, flow elasticity, and stickiness which, like its secretion and morphology, are regulated by the ovarian hormones. The cyclic alterations in the biophysical properties of mucus are accompanied by variations in its carbohydrate composition. The physical and chemical changes in cervical mucus during the menstrual cycle not only influence penetration, but also nutrition and survival of spermatozoa. The principal macromolecular constituent of cervical mucus is a carbohydrate-rich glycoprotein which shares the physical and chemical properties of other epithelial secretions.

An essential event in mammalian fertilization is the passage of sperm through the mucus of the cervix into the uterine cavity. It has been suggested that the ordered glycoprotein-fibril system of periovulatory phase cervical mucus guides sperm penetration, whereas the random fibrillar network structure of luteal phase mucus inhibits this process.[2] A potential means for fertility control involves the induction of changes in the morphology of mucus as well as in its consistency. This communication describes the preparation of an antibody to a cervical mucus glycoprotein, and an immunologically induced alteration in the morphology of the fibrillar channels of periovulatory phase cervical mucus glycoprotein resulting in a network characteristic of luteal phase cervical mucus.

The bonnet monkey, *Macaca radiata*, was chosen for this study not only because of its phylogenetic proximity to man and its 28-day menstrual cycle,[3] but also because its cervix secretes mucus in large amounts.

Received December 26, 1978; revised February 7, 1979; accepted March 7, 1979.

*Supported by Contract NO 1 HD-4-2829 from the National Institute of Child Health and Human Development, National Institutes of Health, United States Public Health Service.

†Cervical glycoproteins, Part VI; for Part V see reference 1. Publication No. 784 of the Robert W. Lovett Memorial Group for the Study of Diseases Causing Deformities, Harvard Medical School and Massachusetts General Hospital.

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MATERIALS AND METHODS

Mucus was aspirated from the cervix with the aid of a suction pump and was stored at −20° C prior to use. The cervical mucus was purified and fractionated by chromatography on Bio-Gel P-200 and then on Sepharose 2B, as previously described.[4] Acrylamide-agarose gel electrophoresis was performed as previously described.[1] Immunoelectrophoresis and electrophoresis were performed in 1% agarose in 50 mM barbital buffer, pH 8.2, at 90 to 100 volts with bromophenol blue as a migrating marker. For periodate-Schiff staining, the plates were treated with 0.2% periodic acid in 7.5% acetic acid for 45 minutes in the dark, and

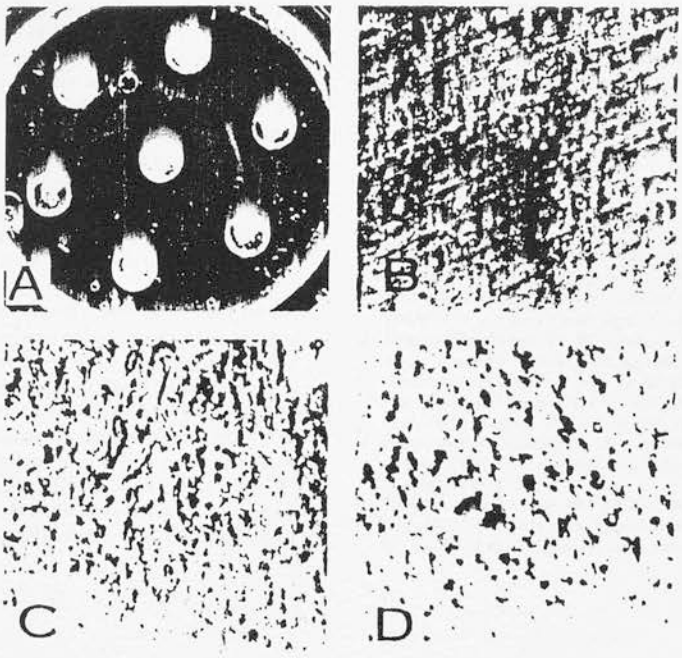

FIG. 1. *A*, Immunodiffusion plate containing fresh cervical mucus from the periovulatory phase. Rabbit antimucus antibody is in the center well, and peripheral wells 1 and 2 contain diluted (10 times) fresh and frozen periovulatory phase cervical mucus; wells 3 and 4 contain diluted (10 times) fresh and frozen luteal phase cervical mucus; well 5 contains bonnet monkey serum, and well 6 contains normal rabbit serum. *B*, Fresh periovulatory phase cervical mucus on a microscope slide. *C*, Fresh luteal phase cervical mucus on a microscope slide. *D*, Complex of fresh periovulatory phase cervical mucus and antiserum on a microscope slide (*B*, *C*, and *D*, ×100).

then treated with Schiff reagent (Fisher Scientific Co., Piscataway, N. J.) for 45 minutes. Protein staining was achieved by fixing the plates in 10% acetic acid (in methanol) and staining with Amido schwarz. For toluidine staining, the plates were treated with 0.2% toluidine blue in 1.5% acetic acid for 30 minutes. Cordis II plates (Cordis, Miami, Fla.) were employed for immunodiffusion, and the plates were developed for 96 to 120 hours. Carbohydrate in the column eluates was determined with the phenol-sulfuric acid method. Bonnet monkey serum and normal rabbit serum were used as controls. Antibodies were raised by immunizing rabbits to purified cervical mucus as follows: Various amounts (0.940 mg, 0.840 mg, 0.740 mg, and 0.190 mg) of glycoprotein were injected into footpads at a concentration of 0.125 mg/ml of saline mixed with Freund's complete adjuvant (v/v, 1:1). After 30 days, the rabbits again received intravenous injections of glycoprotein (0.450 mg, 0.375 mg, 0.325 mg, and 0.105 mg) at a concentration of 0.5 mg/ml of saline. The serum was harvested 7 days later from an ear vein.

RESULTS AND DISCUSSION

The glycoprotein of periovulatory phase cervical mucus was purified by gel filtration on Bio-Gel P-200 and Sepharose 2B as described earlier.[4] Periovulatory glycoprotein, which was eluted as a major, carbohydrate-containing material from the Sepharose 2B column, was homogenous on agarose gel electrophoresis and stained with periodate-Schiff reagent and toluidine blue, but did not react with Amido schwarz. The antibody for this glycoprotein was raised in the rabbit by use of Freund's complete adjuvant, and in immunodiffusion two precipitin lines were observed. The two precipitins observed in immunoelectrophoresis corresponded to the area where glycoprotein was stained with

periodate-Schiff reagent, as well as with toluidine blue. Electrophoresis of the antigen in polyacrylamide[1] and agarose[4] confirmed the absence of contaminating proteins in the antigen. It is possible that the two precipitin lines observed in immunodiffusion and immunoelectrophoresis arose as a result of aggregation of the glycoprotein molecule. The linear fibrillar channels of either fresh or frozen periovulatory mucus and the network of luteal phase mucus was easily seen under a microscope (×100 or ×320) with the technique of Davajan et al.[5] (Fig. 1).

Cervical mucus obtained fresh or stored in the frozen state exhibited a precipitin reaction toward rabbit antimucus antibody raised against purified cervical mucus glycoprotein (Fig. 1), but it did not react to normal rabbit and monkey serum. The antibody reacted with periovulatory mucus, which exhibited fibrillar glycoprotein channels, either on a glass slide or in a tube within 15 to 20 minutes. In either case, the precipitate displayed a morphology which was entirely different from that of periovulatory phase mucus and similar to that of luteal phase mucus (Fig. 1).

In addition to its usefulness in the study of antigen determinants of cervical mucus, the antibody may lead to a functional change in cervical mucus in vivo and provide nonsteroidal control of sperm transport through the cervix.

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CERVICAL MUCUS GLYCOPROTEINS IN REPRODUCTION STUDY OF CERVICAL MUCUS FROM *Macaca radiata*

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The factors that control sperm transport through the cervix into the uterine cavity are of considerable importance to the process of fertilization. It has been demonstrated that cervical mucus, one of these factors, undergoes cyclic alterations in physical behavior and morphology (1) in addition to variations in the carbohydrate compositions (2). The chemical and physical changes in the cervical mucus during the menstrual cycle influence penetration, nutrition, and survival of the sperm. The linear channels of the peri-ovulatory cervical mucus facilitate sperm migration through the cervix, whereas the network of the luteal-phase mucus impedes sperm penetration (1). In spite of considerable interest in the morphology of the cervical mucus, little is known about the nature of the molecules that contribute to the defined morphology of the mucus during the different phases of the menstrual cycle. This report attempts to correlate the structure of glycoproteins with the changing morphology of the cervical mucus during the cycle.

The peri-ovulatory-phase mucus glycoproteins in phosphate buffer (50 mM, pH 7.0) was purified on Bio-Gel P-200. The purified glycoprotein was fractionated on Sepharose 2B into two glycoproteins, a major and a minor component. The major component contained L-fucose, D-galactose, *N*-acetyl-D-glucosamine, *N*-acetyl-D-galactosamine, *N*-acetylneuraminic acid, sulfate, and amino acids. Neither mannose nor *N*-glycolylneuraminic acid was identified. The glycoprotein contained nearly 70% carbohydrate; serine and threonine were the predominant amino acids. On electrophoresis in 1.5% acrylamide and 0.5% agarose, this fraction did not enter the gel; however, electrophoresis in 1% agarose showed the presence of a homogenous, high-molecular-weight component. Alkali-borohydride treatment of this glycoprotein demonstrated the presence of *O*-glycosyl linkage since it was accompanied by partial loss of serine and threonine and increase in alanine residues, and the formation of α-aminobutyric acid and 2-acetamido-2-deoxy-D-galactitol residues. This glycoprotein was treated with insolubilized Pronase, trypsin and chymotrypsin in appropriate buffers at 37°C. The degraded glycoproteins were chromatographed on Sepharose 2B, and the carbohydrate moiety of the Pronase-, trypsin-, and chymotrypsin-resistant glycoproteins was found to contain L-fucose, D-galactose, *N*-acetyl-D-glucosamine, *N*-acetyl-D-galactosamine, and sialic acid. The carbohydrate portion of the degraded glycoprotein represents nearly 90% of the sugars in the starting material, which suggests that only a minimal amount of sugar residues were linked to the peptides eliminated by the proteolytic degradation.

An antibody against the glycoprotein, obtained as a major fraction from Sepharose 2B, was raised in rabbit by the use of Freund's complete adjuvant. A diffuse line as well as a sharp precipitin line were

Glycoconjugates, 1979, R. Schauer et al., (eds.) G. Thieme, Stuttgart.

observed in immunodiffusion. In immunoelectrophoresis, two precipitin lines were observed, and these lines in electrophoresis corresponded to a broad band, which was stained with the periodate Schiff reagent. It is likely that the precipitin observed in immunodiffusion and immunoelectrophoresis was due to aggregation of the glycoprotein molecule. The antiserum in immunodiffusion reacted positively also with the Pronase-, trypsin-, and chymotrypsin-resistant glycoprotein, suggesting that the antigenic determinants were located in the enzyme-resistant portion of the glycoprotein and not in the molecule or in the fragment of the molecule that was susceptible to the proteolytic degradation.

The linear fibrillar channels of peri-ovulatory mucus and the network of luteal-phase mucus obtained fresh from bonnet monkey were observed under the microscope (x100 or 320) with the technique of Davajan *et al.* (3). Cervical mucus, fresh or stored in the frozen state, exhibited precipitin reaction toward rabbit anti-mucus antibody raised against mucus glycoprotein, Sepharose 2B major fraction, but did not react with luteal-phase mucus, and normal rabbit and monkey serum. The antibody reacted with the peri-ovulatory mucus, which exhibited fibrillar glycoprotein channels on a glass slide as well as in a tube within 15 to 20 min. In either case, the precipitate under a microscope displayed a morphology that was different from that of the peri-ovulatory-phase mucus and similar to that of luteal-phase mucus. The morphological change induced by the antibody clearly suggests that the peri-ovulatory mucus channels are lined with glycoprotein.

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Supported by a grant from the National Institute of Child Health and Human Development (HD 12431-01), National Institutes of Health, U.S. Public Health Service.

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Changes in the Cervical Mucus of the Bonnet Monkey (*Macaca radiata*) During the Menstrual Cycle

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SUMMARY

In phylogenetic proximity and physiologic resemblance to the human, the bonnet monkey possesses advantages over the cow, the classical animal model for study of cervical mucus chemistry. Its menstrual cycle, like the human, is 28 days in duration, and it produces a large amount of cervical mucus that exceeds at least by tenfold the quantity produced by women. In the first stage of a program designed to characterize the macromolecular components of bonnet monkey cervical mucus and ultimately control the biosynthetic mechanisms involved in their synthesis, we have obtained the major glycoprotein from the premenstrual mucus and purified it by gel filtration and ion exchange chromatography. The structure of its carbohydrate moiety was investigated by methylation and periodate oxidation, and compared with that of the periovulatory glycoprotein. In order to assess the effects of human seminal plasma and sperm head proteolytic enzymes, purified cervical mucus glycoprotein was treated with pronase.

INTRODUCTION

The secretions of the mammalian cervix mediate and control the key functions of the cervix. The physical, chemical, and morphologic changes in these secretions during the menstrual cycle alter the biophysical behavior of the cervical mucus, including permeability to sperm, quantity, composition, and rheology. The fundamental function of the cervical secretion is to act as a biologic valve controlling sperm entry into the uterus, thereby encouraging or inhibiting sperm penetration during the cycle.

Cervical mucus is a complex substance; the principal constituents are mucin-type, carbohydrate-rich glycoproteins that share the properties of other epithelial secretions and cell surface glycoproteins. They exhibit gellike behavior and have a protein core rich in hydroxyamino acids. Because these glycoproteins represent the major macromolecular component of the mucus, it is likely that they are responsible

Animal models for research on contraception and fertility. 1979. N.J. Alexander (ed.), Harper & Row, Hagerstown.

for its distinctive physiologic and biophysical characteristics. Bovine cervical mucus, which is readily available, has been extensively used for most macromolecular studies.

The chemical investigations on human (Iacobelli *et al.*, 1971; Masson, 1973) and bovine (Gibbons, 1968; Gibbons and Mattner, 1971) cervical mucus have shown compositional differences which may be considered an expression of species variation. The neuraminic acid in the human and bonnet monkey cervical mucus glycoprotein is essentially the usual *N*-acetyl derivative, whereas in bovine it is the *N*-glycolyl type. In addition, the fucose content in human cervical mucus is much higher than in bovine. In this paper we describe the structural changes in cervical glycoproteins during the menstrual cycle of the bonnet monkey and attempt to relate these changes to the morphology, biophysical behavior, and the known functions of the mucus.

The bonnet monkey (*Macaca radiata*), so much closer phylogenetically to the human than the cow is, was chosen because its copious cervical mucus facilitates chemical and enzymatic studies.

At the peak of follicular activity several grams of mucus are secreted, an amount that exceeds by at least ten fold the quantity produced by women. Moreover, McArthur *et al.* (1972) and Bashir-Farahmand *et al.* (1976), in systematic studies on the menstrual cycle, vaginal cytology, and endocervical epithelium of *M. radiata*, showed the physiologic similiarities of this subhuman primate to the human being. They employed a number of indices to evaluate the ovulatory cycle of the adult female bonnet monkey: 1) cervical mucus weight, spinnbarkeit, and arborization, 2) vaginal cytology, 3) sex-skin changes, 4) uterine and ovarian size consistency, and 5) urinary estrogen secretion.

The follicular phase was characterized by a steady rise in cervical mucus weight, gradual reddening of the perianal and perielitoral regions, a rapid increase and a sudden decline in estrogen excretion, and an abrupt midcycle increase in the proportion of superficial cells in the vaginal smear with a reciprocal decrease in the proportion of anucleate squames. The luteal phase was marked by a reduction in the quantity of cervical mucus until the close of the cycle, fading of the sex-skin color, a decrease in urinary estrogen secretion followed by a mild rise late in the cycle, and a postovulatory decline in the proportion of the superficial cells in the vagina. It was further shown that vaginal desquamation was minimal during the follicular phase when ovarian estrogen secretion was rising. An increased desquamation was observed during the postovulatory and luteal phases. Thus it appears that the bonnet monkey has much to commend it as an animal model for studies of the primate cervix from the viewpoint of contraception and fertility. Like the human cervical mucus, the bonnet monkey mucus exhibits distinct cyclic variations in rheologic properties and contains mainly glycoproteins as high-molecular-weight components.

PERIOVULATORY GLYCOPROTEIN

In earlier studies (Nasir-ud-Din *et al.*, 1976, 1977) we have shown that periovulatory mucus contains two glycoproteins, a major and a minor one, which we separated by gel filtration. The major glycoprotein exhibited microheterogeneity with respect to acid functions (sulfate and carboxyl groups) on ion exchange chromatography

and a homogeneous composition in agarose gel electrophoresis. The carbohydrate moiety of the glycoprotein contained fucose, galactose, *N*-acetylglucosamine, *N*-acetylgalactosamine, and *N*-acetylneuraminic acid. The protein moiety of the glycoprotein had a high proportion of serine and threonine residues, a distinct feature of secreted glycoproteins; cysteine was present only in a minor quantity as a glycoprotein component. No alien protein cross-linking fraction, composite of cysteine as in the case of bovine cervical mucus (Gibbons and Mattner, 1971), was isolated. The sulfate groups were located in the terminal galactose and *N*-acetylglucosamine residues.

Alkaliborohydride treatment of the glycoprotein (Iyer and Carlson, 1971) released oligosaccharides with partial loss of serine and threonine residues, and there

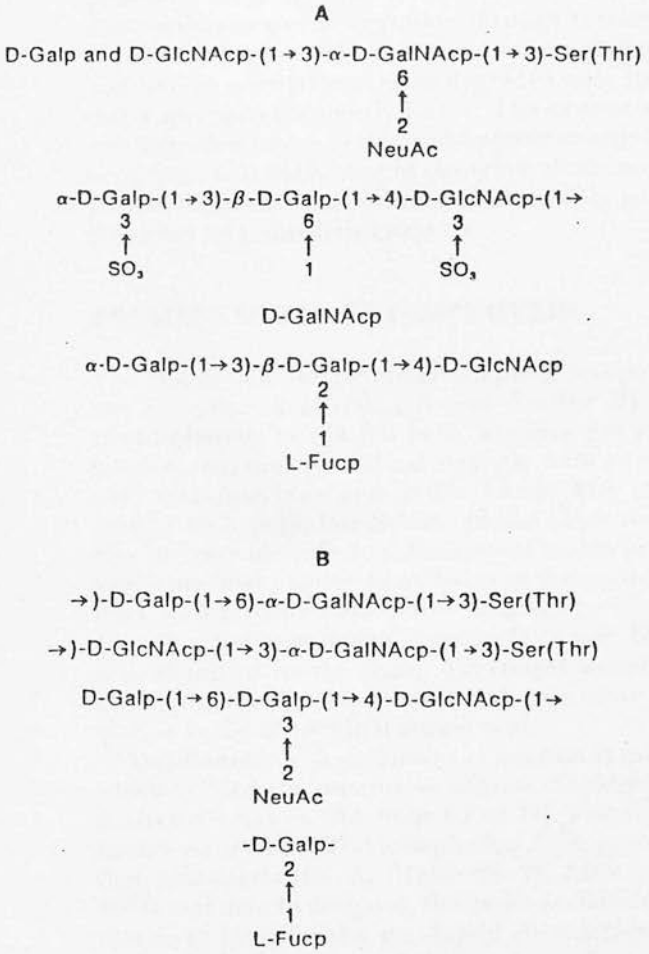

FIG. 28–1. Partial structures of the carbohydrate branches of glycoproteins in the bonnet monkey cervical mucus. A. Periovulatory phase. B. Premenstrual phase.

was a corresponding increase in alanine and formation of α-aminobutyric acid residues; these phenomenon demonstrated the presence of *O*-glycosyl linkages. The oligosaccharides were fractionated by gel filtration. The major oligosaccharide fraction (70%) was further purified by paper chromatography, and the structure of this oligosaccharide was elucidated by methylation and Smith degradation studies. The site of attachment of sialic acid at C-6 of the *N*-acetylgalactosamine residue was determined by selective cleavage of this residue from the methylated oligosaccharide with mild acid and subsequent remethylation with deuterated iodomethane. About 25% of the oligosaccharide chains were devoid of sialic acid residues. The structural studies on the carbohydrate moiety of the major glycoprotein showed that there were at least two types of oligosaccharide chains, which differed in sequence and in the number of sugar residues (Fig. 28–1).

Because it has been suggested that the proteolytic enzymes from human seminal plasma (Stambaugh and Buckley, 1970) and sperm heads (Moghissi and Syner, 1970) enhance sperm migration through cervical mucus, we examined the action of proteolytic enzymes on cervical mucus glycoproteins. Treatment with pronase and bovine seminal peptidase degraded only the minor glycoprotein and left the major glycoprotein mostly intact. The neuraminidase-treated major glycoprotein was degraded into a number of fragments both by pronase and by bovine seminal peptidase. It is likely that in the cervical mucus only those glycoproteins or glycoprotein fragments that either are devoid of or have lesser amounts of sialic acid are degraded by proteolytic enzymes.

PREMENSTRUAL GLYCOPROTEIN

The mucus from the premenstrual phase was purified and further fractionated into two components (fraction A and fraction B) by gel filtration. On agarose gel electrophoresis at pH 8.2 both fractions did partially enter the gels; the major fraction, fraction A, stained strongly with periodic acid-Schiff and very weakly with toluidine blue and amido black. The minor fraction, fraction B, stained weakly with periodate-Schiff, amido black and toluidine blue. Threonine and glycine were identified as *N*-terminal amino acids in the fraction-A glycoprotein, and lysine and alanine in addition to threonine and glycine were identified in the fraction-B glycoprotein. Chromatography of fraction A on DEAE-cellulose gave a single major component, fraction D (Table 28–1). Serine, with traces of glycine, was identified as the main *N*-terminal amino acid. Rechromatography of the glycoprotein on DEAE-cellulose did not remove the trace amounts of protein having glycine as the *N*-terminal amino acid.

Alkaliborohydride treatment of fraction D under strong borohydride conditions, which yielded the mixture of oligosaccharides that was purified by gel filtration, gave two fractions (fractions I and II). Fraction I, obtained in a 65% yield and further purified on DEAE-Sephadex A-25, gave one carbohydrate-containing fraction, oligosaccharide A_1 (Table 28–1). After it had been methylated (Hakomori, 1964) and then hydrolyzed, the monosaccharides were converted to alditol acetates that were identified by gas–liquid chromatography and mass spectrometry. The methylated sugars showed the presence of a terminal fucose residue; of terminal, *O*-2-linked, and *O*-6- and *O*-3-linked D-galactose residues; of an *O*-4-linked *N*-acetylglucosamine residue; and of terminal *O*-6-linked and *O*-3- and *O*-6-linked

TABLE 28–1. **Composition of the Premenstrual Phase Glycoprotein* and of Derived Oligosaccharides†**

Sugar	Fraction D		A_1		A_2		SA_1
	%	Molar Ratio	%	Molar Ratio	%	Molar Ratio	Molar Ratio
L-Fucose	6.5	0.65	10.0	0.90	9.5	0.91	0.00
D-Galactose	26.5	2.35	25.0	2.03	25.5	2.23	1.00
2-Acetamido-2-deoxy-D-glucose	13.6	1.0	15.0	1.00	14.0	1.00	1.00
2-Acetamido-2-deoxy-D-galactose	19.9	1.45	11.2	0.73	10.1	0.72	0.00
N-Acetylneuraminic acid	9.9	0.5	14.2	0.68	3.7	0.19	0.00
2-Acetamido-2-deoxy-D-galactitol			14.5	0.96	13.0	0.92	
2-Acetamido-2-deoxy-threitol							+ +
2-Acetamido-2-deoxy-glycerol							+ + + +

* Obtained from DEAE-cellulose column (D).

† Oligosaccharides (A_1 and A_2) obtained by alkaliborohydride treatment and Smith degradation of oligosaccharides A_1 (SA_1). Molar ratio relative to 2-acetamido-2-deoxy-D-glucose.

N-acetylgalactosamine residues. The point of attachment of sialic acid was at C-3 of an inner D-galactose residue. Periodate oxidation and sodium borohydride reduction, followed by mild acid treatment (Smith degradation), resulted in total destruction of sialic acid, fucose, and *N*-acetylgalactosamine, and in partial degradation of D-galactose and 2-acetamido-2-deoxy-D-galactitol (Table 28–1). There was definite conversion of 2-acetamido-2-deoxy-D-galactitol to 2-acetamido-2-deoxy-glycerol, a fact that confirms an *O*-6 linkage of the *N*-acetylgalactosamine residue that is linked to the protein core.

Fraction II, obtained in a 12% yield and further purified by chromatography on DEAE-Sephadex A-25, gave oligosaccharide A_2 (Table 28–1).

In an additional experiment, the glycoprotein of this phase (fraction A) was extensively degraded with insolubilized protease, and the nondialyzable material was purified by gel filtration on Sepharose 2B. Further chromatography on DEAE-cellulose indicated the presence of a single carbohydrate-containing polymer. On 1% agarose gel electrophoresis this polymer entered the gel and stained with periodic acid-Schiff reagent and toluidine blue, but not with amido black. That fact that determination of *N*-terminal amino acids demonstrated the presence of threonine, glycine, alanine, and lysine suggested extensive heterogeneity.

STRUCTURAL FEATURES AND FUNCTIONS OF PREMENSTRUAL AND PERIOVULATORY GLYCOPROTEINS

The major glycoproteins of the two phases exhibit distinct differences in the chemical structures of their carbohydrate moieties. The essential changes observed during the menstrual cycle concern the linkages of *N*-acetylneuraminic acid, *N*-acetyl-D-galactosamine, and D-galactose residues (Fig. 28–1).

The *N*-acetylneuraminic acid residues of the periovulatory glycoprotein are linked to C-6 of an *N*-acetylgalactosamine residue that is adjacent to the protein core; in the premenstrual phase these residues are linked to C-3 of the D-galactose residues remote from the protein moiety. It has been postulated that because of their mutual repulsion, the *N*-acetylneuraminic acid residues are responsible for the rigidity, coherence, and consistency of the mucin secretion (Masson, 1973). It is likely that the presence of *N*-acetylneuraminic acid in the vicinity of the protein core during the periovulatory phase enhances this rigidity and may be responsible for the parallel alignment of the mucus component that allows channels of least resistance for sperm penetration (Davajan *et al.*, 1971). In the premenstrual glycoprotein, the increased distance of the *N*-acetylneuraminic acid residues from the protein backbone may decrease the interaction between the intramolecular carbohydrate chains and result in a more flexible macromolecule that allows more intermolecular interaction. In addition, the increased distance of the *N*-acetylneuraminic acid residue may explain the increased susceptibility of glycoprotein to proteolytic enzymes, which would result 1) in a network of smaller glycoprotein chains and 2) in the consequent impermeability of premenstrual mucus.

The purified bonnet monkey cervical mucus glycoprotein is a high-molecular-weight material which is similar in composition and relative proportion of sugars and amino acids to the human cervical mucus glycoprotein. The sialic acid residues in the glycoprotein are, as in human, mainly of *N*-acetyl type. The cervical mucus

glycoproteins we have investigated in our studies, using the bonnet monkey as a model, show resemblance to human cervical glycoproteins, although the structural information on human glycoproteins is very scant. Also, the data to indicate how ovarian hormones influence the changes in the mucus secreted during the cycle are limited. It is, however, known that in the bonnet monkey, as in the human, estrogens stimulate the production of copious amounts of cervical mucus, and it is likely that steroids exercise some control in the biosynthesis.

In the study of glycoproteins of periovulatory and premenstrual mucus, distinct structural differences were observed. At the moment, it is not clear whether these changes are restricted to the carbohydrate moiety or whether they also occur in the protein portion. However, it is known that the diversity in the glycoproteins during the menstrual cycle is mediated by the same cells (Bashir-Farahmand *et al.*, 1976). The role of the cervical mucus in fertility, sterility, and contraception has been emphasized (Elstein *et al.*, 1973), and the complexity of the macromolecular component responsible for its behavior during the cycle is becoming better understood.

RESEARCH GOALS

The focus of our current research is to elucidate the total structure of cervical mucus glycoproteins and the structure–function relationship. This will provide a firm basis for the study of biochemical mechanisms. A stage further will be to characterize the transferases responsible for the synthesis of glycoproteins during the cycle in order to establish the biochemical pathways, which will increase the understanding of biochemical mechanisms and may suggest procedures of their control.

In addition, a study of the proteolytic enzymes present in cervical mucus will be undertaken. In one phase of the cycle these substances may be active as mucolytic agents and in another phase may increase the consistency of the mucus by a sequence of events (degradation of the glycoprotein followed by intermolecular reaction) which will impede sperm penetration.

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Carbohydrate Research, 75 (1979) 349–356

Note

Changes in the glycoprotein structure of the cervical mucus of the bonnet monkey during the menstrual cycle. Study of the premenstrual-phase mucus*

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(Received September 5th, 1978; accepted for publication in revised form, December 18th, 1978)

Cervical mucus is a hydrophilic, gel-like, dynamic epithelial secretion, which implements an important role in the mammalian reproductive cycle. It displays distinct biophysical and morphological variations during the menstrual cycle. The mucus glycoproteins, which constitute the totality of the macromolecular components of the mucus, exhibit alterations of their carbohydrate composition during the menstrual cycle[4,5]. An investigation of the chemical structure of the mucus glycoproteins was undertaken in order to understand their contribution to the reproductive cycle and relate changes in their chemical structure to the biophysical properties of the mucus in various phases of the menstrual cycle. The cervical mucus of the bonnet monkey was chosen for this study, as it is produced in large amounts, and the menstrual cycle of the bonnet monkey is very similar to that of the human[6].

The glycoprotein component of the premenstrual-phase cervical mucus was purified[3] (for composition, see Table I) and fractionated, as previously described for the peri-ovulatory phase mucus[7]. The oligosaccharides from the major glycoprotein fraction were prepared by reductive β-elimination, as described by Iyer and Carlson[8]. After separation on a column of Bio-Gel P-4 (Fig. 1), the main oligosaccharide fraction (Fraction B) was obtained in 62% yield, and shown to be homo-

*Cervical Glycoproteins of the Bonnet Monkey, Part V. For Parts I–IV, see refs. 1–4. This is publication No. 781 of the Robert W. Lovett Memorial Group for the Study of Diseases Causing Deformities, Harvard Medical School and Massachusetts General Hospital. This work was supported by a contract from the National Institute of Child Health and Human Development (NO1-HD-4-2829), National Institutes of Health, U.S. Public Health Service.

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geneous by paper electrophoresis in borate buffer and by paper chromatography in two solvent systems. On further purification by ion-exchange chromatography, it gave a single, carbohydrate-containing fraction (Fig. 2, Fraction B-1) in 86% yield (see Table I). This fraction was shown to be homogeneous by paper chromatography in two solvent systems, as well as by the symmetrical appearance of the elution peak. The fraction constitutes ~65, 60, 50, 60, and 55% of the 2-acetamido-2-deoxy-D-galactose, 2-acetamido-2-deoxy-D-glucose, *N*-acetylneuraminic acid, L-fucose, and D-galactose residues, respectively, present in the original glycoprotein. These proportions suggest either a slight loss of *N*-acetylneuraminic acid residues during the purification procedure, or some elimination of oligosaccharides containing these residues. The proportion of 2-acetamido-2-deoxy-D-galactitol indicates the presence of a heptasaccharide or a mixture of hexa-, hepta-, and octa-saccharides, as oligosaccharides of lower mol. wt. could be separated distinctly by the chromatographic procedures used.

Methylation studies of Fraction B-1 (see Table II) showed the presence of terminal L-fucopyranosyl residues; of terminal, 2-linked, and 6- and 3-linked D-galactopyranose residues in the relative proportions of 1:1:1; of 4-linked 2-acetamido-2-deoxy-D-glucopyranose residues; and of 6-linked, and 3- and 6-linked 2-acetamido-2-deoxy-D-galactopyranose residues, in the proportion of 2:1.

Mild acid-treatment of the methylated oligosaccharide with 50mM sulfuric acid for 75 min at 80° removed ~70% of the terminal *N*-acetylneuraminic acid residues. Subsequent trideuteriomethylation introduced a trideuteriomethoxyl group at C-3 of the D-galactopyranose residues linked at O-6, and (in very small proportion) at C-3 of the terminal D-galactopyranose residues. The incorporation of a trideuteriomethoxyl group at C-3 of terminal D-galactopyranose residues arising from partial removal of terminal L-fucopyranose residues is unlikely, as the ratio of L-fucose to terminal D-galactose before and after acid treatment was very similar. The incorporation of the trideuteriomethoxyl group at only C-3 of the D-galactopyranose residues is also indicative of the selectivity of the cleavage of sialic acid residues.

For the Smith degradation of Fraction B-1, the periodate oxidation was conducted for 16 h at 22°. The oxidized fragments were reduced with sodium borohydride, and the resulting polyalcohols were hydrolyzed by mild acid-treatment. The results of the degradation are shown in Table I. As expected from the results of the methylation study, *N*-acetylneuraminic acid, 2-acetamido-2-deoxy-D-galactose, and L-fucose residues were completely degraded, as well as ~50% of the D-galactose residues. The 2-acetamido-2-deoxy-D-galactitol residues were converted into 2-acetamido-2-deoxy-D-threitol and 2-acetamido-2-deoxyglycerol residues. These results suggest that the oligosaccharide fraction has a considerable proportion of D-galactose residues, in addition to L-fucose, 2-acetamido-2-deoxy-D-galactose, and *N*-acetylneuraminic acid residues, as nonreducing, terminal sugars. The formation of 2-acetamido-2-deoxy-D-threitol by Smith degradation suggests that nearly two-fifth of the 2-acetamido-2-deoxy-D-galactose residues linked to the protein core are substituted at both O-3 and O-6, or at O-3 alone.

TABLE I

COMPOSITIONS OF ORIGINAL GLYCOPROTEIN; OF OLIGOSACCHARIDE FRACTIONS A, B, AND C OBTAINED FROM A COLUMN OF BIO-GEL P-4; OF FRACTION B-1 OBTAINED FROM A COLUMN OF DEAE-SEPHADEX; AND OF FRACTION SB-1 OBTAINED BY SMITH DEGRADATION

Components[a]	*Original*		*Fractions*								
			A		*B*		*C*		*B-1*		*SB-1*
	%	*M.r.*[b]	%	*M.r.*[b]	%	*M.r.*[b]	%	*M.r.*[b]	%	*M.r.*[b]	*M.r.*[b]
2-Acetamido-2-deoxy-D-galactitol	0.0	0.00	1.0	0.06	14.5	0.96	16.6	1.18	14.5	0.96	0.00
2-Acetamido-2-deoxy-D-galactose	14.0	1.58	15.7	1.02	11.0	0.73	12.4	0.89	11.2	0.73	0.00
2-Acetamido-2-deoxy-D-glucose	9.0	1.00	15.4	1.00	15.0	1.00	14.0	1.00	15.0	1.00	1.00
2-Acetamido-2-deoxy-glycerol											0.60
2-Acetamido-2-deoxy-D-threitol											+[c]
N-Acetylneuraminic Acid	10.0	0.79	7.0	0.36	11.0	0.52	3.7	0.19	14.2	0.68	0.00
L-Fucose	6.0	0.90	8.0	0.70	8.5	0.76	8.8	0.84	10.0	0.90	0.00
D-Galactose	16.0	2.18	21.0	1.67	25.0	2.05	26.0	2.23	25.0	2.03	1.00

[a]Determined by g.l.c. [b]Molar ratio relative to 2-acetamido-2-deoxy-D-glucose. [c]Present but not determined.

The formation of 2-acetamido-2-deoxyglycerol by the same degradation-method suggests that the remaining 2-acetamido-2-deoxy-D-galactose residues linked to the protein core are either substituted at O-6, or are unsubstituted. The methylation results (Table II), however, show that these residues are substituted at O-6, and at

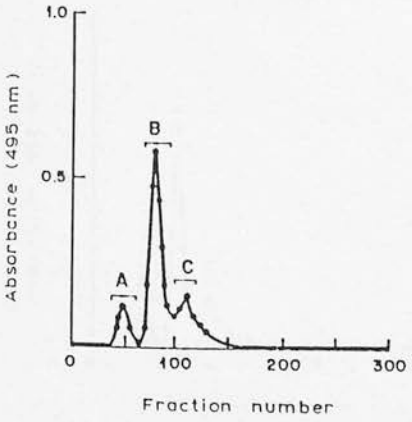

Fig. 1. Elution profile of oligosaccharides from a column of Bio-Gel P-4. The column was eluted with 50mM pyridine–acetic acid (pH 5.4, 750 mL), and fractions of 2.5 mL were collected. Every third fraction was examined for the presence of carbohydrates by the phenol–sulfuric acid method[11]. Fraction A: tubes 39–63; Fraction B: tubes 70–93; and Fraction C: tubes 102–120.

TABLE I

COMPOSITIONS OF ORIGINAL GLYCOPROTEIN; OF OLIGOSACCHARIDE FRACTIONS A, B, AND C OBTAINED FROM A COLUMN OF BIO-GEL P-4; OF FRACTION B-1 OBTAINED FROM A COLUMN OF DEAE-SEPHADEX; AND OF FRACTION SB-1 OBTAINED BY SMITH DEGRADATION

Components[a]	*Original*		*Fractions*								
			A		*B*		*C*		*B-1*		*SB-1*
	%	*M.r.*[b]	%	*M.r.*[b]	%	*M.r.*[b]	%	*M.r.*[b]	%	*M.r.*[b]	*M.r.*[b]
2-Acetamido-2-deoxy-D-galactitol	0.0	0.00	1.0	0.06	14.5	0.96	16.6	1.18	14.5	0.96	0.00
2-Acetamido-2-deoxy-D-galactose	14.0	1.58	15.7	1.02	11.0	0.73	12.4	0.89	11.2	0.73	0.00
2-Acetamido-2-deoxy-D-glucose	9.0	1.00	15.4	1.00	15.0	1.00	14.0	1.00	15.0	1.00	1.00
2-Acetamido-2-deoxy-glycerol											0.60
2-Acetamido-2-deoxy-D-threitol											+[c]
N-Acetylneuraminic Acid	10.0	0.79	7.0	0.36	11.0	0.52	3.7	0.19	14.2	0.68	0.00
L-Fucose	6.0	0.90	8.0	0.70	8.5	0.76	8.8	0.84	10.0	0.90	0.00
D-Galactose	16.0	2.18	21.0	1.67	25.0	2.05	26.0	2.23	25.0	2.03	1.00

[a]Determined by g.l.c. [b]Molar ratio relative to 2-acetamido-2-deoxy-D-glucose. [c]Present but not determined.

The formation of 2-acetamido-2-deoxyglycerol by the same degradation-method suggests that the remaining 2-acetamido-2-deoxy-D-galactose residues linked to the protein core are either substituted at O-6, or are unsubstituted. The methylation results (Table II), however, show that these residues are substituted at O-6, and at

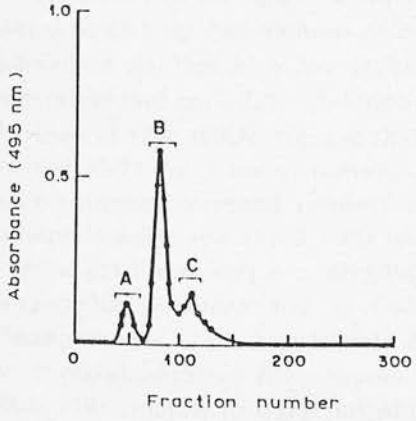

Fig. 1. Elution profile of oligosaccharides from a column of Bio-Gel P-4. The column was eluted with 50mM pyridine-acetic acid (pH 5.4, 750 mL), and fractions of 2.5 mL were collected. Every third fraction was examined for the presence of carbohydrates by the phenol-sulfuric acid method[11]. Fraction A: tubes 39–63; Fraction B: tubes 70–93; and Fraction C: tubes 102–120.

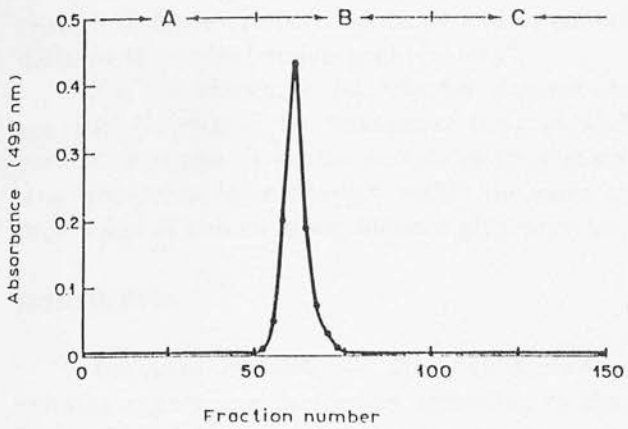

Fig. 2. Elution pattern of Fraction B from a column of DEAE-Sephadex A-25. The column was eluted with: (A) 50mM sodium monophosphate (pH 6.0, 150 mL); (B) a gradient of 0.05–0.5M lithium chloride in 50mM sodium monophosphate (pH 6.0, 150 mL); and (C) 0.5M lithium chloride in 50mM sodium monophosphate (pH 6.0, 150 mL). Fractions of 3 mL were collected, and every third fraction was examined for hexoses by the phenol–sulfuric acid procedure[11]. Fractions 50–75 were combined to give Fraction B-1.

O-3 and O-6. The resistance of the 2-acetamido-2-deoxy-D-glucose residues to periodate oxidation is consistent with the substitution at O-4 established by the methylation studies.

Structural study of the oligosaccharides obtained by degradation of the peri-ovulatory-phase glycoprotein has established that β-D-galactopyranose residues are substituted at O-3 by α-D-galactopyranose and at O-2 by L-fucopyranose, terminal residues[3], or at O-6 by 2-acetamido-2-deoxy-D-galactopyranose and at O-2 by L-fucopyranose terminal residues[7]. In addition, the 2-acetamido-2-deoxy-D-galactose residues linked to the serine or threonine residues of the protein backbone are substituted at O-3 by 2-acetamido-2-deoxy-D-glucopyranose chain residues and at O-6 by terminal residues of α-*N*-acetylneuraminic acid[9]. In contrast, the results of the methylation and periodate-oxidation studies of the pre-menstrual, main glycoprotein described in this article suggest that D-galactopyranose residues are substituted at O-6 (or O-2) by L-fucopyranose, D-galactopyranose, or 2-acetamido-2-deoxy-D-galactopyranose terminal residues and at O-3 by terminal residues of *N*-acetylneuraminic acid; and the 2-acetamido-2-deoxy-α-D-galactopyranose residues linked to serine (or threonine) are substituted at O-6 by a chain residue of 2-acetamido-2-deoxy-D-glucopyranose and at O-3 by terminal residues of L-fucopyranose, D-galactopyranose, or 2-acetamido-2-deoxy-D-galactopyranose. Thus, the major glycoproteins obtained from mucus of the peri-ovulatory and of the pre-menstrual phases differ mainly in the point of linkage of the *N*-acetylneuraminic acid terminal residues and of the substituents of the 2-acetamido-2-deoxy-D-galactopyranose residues linked to the protein chain. In addition, the glycoprotein component from the cervical mucus of the pre-menstrual phase differs from that of the peri-ovulatory

phase in being more heterogeneous and in containing several glycoproteins having different *N*-terminal amino acid residues[9].

It is not known, as yet, whether changes observed during the menstrual cycle are restricted only to the linkages of the carbohydrate residues or also occur in the protein backbone. It is also unclear as to whether the diversity in the carbohydrate structures arises from changes within the same glycoprotein macromolecule or the production of two or more different glycoproteins.

EXPERIMENTAL

Analytical methods. — Gas–liquid chromatography of reducing and non-reducing sugars was performed according to the procedure of Reinhold[10] with a Perkin–Elmer 900 gas chromatograph equipped with a dual-ionization detector. G.l.c.–mass spectrometry of the methylated sugars was performed with an analytical system consisting of an IBM-1800 computer fed with raw data generated by a single-focusing, Hitachi–Perkin–Elmer RMU-6 mass spectrometer interfaced with a Perkin–Elmer 900 gas chromatograph. The hexose content of the eluates from the columns of Bio-Gel P-4, AG 50W-X8, and DEAE-Sephadex was determined by the phenol–sulfuric acid method[11].

Preparation of oligosaccharides from pre-menstrual mucus glycoprotein. — The cervical mucus was collected (by aspiration with a suction pump) at times other than the mid-cycle estrogen surge. It was fractionated on columns of Bio-Gel P-200 and Sepharose 2B, as described for the preparation of peri-ovulatory glycoproteins[3,7]. The major glycoprotein (50 mg; for composition, see Table I), obtained from the Sepharose 2B column, was subjected to reductive β-elimination under conditions similar to those described by Iyer and Carlson[8]. After completion of the reaction, the pH of the solution was adjusted to 5.0 with 4M acetic acid and the solution applied to a column (2.2 × 60 cm) of AG 50W-X8 (H^+, 100–200 mesh) ion-exchange resin. The column was eluted with water (300 mL), and then with 10mM acetic acid (250 mL). The combined eluates were lyophilized, and the remaining boric acid was removed from the residue by repeated addition and evaporation of methanol. The residue (37 mg) was applied to a column (2.2 × 166 cm) of Bio-Gel P-4 (200–400 mesh), and the column was eluted with 50mM pyridine–acetic acid (pH 5.4, 750 mL). The carbohydrate-containing fractions (see Fig. 1) were combined and lyophilized to give Fraction A (6 mg), Fraction B (23 mg), and Fraction C (5 mg) (for composition, see Table I). Electrophoresis of Fraction B in 50mM sodium borate buffer (pH 9.5) at 1500V for 2 h, and staining with periodate–benzidine[12] (after spraying with 6M acetic acid) revealed a single, negatively charged component (2.8 cm). Paper chromatography in 1-butanol–pyridine–water (5:1:4, v/v, upper layer, Solvent A, $R_{Cellobiose}$ 0.13) and 1-butanol–propanol–0.1M hydrochloric acid (1:2:1, Solvent B, $R_{Cellobiose}$ 0.30) showed the presence of a periodate–benzidine-positive component with minimal tailing. A solution of the main oligosaccharide fraction (Fraction B, 21 mg) in 50mM sodium phosphate buffer (pH 6.0, 1 mL) was applied to a column

(1.5 × 40 cm) of DEAE-Sephadex A-25. The column was eluted with 50mM sodium phosphate (pH 6.0, 150 mL), and then with a gradient of 0.05–0.5M lithium chloride in 50mM sodium phosphate (pH 6.0, 150 mL), followed by 0.5M lithium chloride in 50mM sodium phosphate (pH 6.0, 150 mL). The carbohydrate-containing fractions (see Fig. 2) were combined, evaporated, and rechromatographed on a column (2.2 × 38 cm) of Bio-Gel P-2. The carbohydrate-containing fractions were combined and lyophilized to give Fraction B-1 (18 mg; for composition, see Table I). Fraction B-1, on chromatography in solvents A ($R_{Cellobiose}$ 0.15) and B ($R_{Cellobiose}$ 0.32), showed the presence of a single component.

Methylation of oligosaccharide Fraction B-1. — A portion of Fraction B-1 (10 mg) was methylated[13] in dimethyl sulfoxide (1 mL) with methylsulfinylsodium (1 mL) for 2 h at 22°, with subsequent addition of methyl iodide (0.5 mL). The solution was stirred for 3 h, and then diluted with water (25 mL) and lyophilized. The residue was extracted with 9:1 (v/v) chloroform–ethanol, and the solvent was evaporated off. The residue was dried *in vacuo* (phosphorus pentaoxide), and a portion of it (2 mg) was treated with 2M trifluoroacetic acid (1 mL) for 2.5 h at 105°. The solution was cooled and applied to a column (1 × 15 cm) of AG 1-X8 (OAc^-, 100–200 mesh) ion-exchange resin, and the column was eluted with water (15 mL), followed by 10mM acetic acid (10 mL). The combined eluates were lyophilized. The residue was acetylated with pyridine (0.5 mL) and acetic anhydride (0.3 mL), and the solution was evaporated. A solution of the residue in 9:1 (v/v) water–methanol (1 mL) was treated with sodium borohydride (10 mg) for 6 h at 4°. A further amount of sodium borohydride was added, and the solution was kept for 2 h at 22°. The remaining sodium borohydride was eliminated with 4M acetic acid, and the sodium ions were removed by treatment with AG 50W-X8 (H^+, 50–100 mesh) ion-exchange resin. After evaporation, boric acid was removed from the residue by repeated additions and evaporations of methanol (3 × 4 mL). The residue was treated with pyridine (1 mL) and acetic anhydride (0.65 mL) for 4 h at 22°. The resulting *O*-methylalditol acetates were estimated and identified by g.l.c.–m.s.

Mild acid-hydrolysis and remethylation of methylated oligosaccharide Fraction B-1. — The methylated oligosaccharide B-1 (5 mg) was treated with 50mM sulfuric acid for 75 min at 80°. The cooled solution was applied to a column (1 × 15 cm) of AG 1-X8 (OAc^-, 50–100 mesh) ion-exchange resin. The column was eluted with water (15 mL), and then with 5mM acetic acid (10 mL), and the combined eluates were lyophilized. The residue was methylated with iodomethane-d_3 (+99 atom%, Aldrich Chemical Co., Inc., Milwaukee, WI 53233) and methylsulfinylsodium, processed, hydrolyzed, and converted into the alditol acetates, which were identified and estimated by g.l.c.–m.s. (Table II), as just described.

Degradation by periodate oxidation–sodium borohydride reduction of oligosaccharide Fraction B-1. — Oligosaccharide Fraction B-1 (6 mg), in 0.1M sodium acetate (pH 4.5, 1.5 mL) containing 0.1M sodium metaperiodate, was kept in the dark for 16 h at 22°. The excess of sodium periodate was decomposed by the addition of 1,2-ethanediol (0.5 mL), and the salts were removed by gel filtration on a column

TABLE II

O-METHYL DERIVATIVES OBTAINED BY ACID HYDROLYSIS OF METHYLATED FRACTION B-1 OBTAINED FROM THE PREMENSTRUAL-PHASE GLYCOPROTEIN, BEFORE AND AFTER MILD ACID-TREATMENT, FOLLOWED BY DEUTERIOMETHYLATION

O-Methyl derivatives	*Molar ratio*	
	Before acid treatment[a]	*After acid treatment*
L-Fucose[b]		
2,3,4-	1.2	
D-Galactose[b]		
2,4-	0.6	
2,3,4-	Traces	9[d]
3,4,6-	0.6	
2,4,6-	Traces	
2,3,4,6-	0.6	1[d]
2-Acetamido-2-deoxy-D-glucose[c]		
3,6-	1.00	
2-Acetamido-2-deoxy-D-galactose[c]		
3,4,6-	1.00	
2-Acetamido-2-deoxy-D-galactitol[c]		
1,4,5-	0.4	
1,3,4,5-	0.8	
N-Acetylneuraminic acid[e]	1.0	0.3[f]

[a]Molar ratios relative to 2-acetamido-1,4,5-tri-*O*-acetyl-2-deoxy-3,6-di-*O*-methyl-D-glucitol. [b]Determined as alditol acetates and molar ratios calculated by g.l.c.[14]. [c]Determined as alditol acetates and molar ratio calculated by monitoring[15] *m/e* 158 or 116, and *m/e* 130 or 88. [d]Molar ratio of 3-*O*-[$^2H_3$]methyl-2,4,6-tri-*O*-methyl- to 3-*O*-[$^2H_3$]methyl-2,4-di-*O*-methyl-D-galactose. [e]Determined as methyl ester methyl glycosides by g.l.c. [f]Molar ratio relative to permethylated *N*-acetylneuraminic acid before acid treatment, and determined as in footnote *e*.

of Bio-Gel P-2. Carbohydrate-containing fractions were combined and lyophilized. A water solution of the residue was treated with sodium borohydride (8 mg) for 8 h at 4°, the remaining borohydride was eliminated by addition of 4M acetic acid, and the pH of the solution was adjusted to 5.4. The solution was applied to a column (0.8 × 12 cm) of AG 50W-X8 (H^+, 100-200 mesh) ion-exchange resin. The column was eluted with 5mM acetic acid (50 mL), the eluate was lyophilized, and the boric acid was removed from the residues by repeated additions and evaporations of methanol. The residue was treated with 0.5M hydrochloric acid (0.5 mL) for 8 h at 22°. The solution was applied to a column (0.8 × 16 cm) of AG 1-X8 (OAc^-, 200-400 mesh) ion-exchange resin. The column was eluted with water (20 mL) and the eluate lyophilized to give the periodate-oxidized oligosaccharide (for composition, see Table I).

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Carbohydrate Research, 85 (1980) C7–C9
Elsevier Scientific Publishing Company, Amsterdam – Printed in The Netherlands

Preliminary communication

Isolation and identification of spermatozoon-surface glycoproteins from *Macaca radiata* *

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(Received July 17th, 1980; accepted for publication, August 1st, 1980)

The motility of spermatozoa critically affects the process of reproduction. Despite their involvement in immunological infertility[1], the membrane-bound glycoproteins have not been structurally investigated, and their nature and composition remain unclear. Bonnet-monkey semen was obtained by electroejaculation[2] (0.25–0.4 mL), and kept for 1 h at 22° to allow liquefaction. The semen was then washed three times with 4% Tris hydrochloride (pH 7.0, 10 mL), and centrifuged at 500*g* to remove the seminal plasma and sperm-coating antigens. The supernatant liquor was removed, and spermatozoa (1.2×10^8), having a viability of 90% as judged by dye exclusion[3], were recovered from the pellet.

Sialic acid (260 μg/10^9 sperm) was detected[4] after treatment of the sperm with *V. cholerae* neuraminidase (Boehring, 150 U/mL) in 0.1M phosphate-buffered saline (PBS), pH 7.0, for 80 min at 37°. A suspension of the washed sperm in PBS (10 mL) containing TPCK-treated trypsin (Worthington, 20 μg/mL) was shaken for 30 min at 4°, and the sperm were then centrifuged at 500*g*. The procedure was repeated twice, the supernatant liquors were combined and dialyzed, and the retentate was lyophilized. Although human sperm are known to swell in trypsin solution[5], no alteration was detected under the mild conditions used in our experiments.

The residue was dissolved in 20mM phosphate buffer, pH 7.0 (0.5 mL), and treated with D-galactose oxidase (40 U) and peroxidase (150 U) for 12 h at 22°. The solution was then treated with 2M sodium boro[^{3}H]hydride (150 μL, 2 mCi) in mM

*Amino Sugars 125 and Sperm-Surface Glycoproteins I. This is publication No. 828 of the Robert W. Lovett Memorial Group for the Study of Diseases Causing Deformities, Harvard Medical School and Massachusetts General Hospital. This work was supported by a research grant from the National Institute of Child Health and Human Development (HD 12431), National Institutes of Health, U.S. Public Health Service.

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sodium hydroxide for 6 h at 4°, followed by addition of another batch of sodium boro-[^{3}H]hydride, and the mixture was incubated for 2 h at 22°. The pH of the solution was adjusted to 5.4 with 4M acetic acid, and the solution was dialyzed, and lyophilized, to obtain the sperm-surface glycoproteins. These were chromatographed on a column (2.1 × 48 cm) of Bio-Gel P-200 in 0.1M pyridine–acetic acid, pH 5.4 (300 mL). The tritium- and carbohydrate-containing (phenol–sulfuric acid[6]) fractions were combined, and lyophilized, to give the glycoproteins. The polymers contained carbohydrates and amino acids (see Tables I and II).

In a separate experiment, the glycoproteins from the sperm surfaces were isolated by treatment first with D-galactose oxidase–sodium boro[^{3}H]hydride, according to the method of Gahmberg and Hakomori[7], and then processed as described earlier.

TABLE I

CARBOHYDRATE COMPOSITION OF SPERM-SURFACE GLYCOPROTEINS OBTAINED BY CHROMATOGRAPHY ON A COLUMN OF BIO-GEL P-200

Sugar components[a]	*Molar ratio*[b]
L-Fucose	2.35
D-Galactose	1.00
D-Glucose	1.57
D-Mannose	7.14
2-Acetamido-2-deoxy-D-glucose	3.81
2-Acetamido-2-deoxy-D-galactose	0.52
Sialic acid[c]	1.70

[a] Determined by gas–liquid chromatography[12]. [b] Molar ratio relative to D-galactose. [c] Determined by the Warren procedure[4].

TABLE II

AMINO ACID COMPOSITION OF SPERM-SURFACE GLYCOPROTEINS OBTAINED BY CHROMATOGRAPHY ON A COLUMN OF BIO-GEL P-200

Amino Acids	*Residues/1000 residues*
Ala	75
Val	43
Gly	79
Ile	46
Leu	97
Pro	63
Thr	88
Ser	108
Phe	69
Asp	184
Glu	109
Lys	39

The elution profiles of the glycoproteins obtained by use of the alternative procedures (*i.e.*, first trypsinizing and then labeling, or, first labeling and then trypsinizing) were identical on a Bio-Gel P-200 column.

The carbohydrate composition of this glycoprotein is unlike that of either the *N*-glycosyl or the *O*-glycosyl type, as it contains 2-acetamido-2-deoxygalactose, as well as fucose and glucose, in addition to mannose, galactose, 2-acetamido-2-deoxyglucose, and sialic acid residues. However, in the protein moiety, a significant content of asparagine suggests a preponderant proportion of an *N*-glycosylated type of glycoprotein. The structure and function of sperm-surface glycoproteins are still unclear, and their contribution to HL-A antigens is controversial[8], although their significance in immunological infertility in males, as well as females, has been considerably emphasized[1,9–11].

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Biochimica et Biophysica Acta, 678 (1981) 483–496
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BBA 29826

STUDIES ON BONNET MONKEY CERVICAL MUCUS

THE EFFECT OF PROTEASES ON MUCUS GLYCOPROTEINS OF *MACACA RADIATA* *

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(Received March 25th, 1981)
(Revised manuscript received July 27th, 1981)

Key words: *Glycoprotein structure; Proteolysis; Mucin; (Monkey)*

The influence of proteinases on monkey cervical glycoproteins was investigated to assess their effect on cervical mucus and, thereby, on sperm penetration. The major component of periovulatory cervical mucus, a high molecular weight glycoprotein, was treated with Pronase, trypsin, chymotrypsin, papain, and bovine seminal peptidase, and the enzyme-resistant glycoprotein was purified by gel filtration on Sepharose 2B. A macromolecular component in high yield was recovered containing carbohydrate and protein moieties. Asialoglycoprotein, on treatment with Pronase, trypsin, and bovine seminal peptidase released more than one glycoprotein fragment. The carbohydrate and amino acid components of the native and degraded glycoproteins were similar in composition with variations in proportions. The structure of the carbohydrate-rich, pronase-resistant glycoprotein, further purified on Sepharose 2B, was examined. Sequential Smith degradation and methylation of the degraded glycoprotein fragment established a structure that shows some differences to that of the native glycoprotein. The influence of proteinases on cervical-mucus glycoproteins and a possible mechanism of sperm penetration through Pronase-treated glycoproteins is discussed.

Introduction

The mechanism of sperm penetration through the cervical mucus is of great importance in reproductive physiology and depends on the intrinsic motility of the sperm and receptivity of the mucus [2,3]. In addition, the proteolytic activity of the seminal fluid as well as that of spermatozoa has been considered essential for sperm penetration through the cervix [4].

Cervical mucus is a complex milieu produced continually by the endocervical cells. Minor quantities of endometrial, tubal, and possibly follicular fluids, may also contribute to the cervical mucus [5]. The mucus displays a number of rheological properties, such as viscosity, spinnbarkeit, flow elasticity, and stickiness, that are regulated by the ovarium function. The chemical and physical changes in the cervical mucus during the menstrual cycle not only influence penetration, but also nutrition and survival of the sperm. The cyclic alteration of the physical properties of the mucus are accompanied by variations in the carbohydrate composition [6,7].

The human seminal plasma contains a variety of proteolytic enzymes, and the presence of a trypsin-like enzyme in sperm head has been suggested [8]. Treatment of cervical mucus in vitro with commercial pancreatic trypsin or chymotrypsin enhanced sperm

* Cervical glycoproteins, Part VII; for Part VI see Ref. 1. Publication No. 000 of the Robert W. Lovett Memorial Group for the Study of Diseases Causing Deformities, Harvard Medical School, Massachusetts General Hospital, Boston, MA 02114 (U.S.A.).

migration and decreased the ferning, spinnbarkeit, viscosity, and gel structure properties [4]. In addition, the digestion of mid-cycle human cervical mucus with Pronase destroyed the filamentous structure of the mucus, suggesting degradation of the protein backbone or of the bridges between the glycoprotein fibrils [8]. Furthermore, it is likely that the escape of spermatozoa from the cervical crypts may result from the breakdown of the glycoprotein filaments by the sperm-head protease [8].

An understanding of the relationship between the macromolecular component of the cervical mucus and the physiological functions of this secretion, requires, in part, the characterization of the glycoproteins responsible for the biological role and morphological outlay of the mucus, and for their interaction with proteolytic enzymes.

Fractionation of periovulatory cervical mucus by gel-exclusion chromatography on Sepharose 2B produced two high molecular weight components. The major component was a carbohydrate-rich glycoprotein of the mucin type [9,10]. An antibody raised in rabbits against this glycoprotein reacted positively with the periovulatory cervical mucus [11], showing a diffuse as well as a sharp precipitin line. In immunoelectrophoresis, two precipitin lines were observed [12]. The antibody reacted with the periovulatory mucus, which displays fibrillar glycoprotein channels, on a glass slide as well as in a tube [11]. In either case, the precipitate under a microscope exhibited a morphology that was different from that of the periovulatory mucus. The morphological change induced by the antibody clearly suggests that the mucus channels are lines with glycoproteins and, thus, indicates the physiological role played by the glycoproteins in the mucus.

In order to understand the interaction between the cervical mucus and proteolytic enzymes, and their influence on sperm penetration, purified glycoproteins and neuraminidase-treated glycoproteins from the periovulatory phase mucus were treated with Pronase, trypsin, chymotrypsin, papain, and bovine seminal peptidase. The detailed structure of the Pronase-treated glycoprotein was investigated and compared with that of the intact glycoprotein.

Materials and Methods

Collection of cervical mucus. The cervical mucus of the bonnet monkey was collected by aspiration with a suction pump at the time of mid-cycle estrogen surge (periovulatory phase). The secretion was promptly frozen and maintained in the frozen state until use.

Analytical methods. The hexose content was estimated by the phenol-sulfuric acid method, the protein content by measuring the absorbance at 280 nm or by using the procedure of Lowry et al. [13], and the sialic acid content by the thiobarbituric acid method of Warren [14], after acid hydrolysis with 50 mM sulfuric acid, or by GLC [15]. All solutions were dialyzed in hydrogencarbonate-treated cellulose tubing at 4°C.

GLC. GLC determinations of carbohydrate residues of the glycoproteins were performed according to the procedure of Reinhold [15], with a Perkin-Elmer 900 chromatograph, equipped with a dual ionization-detector. GLC-MS of the methylated sugars was performed with an analytical system consisting of an IBM-1800 computer, fed with raw data generated by a single-focusing, Hitachi-Perkin-Elmer RMU-6 mass spectrometer interfaced with a Perkin-Elmer 900 gas chromatograph.

Column chromatography. Bio-Gel P-200 (Bio-Rad Laboratories) and Sepharose 2B (Pharmacia Fine Chemicals) columns were run in 50 mM sodium monophosphate (pH 7.0), and 5 mM Tris-HCl (pH 7.5), respectively. The DEAE-cellulose (Whatman) column was eluted with 50 mM sodium phosphate (pH 6.8, 100 ml), followed by a gradient of 0.1–1 M LiCl (200 ml), and finally with 100 ml of 1 M LiCl containing 10 mM HCl.

Gel electrophoresis. Agarose-gel electrophoresis was performed in 50 mM barbital buffer (pH 8.2) on slides containing 1% agarose gel. Polyacrylamide agarose gel electrophoresis was performed according to the method of Holden et al. [16]. The polyacrylamide gels and agarose slides were stained with Amido black or Coomassie blue, and with the periodate-Schiff reagent. For agarose electrophoresis, approx. 0.3 mg per ml of each substance was used, and each well had 15 µl of the solution.

Purification of the mucus glycoproteins. The crude mucus was partially solubilized in 50 mM sodium

monophosphate (pH 7.0) containing 0.02% sodium azide by stirring for 16 h. The cellular debris and other suspended materials were removed by centrifugation (2500 rev./min), the supernatant was dialyzed, and the nondialyzable material was lyophilized to give the crude mucus glycoproteins. This material (0.5 g) was solubilized in 50 mM sodium monophosphate (150 ml, pH 7.0) containing 0.02% sodium azide by stirring overnight at 4°C. The solution was applied to a column (5 × 80 cm) of Bio-Gel P-200 (50–100 mesh). The carbohydrate- and protein-containing fractions were pooled, the pH was adjusted to 5.5 with 4 M acetic acid, and the solution was extensively dialyzed and then lyophilized to give the purified mucus glycoprotein.

Fractionation of the purified glycoprotein. The purified 20 mg glycoprotein, in 5 ml phosphate buffer was applied to a column (1.5 × 48 cm) of Sepharose 4B. Fractions containing carbohydrates and proteins were combined, dialyzed, and lyophilized. Two fractions, Fraction A and Fraction B, were obtained.

Enzyme degradations

Pronase treatment of the purified glycoprotein (50 mg) was performed in 50 mM sodium monophosphate buffer (15 ml; pH 8.0) containing 0.1% sodium azide with unsolubilized Pronase (15 mg, Enzite) protease, Miles Laboratories, Inc.). The mixture was stirred at 22°C until the glycoprotein was dissolved (2–3 h), and then incubated for 6 days at 37°C with stirring. A further addition of the prewashed enzyme (3 mg) was made and the solution incubated for another 3 days. The suspension was centrifuged, and the residue was washed with the buffer. The pH of the supernatant solution was adjusted to 5.0 with acetic acid and the solution dialyzed against distilled water. The nondialyzable material was lyophilized, and a proportion of the residue (20 mg) was applied to a column (1.5 × 48 cm) of Sepharose 2B. Fractions containing carbohydrate and protein were combined and dialyzed, and the retentate was lyophilized. The Pronase-degraded glycoprotein was chromatographed on a column of DEAE-cellulose, the carbohydrate- and protein-containing fractions eluted with a gradient of lithium chloride were combined and dialyzed, and the nondiffusable solution was lyophilized to give the Pronase-degraded glycoprotein.

Trypsin treatment of the purified mucus glycoprotein (20 mg) was performed in 10 ml 40 mM Tris-HCl buffer, pH 8.2, with 1 mg trypsin (Worthington Biochemical Corp., activity 180–220 units per mg protein). After incubation for 20 h at 37°C, another portion of trypsin (0.2 mg) in 0.5 ml 40 mM Tris-HCl buffer, pH 8.2, was added, and the mixture was further incubated for 10 h. Trypsin was inactivated by the addition of an 8-fold excess of *N*-α-*p*-tosyl-L-lysine chloromethyl ketone (TLCK, Sigma), the solution was acidified to pH 4.5 with 1 M HCl, and dialyzed, and the nondialyzable material was lyophilized. The residue was applied to the Sepharose 2B column.

The purified glycoprotein (20 mg) was treated with 0.5 ng α-chymotrypsin (Worthington Biochemical Corp., 45–70 units per mg protein) in 0.1 M sodium phosphate buffer, pH 7.0, (5 ml) at 37°C for 24 h. The pH was adjusted to 4.5 with 1 M HCl, and the reaction mixture was dialyzed. The nondiffusable solution was lyophilized, and the residue was applied to the Sepharose 2B column. The carbohydrate- and protein-containing fractions were combined, dialyzed, and lyophilized to give the chymotrypsin-degraded glycoprotein.

The purified glycoprotein was treated with papain in 0.1 M sodium acetate buffer, pH 6.1, (5 ml) containing 5 mM cysteine-HCl and 5 mM dithiothreitol, 1 mM EDTA, and 0.5 mg papain (Sigma Chemical Co., 10–15 units per mg protein) for 16 h at 37°C. After a further addition of 0.25 mg enzyme, 13 mg cysteine-HCl, and 11.5 mg dithiothreitol, the mixture was further incubated for 12 h at 37°C. The pH was adjusted to 4.5, the reaction mixture was dialyzed, and the nondialyzable material was lyophilized. The residue was chromatographed on the Sepharose 2B column, and fractions containing both carbohydrate and protein were combined, dialyzed, and lyophilized to give the papain-degraded glycoprotein.

The purified glycoprotein (20 mg) was treated with 0.425 mg bovine seminal peptidase in 50 mM sodium phosphate buffer, pH 6.8, (15 ml) for 16 h at 37°C. The bovine peptidase from seminal plasma was purified by ammonium sulfate precipitation and chromatography on Bio-Gel P-200 (see Fig. 1 and Table I). After another addition of bovine seminal peptidase (0.425 mg), the mixture was incubated for 12 h. The solution was dialyzed, the nondialyzable material was lyophilized, and the residue was

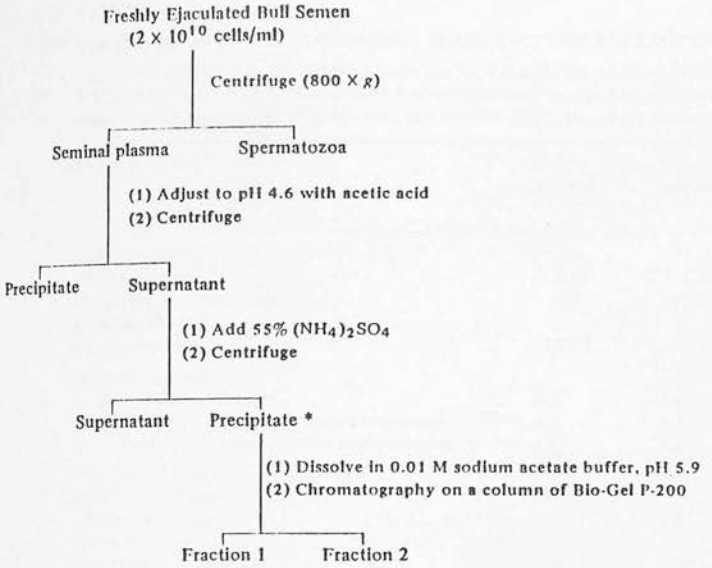

Fig. 1. Purification procedure of bovine seminal peptidase. * Ultrafiltration and pressure dialysis at 4°C using P-10 membranes.

chromatographed on the Sepharose 2B column.

Siliac acid was removed from 50 mg glycoprotein Fraction A by treatment with neuraminidase (*Vibrio cholerae*, 0.5 ml; 250 units, Behring Diagnostics) in 0.1 M sodium acetate (25 ml) containing 0.1% calcium chloride and 0.5% sodium chloride, pH 5.5, for 24 h at 37°C. After a further addition of neuraminidase (100 µl), the solution was incubated for another 10 h. The reaction was terminated by immersing the mixture in a boiling-water bath for 3 min. The mixture was dialyzed, and the nondialyzable material was lyophilized. The residue in 0.1 M sodium acetate (pH 5.0, 15 ml) was incubated at 37°C for 32 h with neuraminidase (2 mg, *Clostridium perfringens*, Sigma Chemical Co.; Type VI). The mixture was dialyzed, and the nondialyzable material was lyophilized to give the asialoglycoprotein. Degradation of the asialoglycoprotein with Pronase, trypsin, and bovine seminal peptidase was performed in a manner similar to that described for the purified glycoprotein.

End-group analysis. The amino-terminal end-group was determined with the dansyl chloride method. Enzyme-degraded glycoproteins (0.4–0.6 mg) were dansylated and hydrolyzed according to the procedure of Gros and Labouesse [17]. The dansylated amino acids were separated and identified by chromatography on polyamide plates.

Periodate oxidation-sodium borohydride reduction of the Pronase-degraded glycoprotein. The Pronase-degraded glycoprotein (35 mg) was oxidized with 0.1 M sodium metaperiodate (6 ml) for 16 h at room temperature in the dark. The excess of periodate was destroyed with 1,2-ethanediol, and the solution was dialyzed against distilled water. The nondialyzable material was lyophilized, and then treated with 15 mg sodium borohydride for 4 h at 4°C. A fresh portion of 10 mg sodium borohydride was added. After the solution had been kept for another 8 h at 4°C, the excess of borohydride was eliminated with 4 M acetic acid (to pH 4.5), the solution was dialyzed, and the nondialyzable material was lyophilized. The residue was treated with 0.25 M sulfuric acid for 2 h at 22°C, then the solution was dialyzed, and the nondiffusable solution was lyophilized to give the periodate-resistant glycoprotein.

Second periodate oxidation. The periodate-resis-

TABLE I

ENZYMATIC ACTIVITIES OF SEMINAL PLASMA PEPTIDASE FRACTIONS

Values are expressed as μM substrate/min per mg protein. Bio-gel fractionation was carried out using 0.01 sodium acetate buffer/0.2 M NaCl, pH 5.9. Fractions 1 and 2 were combined in equal amounts and were used as bovine seminal peptidase. n.d., not done; high blanks prohibited assay. n.a., not assayed. GOT, glutamate oxaloacetate transaminase.

Enzymic activity	Crude seminal	95% Ammonium sulfate fraction	Bio-Gel P-200 fractionation	
			Fraction 1	Fraction 2
α-D-Mannosidase	1.2	2.37	0.1	1.0
β-D-Mannosidase	0.05	0.007	0.0	0.0
N-Acetyl-β-D-glucosaminidase	5.9	42.2	6.1	8.5
β-D-Galactosidase			2.6	8.2
α-L-Fucosidase	0.02	0.1	0.0	0.0
Peptidase substrates				
Bovine serum albumin	n.d.	n.d.	0.0	15.0
N-(Carbobenzyloxy)-L-glutamyl-L-tyrosine	n.d.	n.d.	19.8	15.5
N-(Carbobenzyloxy)-glycyl-L-phenylalanine	n.d.	n.d.	6.7	11.6
GOT (2,4-dinitrophenylhydrazine) [a]	n.d.	n.d.	2.42	0.473
GOT (coupled transaminase)	n.d.	2.3	83.0	n.d.
Alkylphosphatase	n.d.	n.d.	0.38	0.0
Hyaluronidase [a]	0.8	4.3	5.3	18.0
Lysozyme [b]	0.21	4.7	0.0	0.0
Phosphoglucoisomerase	3.1	3.3	0.0	0.0
α-Amylase [c]	n.d.	682	0.2	0.0
Protein [d]	168	20.6	0.891	0.254
Neutral carbohydrate [e]	n.d.	n.d.	0.328	0.44

[a] Units of μg substrate hydrolyzed/min per mg protein.
[b] Units expressed as $(\Delta A \times 0.001)$/min.
[c] Seminal plasma gave peculiarly high background, reducing values.
[d] Maximal absorbance at 250 nm with bovine serum albumin standard ($E^{1\%}_{1cm}$ 6.6).
[e] Phenol/sulfuric acid method [30], mg/ml.

tant glycoprotein (18 mg) was dissolved in 0.1 M sodium metaperiodate (5 ml), and the solution kept for 24 h at 22°C in the dark. The solution was processed as described for the first treatment. The sequentially periodate-treated glycoprotein (5 mg) was methylated as described below.

Methylation analysis. After chromatography on Sepharose 2B the Pronase-degraded glycoprotein (60 mg) was treated in freshly distilled formamide with acetic anhydride for 24 h at 4°C, thereafter dialyzed, and the nondiffusable solution was lyophilized. The Pronase-resistant, acetylated glycoprotein was dissolved in 3 ml dimethyl sulfoxide and methylated with methyl iodide in the presence of methylsulfinyl anion [18]. The methylated product was isolated by dialysis and lyophilization. The residue (55 mg) was methylated again as just described to give the methylated glycoprotein (49 mg).

Analysis. Calculated for a fully methylated polymer: OCH_3 17.0. Found: OCH_3 16.75.

A solution of the methylated glycoprotein (40 mg) in 0.5 M pyridine/acetic acid (20 ml, pH 5.4) was applied to a column (2 × 54 cm) of Bio-Gel P-200 (100–200 mesh). The column was eluted with 0.5 M pyridine/acetic acid (500 ml, pH 5.4). The carbohydrate-containing fractions were combined and lyophilized. A portion of the residue (15 mg) in 2 M trifluoroacetic acid (3 ml) was heated at 110°C for 3 h, diluted with 125 ml water, and lyophilized. A solution of the residue in 20% aqueous methanol was passed through a column of Dowex 1-X8 (acetate). The effluent was evaporated, and the residue was

N-acetylated with 0.5 ml acetic anhydride in 5 ml methanol. The residue in aqueous methanol was treated with 20 mg sodium borohydride for 8 h at 4°C. The excess of borohydride and sodium ions was eliminated by treatment with Dowex 50W-X8 (H^+). After removal of the borate ions as methyl borate, the residue was treated with 4 ml pyridine and 2 ml acetic anhydride for 8 h at 22°C, and the solution was evaporated to dryness under reduced pressure. The alditol acetates were examined by GLC-MS.

Results

Purification of glycoproteins

The mucus glycoproteins were eluted in the void volume of the Bio-Gel P-200 column. The agarose polyacrylamide and agarose electrophoresis indicated the presence of periodate-Schiff and Coomassie blue-positive components, although the components had the minimal entry into the gels. The Bio-Gel P-200-purified mucus afforded two components on Sepharose 2B chromatography, a glycoprotein component (85%), and a mixture of glycoprotein and proteins (10%). The glycoprotein component showed, in agarose-polyacrylamide and agarose electrophoresis, the presence of a single periodate-Schiff and Coomassie blue-staining material, and the absence of contaminating proteins.

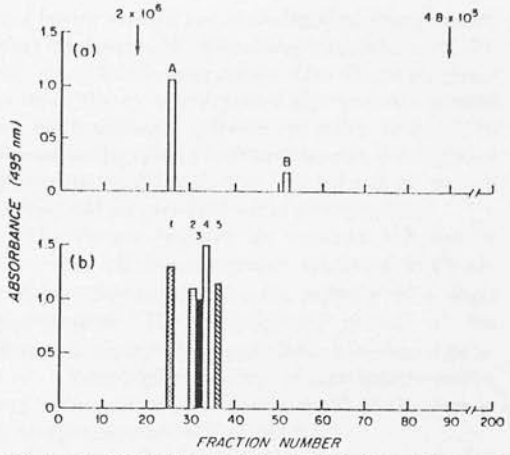

Fig. 2. (a) Fractionation of the purified glycoprotein on a column (1.6 × 46 cm) of Sepharose 2B. Two glycoprotein fractions, A and B, were obtained. (b) Chromatography of the Pronase-, trypsin-, chymotrypsin-, papain-, and bovine seminal peptidase-degraded non-dialyzable material on the Sepharose 2B column. Fractions of 2 ml were collected and every third fraction was examined for carbohydrates by the phenol/sulfuric acid procedure [26]. Bars 1, 2, 3, 4, and 5 show the peak eluted from the Sepharose 2B column that contained the maximum amount of carbohydrate in Pronase-, chymotrypsin-, papain-, trypsin-, and bovine seminal peptidase-treated glycoproteins, respectively. The column was calibrated with Blue Dextran (M_r 2 000 000) and apoferritin (M_r 480 000).

TABLE II

CARBOHYDRATE COMPOSITION OF INTACT CERVICAL MUCUS PERIOVULATORY-PHASE GLYCOPROTEINS, AND PRONASE-, TRYPSIN-, CHYMOTRYPSIN-, PAPAIN-, AND BOVINE SEMINAL PEPTIDASE-TREATED GLYCOPROTEINS, OBTAINED FROM SEPHAROSE 2B COLUMNS

Ratio is relative to the same sugar in the intact glycoprotein.

Carbohydrate	Intact glycoprotein		Glycoprotein fractions			
			A		B	
	(%)	(Ratio)	(%)	(Ratio)	(%)	(Ratio)
L-Fucose	7	1.00	7	1.00	6	0.85
D-Galactose	19	1.00	22	1.15	18	0.94
N-Acetylglucosamine	12	1.00	9	0.75	7	0.58
N-Acetylgalactosamine	17	1.00	17	1.00	14	0.82
N-Acetylneuraminic acid	9	1.00	11	1.22	4	0.44
Sulfate	1.0		1.1			
Total carbohydrate	64		66		49	

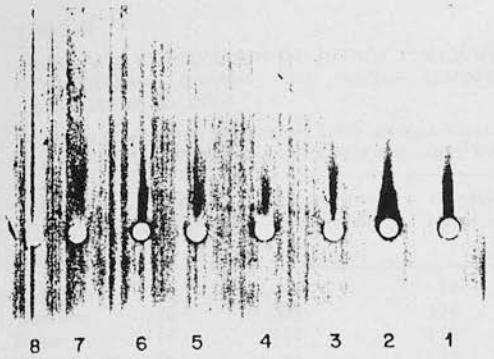

Fig. 3. Electrophoresis, in agarose, of purified and enzyme-degraded glycoproteins stained with the periodate-Schiff reagent. Wells 1 and 2 contained the purified glycoprotein; wells 3, 4, 5, 6 and 7 the Pronase-, trypsin-, chymotrypsin-, papain-, and bovine seminal peptidase-degraded glycoproteins, respectively, and well 8 the Bromphenol Blue marker dye.

Purification, and physical and chemical characterization of enzyme-degraded glycoproteins.

The nondialyzable portion of the digests obtained by Pronase, trypsin, chymotrypsin, papain and bovine seminal peptidase treatment gave a single component on purification by chromatography on a column of Sepharose 2B (Fig. 2), calibrated with native glycoprotein. The material that gave a positive reaction for neutral sugars and protein was eluted from the column (Fig. 2(b)) in the vicinity where Fraction A of the native glycoprotein was eluted. The recoveries of the Pronase-, trypsin-, chymotrypsin-, papain-, and bovine seminal peptidase-degraded glycoproteins, after Sepharose 2B chromatography, where 80, 70, 69, 75, and 74%, respectively. Like the native glycoprotein, the enzyme-degraded glycoproteins showed in electrophoresis minimal mobility (Fig. 3). In agarose-polyacrylamide electrophoresis, the degraded glycoproteins did not enter the gel and no protein component was detected with Coomassie blue.

The Pronase-degraded glycoprotein obtained by Sepharose 2B chromatography exhibited, in DEAE-cellulose chromatography, the presence of a single glycoprotein. The carbohydrate moiety of the degraded glycoprotein contained L-fucose, D-galactose, *N*-acetylglucosamine, *N*-acetylgalactosamine, and sialic acid residues with a significant increase in *N*-acetylneuraminic acid (Table II).

The main action of the various enzymes resulted in a loss of amino acids, as shown by the increase in total carbohydrate content, but the carbohydrate chains lost through protease activity were not the same for all enzymes. The most striking difference was a decrease in the content of fucose after trypsin treatment, and of *N*-acetylneuraminic acid after trypsin and chymotrypsin treatment, and some increase in the content of galactose, *N*-acetylglucosamine, and *N*-acetylgalactosamine after trypsin, chymotrypsin, and bovine seminal peptidase treatment (Table II).

The amino acid composition of the degraded glycoproteins was similar to that of the native glycoprotein, except for variations in the relative ratios of the amino acids (Table III). A significant increase in threonine in the case of Pronase-, trypsin- and chymo-

TABLE II (continued)

Pronase-treated		Trypsin-treated		Chymotrypsin-treated		Papain-treated		Bovine seminal, peptidase-treated	
(%)	(Ratio)	(%)	(Ratio)	(%)	(Ratio)	(%)	(Ratio)	(%)	(Ratio)
8	1.14	6	0.85	9	1.28	8	1.142	7	1.00
21	1.10	27	1.42	27	1.42	22	1.15	22	1.15
9	0.75	13	1.08	13	1.08	12	1.00	11	1.22
19	1.11	24	1.41	24	1.41	18	1.05	17	1.00
16	1.77	8	0.88	7	0.77	12	1.33	12	1.33
1.2		1.1		1.2		1.1		1.1	
73		78		80		72		69	

TABLE III

AMINO ACID COMPOSITION OF INTACT PERIOVULATORY-PHASE GLYCOPROTEINS, AND PRONASE-, TRYPSIN-, CHYMOTRYPSIN-, PAPAIN-, AND BOVINE SEMINAL PEPTIDASE-TREATED GLYCOPROTEIN, OBTAINED FROM SEPHAROSE 2B COLUMN

Results are expressed as residues per 1000 residues. Papain-treated glycoprotein was determined by GLC as *N*-trifluoroacetyl-*n*-butyl ester. All the other glycoproteins were determined by amino acid analyzer using a Beckman 121-MB instrument.

Amino acids	Intact glycoprotein	Pronase-treated glycoprotein	Trypsin-treated glycoprotein	Chymotrypsin-treated glycoprotein	Papain-treated glycoprotein	Bovine seminal peptidase-treated glycoprotein
Aspartic acid	43	42	16	20	72	54
Threonine	226	306	378	367	231	254
Serine	114	117	119	126	127	104
Glutamic acid	86	63	39	43	104	72
Proline	82	92	103	103	73	88
Glycine	83	92	79	85	71	79
Alanine	89	102	102	104	90	95
Cysteine/2	14	–	–	–	–	–
Valine	50	27	26	26	55	47
Methionine	–	17	18	17	–	18
Leucine	73	63	50	51	69	70
Isoleucine	49	51	51	52	32	46
Tyrosine	20	–	–	–	–	–
Phenylalanine	20	–	–	–	24	–
Lysine	21	11	8	3	52	34
Histidine	14	–	3	3	–	15
Arginine	16	17	8	–	–	24

TABLE IV

CARBOHYDRATE COMPOSITION OF PRONASE-, TRYPSIN-, AND BOVINE SEMINAL PEPTIDASE-DEGRADED FRACTIONS OF ASIALOGLYCOPROTEINS FROM SEPHAROSE 2B COLUMNS

Ratio is relative to the same sugar present in the neuraminidase-treated glycoprotein.

Carbohydrate	Cervical glycoprotein after treatment with neuraminidase		Pronase-treated glycoprotein fractions			
			F_1		F_2	
	(%)	(Ratio)	(%)	(Ratio)	(%)	(Ratio)
L-Fucose	7	1.00	9	1.28	8	1.14
D-Galactose	28	1.00	27	0.96	26	0.92
N-Acetylglucosamine	11	1.00	14	1.27	13	1.18
N-acetylgalactosamine	20	1.00	25	1.25	24	1.20
N-acetylneuraminine acid	3	1.00	5	1.66	4	1.33
Sulfate	1.2		1.1		0.98	
Total carbohydrate	69		80		75	

trypsin-degraded glycoprotein was observed, which was concommitant with the increase in the carbohydrate moeity. Also, a fair decrease in valine was noted, indicating that a portion of the peptide moeity containing this amino acid was eliminated. Lysine, glycine, and trace proportions of serine were detected as amino terminal amino acids for the trypsin- and chymotrypsin-degraded glycoproteins; in addition, the former compound showed a trace proportion of threonine. Papain- and bovine seminal peptidase-degraded glycoproteins showed the presence of lysine, valine, and alanine as amino terminal amino acids but alanine (in the case of papain) and valine (in the case of bovine seminal peptidase-degraded glycoprotein) were present only in trace proportions. The Pronase-digested glycoprotein showed glycine and threonine as amino terminal amino acids, but purification by DEAE-cellulose chromatography reduced greatly the proportion of threonine.

Preparation and enzyme degradation of asialoglycoprotein

Glycoprotein Fraction A contained 66% (Table II) carbohydrate and only three-fourths of the sialic acid component could be removed by successive treatments with *Virbrio cholerae* and *Clostridium perfringens* neuraminidase (Table IV). Pronase, bovine seminal peptidase, and trypsin degraded the asialoglycoprotein. The Pronase- and trypsin-degraded materials afforded two asialoglycoprotein fractions on Sepharose 2B chromatography, whereas bovine seminal peptidase released three asialoglycoprotein fractions (Fig. 4). In agarose electrophoresis, the enzyme-degraded asialoglycoprotein fractions entered the gel and reacted positively with the periodate-Schiff reagent (Fig. 5). The Pronase- and bovine seminal peptidase-degraded asialoglycoprotein fractions showed an increase in the carbohydrate content, whereas the trypsin-degraded fractions showed a

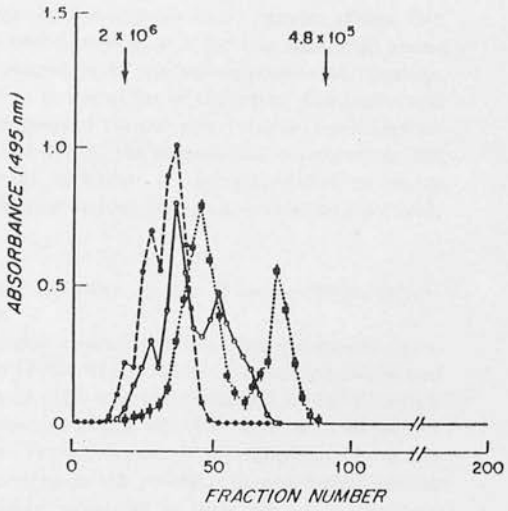

Fig. 4. Gel chromatography of the Pronase- (•------•); trypsin- (∮------∮); and bovine seminal peptidase- (o——o) degraded asialoglycoprotein on the column of Sepharose 2B. Fractions of 2 ml were collected and every third fraction was examined for presence of carbohydrates.

Trypsin-treated glycoprotein fractions				Bovine-seminal peptidase-treated glycoprotein fractions					
F_1		F_2		F_1		F_2		F_3	
(%)	(Ratio)	(%)	(Ratio)	(%)	(Ratio)	(%)	(Ratio)	(%)	(Ratio)
7	1.00	4		7	0.57	8	1.14	5	0.71
21	0.75	16		20	0.71	25	0.89	22	0.78
11	1.00	8	10	0.90	14	1.27	11	1.00	
19	0.95	15		16	0.80	24	1.20	20	1.00
7	2.33	2		3	1.00	5	1.66	4	1.33
1.2		1.1		1.2		1.1		1.0	
65		45		56		76		62	

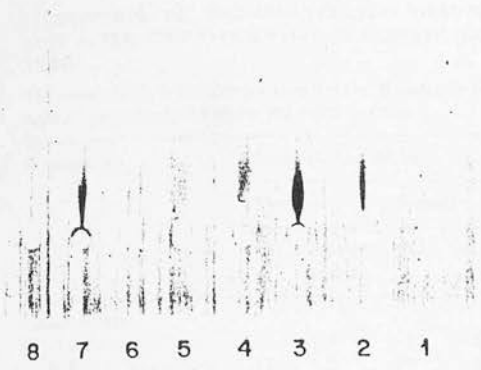

Fig. 5. Electrophoresis, in agarose of Pronase-, trypsin-, and bovine seminal peptidase-degraded asialoglycoprotein. Wells 2 and 3 contained Pronase-treated fractions 1 and 2, wells 4 and 5 trypsin-treated fractions 1 and 2, wells 6 and 7 bovine seminal peptidase-degraded fractions 2 and 3, and wells 1 and 8 the Bromophenol Blue marker dye.

decrease in the carbohydrate content (Table IV). Serine and threonine were the two significant amino acids present in all the asialoglycoprotein fractions, as well as in the native glycoprotein. The amino acid compositions of the asialoglycoprotein fractions were similar to that of the original asialoglycoprotein, but significant variations in the proportions of amino acids among various fractions were observed (Table V).

Chemical structure of the Pronase-degraded glycoprotein

Periodate oxidation of the Pronase-degraded glycoprotein (Table II) eliminated completely L-fucose and sialic acid, 62% of D-galactose, and 53% of *N*-acetylgalactosamine (Table VI). All the amino acids present in the Pronase-degraded glycoprotein (Table III) were present in the periodate-degraded glycoprotein with minor variations in their relative proportions, except for proline which showed a significant

TABLE V

AMINO ACID COMPOSITION OF PRONASE-, TRYPSIN-, AND BOVINE SEMINAL PEPTIDASE-DEGRADED FRACTIONS OF ASIALOGLYCOPROTEINS FROM SEPHAROSE 2B COLUMNS

Results are expressed as Il Residues per 1 000 residues.

Amino acids	Asialo-glycoprotein	Pronase-treated glycoprotein fractions		Trypsin-treated glycoprotein fractions		Bovine seminal peptidase-treated glycoprotein fractions		
		F_1 [a]	F_2 [a]	F_1 [b]	F_2 [a]	F_1 [b]	F_2 [b]	F_3 [b]
Aspartic acid	47	32	51	68	121	18	17	67
Threonine	209	317	263	200	136	289	353	226
Serine	100	116	111	85	133	60	94	130
Glutamic acid	60	63	66	81	100	45	49	67
Proline	86	94	101	103	77	125	112	92
Glycine	68	90	86	65	146	93	57	101
Alanine	81	99	100	124	76	153	124	116
Valine	25	33	43	80	60	55	50	59
Methionine	–	17	17	–	12	–	–	–
Leucine	71	59	62	86	64	61	51	67
Isoleucine	152	54	39	42	49	73	63	37
Phenylalanine	trace	–	–	30	–	8	8	23
Lysine	43	11	26	36	21	20	22	15
Histidine	14	2	15	–	–	–	–	–
Arginine	43	13	20	–	5	–	–	–

[a] Determined by amino acid analyzer using a Beckman 121-MB instrument.
[b] Determined by gas-liquid chromatography as *N*-triflyoroacetyl-*n*-butyl ester.

TABLE VI

COMPOSITION OF PRONASE-TREATED GLYCOPROTEIN AFTER TWO SEQUENTIAL PERIODATE OXIDATIONS

The molar ratio is relative to D-galactose. Results of amino acids are expressed as residues per 1 000 residues.

Components	Sequential oxidation			
	First		Second	
	(%)	(Molar ratio)	(%)	(Molar ratio)
Carbohydrates				
D-Galactose	8	1.0	8	1.0
N-Acetylglucosamine	12	1.2	10	1.0
N-Acetylgalactosamine	9	0.9	9	0.9
Amino acids				
Aspartic acid				39
Threonine				308
Serine				139
Glutamic acid				72
Proline				30
Glycine				72
Alanine				107
Cysteine/2				3
Valine				41
Leucine				54
Isoleucine				53
Tyrosine				7
Phenylalanine				26
Lysine				15
Histidine				14
Arginine				20

decrease. Methylation of the periodate-degraded glycoprotein showed 2,3,4,6-tetra- and 2,4,6-tri-*O*-methylgalactose; 3,4,6-tri- and 3,6-di-*O*-methyl-2-(*N*-methylacetamido)glucose; and 4,6-di-*O*-methyl-2-(*N*-methylacetamido)galactose. Methylation of the Pronase-treated glycoprotein gave a methylated derivative (85% yield) having a high methoxyl content as a single component from Bio-Gel P-200 chromatography. Hydrolysis, followed by reduction of the glycoprotein indicated the presence of 2,3,4-tri-*O*-methylfucitol; 2,3,4,6-tetra-, 2,4,6-tri, 4,6-di-, and 2,6-di-*O*-methylgalactitol; 2-deoxy-3,4,6-tri- and 4-*O*-methyl-2-(*N*-methylacetamido)galactitol; and 2-dexoy-3,6-di-*O*-methyl-2-(*N*-methylacetamido)-glucitol (Table VII).

Discussion

In the present investigation, the mucus was collected from several monkeys at the time of estrogen surge. The chromatographic and electrophoretic behavior of the enzyme-degraded glycoproteins indicate that they represent high molecular weight glycoproteins. The change in the molecular size of the papain-treated glycoprotein, as indicated by gel filtration (Fig. 2(b)), may have arisen because of the mucolytic agents present in the buffer. However, the low concentration of these reagents at the pH used would appear to have no significant effect on the disulfide bonds [19]. On electrophoretic analysis in 1% agarose, the carbohydrate-containing material barely entered the gel, and in 0.5% agarose/1.5% polyacrylamide, it did not enter the gel. Holden et al. [16] have observed a similar behavior for several mucins. The recovery after protease treatment (approx. 70--80%) indicates that all enzymes degraded the original glycoprotein to a similar, limited extent, suggesting that some portions are relatively easily accessible, whereas the remaining are well protected by the carbohydrate chains. Furthermore,

TABLE VII

PRODUCTS OF METHYLATION OF THE PRONASE-TREATED GLYCOPROTEIN

O-Methyl derivatives of:	Methyl group substitution at C-							
	-2, 3, 4, 6	-2, 3, 4	-2, 4, 6	-3, 4, 6	-3, 6	-2, 4	-4, 6	4
L-Fucose		+						
D-Galactose	+		+			+	+	
N-Acetylglucoseamine					+			
N-Acetylgalactosamine				+				+

the size of the macromolecule in gel filtration on Sepharose 2B as well as the carbohydrate analysis suggest some differences in the degraded glycoproteins. Minimal degradation of Fraction A glycoprotein was observed with Pronase (Fig. 2(b)), but other proteinases showed enhanced effects. The carbohydrate component of the degraded glycoproteins contained all the sugars present in the native glycoprotein, and represented nearly 90% of the sugars in the starting material, suggesting that only a small proportion of sugar residues were linked to peptides eliminated by proteolysis. Donald [20] has observed a similar result for the degradation of bloodgroup active glycoproteins by Pronase. Proteolytic degradation of bovine cervical mucus, in addition to human cervical mucus [4,8], has been studied by use of several enzymes after various chemical modifications [21–23]. The data obtained by Gibbons and Selwood [21] by papain digestion of the glycoprotein, after reduction of the disulfide bonds and alkylation, suggest a general increase in the carbohydrate components similar to that observed in the present investigation. The Pronase treatment generated glycoproteins that seemed to differ mainly in the relative proportion of carbohydrate residues, particularly for the sialic acid and *N*-acetylglucosamine content as well as N-terminal amino acids. These observations may be explained by the presence of two glycoprotein molecules differing in the degree of glycosylation (or sialylation), or by a carbohydrate-rich and a carbohydrate-deficient region in the glycoprotein, which could influence their susceptibility to proteolytic degradation. A loss of sialic acid residues may result from the weak neuraminidase activity present in commercial trypsin and chymotrypsin (Nasir-ud-Din and Jeanloz, R.W., unpublished data). A significant change observed after proteolysis of the purified glycoprotein was the absence of a Fraction B glycoprotein having a relatively low carbohydrate content, in particular sialic acid, and a limited loss of the protein moeity of Fraction A glycoprotein.

Proteolysis of the asialoglycoprotein, unlike that of the sialic acid-containing glycoprotein, generated more than one glycoprotein fragment with a lower recovery. The influence of sialic acid residues on the susceptibility of the glycoprotein to proteolytic digestion could not be completely ascertained since only a portion (75%) of the sialic acid could be removed by neuraminidase. This assessment was further complicated by the presence of sulfate groups. The degradation of the asialoglycoprotein into several fragments, however, strongly suggests that the lack of sialic acid residues in the glycoproteins enhances the susceptibility to proteolytic cleavage. Several glycoproteins, serum- as well as secretory-type, have been reported to lose their resistance toward proteinases after treatment with neuraminidase [24–26]. It appears that the peptide fragments, which are in the vicinity of oligosaccharide chains devoid of sialic acid residues, are those that are eliminated. Although the recovery of the Pronase-digested asialoglycoprotein was only 65%, the proportion of the carbohydrate moiety substantially increased from 69% to 80 and 75% in the two fractions obtained, which suggests the elimination of peptide fragments. Trypsin treatment resulted in a significant loss of carbohydrate content in addition to protein, whereas bovine seminal peptidase digestion had little effect on the content of carbohydrate.

Methoxyl group analysis and chromatography of the methylated glycoprotein suggested complete methylation and minimal degradation of the *O*-glycosyl linkages during methylation. A similar resistance of *O*-linkages to the drastic conditions of Hakomori's procedure [18] was observed by Baenziger and Kornfeld [27] during the methylation of *O*-glycosyl-linked oligosaccharides of IgA myeloma protein. Methylation and periodate oxidation data suggest terminal L-fucose, D-galactose, sialic acid, and *N*-acetylgalactosamine residues; C-3-linked-, C-3- and C-4-linked-, and C-2- and C-3-linked D-galactose residues; C-4-linked-*N*-acetylglucosamine residues; and C-3- and C-6-linked *N*-acetylgalactosamine residues. From these results, obtained with the quantitative determination of carbohydrates of the Pronase-treated glycoprotein, partial structures (I) and (II) for the glycoproteins are proposed (Fig. 6). These structures, and the sequence and linkages in the oligosaccharide chains of the glycoprotein are similar to those proposed for the oligosaccharide chains containing sialic acid residues [10], except for the sequence of *N*-acetylglucosamine (Fig. 6), [2]. The structure (II) (Fig. 6) was derived from the results of sequential periodate oxidation followed by methylation analysis of the degraded glycoproteins, which suggests the presence of both structures (I) and (II).

(I) D-Gal*p*-(1 → 3)-D-Gal*p*-(1 → 4)-D-GlcNAc*p*-(1 → 3)-D-Gal*p*-(1 → 3)-D-GalNAc*p*-(1 → 3)-Ser or Thr

6
↑
2
NeuAc

(II) D-Gal*p*-(1 → 3)-D-Gal*p*-(1 → 3)-D-Gal*p*-(1 → 4)-D-GlcNAc*p*-(1 → 3)-D-GalNAc*p*-(1 → 3)-Ser or Thr

6
↑
2
NeuAc

Fig. 6. Partial structures of oligosaccharide chains, with sialic residues, from Pronase-resistant glycoprotein.

The studies on the Pronase-treated glycoprotein indicate that, although the main structural features of the glycoprotein remain unchanged, distinct effects occurred, namely, (a) Fraction B glycoprotein was degraded, (b) this resulted in a glycoprotein containing a high proportion of sialic acid, and (c) all the oligosaccharide chains of the resistant glycoprotein fragment possess sialic acid residues.

It is probable that a mixture of glycoproteins is always present during the various phases of the menstrual cycle. The molecular ratio of the mixture undergoes changes during the cycle and a particular glycoprotein becomes of significance for a specific phase. Thus, Fraction B glycoprotein, a minor glycoprotein-component of the periovulatory phase cervical mucus, may represent a dominant glycoprotein of a different phase as previously suggested by Odeblad [28]. The contribution of the various proteins that are components of the mucus and are degraded by proteases is not known. Alteration of the biophysical and biochemical behavior of cervical mucus by proteolytic enzymes, whether of commercial, seminal plasma, or spermatozoan origin, enchances sperm penetration [5]. This observation may be explained by the present results, which suggest that Pronase, the specificity of which is similar to that of bovine seminal peptidase, degrades the purified glycoprotein, increases the charge of cervical mucus to confer rigidity by mutual repulsion of negative charges, and strengthens the coherence and consistency of the secretion [29]. The increased ionic strength and consistency of the periovulatory mucus offer better mobility to the sperm, and the increased charge favors a higher degree of hydration. This suggestion is supported by preliminary experiments in which treatment of cervical mucus with neuraminidase changed the rheological properties as well as sperm penetrability.

Acknowledgment

Supported by a grant from the National Institute of Child Health and Human Development (HD 12431-01), National Institutes of Health, U.S. Public Health Service.

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BONNET MONKEY CERVICAL MUCUS GLYCOPROTEINS. STUDY OF THE MINOR GLYCOPROTEIN COMPONENTS OF THE PERIOVULATORY PHASE MUCUS

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The uterine cervix is a target organ for sex hormones with significant differences in hormonal action and, thus, different cervical activity during the menstrual cycle. The action of various hormones is markedly distinct; estrogen stimulates the cervical secretion system, whereas the progestational hormones inhibit cervical secretion and stimulate endometrial secretion. The mechanism of these actions is not well defined and is presumably based on the presence of hormone receptors (1).

The principal constituents of the periovulatory-phase, cervical mucus are carbohydrate-rich glycoproteins that share the properties of other epithelial secretions and cell-surface glycoproteins. Cervical mucus displays properties, physical as well as chemical, of diagnostic significance that are regulated by ovarian function, and the contribution of glycoproteins to these properties has recently been suggested. The properties of the preponderant glycoprotein component of the mucus have been characterized, and the present paper describes some of the properties of the minor glycoprotein components.

Periovulatory cervical mucus purified by Bio-Gel P-200 chromatography gave a glycoprotein in addition to proteins. Further fractionation of the glycoprotein on Sepharose 2B resulted into two glycoprotein components, a major (80--90%) and a minor component (5%). On electrophoresis in 1% agarose, the minor glycoprotein showed the presence of glycoproteins and proteins. On ion-exchange chromatography on DEAE-cellulose the minor glycoprotein provided four fractions, *i.e.*, two glycoproteins and two proteins. Upon chromatography on Bio-Gel P-300, the main glycoprotein gave a single component (Fraction A) that contained L-fucose, D-galactose, *N*-acetylglucosamine, *N*-acetylgalactosamine, and neuraminic acid, in addition to amino acids. Upon gel filtration on Bio-Gel P-200, followed by ion-exchange chromatography on Sephadex A-25, the second glycoprotein fraction afforded a single glycoprotein component Fraction B.

Sequential Smith degradation of the glycoprotein Fraction A, inhibition of agglutination of type-O human erythrocytes by *Ricinus communis* and wheat germ agglutinin, as well as methylation showed similarities between the structure of Fraction A and that of the major glycoprotein (2,3), except fc a significantly lower amount of sialic acid. A possible role for this difference is the following: In the hamster system, spermatozoa can penetrate the squamous cells, and these cells become transformed and behave like cancer cells (4). In orde to prevent transformation by spermatozoa, the mucosal glyco-

Glycoconjugates. 1981. T. Yamakawa, T. Osawa & S. Handa (eds.) Japan scientific press. Tokyo.

proteins (mucins) may be desialosylated by mucosal membrane neuraminidase (5), resulting into Fraction A (the minor glycoprotein), which, then, would act as a protective mucin layer for squamous cells. The Fraction B (minor) glycoprotein displays a compositional analysis and structural features similar to those of the premenstrual-phase major glycoprotein (6).

The heterogeneity of normal cervical mucuses has been established by Odeblad (7). Ovulatory mucus consists of type E (E_S and E_I), a thin and watery mucus, characteristic of estrogenic stimulation, and type G mucus, a product of gestagenic stimulus. Ovulatory mucus contains only 3% of type G mucus. Although the occurrence of two varieties of mucus, E and G, under estrogenic stimulus, is now established, the function of G-type mucus in the periovulatory-phase mucus is unclear. The location of type G mucus is mainly in the lower part of the cervical canal (7), which might suggest a protective role against bacterial infection as well as filtration of morphologically atypic spermatozoa. Fraction B glycoprotein, which exhibits the characteristics of premenstrual phase glycoprotein, might represent the type G-mucus in the periovulatory phase of the menstrual cycle.

Table 1. CARBOHYDRATE COMPOSITION OF THE MINOR GLYCOPROTEINS

Carbohydrates	Fraction A (%)	Fraction B (%)
L-Fucose	4.5	5.0
D-Galactose	15.0	15.0
N-Acetylglucosamine	6.5	9.0
N-Acetylgalactosamine	10.5	13.0
N-Acetylneruaminic acid	1.8	5.8

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FERTILITY AND STERILITY
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Vol. 37, No. 3, March 1982
Printed in U.S.A.

Immunologically induced changes in macaque cervical mucus functions: inhibition of sperm penetration*

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The key functions of the uterine cervix in reproductive physiology are mediated by the cervical mucus, which regulates sperm transport in the upper reproductive tract during the menstrual cycle. The authors describe an in vitro procedure that uses an anti-cervical mucus glycoprotein antibody to change the properties and functions of the cervical mucus. Fertil Steril 37:431, 1982

Sperm penetration through the cervical mucus, an epithelial secretion, is of importance in reproductive physiology and depends on sperm motility and the particular phase-related character of the mucus. The biophysical characteristics of the mucus and its form and function during the menstrual cycle are hormonally regulated. As a consequence, sperm can penetrate through the mucus in the periovulatory phase of the cycle but not in the luteal phase.[1] Exogenously administered steroid hormones can induce changes in mucus behavior, but they have multiple sites of action and exert numerous undesirable effects.

The major component of the cervical secretion of the bonnet monkey is a mucin-type glycoprotein, the chemical structure of which, specifically the linkages of *N*-acetylneuraminic acid, D-galactose, and 2-acetamido-2-deoxy-D-galactose residues, changes during the cycle.[2] The mechanism by which glycoproteins participate in the process associated with sperm penetration is unknown. In order to elucidate their role, an antibody to the purified glycoprotein of the periovulatory-phase mucus was prepared[3] and its effect on sperm penetration tested.

MATERIALS AND METHODS

The cervical mucus of *Macaca radiata* was collected during the menstrual cycle as previously described.[4] The mucus was aspirated from the

Received August 7, 1981; revised and accepted October 8, 1981.

*Cervical glycoproteins, Part VIII; for Part VII see reference 7. This is Publication No. 880 of the Robert W. Memorial Group for the Study of Diseases Causing Deformities, Harvard Medical School and Massachusetts General Hospital. This work was supported by a research grant from the National Institute of Child Health and Human Development (HD 12431), National Institutes of Health, U.S. Public Health Service.

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vagina and cervix with the aid of a suction pump and stored at −20° C in preweighed vials.

The periovulatory cervical glycoprotein was purified by gel filtration on Bio-Gel P-200 (Bio-Rad Laboratories, Richmond, Calif.) and then fractionated[5] on Sepharose 2B (Pharmacia Fine Chemicals, Piscataway, N. J.). A glycoprotein, eluted as a major carbohydrate-containing material from the Sepharose 2B column, was homogeneous in agarose electrophoresis and was stained with periodate-Schiff reagent and toluidine blue, but not with amido black. The antibodies were raised[6] by the injection of purified mucus glycoprotein mixed with Freund's complete adjuvant into the footpads of rabbits. An intravenous injection of glycoprotein was administered after 30 days, and the serum harvested from an ear vein 1 week later. The immunogenic glycoprotein was subjected to electrophoresis in 50 mM barbital buffer, pH 8.2, at 90 to 100 volts, and then treated with the antiserum against the purified glycoprotein.

Fern patterns of the periovulatory-phase mucus, luteal-phase mucus, and periovulatory mucus-antibody complex were obtained by allowing the mucus to dry on a glass slide at 37° C for various periods of time. A degraded glycoprotein obtained by proteolysis of the purified glycoprotein[7] was treated with antibodies in Cordis II plates (Cordis, Miami, Fla.). A periodate-degraded (Smith degradation) glycoprotein was obtained from the pronase-degraded glycoprotein as described earlier.[2]

Semen was collected from sexually mature bonnet monkeys sedated with 15 mg/kg ketamine HCl (Ketaset, Bristol Laboratories, Syracuse, N. Y.) administered intramuscularly 10 minutes before electroejaculation. An electrode was connected to an SPE (Standard Precision Electronics, Inc., Denver, Ohio) electroejaculator inserted 7 cm into the rectum. This probe was constructed from four stainless steel rods measuring 6 mm in diameter embedded in an epoxy mold on the corners of the square with a cross-sectional dimension of 2 × 2 cm. Only the outer 5 mm of the circumference of each rod was left bare, for electrical contact with the rectal mucosa. Two opposite electrodes (0°, 180°) were connected across to the output of the electroejaculator. The other two (90°, 270°) were allowed to float. The current was raised linearly to approximately 100 mA or less for 2 seconds, lowered quickly, and a period of 2 seconds of rest was allowed. About 20 such pulses were given before ejaculation occurred. In vitro sperm penetration was performed on the periovulatory-phase mucus and mucus-antiglycoprotein-antibody complex according to the method of Moghissi.[8]

RESULTS

Gel-filtration[5] of periovulatory-phase mucus of the bonnet monkey (*Macaca radiata*) gave, in over 60% yield, a glycoprotein that was further fractionated into two components (major and minor) on Sepharose 2B. An antibody against the major component[6] reacted with the glycoprotein, as well as with the periovulatory cervical mucus, in immunodiffusion and in immunoelectrophoresis (Fig. 1).

The linear fibrillar channels of the periovulatory-phase mucus and the tangled network of the luteal-phase mucus were clearly seen under the microscope (×320) by the technique of Davajan et al.[9] (Fig. 2). The ferning patterns of the phases exhibited distinct differences, rosettelike for the luteal-phase mucus and linear channels for the periovulatory mucus. The periovulatory-phase mucus treated with the antiglycoprotein antibody displayed a precipitin reaction accompanied by a release of droplets, both on a microscope slide as well as in a tube. Both examinations of the antibody-mucus complex, before and after centrifugation at 4000 × *g* to remove the adhering liquid, showed a network that resembled the tangled network of the luteal-phase mucus (Fig. 2). Ferning of the antibody-mucus complex gave rosettelike structures.

The pronase-, trypsin-, papain-, bovine seminal peptidase-, and chymotrypsin-treated glycoproteins gave also a precipitin reaction with the antibody in immunodiffusion. The antibody also reacted with periodate-treated glycoprotein (Fig. 3). The periodate-treated glycoprotein had only 6% of sugar residues, as compared with over 70% in the original pronase-treated glycoprotein.

The semen obtained by electroejaculation was normal with regard to volume, sperm count, vi-

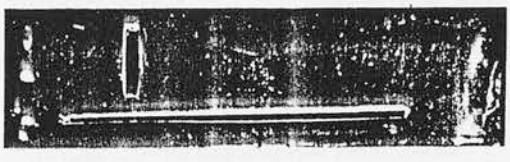

Figure 1
Immunoelectrophoresis of the periovulatory cervical mucus (×3) with antiglycoprotein antibody.

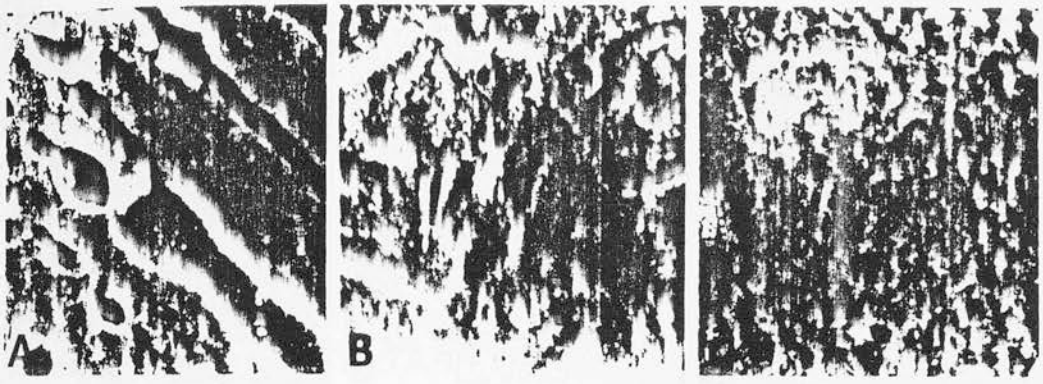

Figure 2
(A), Fresh periovulatory-phase mucus on a microscope slide. A drop of mucus on the slide was held with fine forceps at an angle of 60° and then laid on the microscope slide and covered with a coverslip. (B), Fresh luteal-phase mucus on a microscope slide. (C), Complex of fresh periovulatory-phase mucus and antiserum on a microscope slide (A, B, and C, ×320). The slides in B and C were prepared as described in A.

ability, and structure. The sperm migrated rapidly toward the cervical mucus as soon as contact was established, and a maximum concentration of spermatozoa was observed at the interface of semen and periovulatory-phase mucus. The mucus tended to move toward the seminal plasma,[8] a physical phenomenon resulting from the contact of two fluids differing in surface tension.

Within a few minutes, the spermatozoa penetrated the boundary or phalangeal canal and entered the periovulatory mucus. After the initial contact with the semen, the spermatozoa penetrated the cervical mucus; and, subsequently, a few moved in the reverse direction. After a few minutes, the spermatozoa migrated in an orderly pattern, following linear channels (Fig. 4A). In contrast to their behavior with periovulatory-phase mucus, the spermatozoa did not penetrate and form an orderly pattern and channels with the antibody-mucus complex. Similarly, sperm did not penetrate the luteal-phase mucus, a known phenomenon.[7, 10] After overcoming the initial barrier, a few spermatozoa penetrated the complex, and some migrated further into the mucus and moved at random until they ceased flagellar motion and were immobilized (Fig. 4B and C).

DISCUSSION

The present study confirms our earlier report[6] that the in vitro action of an antibody on cervical mucus changes both its physical and physiologic properties, rendering the mucus-antibody complex similar to luteal-phase mucus. The antibody raised against Sepharose 2B-purified glycoprotein interacted with crude mucus in immunoelectrophoresis, giving two precipitin lines. These two lines may have arisen because of aggregation of macromolecules. The fern pattern of the antibody-mucus complex was similar to that of luteal-phase mucus and different from that of periovulatory-phase mucus. In order to ensure that the

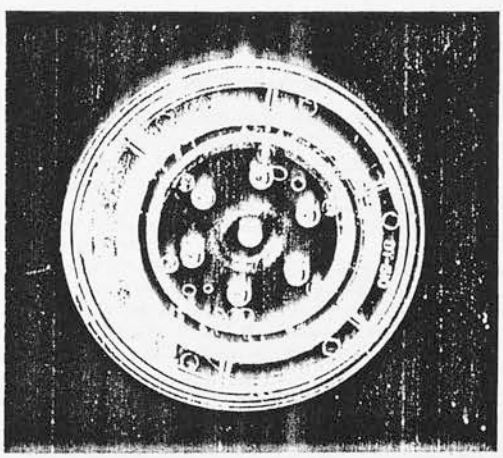

Figure 3
Immunodiffusion plate containing fresh cervical mucus and degraded glycoproteins. Rabbit antiglycoprotein antibody is in the center well; the peripheral wells 2 and 5 contain fresh mucus (×10); wells 1 and 4 contain pronase-degraded periovulatory glycoprotein; well 3 contains pronase-periodate (two Smith degradations)-degraded glycoprotein; and well 6 contains pronase-periodate (one Smith degradation)-degraded glycoprotein.

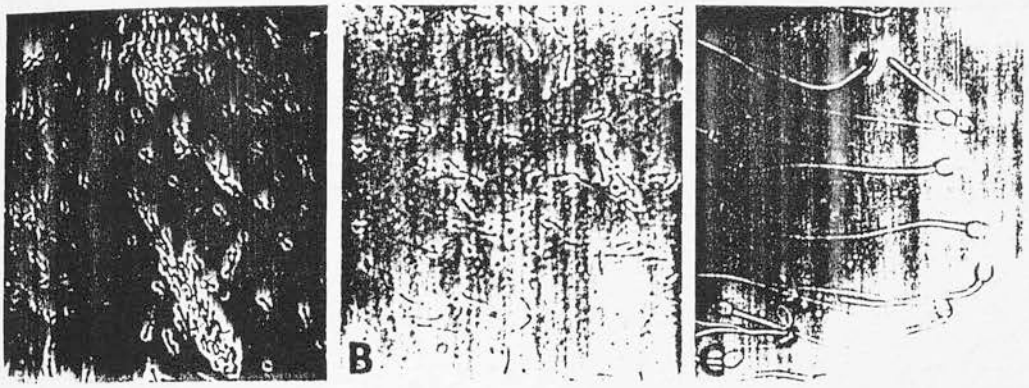

Figure 4
(A), Spermatozoa penetrating periovulatory-phase cervical mucus. Channeling and alignment of sperm begins to appear. (B), Spermatozoa penetrating antiglycoprotein antibody-periovulatory-phase cervical mucus complex. After moving at random for a few minutes, the spermatozoa became immobilized (A and B, ×100; about 5 minutes after the contact of seminal plasma and cervical mucus). (C), Spermatozoa soon after penetrating the antibody-mucus complex are immobilized; some heads are lysed (×500).

antiglycoprotein antibody was reacting with the glycoprotein alone, and not with other components of the mucus, the precipitin reaction was performed with the pronase-, trypsin-, chymotrypsin-, papain-, and bovine seminal peptidase-treated glycoproteins. The results of this treatment indicate that the antibody is specific for the glycoprotein, and that no protein component of the mucus is responsible for the immunoprecipitin reaction. The observation that the pronase- and periodate-treated glycoprotein, which had a low carbohydrate content, reacted with the antibody in immunodiffusion suggests that the antigenic determinants are located in the pronase-periodate-resistant portion of the glycoprotein, i.e., essentially in the protein component.

Inhibition of sperm penetration, in vitro, by the antibody-glycoprotein complex suggests that physical changes in the matrix of cervical mucus during the menstrual cycle regulate sperm transport through the cervix. In addition to inhibition of sperm movement by immobilization, lysis of spermatozoal heads was also observed for the spermatozoa penetrating the glycoprotein-antibody complex, possibly due to lesions of the outer membranes,[11] a phenomenon peculiar to the complex.

In an immunologic approach to fertility regulation, an antiglycoprotein antibody has been used to change the physical and morphologic behavior of the mucus and to alter its function in vitro, illustrating the role of glycoprotein components of the mucus. The observation suggests a tool by which cervical mucus could serve to develop an in vivo, nonsteroidal, localized method of contraception.

Acknowledgment. The authors wish to thank Dr. David H. Frisch, Department of Physics, Massachusetts Institute of Technology, Cambridge, for helpful discussion and the design of a battery-operated electroejaculator.

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REPRINTED FROM
FEDERATION PROCEEDINGS
VOLUME 41, MARCH 1982
PRINTED IN THE U.S.A.

ISOLATION AND CHARACTERIZATION OF BONNET MONKEY SPERM SURFACE GLYCOPROTEINS. Moshe Shalev*, Nasir-ud-Din* and Anwar Joher* (SPON: J.J. Ferguson). Univ. of Pennsylvania, Philadelphia, PA 19104, Harvard Medical School and Harvard School of Public Health, Boston, MA 02115

Bonnet monkey sperm obtained by electroejaculation were freed from seminal plasma and coating antigens by treatment with 4% Tris-HCl (pH 7.0), and spermatozoa were recovered with a viability of 90%. A suspension of sperm in PBS was treated with TPCK-trypsin for 30 min at 4°, and the sperm were removed at 700 g. The process was repeated (x3), the supernatants were combined and dialyzed, and the nondiffusible material was lyophilized. The residue was chromatographed in 50 mM phosphate buffer (pH 7.0) on Bio-Gel P-200. The purified material in SDS-PAGE showed the presence of three coomassie blue-positive components. Purification of this material on Sepharose 6B followed by DEAE-Sephadex A-25 gave a single glycoprotein containing ^{3}H in the galactose residues, label introduced by galactose oxidase-borotritide treatment. The glycoprotein contained fucose, galactose, mannose, N-acetylgalactosamine, N-acetylglucosamine and sialic acid. Sequential Smith degradation removed fucose, sialic acid and in part galactose, mannose and N-acetylglucosamine. Partial structural information was further obtained by inhibition of hemagglutination by various lectins.

Carbohydrate Research, 118 (1983) C1–C4
Elsevier Science Publishers B.V., Amsterdam – Printed in The Netherlands

Preliminary communication

Fractionation of oligosaccharides containing sialic acid by liquid chromatography on amino silicagel

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(Received December 1st, 1982; accepted for publication in revised form, March 2nd, 1983)

Recently, liquid chromatography under elevated pressure (50–100 MPa) (h.p.l.c.) on alkylamine-modified silicas (specially on 5-μm packing material) has been recognized as a convenient method of separation of high-molecular-weight oligosaccharides containing neutral sugars and 2-acetamido-2-deoxyhexoses[1–6]. The mechanism by which the sugars are retained and are eluted from these solid phases has been extensively reported and reviewed[7,8].

We have reported[5] earlier the separation of neutral oligosaccharides from human bronchial-mucus performed with a linear gradient of 17:3 to 3:2 (v/v) acetonitrile–water. The alkylamine-bonded column may function as a weak anion-exchanger, and this could explain the retention on the column of compounds containing an organic acid. H.p.l.c. procedures utilizing an anion-exchange system have recently been developed to perform the separation of sialyloligosaccharides. Various elution procedures, such as isocratic elution[2] with 11:9 (v/v) acetonitrile–sodium acetate buffer, pH 5.8, and linear-gradient elution[9,10] with 4:1 to 2:3 (v/v) acetonitrile–phosphate buffer, pH 5.2, or 25 to 500mM phosphate buffer[11], pH 4.0, have been described for the elution of acidic oligosaccharides. We report now the resolution of sialyloligosaccharides on an amine column by means of a linear gradient of acetonitrile–water–ammonium hydrogencarbonate (pH 7.5). This mobile phase suppressed the ionization of the primary-amine packing and could be removed by evaporation. This method is, therefore, convenient for isolating, from a complex mixture, pure acidic oligosaccharides.

In the present work, sialyloligosaccharides from bonnet monkey cervical-mucus were separated by h.p.l.c. into seven well-separated fractions (Fig. 1A), the molecular composition of which is shown in Table I, and human bronchial sialyloligosaccharides into eleven less well-separated fractions (Fig. 1B); the latter fractions did not always correspond to molecular proportions (Table I). This result is explained by the very high heterogeneity found in bronchial oligosaccharides[12] and shows the limits of this h.p.l.c. technique for very heterogeneous mixtures of sialyloligosaccharides.

0008-6215/83/$03.00

TABLE I

MOLECULAR COMPOSITION[a] OF SIALYLOLIGOSACCHARIDES FRACTIONATED BY LIQUID CHROMATOGRAPHY

Mucin subfraction	*Carbohydrate component*					
	L-*Fuc*	D-*Gal*	D-*GlcNAc*	D-*GalNAc*	*NeuAc*	D-*GalNAcol*
Cervical						
1					0.8	1
2		1			0.8	1
3	0.7	0.9			0.7	1
4[b]						
5	0.7	2.1	0.9	1.1	1	1
6	0.6	1.6	0.8	1.4	0.9	1
7	0.5	3.4	1	1.2	1	1
Bronchial						
1	0.7	1	1		0.8	1
2		0.6	1.2		0.6	1
3	0.9	2.1	1.1		1.3	1
4[c]						
5	0.1	1	2.2		0.5	1
6	0.1	0.5	1		0.4	1
7	0.1	0.5	0.5		0.7	1
8	0.5	1.6	1.3		0.7	1
9		0.3	1.5		0.3	1
10		0.6	0.8		0.6	1
11	0.3	0.8	0.8		0.9	1

[a] Relative to 2-acetamido-2-deoxy-D-galactitol taken as 1. [b] An insufficient amount of this subfraction was obtained to allow determination of the molecular composition. [c] This subfraction was contaminated with noncarbohydrate material.

EXPERIMENTAL

Materials. – The reduced sialyloligosaccharides used in this work were obtained from bonnet monkey, mid-cycle cervical-mucus glycoproteins[13] and from bronchial-mucus glycoproteins[14].

Liquid chromatography. – The chromatograph was equipped with two pumps from Waters Associates Inc. (Milford, MA 01257, U.S.A.), model 6000A; a solvent programmer from Waters Associates Inc., model 660, coupled to a Uvicord detector LKB, model S 2138; a universal injector from Waters Associates, model U6K; an LKB model 2250 recorder; and a column (25 × 0.46 cm i.d., E. Merck) of 5 μ Lichrosorb-NH_2. The elution was performed with a linear gradient of 4:1 to 1:1 acetonitrile–water containing 2.5mM ammonium hydrogencarbonate for 70 min at room temperature, and at a flow rate of 1 mL/min. Water was first de-ionized and then treated with the Milli-q system (Millipore Corp., Bedford, MA 01730, U.S.A.). All solvents were degassed by sonication. Although nonspecific for carbohydrate, the wavelength of detection was 206 nm, and 100 μg of oligosaccharides in water (5 μL) were injected through the loop injector.

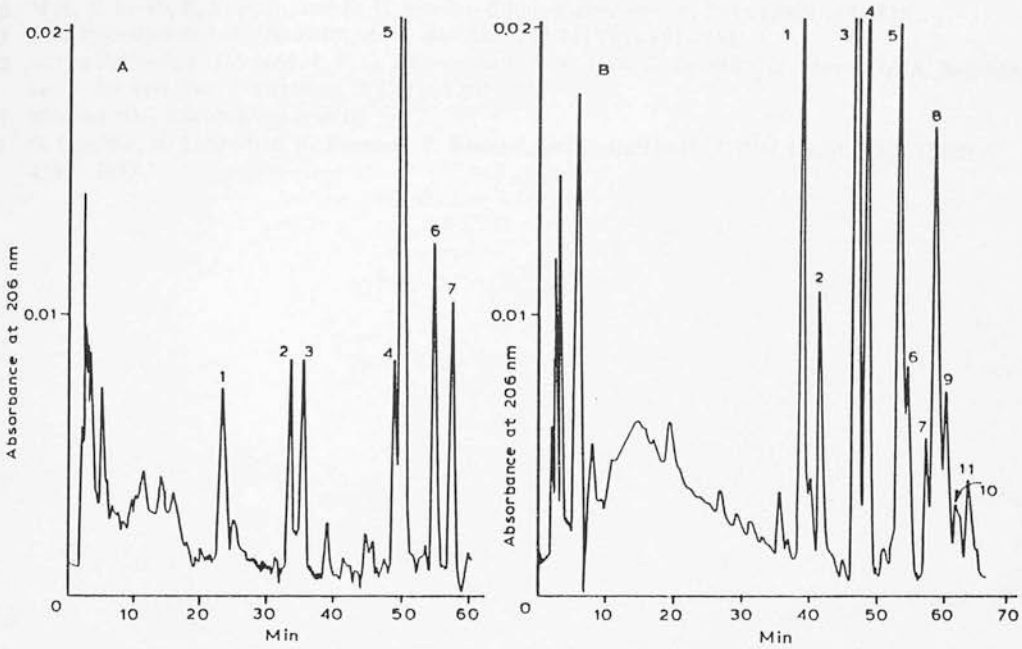

Fig. 1. Separation of sialyloligosaccharides in liquid chromatography under elevated pressure: (A) Bonnet monkey cervical-mucus oligosaccharides. (B) Human bronchial-mucus oligosaccharides. The chromatography was performed as described in the Experimental section.

Elution profiles of both oligosaccharide subfractions are given in Fig. 1, and the molecular composition of the major well-separated fractions relative to the content of 2-acetamido-2-deoxy-D-galactitol (taken as 1) is reported in Table I.

ACKNOWLEDGMENTS

This work was supported by a grant from l'Association Française de Lutte contre la Mucoviscidose, by la Fondation pour la Recherche Médicale Française and by contract INSERM No. 803025. The authors thank Bénédicte Gillet for her technical assistance and Catherine Masson for typing the manuscript.

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BIOLOGY OF REPRODUCTION 28, 1189–1199 (1983)

Bonnet Monkey Cervical Mucus Glycoproteins. Study of the Minor Glycoprotein Components of Periovulatory Phase Mucus[1]

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ABSTRACT

Two glycoproteins present in small quantities, in addition to a main glycoprotein, were isolated from the periovulatory phase mucus. The glycoproteins, obtained in minute amounts, showed distinct variations in the relative proportions of sugar residues. The results of analytical as well as chemical studies suggest the existence of two distinct molecular species differing in structure. The main component of the minor glycoproteins, Fraction A, characterized by Smith degradation and by subsequent methylation and inhibition of hemagglutination, bears similarities to the periovulatory phase main glycoprotein. The other component, Fraction B, characterized by Smith degradation followed by inhibition of hemagglutination, shows structural resemblance to the premenstrual phase glycoprotein.

INTRODUCTION

The uterine cervix is a target organ for sex hormones with appreciable differences in hormonal action and thereby different cervical functions during the menstrual cycle. The response to different hormonal stimuli is markedly distinct: estrogen stimulates the cervical secretion system and proliferation of endometrium, whereas the progestational hormones inhibit cervical secretion and stimulate endometrial secretion. The mechanism by which these processes are regulated is not clear. Presumably it is based on the presence of hormone receptors and on the local feedback mechanism on stimulation and inhibition of biosynthesis or replenishment of receptors (Holt et al., 1979).

Cervical mucus is receptive to sperm for a limited span of time during the periovulatory phase of the menstrual cycle and inhibits its transport during the luteal phase, as is the case in pregnancy. Cervical mucus is a complex mixture of diverse molecular components continuously produced by the endocervical cells. In addition to rheological properties, mucus displays ferning and spinnbarkeit, properties of diagnostic significance, all of which are regulated by the ovarian function.

The principal constituents of the peri-

Accepted January 4, 1983.
Received March 2, 1982.

[1] This is a publication of the Robert W. Lovett Memorial Group for the Study of Diseases Causing Deformities, Harvard Medical School and Massachusetts General Hospital, Boston. This work was supported by a reseach grant from the National Institute of Child Health and Human Development (HD 12431), National Institutes of Health, U.S. Public Health Service. A preliminary communication of this work has been published (Nasir-ud-Din et al., 1981).

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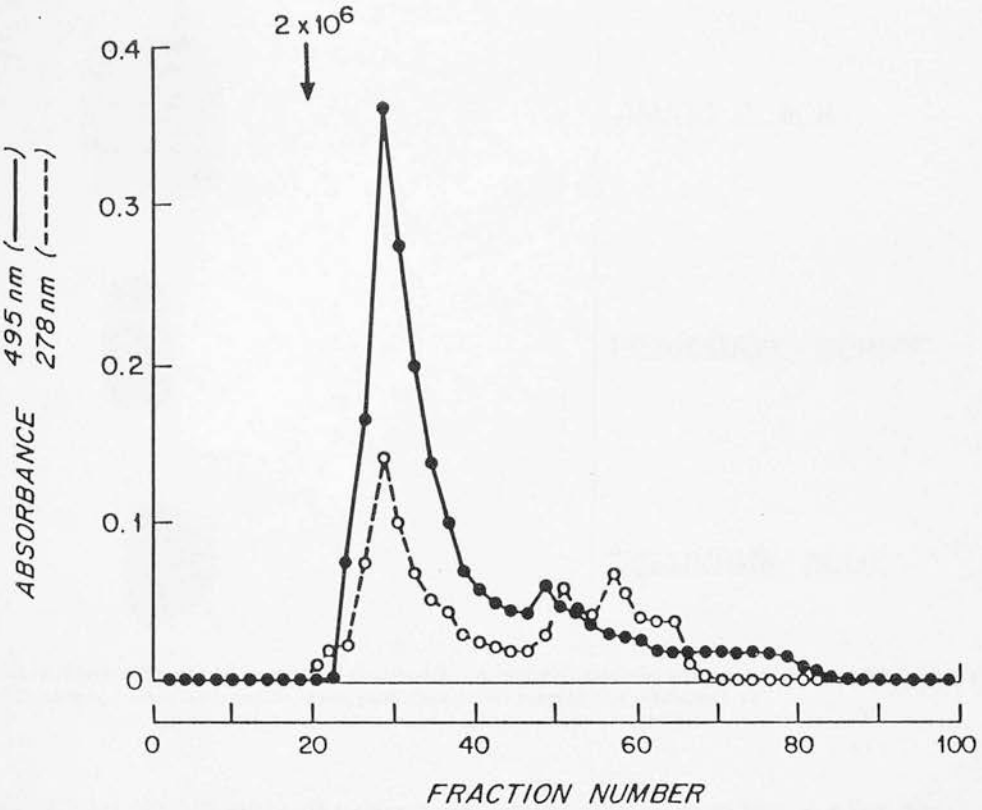

FIG. 1. Fractionation of the Bio-Gel P-200 purified periovulatory mucus on a column of Sepharose 2B. Every third fraction from the column was analyzed for carbohydrate (•—•) and protein (o- - -o). Two glycoprotein fractions were obtained, Fraction 1 (fraction numbers 22–41) and 2 (fractions numbers 45–65).

ovulatory phase mucus are carbohydrate-rich glycoproteins that share the chemical characteristics of other epithelial secretions and cell surface glycoproteins. The periovulatory phase mucus has been shown to contain a dominant glycoprotein component, the physical properties and chemical structure of which have been proposed (Hatcher et al., 1977; Nasir-ud-Din et al., 1979a). This paper describes the structure and possible role played by the other glycoprotein components present in the periovulatory phase mucus in minute quantities.

MATERIALS AND METHODS

Collection of Cervical Mucus

The cervical mucus of the bonnet monkey was collected by aspiration with suction pump at midcycle. The secretion was promptly frozen and maintained in the frozen state until use.

Analytical Methods

Gas-liquid chromatography (GLC) of the sugars was performed according to the procedure of Reinhold (1972) with a Perkin-Elmer gas chromatograph 900. The hexose content of the eluates from the columns of Bio-Gel P-200, Bio-Gel P-300, Sepharose 2B, DEAE-cellulose and DEAE-Sephadex was determined by phenolsulfuric acid method of Dubois et al. (1956), and the protein content by the procedure of Lowry et al. (1951).

Preparation and Fractionation of the Minor Glycoprotein

The crude mucus (0.5 g) was dissolved in 50 mM sodium phosphate, pH 7.0, containing 0.02% sodium azide by stirring for 16 h at 4°C. The cellular debris were removed by centrifuging (2000 × *g*) at 4°C for 30 min, the supernatant was dialyzed, and the nondiffusible material was lyophilized to give the mucus macromolecule. This material was dissolved in 50 mM sodium monophosphate (pH 7.0, 3 mg per ml) containing 0.02% sodium azide by stirring at 4°C for 12 h. The solution was applied to a column (5 × 80 cm)

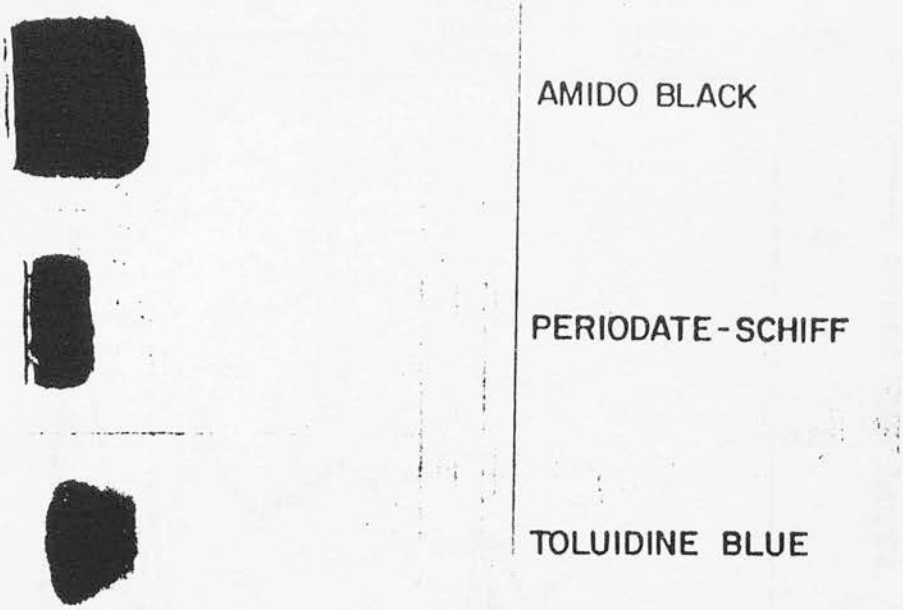

FIG. 2. Electrophoresis in agarose of Fraction 2, a minor glycoprotein component obtained from the Sepharose 2B column, stained with amido black, periodate-Schiff reagent and toluidine blue.

of Bio-Gel P-200 (50–100 mesh). The column was treated with sodium monophosphate buffer (50 mM, pH 7.0) and the carbohydrate and protein-containing fractions were pooled, the pH was adjusted to 5.5 with 4 M acetic acid, and the solution was dialyzed. The retentate was lyophilized to give the purified glycoprotein.

The purified glycoprotein (0.3 g), in phosphate buffer (100 ml), was aplied to a column (3.8 × 80 cm) of Sepharose 2B and it was eluted with phosphate buffer. Fractions containing carbohydrate and protein were combined, dialyzed and lyophilized. Two fractions, Fraction 1 and Fraction 2 were obtained (Fig. 1). Preliminary structural investigations on Fraction 1 have been reported (Nasir-ud-Din et al., 1979a).

Fraction 2 in barbital buffer (50 mM, pH 8.2) was applied to an agarose plate. The material was electrophoresed at 2.6 volts per cm for 1 h. The plates were stained with periodate-Schiff reagent, amido black and toluidine blue as previously described (Nash et al., 1977). Prior to staining, the plates were fixed either in acetic acid (15% v/v) or in 95% ethanol (Fig. 2).

Fraction 2 (12.5 mg), the minor glycoprotein component, was dissolved in 0.1 M sodium monophosphate (pH 6.6, 5 ml), and the solution was applied to a column (2.4 × 60 cm) of DEAE-cellulose. The column was washed with two subsequent gradients of 0.1–1 M sodium monophosphate (pH 6.6, 400 ml) and 0.1–1M lithium chloride (200 ml), and finally with 1 M lithium chloride (200 ml). The fractions containing carbohydrate and protein were combined, dialyzed and lyophilized. Two glycoproteins, Fractions A and B, and two proteins, Fractions C and D, were obtained (Fig. 3) in addition to a very minor component.

Purification and Characterization of Fraction A Glycoprotein

Fraction A glycoprotein (4.3 mg) in 50 mM sodium phosphate (pH 7.0) was applied to a column (1.5 × 40 cm) of Bio-Gel P-300. The column was washed with 50 mM pyridine-acetic acid (pH 5.4) 400 ml. Fractions of 2.5 ml were collected, and fractions containing carbohydrate and protein were combined and lyophilized. The residue was applied to a column (1.2 × 21 cm) of DEAE-Sephadex A-25. The glycoprotein was eluted with a gradient of 10 mM-0.25 M sodium chloride. The carbohydrate-containing fractions were combined and dialyzed. The retentate was lyophlilized to give Fraction A glycoprotein (2 mg). Fraction A glycoprotein, electrophoresed on 1% agarose in barbital buffer as described for Fraction 2, showed a single band.

Fraction A glycoprotein (1.5 mg) was dissolved in 0.1 M sodium periodate (1 ml), and the solution was kept in the dark at 22°C for 22 h. 1,3-Propanediol (100 μl) was added to the mixture, the solution was dialyzed, and the retentate was lyophilized. The residue in water (0.5 ml) was treated with $NaBH_4$ (0.5 ml, 40 mg/ml of 10 mM NaOH), and the mixutre was kept at 4°C for 4 h; 0.25 ml of sodium borohydride solution was further added, and the mixture was kept at 22°C for 2 h. Thereafter the pH of the solution was adjusted to 4.5 with 4 M acetic acid, and the solution was dialyzed. The nondiffusible material was lyoph-

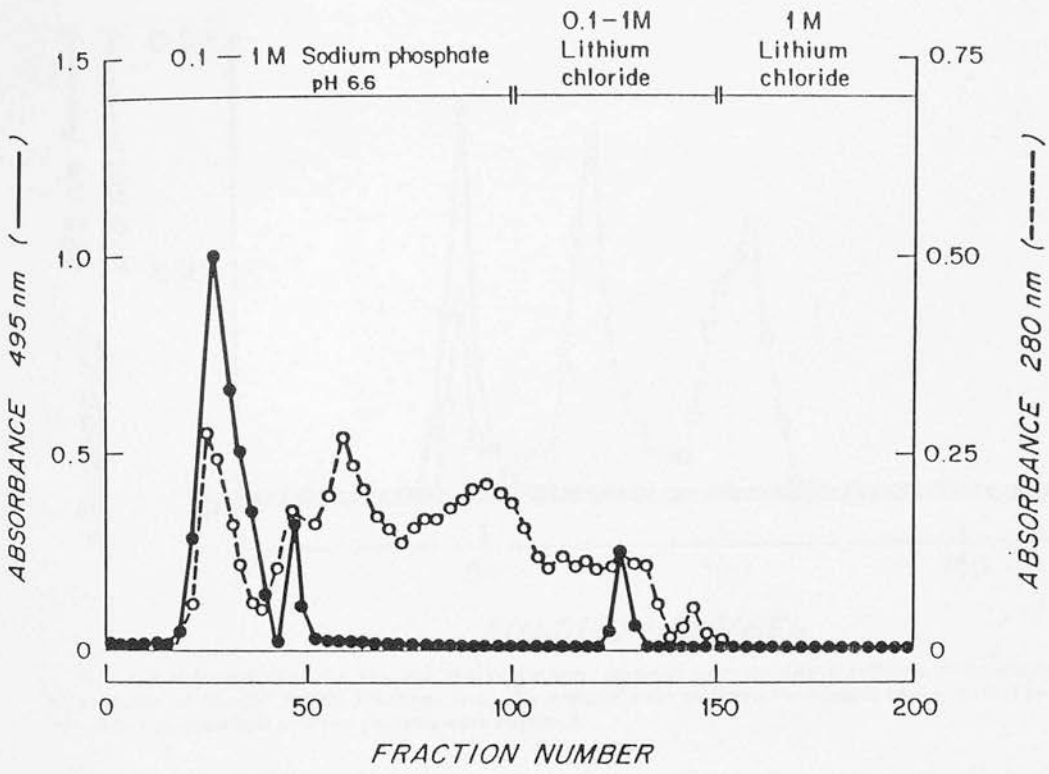

FIG. 3. Fractionation of a minor glycoprotein component, Fraction 2, on a column of DEAE-cellulose. Fractions of 4 ml were collected and every 12 ml the eluate was examined for protein (○- - -○) and hexoses (●——●). Four fractions were obtained, Fractions A (fraction numbers 20- - 42), B (fraction numbers 45—52), C (fraction numbers 54—72) and D (fraction numbers 75—110).

ilized, and the residue was treated with 0.25 M sulfuric acid for 2 h. The solution was dialyzed and the retentate was lyophilized. A portion of the residue was analyzed for carbohydrates. The remaining glycoprotein was treated with periodate and processed as described above.

The sequentially degraded glycoprotein (0.2 mg) was subjected to reductive β-elimination under conditions similar to those described by Nasir-ud-Din et al. (1979b), except that instead of $NaBH_4$, a mixture of sodium borotritide (2 mCi) and sodium borohydride (25 mg) was used in order to label the resulting sugar alcohol with tritium. After completion of the reaction the products were processed as previously described by Nasir-ud-Din et al. (1979b). The oligosaccharides after periodate treatment (Nasir-ud-Din et al., 1979b) were hydrolyzed with 1 M HCl (0.3 ml) for 6 h at 100°C, diluted with water (5 ml) and lyophilized. The residue was applied to a column of Bio-Gel P-2 (1.2 × 15 cm, 100–200 mesh), and the column was washed with 50 mM pyridine-acetic acid, pH 5.4 (200 ml). The fractions containing tritium-labeled sugars were combined and examined by thin-layer chromatography according to the procedure of Van den Eijnden et al. (1976).

Ricinus communis hemagglutinin (120, Boehringer Mannheim, GMBH, WG) and *Triticium vulgaris* hemagglutinin (Sigma Chemical Company, St. Louis, MO 63178) were used as such. The titration and inhibition assays were performed using human type O erythrocytes according to the procedure of Matsumoto and Osawa (1970).

The periodate-treated glycoprotein (0.2 mg) was methylated according to the procedure of Hakomori (1964). The methylated glycoprotein was dialyzed, and the nondiffusible material was lyophilized. The residue was dried in vacuo and treated with 2 M trifluoroacetic acid (400 μl) for 3 h at 105°C. The solution was cooled and applied to a column of AG 1-X8 (0.4 × 6 cm, OAc, 100–200 mesh) ion-exchange resin; and the column was washed with water-methanol (9:1, 5 ml), followed by 10 mM acetic acid (5 ml). The combined eluates were evaporated, the residue was acetylated with pyridine (0.2 ml) and acetic anhydride (0.2 ml), and the solution was evaporated. A solution of the residue in water-methanol (7:3, v/v) was treated with $NaBH_4$ (5 mg) at 4°C for 6 h. A further quantity of $NaBH_4$ (2 mg) was added, and the solution was kept at 22°C for 4 h. The remaining $NaBH_4$ was removed with 4 M acetic acid, and the cations were removed by treatment with AG 50W-X8 (H^+, 100–200 mesh) ion-exchange resin. After evaporation, boric acid was removed by repeated evaporations with methanol (5 × 1 ml). The residue

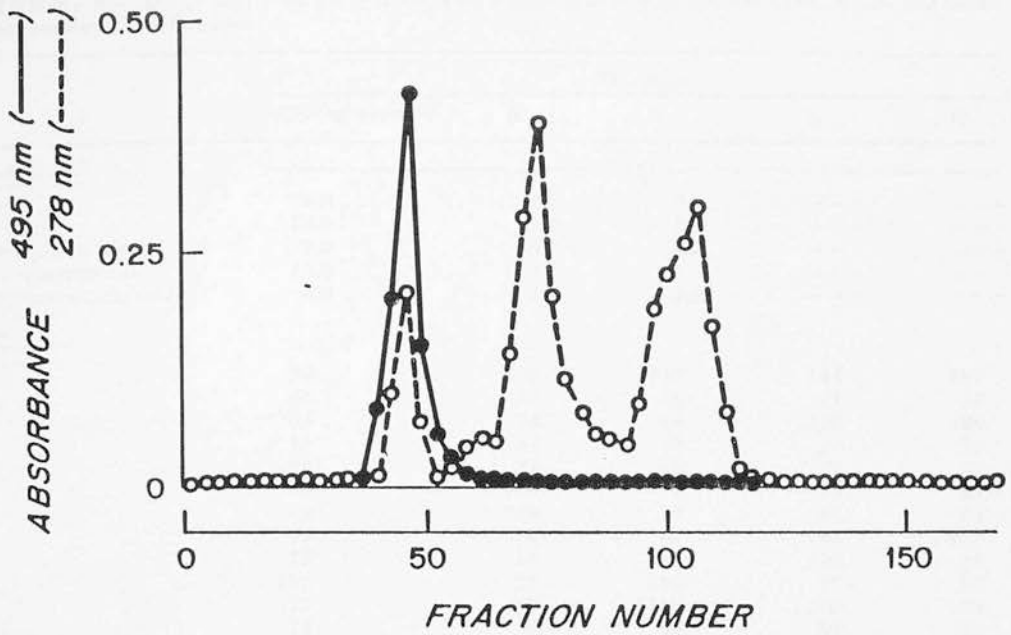

FIG. 4. Gel filtration of the Fraction B glycoprotein, obtained from the DEAE-cellulose chromatography, on a column of Bio-Gel P-200. Fractions from the column were analyzed for hexoses (•——•) and protein (o- - -o). A glycoprotein and two proteins were obtained.

was treated with pyridine (0.2 ml) and acetic anhydride (0.1 ml) for 4 h at 22°C. The resulting 0-methylate-dalditol acetates were identified by GLC-mass spectrometry.

Preparation and Characterization of Fraction B Glycoprotein

Glycoprotein Fraction B (4 mg) in 50 mM sodium phosphate buffer was applied to a column (1.5 × 20 cm) of Bio-Gel P-200. The column was washed with 50 mM sodium phosphate buffer, pH 7.0 (400 ml). Fractions of 2 ml were collected, and aliquots of the fractions were examined for carbohydrate and protein (Fig. 4). A glycoprotein and two proteins were obtained. The Bio-Gel P-200 purified glycoprotein (0.9 mg) in sodium phosphate buffer (0.5 ml, pH 7.0) was applied to a column (0.8 × 16 cm) of DEAE-Sephadex A-25 (40–120 μm), which was eluted with a gradient of 50 mM sodium phosphate (pH 7.0, 200 ml) followed by a gradient of 50 mM NaCl-0.5 M NaCl.The carbohydrate-containing fractions were combined to give a single glycoprotein component (0.5 mg). Fraction B glycoprotein was electrophoresed in 1% agarose and stained with periodate-Schiff reagent and Coomassie blue as described for Fraction 2.

Fraction B glycoprotein (0.3 mg) was treated with periodate-sodium borohydride as described for Fraction A glycoprotein, to give a periodate-treated glycoprotein (0.12 mg). The periodate-degraded glycoprotein (0.10 mg) was subjected to β-elimination under conditions similar to those described for Fraction A glycoprotein, and after acid hydrolysis the tritium-labeled alditols were identified by thin-layer chromatography according to Van den Eijnden et al. (1976).

Inhibition of hemagglutination of type 0 human erythrocytes by the periodate-degraded glycoprotein Fraction B was performed as described for Fraction A.

RESULTS

Purification, Physical and Chemical Characterization of the Minor Glycoprotein

Fractionation of periovulatory phase cervical mucus by gel filtration on Bio-Gel P-200 afforded a glycoprotein in addition to proteins. Further fractionation of the glycoprotein on Sepharose 2B resulted into two glycoprotein components, a major (80–90%) and a minor component (5%, Fig. 1).

The minor component, on electrophoresis in 1% agarose, showed the presence of a glycoprotein in addition to proteins (Fig. 2). The minor glycoprotein (Table 1) on ion-exchange chromatography on DEAE-cellulose (Fig. 3) provided four fractions, two glycoproteins and two proteins (Table 1). The main glycoprotein component obtained from DEAE-cellulose gave a single component. Fraction A, upon filtration

TABLE 1. Fractionation of Sepharose 2B Fraction 2 on a column of DEAE-cellulose. Carbohydrate and amino acid composition of the fractions.

	Fractions				
	Glycoprotein[a]	A	B	C	D
Carbohydrates[b]	(%)				
L-Fucose	6.0	4.2	0.7	– – –	– – –
D-Galactose	18.0	13.8	2.2	– – –	– – –
N-Acetylglucosamine	7.0	6.7	2.3	– – –	– – –
N-Acetylgactosamine	14.0	9.7	1.5	– – –	– – –
N-Acetylneuraminic acid	4.0	2.2	0.5	– – –	– – –
Amino acid[c]					
Ala	78	108	139	162	197
Val	63	67	80	77	73
Gly	64	85	93	158	180
Ileu	23	42	22	27	23
Leu	85	77	96	77	49
Pro	80	148	78	42	30
Thr	68	265	77	63	50
Ser	73	101	87	108	159
Phe	25	14	41	26	15
Asp	101	29	110	89	70
Glu	73	60	119	109	128
Lys	72	4	58	62	26

[a]Glycoprotein refers to Sepharose 2B Fraction 2.

[b]Determined by gas-liquid chromatography.

[c]Residues per 1000 residues.

on Bio-Gel P-300 followed by DEAE-Sephadex chromatography. Fraction A contained L-fucose, D-galactose, N-acetylglucosamine, N-acetylgalactosamine, sialic acid and amino acids with serine and threonine as the major amino acids (Table 2). The second glycoprotein fraction upon gel filtration on Bio-Gel P-200 (Fig. 4) followed by ion exchange chromatography on Sephadex A-25 afforded a single glycoprotein component (Table 4), Fraction B. Fraction A and Fraction B glycoproteins in 1% agarose electrophoresis showed a single diffused band with periodate-Schiff reagent and Coomassie blue.

Periodate Oxidation, Methylation and Inhibition of Lectin Hemagglutination of Fraction A Glycoprotein

The first Smith degradation of Fraction A glycoprotein eliminated 51% of the sugar residues present in the glycoprotein, degrading completely L-fucose and sialic acid, and 69% of the D-galactose residues (Table 3). In addition, a decrease in *N*-acetylgalactosamine also occurred (Table 3). The second Smith degradation removed 20% of the remaining carbohydrates from the glycoprotein (Table 3). The molar ratios of the sugar residues suggest essentially the degradation of N-acetylglucosamine and N-acetylgalactosamine. The degraded glycoprotein was effective in inhibiting the agglutination of type O human erythrocytes by *Ricinus communis* lectin. A weak inhibition with wheat germ agglutinin was also observed. The inhibitory activities of these lectins was not surprising as the methylation results of the degraded glycoprotein suggested the presence of terminal galactose and N-acetylglucosamine residues, and 3-linked N-acetylgalactosamine and D-galactose and 4-linked N-acetylglucosamine were also suggested. In addition, β-elimination of the degraded glycoprotein followed by periodate oxidation and purification showed mainly 2-amino-2-deoxy-threitol as the only sugar arising from the protein-linked N-acetylgalactosamine.

TABLE 2. Carbohydrate and amino acid composition of Fraction A glycoprotein obtained from DEAE-Sephadex column.

	Percent	Molar ratios[a]
Carbohydrates		
L-Fucose	4.5	0.93
D-Galactose	15.0	2.83
N-Acetylglucosamine	6.5	1.00
N-Acetylgalactosamine	10.5	1.61
N-Acetylneuraminic acid	1.8	0.19
Amino acid	Residues per 1000 residues	
Asp	47	
Thr	209	
Ser	100	
Glu	60	
Pro	86	
Gly	68	
Ala	81	
Val	25	
Ileu	152	
Leu	71	
Tyr	Tr	
Phe	Tr	
Lys	43	
His	14	
Arg	43	

[a]Molar ratio relative to *N*-Acetylglucosamine

Smith Degradation, Inhibition of Lectin Hemagglutination and β-Elimination of Fraction B Glycoprotein

Smith degradation of Fraction B glycoprotein, as expected, eliminated completely L-fucose and 48% of the D-galactose residues (Table 4). Changes in the molar ratios of N-acetylglucosamine and N-acetylgalactosamine also occurred (Table 4). Reductive β-elimination of the Smith-degraded glycoprotein, and examination of the eliminated 2-amino-2-deoxyalditols by thin-layer chromatography, showed the presence of 2-amino-2-deoxy-1,3-propanediol and 2-amino-2-deoxy-galactitol in an approximate ratio of 1:1. The detection of 2-amino-2-deoxy-1,3-propanediol suggested the presence of either 6-linked or N-acetyl-galactosamine residues which are linked to serine or threonine of the protein moiety. The

TABLE 3. Carbohydrate composition of sequentially Smith-treated Fraction A glycoprotein.

Carbohydrate	First Smith treatment		Second Smith treatment	
	Percent	Molar ratio[a]	Percent	Molar ratio[a]
L-Fucose	– – –	– – –	– – –	– – –
D-Galactose	4.7	0.68	5.9	1.20
N-Acetylglucosamine	8.5	1.00	5.9	1.00
N-Acetylgalactosamine	10.5	1.23	6.9	1.10
N-Acetylneuraminic acid	– – –	– – –	– – –	– – –

[a]Molar ratio relative to *N*-Acetylglucosamine.

TABLE 4. Carbohydrate composition of intact and Smith-degraded Fraction B glycoprotein.

	Intact glycoprotein		Smith-degraded glycoprotein	
	Percent	Molar ratio[a]	Percent	Molar ratio[a]
Sugars				
L-Fucose	5.0	0.75	– – –	– – –
D-Galactose	15.0	2.05	7.8	0.82
N-Acetylglucosamine	9.0	1.00	8.8	1.00
N-Acetylgalactosamine	13.0	1.44	8.1	0.92
N-Acetylneuraminic acid	5.8	0.46	– – –	– – –
Amino acid[b]		Residues per 1000 residues		
Asp		100		
Thr		128		
Ser		60		
Glu		137		
Pro		53		
Gly		100		
Ala		83		
Val		42		
Met		12		
Ileu		21		
Leu		54		
Tyr		14		
Phe		11		
Trp		68		
Lys		72		
His		11		
Arg		24		

[a]Molar ratio relative to *N*-acetylglucosamine.

[b]Because of very small quantity of Fraction B glycoprotein available obtained from DEAE-Sephadex A-25, amino acid analysis was performed on the material obtained from Bio-Gel P-200 (see text).

POSSIBLE CARBOHYDRATE CORE STRUCTURES FOR FRACTION B GLYCOPROTEIN

(i) D-Gal$\underline{p}$-(1→6)-D-GalNac$\underline{p}$-(1→3)-Ser or Thr

(ii) D-Gal$\underline{p}$-(1→6)-D-GalNAc$\underline{p}$-(1→3)-Ser or Thr
3(4)

(iii) D-Gal$\underline{p}$-(1→3)-D-GalNac$\underline{p}$-(1→3)-Ser or Thr

FIG. 5. Possible carbohydrate core structures of oligosaccharide chains of the periodate-treated Fraction B glycoprotein.

presence of N-acetylgalactosamine as a single sugar linked to the protein moiety appears unlikely as the loss of N-acetylgalactosamine is concomitant with the loss of L-fucose, D-galactose and sialic acid, as well as with a significant loss of carbohydrates in the glycoprotein. The periodate oxidation results (Table 3) thus indicate a 6-linked N-acetylgalactosamine in the oligosaccharide chains, a feature common with the premenstrual phase glycoprotein (Nasir-ud-Din et al., 1979b). The Smith-degraded glycoprotein completely inhibited the agglutination of type O human erythrocytes by *Ricinus communis* lectin. With wheat germ agglutinin, unlike Fraction A, no inhibitory activity was observed. The inhibitory activity, combined with the compositional analysis of the degraded glycoprotein, suggests the presence of terminal D-galactose, and strongly indicates the sugar sequence shown in Fig. 5 for the degraded Fraction B glycoprotein.

DISCUSSION

In the current studies, the mucus was obtained from several monkeys at the periovulatory phase (estrogen stimulation) of the menstrual cycle. The mucus was stored frozen at −20°C until use with no significant change in its morphology (Nasir-ud-Din et al., 1979c). The chromatographic and electrophoretic behavior of the minor mucus glycoprotein components, Fraction A and Fraction B, indicate that they represent high molecular weight glycoproteins. Of significance was the separation, by DEAE-cellulose chromatography, of proteins from mucin-type glycoproteins (Fig. 2 and Table 1) which are known to associate with glycoproteins (Yurewicz and Moghissi, 1981).

The purity of Fraction A and Fraction B glycoproteins was assessed by a combination of gel and ion-exchange chromatography, and agarose electrophoresis. The glycoproteins in agarose electrophoresis behaved as high molecular weight entities in that they barely entered the gel, a feature common to mucin-type glycoproteins (Holden et al., 1971a), and behaved as a single component, albeit polydisperse. In polyacrylamide-SDS or in the presence of deaggregating agents such as urea or SDS, the glycoproteins neither enter the gel nor are subunits of the mucin glycoproteins produced (Holden et al., 1971a,b). The restricted procedures available to assess the purity of the mucus glycoproteins indicated homogeneity of Fraction A and Fraction B glycoproteins.

The amino acid composition of both Fraction A and Fraction B glycoproteins was characteristic of cervical glycoproteins as well as of other glycoproteins obtained from epithelial secretions. Serine and threonine in Fraction A glycoprotein constituted over 30 mol% of the total amino acids, and a somewhat higher isoleucine content than that observed in cervical glycoproteins was present. In the case of Fraction B glycoprotein, in spite of the fact that serine and threonine were in molar ratios characteristic of mucin-type glycoproteins, the content of aspartic acid, glutamic acid and glycine was somewhat different from that reported for simian (Hatcher et al., 1977) and human cervical mucus glycoproteins (Yurewicz and Moghissi, 1981). These differences in amino acids are not peculiar because with the application of better purification procedures, mucin-type glycoproteins show marked differ-

ences in amino acid composition (Woodward et al., 1982).

Carbohydrate composition and structural studies on Fraction A glycoprotein suggest similarities with the major glycoprotein of the periovulatory phase mucus glycoprotein (Hatcher et al., 1977; Nasir-ud-Din et al., 1979a) except for a significantly low amount of sialic acid.

The mechanism by which Fraction A glycoprotein, a small proportion of the total periovulatory glycoprotein, arises is not clear. However, it is known that there exists neuraminidase activity in the mucus, and it is possible that Fraction A is the product of desialylation of glycoprotein by mucosal membrane-bound neuraminidase (Pricer and Ashwell, 1971; Daunter and Counsilman, 1980). Various biological functions have been attributed to desialylated glycoproteins in number of systems (Bendich et al., 1976; Daunter and Counsilman, 1980). However, the role of Fraction A glycoprotein remains to be investigated.

Fraction B glycoprotein displayed a carbohydrate composition similar to that of Fraction A glycoprotein except for a marked increase in sialic acid and variations in relative proportions of the sugar residues. The total quantity of the purified Fraction B glycoprotein in the periovulatory mucus accounted for 0.2%, and showed structural features at variance with the major glycoprotein (Hatcher et al., 1977) as well as with Fraction A glycoprotein, but similar to that of premenstrual phase glycoprotein (Nasir-ud-Din et al., 1979b).

Significant differences between Fraction A and Fraction B glycoproteins arose due to the presence of 2-amino-2-deoxy-threitol in Fraction A, and 2-amino-2-deoxy-galactitol and 2-amino-2-deoxy-propanediol in Fraction B, among the degradation products of Smith-treated glycoprotein. This observation, in addition to compositional analysis and results of hemagglutination inhibition, strongly suggest the existance of two glycoproteins with differences in structure in the periovulatory phase mucus of the menstrual cycle.

It is now established that a normal cervical mucus sample never occurs in pure form. Periovulatory mucus contains type E, a thin watery mucus, characteristic of estrogenic stimulation, as well as type G mucus, a product of gestagenic stimulus (Odeblad, 1978). Type E mucus allows sperm transport between macromolecule assemblies, and type G mucus effectively impedes sperm progression because the macromolecular networks are small (Odeblad, 1976). However, ovulatory mucus is known to contain only 3% of type G mucus (Odeblad, 1978). Although the occurrence of two types of mucuses, E and G, under estrogenic stimulus or in the periovulatory phase is now known, the function of the G-type mucus in this phase is not clear. The location of type G mucus is mainly in the lower part of the cervical canal (Odeblad, 1978), which might suggest a protective role against bacterial infection as well as filtration of morphologically abnormal spermatozoa. Fraction B glycoprotein, which exhibits structural characteristics of premenstrual phase mucus glycoprotein, might represent the type G mucus in the periovulatory phase. The occurrence of Fraction A and Fraction B glycoproteins in periovulatory phase mucus is in line with the physiological regulation of cervical mucus by the ovarian hormones; however, their precise function remains to be clarified.

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BONNET MONKEY CERVICAL MUCUS: ISOLATION AND CHARACTERIZATION OF OLIGOSACCHARIDES FROM THE PRONASE-TREATED PERIOVULATORY PHASE GLYCOPROTEIN

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Epithelial tissues amenable to extraneous and invasive actions secrete a variety of biomolecules to mediate different functions, essentially to protect the tissue. Epithelial secretions are major components of the bronchial, gastrointestinal and cervical systems, and these fluids are secreted by various types of cells, such as goblet, columnar, mucous and serous cells. In some secretory systems more than one type of cells of the epithelium may be involved in secretion, therefore suggesting a heterogeneous mixture of secretory components. The mucus secretion, mucin, of the mammalian cervix is produced solely by the columnar cells (1) and consists of least heterogeneous biopolymers. In addition, the cervical epithelial cells respond to estrogen and progesterone stimulation and produce biophysically different secretions during the menstrual cycle (2). Because of single type of epithelial cells, columnar; involved in cervical secretion and also due to their distinct response to hormonal stimuli, cervical mucus provides an important model for studies on biological functions and their relation to molecular structure. Cervical mucus has been shown to contribute to regulate fertility (3) as well as to protect the cervical tissue (4).
The crude cervical mucus from periovulatory phase was purified by gel filtration on Bio-Gel P-200 and then was extensively treated with insolubilized protease as previously described (5). The Pronase-treated glycoprotein was extensively dialyzed and the nondiffusible material was purified by chromatography on Sepharose 4B and DEAE-cellulose. The purified polymer was homogeneous in agarose and polyacrylamide gel electrophoresis and sedimentation equilibrium, albeit polydisperse. The average molecular weight of the glycoprotein was determined to be 80000. The Pronase-treated and purified glycoprotein was treated with 2M $NaBH_4$ in 50mM NaOH for 22 hours at 48° C and processed as described earlier (6). The residue was separated into neutral and acidic oligosaccharides on a column of AG 1X-2. The oligosaccharides were fractionated on Bio-Gel P-6 (200-400), neutral into five fractions and acidic into seven fractions. Each oligosaccharide fraction, neutral and acidic, was further purified by gel and ion-exchange chromatography. The carbohydrate composition of neutral (N-1 to N-4) and acidic (A-1 to A-4, and S-1 and S-2) oligosaccharides is given in Table 1.

TABLE 1.

Carbohydrate[a]	Oligosaccharide Fractions									
	N-1	N-2	N-3	N-4	A-1	A-2	A-3	A-4	S-1	S-2
L-Fucose			1	1		1	1		1	1
D-Galactose	1	1	1	2	1	1	2	2	2	2
N-Acetylglucosamine	1	1	1	1	1	1	1	1	1	1
N-Acetylgalactosamine		1		1			1		1	1
N-Acetylneuraminic acid					1	1	1	2		
N-Acetylgalactosaminitol	1	1	1	1	1	1	1	1	1	1
Sulfate									1	2

Glycococonjugates. 1983. M. Alan Chester et al., (eds.) Lund-Ronnbey. Rahms, Lund.

[a]Molar ratio relative to N-acetylgalactosaminitol.

The neutral and acidic oligosaccharides were characterized by a combination of enzymic degradation, periodate oxidation-borohydride reduction and methylation studies. The structure of these oligosaccharides are given in Fig. 1.

Figure I

N-1. D-Galp-1$\xrightarrow{\beta}$4-D-GlcNAcp-1$\rightarrow$3-GalNAc-ol

N-2. D-GalNAcp-1$\xrightarrow{\alpha}$3-D-Galp-1$\xrightarrow{\beta}$3-GalNAc-ol
D-GlcNAcp-1$\rightarrow$6

N-3. L-Fucp-1$\xrightarrow{\alpha}$2-D-Galp-1$\xrightarrow{\beta}$4-D-GlcNAcp-1$\rightarrow$3-GalNAc-ol

N-4. L-Fucp-1$\xrightarrow{\alpha}$2-D-Galp-1$\xrightarrow{\beta}$3-GalNAc-ol
D-Galp-1$\xrightarrow{\beta}$4-D-GlcNAcp-1$\rightarrow$6
3$\xleftarrow{\alpha}$1-D-GalNAcp

A-1. D-Galp-1$\xrightarrow{\beta}$3-GalNAc-ol
Neu-2$\xrightarrow{\alpha}$6

A-2. L-Fucp-1$\xrightarrow{\alpha}$2-D-Galp-1$\xrightarrow{\beta}$4-D-GlcNAcp-1$\rightarrow$3-GalNAc-ol
Neu-2$\xrightarrow{\alpha}$6

A-3. Neu-2$\xrightarrow{\alpha}$3-D-Galp-1$\xrightarrow{\beta}$4-D-GlcNAcp-1$\rightarrow$3-GalNAc-ol
L-Fucp-1$\xrightarrow{\alpha}$2-D-Galp-1$\rightarrow$6 D-GalNAcp-1- ?

A-4. Neu-2$\xrightarrow{\alpha}$3-D-Galp-1$\xrightarrow{\beta}$3-GalNAc-ol
Neu-2$\xrightarrow{\alpha}$3-D-Galp-1$\xrightarrow{\beta}$4-D-GlcNAcp-1$\rightarrow$6

S-1. D-Galp-1$\rightarrow$3-D-Galp-1$\xrightarrow{\beta}$4-D-GlcNAcp-1$\rightarrow$3-GalNAc-ol
3$\xleftarrow{}SO_3$ L-Fucp-1$\rightarrow$3 D-GalNAc-1-?

S-2. D-Galp-1$\rightarrow$3-D-Galp-1$\rightarrow$4-D-GlcNAcp-1$\rightarrow$3-GalNAc-ol
3$\xleftarrow{}SO_3$ 2$\xleftarrow{}$1-L-Fucp 3$\xleftarrow{}SO_3$ D-GalNAc-1-?

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Proc. Poly. Sci. Sym. 2, 1983

ROLE OF EPITHELIAL SECRETIONS : GLYCOPROTEIN STRUCTURE-FUNCTION RELATIONSHIP

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Epithelial secretions, mucin, are major components of the bronchial, gastrointestinal and cervical systems. Tracheobronchial secretion is an important part of mucocilliary system which plays significant physiological roles, particularly in the defence mechanism. Similarly secretions in the gastrointestinal tract and in cervix perform number of functions without which regulation of normal physiological processess will be impossible. Mucin is a high molecular weight, carbohydrate-rich protein secreted by epithelial cells of the salivary glands, intestine, trachea and genital tract. All these secretions share biophysical and biochemical properties with minor variations. The biophysical properties of mucin contributes to its ability to form protective coat on the mucosal surface which acts as a physical barrier against invasive agents. This mucosal sheath is of particular importance to bronchial, intestinal as well as to cervical cells where it protects underlying epithelium from enzymes, viruses, becteria and waste products.

Epithelium fluids are secreted by various types of cells such as goblet, columnar, mucus and serous cells, and these different secretary cell types may not be equally distributed over the epithelium, suggesting that the mucin is a heterogenous mixture of secreted components, particularly glycoproteins. In addition, the secretion may also contain components of other origin i.e., bacterial and serum. Besides inherent heterogeneity, the procedures used to collect the secretion may not yield material identical to the physiological secretion. Because of the involvement of the columnar cells only in cervical secretion and since the regulation of this secretion is strictly under ovarian hormones during menstrual cycle, cervical system provides an excellent model for the study of glycoproteins, a major constituent of secretion, structure-function relationship.

The mucus secretion, mucin, of the mammalian cervix mediate and control the key functions in the process of reproduction. Since the cervical secretion in human is available in minute quantity and because of structural variation that may arise due to secretor specificity a primate, bonnet monkey, was choosen for these investigations. Also a phylogentic proximity and physiologic resemblance, the bonnet monkey secretion possesses biochemical similarity to the human.

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Cervical mucus is a complex milieu, the principal constitutents of which are mucin type carbohydrate rich glycoproteins. They display gel like behaviour and have a protein rich in hydroxyamino acids. As these glycoproteins represent the major polymeric component of the mucus, it is logical to assume that they are responsible for its specific physiological and biophysical properties. The mucus displays a number of rheological properties such as spinbarkeit, flow elasticity and stickiness that are regulated by ovarian function. The biochemical and biophysical changes in the cervical mucus during the menstrual cycle not only influence passage of sperm, but also survival and nutrition. In addition, secretion also effect the development and differentiation of fetus. The cyclic alteration of the physical properties of the mucus are accompanied by variations in the carbohydrate composition (1,2) and also the chemical structure, specifically, the linkages of N-acetylneuraminic acid, D-galactose and 2-acetamido-2-deoxy-D-galactose residues (3). The mechanism by which glycoproteins, specifically, and mucus secretion in general participate in the processes of reproduction is unknown. In order to assess the role of glycoproteins, a major and a minor, were isolated from estrogen-stimulated cervical secretion and an antibody to the main glycoprotein (4) was raised to define the function of this glycoprotein. Furthermore, salient chemical structural features of the main glycoprotein were partially identified to translate the biochemical structure into physiological function (5).

Periovulatory Phase Mucus

In previous studies (6, 7) we have shown that midcycle (periovulatory) mucus contains at least two glycoproteins, a major and a minor, which were separated by gel filteration and they were devoid of any lipid contaminents. The lipid contaminents are of common occurance in bronchial secretion (8). The major glycoprotein exhibited the presence of sulfate as well as carboxyl groups (9). Carboxyl groups were identified on a nine carbon sugar, a monosaccharide of common occurance in mammalian tissue, known as neuraminic acid. In addition, microheterogeneity with respect to acidic functions were observed in the glycoprotein on ion-exchange chromatography. In SDS-polyacrylamide gel electrophoresis (9) no contaminating proteins were observed. The carbohydrate moeity of the glycoprotein contained fucose, galactose, N-acetylglucosamine, N-acetylgalactosamine and N-acetylneuraminic acid. The protein portion of the glycoprotein contained a high proportion of serine and threonine residues, a feature common to secretory glycoproteins.

Alkaline-borohydride treatment of the glycoprotein released neutral as well as acidic oligosaccharides, which were characterised using chemicals and enxymatic procedures. The site of attachment of sialic acid at C-6 of the N-acctylgalactosamine residue, ultimate to protein moiety, was determined by a series of complex reactions i.e., enzymic degradation, permethylation and mass-spectrometry. Treatment of the glycoprotein with proteolytic enzymes showed that these glycoproteins of mucus that have lesser amount or were devoid of sialic acid residues were degraded. The major difference between the main and the minor glycoproteins was the quantity of

the sialic acid residues. High resolution, 500 MHz, ^{1}H-NMR spectroscopy revealed the presence of diverse, complex and novel structures (Fig. 1).

The minor glycoprotein which were present in about five percent of the total glycoprotein content of the mucus glycoprotein contained two distinct types of components differing in carbohydrate composition and linkages. The structural features of these glycoproteins are shown in Fig. 1.

Luteal Phase Glycoprotein

The mucus glycoproteins from the luteal phase, gestagenic secretions, was purified and fractionated into two components (Fraction 1 and 2) by gel filtration. On agarose and polyacrylamide-SDS electrophoresis both fractions showed homogeniety, albeit polydisperse, and absence of contaminating lipids and proteins. The major component, Fraction 1, stained strongly with periodic-Schiff reagent and very weakly with toludine blue and amido black. The other fractions, Fraction 2, stained weakly with periodate-Schiff reagent, amido black and toluidine blue. Chromatography of Fraction 1 on DEAE cellulose showed the presence of a homogeneous component. On agarose gel electrophoresis at pH 8.2 the glycoprotein did partially enter the gel and stained with periodate-Schiff reagent and commassie blue. Carbohydrate compositions of Fraction 1 and 2 are given in Table 1. Alkaline borohydride treatment of Fraction 1 yielded a series of neutral and acidic oligosaccharides which were separated on Bio-Gel P-6, followed by chromatography on DEAE-Sephadex A-25. The oligosacoharides were further purified by paper chromatography and their homogeneity was examined by high performance liquid chromatograpy. The purified oligosaccharides were methylated by Hakomori's procedure. In addition, sequences and anomeric configuration of linkages of sugar residues of the oligosaccharides were accomplished by periodate oxidation-borohydride reduction and sequential enzymic degradation. The main structural features of carbohydrate moiety of Fraction 1 glycoprotein is shown in Fig. 2.

The main glycoprotein components of the two phases display distinct features in the chemical structures of their carbohydrate chains. The changes observed during the menstrual cycle concern the sequence and linkages of N-acetylneuraminic acid, D-galactose and N-acetylgalactosamine (Figs. 1 & 2) .

The N-acetylneuraminic acid residues in the periovulatory glycoprotein are predominantly linked to C-6 of an N-acetylgalactosamine that is linked to the protein core. In the luteal phase the neuraminic acid residues are linked to C-3 of the D-galactose residues that are remote from the protein backbone. It has been proposed that because of their mutual repulsion, due to negative charges, the N-acetylneuraminic acid residues are responsible for biophysical properties i.e., rigidity consistancy and coherene, of mucin secretion. *It is possible that the presence of N-acetylneuraminic acid in the close vicinity of the protein backbone in the periovulatory phase enhances the rigidity and is responsible for the parallel alignment of the glycoproteins that form channels of least resistance for sperm penetration (10, 11).*

In the luteal phase, the increased distance between the protein backbone and the N-acetylneuraminic acid may decrease the interaction of the intramolecular carbohydrate branches and result in a flexible macromolecule that allow more intermolecular interaction (10). Furthermore, the remotness of the N-acetylneuraminic acid residue may explain the increased succeptibility of glycoprotein to proteolytic enzymes which result in smaller glycoproteins and in the consequent impermeability of luteal mucus (12). In the periovulatory phase mucus existence of 3-linked N-acetylneuraminic acid away from the protein core is now known (9), their function is not clear, however.

In addition, an antibody to the main periovulatory glycoprotein was raised and *in vitro* action of this antibody on cervical mucus changed both its physical and physiological properties, rendering the mucus similar to luteal phase mucus. The antibody raised against purified glycoprotein interacted with crude mucus in immunoelectrophoresis as well as in immunodiffusion. Inhibition of sperm penetration, *in vitro*, by antibody-glycoprotein, complex suggests that physical changes in the matrix of cervical mucus, from channels (to support sperm enhancement) to network (to inhibit sperm transport), during the menstrual cycle regulate sperm transport through the cervix (10). The role of minor glycoproteins in periovulatory phase is not clear. However, it is very likely that the minor glycoprotein which is nearly devoid of neuraminic acid acts as an acceptor for sperm surface sialic acid. Occurance of sialyltransferase in cervical mucus is known (11). It is possible that because of the presence of sperm surface sialyl acceptor i.e. minor glycoprotein Fraction 1 acts as a protective coat on the epithelium and thereby formation of the diploid cells by interaction of epithelium and spermatozoa and transformation of normal cells, is avoided. Minor glycoprotein Fraction 2, a glycoprotein similar to luteal phase glycoprotein may act as a barrier to morphologically abnormal sperm and bacteria. However, these two phenomenon remains to be varified in human.

In the structures of glycoproteins of periovulatory mucus, distinct differences in the carbohydrate branches were observed. In addition differences between periovulatory and luteal mucuses carbohydrate chains were observed. At the moment, it is not clear whether these differences are limited to the carbohydrate chain or whether they also exist in protein backbone. There is a reasonable suggestion to indicate that the limited difference between the two proteins may exist (10), and this would indicate that the same cells during the menstrual cycle produce different mucus proteins. It is known that the diversity in the glycoproteins or secretion during the cycle is produced by the same cells (13). The functions of cervical mucus in fertility, sterility, regulation of fertility (14) and defence of epithelium is empahsised, and the complexity of the molecules responsible for its behaviour during the menstrual cycle is beginning to be better understood.

Fig. 1 : **Structures and Partial Structures of the Carbohydrate Chains of Glycoproteins in the bonnet monkey cervical secretion of Periovulatory-Phase:**

Major Glycoprotein

(a) α-Neu-(2→6)-GalNac-α-(1→3)-Ser
Neu-(2→6)

(b) α-*L*-Fucp-(1→2)-β-D-Galp-(1→3)-β-D-GalNAcp-(1→3)-Ser or Thr

(c) β-D-Galp-(1→3)-β-D-Galp-(1→3)-D-GalNAcp-(1→3)-Ser or Thr

(d) α-L-Fuc-(1→2)-β-D-Gal-(1→3)-β-D-GalNAc-α-(1→3)-Ser or Thr.
β-D-Gal-(1→4)-β-D-GlcNAc-(1→6)

(e) α-D-Galp-(1→3)-β-D-(1→4)-D-GlcNAcp-(1→3)-D-GalNAcp-(1→3)-Ser or Thr.
3 3
↑ ↑
SO_3 SO_3

Minor Glycoprotein I.

(a) α-L-Fucp-(1→2)-β-D-Galp-(1→4)-β-D-GlcNAcp-(1→3)=D-GalNAcp-(1→3)-Ser or Thr.

(b) α-L-Fucp-(1→2)-β-D-Galp-(1→4)-β-D-GlcNAcp-(1→3)-D-Galp-(1→3)-D-GalNAcp-(1→3)-Ser or Thr.

Minor Glycoprotein II.

(a) α-L-Fuc-(1→2)-β-D-Galp-(1→4)-D-GlcNAc-(1→6)-D-Galp-(1→6)-D-GalNAcp-(1→3)-Ser or Thr.

(b) α-D-Galp-(1→6)-D-Galp-(1→4)-GlcNAcp-(1→3)-D-GalNAcp-(1→3)-Ser or Thr.

3
↑
2
α-NeuAc

Fig. 2. **Structures and Partial Structures of Carbohydrate Moiety of Luteal-Phase Main Glycoprotein**

(a) β-D-Galp-(1→4)-β-D-GlcNAcp-(1→6)-D-Galp-(1→3)-β-D-GalNAcp-α-(1→3)-Ser or Thr.

(b) β-D-Galp-(1→4)-β-D-GlcNAcp-(1→3)-D-Galp-(1→3)-β-D-GalNAcp-α-(1→3)-Ser or Thr.

(c) β-D-Galp-(1→6)-β-D-Galp-(1→4)-β-D-GlcNac-(1→3)-β-D-GalNAc-α-(1→3)-Ser or Thr.

(d)
α-Neu-(2→3)
-D-Galp-
α-L-Fuep-(1→2)

Table-1 .Carbohydrate composition of the Luteal Phase glycoprotein Fractions

Carbohydrate	Fraction 1 (%)	Fraction 2 (%)
L-Fucose.	4.5	5.0
D-Galactose.	15.0	15.0
N-Acetylglucosamine.	6.5	9.0
N-Acetylgalactosamine.	10.5	13.0
N-Acetylneuraminic Acid.	1.8	5.8

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Commentary

MODERN TRENDS: GLYCOPROTEINS

For the past three decades chemistry has seen a pragmatic evolution of its involvement into biological phenomena that have been strictly compartmentalised for the biologists or the physiologists. This rapid evolution or limited revolution was of immense value to basic research as well as to practical applications, in particular in the domain of health care as well as consumer needs. This period of scientific development will certainly be regarded as an era of explosive advance in the application of one area of science to the development of the other, such as application of electronics and computer for health concerns. Similar is the case of chemistry. With the development of many powerful and fine techniques, specifically analytical systems, structure of biologically important complex molecules can be established in days which normally would require years. One example of such a combination of procedures will be the purification of a molecule by high performance liquid chromatography followed by characterisation by 500 MHz proton nuclear magnetic resonance. In order to elucidate the molecular basis of the biological phenomena in which the chemical molecules are involved, information as to the exact chemical structure of the relevant molecules is indispensable.

The recent trends in chemistry have been generally concerned with the molecular understanding of the biological phenomena, with multiple applications, of the human systems. A logical question that every mind raises is whether the necessity of understanding the biological mechanisms has arisen due to the inquisitive and observing instinct or it was self-created due to prevalent hazards generated by man, and mainly circumstantial. The current approaches to understand and unravel the complicated chemical basis of biological

phenomena have unlimited resource of development and consequences. The remarkable feats achieved in the field of medicine, in the immediate past, based on molecular biology and biochemistry have undisputedly lodged medicine into a science.

Necessarily these fundamental changes in the understanding of normal mechanisms and continuous progress to regulate the pathological conditions arose from combination of marvellous advances in the incisive growth in chemical procedures and elaborate development in biological technology. The rapid progress in gene coloning that can be translated at the molecular level or alterations in the chemical residues is likely to provide innovative procedures for regulating many processes which were not possible earlier. The other item which requires special mention is the immunological process of preparing specific antibodies. Furthermore, extensive studies in the early diagnosis and elaborate programme on useful prognosis based on molecular understanding may lead to a comprehensive regulation of the neoplastic cells, i.e. cell oncogenesis. It is extremely helpful for the above-mentioned studies to know the state of art and the chemical basis as applicable to macromolecules. The understanding of the involved chemistry is of immense value in the growth of these processes.

The two significant chemical molecules participating in the human physiological processes are amino acids and carbohydrates, and their polymers, proteins and polysaccharides. More importantly the conjugates of these two polymers, glycoproteins, contribute significantly to human physiology. The synthesis of proteins in the glycoproteins is directly controlled by the genetic material. The synthesis of carbohydrates, a cotranslational step, is indirectly regulated by genes, as the enzymes responsible for the glycan biosynthesis are controlled by specific genes.

Glycoproteins are ubiquitously distributed in nature, and particularly in man. It has been recognised that out of nearly sixty proteins in human serum fifty-eight are glycoproteins,[1] and the remaining two may, as well, in due course of time, be recognised as glycoproteins. It is a common belief now that

most proteins are glycoproteins as are many polysaccharides including starch and glycogen.

Glycoproteins exist in cells both in soluble and membrane-bound forms, as well as in the intercellular matrix and extracellular fluids. This class of macromolecule includes enzymes, immunoglobulins, hormones, lectins toxin and structural proteins, and are major constituents of the cell surface membrane.

The most striking differences between surface macromolecule components of normal and neoplastic cells lie perhaps in the amount of glycoproteins[2] and in their oligosaccharide chains,[3] in the amount of glycosaminoglycans,[4] in the amount and type of sialic acids present in the carbohydrate moity,[5] as well as in the presence, in tumor cells, of short O-glycosyl-linked carbohydrate chains having a structure similar to that of the Thomsen-Firedenreich antigen.[6] The latter is the precursor of both M-and N-blood group substances, and it has been reported to be associated with some malignant human tumors.[7] The involvement of surface sugar receptors in the control of various cell functions is well documented.[8] Intensive studies, chemical, biochemical, as well as immunological and immunochemical, in the area of macromolecular structures at the cell surface, particularly glycoproteins and glycopeptides, are still required in order to unravel the complex sequence of events leading to cancer.

There are sufficient indications to suggest that the carbohydrate portions of the glycoproteins perform important biological roles such as biological recognition, i.e. specification of blood type, control of the half-life of glycoproteins, regulation of glycoprotein uptake by the cells, stage specific differentiation, acceptors for carbohydrates or protein components and protection of tissues exposed to invasive action of organisms and molecules. The molecular basis underlying the functions of glycans and particularly in recognition is the interaction of carbohydrates with the carbohydrate binding proteins. A fascinating concept of heteroglycan function is based on the finding that certain carbohydrates may represent highly specific compounds as carriers of biological information. This property of the

glycan moiety of the glycoprotein arises from a large variety of oligosaccharide structures that can be formed from a relatively few monosaccharides. Considering the differences in the anomeric configuration and position of the glycosidic linkages, for example three molecules of the same hexose (e.g. galactose) can form 176 different trisaccharides, while three molecules of the same amino acid can form only one tripeptide, and three nucleotides containing the same base can form only one coden. The monosaccharides may function as letters in a code of biological specificity comparable to the specific amino acid or nucleotide sequences in proteins or nucleic acids, respectively.

In order to establish the molecular basis of the biological phenomenon in which carbohydrates are involved, information as to the precise structure of the relevant glycoconjugate, particularly the sugar moieties are indispensable. The carbohydrates in the glycoproteins may range from less than one per cent, collagen, to more than eighty per cent, soluble blood group substances, of the total molecule. Glycoproteins also differ in the number of carbohydrate chains present. This may vary from one, in ribonuclease B, to eight hundred, in submaxillary mucin. The oligosaccharide chains in the glycoproteins differ in the different constituent carbohydrates, ranging from one to a dozen or more. Glycoproteins usually contain multiple sugar chains with different structures.

The sugar chains of glycoproteins can preferably be classified only in their physiological properties. The arbitrary classification is based on the type of linkage of sugar residues. In the case where *N*-acetylgalactosamine is linked to serine or threonine through an O-glycosidic linkage the glycoproteins have been called mucin and this type of sugar chains is abundantly found in mucous type proteinous material. The second major type of sugar chains are called asparagine linked. These sugar chains are linked *N*-glycosidically from *N*-acetyglucosamine to the amide nitrogen of asparagine.

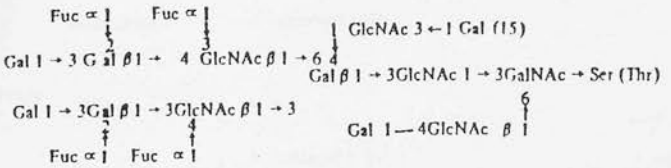
Mucin-type sugars are usually isolated after chemical release, β-elimination and simultaneous reduction, from the protein backbone. Identification of the eliminated sugars

can be enhanced by the incorporation of tritium during the reductive conversion of the peptide-linked N-acetylgalactosamine to N-acetylgalactosaminotol. This sugar residue can be identified after acid hydrolysis of the released sugar chains. The structures of some of mucin-type oligosaccharides, simple to complex, determined are given in Fig. 1.[9-15]

Fig. 1

Sugar chains linked through N-acetylgalactosamine to serine or threonine

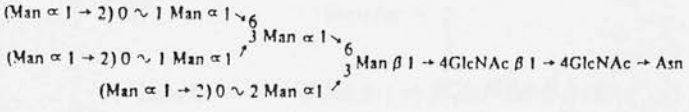
	Structure	Reference
	GalNAc → Ser (Thr) 6 ↑ NeuAc ∝ 2	(9)
I		
II	Gal β 1 → 3GalNAc → Ser (Thr)	(10, 11)
III	Gal β 1 → 2GalNAc → Ser (Thr) 3 ↑ NeuAc ∝ 2	(11, 12)
IV	Gal β 1 → 3GalNAc → Ser (Thr) 3 6 ↑ ↑ NeuAc ∝ 2 2NeuAc ∝	(11, 12)
	Gal β 1 → 3GalNAc → Ser (Thr) 2 ↑ Fuc ∝ 1	(13
V		
VI	Galβ1→3GlcNAcβ1→3Galβ1→3GalNAc→Ser(Thr) 6 ↑ Gal1→4GlcNAc β 1	(14)



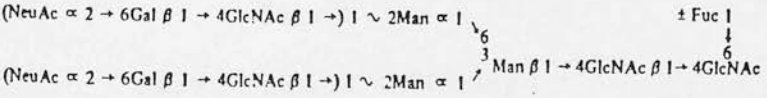
Structural investigation of asparagine-linked sugar chains has been rapidly increased in the last few years due to the development of enzymatic and chemical procedures to release these chains from glycoproteins and glycopeptides. Three structures have evolved on which further enlargement occurs. These structures are shown in Fig. 2. The extension or structural variations found in the outer chain moieties of complex type asparagine-linked sugar chains are shown in Fig. 3.

Fig. 2
General structures of three types of asparagine-linked sugar chains

High mannose type



Complex type



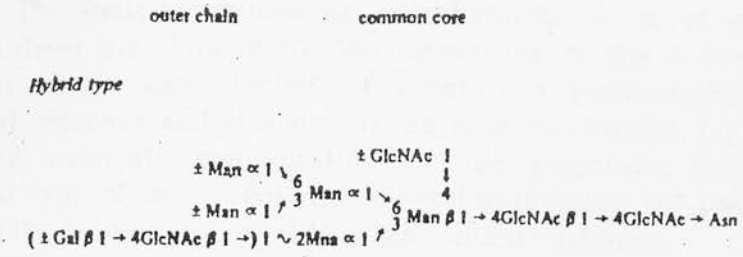


Fig. 3

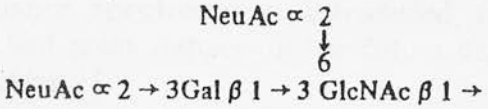
Different types of outer chains found in the outer chains of complex type sugar chains

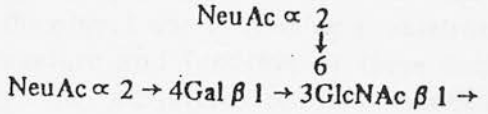
I Gal β 1 → 4GlcNAc β 1 →

II NeuAc α 2 → 6Gal β 1 → 4GlcNAc β 1 →

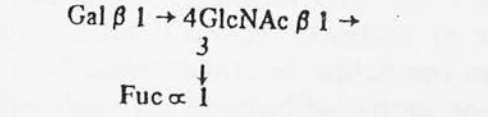
III NeuAc α 2 → 3Gal β 1 → 4GlcNAc β 1 →

IV





VI



VII Fuc α 1 → 2Gal β 1 → 4GlcNAc β 1 →

VIII Gal β 1 → 3Gal β 1 → 4GlcNAc β 1 →

IX $(\text{Gal}\,\beta\,1 \rightarrow 4\text{GlcNAc}\,\beta\,1 \rightarrow 3)_n\ \text{Gal}\,\beta\,1 \rightarrow 4\text{GlcNAc}\,\beta\,1 \rightarrow$

The basic structures of carbohydrate chains of glycoproteins are defined by the assessment of the following: (a) nature and number of constituent monosaccharides; (b) sequence and ring size of the monosaccharides; (c) type and anomeric configuration of the glycosidic linkages; (d) type of the carbohydrate-peptide linkages; (e) position of the evolved amino acids in the protein backbone.

For the establishment of these parameters periodate oxidation, alkaline and enzymatic degradation, in combination with analytical techniques such as paper electrophoresis, thin layer chromatography, get filtration and high performance liquid chromatography have been the method of choice. For the investigation of complex glycan structures as occurring in glycoproteins, these techniques did not provide reliable answers. The real advance in the structural elucidation arose due to the methylation analysis, based on the application of gas-liquid chromotagraphy mass spectrometry and specific chemical derivitisation. [16-19] Finally, the application of high-resolution ^{1}H-nuclear magnetic resonance spectroscopy, introduced in the last few years, has had great impact on the future development in structural analysis.[20]

Our work has mainly been concerned with epithelial glycoproteins and the object was to develop a relation between the chemical structure and function of these secretions. In order to study the structure-function relationship gall bladder and cervical secretions provided excellent models, since these secretions are produced only by a single type of cells, columnar cells. Cervical secretion is a complex milieu, the principal constituents of which are mucin-type glycoproteins, that share the physical behaviour and chemical structure with other epithelial secretions. As the major macromolecular components of the mucus are glycoproteins, it is reasonable to assume that they are responsible for its physiological and biological behaviour.[21]

In order to assess the role of glycoproteins, the structural features of the glycoproteins from the cervical secretion during the mid-cycle were investigated, and the structure of neutral and acidic oligosaccharides was established.[22-23]

Furthermore, an antibody raised against the midcycle glycoprotein and its cross-reaction with mucus effectively altered the morphology and function of the secretion.[24] Understanding of chemical structures and their relation to physiological function was established by *a combination of chemical enzymatic and immunological procedures.*

It must be realised that understanding of the biological mechanisms and, ultimately, regulation of physiological functions in man begin with the identification of the molecules responsible for a particular function. The unfolding of the chemistry of these molecules leads to the recognition of specific portion of the chemical entity; it may be carbohydrates or a protein moiety in the case of the glycoprotein. Furthermore, another conjugate of the glycoprotein may as well impart biological activity. An example of a glycoprotein, where the activity is not generated by either carbohydrate or protein, is rhodopsin. The biological activity in rhodopsin is imparted by the retinal.

It is apparent from these studies that the current trends in chemistry related developments, be it in basic or applied chemistry, engage more than one area of chemistry to explicitly study the complex biological phenomenon. The progress in the understanding of the complex human systems appears to require multidisciplinary approach. Indeed, this will require a close cooperation between chemists, biologists, physiologists, immunologists and cytologists to acquire knowledge of phenomenon related to various mammalian functions.

The beginning of the understanding of the physiological or biological phenomenon depends on the molecular structure and establishment of fine chemical structure of physiologically active sites may initiate the unfolding of the complex molecular systems of man.

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THE CHEMICAL STRUCTURE OF A HIGH MOLECULAR WEIGHT FRAGMENT OF MICROCOCCUS LYSODEIKTICUS CELL WALL

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Summary. The peptidoglycan and a covalently linked antigenic external polysaccharide is a major component of *Micrococcus lysodeikticus* cell wall, and displays distinct composition. The present study describes purification of a lysozyme resistant, nondialysable high molecular-weight fragment of cell wall. The structure of a fragment of cell-wall (CPC_{A-1}) obtained by cetylpyridinium precipitation and gel filtration from the nondialysable portion of the degraded products of egg, white lysozyme was studied by the periodate oxidation and methylation procedures. The fragment consists of a polysaccharide chain composed of approximately 25 repeating (1 → 4) -O-(2-acetamido-2-deoxy-β-D-mannophyranosyluronic acid)-(1 → 6)-O-(α-D-glucopyranonsyl) residues with D-glycopyranosyl residues at both, reducing and non-reducing, ends.

The mode of the attachment of the polysaccharide to the peptidoglycan through a phosphodiester has also been established. The D-glucose residue was found as a point of external polysaccharide attachment to the peptodoglycan through a phosphate diester to a residue of 2-acetamido-2-deoxy-

D-glucose. Kinetic studies for the acid catalysed release of external polysaccharide from the peptidoglycan was performed in parallel with synthetic [methyl-2-acetamide-3-0-(D-1-carboxyethyl)-2- deoxy-α-D-glucopyranoside-6-yl] -α-D-glucopyranosyl phosphate and α-D-glucopyranosyl phosphate and showed the presence of a phosphodiester linkage between external polysaccharide and peptidoglycan.

Introduction. Perkins[1] has isolated in low-yield external polysaccharide chains of *Micrococcus lysodeikticus* cell wall by trichloroacetic acid treatment, and the chemical structure of these chains has been partially elucidated by Hase *et al,*[2] A fragment of the cell wall representing 26% of the total cell wall, 10% of the peptidoglycan, and 50% of the external polysaccharide chains was isolated from *Micrococcus lysodeikticus* cell wall. This fragment, obtained by degradation with egg-white lysozyme, precipitation with cetylpyridinium chloride, Bio-Gel chromatography, and ion-exchange chromatography, was shown to consist of a peptidoglycan moiety composed of four alternating, (1 → 4)-linked 2-acetamido-2-deoxy-β-D-glucopyranosyl and 2-acetamido-3-0 (D-1-carboxyethyl)-2-deoxy-β-D-glucopyranosyl (N-acetylmuramic acid) residues to which is attached an external, polysaccharide chain composed of 4-0-(β-D-manopyranosyluronic acid)-0-(α-D-glucopyranosyl) repeating units linked through a phosphate group to C-6 of one of the muramic acid residues of the peptidoglycan chain; in addition, all the carboxyl groups of the muramic acid residues are substituted by peptide chains.[2]

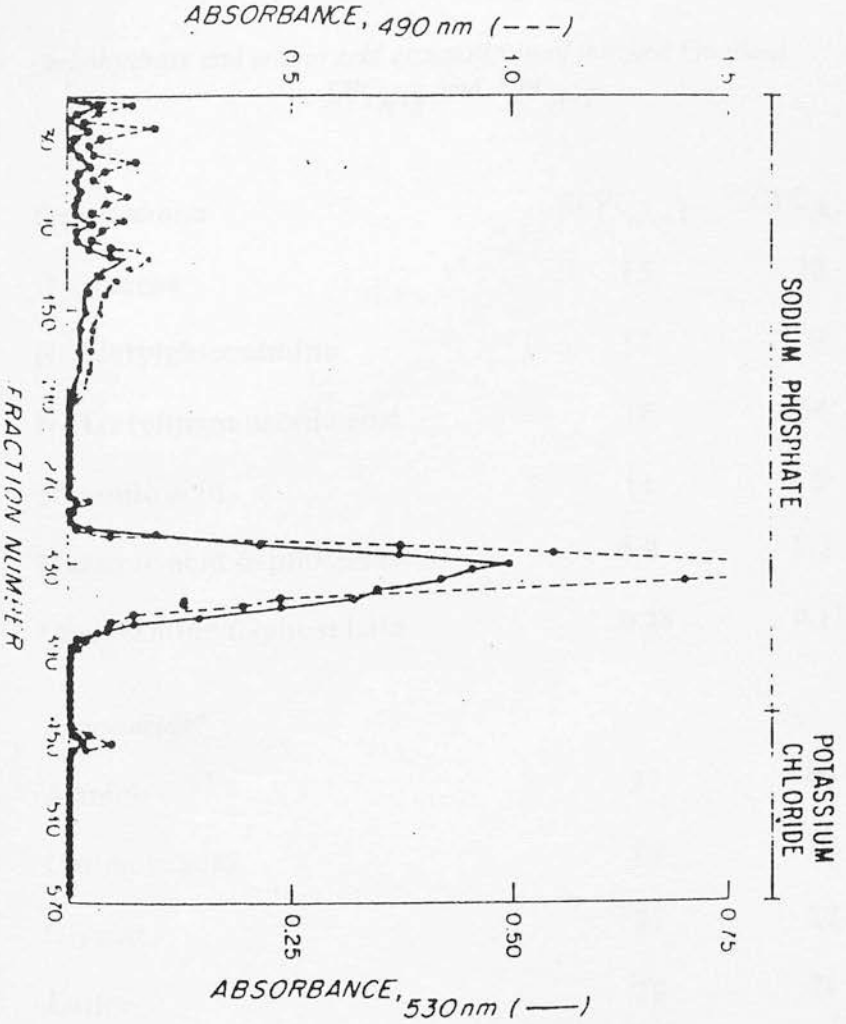
The external antigenic polysaccharide chains, which have a different structure for each micro-organism, are composed in *M. lysodeikticus* of alternate glucose and 2-acetamido-2-deoxy-D-mannuronic acid (N-acetylmannosaminuronic acid) units.[3, 4] It has been suggested that they are linked to the peptidoglycan moiety through a phosphatediester linkage involving some of the muramic acid residues at C-6.[5, 6] The linkage region of the external polysaccharide to the peptido-

glycan in different organisms has various termini involving glucose-1-phosphate.[5,6] N-acetylglucosamine-1-phosphate[7] and the polysaccharide directly linked, without the involvement of phosphodiester, to the peptidoglycan.[8]

In the case of *M. lysodeikticus* the linkage of the acidic polysaccharide to C-6 of the muramic acid 6-phosphate residue, through a phosphodiester linkage, has been proposed to be through a N-acetylglucosamine residue.[7] There was also a suggestion of glucose being involved as a point of linkage.[2,5] Subsequently it was shown that for the biosynthesis of (external) polysaccharide chains a lipid intermediate containing N-acetylglucosamine was required to initiate the elongation of the polymer.[9] In addition, it has been shown that tunicamycin and bacitracin, both, inhibited the synthesis of the polysaccharide.[10]

Results and Discussion. The nondialysable cell wall was fractionated by CPC-precipitation. The cetylpyridinium fraction insoluble in water (Fraction CPC_A) was the major nondiffusible component. On gel filtration it yielded two macromolecules. The high molecular weight fraction, CPC_{A-1}, was further purified by ion-exchange chromatography on ECTEOLA-cellulose (Fig. 1). This was homogenous in SDS-polyacrylamide electrophoresis.

Fig. 1



Separation of the undialysable cell-wall on a column of ECTEOLA-cellulose. Fractions of 4 ml were collected and every third Fraction was examined for the presence of hexoses by phenol-sul-furic acid procedure (490, – – – –) and hexosamines by Gatt-Bermman colorimetric reaction (530 nm, —— ——).

Table 1

Carbohydrate and amino acid composition of purified Fractions CPC_{A-1} and CPC_{A-2}

Carbohydrates	$\%CPC_{A-1}$	$\%CPC_{A-2}$
D-Glucose	15	28
N-Acetylglucosamine	13	7
N-Acetylmannuronic acid	18	34
Muramic acid	11	9
Muramic acid 6-phosphate	0.8	1.2
Glucosamine 6-phosphate	0.28	0.11
*Amino acids**		
Alanine	37	40
Glutamic acid	14	17
Glycine	21	22
Lusine	28	21

*Residues per 100 residues.

Periodate oxidation at 4° in the dark, which has been shown to cause no over-oxidation of 2-acetamido-2-deoxy sugar residues, gave an amount of formic acid (0.8 mol D-glucose residue) suggesting oxidation of all of the D-glucose residues, indicating that these residues are linked at C-6 (see Table II).

The release of formaldehye (60 nmol/mg) corresponds to the oxidation of reducing terminal muramic acid residue of the peptidoglycan fragment. The periodate treated fragment was reduced with sodium borohydride, the resulting polyalcohol hydrolysed by mild hydrolysis with acid, and the hydrolyzate dialysed. The low molecular weight fragments contained only one quarter of the expected amount of glycerol, and the remainder consisted of 2-amino-2-deoxy-D-mannuronic acid. This compound, after treatment by methanolic hydrogen chloride and reduction, was identified by g.l.c as methyl 2-acetamido-2-deoxy-α-D-mannopyranuside.

Table II

Sequential periodate oxidation, sodium borohydride reduction, and hydrolysis (Smith degradation) of fraction CPC_{A-1}

Components reagent, and compounds formed	Fraction CPC_{A-1}	Sequential Smith degradation			
		First		Second	
		DI	Ndl	DI	Ndl
D-Glucose	28		3		0
2-Amino-2-deoxy-D-mannuronic acid	37		12		0
2-Amino-2-deoxy-D-glucose	7		9		12
Muranic acid	9		14		18
Muramic acid 6-phosphate	1.4		2.2		
Glycerol		2.4	6	0	0
Alanine	4		10		12
Glutamic acid	3		8		8
Glycine	2		4		8
Lysine	3		5		10
Periodate consumed (unol/mg)		2.80			
Formic acid released (unol/mg)		0.89			
Formaldehyde released (umol/mg)		85			

Component contents expressed in %. Abbreviations: D, dialysable fraction: Nd nondialysable fraction. Determined by the anthrone calorimetric method. Determined by g.l.c. as 2-amino-2-deoxy-D-mannose, relative to the D-glucose content. Determined with an aminoacid analyser (see Experimental section). Determined by u.v. absorption at 223 nm. Determined by titration with sodium hydorxide. Determined by the chromotropic acid calorimetric method.

The presence of approximately 3% of unoxidised D-glucose and 6% of glycerol in the oxidised, hydrolysed, and nondialysable material indicates the limitation of Smith procedure for structure determination of complex carbohydrate molecules. The resistance of the D-glucose residues to periodate oxidation may suggest a (1 → 3)-linkage, but this observation was not confirmed by the methylation procedure, and it is likely that steric effects were responsible for this resistance to the periodate oxidation. The presence of glycerol shows that the conditions of hydrolysis were not sufficient to hydrolyse the oxidised, reduced, external chains completely, but stronger conditions would have hydrolysed the phosphate bond and, possibly, the 2-acetamido-2-deoxy-D-glycopyranosyl linkages. The total amount of glycerol found in the dialysable and non-dialysable fractions corresponds to about half of the value expected, the loss probably being due to hydrolysis during purification of the reduced fractions. No erythrose was observed, thus confirming that all of the D-glucose residues are linked at C-6. A second periodate oxidation of the oxidised, reduced, nondialysable fraction degraded the remaining D-glucose residues.

Methylation was performed by a modified Haworth procedure,[13] in order to avoid degradation of alkali-sensitive bonds. Methanolysis, followed by acetylation and examination of the derived trimethylsilyl ethers by g.l.c-m.s. showed the presence of D-glucose as 2, 3-di, 2, 3, 4-tri, and 2, 3, 4, 6-tetra-methyl ethers. The ratio of 2, 3, 4-tri to 2, 3, 4, 6-tetra-methyl ether suggested chain-length of 25 repeating units, in good agreement with the value of 30 found by determination of the reducing end linked to the phosphate group. The small amount of 2, 4-di-0-methyl-D-glucose dues suggest a branching at position C-3 of glucose. On reduction of the product of methanolysis, the 3-methyl ether of the corresponding 2-amino-2-dexoy-D-mannose residue was obtained in good yield by g.l.c and was identified by g.l.c-m.s. and comparison with an authentic sample.[11]

The 6-methyl ether of muramic acid 2-amino-3-0-(D-1-carboxyethyl)-2-deoxy-6-0-methyl-D-glucose) was charac-

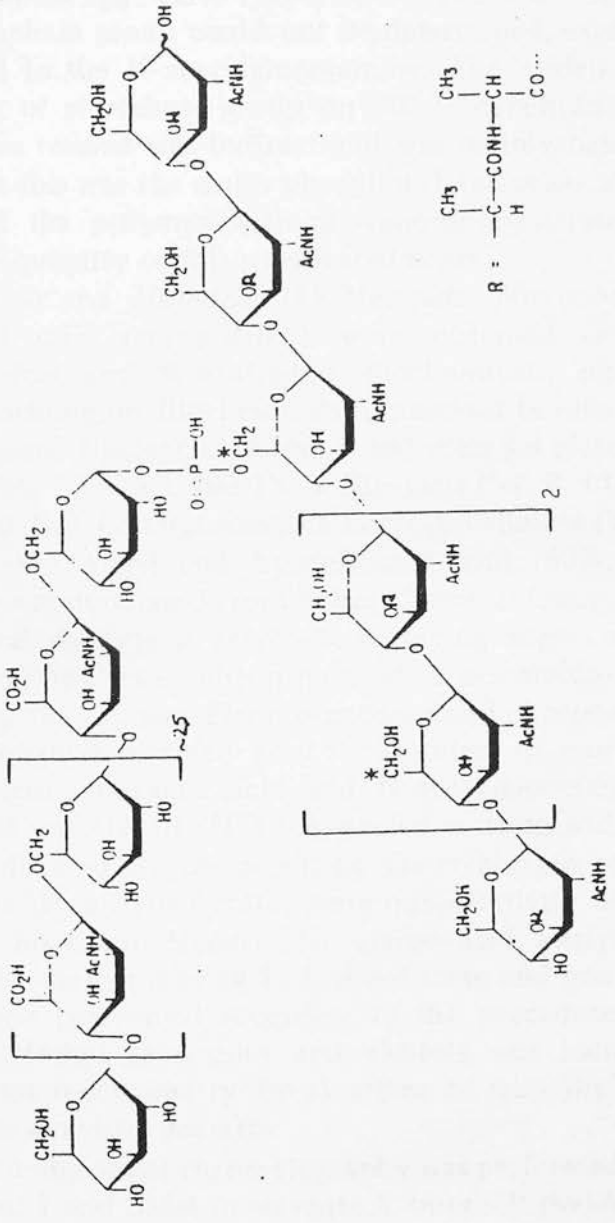
terised by comparison of the g.l.c-m.s. data with those for an authentic sample.[15]

The 2-amino-2-deoxy-D-glucose component was characterised by g.l.c-m.s. mainly, after methanolysis, as 3, 4, 6 and 3, 6-dimethyl ethers. The proportion of 3, 6-di- to 3, 4, 6-tri-methyl ethers indicated a chain-length, for the glycan protein of the peptidoglycan moiety.

The results of the sequential periodate degradation and of the methylation procedure, although not quantitative and complete, are both in agreement with the basic structure shown in Fig. 3. This structure agrees with the "external" polysaccharide chain having the partial structure proposed by Hase *et al*,[3] and Nasir-ud-Din and Jeanloz[4] by methylation and periodate oxidation and also with that proposed for the glycan part of the peptidoglycan moiety based on the isolation of di- and tetra-saccharides.

Fig. 3

Proposed chemical structure for Fraction CPC_{A-2^o}



Removal of the carbohydrate chain by periodate oxidation did not increase the degradation by lysozyme, which suggests that the phosphate group is essentially responsible for lack of lysis and this observation is in agreement with the specificity of egg-white lysozyme.[16] The exact location of the phosphate group could not be determined, except that it is linked to the N-acetylglucosamine. The evidence for the presence of phosphate group on the 2-acetamido-2-deoxy-D-glucose residue was indirect and was mainly based on the fact that this was the major phosphorylated sugar. Also treatment of the polymer with phosphomonoesterase did not alter the quantity of this substituted sugar.

Materials and Methods. (1) *Materials: Micrococcus lysodeikticus* cells (spray-dried) were obtained from Miles Laboratories and Worthington Biochemicals, phosphatase from Worthington Biochemicals, potassium borotritide from New England Nuclear, cellulose F and silica gel plates from E. Merck, AG 50W-X8, AG IX-8, Bio-Gels P-2, P-30 and P-60 from Bio-Rad Laboratories, ECTEOLA-cellulose (Whatman) from Reeve Angel and hydroflouric acid (50%, analysed reagent) was purchased from Baker Chemical Company.

General Analytical Methods. Reducing sugars were estimated by the Park-Johnson method, 2-acetamido-2-deoxy-sugars by the Morgan-Elson procedure and phosphate groups by the method of Chen *et al.*[17] Mixtures of muramic acid 6-phosphate, muramic acid and N-acetylglucosamine were separated on AG 50 (H^+) ion-exchange resin and quantitatively estimated by the modified Elson-Morgan reaction.[18] Amino acids in hydrolyzates were quantitatively determined with a Beckman Model 116 amino-acid analyser. Gas-liquid chromatography (g.l.c) of reducing and non-reducing sugars was performed according to the procedure of Reinhold.[19] Methyl glucosides and alditols wer identified by g.l.c-mass spectrometry (m.s) either as trimethylsilyl derivatives or as alditol acetates.

Descending paper chromatography was performed on Whatman Nos. 1 and 3MM in solvents A-butanol: pyridine: water (6/4/3), B-2 butanone: acetic acid: water (9/1/1), saturated with boric acid and C-butanol: acetic acid: water (4/1/5,

upper layer). Amino sugars and amino acids were detected with ninhydrin,[20] reducing and non-reducing sugars with alkaline silver nitrate[21] and periodate-benzidine,[22] and lactones with hydroxylamine and ferric chloride reagent.[23] Electrophoresis on Whatman No. 1 paper was performed in pyridine: acetic acid buffer (pH 4.3) at 110 V/cm for 3 hrs. The strips were stained with Toluidine Blue,[24] SDS-polyacrylamide (15%) gel electrophoresis was performed according to Laemmli.[25]

Column chromatography on Bio-Gel P-30 was run in water or in water followed by 0.5 M LcCl; the Bio-Gel P-60 column was run in 50 mM pyridine acetic acid (pH 5.4) and the DEAE-cellulose column was run with increasing concentrations of sodium phosphate (25 mM, 50mM, 100mK, 0.25 M and 0.5 M or with a gradient of 25 mM to 1 M pH 6.0) followed by a gradient of 10 mM to IM KCI or with 0.5 M KCI.

Hydrolysis and N-acetylation were performed as previously described.[4] N-acetylation of small samples of amino sugars was performed by dissolution in methanol and addition of a 3-molar excess of acetic anhydride. After 4 hrs at room temperature, the mixture was evaporated under a stream of nitrogen. Small-scale hydrolyses were performed on 1-2 mg of material with 4 M hydrochloric acid for 16 hrs at 100°. The acid was removed by evaporation under a stream of nitrogen, followed by repeated addition and evaporation of methanol and toluene.

Small-scale methanolysis was performed by treating the dried material (1-5 mg) with 0.5-IM methanolic hydrogen chloride (0.5-1.5 ml) for 8-12 hrs at 100° The methanolic hydrogen chloride was removed by evaporation under a stream of nitrogen, followed by repeated addition and evaporation of methanol and toluene.

Ester and aldehyde groups were reduced by treating the cooled, aqueous solution of the substance (2-4 mg in 100-200 ul). The excess of borohydride was decomposed by addition of acetic acid or of Dowex-50 (H^+) ion-exchange resin. The solution was deionised with Dowex 50 ion-exchange resin (when necessary), and the borate ions were

removed as methyl borate by repeated addition and evaporation of methanol. Ninhydrin degradation of hexosamines was performed according to Stoffyn and Jeanloz.[26]

Preparation and fractionation of nondialysable Cell Wall. The cell wall was prepared according to the procedure of Sharon and Jeanloz[27] and was lysed with egg-white lysozyme to obtain nondialysable cell wall as described earlier.[4] The nondialysable cell wall in water (40% w/v) was treated with cetylpyridinium chloride (CPC, 1% w/v) until the precipitation was complete. The precipitate was centrifuged off and washed with aqueous ethanol. The polymer was released from the complex by treatment with 2 M acetic acid for 6 hrs at 22°C, and the polymer was precipitated with ethanol to give Fraction CPC_A.

Fractionation of CPC_A on Bio-Gel P-30. A solution of fraction CPC_A in water (0.09%, w/v) was applied to a column of Bio-Gel P-30. Two fractions CPC_{A-1} and CPC_{A-2} were obtained. The carbohydrate and amino acid composition of fraction CPC_{A-1} is given in Table I.

Sequential periodate oxidation-sodium borohydride reduction of fraction CPC_{A-1}. First treatment. Fraction CPC_{A-1} (0.2 gm in 35 ml of water, PH3.7) was treated with sodium metaperiodate (0.50 g) for 24 hrs at 4° in the dark. The consumption of periodate, measured spectrophotometrically at 223 nm, was constant after 8 hrs. The formic acid released was estimated by titration with 20 nm sodium hydroxide, and the formaldehyde released with the chromotropic acid reagent (2-acetamido-2-deoxy-D-glucitol as the control). The excess of periodate was decomposed with 1, 2-ethanodiol, and the solution was dialysed for three days at 4° against distilled water, to give a nondialysable fraction (0.146 g after lyophilisation). To a portion of this material (0.125 g) in water (25 ml) at 4° was added sodium borohydride (0.10 g) in four portions. An additional amount of sodium borohydride (30 mg) was added and the solution was kept for 24 hrs in the cold. The excess of borohydride was decomposed with M acetic acid, and the solution was dialysed for 30 hrs against distilled water. The nondialysable portion was concentrated and borate ions were removed as methyl borate by repeated

addition and evaporation of methanol (4 X 2 o ml). The residue was dissolved in water, the solution lyophilised (0.10 g), and the product treated with 0.5 M sulfuric acid (5 ml) for 2 hrs at room temperature; the solution was then dialysed against distilled water for 40 hrs. The nondialysable fraction was lyophilised, to give a periodate resistant fraction (94 mg), and kept for further treatment with periodate. The composition of this fraction is reported in Table II.

The dialysable fraction was reduced in volume and the solution passed through a column (0.8 X 6 cm) of Dowex 1 X-8 (AcO^-) ion-exchange resin (200-400 mesh), which was eluted with water (8 ml) and 10 mM acetic acid (8 ml). The eluate was concentrated to 2 ml, and lyophilised (40 mg). Thin-layer chromatography on cellulose, and paper chromatography in Solvents B and C, and detection with alkaline silver nitrate, toluidine Blue, and the hydroxylamine-ferric chloride reagent, indicated the presence of glycerol, 2-acetamido-2-deoxy-D-mannuronic acid and 2-acetamido-2-deoxy-D-mannuronolactone. G.l.c. on cloumns of OV-1 and OV-17 of the derived methyl glycosides showed the presence of glycerol (2.4%), in addition to unidentified compounds.

Hydrolysis of a portion of the dialysable material with M hydrochloric acid for 16 hrs at 100°, and examination by paper chromatography in solvent D, indicated the presence of glycerol and 2-amino-2-deoxy sugars. Another portion of the dialysable fraction was treated with 0.5 M methanolic hydrogen chloride for 6 hrs at 100°. The acid-free residue was N-acetylated, and the product treated in aqueous methanol (1 : 1) with sodium borohydride in the usual way. The reduced material was hydrolysed with M hydrochloric acid for 3 hrs at 100°, and the product N-acetylated. Examination by paper chromatography in solvent D (detection with alkaline silver nitrate), and 0.5 M sodium hydroxide in ethanol (u.v. light), showed the presence of 2-acetamido-2-deoxy-D-mannose and glycerol. This result was confirmed by g.l.c of the methyl glycosides.

The dialysable fraction, remaining, was separated into two major fractions on Whatman paper No. 3MM in solvent C.

The first fraction (1.5 mg) (^{R}GlcNAc - 0.23) of the chromatogram consisted of glycerol, as indicated by t.l.c. The second fraction (16 mg) was composed of two compounds, respectively, having RD-glucuronolactone 0.9 and 1.11, positive to toluidine Blue and to the hydroxylamine-ferric chloride reagent. It was eluted from the chromatogram, glycosidated and esterified with 0.5M methanolic hydrogen chloride (1 ml) for 6 hrs at 100°, and the product acetylated with pyridine and acetic anhydride. The product was reduced with sodium borohydride (2.5 mg), and the product hydrolysed with M hydrochloric acid (1.5 ml) for 6 hrs at 100° The hydrolyzate was adsorbed on a column of Dowex 1 X-8 (AcO^-) ion-exchange resin, and the column was eluted with aqueous methanol. The eluate was evaporated, the residue N-acetylated, and the product crystallised from aqueous ethanol (1 : 1, v/v) and acetone. Recrystallisation from a mixture of aqueous ethanol and acetone gave platelets having m.p. 102-103° and mixed m.p. with authentic 2-acetamido-2-deoxy-D-mannose. 102-105°.

Second degradation. A small portion of the nondialysable material resulting from the first treatment (10.0 mg) was dissolved in 0. IM sodium metaperiodate (5 ml), and the solution kept for 24 hrs at 4°. The uptake of periodate was constant after 4 hrs, and corresponded to 20 mmol D-glucose residue. The solution was processed as described for the first treatment, except that the duration of the acid hydrolysis with 0.5 M sulfuric acid was extended to 3 hrs. The nondialysable material was lyophilised (8.0 mg). Examination of the dialysate by paper chromatography showed the absence of reducing and nonreducing sugars.

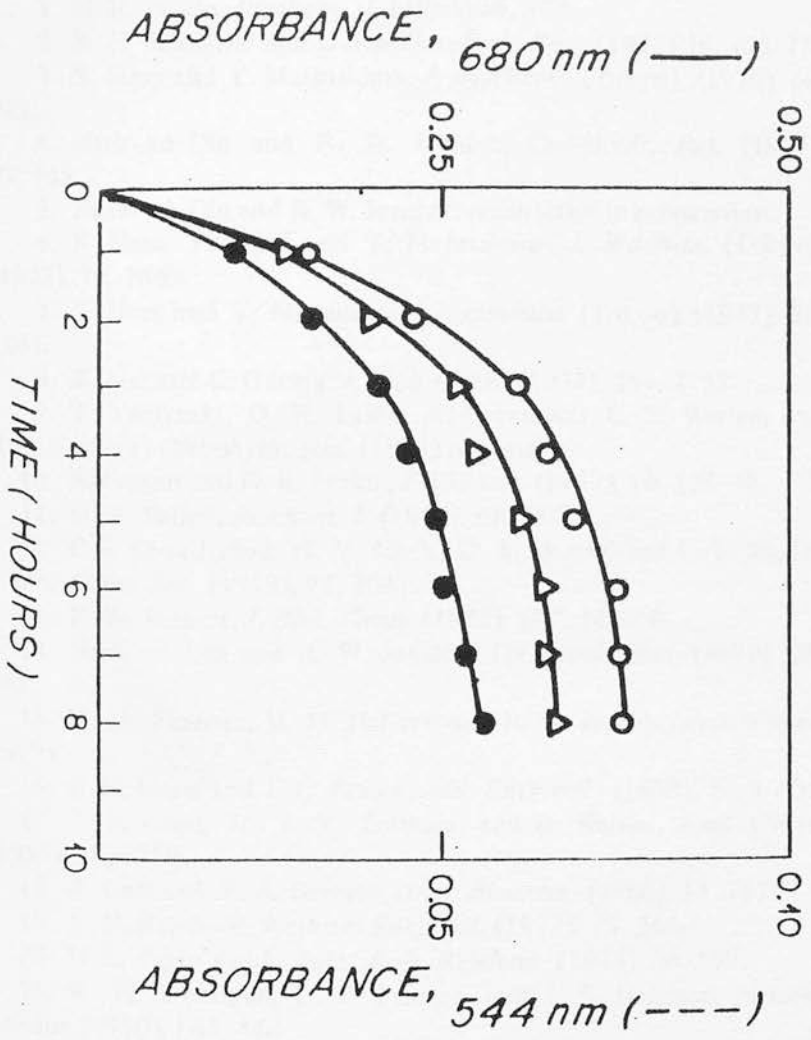
Lysozyme degradation of periodate-degraded CPC_{A-2} *fraction.* Twice periodate treated fraction CPC_{A-1} (4 mg) in 10 mM ammonium acetate (2 ml) was treated with lysozyme (10 mg: General Biochemicals) for 24 hrs at 37° in the presence of a drop of toluene. The resulting solution was dialysed to give a nondialysable fraction (3 mg). Examination of the dialysable fraction by paper chromatrography in solvent D showed the absence of reducing and nonreducing sugars.

Liberation of Polysaccharide Chains from Reduced Pepti-

doglycans. Acid hydrolysis was followed to remove the external polysaccharide from reduced peptodoglycan.

Fraction CPC_{A-1} (10 mg) was dissolved in water (4 ml), and to samples (1 ml), 1.25 mg) in stoppered tubes was added Ag 50W-X8 (H^+, 50-100 mesh, 25 mg). Samples containing methyl-2-acetamido-3-O-(D-I-carboxyethyl)-2-deoxy-D-glucopyranoside-6-yl – D-glucopyranosyl phosphate (1) (Me-Mur-6)-(Glc-I)-P (0.31 mg per ml of water) and glucose-1-phosphate (Glc-I-P) (2.0 mg), were similarly treated. The samples were heated at 65°C, and the release of reducing sugars, amino sugars, and N-acetylated sugars was measured and shown in Fig. 4. Similar aliquots were filtered, the filtrate was evaporated under a stream of nitrogen, and the residue was dissolved in 50 mM sodium-acetate buffer ($_pH$ 5.0, 80 ul) containing acid phosphatase (400 ug per ml). The solution was incubated at 37°C for 4 hrs. The enzyme solution and the solution of reduced peptidoglycan was used as a blank. The phosphate release was measured with Chen's method[17] and is reported in Fig. 2. In addition, samples withdrawn at 3 and 5 hr intervals were examined for the presence of inorganic phosphate.[17] There was no release of reducing sugars by acid phosphatase. Also inorganic phosphate was not detected in hydrolysis samples of 3 and 5 hr intervals, and thereby suggesting absence of pyrophosphate linkage.

Fig. 2



Rate of mild acid hydrolysis of CPC_{A-1} (O), glucose-1-phosphate (0) and (ME-Mur-6)-(Glc-1)-P (Δ). Release of reducing sugars was determined using Park-Johnson (680 nm) and Morgan-Elson (544 nm) procedures.

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STUDY OF ASPARAGINE-LINKED GLYCOPEPTIDE DERIVATIVES BY FIELD DESORPTION MASS SPECTROMETRY*

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Introduction. In N-linked glycoproteins, which include membrane proteins, enzymes, lectins, secretory proteins, hormones and immunoglobulins, the oligosaccharide chains are linked N-glycosidically to the β-amide nitrogen of an asparagine residue in the polypeptide chain. The *core region* of these glycoproteins contains the sequence GlcNAcβ1-4GlcNAcβ-Asn.

Synthetic oligosaccharide-asparagine derivatives are required as model compounds for the study of glycoproteins, for the determination of lectin specificity,[1-3] as intermediates in biosynthetic experiments, such as exogenous substrates for the study of glycosidases,[4] and for the structure elucidation of antigens.[5] Di-N-acetylchitobiose-asparagine[6] and its (1→3) and (1→6) analogs[1-2] have, therefore, been synthesised.

A convenient method was then sought, by which the chemically synthesised key intermediates of the three N-acetylchitobiose isomers could be analysed and differentiated from each other, as they appeared identical by most of the commonly used physicochemical tests. Gas liquid chromatography mass spectrometry has proven to be a most useful

*This was supported in part by a research grant (AM-03564) from the National Institute of Arthritis, Metabolism, and Digestive Diseases, National Institutes of Health (U.S.A.).

tool for structural analysis of carbohydrate derivatives; however, conventional electron impact mass spectrometry is restricted to volatile organic compounds as well as to rather small molecules. Field desorption mass spectrometry,[7] a very mild ionisation technique,[8] was, therefore, used in the present study.

This technique allows the direct analysis of non-volatile glycoconjugates,[9] even of thermally labile or highly polar compounds which cannot be studied by electron impact mass spectrometry, and provides information on their molecular weights. Two principles of the field desoption process make this possible, i.e. the sample is not subjected to a separate evaporation process prior to the formation of ions, and the ions are formed with very little transfer of electronic excitation energy.

Added advantages are the small amounts of material required,[10] in the range of 10^{-8} g for carbohydrates; the fact that there is no need to prepare derivatives prior to analysis, as is the case with conventional mass spectrometry, as well as the simplicity of the spectra obtained. Desorption at increasingly higher emitter currents introduces sufficient energy to cause fragementation at the most labile conjugating groups, thereby also providing structural information. Field desorption mass spectrometry has been used successfully in structure analysis of various biological materials present in minute quantities,[7] in particular of oligosaccharides of the N-glycosidic type.[12]

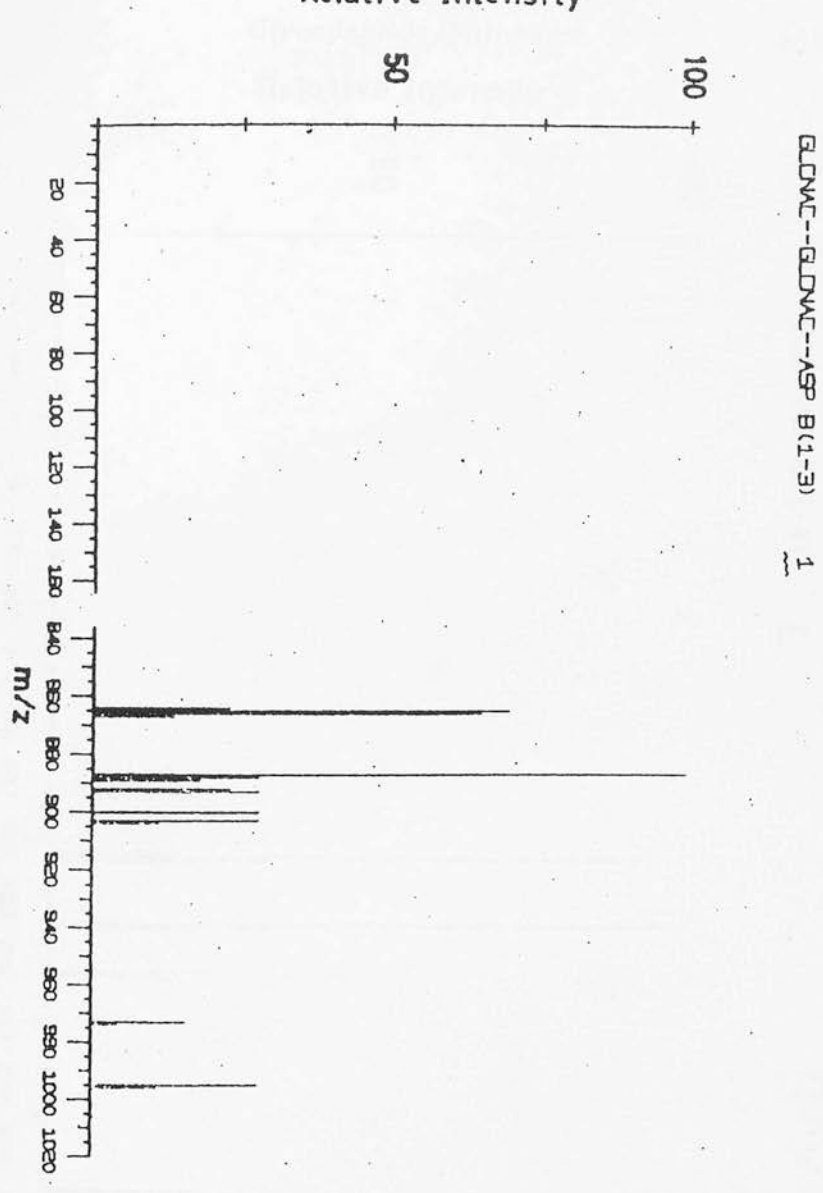
Experimental. 2-Acetamido-3-O-^{2}(1), -4-O-^{6}(2), and -6-O-(2-acetamido-3, 6-tri-O-acetyl-2-deoxy-β-D-glucopyranosyl)-4, 6-di-O-acetyl-N-[benzyl, N-(benzyloxycarbonyl)-L-aspart-4-oyl] -2-deoxy-β-D-glycopyranosylamine[1] (3) were synthesised as previously described.

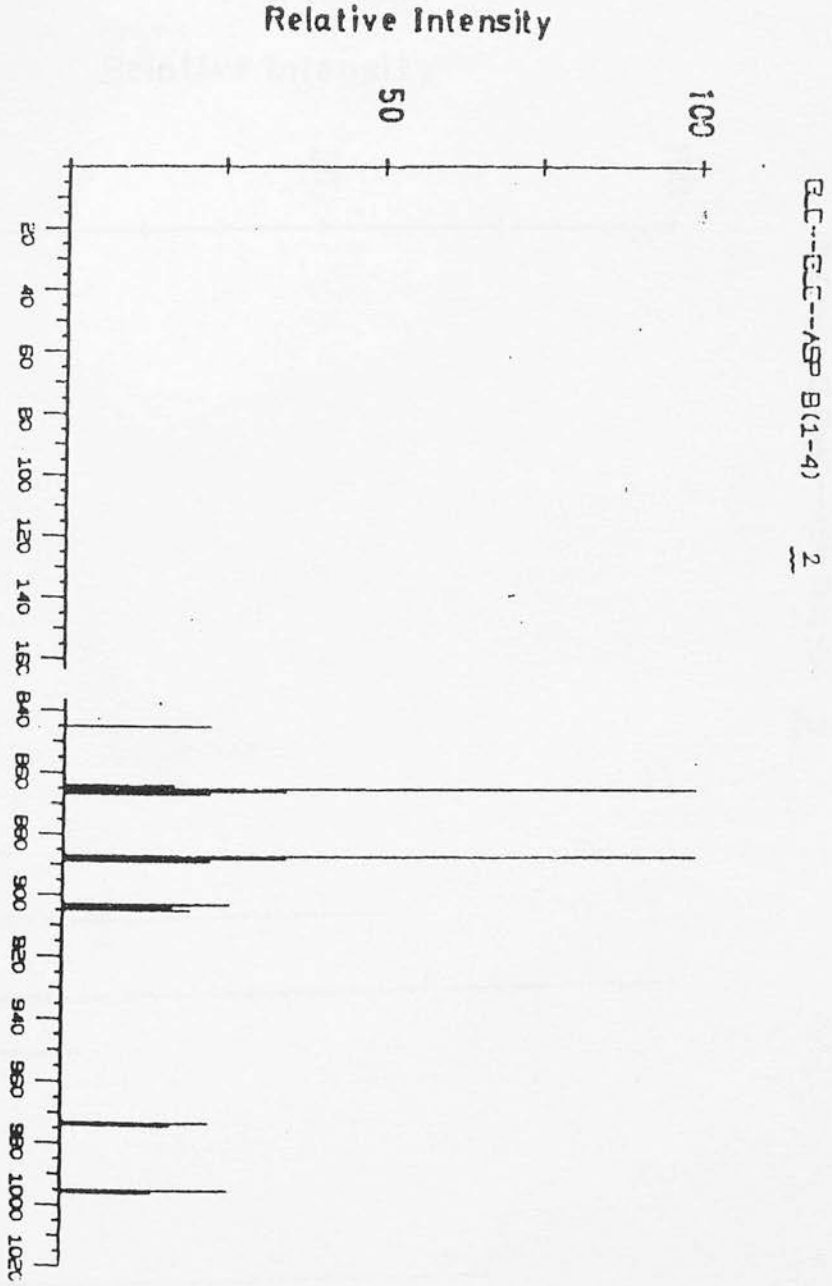
Field desorption – mass spectrometry was performed as described[9] on a Varian MAT 731 instrument fitted with the combined EI/FI/FD ion source. Emitters were prepared according to the method of Schulten and Beckey.[11]

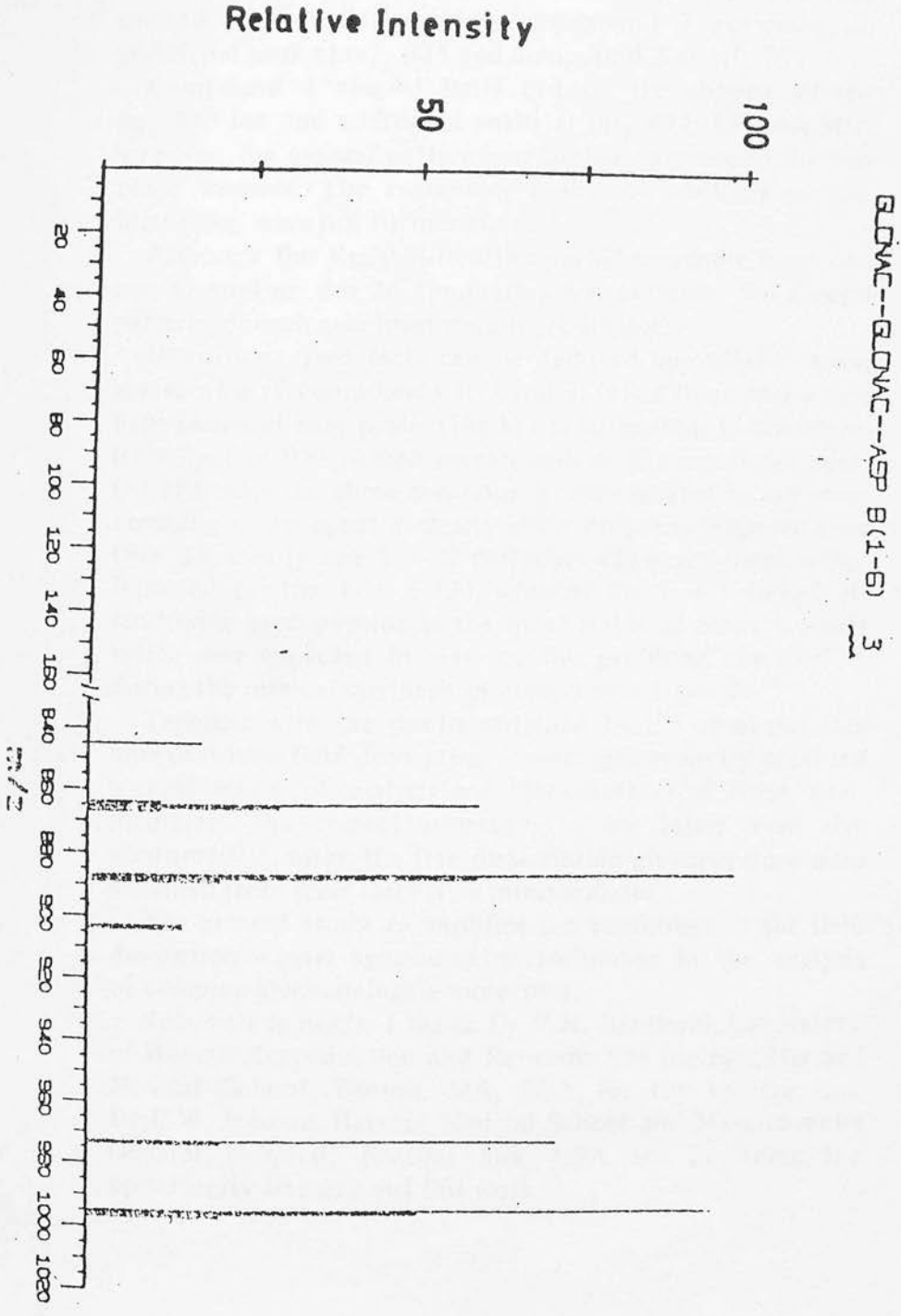
Results. The spectra of the glycopeptide derivatives 1, 2 and 3 showed protonated molecular ions at $m/_z$ 973 of

medium intensity in the case of both the (1→3) and the (1→4) isomers and of high intensity in the (1→6) isomer (Fig.1). "Cationisation,"[9] i.e. attachment of a positively charged metal ion, usually Na or K, to a neutral molecule forming a positively charged complex, was observed in the three spectra, and molecular ions at $m/_z$ 995 (M + Na) with similar relative intensities as the molecular ions were observed.

The three compounds showed common features, and besides the molecular ions at $m/_z$ 995 and 973 (MH^+), ions of high intensities were observed at $m/_z$ 887 (M-85) and 865 (M-Bzl) respectively; clusters of peaks of lower intensities also appeared (Fig.1).


Relative Intensity
50
100
m/z
20
40
60
80
100
120
140
160
840
860
880
900
920
940
960
980
1000
1020
GLCNAC--GLCNAC--ASP B(1-3) 1


Relative Intensity
50
100
20 40 60 80 100 120 140 160 840 860 880 900 920 940 960 980 1000 1020
GLC--GLC--ASP B(1-4) 2


Relative Intensity
50
100
GLCNAC--GLCNAC--ASP B(1-6) 3
m/z
20 40 60 80 100 120 140 160 840 860 880 900 920 940 960 980 1000 1020

The spectra of the (1→4) – and (1→6) – isomers showed very similar patterns, compound 2 possessing an additional peak at $m/_z$ 905 and compound 3 at $m/_z$ 997.

Compound 1 singled itself out by the absence of the $m/_z$ 845 ion and additional peaks at $m/_z$ 892, 893 and 902; however, the general pattern was similar to those of the two other isomers. The remaining peaks, of medium or low intensities, were not further studied.

Although the signal intensities varied somehow from one run to another due to fluctuating ion currents, the general patterns of each spectrum were reproducible.

Discussion. Two facts can be deduced immediately from the spectra of compounds 1, 2 and 3; (a) all three derivatives have identical base peaks (Fig.1) corresponding to a molecular weight of 973, which corresponds to the calculated ones; (b) although the three compounds were treated in the same conditions, the spectra clearly show different fragment ions (Fig. 1); clearly the 1 → 3 derivative (1) is the most labile, followed by the 1 → 6 (3), whereas the 1 → 4 linked disaccharide glycopeptide is the most stable of them, a result which was expected in view of the problems observed[1,2] during the original synthesis of compounds 1 and 3.

Together with the results obtained by the usual physicochemical tests field desorption – mass spectrometry provided a rapid means of analysis and identification of these intermediates. The correct structures of the latter were also confirmed[1,2] when the free disaccharide glycopeptides were obtained from their respective intermediates.

The present study exemplifies the usefulness of the field desorption – mass spectrometry technique in the analysis of complex glycoconjugate molecules.

Acknowledgments. I thank Dr V.N. Reinhold, Laboratory of Human Reproduction and Reproductive Biology, Harvard Medical School, Boston, MA, USA, for the spectra, and Dr R.W. Jeanloz, Harvard Medical School and Massachusetts General Hospital, Boston, MA, USA, for providing the opportunity to carry out this work.

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THE JOURNAL OF BIOLOGICAL CHEMISTRY
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Vol. 260, No. 18, Issue of August 25, pp. 9981-9987, 1985
Printed in U.S.A.

The Phosphate Diester Linkage of the Peptidoglycan Polysaccharide Moieties of *Micrococcus lysodeikticus* Cell Wall*

(Received for publication, November 9, 1984)

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The external polysaccharide is a major component of *Micrococcus lysodeikticus* cell wall and displays distinct composition. The complete structure of the external polysaccharide had been elucidated as a basis for investigation of the cell wall structure-function relation. However, the mode of attachment of the polysaccharide to the peptidoglycan through a phosphodiester was not clear due to limitations in structural and biosynthetic studies. The present study describes purification of a lysozyme-resistant nondialyzable high-molecular-weight fragment of cell wall and identifies the sugar, D-glucose, as the point of external polysaccharide attachment to the peptidoglycan through a phosphate diester. Kinetic studies for the acid-catalyzed release of external polysaccharide from the peptidoglycan were performed in parallel with synthetic [methyl-2-acetamido-3-*O*-(D-1-carboxyethyl)-2-deoxy-α-D-glucopyranoside-6-yl]-α-D-glucopyranosyl phosphate and α-D-glucopyranosyl phosphate and showed the presence of a phosphodiester linkage between external polysaccharide and peptidoglycan. In addition, type of phosphate residue and cross-linking between muramic acid and protein part have been determined.

The cell wall of *Micrococcus lysodeikticus* is composed, in part, of peptidoglycan and external antigenic polysaccharide chains. The peptidoglycan moiety has been shown to include polysaccharide chains composed of alternate *N*-acetylglucosamine and *N*-acetylmuramic acid units linked β(1→4) (2), similar to those of numerous other peptidoglycans bound to peptide chains in microorganisms (3). These peptide chains which interconnect the polysaccharide chains of the peptidoglycan moiety, are linked to the carboxyl group of the *N*-acetylmuramic acid residues. The external polysaccharide chains, which have a different structure for each microorganism, are composed in *M. lysodeikticus* of alternate glucose and 2-acetamido-2-deoxy-D-mannuronic acid (*N*-acetylmannosaminuronic acid) units (4, 5). It has been suggested that they are linked to the peptidoglycan moiety through a phosphate diester linkage involving some of the muramic acid residues at C-6 (6, 7). The linkage region of the external polysaccharide to the peptidoglycan in different organisms has various termini involving glucose 1-phosphate (6, 7), *N*-acetylglucosamine 1-phosphate (8), and the polysaccharide directly linked, without the involvement of phosphodiester, to the peptidoglycan (9).

In the case of *M. lysodeikticus* the linkage of the acidic polysaccharide to C-6 of the muramic acid 6-phosphate residue, through the phosphodiester linkage, has been proposed to be through an *N*-acetylglucosamine residue. These conclusions were based on the results of mild acid cleavage of the phosphate ester linkage that was performed in the absence of control compounds to compare the rate of glycosidic cleavage (8), and thereby rendering the conclusion of limited significance.

There was also a suggestion of glucose being involved as a point of linkage (6). Subsequently it was shown that for the biosynthesis of (external) polysaccharide chains a lipid intermediate containing *N*-acetylglucosamine was required to initiate the elongation of the polymer (10). Furthermore, it was shown that tunicamycin and bacitracin both inhibited the biosynthesis of external polysaccharide (11). In more recent studies, however, there is a good deal of evidence to suggest that a lipid intermediate containing glucose may be involved in the initiation process of external polysaccharide (12). Because of the inconclusiveness as to the point of linkage of the polysaccharide to the peptidoglycan, synthetic and commercially available model compounds were utilized to define optimal conditions for the acid-catalyzed release of polysaccharide from the peptidoglycan. The nature of the carbohydrate residue linked to phosphodiester and the distribution of the phosphate diester linkages along the peptidoglycan chain are the subject of this report.

EXPERIMENTAL PROCEDURES[1]

RESULTS

Fractionation and Properties of Nondialyzable Fraction $CPC_{A\text{-}2}$—The nondiffusible cell wall was fractionated by cetylpyridinium complex fractionation and DEAE-cellulose chromatography (Fig. 1). The cetylpyridinium complex insol-

* This investigation was supported, in part, by Grant AI-06692 from the National Institute of Allergy and Infectious Diseases, National Institutes of Health, and United States Public Health Service. A preliminary communication of this work has been presented (1). This is publication 983 of the Lovett Memorial Group for the Study of Disease Causing Deformities, Harvard Medical School and Massachusetts General Hospital. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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[1] Portions of this paper (including "Experimental Procedures," Tables I to IV, and Figs. 1 to 6) are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are available from the Journal of Biological Chemistry, 9650 Rockville Pike, Bethesda, MD 20814. Request Document No. 84 M-3452, cite the authors, and include a check or money order for $8.00 per set of photocopies. Full size photocopies are also included in the microfilm edition of the Journal that is available from Waverly Press.

uble in water (Fraction CPC_A) represented the major component of undialyzable material, 54% of the nondialyzable material, and 35% of the total cell wall, and corresponded to the second, more acidic, fraction of the DEAE-cellulose chromatography (Table I). The two other fractions of the cetylpyridinium fraction, which were insoluble in ethanol of various concentrations represented 23 and 13% of the undialyzable material; they contained either much larger (Fraction CPC_B) or much lower proportions (Fraction CPC_C) of amino acid components and were not further investigated. Fraction CPC_A was further fractionated by chromatography on Bio-Gel P-30 (Fig. 2) into a minor fraction (Fraction CPC_{A-1}) representing about 10% of CPC_A and a major fraction (Fraction CPC_{A-2}) representing 40% of the total undialyzable material and 26% of the cell wall (Table II). This material was homogeneous on the basis of gel filtration, DEAE-cellulose chromatography, and by paper and SDS[2]-polyacrylamide gel electrophoresis. Its molecular weight was estimated at 25,000. Fraction CPC_{A-2} on reduction with sodium borohydride followed by methanolysis and examination by glc gave *N*-acetylmuramicitol in addition to D-glucose, *N*-acetylglucosamine, *N*-acetylmuramic acid, and *N*-acetylmannosaminuronic acid.

Point of Linkage of External Polysaccharide with Peptidoglycan—In order to ascertain whether the external polysaccharide was linked to the peptidoglycan through a phosphate or pyrophosphate linkage to the muramic acid 6-phosphate residue, Fraction CPC_{A-2} was treated with $NaBH_4$ in order to reduce the terminal sugar which was found to be muramic acid as shown by the results of esterification, reduction followed by acid hydrolysis. The resulting muramic acid-reduced Fraction CPC_{A-2} was treated under mild acid conditions, in parallel with synthetic (Me-Mur-6)-(Glc-1)-P and Glc-1-P. The kinetics of the release of the reducing sugars were similar, for both Fraction CPC_{A-2} and low molecular weight synthetic compounds (Fig. 3). A very small proportion of reducing 2-acetamido-2-deoxy sugars was also liberated, either through degradation of linkages of the GlcNAc or MurNAc residues of the peptidoglycan moiety or of the ManANAc residues of the external polysaccharide. Since no inorganic phosphate was detected, it can be assumed that the acid hydrolysis ruptured specifically the bond between the muramic acid 6-phosphate group and the external polysaccharide. Phosphate was subsequently released from the acid-treated CPC_{A-2} by acid phosphatase (Fig. 4). The gradual increase in the amount of liberated phosphate was concomitant with the release of external polysaccharide by acid treatment.

Four different conditions of acid hydrolysis, AG 50W-X8, 50% hydrofluoric acid, 20 mM HCl, and 10 mM HCl were utilized for the release of external polysaccharide from the peptidoglycan. The results of acid treatment using synthetic compound showed that AG 50W-X8 hydrolysis provided the least degradation and maximum release of the polysaccharide as identified by the combination of kinetic studies and examination of the hydrolysis products by tlc or glc. The released external polysaccharide, after purification on Bio-Gel P-60, showed mainly D-glucose and 2-acetamido-2-deoxymannuronic acid (Table III) as the component sugars.

Identification of the Reducing-end Residue of the External Polysaccharide—The reducing terminal liberated by mild acid hydrolysis was identified as D-glucose, after reduction with potassium borotritide, which gave as major radioactive labeled compound, [1-^{3}H]glucitol. In addition to glucitol tritium, approximately 20% of the total incorporation was also introduced into another sugar component (Fig. 5). This slower moving component appeared to be an oligosaccharide and possibly arose due to incomplete breakdown of Fraction CPC_{A-2}. The proportion of D-glucitol to D-glucose determined, after hydrolysis, by glc or by measurement of radioactivity was found to be 1:40, indicating an average chain length of about 40 Glc-ManANAc residues. In addition, external polysaccharide was released from the peptidoglycan utilizing two different acidic conditions and the reducing terminal residue, which provided linkage to muramic acid 6-phosphate of the peptidoglycan, of the external polysaccharide was identified by glc-mass spectrometry. In both cases glucose was the only sugar present as a reducing terminal residue, and total absence of *N*-acetylhexosamine or *N*-acetylpentosamine (derived from *N*-acetylmannosaminuronic acid as a consequence of decarboxylation) was shown. The proportion of glucitol to glucose was determined to be 1:20. However, the *M. lysodeikticus* cells used in these experiments were obtained from Worthington whereas in all other experiments cells obtained from Miles Laboratories were used (after 1977 *M. lysodeikticus* cells were not available from Miles Laboratories). It is very likely that the variation in the chain length of the external polysaccharide, *i.e.* 40 to 20 disaccharide units, may arise due to different growth conditions.

Reduced Terminal of Peptidoglycan and Reduced Side Chain of Muramic Acid—Hydrolysis of Fraction CPC_{A-2} (reducing terminal reduced) gave 2-amino-3-*O*-(D-1-carboxyethyl)-2-deoxy-D-glucitol in the proportion 1:2.5 relative to unreduced muramic acid, suggesting a peptidoglycan chain composed of 3 to 4 disaccharide Glc-NAc-(1→4)-Mur residues. Hydrolysis of Fraction CPC_{A-2}, (muramic acid side chain reduced) and examination of the hydrolysis products by glc and amino acid analysis showed the absence of muramic acid with reduced side chain, whereas in the control sample the side chain was reduced. This clearly showed that all the carboxyl groups of the muramic acid residues in the polymer are linked to the peptide moiety.

Pyrophosphate Linkage—The presence of pyrophosphate linkage between C-6 of muramic acid moiety and C-1 of the glucose reducing residue of the external polysaccharide was disproved by the negative result of the treatment with ammonium hydroxyde of Fraction CPC_{A-2} followed by chromatography on a calibrated column of Bio-Gel P-60 (Fig. 6); such a treatment had been shown to split off the pyrophosphate bridge (1, 27). The control sample and the alkali-treated material were eluted from the column in close vicinity of each other, suggesting a similarity in molecular size, and contained sugars and amino acids that were identical in composition and comparable in quantity (Table IV).

DISCUSSION

The bacterial cell wall of *M. lysodeikticus* is composed of a peptidoglycan, an external polysaccharide, and a protein (4, 28, 29). The peptidoglycan moiety has been shown to be composed of repeating units of (1→4)-GlcNAc-(1→4)-Mur (2, 30) with partial replacement of Mur residues by the manno analog (31) and of the acetamido group by an amino group, free (32) or engaged in an internal amide formation with the D-1-carboxyethyl group (33). The external polysaccharide is composed of D-glucose and ManANAc residues (4), and its structure has been resolved (5, 6, 34, 35). In the present work,

[2] The abbreviations used are: SDS, sodium dodecyl sulfate; CPC, cetylpyridinium chloride; Glc-1-P, glucose 1-phosphate; Mur, muramic acid; Mur-6-P, muramic acid 6-phosphate; (Me-Mur-6)-(Glc-1)-P, [methyl-2-acetamido-3-*O*-(D-1-carboxyethyl)-2-deoxy-α-D-glucopyranoside-6-yl]-α-D-glucopyranosyl phosphate; MurNAc, *N*-acetylmuramic acid; ManANAc, *N*-acetylmannosaminuronic acid; glc, gas-liquid chromatography.

the mode of linison of the external polysaccharide has been investigated, and a model for the chemical structure of a major bacterial cell wall fragment, Fraction $CPC_{A\text{-}2}$, is proposed.

Previous investigations (30, 32) have shown that degradation with egg-white lysozyme gives fragments of large molecular size that contain the totality of the external polysaccharide linked to fragments of the peptidoglycan outer polysaccharide and represents about 65 to 70% of the total cell wall (Table I). Similar observations have been made during fractionation of nondialyzable material on DEAE-cellulose.

After fractionation by precipitation with cetylpyridinium chloride and gel filtration on Bio-Gel P-30, a homogeneous fraction ($CPC_{A\text{-}2}$) was obtained. The composition (Table II) suggests an average distribution of 1 residue of Mur-P-Glc/four disaccharide units of (1→4)-D-GlcNAc-(1→4)-Mur of the peptidoglycan and approximately 40 disaccharide repeating units of (1→6)-D-Glc-(1→4)-D-ManANAc for the external polysaccharide/residue of Mur-6-P. The content of phosphate of Fraction $CPC_{A\text{-}2}$ is approximately equal to that of Mur-6-P. Although the existence of another *O*-6 sugar phosphate, *i.e.* *N*-acetylglucosamine 6-phosphate in the cell wall of *M. lysodeikticus*, is known (7), muramic acid 6-phosphate was the only sugar detected in this fragment of the cell wall. The ratio of muramic acid to muramicitol does not quite complement the molar ratio of muramic acid to muramic acid 6-phosphate. The ion exchange purification procedure required for determination of muramic acid 6-phosphate is likely to provide lower yields, and this might explain the anomaly between the two complementary experiments. The presence of D-ManANAc residues in the external polysaccharide has been ascertained (5), and this was confirmed by the isolation and characterization of a derivative of ManANAc in the methylation procedure (6). The kinetics of acid hydrolysis of Mur-reduced Fraction $CPC_{A\text{-}2}$ was similar to that of Glc-1-P and of (Me-Mur-6)-(Glc-1)-P, strongly suggesting that the external polysaccharide chain was linked to the phosphate group attached in C-6 of the Mur residue. The sugar residue involved in this linkage was shown to be a D-glucose residue in the proportion of 1:40 in relation to the total amount of D-glucose residues, which suggests a chain length of about 40 (1→6)-D-Glc repeating units. This is in good agreement with the results of the methylation procedure which indicated a ratio of one nonreducing terminal D-glucose residue to 49 internal D-glucose residues corresponding to (1→6)-D-Glc-(1→4)-D-ManANAc repeating units. The chain length at the peptidoglycan moiety was found to be 3 to 4 repeating units of (1→4)-Mur by determination of the reducing end as reduced muramic acid, whereas the methylation procedure (6) had indicated 4 repeating units on the basis of the isolation of 3,4,6-tri-*O*- and 3,6-di-*O*-methyl ethers of *N*-acetylglucosamine. As shown by the result of the methylation procedure (6) this component has the same β-D-(1→4) linkage as found in the portions of the chain susceptible to egg-white lysozyme degradation. Since this enzyme degradation led to the formation of peptidoglycan fragments containing peptide moieties (32), it is clear that the presence of external polysaccharide chain is one of the main causes of inhibition to egg-white lysozyme degradation, in agreement with the studies made on the specificity of egg-white lysozyme (35). The amino acid content of Fraction CPC_B and CPC_C suggests that resistance to lysozyme degradation may also depend upon a high degree of substitution with peptide linkage (Fraction CPC_B) or only from substitution with the external polysaccharide (CPC_C). The resistance to egg-white lysozyme of the periodate-degraded Fraction $CPC_{A\text{-}2}$ (6) indicates that substitution with a long-chain polysaccharide phosphate at C-6 of the muramic acid component is not necessary and that a phosphate group alone is sufficient to block the degradation. Thus, it is possible that the resistance of Fraction CPC_C is due to phosphate esters not bound to a large-chain polysaccharide.

These data and the results of the methylation and the periodate degradation procedures (6) suggest the structure depicted in Fig. 7. The mode of linkage of the external polysaccharide chain is identical with that suggested by Hase and Matsushima (5, 34). The linkage of the external polysaccharide has been suggested to be through *N*-acetylglucosamine (8) based on the acid-catalyzed release of reducing sugar of the external polysaccharide. This conclusion was mainly based on the results of mild acid hydrolysis and in the absence of controls, *i.e.* model compounds. In the present studies acid-catalyzed release of external polysaccharide under four conditions, *i.e.* AG 50W-X8, 20 mM HCl, 10 mM HCl, and 50% hydrofluoric acid was performed, with (Me-Mur-6)-(Glc-1)-P and Glc-1-P as controls, and in all cases D-glucose was found as the reducing sugar after acid treatments and, as a consequence, was assigned as the point of linkage of the polysaccharide to the peptidoglycan. Definitive evidence for this residue as the linkage sugar was provided by kinetic studies and glc-mass spectrometry. The kinetics of hydrolysis favor an α-D-glucopyranosyl phosphate linkage, since a β-D-linkage would be more susceptible to acid hydrolysis (39).

On the basis of the molecular weight determination, it is evident that the basic structure consists of an external polysaccharide chain, one phosphate group, and a peptidoglycan chain composed of 8 carbohydrate units and an average of 20

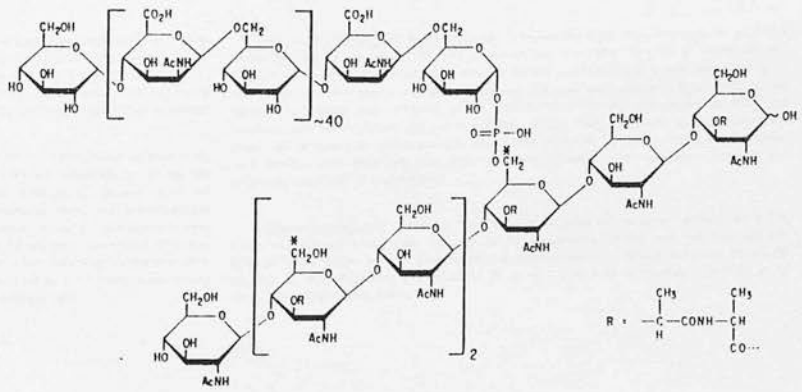

FIG. 7. **Proposed chemical structure for Fraction $CPC_{A\text{-}2}$.**

amino acids. The results of mild alkaline hydrolysis (Fig. 6) do not support the hypothesis of a pyrophosphate linkage. It is of interest that the external polysaccharide chains start and terminate with a D-glucose residue. From the data on the homogeneity of Fraction $CPC_{A\text{-}2}$, it is not possible to ascertain whether all the chains have the same length, but they do not seem to vary greatly in length. In view of the known mechanism of biosynthesis of bacterial polysaccharide through a lipid intermediate (36) and still undefined mechanism of external polysaccharide synthesis (10, 12, 37, 38) it is possible that the transfer of the external polysaccharide chain to the peptidoglycan takes place through an intermediate containing only one D-glucose residue instead of the usual disaccharide unit.

All the carboxylic groups of the muramic acid component in Fraction $CPC_{A\text{-}2}$ are bound to the peptide chains and it is not possible, at the present time, to give their detailed structure and distribution along the glycan chains of the peptidoglycan in the cell wall.

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SUPPLEMENTARY MATERIAL TO

THE PHOSPHATE DIESTER LINKAGE OF THE PEPTIDOGLYCAN POLYSACCHARIDE MOIETIES OF MICROCOCCUS LYSODEIKTICUS CELL WALLS

by Nasir-ud-Din, Michel Lhermitte, Geneviève Lamblin and Roger W. Jeanloz

EXPERIMENTAL PROCEDURES

Materials - Micrococcus lysodeikticus cells (spray-dried) were obtained from Miles Laboratories and Worthington Biochemicals, phosphatase from Worthington Biochemicals, potassium borotritide from New England Nuclear, cellulose F and silica gel plates from E. Merck, AG 50W-X8, AG 1X-8, Bio-Gels P-2, P-30 and P-60 from Bio-Rad Laboratories, DEAE-cellulose (Whatman DE-32) from Reeve Angel and hydrofluoric acid (50%, analyzed reagent) was purchased from Baker Chemical Company.

Analytical Methods - Reducing sugars were estimated by the Park-Jonhson method (13), 2-acetamido-2-deoxy-sugars by the Morgan-Elson procedure (14) and phosphate groups by the method of Chen et al. (15). Mixtures of muramic acid 6-phosphate, muramic acid and N-acetylglucosamine were separated on AG 50 (H^+) ion-exchange resin and quantitatively estimated by the modified Elson-Morgan reaction (16). Amino acids in hydrolyzates were quantitatively determined with a Beckman Model 116 amino acid analyzer. Gas-Liquid chromatography (g.l.c.) of reducing and nonreducing sugars was performed according to the procedure of Reinhold (17). Methyl glycosides and alditols were identified by g.l.c.-mass spectrometry (m.s.) either as trimethylsilyl derivatives or as alditol acetates (18).

Paper Chromatography and Electrophoresis - Descending paper chromatography was performed on Whatman Nos. 1 and 3MM in solvents A, butanol:pyridine:water (6/4/3), B, 2-butanone:acetic acid:water (9/1/1, saturated with boric acid) and C, butanol:acetic acid:water (4/1/5, upper layer). Amino sugars and amino acids were detected with ninhydrin (19), reducing and nonreducing sugars with alkaline silver nitrate (20) and periodate/benzidine (21), and lactones with hydroxylamine and ferric chloride reagent (22). Electrophoresis on Whatman No1 paper was performed in pyridine:acetic acid buffer (pH 4.3) at 110V/cm for 3 h. The strips were stained with toluidine blue (23). SDS-polyacrylamide (15%) gel electrophoresis was performed according to Laemmli (24).

Column Chromatography - Bio-Gel P-30 was run in water or in water followed by 0.5 M LiCl; the Bio-Gel P-60 column was run in 50 mM pyridine acetic acid (pH 5.4) and the DEAE-cellulose column was run with increasing concentrations of sodium phosphate (25 mM, 50 mM, 100 mM, 0.25 M or with a gradient of 25 mM to 1 M pH 6.0) followed by a gradient of 10 mM to 1 M KCl or with 0.5 M KCl.

Acid Hydrolysis and N-Acetylation - Hydrolysis and N-acetylation were performed as previously described (6).

Preparation and Fractionation of Nondialyzable Cell Wall - The cell wall was prepared according to the procedure of Sharon and Jeanloz (25) and was lysed with egg-white lysozyme to obtain nondialyzable cell wall as described earlier (2). The nondialyzable cell wall in water (40%, w/v) was treated with cetylpyridinium chloride (CPC, 1% w/v) until the precipitation was complete. The precipitate was centrifuged off and washed with aqueous ethanol. The polymer was released from the complex by treatment with 2 M acetic acid for 6 h at 22°C and was precipitated with ethanol to give Fraction CPC_A. The supernatant solution and the washings were combined, reduced in volume, and ethanol (80%) was added until complete precipitation. The precipitate was washed with aqueous ethanol to remove CPC. The insoluble complex was treated in a manner similar to that described for CPC_A to give Fraction CPC_B. The washings and supernatant were combined, reduced in volume, and absolute ethanol (5 volumes) was added to give a precipitate. The free polymer was recovered by the procedure described for CPC_A, to give Fraction CPC_C. The composition of Fractions CPC_A, CPC_B and CPC_C is given in Table I.

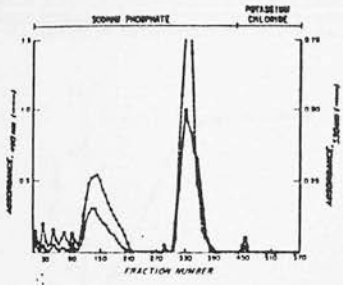

Fig. 1. Separation of undialyzable cell-wall (250 mg) on a column(2.5 x 64 cm) of diethylaminoethylcellulose. Fractions of 8 ml were collected and every third fraction was examined for the presence of hexoses (40) by the phenol-sulphuric acid procedure (490 nm, ---) and hexosamines by Gatt-Berman colorimetric reaction (530 nm, ——). The column was washed with an increasing concentration of sodium phosphate buffer, pH 6.8 (25 mM, 600 ml ; 50 mM, 600 ml ; 100 mM, 600 ml ; 0.25 M, 600 ml ; 0.50 M, 600 ml) followed by a gradient of 10 mM (700 ml) to 1M KCl (700 ml).

Fractionation of Nondialyzable (Lysozyme-Resistant) Cell Wall on DEAE-Cellulose - The nondialyzable cell wall was fractionated on DEAE-cellulose into two main fractions (Fig. 1). The composition of the fractions is given in Table I.

Table I : Carbohydrate and amino acid composition of lysozyme-resistant (nondialyzable) cell wall, and of fractions obtained by cetylpyridinium chloride and DEAE-cellulose fractionation.

	Nondialyzable cell wall		CPC_A		CPC_B		CPC_C		DEAE-1		DEAE-2	
Carbohydrates[a]	%	MR	%	MR	%	MR	%	MR	%	MR	%	MR
D-Glucose	30	3.7	32	4.3	9	1.2	40	6.1	22	2.7	34	5.9
N-Acetylglucosamine	10	1.0	9	1.0	9	1.0	8	1.0	10	1.0	7	1.0
N-Acetylmuramuronic acid	33	3.1	39	4.1	14	1.4	44	5.1	28	2.6	38	5.1
Muramic acid	7	0.5	7	0.6	4	0.3	5	0.5	12	0.9	7	0.8
Muramic acid 6-phosphate	0.8	0.05	1.1	0.07	1.2	0.08	0.4	0.03	1.1	0.06	1.2	0.1
Glucosamine 6-phosphate	0.16	0.01	0.18	0.01	0.11	0.008			0.19	0.01	0.18	0.02
Amino acid[b]												
Alanine		43		43		40		36		40		44
Glutamic acid		19		19		19		27		21		14
Glycine		19		20		21		18		20		24
Lysine		19		18		20		19		19		18

[a]Molar ratio (MR) relative to N-Acetylglucosamine. [b]Residues per 100 residues

Fractionation of CPC_A on Bio-Gel P-30 - A solution of Fraction CPC_A in water (0.09%, w/v) was applied to a column of Bio-Gel P-30. Two fractions CPC_{A-1} and CPC_{A-2} were obtained (Fig. 2). The properties of these fractions are reported in Table II.

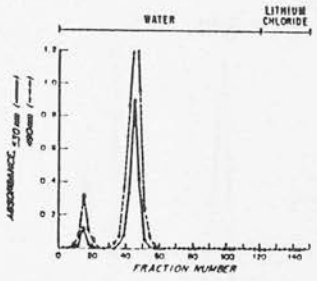

Fig. 2. Separation of Fraction CPC_A (0.35 g) on a column (2.8 x 72 cm) of Bio-Gel P-30. Fractions of 4 ml were collected and examined by phenol-sulphuric acid (490 nm) for hexoses and by Gatt-Berman (530 nm) for hexosamines. The column was washed with water (500 ml) followed by 0.8 M LiCl (500 ml).

Table II : Carbohydrate and amino acid composition of Bio-Gel P-30 purified Fraction CPC_A and DEAE-cellulose purified Fraction CPC_{A-2}.

	CPC_{A-1}		CPC_{A-2}		DEAE F1	
Carbohydrates[a]	%	MR	%	MR	%	MR
D-Glucose	16	1.4	28	4.9	29	3.9
N-Acetylglucosamine	14	1.0	7	1.0	9	1.0
N-Acetylmuramuronic acid	18	1.2	34	4.5	36	3.8
Muramic acid	11	0.6	9	1.0	10	0.8
Muramic acid 6-phosphate	1.9	0.08	1.2	0.1	1.1	0.07
Glucosamine 6-phosphate	0.13	0.007	0.11	0.01	0.12	0.009
Amino acids[b]						
Alanine	38		40		40	
Glutamic acid	14		17		16	
Glycine	21		22		25	
Lysine	27		21		19	

[a]Molar ratio (MR) relative to N-Acetylglucosamine [b]Residues per 100 residues

Chromatography of Fraction CPC_{A-2} on DEAE-cellulose - Fraction CPC_{A-2} was chromatographed on a column of DEAE-cellulose and the polymer was eluted with a gradient of 25 mM sodium phosphate (pH 6.0) followed by 0.5 M KCl. A single component was eluted from the column (Table II).

Molecular Weight Determination by Sedimentation Equilibrium of Fraction CPC_{A-2} - A sample of CPC_{A-2} (1.5 mg) in 6 M guanidine hydrochloride-10 mM EDTA (1 ml) was dialyzed at 4°C against the same solution for 36 h and analyzed on a Beckman Model E analytical ultracentrifuge by the method of Yphantis (28). The partial specific volume of 0.65 was used for calculation of the molecular weight.

Reducing Carbohydrate Residue of Fraction CPC_{A-2} - A solution of Fraction CPC_{A-2} (100 mg) in water (5 ml) was cooled to 4°C and treated with sodium borohydride for 12 h at 4°C. The reduced polymer was precipitated by the addition of ethanol. The precipitate was washed and repeatedly evaporated with methanol (4 x 25 ml) to give the reduced polymer (85 mg). A portion of the reduced polymer was methanolysed and examined by g.l.c. (17).

Scission of External Polysaccharide Chains from Reduced Peptidoglycans - Three different conditions of acid hydrolysis were followed to remove the external polysaccharide from the reduced peptidoglycan :

(a) Fraction CPC_{A-2} (20 mg) was dissolved in water (8 ml), and to aliquots (1 ml, 2.5 mg) in stoppered tubes was added AG 50W-X8 (H^+, 50-100 mesh, 100 mg). Samples containing [methyl-2-acetamido-3-O-(D-1-carboxyethyl)-2-deoxy-α-D-glucopyranoside-6-yl]-α-D-glucopyranosyl phosphate (1), (Me-Mur-6)-(Glc-1)-P, (0.31 mg per ml of water) and glucose-1-phosphate (Glc-1-P) (0.6 mg per ml of water) were similarly treated. The samples were heated at 65°C and the release of reducing sugars, amino sugars and N-acetylated sugars was measured as shown in Fig. 3. Similar aliquots were filtered ; the filtrate was evaporated under a stream of nitrogen, and the residue was dissolved in 50 mM sodium acetate buffer (pH 5.0, 80 µl) containing acid phosphatase (400 µg per ml). The solution was incubated at 37°C for 4 h. The enzyme solution and the solution of reduced peptidoglycan was used as a blank. The phosphate released was measured with Chen's method (15) and is reported in Fig. 4. In addition, samples withdrawn at 3 and 5 hour intervals were examined for the presence of pyrophosphate (15).

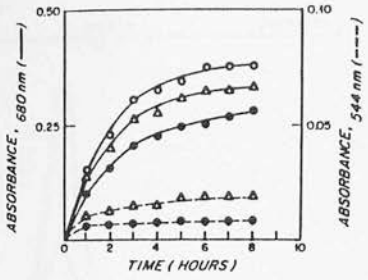

Fig. 3. Rate of mild acid hydrolysis of CPC_{A-2} (Δ), glucose-1-phosphate (○) and (Me-Mur-6)-(Glc-1)-P (●). Release of reducing sugars was determined using Park-Johnson (680 nm) and Morgan-Elson (544 nm) procedures.

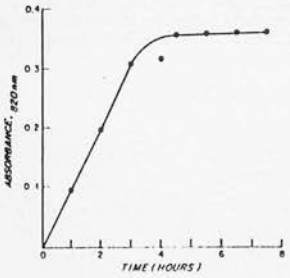

Fig. 4. Measurement of the release of phosphate by acid phosphatase from the aliquots of mild acid hydrolysate. Inorganic phosphate was determined by Chen's procedure (15).

(b) Fraction CPC_{A-2}, reducing terminal reduced (10 mg), in a polyethylene tube was treated with 50% hydrofluoric acid (200 µl) for 90 min at 4°C. (Me-Mur-6)-(Glc-1)-P (50 µg) and Glc-1-P (50 µg) were similarly treated. The reaction was quenched by adding a saturated solution of lithium hydroxide, and finally the solution was adjusted to pH 7.0 with solid lithium carbonate. Lithium fluoride was removed at the centrifuge, and the supernatant was adjusted to pH 5.0 with 4 M acetic acid. The solution was deionized by gel filtration on a column of Bio-Gel P-2, to give a carbohydrate-containing fraction 8 mg).

In the case of the reference compounds the neutral solution was evaporated. The residue was dispersed in methanol (200 µl) and a portion of this solution was examined by t.l.c. in solvent A. The remaining solution was treated with acetic anhydride (150 µl) for 6 h at 22°C. The solution was evaporated ; the residue in water was treated with AG 50W-X8 to remove lithium ions. The filtrate was lyophilized, the residue dissolved in water and examined by t.l.c. (solvents A and B) and Park-Jonhson (13) reaction for the presence of reducing sugars.

A portion of the hydrofluoric acid-treated Fraction CPC_{A-2} was examined by the Park-Johnson (13) procedure for the presence of reducing sugars ; borohydride treated Fraction CPC_{A-2} was used as blank and glucose was used as a standard of reducing sugar. Another portion of the acid-treated Fraction CPC_{A-2} was treated with acid phosphatase (500 µl, same concentration as described above) for 6 h at 37°C. The enzyme solution and fraction CPC_{A-2} were used as control.

(c) Fraction CPC_{A-2} (10 mg) in 20 mM HCl (3 ml) was heated at 100°C for 20 min in a sealed tube. The solution was deionized on a column of Bio-Gel P-2 and the carbohydrate-containing material was lyophilized. The residue in water was examined for the presence of reducing sugars (13).

Identification of the Reducing End Group of the External Polysaccharide

(a) To a cooled solution of external polysaccharide, described in the preceding section under (a), in water was added sodium borohydride, and the solution was processed to give a reduced polymer. A portion of the reduced polymer was methanolyzed and the products were examined by g.l.c.

(b) A solution of external polysaccharide (12 mg), described in the preceding paragraph under (a), in water (1 ml) was treated with potassium borotritide (14 µCi/ mol ; ca. 0.5 mg) for 4 h at 0°C and then for 2 h at 22°C. A sample of glucose was reduced with $KB[^3H]_4$ and H-labelled glucose was used as a control. The radioactivity in the acid liberated external polysaccharide corresponded to 7.3 g of [^{3}H-1-] glucitol per 1 mg of the liberated polysaccharide in Fraction CPC_{A-2}.

The ^{3}H-labelled reduced polymer (2 mg) was acid hydrolyzed (6) and a measured quantity of the hydrolyzate, ^{3}H-glucitol and sorbitol, were deposited on a paper chromatogram and developed in solvent B. The sugars corresponding to glucitol were eluted from the paper and the radioactivity in the isolated sugar corresponded to 0.69 mg of [^{3}H-1-]-glucitol per 100 mg of liberated, reduced polysaccharide. Another sample of the hydrolysate were deposited on the paper, and the chromatogram was developed in solvent B. The paper was cut into strips, the strips dispersed into 0.2 ml of water and 10 ml of Aquasol, and counted for radioactivity. The elution profile is shown in Fig. 5.

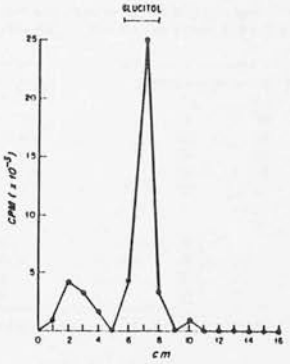

Fig. 5. The elution profile of ^{3}H-labelled sugars from the paper chromatograms of the potassium borotritide treated and acid hydrolyzed outer polysaccharide.

(c) 1. Fraction CPC_{A-2} (reducing terminal reduced, 5 mg) was treated with AG 50W-X8 (25 mg) in 0.5 ml of water at 65°C for 3 h ; after filtration, the filtrate was evaporated and the residue was acetylated with pyridine (400 µl) and acetic anhydride (300µl). After evaporation of the solvents, the residue in aqueous methanol (30%, 0.5 ml) was treated with sodium borodeuteride (20 mg). The mixture was kept at 4°C for 12 h. The solution was further treated with $NaB[^2H]_4$ (15 mg) and the reaction mixture was allowed to stand at 22°C for 6 h. After removal of the sodium and borate ions, the solution was evaporated to dryness. The residue was methanolyzed, and the resulting sugars were examined by g.l.c.-mass spectrometry.

2. Fraction CPC_{A-2} (5 mg, reducing terminal reduced) was treated with 10 mM HCl (100 µl) at 100°C for 1 h (8). The resulting solution was diluted with water (× 20) and lyophilized ; the residue was acetylated with pyridine (300µl) and acetic anhydride (200µl). After removal of the acetylating reagents the residue was treated with $NaB[^2H]_4$ as described above. The reduced material was acid hydrolyzed and the chloride ions were removed from the hydrolyzate by passage through a column of AG-1X8 (OAc^-). The column was washed with water followed by acetic acid (1 M). The washings were combined and lyophilized, the residue was acetylated as just described and the acetylated sugars were examined by g.l.c.-mass spectrometry.

Isolation and Purification of External Polysaccharide - The acid-treated (AG 50W-X8) Fraction CPC_{A-2} was applied to a column of Bio-Gel P-60. The column was washed with water, the carbohydrate-containing fractions were combined and lyophilized. The residue was rechromatographed on Bio-Gel P-60 to give a single component (Table III). A portion (2 mg) was reduced with sodium borohydride ; the product was hydrolyzed ; the hydrolyzate was acetylated, methanolyzed, and examined by g.l.c.

Table III : Carbohydrate and amino acid composition of Bio-Gel P-60 purified external polysaccharide.

	First Treatment %	Second Treatment %
Carbohydrates		
D-Glucose	38	42
N-Acetylmannuronic acid	45	48
N-Acetylglucosamine	1.2	tr
Muramic acid	0.4	tr
Amino Acids		
Alanine	0.6	tr
Glycine	0.3	tr
Glutamic acid	0.6	tr
Lysine	0.9	tr

tr : trace

Presence of Orthophosphate as Diester Linkage Between Glucose and Muramic Acid in Fraction CPC_{A-2} - Solutions containing fraction CPC_{A-2} (25 mg, reducing end converted to alditol) and [methyl 2-acetamido-3-O-(D-1-carboxyethyl)-2-deoxy-α-D-gluropyranoside-6-yl]-α-D-glucopyranosyl phosphate (2.5 mg), respectively, in 2 M ammonium hydroxide (2 ml and 0.4 ml) were heated at 37°C for 20 min. After being cooled, the solutions were diluted and lyophilized. The polymer was applied to a column of Bio-Gel P-60. As a control, untreated Fraction CPC_{A-2} was chromatographed on the same column. The carbohydrate and amino acid compositions of the ammonia treated and control samples are given in Table IV.

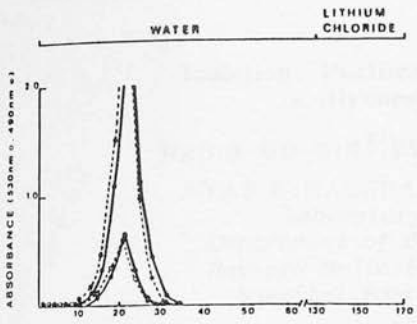

Fig. 5. Fraction CPC_{A-2} (19 mg) (----) and ammonia treated (17 mg) (———) was chromatographed on the same column (1.2 x 40 cm) of Bio-Gel P-60. Fractions of 2ml were collected and every third fraction was examined for hexoses and hexosamines. The column was washed with water (250 ml) followed by 0.5 M LiCl (100 ml).

Table IV : Carbohydrate and amino acid composition of Bio-Gel P-60 purified Fraction CPC_{A-2} and ammonia treated Fraction CPA_{A-2}. (from the same Bio-Gel P-60 column).

	Fraction CPC_{A-2}		Ammonia treated Fraction CPC_{A-2}	
Carbohydrates[a]	%	MR	%	MR
D-Glucose	17	1.5	18	1.7
N-Acetylglucosamine	14	1.0	13	1.0
N-Acetylmannuronic acid	19	1.3	18	1.3
Muramic acid	12	0.6	12	0.7
Amino acids[b]				
Alanine	41		38	
Glutamic acid	12		15	
Glycine	22		21	
Lysine	25		24	

[a]Molar ratio (MR) relative to N-Acetylglucosamine [b]Residues per 100 residues

Determination of Free Carboxyl Functions of 2-Acetamido-3-O-(D-1-carboxyethyl)-2-deoxy-D-glucose residues in Fraction CPC_{A-2} - Fraction CPC_{A-2} (50 mg) in 10 mM methanolic HCl (100 ml) was stirred for 48 h at 4°C. Methanol (250 ml) was added and then evaporated to dryness at 20°C. The esterification was repeated three times. The esterified fraction CPC_{A-2} was dissolved in aqueous methanol (20 ml, 10% v/v) and treated with $NaBH_4$ (150 mg) for 12 h at 4°C. The solution was adjusted to pH 5.0 with 4 M acetic acid and dialyzed at 4°C. The nondialyzable material was esterified as described above, dispersed in tetrahydrofuran (25 ml) and treated with lithium aluminium hydride (100 mg) for 8 h and at reflux for 1 h. The cooled mixture was treated with acetic acid and ethyl acetate and dialyzed at 4°C. Methyl 2-acetamido-3-O-(D-1-carboxyethyl)-2-deoxy-α-D-glucopyranoside (2 mg) was processed in a way similar to that described for Fraction CPC_{A-2}. The carboxyl function of this compound was converted to give muramic acid with reduced side chain (methyl 2-acetamido-2-deoxy-3-O-[(R)-1-(hydroxymethyl)-ethyl]-α-D-glucopyranoside. The carboxyl-reduced Fraction CPC_{A-2} and muramic acid with reduced side chain were acid hydrolyzed. The acid free hydrolysates were examined by g.l.c. and amino acid analyzer for the presence of muramic acid with reduced side chain.

J.Chem.Soc.Pak. Vol. 7, No. 3, 1985

Isolation, Purification and Partial Characterization of a Glycoprotein from Plasma Secretion

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(Received 9th October, 1983, Revised 28th February, 1984)

Summary: The antigenic polymers present in seminal plasma play a key role in the processes of reproduction, survival of cells and provide genetic markers. A high molecular-weight glycoprotein component has been isolated from seminal plasma, purified and its partial structure has been investigated by lectin affinity and haemagglutination inhibition. A possible function of this glycoprotein has been discussed.

Introduction

The antigenic macromolecules present in seminal plasma as well as on the sperm surface critically influence the morbidity and mortality of the sperm and the process of reproduction. A number of macromolecules, most of which originate from seminal plasma, adhere to the surface of spermatozoa. Human seminal plasma contains various proteins, amino acids, lipids and ions in high concentration [1]. In addition, secretion from the accessory glands which have different effects on sperm motility have also been detected in seminal plasma [1]. The initial portion of the secretion contains prostatic and Cowper's gland fluid whereas additional component arise from seminal vesicles. Human seminal plasma also contains IgG, IgA and fragments of ß-globulin in addition to different antigenic glycoproteins. A water-soluble glycoprotein has been isolated from seminal plasma and was shown by immunodiffusion studies to be secretory product of seminal vesicles. It contained sialic acid as well as galactose residues as nonreducing terminal sugars [2].

Although glycoproteins, which are related to autoimmune interference in male fertility [3] and are of diagnostic value, are known to occur in seminal plasma [4], the relationship between the composition and structure of glycoprotein and function of seminal plasma has not been studied. The present study pertaining to the isolation, purification and partial characterization of seminal plasma glycoprotein was undertaken in an attempt to comprehend the role of macromolecules in the secretion.

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Materials and Methods

Secretion was collected from sexually mature and normal type-A secretor donor, and was normal in terms of sperm count and morphology. The semen (2 ml) was diluted with phosphate buffer-saline (PBS) (Dulbecco, 5 ml). The suspension was mildly centrifuged (750 g) for 30 minutes at 4°C in order to avoid injury to sperm. The sperm was washed four times with PBS to remove adhering materials. Finally the supernatants were combined and centrifuged at 2500g for 20 minutes at 4°C. The supernatant was dialysed and lyophilized to give the seminal plasma macromolecules.

The lyophilized material (7 mg) in PBS was treated with galactose oxidase (250 units, Worthington) at 37°C for 8 hours. The mixture was treated with sodium borotritide (0.5 ml solution in 0.005-M sodium hydroxide containing 10 mCi) for 2 hours followed by treatment with sodium borohydride (25 mg) at 4°C for 8 hours. The mixture was treated with 4 ml acetic acid to pH 5.5 and then dialysed against distilled water at 4°C. The retentate was lyophilized to give the tritium-labeled seminal plasma glycoprotein.

The lyophilized seminal plasma glycoprotein was applied to a column (0.8 x 32 cm) of Bio-Gel P-200 in 0.05-M pyridine-acetic acid (0.5 ml) pH 5.4. Fractions containing carbohydrates and tritium were combined and lyophilized. The carbohydrate and amino acid composition of this glycoprotein is given in Table 1. The glycoprotein was further purified by gel filtration on a column of Sepharose 4B under conditions similar to those described for the P-200 column. Fractions containing tritium, amino acids and carbohydrates were combined (Table 1 and Fig.1) to give a glycoprotein component. The glycoprotein was examined for homogeneity in agarose

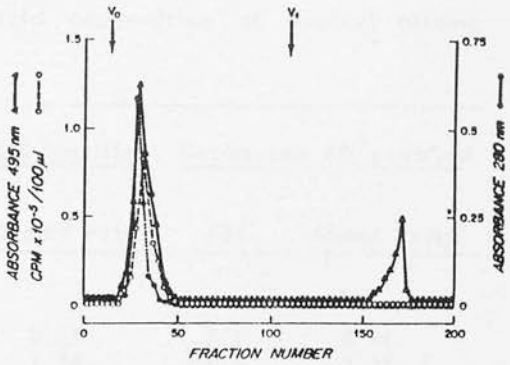

Fig.1: Fraction of Bio-Gel p-200 purified glucoprotein from human seminal plasma on a column of Sepharose 4B. Fractions of 2 ml were collected, and every third fraction was examined for the presence of tritium, hexose [18] and for amino acids (absorption at 280 nm). The column was calibrated with Blue Dextran (V0, M_r 2000000) and apoferritin (Vt, M_r 480000).

[5] and SDS-polyacrylamide gel electrophoresis (PAGE) [6]. Ricinus communis haemagglutinin (120, Boehringer), Triticum vulgaris and Concanavalin A (Sigma) were used as such. The inhibition assays were performed according to the procedure of Matsumoto and Osawa [7] using type-O erythrocytes. The glycoprotein was treated with neuraminidase [5] and the sialic acid free glycoprotein was examined for inhibition of haemagglutination.

The affinity assays in double diffusion were performed in microscope slides in 1% agarose gel in Veronal buffer (pH 8.2). The outer wells were filled with 10 µl solution of lectin (500 µg per ml) for Ricinus communis 120 and 300 µg per ml for Triticum vulgaris, Concanavalin A and Helix pomatia, from Sigma) and the center well was filled with 10 µl of a solution of seminal plasma glycoprotein(100 µg per 100 µl). The slides were incubated in a humid chamber at 4°C.

Table-1: Carbohydrate and amino acid composition of seminal plasma glycoproteins

	Biol-Gel P-200 purified		Sepharose 4B purified	
	(%)	Molar ratio[a]	(%)	Molar ratio[a]
Carbohydrates[b]				
L-Fucose	1.4	0.61	2.1	0.84
D-Galactose	4.6	1.84	6.1	2.21
D-Mannose	tr		tr	
N-Acetylglucosamine	3.1	1.00	3.4	1.00
N-Acetylgalactosamine	1.3	0.42	2.2	0.65
N-Acetylneuraminic acid	2.6	0.72	3.3	0.72
Amino acids[b,c]				
Alanine		39		60
Valine		47		48
Glycine		33		54
Isoleucine		32		58
Leucine		70		79
Proline		119		86
Threonine		182		210
Serine		118		104
Phenylalanine		51		51
Aspartic acid		76		70
Glutamic acid		142		110
Lysine		91		90

[a] Molar ratio related to N-acetylglucosamine.

[b] Determined by gas-liquid chromatography; carbohydrates according to the procedure of Reinhold[17].

[c] Residues per 1000 residues.

tr: Trace

Results

Mild treatment of plasma by repeated washing and centrifugations removed the maximum quantity of macromolecules as well as sperm coating antigens without injury to sperm. Galactose oxidase treatment followed by reduction of the resulting aldehyde residues with sodium borotritide resulted in a high yield of tritium incorporation into glycoprotein. The tritium-labelled component purified on Bio-Gel P-200 and fractionated on Sepharose 4B (Fig.1) gave a glycoprotein with a high amount of tritium incorporated into the galactose or N-acetylgalactosamine residues. The purified glycoprotein (Table 1)

showed a marked increase in the relative amount of sugar residues, in particular those of galactose, N-acetylglucosamine and N-acetylneuraminic acid. The high molecular-weight glycoprotein barely entered the gel and was homogeneous in agarose electrophoresis. In SDS-polyacrylamide (5%) electrophoresis the glycoprotein did not enter the gel.

Inhibition of haemagglutination of type-O erythrocytes with Ricinus communis, by the glycoprotein was observed, suggesting the presence of the terminal D-galactose residue linked, ß(1→4), to N-acetylglucosamine. Treatment of the glycoprotein with neuraminidase resulted in a significant increase in the inhibition of haemagglutination of type-O erythrocytes with Ricinus communis, suggesting the linkage Neu Gal. The absence of inhibition against wheat germ agglutinin despite the significant amount of N-acetylglucosamine residue suggests that this sugar resides in the glycoprotein, either in a configuration or sequence which would be expected to be inactive. Weak inhibitory activity against Concanavalin A is in agreement with the compositional analysis of the glycoprotein. In double diffusion Ricinus communis showed a strong reaction whereas Concanavalin A, wheat germ and Helix pomatia agglutinins had a very weak precipitin reaction, confirming the results of haemagglutination inhibition.

Discussion

The present studies clearly demonstrated the presence of a high molecular weight glycoprotein in seminal plasma. The glycoprotein was homogeneous in agarose electrophoresis but did not enter the gel in SDS-PAGE, a feature common to high molecular weight secretory glycoproteins [8]. Due to limited amount of glycoprotein and the restricted procedures available to asses the purity of macromolecule, it was only possible to suggest that the glycoprotein was homogeneous, albeit polydisperse. The microheterogeneity of the carbohydrate chains, a common feature of secretory glycoproteins, may account for polydispersity of the macromolecules. Compositional analysis of the purified glycoprotein showed the presence of N-acetylglucosamine and N-acetylgalactosamine in a molar ratio appropriate for a secretory glycoprotein. Furthermore, the ratio of serine to threonine in the glycoprotein also suggested a secretory-type and homogeneous glycoprotein. Lectins were utilized to obtain structural information mainly because of minute quantity of the glycoprotein available. In addition some lectins may preferentially bind to mucin-type or serum-type carbohydrate chains of glycoproteins, and therefore may provide information as to the basic mode of structure. Wheat germ agglutinin has been reported to react with both types of glycoproteins [9]. The reaction of wheat germ agglutinin with the carbohydrate moiety of glycoprotein are complex. It may react with the inner sequence N-acetylglucosamine ß(1→4) N-acetylglucosamine of N-linked glycoproteins if this sequence is accessible to the lectin [10,11]. It reacts also with N-acetylneuraminic acid and to a lesser extent with N-acetylgalactosamine [12,13]. However, it reacts intensely with terminal, non-reducing N-acetylglucosamine residues [12,13]. Helix pomatia lectin reacts with blood group A substances or molecules containing an -linked N-acetylgalactosamine residue [11,14]. Since the plasma glycoprotein was obtained from secretor type-A donors reaction with Helix pomatia was expected. The inability of the glycoprotein to exhibit a positive reaction could be attributed to the absence of the required sequence or anomery in the linkage of N-acetylgalactosamine. Ricinus communis 120 has shown to have high

(i) α-Neu-(2→?)-Gal-1→

(ii) β-Gal-(1→4)-NAcGlc-1→

(iii) β-Gal-1→

Fig.2 Partial structure of oligosaccharide chains in the glycoprotein.

affinity for -galactose, and to react with secretory and cell surface glycoproteins [11,15].

The reaction of the seminal plasma glycoprotein with lectins suggests the presence of structural features shown in Fig. 2 as well as N-acetylglucosamine linked in the inner core and N-acetylgalactosamine either -linked in the inner core or -linked in non-reducing terminal position in the glycoprotein. The partial structural information obtained clearly suggests that it is a secretory-type glycoprotein and might be similar to the uncharacterized seminal plasma glycoproteins reported by Li et al. [3] and Uhlenbruck et al.[2].

In addition to its usefulness in the regulation of fertilization a completely characterized glycoprotein may provide a genetic marker in human seminal plasma [16].

Acknowledgement

One of the authors (N.D) wishes to thank Dr. R.W.Jeanloz, Laboratory for Carbohydrate Research, Massachusetts General Hospital, Boston, for helpful discussion.

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GLYCOPROTEINS IN REPRODUCTION SPERM SURFACE AND SEMINAL PLASMA GLYCOPROTEINS

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Glycoconjugates in general and glycoproteins in particular are actively involved in reproduction[1-3]. The sperm surface antigens, components of seminal plasma and cervical mucus which all contain glycoproteins, significantly contribute to the process of reproduction, a complex phenomenon that involves a cascade of events. Various approaches have been adopted to tackle the regulation of this process with limited utility. One approach to regulate human reproduction is to develop comprehensive knowledge of the glycoconjugates in general and glycoproteins in particular that are involved in the processes of reproduction i.e., at the surface of the sperm and in seminal plasma, and thereafter regulate the process of reproduction by controlling the biochemistry of these glycoconjugates.

Autoimmune interference in male fertility had been induced in humans by male gonad preparation[4] suggesting that azoo-or oligospermia may be attributed to autoimmunity. The occurrence of cell mediated immunity in spermatozoa was of higher incidence in males with azoo- or oligospermia as compared with that in normal men[5-6]. Complements and complement dependent antibodies found in the reproductive fluids of the female have been implicated in the failure of the sperm to fertilize[7-9]. The presence

of such antibodies is attributed to sperm antigens. Also a number of molecules, most of which originate from seminal plasma, adhere to the surface of spermatozoa. Spermsurface antigens have been classified into two categories, either intrinsic or coating[10]. Sperm-coating antigens are weakly associated with the membrane and are often the product of the male genital tract. Intrinsic sperm surface antigens, as in the case of membrane proteins, are an integral part of the sperm plasma membrane, and include glycoproteins, enzymes, structural proteins and perhaps glycolipids[11]. The existence of carbohydrate containing material on the surface of sperm[12,13] as well as of sialic acid has been demonstrated[14]. Various lectins have been used to map the carbohydrate along the surface of the sperm. Human seminal plasma contains various proteins, amino acids carbohydrates, lipids, ions, IgG, IgA and fragments of β-globulins[15]. Different antigenic glycoproteins have been shown to exist in human seminal plasma[16, 17] and these lacked characterization. Sperm-coating antigen glycoproteins and sperm-decapacitating glycoproteins have been isolated from seminal plasma.[18]

Human seminal plasma also contains a glycoprotein that shares physicochemical, immunochemical and immunosuppressive properties with the pregnancy-associated plasma protein-A[19,20]

Cervical mucus is a complex millieu secreted continuously by endocervical cells. Small quantities of endometrial, tubal, and possibly follicular fluids, may also contribute to the cervical mucus[21]. The cervical secretion, mucus, displays a number of rheological properties, such as viscosity, flow elasticity, stickness and spinnbarkeit, that are regulated by the ovarian function. The chemical and physical changes in the cervical mucus during the menstrual cycle influence migration, survival and nutrition of sperm, and are accompanied by alteration in carbohydrate composition and structure[22,23]

Sperm-Surface Glycoproteins: Sperm surface glycoproteins antigens have been intensively investigated[24,25] mainly due to their immunological relevance. The immunological significance of these compounds has been reported but

neither these have been isolated in sufficient quantity nor useful identification and characterization have been accomplished. The present study describes sperm surface and seminal plasma glycoproteins that have been purified and partially characterized.

The major difficulty in the isolation of sperm surface glycoprotein antigens has been the purification from seminal fluid and the small amounts of purified glycoproteins that can be obtained from sperm for structural studies. The problem of purification has been approached either by trypsin digests of sperm or sperm membrane preparations of glycoproteins and glycopeptides. Both procedures of isolation i.e., trypsinization as well as membrane preparation are known not to be compeltely satisfactory. Although human sperm is known to swell in trypsin solution[33], no significant changes were observed in our experiments at 4°C. The glycoproteins from bonnet monkey, (a primate with close similarity to human) sperm were isolated by trypsinization as previously described[34]. In addition to a main glycoprotein, number of proteins in small quantity were detected in PAGE. The glycoprotein component was purified by gel filtration on Sepharose 6B, followed by ion-exchange chromatography (Fig. 2). The main glycoprotein component obtained after prufication by ion-exchange chromatography contained fucose, galactose, N-acetylglucosamine, N-acetylgalactosamine and sialic acid in addition to a minor quantity of glucose (Table-1). The protein moiety contained glycine, serine, threonine, aspartic and glutamic acids as major components in addition to other amino acids. This glycoprotein was further purified by affinity chromatography on Concanavalin A. Affinity chromatography removed the contaminating glucose with a concurrent limited loss of N-acetylgalactosamine, suggesting that glucose is attached to a molecule containing N-acetylgalactosamine. Amongst the membrane glycoproteins a similar observation has been made in the case of epiglycanin, a membrane glycoprotein of transformed mucuse TA3 Haascilis cells[35,36].

The glycoprotein was methylated twice using Hakomori's procedure,[37] and the points of the sugar linkages were

identified using gas liquid chromatography–mass spectrometry (g.l.c.–m.s.). The identified sugars are listed in Table 2.

In affinity assays in double diffusion on microscope slides Ricinus communis, wheat germ and Helix pomatia showed a positive reaction where as concanavalin A did not react with the purified glycoprotein.

Inhibition of hemagglutination of type-O erythrocytes showed strong inhibition with low concentration of glycoprotein in the case of Ricinus communis whereas weak inhibitory activity was observed with Helix, pomatia and Ulex europeus.

A small amount of the glycoprotein (250 μg) was subjected to alkaline borotritide treatment to obtain the ^{3}H-labelled-oligosaccharide component of the glycoprotein. The oligosaccharides were purified by gel chromatography and by high performance liquid chromatography. Five purified and tritium-containing oligosaccharides (two sialylated and three neutral) were obtained in minute quantities. The sugar components of these oligosaccharides are listed in Table 3. The oligosaccharides contained N-acetylgalactosaminitol, N-acetylglucosamine, N-acetylgalactosamine, galactose, fucose and sialic acid residues, and ranged from disaccharide to hexasaccharide.

The *oligosaccharide P-1* contained N-acetylgalactosaminitol and a sialic acid residue. Methylation of this oligosaccharide showed that the sialic acid was linked to C-6 of N-acetylgalactosaminitol.

The second *oligosaccharide, P-2* consisted of N-acetylgalactosaminitol and galactose residues. Methylation studies showed the linkage between galactose and N-acetylgalactosaminitol to be 1–3 (Fig. 2).

The *trisaccharide, P-3*, contained sialic acid in addition to galactose and N-acetylgalactosaminitol residues. Methylation of this oligosaccharide suggested that the N-acetylgalactosaminitol residue was linked in C-3 and C-6. The minute amount of the purified oligosaccharide available for investigations requires further structural investigations. The point of linkages points in N-acetylgalactosaminitol for sialic acid

and galactose residues need be clarified. It is, however, clear that N-acetylgalactosaminitol at 0-3 and 0-6. It is very possible that this oligosaccharide is an extension of the disaccharide in which galactose is linked to N-acetylgalactosaminitol in C-3, and this would suggest that sialic acid is linked in position C-6 (Fig. 2).

The *tetrasaccharide, P-4,* contained N-acetylgalactosaminitol, N-acetylglucosamine and two galactose residues. Methylation of the oligosaccharide suggested terminal galactose residues, a 4-linked N-acetylglucosamine and a 3,6-linked N-acetylgalactosaminitol. Treatment of the oligosaccharide with β-galactosidase and subsequent methylation suggested that a galactose residue was linked to N-acetylglucosamine in position C-4. From these studies it evolves that the tetrasaccharide has the structure shown in Fig. 2.

The *oligosaccharide P-5* in carbohydrate analysis was showed to be a hexasaccharide consisting of N-acetylgalactosaminitol, N-acetylglactosamine, N-acetylglucosamine, two galactose residues and a fucose residue. Methylation of the oligosaccharide showed the presence of terminal fucose, galactose and N-acetylgalactosamine; 2,3-linked galactose, 4-linked N-acetylglucosamine and 3,6-linked N-acetylgalactosaminitol. Sequential treatment of the oligosaccharide with α-Nacetylgalactosaminidase and β-galactosidase removed an N-acetylgalactosamine and a galactose residue. Subsequent methylation of the enzyme-treated oligosaccharide and identification of the products suggested the presence of terminal fucose and N-acetylglucosamine; 2-linked galactose and 3,6-linked-N-acetylgalactosaminitol. From these studies the structure for oligosaccharide P-5, as shown in Fig. 2, is proposed.

A diverse description of sperm surface carbohydrates involvement in the processes of reproduction has been proposed. Progress in the biochemistry of the sperm surface glycoconjugates lacked due to the minute quantity of the material available for investigations and also because of the low sensivity of the methods. In addition, the interest of the investigators had focussed only on monitoring the various behaviours of sperm[4,29].

Seminal Plasma Glycoproteins: Mild treatment of human seminal plasma by repeated washings and centrifugation in PBS recovered most of the macromolecules and sperm coating antigens without injury to sperm. The sugar containing macromolecules were lebelled with ^3H by treatment with galactose oxidase followed by reduction of the resulting aldehyde with NaB $[^3H]_4$.

The tritium incorporated polymer was purified by chromatography on Biogel P-200 and fractionated on Sepharose 2B. A tritium containing glycoprotein was obtained that was homogeneous in PAGE and contained fucose, galactose, N-acetylglucosamine, N-acetylgalactosamine, sialic acid and traces of mannose. The polymer was further purified by affinity chromatograph on Concanavalin A--Sepharose 4B. A mannose containing component was removed and a purified glycoprotein was obtained. The glycoprotein contained sugars and amino acids (Table 4).

Inhibition of hemagglutination of type-0 erythrocytes with *Ricinus communis* by the glycoprotein was observed, suggesting the presence of a terminal galactose residue linked to an N-acetylglucosamine. Neuraminidase treatment of the glycoprotein resulted in a substantial increase in the inhibition of hemagglutination of type-0 erythrocytes, indicating that a sialic acid residue is linked to a galactose residue. In double diffusion *Helix pomatia* showed a precipitin reaction with the glycoprotein, whereas Concanavalin A and wheat germ agglutiniins did not react with the glycoprotein in double diffusion.

Reductive β-elimination of the carbohydrate moiety of the glycoprotein yielded oligosaccharides. Purification of the oligosaccharides by gel filtration and ion-exchange chromatography provided four oligosaccharides (S_1 to S_4). The carbohydrate composition of these oligosaccharides is given in Table 5.

Oligosaccharide S-1 contained N-acetylgalactosaminitol, galactose and sialic acid. Neuraminidase treatment and subsequent carbohydrate analysis suggested the loss of sialic acid. Methylation of the enzyme treated oligosaccharide showed the presence of terminal galactose and 3–linked N-

acetylgalactosaminitol. Because of minute quantity of oligosaccharide available no further investigation was possible. From these results it is possible that sialic acid is either linked to C-6 of N-acetylgalactosaminital or to galactose (Fig. 3).

Oligosaccharide S-2 contained fucose, galactose, N-acetylgalucosamine and N-acetylgalactosaminitol in a ratio of 1:2:1:1, suggesting it to be a pentasaccharide. Methylation studies suggested the presence of terminal fucose and galactose, 2-linked galactose, 4-linked N-acetylgalucosamine and 3,6-linked N-acetylgalactosaminitol. From these partial structural studies and because of similarities between secretory and O-glycosylated oligosaccharides a partial structure for this oligosaccharide is proposed and is shown in Fig. 3.

Oligosaccharide S-3 contained fucose, galactose, N-acetylgalucosamine and N-acetylgalactosaminitol in a ratio of 1:3:1:1. Methylation studies indicated the presence of terminal fucose and galactose, 2,3-linked galactose, 4-linked N-acetylgalucosamine and 3,6-linked N-acetylgalactosaminitol. Sequential treatment of the oligosaccharide with β-galactosidase and α-fucosidase and subsequent methylation of the oligosaccharide showed the presence of a terminal galactose and N-acetylglucosamine and a 3,6-linked N-acetylgalactosaminitol. From these studies a pertial structure for this oligosaccharide is proposed (Fig. 3).

Oligosaccharide S-4 contained sialic acid, galactose, N-acetylglucosamine, N-acetylgalactosamine and N-acetylgalactosaminitol. Methylation of this oligosaccharide suggested terminal N-acetylgalactosaminitol, 3-linked galactose, 4-linked N-acetylgalucosamine and 3,6-linked N-acetylgalactosaminitol. Treatment of the oligosaccharide with neuraminidase and subsequent methylation suggested that sialic acid is linked to C-3 position of a galactose residue. From these results a structure can be evolved and that is shown in Fig. 3.

Structural studies on these glycoproteins, sperm surface and seminal plasma, are preliminary and need be completed document the complete structure of the glycoproteins that may perform a significant physiological function. The current study suggests that these glycoproteins are mainly O-glyco-

sylated, and the carbohydrate moiety in these glycoproteins bears diversity of chain length and charge.

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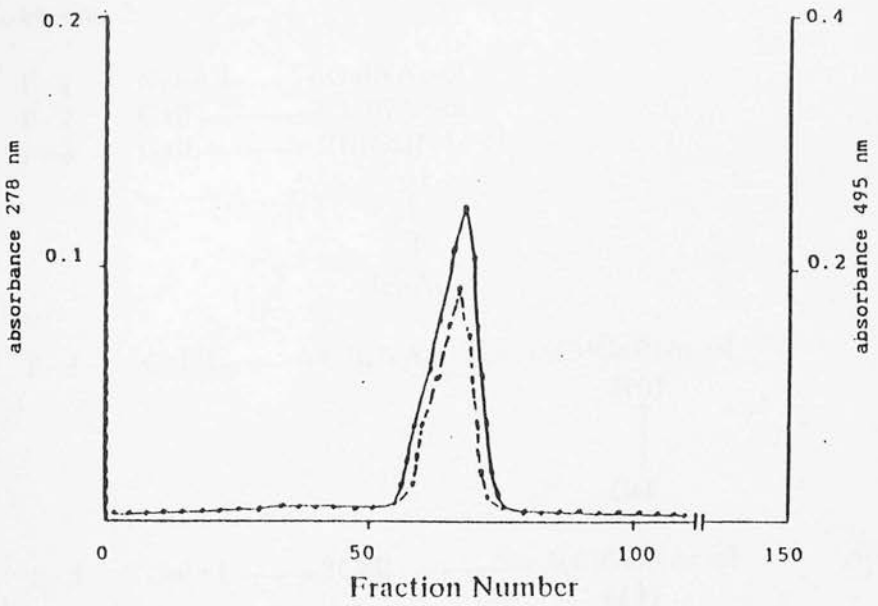
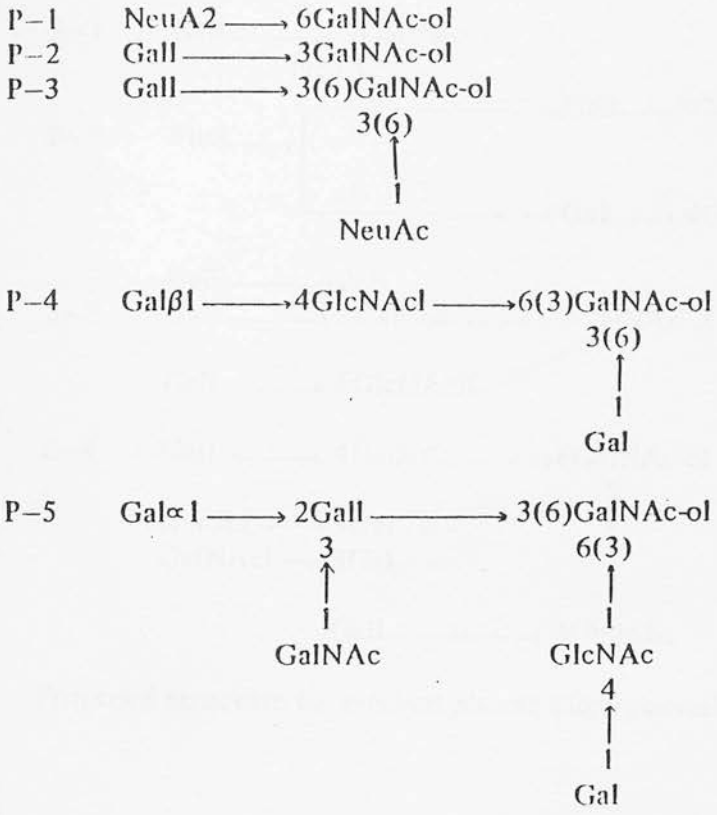


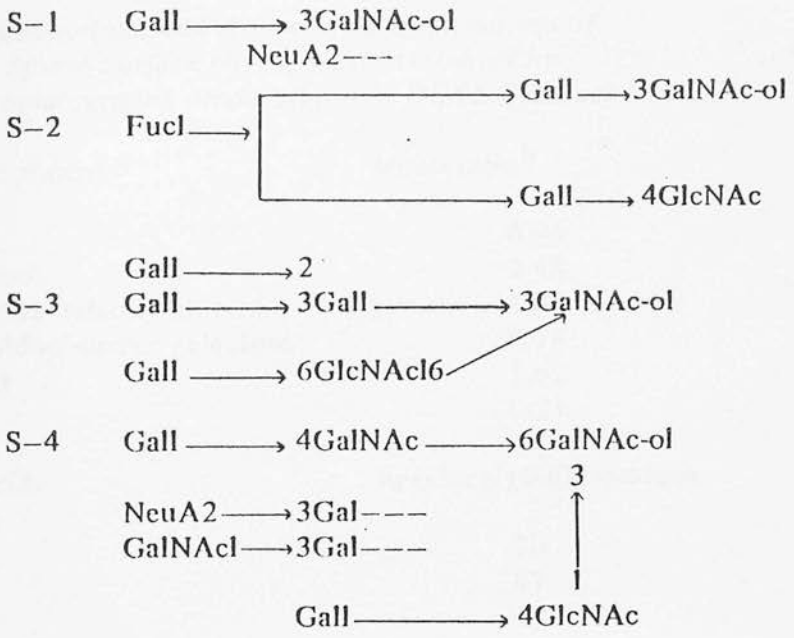
Figure 1: Purification of sperm-surface glycoprotein on a column of DEAE-cellulose. The column was eluted with 0.1 M NaCl. Fractions of 2ml were collected and every third fraction was examined for the presence of carbohydrates (495 nm) and proteins (278 nm).

Figure. 2



Proposed structures for sperm surface oligosaccharides.

Figure 3.



Proposed structure fo seminal plasma oligosaccharides.

Table 1

Carbohydrate and Amino Acid Composition of Sperm-Surface Glycoprotein Obtained by Chromatography on a Column of DEAE-Cellulose

Sugar components[a]	Moarl ratio[b]
L-Fucos	0.96
D-Galactose	2.48
2-Acetamido-2-deoxy-glucose	1.00
2-Acetamido-2-deoxy-galactose	0.98
Sialic acid	1.62
Mannose	0.21
Amino acids	**Residues/1000 residues**
Ala	70
Val	47
Ileu	49
Leu	78
Pro	70
Thr	158
Ser	206
Phe	43
Asp	57
Glu	49
Lys	28
Gly	146

[a]Determined by gas-liquid chromatography.
[b]Molar ratio relative to 2-acetamido-2-deoxyglucose.

Table 2

Methylated Sugars Identified From the Methylated Sperm-Surface Glycoprotein

METOXYL GROUP AT	Fuc	Gal	GalNAc	GlcNAc	GalNac-ol
2,3,4,6-		+			
2,3,4-	+				
3,4,6-	+				
2,4,6-		+			
3,6-		+		+	+
4,6-		+			+
4-					

Table – 3

Carbohydrate composition of purified sperm surface oligosaccharides

Oligosaccharides	Fuc		Gal		BeyA		GlcNAc		GalNAc		GalNAcol	
	%	MR[a]	%	MR[a]	%	MR[a]	%	MR[a]	%	MR[a]	%	MR[a]
P–1					51	0.94					39	1.00
P–2			38	0.96							49	1.00
P–3			22	0.94	36	0.89					29	1.00
P–4			37	0.95			22	0.92			24	1.00
P–5	12	0.81	15	0.92			18	0.90	16	0.80	20	1.00

[a]Molar ratio relative to 2-acetamido-2-deoxy-galactitol.

Table – 4

Carbohydrate and amino acid composition of seminal plasma glycoprotein from Concanavalin A-Sepharose 4B column

Sugar Components	%	Molar ratio[a]
Fucose	7	1.04
Galactose	15	2.05
2-Acetamido-2-deoxy–glucose	9	1.00
2-Acetamido-2-deoxy–galactose	8	0.88
Neuraminic acid	11	0.88
Amino acids[b]		
Ala	70	
Val	41	
Gly	36	
Ileu	44	
Leu	51	
Pro	82	
Thr	218	
Ser	128	
Phe	41	
Asp	82	
Glu	88	
Lys	119	

[a]Molar ratio relative to 2-acetamido-2-deoxyglucose.
[b]Residues per 1000 residues.

Table –5
Carbohydrate composition of purified seminal plasma oligosaccharides

Oligosaccharides	Molar ratio of sugars[a]					
	Fuc	Gal	GlcNAc	GalNAc	NeuA	GalNAcIol
S–1		1		1		1
S–2	1	2	1			1
S–3	1	3	1			1
S–4		2	1	1	1	1

[a]Molar ratio relative to 2-acetamido-2-deoxy-glactitol.

Glycoprotein Structure Function Relationship: Study of Epithelial Cell Secretion

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Glycoproteins are polymers in which proteins and carbohydrates are covalently linked, and in which carbohydrates contribute to major biological functions. Glycoproteins are abundantly distributed in microorganisms, plants, animals and humans. There are sufficient indications to suggest that the carbohydrate moiety of the glycoprotein performs significant biological roles such as biological recognition. i.e., specification of blood type, control of the lifetime of glycoproteins in the circulatory system, stage specific differentiation, control of glycoprotein uptake by the cells, acceptors for carbohydrate or protein components and protection of exposed tissue from invasive organisms or molecules. This capability of the carbohydrate arises from the great variety of oligosaccharides that can be formed from a relatively small number of monosaccharides. Oligosaccharide chains can influence the ability of the protein moiety of the glycoprotein to fold properly. The transfer, cotranslational event, of oligosaccharide having the structure $(Glc)_3\ (Man)_9(GlcNAc)_2$ to an aspargine residue of the protein during the biosynthesis in the endoplasmic reticulum may be essential for the protein to acquire correct tertiary structure. Inhibition studies of protein glycosylation support the concept that carbohydrate chains influence the conformation of proteins. The consequence of glycosy-

Abbreviations: N-Acetylglucosamine: GlcNAc; N-acetylgalactosamine: GalNAc: N-acetylgalactosaminitol: GalNAc-ol

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Ed. by Atta-ur-Rahman

lation for protein folding and assembly may be examplified by the synthesis of virus glycoprotein, the G protein of vesicular stomatitis virus (VSV), initially described by Gibson et al., [1]. The main molecular basis underlying the function of carbohydrates in biological recognition is the interaction of oligosaccharides with the carbohydrate binding proteins. The specific determinant formed by a saccharide sequence has affinity to complementary structures in other molecules. Furthermore cell surface or membrane glycoproteins possibly stabilize cell surface structures, particularly in eukaryotes. It has been proposed that the carbohydrate component of the major sialoglycoproteins of erythrocytes, such as glycophorin, interact to form a lattice over the internal cell membranes and thus provide the membrane with greater rigidity [2].

The carbohydrates in the glycoproteins range from less than one per cent, collagen, to eighty five per cent, soluble blood group substances, of the total macromolecule. Glycoproteins also differ in the number of carbohydrate chains present. This may vary from one, in ribonuclease B, to eight hundred, in submaxillary mucin. Furthermore the oligosaccharide chains in glycoprotein differ in the number of different constituting monosaccharides, ranging from one to a dozen or more.

Carbohydrate-peptide linkage

The oligosaccharide linkages in the glycoproteins can be classified according to the type of bond to the protein moiety as being N- or O-glycosidic. The N-glycosidically linked oligosaccharide chains are joined through N-acetylglucosamine to the amide nitrogen of asparagine in the protein. The O-glycosidic chains may be linked to the hydroxyl group of L-serine, L-threonine, L-hydroxylysine or L-hydroxyproline in the protein moiety. Glycoproteins from epithelial secretions mainly contain O-glycosidic type linkages between carbohydrate chains and protein backbone. Amongst the carbohydrate residues the joining site is provided by N-acetylgalactosamine whereas L-serine and L-threonine from the protein moiety is involved in the covalent linkage.

Epithelial secretions

Mucins, epithelial glycoproteins, are dominant components of bronchial, gastrointestinal and cervical secretions. Epithelial fluids are produced by a variety of cells such as goblet, columnar, mucous and serous cells. In systems like tracheobronchial and gastrointestinal, mucus is secreted by different types of cells, whereas in cervical system [3] and gall bladder [4], a component of the gastrointestinal system, mucins are produced only by columnar cells. In the case of tracheobronchial and gastrointestinal mucin fluids are secreted by a variety of cells, suggesting that the mucus may be a heterogeneous milieu of secreted components, including glycoproteins. In order to study structure function relationship cervical and gall baldder secretions provide excellent systems.

Cervical mucus is a complex mixture, the principal constituents of which are mucin type glycoproteins that share the chemical composition and physical behaviour with other epithelial secretions. As the glycoproteins in the mucus represent the major macromolecular components, it is reasonable to assert that they are responsible for its physiological and biophysical properties [5]. The secretion regulated during the menstrual cycle to influence sperm passage, its survival and nutrition. Treatment of cervical mucus in vitro with proteolytic enzymes enhanced sperm migration [6] and altered the biophysical behaviour of cervical mucus. In addition, treatment of the midcycle human cervical mucus with Pronase destroyed the protein backbone or bridges between the glycoprotein fibrils. The cyclic changes in mucus behavior are accompanied by alterations in carbohydrate composition [7,8] and in chemical structure [9]. The mechanism by which glycoproteins, specifically, and mucus, in general, function in above described processes is unknown. In order to assess their role the structural features of a main glycoprotein from midcycle mucus was investigated, and the structures of neutral oligosaccharides are discussed. Understanding of chemical structures and their relation to physiological functions will provide a biochemical and immunological procedure to regulate glycoprotein functions.

TABLE I

COMPOSITION OF GLYCOPROTEINS FRACTIONATED ON SEPHAROSE 2B

Carbohydrates	Fraction-1		Fraction-2	
	%	Molar Ratio[1]	%	Molar Ratio[1]
Fucose	7	1.0	6	1.1
Galactose	22	3.0	18	3.1
N-Acetylglucosamine	9	1.0	7	1.0
N-Acetylgalactosamine	17	1.9	14	2.0
N-Acetylneuraminic acid	11	0.9	10	1.0
Sulphate	1			
Amino acids[2]	Fraction-1		Fraction-2	
Aspartic acid	42		101	
Threonine	228		54	
Serine	112		78	
Glutamic acid	62		111	
Proline	125		98	
Glycine	75		67	
Alanine	73		82	
Cysteine	22		31	
Isoleucine	41		18	
Leucine	50		87	
Tyrosine	31		30	
Phenylalanine	22		24	
Lysine	29		64	
Histidine	21		23	
Arginine	31		72	
Valine	36		60	

[1]Molar ratio relative to N-acetylglucosamine

[2]Residues per 1000 residues

Periovulatory-phase cervical mucus

Cervical secretion obtained from bonnet monkey (Macaca radiata), a primate of close proximity to man, as also shown by the presence of N-acetylneuraminic acid mainly in the glycoprotein [10], of similar blood type was pooled lyophilized after removal of cellular debris by mild centrifugation. The crude material was purified by gel filtration on Bio-Gel P-200, mainly to remove immunoglobulins and other contaminating molecules of low molecular size. The cervical glycoproteins were eluted in the void volume of the column. The partially purified glycoproteins were fractionated on Sepharose 2B, and their composition is listed in Table I. An antibody raised in rabbits against the main glycoprotein reacted positively with the periovulatory cervical mucus [11], which displays fibrillar channels. The precipitate, antibody-mucus complex, exhibited a morphology that was different from that of the periovulatory mucus. The morphological alteration induced by the antibody clearly indicates that the mucus channels are lined with glycoproteins.

The main glycoprotein fraction obtained from the Sepharose 2B column was further fractionated on a column of DEAE-Cellulose (Fig.1) into two fractions. The composition, carbohydrates and amino acids, of these fractions is given in Table II. The recovery of the glycoprotein from the ion-exchange column was not satisfactory, a feature common to mucin-type glycoproteins. The main difference between the two fractions was in the quantity of sulfate and minor variations in sugar composition. In agarose (1%) and in polyacrylamide-agarose (1.5:0.5%) gel electrophoresis a homogeneous glycoprotein, albeit polydisperse, was observed. Efforts to reduce, completely, the disulfide bonds using tributyl phosphine [12] and alkylate with 1,3-propanesultone [13] was not successful. The presence of cysteine after repeated reduction and alkylation was observed, suggesting that the reducing as well as alkylating agent failed to approach the site of the disulfide bond location. Also it is possible that a mechanism other than the disulfide phenomenon may operate which would result in incomplete reduction and alkylation. Furthermore, the Sepharose 2B-purified glycoprotein was extensively treated with Pronase, papain and pepsin, as previously described [10], and then treated with reducing and alkylating

TABLE II

COMPOSITION OF GLYCOPROTEIN SEPARATED ON DEAE-CELLULOSE

Carbohydrate	Fraction-A		Fraction-B	
	%	Molar Ratio[1]	%	Molar Ratio[1]
Fucose	8	0.8	7	0.9
Galactose	25	2.2	18	2.2
N-Acetylglucosamine	14	1.0	10	1.0
N-Acetylgalactosamine	22	1.6	12	1.2
N-Acetylneuraminic acid	17	0.6	11	0.6
Sulfate	1.4	0.2	3.8	0.9

Amino acids[2]	Fraction A		Fraction B
	Original glycoprotein	Difference after treatment with alkali	
Aspartic acid	74		68
Threonine	234	- 148	264
Serine	140	- 62	124
Glutamic acid	58		51
Proline	48		70
Glycine	70		69
Alanine	90	+ 49	99
α-Aminobutyric acid		+ 120	
Cysteine	16		9
Valine	51		48
Isoleucine	48		39
Leucine	51		58
Tyrosine	20		18
Phenylalanine	30		20
Lysine	33		30
Histidine	14		12
Arginine	23		21

[1] Molar ratio relative to N-acetylglucosamine

[2] Residues per 1000 residues

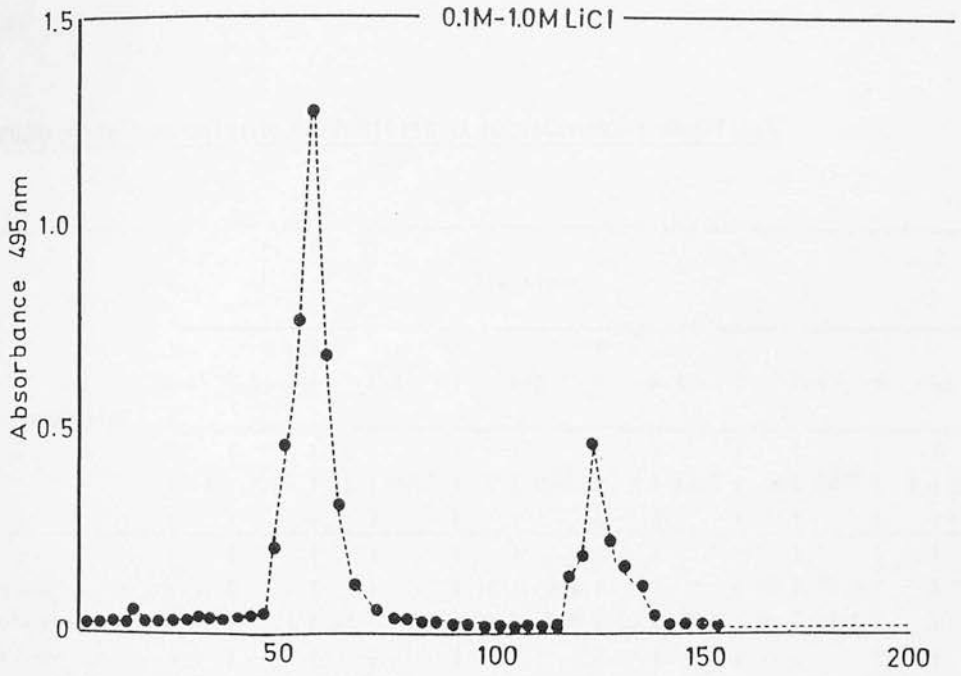


Fig. 1. Fractionation of the Sepharose 2B purified glycoprotein on a column (1.8 x 52 cm) of DEAE-cellulose. The glycoproteins from the column were eluted with a gradient of 0.1M to 1M lithium chloride. Fractions of 2.5 ml were collected and every third fraction was examined for the presence of carbohydrates by the phenol-sulfuric acid procedure (33).

agents i.e., dithiothreitol and tributyl phosphine and iodoacetamide and 1,3-propanesulthone respectively. In both the conditions utilized the alkylation was incomplete. In the event of an external protein constituting a bridge between the glycoprotein fragments [14] the extensive proteolytic enzyme treatment and subsequent alkylation would remove cysteine. In these circumstances it will be relevant to consider that there exists no link protein and cysteine form a component part of the glycoprotein. It is known that the presence of excessive amounts of neuraminic acid and sulfate groups in the glycoprotein will limit the approach of degrading enzymes as well as some reducing and alkylating reagents. This phenomenon may explain the incomplete reduction and alkylation, as has been observed for the luteal phase glycoprotein too [15].

TABLE III

CARBOHYDRATE COMPOSITION OF PURIFIED OLIGOSACCHARIDE FRACTIONS

Carbohydrates	Fractions											
	F-1		F-2		F-3		F-4		F-5		F-6	
	%	M/R[b]	%	M/R[b]	%	M/R[b]	%	M/R[b]	%	M/R[b]	%	M/R[a]
Fucose					18	0.9			12	0.9		
Galactose	26	1.1	24	1.0	22	1.0	50	2.9	30	2.1		
Mannose											41	1.0
N-Acetyl-glucosamine	34	1.1			28	1.1	22	1.1	16	0.9	43	0.9
N-Acetyl-galactosamine			35	1.1					19	1.1		
N-Ace tylgalac-tosaminitol	32	1.0	31	1.0	26	1.0	21	1.0	18	1.0		

[a]Molar ratio relative to mannose

[b]Molar ratio relative to N-acetylgalactosaminitol

Reductive β-elimination and preparation of oligosaccharides

The oligosaccharides from DEAE-cellulose column fraction one, the main purified glycoprotein, were isolated as previously described [10] with minor modification. The glycoprotein was incubated at 48°C for 16 hours in a solution prepared by dissolving $NaBH_4$-$NaB[^3H_4]$ (50 mCi) in 0.05M NaOH to give a final concentration of 2M $NaBH_4$. An aliquot from the reaction mixture was withdrawn and after, reduction, followed by acid hydrolysis showed an appropriate increase in alanine and appearance of α-aminobutyric acid (Table II). The reaction mixture was then cooled to 4°C, adjusted to pH 5.4 with 4M acetic acid. The mixture was applied to a column of AG 50-X8 (H^+) ion-exchange resin. Reduced oligosaccharides were eluted from the column with water and then with 0.1M acetic acid. The water and acid eluates were freed from borate ions separately by repeated evaporations with methanol. The β-eliminated material, obtained by water elution from the ion-exchange column was separated into neutral and acidic oligosaccharides on a column of AG 1X2 ion-exchange resin. The water washings were combined and lyophilized. The residue in pyridine-acetic acid buffer, pH 5.4, was fractionated on a column of Bio-Gel P-6 equilibrated with 0.1M pyridine-acetic acid. The elution profile of the oligosaccharides is shown in Fig.2. The carbohydrate composition of the oligosaccharide fractions, determined by gas liquid chromatography (glc,15), is given in Table III.

Characterization of oligosaccharide Fractions

Oligosaccharide Fraction-1: This fraction was further purified by ion-exchange chromatography on DEAE-Sephadex A-25 and by high performance liquid chromatography (HPLC) on Lichosorb-NH_2 (Merck). The Lichosorb-NH_2 column was washed with a linear gradient of acetonitrile-water. Oligosaccharide F-1, a trisaccharide consisting of a galactose, an N-acetylglucosamine and an N-acetylgalactosaminitol residue was one of the major oligosaccharides. Upon periodate oxidation-borohydride reduction all of the galactose was destroyed, whereas N-acetylglucosamine was recovered unchanged. The N-galactosaminitol was quantitatively converted to N-acetylthreosaminitol.

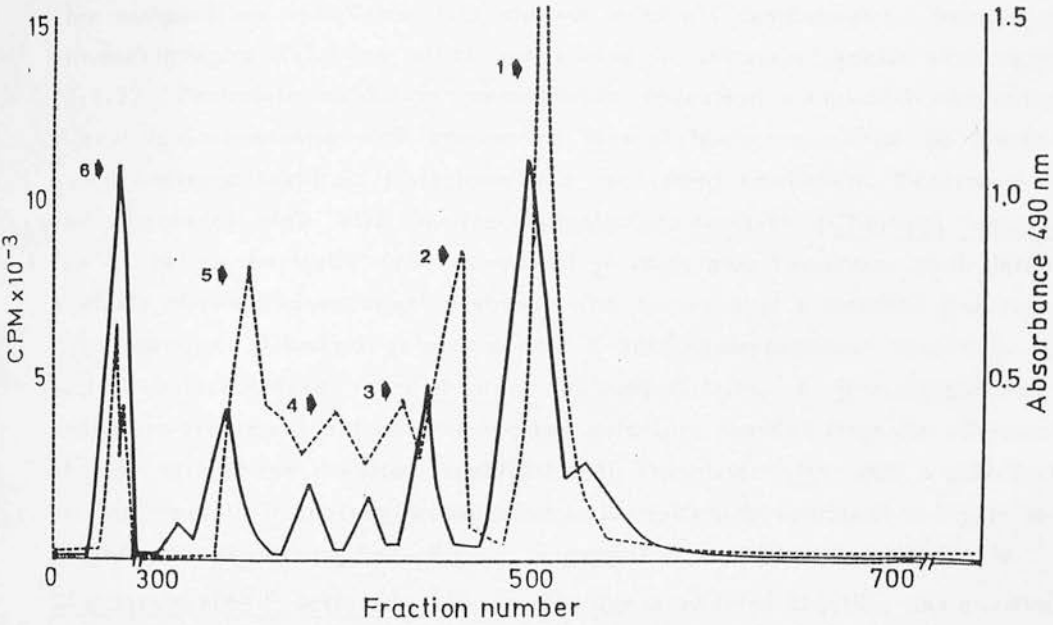


Fig. 2. Gel filtration of the neutral oligosaccharides on a column (2.4 x 190 cm) of Bio-Gel P-6. The oligosaccharides were eluted from the column with 0.1M pyridine-acetic acid (pH 5.4). Fractions of 1.8 ml were collected and every fourth fraction was examined for the presence of carbohydrates (●——●, pehnol-sulfuric acid) and tritium (●——●).

Methylation of the oligosaccharide F-1 using Hakomori procedure [16], and subsequent glc-mass spectrometry showed the presence of 2,3, 4,6-tetra-O-methyl-galactose, 2-deoxy-3,6-di-O-methyl-2-(N-methylacetamido)-glucose and 2-deoxy-1,4,5,6-tetra-O-methyl-2-(N-methylacetamido) -galactitol. Extensive treatment of the oligosaccharide with β-galactosidase (Aspergillus niger, Calbiochem) removed approximately 60% of the galactose, whereas treatment with α-galactosidase (A.niger) had no effect on the oligosaccharide. These combined results suggest the presence of structure F-1 proposed in Fig.3.

Oligosaccharide Fraction-2: Also a trisaccharide, consisting of a galactose, an N-acetylgalactosamine and an N-acetylgalactosaminitol residue, was obtained from Bio-Gel P-6 column in very close proximity to oligosaccharide F-1. This oligosaccharide was further purified on DEAE-Sephadex, Bio-Gel P-4, followed by HPLC on Lichosorb-NH_2. The purified oligosaccharide

(for composition see Table III) showed a single component on thin layer chromatography (tlc) on silica gel plates in n-butanol-acetic acid-water (3:3:2). Periodate oxidation-borohydride reduction completely eliminated N-acetylgalactosamine and converted N-acetylgalactosaminitol to 2-acetamido-2-deoxy threitol. Galactose was recovered unaltered. Treatment of the oligosaccharide with α-N-acetylgalactosaminidase (Charonia lampas) for 64 hours partially (65%) removed N-acetylgalactosamine. Methylation analysis of the oligosaccharide showed the presence of a terminal N-acetylgalactosamine, 3-linked galactose and N-acetylgalactosamine residues. α- and ß-Galactosidase from A.niger subsequent to α-N-acetylgalactosaminidase treatment did not remove the galactose residue from the oligosaccharide with these results, combined with the observation that a galactose residue next to N-acetylgalactosamine in ß-position is resistant to ß-galactosaminidase, structure F-2. Fig.3, is proposed for this oligosaccharide.

Oligosaccharide Fraction-3: The crude oligosaccharide fraction was purified on DEAE-Sephadex followed by paper chromatography in ethyl acetate-pyridine-acetic acid-water (5:5:3:1). The isolated oligosaccharide was purified by HPLC as described for oligosaccharide Fraction-1. The carbohydrate composition of the oligosaccharide (Table III) suggested the presence of a tetrasaccharide. Permethylation of the oligosaccharide and characterization of the hydrolytic products as alditol acetates by glc-mass spectrometry showed the presence of a terminal fucose, a 2-linked galactose residue, a 4-linked-N-acetylglucosamine and a 6-linked N-acetylgalactosaminitol. Treatment of the oligosaccharide with α-L-fucosidase (beef epididymis, Sigma) removed a focuse residue. Subsequent treatment of the fucosidase-treated oligosaccharide first with ß-galactosidase (Escherichia coli, Boehringer) and then a second treatment with purified ß-galactosidase (A.niger, Calbiochem) removed a substantial amount (75%) of the galactose, leaving behind mainly N-acetylglucosamine and N-acetylgalactosaminitol. Periodate oxidation-borohydride reduction destroyed the fucose and galactose residues, converted N-acetylgalactosaminitol to 2-acetamido-2-deoxyglycerol, and N-acetylglucosamine was recovered unchanged. Methylation analysis and enzyme degradations confirm the results of periodate oxidation, suggesting the N-acetylglucosamine linkage to 0-6 of N-acetylgalactosaminitol. These data support the structure, F-3, for the oligosaccharide proposed in Fig. 3.

PROPOSED STRUCTURES FOR OLIGOSACCGARIDE FRACTIONS

F-1

D-Galpβ-(1→4)-D-GlcNAcp-(1→3)-D-GalNAc-ol

F-2

D-GalNAcpα-(1→3)-D-Galpβ-(1→3)-D-GalNAc-ol

F-5

L-Fucpα-(1→2)-D-Galpβ-(1→3)-D-GalNAc-ol
D-GalNAcpα-(1→3)-D-Galpβ-(1→4)-D-GlcNAcp-(1→6)/

F-3

L-Fucpα-(1→2)-D-Galpβ-(1→4)-D-GlcNAcp-(1→6)-D-GalNAc-ol

F-4

D-Galpβ-(1→3)-D-Galpβ-(1→3)-D-GalNAc-ol
D-Galpβ-(1→4)-D-GlcNAcp-(1→6)/

F-6

D-Manpα-(1→6)
\
D-Manpβ-(1→4)-D-GlcNAcpβ-(1→4)-D-GlcNAc
/
D-Manpα-(1→3)

D-GlcNAcp ?

Fig. 3. Proposed structures for neutral oligosaccharides of the glycoprotein.

Oligosaccharide Fraction-4: This oligosaccharide was purified on Bio-Gel P-6 followed by paper chromatography in n-butanol-acetic acid-water (4:1:5). The partially purified oligosaccharide was further cleaned by HPLC, and was found to be homogeneous on silica gel tlc in n-butanol-acetic acid-water (3:3:2). The carbohydrate composition of this oligosaccharide suggested it to be a pentasaccharide with three residues of galactose, an N-acetylglucosamine and an N-acetylgalactosaminitol (Table III). Periodate oxidation-borohydride reduction degraded two galactose residues and converted N-acetylgalactosaminitol to 2-acetamido-2-deoxy-threitol. A galactose and an N-acetylglucosamine residue was recovered unchanged. Among the degraded products were isolated galactose linked to 2-acetamido-2-

deoxy-threitol and free N-acetylglucosamine. Methylation of the disaccharide galactose-2-acetamido-2-deoxy-threitol showed the presence of a terminal galactose and a substituted 2-acetamido-2-deoxy-threitol. The mass spectrum of methylated 2-acetamido-2-deoxy-threitol (Fig.4) was characteristic of 1,4-di-O-acetyl-2-deoxy-3-O-methyl-2-(N-methylacetamido)-fragment clearly suggests that a galactose residue in the oligosaccharide and not the N-acetylglucosamine is linked to the O-3 of N-acetylgalactosaminitol.

Methylation of the oligosaccharide and characterization of the resulting methylated alditol acetates showed the presence of two terminal galactose residues, a 3-substituted galactose residue, a 4-linked N-acetylglucosamine and an N-acetylgalactosamine residue linked in O-3 and O-6. Consecutive

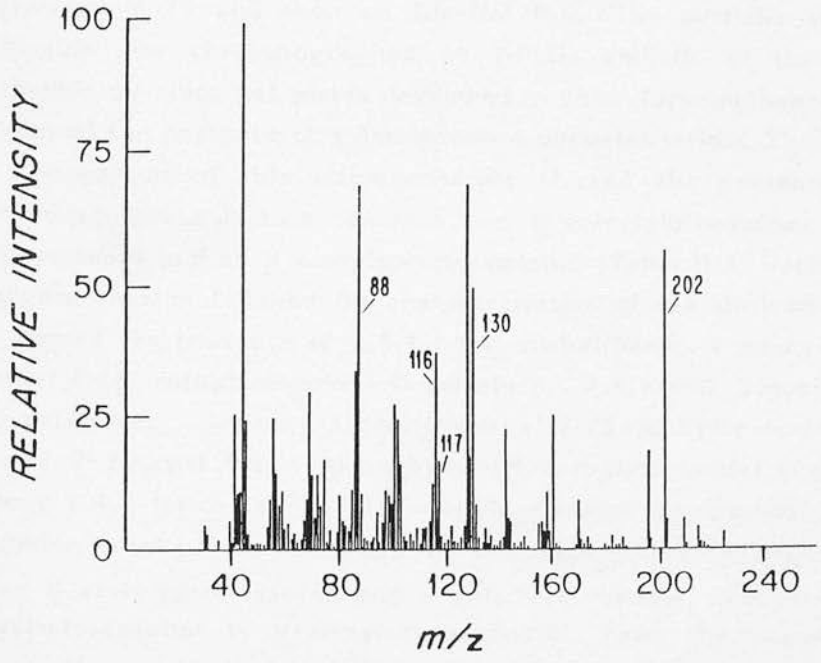


Fig. 4. Mass spectrum of 1,4-di-O-acetyl-2-deoxy-3-O-methyl-2-(N-methylacetamido)-threitol. Glc-mass spectrometry was performed with an analytical system consisting of an IBM computer fed with raw data generated by single-focusing Hitachi-Perkin Elmer-RMU-6 mass spectrometer interfaced with a Perkin Elmer gas chromatograph.

treatment of the oligosaccharide with β-galactosidase from C.lampas and from E.coli removed two residues of galactose, and subsequent methylation analysis of the remaining oligosaccharide provided a terminal galactose, terminal N-acetylglucosamine and an N-acetylgalactosaminitol residue linked in O-3 and O-6. This confirmed the sequence of the sugar residues as well as the branching at N-acetylgalactosaminitol, in addition to the anomery of terminal residues. The β -anomeric configuration of the linkage of the galactose residue linked to N-acetylgalactosaminitol has been assigned due to the fact that only a β-linkage for this galactose residue is known [17-21] and also due to the fact that among the acidic oligosaccharides of this glycoprotein this anomeric linkage has been identified by 500-MHz ^{1}H-NMR studies [22]. From the combination of these results structure for oligosaccharide F-4 is proposed in Fig.3.

Oligosaccharide Fraction-5: This oligosaccharide fraction was purified on DEAE-Sephadex A-25 and then on Bio-Gel P-6. The partially purified oligosaccharide was chromatographed on HPLC, and tlc of the eluted oligosaccharide on silica gel plates developed in chloroform-methanol-water (3:2:1) showed the presence of a homogeneous oligosaccharide. The carbohydrate composition of this oligosaccharide showed the presence of a fucose residue, two galactose residues, an N-acetylglucosamine, an N-acetylgalactosamine and an N-acetylgalactosaminitol (Table III). Methylation of the oligosaccharide followed by characterization of the derived alditol acetates showed the presence of 2,3,4-tri-O-methylfucose, 2-deoxy-3,4,6-tri-O-methyl-2-(N-methylacetamido)-D-galactose, 2,4,6-and 3,4,6-tri-O-methyl-D-galactose, 2-deoxy-3,6-di-O-methyl-2-(N-methylacetamido)-D-glucose and 2-deoxy-1,4,5-tri-O-methyl-2-(N-methylacetamido)-D-glucose and 2-deoxy-1,4,5-tri-O-methyl-2-(N-methylacetamido)-D-galactitol. Periodate oxidation-borohydride reduction of the oligosaccharide degraded a fucose, an N-acetylgalactosamine and a galactose residue, and converted N-acetylgalactosaminitol to N-acetylthreosaminitol. From the degradation products an oligomer of oligosaccharide F-4, was isolated and characterized as described for F-4. Treatment of the oligosaccharide with α-L-fucosidase (beef kidney, Boehringer) and subsequent methylation of the fucose-free oligosaccharide followed by characterization of the methylated products showed that the fucose was linked to a galactose residue at O-2. The

F-5 oligosaccharide was treated with α-N-acetylgalactosaminidase (Charonia lampas, Miles) and then with β-galactosidase (Aspergillus niger, Calbiochem). The residual oligosaccharide was purified on Bio Gel P-6 and methylated in the usual manner.. Characterization of the methylated alditol acetates suggested the presence of a terminal fucose and a galactose, a 2-linked galactose, a 4-linked N-acetylglucosamine and a 3,6-linked N-acetylgalactosaminitol. The results of periodate oxidation, methylation and enzymic degradation studies lead to structure F-5 (Fig.3) for this oligosaccharide.

Oligosaccharide Fraction-6: This oligosaccharide fragment had limited tritium incorporation, and methanolysis of the oligosaccharide suggested the presence of mannose and N-acetylglucosamine in addition to small quantities of galactose, fucose, N-acetylgalactosamine and few amino acids. The oligosaccharide was purified on a column of Cocanavalin-A-conjugate into at least two fragments. The major tritium-containing component was an oligosaccharide with tritium incorporated in N-acetylglucosaminitol. The other fraction was treated with endo- β-N-acetylglucosaminidase [20], and subsequent carbohydrate analysis showed only a minor increase in N-acetylglucosamine. The two fractions were combined and rechromatographed on Bio-Gel P-6 and then Concanavalin-A-Sephadex-G-25. The partially purified oligosaccharide was further chromatographed on HPLC. The purified oligosaccharide, isolated in very small quantity, showed the presence of mannose and N-acetylglucosamine in almost equimolar quantity (Table III). Methylation of the oligosaccharide followed by characterization of the derived alditol acetates showed therein the presence of terminal mannose, 3,6-linked mannose, terminal N-acetylglucosamine, 4-linked N-acetylglucosamine and 4-linked N-acetylglucosaminitol. Periodate oxidation-borohydride reduction and subsequent analysis of the carbohydrates of the degraded oligosaccharide suggested it to be a trisaccharide, consisting of a mannose, an N-acetylglucosamine and an N-acetylglucosaminitol residue. Extensive treatment of the oligosaccharide with purified α-mannosidase (Jack bean, Sigma) removed two mannose residues. The residual oligosaccharide was again treated with α-mannosidase with no appreciable effect. Methylation of the mannosidase-degraded oligosaccharide showed the presence of a terminal mannose, a 4-linked N-acetylglucosamine and

TABLE IV

CARBOHYDRATE COMPOSITION OF PURIFIED BOVINE MUCIN OLIGOSACCHARIDES

Sugars[a]	Neutral oligosaccharides Fractions				Acidic oligosaccharides Fractions	
	F-1	F-2	F-3	F-4	F-1	F-2
Fucose	1.0	1.0			1.0	2.0
Galactose	3.8	1.7	1.9	2.0	3.0	7.5
N-Acetyl-glucosamine	3.0	1.0		1.3	2.3	2.0
N-Acetyl-galactosamine	1.5	1.0		1.0	1.0	0.5
Neuraminic acid					1.0	0.3
N-Acetylgalac-tosaminitol	1.0	1.0	1.0	1.0	1.0	1.0

[a]Molar ratio relative to N-acetylgalactosaminitol

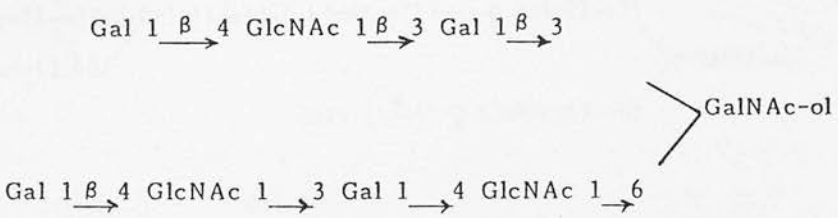
a derivative of 4-linked N-acetylglucosaminitol (N-acetylpentosamine derivative). With this limited information, because of very small amount of purified oligosaccharide available for structural studies, the position of the N-acetylglucosamine residue was unclear, and only a partial structure for this oligosaccharide-glycopeptide is proposed in F-6 (Fig.3). It is not only unlikely that this oligosaccharide fraction was a mixture of a large size secretory-type oligosaccharide and a minor quantity of mannose-containing N-acetylglucosamine-asparagine type oligosaccharides.

Gall bladder glycoprotein and oligosaccharides

Gall bladder glycoprotein was purified in a manner similar to that described for the cervical glycoproteins, and oligosaccharides were prepared by reductive beta elimination under conditions similar to those described for cervical oligosaccharides. After separation of neutral oligosaccharides from acidic one on AG 1X2 ion-exchange resin (CH_3COO^-), the neutral oligosaccharides were separated on a column of Bio-Gel P-6 into at least three oligosaccharides.

Oligosaccharide Fraction-1: This fraction was rechromatographed on DEAE-Sephadex and then on Bio-Gel P-6. A single carbohydrate-containing component was eluted from P-6 and it was further purified by HPLC. Oligosaccharide F-1 had a carbohydrate composition that suggested it to be a decassaccharide (Table IV). Methylation analysis of the oligosaccharide, and characterization of the products reduction with borohydride and acetylation showed the presence therein of a terminal N-acetylgalactosamine, a terminal- and 4-linked N-acetylglucosamine, a terminal galactose residue, a galactose residue linked at O-2 and O-3, and an N-acetylgalactosamine residue linked at O-3 and O-6. Sequential Smith degradation, two cycles, destroyed three residues of galactose, two residues of N-acetylglucosamine, an N-acetylgalactosamine and a fucose, and converted N-acetylgalactosaminitol to N-acetylthreosaminitol. The degraded oligosaccharide was purified on Bio-Gel P-2 and then methylated. Among the methylated products 2-deoxy-3,4,6-tri-O-methyl-2-(N-methylacetamido)-glucose, 2,4,6-tri-O-methyl-galactose and 2-deoxy-1,4-di-O-methyl-2-(N-methylacetamido)-threitol were identified. After the first Smith treatment the degraded

oligosaccharide was extensively treated with β-galactosidase. The carbohydrate analysis of the enzyme-treated oligosaccharide suggested a loss of a galactose residue, indicating a terminal, β-linked residue. Consecutive treatment of the oligosaccharide with α-N-acetylgalactosaminidase and then with α-L-fucosidase removed from the oligosaccharide an N-acetylgalactosamine and a fucose residue. Methylation analysis of this oligosaccharide fragment, enzyme degraded product, cleared the location of N-acetylgalactosamine and fucose as a galactose that was linked in O-2 and O-3. Treatment of the residual oligosaccharide with α-galactosidase suggested the removal of two galactose residues, and subsequent methylation of the remaining oligosaccharide showed the presence of terminal N-acetylglucosamine residues and 3-linked galactose residues in addition to a 3,6-linked N-acetylgalactosamine and a 3-linked N-acetylglucosamine. However, the molar ratios of the methylated sugars indicated two residues each of terminal N-acetylglucosamine and 3-linked galactose for one residue of 3,6-N-acetylgalactosaminitol. Based on the results of enzymic degradation and on the molar ratios of the methylated sugars [23] the structure given below for the oligosaccharide fragment, free of fucose and N-acetylgalactosamine, is proposed.



However, this structure does not conclusively identify the position of a terminal galactose and 4-linked N-acetylglucosamine residue in the original oligosaccharide due to the limited value of the response factor [23] used to obtain the molar ratio of the methylated sugars in glc-mass spectrometry.

Periodate oxidation-borohydride reduction of the enzyme-treated oligosaccharide, a hexasaccharide, and characterization of the degraded fragments by compositional analysis and methylation studies suggested the

presence of two fragments:(a) galactose linked N-acetylthreosaminitol and (b) N-acetylglucosamine linked to galactose. These findings do support the partial structure shown above; however, complete study of this oligosaccharide does not clearly indicate the linkage of a galactose and an N-acetygalactosamine. Therefore, a partial structure for this oligosaccharide, F-1, is proposed in Fig.5.

Oligosaccharide Fraction-2: This oligosaccharide was purified by chromatography on HPLC, and carbohydrate composition (Table IV) suggested it to be a pentasaccharide consisting of a galactose, a fucose, an N-acetylglucosamine, an N-acetylgalactosamine and an N-acetylgalactosaminitol. Periodate oxidation and characterization of the degraded products, by analysis of the carbohydrate composition and methylation, established the presence of a fragment (i) and N-acetylglucosamine.

(i) Gal $1 \rightarrow 3$ ThrNAc-ol

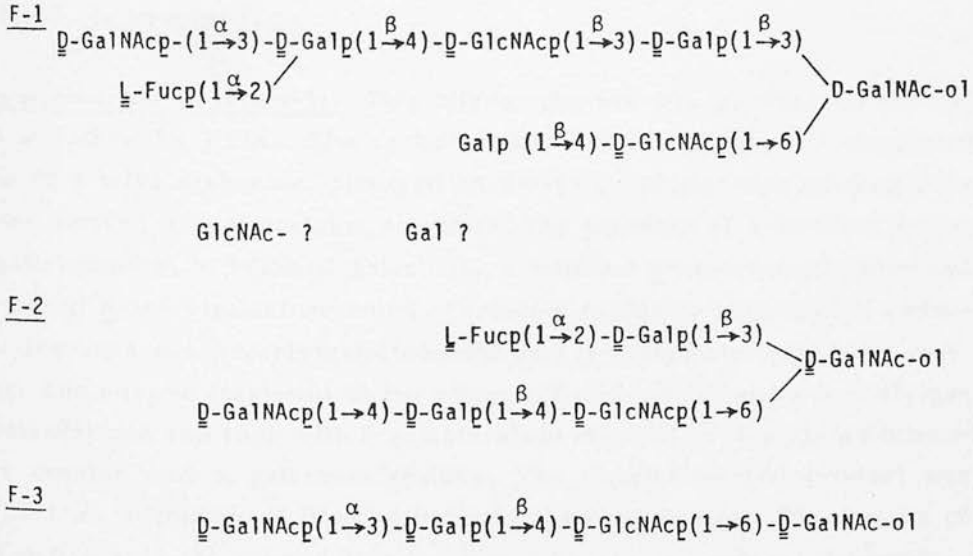
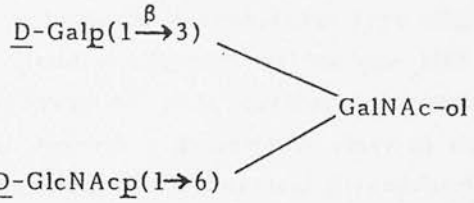


Fig. 5. Proposed partial structures for bovine gall bladder oligosaccharides.

Methylation of the oligosaccharide and characterization of the derived methylated alditol acetates suggested therein the presence of terminal N-acetylgalactosamine, terminal fucose, 2-linked and 4-linked galactose residues, 4-linked N-acetylglucosamine and 3,6-linked N-acetylgalactosaminitol. The oligosaccharide was treated with α-L-fucosidase, α-N-acetylgalactosaminidase and then with β-galactosidase. The enzyme-treated oligosaccharide (residual fragment) was subjected to methylation analysis. These studies on the oligosaccharide suggested therein the presence of a basic structure:



The terminal β-galactose residue was not eliminated by β-galactosidase; however, it is not unusual that a galactose residue in the immediate vicinity of N-acetylgalactosaminitol is not cleaved by the enzyme used (21). From the combined results of these studies structure F-2, as shown in Fig.5, is proposed.

Oligosaccharide Fraction-3: This oligosaccharide was purified on Bio-Gel P-6 and then by HPLC. The carbohydrate analysis (Table IV) suggested it to be a tetrasaccharide. Methylation analysis and glc-mass spectrometry of the derived alditol acetates suggested the presence of a terminal N-acetylgalactosamine, a 3-linked galactose, a 4-linked N-acetylglucosamine and a 6-linked N-acetylgalactosaminitol. Periodate oxidation-borohydride reduction degraded the N-acetylgalactosamine and N-acetylgalactosaminitol residues. The enzyme treatment of the oligosaccharide, first with α-N-acetylgalactosaminidase and then with β-galactosidase removed an N-acetylgalactosamine residue and a galactose residue. The enzyme-treated product was purified on a column of Bio-Gel P-2 and then methylated. The results of methylation analysis showed the presence of traces of terminal N-acetylgalactosamine and a galactose residue, a terminal N-acetylglucosamine and a 6-linked N-acetylgalactosaminitol. The trace amounts of terminal N-acetylga-

lactosamine and galactose residues probably due to incomplete removal of the sugars by α-N-acetylgalactosaminidase and -galactosidase respectively. The results of methylation analysis and enzymic studies suggest structure F-3 for this oligosaccharide, Fig.5.

The cervical glycoprotein exhibits a similarity to gall bladder glycoprotein in containing neutral oligosaccharide chains that possess both GlcNAc (1--6) GalNAc-ol and Gal (1--3) GalNAc-ol base structures, and N-acetylgalactosaminitol linked in position 0-3 and 0-6. The cervical oligosaccharides display a variety of chain lengths, and more importantly appear to contain a minute quantity of N-acetylglucosamine-asparagine type oligosaccharides. The investigation on gall bladder oligosaccharides are still preliminary, more complete studies are likely to yield further information regarding size and types of linkages. However, it remains clear at this stage that dominant neutral oligosaccharides in the cervical glycoprotein have short carbohydrate chains, whereas in gall bladder glycoprotein there are large carbohydrate chain oligosaccharides.

Structural features and functions of glycoprotein

The cervical glycoproteins of the different phase exhibit distinct differences in physiological properties (24) as well as in the chemical structure of their acidic oligosaccharides (9,22). The major variations observed during the cycle concern the quantity and linkages of carbohydrates, in particular N-acetylneuraminic acid, N-acetylgalactosamine and galactose (7-9,25). The N-acetylneuraminic acid residues of the periovulatory glycoprotein are linked to O-6 of an N-acetylgalactosamine residue that is adjacent to the protein core as well as to the O-3 of the outer galactose (8,22); in the premenstrual phase these residues are mainly linked to the O-3 of the galactose residue remote from the protein moiety (8). It has been postulated that because of their mutual repulsion the N-acetylneuraminic acid residue are responsible for the coherence, rigidity and consistency of the mucin secretion (26). It is very likely that the presence of N-acetylneuraminic acid in the vicinity of the protein core during the periovulatory phase enhances the rigidity of the glycoprotein

that is responsible for the parallel alignment of the mucus, which allows channels of least resistance for sperm migration. The involvement of the glycoprotein in the maintenance of channels has been demonstrated by cross reaction of the mucus with anti-glycoprotein antibody (11). It is probable that carbohydrate chains that have N-acetylneuraminic acid in close proximity to the protein moiety cause parallel alignment of the glycoprotein, whereas those with N-acetylneuraminic acid remote from the protein core due to continous movement caused by ionic repulsion directs sperm migration.

Extensive treatment of partially purified cervical glycoprotein with proteolytic enzymes caused only a minor change in the molecular size of the major glycoprotein (10). The change in molecular size indicates that the enzymes degraded the original glycoprotein to a similar extent but to a limited degree. This suggests that only some portions of the glycoprotein are relatively easily accessible to enzymes, whereas the remaining are well protected by the carbohydrate chains, particularly those chains that contain sialic acid (10). It has been reported that several glycoproteins, serum as well as secretory type, lose their resistance towards proteinases after treatment with neuraminidase (27-29). It appears that N-acetylneuraminic acid, whether in vicinity or remote from the protein moiety, provides a necessary chemical structure for an important physiological function.

The glycoproteins with neutral oligosaccharide carbohydrate chains or the neutral oligosaccharides within a glycoprotein with hybrid chains (acidic as well as neutral) may contribute to two functions. The neutral carbohydrate chains of the glycoprotein may have been involved in retaining definite protein conformation that imparts to the glycoprotein molecule a physiological activity as well as to give rigidity to the protein. Similar functions have been attributed to the carbohydrate chains of vesicular stomatitis virus G protein (1) and to erythrocytes (2). This assumption need be further verified and possibly a procedure similar to that applied to the virus protein, i.e. inhibition of glycosylation, will be the method of choice.

The other function for the glycoprotein with the neutral oligosaccharide chains which has been proposed is to protect the cervical tissue from invasive action by acting as an acceptor for N-acetylneuraminic acid. It had been demonstrated that spermatozoa will penetrate squamous cells, and that these cells take on characteristics of cancer cells (30). If sperm have the ability to penetrate diploid cells and induce transformation, then some mechanism in the female reproductive tract must function to prevent this phenomenon. It has been proposed that the layer of mucin in immediate contact with mucosal cells of the female genital tract is freed from N-acetylneuraminic acid by mucosal membrane-bound N-acetylneuraminidase (31), thus exposing mucin acceptor molecules for sperm surface N-acetylneuraminic acid, a molecule that endows the sperm with the ability to penetrate cells (32). The fact that cervical carcinoma is more frequently observed at the squamo-columnar cells junction, a place where mucus is scant with excess of bacteria producing a variety of enzymes, including N-acetylneuraminidase, indicates that the neutral oligosaccharide-containing glycoproteins may provide a natural protection to the exposed tissue.

The natural or biological phenomenon involves a cascade of events. The begining of the understanding of these events focusses on the molecular structure, and establishment of chemical structure may initiate the unravelling of the phenomenon.

Acknowledgements

The authors wish to thank Dr. Vernon N. Reinhold, Harvard Medical School, Boston, MA., for the mass spectrum. Part of this investigation (gall bladder) was spported by a research grant from the Pakistan Science Foundation (Grant B-BU/MED 89).

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ISOLATION AND PARTIAL CHARACTERIZATION OF HUMAN GALLBLADDER MUCIN GLYCOPROTEINS

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The contribution of gallbladder mucus secretion in the gallstone formation is well known (1,2). The mechanism of gallstone formation have been the subject of investigation for sometime and is now being learned (3-4). Supersaturation of cholesterol in bile was considered an important factor in the gallstone formation (3). More recently, however, it has been observed that the supersaturation of cholesterol in the bile alone does not explain the gallstone formation (4). It has been proposed that gallbladder mucin promotes nucleation and thereby gallstone formation. The structure of normal gallbladder mucin has been investigated to study the structure-function relationship.

Normal human bile was withdrawn from known secretor type, and secretion from similar blood type was combined and purified by gel filtration on Bio Gel P-200. The excluded material from the column containing carbohydrate and protein was further purified by gel filtration on Sepharose 2B. The carbohydrate and protein containing fractions were combined and dialysed, and the retentate was freeze dried. The freeze dried material in potassium thiocyanate was mixed with chloroform (5:1) and stirred for twenty-four hour at 40°. The solution was repeatedly dialysed, retentate centrifuged and aqueous layer lyophilized. This material was chromatographed on a column of DEAE-cellulose and the glycoprotein was further purified by gel filtration on sepharose 4B. Gradient polyacrylamide gel electrophoresis (3-15%) showed the absence of contaminating protein and lipids. Carbohydrate compositional analysis showed the presence of fucose, galactose, N-acetylglucosamine, N-acetylgalactosamine and N-acetylneuraminic acid. Reductive β-elimination afforded a mixture of acidic and neutral oligosaccharide. The mixture was separated into neutral and acidic oligosaccharides by ion-exchange chromatography. The neutral oligosaccharide were further separated into six component oligosaccharides by a combination of gel filtration and ion-exchange chromatography followed by high performance liquid chromatography. The structures of these oligosaccharides has been partially established using enzymic and chemical degradations, and permethylation. The longer oligosaccharides were branched and had blood group determinants. There also appeared to be oligosaccharide chains with N-glycosidic linkage in the glycoprotein.

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Supported by Pakistan Science Foundation Grant Number B-BU/MED(89).

Dedicated to Professor Roger W. Jeanloz

THE JOURNAL OF BIOLOGICAL CHEMISTRY
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Vol. 261, No. 5, Issue of February 15, pp. 1992-1997, 1986
Printed in U.S.A.

Structure of Sialyloligosaccharides Isolated from Bonnet Monkey (*Macaca radiata*) Cervical Mucus Glycoproteins Exhibiting Multiple Blood Group Activities*

(Received for publication, July 17, 1985)

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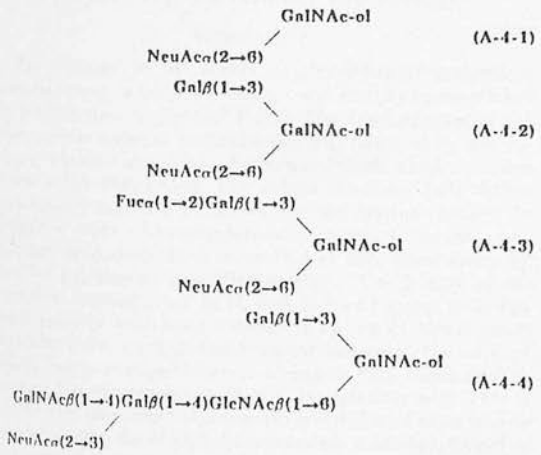
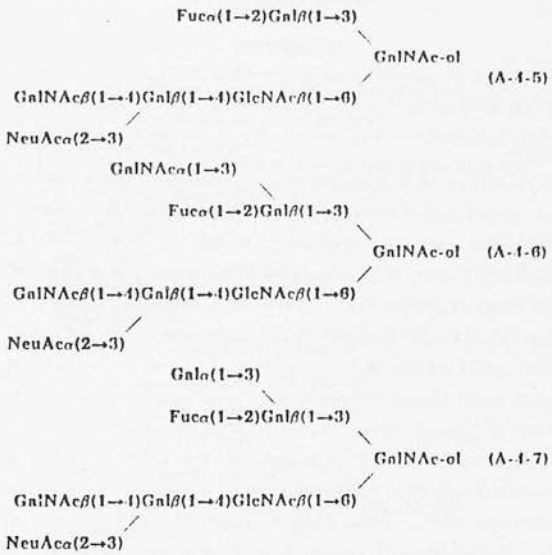
Mucin glycoproteins purified from cervical epithelial secretion of the bonnet monkey (*Macaca radiata*) exhibit multiple blood group activities. Alkaline borohydride reductive cleavage resulted in a mixture of neutral and acidic oligosaccharide-alditols. By high-performance liquid chromatography, seven oligosaccharides (A-4-1 to A-4-7) have been purified from the monosialyloligosaccharide fraction (A-4). Based on the results of 500-MHz ^{1}H NMR spectroscopy, in conjunction with sugar analysis and immunological assays, we propose the following structures for these oligosaccharides.





These structures imply that either the A, B, or H determinant may be found in combination with the Cad/Sd^a determinant; the oligosaccharides identified, together, account for the blood group activities exhibited by the cervical mucus.

Cervical mucus is a gel-like, hydrophilic epithelial secretion, playing a significant role in reproductive physiology (1). The physical and chemical properties of human (2) and bovine (3) cervical secretions change with the state of ovarian function.

*This investigation was supported in part, by Grant AM-03564 from the National Institute of Arthritis, Diabetes, Digestive and Kidney Diseases, by Grant HD-12431 from the National Institute of Child Health and Human Development, National Institutes of Health, by the Netherlands Foundation for Chemical Research with financial aid from the Netherlands Organization for the Advancement of Pure Research, and by Grant UUKC 83-13 from the Netherlands Foundation for Cancer Research. This is publication 989 of the Robert W. Lovett Memorial Group for the Study of Diseases Causing Deformities, Harvard Medical School and Massachusetts General Hospital. A preliminary account of this study was presented at the XIIth International Carbohydrate Symposium, Utrecht, The Netherlands, July 1-7, 1984. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Dedicated to Luis Leloir on the occasion of his 80th birthday.

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Near the time of ovulation, the production of mucus increases, and the secretion becomes profuse, thin, and receptive to sperm. In the postovulatory phase, the mucus becomes thick and scanty, and impedes sperm penetration. Abnormalities in this secretion may be a factor in infertility. Furthermore, it has been shown that the receptors for various steroid hormones exist in cervical tissues (4). The rheological properties of the cervical mucus are largely determined by high-molecular-weight glycoproteins (5).

For the understanding of the physiological role of the glycoproteins of the cervical secretion during the menstrual cycle, knowledge of the carbohydrate structure is essential in relation to the biophysical properties and physiological function of the mucus at various phases of the ovulatory cycle (6, 7). For human cervical proteins, the structure of a few carbohydrate chains has been determined (8, 9). This kind of investigation is hampered by the fact that mucus of human origin is scarce and difficult to obtain. The bonnet monkey secretes large amounts of mucus, the rheological properties of which resemble those of human mucus (10); moreover, the simian menstrual cycle is similar to the human cycle. This makes the mucus of the bonnet monkey a suitable model system.

Fractionation of midcycle (periovulatory) cervical mucus provides two high-molecular-weight glycoproteins having a chemical composition characteristic of mucin-type structure (10-14). This paper describes further characterization of midcycle mucus glycoproteins, in particular the structure determination of their sialyloligosaccharides.

EXPERIMENTAL PROCEDURES AND RESULTS[1]

DISCUSSION

The isolation of the secretory glycoproteins present in cervical mucus of bonnet monkeys was readily accomplished by gel filtration on Bio-Gel P-200. The fractionation of the glycoprotein material by Sepharose 2B, followed by ion exchange chromatography on Ecteola-cellulose, yielded a main fraction 1-A (80%) (14). The minor fraction (1-B) differs significantly from 1-A in its sulfate and cystine content. In inhibition tests of hemagglutination against human anti-blood group A, anti-blood group B, and anti-blood group H, fraction 1-A showed a positive reaction. The feature of the combined occurrence of A, B, and H blood group activities could possibly have been avoided if typing of blood group activities of the monkeys had been carried out on the salivary mucin, before pooling the cervical mucus (37). Sialic acid in fraction 1-A was present as *N*-acetylneuraminic acid. This is similar to human cervical mucus (8) and different from bovine which contains the *N*-glycolylneuraminic acid (38). Cysteine was present only in a small amount as a component of glycoprotein 1-A. No cross-linking fraction containing cystine, as in the case of bovine cervical mucus (39, 40) was isolated.

Alkaline borohydride treatment of fraction 1-A resulted in a mixture of oligosaccharide-alditols which was subsequently fractionated on Bio-Gel P-4. The main fraction A-4 was further separated on hplc,[2] affording 7 subfractions (A-4-1 to A-4-7). The structures elucidated can be divided into two groups on the basis of the structure of the core, namely Galβ(1→3)[NeuAcα(2→6)]GalNAc-ol (A-4-1 to A-4-3) and Galβ(1→3)[GlcNAcβ(1→6)]GalNAc-ol (A-4-4 to A-4-7). The larger structures (A-4-5 to A-4-7) exhibit multiple blood group determinants.

The occurrence of a terminal nonreducing sequence GalNAcβ(1→4)[NeuAcα(2→3)]Gal is a feature already described in oligosaccharides from glycophorins with blood group Cad specificity (31) and from Tamm and Horsfall urinary glycoproteins with Sd^a activity (32, 41). Sd^a activity has also been detected in urinary mucin (42) and meconium (43). GalNAcβ(1→4)[NeuGlα(2→3)]Gal sequences have been observed in fish egg glycoproteins (44) and GalNAcβ(1→4)Gal in a cloned murine cytotoxic T lymphocyte line (45). In oligosaccharides (A-4-5 to A-4-7) of cervical mucins from the bonnet monkey, the Cad blood group determinant can occur together with an A, B, or H determinant.

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[1] Portions of this paper (including "Experimental Procedures," "Results," Figs. 1-7, and Tables I-IV) are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are available from the Journal of Biological Chemistry, 9650 Rockville Pike, Bethesda, MD 20814. Request Document No. 85 M-2359, cite the authors, and include a check or money order for $9.20 per set of photocopies. Full size photocopies are also included in the microfilm edition of the Journal that is available from Waverly Press.

[2] The abbreviations used are: hplc, high-performance liquid chromatography; glc, gas-liquid chromatography; GalNAc-ol, *N*-acetylgalactosaminitol; Fuc, fucose.

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SUPPLEMENTARY MATERIAL TO

"STRUCTURE OF SIALYL-OLIGOSACCHARIDES ISOLATED FROM BONNET-MONKEY (MACACA RADIATA) CERVICAL MUCUS GLYCOPROTEINS EXHIBITING MULTIPLE BLOOD-GROUP ACTIVITIES"

by Nasir-ud-din, Roger W. Jeanloz, Geneviève Lamblin, Philippe Roussel, Herman van Halbeek, Johanna H.G.M. Mutsaers and Johannes F.G. Vliegenthart

EXPERIMENTAL PROCEDURES

Materials : Bio-Gel P-200, Bio-Gel P-4, AG50W-X8 (100-200 mesh), AG1-X2 ion-exchange resins and Eckeola-cellulose (Cellex C) were purchased from Bio-Rad Laboratories, Sepharose 2B from Pharmacia Fine Chemicals Inc., agarose from MCI Biomedical Division of Marine Colloids Inc. and L'Industrie Biologique Francaise. Ulex europeus, Triticum vulgaris, Helix pomatia and Ricinus communis IgG lectins were purchased from Sigma.

Analytical methods. The hexose content of column eluates was measured with the phenol-sulfuric acid method (15), and the protein content by determining the absorbance at 280 nm. Amino acid analysis was performed with a Beckman Model 116 amino acid analyzer, after hydrolysis of the sample with 6 M HCl at 105°C for 20 h in an atmosphere of N_2, followed by dilution with water and lyophilization.

Polyacrylamide gel electrophoresis was performed according to (16), agarose gel electrophoresis in veronal buffer at pH 8.2 (ionic strength 0.1) according to (17). Gels were stained with periodate-Schiff reagent, amido black, Coomassie blue, toluidine blue or Sudan black (16,17).

Quantitative analysis of carbohydrates by gas-liquid chromatography (glc)[1] was performed according to (18). The samples were per-O-(trimethyl) silylated with Sylon HTP (Supelco) ; epi-inositol was used as internal standard. The glc analyses were performed with a Carlo-Erba Model 900 gas chromatograph, on a column (180 × 0.3 cm) containing 3 % OV 17 on Chromosorb WHP PD-100 mesh (Supelco, Bellefonte, Pa).

Hplc was performed on a 5 µ Lichrosorb-NH_2 column. The elution was performed with a linear gradient of 4:1 to 1:1 acetonitrile-water containing 2.5 mM ammonium hydrogencarbonate for 70 min at room temperature and at a flow rate of 1 ml/min (19). Prior to ^{1}H-nmr spectroscopic analysis, samples were repeatedly exchanged in D_2O (99.96 mol% D, Aldrich) with intermediate lyophilization. The pD of the solution was adjusted to 7. ^{1}H-NMR spectroscopic analysis was performed on a Bruker WM-500 spectrometer (SON hf-NMR facility, Department of Biophysical Chemistry, Nijmegen University, the Netherlands) operating at 500 MHz in the Fourier transform mode at probe temperatures of 5, 10 or 27°C (20). Chemical shifts are given for neutral solutions at 27°C, relative to internal sodium 4,4-dimethyl-4-silapentane-1-sulfonate, but were actually measured by reference to internal acetone (δ 2.225) with an accuracy of 0.002 ppm.

Isolation and purification of mucus glycoproteins. The crude pooled mucus obtained from 4 bonnet monkeys at the mid-cycle (12 to 15 days of the menstrual cycle) was partially solubilized in 50 mM sodium phosphate buffer, pH 7.0, containing 0.02 % NaN_3 by stirring at 4°C for 16 h. The cellular debris and other insoluble materials were removed by centrifugation (2 000 × g) ; the supernatant was dialyzed against distilled water and the retentate was lyophilized affording a mixture of (glyco)proteins. The residue was solubilized in 50 mM sodium phosphate buffer, pH 7.0, containing 0.02 % NaN_3 by stirring at 4°C overnight. The complex mixture of macromolecules was purified by filtration over Bio-Gel P-200 followed by fractionation on Sepharose-2B (11,12). The main fraction (100 mg) was subfractionated on an Eckeola-cellulose column (2.7 × 65 cm). The column was eluted with 0.1 M NaCl (200 ml) followed by a gradient of 0.1 M to 1 M NaCl in 10 mM HCl (Fig. 1).

End group analysis. The amino-terminal end-group was determined by dansylation of the major glycoprotein fraction (I-A, see below) (0.5 mg) and hydrolysis (21). The dansylated amino acids were separated and identified by thin layer chromatography on polyamide plates.

Blood-group activity and inhibition of lectin-induced hemagglutination. Hemagglutination inhibition tests were performed according to Watkins and Morgan (22) using anti-A, anti-B and anti-H immune sera (CRTS, Lille, France). The tests were performed by mixing one volume of mucin solution (25 µl) and one volume of antiserum representing 2 complete agglutinating doses. The mixtures were incubated for 15 min at room temperature and examined for agglutination.

Inhibitory activity towards lectins was assayed for glycoprotein fraction I-A. Type O human red blood cells treated with neuraminidase (3.0×10^8 cells with 150 µl neuraminidase, 25 units from *V. cholerae*) were used with peanut agglutinin and Ricinus communis, whereas untreated cells were used with Ulex europeus, Ricinus communis, Helix pomatia and wheat germ agglutinins. The titration and inhibition assays were performed according to (23).

Alkaline borohydride treatment. The major fraction eluted from the Eckeola column was treated with 2 M $NaBH_4$ in 50 mM NaOH for 18 h at 45°C according to (24). Then the mixture was adjusted to pH 5.4 with 4 M acetic acid. For preparation of ^{3}H-labeled oligosaccharides, β-elimination on a portion of glycoprotein (5 mg) was performed using NaB^3H_4 (5 mCi) under the conditions described above. The two reaction mixtures were combined and desalted on a column of AG50W-X8 (H^+, 100-200 mesh). Reduced oligosaccharides were separated into neutral (25 %) and acidic compounds (75 %) on a column (3.4 × 70 cm) of AG1-X2 (OAc^-, 200-400 mesh). The column was eluted with water, 0.5 M pyridine-acetic acid (pH 5.4) and then with 1 M acetic acid. The acidic oligosaccharides were further purified by filtration on Bio-Gel P-4 (200-400 mesh) in pyridine-acetic acid.

RESULTS

Purification and characterization of cervical mucus glycoproteins. The mucus glycoproteins obtained from bonnet monkey at menstrual mid-cycle were purified by gel filtration on Bio-Gel P-200 followed by fractionation on Sepharose 2B (12). The main fraction (85 %) after Sepharose 2B chromatography (denoted fraction I) was analyzed by polyacrylamide (15 %) gel electrophoresis. The glycoprotein did not enter the gel and no contaminating (glyco)lipids were observed by staining with Coomassie blue, periodate-Schiff reagent or Sudan black. Eckeola-cellulose chromatography of fraction I afforded two subfractions, denoted I-A (80 % by weight) and I-B (fig. 1). The carbohydrate and amino acid compositions and the sulfate contents of these fractions are reported in Table I.

Agarose electrophoresis of fraction I-A showed a single band with periodate-Schiff reagent and toluidine blue. The carbohydrate chains consisted of fucose, galactose, N-acetylglucosamine, N-acetylgalactosamine and sialic acid. The glycoprotein contained a relatively high proportion of threonine, serine, alanine and glycine residues, while cysteine was detected in only small amounts (Table I). Threonine and a trace of glycine were found as amino terminal end groups.

Blood-group activity and inhibition of lectin hemagglutination. Fraction I-A had blood group A, B and H activities. Under our experimental conditions, inhibition of anti-A serum was obtained with 31 µg of fraction I-A, inhibition of anti-B and anti-H immunosera was obtained with 250 µg of fraction I-A.

The hemagglutination inhibition assays using glycoprotein fraction I-A showed no activity with wheat germ and peanut agglutinins. A weak inhibitory activity using relatively high concentration of glycoprotein (100 mg/ml, 50 µl solution used) was observed with *H. pomatia* and *U. europeus* agglutinins. With *R. communis* low concentrations of glycoproteins demonstrated inhibition.

Preparation of sialyl oligosaccharide-alditols. Fraction I-A (70 mg) was subjected to alkaline borohydride reductive cleavage (24) yielding a mixture of oligosaccharide-alditols. A decrease of serine and threonine and a corresponding increase of alanine and appearance of α-aminobutyric acid were noticed, concomitant with conversion of part of the GalNAc residues into GalNAc-ol (see Table I). The acidic oligosaccharides, eluted from the column of AG1-X2 by 0.5 M pyridine-acetic acid, pH 5.4, were separated on a Bio-Gel P-4 column into five fractions (Fig. 2). Fractions A-1 and A-2 represent mainly glycopeptide material as indicated by very low percentage of N-acetylgalactosaminitol (Table II) and the presence of hydroxylated amino acids (data not shown). The major fraction A-4 (12 mg) was further investigated. The molar carbohydrate composition of fraction A-4 is included in Table II. A-4 was fractionated by hplc into seven subfractions (A-4-1 to A-4-7) (Fig. 3).

Structure determination of sialyl oligosaccharides. The molar carbohydrate composition of A-4-1 to A-4-7 is given in Table III. Starting from these data, the complete primary structures of the oligosaccharides could be elucidated by employment of high-resolution (500-MHz) ^{1}H-NMR spectroscopy. The chemical shifts of the structural-reporter groups of these compounds are compiled in Table IV.

Compound A-4-1 could be identified as the disaccharide-alditol NeuAcα(2-6) GalNAc-ol (cf. Table III). Its NMR characteristics (Table IV) match exactly those described for this compound, obtained from other sources (20,25,26). Similarly, comparison of the NMR data of A-4-2 with those of the branched trisaccharide Galβ(1-3)[NeuAcα(2-6)]GalNAc-ol obtained from cow κ-casein (25,26) revealed that their structures are identical.

Compound A-4-3 was found to be the extension of A-4-2 with a fuc residue in α(1-2) linkage to Gal[3] (for explanation of superscript notation, see Table IV, footnote a). This structure is essentially identical to the acidic tetrasaccharide Fucα(1-2)Galβ[N(1-3)][NeuGlα(2-6)]GalNAc-ol (27), except for the type of sialic acid in A-4-3 being NeuAc instead of NeuGl.

For compound A-4-4, the positions of the signals of H-2 (δ 4.392) and H-5 (δ 4.240) of GalNAc-ol in the ^{1}H-NMR spectrum point to the presence of a Galβ(1-3)[GlcNAcβ(1-6)]GalNAc-ol type of core (18,28,29). The H-1 signal of Gal[3] found at δ 4.467 ($J_{1,2}$ = 7.8 Hz), together with the H-4 signal at δ 3.907, indicate that A-4-4 contains this core-Gal residue in terminal position (20,28,30). The (1-6)-linked branch in A-4-4 could be identified to contain the Cad blood-group structure, by comparing the NMR data of A-4-4(Table IV) with those of the Cad pentasaccharide structure

TABLE I : Carbohydrate and amino acid composition of cervical-mucus glycoprotein fractions I-A and I-B from bonnet monkey separated on Ectola-cellulose, and amino acid composition of alkali-treated fraction I-A glycoprotein.

	I-A		I-B	
	%	Molar ratio[a]	%	Molar ratio[a]
Fuc	7	0.9	6	1.1
Gal	24	3.0	15	2.8
GlcNAc	10	1.0	7	1.0
GalNAc	18	1.8	13	1.8
NeuAc	15	1.1	10	1.0
Total carbohydrate	74		51	
Sulfate	1		5	
Amino acid	14			n.d.[c]

	I-A	I-A after treatment with alkali	I-B
Asp[b]	44		48
Thr	228	-123	250
Ser	117	- 51	118
Glu	64		60
Pro	58		87
Gly	84		79
Ala	101	+ 41	99
Aba		+111	
Cys	14		3
Val	61		53
Ileu	59		44
Leu	62		62
Tyr	14		7
Phe	19		17
Lys	31		32
His	16		13
Arg	28		28

[a] Molar ratio relative to GlcNAc

[b] Residues per 1 000 residues

[c] not determined

TABLE II : Carbohydrate composition of acidic oligosaccharide fractions separated by Bio-Gel P 4 filtration of the β-elimination cleavage product of fraction I-A glycoprotein from bonnet monkey cervical mucus.

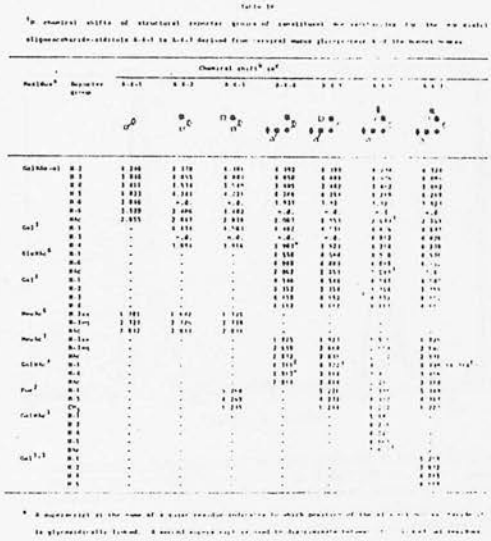
	Content in fraction							
Monosaccharide	A-1	A-2	A-3		A-4		A-5	
	%	%	%	Molar[a] ratio	%	Molar[a] ratio	%	Molar[a] ratio
Fuc	6.8	6.9	6.4	0.11	6.3	0.59	4.5	0.25
Gal	18.9	14.0	17.4	4.50	23.4	1.99	20.4	1.00
GlcNAc	16.8	15.7	13.9	2.76	13.9	0.99	4.9	0.20
GalNAc	18.6	18.0	17.3	3.62	16.1	1.12	5.0	0.2
NeuAc	19.8	18.9	21.1	3.13	19.1	0.95	27.9	0.82
GalNAc-ol	1.8	1.0	4.8	1.00	14.5	1.00	24.6	1.00

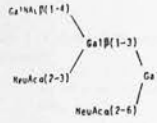
[a] Molar ratio relative to GalNAc-ol.

TABLE III : Molar composition of sialyl oligosaccharides obtained by fractionation of cervical mucus glycan fraction of A-4 by hplc.

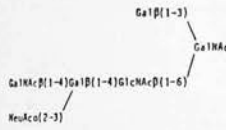
A-4 subfraction	Molar ratio[a] of monosaccharides					
	Fuc	Gal	GlcNAc	GalNAc	NeuAc	GalNAc-ol
A-4-1	-	-	-	-	0.8	1
A-4-2	-	1.0	-	-	0.8	1
A-4-3	0.7	0.9	-	-	0.7	1
A-4-4	-	2.0	0.8	1.2	0.8	1
A-4-5	0.7	2.1	0.9	1.1	1.0	1
A-4-6	0.6	1.6	0.8	1.4	0.9	1
A-4-7	0.5	3.4	1.0	1.2	1.0	1

[a] relative to GalNAc-ol





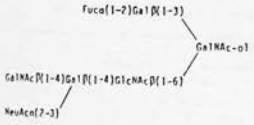
obtained from glycophorin-A of erythrocytes with blood-group Cad activity (31). The set of chemical shifts observed in A-4-4 for NeuAc H-3ax and H-3eq (δ1.93 and δ2.66, respectively) is known to be typical for the occurrence of a so-called internal sialic acid residue (31,32). Such a NeuAc residue is α(2-3)-linked to a Gal residue which also bears a sugar residue at C-4. The occurrence of NeuAc in α(2-3)-linkage to Gal is corroborated by the appearance of the Gal H-3 signal at 4.15. The substituent at C-4 of Gal is β-linked GalNAc (compare Table III), characterized by its H-1 doublet at 4.72 ($J_{1,2}$ = 8.1 Hz) and its N-acetyl signal at δ2.014, the former signal is partly hidden under the HOD-line when recording the NMR spectrum at room temperature, but could be visualized by lowering the sample temperature to 5-10°C (compare inset Fig. 4). The C-3,C-4 disubstituted Gal residue itself is β(1-4)-linked to the core GlcNAc[6]. This can be derived from the chemical shifts of H-1, H-6 and the N-acetyl signal of GlcNAc[6], being 4.550, 4.000 and 2.062 respectively (28-33). Therefore, the structure of A-4-4 is the following :



The small but significant difference in chemical shift of the N-acetyl protons of β-GalNAc in A-4-4 compared to the Cad pentasaccharide (2.014 vs. 2.025 respectively) must be attributed to the difference in branch location of the Cad determinant. Analogously, the set of H-2, H-3 and H-4 resonances of the disubstituted β-Gal in this sequence appear to be slightly different for the two compounds (δ3.35/4.15/4.11 vs.δ3.42/4.16/4.09). Therefore, the combination of the chemical shifts of these structural-reporter groups seems to be suitable for branch localization of the Cad determinant in more complex oligosaccharides.

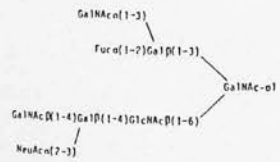
The application of this finding to the spectra of the remaining three compounds (A-4-5 to A-4-7) allows the recognition of the Cad sequence in the (1-6)-linked branch. By consequence, the structural differences between compounds A-4-4 to A-4-7 must be limited to the (1-3) branch. The carbohydrate composition of the oligosaccharides (Table III) suggests that A-4-5 may be an extension of A-4-4 with a Fuc residue whereas A-4-6 and A-4-7 have, in addition to Fuc, an extra GalNAc or Gal residue, respectively.

In the 500-MHz ^{1}H-NMR spectrum of A-4-5 (Fig. 4) the structural-reporter-group signals characteristic of Fucα(1-2) linked to Gal[3] of the core (δH-1 5.23, δH-5 4.27 and δCH_3 1.24) are readily recognized (28-30). The α(1-2)-linkage to Gal[3] is confirmed by the H-1 signal of Gal[3] at δ4.57 (compare A-4-3). Therefore, the structure of A-4-5 is :



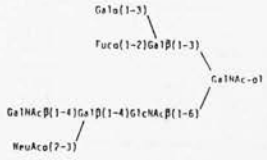
This implies that this oligosaccharide contains both the H and the Cad blood-group determinant. Comparison of the NMR spectra of A-4-4 and A-4-5 shows that the apparent extension of Gal[3] with Fuc in α(1-2) linkage causes some remarkable effects on the chemical shifts of other structural-reporter groups. The H-5 signal of GalNAc-ol was found in shift from δ4.280 to δ4.254 (Table IV). The N-acetyl signals of GalNAc-ol and GlcNAc[6] have shifted from δ2.067 and δ2.062, respectively, both to 2.053 in A-4-5. The effects are in line with those observed in the step from A-4-2 to A-4-3 as far as the GalNAc-ol signals are concerned (compare also ref. 30, in particular the step from compound 11 to 12 therein).

In A-4-6 (for spectrum see Fig. 5), identification of the (1-3)-linked branch is readily achieved by comparison of its 500-MHz ^{1}H-NMR parameters with those of the tetrasaccharide GalNAcβ(1-3)[Fucα(1-2)]Galβ(1-4)Glc (34) and with those of the acidic pentasaccharide-alditol GalNAcβ(1-3)[Fucα(1-2)]Galβ(1-3) [NeuGlα(2-6)]GalNAc-ol given in (27) (it should be noted that the spectrum of the latter compound (Fig. 6) contains a couple of corrected assignments in comparison with earlier 360-MHz data (cf. refs 34-36)). The simultaneous occurrence in the spectrum of A-4-6 of two H-1 signals of α-linked residues (δ5.378, Fuc[2] ; δ5.185, GalNAc[3]) in conjunction with the presence of Fuc H-5 at δ4.322 and of GalNAc[3] H-2, H-4 and H-5 at δ4.251, δ4.020 and δ4.160 respectively, point to the occurrence of the A-determinant sequence in A-4-6. Therefore, the structure of A-4-6 is :



Conceiving A-4-6 as an extension of A-4-5 with an α-GalNAc, profound shift effects are observed on the signals of H-2 and H-5 of GalNAc-ol, H-1 and H-4 of Gal[3] and H-1, H-5 and CH_3 of Fuc (Table IV). These effects are essentially identical to those observed in the step from the acidic H[4] tetrasaccharide Fucα(1-2)Galβ[1-3]‖ NeuGlα(2-6)‖GalNAc-ol to the acidic A[4] pentasaccharide (27,35).

The ^{1}H-NMR spectrum of A-4-7 (Fig. 7) shows, like that of A-4-6, two α-anomeric signals, but now at δ5.349 and δ5.259. In combination with the knowledge of the presence of an additional Gal residue as compared to A-4-5 (Table III), these are assigned to α-linked Fuc and Gal, respectively. Comparison with literature ^{1}H-NMR data on the blood-group B determinant (29,35) learns that A-4-7 possesses this sequence in its (1-3)-branch. So it can be concluded that A-4-7 possesses a blood-group Cad and blood-group B determinant jointly in one oligosaccharide as follows :



The apparent extension of A-4-5 (blood-group H determinant) with an α-linked Gal residue to A-4-7 (blood-group B determinant) causes profound shift effects on H-2 and H-5 of GalNAc-ol, H-1 and H-4 of Gal[3] and on H-1, H-5 and CH_3 of Fuc (Table IV) (cf. 35).

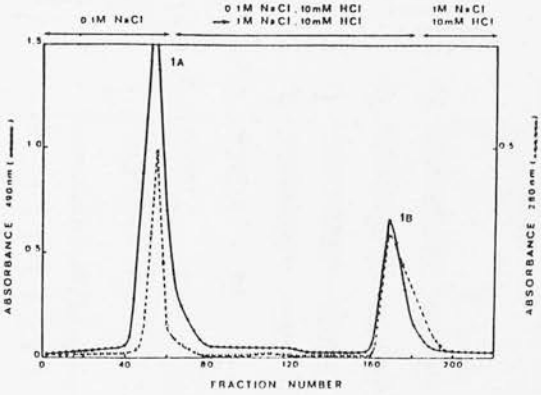


Fig.1 - Fractionation of Sepharose 2B purified glycoprotein 1 on an Ecteola-cellulose column (65 x 2.7 cm). The column was eluted with 0.1 M NaCl (200 ml), with a gradient of 0.1 M NaCl in 10 mM HCl/1 M NaCl in 10 mM HCl (450 ml) followed in 1 M NaCl in 10 mM HCl (200 ml). Fractions of 5 ml were collected and every third fraction was examined for the presence of hexoses and for protein. 100 mg of glycoprotein was applied to the column.

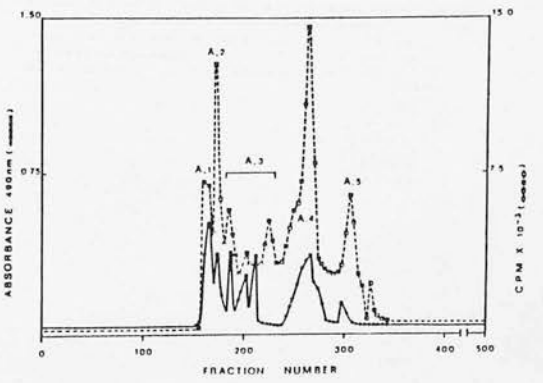


Fig. 2 - Fractionation of acidic oligosaccharides on a column (175 x 3.5 cm) of Bio-Gel P-4. The column was eluted with 50 mM pyridine acetic acid (pH 5.0, 800 ml), followed by a gradient of 0.1 to 1 M acetic acid. Fractions of 2.5 ml were collected and every third fraction was examined for hexoses and tritium. Five main fractions A1-A5 were pooled according to the bars (A-1, 161-168, 1.4 mg ; A-2, 170-184, 3 mg ; A-3, 191-236, 7 mg ; A-4, 240-294, 12 mg and A-5, 296-315, 2 mg).

TABLE I : Carbohydrate and amino acid composition of cervical-mucus glycoprotein fractions I-A and I-B from bonnet monkey separated on Ecteola-cellulose, and amino acid composition of alkali-treated fraction I-A glycoprotein.

	I-A		I-B	
	%	Molar ratio[a]	%	Molar ratio[a]
Fuc	7	0.9	6	1.1
Gal	24	3.0	15	2.6
GlcNAc	10	1.0	7	1.0
GalNAc	18	1.8	13	1.8
NeuAc	15	1.1	10	1.0
Total carbohydrate	74		51	
Sulfate	1		5	
Amino acid	14			n.d.[c]

	I-A	I-A after treatment with alkali	I-B
Asp[b]	44		48
Thr	228	-123	250
Ser	117	- 51	118
Glu	64		60
Pro	58		87
Gly	84		79
Ala	101	+ 41	99
Abu		+111	
Cys	14		3
Val	61		53
Ileu	59		44
Leu	62		62
Tyr	14		7
Phe	19		17
Lys	31		32
His	16		13
Arg	28		28

[a] Molar ratio relative to GlcNAc

[b] Residues per 1 000 residues

[c] not determined

TABLE II : Carbohydrate composition of acidic oligosaccharide fractions separated by Bio-Gel P-4 filtration of the β-eliminative cleavage product of fraction I-A glycoprotein from bonnet monkey cervical mucus.

	Content in fraction							
Monosaccharide	A-1	A-2	A-3		A-4		A-5	
	%	%	%	Molar[a] ratio	%	Molar[a] ratio	%	Molar[a] ratio
Fuc	6.8	6.9	6.4	0.11	6.3	0.59	4.5	0.25
Gal	18.9	14.0	17.4	4.50	23.4	1.99	20.4	1.00
GlcNAc	16.8	15.7	13.9	2.76	13.9	0.99	4.9	0.20
GalNAc	18.6	18.0	17.3	3.62	16.1	1.12	5.0	0.2
NeuAc	19.8	18.9	21.1	3.13	19.1	0.95	27.9	0.82
GalNAc-ol	1.8	1.0	4.8	1.00	14.5	1.00	24.8	1.00

[a] Molar ratio relative to GalNAc-ol.

TABLE III : Molar composition of sialyl oligosaccharides obtained by fractionation of cervical mucus glycan fraction of A-4 by hplc.

	Molar ratio[a] of monosaccharides					
A-4 subfraction	Fuc	Gal	GlcNAc	GalNAc	NeuAc	GalNAc-ol
A-4-1	-	-	-	-	0.8	1
A-4-2	-	1.0	-	-	0.8	1
A-4-3	0.7	0.9	-	-	0.7	1
A-4-4	-	2.0	0.8	1.2	0.8	1
A-4-5	0.7	2.1	0.9	1.1	1.0	1
A-4-6	0.6	1.6	0.8	1.4	0.9	1
A-4-7	0.5	3.4	1.0	1.2	1.0	1

[a] relative to GalNAc-ol

Table IV

[illegible]

[illegible]

[illegible]

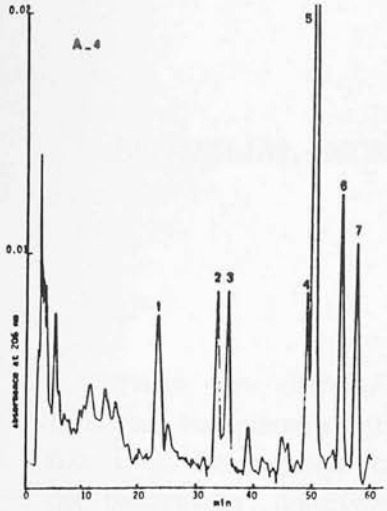


Fig. 3 - Hplc separation of sialyl-oligosaccharides A-4-1 to A-4-7 (peaks 1 to 7) of fraction A-4 on a Lichrosorb-NH_2 column eluted with a linear gradient of 4:1 to 1:1 (v/v) acetonitrile-water containing 2.5 mM ammonium hydrogen carbonate for 70 min at room temperature and at a flow rate of 1 ml/min (19). The relative yields of the recovered fractions were : A-4-1, 9.3 % ; A-4-2, 9.6 % ; A-4-3, 10.5 % ; A-4-4, 6.8 % ; A-4-5, 33.6 % ; A-4-6, 15.7 % ; A-4-7, 14.5 %.

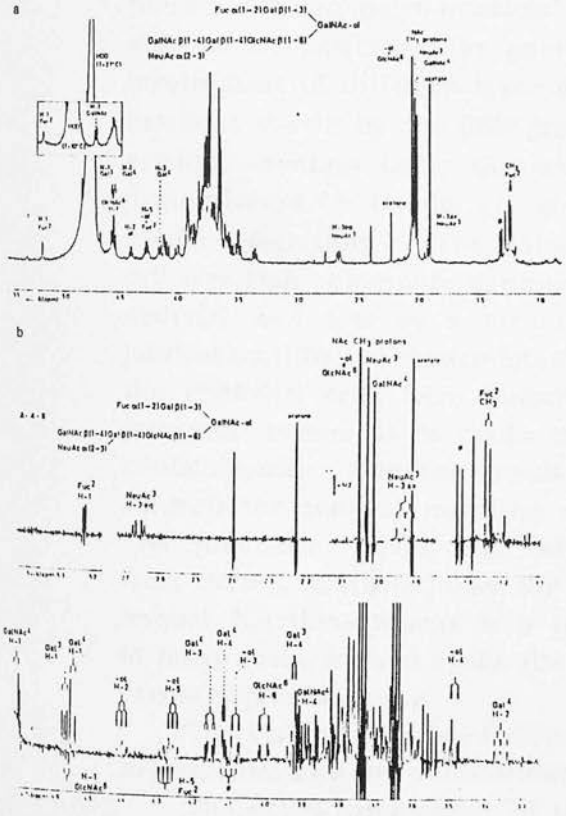


Fig. 4 - (a) 500-MHz ^{1}H-NMR spectrum of compound A-4-5 in D_2O at 27°C. The inset shows the H-1 signal of GalNAc which is visualized by lowering the temperature to 10°C. The signal marked by * stems from a frequently occurring, non-carbohydrate contaminant. (b) Resolution-enhanced 500-MHz ^{1}H-NMR spectrum of the oligosaccharide-alditol A-4-5 in D_2O at 27°C. The relative-intensity scale of the N-acetyl proton region of the spectrum differs from that of the other parts, as indicated.

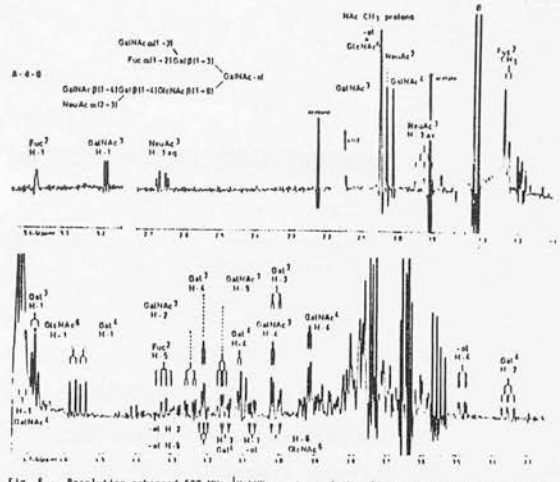


Fig. 5 - Resolution-enhanced 500-MHz ^{1}H-NMR spectrum of the oligosaccharide alditol A-4-6 in D_2O at 27°C. The relative-intensity scale of the N-acetyl proton region of the spectrum differs from that of the other parts, as indicated (* spinning side band).

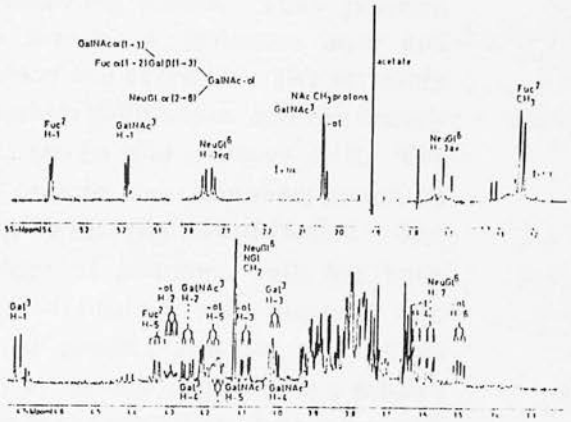


Fig. 6 - Resolution-enhanced 500-MHz ^{1}H-NMR spectrum of the acidic pentasaccharide-alditol with blood-group A-determinant supplied with the corrected (compare ref. 7?) assignments. The spectrum is recorded in D_2O at 20°C.

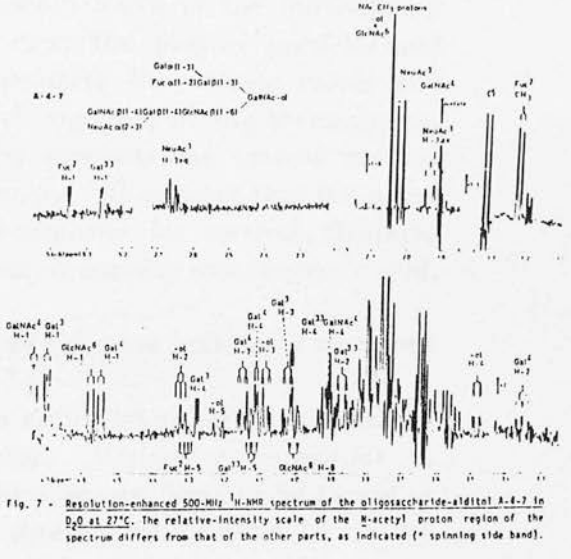


Fig. 7 - Resolution-enhanced 500-MHz ^{1}H-NMR spectrum of the oligosaccharide-alditol A-4-7 in D_2O at 27°C. The relative-intensity scale of the N-acetyl proton region of the spectrum differs from that of the other parts, as indicated (* spinning side band).

EPITHELIAL SECRETION: MUCUS GLYCOPROTEINS

NASIR-UD-DIN

There are different types of epithelial cells and various functions have been attributed to them. Epithelial cells are commonly found on the external surface of the skin, the internal surface of the respiratory, digestive, and genito-urinary tracts, and the acini and ducts of all secreting and excreting glands. They perform various functions, in some cases forming a protective layer and in others acting as a source of secretion and excretion. The secretory functions in the gastro-intestinal besides gallbladder, and respiratory systems are performed by goblet, serous and *mucous* cells. The involvement of different types of cells in the pulmonary secretion has been shown by the Lille group[1] with the use of lectins. The cervical secretion being the product of columnar cells has been demonstrated by Ovadia *et al*[2]. Gall-bladder columnar cells also produce secretion[3]. The columnar-,goblet-, *mucous* and serous cell secretion commonly expressed as mucus or mucin, is a gel-like material and acts as a medium for protection, transport and lubrication. In the gastro-intestinal system a mucus layer prevents the epithelial cells from autodigestion while in the mucocilliary transport system it is made to clear the inhaled particles and contaminants. Cervical mucus protects the uterine cervix and controls the survival, nutrition and migration of the spermatozoa. The preocular liquid film, which prevents the corneal surface from drying, is stablized by the mucus. It appears that for many normal functions mucus is a pre-requisite for survival. However in many cases, such as cystic fibrosis, bronchitis and common cold, excess of it is damaging.

The various aspects of different mucuses have been described in several reviews and publications[4-9].

The active component of the epithelial secretion is generally referred to as mucus glycoproteins. Various examinations of mucuses, respiratory and cervical have shown these to be thread,or stand-like structures[10-12]. These threads have proven to be the

Essays on Science. 1986. H.M. Saeed (ed.) H.F. Press. Karachi, Pakistan.

largest ($M_r \sim 1x10^6$ x 5 x 10^6) and consist of the most complex glycoproteins. The glycoprotein macromolecule consists of a peptide core and carbohydrate side chains (Fig. 1) varying in size, sequence and anomery. The carbohydrate chains are attached to the peptide core through N-acetylgalactosamine O-glycosically to serine or threonine (Fig. 2). Carbohydrate, 70-80% by weight, occurs as neutral and acidic oligosaccharides that are assembled from various monosaccharides in linear and branched sequences. Sugars of common occurrence in mucus glycoproteins are fucose, galactose, N-acetyglucosamine, N-acetylneuraminic acid (sialic acid) and N-acetylgalactosamine. In addition to sialic acid, ester sulfate is often found.

PURIFICATION OF MUCUS GLYCOPROTEINS

Purification of glycoproteins has posed major problems to the investigators because of the size of the macromolecule as well as the inherent heterogeneity arising due to the varying size of carbohydrate chains. Mucus glycoproteins can occur in gel as well as in soluble form. Dilution with buffers or salt solutions in the case of normal secretion often results in the solubilization of gel. In the case of pulmonary pathological secretion, cystic fibrosis and bronchitic samples, reducing agents, mercaptoethanol or dithiothreitol, as well as treatment with Pronase help solubilize the gel-phase. Strong binding of glycoproteins with lipids and proteins results in further complication for the process of purification[12-15].

Purification and fractionation of glycoproteins thus involve special care and the methods used to obtain the pure material can influence the size and properties of the isolated material. Slow agitation in nonreducing environment[16,17], high speed homogenization[18-20], disulfide cleavage[21-24] and proteolytic digestion[25-27] are the commonly used procedures for purification and isolation of glycoproteins. Treatment of the mucus glycoproteins with proteolytic enzymes and disulfide bond-cleaving reagents generally result in fragments of glycoproteins that are large in size.. Such fragments are less relevant for studies on the polymeric structure of the glycoprotein but provide a useful source to isolate oligosaccharides and characterize the chemical structure of the carbohydrate moiety of the glycoproteins.

The microheterogeneity of carbohydrate chains arising due to differences in chain lengths and branching, a dominant feature in mucus glycoproteins, results in variations in molecular size. The presence of sulfate groups and sialic acid in the carbohydrate chains of the glycoprotein endows the polymer with difference of charge. Combination of gel filtration and ion-exchange chromatography in appropriate conditions remains the commonly used procedure of purification. In addition to gel- and ion-exchange chromatography isopyrine density gradient centrifugation is a useful method to remove adhering lipids, proteins and other contaminating or binding molecules[18].

The purity and homogeneity of mucus glycoproteins is usually assessed by demonstrating the *absence* of contaminating macromolecules. A glycoprotein preparation is rarely monodisperse i.e. all molecules do not exhibit identical properties of charge and size. Gradient gel electrophoresis in the presence of a denaturing agent is a method that convenienty displays the purity or the presence of contaminating molecules, such as lipids, proteins and nucleic acids. Isopyrine density gradient centrifugation in caesium chloride or bromide was applied to mucus-like glycoproteins by a number of investigators to remove adhering proteins, lipids[28], bilirubin[18] and other contaminating materials [14, 29-31].

The viscous properties of the mucus glycoproteins which are essential for its function are related to its chemical composition and structure. The mucus displays rheological properties[32,33]; it is viscous but its viscosity varies with the shear rate applied, which is characteristic of a non-newtonian fluid resembling a pseudoplastic material. Mucus also exhibits elastic properties, being able to store energy and to dissipate this energy during flow [39].

Because of its high viscosity mucus exhibits stickiness, and the procedures applied to study the physical properties of these molecules are hampered. Gel filtration, that offers information on the size and polydispersity of the glycoprotein, is performed with difficulty of retardation of and sometimes complete blockage of the gel. Extreme care in the concentration of the solution is required. Often 1-3x 10^{-4} g per ml of the glycoprotein in an appropriate buffer on lg of gel (dry weight) can provide useful information of the polymer in non-reducing and dissociating conditions.

The intrinsic viscosity ($[\eta]$) obtained when extrapolating the

reduced viscosity $[(\eta-1)/C]_{ref.}$ to zero concentration provide information on the size of the polymer in solution. For large assymmetric glycoproteins the data must be extrapolated to zero shear rate.

Sedimentation velocity analyses allow the assessment of polydispersity and heterogeneity. The sedimentation constant should be extrapolated to zero concentration (or the concentration at which single measurements are performed) and corrected to standard conditions (water 20°C). Along with the diffusion coefficient (Svedberg equation) or the intrinsic viscosity equilibrium (Scheraga Mandelkern equation), an estimate of M_r can be calculated. The sedimentation equilibrium (s) study leads to the direct determination of M_r, but data obtained with this method for large molecules, such as glycoproteins, must be interpreted with the view that the polydispersity is commonly prevalent in these molecules. Special care must be exercised when applying the meniscus depletion procedure [35], the limiting value near the meniscus will reflect only the smallest molecules of a polydisperse distribution, and the existence of larger components can be obscured. The value of the partial specific volume ($\bar{v}$) is required; the appropriate conditions for measuring this parameter have been described [23,36].

Light scattering carried out as total intensity measurements yields M_r and the radius of gyration (R_G) whereas quasi-elastic light scattering, by photon correlation spectroscopy, is used to obtain the translational diffusion coefficient (D_T). Both procedures are very sensitive and require very low concentration, in the range of 50 to 100 ug per ml of mucus glycoproteins. Obviously there is an advantage in light scattering over sedimentation analysis where often a higher concentration is required, and often difficulty may be encountered to dissolve one mg/ml of the glycoprotein. Light scattering offers a major tool to study the intact glycoproteins.

MUCUS GEL

The major component of mucus is water, > 90%, and glycoproteins only represent between 0.5 to 5%. In addition to water and glycoproteins, proteins, lipids, nucleic acid and inorganic salts are found. In cervical mucus glycoproteins vary between 0.7 to 2.8% (Table 1) during the menstrual cycle, and bronchial glycoproteins (Table 2) also vary depending upon the source or pathology.

The formation of hydrated, cohesive, gel structures implies a fine balance between polymer-polymer and polymer-solvent interactions. Application of an external force will first deform the mucus, and thereafter will exhibit elastic properties. The tendency of mucus to regain its original form is an elastic property. These two behaviors endow mucus with the characteristics of a viscoelastic gel. Gels with rheological properties similar to those of mucus can be reconstituted with purified glycoproteins without the addition of the proteins removed during purification. Furthermore, anti-cervical mucus glycoproteins antibody has been used to change properties[12] and functions of the cervical mucus[37] It is thus very likely that glycoproteins alone are responsible for the properties and functions of mucus[12,37-39]. Physical interactions may be considered an important factor in the formation of gel as the solvent domain of the individual glycoprotein will be at a concentration much lower than that found in mucus.

CARBOHYDRATE CHAINS

1. *Composition and Structure*

The complete structural analysis of the complex carbohydrate moiety typically requires liberation of the carbohydrate moiety from the protein core and determination of the following structural aspects of the carbohydrate: 1) carbohydrate composition; 2) configuration of each of the sugar residues; 3) conformation of the ring; 4) the sequence of the sugars; 5) the position of glycosidic linkages between the sugars; 6) the anomeric configuration of the intersugar glycosidic linkage.

Of the abundantly common sugar residues only five are known in the mucus glycoproteins, but these five sugars provide a complex and diverse structural assembly. The structural variations are known to occur in composition, branching, substitution (presence of sulfate ester groups) and acidity.

The oligosaccharides are released from the protein core mainly by alkaline borohydride cleavage[40,41] and the oligosaccharide alditols separated into neutral and acidic fractions. Each of the fractions, neutral and acidic oligosaccharides, is separated into discrete populations. The usual scheme of separation and fractionation included gel filtration and ionexchange chromatography. The advent of high performance chromatography

and its successful application to carbohydrate chemistry[42, 43] has proved to be very helpful in resolving a variety of different and complex oligosaccharides [17,44].

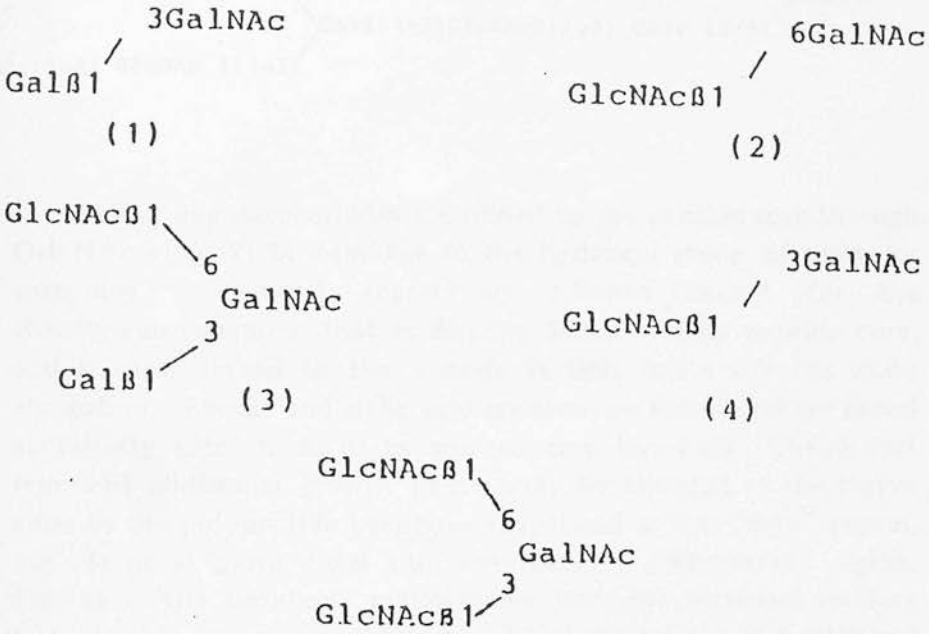
Methylation analysis is the traditional procedure applied to establish the position of the glycosidic linkages and the ring size of sugar substitutents [45-49]. The permethylated polymer is acid hydrolyzed[50,51], yielding a mixture of partially methylated fragments. In order to avoid ambiguities arising due to the mixture of anomers, the methylated sugars are reduced with sodium borohydride to methylated alditols. The partially methylated alditols are acetylated with acetic anhydride, and these products are readily analyzed by gas-chromatography-mass spectrometry (Gc-Ms). For example the electron mass spectrum of the partially methylated alditol acetates (Fig. 3) obtained from the fucose at the terminal non-reducing end of the trisaccharide would be expected to have fragment ions m/z 175, 161, 131 and 117 because fission occurs predominantly between carbon atoms that are methoxylated. The N-acetylglucosamine residue yields a product (Fig. 3-b) that can be assumed to be derived from the 3-substituted pyranose form of N-acetylglucosamine by the characteristic fragment ions at m/z 45, 161 and 274 which arise by fission between carbon atoms containing one methoxyl group and one acetoxyl group with charge retention on the fragment with the methoxyl group [45]. Mass spectra of products of N-acetylhexosamines have a characteristic ion at m/z 158, derived from C-1 and C-2 of the partially methylated alditol acetate, and a companion at m/z 116, arising from m/z 158 by the elimination of ketene [50,52]. The determination of the reducing terminal or C-1 of the reducing terminals is usually apparent. Ambiguities can be readily resolved by reduction of the methylated monosaccharide with sodium borodeuteride, which places a deuterium atom on C-1 and increases the m/z values of fragment ions containing C-1 by one mass unit (Fig. 3-C).

The sequence or arrangement of the sugar residues in an oligosaccharide alditol is best studied by combining the results of enzymic degradation using specific glycosidases and smiths degradation [48,49,53-57]. The anomeric configuration of the inter-glycosidic linkage is best arrived at by using anomery-specific glycosidases [49,55-57].

Due to the complexities of the glycan chains, and because of the scant availability of the purified materials and also the

involvement of cumbersome procedures of purification and structural investigations the progress was slow. With the availability of HPLC and 500-MHz ^{1}H-NMR spectroscopy[17,44] GC-MS spectrometry as well as of carbohydrate-binding monoclonal antibodies[58-59], steady and rapid progress is stipulated.

The structures thus far established suggest that there exist at least three groups of common core structures i.e. the sugar linkages in the vicinity of the N-acetylgalactosamine residue that is linked to serine or threonine (structures 1 to 3). These types of core have been found in mucus glycoproteins[17,44].



The structure types 4 and 5 are less common[59], they may coexist in mucous glycoproteins[34].

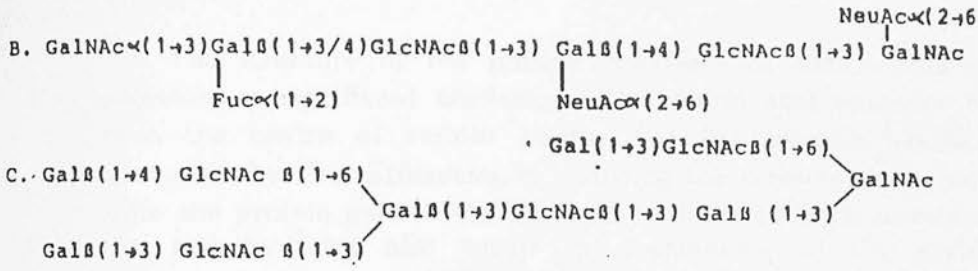
2. *Biosynthesis*

Comprehensive reviews dealing with the biosynthetic pathways of the carbohydrates have been published [60-62]. The carbohydrate chains, oligosaccharides, are synthesized by concerted action of specific glycosyl transferases, and the biosynthetic process appears to proceed in a well-defined order.

Orderly pattern of biosynthesis has been suggested by the restricted structural variability. Structures A-C are commonly

found in mucin-type sugar sequences and are used to explain those few principles that can be discerned.

A. NeuAc ∝ (2→6)GalNAc



Mucin oligosaccharidde are joined to the peptide core through Gal NAc (Fig. 2) in α-linkage to the hydroxyl group of serine or theronine. Internal sugars are β-linked except for the N-acetylgalactosamine that is directly linked to the peptide core, and a sugar linked in the α-mode is thus not a site for chain elongation. Fucose and sialic acid are always α linked and are found exclusively (Str, A & B) as non-reducing terminals. Chains that represent additional growth points may be initiated in the region close to the polypeptide backbone (expressed as the "core" region, Fig. 4) or at more distal sites (expressed as "backbone" region, Fig. 4) The backbone region stems from the galactose residues whereas the core region originates at N-acetylgalactosamine (linked to peptide, Str. C, ref. 59). Sugars that form these branched structures (Gal and Glc NAc) are always joined to C-3 and C-6 hydroxyls of the initiating sugars. Sugars in β(1→6) linkage occur only at branch points. Two such β (1→6) linkages occur in the doubly-branched structure (C), whereas in the linear portion of the sequence (B) the sugars are always in β(1→3) or β(1→4) linkage. The synthesis of oligosaccharide chains will involve the formation of core structures, backbone and peripheral regions as well as terminal sugars. In addition, quite a few carbohydrate chains contain sulfate groups. Sulfate groups have been identified on galactose, N-acetylglucosamine and N-acetylgalactosamine residues. The biosynthetic pathways of oligosaccharide chains have been worked

out with better success than the structural aspect of the oligosaccharides. The progress has been rapid and the understanding of the complicated structures based on the synthetic pathways is begining to evolve.

PROTEIN MOIETY

The structure of the protein backbone of the mucins has presented a significant challenge. The amino acid sequence has been the centre of various studies but the progress has been hampered by the difficulties in removing the carbohydrate chains from the protein core. The chemical methods available to remove the carbohydrates also result in degradation of the protein moiety. The endo-N-acetylgalactosaminidase prepared by Bhavanandan[63] is active to a limited degree in removing the shorter chains but the longer chains are resistant to the enzyme. Currently no enzymic procedure is available that can remove carbohydrate chains.

Also little is known about the biosynthesis of the core protein in the mucins although the process would appear to conform to that of traditional proteins.

MUCUS GLYCOPROTEINS IN MALIGNANCY

In many gastric carcinomas the expression of antigenic determinants in mucus glycoproteins vary from that anticipated from the ABO genotype. For example, blood group A patients may secrete blood group B-active mucins, possibly due to the alteration of the specificity of the A-forming enzymes. Human colon carcinomas may secrete blood group-active mucins, whereas the normal human colon produces mucin that is devoid of determinants[59]. Peanut lectin reactivity is specific to transformed mucus cells of rat intestinal tumours[64]. This lectin reacts only with the unsubstituted Gal-GalNAc sequence that is found in the core region of the oligosaccharide. Also there is a suggestion that a decrease in sulfation of colonic and other carcinomas occurs during malignancy[65]

CONCLUSIONS

The study of mucus glycoproteins impinge on many areas of

biochemistry and cell biology, and will continue to pose challenges of fundamental significance in relation to protein moiety and diversity of carbohydrate moiety. Mucins help to perform numerous normal functions, and mucus is also a major source of distress in diseases of the respiratory and gastrointestinal tracts and may influence clinical conditions in infertility. Mucus glycoproteins will continue to test our skills, patience and ingenuity for many years to come, for these polymers are so well endowed with the possibility to alter their behaviour in various biological environments.

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(Article continued...)

TABLE-1

CHEMICAL COMPOSITION OF HUMAN CERVICAL MUCUS

Constituents[a]	Type of Mucus		Pooled Specimen
	Mid-Cycle	Luteal Phase	
Water	98.0	92.0-95.0	—
Inorganic salts	c. 1.0	c. 1.0	—
NaC	0.9	0.9	—
Others (K, Mg, Ca, Cu Po_4, SO_4, $HCO)_3$	0.1	0.1	—
Total proteins including glycosylated proteins	c. 1.0	c. 4.0-7.0	—
Glycoproteins[b]	0.7	1.6- 2.8	—
	70.0	40.0	—
Globulins[b]	0.2	1.0- 1.7	—
	22.0	8.0	—
Albumins[b]	0.1	1.5- 2.5	—
	8.0	35.0	—
Total lipids[c]	2.0	5.0	—
Hydrocarbons[d]	—	—	7.4
Cholesterol Esters[d]	—	—	12.5
Cholesterol[d]	—	—	19.5
Triglycerides[d]	-	—	21.8
Diglycerides[d]	-	—	6.3
Monoglycerides[d]	—	—	5.0
Free Fatty acids	—	—	10.5
Phospholipids[d]	-	—	17.0

[a]Gram per 100 gram.

[b]Percent of total proteins(TP)

[c]Milligram per 100 milligram.

[d]Percent of total lipids (TL)

TABLE 2

CHEMICAL COMPOSITION OF PULMONARY SECRETION (7).

Constituents[a]	Cystic fibrosis	Bronchiectasis	Laryngectomized patients
Water	89.36	94.79	94.79
ASh	0.73	0.88	1.13
DNA	0.408	0.084	0.028
Carbohydrate	1.137	1.050	0.951
Protein	5.570	2.040	1.000
Lipid	3.140	1.170	0.840

[a]Gram per 100 gram wet weight.

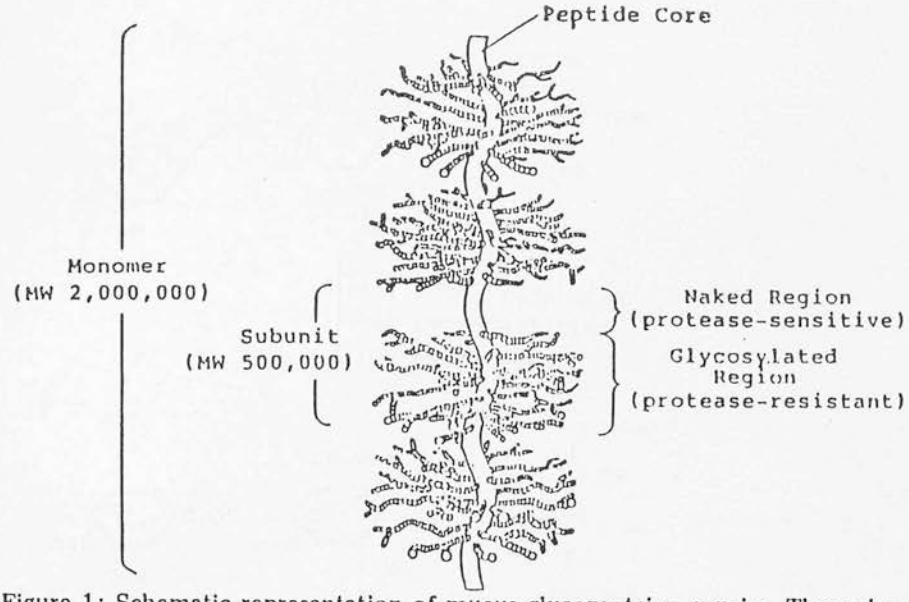

Figure 1: Schematic representation of mucus glycoproteins, mucins. The carbohydrates are radially arranged on a peptide core. The protease-sensitive region is capable of binding lipophilic compounds or bilirubin (18).

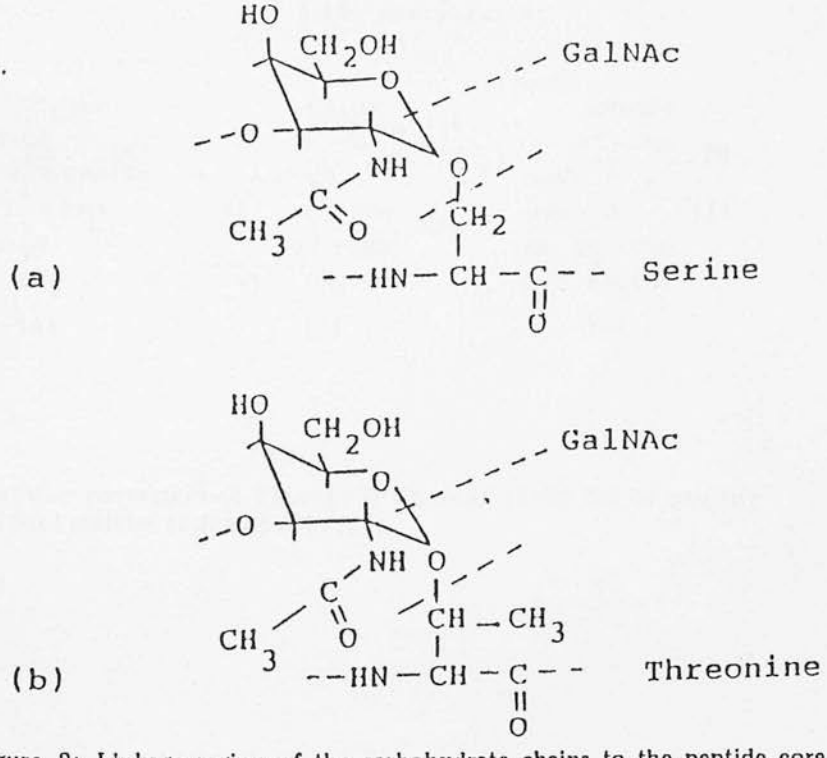

Figure 2: Linkage region of the carbohydrate chains to the peptide core. N-Acetylgalactosamine is the sugar residue which is linked to serine (a) or threonine (b) in the mucus glycoprotein.

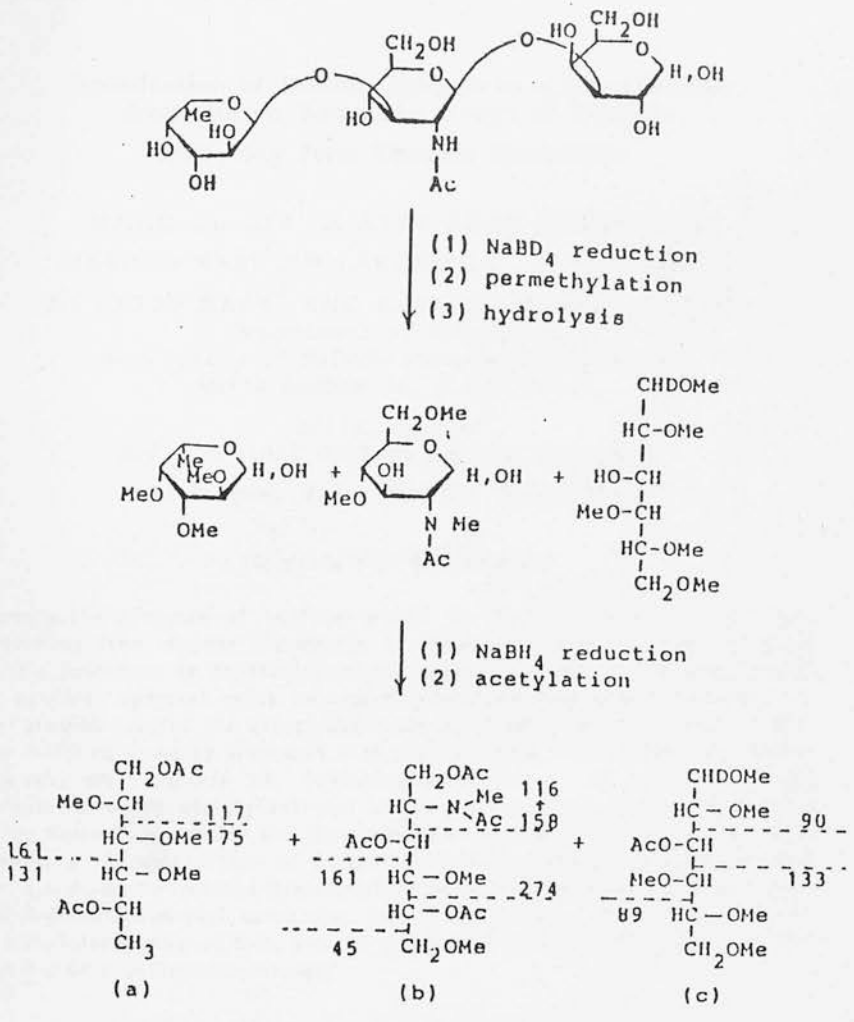

Figure 3: Stepwise conversion of Fucα(1→3) Glc NAc (1→3) Gal to partially methylated alditol acetates as described in (48).

J.Chem.Soc.Pak. Vol. 8, No. 2, 1986.

Localization of Sulfate Groups in a Glycoprotein from Human Bronchial Mucus of Patients Suffering from Chronic Bronchitis*

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(Received 10th October, 1984)

Summary: The presence of sulfate groups in the glycoproteins of patients suffering from chronic bronchitis is known. The exact location of these acidic functions on particular sugar residues as well as the substitution of specific hydroxyl group on a particular sugar have been obliterated. In the present studies the glycoprotein was purified by gel filtration on Bio-Gel P-200 followed by treatment with insolubilized Pronase and then chromatography on Sepharose 2B. Following purification, the glycoprotein was methylated using dimethylsulfinyl carbanoin and methyl iodide, desulfated using Kantor's procedure and then remethylated with carbanion and deuteriodomethane. Identification of deuteriomethylated sugars, 3-O-deuteriomethyl and 3,6-di-deuteriomethyl-N-acetylglucosamine, 3-O-deuteriomethyl galactose and 6-O-deuteriomethyl galactose, showed the presence of sulfate groups on N-acetylglucosamine at O-3, and O-3 and O-6 of N-acetylglucosamine, and O-3 and O-6 of D-galactose residues.

Introduction

The tracheobronchial secretion is an important constituent of the mucusilliary system which provides airways with necessary protection by removing invasive materials that are inhaled. The mucus exhibits rheological behaviour which is important for its effective functioning. The rheological properties to the mucus are necessarily imparted by the mucin glycoprotein, and the viscous characteristics of the glycoprotein are largely due to carbohydrate components. Under the pathological conditions characterized by bronchial hypersecretion like chronic bronchitis, the functioning of mucocilliary system is disturbed, resulting in bronchial blockage of airways that may lead to severity of the disease.

Important constituents of glycoprotein are neuraminic acid and sugars with sulfate groups. Neuraminic acid

and sulfate containing sugars impart electronegative environment in the macromolecule. It has been proposed that several glycoproteins, serum as well as secretary type, lose their resistance towards proteinases after treatment with neuraminidase [1-4]. It would appear from this behaviour of glycoproteins that acid functions in the macromolecule imparts resistance to proteolytic degradation that may directly or indirectly effect the efficiency of mucocilliary system. In order to assess whether these phenomena are related to the presence and alteration of the structure of the sulfate containing carbohydrates in the mucin glycoprotein, it was considered desirable to investigate the exact sugar residue on which the sulfate group is located in addition to the precise position of sulfate substitution.

Material and Methods

Analytical methods

The hexose content was estimated by the phenol sulfuric acid method [5], the protein content by measuring the absorbance at 280 nm or by using the procedure of Lowry et al. [6], and the sialic acid content by the thiobarbituric acid method of Warren [7], after acid hydrolysis with 50 mM sulfuric acid, or by gas liquid chromatography. All solutions were dialyzed in hydrogencarbonate treated cellulose tubing at 4°C.

Gas-Liquid Chromatography

Gas liquid chromatography (GLC) determinations of carbohydrate residues of glycoprotein were performed according to the procedure of Reinhold [8]. Gas-liquid chromatography-mass spectrometry (GLC-MS) of the methylated sugars was performed on the alditol derivatives prepared as described previously [9].

Column Chromatography

Bio-Gel P-200 (Bio-Rad Laboratories) and Sepharose 2B (Pharmacia Fine Chemicals) were run in 50 mM sodium phosphate (pH 7.0), and 5 mM tris-HCl (pH 7.5) respectively.

Gel electrophoresis

Agarose-gel electrophoresis was performed in 50 mM barbital buffer (pH 8.2) on slides containing 1% agarose. The agarose slides were stained with Coomassie blue, and with periodate-Schiff reagent. For agarose electrophoresis, approximately 0.4 mg per ml of the material was used, and each well had 15 µl of the solution.

Purification of the mucus glycoproteins

The crude mucus (100 mg) was partially solubilized in sodium monophosphate (25 ml) containing 0.02% sodium azide by stirring for 16 hours. The cellular debris and other suspended materials were removed by centrifugation (2500 rev./min), the supernatant was dialyzed, and the dialyzable material was lyophilized to give the crude mucus glycoprotein. This material (65 mg) was solubilized in 50 mM sodium monophosphate (10 ml, pH 6.8) containing 0.02% sodium azide by stirring overnight at 4°C. The solution was applied to a column (2.2 x 60 cm) of Bio-Gel P-200 (50-100 mesh). The carbohydrate and protein containing fractions were pooled, the pH was adjusted to 5.0 with 4 M acetic acid and the solution was extensively dialyzed and then lyophilized to give the partially purified glycoprotein. The partially purified (45 mg) glycoprotein in 6 ml of phos-

phate buffer was applied to a column (1.5 x 60 cm) of sepharose 2B. The column was washed with phosphate buffer, and fractions containing carbohydrate and protein were combined, dialyzed, and lyophilized to give purified glycoprotein.

Enzyme degradation

Pronase treatment of purified glycoprotein (30 mg) was performed in 50 mM sodium monophosphate buffer (10 ml. pH 8.0) containing 0.1% sodium azide with unsolubilized Pronase (10 mg, Enzite protease, Miles Laboratories, Inc.). The mixture was stirred at 22°C until the glycoprotein was dissolved (3 hours), and then incubated for six days at 37°C with stirring. A further addition of prewashed enzyme (2 mg) was made and the solution incubated for 2 days. The suspension was centrifuged, and the residue was washed with the buffer. The pH of the supernatant solution was adjusted to 5.0 with acetic acid and the solution dialyzed against distilled water. The nondialyzable material was lyophilized and the residue (22 mg) was applied to a column (1.5 x 48 cm) of sepharose 2B. Fractions containing protein and carbohydrate were combined and dialyzed and the retentate was freeze dried to give the pronase-treated glycoprotein.

Methylation analysis

After chromatography on sepharose 2B the Pronase-degraded glycoprotein (20 mg) was treated in freshly distilled formamide with acetic anhydride for 24 hours, thereafter dialyzed, and nondialyzable solution lyophilized. The Pronase-resistant, acetylated glycoprotein was dissolved in dimethyl sulfoxide (2 ml) and methylated with iodomethane in the presence of methylsulfinyl carbanion [10]. The methylated product was isolated by dialysis and freezedrying. The residue (16 mg) was methylated again as just described to give the methylated glycoprotein (12 mg).

A solution of the methylated glycoprotein (2 mg) in 0.5 M pyridine acetic acid (pH 0.5,5.4 ml) was applied to a column of Bio-Gel P-200. The column was washed with 0.5 M pyridine-acetic acid (pH 5.4, 50 ml). The carbohydrate containing fractions were combined and lyophilized. A portion of the residue (4 mg) in 2 M trifluoroacetic acid I (1 ml) was heated at 110° for 3 hours, diluted with water and lyophilized. A solution of the residue in 20% aqueous methanol was passed through a column of AG1-X8 (CH_3COO^-). The effluent was evaporated, and the residue containing sugars was converted into alditol acetates [9]. The alditol acetates were examined by GLC-MS.

Desulfation of the methylated glycoprotein and remethylation.

The methylated glycoprotein (8 mg) was dissolved in 60mM methanolic hydrogen chloride (1 ml) and the solution was allowed to stir for 8 hours at 22°C [11]. The solution was diluted with methyl alcohol (4 ml) and toluene (2 ml), and evaporated under reduced pressure. The residue in aqueous methyl alcohol (2 ml; 50 %) was dialyzed against distilled water. The nondiffusible material was lyophilized to give desulfated methylated glycoprotein. The desulfated polymer was remethylated using carbanion and iodomethane-d3 (+ 99 atom % from Aldrich chemical Co. In., Milwaukee, Wisconsin 532233). The methylated polymer was depolymerized with 2M trifluoroacetic acid, the

resulting methylated sugars reduced with $NaBH_4$ and acetylated with acetic anhydride in pyridine. The alditol acetates were identified by GLC-MS.

Results

Purification and Enzyme Degradation of the Glycoprotein

The mucus glycoproteins were eluted in the void volume of the Bio-Gel P-200. The Bio-Gel P-200 purified mucus afforded a single major glycoprotein component on Sepharose 2B chromatography. The carbohydrate component of the glycoprotein contained fucose, galactose, N-acetylglucosamine, N-acetylgalactosamine and N-acetylneuraminic acid. The glycoprotein component showed, in agarose electrophoresis, the presence of a single periodate-Schiff and Coomassie blue-staining material. The glycoprotein, in agarose, did enter the gel and showed a diffused band, however. The Sepharose 2B purified material after degradation with Pronase afforded a high molecular weight, nondiffusable in dialysis, glycoprotein.

Purification and physical and chemical characterization of Pronase-treated glycoprotein

The nondiffusible portion of the digest obtained by Pronase gave a single component on purification by chromatography on a column of Sepharose 2B. In agarose electrophoresis the degraded glycoprotein did enter the gel and no additional protein component was detected with Coomassie blue. The carbohydrate moiety of the Pronase-treated glycoprotein contained L-fucose, D-galactose, N-acetylglucosamine, N-acetylgalactosamine and neuraminic acid residues with some increase in sialic acid (Table II). The amino acid composition of the degraded glycoprotein was similar to that of the Sepharose 2B purified or native glycoprotein, except for significant variations in the relative ratios of the amino acids (Table II).

Methylation and desulfation of methylated glycoprotein

Methylation of the Pronase-treated glycoprotein gave a methylated derivative that eluted as a single component from Bio-Gel P-200 chromatography. Hydrolysis, followed by identification of the products showed the presence of terminal fucopyranosyl residues; of terminal, 6-linked, 3-linked, 2-linked, 6 and 3-linked, 2 and 3-linked and 2 and 6-linked galactose residues; of 4-linked, 6-linked and 4 and 6-linked 2-acetamido-2-deoxy-D-glucose residues, and of terminal, 6-linked and 3- and 6-linked 2-acetamido-2-deoxy-D-galactopyranose residues. Mild acid treatment of the methylated glycoprotein with 60 mM methanolic hydrogen chloride for 8 hours at 22° removed nearly 80% of the sulfate groups.

Remethylation of desulfated permethylated glycoprotein

Subsequent trideuteriomethylation of the desulfated polymer introduced trideuteriomethoxyl group at C-5 of the galactose residues linked at O-2 and O-6 and C-6 of the terminal galactose and C-3 of N-acetylglucosamine residues linked at O-4 (Table III).

Discussion

In the present investigation mucus was collected from several patients of similar blood type suffering from

Table-1: Carbohydrate and amino acid composition of Sepharose 2B purified bronchial glycoprotein

Carbohydrate[a]	Percent	Molar ratio
L-Fucose	7.6	0.43
D-Galactose	24.3	1.25
N-Acetylglucosamine	23.8	1.00
N-Acetylgalactosamine	16.9	0.71
N-Acetylneuraminic acid	4.8	0.14
Sulfate	1.9	

Amino acids[a.c]	
Alanine	90
Valine	42
Glycine	88
Isoleucine	11
Leucine	28
Proline	78
Threonine	298
Serine	178
Phenylalanine	14
Aspartic acid	28
Glutamic acid	120
Lysine	25

[a] Molar ratio relative to N-acetylglucosamine

[b] Determined by gas-liquid chromatography

[c] Residues per 1000 residues.

Table-2: Carbohydrate and amino acid composition of Sepharose 2B purified pronase-treated bronchial glycoprotein

Carbohydrate[a]	Percent	Molar ratio
L-Fucose	8.2	0.48
D-Galactose	26.2	1.40
N-Acetylglucosamine	22.9	1.00
N-Acetylgalactosamine	15.8	0.68
N-Acetylneuraminic acid	6.8	0.21
Sulfate	1.8	

Amino acids[a.c]	
Alanine	80
Valine	38
Glycine	101
Isoleucine	18
Leucine	22
Proline	58
Threonine	306
Serine	192
Phenylalanine	31
Aspartic acid	46
Glutamic acid	94
Lysine	14

[a] Molar ratio relative to N-acetylglucosamine.

[b] Determined by gas-liquid chromatography.

[c] Residues per 1000 residues.

chronic bronchitis. The chromatographic, gel filtration, and electrophoretic behaviour of the purified glycoprotein indicated that it represent a high molecular weight glycoprotein. Further purification of this glycoprotein on charge basis was considered unnecessary due to the known microheterogeneity of glycoprotein [14], to avoid heavy losses of the glycoprotein during ion-exchange purification and also because the glycoprotein was mainly investigated for the specific positions of sulfate groups in the sugar residues. The bronchial mucus glycoproteins are known to be contaminated with proteins, nucleic acids and lipids. The glycoprotein had a minimal entry in the gel in electrophoresis but showed the absence of low molecular weight contaminating proteins and glycoprotein, an observation similar to that of Feldhoff et al. [13]. The Pronase-treated glycoprotein in gel filtration and in electrophoresis indicated the presence of a homogenous, high molecular weight component. There was no significant change in the molecular size between the enzyme-treated and native glycoprotein as indicated by gel filtration. The recovery after protease treatment (approximately 75%) indicated that the enzyme degraded the polymer to a limited extent (25%), suggesting that some portions are readily accessible, whereas the others are well protected by carbohydrate chains. It is also possible that proteins that existed in association with the glycoprotein were degraded by the enzyme. It appears that minimal degradation of glycoprotein containing sulfate groups occured as shown by minor variation in sulfate composition between the native and enzyme-treated glycoprotein (Table I and II). The carbohydrate component of the Pronase-treated glycoprotein contained all the sugars present in native glycoprotein and represented nearly 90% of the sugars in the starting material.

The Pronase treatment produced glycoprotein that appears to differ mainly in the relative proportion of sugar residues, particularly for the sialic acid and galactose residues, as well as some variations in relative proportion of amino acids. The observations may be explained by the presence of different glycoprotein molecules differing in the degree of glycosylation, a carbohydrate rich and a carbohydrate deficient region in the glycoprotein or different degree of sulfation of carbohydrate moiety in the glycoprotein, which could influence their suceptibility to proteolytic treatment. It appears that the Pronase treatment resulted in insignificant change in the glycoprotein and, as desired, mainly removed contaminating proteins, an observation similar to that observed earlier [4,18].

Methylation of the glycoprotein (twice), methoxyl analysis and chromatography of the methylated glycoprotein indicated complete methylation and minimum degradation of the O-glycosyl linkages during methylation. A similar stability of O-glycosyl linkages to Hakomori's method [10] was observed by Benziger and Kornfeld [19].

Mild acid treatment of the methylated glycoprotein and subsequent remethylation of the desulfated glycoprotein localized the position of the sulfate groups. Removal of the sulfate groups was fiarly complete with least degradation of the glycoprotein as the molar ratio of terminal fucose to terminal galactose before and after acid treatment was very similar. Furthermore, the conditions used for desulfation was too weak to cause demethylation. It, therefore, appears that

the incorporated trideuteriomethyl groups represents the location of sulfate groups on the sugar residues.

Sulfate groups on the sugar residues in the secretory glycoprotein are of common occurance. The function of these ionic groups may be in part, like that of sialic acid, be attributed to maintain the integrity of the glycoprotein, particularly from the proteolytic enzymes. Several glycoproteins are known to loose their resistance towards proteinases after treatment with neuraminidase [1-3]. The role of ionic strenght of the glycoprotein in the pathological (bronchial) conditions i.e., chronic bronchitis and cystic fibrosis, is unknown as the studies on the functions of sulfate groups as well as sialic acid residues in the normal human bronchial secretion are still not available to compare the contribution of sulfate groups to the function of glycoprotein obtained from pathological conditions.

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Carbohydrate Research, 159 (1987) 95–107
Elsevier Science Publishers B.V., Amsterdam – Printed in The Netherlands

STRUCTURE OF ACIDIC OLIGOSACCHARIDES ISOLATED FROM PRONASE-TREATED GLYCOPROTEIN OF BONNET-MONKEY (*Macaca radiata*) CERVICAL MUCUS*,†

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(Received November 27th, 1985; accepted for publication in revised form, April 1st, 1986)

ABSTRACT

The major glycoprotein component of cervical mucus of bonnet monkey was treated with Pronase, and the enzyme-resistant glycoprotein purified by gel filtration on Sepharose 4B followed by DEAE-cellulose chromatography. Alkaline-borohydride cleavage of the carbohydrate chains gave a mixture of neutral and acidic oligosaccharides. Seven acidic oligosaccharides were characterized by chemical and enzymic procedures; their proposed structures are: αNeuAc(2→3)-[βGalNAc(1→4)]βGal(1→4)GlcNAc(1→6)[αFuc(1→2)βGal(1→3)]GalNAc-ol; αFuc(1→2)βGal(1→3)/6)[αNeuAc(2→3)βGal(1→4)GlcNAc(1→3)/6)]GalNAc-ol; αGalNAc(1→3)βGal(1→3)[αNeuAc(2→3)βGal(1→4)GlcNAc(1→6)]GalNAc-ol; βGlcNAc(1→3)[αFuc(1→2)]βGal(1→3)[αNeuAc(2→6)]GalNAc-ol; βGal(1→3)-[αNeuAc(2→6)]GalNAc-ol; αNeuAc(2→6)GalNAc-ol; and βGal3SO_3(1→4)GlcNAc(1→6)[αFuc(1→2)βGal(1→3)]GalNAc-ol.

INTRODUCTION

Cervical mucus is an exceedingly complex mixture of epithelial secretions, the principal constituents of which are mucin-type, carbohydrate-rich glycoproteins that share the chemical and physical properties of other epithelial secretions. The biochemical and biophysical changes in cervical mucus during the menstrual cycle influence the survival, nutrition, and passage of sperm. The cyclic alteration of the physical properties of the mucus are accompanied by variations in the carbohydrate composition[2,3] and also the chemical structure, specifically the linkage of *N*-acetylneuraminic acid, galactose, and 2-acetamido-2-deoxygalactose residues[4]. The mechanism whereby mucus secretion and glycoproteins participate in the process of reproduction is unknown. The role of glycoproteins could be assessed, because a major and a minor glycoprotein have been isolated from estrogen-stimulated cervical secretions, and antibody to the main glycoprotein[5] was raised to define the

*Dedicated to Roger W. Jeanloz.
†For a preliminary report, see ref. 1.

0008-6215/87/$ 03.50

function of this glycoprotein[6]. In addition, salient features of the chemical structure were identified[4,7,8] that related biochemical structure to physiological function. The current study describes the structural features of the acidic oligosaccharide moiety of the main glycoprotein of the periovulatory phase cervical mucus, as purified by Pronase treatment.

EXPERIMENTAL

Collection of cervical mucus. — The cervical secretion of the bonnet monkey was collected by aspiration with a suction pump at the time of midcycle. The mucus was frozen and so maintained prior to use.

Analytical methods. — The hexose content of the glycoprotein was estimated by the phenol–sulfuric acid method[9]. Protein was assayed by absorbance at 280 nm. The neuraminic acid content was determined either by the thiobarbituric acid procedure of Warren[10] (after acid hydrolysis with 50mM sulfuric acid), or by g.l.c.

Gas–liquid chromatography. — G.l.c. determinations of the carbohydrate moiety of the glycoproteins were performed according to the procedure of Reinhold[11]. G.l.c.–m.s. of the methylated sugars was performed with a Varian MAT 731 instrument fitted with a combined c.i., e.i., and f.d. ion-source.

Column chromatography. — Columns of Bio-Gel P-200 (Bio-Rad Laboratories) and Sepharose 4B (Pharmacia Fine Chemicals) were eluted with 50mM sodium phosphate (pH 7.0) containing 0.2% sodium azide. The column of DEAE-cellulose (Whatman) was washed with 0.1M NaCl followed by a gradient of 0.1–1.0M NaCl containing 10mM HCl. Chromatography on Bio-Gel P-4 and Bio-Gel P-6 (200–400 mesh) was performed in 50mM pyridine–acetic acid (pH 5.4). The fractions containing sugar, detected by the phenol–sulfuric acid procedure and by counting tritium, were combined and lyophilized. Columns of DEAE-Sephadex A-50 were eluted with 50mM–0.5M phosphate buffer (pH 7.0) followed by 0.1–0.5M LiCl. Fractions containing carbohydrates were combined and desalted on a column of Bio-Gel P-2.

Gel electrophoresis. — Gel electrophoresis was performed as described previously[8].

Purification and Pronase treatment of the mucus glycoprotein. — The mucus glycoprotein was isolated as described earlier[7]. The lyophilized glycoprotein (99 mg) obtained after chromatography on Bio-Gel P-200 was treated with immobilized Pronase and purified on Sepharose 4B and DEAE-cellulose as described before[8]. A sodium chloride gradient was used to elute the glycoprotein from the DEAE-cellulose column. Carbohydrate- and protein-containing fractions were combined, dialyzed, and the retentate lyophilized to give the Pronase-treated glycoprotein (40 mg). The glycoprotein was examined by electrophoresis on agarose and polyacrylamide gels.

Sedimentation-equilibrium studies. — The sedimentation-equilibrium study was performed with a solution of Pronase-treated glycoprotein (1.5 mg) in one mL

of 6M guanidine hydrochloride (Heico)–0.05M tris(hydroxymethyl)aminomethane–HCl), pH 7.0, dialyzed for 48 h against the same buffer, with the meniscus-depletion sedimentation method of Yphantis[12] on a Model E ultracentrifuge. A value of 0.637 for the partial specific volume was used[13]. The molecular weight was calculated by extrapolation of the point-average molecular weights at infinite dilution.

Alkaline-borohydride treatment. — The Pronase-treated glycoprotein (30 mg) was treated with 2M sodium borohydride in 50mM sodium hydroxide according to the procedure of Iyer and Carlson[14]. A 0.2% solution of the glycoprotein in 2M sodium borohydride, containing 5 mCi of sodium [^{3}H]borohydride, in 50mM sodium hydroxide, was incubated for 18 h at 45°. After alkaline-borohydride treatment, the mixture was adjusted with 4M acetic acid to pH 5.4. The mixture was applied to a column (2.5 × 68 cm) of AG 50W-X8 (100–200 mesh) ion-exchange resin containing a 150-fold excess with respect to the sodium ions from NaOH and $NaBH_4$. Oligosaccharides, oligopeptides, and glycopeptides were eluted with water and 50mM acetic acid. Reduced oligosaccharides were separated into neutral and acidic oligosaccharides on a column (3.4 × 70 cm) of AG 1-X2 (OAc^-, 200–400 mesh) resin. The column was washed with water, 0.5M pyridine–acetic acid buffer (pH 5.4), and then with 0.1–1.5M acetic acid in the cold. The acidic oligosaccharides were further chromatographed on Bio-Gel P-6 (200–400 mesh) in 10mM pyridine–acetic acid (pH 5.4), with subsequent paper chromatography or paper electrophoresis. Paper chromatography was performed in solvents (*A*) 5:5:1:3 ethyl acetate–pyridine–acetic acid–water, and (*B*) 1:2:1 butanol–1-propanol–0.1M acetic acid. Paper electrophoresis was performed on Whatman No. 1 paper in 50mM pyridine–acetic acid, pH 5.8, for 2 h at 4°. A potential of 50 V per cm was applied.

Enzyme degradation. — Oligosaccharides were digested with some or all of the following enzymes: (*i*) α-L-fucosidase from beef epididymis (Sigma, 10mM sodium citrate buffer, pH 6.0 for 50 h at 37° and from emulsin (50mM sodium citrate buffer, pH 5.0, for 50 h at 37°; (*ii*) α-D-galactosidase from *Aspergillus niger* (Sigma, 50mM sodium citrate, pH 4.1, 70 h at 37°); (*iii*) β-D-galactosidase from *Escherichia coli* (Boehringer, 50mM sodium phosphate, pH 7.0, 48 h at 37°) and β-D-galactosidase from *Charonia lampas* (Miles, 50mM sodium citrate buffer, pH 4.0, 48 h at 37°); (*iv*) *N*-acetyl-β-glucosaminidase from jack-bean (Sigma, 50mM sodium citrate buffer, pH 4.5, 40 h at 37°); and (*v*) *N*-acetyl-α-galactosaminidase from *Charonia lampas* (Miles, 50mM sodium citrate–phosphate buffer, pH 4.1, 42 h at 37°).

Methylation analysis. — Oligosaccharides were methylated by the procedure of Hakomori[15], using iodomethane in the presence of methylsulfinyl carbanion. The methylated oligosaccharides were recovered by partition between chloroform and water, and depolymerized by treatment with 2M trifluoroacetic acid for 3 h at 105°. The cooled solution was diluted tenfold and freeze-dried. The residue in 4:1 water–methanol was treated with $NaBH_4$ (25 mg) for 12 h at 4° and for 4 h at 22°. The excess of $NaBH_4$ and sodium ions were removed simultaneously by treatment with AG 50 W-X8 (100–200 mesh) ion-exchange resin, and the reduced sugars were

TABLE I

O-METHYL DERIVATIVES OBTAINED BY ACID HYDROLYSIS OF OLIGOSACCHARIDES BEFORE AND AFTER ENZYME TREATMENT OR CHEMICAL TREATMENT

Oligosaccharides	*O-Methyl derivatives*															
	Fuc	*Gal*						*GlcNAc*		*GalNAc*	*GalNAc-ol*			*NeuAc*	*ThrNAc-ol*	
	2,3,4-	*2,6-,*	*4,6-,*	*2,3,6-,*	*2,4,6-,*	*3,4,6-,*	*2,3,4,6-*	*3,6-,*	*3,4,6-*	*3,4,6-*	*1,4,5-,*	*1,4,5,6-,*	*1,3,4,5-*	*4,7,8,9-*	*1,4-*	
A-3	+	+		+		+		+		+	+			+		
A-3[a]	+			+		+		+		+	+					
A-3[b]		+						+		+	+			+		
A-3[c]				+			[d]	+		+	[d]	+				
A-3[e]							+	+								
A-4	+				+	+		+			+			+		
A-4[f]					+											
A-4[g]							+	+			+					
A-4[e]							+	+							+	
A-5					+			+		+	+			+		
A-5[a]					+		+	+		+	+					
A-5[g]					+		+	+			+			+		
A-5[h]					+				+	+	+					
A-6	+	+							+		+			+		
A-6[a]	+	+							+			+				
A-6[f]					+				+		+			+		
A-7							+				+			+		
A-7[a]							+					+				
A-8													+	+		
S-1	+				+	+		+			+					
S-1[i]	+					+	+	+			+					
S-1[b,i]							[d]		+		[d]		+			

[a]Neuraminidase-treated. [b]α-L-Fucosidase- and β-D-galactosidase-treated. [c]Sequentially neuraminidase-, α-fucosidase- and β-galactosidase-treated. [d]Low proportion. [e]Periodate degraded. [f]α-L-Fucosidase-treated. [g]*N*-acetyl-α-D-galactosaminidase-treated. [h]Neuraminidase- and β-D-galactosidase-treated. [i]-Desulfated oligosaccharide.

eluted with water, methanol, and methanolic NH_3. The combined washings were evaporated, and the residue in methanol was repeatedly evaporated to remove boric acid. Finally, the residue was acetylated with pyridine (0.5 mL) and acetic anhydride (0.4 mL) for 12 h at 22° and the methylated alditols were examined by g.l.c. and g.l.c.–m.s. (see Table I).

Periodate oxidation–sodium borohydride reduction. — Reduced oligosaccharides (1–3 mg) were oxidized with 0.1M periodate for 12 h at 4° and then for 8 h at room temperature. The reaction was processed conventionally and the products treated with $NaBH_4$ (25 mg/mg oligosaccharide for 12 h at 4° followed by 5 mg/mg for 6 h at temperature). Sodium ions were removed with AG 50W-X8 (100–200 mesh) ion-exchange resin and boric acid by evaporation with methanol. The residue was treated with 0.25M H_2SO_4 for 2.5 h and deionized with AG 1-X8 (OAc^-, 100–200 mesh) resin. After methanolysis with 0.5M methanolic hydrogen chloride for 20 h at 80°, the products were examined by g.l.c. in comparison with reference standards. Samples of 2-acetamido-2-deoxythreitol, 2-acetamido-2-deoxyarabinitol, and *N*-acetylserinol were obtained by periodate oxidation (10mM) of 2-acetamido-2-deoxy-*O*-galactitol for 30 min at 22°.

Removal of sialic acid. — Sialic acid was removed from oligosaccharides (2–3 mg) by treatment with neuraminidase (*Vibrio cholerae*, 100–200 μL; 50–100 units, Behring Diagnostics) in 0.1% $CaCl_2$ and 0.5% NaCl, pH 5.5, for 24 h at 37°. The reaction was terminated by immersing the vessel in a boiling-water bath for 3 min. The mixture was chromatographed and the eluates containing oligosaccharides were combined and lyophilized. Neuraminidase-treated oligosaccharides were methylated, and the methylated sugars were identified as already described.

RESULTS

Purification and characterization of Pronase-degraded cervical mucus glycoprotein. — The mucus glycoproteins obtained from bonnet monkeys at the periovulatory phase of the menstrual cycle were purified by gel filtration on Bio-Gel P-200 followed by treatment with Pronase. Pronase-treated glycoproteins were fractionated on Sepharose 4B. The main fraction (80%) showed in DEAE-cellulose chromatography the presence of a single glycoprotein (Fig. 1). The glycoprotein, in polyacrylamide gel electrophoresis, did not enter the gel, and no contaminating proteins or glycoproteins were observed. In agarose (1%), the glycoprotein entered the gel and exhibited a single component. The purity of the glycoprotein was further assessed by sedimentation equilibrium; a molecular mass of 1×10^5 was calculated. The carbohydrate and amino acid composition, and the sulfate content of the glycoprotein are given in Table II.

Preparation of acidic oligosaccharide-alditols. — The glycoprotein was subjected to reductive cleavage by alkaline borohydride yielding a mixture of oligosaccharide-alditols. A decrease of serine and threonine and a corresponding increase of alanine and the appearance of 2-aminobutanoic acid were detected in

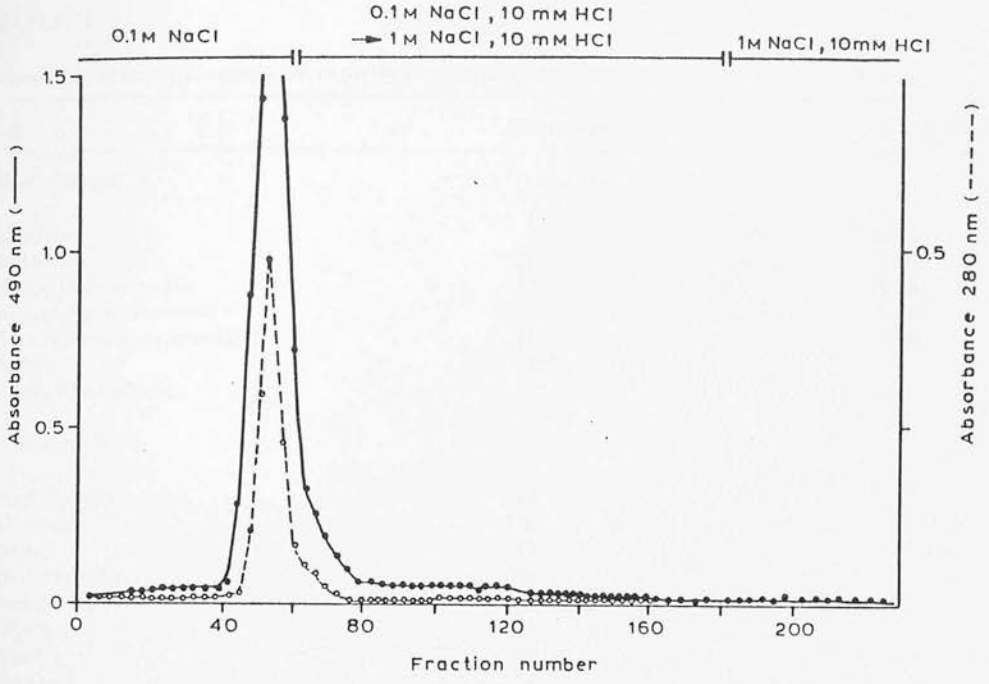

Fig. 1. DEAE-cellulose chromatography of Pronase-treated major glycoprotein fraction from Sepharose 4B. Fractions of 3 mL were collected and every third fraction was examined for hexoses by phenol–sulfuric acid and for amino acids by absorbance at 280 nm.

hydrolyzates of the protein. The acidic oligosaccharides eluted from the column of AG 1-X2 were separated on a column of Bio-Gel P-6 into seven fractions (Table III, Fig. 2). The acetic acid (0.1–0.5M) washing from the column of AG 1-X2 afforded an acidic oligosaccharide (S-1) that was purified on a cellulose plate (Table III). Fractions A-1 and A-2 constituted mainly glycopeptides, as indicated by the low percentage of 2-acetamido-2-deoxygalactitol and the presence of hydroxylated amino acids.

Desulfation of oligosaccharides. — The oligosaccharides (0.3–0.7 mg) were desulfated according to the procedure of Kantor and Schubert[16].

Oligosaccharide fraction A-3. — This fraction was further purified by chromatography on DEAE-Sephadex A-50 and was homogeneous in paper chromatography in solvents *A* and *B*. Its sequential treatment with neuraminidase, α-L-fucosidase, and with β-D-galactosidase removed residues of fucose, galactose, and *N*-acetylneuraminic acid. Methylation of the residual oligosaccharide showed the presence of terminal *N*-acetylgalactosamine, a terminal galactose (small proportion), 4-linked galactose, 4-linked *N*-acetylglucosamine, and 6-linked and 3,6-linked (small proportion) 2-acetamido-2-deoxygalactitol. These results suggest incomplete removal of a galactose residue. Incomplete removal of terminal galactose linked to 2-acetamido-2-deoxygalactitol that is substituted by a chain at

TABLE II

CARBOHYDRATE AND AMINO ACID COMPOSITION OF GLYCOPROTEIN PURIFIED ON DEAE-CELLULOSE

	Percentage	*Molar ratio*[a]
Carbohydrate		
L-Fucose	8	0.83
D-Galactose	21	2.00
N-Acetylglucosamine	13	1.00
N-Acetylgalactosamine	19	1.46
N-Acetylneuraminic acid	11	0.60
Sulfate	1.3	—
Total carbohydrate	72	—
Amino acids[b]		
Aspartic acid	44	
Threonine	210	
Serine	99	
Glutamic acid	84	
Proline	88	
Glycine	71	
Alanine	86	
Cystine/2	11	
Valine	72	
Methione	—	
Leucine	61	
Isoleucine	48	
Tyrosine	29	
Phenylalanine	31	
Lysine	32	
Histidine	16	
Arginine	18	

[a]Molar ratio relative to *N*-acetylglucosamine. [b]Residues per 1000 residues.

TABLE III

CARBOHYDRATE COMPOSITION OF PURIFIED OLIGOSACCHARIDES

Oligo-saccharides	*Fucose*		*Galactose*		N-*Acetyl glucosamine*		N-*Acetyl galactosamine*		N-*Acetyl neuraminic acid*		*2-Acetamido-2-deoxy-galactitol*	
	%	*MR*[a]	%	*MR*[a]	%	*MR*[a]	%	*MR*[a]	%	*MR*[a]	%	*MR*[a]
A-3	8	0.67	22	1.708	13	0.81	14	0.88	19	0.85	16	1.00
A-4	8	0.54	26	1.60	15	0.75			24	0.86	20	1.00
A-5			24	1.65	15	0.83	14	0.78	20	0.80	18	1.00
A-6	12	0.77	15	0.88	17	0.81			26	0.89	21	1.00
A-7			18	0.71					40	0.92	31	1.00
A-8									47	0.80	42	1.00
S-1[b]	10	0.56	30	1.61	18	0.75					24	1.00

[a]Molar ratio relative to 2-acetamido-2-deoxygalactitol. [b]Sulfated oligosaccharide. Sulfate not determined.

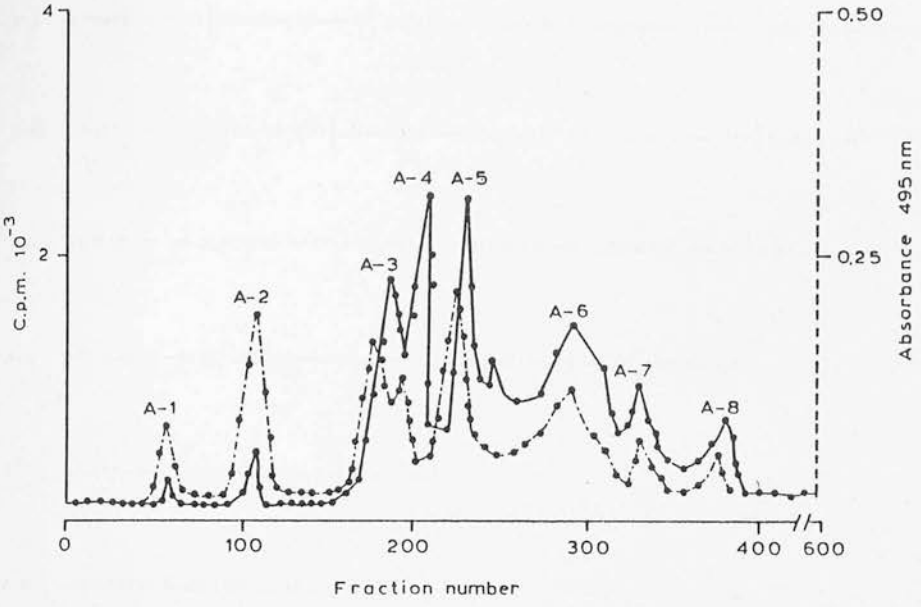

Fig. 2. Separation of oligosaccharides on a column of Bio-Gel P-6. Eight fractions (A-1, 2 mg; A-2, 4 mg; A-3, 6 mg; A-4, 7 mg; A-5, 8 mg; A-6, 4 mg; A-7, 3 mg; and A-8, 2 mg) were obtained by elution with 50mM pyridine–acetic acid. Hexoses were monitored by the phenol–sulfuric acid assay.

O-6 has been observed[17]. Methylation of the oligosaccharide after treatment with neuraminidase alone showed the presence of terminal *N*-acetylgalactosamine, terminal fucose, 4-linked and 2-linked galactose, 4-linked *N*-acetylglucosamine, and 3,6-linked *N*-acetylgalactosaminitol; these results suggest that sialic acid is linked to O-3 of a galactose residue. Methylation of the oligosaccharides after sequential treatment with α-L-fucosidase and β-D-galactosidase showed the presence of 3,4-linked galactose, 4-linked *N*-acetylglucosamine, terminal *N*-acetylgalactosamine, and 6-linked 2-acetamido-2-deoxygalactitol. These results suggest that fucose is linked to O-2 of a galactose residue that is adjacent to 2-acetamido-2-deoxygalactitol. Treatment of the oligosaccharide with α-*N*-acetylgalactosaminidase did not remove *N*-acetylgalactosamine.

Periodate oxidation–borohydride reduction followed by methylation of the degraded oligosaccharide showed the presence of a terminal galactose group and 4-linked *N*-acetylglucosamine.

Methylation of the oligosaccharide showed the presence of terminal *N*-acetylgalactosamine, sialic acid, and fucose groups; 3,4-linked and 2-linked galactose; 4-linked *N*-acetylglucosamine, and 3,6-linked 2-acetamido-2-deoxygalactitol. The results of these experiments showed that oligosaccharide A-3 is a heptasaccharide having the sequence, linkages and anomeric configurations as shown in Fig. 3.

Oligosaccharide fraction A-4. — Oligosaccharide A-4 was purified on DEAE-Sephadex A-50 and was homogeneous in p.c. (solvents *A* and *B*) and in p.e.

Periodate oxidation–borohydride reduction completely removed fucose,

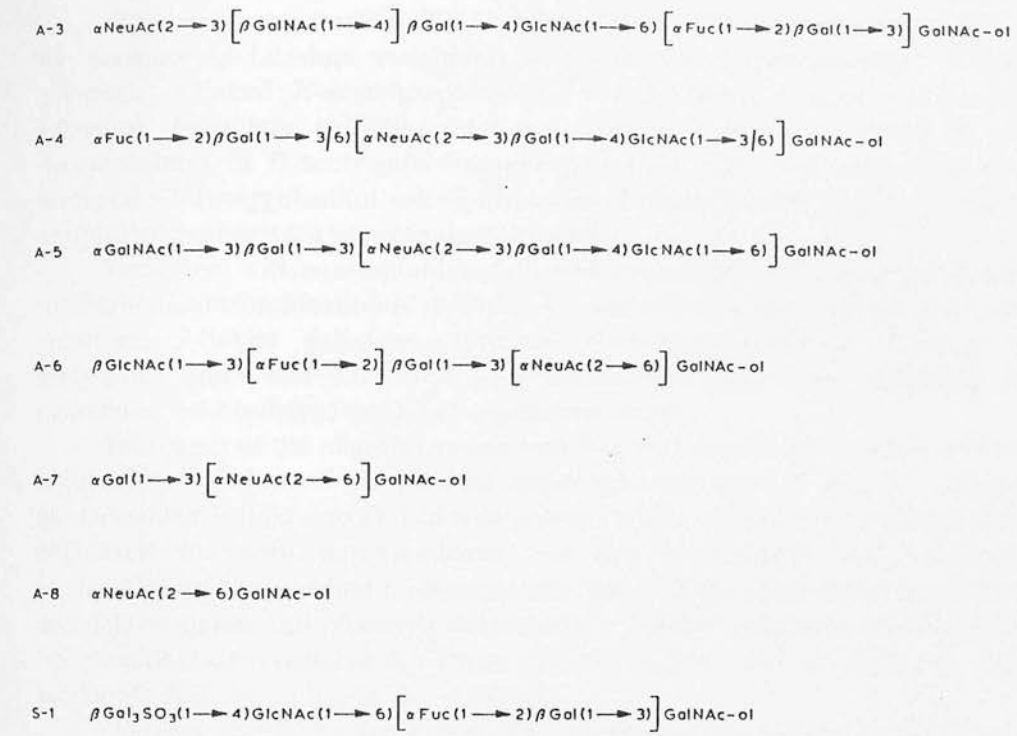

Fig. 3. Proposed structure for acidic oligosaccharides.

sialic acid, and a galactose residue, and converted 2-acetamido-2-deoxygalactitol into 2-acetamido-2-deoxythreitol. Methylation of the sugar components of the degraded oligosaccharide showed the presence of terminal galactose, 4-linked *N*-acetylglucosamine, and 3-linked 2-acetamido-2-deoxythreitol. Sequential treatment of oligosaccharide with α-L-fucosidase and β-D-galactosidase (*C. lampas*) showed the loss of a fucose and a galactose residue. Methylation of the oligosaccharide after treatment with α-L-fucosidase showed the presence of terminal galactose and *N*-acetylneuraminic acid, 3-linked galactose, 4-linked *N*-acetylglucosamine, and 3,6-linked 2-acetamido-2-deoxygalactitol. Methylation of the sequentially α-L-fucosidase- and neuraminidase-treated oligosaccharide showed the presence of terminal galactose, 4-linked *N*-acetylglucosamine, and 3,6-linked 2-acetamido-2-deoxygalactitol. Methylation of the native oligosaccharide showed the presence of terminal neuraminic acid and fucose, 3-linked and 2-linked galactose, 4-linked *N*-acetylglucosamine, and 3,6-linked 2-acetamido-2-deoxygalactitol. From these studies the linkages, sequences, and anomeric configurations of the sugar residues present in the oligosaccharide is defined. However, the identity of the sugars that are linked to 2-acetamido-2-deoxygalactitol at O-3 and O-6 is not clear. It is possible that periodate oxidation, which was performed at 4°, was not complete or that oligosaccharide may be a mixture of two similar components differing only in substitution on 2-acetamido-2-deoxygalactitol. Based on these results, the structure shown in Fig. 3 is proposed for oligosaccharide A-4.

Oligosaccharide fraction A-5. — Methylation of the oligosaccharide showed the presence of terminal neuraminic acid and *N*-acetylgalactosamine, 3-linked galactose, 4-linked *N*-acetylglucosamine, and 3,6-linked 2-acetamido-2-deoxygalactitol. Periodate oxidation and borohydride reduction resulted in total decomposition of *N*-acetylgalactosamine and sialic acid, and conversion of 2-acetamido-2-deoxygalactitol into 2-acetamido-2-deoxythreitol. Galactose and *N*-acetylglucosamine were recovered unchanged.

Treatment with neuraminidase followed by methylation of the residual oligosaccharide and identification of methylated sugars showed the presence of terminal galactose, 3-linked galactose, terminal *N*-acetylgalactosamine, 4-linked *N*-acetylglucosamine and 3,6-linked 2-acetamido-2-deoxygalactitol, suggesting that neuraminic acid is linked to O-3 of a galactose residue.

Treatment of the oligosaccharide with *N*-acetyl-α-galactosaminidase and subsequent methylation of the residual oligosaccharide showed that the *N*-acetylgalactosamine is linked to O-3 of a galactose residue. Sequential treatment of the oligosaccharide with neuraminidase and β-D-galactosidase, and subsequent methylation of the residual oligosaccharide, showed the presence of terminal *N*-acetylglucosamine and *N*-acetylgalactosamine, 3-linked galactose, and 3,6-linked 2-acetamido-2-deoxygalactitol. These results suggest the structure of oligosaccharide A-5 shown in Fig. 3.

Oligosaccharide fraction A-6. — The oligosaccharide A-6 was purified by p.c. in solvent *A* followed by chromatography on DEAE-Sephadex A-50, and was homogeneous in p.c. in solvents *A* and *B*. Periodate oxidation–borohydride reduction decomposed fucose, *N*-acetylglucosamine, and neuraminic acid, and converted 2-acetamido-2-deoxygalactitol into 2-acetamido-2-deoxythreitol: galactose was recovered unchanged. Methylation of the oligosaccharide showed the presence of terminal fucose, sialic acid, and *N*-acetylglucosamine, 2,3-linked galactose, and 3,6-linked 2-acetamido-2-deoxygalactitol. Treatment of the oligosaccharide with α-L-fucosidase and subsequent methylation of the residual oligosaccharide showed the presence of terminal *N*-acetylglucosamine and neuraminic acid, 3-linked galactose, and 3,6-linked 2-acetamido-2-deoxygalactitol. Treatment of the oligosaccharide with neuraminidase followed by methylation showed the presence of terminal fucose and *N*-acetylglucosamine, 2,3-linked galactose, and 3-linked 2-acetamido-2-deoxygalactitol. These results provide evidence that oligosaccharide A-6 is a pentasaccharide with the structure shown in Fig. 3.

Oligosaccharide fraction A-7. — The oligosaccharide was purified on Bio-Gel P-6 and was a single component in p.c. in solvents *A* and *B*. Methylation of the oligosaccharide and identification of the products showed terminal galactose and neuraminic acid, and 3,6-linked 2-acetamido-2-deoxygalactitol. Treatment of the oligosaccharide with neuraminidase and subsequent methylation of the remaining oligosaccharide showed that sialic acid was linked to O-6 of 2-acetamido-2-deoxygalactitol. These data suggest the structure of oligosaccharide A-7 as shown in Fig. 3.

Oligosaccharide fraction A-8. — Methylation of the oligosaccharide and identification of the product showed the presence of a terminal sialic acid and 6-linked 2-acetamido-2-deoxygalactitol. These data suggest the structure shown in Fig. 3 for oligosaccharide A-8.

Oligosaccharide fraction S-1. — This fraction, obtained by washing the AG-1 column with acetic acid, was purified on cellulose plates in solvent *A*: it was obtained in very small amount and underwent degradation at room temperature. The carbohydrate composition of the oligosaccharide did not change after desulfation. Methylation of the desulfated oligosaccharide and identification of the methylated sugars showed therein the presence of terminal galactose and fucose, 2-linked galactose, 4-linked *N*-acetylglucosamine, and 3,6-linked 2-acetamido-2-deoxygalactitol. Methylation of the intact oligosaccharide and identification of the methylated sugars showed the presence of terminal fucose, 2-linked and 3-linked galactose residues, 4-linked *N*-acetylglucosamine and 3,6-linked 2-acetamido-2-deoxygalactitol. Sequential treatment of the desulfated oligosaccharide with α-D-galactosidase and α-L-fucosidase removed a fucose residue. Treatment of the desulfated oligosaccharide with α-L-fucosidase and β-D-galactosidase removed a fucose and partially removed two galactose residues. Methylation of the α-L-fucosidase- and β-D-galactosidase-treated oligosaccharide showed the presence of terminal *N*-acetylglucosamine, a small amount of terminal galactose, and 6-linked and 3,6-linked (small amounts) 2-acetamido-2-deoxygalactitol. The presence of terminal galactose and 3,6-linked 2-acetamido-2-deoxygalactitol in the products of methylation, after treatment with α-L-fucosidase and β-D-galactosidase, suggests that β-galactosidase did not remove the galactose residues completely. It is known that galactose residues linked to 2-acetamido-2-deoxygalactitol, bearing a chain at O-6, are not removed completely by β-D-galactosidase[17]. It is, therefore, unlikely that the oligosaccharide was impure; evidently the glycosidase did not remove hexose completely. As a consequence, terminal galactose and 3,6-linked 2-acetamido-2-deoxygalactitol were observed. Based on these results, the structure shown in Fig. 3 is proposed for oligosaccharide S-1.

DISCUSSION

The separation of secreted glycoprotein from the remaining polymeric materials, namely serum glycoproteins, proteins, and enzymes, was readily accomplished by gel filtration on Bio-Gel P-200. The glycoprotein component from the column of Bio-Gel P-200 was treated with Pronase to remove any contaminating proteins and glycoproteins. It is known that proteolytic enzymes degrade a minor glycoprotein component of cervical mucus glycoproteins[8], and remove the hydrophobic-binding region from the protein core[18]. Fractionation of Pronase-treated glycoprotein on Sepharose 4B resulted in two fractions. The major fraction in ion-exchange chromatography resulted in a single component of high molecular weight. Despite the fact that glycoproteins are known to be degraded by Pronase[8,18], a

high-molecular-weight (1×10^5) glycoprotein was obtained that was homogeneous, albeit polydisperse, in agarose electrophoresis and free of contaminating proteins. Sialic acid in the glycoprotein was present as *N*-acetylneuraminic acid. This is similar to observations with human cervical mucus[19]; bovine material contains *N*-glycosylneuraminic acid[20]. The glycoprotein was free of the cross-linked protein fractions containing cystine that are, observed with bovine cervical mucus[21].

Treatment of the glycoprotein with alkaline borohydride gave a mixture of oligosaccharides that was fractionated on Bio Gel P-6. The saccharides obtained from Bio-Gel P-6 were further purified by ion-exchange and paper chromatography, affording six sialylated oligosaccharides. A sulfated oligosaccharide was obtained by washing the AG-1 column with acid. The oligosaccharides characterized may be considered to have the following core structures:

(i) βGal(1→3)GalNAc-ol
(ii) βGal(1→4)βGlcNAc(1→6)GalNAc-ol

Chain elongation on these two core residues takes place, resulting in the structures here characterized and shown in Fig. 3. The partial structures of few carbohydrate chains have been identified for human cervical glycoprotein[17], and these bear similarity to the core structures of the bonnet-monkey oligosaccharides. Monosialylated oligosaccharides from bonnet-monkey cervical glycoprotein obtained at midcycle have been characterized by n.m.r. spectroscopy[22]. These structures, like those of human cervical oligosaccharides, have common core-residues and also share some chain structures.

A variety of carbohydrate chain-lengths in mucins[23,24] and in blood group-active glycoproteins[25–27] is known, and the heterogeneity of cervical mucus glycoprotein could be even wider because of changing physicochemical behavior with the menstrual cycle.

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performing TLC against an authentic sample of pyridine-2-aldehyde. The reflux of the reaction mixture was continued till such time that the spot due to pyridine-2-carboxylic acid hydrazide on the TLC plate was no more visible. This required a total reflux time of forty eight hours. At this stage, most of n-butylamine was removed from the reaction mixture by distillation. Vacuum distillation of the concentrated reaction mixture, thus obtained, yielded pyridine-2-aldehyde at 38-39°/7 mm as a viscous oil (4.2g, 0.039 mole) in 97.9% yield with respect to pyridine-2-carboxylic acid hydrazide.

Pyridine-2-carboxylic acid hydrazide was prepared from pyridine-2-carboxylic acid by the same method as described [10] for preparation of pyridine-4-carboxylic acid hydrazide from pyridine-4-carboxylic acid. It was recrystallised from methanol, m.p. obs. 100°, m.p. lit. [11] 100-101°.

Further work on the reaction of some other heterocyclic carboxylic acid hydrazides with n-butylamine is in progress.

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Review

Glycoproteins: Biologically Vital Macromolecules

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(Received 19th January, 1986, Revised 25th September, 1988)

Amongst the glycoconjugates, the group of glycoproteins represent biologically abundant and clinically vital molecules. From immunoglobulines to hormones, membrane constituents of normal and transformed cells, as well as in the processes of reporduction, glycoproteins play a significant role.

Quite recently, it has been recognised that living organisms form a large number of biologically important polymers [1-6], in which proteins and carbohydrates are covalently linked and in which the sugar moiety has a significant function. In fact not long ago a concerted effort was applied to remove carbohydrates, utilizing even the most drastic alkaline conditions in order to obtain pure proteins. However, now it seems that most proteins are glycoproteins as are many polysaccharides, inlcuding starch and glycogen. Furthermore, there are sufficient suggestions that the sugar chains of glycoproteins contribute to important biological functions. These include biological recognition, such as specification of blood types [5], regulation of the half-life of glycoproteins in the circulatory system [7-14], stabilization protein conformation [6], protection of exposed tissue, bronchial, gastrointestinal and cervical [1], as well as glycoprotein uptake by cell [15]. The glycoprotein uptake by the cells [15] and the presence in the circulatory system of these macromolecules has opened possibilities for directing chemotherapy to specific sites in the body and for enzyme replacement therapy for the treatment of genetic disorders. Of particularly significance is the possibility that the carbohydrates of cell membrane glycoproteins play a dominant role in differentiation [16], growth [17] and intercellular recognition [18,19] and in many pathological processes including malignancy [20]. Glycoprotein may also function (Tamm Horsfall) to regulate circulatory activity of cytokines [21]. Carbohydrate moiety in this glycoprotein, (Uromodulin) is responsible for its biological activity [22]. Currently the progress rate in the biochemistry, molecular biology and biological aspects of glycoproteins is unfolding, and there is every suggestion that implications of these macro-molecules will gain in importance.

Glycoproteins exist in cells both in soluble and membrane bound forms in addition to being present in the intercellular matrix and in extracellular s ecretory fluids. Human serum is a rich source of glycoproteins where nearly all the proteins except two, albumin and proalbumin, contain sugars. The monosaccharides of common occurence in glycoproteins are hexoses, N-acetylhexosamines, uronic acids, a deoxyhexose and two pentoses. In addition, a complex monosaccharide derived from a nine carbon straight-chain sugar known as neuraminic acid is a common component of mammalian glycoproteins [1]. Neuraminic acid has been known to exist in man and primates as N-acetylneuraminic acid and in other animal species as N-glycolyneuraminic acid [23]. The hydroxyl groups of sugars in glycoproteins are substituted by other groups, particularly in secretory fluids, such as bronchial, intestinal and cervical mucuses, and in viral glycoproteins by sulfate groups [24], or in lysosomal hydrolases and yeast mannans, by phosphate groups [25,26].

A singificant feature of glycoproteins is the carbohydrates-protein linkage. Two distinct types of linkage dominate: an N-glycosidic linkage between the anomeric carbon of N-acetylglucosamine and nitrogen of the amide group asparagine and the other involving the O-glycosidic linkage between N-acetylgalactosamine, galactose and xylose of the carbohydrate chains to the hydroxyl groups serine, threonine, hydroxylysine, and hydroxyproline of the protein moeity (Fig.1). Of rare occurrence are the O-glycosidic linkage such as that between mannose and serine or between L-fucose and threonine. However, N-acetylglucosamine-asparagine contains the only known N-glycosidic linkage in the glycoproteins. A single glycoprotein such as immunoglobulin and glycoprotein hormones may contain more than one type

				Source
	N-Glycosyl Linkage			
	N-Acetylglucosamine		Asparagine	Serum glycoproteins, glycoprotein hormones, enzymes and cell-membrane glycoproteins
	O-Glycosyl Linkage			
1.	N-Acetylgalactosamine	⟶	Serine or Threonine	Mucins, Fetuin, Epiglycanin, Antifreeze glycoprotein
2.	Xylose	⟶	Serine or Threonine	Proteoglycans
3.	Fucose	⟶	Serine or Threonine	Normal human urine and rat tissue
4.	Galactose	⟶	Serine or Threonine	Cuticle collagen
5.	Mannose	⟶	Serine or Threonine	Yeast and Fungal glycoproteins
6.	Galactose	⟶	Hydroxylysine	Glomerular basement membrane and tropocollagen
7.	Galactose	⟶	Hydroxyproline	Plant cells
8.	Arabinose	⟶	Hydroxyproline	Potato lectin

Fig.1: Attachment of oligosaccharide to proteins.

of carbohydrate-protein linkage. More recently it has been recognized that O-glycosidically linked oligosaccharide present complexity by providing diversity of sugar linkages at the ultimate sugar residue linked to protein.

Monosaccharides are multi-functional organic compounds, usually with three or more free hydroxyl groups of rather similar chemical reactivity, in addition to a primary hydroxyl group of different chemical reactivities. Furthermore, there is the problem of anomery, since glycosidic linkages may be either α or β i.e., the oxygen atom on carbon number one can assume different planes of the sugar ring. Diverse reactivity of functional groups in a single sugar unit requires a meticulous design and the explicit execution of a complex series of reactions to introduce a carbohydrate chain in the glycoprotein. The synthesis of oligosaccharides in vivo required therefore, considerable enzyme specificity; in vitro it is a complex task, and there are few reports on such syntheses. However, a consequence of this structural complexity and variability is the capability of the sugars to serve as carriers of biological information. In contrast to peptides and oligonucleoitides, in which the information is based entirely on the number of different monomeric units, in oligosaccharides the information rests also in the position and anomeric configuration of the glycosidic unit and in the branching points. As a consequence sugar macromolecules can store substantially more information per unit weight than proteins and nucleic acids.

Protein moiety

The protein backbone of glycoproteins may have different peptide sequences although the carbohydrate

chains may show some similarity in structures. The biosynthesis of the protein moiety of the glycoproteins is analogous to that of nonglycosylated proteins, so that their structure is under regulation of the genetic code. As a consequence, all molecules of a given protein are identical. Glycosylation of proteins is not primarily regulated by genes, and the biosynthesis of glycoproteins is controlled by the transferases in the absence of template. Furthermore, the sugars may be added to peptide chains in vivo, nonenzymatically. The best example of this type of glycosylation is in haemoglobin A_{1c}, present in minute amounts in the red blood cells of humans.

Haemoglobin A_{1c} is formed by nonenzymic attachment of glucose to the αamino groups of the aminoterminal valine of the β-chain in haemoglobin A. Higher levels of haemoglobin A_{1c} and glycosylated serum albumin are present in diabetic patients. There is a strong suggestion that quantitation of these glycoproteins may be a better test than glucose analysis for evaluating carbohydrate metabolism in normal and diabetic individuals.

Glycoprotein structure

Recent advances in the knowledge of the structure and metabolism of glycoproteins and insight into the biosynthesis and function would be impossible without the development of new and sophisticated techniques for their isolation, purification and structural characterization. Essentially, the purification of glycoproteins is performed by methods commonly used in protein and polysaccharide chemistry such as gel filtration, ion-exchange chromatography, agarose and sodium dodecyl sulfate polyacrylamide gel electrophoresis and isoelectric focusing. Among the newer techniques now more commonly utilized is the purification of glycoproteins by the use of lectins i.e. affinity chromatography. Lectins are a group of sugar-specific proteins of nonimmune origin that are common in plant but are also found in microorganisms and animals. These proteins bind to specific sugars, essentially with specific structure, and hence can be effectively used to purify a mixture of glycoproteins. In addition, this binding property imparts an important functional behaviour of lectins, i.e., agglutination of cells which have glycoproteins and glycolipids on the cell surface. Amongst the commonest lectins are concanavalin A (specific for αmannose and α-glucose), peanut lectin (specific for galactose), wheat germ agglutinin (specific for β-N-acetylglucosamine and N-acetylneuraminic acid), and soybean agglutinin (specific for N-acetylgalactosamine). Because the glycoproteins bind non-covalently to either solubilized or immobilized lectins, the complexes thus formed can be dissociated by adding the sugar for which the lectin is specific. Due to this behaviour a pure glycoprotein can be isolated from a crude biological extract, or a mixture of glycoproteins that differ in sugar composition or in the structure of the carbohydrate moiety can be resolved.

Largely glycoproteins contain several monosaccharides and different sugar chains. Because of microheterogeneity there are variations in the number and type of carbohydrate units attached to the protein backbone of glycoproteins. Due to the multifunctional character of the carbohydrate chains as well as protein moiety, the macromolecules assume a distinct chemical behaviour. In addition, the capability of these macromolecules to form aggregated structures due to intra- and intermolecular activity and the physical diversity in behaviour makes their handling and the study of their physical properties difficult. Therefore, due to the complexity of these organic

Table-1:

Few natural glycoproteins and their origin

Glycoprotein	Source	Size (Molecular weight)	Carbohydrate (Content percent)
Serum glycoproteins			
IgG immunoglobulin	Human	150,000	10
Thyroglobulin	Calf	670,000	8
Prothrombin	Human	72,000	8
Hormones			
Human chorionic gonadotropin	Urine	38,000	31
Erythropoietin	Urine	34,000	29
Membrane constituents			
Human glycophorin	Erythrocytes	31,000	61
Bovine rhodopsin	Retina	40,000	7
Enzymes			
Alkaline phosphatase	Mouse liver	130,000	18
Bromelain	Pineapple	33,000	36
Other			
Human interferon	Leucocytes	26,000	26

molecules the characterization of glycoproteins , is a difficult task. The nature of the carbohydrate units and the microheterogeneity present in the glycoproteins permit, with great difficulty, structural elucidation on an intact molecule. Traditionally, the glycopeptides isolated from proteolytic digests of a glycoprotein or β-eliminatedborohydride reduced olgiosaccharides are the starting materials for structural studied of carbohydrates. For the study of the protein moiety various reactions are used depending upon the amount of carbohydrate in the glycoprotein.

There is, however, still continuous progress being made in developing microanalytical techniques, since most glycoproteins of value are available in homogeneous form in small quantities. However, refinement of carbohydrate and amino acid methodology and the development of new techniques have almost made it possible to do a reasonably complete structural study in a short time on as little as 0.5 mg of material. The easier structural feature in the glycoproteins to indentify is the carbohydrate peptide linkage, since the various linkages have different stabilities towards acid and alkali. The N-glycosidic linkage is relatively stable to mild acid but is hydrolyzed under stronger conditions. The O-glycosidic linkages vary markedly in their sensitivity to alkali. The galactosylhydroxylysine linkage is highly stable whereas the O-glycosidic linkage to serine and threonine is readily cleaved by alkali. The common procedure in the structural investigation of glycoproteins is the elimination of carbohydrate chains from the proteins by mild alkaline treatment in the presence of sodium borohydride. Under these conditions the glycosidic

bonds are cleaved, and serine and threonine residues are converted into alanine and α-aminobutyric acid respectively, whereas the sugars involved in the glycosidic linkage are converted into the corresponding sugar alcohols. The sugar commonly involved in such type of linkage in the secretory glycoproteins is N-acetyl-galactosaminitol. Quantitative assay in the loss of serine and theornine, increase in the amounts of alanine and α-aminobutyric acid and indentification of the sugar alcohol formed provide information as to the nature of the carbohydrate-peptide linkage. In addition it also suggests an approximate number of O-glycosidically linked residue in the glycoprotein.

For the structural analysis of carbohydrates the information of importance pertains to the sequence and linkage of sugar residues. Methylation analysis [27,28], which involves the substitution of free hydroxyl groups by methoxyl groups, followed by acid hydrolysis [29,30], is the most important and frequently used method in the structural investigations of complex carbohydrates. The partially methylated sugar, after reduction with sodium borohydride and acetylation with acetic anhydride, are analyzed by gas-liquid chromatography - mass spectrometry [31-36]. In order to avoid possible mass symmetries in the alditol derivatives, it is useful to use borodeuteride instead of borohydride [37] for the reduction. Also oximes [37] and aldonitriles [38-40] have been used to avoid mass symmetries. The position of O-methyl and O-acetyl groups in these derivatives can be ascertained from the specific fragmentation observed in the highly specific electron impact mass spectra. The sequence of sugars in the oligomers and their anomeric configuration is best analyzed by the use of glycosidases. Glycosidases are now available in highly purified form, and they display strict stereochemical and in some cases linkage specificity [41,42]. Furthermore, information on the anomeric linkages as well as on the sequence of sugars and their linkage by high-resolution protein nuclear magnetic resonance (NMR) spectroscopy has been in recent years a subject of extensive studies. Beyond doubt NMR spectroscopy of carbohydrate chains in the glycoproteins, secretory as well as of serum origin, has added a new dimension in the study of structure-function relationship with the combined application of high performance liquid chromatography and 500 MHz NMR spectroscopy, a number of oligosaccharide structures have been characterized which otherwise could not be identified [43-47].

The protein moiety of glycoprotein, as described earlier, is synthesized by the same mechanism that produces nonglycosylated proteins. Synthesis of the protein takes place on polyribosomes, although initiated on free ribosomes. Attachment of the free ribosome to the membrane of the endoplasmic reticulum is initiated by a signal sequence of the nascent peptide chain. Addition of sugar to proteins is a cotranslational event, involving alternation of amino acids already in existence in the polypeptide. Glycosyltransferases catalyze the stepwise synthesis of both O-linked and N-linked oligosaccharides. The enzymes that transfer sugars from their nucleotide and catalyze the transfer to sugars to the proteins [48] in the case of O-linked oligossachardes begins with the transfer of N-acetyl-galactosamine to the hydroxyl group of threonine or serine. Biosynthesis of the oligosaccharides sequence in the glycoproteins is regulated by the availability of transferases, and is, therefore, achieved without the involvement of RNA or DNA. The sequence is, however, indirectly regulated by the transferases, which are the primary gene products. In

Table-2: Lectin sources and some of their characteristics

No.	Lectin source	Molecular weight	Blood group specificity	Carbohydrate specificity
1.	Peanut	110,000	Neuraminidase-digested A, B, O or T antigen	β-D-Galp-(1→3)-D-GalNAcp
2.	Jack Bean (concanavalin A)	104,000	Non specific	α-D-Manp>α-D-Glcp>α-D-GlcNAcp
3.	Horse Gram A	113,000	A >>Az	α-D-GalNAcp
	B	109,000		
4.	Soybean	120,000 - 122,000	A >>O B	α-D-GalNAcp, β-D-GalNAcp
5.	Lentil	42,000 - 63,000	Non specific	α-D-Manp>α -D-Glcp, α-D-GlcNacp
6.	Asparagus pea A	120,000	O A	α-L-Fucp, 2-O-Me-D-Fucp
	B	58,000		
	C	117,000		
7.	Red Kidney bean	126,000 - 136,000	Non specific	
8.	Pea	49,000 - 53,000	Non specific	α-D-Manp>α-D-Glcp α-D-GlcNac
9.	Castor bean	120,000	Non specific	β-D-Galp>α-D-Galp
10.	Potato	100,00	Non specific	β-D-GalNacp-(1→4)-[β-D-GlcNAcp-(1→4)] β-D-GlcNAc
11.	Wheat gum	36,000	Non specific	β-D-GleNAcp(1→4)β-D-GlcNAcp-(1→4)-β-D-GlcNAcp>β-D-GlcNAcp-(1→4)-β-D-GlcNAc; NANA

serum type glycoproteins containing an N-glycosyl linkage between asparagine and N-acetylglucosamine lipid linked oligosaccharide precursors are formed in animals [49], yeast and plants for the enbloc transfer to the amide group of an asparaginyl residue that is part of the sequence Asn-X-Ser/Thr in a growing peptide. The transfer of the oligosaccharide from the lipid-linked oligosaccharide to the growing peptide probably takes place on the inner side of the endoplasmic reticulum, and the completion of the complex oligosaccharide units occurs in the Golgi apparatus, a sub- cellular organelle that consists of membrane sacs. A recent development of significant consequence was the discovery of glucose residues in N-glycosylated glycoproteins, since this sugar had not been encountered before. It is now established that the glucose residues in the glycoprotein are present as a means to introduce further sugar residues and not as final components of the macromolecule.

Glycoconjugates in pathogenesis

Since biologically important glycoproteins are very widely distributed in nature and all cell membranes are coated with sugar-containing molecules it is evident that the contirbution of the sugar must be significant. The readers are referred to reference t for a comprehensive review of functions of the sugar moiety in glyco-conjugates.

The surface of mammalian cells posesses a variety of antigentic molecules, that may characterize the cell with regard to individual [50,51], and tissue [52] of origin. Definite antigens at the cell surface may reflect the stage of embryonic development [53], and antigens may develop from envi-

ronmental factors i.e., exposure to viruses [54], chemicals [55], or radiation [56]. Neoplasia can result from these factors, and the cell surface of neoplastic cells possesses antigens which have arisen from these environmental factors [57-58] or they may possess suppressed embryonic or fetal antigens [59]. These molecules, antigens, known as tumor associated or tumor specific antigens, can not usually be identified at the surface of normal cells similar to those from which the neoplasm was derived.

In the past decade, attempt to correlate transformation properties with macromolecular structure at the cell surface have not been conclusive. Some researchers have reported a correlation between metastatic potential and low concentrations of cell surface glycoproteins in mammary tumor cells, whereas others have observed large quantities of high-molecular weight, endogenous O-glycosylated glycoproteins in metastatic carcinoma cells grown in vitro [60-62]. There have been different reports concerning cancer-related cell surface carbohydrates and invasiveness. An increased degree of branching and sialic acid density in surface carbohydrates from tumor cells is generally observed. Studies in human malignancies as well as in a wide variety of cell systems have revealed that the presence of cancer-related surface carbohydrates correlates with the manifestation of the malignant phenotype of cells rather than with the transformed mode of growth in vivo or in vitro [63-65]. As the intrinsic factor of malignant cells is to invade neighbouring tissue it is expected that alterations in the surface carbohydrates may be responsible for difficient tissue control leading to invasion. Recently a report using an organ culture model [66] has been recorded [67]. Oncogenes and oncoviruses are known, their influence on the alteration as well as transformation of cell-surface glycoproteins is yet to be clearly defined, however. Among other glycoconjugates, heparin, a polymer of 2-acetamido-2-deoxy-D-glucose, glucouronic acid and idouronic acid residues linked to a protein through a linkage region of $(Gal)_2$-xyl, a trisaccharide, linked to a serine residue to the protein presents a delicate case of interaction with protein. Heparin is synthesized and stored in mast cells in close vicinity of blood vessels and released from mast cells when desired. The blood anticogulant activity of heparin rests on its ability to interact with high affinity to antithrombin, a plasma protein that inhibits the proteases participating in the coagulation mechanism. The main antithrombin (AT) binding region in heparin is contained in the octasaccharide (Fig. 2). The vital and shortest oligosaccharide sequence in heparin, able to bind to antithrombin and to selectively reinforce its activity towards blood coagulation factor Xa, was at most of the size of a pentasacc-

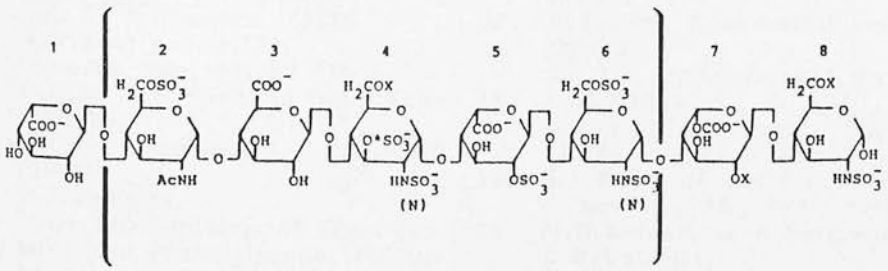

Fig.2: Structure of the AT-binding Octasaccharide.

haride [88,71], units 2-6 within brackets. There are structural variants within the pentasaccharide, the actual AT-binding unit, and these are indicated by X (H or SO_3, Fig. 2) or by the sugar unit, glucouronic acid or idouronic acid. The 3-O-sulfate group of sugar unit 4, marked by an asterisk, is unique to the AT-binding region of the heparin molecule. In addition, each of the sulfate group marked by (N) in Fig. 2 is essential to the high affinity binding of antithrombin. Thrombosis, i.e., the formation of clots inside the blood vessels, could occur as a result of transformed binding sites or disfunction of the heparin-antithrombin complex. Transformation or metastatic potential may be caused by a variety of mechanism, however. There exists scope of more intensive investigations in this area to define the exact mechanism of cell-transformation.

A variety of disorders are generated by disfunction of glyco-conjugates and particularly glycoproteins; only two i.e., neoplasia and thrombosis have been considered in this article. The precedence of these disorders over others is given in this review because of the preeminence of the pathology of these vitally significant disorders.

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J.Chem.Soc.Pak. Vol. 10, No. 1, 1988.

Isolation, Purification and Partial Characterization of Neutral oligosaccharides from Bovine Gallbladder Mucin Glycoprotein

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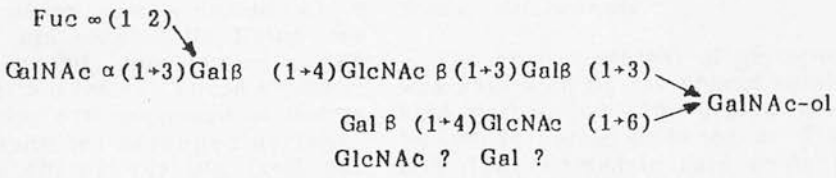
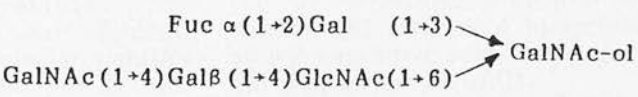
(Received 5th January,1986)

Summary:A glycoprotein was isolated from the bovine gallbladder secretion. The glycoprotein was purified on Bio-Gel P-200 and by subsequent mild treatment with Pronase. The Pronase treated glycoprotein was further purified by gel chromarography on Sepharose 4B and by ion-exchange chromatography. The purified glycoprotein was subjected to alkaline degradation-borohydride reduction. The liberated oligosaccharide alditols were purified by gel filtration, and separated into neutral and acidic oligosaccharides. Four neutral oligosaccharides, after purification, were characterized by chemical and enzymatic studies and were assigned the following structures and partial structure.

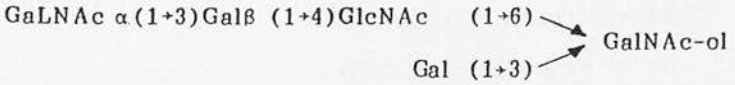
N-3:



N-4:



N-5:



N-6:

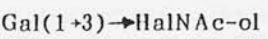


Fig.1: Proposed structures for neutral oligosaccharides.

*Supported in part by Pakistan Science Foundation Research Grant B-BU/MED (89).

Introduction

The gallbladder secretion, mucus, is of vital importance in the pathogenesis of gallstones. It has been observed that gallbladder hypersecretion preceeded gallstone formation [1]. It was further observed that cholesterol crystals first formed in aggregates of mucus rather than in the liquid phase of gallbladder bile and thereby it was implied that mucus was essential not only for development of cholestrol type gallstones but important in the pathophysiology of other types of gallstones [1]. Purified human gallbladder mucin glycoproteins have been shown to induce nucleation of lecithin-cholesterol crytals from supersaturated hepatic bile. These in turn resulted in formation of cholesterol monohydrate crystals within short period. In addition, it has been shown that gastric [2,3] and bronchial [4] mucins exhibit lipid-binding properties.

Mucins are highly glycosylated proteins, which are secreted by a variety of epithelial cells lining the trachaeobronchial, genitourinary and gastrointestinal tracts. These glycosylated proteins are composed of nearly 75% carbohydrates arranged as branching side chains varying from two to twelve sugar residues. The hydroxyl functions in carbohydrate moiety of the glycoproteins in solution are extensively hydrated and contribute to the rheologic, viscoelastic and gel-forming properties of the macromolecule.

The mechanism by which glycoproteins participate in the process of gallstone formation is still unclear. In order to understand the role of the carbohydrates a glycoprotein was isolated and structures of neutral oligosaccharides have been partially characterized to translate the biochemical structure into the physiological functions.

Material and Methods

Isolation of gallbladder mucus

Bovine gallbladders were obtained at a local abatoir, drained of bile, and transported to the laboratory on ice. The gallbladders were opened and the mucosal surface washed with iced saline to remove blood and debris. The mucosal surface was then gently scraped with a glass slide to remove the adherent mucus gel, after which the mucosa was removed by sharp dissection. The mucosa was minced with a scalpel, combined with the mucus gel in 0.2 m NaCl, 0.04% NaN_3 azine (10 ml/g of tissue) and homogenized. The bile and mucosal gel were processed for isolation of glycoproteins in the same manner but separately.

Analytical Methods

The hexose content of glycoprotein was estimated by the phenol sulphuric acid method [5]. The protein content by measuring absorbance at 278 nm and the neuraminic acid content by the thiobarbituric acid procedure of Warren [6] after acid hydrolysis with 50 mM sulphuric acid, or by gas-liquid chromatography (GLC).

Gas Liquid Chromatography (GLC)

GLC determinations of the carbohydrate moiety of the glycoprotein were performed according to the procedure of Reinhold [7]. GLC-MS (mass spectrometry) of the methylated sugars was performed on a varian MAT 731 instrument fitted with the combined source EI, CI/FD ion source.

Column chromatography

Bio-Gel P-200 (Bio-Rad Laboratories) and Sepharose 4B (Pharmacia Fine Chemicals) column were run in 50mM sodium phosphate (pH 7.0) containing 0.02% sodium azide. The DEAE-cellulose (Whatman) column was eluted with 0.1M NaCl followed by a gradient of 0.1M to 1M sodium chloride containing 10mM HCl. Bio-Gel P-4 and Bio-Gel P-6 (200-400 mesh) chromatography was performed in 50mM pyridine-acetic acid. The sugar containing fractions, detected by phenol-sulfuric acid procedure or by counting tritium, were combined and lyophilized. DEAE-Sephadex A-50 columns were run with 50mM 0.5M phosphate buffer (pH 7.0) followed by 0.1M-0.5M LiCl, portions containing carbohydrates were combined and desalted on a column of Bio-Gel P-2.

Gel Electrophoresis

Agarose gel electrophorjesis was performed in 50 mM barbital buffer (pH 8.2) on glass slides containing 1% agarose. Polyacrylamide and agarose gel electrophoresis was carried out according to the method of Holden et al. [8]. The agarose and polyacrylamide slides were stained with Amido black or Coomassie blue, and with periodate-Schiff reagent. For agarose and polyacrylamide electrophoresis nearly 0.1 mg of each substance per ml was used and each well had 25 ul of the solution.

Purification and Protease Treatment of the Mucus Glycoprotein

The crude mucus was solubilized in 50mM sodium monophosphate (pH 7.0) containing 0.02% sodium azide by stirring for 24 hours. The cellular debris and other insoluble materials were removed by centrifugration (2500 rev./min), the supernatant was dialyzed, and the retentate was lyophilized to give the crude mucus glycoprotein. The residue (80 mg) was dissolved in 50mM sodium monophosphate (10 ml, pH 7.0) containing 0.02% sodium azide by stirring for 16 hours at 4°. The solution was applied to a column (2.0 x 60 cm) of Bio-Gel P-200 (50-100 mesh). The carbohydrate and protein containing fractions were pooled, the pH was adjusted to 5.5 with 4M acetic acid, and the solution was dialyzed and then lyophilized to give the purified mucus glycoprotein (20 mg). The purified glycoprotein (19 mg) was treated with insolubilized Pronase (10 mg, Enzite protease, Miles Laboratories, Inc.) in 50mM sodium monophosphate buffer 30 ml, pH 8.0) containing 0.1% sodium azide. The mixture was stirred at 22° until solubilized and then incubated for 48 hours at 37° with stirring. Another addition of prewashed enzyme (2 mg) was made and the solution incubated for another 8 hours. The suspension was centrifuged, and the residue was washed with buffer. The pH of the supernatant was adjusted to pH 5.0 with acetic acid and the solution dialyzed against distilled water. The non-dialyzable material was lyophilized and the residue (8 mg) was applied to a column (1.5 x 48 cm) of Sepharose 4B. Fractions containing carbohydrate and protein were combined and dialyzed, and the non diffusible material was lyophilized. The sepharose 4B purified carbohydrate containing material was further chromatographed on a column of DEAE-cellulose, the carbohydrate and protein containing fractions eluted with a gradient of lithium chloride were combined and dialyzed. The retentate was lyphilized to give the Pronase-treated glycoprotein (5 mg). The glycoprotein was examined by agarose and polyacrylamide electrophoresis.

Alkaline Borohydride Treatment

The Pronase-treated glycoprotein (4 mg) was treated with 2M sodium borohydride in 50mM sodium hydroxide according to the procedure of Ilyer and Carlson [9]. A 0.2% solution of the glycoprotein in 2M sodium borohydride in 0.05M sodium hydroxide was incubated for 18 hours at 45°. Following alkaline-borohydride treatment, mixture was adjusted with 4M acetic acid to pH 5.4.

The reaction mixture was applied to a column (2.5 x 68 cm) of Ag 50+W-X8 (H^+, 100-200 mesh) ion-exchange resin containing 150 fold excess with respect to the sodium ions from NaOH and $NaBH_4$. Oligosaccharides, oligopeptides and glycopeptides were eluted with water and 50mM acetic acid. Reduced oligosaccharides were separated into neutral and acidic oligosaccharides on a column (3.4 x 70 cm) of AG 1-X2 (OAc^-, 200-400 mesh). The column was washed with water 0.5M pyridine-acetic acid (pH 5.4) and then with 0.1M-1.5M acetic acid at 4°. The acidic oligosaccharides were further chromatographed on Bio-Gel P-6 (200-400 mesh) in 10mM pyridine-acetic acid (pH 5.4) followed by paper chromatography or paper electrophoresis. Paper chromatography was performed in solvents (A) ethyl acetate-pyridine-acidic acid-water (5:5:1:3) (B) butanol-propanol-0.1M acetic acid (1:2:1,v/v).

Enzyme Degradation

Oligosaccharides were digested with some or all of the following enzymes:

i) α-L-fucosidase from beef epididymis (Sigma, 10mM sodium cirate buffer, pH 6.0 at 37° for 50 hrs) and from emulsin (50 mM sodium citrate buffer, pH 5.0, 37° for 50 hrs).

ii) β-galactosidase from Aspergillus niger (Sigma, 50mM, sodium citrate, pH 4.1, 70 hrs at 37°).

iii) β-galactosidase from Escherichia coli (Boehringer, 50mM sodium phosphate, pH 7.0, 48 hrs at 37°) and -galactosidase from Charonia lamps (Miles, 50 mM sodium cirate buffer, pH 4.0, 48 hrs 37°).

iv) β-N-acetylglucosaminidase from Jack Bean (Sigma, 5mM sodium citrate buffer, pH 4.5, 40 hrs).

v) α-N-acetylgalactosaminidase from Charonia lampas (Miles, 50mM sodium citrate-phosphate buffer, pH 4.1, 42 hrs at 37°).

Methylation Analysis

Methylation of oligosaccharides was carried out according to the procedure of Hakomori [10]. The methylation was achieved with iodomethane in the presence of methylsulphinyl carbanion. The methylated oligosaccharides were recovered by dissolution in chloroform. The methylated product was converted into monomers by treatment with 2M trifluoroacetic acid at 105° for 3 hrs. The solution was diluted (x10), and freeze-dried. The residue in water-methanol (4:1) was treated with $NaBH_4$ (25 mg) for 12 hrs at 4° and for 4 hrs at 22°. The excess of $NaBH_4$ and sodium ions was removed with water, methanol and methanolic-NH_3. The combined washings were evaporated, and the residue in methanol was repeatedly evaporated to remove boric acid. Finally, the residue was acetylated with pyridine (0.5 ml) and acetic anhydride (0.4ml) for 12 hrs at 22° and the methylated alditols were examined by g.l.c. and g.l.c. -m.s.

Periodate Oxidation-Sodium Borohydride Reduction

In order to establish the sequence and linkages of the sugar residues in oligosaccharides, reduced oligosaccharide (0.1 to 0.3 mg) were oxidized with periodate (0.1M) at 4° for 12 hrs, then at room temperature for 8 hrs. The excess of periodate was destroyed by addition of 1,2-ethandiol and the oxidized material was reduced with $NaBH_4$ (5 mg per mg of the starting material) for 12 hrs at 4°, followed by another addition of $NaBH_4$ (2 mg per mg of oligosaccharide), and the reduction was allowed to proceed at room temperature for 6 hrs. Excess borohydride and sodium ions were removed by adding an excess of AG 50W-X8 (100-200 mesh) ion-exchange resin. The resin was filtered off, and boric acid was removed by repeated evaporation with methanol. The residue was treated with 0.25M H_2SO_4 for 2.5 hrs, the acidic solution was treated with 0.25M AG1-X8 (OAc^-, 100-200 mesh). After methanolysis with 0.5M methanolic hydrogen chloride for 20 hrs at 80°, the oxidized oligosaccharides were examined by g.l.c. for the presence of sugars. A sample of N-acetylgalactosaminitol was obtained by reduction of N-acetylgalactosamine; 2-acetamido-2-deoxyserinol was obtained by N-acetylation of commercially available 2-amino-2-deoxy-serinol (Sigma). Samples of N-acetylthreosaminitol and N-acetylarabinosaminitol alongwith N-acetylated serinol were obtained by mild (10mM) periodate oxidation of N-acetyl-galactosaminitol for 30 min at 220°.

Results

Purification and characterization of the Pronase-degraded mucus and the gallbladder glycoproteins

The mucus glycoproteins obtained from the bile were purified by gel filtration on Bio-Gel P-200 followed by treatment with the Pronase. The Pronase-treated glycoproteins were fractionated on Sepharose 4B. The major fraction (70%) showed the presence of a single glycoprotein in DEAE-cellulose chromatography. The glycoprotein in gel electrophoresis barely entered the gel and exhibited a single component.

Table-1: Carbohydrate Composition of Purified Oligosaccharide Fractions

Oligosaccharides	Fucose		Galactose		N-Acetyl-glucosamine		N-Acetyl-galactosamine		N-Acetyl-galactos aminitol	
	%	MR[a]	%	MR[a]	%	MR[a]	%	MR[a]	%	MR[a]
N-3	8	0.90	29	2.90	31	2.60	10	0.84	12	1.00
N-4	12	0.90	28	0.96	17	0.94	16	0.89	18	1.00
N-5			31	1.74	20	0.91	19	0.86	22	1.00
N-6			40	0.96					51	1.00

[a]Molar ratio relative to N-acetylgalactosaminitol.

Preparation of oligosaccharide-alditols

The glycoprotein was subjected to alkaline borohydride-reductive cleavage yielding a mixture of oligosaccharidealditols. A decrease of serine and threoine and a corresponding increase of alanine and appearance of -aminobutyric acid were dectected. The neutral oligosaccharides eluted from the column of AG 1-X2 were separated on a column of Bio-Gel P-6 into six fractions (see Table 1 and Fig. 1). Fractions N-1 and N-2 represent mainly glycopeptides as indicated by low percentage of N-acetylgalactosaminitol and the presence of hydroxylated amino acids.

Oligosaccharide fraction N-3.

This fraction was further purified by chromatography on DEAE-Sephadex A-50 and was homogeneous in paper chromatography in solvents A and B. Carbohydrate analysis of the oligosaccharide suggested this fraction to be a decasacharide. Sequential treatment of the oligosaccharide with -fucosidase and with β-galactosidase removed the fucose and a galactose residue. Methylation of the residual oligosaccharide showed the presence of a terminal N-acetylglucosamine and a N-acetylgalactosamine 2,3- and 3-linked galactose, 4-linked N-acetylglucosamine, and 3,6-linked N-acetylgalactosaminitol.

Periodate oxidation-borohydride reduction followed by methylation of the degraded oligosaccharide showed the presence of a terminal and 3-linked galactose, 4-linked N-acetylgluco, samine and 3-linked N-acetylthreosaminitol

Methylation of the oligosaccharide showed the presence of a terminal N-acetylglucosamine, N-acetylgalactosamine, galactose, and a fucose residue; 3-linked and 2,3-linked galactose; a 4-linked N-acetylglucosamine and 3,6-linked N-acetylgalactosaminitol. The results of these experiments showed that oligosaccharide N-3 is a decasaccharide with the possible sequence, linkage and anomery as shown in Fig. 2.

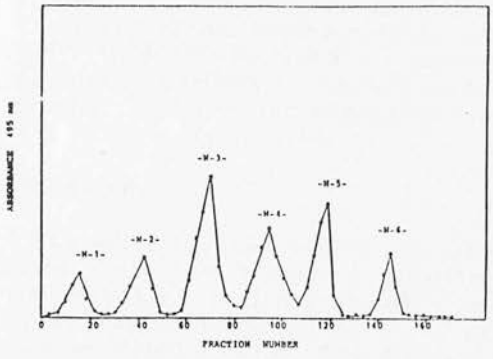


Fig. 2: Separation of oligosaccharides on a column of Bio-Gel P-6. Six fractions (N-1, 0.2 mg; N-2, 0.3 mg; N-3, 1.2 mg; N-4, 1.4mg; N-5, 1.8 mg; N-6, 0.6 mg) were obtained.

Oligosaccharide N-4

Oligosaccharide N-4 was purified on DEAE-Sephadex A-50 and was homogeneous in paper chromatography (solvent A and B).

Periodate oxidation-borohydride reduction completely removed fucose, N-acetylgalactosamine and galactose residues, and converted N-acetylgalactosaminitol to 2-acetyamido-2-deoxythreitol and N-acetylglucosamine was receoved unchanged. Sequential treatment of the oligosaccharide with αfucosidase and β-galactosidase (C. lampas) showed the loss of only fucose.

Methylation of the oligosaccharide after α-fucosidase treatment showed the presence of terminal galactose and

N-acetylgalactosamine, 4-linked galactose, 4-linked N-acetylglucosamine and 3,6-linked N-acetylgalactosaminitol. Methylation of the sequentially α-fucosidase and -N-acetylgalactosaminidase treated oligosaccharide showed the presence of terminal galactose, terminal N-acetylgalactosamine, 4-linked galactose, 4-linked N-acetylglucosamine and 3,6-linked N-acetylgalactosaminitol. Methylation of the native oligosaccharide showed the presence of a terminal fucose and N-acetylgalactosamine, 2-linked and linked galactose, 4-linked N-acetylglucosamine, and 3,6-linked N-acetylgalatosaminitol. These results suggest that oligosaccharide N-4 is a hexasaccharide with the structure given in Fig.2. It appeared that terminal N-acetylgalactosamine is linked β or was resistant to -N-acetylgalactosaminidase due to conformational reasons.

Oligosaccharide N-5

Methylation of the oligosaccharide showed the presence of terminal galactose and N-acetylgalactosamine, 3-linked galactose, 4-linked N-acetylglucosamine and 3,6-linked N-acetylgalactosamine. Periodate oxidation-borohydride reduction resulted in total destruction of N-acetylgalactosamine and a residue of galactose and conversion of N-acetylgalactosaminitol to N-acetylthreosaminitol. N-acetylglucosamine and a galactose residue were recovered unchanged. Methylation of the residual oligosaccharide showed the presence of terminal galactose and 4-linked N-acetylglucosamine, suggesting that a galactose in the parent oligosaccaride is linked at C-3 to N-acetylgalactosamine. These results suggest the structure oligosaccharide N-5 as shown in Fig. 2.

Oligosaccharide N-6

The oligosaccharide N-6 was purified by paper chromatography in solvent A followed by chromatography on DEAE-Sephadex A-50, and was homogenous in solvents A and B. Methylation of the oligosaccharide showed the presence of a terminal galactose and 3-linked N-acetylgalactosaminitol. Treatment of the oligosaccharide with β-galactosidase (C.lampas) removed galactose partially. These results provide evidence for oligosaccharide N-6 to be a disaccharide.

Discussion

The separation of secreted glycoproteins from the remaining polymeric materials i.e., proteins, enzymes and lipids was accomplished by gel filtration on Bio-Gel P-200. The glycoprotein component from the Bio-Gel P-200 column was treated with Pronase to remove any contaminating proteins, glycoprotein and bilirubin. It is known that proteolytic enzymes degrade a minor glycoprotein component of mucus glycoprotein [11]. Secreted glycoproteins are known to undergo proteolysis when treated with the Pronase, particularly in the hydrophobic region of the protein moiety [12]. A major breakdown of the glycoprotein is unlikely with the mild Pronase treatment that was given in this investigation, however. Fractionation of the Pronase-treated glcoprotein on the Sepharose 4B resulted in two fractions. The major fraction in ion-exchange chromatography resulted in a single high molecular weight component. Despite the fact that the glycoprotein are known to be degraded [12] by the Pronase, a high molecular weight glycoprotein, homogeneous, albeit polydisperse, in agarose electro-

phoresis and free of contaminting proteins was obtained. A glycoprotein, free of cross-linked protein fraction containing cystine, as is observed in the case of bovine cervical mucus [13,14], was obtained.

Treatment of the glycoprotein with alkaline borohydride resulted in a mixture of oligosaccharide alditols which was fractionated on Bio-Gel P-6 and was further purified by ion-exchange and paper chromatography affording four neutral oligosaccharides. The oligosaccharides structures characterized can be divided into two groups on the basis of the known core structures:

(i) Galβ (1→3) Gal NAc-ol
(ii) Gal (1→3) Glc NAcβ(1→6)Gal NAc-ol

The chain elongation of carbohydrate occurs on these two structures resulting in structures that has been characterized and is shown in Fig. 2. For gallabladder glycoproteins the structure of carbohydrates chains has not been completely identified and these pontial structures bear similarly to the secreted glycoprotein oligosaccharides core structures.

A variety of carbohydrate chain lengths in mucins [15] and in blood group active glycoproteins [16-18] are known, and the heterogeneity of gallabladder glycoprotein could be even complex because of the changing physicochemical behaviour.

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J.Chem.Soc.Pak. Vol. 10, No. 1, 1988.

Partial Characterization of Neutral Oligosaccharides from a Normal Human Bronchial Secretion

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(Received 14th December, 1985, Revised 25th December, 1986)

Summary: Normal human bronchial secretion was obtained by washing the lungs with normal saline. The lavage was midly centrifuged to remove the cellular debris, dialyzed and lyophilized. The residue was chromatographed on Bio-Gel P-200 and then on Sepharose 2B. The major oligosaccharides. The neutral oligosaccharides, after separation from acidic oligosaccharides, were further purified by gel filtration and ion-exchange chromatography. Five neutral oligosaccharides were characterized using enzymic and chemical procedures, and - following structures are proposed:

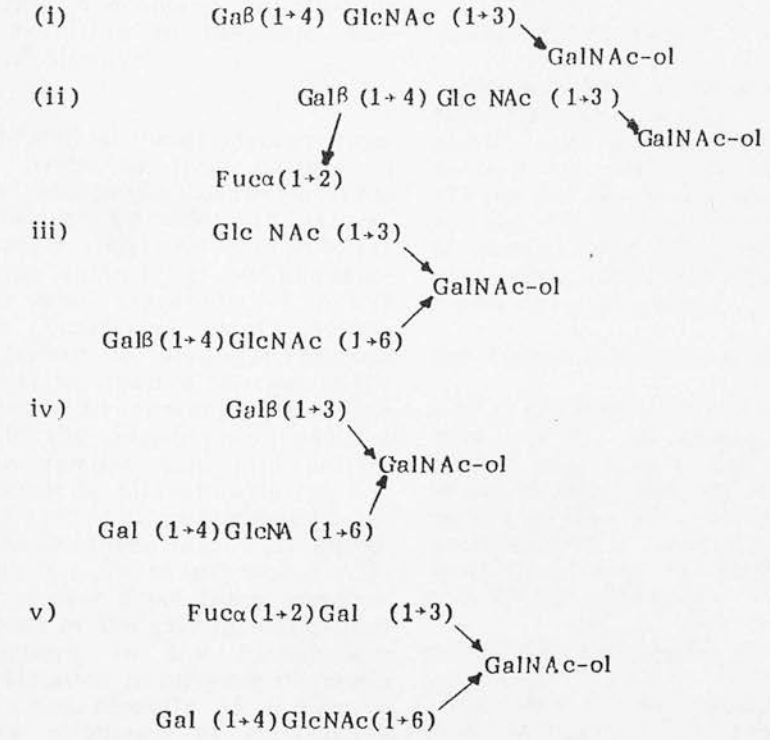

Fig.1: Proposed structure for neutral oligo- saccharides.

This work was supported by Pakistan Science Foundation Grant B-BU/MED (93).

Introduction

The tracheobronchial system is an important component of mucociliary function. This system provides airways with necessary protection by removing pathogenic materials that are inhaled. An important constituent of the tracheobronchial system is the epithelial secretion, mucus, that consists of glycoproteins, protein and lipids. The mucus possesses rheological properties which are important for its physiological functions. The rheological properties are necessarily imparted to the secretion by the glycoproteins and are mainly due to carbohydrates.

In pathological conditions, characterized by hypersecretion and changes in the rheological behaviour of the mucus, the mucociliary function is altered, resulting in bronchial disfunction of airways.

Human bronchial mucus glycoproteins (mucins) prepared from lavages of macroscopically healthy bronchial areas have shown to be acidic in nature, predominantly sialylated, and in which the average chain length of oligosaccharide is relatively small [1]. Acidic functions, carboxyl and sulfate groups, present in the sugar residues provide electronegative charges to the glycoproteins. In several glycoproteins changes in the quantity and position of the neuraminic acid and sulfate groups result in alteration in the behaviour of these macromolecules [2-5]. Also the asialoglycoproteins are knwon to be susceptible to proteases [2]. It would appear from these chemical modifications in the glycoproteins that acid functions in the biopolymers impart resistance to enzymic degradation that may directly or indirectly effect the efficiency of mucociliary system.

Despite various studies performed on human secretion no detailed structural characterization of carbohydrates, so necessary for mucocilary function, have been achieved; yet because of the difficulty of obtaining workable amounts of normal human bronchial mucin glycoproteins. Most studies related to human bronchial mucins have mainly concerned material isolated from sputum of patients suffering from bronchial hypersecretion [6-8].

Materials and Methods

Collection of bronchal secretion

The secretion was obtained by aspirating bronchus with normal saline. The secretion was frozen and maintained in the frozen state prior to use.

Analytical methods

The hexose content of the glycoproteins was estimated by the phenol sulphuric acid method [9] the protein content by measuring absorbance at 278 nm and the neuraminic acid content by the thiobarbituric acid procedure of Warren [10] after acid hydrolysis with 50mM sulphuric acid, or by gas liquid chromatography.

Gas-liquid chromatography (GLC)

GLC determinations of carbohydrate moiety of the glycoproteins were performed according to the procedure of Reinhold [11]. GLC-MS (mass spectrometry) of the methylated sugars was performed on a Varian MAT 731 instrument fitted with the combined EI, CI and FD ion source.

Column chromatography

Bio-Gel P-200 (Bio-Rad laboratories) and Sepharose 4B (Pharmacia fine chemicals) columns were run in 50mM

sodium phosphate (pH 7.0) containing 0.02% sodium azide. The DEAE-cellulose (Whatman) column was eluted with 0.1M NaCl followed by a gradient of 0.1M to 1M sodium chloride containing 10mM HCl. Bio-Gel P-4 and Bio-Gel P-6 (200-400 mesh) chromatography was performed in 50mM pyridine-acetic acid. The sugar containing fractions, detected by phenol sulphuric acid procedure and/or by counting tritium, were combined and lyophilized. DEAE-Sephadex A-50 columns were run with 50mM - 0.5M phosphate buffer (pH 7.0) followed by 0.1M - 0.5M LiCl, portions containing carbohydrates were combined and desalted on a column of Bio-Gel P-2. Agarose gel electrophoresis was performed in 50mM barbital buffer (pH 8.2) on glass slides containing 1% agrose. Polyacrylamide and agrose gel electrophoresis was carried out according to the method of Holden et al., [12]. The agarose and polyacrylamide slides were stained with Amido black or Coomassie blue, and with periodate-Schiff reagent. For agrose and polyacrylamide electrophoresis nearly 0.3mg per ml of each substance was used and each well had 25 µl of the solution.

Purification and protease treatment of the mucus glycoprotein

The crude mucus was solubilized in 50mM sodium monophosphate (pH 7.0) containing 0.02% sodium azide by stirring for 24 hours. The cellular debris and other insoluble materials were removed by centrifugation (2500 rev./min.), the supernatant was dialyzed and the nondiffusible material was lyophilized to give the crude mucus glycoprotein. The residue (50 mg) was dissolved in 50mM sodium monophosphate (60 ml, pH 7.0) containing 0.02% sodium azide by stirring for 16 hours at 4°C. The solution was applied to a column (2.0 x 60 cm) of Bio-Gel P-200 (50-100 mesh). The carbohydrate and protein containing fractions were pooled, the pH was adjusted to 5.5 with 4M acetic acid, and the solution was dialyzed and lyophilized to give the purified mucus glycoprotein (20 mg). The purified glycoprotein (15mg) in 50mM sodium monophosphate buffer (30 ml, pH 8.0) containing 0.1% sodium azide was stirred at 22°C until solubilized and then treated with insolubilized Pronase (20mg, Enzite protease, Miles laboratories, Inc.). The mixture was incubated for one day at 37°C with stirring. Another addition of prewashed enzyme (8mg) was made and the solution was incubated for another three days. The suspension was centrifuged, and the residue was washed with buffer. The pH of the supernatant was adjusted to pH 5.0 with acetic acid and the solution dialyzed against distilled water. The nondialyzable material was lyophilized and the residue (12 mg) was applied to a column (2.5 x 60 cm) of Sepharose 2B. Fractions containing carbohydrate and protein were combined and dialyzed and the nondiffusible material was lyophilized. The Sepharose 2B purified carbohydrate containing material was further chromatographed on a column of DEAE-celluse, the carbohydrate and protein containing fractions eluted with a gradient of lithium chloride were combined and dialyzed, and the retentate was lyophilized to give the Pronase-treated glycoprotein (8mg). The glycoprotein was examined by agarose and polyacrylamide gel electrophoresis.

Sedimentation equilibrium studies

The sedimentation equilibrium study was performed on a solution of Pronase-treated glycoprotein (1.5 mg) in a ml of 6M guanidine hydrochloride -0.05M tris (pH 7.0), dialyzed for 48 hours against the same buffer, with

the meniscus - depletion sedimentation method of Yphantis [13] on a model E Ultracentrifuge. A value of 0.637 for partial specific volume was used. The molecular weight was calculated by extrapolation of the point average molecular weights of infinite dilutions.

Alkaline borohydride treatment

The Pronase-treated glycoprotein (7 mg) was treated with 2M sodium borohydride in 50mM NaOH according to the procedure of Iyer and Carlson [14].

A 0.2% solution of the glycoprotein in 2M sodium borohydride containing 5mCi of sodium borotritide in 0.05M NaOH was incubated for 18 hours at 45°C. Following alkaline borohydride treatment the mixture was adjusted with 4M acetic acid to pH 5.4. The reaction mixture was applied to a column (2.5 x 68 cm) of AG 50 W-X8 (H, 100-200 mesh) ion exchange resin containing 150 fold excess with respect to the sodium ions from NaOH and $NaHB_4$. Oligosaccharides, oligopeptides and glycopeptides were eluted with water and 50 mM acetic acid. Reduced oligosaccaride were separated into neutral and acidic oligosaccharides on a column (3.4 x 70 cm) of AG 1-X2 OAc-(200-400 mesh). The column was washed with water, 0.5mM pridine -acetic acid (pH 5.4) and then with 0.1M - 1.5M acetic acid in the cold. The acidic oligosaccharides were further chromatographed on Bio-Gel P-6 (200-400 mesh) in 10mM pyridine-acetic acid (pH 5.4) followed by paper chromatography or paper electrophoresis. Paper chromatography was performed in solvents: (A) ethyl acetate-pyridine-acetic acid-water (5:5:1:3) (B) butanol-propanol-1.10M acetic acid (1:2:1 V/V).

Enzyme degradation

Oligosaccharides were digested with some or all of the following enzyme:

i) α-L-fucosidase from beef epididymis (Sigma, 10mM sodium citrate buffer, pH 6.0 at 37°C for 50 hours) and from emulsin (50 mM sodium citrate buffer, pH 5.0, 50 hours at 37°C).

ii) β-galactosidase from Aspergillus niger (Sigma, 50mM sodium-citrate, pH 4.1, 70 hours at 37°C).

iii) β-galactosidase from Escherichia coli (Boehringer, 50mM sodium phosphate, pH 7.0, 48 hours at 37°C), and β-galactosidase form Charonia lampas (Miles, 50mM sodium citrate buffer, pH 4.0, 48 hours 37°C).

iv) β-N-acetylglucosaminidase from jack bean (Sigma, 50mM sodium citrate buffer, pH 4.5, 48 hours at 37°C.

Methylation analysis

Methylation of oligosaccharides was performed according to the procedure of Hakomori [15]. The methylated oligosaccharides were recovered by partition between chloroform and water. The methylated product was converted into monomers by treatment with 2M trifluoroacetic acid at 105°C for three hours. The solution was diluted (x10) after cooling, and freeze-dried. The residue in water-methanol (4:1) was treated with sodium borohydride (25 mg) for 12 hours at 4°C and for 3 hours at 22°C. The excess of sodium borohydride and sodium ions were removed simultaneously by treatment with AG 50W-X8 (H, 100-200 mesh) ion-exchange resin, reduced sugars were eluted with water, methanol and methanolic-

ammonia. The combined washings were evaporated, and the residue in methanol was repeatedly evaporated to remove boric acid. Finally, the residue was acetylated with pyridine (0.5 ml) and acetic anhydride (0.4 ml) for 12 hours at 22°C and the methylated alditols were examined by g.l.c. and g.l.c. - m.s.

Periodate oxidation-sodium borohydride reduction

The reduced oligosaccharides (300 µg)were oxidized with periodate (0.1M) at 4°C for 12 hours, then at room temperature for 8 hours.

The excess of periodate was destroyed by addition of 1,2-ethanediol and the oxidized material was reduced by treatment with sodium borohyride (25 mg per mg of the starting material) for 12 hours at 4°C, followed by another addition of sodium-borohydride (5 mg per mg of oligosaccharide), and the reduction was allowed to proceed at room temeprature for 6 hours. Excess borohyride and sodium ions were removed by adding an excess of AG 50W-X8 (100-200 mesh) ion-exchange resin. The resin was filtered off, and boric acid was removed by repeated evaporation with methanol. The residue was treated with 0.25M sulfuric acid for 2.5 hours, the acidic solution was treated with AG1-X8 (OAc-100-200 mesh). After methanolysis with 0.5M methanolic hydrogen chloride for 20 hours at 80°C the oxidized oligosaccharides were examined by g.l.c. for the presence of sugars. A sample of 2-acetamido-2-deoxyserinol was obtained by N-acetylation of commercially available 2-amino-2-deoxy-serinol (Sigma).

Samples of N-acetylthreosaminitol and 2-N-acetylarabinosaminitol alongwith N-acetylated serinol were obtained by mild (10mM) periodate oxidation of N-acetylgalactosaminitol for 30 minutes at 220°C.

Results

Purification and Characterization of Pronase-degraded cervical mucus glycoproteins

The bronchial glycoproteins obtained from normal human beings were purified by gel filtration on Bio-Gel P-200 followed by very mild treatment with Pronase. Degeraded normal human bronchial mucus glycoproteins.

Pronase-treated glycoproteins were fractionated on Sepharose 4 B. The main fraction (60%) showed the presence of a single glycoprtein and in gel electrophoresis barely entered the polyacrylamide gel. In agarose (1%) the glycoprotein entered the gel and exhibited a single dispersed component.

Preparation of oligosaccharide-alditols

The glycoprotein was subjected to alkaline borohydride-reductive cleavage yielding a mixture of oligosaccharide-alditols. A decrease of serine and threonine and a corresponding increase of alanine and the appearance of γ-aminobutyric acid were detected. The neutral oligosaccharides eluted from the column of AG 1-X2 were separated on a column of Bio-Gel P-6 into five oligosaccharide fractions (see Table-1, Fig. 1).

Oligosaccharide N-1

The oligosaccharide purified on Bio-Gel-P-6 was identified as a single component in solvent systems A and B. Methylation of the oligosaccharide and identification of the products showed

Table-1: Carbohydrate composition of purified oligosaccharides

Oligosaccharides	Fucose		Galactose		N-Acetyl-Glucosamine		N-Acetyl-Galactosaminitol	
	%	MR[a]	%	MR[a]	%	MR[a]	%	MR[a]
N-1			26	0.94	30	0.88	34	1.0
N-2	15	0.67	20	0.86	26	0.86	30	1.00
N-3			21	0.99	49	0.94	26	1.00
N-4			38	1.60	24	0.90	29	
N-5	14	0.75	34	1.82	19	0.83	23	

[a]MR : Molar ratio is releative to N-acetylgalactosaminitol

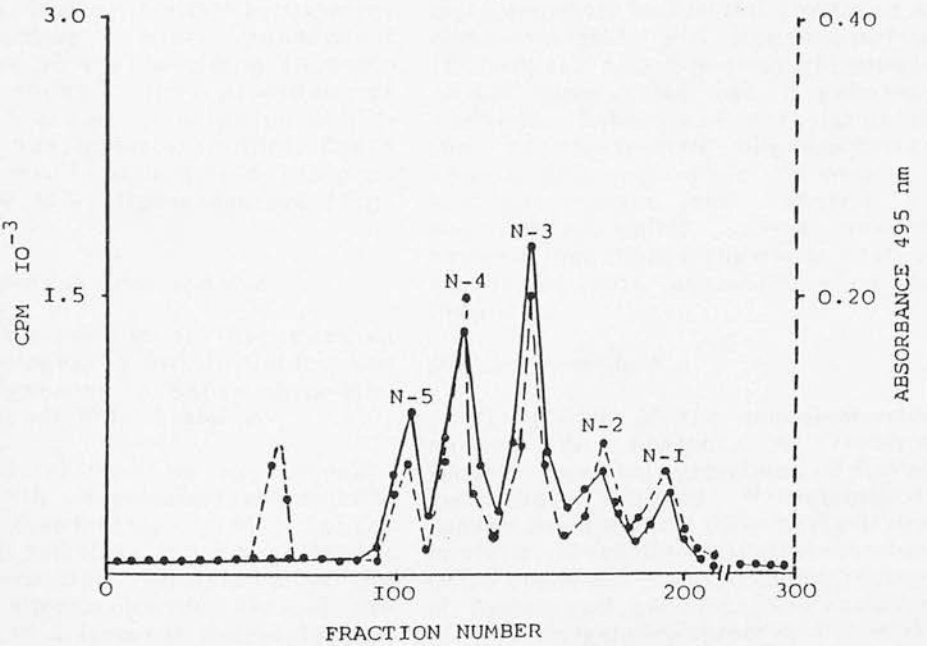

Fig.2: Separation of oligosaccharides on a column of Bio-Gel P-6. Five fractions (N-1, 1.2 mg;N-2, 1.4 mg; N-3, 1.6 mg; N-4, 1.7 mg; N-5, 1.2 mg) were obtained.

a terminal galactose, 4-linked N-acetylglucosamine and a 3-linked N-acetylgalactosaminitol. Periodate oxidation-borohydride reduction and identification of the resistant products showed the presence of N-acetylglucosamine and N-acetylthereosaminitol. Treatment of the oligosaccharide with ß-galactosidase removed a galactose residue. These results suggest the structure N-1 as shown in figure 2.

Oligosaccharide N-2

The oligosaccharide N-2 was purified by chromatography on DEAE Sephadex A-50 and was homogeneous in solvents A and B. The oligosaccharide on methylation showed the presence of terminal fucose, 2-linked galactose, 4-linked N-acetylglucosamine and 3-linked N-acetyl galactosaminitol. Sequential treatment with α-fucosidase, ß-galactosidase, and subsequent methylation of the remaining oligosaccharide showed the presence of terminal N-acetyl glucosamine and 3-linked N-acetylgalactosaminitol. Based on these results structure N-2 is proposed for this oligosaccharide (Fig. 2).

Oligosaccharide fraction N-3

This fraction was further purified by chromatography on Bio-Gel P-4 and was homogeneous in paper chromatography in solvents A and B.

Sequential treatment of the oligosaccharide with ß-galactosidase and ßN-acetylglucosaminidase (96 hours) removed a galactose and a N-acetylglucosamine residue. Methylation of the residual oligosaccharide showed the presence of a terminal N-acetylglucosamine, and 3,6-linked N-acetylgalactosaninitol residues. Methylation of the oligosaccharide showed the presence of a terminal N-acetylglucosamine, a terminal galactose, a 4-linked N-acetylglucosamine and 3,6-linked N-acetylgalactosaminitol. The results of these experiment showed that oligosaccharide N-3 is a tetrasaccharide with sequence, linkage and anomery as shown in Figure 2.

Oligosaccharide N-4

Oligosaccharide N-4 was purified on DEAE Sephadex A-50, and was homogeneous in paper chromatography (solvents A and B) and electrophoresis. Periodate oxidation-borohydride reduction completely removed galactose residues, and converted N-acetylgalactosaminitol to 2-acetamido-2-deoxythreitol. Methylation of the oligosaccharide showed the presence of terminal galactose, 4-linked N-acetylglucosamne and 3- and 6-linked N-acetylgalactosaminitol. Sequential treatment of oligosac-charide with ß-galactosidase (C.Lampas) and ß-N-acetylglucosaminidase showed the loss of galactose residues. Subsequent methylation of the enzyme-treated oligosaccharide showed the presence of terminal N-acetylglucosamine and 3-linked N-acetyl-thresaminitol. These results suggest that oligosaccharide is a tetrasaccharide with structure given in Figure 2.

Oligosaccharide N-5

Methylation of the oligosaccharide showed the presence of terminal fucose, terminal galactose, 2-linked galactose, 4-linked N-acetylglucosamine and 3,6-linked N-acetylgalactosaminitol. Periodate oxidation-borohydride reduction resulted in destruction of fucose and galactose and residues of N-acetylgalactosamine to N-acetylthreosaminitol. N-acetylglucosamine was recovered unchanged. Treatment of the oligosaccharide with α-fucosidase and subsequent methylation of

the residual oligosaccharide showed that the fucose is linked to C-2 of galactose. Sequential treatment of the oligosaccharide with α-fucosidase and βgalactosidase and subsequent methylation of the residual oligosaccharide showed the presence of terminal N-acetylglucosamine and 3,6-linked N-acetylgalactosaminitol. A small amount of terminal galactose was also identified. These results suggest the structure of oligosaccharide A-5 as shown in Figure 2.

Discussion

The separation of bronchial glycoprotein from the remaining polymeric materials i.e., protein and lipids was readily accomplished by gel filtration on Bio-Gel P-200. The glycoprotein component from the Bio-Gel P-200 column was treated with Pronase to remove any contaminating proteins and glycoproteins. it is known that proteolytic enzymes degrade a minor glycoprotein component of cervical mucus glycoproteins [2]. Secreted glycoproteins are known to undergo proteolysis when treated with Pronase, particularly in the hydrophobic regions of the protein moiety [16]. Fractionation of Pronase treated glycoprotein on Sepharose 4B resulted in two fractions. The main fraction in ion-exchange chromatography resulted in a single high molecular weight component. Despite the fact that glycoproteins are known to be degraded [16] by Pronase, a high molecular weight ($1x10^5$ to $1x10^6$) glycoprotein homogeneous, albeit polydisperse, in agarose electrophoresis and free of contaminating proteins was obtained. Sialic acid in the glycoprotein was present as N-acetylneuraminic acid. This is similar to human mucus [18] and different from bovine which contains N-glycolyneuraminic acid. The glycoprotein was free of cross-linked protein fraction containing cystine, as is observed in the case of bovine mucus [19,20].

Alkaline-borohydride treatment of the glycoprotein resulted in a mixture of oligosaccharide alditols that were fractionated on Bio-Gel P-6 and further purified by ion-exchange and paper chromatography affording five neutral oligosaccharides. The oligosaccharides structures characterized can be divided into three groups on the basis of the core structures:

i) Galß(1→3)Gal-NAc-ol
ii) GlcNAcß(1→3)Gal-NAc-ol
iii) HlcNacß(1→6)Gal-Nac-ol

It appears that the biosynthesis or the chain elongation of the oligosaccharides occurs in core structures resulting in the structure that has been characterized and shown in Figure 2. The glycoprotein obtained from the normal bronchial secretion was isolated in very small quantity and a large number of secretions from different secretor types were combined. The heterogeneity observed in the carbohydrate chains perhaps, arises, due to mixing of secretions. A variety of carbohydrate chain lengths in mucins [6,8,21] and in blood group active glycoproteins [22-24] is known, and the heterogeneity of bronchial mucus glycoproteins could be even wider.

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Chemical Structure of Shark Cartilage Chondroitin Sulfate[#]

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Received 14 December, 1988, revised 2nd February, 1989.

Summary: The chemical structure of chondroitin sulfate isolated from shark cartilage was studied by the methylation procedure. Reduction of the fully methylated polysaccharide, followed by hydrolysis, gave, as the main amino sugar component, 2-amino-2-deoxy-1-O-methyl-D-galactose, characterized by the crystalline hydrochloride, 2-acetamido- and methyl 2-acetamido-2-deoxy-D-glycopyranoside derivatives, and 2,3-di-O-methyl-D-glucose, characterized by the crystalline 2,3-di-O-methyl-1,4,5,6-tetra-O-p- phenylazobenoyl-D-glucitol derivative. A similar procedure, applied to the desulfated chondroitin sulfate, gave, as one of the main components, 2-amino-2-deoxy-4,6-di-O-methyl-D-galactose, similarly characterized as the 4-methyl derivative. Gas-liquid chromatography of the hydrolyzate of the desulfated, methylated, and reduced polysaccharide indicated the presence of the 2,3,1-trimethylether of D-glucose in the proportion 1:6, relative to the 2,3,6-trimethyl ether. The neutral sugar fraction contained mainly 2,3-di-O-methyl- glucose, but also some of the 2-O- and 3-O-monomethyl derivatives. These results establish the basic structure of the polysaccharide as chondroitin 6-sulfate, i.e. (1 3) - (2-acetamido-2-deoxy D- galactopyrano 6-sulfate) (1 4)-β-D-glucopyranuronoglycan.

Introdcution

Meyer et al. [1,2] reported the isolation, after alcohol fractionation, of three isomers of chondroitin sulfate that were designated A,B, and C**. Chondroitin 6-sulfate (Chondroitin sulfate C), was found to be a minor component of such various connective tissues as umbilical cord, skin, costal cartilage, heart valves, and aorta, and the main polysaccharide of a chordoma tumor [2]. A polysaccharide, showing the same i.r. spectrum as chondroitin 6- sulfate, was found to be the main polysaccharide present in nucleus pulposus [4,5] and shark cartilage [6]. From indirect evidence [5,7], later supported by the study of the i.r. spectra of synthetic ester sulfate drivatives [8,9] and of the products of enzymic degradation [3], the structure (1 3)-O-(2-acetamido-2-deoxy-β-D-galactopyrano 6- sulfate)- (4)-O-β-D-glucopyranuronoglycan was suggested for chondroitin 6-sulfate.

With the object of establishing unequivocally the detailed structure of the chondroitin 6-sulfate molecule***, we have applied the methylation technique to a chondroitin sulfate, isolated from the cartilage of shark head that had the typical absorption in the i.r. spectrum at 820 cm^{-1}. The methylation procedure applied in the present work was similar to that which established the chemical structures of chondroitin 4-sulfate [11], and dermatan sulfate [12], namely, methylation of the polysaccharide before and after desulfation [13] with methanolic hydrogen chloride.

Results and Discussion

The chondroitin 6-sulfate isolated from shark head cartilage was found to be homogeneous by gel chromatography (Fig. 1), by elution with a salt

[#]This investigation was supported by grant AM-03564 from the National Institute of Arthritis, Metabolism, and Digestive Diseases.

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**These names correspond, in the nomenclature generally used at the present time, to chondroitin 4-sulfate, dermatan sulfate, and chondroitin 6-sulfate, respectively. The name of "chondroitin sulfate D" has been proposed to describe a chondroitin sulfate isolated from shark cartilage[3]; in view of the results described in this paper, the name "oversulfated chondroitin 6- sulfate" is proposed instead.

***The name chondroitin 6-sulfate is applied solely to the polysaccharide chain obtained by mild alkali degradation and not to the proteoglycan molecule from which it derives[10].

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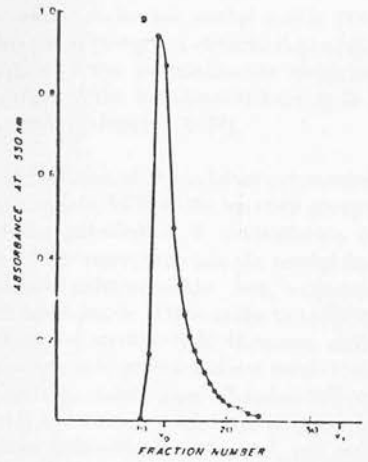

Fig. 1: Gel chromatography of chondroitin 6-sulfate (0.3 g), isolated from shark head cartilage, on a column (0.8 x 64 cm) of Sephadex G 200 in 0.2M sodium chloride. The fractions were analyzed with the carbazole reagent [14]. V_o, void volume: V_T, total volume.

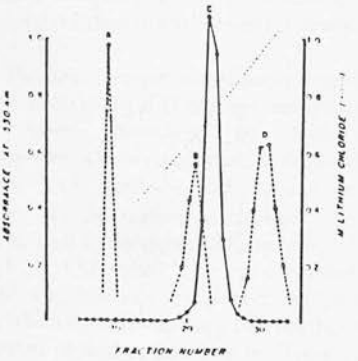

Fig. 2: Elution at 60° from a column of O-diethylaminoethyl- cellulose DE-52 (1x5 cm) with a gradient of lithium chloride buffered at pH 4. Two chromatograms (o and o) super imposed. A, hyaluoronic acid; B, chondroitin 4-sulfate from bovine nasal septa; C, chondroitin 6-sulfate (1.3 mg) from shark head cartilage; D, hog intestinal heparin. The fractions (2.5 ml) were analyzed with the carbazole reagent [14].

gradient from an ion-exchange cellulose column (Fig. 2), by a cetylpyridinum precipitation, and by electrophoresis. The elementary analysis showed the presence of approximately equimolar proportions of uronic acid, nitrogen, sulfate, and acetyl groups, and an ash content corresponding to two molecules of sodium per repeating unit. A molecular mass superior to 40 000 daltons was estimated by gel chromatography on Sephadex G-200. On the basis of the optical rotation and i.r. spectrum (Fig. 3), the shark cartilage chondroitin sulfate was found to be identical with the chondroitin 6- sulfates isolated from chordoma tumor [2], umbilical cord [2,5], and intervertebral disc [4,5].

Strong acid hydrolysis gave only 2-amino-2-deoxy-D-galactose, whereas mild acid hydrolysis gave the disaccharide chondrosin [15,16] [2-acetamido-2-deoxy-3-O-(β-D-glucopyranosyluronic acid)-D-galactose]. Enzymic degradation with a *Proteus vulgaris* chondroitinase gave a compound identified by color reaction and electrophoresis as an unsaturated, monosulfate ester of a disaccharide. Only a trace-amount of a compound similar to the disaccharide disulfate obtained by Suzuki [3] was observed, but no disacharide was devoid of sulfate groups. This observation rules out the possibility of a structure composed of a mixture of disaccharide units possessing more than one and less than one sulfate group. The presence of two or more sulfate groups located on some of the disaccharide units of the shark cartilage presently studied is negligible (about 1 disaccharide disulfate unit per 20 disaccharide monosulfate units).

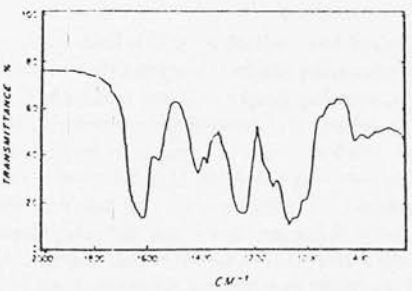

Fig. 3: Infrared spectrum of chondroitin 6-sulfate, isolated from shark head cartilage.

The detailed chemical structure of the polysaccharide was examined by the methylation procedure previously described for hyaluronic acid [17], dermatan sulfate [12], and chondroitin 4-sulfate [11]. Although this procedure required more time and manipulation than the more recent procedures based on the permethylation with sodium

methylsulfinyl carbanion methyl iodide [18-20], it presents the advantage of minimal degradation and desulfation of the polysaccharide chain, and N-methylation of the 2-acetamido-2-deoxy-D- galactopyranosyl residues [17,20-22].

Methylation of the original polysaccharide is nearly complete, 96% of the hydroxyl groups available being substituted, if oversulfation is considered. After conversion into the methyl ester, the methylated polysaccharide was reduced with sodium borohydride [23] in order to facilitate both hydrolysis and methanolysis. However, about 30% of the uronic acid groups did not react. Hydrolysis with sulfuric acid gave 2-amino-2-deoxy-4-O-methyl-D-galactose, which was characterized by crystalline hydrochloride, N-acetyl, and methyl N-acetyl-α-D- glycopyranoside derivatives. On paper chromatography, in addition to the spot corresponding to the 4-methyl ether of the 2-amino 2-deoxyhexose residues, faint spots corresponding to a nomethylated and to a di-O-methyl derivative of 2-amino-2-deoxyhexoses were observed, but no spot corresponding to a tri-O-methyl derivative.

The neutral sugar components resulting from the reduction of the β-D-glucopyranosyluronic acid units were identified, by high voltage electrophoresis in borate buffer, as D glucose, 3-O-methyl-, 2-O- methyl-, and 2,3-di-O-methyl-D-glucose. The last-named compound was further characterized by formation of crystalline 2,3-di-O-methyl- 1,4,5,6-tetra O-p-phenyllazobenzoyl-D-glucitol. The relatively low yield of the 2,3-dimethyl ether, which represented only two-third of the total mixture of neutral sugars, can be interpreted only as the result of incomplete methylation, or possibly of oversulfation, and not of branching, in view of the proportion of nonreducing end groups determined (see subsequent paragraphs). Similar results have been observed in the study by methylation of dermatan sulfate [12] and chondroitin 4-sulfate [11].

Gas-liquid chromatography of the product of extensive methanolysis [24] of the methylated, reduced polysaccharide showed peaks corresponding to methyl 2-acetamido-2-deoxy-4-O-methyl-αβ-D- galactopyranoside and of methyl 2,3-di-O-methyl- and 2,3,4-tri-O- methyl-αβ-D-glucopyranoside, the last-named compound being present in trace amounts only. These results were confirmed by g.l.c. of the product of trifluoroacetic acid hydrolysis, after its conversion into the glycoside, as well as by g.l.c. of the alditol acetates of the neutral sugars. The presence, in trace proportion, of non-methylated galactosamine observed in paper chromatography, but not in g.l.c. may be the result of hydrolysis of the 4-O-methyl group. No evidence was found by g.l.c. for the presence of derivatives of L-iduronic acid. Admittedly, one-third of the uronic acid units have not been reduced, but previous studies with dermatan sulfate [23] have shown that the L-iduronic acid units are reduced as easily as the D-glucuronic acid units.

The shark cartilage chondroitin sulfate was desulfated by the method of Kantor and Schubert [13]. Despite extensive treatment, about 20% of the sulfate groups were not hydrolyzed, and no further removal was attempted in order to avoid degradation of the glycosidic bonds. The observation that the hydrolysis or methanolysis of a sulfate ester linked to a primary hydroxyl group proceeds slower than that of the ester linked to a secondary hydroxyl group has been previously reported [25], but we do not feel that this different behaviour presents, for this study, a serious limitation to the desulfation method of Kantor and Schubert [13].

After methylation, reduction, and hydrolysis with sulfuric acid of the desulfated polysaccharide, as just described for the original polysaccharide, paper chromatography indicated the presence of a main component having the same mobility as 2-amino-2-deoxy-4,6 di-O- methyl-D-galactose and of minor components corresponding to 2-amino-2-deoxy-D-galactose and its monomethyl ether; no spot corresponding to the 3,4,6-tri-O-methyl derivative of the hexosamine residue was observed. The 4,6 dimethyl ether was identified by crystalline hydrochloride, N acetyl, and methyl N-acetyl-α-D-glycoside derivatives.

After extensive methanolysis, g.l.c. showed peaks corresponding to methyl 2-acetamido-2-deoxy-4,6-di-O-methyl-αβ-D galactopyranoside, and methyl 2,3-di-O-methyl- and 2,3,4 tri-O-methyl-αβ-D- glucopyranoside. These results were confirmed by g.l.c. of the product of hydrolysis with trifluoroacetic acid followed by glycosidation.

In order to ascertain the identity of the terminal, nonreducing residues of the polysaccharide chains, the product of methanolysis was examined by gas-liquid chromatography combined with mass spectrometry. Thus, the presence of the 4,6-di-O-methyl and the absence of the 3,4,6 tri-O-methyl derivatives of 2-amino-2-deoxy-D- galactose were confirmed. The neutral sugars were converted into alditol acetates, and g.l.c. m.s. confirmed that the 2,3 dimethyl ether was the main component. A minor peak at t_R 10.0 showed ions at m/z 233, 189, 173, 161, 117, 73, and 43, characteristic for 1,5,6- tri-O-acetyl-2,3,4-tri-O-methyl-D-hexose [26]. The approximate ratio of 2,3,4-tri to 2,3-di-O-methyl derivative was 1:60. Since the results of the methylation of both original and desulfated polysaccharides indicate that the nonreducing end-groups are not sulfated, the proportion of 1:60 indicates a minimum chain-length of 60 disaccharide units. The evaluation of the size of the chain is very approximate, because parts of the polymer chain may be only partially split off by methanolysis [27]. However, this size is comparable to the minimum molecular-weight of 40,000 daltons estimated by gel-chromatography on Sephadex G-200, and it suggests that the chains are not branched.

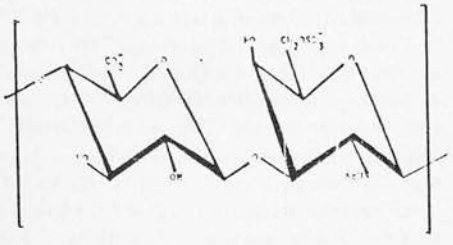

Fig. 4: Repeating unit of chondroitin 6-sulfate.

From the results just described, it is possible to ascribe a repeating structure of (1-3)-O-(2-acetamido-2-deoxy-β-D-galactopyranosyl 6-sulfate)-(1-4)-O-β-D-glucopyranosyl (Fig. 4) to the polysaccharide chain, no microheterogeneity of composition or of structure being evident. The nature of the nonreducing end-groups is difficult to ascertain, because of the small proportion of these groups and the presence of the neutral sugars of the trisaccharide "linking" the polysaccharide chain to the protein backbone. Roden [10] and, also, Otsu et al [27] have proposed that the non-reducing group in the polymer is composed of 2-acetamido-2-deoxy-D-galactose residues. These observations [27] are based on the results of the controlled biosynthesis. Our results disagree with this suggestion, since no 3,4,6-trimethyl ether of the amino sugar could be detected, either by paper chromatography or by g.l.c. -m.s., in the methanolyzates and hydrolyzates of the original, methylated polysaccharide, or of the desulfated, methylated polymer.The trace of dimethyl ether deriving from the original, methylated polymer observed by paper chromatography and g.l.c. could not be ascertained by g.l.c. -m.s. and may have derived from desulfation during methylation. Identification, as the alditol acetate, of 2,3,4- tri-O- methyl glucose derived from the methylated and reduced D-glucopyranuronosyl end-groups is complicated by the possible presence of 2,4,6-tri-O-methyl-D-galactose and 2,3, di-o-methyl-D-xylose derived from the "linking" trisaccharide and was ascertained [25] solely by the presence of the ion at m/z 189, which is absent in 2,4,6-trimethyl ethers. However, this ion may also have derived from 2,3-di-O-methyl-D-xylose, although, in this case, the g.l.c. peak of the corresponding alditol acetate is eluted at a time very different from those of the alditol acetates of 2,3,4-tri-O-methyl-D-glucose and 2,4,6-tri-O-methyl-D-galactose. The possibility that the nonreducing, terminal 2-acetamido-2-deoxy-D-galactopyranosyl residues may be removed by the isolation or methylation procedures [10] is very unlikely, since the glycosidic linkage of a derivative of 2- acetamido-2-deoxy-D-glucose was found [28] to be resistant to 86% potassium hydroxide at 120°. However, nonreducing D-glucopyranosyl end-groups, substituted by a sulfate group at C-4, may be split off under mild alkaline conditions [29], but this structure is not present in chondroitin 6-sulfate.

It is probable that the chondroitin 6-sulfate isolated from various other sources [2,4,5] has a chemical structure similar to or identical with the structure established for the chondroitin sulfate isolated from shark cartilage. No attempt was made, in the present study, to detect and isolate the methylated derivatives of the neutral sugars that link the carbohydrate chain to the protein backbone [10,30]; it is probable that a large proportion of these sugars was degraded during the isolation in alkaline medium. In view of the incomplete methylation, it was not possible to ascertain the location of the small proportion of "additional" sulfate groups, suggested by Suzuki [3] to be linked to the D-glucuronic residues.

Experimental

General methods. Melting points were taken on a hot stage equipped with a microscope, and correspond to "corrected melting points". Optical rotations were determined in semimicro tubes with length of 100 or 200 nm., using a Rudolph photoelectric polarimeter attachment, model 200. Infrared spectra were recorded on a Perkin Elmer infrared spectrophotometer, model 237; the sample (about 1 mg) was dried at 60° in a high vacuum over phosphorus pentaoxide for a minimum of 3 h. and dispersed in a pellet of dry potassium bromide (200 mg). Evaporations were carried out in vacuo with an outside bath temperature kept below 45°. Amounts of volatile solvents smaller than 20 ml were evaporated under a stream of dry nitrogen, with an outside temperature kept below 45°. The microanalyses were performed by Dr. W. Manser, Zurich (Switzerland).

Gas-liquid chromatography and mass spectrometry. Gas-liquid chromatography was performed with a Perkin-Elmer model 900 chromatograph, equipped with a dual ionization detector in a differential mode. The methyl ethers of 2-amino-2-deoxy-D-galactose were analyzed [24] on stainless-steel columns (280cm x 0.3cm) containing: (a) 3% OV-25 on Gas-Chrom Q (60-80 mesh) operated at 195° for 5 min. followed by a rise of 2° per min to 240°; and (b) 3% HI- Eff-8BP on Chromosorb W (AW) (80-100 mesh) operated at 245°. The methyl ethers of D-glucose were analyzed on stainless-steel columns (300cm x 0.3cm) containing: (a) 1% OV-11 (coated on Chromosorb G, high purity), and (b) 3% OV. 17 (coated on Gas-Chrom Q, 80-100 mesh); with an increase of 6.5° per min. The methyl ethers of D-glucose were analyzed as alditol acetates on a stainless steel column (150 x 0.3cm) containing OV-225 (3% on Gas Chrom Q, 100-120 mesh) at a temperature programmed from 150-250°, with an increase of 8° per min. Helium was the carrier gas for all determinations. The liquid phases on the solid supports were obtained from Applied Science Labs., Inc. (State College, Pa 16801).

G.l.c-m.s. was performed on an analytical system consisting of an IBM 1800 computer which was fed raw data generated by a single focusing mass spectrometer (Hitach Perkin Elmer RMU 6), interfaced with a gas chromatograph (Aerograph Hy Fi).

Methanolysis. The methylated polysaccharides (4-5mg), dried to constant weight at 110° in a high vacuum, were heated in a tube closed with a Teflon lined cap with M methanolic hydrogen chloride (1.5 ml) for 16 h at 100°. The solution was evaporated under a stream of nitrogen at 35°. The residue was acetylated by pyridine (0.4 ml) and acetic anhydride (0.25 ml). After 1 h at room temperature, the reaction mixture was evaporated under nitrogen and the residue O-deacetylated by addition of a saturated solution of methanolic ammonia (0.5 ml). After 1 h at 65°, the solution was evaporated under nitrogen at 35°. The residue was dissolved in chloroform and injected directly into the chromatograph. For examination by g.l.c. m.s., the residue was per (trimethyl) silylated according to Reinhold [31].

Hydrolysis with trifluoroacetic acid. The dried, methylated polysaccharide (4-5 mg) was dissolved in 2M trifluoroacetic acid (1 ml), in a tube with a Teflon-lined cap, and the solution heated for 4 h at 100°. The acid was evaporated under a stream of nitrogen at 35° by repeated addition of ethanol and toluene, and the residue acetylated with pyridine (0.5 ml) and acetic anhydride (0.3 ml). After 1 h at room temperature, the reaction mixture was evaporated under nitrogen, and the residue methanolyzed, acetylated, and O-deacetylated, as described in the preceding paragraph.

Alditol acetates. - For the identification of the methyl ethers of D- glucose as alditol acetates, the residues of hydrolysis and methanolysis were treated with an excess of Dowex 50W (X 8, H^+) ion- exchange resin in order to remove the methylated hexosamines (part of the methylated hexosamines are not N-deacetylated during methanolysis, but they do not interfere with the identification of the methyl ethers of D-glucose). The fraction obtained by methanolysis was hydrolyzed with M sulfuric acid for 4 h at 100°, and then neutralized with Dowex 1 (X-8, OH^-) ion-exchange resin. This solution or the solution obtained by Dowex-50 treatment of trichloroacetic acid hydrolyzate was treated with an excess of sodium borohydride at room temperature. The sodium ions were removed by treatment with Dowex 50 (X-8, H^+) ion-exchange resin, and the borate ions as methyl borate with methanol. The reduced sugars were acetylated in the usual manner.

Characterization of chondroitin 6-sulfate. - The sodium salt of shark cartilage chondroitin 6-sulfate was obtained from the Kaken Yaku Pharmaceutical Co., Ltd., Tokyo (Japan). The sample studied (N 556 B) was prepared by Dr. T. Furuhashi from the nasal cartilage of the shark *Prionace glaucus* (Linne), according to the method of Masamune and Osaki [32]. The extraction is based on treatment with 5° sodium carbonate for several hours at 60° in the presence of formaldehyde, followed by adsorption of the proteins on kaolin, and then a Sevag's treatment [33]; $[\alpha]_D^{15}$-12° (sodium salt. c 1.0. water). mol. wt. 50-60,000 (by reduction [34]) lit [7]; $[\alpha]_D$-12° (water).

Anal. Calc. for $C_{14}H_{19}NNa_2O_{14}S$; N, 2.77; S. 6.55: hexosamine, 35.6: uronic acid, 38.6: Na(as sulfate). 28.2 Found; N (Kjeldahl). 2.77: S. 6.55; hexosamine (Elson-Morgan [35]). 35.4: uronic acid (decarboxylation [36]). 38.4: ash., 25.9.

Identical properties were observed after extensive dialysis against distilled water, followed by lyophilization: the i.r. spectrum is reported in Fig. 3.

A sample (0.3 mg), chromatographed on a column (0.8 x 64 cm) of Sephadex G-200 gel, according to the procedure of Wasteson [37], was found in the nonretarded fraction (Fig. 1). A sample (1.3 mg) chromatographed on a column (1 x 5cm) of O-diethylaminoethylcellulose DF 52 at 60° with a gradient of lithium chloride buffered at pH 4 as described by Hallen [38], showed a homogeneous peak at 0.75 M lithium chloride (Fig. 2), as expected for a high molecular weight, slightly oversulfated chondroitin sulfate.

Addition of ethanol to a 1% solution of the calcium salt, obtained by passage of the sodium salt through a Dowex 50 column (Ca^{2+}), precipitated almost all the compound at an ethanol concentration of 40%. Thus, the calcium salt of shark cartilage chondroitin sulfate is less soluble than the calcium salt of mammalian chondroitin 6-sulfate [2], in agreement with the report of Dodgson *et al.* [9].

On paper electrophoresis in barium acetate buffer, according to the procedure of Wessler [39] or in calcium acetate buffer, according to the procedure of Seno *et al* [40], the polysaccharide had the same mobility as that of mammalian chondroitin 6-sulfate. However on electrophoresis on a strip of cellulose acetate in 0.01 M hydrochloric acid immersed in Varsol [41] (Esso Standard Div., Humble Oil and Refining Co., New York, N.Y. 10019), the mobility of the polysaccharide was 10% higher than that of mammalian chondroitin sulfates considered to have one sulfate group per disaccharide unit. Under similar conditions in 0.1M hydrochloric acid, the mobility of the shark chondroitin 6-sulfate was slightly higher than that of mammalian chondroitin 6-sulfate.

After hydrolysis with 0.6 M hydrochloric acid for 2.5 h at 100° and with 6 M hydrochloric acid for 4 h at 100°, only 2 acetamido 2-deoxy- 3-O-(β-D-glucopyranosyluronic acid)-D-galactose and 2-amino-2-deoxy- D-galactose, respectively, could be observed on chromatography on a sulfonated ion-exchange column, as described by Hallen [42].

As 1% solution of the polysaccharide in 20m M sodium phosphate buffer (pH 7.9) containing 10mM sodium fluoride was degraded with a *Proteus vulgaris* chondroitinase [43] for 24 h. at 35°, in parallel with a sample of mammalian chondroitin 4-sulfate. The color formed in the Morgan-Elson test [44] by the digest of the shark cartilage chondroitin sulfate was about 6 times higher than that of the digest of the chondroitin 4-sulfate, although the u.v. absorptions due to formation of a double bond were similar. The digest of the shark cartilage chondroitin sulfate was lyophilized and the residue examined by high voltage electrophoresis according to the procedure of Suzuki [3], the main component corresponded to a disaccharide monosulfate, and only a very small trace of a faster moving component that might correspond to a disaccharide disulfate [3] was observed.

Methylation of chondroitin 6-sulfate. A solution of the polysaccharide (5 g), corresponding to 4.7 g of dry material, in distilled water (200 ml) that had been boiled and cooled under nitrogen, was prepared in a 500 ml glass bottle closed with a rubber stopper. The solution and the 30% sodium hydroxide subsequently added were always kept under nitrogen at 5° and all reagents were added under nitrogen. After the addition of 30% sodium hydroxide (1 ml), dimethyl sulfate (3.6 ml) and 30% sodium hydroxide (5 ml) were added, and the closed bottle was vigorously shaken until all the dimethyl sulfate had dissolved. The addition of

identical amounts of dimethyl sulfate and sodium hydroxide was repeated at intervals of a minimum of 3 h. The pH was frequently determined with litmus paper and, if necessary, additional 30% sodium hydroxide was added to keep the solution alkaline. After 40 additions, the solution was dialyzed for several days at 5° against distilled water (which was frequently changed) and lyophilized to give a partly methylated product (Anal. Found: OMe 13.2). The procedure was repeated once, since preliminary experiments had shown that futher repetitions of the methylation process decreased the yield of the product without increasing the degree of methylation. A total weight of 3.7 g (about 70% yield) was obtained. These analytical results indicate a degree of methylation of at least 90%. Two more samples were similarly methylated with the same results.

Anal: Calc. for $C_{17}H_{25}NNa_2O_{14}S$ (fully methylated sodium chondroitin sulfate), N, 2.44; OMe, 16.40; ash (as sodium sulfate). 25.8 Found: N, 2.80; OMe, 15.27 ash, 23.8.

Reduction of methylated chondroitin 6-sulfate. A suspension of the methylated polysaccharide (2.1 g, dried in vacuo at 60° in the presence of phosphorus pentaoxide) in dry 60mM hydrogen chloride in methanol (500 ml) was shaken overnight. The residue was centrifuged off, washed with methanol and ether, and then dissolved in water (60 ml) containing a few mg of sodium hydrogen carbonate. A solution of sodium borohydride (1.5 g in 15 ml of water) was added, and the solution was kept for two days at room temperature, and then dialyzed against distilled water and lyophilized. The treatment with hydrogen chloride and with sodium borohydride was repeated once to give 1.0 g (ca. 60% yield) of dry material. The analytical results indicate that almost 30% of the carboxyl groups were not reduced.

Anal: Calc. for $C_{17}H_{29}NO_{10}$ (fully reduced, methylated, chondroitin): N, 3.44; OMe, 20.4; S, 0.0; ash, 0.0; uronic acid, 0.0. Found: N, 3.96; OMe, 19.5; S, 0.1; ash (as sodium sulfate), 3.2; uronic acid [36], 12.0.

Identification of the products of hyrrolysis and methanolysis of methylated, reduced chondroitin 6-sulfate. - A Preparative isolation of methyl ethers of 2-amino-2-deoxy-D-galactose. The methylated, reduced polysaccharide (530 mg, after drying) was hydrolyzed with 2M sulfuric acid (50 ml) for 15 h at 100°. After being cooled, the solution was passed through a column (90 ml) of Amberlite IR-45 (AcO^-) and then a column of Dowex 50 (40-60 mesh, H^+) ion-exchange resins. The columns were washed with distilled water and the eluate was lyophilized to give a neutral hexose fraction (193 mg). The column of Dowex 50 was eluted with M hydrochloric acid (400 ml), and the solution was evaporated to give a semicrystalline residue (420 mg), which could not be recrystallized. It was chromatographed on a column of Dowex-50 ion-exchange resin with 0.3 M hydrochloric acid, according to the procedure of Gardell [45], to give four ninhydrin positive syrupy fractions (19,44,213, and 15 mg). Crystallization of the third fraction from methanol-acetone gave 177 mg (44% yield calc. on the basis of the methylated reduced polysaccharide) of 2 amino 2 deoxy 4-O-methyl-D-galctose hydrochloride, dec. ca. 180° $[\alpha]^{20}_D$ + 111 - + 94° (at equilibrium, c. 1.1 water) : lit. [46]: dec. above 178°, $[\alpha]^{20}_D$ + 125° - + 100°; the i.r. spectrum was identical with that of an authentic sample, but different from that of the 6-methyl ether.

Anal. Calc. for $C_7H_{16}ClNO_5$: C, 36.61; H, 7.02; Cl, 15.44; OMe, 13.51. Found; C, 36.42; H, 7.07; Cl, 15.55; OMe, 13.46.

The first two syrupy fractions were further fractioned by paper chromatography in the solvent system described by Fischer and Nebel [47]. Some of the spots gave a positive carbazole reaction [14] that indicated the presence of uronic acid containing methylated disaccharides and two faint spots having the mobility of 2-amino-2-deoxy-D-galactose and of its 4,6-dimethyl ethers were detected, but no spot corresponded to the trimethyl ether; the last crystalline fraction was probably ammonium chloride.

The third crystalline fraction was further characterized: (a) By N-acetylation in the usual manner to give, in low yield, 2 acetamido-2-deoxy-4-O-methyl-β-D galactose, after recrystallization from methanol acetone, m.p. 198°, $[\alpha]^{20}_D$ + 102 - + 85° (at equilibrium, c. 0.6 water): the compound showed no depression of m.p. in admixture with authentic material and the same low yield of color (2%) in the Morgan Elson test [48]: the i.r. spectrum was the same as that of an authentic

sample, but different from that of the 3 and 6-methyl ethers: lit [24]: m.p. 197-199°, $[\alpha]^{20}_D$ + 102 → + 85° (at equilibrium, water).

Anal. Calc. for $C_9H_{17}NO_6$: C, 45.95, H, 7.28. Found: C, 45.93; H, 7.34.

(b) By acetylation with acetic anhydride pyridine in the usual way, followed by heating for 2 h at reflux with 0.5 M hydrogen chloride in methanol to give, in low yield after recrystallization from methanol-ether, methyl 2 acetamide-2-deoxy 4-O-methyl-α-D-galactopyranoside, m.p. 225-226°, showing no depression of the m.p. in admixture with authentic compound: lit [46] m.p. 241-242° (new determination showed m.p. 225-227°).

Anal. Calc. for $C_{10}H_{19}NO_6$: C, 48.18; H, 7.68. Found; C, 48.10; H, 7.66.

B. Preparative isolation of methyl ethers of D-glucose. The neutral hexose fraction (190 mg. see A) was chromatographed on a column (1.8 x 30 cm) of charcoal (Dacro G-60) - Celite with a gradient of borate buffer ethanol-water according to the procedure of Barker *et al* [49], to give D-glucose (6 mg), 3-O-methyl-D-glucose (16 mg), 2-O-methyl-D-glucose (39 mg), and 2,3-di-O-methyl-D- glucose (10 mg) identified by high voltage electrophoresis [50] and paper chromatography in the solvent system (4:1:5 v/v, top phase) 1-butanol-ethanol-water with 1% ammonia [51], 2,3-di-O- methyl-D-glucose (98 mg) was characterized by reduction to the glucitol derivative and preparation of the crystalline 1,4,5,6- tetra-O-(p-phenylazobenzoyl) derivatives [52], m.p. 160-170° (260 mg. after crystallization from benzene-hexane, 30% yield calc. from fully methylated polysaccharide with 70% reduction of uronic acid residues); further recrystallization from the same solvent mixture and from ethyl acetate raised the m.p. to 178-180°: $[\alpha]^{20}_D$ + 102° (c 0.8 benzene); the compound showed no depression of m.p. in admixture with authentic material and the same i.r. spectrum as that of the authentic material: lit [52]: m.p. 180°, $[\alpha]_D$ + 104° (benzene).

Anal. Calc for $C_{60}H_{50}N_8O_{10}$: C, 69.09; H, 4.83; N, 10.74; OMe. 5.95. Found; C, 68.98; H, 4.86; N, 10.71; OMe. 5.94.

C. *G.l.c. identification.* The products of methanolysis, examined on HI EFF 8BP, showed the presence of (a) traces of a compound eluted at t_R 1.00: (b) of a minor component corresponding to methyl 2-acetamido-2-deoxy-4,6-di-O methyl-α, β-D- galactopyranoside (t_R 1.33): and (c) a major component corresponding to methyl 2-acetamido-2-deoxy-4-O-methyl-D- galactopyranoside (t_R 2.98): the t_R are relative to methyl 2- acetamido-2-deoxy 3,4,6-tri-O-methyl-α-D galactopyranoside. Chromatography on OV-25 indicated the same compounds.

The products of trifluoroacetic acid hydrolysis, examined as glycosides on OV 11, showed the presence of methyl 2,3,4-tri-O-methyl-αβ-D-glucopyranoside (t_R 0.76), methyl 2,3-di-O-methyl-β- D glucopyranoside (t_R 0.91), and methyl 2,3-di-O-methyl- α-D glucopyranoside (t_R 1.00); the t_R are given relative to that of the last named compound and the proportion of 2,3,4 trimethyl ether to 2,3 dimethyl ethers was ca. 1 to 60.

Chromatography on OV-17 indicated similar results. The products of the same hydrolysis, examined as alditol acetates on OV-225, showed the presence of 1,5,6-tri-O-acetyl-2,3,4-tri-O-methyl-D-glucitol (T_R 0.91) and of 1,4,5,6-tetra-O-acetyl-2,3-di-O-methyl- D-glucitol (t_R 1.00) (t_R relative to that of the latter compound in the same proportion as that reported for the glycosides.

Desulfation of chondroitin 6-sulfate. The polysaccharide (5.0 g. after drying) was shaken three times with 0.06M hydrogen chloride in methanol (1 liter), each time for one day [13]. The insoluble product was washed extensively with methanol and ether, and then dissolved in a dilute sodium hydrogen carbonate solution. The solution was extensively dialyzed against distilled water and lyophilized to give the methyl ester of desulfated polysaccharide (2.2 g, ca. 55% yield). The analytical results indicate a removal of about 80% of the sulfate groups.

Anal. Calc. for $C_{15}H_{23}NO_{11}$: N, 3.56; OMe, 7.88; S, 0.0; ash, 0.0. Found N, 4.14; OMe, 6.45; S, 1.60; ash (as sodium sulfate), 2.70.

Methylation and reduction of desulfated chondroitin 6-sulfate. The desulfated polysaccharide (2.0 g) was methylated, as just described for the original material to give a product containing 22.6% of methoxyl groups. Repetition of the methylation, followed by dialysis and freeze-drying, gave 1.3 g (ca. 54% yield) of a compound soluble in methanol

but sparingly soluble in water. In view of the presence of 24% of sulfate groups, the polysaccharide was considered fully methylated (OMe theor. 24.6).

Anal. Calc. for $C_{18}H_{28}NNaO_{11}$ (fully methylated chondroitin): N, 3.06; OMe, 27.1; ash (as sodium sulfate), 15.5. Found: N, 3.15; OMe, 24.6; ash, 13.7.

The desulfated, methylated polysaccharide was reduced with sodium borohydride after treatment with methanolic hydrogen chloride, as described earlier. The reduction was incomplete after one treatment, probably because of the low solubility in water, and the procedure was repeated twice.

Identification of the products of hydrolysis and methanolysis of desulfated, methylated, and reduced chondroitin 6-sulfate. A. By isolation of the methyl ethers of 2-amino-2-deoxy-D-galactose. The material obtained after only one reduction (1.2 g) was hydrolyzed with M sulfuric acid (100 ml) for 20 h at 100°, separated into a hexosamine fraction (0.92 g) and a neutral sugar fraction, as described earlier. Paper chromatography indicated the presence of 2-amino-2-deoxy-4,6-di-O-methyl-D-galactose, as main component, and 2-amino-2-deoxy-D-galactose and its monomethyl ether(s) as minor component, but no spot corresponding to the trimethyl ether was present.

Chromatography of the hexosamine fraction (138 mg) on a column (40 x 1 cm) of a sulfonated cation-exchange resin (Rexyn) with 0.3M hydrochloric acid, according to the procedure of Gardell [45], gave some unidentified compounds (31 mg), 2-amino-2-deoxy- D-galactose (2 mg), 2-amino-2-deoxy-O-methyl-D-galactose (16 mg), and 2-amino-2-deoxy-4,6-di-O-methyl-D-galactose (32 mg., ca. 46% yield), identified by paper chromatography and by i.r. spectrum as described earlier.

The hexosamine fraction (421 mg) was N-acetylated, as described earlier, and chromatographed on silica gel (Davison). The semicrystalline fractions eluted with acetone were fractionated on a column (9x2 cm) of charcoal (Darco G-60)-Celite with a gradient of water and ethanol, according to the procedure of Whistler and Durso [53]. The main, crystalline compound (78 mg, 36% yield) was recrystallized from methanol, m.p. 224-225° (lit [24]: m.p. 226-227°), showed no depression of m.p. in admixture with authentic 2-acetamido-2-deoxy-4,6-di-O-methyl-D-galactose [54] and the i.r. spectra of both compounds were identical.

Anal. Calc. for $C_{10}H_{19}NO_6$: C, 48.19; H, 7.63. Found: C, 48.13; H, 7.70.

As described earlier for the 4-methyl ether the 4,6-dimethyl ether was transformed into the methyl D-glycopyranoside showing properties identical with those of an authentic sample [54]

B. By g.l.c. -m.s. identification HI EFF 8BP. The products of methanolysis, examined on HI-EFF-8BF, showed the presence of (a) traces of a compound eluted at t_R 1.00: (b) a major component corresponding to methyl 2-acetamido-2-deoxy-4,6-di-O-methyl-α,β-D-galactopyranoside (t_R 1.33); the t_R are relative to methyl 2- acetamido-2-deoxy-3,4,6-tri-O-methyl-α-D-galacto-pyranoside. Chromatography on OV-25 indicated the same compound. The peak (b) was further identified as the 4,6-dimethyl ether by m.s.[24]. The same technique clearly indicated that no 3,4,6-trimethyl ether was present in peak (a), since the typical ions[24] due to the presence of methoxyl groups at C-3 and C-4 were absent.

Examination of the products of trifluoroacetic acid hydrolysis as glycosides on OV-11 and OV-17 and as alditol acetates on OV-225 gave results identical with those described earlier for the original (not desulfated) chondroitin 6-sulfate. In addition, both peaks of the alditol acetates were examined by m.s., which conclusively established [26] the presence of 1,5,6-tri-O-acetyl- 2,3,4, -tri-O-methyl-D-glucitol.

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From these studies it is concluded that (+)-$[Ni(phen)_2(bpy)]^{2+}$, (+)-$[Ni(phen)(bpy)_2]^{2+}$, (+)-$[Ni(phen)_2gly]^{+}$, $[Ni(phen)_2pyca]^{+}$ and (+)-$[Ni(bpy)_2pyca]^{+}$ ions have Δ configuration of ligands around the metal ion.

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Carbohydrate Moiety of *Plasmodium falciparum*

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(Received 5th August, 1990, revised 11th November, 1990)

Summary: Malaria is widespread disease and its pathological consequences are due to the development and proliferation of erythrocytic stages in the host. Metabolic labeling of the Plasmodium falciparum parasites with [^{3}H]-glucosamine, [^{3}H]-galactose and [^{3}H]-mannose and subsequent purification in gel filteration or SDS-PAGE yielded labeled glycoproteins. Reductive -elimination of the glycoproteins released molecules containing labeled sugars. Processing of the reaction products and acid hydrolysis of the derived sugars followed by paper chromatography suggested the presence of N-acetylglucosaminitol, N-acetylgalactosaminitol, N-acetylglucosamine and galactose. Following reductive β-elimination with NaOH-$NaBH_4$ and acid hydrolysis, the products in paper chromatography showed the presence of labeled components that migrated with standard N- acetylglucosaminitol and serine. Enzymes treatment with hexosaminidase and subsequent paper chromatography suggested the release of N-acetylglucosamine. It is unknown at this stage, whether other glycoproteins of P. falciparum have similar types of glycoproteins or bear different types of linkages between the carbohydrate moiety and the protein core. This study represents the first direct investigation into the composition and structure of P. falciparum glycoprotein. In addition we have demonstrated the existence of terminal O- linked GlcNAc in the parasitic glycoprotein.

The membrane anchor glycoproteins that are known to be present in falciparum contain phosphotidylionisitol complex (PIC). The PIC are under investigation as synthetic model compounds for chemical and immunological studies.

Introduction

Malaria is a major parasitic disease of the developing world and is caused by the unicellular protozoan of the genus *Plasmodium*. Evidence has accumulated to suggest that the pathological consequences of malaria are due to the development and proliferation of asexual stages in the host. In the erythrocytic stages of the parasites development, high molecular weight antigens have been described [1-3]. These high molecular weight antigens, proteins and glycoproteins, are considered to mediate merozoite invasion of erythrocytes [4-5], and host immune response after repeated exposure

have resited in reasonable protection [6-7]. There has been concerted effort to develop a viable vaccine against malaria [3], and researches have focussed on plasmodial antigens. The parasite proceeds through various developmental stages in a complex manner resulting in different antigens that are presented to the host. The immune response elicited are equally complex, stage specific and engage both cell mediated and humoral mechanism [8-10].

An extensive interest has developed to investigate the role of the carbohydrate moiety of the glycoproteins that are synthetized by *Plasmodium falciparum* [11-12]. Although these proteins are likely to be associated with the surface of the merozoites, it is not unlikely that some may be part of the surface of the erythrocytes attacked by the parasite [13-14]. The carbohydrate moieties of the glycoproteins of the asexual blood stages are potentially artigenic [15] and are a possible target for protective immune response. Comprehensive studies on the structure and functions of carbohydrates is vital since the subunit vaccines synthetized by recombinant DNA methods in bacteria and peptides, prepared by chemical procedures lack oligosaccharide chains of native molecules [16].Because of the ineffectiveness of many peptide immunogens that are being tested as potential candidates for vaccines, the significance and determination of the carbohydrates is increasing.The outer surface molecules of *Plasmodium falciparum* merozoites are the site of the first contact between extracellular invasion of the parasite and its host erythrocyte [17]. Since the surface components, proteins and glycoproteins, are thought to participate in the complex process of invasion, the analysis of the merozoite surface components is of immense interest. Because of the known significance of the carbohydrate moiety of the merozoite surface glycoproteins [15-16,18-19] and as practically there is no information about the composition and structure, except for the recent findings of sugar components and the presence of distinct o-glycosyl linkage between N-acetylglucosamine and serine [20], as well as the role of the carbohydrate component of the merozoite surface glycoproteins, the present investigation was conducted.

Materials and Methods

[^{3}H]-Galactose, [^{3}H]-mannose, [^{3}H]-Glucosamine hydrochloride and sodium borotritide were purchased from Amersham International, PTC, Amersham, UK.

In vitro metabolic radio-labelling of P.falciparum and preparation of soluble glycopeptides

The parasite cells of strain M25/ZAIRE were used for the experiments. Asexual blood stages were cultured in asynchronous mode in RPMI 1640 medium supplemented with 10% normal human serum, 0.1% glucose and 50 uCi ml of the radiolabeled sugasr i.e., [^{3}H]-glucosamine hydrochloride, [^{3}H]-mannose and [^{3}H]-galactose separately as well as in mixture. Labeled parasites wereharvested after 6 h of incubation at 37°C in a candle jar [21], resuspended in RPMI medium and layered on to a 60% Percoll solution. The Percoll treated material was centrifuged for 15 min at 2700 x g. The tropozoites and erythro- cytes, in pellet, were washed with complete RPMI, RPMI containing 10% normal human serum and 0.1% glucose, and resuspended in partly consumed medium, obtained after harvesting of parasites incubated for 6 h and containing labeled sugars, and 5 ml of fresh RPMI containing 0.2% glucose. The suspension was returned to the candle jar for overnight incubation. The parasites after 18 h labelling were centrifuged at 1500 x g for 7min. The parasite pellet was immediately resuspended in fresh medium and loaded onto 60% isotoni Percoll solution. The Percoll containing material was centrifuged for 15 min at 4°C and at 1500 g. The Percoll enriched pellet, containing infected erythrocytes remained on top of the percoll. This cell fraction was repeatedly washed with cold PBS. The washed cells were treated with 10 μl of TPCK- trypsin (20 μg/ml of PBS, pH 6.8) for 10 min. at 4°C. The suspension was centrifuged at 1500 rpm at4°C and supernatant was removed. The process was repeated three times. The residue in PBS (200 μl) was sonicated for 15 seconds at 0°C. After an interval of 30 seconds the cycle was repeated three times, the suspension was centrifuged and the supernatant was treated with TPCK-trypsin (100 μ) as described above. The

combined supernatant of TPCK-trypsin treated material was deionized by gel filteration and lyophilized.

SDS-PAGE

The Percoll enriched pellet of the metabolically labelled cells was lysed with TNE (10 volumes) containing 2% NP-40 and protease inhibitors (leupeptin, 0.19 chymostatin 0.17 mM; TLCK, 2 mM and a mixture of TPCK, PMSFand phenanthroline in 0.2 mM, 1. mM and 2 mM concentration respectively. The lysate was centrifuged at 800 x g for 5 min followed by 100000 g for 1 h. The supernatant was used to examine the labeled glycoproteins by SDS-PAGE. Electrophoresis was performed using the discontinuous system of Laemmli [22] in 1.5 mm slab gels. The concentration of acrylamide in the stacking gel was 3% that of the running gel was a linear gradient of acrylamide from 6 to 20%. Samples were electrophoresed for 3 to 4h at 40 to 45 mAmp. Prior tc autoradiography, gels were treated with Amplify (Amersham). Gels were dried and autoradiographed with Kodak AR-5 for 4 to 25 days.

Column Chromatography

Bio-Gel P-2, Bio-Gel P-30 (Bio-Rad Laboratories) and Sepharose 6 B (Pharmacia Fine Chemicals) columns (1 x 6 cm) were run in 50 mM pyridine-acetic acid buffer (pH 6.4). Fraction containing radiolabel components were combined and lyophilized.

Alkaline borohydride treatment

The glycopeptides obtained from chromatographic procedures were incubated for 20 h in 7 ml of 50 mM sodium hydroxide containing 1M sodium borohydride at 45°C.The excess borohydride was removed with 4 M acetic acid, and the pH of the solution was adjusted to 5.5 with 4 M acetic acid. The solution was passed through a column of Dowex 50 X-8 H^+ equiliberated with 10 mM acetic acid. The column was washed with water and then with 10 mM acetic acid. The column was washed with water then 10 mM acetic acid. The combined elutes were lyophilized. The residue was repeatedly evaporated with methanol (5 x 4 ml) to remove boric acid as methyl borate. The residue in water (2 ml) was lyopholized rpeatedly (3 x 2 ml) to remove the exchangeable radioactivity. In some experiments mixture of sodium borohydride and borotritide was used.

Depolymerization of glycopeptides and oligosaccharides

The glycoproteins and glycopeptides were acid hydrolyzed with 3 M HCl for 16 h at 100°C. The mixture was cooled and passed through a column of Dowex 1 in AcO^- form, the eluate was evaporated under nitrogen at 40°C. The residue was N-acetylated in methanol with acetic anhydride, and the products examined in pc.

Enzyme degradation

Glycopeptides were digested with some or all of the following enzymes:

(i) α-D galactosidase from *Aspergillus niger* (Sigma, 50 mM sodium citrate, pH 4.1, 30 h at 37°C); (ii) α-D-galactosidase from *Escherichia coli* (Sigma, pH 4.2, 20 h at 37°C); (iii) β-D-galactosidase from *Escherichia coli* (Boehringer, 50 mM sodium phosphate, pH 7.0, 36 h at 37°C) (iv) β-D-Galactosidase from *Charonia lampas* (Miles, 50 mM sodium citrate buffer, pH 4.0, 30 h at 37°C); (v) α-mannosidase from Jack Bean (Sigma, sodium citrate pH 4.4, 24 h at 37°C); (vi) α-mannosidase from almonds (Sigma, 50 mM sodium citrate 4.4, 20 h at 37°C); (vii) β-D-N- acetylglucosaminidase (hexosaminidase) from Sigma (10 mM sodium cacodylate, pH 5.0 at 37° for 20 h.

Ninhydrin degradation of hexosamines

Hexosamine hydrochlorides obtained after acid hydrolysis of the glycoproteins were converted to free amines by treatment with Dowex 1 in OH-form. The amino sugars were treated with ninhydrin according to the procedure of Stoffyn and Jeanloz [23]. The products were examined in solvent (c).

Paper chromatography

Descending paper chromatography was performed on Whatman No.1 paper for 16 h using solvent systems (a) ethyl acetate-pyridine- acetic acid-water (5:5:1:3, v/v hexosamines, hexosa minitols, hexoses, pentoses and glycerol), (b) 1-butanol-acetic acid-water (4:1:5, v/v upper phase,

hexosamines, hexosaminitol and hexoses) (c) 1-butanol-pyridine-water (6:4:3, v/v, hexosamines, hexosaminitols and hexoses) (d) ethyl acetate-pyridine-water (4:1:1, hexosamines and pentoses). Standard sugars were visualized with silver nitrate. One centimeter strips, of the radiolabeled sample, were cut to measure the radioactivity.

Results

Metabolic labeling and SDS-PAGE

Malarial parasites prepared for the experiments were free of erythrocytic contamination [23] and were in good morphological condition. In particular, the surface coat was preserved since protease inhibitors were present throughout the extraction and isolation procedures. The parasites were harvested after continuous labeling of the cultures with [^{3}H]-glucosamine, [^{3}H]-mannose and [^{3}H]-galactose, and analyzed by SDS-PAGE followed by autoradiography. The gel pattern showed significant incorporation of [^{3}H]-glucosamine in a variety of proteins, (Fig. 1), of different molecular sizes. The labeled protein pattern for [^{3}H]-glucosamine and ^{3}H-mannose was identical. For [^{3}H]-galactose only selected proteins were labeled. The activities of incorporated [^{3}H]-glucosamine were high, whereas those of [^{3}H]-mannose and [^{3}H]- galactose were low.

Fig.1: SDS-PAGE of ^{3}H-glucosamine (lane 1), ^{3}H-galactose (lane 2) and ^{3}H-mannose (lane 3) of glycoproteins of P.falciparum Metabolically labeled parasites were enriched on Percoll gradient and lysed with NP-40. Equal volumes of the lysate were applied to the gel for electrophoresis.

Release of labeled surface glycoproteins

The labeled glycoproteins from *P. falciparum* were released from the surface of the cells by very mild tryptic treatment with TPCK-trypsin. The trypsin-treated cells were separated from the liberated glycoproteins by centrifugation and the intracellular as well as surface glycoproteins were further released by sonfication in the cold and subsequent tryptic treatment. The completeness of the release of the labeled material was assessed by counting the radioactivity in the residual, disintegrated, cells and cellular debris. Nigligible radioactivity was observed in the processed cells.

Purification of the labeled glycoproteins

The glycoproteins obtained after tryptic-treatment of the labeled cells were deionized by chromatography on Bio-Gel P-2, and then the labeled glycoproteins were separated by gel filtrations on Bio-Gel P-30. Two fractions, GP-1 and GP-2 (Fig. 2) were obtained. The main fraction (GP-1, 65%) in Sepharose 6B chromatography showed the presence of a major labeled component GP-1S (Fig. 3). The second fraction from Bio-Gel P-30 column (GP- 2,20%) showed in Sepharose 6B chromatography the presence of two components, GP-2S1 and GP2S2 (Fig. 4).

Preparation of oligosaccharide alditols

The glycopeptides obtained from Bio-Gel P-30 column were subjected to reductive cleavage by alkaline borohydride yielding a mixture of labeled

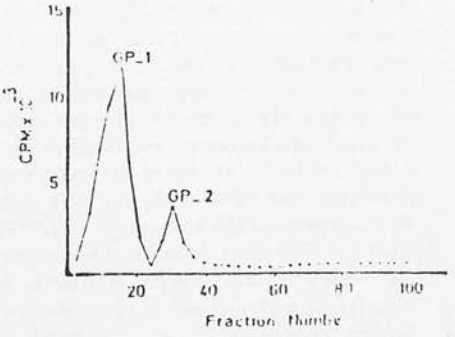

Fig.2: Gel filtration of the TPCK trypsin-treated P.falciparum cells on a column (1 x 6 cm) of Bio-Gel P- 30. Fractions of 0.2 ml were collected and every third fraction was examined for the presence of labeled material.

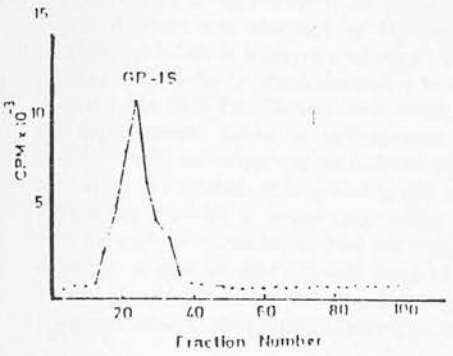

Fig.3: Gel filtration of Bio-Gel P-30 purified glycoprotein fraction, Gp-1, on a column of Sepharose 6B. Fractions of 0.15 ml were collected and every third fraction was examined for the presence of labeled polymer.

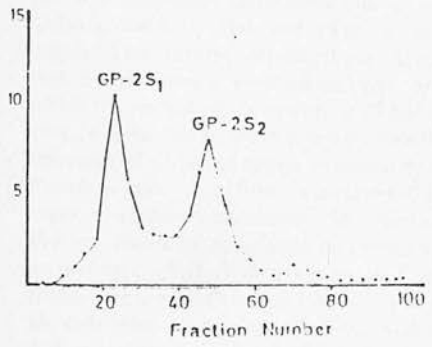

Fig. 4: Gel chromatography of Bio Gel P-30 purified fraction, GP-20,on a column of Sepharose 6B. Fraction of 0.15 ml were collected and every third fraction was examined for the presence of radiolabeled glycoproteins.

oligosaccharide/sugar alditols. A derived serine residue i.e. labeled alanine was detected in hydrolyzates of glycopeptides. The liberated labeled oligosaccharides/sugars in pc showed the presence of mono- and disaccharides as well as few slower moving labeled oligosaccharides. The monosaccharide, N-acetylglucosaminitol, and disaccharide were identified by the available radioactivity i.e. comigrating with the known sugars in pc in solvents, a,b and c. Acid hydrolysis of the mixture, mono- and disaccharides, showed the presence of N-acetylglucosaminitol, and in small quantities, galactose and N-acetylgalactosaminitol.

Enzyme treatment

Bio-Gel P-30 Fraction 1 (GP-1)

Cells labeled in admixture, i.e., with $GlcNH_2$, Man and Gal, were used for this study. This fraction in pc in solvents, a,b and c, showed the presence of a slow moving labeled components that barely moved in pc. Sequential treatment with β-galactosidase and hexosaminidase (β-N-acetylglucosaminidase) removed residues of galactose and N-acetylglucosamine. Treatment of the residual material bearing radioactivity with α-mannosidase released minor amount of labeled components. A component bearing small radioactivity in pc corresponded to mannose. Treatment of this fraction with β-galactosidase and subsequently with β-mannosidase released components bearing small radioactivity, and these labeled material in pc comigrated with galactose. β-N-acetylglucosaminidase treatment of the $GlNH_2$ labeled glycopeptides released labeled components that comigrated in pc with N-acetylglucosamine.

Bio-Gel P-30 Fraction 2 (GP-2).

The mixture of glycopeptides obtained from the cells labeled in admixture i.e. $GlcNH_2$, Man and Gal, in treatment with β-galactosidase and β-N-acetylglucosaminidase in sequence released major radiolabeled sugars. Because of the small amounts of the material available further enzyme degradative studies were not pursued.

Discussion

In the present studies, parasites in culture incorporated [^{3}H] - glucosamine, [^{3}H]-mannose and [^{3}H]-galactose as has been reported previously [11,24,25]. The isolated parasites were highly pure. Erythrocytes, infected erythrocytes or erythrocyte membranes were not observed. The radiolabeled proteins are the known biosynthetic products, and also the erythrocytes in culture do not incorporate radiolabel [25]. The glucosamine incorporation in proteins was significant, and this sugar was metabolically converted to GlcNAc and GalNAc. Large number of proteins incorporated glucosamine and mannose, whereas the incorporation of galactose was restricted to fewer proteins (Fig. 1). The differences in incorporation of the labeled sugars into glycoproteins were accompanied by variations in the activities of the radiolabel. Similar observations

i.e., differences in the incorporated activities of the sugar residues was observed by Heidrich et al. [25].This variation in incorporated sugars and the number of proteins in which the sugars are added, as noticed by SDS-PAGE, may have arisen due to the asynchronous mode of culture used in the present study. Investigations on isolated glycoproteins from the labeled cells grown in this mode of culture has provided a broad range of the labeled proteins and glycoproteins synthesized, probably, in different stages of development involved in the asexual blood cycle of the parasite, and may reflect a general biosynthetic pattern of glycoproteins.

The study was performed for the known reason that both the precursor glycoproteins. 195 kDa, and their processed product contain carbohydrates [26], and that the precursor protein [27] and the processed fragments [27] may provide effective blood stage vaccine. Glycoproteins of *Plasmodium falciparum* have been shown to contain GlNAc, GalNAc, Gal and Man as component sugars. The current investigations demonstrates that *P. falciparum* synthesizes glycoproteins in which the carbohydrate moiety is *O*-linked to the protein core, and in these glycoproteins there are two types of *O*-linked sugars as shown by reductive β-elimination. The first contained GalNAc, a sugar of common occurence in *O*-linked chains that are found in abundance in animal cells. The second type of *O*- linked chains in *P.falciparum* contain the unsual *O*-linked GlcNAc. The *O*-linked glycoproteins containing GlcNAc, GalNAc and Gal are present in the cell. The GlcNAc and GalNAc residues were characterized in glycoproteins that have been investigated for carbohydrate composition and to identify the type of linkage between the carbohydrate moiety and the protein core. Whether these two types of *O*-linked residues occur only in glycoproteins or also in anchor region is not known. Also, the existence of N- linked oligosaccharides in glycoproteins bearing *O*-linked chains need be clarified. [^{3}H]-Mannose residues were incorporated in glycoproteins, and are shown to be present in these polymers. The presence of this sugar in the N-linked chains or as a component sugar of glycans in the anchor region, that is present in the 195 kDa glycoprotein [28], will require further studies.

The enzymic studies suggest the presence of terminal galactose and N-acetylglucosamine residues. The sequential enzyme degradation indicate the presence of oligosaccharide chains. It is not possible, at this stage, to assess whether the enzymes are eliminating radiolabel sugars from the oligosaccharide chains of the glycoproteins or from the anchor region glycans of the proteins. The presence of terminal GlcNAc in animal cell glycoproteins has been described by Torres and Hart [29] from the surface glycoproteins of intact mouse lymphocytes. More recently, terminal *O*-linked GlcNAc residues have been found in glycoproteins associated with subcellular organelles in animal cells [30], and also in a human blood fluke *Schistosoma mansoni* [31].

This study of the *O*-linked sugars in glycoproteins synthesized by *P.falciparum* is relevant to previous studies. Lectin binding and enzyme cleavage studies [18] suggested the presence of GlcNAc, terminal Gal and Man residues in the glycoproteins and the absence of N-glycosidic linkage [15]. The biological and immunological significance of the carbohydrate moiety in the glycoproteins of *P.falciparum* is not known. It is, however, known that enzymic treatment of parasitic glycoproteins diminishes their antigenicity [16]. The importance of *O*-linked GlcNAc in glycoproteins is not yet known, and its role in *Plasmodium* glycoproteins will need be investigated. it is, however, postulated that these polymers may be involved in the transport of macromolecules [32] appropriate assembly of multimeric protein complexes [32], might prevent proteolysis and reversibly block sites of phosphorylation on proteins [32].

This study represents direct examination into the composition and structure of *P. falciparum* glycoproteins. In addition we have demonstrated the existence of terminal O-linked GlcNAc in the parasitic glycoproteins. Because of the minute quantities of the parasitic material available the detailed studies on individual glycoproteins as well as structural studies on oligosaccharides was not possible. We are currently examining the distribution of *O*-linked GlcNAc.

Acknowledgement

Dr. Nasir-ud-din acknowledges partial financial support for this investigation from UNDP/Word Bank/WHO special programme for research and training in tropical diseases.

Abbreviations: GlcNH$_2$, glucosamine; GlcNAc, N-acetylglucosamine GlcNAc-ol, N-acetylglucosaminitol; GalNH$_2$, galactosamine; GalNAc, N-acetylgalactosamine; GalNAc-ol, N-acetylgalactosaminitol; Gal, galactose; Man, mannose; TLCK, tosyl-L-lysine chloromethyl ketone; TPCK, N-tosyl-L-phenylalanine chloromethyl ketone; NP-40, Nonidet P-40; PBS, phosphate buffered saline; TBS, Tris buffered saline. SDS-PAGE, sodium dodecylsulfatepolyacrylamide gel electrophoresis; kDa, kiloDaltons; pc, paper chromatography.

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Carbohydrate Research, 205 (1990) 444–452
Elsevier Science Publishers B.V., Amsterdam

Note

Studies on cervical glycoproteins. Isolation and characterization of neutral oligosaccharides from Pronase-treated glycoproteins of bonnet monkey (*Macacca radiata*)*,†

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(Received December 29th, 1989; accepted for publication April 16th, 1990)

In the human cervix the columnar and gbolet cells[2], or only the columnar cells[3], perform the function of mucus secretion, whereas in the bonnet monkey only columnar cells are involved[1]. The cervical mucus of the bonnet monkey was chosen for glycoprotein studies because it is produced by a homogenous, single type of cells in copious amounts, and because the bonnet monkey is phylogenetically closely related to man[5–10]. Cervical mucus is a complex mixture of epithelial secretions exhibiting several rheological properties, such as viscosity, flow elasticity, "spinnbarkeit", and stickiness, which are regulated by ovarian functions. The alteration of these properties during the menstrual cycle is accompanied by alterations of the carbohydrate composition[11] and the chemical structure of the glycoproteins[6]. Thus, it is of interest to relate the carbohydrate structure of the glycoprotein with the biological functions and the morphological setting of the mucus. In a previous communication[10], one of us described the chemical structure of seven acidic oligosaccharides isolated from mucus of the periovulatory phase after purification by Pronase treatment. We describe herein the structure of five neutral oligosaccharides isolated at the same phase after an identical treatment.

* Dedicated to Professor Leslie Hough in the year of his 65th birthday.

† This is publication No. 1057 of the Robert W. Lovett Memorial Group for the Study of Diseases Causing Deformities, Harvard Medical School and Massachusetts General Hospital, Boston, Massachusetts. This investigation was supported, in part, by grants AM-03564 and HD-12431 (to R.W.J.) from the National Institutes of Health. For a preliminary communication, see ref. 1.

0008-6215/90/$03.50

EXPERIMENTAL

Materials. — Bio-Gel P-200, P-6, P-4, and P-2 gels, and AG 50W-X8 (100–200 mesh) and AG 1-X2 (100–200 mesh) ion-exchange resins were purchased from Bio-Rad Laboratories, Richmond, CA. Sepharose 2B was purchased from Pharmacia LKB Biotechnology Inc., Piscataway, NJ, and insolubilized Pronase (Enzite protease) from Miles Inc., Kankakee, IL.

Collection of cervical mucus. — The cervical mucus from the bonnet monkey was collected by aspiration with a suction pump at the estrogen-stimulated phase. The secretion was promptly frozen and maintained in the frozen state until use.

General methods. — The hexose content of the column-eluate fractions was determined by the phenol–H_2SO_4 method[12], and the protein content by measuring the absorbance at 278 nm. Quantitative analysis of carbohydrates by g.l.c. was performed according to the procedure of Reinhold[13]. 2-Acetamido-2-deoxythreitol was determined by g.l.c. as described previously[6]. G.l.c.–m.s. was performed with an analytical system consisting of a Varian MAT 731 instrument fitted with a combined c.i., e.i., and f.d. ion source. Columns of Bio-Gel P-200 and Sepharose 2B were washed with 50mM sodium phosphate (pH 7.0) containing 0.2% NaN_3. Chromatography on columns of Bio-Gel P-4 and P-6 was performed in 50mM pyridine–acetic acid, pH 5.4, and the column eluates were examined by counting tritium radioactivity or by the phenol–H_2SO_4 procedure for hexoses[12]. Chromatography on DEAE-Sephadex columns was performed as previously described[10].

Purification, Pronase treatment, and alkali borohydride treatment of mucus glycoproteins. — The glycoproteins were purified on a Bio-Gel P-200 column, the eluted glycoprotein was treated with Pronase, and then purified by gel filtration on Sepharose 2B as described earlier[8]. The purified glycoprotein (30 mg) was treated with 2M $NaBH_4$ in 50mM NaOH according to the method of Iyer and Carlson[14], and with sodium [^{3}H]borohydride as previously described[10]. The neutral oligosaccharides were separated from the acidic oligosaccharides on a column of AG 1-X2 anion-exchange resin (AcO$^-$). The column was washed successively with water, 5mM acetic acid, and a gradient of 50mM–0.2M acetic acid. The water and 5mM acetic acid eluates contained the neutral oligosaccharides, which were further purified by chromatography on a Bio-Gel P-6 column (100–200 mesh), and subsequent paper chromatography or paper electrophoresis[10]. Paper chromatography (p.c.) was performed in *A*, 1:2:1 (v/v) butanol–propanol–acetic acid, and *B*, 7:7:6 (v/v) pentanol–pyridine–water.

Enzymic degradation of oligosaccharides. — Oligosaccharides were digested with the following enzymes: (*a*) α-L-fucosidase (EC 3.2.1.51) from beef epididymis (Sigma Chemical Co., St. Louis, MO), in 10mM sodium citrate buffer, pH 6.0, for 48 h at 37°; (*b*) α-D-galactosidase (EC 3.2.1.22) from *Aspergillus niger* (Sigma), in 50mM sodium citrate, pH 5.0, for 48 h at 37°; (*c*) β-D-galactosidase (EC 3.2.1.23) from *Charonia lampas* (Miles), in 50mM sodium citrate, pH 4.0, 48 h at 37°; (*d*) β-D-galactosidase from *Escherichia coli* (Boehringer Mannheim Corp., Indianapolis, IN), in 50mM sodium phosphate, pH 7.0, 48 h at 37°; (*e*) *N*-acetyl-β-D-glucosaminidase (EC 3.2.1.30) from

jack bean (Sigma), in 50mM sodium citrate, pH 4.5, 48 h at 37°; (*f*) *N*-acetyl-α-D-galactosaminidase (EC 3.2.1.49) from *C. lampas* (Miles), in McIlvaine sodium citrate–phosphate buffer, pH 4.1, 42 h at 37°; and (*g*) *N*-acetyl-β-D-glucosaminidase, also referred as *N*-acetyl-β-D-hexosaminidase (EC 3.2.1.30; Sigma), from jack bean, sodium citrate, pH 4.0, 76 h at 37°, to act also as *N*-acetyl-β-D galactosaminidase.

Methylation analysis and periodate oxidation–sodium borohydride reduction (Smith degradation) of oligosaccharides. — Neutral oligosaccharides (as alditols) were methylated by Hakomori's procedure[15], and the methylated oligosaccharides processed as previously described[10]. The methylated oligosaccharides were hydrolyzed with trifluoroacetic acid, and the methylated sugars identified by g.l.c.–m.s.[10].

Reduced, neutral oligosaccharides (1–2 mg) were treated with $NaIO_4$, and subsequently reduced with $NaBH_4$, treated with acid, and processed as described earlier[10].

RESULTS

Alkali–borohydride treatment of the Pronase-treated, purified glycoprotein gave a mixture of oligosaccharide alditols. The neutral oligosaccharides were separated on a column of Bio-Gel P-6 into seven fractions, of which five contained oligosaccharides (Table I and Fig. 1), and the remaining two fractions glycopeptides.

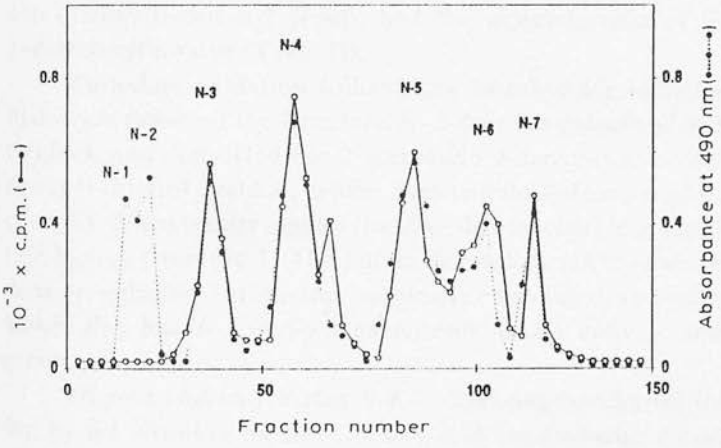

Fig. 1. Fractionation of neutral oligosaccharides on a column (125 × 1.2 cm) of Bio-Gel P-6. Two glycopeptides (N-1 and N-2) and five oligosaccharides (N-3, 5.0 mg; N-4, 4.0 mg; N-5, 3.0 mg; N-6, 4.6 mg; and N-7, 3.0 mg) were obtained. Hexoses were monitored by the phenol–sulfuric acid procedure (o–o) and 2-acetamido-2-deoxy-galactitol by counting tritium radioactivity (•·····•).

Oligosaccharide fraction N-3. — This fraction was further purified by Bio-Gel P-6 chromatography and was homogeneous on p.c. in solvents *A* and *B*. Methylation of the oligosaccharide showed the presence of a terminal 2-acetamido-2-deoxy-D-galactopyranosyl group and of 4-linked 2-acetamido-2-deoxy-D-glucopyranosyl, 3- and 4-linked D-galactopyranosyl, and 3,6-linked 2-acetamido-2-deoxy-D-galactitol residues (Table II). Treatment of the oligosaccharide with *N*-acetyl-α-D-galactosaminidase and sub-

TABLE I

Sugar composition of purified neutral oligosaccharides

Fraction	*Component*									
	Fucose		*Galactose*		*2-Acetamido-2-deoxyglucose*		*2-Acetamido-2-deoxygalactose*		*2-Acetamido-2-deoxygalactitol*	
	%	*MR*[a]	%	*MR*[a]	%	*MR*[a]	%	*MR*[a]	%	*MR*
N-3			25.4	1.95	16.4	1.05	31.2	1.95	16.0	1.00
N-4			32.0	2.0	20.5	1.05	19.8	1.0	20.0	1.00
N-5	15.6	1.0	33.0	1.9	21.8	1.0			20.9	1.00
N-6	15.5	1.0	34.2	2.0	22.0	1.0			21.0	1.00
N-7			41.0	2.05	27.4	1.1			25.0	1.00

[a] Molar ratio relative to 2-acetamido-2-deoxygalactitol.

sequent methylation showed the presence of the same group and residues, whereas a prolonged treatment with *N*-acetyl-*β*-D-hexosaminidase, followed by methylation, showed the appearance of a terminal D-galactopyranosyl group. Finally, sequential treatment of the oligosaccharide with *N*-acetyl-*β*-D-hexosaminidase and *β*-D-galactosidase, and subsequent methylation indicated the appearance of a terminal 2-acetamido-2-deoxy-D-glucosyl group, and the disappearance of the subterminal 4-linked D-galactosyl residue (Table II).

Periodate oxidation followed by borohydride reduction and subsequent acid hydrolysis removed the 2-acetamido-2-deoxy-D-galactosyl and partly the D-galactosyl residues, and converted the 2-acetamido-2-deoxy-D-galactitol into a 2-acetamido-2-deoxy-D-threitol residue, while 2-acetamido-2-deoxy-D-glucose was recovered unchanged. These results suggest that the oligosaccharide of fraction N-3 is a hexasaccharide having structure **I**. The anomeric configuration of the terminal 2-acetamido-2-deoxy-D-galactosyl group was not clearly established, as *N*-acetyl-*β*-D-hexosaminidase, which also has *N*-acetyl-*β*-D-galactosaminidase activity, only partly removed this group.

Oligosaccharide fraction N-4. — This oligosaccharide fraction was further purified by gel filtration on Bio-Gel P-4, and ion-exchange chromatography on DEAE-Sephadex, and was homogeneous on p.c. in solvents *A* and *B*. Methylation of the oligosaccharide and identification of the methylation products by g.l.c.–m.s. showed the presence of terminal, nonreducing 2-acetamido-2-deoxy-D-galactosyl and D-galactosyl groups, and 3-linked D-galactosyl, 4-linked 2-acetamido-2-deoxy-D-glucosyl, and 3,6-linked 2-acetamido-2-deoxy-D-galactitol residues (Table II). Treatment of the oligosaccharide with *N*-acetyl-*α*-D-galactosaminidase and subsequent methylation showed the disappearance of the 2-acetamido-2-deoxy-D-galactosyl nonreducing group and the 3-linked D-galactosyl group (Table II). Smith degradation of this oligosaccharide removed the D-galactosyl (in part) and 2-acetamido-2-deoxy-D-galactosyl groups and converted the 2-acetamido-2-deoxy-D-galactitol into a 2-acetamido-2-deoxy-D-threitol

TABLE II

O-Methyl derivatives obtained by acid hydrolysis of methylated oligosaccharides before and after enzyme treatment

Oligo-saccharides	*Enzyme treatment*[a]	O-*Methyl derivatives*									
		Fuc	*Gal*				*GlcNAc*		*GalNAc*	*GalNAc-ol*	
		2,3,4	*2,3,6*	*2,4,6*	*3,4,6*	*2,3,4,6*	*3,6*	*3,4,6*	*3,4,6*	*1,4,5*	*1,3,4,5*
N-3	None		+	+			+		+	+	
	α-GalNAc-ase		+	+			+		+	+	
	β-HexNAc-ase		+			+	+		+	+	
	β-HexNAc-ase, then										
	β-Gal-ase			+				+	+	+	
N-4	None			+		+	+		+	+	
	α-GalNAc-ase					+	+			+	
N-5	None	+			+	+	+			+	
	β-Gal-ase	+			+			+		+	
	β-Gal-ase, then										
	α-Fuc-ase					+	+			+	
N-6	None	+			+	+	+			+	
	α-Fuc-ase					+	+			+	
	β-Gal-ases	+			+	+	+			+	
	α-Fuc-ase, then										
	β-Gal-ase					[b]		+		[b]	+
N-7	None					+	+			+	
	β-Gal-ase					+		+		+	

[a] α-GalNAc-ase, *N*-acetyl-α-D-galactosaminidase; β-HexNAc-ase, *N*-acetyl-β-D-hexosaminidase; β-Gal-ase, β-D-galactosidase; and α-Fuc-ase, α-L-fucosidase.
[b] Trace.

residue, whereas 2-acetamido-2-deoxy-D-glucose was unchanged. The results of these experiments suggest that the oligosaccharide of fraction N-4 is a pentasaccharide having structure **2**.

Oligosaccharide fraction N-5. — This oligosaccharide was purified by chromatography on Bio-Gel P-4 and was homogeneous on p.c. in solvents *A* and *B*, and on paper electrophoresis. Methylation of the oligosaccharide of fraction N-5 and characterization of the products of hydrolysis by g.l.c.–mass spectrometry showed the presence of terminal L-fucosyl and D-galactosyl groups, and 2-linked D-galactosyl, 4-linked 2-acetamido-2-deoxy-D-glucosyl, and 3,6-linked 2-acetamido-2-deoxy-D-galactitol residues (Table II).

Treatment of the oligosaccharide with β-D-galactosidase and subsequent methylation of the enzyme-treated oligosaccharide showed the presence of terminal, nonreducing L-fucosyl and 2-acetamido-2-deoxy-D-glucosyl groups, and 2-linked D-galactosyl and 3,6-linked 2-acetamido-2-deoxy-D-galactitol residues. α-L-Fucosidase degradation of the β-D-galactosidase-treated oligosaccharide showed, by methylation analysis (Table II), the presence of terminal, nonreducing D-galactosyl and 2-acetamido-2-deoxy-D-glucosyl groups, and a 3,6-linked 2-acetamido-2-deoxy-D-galactitol residue.

Periodate oxidation–borohydride reduction of the oligosaccharide removed the terminal nonreducing L-fucosyl and D-galactosyl groups and the internal D-galactosyl residue, and converted the 2-acetamido-2-deoxy-D-galactitol into a 2-acetamido-2-deoxy-D-threitol residue, but left 2-acetamido-2-deoxy-D-glucose unchanged. On the basis of these experiments, the structure of pentasaccharide **3** is proposed for the oligosaccharide of fraction N-5.

Oligosaccharide fraction N-6. — This fraction was purified to homogeneity by chromatography on Bio-Gel P-4. Methylation analysis of the oligosaccharide showed the presence of terminal, nonreducing L-fucosyl and D-galactosyl groups, and 2-linked D-galactosyl, 4-linked 2-acetamido-2-deoxy-D-glucosyl, and 3,6-linked 2-acetamido-2-deoxy-D-galactitol residues (Table II). Treatment of the oligosaccharide with α-L-fucosidase followed by methylation analysis showed the presence of terminal, nonreducing D-galactosyl groups, a 4-linked 2-acetamido-2-deoxy-D-glucosyl, and a 3,6-linked 2-acetamido-2-deoxy-D-galactitol residue (Table II). Extensive treatment of the oligosaccharide with β-D-galactosidases from *C. lampas* and *E. coli* indicated only a partial removal of the D-galactosyl groups as shown by methylation (Table II), but sequential treatment of the oligosaccharide with α-L-fucosidase and β-D-galactosidase removed both the L-fucosyl and β-D-galactosyl groups. Methylation analysis of this enzyme-treated oligosaccharide showed the presence of a terminal, nonreducing 2-acetamido-2-deoxy-D-glucosyl group and a trace of D-galactosyl group, and 6- and traces of 3,6-linked 2-acetamido-2-deoxy-D-galactitol residues (Table II).

Smith degradation of the oligosaccharide completely removed the terminal, nonreducing L-fucosyl and D-galactosyl groups and the internal D-galactosyl residue, and converted the 2-acetamido-2-deoxy-D-galactitol into a 2-acetamido-2-deoxy-D-threitol residue, while 2-acetamido-2-deoxy-D-glucose was unchanged. These results

suggested pentasaccharide structure **4** for the oligosaccharide of fraction N-6.

Oligosaccharide fraction N-7. — This oligosaccharide was homogeneous after gel filtration through Bio-Gel P-2 (p.c. in solvents *A* and *B*). Carbohydrate analysis of the purified oligosaccharide suggested a tetrasaccharide structure. Methylation analysis (Table II) showed the presence of terminal, nonreducing D-galactosyl groups, and 4-linked 2-acetamido-2-deoxy-D-glucosyl and 3,6-linked 2-acetamido-2-deoxy-D-galactitol residues. β-D-Galactosidase treatment of the oligosaccharide, followed by methylation analysis of the enzyme-treated remainder, showed the presence of terminal, nonreducing 2-acetamido-2-deoxy-D-glucosyl and D-galactosyl groups, and a 3,6-linked 2-acetamido-2-deoxy-D-galactitol residue (Table II).

Smith degradation removed the terminal, nonreducing D-galactosyl groups and converted the 2-acetamido-2-deoxy-D-galactitol into a 2-acetamido-2-deoxy-D-threitol residue, while 2-acetamido-2-deoxy-D-glucose was unchanged. These results suggested structure **5** for the oligosaccharide of fraction N-7.

D-Gal*p*NAc-(1→4)-β-D-Gal*p*-(1→4)-β-D-Glc*p*NAc-(1→6 or 3)-D-GalNAc-ol
3 or 6
↑
1

1 D-Gal*p*NAc-(1→3)-β-D-Gal*p*

β-D-Gal*p*-(1→4)-β-D-Glc*p*NAc-(1→6 or 3)-D-GalNAc-ol
3 or 6
↑
1

2 α-D-Gal*p*NAc-(1→3)-β-D-Gal*p*

β-D-Gal*p*-(1→4)-β-D-Glc*p*NAc-(1→6 or 3)-D-GalNAc-ol
3 or 6
↑
1

3 α-L-Fuc*p*-(1→2)-β-D-Gal*p*

α-L-Fuc*p*-(1→2)-β-D-Gal*p*-(1→4)-β-D-Glc*p*NAc-(1→6 or 3)-D-GalNAc-ol
3 or 6
↑
1

4 β-D-Gal*p*

β-D-Gal*p*-(1→4)-β-D-Glc*p*NAc-(1→6 or 3)-D-GalNAc-ol
3 or 6
↑
1

5 β-D-Gal*p*

Chart 1. Proposed structures for oligosaccharides of fractions N-3–N-7.

DISCUSSION

In the present study, mucus was collected from several monkeys at the time of estrogen surge. The mucus was purified, and the resulting glycoproteins were treated with Pronase and purified to homogeneity on Sepharose 2B, as shown by chromatography and electrophoresis[10]. It is likely that, after Pronase treatment, these purified glycoproteins differed only in molecular size. On treatment of the glycoproteins with alkali-borohydride, a mixture of neutral and acidic oligosaccharides was obtained that was separated by ion-exchange chromatography[10], and the neutral oligosaccharides were further fractionated on Bio-Gel P-6 (Fig. 1). Each oligosaccharide obtained from this chromatography was subsequently purified by paper chromatography or electrophoresis to provide five neutral oligosaccharide fractions (N-3–N-7). The structure of each oligosaccharide was studied by chemical and enzymic procedures. The main limitations of this study were the availability of specific enzymes and the amounts of available oligosaccharides. Consequently, it was not possible to ascertain definitely the anomeric configurations of the 2-acetamido-2-deoxy-D-glucosyl and -D-galactosyl residues linked to the 2-acetamido-2-deoxy-D-galactitol residue, and they were attributed in analogy with the results obtained for the acidic oligosaccharides[9,10]. In the case of oligosaccharide fraction N-3, the anomeric configuration between the terminal, nonreducing 2-acetamido-2-deoxy-D-galactosyl group and the D-galactosyl residue was not definitely established, as *N*-acetyl-α-D-galactosaminidase did not split off this group, but *N*-acetyl-β-D-hexosaminidase was also not able to completely remove it. These results suggested that either the latter enzyme cleaves off some 2-acetamido-2-deoxy-β-D-galactosyl groups with difficulty, or that some α-D-anomeric groups were also present. 2-Acetamido-2-deoxy-β-D-galactopyranosyl groups have been characterized in the acidic oligosaccharides of the intact glycoprotein[9], and therefore it is probable that they are also present in the neutral oligosaccharides.

The precise linkages of the penultimate 2-acetamido-2-deoxy-β-D-glucosyl and D-galactosyl residues to the 2-acetamido-2-deoxy-D-galactosyl residue involved in the carbohydrate–protein linkage were also not completely defined, as the methods used could only indicate that both O-3 and O-6 of this residue are substituted. As the 2-acetamido-2-deoxy-β-D-glucose residue is linked (1→6) and the β-D-galactosyl group is linked (1→3) to the 2-acetamido-2-deoxy-D-galactose residue in the acidic oligosaccharides of the bonnet monkey glycoprotein[9], it is very likely that identical linkages exist in the neutral oligosaccharides of the periovulatory-phase mucus. These two linkages are also the preponderant ones in mucins from other sources[16,17]. The oligosaccharides presently characterized bear chemical structures similar to those of the acidic oligosaccharides[9,10], all having the core structure, β-D-Gal*p*-(1→3)-[β-D-Glc*p*NAc-(1→6)]-D-GalNAc. The same common core was identified in the partial chemical structures of four carbohydrate chains obtained from human cervical glycoproteins[18]. The great heterogeneity of the carbohydrate chains of cervical mucus glycoproteins, as compared with those of other mucins, may be related to the changing physiological functions and biophysical properties of the mucus.

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Carbohydrate Research, 209 (1991) c5–c8
Elsevier Science Publishers B.V., Amsterdam

Preliminary communication

Presence of *O*-glycosylated glycoproteins in the *Plasmodium falciparum* parasite*

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(Received April 25th, 1990; accepted for publication, in revised form, October 17th, 1990)

Malaria is a widespread disease caused by the protozoan parasite Plasmodium. About one third of the world's population lives in malaria endemic areas. Of the four species that infect man, *Plasmodium falciparum* is the most virulent, causing considerable morbidity and high mortality, particularly among children[1]. The asexual erythrocytic stage is responsible for the disease and associated pathology. Protein and glycoprotein antigens of asexual erythrocytic development forms have been indentified[1,2]. The presence of carbohydrates on the surface of *P. falciparum* cells, particularly merozoites, has been ascertained by *in vitro* incorporation of radiolabeled carbohydrates[3,4], enzymic release of radiolabeled sugars[5], lectin binding[6], and inhibition studies[7]. Because of failure to produce a plasmodial antigen-based malarial vaccine by recombinant DNA technology[8], growing interest is being focused on the carbohydrates of integral antigens[5]. The glycoproteins of the parasite have not been structurally investigated yet, and their composition and nature still remain unclear.

In the present study, *P. falciparum* strain M25/Zaire was cultured asynchronously in human A^+ erythrocytes in RPMI medium containing human serum from A^+ donors. The cells were labeled with D-[6-^{3}H]- or D-[1-^{14}C]-glucosamine, D-[6-^{3}H]galactose, and D-[2,6-^{3}H]mannose, separately as well as in a mixture. Labeled parasites were harvested after 6- and 16-h incubations at 37° in a candle jar[9]. Infected-erythrocyte-containing parasites were enriched on 60% Percoll. This cell fraction was lysed in 20 vols. of 2% NP-40 in TNE (50mM Tris·HCl, 100mM NaCl, and 5 mM EDTA) containing protease inhibitors [leupeptin, chymostatin, *N*-α-*p*-tosyl-L-lysine chloromethyl ketone (TLCK) L-1-*p*-tosylamino-2-phenylethyl chloromethyl ketone (TPCK), and α-toluenesulfonyl fluoride (PMSF)]. The lysate was centrifuged at 100 000*g* and the supernatant was

* This work was supported by UNDP World Bank WHO Special Programme for Research and Training in Tropical Diseases, and a Visiting Scientist Award from the same source to Nasir-ud-Din.

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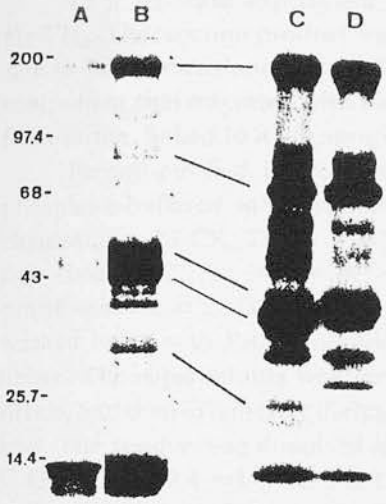

Fig. 1. Biosynthetic labeling of *P. falciparum* glycoproteins and immunogenicity in human hosts. Autoradiographic analysis of D-[^{3}H]mannose (A) and D-[^{3}H]glucosamine-labeled glycoproteins (B, C, and D). A and B: total labeled glycoproteins; C and D: immunoprecepitates with human immune sera. Lanes A and B are 10% poly(acrylamide) gels, and lanes C and D are 6–20% poly(acrylamide)-gradient gels. Mobilities of bands in lanes C and D have been aligned to those in lanes A and B on the basis of mobilities of standard molecular-weight markers shown on the left of the figure.

examined by sodium dodecyl sulfate–poly(acrylamide) gel electrophoresis (SDS-PAGE) and by immunoprecipitation. The labeled components in SDS-PAGE (Fig. 1) showed the presence of glycosylated proteins of molecular sizes between 195 and 10 kDa, labeled either with D-[^{3}H]mannose (lane A) or D-[^{3}H]glucosamine (lane B). The major labeled species were also immunoprecipitated by two different immune human sera from adults living in malaria-endemic areas of West Africa (Fig. 1, lanes C and D), indicating that these species are immunogenic in humans and represent antigenic targets for the human immune response against *P. falciparum*. Nonimmune human sera do not recognize any of these glycoproteins (not shown).

Because of low uptake of sugars into *P. falciparum*-glycosylated proteins and the small amounts of purified infected cells available after labelling, surface glycoproteins were isolated by use of TPCK-trypsin[10] (20 μg/mL, shaken for 15 min at 0°). Several glycopeptides were separated by gel chromatography on Bio-Gel P-30 and Bio-Gel P-60. Attempts to purify these glycopeptides were limited owing to the very minute quantity of isolated material. Chemical and enzyme studies were, therefore, performed on the mixture of glycopeptides obtained by TPCK-trypsin treatment, followed by chromatography on Bio-Gel P-30. These glycopeptides were treated with NaOH–$NaBH_4$, and then processed as described earlier[11]. After acid hydrolysis in 2M HCl for 16 h at 98°, and separation by p.c. [solvents: (*a*) 5:5:1:1 ethyl acetate–pyridine–acetic acid–water; (*b*) 4:1:5, upper layer butanol–ethanol–water], radiolabeled components having the mobility of 2-acetamido-2-deoxyglucitol, galactose, and a small amount of 2-acetamido-2-deoxygalactitol were observed.

In a separate experiment, glycopeptides were incubated in $NaOH–NaBH_4–NaB^3H_4$. The reaction product was processed as described above, and the acid hydrolyzate in two-dimensional p.c., solvents (*a*) and (*b*), showed the presence of a labeled component that migrated with alanine. This suggested that derived alanine had arisen from serine, linked to a hexosamine, during reductive β-elimination.

Percoll-purified, labeled, and parasitized cells (10^9 cells/mL) were resuspended in phosphate-buffered saline solution (PBS) containing protease inhibitors (leupeptin, chymostatin, TLCK, TPCK, PMSF, and phenanthroline), and disrupted by sonication four times at 0° for 30 s with 30-s intervals betweeen each sonication cycle. After centrifugation at 20 000*g* for 30 min, the supernatant was removed, and the pellet washed twice with PBS containing protease inhibitors and centrifuged as described above. The supernatants were mixed and centrifuged at 100 000*g* to remove cellular debris, and then extensively dialyzed to remove exchangeable radioactivity and lyophilized. The residue was dissolved in 10mM phosphate buffer, pH 6.4, and treated with O-Glycanase (2.4 mU; EC 3.2.1.97; Genzyme, Boston, MA) for 20 h at 37°. The enzyme-treated product was extensively dialyzed at 4° and freeze-dried. The residue was dissolved in water and de-ionized by chromatography on AG 50 (H^+) and AG 1 (AcO^-) ion-exchange resins. The column washings (water) were combined and freeze-dried. The residual material showed in p.c., in solvent (*a*), the presence of radiolabeled components that migrated with standard *N*-acetylglucosamine and *N*-acetylgalactosamine. The dialyzate contained over 60% of the labeled material, whereas, in the control-treated sample, the dialyzable radioactively-labeled material did not exceed 10%. These results suggested that the O-Glycanase cleaved *O*-glycosyl-linked sugars from the protein core. The O-Glycanase is known to cleave the *O*-(2-acetamido-2-deoxy-D-galactopyranosyl)-(1→3)-L-serine or -L-threonine linkage[12] and its activity towards the *O*-(2-acetamido-2-deoxy-D-glucopyranosyl) linkage has not yet been explored.

Labeled glycopeptides were incubated with N-Glycanase (0.25 U; EC 3.5.1.52; Genzyme, Boston, MA) in 0.2M sodium phosphate buffer, pH 8.5, at 37° for 16 h. The enzyme-treated material was chromatographed on a column of Bio-Gel P-30, calibrated with labeled glycopeptides. The N-Glycanase-treated glycopeptides were recovered unchanged, with only a slight decrease in c.p.m., suggesting the resistance of these glycopeptides to N-Glycanase and the lack of asparagine-linked oligosaccharides in the malarial glycoproteins. A similar observation, *i.e.*, absence of asparagine-linked oligosaccharides, has been reported earlier[6], though the studies were performed under different conditions.

The carbohydrate composition of the malarial glycopeptides, as determined by β-elimination–reduction, and O-Glycanase and N-Glycanase treatments, suggested the presence of an *O*-glycosyl-type linkage in the *P. falciparum* asexual-form glycoproteins. The labeled glycoproteins with *O*-glycosyl linkage are the biosynthetic products of the parasite and not of erythrocytes, since the latter do not incorporate radiolabeled compounds[4] in culture.

To our knowledge, this is the first report of the existence of *O*-glycosylated glycoproteins in the malaria parasite. The structure and function of plasmodial gly-

coproteins are not known, and their contribution to the immunogenicity and antigenicity of plasmodial antigens is unclear, although their significance towards the development of a malaria vaccine has been emphasized[13].

ACKNOWLEDGMENT

The authors thank Prof. P. Roussel, Lille, France, for helpful discussions.

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Vol. 27, No. 1, June 1992 BIOCHEMISTRY INTERNATIONAL
Pages 55-64

PLASMODIUM FALCIPARUM SYNTHESIZES O-GLYCOSYLATED GLYCOPROTEINS CONTAINING O-LINKED N-ACETYLGLUCOSAMINE

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Received February 14, 1992
Received after revision, March 16, 1992

SUMMARY Asexual blood forms of the human malaria parasite, *Plasmodium falciparum*, synthesize a major glycosylated 195 kDa protein that has been considered for the development of a vaccine. β-Elimination-borohydride reduction of the 195 kDa glycoprotein and its 16 kDa processed product after metabolic labeling of their carbohydrates, showed the presence of derived, labeled glucosaminitol and alanine. This suggests that the 195 and 16 kDa glycoproteins contain distinct O-glycosyl linkages and that N-acetylglucosamine and serine residues are involved in the attachment of carbohydrate moieties to the protein core. Endo-O-glycanase treatment of total glycoproteins shows that O-glycosidycally-linked sugars represent a major carbohydrate moiety in *P. falciparum* glycoproteins.

INTRODUCTION

Malaria induced by *P. falciparum* still remains one of the most prevalent diseases in tropical and subtropical areas. To develop an efficacious vaccine against this parasite [1], much of the recent research has been devoted to the identificatiion and characterization of specific protein antigens expressed by the parasite in its human host. The plasmodial infection progresses through distinct stages in the human and arthropod hosts and immune responses are elicited against stage-specific antigens that involve both cell and antibody mediated mechanisms [2, 3].

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Among the antigens characteristic of the asexual erythrocytic stages of *P. falciparum* infection [4, 5], the 195 kDa glycoprotein is expressed by merozoites invading human erythrocytes [6, 7]. Several lines of evidence suggest that this antigen can induce protective immune responses [8, 9].

The biological and structural roles played by the carbohydrate moiety of glycoprotein antigens of *P. falciparum* has long been recognized [10, 11]. Evidence for the presence of carbohydrates on surface proteins of the asexual stages of the parasite has been obtained by metabolic incorporation of radiolabelled sugars into these proteins followed by their enzymic release [12, 13] and by lectin binding studies [14]. As there is still only scant information on the composition and structure of carbohydrate moieties of plasmodial glycoproteins, despite their well documented immunogenicity [13], the current research was initiated to gather further structural information on the carbohydrate moieties of immunodominant parasite glycoproteins.

MATERIALS AND METHODS

Metabolic radiolabeling of *P. falciparum* and preparation of detergent extracts: the parasite cells of strain M25/ZAIRE were used. Asexual blood stages were cultured in asynchronous mode in RPMI medium supplemented with 10% normal human serum, 0.1% glucose and 50 μCi ml^{-1} of the radiolabelled sugars (Amersham, UK) i.e. D-[6-^{3}H] (40 Ci/mmole) or D-[1-^{14}C] (60 Ci/mmole)-glucosamine hydrochloride, D-[2,6^{3}H]-mannose (60 Ci/mmole), D-[6-^{3}H]-galactose (40 Ci/mmole) separately as well as in admixture. Labeled parasites were harvested after 6 h of incubation at 37^{0}C in a candle jar [15], resuspended in RPMI medium and layered onto 60% isotonic Percoll (Pharmacia). Infected erythrocytes containing multinucleated parasites (segmented schizonts and merozoites) remained on top of the Percoll layer and were highly enriched in cell content, i.e 90%. This cell fraction was washed twice with cold phosphate buffered saline (PBS) and lysed with TNE (50 mM Tris-HCl pH 8.8, 100 mM NaCl and 5 mM EDTA) containing 2% NP-40 and protease inhibitors (leupeptin, 0.19 mM, chymostatin, 0.17 mM, TLCK, 2 mM, TPCK, 0.2 mM, phenyl-methyl-sulfonyl-fluoride (PMSF) 1 mM and phenanthroline 2 mM).
The lysate was centrifuged once at 800 x g for 5 min and then again at 100'000 x g for 1 h, and the supernatant processed for immunoprecipitation.

Immunoprecipitation: NP-40 extracts of cells metabolically labelled with [^{3}H]-GlcN, [^{3}H]-Man and [^{3}H]-Gal were immunoprecipitated with a mouse mAb against the 195 kDa glycoprotein (mAb 3B10, ref 16), a human immune serum against asexual blood stage antigens and normal mouse serum as control. Aliquots of the extracts containing 5-20 x 10^4 cpm were incubated overnight in excess antibody at 4^{0}C. Antigen-antibody complexes were recovered with an anti-mouse immunoglobulin-Sepharose 4B matrix in TNE containing 0.5% NP-40 and 5% defatted milk. The Sepharose beads were centrifuged at 2500 x g for 5 min and washed 3 times with TNE-NP-40-milk and 3 more times with TNE-NP-40 to remove non-specifically bound material. The antigen-antibody complexes were eluted in 50 mM Tris-HCl pH 6.8 sample buffer containing 2% SDS and 3% 2-mercaptoethanol. These samples were boiled for 3 min and analyzed by SDS-PAGE.

SDS-PAGE: electrophoresis was performed using the discontinuous Laemmli system with molecular weight standards. Gels were treated with Amplify (Amersham), dried and exposed to Kodak (Rochester, NY) AR-5 films for 4-25 days.

Alkaline borohydride treatment: the 195 and 16 kDa proteins revealed by autoradiography were excised from the gel and incubated for 20 h in 5 ml of 50 mM NaOH containing 1 M $NaBH_4$ at 45^{0}C [15]. In some experiments, a mixture of $NaB[^3H]_4$-labelled and unlabelled $NaBH_4$ was used. The reaction mixture was cooled to 4^{0}C, the excess borohydride was removed by treatment with 4 M acetic acid. The liquid phase was removed and saved, the gel and the paper were repeatedly washed with 10 mM acetic acid (3 x 2 ml). The washings and the liquid phase were combined and the solution passed through a column of Dowex (BioRad) 50X-8 H^+ equilibrated with 10 mM acetic acid. The column was washed with H_2O (15 ml) followed by 10 mM acetic acid (10 ml). The combined eluates were lyophilized. The residue was repeatedly evaporated with methanol (5 x 4 ml) to remove boric acid as methyl borate. The residue in H_2O (2 ml) was lyophilized repeatedly (3 x 2 ml) to remove the exchangeable radioactivity.

Acid hydrolysis of glycoproteins and oligosaccharides: the 195 and 16 kDa glycoproteins excised from the gel were treated with 3 M HCl for 16 h at 100^{0}C. The mixture was cooled and passed through a column of Dowex 1 in AcO^- form, the eluate was evaporated under nitrogen at 40^{0}C. The residue was N-acetylated in methanol with acetic anhydride.

Paper chromatography (pc): descending paper chromatography was performed on Whatman No 1 paper for 16 h using solvent system (a): ethyl acetate-pyridine-acetic acid-water (5:5:1:3 v/v) for hexosaminitols, hexoses, pentoses and glycerol, (b): 1-butanol-acetic acid-water (4:1:5 v/v, upper phase) for hexosamines, hexosaminitol and hexoses, (c) 1-butanol-pyridine-water (6:4:3 v/v) for hexosamines, hexosaminitols and hexoses, (d) ethyl-acetate-pyridine-water (8:2:1 v/v) for hexosaminitols, hexosamines and hexoses and (e) 1-butanol-ethanol-water (4:1:1 v/v) for hexosamines and pentoses. Standard sugars were visualized with silver nitrate. One cm strips of the chromatogram were cut to measure the radioactivity. Two-dimensional pc was performed in solvents (a) and (c) for sugars and in solvents (a) and (b) for amino acids.

Ninhydrin degradation of hexosamines: hexosamine hydrochlorides obtained after acid hydrolysis of the glycoproteins were converted to free amines by treatment with Dowex 1 in OH-form. The amino sugars were treated with ninhydrin according to the procedure of Stoffyn and Jeanloz [17]. The products were examined by pc in solvent (e).
Hexosaminidase digestion: the 195 and 16 kDa bands in acrylamide were sonicated in Na cacodylate, pH 5.0 and then suspended in 10 µl of the same buffer. The sample were again sonicated briefly. Five µl of a protease inhibitor cocktail (leupeptin, chymostatin, TLCK, TPCK, PMSF and phenanthroline) was added to the same final concentration as in the NP-40 extract. Deglycosylation was performed at 37^{0}C for 20 h and the reaction stopped by boiling for 20 s at 80^{0}C. The sample solutions were centrifuged at 14'000 rpm. The residue washed with buffer and the combined supernatants dialyzed. The dialysable material and non-dialysable materials after acid hydrolysis were examined by paper chromatography.
Endo-O-glycanase treatment: the labelled cells were sonicated at 4^{0}C in the presence of protease inhibitors and the suspension centrifuged at 100'000 x g and the lyophilized. The residue in 10 mM Na phosphate buffer pH 6.4 was incubated with endo-α-N-acetylgalactosaminidase, 2.4 mU (EC 3.2.1.97 ref, 18; O-glycanase^{TM}, Genzyme, Boston, MA) for 20 h at 37^{0}C in the presence of PMSF 5 mM. The enzyme-treated and untreated samples were extensively dialysed and the dialysable oligosaccharides hydrolyzed, N-acetylated and further analyzed by pc.

RESULTS AND DISCUSSION

The metabolically labeled malarial parasites used were in good morphological condition as seen in Giemsa-stained blood smears. In particular, the surface coat was preserved, owing to the presence of protease inhibitors throughout the extraction and isolation procedures. The parasites were harvested after continuous labeling of the cultures with [^{3}H]-GlcN, [^{3}H]-Man and [^{3}H]-Gal. The radiolabeled glycoproteins were extracted in non-ionic detergent and analysed by SDS-PAGE and autoradiography. The gel pattern (Fig. 1A) shows significant incorporation of [^{3}H]-GlcN in a variety of proteins of different M_r. The labeled protein pattern for [^{3}H]-GlcN (lane 1) and [^{3}H]-Man (lane 3) was identical. Only selected proteins were labeled with [^{3}H]-Gal (lane 2). The activities of incorporated [^{3}H]-GlcN were high, whereas those for [^{3}H]-Man and [^{3}H]-Gal were lower, as observed previously [19].

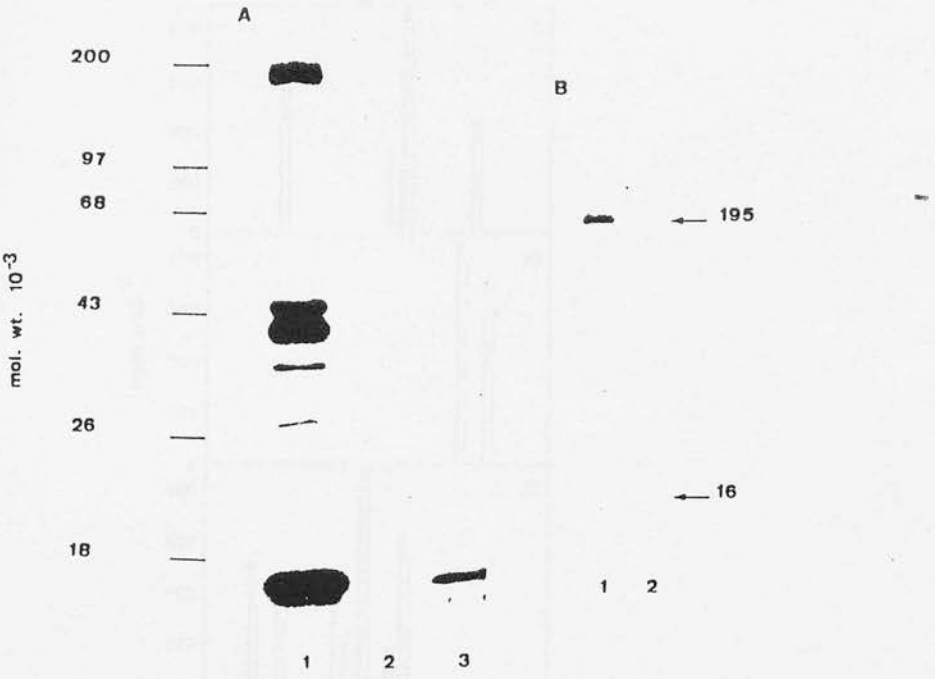

Figure 1. Biosynthetic incorporation of radioactive sugars into *P. falciparum* glycoproteins. A. SDS-PAGE of [^{3}H]-GlcN (lane 1), [^{3}H]-Gal (lane 2) and [^{3}H]-Man (lane 3) labelled glycoproteins of *P. falciparum*. B. SDS-PAGE of the immunoprecipitates of [^{3}H]-GlcN labelled proteins. Lane 1: mAb 3B10 immunoprecipitate. Lane 2: normal mouse serum control immunoprecipitate.

All metabolically labeled glycoproteins extracted in non-ionic detergent NP-40 were precipitated by human immune serum (not shown) and the mAb 3B10 [16] was used to immunoprecipitate the major 195 kDa glycoprotein and its 16 kDa processed product (Fig. 1B, lane 1) from NP-40 extracts of metabolically-labeled parasites.

Upon acid hydrolysis of both the 195 and 16 kDa glycoproteins, radiolabeled molecules migrating with standard Gal, GalNAc, GlcNAc and Man were released (Fig. 2A). The presence of GlcNAc and GalNAc in the glycoproteins was shown by conversion of the acid-treated hexosamines (2-amino-2-deoxyglucose and 2-amino-2-deoxy-galactose) by ninhydrin treatment [17] to respectively

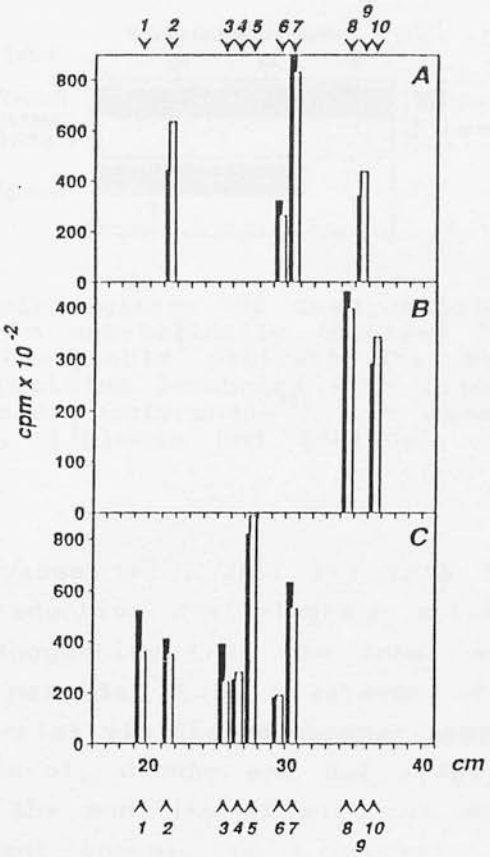

Figure 2. Biochemical characterization of biosynthetically labelled 195 and 16 kDa glycoproteins and analysis of the degradation products by paper chromatography. A: acid hydrolysis. B: acid hydrolysis followed by ninhydrin degradation. C: β-elimination followed by acid hydrolysis. The markers (arrows at top and bottom of figure) are 1: alanine; 2: Gal; 3: α-amino-butyric acid; 4: GlcNAc-ol; 5: GalNAc-ol; 6: GalNAc; 7: GlcNAc; 8: arabinose; 9: Man and 10: lyxose. The mobility of markers 1-7 and 9 is relative to GlcNAc, that of markers 8 and 10 relative to GlcN-HCl. Open bars: 195 kDa, filled bars: 16 kDa glycoprotein.

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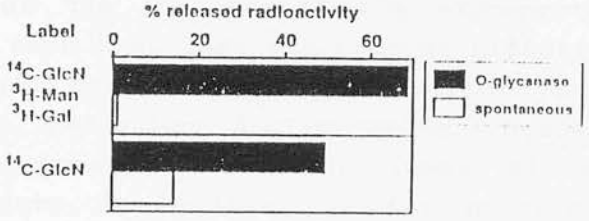

Figure 3. Enzymic release of O-glycosidically linked carbohydrates from metabolically labelled *P. falciparum* glycoproteins. Dialyzable radioactivity released from total labelled proteins incubated with (closed bars) or without (open bars) O-glycanase™. Top panel: labelling with [^{14}C]-GlcN, [^{3}H]-Man and [^{3}H]-Gal. Lower panel: [^{14}C]-GlcN.

arabinose and lyxose (Fig. 2B). For both the 195 and 16 kDa glycoproteins, reductive β-elimination solubilized 80% of the radioactivity, suggesting that the label was O-glycosidically linked to the peptide. Acid preatment of the β-eliminated, radiolabeled material yielded coumpounds migrating with standard GlcNAc-ol, GalNAc-ol, GlcNAc and Gal (Fig. 2C). In addition, components with the mobility of glycerol and faster were also detected (data not shown). In a separate experiment, the β-eliminated products were reduced *in situ* with $NaBH_4$-$NaB[^3H]_4$ and subsequent acid hydrolysis of the mixture showed therein the presence of [^{3}H]-labeled alanine and α-aminobutyric acid derived from serine and threonine respectively (Fig. 2C). Hexosaminidase treatment of the 195 and 16 kDa glycoproteins released a radiolabeled component moving as GlcNAc (data not shown).

As shown in Figure 3, O-glycanase treatment of total radiolabeled glycoproteins with endo-α-N-acetylgalactosaminidase released 68.75% and 49.6% respectively of the incorporated sugars. The released, dialysable carbohydrate moieties consisted of sugars comigrating with GalNAc and GlcNAc by pc (data not shown).

Investigations of the 195 and 16 kDa glycoproteins [7] were prompted by the fact that they may provide effective blood stage vaccines and that they contain carbohydrate [20]. This study demonstrates by reductive β-elimination, hexosaminidase and endo-O-glycanase treatment that in these glycoproteins, the carbohydrate moiety is O-linked to the protein core by two different linkages. The first contains GalNAc, a common sugar in O-linked chains. The second type of O-linked chains contain the unusual O-linked GlcNAc, which was shown to be O-linked to serine or threonine by reductive β-elimination. The derived GlcNAc-ol that was identified arose mainly from GlcNAc O-linked to amino acids. The possibility of GlcNAc-ol arising from N-linked carbohydrate chains in the mildly alkaline conditions of elimination-reduction is remote. Furthermore, it has been shown by N-glycanase treatment that carbohydrates of parasite are linked in non-N-glycosydic mode [14].

The O-linked GlcNAc and GalNAc are present in both the 195 and 16 kDa glycoproteins. Whether these two types of O-linked residues occur in the other glycoproteins of *P. falciparum* is not known. Also, the coexistence of N-linked oligosaccharides in glycoproteins bearing O-linked chains needs be clarified. [^{3}H]-Mannose residues were incorporated in the 195 and 16 kDa glycoproteins, but the presence of Man in the N-linked chains or as part the 195 kDa glycoprotein anchor region [21, 22] will require further studies.

The presence of terminal GlcNAc in animal cell glycoproteins was initially described by Torres and Hart [23] who identified GlcNAc residues in surface glycoproteins of mouse lymphocytes labeled with [^{3}H]-Gal. More recently, terminal O-linked GlcNAc residues have been found in glycoproteins associated with subcellular organelles [24], and also in the helminth *Schistosoma mansoni* [25].

This investigation of the O-linked sugars in glycoproteins synthesized by *P. falciparum* is pertinent to previous studies. Lectin binding and enzyme cleavage studies suggested the presence of GlcNAc, terminal Gal and Man residues in the glycoproteins and the absence of N-glycosidic linkage [14]. It has, however, been reported that enzymic digestion of parasitic

glycoproteins diminishes their antigenicity [26]. The biological and immunological significance of O-linked GlcNAc in *P. falciparum* glycoproteins is not yet known. It is, however, postulated that these polymers may be involved in the transport of macromolecules, proper assembly of multimeric proteins and reversibly block phosphorylation sites on proteins [27].

This study represents the first detailed investigation of the composition and structure of *P. falciparun* glycoproteins. In addition to showing the presence of terminal O-linked GlcN in the 195 and 16 kDa glycoproteins, we have also shown that over 60% of radiolabeled GlcN, Man and Gal incorporated into total parasite glycoproteins are released by O-glycanase treatment. This further supports that fact that O-glycosidically-linked sugars represent a major carbohydrate moiety in *P. falciparum* glycoproteins.

Acknowledgements. Dr. Nasir-ud-Din was supported by a Visiting Scientist Award from UNDP/World Bank/WHO Special Programme for Research and Training in Tropical Diseases. Dr. D. Hoessli was supported by Swiss National Science Foundation grant no 31-28632.90

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A water soluble peptidoglycan-containing polymer from the *Micrococcus lysodeikticus* cell walls

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The cell wall of *Micrococcus lysodeikticus* consists, in part, of peptidoglycan and external polysaccharide chains. The peptidoglycan moiety has been shown to contain polysaccharide chains composed of alternate N-acetylgluconsamine and N-acetylmuramic acid units linked (1--->4) [1], similar to those of numerous other peptidoglycans bound to peptide chains in micro-organisms [2]. These peptide chains which join the polysaccharide chains of the peptidoglycan moiety, are linked to the carboxyl group of the N-acetylmuramic acid residues. The external antigenic polysaccharide chains, which have a different structure for each microorganism, are composed in *M. lysodeikticus* of alternate glucose and 2-acetamido-2-deoxy-D-mannuronic acid (N-acetylmannosaminuronic acid) units [3,4]. It has been suggested that they are linked to the peptidoglycan moiety through a phosphate diester linkage involving some of the muramic acid residues at C-6 [5,6]. The linkage region of the external polysaccharide to the peptidoglycan in different organisms has various termini involving glucose 1-phosphate (5,6), N-acetylglucosamine 1-phosphate [7], and the polysaccharide directly linked, without the involvement of phosphodiester, to the peptidoglycan [8].

A water soluble polymer was obtained from *Micrococcus lysodeikticus* cell walls prepared according to the method of Sharon and Jeanloz [9]. The cell walls were prepared from trypsinized and nontrypsinized cells, and the autolytic enzymes were inactivated as described. The water soluble polymer was obtained from both, trypsinized and nontrypsinized, preparations at room temperature. The washings obtained at various stages of cell wall preparation contained a water soluble peptidoglycan polymer. This polymer, like cell wall peptidoglycan [5], contained glucose, 2-acetamido-2-deoxy-glucose, muramic acid, 2-acetamido-2-deoxy-mannuronic acid, glycine, alanine, lysine, glutamic acid and minor amounts of other amino acids. [Table 1]. A small amount of N-acetylglucosamine 6-phosphate, in addition to muramic acid 6-phosphate, was recovered from the acid hydrolysate of the polymer. The polymer was purified by gel filtration (Bio-Gel P-200 and Sepharose 6-B) and ion-exchange (DEAE-cellulose) chromatography.

Methylation of the purified polymer was performed as described earlier (5). The methylated polymer on depolymerization and examination of the liberated methylated sugars by gas liquid chromatography-mass spectrometry showed the presence of terminal- and 6-linked glucose, terminal- and 4-linked N-acetylglucosamine, 4-linked muramic acid and 4-linked N-acetylmannosaminuronic acid in addition to quantities of 2,3- & 3-O-methylglucose derivatives and 3-O-methyl N-acetylglucosamine. Sequential periodate oxidation at 4° in the dark gave an amount of formic acid corresponding to the oxidation of all the glucose, confirming the methylation results. Periodate oxidation results also indicated the presence of terminal reducing and non-reducing muramic acid and N-acetylglucosamine residues respectively. N-Acetylglucosamine and muramic acid was recovered unchanged in large amounts showing that these residues were 4-linked. The structural studies on this polymer suggested its similarity to the cell wall peptidoglycan. The sugar involved in the phosphodiester linkage between the external polysaccharide and the peptidoglycan was similar to the cell wall [10]. A small amount of N-acetyl-glucosamine in addition in addition to glucose may also be involved as the linkage point. The mechanism of formation of the cell wall-associated polymer and its function is not clear. It is very likely that this polymer may arise as a consequence of proteoplysis, autolysis or incomplete incorporation of this fragment in the cell wall during biosynthesis.

Table 1. Carbohydrate and amino acid composition of the purified soluble cell wall

Carbohydrates[a]	%	Molar ratio
Glucose	24.0	2.1
2-Acetamido-2-deoxy mannuronic acid	27.0	1.8
2-Acetamido-2-deoxyglucose	14.0	1.0
Muramic acid	15.0	0.96
Muramic acid 6-phosphate	0.6	
Amino acids[b]		
Alanine	22.0	
Glutamic acid	14.0	
Glycine	25.0	
Lysine	7.0	
Serine	19.0	

[a]Molar ratio relative to N-acetylglucosamine.
[b]Residues per 100 residues.

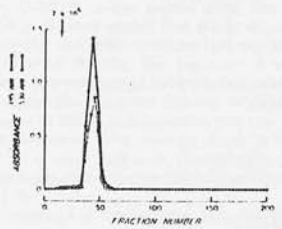

Fig. 1 Bio-Gel P-200 chromatography of cell wall extract. The column was washed with pyridine-acetic acid (pH 5.4). Fractions of were examined for hexoses hexosamines.

Nasir-ud-Din thanks UGC for financial support of bacterial cell wall programme.

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Plasmodium falciparum synthesizes 43000 daltons protein containing O-linked glucosamine.

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Malaria is a major parasitic disease of tropical and sub-tropical countries and occurs in a large part of the population each year with a considerable toll of debilitating morbidity and mortality. The plasmodial infection progresses through various developmental stages resulting in the expression of distinct antigens. The immune responses elicited against these antigens are stage-specific and involve both cell-and antibody-mediated mechanisms [1]. In the asexual erythrocytic stages of P. falciparum infection, different molecular weight glycoproteins antigens expressed by merozoites have been recognized and identified [2].

The biological role played by the carbohydrate moiety of glycoprotein antigens of P. falciparum has long been recognized [3]. Evidenc for the presence of carbohydrates on the surface glycoproteins of the asexual red cell stages of the parasite was obtained by in vitro incorporation of radiolabeled carbohydrate [4,5] and lectin binding [6]. The asexual blood stage of strain M25/ZAIRE were cultured in asynchronous node as described earlier [7]. The parasites were harvested after continuous labeling of the cultures with [^{3}H]-GlcN, [^{3}H]-Man and [^{3}H]-Gal. The radiolabeled gly-coproteins were extracted in non-ionic detergent and analysed by SDS-PAGE and autoradiography. The gel pattern [7-9] showed significant incorporation of [^{3}H]-glucosamine in a variety of proteins of different molecular masses. The labeled protein pattern of [^{3}H]-Glcn and [^{3}H] -Man was identical. For [^{3}H]-Gal only selected proteins were labeled. The activities of incorporated [^{3}H]-GlcN were high, whereas those of [^{3}H]-Man and [^{3}H]-Gal were lower.

The 43 KDa proteins seperated in SDS-PAGE [7] and revealed by autoradiography were excised from the gel and slices were treated with 50 M sodium hydroxide containing 1 M sodium borohydride at 45^{o}C and processed as described before [8]. The sugars were identified in paper chromatography (pc) [9]. The amino sugars were treated with ninhydrin according to the procedure of Stoffyn and Jeanloz [10]. The products were examined by pc. [9,10].

The 43 kDa excised gel fragment released over 70% of the radioactivity after acid hydrolysis, and the radiolabeled molecules migrated in pc with standard GlcNAc, and Gal; the latter was present in trace amounts. The presence of GlcNAc in the glycoprotein was further confirmed by conversion of GlcNAc of arabinose by treatment with ninhydrin [11].

The 43 kDa glycoprotein excised from the gel chromatogram was subjected to reductive β-elimination. This procedure solubilized 75% of the radioactivity in the gel, suggesting that the radio-active label was in a sugar, which was O-dycosidically-linked to the peptide. The radiolabeled solube material was then subjected to pc with standard galactose, GlcNAc, GalNAc, GlcNAc-ol and GalNAc-ol. The radioactive sugars in pc migrated with standard GlcNAc-ol, and in addition contained slower moving components. Subsequent to acid hydrolysis and N-acetylation, the β-eliminated radiolabeled sugar-containing materials in pc migrated with standard GlcNAc-ol and GlcNAc. In addition, radiolabeled components having the mobility of faster then glycerol were also detected. Following reductive elimination and hydrolysis, the products in two dimensional paper chromatography showed the presence of a radiolabeled component that migrated with standard alanine and trace amount aminobutyric acid.

In the current investigation, parasites in culture incorporated radiolabled $GlcNH_2$. Man, Gal, Ethanolamine and myristic acid as has been reported previously [3-6] and were isolated in high yield. Erythrocytes, infected erythrocytes or erythrocyte membranes were not detected in the parasite preparations of P. falciparum. Furthermore, the radiolabeled proteins are the known biosynthetic products, and also the erythrocytes in culture do not incorporate radiolabele [12]. Numerous proteins incorporated GlcNAc and Man, whereas the incorporation of galactose was restricted to fewer proteins. The diversity in incorporation of the labeled sugars into proteins was accompanied by variations in the activities of the radiolabel. This difference in quantities of incorporated sugars and the number of proteins in which the sugars are added, as noticed by SDS-PAGE, may have arisen due to the asynchronous mode of culture used.

Studies on this glycoprotein were performed for the known fact that 43 kDa glycoprotein contain carbohydrates. This glycoprotein contain GlNAc, Gal and Man as component sugars. This study demonstrate by reductive β-elimination that P. falciparum synthesizes glycoproteins in which the carbohydrate molety is O-linked to the protein core. The sugar of usual occurence in O-linked chains that are in abundance in animal cells is GalNAc. The O-linked chains in P. falciparum contain the unusual O-linked GlcNAc. The presence of terminal O-linked GlcNAc in animal cell glycoproteins has been described by Torres and Hart [13] who identified GlcNAc residues in the surface glycoproteins of intact mouse lymphocytes that could be labeled with [3H]-galactose. More recently, terminal O-linked GlcNAc residues have been found in glycoproteins associated with subcellular organelles in animal cell [14] and also in Schistosoma mansoni [15].

The significance of O-linked GlcNAc in glycoproteins is not yet known, and its role in plasmodial glycoproteins will need be explored. It is, however, postulated that these polymers may be involved in the transport of macromolecules [16], proper assembly of multimeric protein complexes [16], might prevent proteolysis and reversibly block sites of phosphorylation on proteins [16].

Dr. Nasir-ud-Din acknowledges financial support from NSRDB and WHO for malaria research programme.

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STRUCTURE OF A MICROCOCCUS LYSODEIKTICUS CELL WALL FRAGMENT CONTAINING PHOSPHORYLATED SUGARS

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Received February 13, 1992

SUMMARY

A polysaccharide-peptidoglycan complex containing different phosphorylated sugars from Micrococcus lysodeikticus cell wall has been isolated and purified. The peptidoglycan contained muramic acid 6-phosphate and N-acetylglucosamine 6-phosphate as phosphorylated sugars in additon to other sugar residues. Mild acid hydrolysis of the peptidoglycan and subsequent reduction of the released polysaccharide showed therein the presence of glucose and N-acetyl-glucosamine in the linkage of the external polysaccharide residues to the peptidoglycan through phosphodiester linkage. These data suggest the presence of polysaccharide chains linked to a peptidoglycan core through two phosphorylated sugars via two different terminal carbohydrate residues of the external polysaccharide chains in a same polymer.

INTRODUCTION

It is well known that peptidoglycan is the major two or three dimensional polymer responsible for strength, rigidity, shape and protective properties of walls of bacteria (1). The cell wall of Micrococcus lysodeikticus contains peptidoglycan and external antigenic polysaccharide. The peptidoglycan part has been shown to contain polysaccharide chains composed of N-acetylglucosamine and N-acetylmuramic acid residues linked β 1→4

Abbreviations: (Me-Mur-6)-(Glc-1)-P, methyl-2-acetamido-3-O-(D-1-carboxyethyl)-2-deoxy-α-D-glucopyranoside-6-yl)-α-D-glucopyranosyl phosphate; Glc-1-P, glucose-1-phosphate; Mur-6-P, Muramic acid 6-phosphate; Mur: Muramic acid; ManANAc, N-Acetylmannosaminuronic acid; glc, gas-liquid chromatography; pc, paper chromatography; PAGE, polyacrylamide gel electrophoresis; SDS: sodium dodecyl sulphate and CPC: cetylpyridinium chloride.

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0158-5231/92/030509-11$01.00/0

(2) similar to those of other peptidoglycans bound to peptide moiety in microorganisms (3). The external antigenic polysaccharide chains consist of alternate glucose and 2-acetamido-2-deoxy-D-mannuronic residues (4,5). It has been proposed that the antigenic polysaccharide chains are linked to the peptidoglycan backbone through a phosphate diester linkage involving muramic acid residues (6,7) at C-6. The linkage region of the antigenic polysaccharide to the peptidoglycan in various microorganisms has different termini such as N-acetylglucosamine 1-phosphate (8), glucose 1-phosphate (6,7), and the polysaccharide linked directly to the peptidoglycan i.e., with no phosphate involvement (9).

In Micrococcus lysodeikticus the linkage of acidic antigenic polysaccharide to C-6 of the muramic acid 6-phosphate unit has been initially proposed to be through N-acetylglucosamine residue involving a phosphodiester linkage (8). More recently it has been comprehensively shown that glucose is involved as a point of linkage (10). From recent investigations, there is evidence to suggest that a lipid intermediate containing glucose residues may be involved in the initiation process of external polysaccharide biosynthesis (11). The wall polymers and particularly the external polysaccharides are of importance as they provide reactive centres for adhesion to mammalian cells involved in recognition process that precedes stimulation of the synthesis of antibodies and modulation of immune responses. Comprehensive studies are required to delineate the linkage between external polysaccharide and peptidoglycan. Furthermore, in many cases extracted and purified wall and other surface polymers are not immunogenic by themselves, they are highly so as an integral component of wall or whole cell (1).

METHODS

Materials: Micrococcus lysodeikticus cells were obtained from Miles Laboratories and Worthington Biochemicals, acid (EC 3.1.3.2) and alkaline (EC 3.1.3.1) phosphatases from Sigma Chemical Company, and sodium borotritide from New England Nuclear.

Analytical Methods: Reducing sugars were estimated by the Park-Johnson method (12), 2-acetamido-2-deoxy-sugars by the Morgan-Elson method (13) and the phosphate sugars by the procedure of Chen et al. (14). Mixtures of glucosamine, muramic acid, glucosamine 6-phosphate and muramic acid 6-phosphate were resolved on AG50 (H^+) ion exchange resin and quantitatively estimated by the modified Elson-Morgan reaction (15). Amino acids in hydrolyzates were quantitatively determined in a high performance liquid chromatograph based on Beckman system. Gas liquid chromatography of reducing and non-reducing sugars were carried out using the Reinhold procedure (16) or according to Boersma et al. (17).

Paper and Thin Layer Chromatography, and Electrophoresis: Descending paper chromatography in Whatman No. 1 and 3 MM papers was performed in solvents (a) butanol-pyridine-water (6:4:3) and (b) butanol-acetic acid-water (4:1:5, upper layer). Amino sugars and amino acids were detected with ninhydrin, reducing and non-reducing sugars with silver nitrate and periodate-benzidine, and lactones with hydroxylamine and ferric chloride reagent as previously described (10). Electrophoresis on Whatmann No. 1 paper and thin layer silica plates were performed in pyridine-acetic acid buffer (pH 4.3) at 110v/cm for 3h and 10v/cm for 2h respectively. The strips of paper were stained with toluidine blue (18), and tlc plates were developed with anisaldehyde-sulfuric acid reagent. The polyacrylamide gel electrophoresis (PAGE) was performed as described earlier (19).

Colum Chromatography: Bio-Gel P-30 columns were run in water followed by 0.1M to 1M LiCl, the Bio-Gel P-60 columns were run in 50 mM pyridine-acetic acid (pH 5.4). DEAE-Sephadex column were run with a gradient of sodium phosphate (25 mM to 0.5M, pH 6.5), and or a gradient of LiCl. Amino sugars and phosphorylated sugars were resolved in DEAE-Sephadex and Dowex 50 ion-exchange resins.

Separation and Fractionation of Non-Dialyzable Cell Wall: The cell wall was prepared as previously described (24) and was lysed with egg-white lysozyme to obtain non-dialyzable cell wall es described earlier (6,10). The non-dialyzable cell wall in water (60% w/v) was treated with cetylpyridinium chloride (CPC 1% w/v) until the precipitation was complete. The precipitate was centrifuged and washed with aqueous ethanol. The polymer-cetylpyridinium complex was treated with 2M acetic acid for 6h at 22° C to release the polymer that was precipitated with ethanol to give fraction CPC-A. The supernatant and the washings were combined, reduced in volume and a mixture of ethanol (80%) and CPC (1% w/v), in the ratio of 4:1, was added until complete precipitation. The precipitate was washed with aqueous ethanol to remove CPC. The precipitated complex was treated with 2M acetic acid as described above for CPC-A to give fraction CPC-B. The washings and supernatant were combined, reduced in volume and treated with ethanol, as previously described, to give fraction CPC-C. The composition of fractions CPC-A, CPC-B, and CPC-C very similar but different from that previously described (10), is given in Table I.

Chromatography of Fraction CPC-C on Bio-Gel and DEAE-Sephadex: Fraction CPC-C was chromatographed on a column of Bio-Gel P-60. A single component was obtained with the composition given in Table II. This fraction was further chromatographed on a column of DEAE-Sephadex A-50 and the polymer was eluted with a gradient of 25 mM to 0.5 M sodium phosphate (pH 6.4) followed by 0.5 M LiCl. Two components, CPC-C-1 and CPC-C-2, were eluted from the column (Table II and Fig. 1). Fraction CPC-C-1 (20mg) was applied to a column of Bio-Gel P-30 and the column was eluted with 50mM to 0.1M pyridine-acetic acid, pH 5.4. A single carbohydrate containing polymer was eluted. The polymer was examined in PAGE.

Reducing Carbohydrate Residue of Fraction CPC-C-1: A sample of CPC-C-1 (40 mg) in water was cooled to 2° C and treated with sodium borohydride (150 mg) for 6h at 2-4° C. A further addition of sodium borohydride (40 mg) was made, and the solution was kept at 4° C for 4h. The reduced polymer was precipitated by the addition of ethanol. The precipitate was washed and repeatedly evaporated with methanol (4x15 ml) to give the reduced CPC-C-1. A portion of the reduced polymer was methanolysed and examined by glc.

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Table I

Carbohydrate and amino acid composition of non-diffusible cell wall, and of different fractions obtained by cetylpyridinium chloride fractionation.

Carbohydrate	Non-diffusible Cell Wall %	CPC-A %	CPC-B %	CPC-C %
D-Glucose	27	28	11	45
N-Acetylglucosamine	12	11	9	11
N-Acetylmannosaminuronic Acid	31	38	15	47
Muramic Acid	9	11	13	12
Muramic Acid 6-Phosphate	1.0	1.2	1.0	1.2
Glucosamine 6-Phosphate	0.14	0.16	0.10	0.08
Amino Acids[a]				
Alanine	47	44	40	38
Glutamic Acid	21	20	19	18
Glycine	19	21	21	20
Lysine	19	18	19	17

[a]Residues per 100 residues.

Table II

Carbohydrate and amino acid composition of CPC-C fractions obtained from DEAE-Sephadex A-50 purification.

Carbohydrate	CPC-C-1 %	CPC-C-2 %
D-Glucose	22	32
N-Acetylglucosamine	12	7
N-Acetylmannosaminuronic Acid	29	31
Muramic Acid	17	8
Muramic Acid 6-Phosphate	2.8	0.3
Glucosamine 6-Phosphate	0.4	-
Amino Acids[a]		
Alanine	44	48
Glutamic Acid	21	19
Glycine	18	17
Lysine	20	17

[a]Residues per 100 residues.

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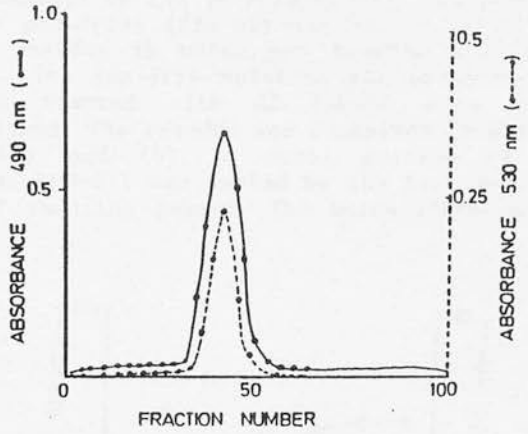

Fig. 1. Separation of non-dialyzable fraction CPC-C (50 mg) on a column (1.87 x 58 cm) of DEAE-Sphadex A-50. Fractions of 3 ml were collected and every third fraction was examined for the presence of hexoses by phenol-sulfuric acid reaction (490 nm, o-o) and hexosamines by Gatt-Berman colorimetric procedure (530 nm, o–o).

Cleavage of External Polysaccharide Chains from Reduced Peptidoglycan: Two different conditions of acid hydrolysis were followed to remove the external polysaccharide from the reduced peptidoglycan.

i) Fraction CPC-C-1 (12 mg) was dissolved in water (5 ml), and to aliquots (1 ml) in stoppered tubes was added AG 50W-X8 (H^+, 50-100 mesh, 50 mg). Samples containing (Me-Mur-6)-(Glc-1)P, and (Glc-1-P), (0.6 mg/ml, H_2O) were similarly treated. The samples were heated to 60° C and release of reducing sugars, amino sugars and N-acetylated sugars was measured as shown in Fig 2. Similar aliquots were filtered; the filtrate was evaporated under a stream of nitrogen, and the residue was dissolved in 50mg sodium acetate (pH 5.0) containing acid phosphatase (400 µg/ml). The solution was incubated at 37° C for 4h. The enzyme solution and the solution of reduced peptidoglycan was used as the control, and the phosphate released was meassured with Chen's method (14) as shwon in Fig 3. In addition, samples withdrawn at 4 hour interval were examined for the presence of pyrophosphate (14).

ii) Fraction CPC-C-1 (reducing terminal reduced, 8 mg), in a polyethylene tube was treated with 50% hydrofluoric acid (160 µl) for 90 min. at 4° C. (Me-Mur-6)-(Glc-1)-P (30 µg) and Glc-1-P (30 µg) were similarly treated. The reaction was quenched by adding a saturated solution of lithium carbonate. Lithium fluoride was removed at the centrifuge, and the supernatant solution was adjusted to pH 5.0 with 4M acetic acid. The solution was deionised by gel filtration on a column of Bio-Gel P-2, to give a carbohydrate containing fraction.

In the case of reference compounds the neutral solution was evaporated. The residue was dispersed in methanol (150 µl), and a portion of

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this solution was examined by tlc in solvent (a). The remaining solution was treated with acetic anhydride (150 µl) for 6h at 22° C. The solution was evaporated and the residue in water was treated with AG 50W-X8 (H^+) to remove lithium ions. The ion-free solution was evaporated. The residue in water (300 µl) was treated with AG 50W-X8 (H^+) filtered, and the filtrate was lyophilized. The residue was dissolved in water and examined by tlc in solvents (a) and (b). A small portion of the hydrofluoric acid-treated fraction CPC-C-1 was tested by the Park-Johnson (12) procedure for the presence of reducing sugars. The borohydride-reduced Fraction CPC

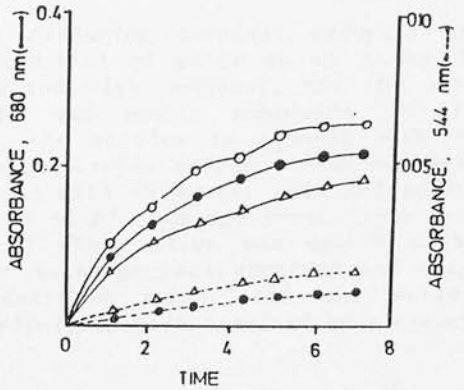

Fig. 2. Rate of acid hydrolysis of CPC-C-1 (o-o), glucose 1-phosphate (Δ-Δ) and (Me-Mur-6)-(Glc-1)-P (o-o). Release of reducing sugars was established using Park-Johnson (680 nm) and Morgan-Elson (544 nm) reactions.

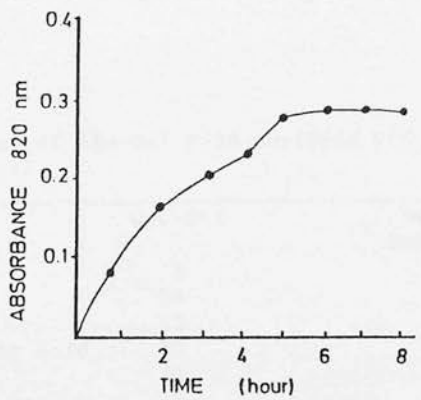

Fig. 3. Measurement of the release of phosphate by acid phosphatase from the aliquots of mild acid hydrolysis of CPC-C-1. Inorganic phosphate was measured by Chen's method.

was used as a blank, and glucose was used as a standard sugar. Another portion of the acid-treated CPC-C-1 was treated with acid and alkaline phosphatase (400 µl, same concentration as described above) for 8h at 37° C. The enzyme solutions were used as controls.

Identification of the Reducing End Group of the External Polysaccharide:

i) External polysaccharide, obtained by AG 50W-X8 (H^+) treatment of CPC-C-1 as described in the preceding section under (1), in water was treated with sodium borohydride, and the solution was deionised with AG 50W-X8 (H^+) to give a reduced polymer. A portion of the reduced polymer was methanolysed, and the products were examined by glc-mass spectrometry.

ii) Fraction CPC-C-1 (reducing terminal reduced) was treated with AG 50W-X8 (H^+) (20 mg) in 0.4 ml of water at 60° C for 3h. After filtration the residue was evaporated with methanol, and the residue was acetylated with pyridine (250 µl) and acetic anhydride (20 µl). The acetylating reagents were removed, the residue in aqueous methanol (30% 0.4 ml) was treated with sodium borodeuteride and/or sodium borotritide. The excess of borohydride was destroyed with 4M acetic acid and sodium ions were removed on a column of AG 50W-X8 in H^+ form and borate ions were removed by treatment with AG-1X8 (OAc^-). The column was washed with water followed by acetic acid (0.5M). The washings were combined and evaporated, the residue was acetylated as described above, and the acetylated sugars after methanolysis or acid hydrolysis were examined by glc-mass spectrometry or in pc.

Isolation and Examination of External Polysaccharide: The acid treated (AG 50W-X8) fraction CPC-C-1 (8 mg) was applied to a column (1.5x15 cm) of Bio-Gel P-30. The column was washed with water. The carbohydrate containing fractions were combined and lyophilized. The residue was rechromatographed on a column of DEAE-Sephadex A-50.

A major and a minor components were obtained (Table III, Fig. 4). A small portion of each component (0.2 mg and 0.1 mg respectively) was reduced with sodium borohydride and the product was acid hydrolyzed. The hydrolyzate was acetylated, methanolyzed and examined by glc.

Table III

Carbohydrate composition of Bio-Gel P-30 purified CPC-C-1 and ammonia treated Fraction CPC-C-1.

Carbohydrate	CPC-C-1	Ammonia Treated Fraction CPC-C-1
	%	%
D-Glucose	28	27
N-Acetylglucosamine	12	14
N-Acetylmannosaminuronic Acid	39	36
Muramic Acid	10	11
Amino Acids	ND	ND

ND: Not determined.

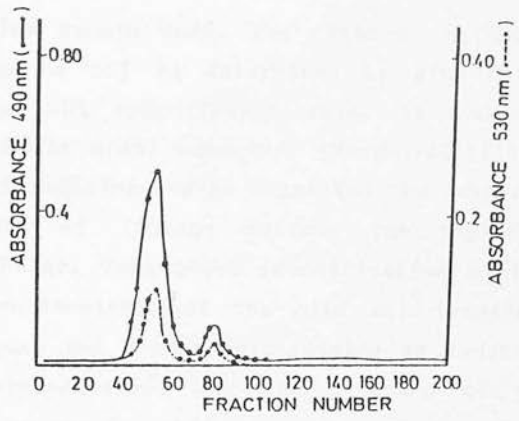

Fig. 4. Separation of the mild acid treated CPC-C-1 on a column (1.4 x 40 cm) of DEAE-Sephadex A-50. Fractions of 1.5 ml were collected and every third fraction was examined for the presence of hexoses (490 nm, o–o) and hexosamines (530 nm, o–o).

Presence of Orthophosphate Linkage between External Polysaccharide and Peptidoglycan Moiety in CPC-C-1: A solution containing CPC-C-1 (8 mg) in 2M ammonium hydroxyde (0.5 ml) was heated at 38° C for 20 minutes. The solution was cooled and chromatographed in a column of Bio-Gel P-30. CPC-C-1 was used as a control and chromatographed on the column. The alkali treated and P-30 purified CPC-C-1 material was treated with acid phosphatase as described earlier (8) with no release of phosphate.

RESULTS AND DISCUSSION

The non-diffusible cell wall was fractionated by CPC-complex fractionation into three fractions i.e., CPC-A, CPC-B and CPC-C, as previously described (10). Fraction CPC-C was purified by gel filtration in Bio-Gel P-60 and Sephadex A-50 into two fractions, CPC-C-1 and CPC-C-2. The CPC-C-1, a major fraction of CPC-C, representing 70% of CPC-C and 11% of the non-dialyzable cell wall, contained sugars, including two phosphorylated sugars; i.e., muramic acid 6-phosphate and glucosamine 6-phosphate; and amino acids similar to non-dialyzable cell wall in relatively lower proportion (Table II). The CPC-C-1 was homogeneous in gel filtration and in PAGE and its molecular weight was estimated at 14 k daltons. This fraction on reduction with sodium borohydride and subsequent glc analysis showed therein the presence of muramicitol and minor components with the mobility of N-acetyl-glucosaminitol, N-acetylmuramic acid, N-acetylglucosamine, glucose and

N-acetylmannosamine uronic acid. The ratio of muramic acid to muramicitol was estimated to be 3:1 as determined by glc. Mild acid treatment, AG 50-W-X8 (H^+) and 50% hydrofluoric acid, of sodium borohydride reduced CPC-C-1 and synthetic model compound, (Me-Mur-6)-(Glc-1)-P, and measurement of the release of reducing sugars suggested the involvement of different, at least two, types of linkage between the peptidoglycan and external polysaccharide chains. Subsequent identification of the products from acid hydrolysis and methanolysis of the mild acid-treated products showed the presence of glucose and N-acetylglucosamine as reducing terminal sugars in the released polysaccharide and the presence of orthophosphate, as no inorganic phosphate was detected.

Also muramic acid 6-phosphate and glucosamine 6-phosphate were the two identified phosphorylated sugars in the peptidoglycan. From these data it can be assumed that the mild acid treatment selectively ruptured the bonds between muramic acid 6-phosphate and/or glucosamine 6-phosphate residues and the external polysaccharide chains.

The phosphate from acid treated CPC-C-1 was subsequently released by treatment with acid phosphatase. The gradual increase with a short lag, between two and four hours, in the amount of phosphate release was concomitant with the release of reducing sugars, delayed increase in reducing power at four hours, and by implication external polysaccharide. Amongst the two conditions of mild acid hydrolysis, the AG50W-X8(H^+) treatment, as reported earlier (10), provided the maximum release of external polysaccharide with minimum degradation as examined and identified by kinetic studies and tlc or glc. The released external polysaccharide contained mainly 2-amino-2-deoxy-mannosaminuronic acid and glucose as the component sugars with a very minor quantity of N-acetylglucosamine.

The reducing terminal residues released by mild acid treatment were identified as glucose and to a lesser extent N-acetylglucosamine. The relative ratio of reducing terminal glucose to N-acetylglucosamine was estimated to be 6:1 as determined by the relative ratio of tritium incorporation into the two sugars. Tritium was incorporated into other sugar components as well with relatively slower mobility in pc, suggesting oligomers of sugars that possibly arose as a consequence of incomplete breakdown of the polymer under hydrolysis conditions. The proportion of D-glucose to D-glucitol was estimated to be 8:1. The kinetics of acid degradation of reducing terminal reduced Fraction CPC-C-1 was similar to that of (Me-Mur-6)-(Glc-1)-P, except for a minor lag in acid hydrolysis,

strongly suggesting that the external polysaccharide chains were linked to the phosphate attached in C-6 of the Mur residues or to a sugar bearing major similarity. The sugar residue involved in the linkage to Mur was shwon to be mainly D-glucose, in the proportion of 1:8 in relation to the total amount of glucose, which suggests a chain length of about 8 (1→6) D-glucose-(1→4)ManANAc repeating units. The chain length of the peptidoglycan moiety was found to be 3 to 4 repeating units of (1→4)D-GlcNAc-(1→4)-Mur by determination of the reducing terminal as reduced muramic acid. The composition of CPC-C-1 (Table II) suggests, in addition to the major phosphorylated sugar muramic acid 6-phosphate, glucosamine 6-phosphate as a minor phosphorylated sugar, and average distribution of 1 residue of Mur-P-Glc / 3-4 disaccharide units of (1→4)-D-GlcNAc-(1→4)-Mur of the peptidoglycan and approximately 8 disaccharide repeating units of (1→6)-D-Glc-(1→4)ManANAc for the external polysaccharide/ residue of Mur-6-P. The presence of glucosmaine 6-phosphate as a linkage point and N-acetylglucosaminitol derived from GlcNAc, after acid treatment and subsequent borohydride reduction, suggest the presence of additional sugars in the linkage region. The proportion of these sugars in the linkage region, GlcNAc-P-Glc-NAc, is unclear. The Micrococcus lysodeikticus cell wall consists of a complex molecular structure composed of a peptidoglycan, an external polysaccharide and a peptide (4, 18-19). The chemical structure of the peptidoglycan moiety (2, 20-22) and that of external polysaccharide have been described (4-6, 22-23).

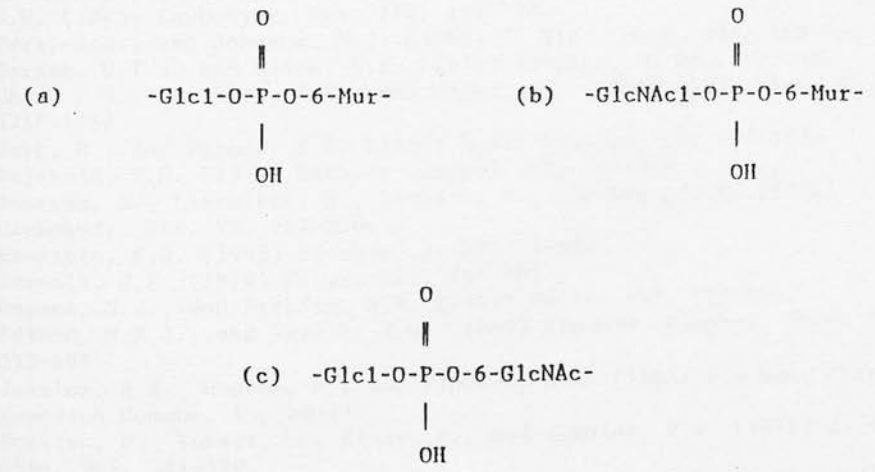

Fig. 5. Proposed structure of the cell wall linkage region.

In the current work the linkage between the external polysaccharide and the peptidoglycan has been further investigated, and a model for the chemical structure of the linkage region (Fig.5) of the carbohydrate rich cell wall fragment, Fraction CPC-C-1, is proposed. The evidence for structures (a) and (b) in Fig. 5 has been obtained in the current studies and this structure had been proposed in earlier studies (8,10). There is sufficient evidence in this study to suggest the presence of structure (c) in the cell wall.

ACKNOWLEDGEMENT: Dr. Nasir-ud-Din thanks the University Grants Commission for financial support for the cell wall research programme.

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Further studies on cervical glycoproteins: isolation and characterization of oligosaccharides from follicular phase cervical glycoproteins of bonnet monkey

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Cervical mucus is a complex mixture of epithelial secretions, the major constituents of which are mucin-type, carbohydrate-rich glycoproteins that share the physical and chemical properties of other epithelial secretions (1). The biochemical and biophysical changes in cervical mucus during the ovarian cycle influence the survival, nutrition and passage of sperm. The cyclic alteration of the physical properties of the mucus are accompanied by variations in the carbohydrate composition (1,2) and also the chemical structure, particularly the linkage of N-acetylneuraminic acid, galactose and 2-acetamido-2-deoxygalactose residues (3). The current study describes the structural features of the acidic oligosaccharide moiety of the main glycoprotein of the follicular phase cervical mucus. The mucus glycoproteins obtained from bonnet monkeys at the phase of the cycle was purified by gel filtration on Bio-Gel P-200 followed by fractionation on sepharose 4B. The main fraction (70%) showed in DEAE-cellulose chromatography the presence of single glycoprotein. The glycoprotein, in polyacrylamide gel electrophoresis, did not enter the gel, and no contaminating proteins or glycoproteins were observed. The glycoprotein was subjected to reductive cleavage by alkaline borohydride yielding a mixture of oligosaccharide-alditols. A decrease of serine and threonine and a corresponding increase of alanine and the appearance of 2-aminobutanoic acid were observed in hydrolyzates of the protein. The acidic oligosaccharides eluted from the column of AG 1-X2 were separated on a column of Bio-Gel P-6 into four fractions.

Oligosaccharide fraction A-1: This fraction was purified by DEAE-sephadex A-50 and was homogeneous in paper chromatography. Its sequential treatment with neuraminidase, α-L-fucosidase, and with β-D-galactosidase removed residues of fucose, galactose, and N-acetylneuraminic acid. Methylation of the residual oligosaccharide showed the presence of terminal N-acetylglucosamine, a terminal galactose (small proportion), 4-linked galactose, 4-linked N-acetylglucosamine, and 6-linked and 3,6-linked (small proportion) 2-acetamido-2-deoxygalactitol. These results suggest incomplete removal of a galactose residue. The results of methylation of the oligosaccharide after treatment with neuraminidase alone and that of native oligosaccharide showed that oligosaccharide A-1 is a heptasaccharide having the sequence, linkage and anomeric configurations as shown in Fig.1.

Oligosaccharide fraction A-2: Methylation of the oligosaccharide showed the presence of terminal neuraminic acid and N-acetylgalactosamine, 3-linked galactose, 4-linked N-acetylglucosamine, and 3,6-linked 2-acetamido-2-deoxygalactitol. Periodate oxidation and borohydride reduction resulted in total decomposition of N-acetyl-galactosamine and sialic acid, and conversion of 2-acetylglucosamine were recovered unchanged. The results of methylation of native-, N-acetyl-α-galactosaminidase and neuraminidase-treated oligosaccharide suggest the structure of oligosaccharide A-2 shown in Fig.1.

Oligosaccharide fraction A-3: The oligosaccharide A-3 was purified by chromatography on DEAE-Sephadex A-50. Periodate oxidation-borohydride reduction decomposed fucose, and neuraminic acid, and converted 2-acetamido-2-deoxygalactitol into 2-acetamido-2-deoxythreitol: galactose was recovered unchanged. The methylation results of the native-, α-L-fucosidase-and neuraminidase-treated oligosaccharides provide evidence that oligosaccharide A-3 is a pentasaccharide with the structure shown in Fig.1.

Methylation of the oligosaccharide and identification of the products showed terminal galactose and neuraminic acid, and 3,6-linked 2-aectamido-2-deoxygalactitol. Treatment of the oligosaccharide showed that sialic acid was linked to 0-6 of 2-acetamido-2-deoxygalactitol. These data suggest the structure of oligosaccharide A-4 as shown in Fig.1.

A variety carbohydrate chain-lengths in mucins and in blood group-active glycoproteins is known, and the heterogeneity of cervical mucus glycoprotein could be even wider because of changing physicochemical behaviour with the ovarian cycle. The follicular phase oligosaccharide structures shows distinct similarities with the periovalatory phase oligosaccharides, A1, A2 and A4, and some difference as in A3.

A-1αNeuAc(2-->3)[βGalNAc(1-->4)]βGal(1-->4)GlcNAc(1-->6)[αFuc(1-->2)βGal(1-->3)]GalNAc-01

A-2αGalNAc(1-->3)βGal(1-->3)[αNeuNAc(2-->3)βGal(1-->4)GlcNAc(1-->6)]GalNAc-01

A-3αFuc(1-->3)βGlcNAc(1-->3)βGal(1-->3)[αNeuAc(2-->6)]GalNAc-01

A-4αGal(1-->3)[αNeuAc(2-->)]GalNAc-01

Fig.1. Proposed structures for oligosaccharide

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The Chemical Structure of a High Molecular Weight Fragment of *Micrococcus lysodeikticus* Cell Wall

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(Received 7th August, 1992, revised 9th January, 1993)

Summary: A fragment of cell wall containing the external polysaccharide was isolated from the Micrococcus lysodeikticus cell wall. This fragment obtained by lysozyme degradation, cetylpyridinium chloride (CPC) precipitation, gel filtration and ionexchange chromatography was homogeneous in sodiumdodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). It was shown to consist a peptidoglycan moiety containing alternating β1,4 linked 2-acetamido-2-deoxy-D-glucose and 2-acetamido-3-0 (D-1-carboxyethyl)-2-deoxy-D-glucose units to which was attached an external polysaccharide chain consisting of approximately 20 repeating (1-4)-D-(2-acatamido-2-deoxy-β-D-mannopyranosyluronic acid)-(1-6)-0-(α-D-glucopyranosyl) residues with D-glucopyranosyl residues at reducing and non-reducing ends. The mode of linkage of the polysaccharide to the peptidoglycan has also been established. The point of linkage of the external polysaccharide, through a phosphodiester, to the peptidoglycan involved a glucose residue and 2-acetamido-2-deoxy-D glucose respectively. Kinetic studies for the acid catalysed release of external polysaccharide from the peptidoglycan was performed in parallel with synthetic [methyl-2-acetamido-3-0-(D-1-carboxyethyl)-2-deoxy- α-D glucopyranoside-6-yl)- α-D-glucopyranosyl phosphate and α-D-glucopyranosyl phosphate and showed the presence of a phosphodiester linkage between the external polysaccharide and the peptidoglycan. In addition, there is evidence to suggest that a small amount of N-acetylglucosamine is involved in the linkage of the polysaccharide in lieu of D-glucose.

Introduction

Peptidoglycan convalently linked to polysaccharide chains is a major component of *M. lysodeikticus* cell wall that displays distinct properties and composition. The mode of attachment of the antigenic external polysaccharide chains to the peptidoglycan through a phosphodiester linkage has been investigated [1,2]. Glucose and N-acetylglucosamine residues have been implicated as points of external polysaccharide attachment to the peptidoglycan through this type of linkage to residues of muramic acid or N-acetylglucosamine. External polysaccharide consisting of glucose and N-acetylmannosaminuronic acid has been isolated by trichloroacetic acid treatment [3], and the chemical structure of the polysaccharide and its attachment to the peptidoglycan has been partially elucidated [4,5].

The external polysaccharide chains, which have a different structure for different microorganisms, are composed of alternating glucose and 2-acetamido-2-deoxy-D-mannuronic acid. The linkage region of the external polysaccharide to the peptidoglycan in different organisms has various termini involving glucose-1-phosphate [6], N-acetylglucosamine-1-phosphate [7], and the polysaccharide directly linked, without involving phosphate, to the peptidoglycan [8].

For *M. lysodeikticus* the linkage of the acidic polysaccharide to C-6 of the muramic acid residue has been proposed to be through a phosphodiester linkage to an N-acetylglucosamine residue [9]. There was also strong evidence to suggest glucose as a point of linkage [5,9]. In later investigations

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[10] it has been observed that for the biosynthesis of external polysaccharide a lipid intermediate containing N-acetylglucosamine was required to initiate the elongation of the polymer [10]. Furthermore, it has been shown that tunicamycin and bacitracin both, inhibited the synthesis of the polysaccharide [11].

The present studies detail the chemical structure including the phosphodiester linkage of a cell wall fragment containing peptidoglycan and external polysaccharide chains.

Results and Discussion

The lysozyme degraded nondiffusible cell wall was fractionated by CPC-precipitation. The cetylpyridinium complex insoluble in water (Fraction CPC-A) was the major nondialysable component. On gel filtration it provided two macromolecules. The high molecular weight fraction, CPC-A-1, was further purified by ion-exchange chromatography on DEAE-cellulose (Fig. 1). This was homogeneous in SDS-polyacrylamide gel electrophoresis.

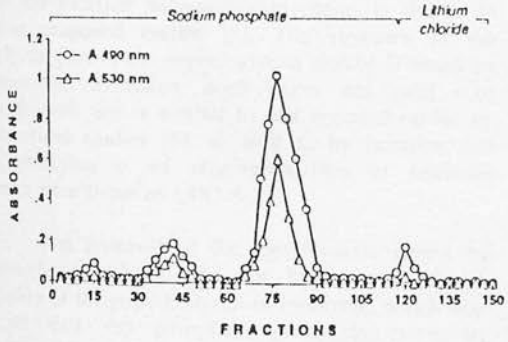

Fig. 1: Fractionation of CPC-A-1 on a column of DEAE-cellulose. Fraction of 2.5 ml were collected and every third fraction was examined for the presence of hexoses by phenol-sulphuric acid procedure (490 nm) and hexosamines by Gatt-Berman [13] (530 nm) method.

Periodate oxidation at 4°C in the dark, that has been shown to cause no over-oxidation of 2-acetamido-2-deoxy-sugar residues [6], provided an amount of formic acid (0.8 mol) suggesting oxidation of all of the glucose residue, indicating that these are linked at C-6.

The release of formaldehyde (nearly 50 nmol/mg) corresponded to the oxidation of reducing terminal muramic acid residues of the peptidoglycan fragment. The periodate treated fragment was reduced with sodium borohydride, the resulting polyalcohols on subsequent mild acid hydrolysis yielded low molecular weight fragments that contained only one quarter of the expected amount of glycerol and 2-amino-2-deoxy-mannuronic acid. This compound, after conversion into ester by treatment with methanolic hydrogen chloride and reduction with sodium borohydride, was identified by GLC as methyl 2-acetamido-2-deoxy-D-mannopyranoside. The presence of approximately 2% of nonoxidised glucose and 4% of glycerol in the oxidised, hydrolysed and non-dialysable material indicated the limitation of the Smith procedure [5] for the structure determination of complex carbohydrate molecules. The resistance of the glucose residues to periodate oxidation may suggest the presence of (1→3) linkage but this observation was not confirmed by the methylation procedure, and it is likely that steric effects were responsible for this resistance to the periodate oxidation. The presence of glycerol shows that the conditions of hydrolysis were not sufficient to hydrolyse the oxidised reduced, external chains completely; stronger conditions would hydrolyse the phosphate bond and, possibly, the 2-acetamido-2-deoxy-D-glucopyranosyl linkages. The total amount of glycerol found in the dialysable fraction corresponded to about one half of the value expected, the loss in glycerol probably being due to hydrolysis during purification of the reduced fractions. No erythrose was observed, thus confirming that all of the D-glucose residues are linkage at C-6. A second periodate oxidation of the oxidised, reduced non- dialysable fraction degraded the remaining D-glucose residues.

Methylation was performed by a modified Haworth procedure [5] in order to avoid degradation of alkali-sensitive bonds. Methanolysis followed by acetylation and examination by GLC-MS showed the presence of glucose as 2,3-di-, 2,3,4-tri- and 2,3,4,6-tetramethyl ether. The ratio of 2,3,4-tri- to 2,3,4,6-tetramethyl ethers was 17:1. The ratio of 2,3,4,6-tetramethyl ether suggested a chain length of 18 repeating units, in good agreement with the value of 20 found by determination of the reducing end linked to the phosphate group. The small

amount of 2,3,-di-*o*-methyl-D-glucose does suggest a branching at position C-4 of glucose. On reduction of the product of methanolysis, the 3-*o*-methyl ether of the corresponding 2-acetamido-2-deoxy-D-mannose residue was obtained in good yield by GLC and was identified by GLC-MS by comparison with an authentic sample [5].

The 6-methyl ether of muramic acid (2-acetamido- 3-(D-1-carboxyethyl)-2-deoxy-6-*o*-methyl-D-glucose) was characterised by compari son of the GLC-MS data with those of an authentic sample.

The 2-acetamido-2-deoxy-D-glucose component was characterised by GLC-MS, mainly after methanolysis as 3,4,6-tri and 3,6-dimethyl ethers. The proportion of 3,6-di- to 3,4,6-trimethyl ethers indicated the chain length of the glycan portion of the peptidoglycan moiety.

The results of the sequential periodate degradation, methylation and acid hydrolysis allowed to deduce the structure of the external polysaccharide moiety, the peptidoglycan portion and the linkage of the external polysaccharide to the peptidoglycan, as shown in Fig. 3. The structure of the external polysaccharide chains is similar to that proposed earlier [5]. The structure of the glycan part of the peptidoglycan moiety is based on periodate oxidation, methylation and mild acid hydrolysis and is similar to that reported earlier by structural studies [5], as well as by isolation and characterisation of oligosaccharides of fractions other than those of CPC-A-1.

The removal of the carbohydrate chains by periodate oxidation did not increase the suceptibility of the peptidoglycan to lysozyme, which suggests that the phosphate group is essentially resposnible for lack of lysis and this observation is in agreement with the specifity of egg-white lysozyme. The exact location of the phosphate group could not be determined. It was established that it is linked to an N-acetylglucosamine residue. The major phosphorylated sugar was 2-acetamido-2-deoxy-D-glucose, and this residue is considered to be the main sugar involved in the linkage region of the polysaccharide to the peptidoglycan through a phosphodiester linkage. Also, treatment of the polymer with phosphomonoesterase did not alter the quantity of this substituted sugar. The evidence for the presence of D-glucose as the terminal

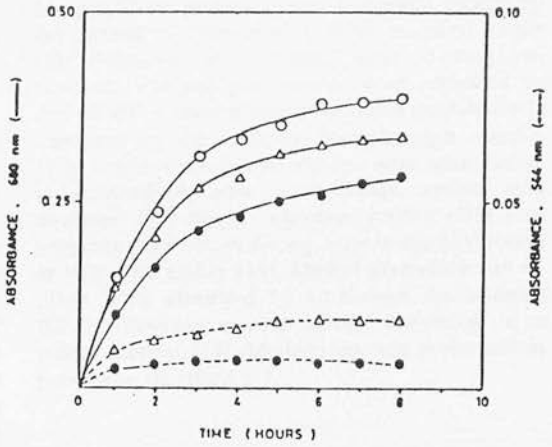

Fig. 2: Rate of mild acid hydrolysis of CPC-A-1 (Δ), glucose-1-phosphate (O) and (Me-Mur-6)-(Glc- 1)-P (●). Release of reducing sugars was measured using Park-Johnson [23] method (680 nm,—) and Morgan-Elson (544 nm,- - -) procedure.

reducing sugar of the external polysaccharide was indirect and based on the fact that this was the major reduced residue, glucitol, obtained after mild acid hydrolysis and subsequent borohydride reduction.

The proportion of chemical constituents, sugars and amino acids, of fraction CPC-A-1 is quite different from that of fraction CPC- A-2 [5]. The sugar content of fraction CPC-A-1 is only 65% of that of fraction CPC-A-2. Glucose and N-acetylmannosaminuronic acid represented only 62% of these residues in CPC-A-2, whereas GlcNAc and Mur were quite similar in quantity to CPC-A-2. The main chemical structural features of peptidoglycan -polysaccharide fractions obtained by CPC fractionation of the nondialyzable cell wall [4-6,9] and other cell wall components have been described [2-3,7]. The major structural features of these fractions are quite similar. The involvement of different sugars in the linkage region of external polysaccharide to peptidoglycan in *M. lysodeikticus* has been investigated. Glucose [5] and GlcNAc [7] have been identified as the terminal reducing sugars and components of the external polysaccharide. By implication these sugars are involved in the linkage of the external polysaccharide, via a phosphate residue, to the peptidoglycan. In the peptidoglycan both Mur-6-P [5,7] and GlcNAc-6-P [4,9,22] have

been identified. The involvement of both, GlcNAc-6-P and Mur-6-P, sugar derivatives in the linkage of peptidoglycan to the polysaccharide [4,9] have been described.

In the present study it has been shown that glucose, as the terminal sugar of the external polysaccharide, and GlcNAc-6-P, as a component of the peptidoglycan, are the sugar residue of CPC-A-1, that along with a phosphate residues, provide the linkage region. The kinetics of acid hydrolysis of the peptidoglycan-polysaccharide and identification of minor quantities of GlcNAc-ol, after acid hydrolysis and subsequent reduction, suggest that GlcNAc may also be involved in the linkage region as a component of the polysaccharide. Based on these data the linkage regions of the polymer are shown in Fig. 3.

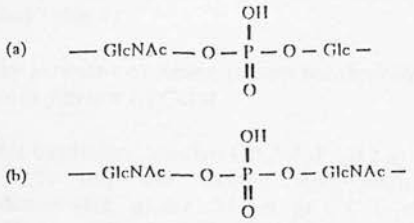

Fig. 3: Proposed linkage for CPC-A-1. (a) GlcNAc-6-P [22] of the peptidoglycan is linked to the glucose residue of the external polysaccharide. (b) GlcNAc-6-P of the peptdoglycan is linked to the GlcNAc of the external polysaccharide.

Experimental

Micrococcus lysodeikticus cells (spray-dried) were purchased from Miles Laboratories and Worthington Biochemicals; acid phosphatase from Worthington Biochemicals; potassium borotritide from New England Nuclear; cellulose F and silica gel plates from E. Merck; AG 50W-X8, AGIX-8, Bio-Gel P-2, Bio-Gel P-30 and Bio-Gel P-60 from Bio-Rad Laboratories; DEAE-cellulose (Whatman) from Reeve Angel; hydrfluoric acid (50% analytical reagent) from Baker Chemical Company. Egg-white lysozyme was purchased from General Biochemicals.

General analytical methods

Reducing sugars were estimated by Park-Johnson method, 2-acetamido-2-deoxysugars by the Morgan-Elson procedure and phosphate groups by the method of Chen *et al.* [12] as described earlier [5]. Mixtures of muramic acid 6-phosphate, muramic acid and glucosamine were separated on AG 50 (H^+) ion-exchange resin and quantitatively estimated by the modified Elson-Morgan reaction [13]. Amino acids in hydrolysates were quantitatively determined with a Beckman amino acid analyzer. Gas liquid chromatography (GLC) of reducing and non-reducing sugars was performed as described earlier [14]. Methyl glycosides and alditols were identified by GLC-mass spectrometry (GLC-MS) either as trimethylsilyl derivatives or as alditol acetates [14]. Methylation was performed as previously described [6].

Descending paper chromatography (PC) was performed on Whatman Nos. 1 and 3MM papers in solvents (a) butanol-pyridine-water (6:4:3, v/v), (b) 2-butanone-acetic acid-water (9:1:1, v/v) saturated with boric acid and (c) butanol-acetic acid-water (4:1:5, upper layer). Amino sugars and amino acids were detected with ninhydrin [15], reducing and non-reducing sugars with silver nitrate [16] and periodate-benzidine [17], and lactones with hydroxylamine and ferric chloride reagent [18]. Electrophoresis on Whatman No.1 paper was performed in pyridine-acetic acid buffer (Ph 4.3) at 110 v/cm for 3 hrs. The strips were stained with toluidine blue [19]. SDS-polyacrylamide (15%) gel electrophoresis was performed according to Laemmli [20]. Column chromatography on Bio-Gel P-30, Bio-Gel P-60 and DEAE-cellulose was performed as described earlier [5]. Hydrolysis and N-acetylation were performed as previously described [6]. N-acetylation of small samples of amino sugars was performed by dissolution in methanol and addition of 3 molar excess of acetic anhydride. After 4 hrs at 22°C, the mixture was evaporated under a stream of nitrogen. Small scale hydrolyses were performed on 1-2 mg of material with 4M hydrochloric acid for 16 hrs at 100°C. Small scale methanolyses were performed by treating the dried material (1-3 mg) with 0.5-1M methanolic hydrogen chloride for 12-16 hrs at 100°C. Esters and aldehyde groups were reduced by treating the cooled aqueous solution of the substance (2-4 mg) with $NaBH_4$. Ninhydrin degradation of hexosamines was performed according to Stoffyn and Jeanloz [21].

Preparation and fractionation of non-dialyzable cell wall

Cell wall was prepared and lysed with egg-white lysozyme to obtained non-dialyzable cell wall as described earlier [5]. The non-dialyzable cell wall in water was fractionated with CPC into three fractions, CPC-A, CPC-B and CPC-C, as previously described [5,9].

Fractionation of CPC-A on Bio-Gel P-30

A solution of CPC-A in water (0.1 %, w/v) was applied to a column (1.8 x 50 cm) of P-30. The column was washed with pyridine-acetic acid (pH 6.2). Two fractions, CPC-A-1 and CPC-A-2, were obtained. The carbohydrate and amino acid compostion of CPC-A-1 is given in Table 1. Fraction CPC-A-1 was further purified on DEAE-cellulose (Fig. 1 and Table 1).

Sequential periodate oxidation-sodium borohydride reduction of fraction CPC-A-1

First treatment; fraction CPC-A-1 (0.1 g) in water (20 ml) was treated with sodium metaperiodate (0.2 g) for 24 hrs at 4°C in the dark. The consumption of periodate measured spectrophotometrically (223 nm) and was constant after 8 hrs. The formic acid release was estimated by titration with NaOH, and the formaldehyde with the chromotropic acid reagent [6]. The excess of $NaIO_4$ was decomposed with 1,2-ethanediol, and the solution was dialysed for 3 days at 4°C against distilled water to give a non-dialyzable fraction (0.68 g). To a portion of this material (50 mg) in water (5 ml) at 4°C was added $NaBH_4$ (200 mg) in 4 portions, and the solution was kept at 4°C for 24 hrs. The excess borohydride was decomposed with 4M acetic acid, and the solution was dialyzed for 30 hrs against distilled water at 4°C. The non-dialyzable portion was concentrated, and borate ions were removed as methyl borate. The residue was dissolved in water, the solution lyophilized and the product treated with 0.5M sulphuric acid (1.5 ml) for 2 hrs at room temperature; the solution was then dialysed against water for 40 hrs. The nondialysable fraction was lyophilised, to give a periodate resistant fraction (38 mg), and retained for further treatment with periodate. The composition of this fraction is reported in Table 2.

Table 1: Carbohydrate and amino acid composition of fractions CPC-A-1 purified on DEAE-cellulose.

Carbohydrates	%
D-glucose	18
N-Acetylglucosamine	14
N-Acetylmannuronic acid	21
Muramic acid	13
Muramic acid 6-phosphate	0.6
Glucosamine 6-phosphate	0.11
Amino acids*	
Alanine	36
Glutamic acid	16
Glycine	22
Lysine	26

*Residues per 100 residues.

Table 2: Carbohydrate and amino acid composition of periodate treated CPC-A-1.

Components and compounds formed from fraction CPC-A-1	Sequential Smith degradation			
	First		Second	
	D	ND	D	ND
D-Glucose[a]		2		0
2-Amino-2-deoxy-D-mannuronic acid[a]		12		0
2-Amino-2-deoxy-D-glucose[a]		9		12
Muramic acid[a]		14		18
Glycerol	2.4	4	0	0
Alanine[b]		10		12
Glutamic acid[b]		8		8
Glycine[b]		4		8
Lysine[b]		5		10
Periodate consumed (unol/mg)	2.25			
Formic acid released (unol/mg)	0.64			
Formaldehyde released (umol/mg)	68.0			

[a]Component contents expressed in %, [b] Residues per 100 residues. Abbreviations: D, dialysable fraction: ND, nondialysable.

The dialysable fraction was reduced in volume and the solution passed through a column (0.8 x 16 cm) of AGIX-8 (AcO^-) ionexchange resin (200-400 mesh) the column was washed with water (12 ml) and 10mM acetic acid (12 ml). The eluate was concentrated to 2 ml, and lyophilised (9 mg). Thin layer chromatography on cellulose and paper chromatography in solvents b and c, and detection with silver nitrate, toluidine blue and hydroxylamine-ferric chloride reagents indicated the presence of glycerol, 2-acetamido-2-deoxy-man-

nuronic acid and 2-acetamido-2-deoxy-mannuronolactone.

Hydrolysis of a portion of the dialysable material with M HCl for 16 hrs at 100°C and examination of the products in PC in solvent c indicated the presence of glycerol and 2-amino-2-deoxy sugars. Another portion of the dialysable fraction was treated with 0.5M methanolic hydrogen chloride for 6 hrs at 100°C. The acid-free residue was N-acetylated and the product was treated in aqueous methanol (1:1) with sodium borohydride in the usual way. The reduced material was hydrolysed with M HCl for 3 hrs at 100°C, and the product was N-acetylated. Examination of the products in solvent c and subsequent detection of the sugars with silver nitrate and 0.5M NaOH in ethanol showed the presence of 2-actamido-2-deoxymannose and glycerol. This was confirmed by thin layer chromatography (TLC) of the methyl glycosides.

The remaining dialysable material was separated into two major fractions on 3MM paper in solvent c. The first fraction (0.6 mg, R_{GlcNAc} 0.22) of the chromatogram contained glycerol as indicated by t.l.c. The second fraction (3 mg) consisted of two compounds having R glucouronolactone 0.9 and 1.11. The two compounds were eluted from the chromatogram, glycosylatd and esterified with methanolic hydrogen chloride. The products were acetylated and reduced with $NaBH_4$. The reduced products were hydrolysed with M HCl (1.5 ml) for 6 hrs at 100°C. The hydrolysate was adsorbed on AG IX-8 (AcO^-) ion-exchange resin, and the column was washed with aqueous methanol. The eluate was evaporated, the residue N-acetylated and the product crystallised from aqueous ethanol (1:1, v/v) and acetone . Recrystallisation from a mixture of aqueous ethanol and acetone gave platelets having melting point (MP) 102-103°C and mixed MP with N- acetylmannosamine 102-105°C.

Second degradation

A small portion of the nondialysable material resulting from the first $NaIO_4$ treatment (5 mg) was dissolved in 0.1 M $NaIO_4$ (3 ml), and the solution was kept for 24 hrs at 4°C in the dark. The consumption of periodate corresponded to 20 nmol glucose residue. The solution was processed as described for the first treatment of CPC-A-1, except that the duration of the acid hydrolysis with 0.5M H_2SO_4 was extended to 3 hrs. The nondialysable material was lyophilised (0.6 mg). Examination of the dialysable material by p.c. showed the absence of reducing and nonreducing sugars.

Lysozyme degradation of periodate-degraded CPC-A-1

Twice periodate-treated fraction CPC-A-1 (2 mg) in 5mM ammonium acetate (2 ml) was treated with lysozyme (3 mg) for 2 hrs at 37°C in the presence of a drop of toluene. The resulting solution was dialysed to give a non dialysable fraction. Examination of the dialysate, after processing as described under first treatment of CPC-A-1 by periodate, in PC in solvent d showed the absence of reducing and nonreducing sugars.

Liberation of external polysaccharide chains from reduced peptidoglycan-polysaccharide

Acid hydrolysis was performed to remove the polysaccharide from the reduced peptidoglycan-polysaccharide. Fraction CPC-A-1 (8 mg) was dissolved in water (4 ml), and to samples (0.5 ml, 1 mg) in stoppered tubes was added AG 50-x8 (H^+, 50-100 mesh, 15 mg). Samples containing (methyl-2-acetamido -3-0-(D-1-carboxyethyl)-2-deoxy-α-D-glucopyranoside-6-yl]-D-glucopyranosylphosphate and glu- coe-1-phosphate were similarly treated. The samp les were heated at 65°C, and the release of reducing sugars and N-acetylated sugars measured is shown in Fig. 2. Similar aliquots were filtered, the filtrate was evaporated under a stream of nitrogen and the residue was dissolved in 50 mM sodium acetate buffer (pH 5.0, 80 µl) containing acid phosphates (400 µg per ml). The solution was incubated for 4 hrs at 37°C. The enzyme solution and the solution of the peptidoglycan were used as blanks. The phosphate release was measured by the method of Chen [13]. In addition, the samples withdrawn at 3 and 5 hrs intervals were examined for the presence of inorganic phosphate. Phosphate was not detected in hydrolysis samples of 3 and 5 hrs intervals, thereby suggesting the absence of a pyrophosphate linkage.

Acknowledgement

Dr. Nasir-ud-Din thanks the University Grants Commission, Pakistan for financial support for the bacterial cell wall research programme.

Abbreviations

The abbreviations used are: P.C. paper chromatography, G.L.C.- M.S., gas liquid chromatography-mass spectrometry, SDS, sodium dodecyl sulphate, CPC, cetylpyridinium chloride, (Me-Mur-6)-(Glc- 1)-6-P, [Methyl-2-acetamido-3-0-(D-1-carboxyethyl)-2-deoxy- α-D-glucopyranoside-6-yl]- α-D-glucopyranosyphosphate.

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Further Studies on Plasmodium falciparum Glycoproteins: Characterization of 195 kDa Glycoprotein:

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Malaria is a major parasitic disease of the developing world and is caused by the unicellular protozoan of the genus Plasmodium. There is a major interest in the possibility of developing viable vaccines against malaria. The development of antimalarial vaccine has necessarily focused on the identification and characterization of plasmodial antigens. The parasitic infection progress through various developmental stages resulting in complex and different antigens presented to the host. The immune responses elicited are equally complex, stage specific and involve both cell mediated and humoral mechanisms (1-3). An extensive interest is developing to investigate the role of the carbohydrate moiety of the glycoproteins that are synthetized by Plasmodium falciparum (4,5). Although the proteins investigated so far are likely to be associated with the surface of the merozoites, it is not unlikely that some may be part of the surface of the erythrocytes attacked by the parasite (6,7). The carbohydrate moieties of the glycoproteins of the asexual blood stages are potentially antigenic (8) and are possible target for protective immune response. It has been observed that antibodies against the high molecular weight glycoproteins in particular 195 kDa, inhibit cell growth in culture. The current studies involving metabolic incorporation of sugars, an amino alcohol and a fatty acid in the 195 kDa glycoprotein suggest the anchor region containing myristic acid, ethanolamine and sugars.

The parasitic cells of strain M25/ZAIRE were used in this investigation. Asexual blood stages were cultured in asynchronous mode in RPMI medium supplemented with 10% glucose and 50 Ci ml of the radiolabelled sugars (Amersham, UK) i.e., D- [6- ^{3}H] or D-[1- ^{14}C]-glucosamine hydrochloride, D-[26 ^{3}H]-mannose, D-[6- ^{3}H]-galactose, [^{3}H]-ethanolamine, [H]-myristic acid, separately as well as in admixture. Labelled parasites were harvested after 6 h of incubation at 37°C in a candle jar (9). NP-40 extracts of metabolically labelled cell were immunoprecipitated with a mouse mAb against the 195 kDa glycoprotein, a human immune serum against asexual blood stage antigens and normal mouse serum as control. Aliquots of the extracts containing 5-20 x 10 cpm were incubated overnight in excess antibody at 4°C. Antigen-antibody complexes were recovered with an anti-mouse immunoglobulin-Sepharose 4B matrix in TNE containing 0.5% NP-40 and 5 defatted milk. The Sepharose beads were centrifuged at 2500 x g for 5 min and washed 3 times with TNE-NP-40-Milk and 3 more times with TNE-NP-40 to remove non-specifically bound material. The antigen-antibody complexes were eluted in 50 mM Tris-HCl pH 6.8 sample buffer containing 2% SDS and 3% 2-mercaptoethanol. These samples were boiled for 3 min and analyzed by SDS-PAGE. The labeled Glc NAc, Man, Gal, ethanolamine and myristic acid were incorporated in the 195 kDa protein. The 195 kDa protein revealed by autoradiography were excised from the gel and incubated for 20 h in 5 ml of 50 mM NaOH containing 1 M $NaBH_4$ at 45°C (10). Paper chromatography of the processed products showed the presence of N-acetylglucosaminitol, trace amounts of N-acetylgalactosaminitol, glactose and mannose. The 195 kDa glycoproteins excised from the gel were treated with 3 M HCl for 16 h at 100°C and the hydrolyzate in paper chromatography showed the presence of GlcN, Gal NAc (trace) Gal and Man. Further investigations on 195 kDa glycoprotein were performed to identify the locations of glycans in this protein. This glycoprotein contains Glc NAc, Man, Gal NAc (Trace), ethanolamine and myristic in addition to amino acids, incorporation of ethanolamine and myristic acid has been accomplished and identification of these incorporated molecules need be performed. The presence of O-linked glycans in this protein is known (10) and this study suggest the presence of glycans also in the anchor region.

Dr. Nasir-ud-Din acknowledges support from WHO-TDR and NSRDB for malaria programme. Professor Qazi thanks NSRDB for financial support for this study.

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Vol. 32, No. 6, April 1994 BIOCHEMISTRY *and* MOLECULAR BIOLOGY INTERNATIONAL
Pages 1129–1137

A WATER-SOLUBLE FRAGMENT OF *Micrococcus lysodeikticus* CELL WALL

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Received October 15, 1993
Received after revision, February 28, 1994

SUMMARY

A fragment of *Micrococcus lysodeikticus* cell wall was obtained by extraction of walls with water, dimethylformamide or dimethyl sulfoxide. The water-soluble polymer was obtained from the cell walls prepared either with or without trypsin treatment of the cell. This fragment was studied by the Smith periodate oxidation, methylation, mild acid treatment and enzymic procedures. The polymer consists of polysaccharide chains composed of (1→4)-*O*-(2-acetamido-2-deoxy-β-D-manno-pyranosyluronic acid)- (1→6)-*O*-α-D-glucopyranosyl residues. The polysaccharide chain is linked to C-6 of a 2-acetamido-3-*O*-(D-1-carboxyethyl)-2-deoxy-β-D-gluco-pyranosyl residue of a peptido-glycan chain composed of repeating (1→4)–*O*–(2-acetamido-2-deoxy-β-D-glucopyranosyl)–(1→4)–[2-acetamido-3-*O*-(D-1-carboxyethyl)–2–deoxy-β-D-glucopyranosyl] residues. The water-soluble cell-wall fragment was also observed in the-culture medium of *Micrococcus lysodeikticus* and was also extractable from the cells in minor quantity.

INTRODUCTION

The cell wall of *Micrococcus lysodeikticus* consists of peptidoglycan and external polysaccharide chains that are responsible for strength, rigidity, shape and protective properties of bacterial cell (1) and for their antigenicity. Perkins (2), has described isolation of external polysaccharide chains of *Micrococcus lysodeikticus* cell wall by trichloroacetic acid treatment, and the chemical structure of these chains has been investigated (3,4) and elaborated (5,6). The antigenic polysaccharide chains contain repeating (1→4)-*O*-(2-acetamido-2-deoxy-β-D-mannopyranosyluronic acid) (1→6)-*O*-(D-glucopyranosyl residues (3,4). The peptidoglycan moiety is known to consist of alternating (1→4)-linked 2-acetamido-2-deoxy-β-D-glucopyranosyl and 2-acetamido-3-*O*-(D-1-carboxyethyl)-2-deoxy-β-D-glycopyranosyl residues to which the polysaccharide chain is attached through a phosphate group at C-6 to one of muramic acid residues.

The linkage region of the polysaccharide to the peptidoglycan in the cell wall of different microorganisms has variable termini, such as *N*-acetylglucosamine 1-phosphate (7), glucose 1-phosphate (8) and the polysaccharide linked directly to the peptidoglycan, i.e., with no phosphate involvement (9). In *Micrococcus lysodeikticus* the linkage of the antigenic polysaccharide to the C-6 of

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muramic acid of peptidoglycan has been shown to involve both glucose 1-phosphate and *N*-acetyl-glucosamine 1-phosphate. The nature of the water-soluble fragment isolated from the *Micrococcus lysodeikticus* cell wall and the chemistry of this fragment are the subject of this report.

EXPERIMENTAL PROCEDURE

Materials: Micrococcus lysodeikticus cells were obtained from Worthington Biochemicals and Miles Laboratories or grown in culture as previously described (10); acid (E.C. 1.3.2) and alkaline (E.C. 3.1.3.1) phosphatase from Sigma Chemical Company, and sodium borotritide from New England Nuclear.

Analytical methods: Reducing sugars, 2-acetamido-2-deoxy sugars and phosphorylated sugars were estimated as described earlier (11). Mixtures of glucosamine, muramic acid, glucosamine-6-phosphate and muramic acid 6-phosphate were separated as described previously (11). Amino acids, reducing and nonreducing sugars in hydrolyzates and methanolyzates were determined as described earlier (11). Gas-liquid chromatography (GLC) and glc-mass spectrometry (GLC-MS) was per-formed as previously described (12).

Paper and thin layer-chromatography and electrophoresis: Paper chromatography (PC), thin-layer chromatography (TLC), paper electrophoresis and polyacrylamide gel electrophoresis (PAGE) were performed as reported previously (7). PC was done in solvents as described earlier (11).

Column chromatography: Bio-Gel P-60, Bio-Gel P-200 and Sepharose 6-B columns were run in 50 mM pyridine acetate (pH 5.8). DEAE-cellulose column was run with a gradient of sodium phosphate (25 mM to 0.5 M) followed by 0.5 M LiCl.

Extraction of cell-wall fragment: Cell walls (2.0 g) prepared according to the procedure of Sharon and Jeanloz (12), trypsinized or nontrypsinized, were dispersed in water (100 ml) and the solution stirred at room temperature for 48 h in the presence of a drop of toluene at 22 or 37 °C. In a few experiments the cell walls were extracted with a mixture of dimethyl sulfoxide (DMSO)–water (1:1) or dimethylformamide (DMF)–water (1:1). These experiments were also performed at 37 °C. The insoluble cell walls were removed by centrifugation at 4 °C at 8000 rpm. The supernatant was removed from the pellet and dialyzed against distilled water (2 × 400 ml). The retentate was lyophilized to give soluble cell-wall polymer (180 mg). The composition of this fragment is given in Table I.

Chromatography of the soluble cell wall (SCW): SCW (150 mg) was chromatographed on a column (0.6 × 45 cm) of Bio-Gel P-200. A single carbohydrate containing component was eluted from the column with the composition given in Table I. This fraction (120 mg) was further chromatographed in a column (0.6 × 24 cm) of Sepharose 6-B. On washing the column with pyridine acetate (50 mM, pH 5.8) two components, a major (SCW-2, 90 mg) and a minor (SCW-1, 9 mg), were eluted from the column (Table II). SCW-2 (60 mg) was applied to a column of Bio-Gel P-60 and the column washed with 50 mM to 1 M pyridine-acetic acid (pH 5.8). A single carbohydrate containing material was eluted from the column. The composition of this material is given in Table II.

DEAE-Cellulose chromatography of the Sepharose purified SCW-2: Polymer SCW-2 was chromatographed on a column (0.4 × 25 cm) of DEAE-cellulose and the material was eluted with a gradient of 25 mM to 0.5 M sodium phosphate (pH 6.5) followed by 0.5 M LiCl. The composition of this material is given in Table II. The polymer was examined in PAGE.

Treatment of Micrococcus lysodeikticus *cells with water, DMSO and DMF:* The *Micrococcus lysodeikticus* cells (12 g) were extracted with water, DMSO and DMF as described for the cell wall. The extracted material was processed as described for the cell-wall to provide a polymer (80 mg). This material was chromatographed on a column of Bio-Gel P-200. A single component was isolated (Table I).

Co-chromatography of SCW and consumed Micrococcus lysodeikticus *medium: Micrococcus lysodeikticus* cells were grown in Difco Bactopeptone as previously described (10). The SCW and consumed medium after lyophilization and resolubilization were chromatographed and co-

TABLE I: Carbohydrate and amino acid composition of soluble cell fragment and soluble fragment of *M. lysodeikticus* cell component.

Component	Soluble cell wall fragment %	*M. lysodeikticus* soluble cell fragment %
D-Glucose	41	38
N-Acetylglucosamine	12	13
N-Acetylmannosamine uronic acid	31	27
Muramic acid	92	8
Muramic acid 6-phosphate	1.2	0.8
Glucosamine 6-phosphate	0.16	0.06
Alanine[a]	36	38
Glutamic acid[a]	21	20
Glycine[a]	22	19
Lysine[a]	21	23

[a] Residues per 100 residues.

TABLE II: Carbohydrate and amino acids composition of Sepharose 6B purified soluble cell wall (SCW) fractions, SCW-1 and SCW-2, Bio-Gel P-60 and DEAE Cellulose purified SCW-2, and peptidoglycans, MF-1 and MF-2, from *M. lysodeikticus* culture medium (consumed):

Component	SCW-1 %	SCW-2 %	P-60 purified SCW-2 %	DEAE cellulose SCW-2 %	MF-1 %	M2 %
D-Glucose	14	30	32	31	21	33
2-Acetamido-2-deoxy-D-glucose	11	12	14	15	12	14
2-Acetamido-2-deoxy-D-mannuronic acid	17	34	36	37	16	37
Muramic acid	9	11	12	14	14	10
Muramic-acid 6-phosphate	0.4	0.7	0.8	0.9	0.32	0.36
N-Acetylglucosamine 6-phosphate	0.08	0.12	0.11	0.10	0.06	0.12
Alanine[a]	41	44	43	39	41	39
Glutamic acid[a]	18	21	20	24	19	21
Glycine[a]	20	18	19	21	19	21
Lysine[a]	21	17	18	16	21	19

[a] Residues per 100 residues.

chromatographed on a column of Sepharose 6B. The consumed medium contained Fraction 1 (MF-1), which co-chromatographed with SCW-1, and another component, Fraction 2 (MF-2) containing reducing sugars which co-chromatographed with SCW-2, in addition to other sugar-containing molecules (Fig. 1). The components MF-1 and MF-2 were rechromatographed on a column of Bio-Gel P-60 and their composition is given in Table II.

Sequential periodate oxidation-sodium borohydride reduction of fraction SCW-2: Fraction SCW-2 (90 mg in 2 ml of water, pH 4.0) was treated with sodium meta-periodate (0.30 g) for 24 h at 4 °C in the dark. The consumption of periodate, measured spectrophotometrically at 222 nm, was constant after 8 h. The re-leased formic acid was estimated by titration with 20 nM sodium hydroxide, and the released formaldehyde with the chromotropic acid reagent (2-acetamido-2-deoxy-D-glucitol) as the control. The excess of periodate was decomposed with 1, 2-ethanediol and the solution was dialyzed for two days at 4 °C against distilled water to give a non-dialyzable fraction (64 mg after lyophilization). To a portion of this material (50 mg) in water (1.5 ml) at 4 °C was added sodium borohydride (60 mg) in four portions. An additional amount of sodium borohydride (15 mg) was added and the solution was kept for 24 h in the cold. The excess of borohydride was decomposed with 0.5 M acetic acid and the solution was dialyzed for 30 h against distilled water. The non-dialyzable portion was concentrated and borate ions were removed as methyl borate by repeated addition and evaporation of methanol (4 × 10 ml). The residue was dissolved in water, the solution lyophilized (40 mg) and the product treated with 0.5 M sulfuric acid (500 ml) for 2 h at room temperature, the solution was then dialyzed against distilled water for 30 h. The nondiffusible material was lyophilized to give a periodate-resistant fraction (34 mg) and kept for further treatment with periodate. The composition of this fraction is shown in Table III.

The dialyzable fraction was reduced in volume and the solution passed through a column (0.4 × 8 cm) of Dowex 1 × 2 (AcO) ion-exchange resin (200-400 mesh). The column was washed with water (4 ml) and 10 mM acetic acid (4 ml). Thin-layer chromatography on cellulose and paper chromatography in solvents B and C and detection with alkaline silver nitrate, toluidine blue and the hydroxylamine–ferric-chloride reagent indicated the presence of glycerol, 2-acetamido-2-D-mannuronic acid

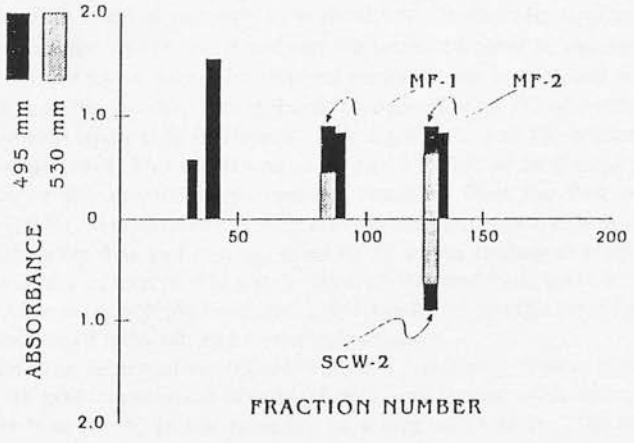

Fig. 1: Co-chromatography of the consumed medium of *Micrococcus lysodeikticus* and the peptidoglycan-polysaccharide, SCW-2, isolated from *Micrococcus lysodeikticus* cell walls on a column of Sepharose 6B. The column was washed with 50 mM pyridine–acetic acid buffer (pH 5.8).

TABLE III: Sequential periodate oxidation, sodium borohydride reduction, and hydrolysis of fraction SCW[a].

Components[b], reagent and compounds formed	Fraction SCW-2	Sequential degradation			
		First		Second	
		Dl[c]	Ndl[c]	Dl[c]	Ndl[c]
D-Glucose[e]	30		1.5		0
2-Amino-2-deoxy-D-mannuronic acid[d]	34		14.0		0
2-Amino-2-deoxy-D-glucose[e]	12		8.0		14
Muramic acid[e]	11		12.0		19
Muramic acid 6-phosphate[e]	1.2		1.8		–
Glycerol[e]		3.2[e]	8.0	0	–
Alanine[e]	44		36		42
Glutamic acid[e]	21		29		20
Glycine[e]	18		16		21
Lysine[e]	17		19		17
Formic acid released[f] (μmol/mg)		1.2			
Formaldehyde released[g] (nmol/mg)		58			

[a] For conditions, see experimental section.
[b] Component contents expressed in %.
[c] Determined by GLC.
[d] Determined by GLC as 2-acetamido-2-D-mannose.
[e] Determined with as amino analyzer (see experimental section), Residues per 100 residues.
[f] Determined by titration with sodium hydroxide.
[g] Determined by the chromotrophic acid, colorimetric method.

and 2-acetamido-2-deoxy-D-mannuronolactone. GLC on columns of OV-1 and OV-17 of the derived methyl glycosides showed the presence of glycerol in addition to unidentified compounds.

Hydrolysis of a portion of the dialyzable material with 1 M HCl for 16 h at 100 °C and examination in PC in solvent D, indicated the presence of glycerol and 2-amino-2-deoxy sugars. Another portion of the dialyzable fraction was treated with 0.5 M methanolic hydrogen chloride for 6 h at 100 °C. The acid free residue was *N*-acetylated and the product treated in aqueous methanol (1:1) with sodium borohydride in the usual way. The reduced material was hydrolyzed with 1 M hydrochloric acid for 3 h at 100 °C and the product *N*-acetylated. Examination by PC (detection with alkaline silver nitrate and 0.5 M sodium hydroxide in ethanol, (UV light), showed the presence of 2-acetamido-2-deoxy-D-mannose and glycerol. This result was confirmed by GLC of the methyl glycosides.

A small portion of the nondialyzable material resulting from the first treatment (6 mg) was dissolved in 0.1 M sodium metaperiodate (1 ml), and the solution kept for 24 h at 4 °C. The uptake of periodate was constant after 4 h and corresponded to 18 mmol D-glucose residue. The solution was processed as described above, except that the duration of the acid hydrolysis with 0.5 M sulfuric acid was extended to 4 h. The nondialyzable material was lyophilized (6 mg). Examination of the dialyzate by PC showed the absence of reducing and nonreducing sugars.

Lysozyme degradation of periodate-degraded SCW-2 fraction: Twice periodate-treated fraction SCW-2 (2 mg) in 10 mM ammonium acetate (2 ml) was treated with lysozyme (6 mg; General Biochemicals) for 24 h at 37 °C in the presence of a drop of toluene. The resulting solution was dialyzed to give nondialyzable fraction (1.8 mg). Examination of the dialyzable fraction by PC in solvent D showed the absence of reducing and nonreducing sugars.

Methylation of SCW-2: A cooled solution of fraction SCW-2 in water (10 mg) was treated at 4 °C with 1 M sodium hydroxide (1 ml) and dimethyl sulfate (1 ml), added in small portions while the mixture was vigorously stirred under an atmosphere of nitrogen. Sodium hydroxide (2 ml, 30%) and

1 ml of dimethyl sulfate was further added and the mixture stirred for 24 h and then dialyzed against distilled water, the nondialyzable material was further methylated by three repetitions of the whole procedure just described, to give 6.5 mg of the methylated SCW-2.

Attempts to raise the methoxyl content further by methylation with silver oxide and methyl iodide, or with barium oxide–barium hydroxide–methyl iodide in *N,N*-dimethylformamide showed no increase in methoxyl content, but excessive degradation took place. The IR spectrum indicated complete methylation.

Identification of methylated sugars: A portion (5 mg) of the methylated material was dissolved in 1 M methanolic hydrogen chloride (2 ml) and the mixture was boiled under reflux for 16 h and evaporated. The residue was freed of hydrochloric acid by repeated addition and evaporation of methanol and toluene, and treated with anhydrous pyridine (0.5 ml) and acetic an-hydride (1 ml) at 4 °C and then for 2 h at room temperature. The mixture of methyl glycosides of the sugar residue was examined by GLC and GLC-MS. The sugars identified are listed in Table IV. A portion of the glycoside was hydrolyzed with 1M hydrochloric acid (100 µl) for 6 h at 100 °C, and the solution was diluted with water (to 3 ml) and applied to a column (0.45 × 5 cm) of Dowex 1X-8 (AcO^-) ion-exchange resin. Elution with water (4 ml) and then with 0.1 M acetic acid (6 ml) gave, after evaporation of water and repeated additions of methanol and toluene, followed by evaporations, methylated sugars .The sugars were identified by TLC or PC.

RESULTS AND DISCUSSION

The soluble cell-wall fragment was obtained by extraction of the cell walls with water, DMSO or DMF in a 9 % yield. The recovery of the soluble polymer at 37 °C was slightly better than that performed at 22 °C. The SCW was purified by gel filtration in Bio-Gel P-200 and Sepharose 6-B. In Sepharose chromatography the Bio-Gel P-200 purified polymer was fractionated in two components, a major (75%) and a minor (7.5%) . The minor component contained more glucose and less amino acids than the major component. The major fraction from the Sepharose column, SCW-2, representing 6.75 % of the total cell-wall contained sugars including phosphorylated sugars, i.e., muramic acid 6-phosphate and glucosamine 6-phosphate, and amino acids similar to nondialyzable cell-wall constituents (Table II). The SCW-2 was homogeneous in gel filtration, ion-exchange chromatography and in PAGE. The purified SCW-2 contained muramic acid, *N*-acetylglucosamine, *N*-acetylmannosamineuronic acid, glucose, *N*-acetyl-glucosamine-6-phosphate, muramic acid 6-phosphate, alanine, glycine, glutamic acid and lysine.

TABLE IV: Methylated derivatives obtained after methylation of SCW-2. The methylated derivatives were identified as methylglycosides.

Sugars	Methylated derivatives						
	2,3,4,6-	2,3,4-	3,4,6-	2,3-	3,6-	6-	3-
Glucose	+	+	–	+	–	–	–
N-Acetylglucosamine	–	–	+	–	+	–	–
N-Acetylmannosamine	–	–	–	–	–	–	+
Muramic acid	–	–	–	–	–	+	–

Periodate oxidation at 4 °C in the dark gave an amount of formic acid corresponding to the oxidation of all the glucose residues, suggesting that these residues are linked at C-6. The release of formaldehyde corresponds to the oxidation of the reducing terminal muramic acid residue of the fragment. The oxidized fragment was reduced with sodium borohyride, the reduced polymer was hydrolyzed with mild acid treatment, and the hydrolyzate was dialyzed. The dialyzate fraction contained only one-fifth of the calculated amount of glycerol, and the remainder consisted of *N*-acetylmannosamine uronic acid. This compound, after conversion to methyl ester and reduction, was identified by GLC as methyl-2-acetamido-2-deoxy-D-mannopyranoside. The presence of about 1.5% unoxidized glucose and 7% glycerol in the oxidized non-dialyzable SCW-2 material was observed as in the case of cell-wall fragment (4). The resistance of the D-glucose residues to periodate oxidation may indicate the presence of (1→3)-linkage in the polymer but this suggestion was not confirmed by methylation and this resistance to periodate oxidation may probably arise due to steric effects. This phenomenon was also observed in the case of cell wall studies (4). The presence of glycerol suggests that the conditions of acid hydrolysis were not sufficient to hydrolyze the oxidised, reduced external chains completely, the stronger conditions would have hydrolyzed the phosphate bonds and, the glycosidic linkages. The total amount of glycerol found in the dialyzable and nondialyzable fractions was nearly one-half of the value expected, the loss probably arose due to hydrolysis during purification of the reduced material.

Methylation was done by a modified Haworth method (4), in order to avoid degradation of alkali-labile bonds. Methanolysis followed by acetylation gave a mixture of depolymerized sugars that were identified by GLC-MS. The glucose derivatives, 2,3,4-tri- and 2,3,4,6-tetra-*O*-methyl ethers, were identified by direct methanolysis and GLC, and GLC-MS. The presence of a very minor amount of 2,3-dimethyl ether was detected in GLC, and probably arose due to de-*O*-methylation of glucose derivatives. The ratio of 2,3,4-trimethyl ether to 2,3,4,6-tetra-methyl ether suggested a chain length of ≈ 30 repeating units.

Methyl ether of 2-acetamido-2-deoxymannuronic acid was identified, by GLC and GLC-MS, as derived methyl 2-acetamido-2-deoxy-3-*O*-methyl-mannopyranoside, after methanolysis and subsequent reduction with sodium borohydride and by comparison with an authentic compound (13). The 6-*O*-methyl ether of muramic acid [2-acetamido-3-*O*-(D-1-carboxyethyl)-2-deoxy-6-*O*-methyl-D-glucose] was identified by GLC and GLC-MS as methyl glycoside and by comparison with an authentic sample (6). The 2-acetamido-2-deoxy-D-glucose components were identified after methanolysis as 3,6-di- and 3,4,6-tri-*O*-methyl derivatives by GLC and GLC-MS. The proportion of 3,6-di- to 3,4,6-trimethyl ethers suggested a chain length, for the glycan portion of the peptidoglycan moiety, of three disaccharide units.

The results of the sequential periodate oxidation and of the methylation studies, although not quantitative, are in agreement with the chemical structure proposed for the cell wall fragment (4). These structures also bear similarities with the partial structures proposed by Hase *et al.* (14).

Extensive removal of the carbohydrate chains by the periodate oxidation and subsequent mild acid hydrolysis did not lead to lysozyme degradation which suggest that the phosphate group is responsible for the resistance to lysozyme, as was observed in the case of cell wall studies (4). This observation concurs with the specificity of egg-white lysozyme (15).

The soluble cell wall possesses chemical and structural similarities to the cell wall peptidoglycan-polysaccharide complex and the extraction of this polymer from the cell wall is time- and temperature-dependent. This polymer was also obtained in minute quantities from *M. lysodeikticus* cells and was also isolated from the culture medium of *M. lysodeikticus*. There is no known example of bacterial cell-wall components that are excreted or are associated with extracellular components. Extracellular polysaccharides in the case of yeast are known (16). This cell wall fragment may arise due to the presence of autolytic enzymes that are known to be associated with the walls and that are not deactivated during preparation of cell walls. There are several cell walls that are known to possess *N*-acetylmuramidase activity (17-19) and *N*-acetyl-glucosaminidase activity (20-21). Also, anionic charges arising due to the presence of muramic acid, *N*-acetyl-mannosamineuronic acid and phosphate groups may cause autohydrolysis of the cell wall during the extraction process, which is time- and temperature-dependent. Finally, there is a possibility that the soluble cell wall fragment forms a part of the cell wall that was synthesized but was not incorporated in the wall and existed as a cell-wall-associated molecule. The exact nature and function of this molecule remains unclear and requires further studies.

ACKNOWLEDGMENT: Dr. Nasir-ud-Din thanks the University Grants Commission for partial financial support for the bacterial cell wall research programme.

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Galactose residues in Plasmodium falciparum glycoproteins: Incorporation and elimination.

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Malaria is a widespread disease of the developing world where a large part of the population suffer with a considerable toll of life. It is caused by protozoa of the genus Plasmodium. The plasmodial infection progresses through distinct stages in the human host, and immune responses are elicited against stage-specific antigens that involve both cell and antibody mediated mechanisms (1,2).

In order to develop an efficacious vaccine against malaria much of the recent research has centred on the identification and characterization of specific protein antigens expressed by the parasite in its human host (3).

The significant antigens of the asexual erythrocytic stages of P.falciparum, the 195 KDa glycoprotein and its processed products, are expressed by merozoites invading human erythrocytes (4,5). Several lines of direct and indirect evidence indicate that these antigens can induce protective immune responses.

The biological and structural role played by the carbohydrate component of the glycoprotein antigens of P.falciparum has long been recognised (6) and their contribution to immunogenicity is well documented (7). In the current study information was obtained as to the contribution of galactose residues in the immunodominant plasmodial glycoproteins.

Asexual blood stage parasites were metabolically labelled by culture in asynchronous mode in human erythrocytes in RPMI medium supplemented with 10% normal human serum, 0.1% glucose and 50 uCi of D-[6-^{3}H]-galactose separately as well as in admixture with [^{3}H]-mannose and [^{3}H]-N-acetylglucosamine. After 6 and 18 hours of incubation at 37°C in a candle jar, culture erythrocytes were resuspended in RPMI medium, layered onto 60% isotonic Percoll and centrifuged. Infected erythrocytes were so enriched and then washed with PBS and lysed with TNE. The lysate was centrifuged and the supernatant subjected to SDS-PAGE in a discontinuous system.

Galactosyl transferase treatment of 195 and 43 kDa glycoproteins. The 195 and 43 kDa glycoproteins from the gel strips containing [^{3}H]-N-acetylglucosamine were eluted and galactose residues from UDP-[^{3}H]-galactose were transferred using galactosyl transferase. The galactose residues were transferred to the 195 and 43 kDa glycoproteins, as shown by the increase in CPM in both glycoproteins after the transfer reaction performed as described earlier (8). Incorporation of galactose residues was significantly more in 195 kDa glycoprotein compared to that of 43 kDa glycoprotein. For the transferase reaction an excess of galactosyl transferase was used in order to avoid deactivation that may arise due to the presence of minute amounts of detergents that may elute from the gel strips.

Galactosidase treatment of 195 and 43 kDa glycoproteins. Surface glycoproteins isolated from 195 and 43 kDa gel strips were treated with alpha and beta galactosidase separately. The alpha galactosidase treatment was carried out in 50 mM phosphate buffer containing 50 mM of $MgSO_4$, pH 6.5. Beta galactosidase treatment was performed in the same buffer but adjusted at pH 7.3. The reaction with alpha galactosidase was performed for 14 hours at 25°C, whereas the one with beta galactosidase for 14 hours at 37°C.

The 195 and 43 kDa glycoproteins metabolically incorporated sufficient [^{3}H]-N-acetylglucosamine, and the incorporation of galactose was minimal in 195 kDa and negligible in 43 kDa glycoprotein. Galactose incorporation using UDP-galactose was sufficient in both glycoproteins. Alpha galactosidase removed galactose residues from the glycoproteins. Beta galactosidase removed more galactose from the 195 kDa glycoprotein compared to that of 43 kDa glycoprotein. It appeared that galactose residues were transferred to the cell surface sugar residues and perhaps in the glycosyl region of the glycosylinositolphosphatidyl anchor, too. From the amount of galactose eliminated by alpha and beta galactosidases from the 43 kDa glycoprotein, it appeared that there was incorporation of the galactose residues by the transferase reaction in both the surface and the anchor region, but little amount was introduced in the anchor region. These experiments are suggestive of the possible nature of the galactose in the glycoproteins.

The metabolically labelled malarial parasites were in good morphological conditions, the surface coat was preserved. The lysed and purified glycoproteins were treated with galactosyl transferase for the incorporation of galactose in the surface as well as in the anchor region of the glycoprotein. From the known biochemistry of the surface glycoproteins (9) it appeared that, on the surface, galactose residues were transferred to N-acetylglucosamine residues. The detailed biochemistry and the immunogenicity of these molecules need to be further studied.

This work was supported by a grant from the UNDP/WORLD BANK/WHO TDR-IMMAL.

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Biochemical Society Transactions (1994) 22 363S

Sialyltransferases of the bonnet monkey cervical epithelium: Isolation and characterization.

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The secretion of the mammalian cervix mediates and controls the major functions of the cervix. The morphological, physical and chemical changes in these secretion during the ovarian cycle alter the biophysical behaviour of the cervical secretion, including permeability to sperm, quantitity, composition and rheology. The fundamental function of the cervical mucus is to act as a biological valve controlling sperm entry into the uterus, thereby encouraging or inhibiting sperm migration or penetration during the cycle. Cervical mucus is a complex mixture, the principal constituents of which are mucin-type carbohydrate-rich glycoproteins that share the properties of other epithelial secretions and cell surface glycoproteins. These exhibit gel-like behaviour and have a protein core rich in hydroxyamino-acids. Because these glycoproteins are the major components of the mucus, it is possible that they are resposnsible for its distinctive physiological and biophysical characteristics.

The major glycoproteins of estrogenic and gestagenic phases exhibit distinct differences in the chemical structure of their carbohydrate moieties. The essential changes observed during the menstrual cycle concern the linkages of N-acetylneuraminic acid, N-acetylgalactosamine and galactose residues (1).

The N-acetylneuraminic acid residues of different phases take residence in vicinity of the protein core, and because of the mutual repulsion arising due to the anionic charges, N-acetylneuraminic acid is responsible for the rigidity, coherence and consistency of the mucin secretion (1). It is likely that the presence of N-acetylneuraminic acid in the vicinity of the protein core during the ovulatory phase enhances the rigidity and may be responsible for the parallel alignment of the mucus component that allows channels of least resistance for sperm penetration (2). In the gestagenic phase glycoprotein, the distance between N-acetylneuraminic acid and the protein core may increase and thereby decrease the interaction and inhibition of sperm penetration. It is, therefore, significantly important to investigate the available sialyltransferase during the cycle. This investigation describes the presence of different sialyltransferases in the estrogenic phase.

Asialofetuin and asialo-cervival glycoprotein were prepared by treatment with neuraminidase (V.cholerae) in 0.1 sodium acetate containing 0.1% calcium chloride and 0.5% sodium chloride, pH 5.5, for 24 hours at 37°C. After a further addition of neuraminidase and additional incubation for 12 hours, the mixture was dialysed, and the non-dialysable material was lyophillised to give asialoglycoproteins. Asialo-agalactoglycoprotein was prepared by treating the asialo glycoprotein sequentially with ß-galactosidase from E.coli and C.lampas. The reactions were performed in sodium phosphate and sodium citrate, respectively.

Asialo- and asialo-agalacto glycoproteins were treated with trypsin in 40 mM Tris-HCl buffer, pH 8.2, for 8 hours at 37°C. After incubation the material was dialysed following inactivation of trypsin with an excess of TLCK, the solution was acidified to pH 4.5 with 1 M HCl and dialysed, and the non-dialysable material was lyophilized to give asialo- and asialo-agalactoglycopeptides.

Nearly 300 mg of cervical epithelium scrapings were extensively washed with 0.15 M NaCl, trimmed of membranes and other contaminants, and homogenized at 16,000 g. The homogenate was filtered through a sieve and centrifuged for 30 min at 20,000 g. The pellet was resuspended in water, and Triton-100 and solid NaCl were then added to the suspension to a concentration of 0.5%. After stirring for 60 min the suspension was centrifuged at 20.000 g to yield Triton extract. The Triton extract was purified on a Con A-Sepharose column and used as enzyme.

The standard incubation system for CMP-(14C)-NeuAc transfer in Tris maleate, pH 8.0, contained bovine serum, $MnCl_2$ acceptor and enzyme containing Triton X-100. Incubation was performed for 1-2 hours at 37°C. Reactions were stopped by the addition of 1.5 ml of 0.5 M HCl containing 0.8% phosphotungstic acid. The precipitate was washed and the radioactivity was counted.

The molecules containing radiolabeled sialic acid were treated with neuraminidase. The glycopeptides in general released most of the radioactivity, whereas the glycoproteins, particularly asialoglycoproteins released only about 70% of the incorporated radioactivity. In the case of asialo-agalactoglycoproteins nearly 80% of incorporated radioactivity using different neuroaminidases was eliminated. It appeared that differently linked sialic acid residues were present in the glycoprotein. Also, it is likely that during the cycle, different sialyltransferase activities are present which transfer differently linked sialyl residues.

The presence of sialyltransferase in an outer membrane of mitochondria capable of transferring sialic acid from CMP-Neu5Ac to an exogenous asialoglycoprotein acceptor at different location is known (3). Also, sialyltransferase responsible for the formation of different sugar chains in glycoproteins have been described in rat hepatoma (4). In the bonnet monkey estrogen stimulated cervical mucus glycoprotein sialic acid residues in different linkages are known (5).

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Pure & Appl. Chem., Vol. 66, Nos 10/11, pp. 2259–2262, 1994.
Printed in Great Britain.

Role of cell surface carbohydrates in malaria

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Abstract. Metabolic labelling of Plasmodium falciparum malarial parasites with [^{3}H]-glucosamine, [^{3}H]-galactose, [^{3}H]-mannose, [^{3}H]-ethanolamine and [^{3}H]-myristic acid and subsequent purification in SDS-PAGE yielded, amongst other proteins, the 195 KDa glycoprotein, which is the major merozoite surface antigen. Reductive ß-elimination of the glycoprotein in the gel released labelled sugars. Processing of the reaction products and acid hydrolysis of the derived sugars suggested the presence of N-acetylglucosaminitol, N-acetylglucosamine and other components. Acid hydrolysis of the labelled glycoprotein and examination of the products by chromatography indicated the presence of glucosamine, galactose, mannose, ethanolamine and myristic acid. The 195 KDa glycoprotein was adsorbed by wheat germ agglutinin and desorbed with N-acetylglucosamine. Labelled galactose was incorporated in the glycoprotein by treatment with [^{3}H]-UDP-galactose and bovine milk galactosyl tranferase. The externally glycosylated glycoprotein released labelled galactose on treatment with ß-galactosidase. The carbohydrate chains in the 195 KDa glycoprotein are linked to the protein core through O-glycosyl linkage and N-acetylglucosamine and serine residues are involved in the linkage region.

Malaria is an important parasitic disease of tropical and subtropical countries with an increasing toll of morbidity and mortality each year. Plasmodium falciparum is the protozoan parasite which causes malignant tertian malaria of major medical concern. Malarial infection progresses through various stages of plasmodial development along a complex life cycle with the expression of distinct antigenic proteins and glycoproteins. The immune responses elicited against these antigens are stage-specific and involve both cell and antibody mediated mechanisms (1). High molecular weight antigens have been recognised on the surface of the merozoite stage of development (2-4). These macromolecules mediate merozoite invasion of erythrocytes (5,6). It has been observed that antibodies against some of the high molecular weight

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glycoproteins inhibit cell growth in culture. There is considerable interest in developing a vaccine against malaria (7). However, the efforts have been hampered because of diversity of the parasite antigens.

Recently, extensive interest has been developed to investigate the role of sugar residues present in the glycoprotein antigens that are synthesized by P.falciparum and exposed on merozoite surface (8,9). The presence of carbohydrates on these surface antigens has been demonstrated by in vitro labelling and enzymic release of sugar residues from the parasite cells (10,11), lectin binding (12) and inhibition studies (13). It has also been shown that intact carbohydrate chains help maintain the antigenicity of the glycoprotein (10). Because of the strong immunological behaviour of the sugar incorporated molecules and lack of information as to the nature of the incorporated carbohydrates, investigations to explore the role of carbohydrates in malaria were initiated.

The parasite cells of strain M25/ZAIRE were cultured in asynchronous mode in RPMI medium containing 10% normal human serum, 0.1% glucose and 50 mCi/ml of the radiolabelled sugars, amino alcohol and myristic acid, separately or in admixture. The metabolically labelled parasites were in good morphological condition and the surface coat was preserved. The labelled proteins and glycoproteins were extracted from the parasite cells in the non-ionic NP-40 detergent. From the NP-40 extract the labelled 195 KDa glycoprotein was immunoprecipitated using the monoclonal antibody 310 (14). The NP-40 extracted and/or immunoprecipitated glycoprotein was analysed by SDS-PAGE (15) and by autoradiography. The gel pattern in autoradiography suggested predominant incorporation of glucosamine in comparison with mannose and galactose in the 195 KDa glycoprotein.

Depolymerization of the 195 KDa glycoprotein with acid and subsequent chromatography of the products of acid hydrolysis showed the presence of labelled molecules comigrating with glucosamine, mannose, galactose, ethanolamine and myristic acid. The presence of glucosamine in the glycoprotein was further confirmed by conversion of glucosamine with ninhydrin to arabinose (16). Reductive alkali treatment of the material in the gel strip corresponding to the 195 KDa glycoprotein solubilized 80% of the radioactivity suggesting that the oligosaccharides which contain labelled sugars are O-glycosidically linked to the peptide. Acid hydrolysis of the ß-eliminated labelled material yielded compounds comigrating with N-acetylglucosaminitol, N-acetylglucosamine, mannose, galactose and trace amounts of N-acetylgalactosaminitol. In an accompanying experiment the ß-eliminated products were reduced in situ with sodium borotritide, and subsequent acid treatment showed therein the presence of labelled alanine arising from serine, suggesting O-glycosidic linkage between glucosamine and serine. Hexosaminidase treatment of 195 KDa glycoprotein liberated a sugar that migrated as N-acetylglucosamine.

The 195 KDa glycoprotein, isolated from the gel in phosphate buffer, was

treated with [^{3}H]-UDP-galactose and bovine milk galactosyl transferase to obtain externally galactosylated glycoprotein. The galactosylated glycoprotein released labelled galactose residues on sequential treatment with alpha- and ß-galactosidases suggesting alpha- and ß-anomery of the galactose residues transferred to the glycoprotein. ß-linked galactose residues were in abundance. The glycoprotein appears to possess two sites to accept galactose residues, in particular terminal N-acetylglucosamine residues. This observation was further supported when this glycoprotein was adsorbed by wheat germ agglutinin and desorbed by N-acetylglucosamine. The enzymic studies on the labelled glycoprotein suggested the presence of terminal galactose residues in addition to N-acetylglucosamine. The sequential degradation with enzymes indicated the presence of oligosaccharide chains. However, it is not possible to assess at this stage whether the enzymes are eliminating radiolabelled sugars from the surface of the protein or from the anchor region glycan.

The O-linked terminal N-acetylglucosamine is now clearly shown to be present in the 195 KDa glycoprotein. The glycoprotein also possesses galactose and mannose residues in addition to N-acetylglucosamine. The evidence available so far suggests only the presence of O-glycosyl linkage in the glycoprotein. This finding is consistent with our earlier observations (17,18). Evidence for the absence of N-glycosyl linkage between the sugar moiety and the protein core has been proposed. The coexistence of N-linked oligosaccharides or N-linked N-acetylglucosamine in a glycoprotein bearing O-linked chains or O-linked N-acetylglucosamine needs to be clarified. Mannose residues were incorporated in the glycoprotein. To establish whether these residues are present in the glycoprotein in N-linked chains or in the anchor glycan (19,20) will require further studies. However, it has been observed that the incorporation of glucosamine in the 195 KDa glycoprotein is much more intense compared to that of mannose.

The 195 KDa glycoprotein and its processed products, the 14 and 16 KDa glycoproteins, under conditions of metabolic labelling similar to those used for glucosamine, mannose and galactose, incorporate glucosamine with much more specific activity as compared to mannose and galactose. Galactose was incorporated in the glycoprotein with least abundance under the metabolic condition used (17). From the results of metabolic labelling experiments, wheat germ agglutination, reductive ß-elimination and galactose incorporation, it is clear that in the glycoprotein the carbohydrate chains mainly contain N-acetylglucosamine residues linked to serine and, as described for other glycoproteins (20), glucosamine, mannose and galactose, in addition to ethanolamine and myristic acid in the anchor region. The presence of O-linked N-acetylglucosamine in cell surface glycoproteins (21), subcellular organelles (21) and in Schistosoma mansoni (23) is known. The biological and immunological role of O-linked glycoproteins in P.falciparum is not yet known. However, this study

demonstrates that the carbohydrate chains of the immunodominant 195 KDa glycoprotein are O-linked, that N-acetylglucosamine is present in it as terminal sugar and that ethanolamine and myristic acid are among its components.

Acknowledgment. This investigation received, in part, financial support from the UNDP/World Bank/WHO Special Programme for Research and Training in Tropical Diseases.

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Integration of Mycobacterial Lipoarabinomannans into Glycosylphosphatidylinositol-Rich Domains of Lymphomonocytic Cell Plasma Membranes[1]

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Lipoarabinomannans (LAMs) are major Ags of the mycobacterial cell envelope where they apparently insert through a glycosylphosphatidylinositol (GPI) anchoring structure. LAMs induce host macrophages to secrete TNF-α, IL-1, and IL-6 and inhibit T cell proliferative responses. The mechanisms by which LAMs mediate these effects remain poorly understood. We show that LAMs were efficiently inserted into the plasma membranes of human and murine lymphomonocytic cells through their GPI anchor. Prior deacylation of LAMs abrogated this event. Phosphatidylinositol hexamannoside (PIM_6), the GPI anchor of all LAMs, competitively inhibited LAM insertion. Deacylated PIM_6 was not inhibitory. The hexamannoside glycan of PIM_6 appears to be important for LAM insertion, since phosphatidylinositol from soybean, lacking the glycan core, was not as efficient an inhibitor. Interaction of LAM with target cells was influenced by the gel/fluid phase distribution of membrane lipids, suggesting a direct interaction of the LAM-GPI anchor with the membrane bilayer. The inserted LAMs were mobile in the plane of the membrane and interfered with Ab-mediated mobilization of the GPI-anchored Thy-1 molecules. Further, LAMs were preferentially incorporated into isolated plasma membrane vesicles enriched in Thy-1. Our results strongly suggest that 1) interaction of LAMs with host lymphomonocytic cells is mediated through a preferential integration of LAM-GPI anchor into specialized plasma membrane domains enriched in endogenous GPI-anchored molecules, and 2) both the acyl chains and the mannoside core glycan of the LAM-GPI anchor contribute to the specificity of integration. *The Journal of Immunology*, 1995, 155: 1334–1342.

Lipoarabinomannans (LAMs)[4] are the major antigenic lipoglycans of *Mycobacterium tuberculosis* and *Mycobacterium leprae* (1). They consist of highly branched carbohydrate polymers of mannose and arabinose terminated by a glycosylphosphatidylinositol (GPI) anchor made of a phosphatidylinositol (PI) moiety linked to an oligomannose core glycan (2). This PI terminus of LAMs is related to the GPI structures synthesized by protozoan and metazoan cells that anchor glycoproteins or complex carbohydrates into membrane bilayers (3). The structure of the most widespread GPI anchor consists of a PI linked to a linear polymer of glucosamine-trimannoside-ethanolamine (3). The LAM-GPI differs from this structure in that PI is directly linked to an oligomannoside, which in turn is linked to the main carbohydrate moiety (4). The antigenic determinants of this membrane-associated molecule are extensively shared among different mycobacteria (5). Purified LAMs have been shown to exert profound biologic effects on lymphocytes and mononuclear phagocytes (6–18).

LAMs isolated from *M. tuberculosis* and *M. leprae* were shown to inhibit 1) T cell proliferative responses (6–9), 2) the expression of IL-2, IL-5, and GM-CSF genes in human T cells (10), and 3) the IFN-γ-mediated activation of macrophages (11–13). On the other hand, LAMs induced expression of the TNF-α gene and cytokine release from macrophages (9, 14). Induction of the TNF-α gene

Received for publication February 1, 1995. Accepted for publication May 22, 1995.

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[1] This work was supported by the Swiss National Science Foundation (Grant #31-39709.93) and contract (NoI-AI-25147) from NIH, NIAID.

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[4] Abbreviations used in this paper: LAM, lipoarabinomannan; GPI, glycosylphosphatidylinositol; PI, phosphatidylinositol; manLAM, mannose-capped LAM; araLAM, unsubstituted LAM; PIM_6, phosphatidylinositol hexamannoside; dLAM, alkali-treated deacylated LAM; $dPIM_6$, alkali-treated deacylated PIM_6; GAM, goat anti-mouse; GAR, goat anti-rat; TBS, Tris-buffered saline; TBST, TBS containing Tween-20; MP, milk powder.

0022-1767/95/$02.00

occurred concomitantly with those of other immediate early genes (15). LAMs also induced the expression of IL-1β, IL-6, IL-8, and IL-10 genes in human mononuclear cells (9, 16–18).

Despite these well documented biologic activities of LAMs on cells of lymphomonocytic origin, detailed investigation into mechanisms by which LAMs mediate these effects at the plasma membrane level has not been reported. Much of the emphasis has been on the nature of the carbohydrate head group structures of the molecule, which may be mannose-capped (manLAM) or unsubstituted (araLAM) (19). Understanding how GPI-linked LAM molecules interact with the plasma membrane of host cells should help define the transmembrane signaling pathways that are modulated by mycobacterial LAMs. Here we report that LAMs integrate directly into the host cell plasma membrane through their GPI anchor, an event that is competitively inhibited by purified LAM-GPI lacking the main carbohydrate moiety (phosphatidylinositol hexamannoside, PIM_6). Furthermore, we show that LAMs are preferentially incorporated into plasma membrane domains enriched in endogenous GPI-linked proteins and interfere with the lateral mobility of the GPI-linked Thy-1 surface glycoprotein in the plane of the membrane.

Materials and Methods

Glycolipids and glycans

LAMs from a rapidly growing unclassified *Mycobacterium* species (araLAM) and a virulent strain (Erdman) of *M. tuberculosis* (manLAM), and generic PIM_6 were isolated as detailed previously (4). The difference between araLAM and manLAM is the presence in the latter of mannose caps on the terminal residues of the arabinan side chains (19, 20). The freeze-dried materials were reconstituted with endotoxin-free deionized water at a concentration of 1 mg per ml, aliquoted, vacuum-dried and stored at −20°C. Soybean PI and mannan (from *Saccharomyces cerevisiae*) were purchased from Sigma Chemical Co., St. Louis, MO. PI was dissolved in ethanol and then diluted in RPMI 1640 for addition to the cells. Deacylated derivatives of LAM (dLAM) and PIM_6 ($dPIM_6$) were prepared by mild alkaline hydrolysis (2) as follows. One hundred μg of LAM or PIM_6 were suspended in 100 μl of 0.1 N NaOH and incubated at 37°C for 2 h. The mixture was neutralized with CH_3COOH and desalted through Bio-Gel P-10 (Bio-Rad Labs, Hercules, CA) in PBS. The eluted deacylated material was lyophilized and reconstituted with PBS. Mock deacylated derivatives of LAM and PIM_6 underwent the same procedure, but were incubated in water instead of 0.1 N NaOH.

Antibodies

Ascitic fluid of anti-LAM mAb CS35 (IgG3) reactive to both araLAM and manLAM (20) was used at 1:500 dilution for immunofluorescence and at 1:2000 for dot blot assays. Isotype-matched anti-TNP mAb (CB1) was used as a control. Rat mAb against mouse T cell markers Thy-1 (anti-Thy-1.2, mAb 30-H12) and CD45 (anti-CD45, mAb M1/9.3.4HL.2) were from the American Type Culture Collection, Rockville, MD. These Abs were used as culture supernatants at 1:20 dilution for flow cytometry, immunofluorescence, and dot blot assays. An irrelevant rat mAb against mouse Ig κ chain was used as control for these mAb. mAbs against human CD2, CD3, CD4, CD8, CD14, CD45, and HLA-DR were purchased from Becton Dickinson, San Jose, CA and used at the recommended dilution. Goat anti-mouse (GAM) IgG-FITC (Caltag Labs, San Francisco, CA), donkey anti-mouse and goat anti-rat (GAR) IgG-Texas Red (Jackson Immunochemicals, Bar Harbor, ME) were used at 1:200 dilution. Horseradish peroxidase-conjugated GAM Ig (Zymed, San Francisco, CA) and GAR IgG (Cappel, West Chester, PA) were used at 1:4000 and 1:500, respectively.

Cell culture

Human PBMC were isolated by density gradient centrifugation over Ficoll-Paque (Pharmacia LKB, Uppsala, Sweden) from venous blood collected from healthy donors. BALB/c T-lymphoma cell line P1798 (Litton Bionetics, Bethesda, MD) was passaged i.p. in syngeneic mice. Cells recovered from ascitic fluid were washed in PBS and suspended in RPMI 1640 medium (Sigma Chemical Co.). Human myelomonocytic cell line THP-1 and murine cytotoxic T cell line CTLL-2 were cultured in RPMI 1640 containing 10% FCS.

LAM incorporation and flow cytometric detection

Cells were washed and suspended in RPMI 1640 at a concentration of 1×10^6 cells per ml. LAMs and dLAMs were resuspended at required concentrations in 50 μl of RPMI 1640 containing 10 mM HEPES and added to the cell pellet. After incubation at 37°C for 30 min, cells were washed in ice-cold PBS containing 0.1% BSA and 0.01% sodium azide. Following incubation with anti-LAM mAb CS35 diluted in the wash buffer for 30 min on ice, cells were washed and incubated with GAM IgG-FITC for a further 30 min. The cells were washed thoroughly and suspended in 300 μl of PBS for FACS analysis.

Immunofluorescence microscopy

Control and LAM-incorporated P1798 cells were incubated with anti-Thy-1 or anti-CD45 rat mAb for 1 h followed by GAR IgG Texas Red (Jackson Immunochemicals) for 30 min on ice. The Abs were diluted in Tris-buffered saline (TBS) containing 1% BSA. Cells were then incubated at 37°C for 15 min and fixed immediately with 2% paraformaldehyde. After thorough washing, cells were suspended in mounting medium containing glycerol, 100 mM Tris-HCl pH 9.5, *n* propyl gallate (Sigma Chemical Co.) in the ratio of 70:30:5 and examined with a Zeiss Axiophot (Carl Zeiss, Oberkochen, Germany) fluorescence microscope. The staining pattern was classified as homogeneous, patched, or capped, according to the surface distribution of fluorescence. A minimum of 100 cells were counted by at least two persons independently.

Confocal laser microscopy

After LAM incorporation, cells were washed in TBS containing 1% BSA and incubated with CS35 mAb followed by donkey anti-mouse Texas Red for 30 min each at 4°C. To demonstrate LAM capping, cells were then shifted to 37°C for 15 min before fixation with 2% paraformaldehyde in PBS. Control noncapped cells were fixed before the temperature shift. Labeled cells were washed and suspended in buffered polyvinyl alcohol (21) and mounted on glass slides. Optical sections were generated in a Zeiss 410 Axiovert Laser Scan Microscope.

LAM incorporation into isolated plasma membrane vesicles and dot blot assay

Membrane fractions from murine P1798 cells were prepared as described previously (22). The membrane fractions recovered at the interfaces between 10% and 22.5% sucrose (light membranes, specific gravity 1.090 g/ml) and 22.5% and 35% sucrose (heavy membranes, specific gravity 1.136 g/ml) were diluted and pelleted by ultracentrifugation. The lipid to protein ratio is 2:1 for light density membranes and 1:1 for heavy density membranes (22). The light and heavy membrane fractions resuspended in 250 μl PBS were incubated with manLAM at a final concentration of 100 μg/ml at 37°C for 30 min, brought to 40% sucrose in 3 ml of Tris-HCl, pH 7.4, 25 mM KCl, 5 mM $MgCl_2$, 1 mM EGTA, and refloated to their respective densities by ultracentrifugation. As a control, the same amount of manLAM without membrane was subjected to the above procedure. Bands corresponding to light and heavy membrane densities were collected from each tube, diluted 10-fold with PBS, ultracentrifuged at 100,000 × *g* and finally resuspended in 250 μl PBS containing the protease inhibitors Pefabloc (1 mM), leupeptin (100 μM), and aprotinin (2 μM) (all from Boehringer-Mannheim, Mannheim, Germany). The total protein contents of membrane fractions were estimated using bicinchoninic acid protein assay reagent (Pierce, Rockford, IL).

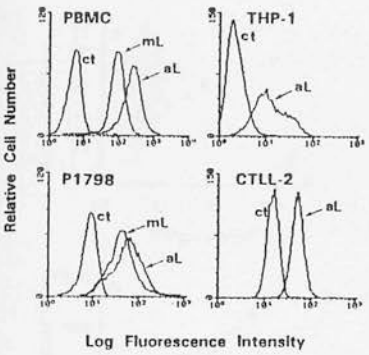

FIGURE 1. Anti-LAM staining of lymphocytes and monocytes incubated with LAMs and analysis by flow cytometry. One × 10^6 PBMC, THP-1, P1798, or CTLL-2 cells were incubated with 100 μg/ml of either araLAM (aL) or manLAM (mL) in 50 μl RPMI-1640 at 37°C for 30 min, followed by anti-LAM mAb CS35 and labeled with GAM IgG-FITC and analyzed by flow cytometry. ct, control with isotype-matched irrelevant first Ab. Each experiment was carried out at least five times and the representative results are shown.

Serial dilutions of light and heavy membrane fractions were blotted onto nitrocellulose filters using a Bio-Rad dot blot apparatus and assayed for the presence of cell surface molecules or incorporated LAM by a modification of the procedure described for native membrane proteins (23). The strips were blocked with 5% nonfat milk powder in TBS containing 0.05% Tween-20 (TBST-MP) and then incubated with mouse anti-LAM mAb CS35 or rat monoclonal anti-Thy-1 or anti-CD45 diluted in TBST-0.5% MP for 1 h at room temperature with agitation. The strips were washed with TBST and incubated with appropriate horseradish peroxidase-conjugated second Abs, diluted in TBST-0.5% MP for 1 h. After thorough washing, the blots were developed with Amersham ECL reagent and exposed to DuPont Reflection autoradiography film (DuPont de Nemours International S. A., Regensdorf, Switzerland). The spots on the film were quantitated using a laser scanning densitometer.

Results

Mycobacterial LAMs insert into the plasma membranes of lymphomonocytic cells through their GPI anchor

Flow cytometric analysis of PBMC incubated with LAMs at 37°C for 30 min in RPMI 1640 resulted in the detection of LAM on the cell surface with the anti-LAM mAb CS35 (Fig. 1). A human myelomonocytic cell line (THP-1), murine T lymphoma P1798 and CTLL-2 cells were also labeled with CS35 mAb after incubation with LAM. The difference in the fluorescence intensity among the various cell types for the same concentration of LAM showed an inverse relationship to the cell size. Simultaneous labeling of LAM-loaded PBMC with anti-LAM mAb and Abs against CD2, CD3, or HLA-DR showed that anti-LAM staining was detectable on lymphocytes, monocytes, and other mononuclear cells (data not shown). These results suggest that LAMs interact equally well with different hematopoietic cell types.

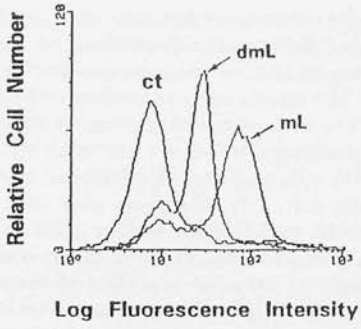

FIGURE 2. Removal of acyl groups abrogates the interaction of LAM with the cell surface. One × 10^6 P1798 cells were incubated with 100 μg/ml of manLAM (mL) or an equivalent amount of deacylated manLAM (dmL) for 30 min at 37°C and labeled for LAM incorporation as in Figure 1. The results shown are representative of three experiments.

Despite extensive mannose capping of the arabinose side chains of manLAM, both manLAM and uncapped araLAM were incorporated efficiently (Fig. 1). Since LAMs contain a GPI anchor, we investigated the possibility of its involvement in the interaction of LAMs with the target cell plasma membrane. Treatment of LAM-loaded cells with PI-specific phospholipase C did not affect anti-LAM Ab binding (data not shown). This was not unexpected because the presence of an additional mannose residue at position 2 of the inositol ring would apparently render the GPI of LAMs resistant to PI-specific phospholipase C digestion (2, 24). However, deacylation by mild alkaline hydrolysis markedly reduced the incorporation of manLAM into P1798 cells (Fig. 2), while control mock deacylated manLAM was incorporated to the same extent as untreated manLAM (data not shown). Similar results were obtained for araLAM (data not shown). These results indicate that the acyl chains are critical for LAMs to interact with the target cell membrane.

Specificity of LAM incorporation into the plasma membrane

Further evidence for involvement of the acyl chains of the GPI anchor in LAM incorporation was sought from competition experiments using PIM_6. It was demonstrated that PIM_6, the core phosphatidylinositol glycan anchor of all LAMs, efficiently competed with LAMs for membrane incorporation in a dose-dependent manner, reaching a maximum of about 80% inhibition at a concentration of 100 μg/ml (Fig. 3A). Soybean-derived PI, which is structurally similar to PIM_6 except for the lack of the hexamannoside core glycan and the mannose residue at position 2 of the inositol ring, was used as a control. A modest inhibition brought about by the highest concentration of PI used in these experiments (200 μg/ml) suggests that the acyl

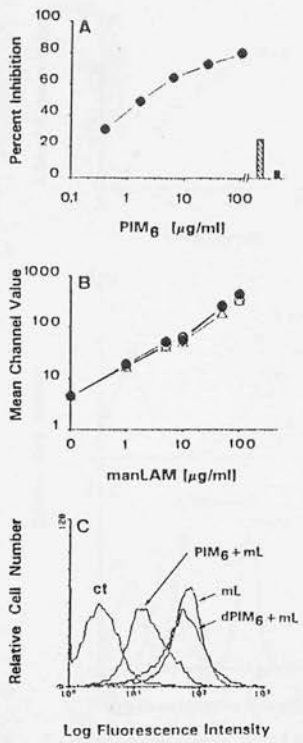

FIGURE 3. *A*, PIM_6 competitively inhibits LAM incorporation into cell membranes. One × 10^6 P1798 cells were incubated with 50 μg/ml of araLAM either alone or together with indicated concentrations of PIM_6 in 100 μl RPMI-1640 for 30 min at 37°C and labeled for LAM as in Figure 1. The percentage of inhibition was calculated using the mean channel value (MCV) for LAM staining by the formula: (1 − (MCV of cells incubated with LAM + PIM_6/MCV of cells incubated with LAM alone)) × 100. The percentage of inhibition of the incorporation of 50 μg/ml of manLAM into PBMC by 200 μg/ml of soybean PI (hatched bar) and 400 μg/ml of yeast mannan (solid bar) are shown. *B*, Dose titration of mannan and soybean PI on LAM incorporation. 400 μg/ml of yeast mannan (open circles) or 200 μg/ml of soybean PI (open triangles) was added to 1 × 10^6 PBMC together with different concentrations of manLAM and incubated for 30 min at 37°C before analysis for incorporated LAM. Filled circles represent manLAM alone without competitor. *C*, Deacylation of PIM_6 abrogates its inhibitory effect on LAM incorporation. One × 10^6 PBMC were incubated with 100 μg/ml of manLAM (mL) together with an equivalent amount of PIM_6 or $dPIM_6$ for 30 min at 37°C and labeled for LAM as in Figure 1. Deacylation of PIM_6 was carried out as for LAM in Figure 2. Representative results from three independent experiments are shown.

chains of PI could interfere with LAM incorporation only to a limited extent. However, mannan alone, even in the presence of a vast excess of 400 μg/ml, did not significantly decrease the manLAM incorporation (Fig. 3*A*). To investigate the possibility that PI or mannan could be inhibitory at lower concentrations of LAM, they were tested against a wide concentration range of manLAM. As shown in Figure 3*B*, the maximal inhibitory effect of PI or mannan (at 200 μg/ml and 400 μg/ml respectively) did not exceed 25%. In addition, the inhibitory effect of PIM_6 was abrogated by prior deacylation (Fig. 3*C*) while mock deacylated PIM_6 retained the inhibitory effect. Collectively, these results argue strongly in favor of a specific integration of the LAM acyl chains into the plasma membrane and also suggest that the core glycan structure of the LAM-GPI is important in determining the specificity of this integration process.

Kinetics of LAM incorporation into the cell membrane

LAM was incorporated into the plasma membrane of PBMC in a dose-dependent manner (Fig. 4*A*). The incorporation was detectable by flow cytometry after incubation with 1 μg/ml of LAM and increased linearly up to 200 μg/ml. This incorporation occurred in aqueous medium and did not require a liposome vehicle. LAM incorporation was already maximal after 5 min of incubation and did not increase significantly after 30 min (Fig. 4*B*). Similar results were obtained with THP-1 cells (data not shown). The incorporated LAM was detectable on the surface of P1798 cells up to 24 h tested (data not shown), suggesting a stable integration into the plasma membrane.

Integration of LAM was temperature dependent, as it was greatly reduced when the cells were incubated at 4°C (Fig. 4*C*), favoring the notion that a physiologic distribution of plasma membrane lipids into gel and fluid phases is required for this process. However, to test the possibility that the integration of LAM could be secondary to its binding to cell surface molecules, cells incubated with LAM were washed briefly twice at 4°C and then incubated at 37°C for a further 30 min. As shown in Figure 4*C*, this protocol resulted in less than 10% of LAM incorporation achieved at 37°C. These results demonstrate that the membrane association of LAMs is dependent on gel/fluid phase distribution of lipids in the plasma membrane bilayer, rather than binding to a lectin-like membrane receptor, an event only minimally reduced at 4°C (25).

The distribution of incorporated LAM on the cell surface was studied by confocal fluorescence microscopy. Figure 5*A* shows a peripheral localization of the incorporated LAM molecules on P1798 cells. Cross-linking of the LAM molecules on the cell surface with appropriate Abs and further incubation under conditions that allowed the movement of cell surface molecules (37°C, 15 min) resulted in patching and capping (Fig. 5*B*), indicating that the inserted LAM was mobile on the cell surface.

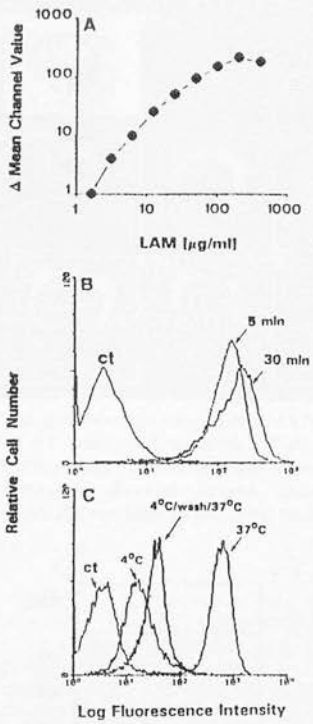

FIGURE 4. *A*, Dose dependence of LAM incorporation. One × 10^6 PBMC were incubated with indicated concentrations of araLAM in 50 μl of RPMI-1640 at 37°C for 30 min and analyzed for LAM incorporation as in Figure 1. The results are expressed as the difference in MCV between control and LAM-incorporated cells. *B*, LAM incorporation is rapid. One × 10^6 PBMC were incubated with 100 μg/ml of araLAM at 37°C and analyzed for the incorporated LAM at various time points between 5 min and 1 h. Only the data points corresponding to 5 min and 30 min are shown. *C*, Temperature dependence of LAM incorporation. One × 10^6 PBMC were incubated with 100 μg/ml of araLAM for 30 min either at 4°C or at 37°C and analyzed for LAM incorporation. An aliquot of cells incubated at 4°C were washed briefly twice in cold RPMI and incubated further at 37°C for 15 min before analysis. The results shown are representative of three independent experiments.

Effects of the incorporated LAMs on the cell surface

Insertion of LAMs into the plasma membrane did not interfere with the expression of CD2, CD3, CD4, CD8, CD20, CD45, or HLA-DR on human PBMC, or the GPI-linked CD14 molecule on THP-1 cells activated with vitamin D3 (data not shown). Since the GPI anchor of LAMs shares structural features with those of endogenous cell surface proteins, the effect of LAM incorporation on the expression of the GPI-linked protein Thy-1 was studied. Thy-1 expression on CTLL and P1798 cells remained unaffected after LAM treatment as measured by flow cytometry (data not shown). However, the mobility of Thy-1 at the surface of P1798 cells, as evaluated by fluorescence microscopy, was significantly modulated following LAM treatment and led to more than 50% reduction in the proportion of cells showing patches and caps at 37°C (Fig. 6). The mobility of the transmembrane CD45 glycoprotein was affected only minimally (Fig. 6).

LAM incorporates preferentially into Thy-1-enriched plasma membrane vesicles

Incubation of LAM with membrane fractions isolated from P1798 lymphoma cells resulted in LAM incorporation into light and heavy density membrane vesicles (Fig. 7*A*). Preincubation with LAM did not affect the flotation properties of these fractions, and LAM alone did not float up to the densities corresponding to the light or heavy membranes. In fact, all LAM in the control tube without any membrane fraction, and unincorporated LAM in tubes incubated with membrane fractions were pelleted (data not shown). When the amount of LAM incorporated into the membrane fractions was quantitated by densitometry and expressed as arbitrary units per unit quantity of membrane protein (Fig. 7*B*), it was readily apparent that LAM was more efficiently incorporated into light membrane than into heavy membrane fraction. Light membranes are also highly enriched for the endogenous GPI-linked protein Thy-1, and interestingly, the ratio of the incorporated LAM to Thy-1 content is very similar in both light and heavy membrane fractions. By contrast, the content of transmembrane glycoprotein CD45 is slightly more abundant in heavy membrane fractions for equal amounts of protein (Fig. 7*B*). Since the lipid-to-protein ratio is 2:1 for the light density membranes, and 1:1 for the heavy membranes (22), comparison of the relative amounts of cell surface markers and the incorporated LAM, expressed per unit amount of lipids, showed again a preferential distribution of LAM and Thy-1 in the light membranes, and of CD45 in the heavy membranes (Fig. 7*C*).

Discussion

Several reports point to the importance of the GPI anchor in mediating the biologic effects of the LAM molecule. Deacylation of LAM not only abrogated its inhibitory effects on T cell proliferative responses (8, 9), but also its capacity to induce TNF-α secretion by macrophages (14, 26). Likewise, dLAM no longer inhibited the IFN-γ-mediated activation of macrophages (11). Furthermore, it was observed that smaller versions of LAM such as lipomannans and PIMs also induced transcription of cytokine mRNA, elicited cytokine secretion, and suppressed Ag-induced T cell response (9). These observations prompted the speculation that LAMs could mediate their effects by inserting into the plasma membranes of target cells (2, 14,

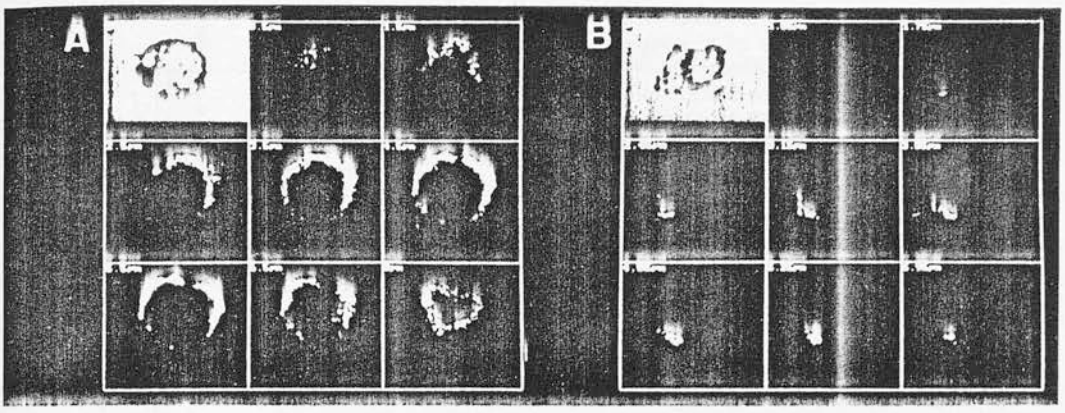

FIGURE 5. Confocal imaging of incorporated LAM. One × 10^6 araLAM-incorporated P1798 cells were washed in TBS-BSA and incubated at 4°C with anti-LAM mAb followed by donkey anti-mouse IgG coupled to Texas Red for 30 min each. Cells were fixed with 2% paraformaldehyde either immediately (*A*) or after incubation at 37°C for 15 min (*B*). The top left corner of each figure shows an individual cell under phase contrast. The depth of the optical sections are indicated in μm. Isotype-matched control Ab did not bind to the cells. Results shown are the representatives of three experiments.

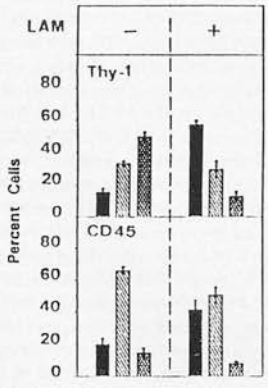

FIGURE 6. Effect of LAM incorporation on the mobility of Thy-1 and CD45. Control and araLAM-incorporated P1798 cells were incubated at 4°C with anti-Thy1 or anti-CD45 Ab for 1 h, followed by GAR IgG conjugated to Texas Red for 30 min. The cells were shifted to 37°C and incubated for a further 15 min before fixation by paraformaldehyde. The cells were examined by fluorescence microscopy for surface staining pattern: solid bars, homogeneous staining; hatched bars, patchy staining; cross-hatched bars, single-cap staining. The results shown are mean + SE of three experiments.

26), but the demonstration of such an interaction has not been provided so far, nor have the transmembrane signaling pathways modulated by LAMs been characterized.

In this report, we show that LAMs are inserted directly into the plasma membrane bilayer of target cells through the acyl chains of their GPI anchor without apparent involvement of surface receptors. LAM incorporation was linear over a broad concentration range between 1 μg/ml and 200 μg/ml. These LAM concentrations covered the wide range used to elicit biologic responses (6, 7, 9–14), and were not toxic to the cells. Spontaneous incorporation of certain purified GPI-linked proteins into plasma membranes has already been reported. Decay-accelerating factor inserts into erythrocyte membranes (27), and the variable surface glycoprotein of *Trypanosoma brucei* is incorporated into cultured cells (28) and erythrocytes (29). Purified Thy-1 molecules have been shown to be incorporated into plasma membranes of Thy-1 negative cells and exhibit lateral mobilities comparable to those of endogenous Thy-1 (30). All these GPI-linked surface glycoproteins share the same core glycan structure (PI-glucosamine-trimannoside-ethanolamine). By contrast, LAMs contain an oligomannosyl glycan core directly linked to the PI moiety without the intervening glucosamine (3, 4). Moreover, LAM-GPI contains an additional mannose residue at position 2 of the inositol moiety (2). The LAM GPI-glycan structure is therefore unique to mycobacteria. We provide several lines of evidence to show that the interaction of purified mycobacterial LAMs with host cell plasma membrane is mediated primarily through a direct integration of the acyl chains of the LAM GPI anchor, rather than via binding of the carbohydrate head groups to lectin-like receptors: 1) both araLAM and manLAM, which differ in the carbohydrate head groups insert efficiently into a variety of cell types; 2) this interaction is abrogated by prior deacylation of LAMs; 3) LAM incorporation is inhibited by PIM_6 (the LAM-GPI anchor), but not by deacylated PIM_6; and 4) LAM incorporation is negligible at 4°C when the plasma membrane fluidity is decreased. In addition, lack of inhibition by excess mannan of manLAM incorporation even into PBMC which do not

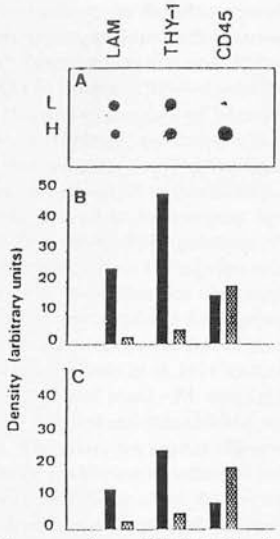

FIGURE 7. Incorporation of LAM into isolated plasma membrane fractions. Light (L) and heavy (H) density membranes isolated from one × 10^7 P1798 murine T lymphoma cells were incubated with 100 μg/ml manLAM in 250 μl PBS at 37°C for 30 min and refloated to their respective densities again by isopycnic density gradient centrifugation. The bands were collected, diluted 10 fold, ultracentrifuged, and resuspended in 250 μl of PBS. Serial dilutions of L and H membrane fractions were dot blotted onto nitrocellulose filters in triplicates and probed with anti LAM, anti-Thy-1, anti CD45 or control mAb followed by appropriate second Abs conjugated to peroxidase. The blots were developed with ECL reagent. The blots shown (*A*) correspond to 1 μl of light and heavy membranes containing 108 ng and 702 ng of total proteins, respectively. Relative quantities of LAM, Thy-1, and CD45 in light and heavy density fractions were quantitated by laser scanning densitometry and were corrected for equivalent protein (*B*) or lipid (*C*) concentration. Solid bars, light membranes; cross-hatched bars, heavy membranes. Results shown are the representatives of two independent assays.

express the mannose receptor (31) argues against a carbohydrate-lectin type of interaction in the insertion of LAMs into host cell plasma membrane. Therefore, we suggest that the direct interaction of purified LAMs with the plasma membrane bilayer is quite distinct from the interaction of manLAM with macrophage mannose receptors involved in phagocytosis of LAM-coated microspheres (32).

Interestingly, the incorporation of LAMs appears to be selective, as it is inhibited almost completely by PIM_6, the GPI of LAMs, while PI from soybean, lacking the hexamannoside glycan core and the mannose residue at C-2 of inositol, resulted in only a marginal inhibition. This observation suggests a requirement for specific membrane environment for LAM insertion and points to the importance of the glycan core of the LAM-GPI anchor in determining this specificity. However, lack of inhibition of LAM incorporation by $dPIM_6$ suggests that the glycan core of LAM-GPI is unlikely to be involved in the primary interaction of LAM with the cell surface. It will be of interest to evaluate the effects of other GPI-anchored molecules containing the widespread PI-glucosamine-trimannosyl glycan on LAM incorporation into cell membranes. The notion of preferred membrane environment for LAM insertion is further supported by our finding that LAMs interact more efficiently with plasma membrane fractions enriched in endogenous GPI-anchored Thy-1 glycoproteins (22). In addition, the marked interference with Ab-mediated lateral mobilization of Thy-1 in intact cells by incorporated LAM suggests that LAMs may preferentially accumulate in plasma membrane domains already enriched in endogenous GPI-linked molecules. It is not known whether Thy-1 also binds to glycosphingolipids in the lymphocyte plasma membrane, as do GPI-linked glycoproteins in the apical plasma membrane of polarized epithelial cells (33), but Thy-1 expressed in fibroblasts (34) or nonpolarized colon cancer cells (35) accumulates in GPI-rich specialized domains. Such behavior of Thy-1 suggests that it is capable of interacting with the sphingolipids and gangliosides in an epithelial cell and so become targeted to specialized membrane domains. It is not unlikely that LAMs could also incorporate into sphingolipid and ganglioside-rich domains of lymphomonocytic cells.

Our observation that inserted LAMs markedly affect the Ab-mediated lateral mobility of Thy-1 suggests that LAMs could perturb the short-range interactions between surface molecules and modulate proximal events in transmembrane signaling pathways. Several GPI-anchored molecules on the surface of thymocytes, T cells, B cells, and monocytes have been implicated in signal transduction leading to intracellular calcium flux, proliferation, and cytokine production (36). Specific immunoprecipitates of many such GPI-linked molecules have been shown to contain a number of intracytoplasmic *src* family tyrosine kinases (37, 38), although the precise nature of this association remains unresolved (39). It is quite conceivable that LAMs incorporated into the GPI-rich domains in the plasma membrane might modulate the signal transduction machinery utilized by endogenous GPI-linked molecules.

The highly branched arabinosyl side chain linked to the mannan core was shown to differ at the termini in different mycobacterial strains. LAM isolated from an unclassified, rapidly growing *Mycobacterium* species (once thought to be an attenuated strain of *M. tuberculosis* H37Rv) contains only β-*d*-arabinofuranosyl residues at the nonreducing ends (4). Later studies revealed that the majority of the terminal arabinan motifs are capped with either a single mannopyranosyl residue, a dimannoside or a trimannoside in LAM isolated from the virulent (Erdman) strain of *M. tuberculosis* (20). The former was designated as araLAM and the latter as manLAM (19). Recently, it has been demonstrated that the mannose residues of Erdman LAM are

important determinants in the phagocytosis of *M. tuberculosis* by macrophages through the macrophage mannose receptor (31). Observations that araLAM was more potent than manLAM in inducing TNF-α secretion from macrophages (14, 26) and expression of immediate early genes involved in macrophage activation (15) prompted the speculation that mannose capping would represent a virulence factor of pathogenic mycobacteria. However, this prediction was proved to be incorrect by recent studies (19, 40) that demonstrated the presence of mannose caps also in LAMs isolated from the vaccine strains of *M. bovis* BCG. Our results show that the structural and antigenic differences in the carbohydrate head group structures of LAM have little influence on the insertion of LAM into the plasma membrane. Adams et al. (41) have observed as well that both araLAM and manLAM were able to block the IFN-γ and LPS induced anti-microbicidal activity in murine macrophages. Therefore, we expect differences in terminal sugar residues of LAMs not to influence the membrane integration process but rather affect the subsequent lateral interactions with surface molecules of lymphomonocytic cells, and thus be responsible for the observed differences in biologic effects of ara- and manLAMs.

Our results strongly suggest that membrane insertion of LAMs through the acyl chains is an early and crucial event in their interaction with target cells, and that the GPI core glycan is contributing specific information regarding the site of insertion into domains enriched in endogenous GPI-anchored molecules. Later perturbations of cellular functions by inserted LAMs probably depend on the interactions of LAM carbohydrate head groups with endogenous cell-surface molecules. Delineating the fine specificities of these lateral interactions will help understanding how LAMs could modulate signaling through surface receptors. Binding of LAM to CD14 (42) could represent one such interaction. Several GPI-linked virulence factors of protozoan parasites such as *Leishmania* (43) and *Plasmodia* (44) also modulate proinflammatory cytokine production by macrophages and influence T lymphocyte functions. It could be envisaged that GPI-linked molecules from pathogenic organisms all function similarly by inserting into plasma membrane domains rich in endogenous GPI-linked molecules and subsequently perturbing cellular functions and responses to environmental stimuli.

Acknowledgment

We thank Dr. E. Rungger-Brändle for her advice in the fluorescence microscopy experiments and for critical reading of the manuscript.

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Structural studies on sialylated oligosaccharides of bonnet monkey (*Macaca radiata*) luteal phase cervical mucus

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Key words: Glycoprotein structure, mucin oligosaccharides, mucus, monkey.

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Abstract

Mucin glycoproteins were purified and fractionated from cervical epithelial secretion in luteal phase of the bonnet monkey (*Macaca radiata*). Alkaline borohydride cleavage of a purified glycoprotein resulted in a mixture of acidic and neutral oligosaccharide alditols. Utilizing high performance chromatography, six oligosaccharide fractions (b-1 to b-6) have been purified from the sialylated oligosaccharide fraction b. Based on the results of enzymic and chemical studies, structures are proposed for these oligosaccharides that bear similarities with those of the midcycle oligosaccharides structures.

b-1-a	NeuAcα(2-6)GalNAc-ol
b-1-b	NeuAcα(2-6)[GlcNAcß(1-3)]GalNAc-ol
b-2	Galß(1-3)GlcNAcα/ß(1-3)[NeuAcα(2-6)]GalNAc-ol
b-3	GlcNAcß(1-3)Galα/ß(1-3)[NeuAcα(2-6)]GalNAc-ol
b-4	Fucα(1-3)GlcNAcß(1-6)[NeuAcα(2->3)Galß(1-3)]GalNAc-ol
b-5	Galß(1-3/4)[Fucα(1-3/4)]GlcNAcα/ß(1-3)[NeuAcα(2-6)]GalNAc-ol
b-6	NeuAcα(2-3)Galß(1-4)GlcNAcß(1-6)[Fucα(1-2)Galß(1-3)]GalNAc-ol

Introduction

The variable biophysical behavior of the cervical mucus during the ovulatory cycle is of great importance in reproductive physiology. The mechanism of sperm penetration through the cervix and cervical mucus depends on the intrinsic motility of the sperm and receptivity of the mucus [1,2].

Cervical mucus is a complex mixture produced continuously by endocervical cells. Minor quantities of endometrial, tubal and follicular fluids may also contribute to the cervical mucus [3]. The mucus exhibits a number of rheological properties, such as viscosity, spinnbarkeit, flow elasticity, and stickiness that are regulated by the ovarian function. The physical and chemical changes in the cervical mucus during the ovulatory cycle influence penetration as well as survival of the sperm. The cyclic changes in the physical properties of the mucus are accompanied by variations in carbohydrate composition [4,5].

Cervical mucus consists of high molecular weight, heavily glycosylated proteins, enzymes, metallic ions and low molecular weight proteins. Glycoproteins are the major constituents of the mucus. The results of physical studies on cervical glycoproteins suggest the presence of linear flexible chains that yield subunits following reduction of disulfide bonds [6,7]. Carbohydrate chains, which account for 50-75% of the molecular mass of the purified cervical glycoproteins in different ovulatory phases, are present in a heterogeneous population of *O*-linked neutral, acidic and sulfated oligosaccharides. The oligosaccharide chains on the protein core are asymetrically distributed, resulting in protease suceptible and protease resistant domains of the glycoprotein [6,9]. The changes in biophysical properties of the mucus are accompanied by alteration in chemical structure; in particular the chemical structure of oligosaccharides is as yet to be clearly defined. In our earlier studies [10,11] it has been shown that the periovulatory and premenstrual

glycoproteins show diversity in the attachment of NeuAc to sugar residues. It was also proposed [11] that this diversity in NeuAc linkage may provide rigidity as well as resistance against proteases to mucus glycoproteins, thereby enabling mucins to perform different functions during the ovulatory cycle.

In order to understand the relationship between the glycoprotein component of the mucus and function of this secretion, it is necessary to characterize the glycoproteins from different phases of the ovulatory cycle responsible for the physiological role and the morphological outlay of the mucus. Purification and fractionation of periovulatory cervical mucus by gel-exclusion chromatography afforded two high molecular weight glycoproteins. The major component is a carbohydrate rich mucin type glycoprotein [12,13]. An antibody against this glycoprotein in immunodiffusion and in immunoelectrophoresis showed sharp and diffused precipitin lines [12]. Under the microscope, the antigen-antibody complex exhibited a morphology different from that of fibrillar channels of the periovulatory mucus [12]. The alteration induced in the morphological outlay by the antibody clearly suggests that the mucus channels are lined with glycoproteins and, thus, suggests the physiologically significant role played by the mucus in sperm migration and penetration [13].

In a previous study, we had reported changes in the glycoprotein structure of the cervical mucus of the bonnet monkey, during the menstrual cycle, based on the oligosaccharide structures. Purification and fractionation of the glycoprotein of the bonnet monkey luteal phase mucus, raising antibody against a purified glycoprotein, liberation of oligosaccharides following treatment with alkaline borohydride and subsequent purification and characterization of the acidic oligosaccharides is the subject of this report.

The Sepharose 2B fractionated glycoprotein, Fraction 1, that was homogeneous in ion-exchange chromatography on DEAE-cellulose has been

homogeneous in ion-exchange chromatography on DEAE-cellulose has been investigated in this study.

This study was pursued on *Macaca radiata* as this monkey produces copious amounts of cervical mucus and because of its phylogenetic closeness to the human.

Materials and methods

Materials

Bio-Gel P-2, Bio-Gel P-4, Bio-Gel P-200, AG50W-X8, AG1-X2 ion exchange resins were purchased from Bio-Rad Laboratories, Sepharose 2B from Pharmacia Fine Chemicals, and exoglycosidases were purchased from Sigma Chemical (α-L-fucosidase from beef epididymis and emulsin; α-D-galactosidase from *Aspergillus niger*; ß-N-acetylglucosaminidase from jack-bean) and Boehringer-Mannheim (neuraminidase from *Vibrio cholerae* and ß-D-galactosidase from *E. coli*).

Collection of cervical mucus

The cervical mucus of the bonnet monkey was collected by aspiration with a suction pump during the cycle. The bonnet monkey menstrual cycle was regular and similar to human [14,15]. The secretion was promptly frozen and was retained in the frozen state until use.

Analytical methods

The hexose content from the column eluates was estimated by the phenol-sulfuric acid method, the protein content by measuring the absorbance at 280 nm or by using the procedure of Lowry *et al.* [16], and sialic acid by the thiobarbituric acid method [17], after mild acid hydrolysis with 50 mM sulfuric acid or by glc [18]. Pc was performed in Whatman No. 1 paper in ethyl acetate:pyridine:acetic acid:water (5:5:1:3). All solutions were dialyzed in hydrogen carbonate-treated cellulose tubing at 4 °C.

Standard oligosaccharides

The oligosaccharides NeuAcα(2-3)Galß(1-3)GalNAc-ol and

NeuAcα(2-3)Galß(1-3)[NeuAcα(2-6)]GalNAc-ol were prepared from fetuin as previously described [19]. The oligosaccharides NeuAcα(2-6)GalNAc-ol, Galß(1-3)[NeuAcα(2-6)]GalNAc-ol and Fucα(1-2)Galß(1-3)[NeuAcα(2-6)]GalNAc-ol were prepared from bonnet monkey cervical mucus [20].

Isolation and purification of luteal phase mucus glycoproteins

The crude luteal phase mucus was partially solubilized in 50 mM sodium phosphate, pH 7.0, containing 0.02% NaN_3. The cellular debris and other insoluble materials were removed by centrifugation (2500 rpm) at 4 °C, the supernatant was dialyzed and the non diffusible material was lyophilized to give the crude mucus. This material (0.4 g) was dissolved in 50 mM sodium phosphate, pH 7.0, 120 ml, containing sodium azide (0.02%) by stirring overnight, at 4 °C. The solution was applied to a column (5x80 cm) of Bio-Gel P-200 (50-100 mesh). The column was washed with phosphate buffer (pH 7.0, 50 mM). The carbohydrate and protein containing fractions were pooled, the pH was adjusted to 5.5 with 4 M acetic acid, the solution was extensively dialyzed and lyophilized to give the purified mucus glycoproteins and proteins (Fig. 1). The glycoprotein fractions were examined in agarose gel electrophoresis as previously described [9]. The purified glycoprotein (Fraction 1, 180 mg) in phosphate buffer (40 ml, pH 7.0) was applied to a column (2.5x70 cm) of Sepharose 2B. The column was washed with the phosphate buffer and fractions containing carbohydrates and proteins were combined and lyophilized. Two fractions, Fraction 1 and Fraction 2, were obtained (Fig. 2). These fractions were examined in polyacrylamide-agarose gel electrophoresis as previously described [9]. The main fraction, Fraction 1 (100 mg), was further purified on a column (1.8x65 cm) of DEAE-cellulose. The column was washed with 100 mM NaCl followed by a gradient of 0.1 M to 1 M NaCl in 10 mM HCl. Antibodies to Fraction 1 glycoprotein were prepared as previously described [12].

Alkaline borohydride (borotritide) treatment

The major glycoprotein fraction (Fraction 1, 60 mg) eluted from the Sepharose 2B column was treated with 2 M sodium borohydride in 50 mM sodium hydroxide (8 ml) for 18 h at 45 °C according to Iyer and Carlson [21]. The reaction mixture was adjusted to pH 5.4 with 4 M acetic acid. The [^{3}H]-labeled oligosaccharides were prepared by subjecting a portion of glycoprotein (7 mg) to ß-elimination using NaB[^{3}H]$_4$ (5 mCi) under conditions described above. The two reaction mixtures were combined and desalted on a column (1.4x60 cm) of AG50W-X8 (H^+, 50-100 mesh). The oligosaccharide alditols were separated into neutral and acidic oligosaccharides on a column of AG1-X2 (OAc^-, 200-400 mesh). The column was washed with water, 50 mM pyridine-acetic acid, pH 5.4, and then 100 mM acetic acid. The acidic oligosaccharides were further fractionated on a column (1.8x90 cm) of Bio-Gel P-4 (200-400 mesh). Hplc of oligosaccharide fraction b, obtained from the Bio-Gel P-4 column, was performed on a Lichosorb-NH_2 column. The oligosaccharides from the column were eluted with a gradient of acetonitrile-water (4:1 to 1:1) containing 2.5 mM ammonium hydrogen carbonate, for 60 min at a flow rate of 1.2 ml/min.

Treatment with glycosidases

[^{3}H]-Labeled oligosaccharides were digested in 100 mM pyridine-acetic acid or 50 mM sodium citrate, pH 5.0, at 37 °C for 24-72 h under a toluene atmosphere. Reactions were terminated by heating the enzyme containing solution in a boiling water-bath for 3 min, and the solutions were dried under vacuum. In the case of sodium citrate buffer, the solutions were desalted by gel filtration on Bio-Gel P-2.

Methylation analysis

The oligosaccharide alcohols were methylated by the modified

procedure of Hakomori [24]. The methylated oligosaccharide alditols were processed, and the methylated sugars were acetylated and identified by glc-ms as previously reported [24].

Periodate oxidation-borohydride reduction

Oligosaccharide alcohols were oxidized in 50 mM sodium acetate (100 µl, pH 4.6) containing 50 mM sodium periodate. The reaction was performed for 6-10 h at 22 °C in the dark. Excess of periodate was consumed by the addition of ethylene glycol. Samples were treated with $NaBH_4$ at 22 °C for 30 min. The excess of borohydride was destroyed and the mixture was desalted simultaneously by the addition of AG50W-X8 (H^+) resin. The solution was filtered, the resin washed with methanol. The combined eluates were dried in vacuo. The residue was treated with 0.25 M H_2SO_4 for 2.5 h and deionized with AG1-X8 (OAc^-, 100-200 mesh) resin. The products were examined by glc in comparison with standards as reported earlier [24].

Results

Purification of glycoproteins

The luteal phase glycoproteins were eluted from the Bio-Gel P-200 column in the void volume. There were overlapping components that were eluted in the void volume (Fig. 1) and contained carbohydrates and proteins. Two major, heterogeneous glycoprotein components in addition to five proteins were obtained. The carbohydrate and amino acid composition of the two glycoproteins were similar (Table 1). The two glycoprotein fractions in agarose gel electrophoresis showed the presence of periodate-Schiff and Coomassie-blue staining materials, although these components had the minimal entry into the gel. The Bio-Gel P-200 purified glycoprotein, Fraction 1, afforded two components in Sepharose 2B chromatography (Fig. 2). The two fractions, Fraction 1 (60%) and Fraction 2 (40%) contained sugars and amino acids (Table 1). The major glycoprotein fraction, Fraction 1, in DEAE-cellulose

chromatography provided a single glycoprotein. Fraction 1 glycoprotein in polyacrylamide-agarose electrophoresis barely entered the gel (data not shown), although it reacted positively with Coomassie-blue and periodate-Schiff stains. Antibody against Fraction 1 glycoprotein in immunodiffusion reacted with crude luteal phase mucus, Bio-Gel P-200 and Sepharose 2B purified glycoprotein fractions. The antibody also weakly reacted with midcycle and follicular phase mucuses, and Bio-Gel P-200 and Sepharose 2B purified glycoproteins from the periovulatory phase.

Preparation of sialylated oligosaccharide alditols

Fraction 1 glycoprotein (54 mg) from the Sepharose 2B column was subjected to alkaline borohydride treatment [21], yielding a mixture of oligosaccharide alditols. A decrease of serine and threonine and a corresponding increase of alanine and the appearance of α-aminobutyric acid were observed, concomitant with partial conversion of GalNAc residues to GalNAc-ol. The acidic oligosaccharides, obtained from the column of AG1-X2, were separated on a Bio-Gel P-4 column into two main components, Fraction a and Fraction b (Fig. 3), in addition to overlapping fractions. The carbohydrate composition of the oligosaccharide fractions is given in Table 2. The oligosaccharide Fraction b was separated by hplc into six fractions (b-1 to b-6, Fig. 4). The molar ratios of the oligosaccharide b-1 to b-6 sugars are given in Table 2. Because of the limited amount of purified oligosaccharides available for structural analyses, characterization was performed on labeled oligosaccharides.

Oligosaccharide b-1

Oligosaccharide b-1, a major oligosaccharide component, was homogeneous in pc and has the composition NeuAc-GlcNAc-GalNAc-ol (1.5:0.6:1; relative to GalNAc-ol). This oligosaccharide in pc moved slower than NeuAcα2-6GalNAc-ol and Neuα(2-3)Galβ(1-3)GalNAc-ol. Treatment of the

chromatography provided a single glycoprotein. Fraction 1 glycoprotein in polyacrylamide-agarose electrophoresis barely entered the gel, although it reacted positively with Coomassie-blue and periodate-Schiff stains. Antibody against Fraction 1 glycoprotein in immunodiffusion reacted with crude luteal phase mucus, Bio-Gel P-200 and Sepharose 2B purified glycoprotein fractions. The antibody also weakly reacted with midcycle and follicular phase mucuses, and Bio-Gel P-200 and Sepharose 2B purified glycoproteins from the periovulatory phase.

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oligosaccharide with neuraminidase resulted in cleavage of NeuAc and release of GalNAc-ol and a component similar to the standard disaccharide, NeuAcα(2-6)GalNAc-ol in pc. Hydrolysis of the neuraminidase treated material obtained from pc showed the presence of GlcNAc and GalNAc-ol. Digestion of b-1 with N-acetylglucosaminidase removed GlcNAc. Periodate oxidation-borohydride reduction, acid hydrolysis and re-N-acetylation of b-1 showed therein the presence of SerNAc-ol and ThrNAc-ol. Methylation results (Table 3) combined with the above data suggested that oligosaccharide Fraction b-1 was a mixture of a disaccharide, and a trisaccharide with the structures b-1-a and b-1-b shown in Fig. 5.

Oligosaccharide b-2

Oligosaccharide b-2 was a tetrasaccharide with the molar composition NeuAc:Gal:GlcNAc:GalNAc-ol (0.7:1:0.9:1, relative to GalNAc-ol). Digestion of the oligosaccharide with neuraminidase removed NeuAc. Methylation analysis of sialo and asialo oligosaccharide showed methylated sugars (Table 3) that suggested the presence of 3,6-disubstituted GalNAc-ol and sialic acid linked to C-6 of GalNAc-ol in the oligosaccharide. In addition, the presence of a terminal galactose residue and a 3-substituted GlcNAc residue was observed. Digestion of b-2 with α-galactosidase had no effect on the oligosaccharide, and treatment with ß-galactosidase removed the galactose.residue. Periodate oxidation-borohydride reduction resulted in destruction of Gal and NeuAc residues, conversion of GalNAc-ol to ThrNAc-ol and GlcNAc was recovered unchanged. Based on these results the b-2 tetrasaccharide was assigned the structure b-2 shown in Fig. 5.

Oligosaccharide b-3

Oligosaccharide b-3 had the molar composition NeuAc:Gal:GlcNAc:GalNAc-ol (0.7:0.9:0.8:1., relative to GalNAc-ol). Treatment

of the oligosaccharide with neuraminidase removed NeuAc. Methylation analyses of the methylated sugars of sialo and asialo oligosaccharides showed derivatives in these oligosaccharides (Table 3) that suggested the presence of terminal GlcNAc, 3-linked Gal, 3,6-linked GalNAc-ol and NeuAc linked to C-6 of GalNAc-ol. Digestion of b-3 with ß-N-acetylglucosaminidase removed GlcNAc residue. Periodate oxidation-borohydride reduction of b-3 eliminated NeuAC and GlcNAc, GalNAc-ol was converted to ThrNAc-ol and Gal was not affected. These results suggested that b-3 was a tetrasaccharide with the structure shown in Fig. 5.

Oligosaccharide b-4

Oligosaccharide b-4 had the molar sugar composition NeuAc:Fuc:Gal:GlcNAc:GalNAc-ol (0.8:0.7:0.9:0.8:1, relative to GalNAc-ol). Treatment of b-4 with neuraminidase removed sialic acid. Analysis of methylated sialo and asialo oligosaccharides showed the presence of terminal Fuc, 3-linked Gal, 3-linked GlcNAc, 3,6-substituted GalNAc-ol (Table 3), and NeuAc linked to C-3 of Gal. Sequential digestion of the oligosaccharide with neuraminidase, α-fucosidase and ß-galactosidase removed residues of NeuAc, Fuc and Gal. Methylation analysis of enzyme-treated oligosaccharide suggested therein the presence (Table 3) of terminal GlcNAc and 6-linked GalNAc-ol. Periodate oxidation-borohydride-reduction of b-4 removed Fuc and NeuA, converted GalNAc-ol to ThrNAc-ol, Gal and GlcNAc were recovered unchanged. These data revealed that b-4 was a pentasaccharide with the structure shown in Fig. 5.

Oligosaccharide b-5

Oligosaccharide b-5 was homogeneous in pc and had the molar composition NeuAc:Fuc:Gal:GlcNAc:GalNAc-ol (0.7:0.8:0.9:0.8:1, relative to GalNAc-ol). Periodate oxidation-borohydride reduction eliminated NeuAc, Fuc

and Gal, GalNAc-ol was converted to ThrNAc-ol and GlcNAc was not affected. Methylation of the oligosaccharide showed the presence of terminal Fuc, NeuAc and Gal, 3,4-linked GlcNAc and 3,6-linked GalNAc-ol (Table 3). Treatment of b-5 with neuraminidase removed NeuAc, and subsequent methylation of the asialo oligosaccharide showed that NeuAc was linked to C-6 of GalNAc-ol. Treatment of the asialo oligosaccharide with ß-galactosidase removed a galactose residue. These results provided evidence that oligosaccharide b-5 was a pentasaccharide with the structure given in Fig. 5.

Oligosaccaride b-6

Oligosaccharide b-6 had the molar composition NeuAc:Fuc:Gal:GlcNAc:GalNAc-ol (0.7:0.6:1.7:0.8:1, relative to GalNAc-ol). Periodate oxidation-borohydride reduction degraded NeuAc, Fuc and a Gal residue, GalNAc-ol was converted to ThrNAc-ol, and a Gal and GlcNAc residues were resistant to oxidation. Methylation of b-6 showed the presence of terminal NeuAc and Fuc, 2-linked and 3-linked Gal, 4-linked GlcNAc and 3,6-linked GalNAc-ol. Treatment of the oligosaccharide with α-fucosidase- and then with ß-galactosidase removed fucose and Gal residues. Methylation of the residual oligosaccharide showed the presence of terminal NeuAc, 3-linked Gal, 4-linked GlcNAc and 6-linked GalNAc-ol. Treatment of the α-fucosidase and ß-galactosidase-treated oligosaccharide with neuraminidase removed NeuAc, and subsequent methylation of the residual oligosaccharide showed therein the presence of terminal Gal, 4-linked GlcNAc and 6-linked GalNAc-ol. Treatment of the asialo oligosaccharide with ß-galactosidase removed a galactose residue. These results suggested the oligosaccharide b-6 to be a hexasaccharide with the structure shown in Fig. 5.

Discussion

In the present study the mucus was collected from several monkeys in the luteal phase of the menstrual cycle. The isolation of the secreted glycoproteins and their separation from other polymeric materials, namely enzymes and serum proteins, was readily accomplished by gel filtration on Bio-gel P-200. The glycoprotein component in the mucus was accompanied by several proteins as shown in the gel chromatography. The composition analysis of the proteins (data not shown) indicated variable amounts of cystine residues in these proteins, suggesting possibilities of inter- or intra-molecular disulfide linkages. The purified glycoprotein fractions had similar carbohydrate and amino acid composition. The main glycoprotein fraction in Sepharose chromatography afforded two glycoproteins with similar carbohydrate and amino acid composition. The carbohydrate moiety of the two glycoproteins differred in Fuc, GalNAc, NeuAc and sulfate contents. Amongst the amino acids there was a noticeable similarity in the hydroxy amino acids. Fraction-2 glycoprotein exhibited a slight increase in cystine and basic amino acids. The main glycoprotein fraction, Fraction-1, was homogeneous in DEAE-cellulose chromatography. In gel electrophoresis it had the minimal entry, like the periovulatory phase glycoprotein, and showed the absence of contaminating proteins. The antibody against the main luteal phase glycoprotein, Sepharose Fraction-1, reacted with luteal phase mucus, Bio-Gel P-200 purified glycoproteins, Sepharose 2B glycoprotein fractions as well as DEAE--cellulose purified glycoprotein. In addition, this antibody also weakly reacted with ovulatory phase mucus, Bio-Gel P-200 and Sepharose 2B purified glycoproteins from the ovulatory phase, suggesting some common epitopes in glycoproteins of different phases of the ovulatory cycle, interalia the presence of molecules with similar structures during the cycle.

Despite the fact that there are morphological and rheological differences between the ovulatory phase and the luteal phase mucuses, the carbohydrate

and amino acid composition of the glycoproteins from both mucuses were similar. There are minor variations in the amounts of carbohydrates as well as of amino acids. These minor variations in carbohydrates during the cycle have been observed in human mucus as well [4]. It has been proposed that function, viscoelasticity and morphology of the mucus are reflected on the carbohydrate composition and /or in their structure, and that this relationship is unique for each individual [25]. Furthermore, it has been proposed [25] that mucin concentration and not composition changes in response to hormonal alteration during the menstrual cycle. The current findings on the purified and fractionated glycoprotein components are in line with these observations. There are number of proteins in the mucus in addition to glycoproteins in each phase of the menstrual cycle, and their functional role in conjunction with the glycoproteins has yet to be investigated. Variations in the protein contents, such as cystine and basic amino acids, may contribute significantly to the functions of the glycoproteins.

Alkaline-borohydride treatment of the glycoprotein, Sepharose Fraction 1, gave a mixture of oligosaccharides that was fractionated by gel filtration. An oligosaccharide fraction, Fraction b, obtained from Bio-Gel P-4, was further separated by hplc into six oligosaccharide fractions. Seven oligosaccharides, disaccharides to hexasaccharides, were characterized. The oligosaccharides had the following core structures:

i) Galß(1-3)GalNAc-ol

ii) Galß(1-4)GlcNAcß(1-6)GalNAc-ol

iii) GlcNAcß(1-3)GalNAc-ol

Elongation of the carbohydrate chains takes place on these core residues, resulting in the structures characterized and shown in Fig.5. The structures of sialylated oligosaccharide chains have been identified from human midcycle cervical glycoproteins [26], and these bear similarities to the core structure of the bonnet monkey midcycle oligosaccharides [20,24]. Sialylated

oligosaccharides from bonnet monkey midcycle cervical glycoproteins have been characterized [24]. The sialylated oligosaccharides from the luteal phase glycoprotein share core structures as well as some structural features with bonnet monkey and human midcycle glycoproteins. There are structural variations in the oligosaccharides of the luteal phase and midcycle glycoproteins. The core structure iii has neither been observed in the midcycle bonnet monkey glycoprotein oligosaccharide chains nor in human midcycle glycoproteins. Similarly, Fuc residues in the sialylated oligosaccharide chains of midcycle bonnet monkey mucus are mainly reported to be linked 1->2 to Gal [20], whereas the luteal phase oligosaccharide chains have Fuc linked 1->2 to Gal as well as 1->3 to GlcNAc residues. The Fuc residues in sialylated oligosaccharides from human midcycle glycoprotein are solely linked 1->3/4 to GlcNAc [26]. The fucosyltransferases responsible for the transfer of fucose residues to position C-2 of Gal and C-3 of GlcNAC from the human cervical epithelium have been characterized [27]. The NeuAc residues in the luteal phase oligosaccharides have α2->3 linkage to Gal and α2->6 linkage to GalNAc-ol, a feature common with midcycle oligosaccharide chains [20,24] of the bonnet monkey as well as those of human [26].

Diverse core structures and variety of oligosaccharide chain lengths in mucin glycoproteins are known [28-30], and the heterogeneity in the oligosacharide structure and chain length in the cervical mucus glycoproteins could be even wider because of changing physicochemical behavior during the menstrual cycle.

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Table 1

Carbohydrate and amino acid composition of glycoproteins purified on Bio-Gel P-200, fractionated on Sepharose 2B and DEAE--cellulose

	Bio-Gel P-200 Fractions				Sepharose 2B Fractions				DEAE--cellulose	
	1		2		1		2			
	%	M/R*	%	M/R*	%	M/R*	%	M/R*	%	M/R*
Carbohydrates										
Fuc	7.0	1.06	6.8	1.04	5.7	0.83	6.2	1.03	6.3	0.82
Gal	16.0	2.19	14.9	2.08	15.8	2.1	15.1	2.29	21.0	2.48
GlcNAc	9.0	1.00	8.8	1.00	9.3	1.00	8.1	1.00	10.4	1.00
GalNAc	14.0	1.55	13.6	1.54	13.2	1.42	10.9	1.34	15.6	1.50
NeuAc	9.0	0.71	9.4	0.76	10.3	0.79	7.9	0.60	11.3	0.78
Total	55.0		50.0		54.3		48.2		64.6	
Sulfate					0.4		0.5			
Amino acids§						¶				
Asp		5.1		4.8	5.3		5.2		5.6	
Thr		18.2		17.1	19.6	-10.4	18.1		19.4	
Ser		12.1		12.9	11.9	- 4.1	12.3		11.7	
Glu		7.1		6.9	6.8		7.1		5.8	
Pro		5.6		7.1	6.4		6.9		6.8	
Gly		9.0		8.5	8.2		9.2		7.8	
Ala		10.2		11.1	10.2	+4.1	10.3		9.6	
Aba						+9.6				
Cys/2		1.8		2.4	1.6		1.9		1.4	
Val		6.8		5.9	6.4		5.7		6.0	
Ileu		5.8		5.1	5.6		4.9		5.8	
Leu		5.8		4.9	5.9		5.4		6.7	
Tyr		2.1		1.9	2.2		1.8		2.3	
Phe		2.3		1.8	2.1		1.9		2.6	
Lys		2.6		3.1	2.9		3.4		3.4	
His		2.2		2.8	2.1		2.7		2.4	
Arg		3.3		3.7	2.8		3.2		2.7	

*M/R (molar ratio) relative to GlcNAc.

§Residues per 100 residues.

¶Fraction 1 after treatment with alkali.

Table 2

Molar composition of oligosaccharides obtained by fractionation of luteal phase cervical mucus glycan

Oligosaccharide Bio-Gel P-4	hplc of Fraction b	Molar ratio* of monosaccharides					
		NeuAc	Fuc	Gal	GlcNAc	GalNAc	GalNac-ol
a		0.58	0.59	2.14	0.79	0.56	1.0
b		0.68	0.61	1.40	0.82	0.30	1.00
	b-1	1.50			0.60		1.00
	b-2	0.70		1.00	0.90		1.00
	b-3	0.70		0.90	0.80		1.00
	b-4	0.80	0.70	0.90	0.80		1.00
	b-5	0.70	0.80	0.90	0.80		1.00
	b-6	0.70	0.60	1.70	0.80		1.00

*Molar ratio relative to GalNAc-ol.

Table 3

O-Methyl derivatives obtained by acid hydrolysis of methylated oligosaccharides before and after enzyme treatment

Oligosaccharide	*O-Methyl derivative*											
	Fuc	*Gal*			*GlcNAc*						*GalNAc-ol*	
	2,3,4-	2,3,4,6-	2,4,6-	3,4,6-	2,3,6-	3,4,6-	3,4-	4,6-	3,6-	6-	3,4-	4-
b-1						+					+	+
b-2		+						+				+
b-2[a]		+						+			+	
b-3			+			+						+
b-3[a]			+			+					+	
b-4	+		+					+				+
b-4[a]	+	+						+				+
b-4[b]						+					+	
b-5	+	+								+		+
b-5[a]	+	+								+	+	
b-6	+		+	+				+				+
b-6[c]			+					+			+	
b-6[d]		+						+			+	

[a]Neuraminidase treated.

[b]Sequentially neuraminidase, α-fucosidase and ß-galactosidase treated.

[c]α-L-Fucosidase and ß-D-galactosidase treated.

[d]Neuraminidase treatment of α-L-fucosidase and ß-galactosidase treated oligosaccharide.

Figure Legends

Fig.1.

Purification of crude luteal phase cervical mucus in Bio Gel P-200. The column was washed with 50 mM sodium phosphate buffer, pH 7.0, containing 0.02% sodium azide. Fractions of 2 ml were collected and every third fraction was examined for the presence of carbohydrates (495 nm) and proteins (750 nm).

Fig. 2.

Gel chromatography of the Bio-Gel P-200 purified Fraction 1 glycoprotein on Sepharose 2B. The glycoproteins were eluted with from the column 50 mM sodium phosphate buffer, pH 7.0, containing 0.02% sodium azide. Fractions of 1.5 ml were collected and every third fraction was examined for the presence of carbohydrates (495 nm) and proteins (290 nm).

Fig. 3.

Separation of acidic oligosaccharides on a column of Bio-Gel P-4. Two main fractions were obtained by elution with 50 mM pyridine acetic acid, pH 5.4. Oligosaccharides were monitored by the presence of tritium and hexoses (495 nm).

Fig. 4.

Hplc separation of acidic oligosaccharides of Fraction b oligosaccharide on a column of Lichsorob-NH_2 with a linear gradient of 4:1 to 1:1(v/v) acetonitrile-water containing 2.5 mM ammonium hydrogen carbonate for 60 min at a flow rate of 1.2 ml/min.

Fig. 5.

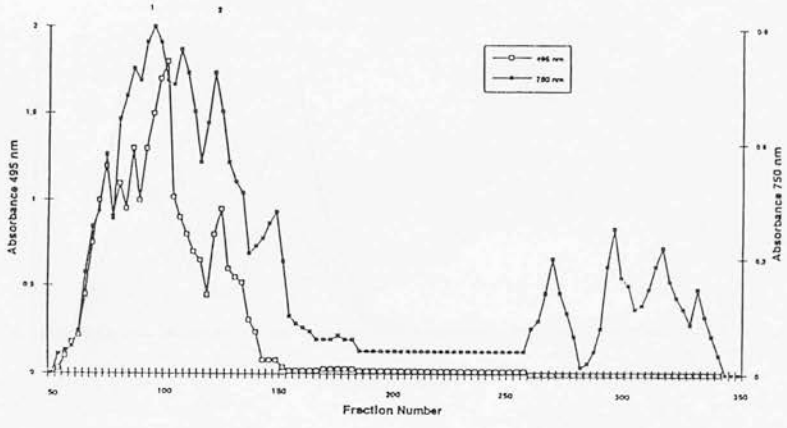
Proposed structures for oligosaccharides b-1 to b-6.

Abbreviations

Abbreviations used are Fuc, fucose; Gal, galactose; NeuAc, N-acetylneuraminic acid; GlcNAc, N-acetylglucosamine; GalNAc, N-acetylgalactosamine; glc, gas liquid chromatography; hplc, high performance liquid chromatography; pc, paper chromatography.

Acknowledgement

Nasir-ud-Din thanks the National Scientific Research Development Board, Pakistan for partial financial support.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Absorbance 750 nm
Fraction Number
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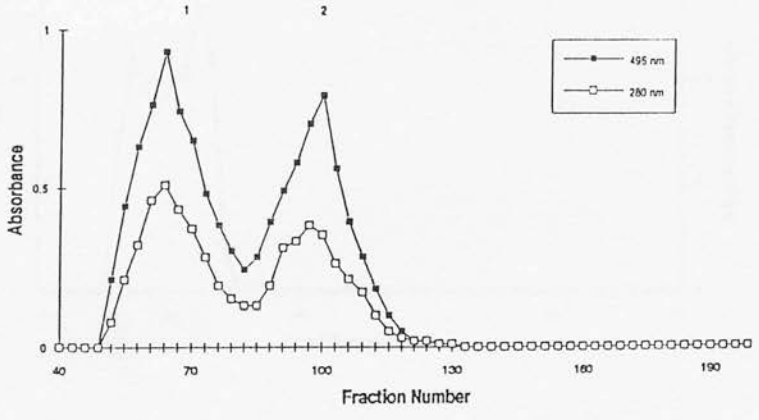
100
150
200
250
300
350

Figure 1.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Absorbance
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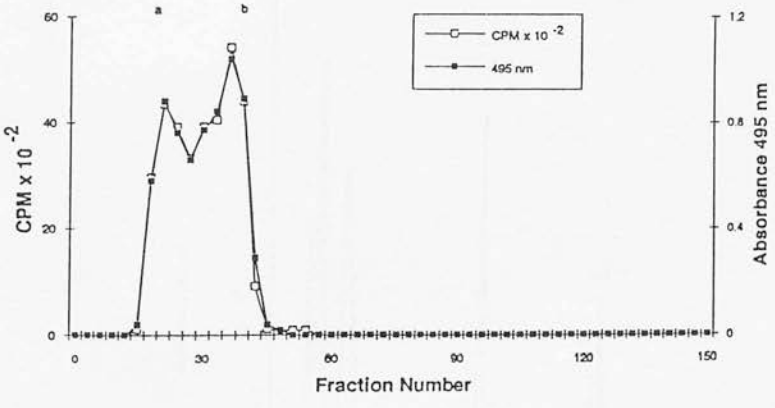
280 nm
40
70
100
130
160
190
Fraction Number

Figure 2.


a
b
CPM x 10 -2
495 nm
CPM x 10 -2
Absorbance 495 nm
Fraction Number
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40
60
0
0.4
0.8
1.2
0
30
60
90
120
150

Figure 3.

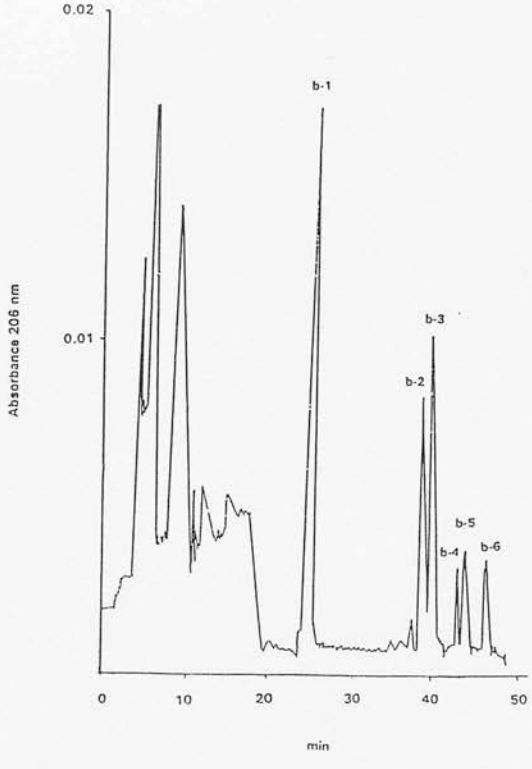

Figure 4.

b-1-a NeuAcα(2->6)GalNAc-ol-

b-1-b NeuAcα(2->6)[GlcNAcß(1->3)]GalNAc-ol

b-2 Galß(1->3)GlcNAcα/ß(1->3)[NeuAcα(2->6)]GalNAc-ol

b-3 GlcNacß(1->3)Galα/ß(1->3)[NeuAcα(2->6)]GalNAc-ol

b-4 Fucα(1->3)GlcNAcß(1->6)[NeuAcα(2->3)Galß(1->3)]GalNAc-ol

b-5 Galß(1->3/4)[Fucα(1->3/4)]GlcNAcα/ß(1->3)[NeuAcα(2->6)]GalNAc-ol

b-6 NeuAcα(2->3)Galß(1->4)GlcNAcß(1->6)[Fucα(1->2)Galß(1->3)]GalNAc-ol

Figure 5.

Carbohydrate Moiety of *Plasmodium falciparum* Glycoproteins: THE NATURE OF THE CARBOHYDRATE-PEPTIDE LINKAGE IN THE 42-53 kDa GLYCOPROTEIN

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Subdivision: (5) Carbohydrates, lipids and other natural products

Running Title: Carbohydrate moiety in *Plasmodium falciparum* 42-53 kDa glycoprotein

Enzymes used in this study:

α-mannosidase [EC 3.2.1.24]

α-galactosidase [EC 3.2.1.22]

β-galactosidase [EC 3.2.1.23]

β-*N*-acetylglucosaminidase [EC 3.2.1.30]

endo-α-*N*-acetylgalactosaminidase [EC 3.2.1.97]

galactosyltransferase [EC 2.4.1.22]

The abbreviations used are: GPI, glycosylphosphatidylinositol; SDS-PAGE, sodium dodecylsulfate polyacrylamide gel electrophoresis; pc, paper chromatography; Ara, arabinose; GalN, 2-amino-2-deoxygalactose; GlcNAc-ol, *N*-acetylglucosaminitol; GalNAc-ol, *N*-acetylgalactosaminitol and sialic acid, Sia.

SUMMARY

Metabolic labelling of *Plasmodium falciparum* parasites with [^{3}H]GlcN, [^{3}H]Man, [^{3}H]Gal and [^{3}H]ethanolamine, and subsequent purification by SDS-PAGE of the labelled material provided effective labelling of the MSP-1, 195 kDA, and MSP-2, 42-53 kDa, glycoproteins. Reductive β-elimination of the 42-53 kDa glycoprotein released from the gel consisted of glycopeptides containing labelled sugars. Processing of the eliminated components and identification of the sugar residues demonstrated the presence of *N*-acetylglucosaminitol and *N*-acetylgalactosaminitol amongst other labelled sugars. Reductive β-elimination with sodium hydroxide-sodium borotritide-borohydride showed the presence of glucosaminitol and alanine in the hydrolysis products. The 42-53 kDa glycoprotein was retained on solid phase wheat-germ agglutinin and was released from the lectin by treatment with GlcNAc. Upon treatment with exoglycosidases the 42-53 kDa glycoprotein and derived oligosaccharides released labelled components corresponding to the metabolically incorporated sugars. Labelled Gal was incorporated into the 42-53 kDa glycoprotein using [^{3}H]UDP-Gal and galactosyltransferase. The galactosylated glycoprotein released labelled Gal upon treatment with β-galactosidase. The results of the present study suggest that the carbohydrate chains of the 42-53 kDa glycoprotein are attached to the protein backbone via GlcNAc- and GalNac-serine/threonine in *O*-glycosyl linkage and the glycoprotein has terminal GlcNAc and Gal residues. The carbohydrate moieties of 42-53 kDa, MSP-2, glycoprotein consist mainly of short chains linked to the protein core.

Keywords: Plasmodium falciparum, merozoite, surface glycoproteins, *O*-glycans.

INTRODUCTION

Malaria is one of the most prevalent disease of tropical and sub-tropical countries. In 103 countries of the world malaria causes 400 million clinical cases with an estimated mortality toll of one to three million [1]. Despite concerted efforts to limit malaria by vector control, development of chemotherapeutics and formulation of vaccines, the disease is on the increase [2].

Malaria results from infection by Protozoa of the genus *Plasmodium*, whose life cycle is complex and involves different stages. *Plasmodium falciparum* causes malignant tertian malaria of major concern. Accumulated evidence suggests that the pathology is due to the development and proliferation of asexual stages in the host blood. The erythrocytic stages of the parasite synthesize high molecular weight glycoproteins [3, 4], which are known to mediate merozoite invasion of erythrocytes [4, 5] and to be antigenic [6, 7]. Amongst the antigens displayed by the asexual blood stages of *P. falciparum* infection [8, 9], the MSP-1 and the MSP-2 glycoproteins are considered vaccine candidates able to induce protective immune responses [10, 11].

Although many proteins of *P. falciparum* have been considered to be glycosylated [12, 13], information about the nature of the carbohydrates present in the malarial glycoproteins and of the carbohydrate-peptide linkage was scant until recently [14-16]. The low yields of malaria parasite in culture and the large variety of proteins being glycosylated during metabolic labelling have rendered glycobiological studies of *P. falciparum* difficult to carry out at the structural level. Structural characteristics of different proteins and glycoproteins have been investigated utilizing lectin binding and enzyme cleavage [17, 18]. The existence of glycosylphosphatidylinositol (GPI) anchored proteins has also been shown using metabolic labelling and phospholipase C digestion [19-21]. It has also been shown that sugar moieties in *P. falciparum* glycoproteins, either as surface glycoproteins or as components of the GPI anchor, may contribute significantly to the antigenicity of the glycoprotein [18].

A 42-53 kDa glycoprotein antigen has been identified on *Plasmodium falciparum* merozoites [22]. This glycoprotein, which appears to be synthesized early in schizogony and to remain on the surface of released merozoites, was shown to mediate invasion of erythrocytes *in vivo* [23]. Studies of this antigen have been complicated because of its polymorphism. Fenton *et al.* have suggested [24] that the polymorphism of the molecule may be of significance in enabling the parasite to avoid immune response.

This study was undertaken to identify the nature of the carbohydrate moiety of the 42-56 kDa glycoprotein from *P. falciparum* asexual blood stages, a form of the 43-56 kDa, MSP-2, glycoprotein. The occurrence of *O*-glycosylation in this glycoprotein has been documented by the use of metabolic labelling, reductive cleavage of sugar chains, enzymic and lectin binding studies. Evidence is presented here for *O*-glycosylation of a novel nature in *P. falciparum* [25], analogous to that in nuclear pore and cytoplasmic glycoproteins which display *O*-linked GlcNAc residues [26].

MATERIALS AND METHODS

Materials Labelled materials: [^{3}H]GlcN hydrochloride, [^{3}H]mannose, [^{3}H]galactose, [^{3}H]ethanolamine, sodium borotritide and [^{3}H]UDP-galactose were purchased from Amersham (Little Chalfont, Bucks, U.K.). Galactosyltransferase and exoglycosidases were obtained from Sigma (St. Louis, MO), Miles (Eikhavel, IN) and Boehringer-Mannheim (Mannheim, Germany).

In vitro *labelling of* P. falciparum *and preparation of parasite lysate* The M25/ZAIRE strain of *P. falciparum* was used in the current studies. Asexual blood stages were cultured in asynchronous mode in RPMI medium supplemented with 10% normal human serum, 0.1% Glc and 50 μCi/ml of [^{3}H]GlcN, [^{3}H]Gal, [^{3}H]Man and [^{3}H]ethanolamine, either separately or in admixture. Labelled parasites were harvested and the lysate containing soluble proteins and glycoproteins was prepared using NP-40 detergent as previously described [16].

Immunoprecipitation of parasite lysate NP-40 extracts of labelled parasite were immunoprecipitated using a mixture of monoclonal antibodies (gift from Dr. I. Shrivastava) directed against surface epitopes on *P. falciparum* erythrocytic stages. Immunoprecipitation and resolution of precipitates in SDS-PAGE were performed as previously described [16]. Gels were treated with Amplify (Amersham), dried and autoradiographed for 4-8 days using Amersham's Hyperfilm.

Thin layer chromatography Ethanolamine was separated on cellulose plates (Merck, Darmstadt), developed in butanol: acetic acid:water (5:2:3, v/v). The plates were dried and the radioactivity distribution was analyzed by autoradiography or by counting of the cellulose fractions that co-migrated with standards.

Paper chromatography The chromatographic analysis of products of β-elimination, oligosaccharides, monosaccharides, amino acids and that of acid hydrolysates of glycoproteins and oligosaccharides were performed by descending paper chromatography (pc) on Whatman No. 1 paper for 16 h in solvents, (a) 1-

butanol-pyridine-water (6:4:3), (b) ethyl acetate-pyridine-water (8:2:1) and as earlier reported [16]. The mobility of sugars is given relative to GlcN (R_g).

Acid hydrolysis of the 42-53 kD glycoprotein and oligosaccharides The glycoprotein was excised from the gel and treated with 4 M HCl at 100 °C for 16 h. The mixture was evaporated under nitrogen at 40°C and the residue examined by pc after *N*-acetylation in methanol with acetic anhydride. The oligopeptides and oligosaccharides were depolymerised with 2 M HCl at 100 °C for 6 h as described [16].

Deglycosylation of 42-53 kDa glycoprotein The 42-53 kDa glycoprotein revealed by autoradiography was excised from the gel and treated for 20 h in 2 ml of 50 mM sodium hydroxide containing 1M sodium borohydride at 45°C. In some experiments, a mixture of sodium borohydride and sodium borotritide was used. The reaction mixture was processed for analyses as described earlier [16]. The reaction products of β-elimination were applied to a column (0.8 x 6 cm) of Bio-Gel P-4. The column was washed with 50 mM pyridine-acetic acid (pH 5.4). The eluted fractions containing radiolabelled material were combined, reduced in volume and portions were examined in pc as such or after acid hydrolysis, by lectin affinity, enzyme treatment or by ninhydrin degradation.

Ninhydrin degradation of amino sugars Hexosamine hydrochlorides obtained after acid hydrolysis of the glycoprotein were converted to free amines by treatment with Dowex-1 OH^- resin. The amino sugars were treated with ninhydrin as previously described [16], and degradation products were examined in pc.

Enzyme treatment of the 42-53 kDa glycoprotein, glycopeptides and oligosaccharides The 42-53 kDa glycoprotein, oligosaccharides or glycopeptides were digested with the following enzymes: (i) α-mannosidase from almonds (Sigma) in 50 mM citrate buffer, pH 4.4, for 20 h at 37 °C; α-mannosidase from Jack bean (Sigma) in 50 mM citrate buffer, pH 4.4, for 24 h at 37°C; (ii) α-galactosidase from *Aspergillus niger* (Sigma) in 50 mM citrate buffer, pH 4.1, for 30 h

at 37°C; α-galactosidase from *E. coli* (Sigma) in 50 mM phosphate buffer, pH 5.0, for 24 h at 37°C; and α-galactosidase from coffee beans (Sigma) in 50 mM phosphate buffer, pH 5.5, for 20 h at 37°C; (iii) β-galactosidase from *Charonia lampas* (Miles) in 50 mM citrate buffer, pH 4.0, for 30 h at 37°C; β-galactosidase from *E. coli* (Sigma) in 50 mM phosphate buffer, pH 7.0, for 36 h at 37°C; (iv) β-*N*-acetylglucosaminidase (hexosaminidase, Sigma) in 10 mM cacodylate buffer, pH 5.5, for 20 h at 37°C and (v) endo-*O*-glycanase (endo-α–*N*-acetylgalactosaminidase, Genzyme, Boston, MA) for 20 h at 37 °C in 10 mM phosphate buffer.

Wheat-germ agglutinin binding of the 42-53 kDa glycoprotein The 42-53 kDa glycoprotein was eluted from the gel strips in 50 mM phosphate buffer, pH 6.8. The gel strips were cut into small fragments, sonicated in buffer at 4 °C for 30 sec five times with an interval of 30 sec between each sonication. The gel suspension was centrifuged at 5000 x g for 30 min at 4 °C, and the supernatant, a small part of which was counted for the presence of tritium, was applied to a prewashed column (0.3 x 1.5 cm) of wheat germ agglutinin bound to agarose (Sigma). The column was washed with water followed by a solution of GlcNAc (20 mM, 200 µl). Tritium containing fractions were combined, dialyzed and lyophilyzed.

Exogalactose labelling of 42-53 kDa glycoprotein using galactosyltransferase and UDP-[^{3}H]-galactose The 42-53 kDa glycoprotein, obtained from the gel strips in 50 mM phosphate buffer, pH 8.4, containing 50 mM Mn^{2+} was treated with 0.5 units of previously autogalactosylated galactosyltransferase (Sigma) in the presence of [^{3}H]UDP-galactose. The galactosylation was terminated by cooling the reaction mixture to 4°C and addition of 50 mM ammonium formate. The solution was dialyzed against distilled water for 16 h at 4°C. After counting for the presence of tritium, the non-dialysable material was reduced in volume and applied to a column (0.3 x 1.2 cm) of Bio-Gel P-60. The column was washed with 50 mM pyridine-acetic acid buffer, pH 5.4, and tritium containing fractions were combined and lyophilyzed. The exogalactosylated

glycoprotein was subjected to reductive β-elimination and the product was examined in pc for galactosylated oligosaccharides and monosaccharides.

Treatment with galactosidases of the exogalactosylated 42-53 kDa glycoprotein The exogalactosylated 42-53 kDa glycoprotein was treated with β-galactosidase (Sigma) in 50 mM phosphate buffer containing 50 mM magnesium sulfate, pH 7.3, for 12 h at 37°C. The galactosidase treatment was stopped by cooling the reaction mixture to 4°C and dialysis against distilled water at 4°C. The dialysate and the retentate were examined for the presence of tritiated galactose. Similarly, the galactosylated 42-53 kDa glycoprotein was treated with α-galactosidase in 50 mM phosphate buffer containing 50 mM magnesium sulfate, pH 6.5, at 25 °C for 12 h. The reaction mixture was processed as described above for β-galactosidase treatment.

RESULTS

The malarial glycoproteins of the asexual blood stages, i.e., trophozoites, schizonts and merozoites, were metabolically labelled with [^{3}H]GlcN, [^{3}H]Man, [^{3}H]Gal and [^{3}H]ethanolamine. The labelling of the parasitic glycoproteins was performed for 6 h as well as for 18 h. The incorporation of the sugar residues was similar under both conditions. The 6 h incorporation was preferred to ensure morphological integrity of the parasites. The labelled glycoproteins were extracted from the cell lysates in non-ionic detergents and examined by SDS-PAGE under non-reducing conditions followed by autoradiography. Figure 1 shows that several glycoproteins were labelled with GlcN (lane B: 195, 100, 85, 42-53, 39, 30 and 12-16 kDa), Man (lane A: 195, 45, 53 and 12-16 kDa) and with ethanolamine (lane C: 195, 100, 80, and 42-56 kDa), Few proteins were labelled with Gal (data not shown). The incorporation of GlcN was much more intense compared to that of Man, Gal and ethanolamine; Man incorporation was abundant relative to Gal.

The 42-53 glycoprotein incorporated significantly more GlcN than Man or ethanolamine (Fig. 1), while Gal incorporation was very weak (data not shown). By electroelution from gel strips at room temperature or at 37°C a very small quantity of labelled material was obtained, and harsh treatment at higher temperatures was not feasible due to observed degradation. It was therefore considered adequate to perform studies either on the gel strips of the glycoprotein or on the sonicated gel extract, which provided sufficient labelled material to work with.

Monosaccharide analysis of the glycoprotein, after acid hydrolysis, showed the presence of GlcN, Gal, Man and a minute quantity of a component with the mobility of GalN. It was not possible to develop a molar ratio from the radioactivity revealed from the paper chromatograms, although a comparison was possible (Fig. 2, Table 1). Ethanolamine was incorporated into the 42-53 kDa glycoprotein (Fig.1, lane C).

To identify the sugar residues and amino acids involved in the glycan-peptide linkage, the 42-53 kDa gel strips were subjected to alkaline borohydride treatment. The reaction was performed for 20 h at 45 °C to allow an efficient release of sugars from the glycoprotein in the gel strips. After 20 h of reaction, approximately 80% of the radioactivity was in solution and about 15% of the radioactivity was present in glycopeptides that were eluted in the void volume of the Bio-Gel P-4 column. Six fractions containing radioactivity were obtained from Bio-Gel P-4 column (Fig. 3, Table 1).

The fraction a in pc showed slow moving components that upon acid hydrolysis and subsequent chromatography in pc, showed the presence of GlcN, a minor amount of Gal and traces of Man (Table I). Treatment of this fraction with β-*N*-acetylglucosaminidase released GlcNAc. Galactose was released from this fraction upon treatment with α-galactosidase (Table II).

The fraction b also contained slow moving radiolabelled material. The main sugar in this fraction was GlcN with traces of Man and no sugar alcohol (Table I). Treatment of this fraction with β-*N*-acetylglucosaminidase released GlcNAc. Sequential treatment with α-galactosidase and β-*N*-acetylglucosaminidase released components with mobility of Gal and GlcNAc in pc (Table II).

After acid hydrolysis, fraction c revealed components with mobility of glucosaminitol, GlcN and a small amount of Gal; a fast moving unidentified component with R_g 1.3 was also observed. This fraction on treatment with α-galactosidase (coffee beans, and a mixture of three α-galactosidases) released a small quantity of a labelled material with the mobility of Gal. Minor amount of GlcNAc was released on treatment of this fraction with β-*N*-acetylglucosaminidase (Table II).

The fraction d in pc showed the presence of a major component with the mobility of N-acetyl-glucosaminitol and minor components. After acid treatment and subsequent pc, the presence of glucosaminitol, a minor amount of GlcN and a faster moving

component could be shown (Table I). Treatment of this fraction with β-*N*-acetylglucosaminidase released a component with the mobility of GlcNAc (Table II).

The presence of N-acetyl-glucosaminitol was suggested in fraction e after acid hydrolysis. Fraction f in pc and after acid hydrolysis showed the presence of components with the mobility of N-acetyl-galactosaminitol and small amount of Gal (Fig. 3), and an unidentified fast moving component of R_g 1.6.

Hexosamines present in different fractions were treated with ninhydrin and corresponding pentoses were analyzed in pc (Table 1.).

The exogalactosylation of the 42-53 kDa glycoprotein with [^{3}H]UDP-galactose and galactosyltransferase was efficient as suggested by tritium incorporation. The transferase, previously autogalactosylated, was used in excess to overcome any inactivation caused by gel contents eluted during isolation of the glycoprotein. The exogalactosylated glycoprotein was freed from UDP-[^{3}H]galactose and other small molecules by dialysis and subsequent gel filtration on Bio-Gel P-60. The exogalactosylated glycoprotein showed a substantial increase in tritium label, and treatment with β-galactosidase resulted in a significant loss of label. The released, ^{3}H-labelled component, obtained by dialysis of the galactosidase-treated glycoprotein, was purified by gel filtration on Bio-Gel P-2, and subsequent analysis in pc showed that it had the the mobility of Gal. The substantial release of labelled Gal residues by β-galactosidase demonstrated the highly selective nature of galactosyltransferase reactivity and the presence of terminal GlcNAc residues. Galactose transfer to the glycoprotein was further confirmed by deglycosylation of the glycoprotein with alkaline borohydride. The eliminated products were analyzed by gel filtration on Bio-Gel P-4. An oligosaccharide with high tritium content was obtained from the Bio-Gel P-4 column. After acid treatment, the products of hydrolysis showed the presence of labelled components with the mobility of Gal and N-acetyl-glucosaminitol. A faster moving,

unidentified component was also observed. These results suggest the presence, in the glycoprotein, of terminal GlcNAc residues linked to serine or threonine.

To obtain further information on the nature and location of GlcN, the 42-53 kDa glycoprotein was incubated with wheat-germ agglutinin-agarose beads. The 42-53 kDa glycoprotein was retained on the wheat-germ agglutinin column, and about 80% of the radioactivity in the glycoprotein was eluted with GlcNAc.

The 42-53 kDa glycoprotein labelled in admixture with GlcN, Man and Gal were used for studies with glycosidases. Sequential digestion of the 42-53 kDa glycoprotein with β-galactosidase and β-*N*-acetylglucosaminidase removed sugars that comigrated in pc with Gal and GlcNAc. Treatment of the residual material with α–galactosidase and then with α-mannosidase removed labelled components that comigrated in pc with Gal and Man. A separate treatment of the glycoprotein with α-galactosidase and subsequently with α-mannosidase and β-*N*-acetylglucosaminidase released components that comigrated in pc with Gal, Man and GlcNAc. The endo-*O*-glycanase treatment of the glycoprotein released labelled material that migrated in pc with GalNAc. The results of the enzymic studies are summarized in Table II.

DISCUSSION

Previous studies showed the presence of carbohydrates in *P. falciparum* erythrocytic stage proteins, particularly the MSP-1 and MSP-2 proteins, that are antigenic and have been considered as vaccine candidates [10, 18]. It has also been observed that the immunogenicity of these molecules depends on the integrity of their carbohydrate moieties [18]. Recent studies have shown the presence of *O*-glycosidically linked GlcNAc residues in erythrocytic stages of malarial glycoproteins [14-16, 27]. In addition, the absence of *N*-glycosidic linkages in erythrocytic malarial glycoproteins had been reported [18] and recently confirmed [15, 27, 28]. Furthermore, it has been found that the alditols derived from the linkage region sugars of the 195 kDa (MSP-1) and 16 kDa (the C-terminal, natural proteolytic product of MSP-1) glycoproteins are similar in structure [16].

The current study was performed for the known reason that the 42-53 kDa glycoprotein, which is a form of the polymorphic MSP-2 glycoprotein derived from the *P. falciparum* strain used [24], in natural form may provide an effective blood stage vaccine, similar to the 195 kDa, MSP-1 glycoprotein [8-10]. Studies on the nature and structure of the carbohydrate components and on the contribution of the sugar residues to the immunogenicity of this protein were warranted by the vaccine potential of the two glycoproteins. Moreover, it was recently shown that GlcNAc residues, *O*-linked to serine and presented by major histocompatibility class I molecules, were important for glycopeptide recognition by cytolytic T lymphocytes [29].

The parasite cells were labelled in asynchronous mode to obtain an overall profile of sugar incorporation into proteins of different stages. There was diversity of sugar incorporation in the different proteins (Fig. 1). Ethanolamine was incorporated into the proteins with selectivity. Similar observations of variable incorporation of different sugars have been reported [13, 16]. The variation in the amounts of sugar incorporation in different glycoproteins and the number of proteins to which sugars were added may

have arisen because of the asynchronous mode of parasite culture used in this study. This mode of culture inevitably provide a broad range of labelled glycoproteins synthesized in different stages of development of the asexual blood cycle of the parasite. The metabolic labelling of *P. falciparum* cells using GlcN resulted in the incorporation of GlcNAc and GalNAc. It appears that the proportions of these two amino sugars in parasitic glycoproteins is stage dependent.

The major sugar of the carbohydrate moiety in the *P. falciparum* 42-53 kDa glycoprotein is GlcNAc mainly occurring as *O*-glycosidically linked to serine or threonine. The linkage configuration of the hydroxyamino acid bound to GlcNAc is not yet known. The removal of GlcNAc with β-*N*-acetylglucosaminidase indicates a β-linkage between GlcNAc and the hydroxyamino acid of the protein core or may suggest β-linkage between the two GlcNAc residues. The existence of the GlcNAc - hydroxyamino acid linkage has been reported in nuclear specific proteins [30], lymphocyte cell surface proteins [31], nuclear membrane proteins [26] and proteins from the human liver fluke, *Schistosoma mansoni* [32].

From the sugar incorporation studies and monosaccharide analysis of the 42-53 kDa glycoprotein, it is evident that this glycoprotein resembles the 195 kDa glycoprotein. The enzymic studies on the glycoprotein revealed the presence of terminal GlcNAc and Gal residues. The sequential enzymic digestion suggested the presence of oligosaccaride chains in addition to single sugar residues on the protein core. In a previous study on the 195 kDa glycoprotein terminal α-linked Gal was identified, a residue responsible for the antigenicity of the glycoprotein [18]. It is not possible to define, at this stage whether the glycosidases were cleaving sugars from the surface of the protein alone or from the anchor region glycans of the 42-53 kDa glycoprotein as well. Wheat-germ agglutinin binds the glycoprotein, suggesting the presence of terminal GlcNAc, an observation similar to that reported for the erythrocytic

stages of malarial glycoprotein [18]. The presence of terminal GlcNAc in animal cell glycoproteins has been described [31].

The sugar residues in *P. falciparum* glycoproteins are present on the peptide backbone as well as in the anchor region. The data of this study suggest the following as salient features of the 42-53 kDa glycoprotein: (a) GlcNAc is incorporated in the glycoprotein more abundantly than is Man or Gal; (b) GlcNAc and GalNac are linked to serine or threonine of the protein core via an *O*-glycosyl linkage; (c) GlcNAc is present as a terminal sugar; (d) α-linked Gal is present as a terminal sugar in the glycoprotein; (e) Man residues are not present in alkaline borohydride-liberated oligosaccharides or in alditol-containing products; (f) *N*-acetylglucosaminitol is the major sugar component amongst the β-eliminated sugar chains; and (g) the glycoprotein contains ethanolamine.

These observations provide clues to the basic structural features of the glycans of the 42-53 kDa glycoprotein, and it is clear from our current and earlier studies [14-16] that the majority of the GlcNAc residues are linked to the protein core through *O*-glycosyl linkage. Our data for the 195 kDa glycoprotein [18] supports the finding that GlcNAc and Gal residues provide termini for carbohydrate chains in this glycoprotein. These findings are consistent with earlier observations [18] that malarial asexual glycoproteins react with wheat-germ agglutinin, Concanavalin A, *Bandeira simplicifolia* and *Ricinus communis* lectins. It has also been observed that the malarial glycoproteins are devoid of *N*-glycosyl linkage [15, 16, 27]. Furthermore, the presence of non-reducing terminal α-linked hexoses, Man or Glc was reported by Concanavalin A binding [17]. In these circumstances it will be appropriate to consider that Man, a sugar incorporated in minor quantity, relative to GlcNAc, in malarial glycoproteins is necessarily located in the protein anchor region [33] as a terminal residue. From the results of this study it is clear that the sugar chains have the following core structure:

GlcNAcβ1---3Ser/Thr

and the chains may elongate on this core. Based on enzymic studies on the glycoprotein and the oligomers (Table II) obtained by reductive β-elimination, wheat germ agglutinin-binding and exogalactosylation, the following structures for the oligosaccharide chains are proposed:

(i) GlcNAcβ---Ser/Thr

(ii) GlcNAcβ---GlcNAcβ---Ser/Thr

(iii) Galα---GlcNAcβ---GlcNAcβ---Ser/Thr

The linkage points between the sugar residues in saccharide chains have not yet been examined, due to the minute quantity of materials available and need to be confirmed. Presence of GalNAc-ol in the β-elimination products, particularly in the fractions obtained from Bio-Gel P-4 column has been indicated.

From the incorporation of precursor sugars in MSP-2 and the the quantities of GlcNAc and Gal recovered it is clear that the core structure GlcNAcβ----Ser/Thr appeared to be dominant saccharide chain. Also, from the radioactivity recovered in the β-eliminated products it appeared that this structure is the major saccharide chain. The 42-53 kDa glycoprotein bears similarities with the already described 35-48 kDa [24] and 46-53 kDa [22] glycoproteins. It is known that these molecules belong to the surface of merozoites and are distinct from the 195 kDa glycoprotein and its naturally derived polypeptides [23]. Antibodies to these molecules inhibited merozoite invasion of erythrocytes [22, 24]. Glycoproteins which appear to be homologous to 42-53 kDa antigen of strains used by us have been described [22, 24]. These glycoproteins were present in schizonts and merozoites that were not processed or recognized by the anti-MSP-1 kDa monoclonal antibody. These characteristics of the molecule may contribute to the parasite ability to avoid host immune responses [24].

The function of glycoproteins containing *O*-linked GlcNAc moieties is not yet known. It has been postulated that these residues have structural and functional roles,

including transport of molecules, proper assembly of proteins and control of phosphorylation [26]. The glycosylation may control the phosphorylation state of the protein by blocking the site of phosphorylation or by hindering kinases, and it is now considered that *O*-GlcNAc is a regulatory modification [34-36]. In *P. falciparum* the existence of such glycoproteins has recently been recognized [14-16], and the preponderance of *O*-glycosyl linkage between sugar moiety and protein core demonstrated [15, 16, 27]. The function of these malarial glycoproteins has not been investigated, although it is known that these are antigenic and can bind to erythrocytes, in particular to glycophorins A and B [37, 38]. It is very likely that some of the merozoite surface antigens may act in biological environment as cell adhesion proteins for Sia-containing oligosaccharides or Sia-oligosaccharide-peptide complex that exist on erythrocytes.

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Table I

Carbohydrate composition of the 42-53 kDa glycoprotein and its derived products after chemical treatments

SUGARS	GLYCOPROTEIN FRACTIONS						
	Gly	a	b	c	d	e	f
N-acetylglucosamine	+	+	+	+	+	-	-
N-acetylgalactosamine	trace	-	-	-	-	-	-
N-acetylglucosaminitol	-	-	-	+	+	+	-
N-acetylgalactosaminitol	-	-	-	-	-	-	+
Mannose	+	trace	trace	-	-	-	-
Galactose	+	+	-	+	-	-	+
Arabinose*	+	+	+	+	-	-	-
Lyxose*	+	-	-	-	-	-	-

Gly: acid hydrolysis products of the 42-53 kDa glycoprotein.
a to f: fractions 1 to 6 of the β-eliminated, alkaline borohydride treatment, products of the 42-53 kDa glycoprotein obtained from Bio-Gel P-4 column
* sugars obtained after ninhydrin treatment of amino sugars

Table II

Sugars released by glycosidases from the 42-53 kDa glycoprotein (Gly) and its ß-eliminated products, fractions a to f, obtained from the Bio-Gel P-4 column.

Glycoprotein Fraction	SUGARS			
	Gal	Man	GlcNAc	GalNac
Gly[1]	+		+	
Gly[2]	+	trace		
Gly[3]	+	trace	+	
Gly[4]				+
a[5]	+			
a[6]			+	
b[5]	+			
b[6]			+	
c[5]	+			
c[6]			+	
d[6]			+	
e				
f	+			

1. Sequentially ß-galactosidase- and ß-*N*-acetylglucosaminidase-treated.
2. Sequentially α-galactosidase- and α-mannosidase-treated after treatment as in 1.
3. Sequentially α-galactosidase-, α-mannosidase and ß-*N*-acetylglucosaminidase-treated.
4. *O*-Endoglycanase treated
5. α-Galactosidase-treated.
6. ß-*N*-Acetylglucosaminidase-treated.

FIGURE LEGENDS

FIGURE 1. SDS-PAGE of *P. falciparum* glycoproteins metabolically labelled with [^{3}H]sugars and [^{3}H]ethanolamine. Lanes *A* and *B*: SDS-PAGE of parasite lysates labelled with [^{3}H]Man and [^{3}H]GlcN. Lane *C*: SDS-PAGE of immunoprecipitated lysate labelled with [^{3}H]ethanolamine.

FIGURE 2. Paper chromatographic analyses of the products of metabolically labelled *Plasmodium falciparum* 42-53 kDa glycoprotein. A: acid hydrolysis. B: Acid hydrolysis followed by ninhydrin degradation. C: β-Elimination products. D: β-Elimination followed by acid hydrolysis. The markers (arrows at the top and the bottom of the figure) are:
1 = alanine; 2 = Gal; 3 = α-aminobutyric acid; 4 = GalNAc-ol; 5 = GlcNAc-ol;
6 = GalNAc; 7 = GlcNAc; 8 = Ara; 9 = Man; 10 = lyxose. Paper chromatography was performed in solvents as described in Ref. 16. The mobility of markers 1-7 and 9 is relative to GlcNAc, that of 8 and 10 is relative to GlcN hydrochloride.

FIGURE 3. Bio-Gel P-4 chromatography of β-eliminated products of metabolically labelled 42-53 kDa glycoprotein. The deglycosylation products were eluted from the Bio-Gel P-4 (100-200 mesh) column (1.2 x 92 cm) in 50 mM pyridine-acetic acid buffer, pH 5.4. The content of each peak was analyzed by pc after acid hydrolysis.

ACKNOWLEDGMENTS

We are grateful to Dr. I. Shrivastava for providing monoclonal antibodies. AMQ, DCH and ND wish to thank the UNDP/World Bank /WHO Special Program for Research and Training in Tropical Diseases for financial support. MHQ and ND wish to thank the Pakistan National Scientific and Research Development Board for partial financial support. DCH acknowledges support from the Swiss National Science Foundation Grant # 31-39709.93.

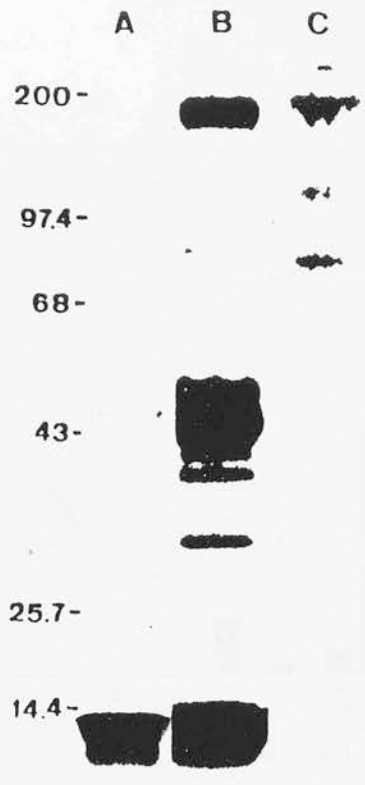

Fig. 1

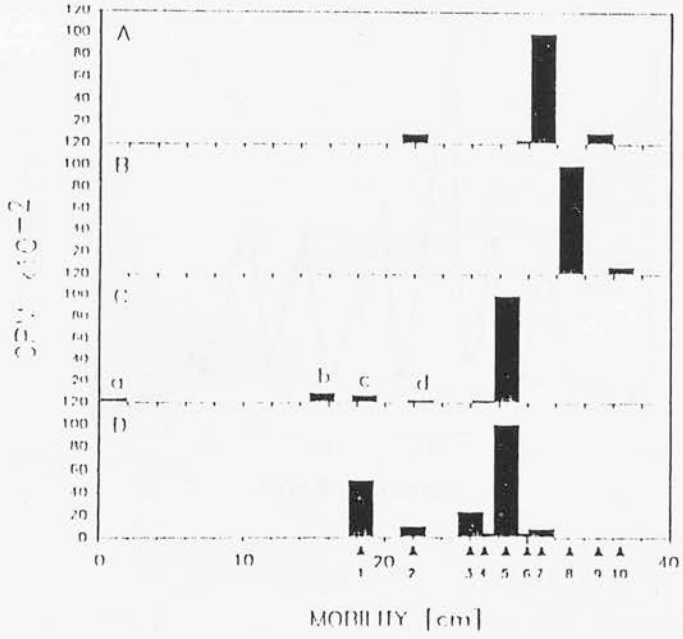

Fig. 2

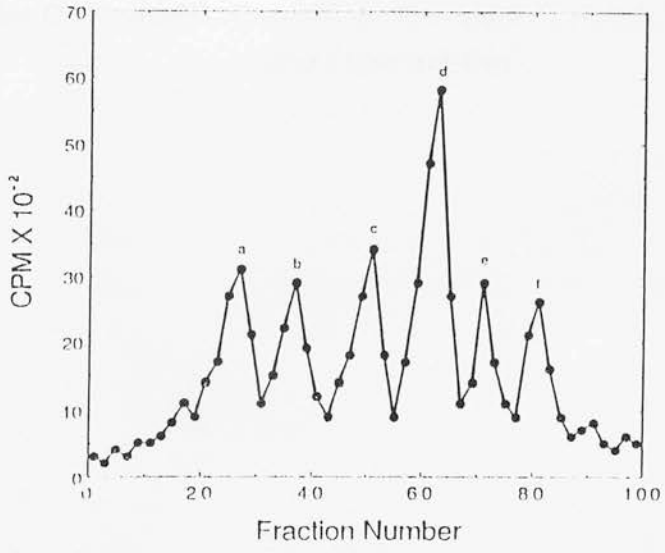
CPM X 10⁻²
70
60
50
40
30
20
10
0
a
b
c
d
e
f
0
20
40
60
80
100
Fraction Number

Fig. 3

Glycobiology of *Plasmodium falciparum*: an emerging area of research

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Is there room for carbohydrates in malaria vaccine design ?

Malaria is a dominant parasitic disease of man that causes more than 400 million clinical cases every year with an estimated mortality toll of between 1 and 3 million. Among the strategies elaborated to combat the disease, vaccines based upon asexual blood stage antigens are of special value as they mimic the natural immunity and offer long-term protection. However, it has become apparent that the protection against human malaria achieved through vaccines currently tested in high endemicity areas is still insufficient (Alonso *et al.*, 1994) compared to that induced by efficacious vaccines against other infectious agents (e.g. smallpox, poliomyelitis) and that improvements of existing malaria vaccines are necessary.

Immunization with proteins purified from asexual blood stages of the human malarial parasite *P. falciparum* confers significantly more protective immunity in experimental animals than related recombinant proteins (Etlinger *et al.*, 1991). This has indeed been observed with many pathogenic parasites and suggests that the native proteins constitute better immunogens. The immunogenicity of malarial and other proteins is dependent on their proper proteolytic processing by host phagocytes and presentation as peptides bound to the cell-surface molecules of the major histocompatibility complex (Neefjies and Momburg, 1993). Such processing and presentation is governed by the proteolytic apparatus of the host phagocytes and the specificity of their peptide-binding surface proteins, but also by the conformation and post-translational modifications of the antigen (Neefjies and Momburg, 1993).

The ***m***erozoite ***s***urface glyco***p***roteins antigens MSP1 and MSP2 are of particular value for immunization because of their known immunogenic properties, their location at the surface of merozoites and involvement in binding of the merozoite to the host erythrocyte (Gratzer and Dluzewski, 1993; Su *et al.*, 1993). Moreover, the glycosylation patterns of MSP glycoproteins has revealed interesting properties (Dayal-Drager *et al.*, 1991; Nasir-ud-Din *et al.*, 1992; Nasir-ud-Din *et al.*, 1990; Dieckmann-Schuppert *et al.*, 1992; Dieckmann-Schuppert *et al.*, 1993) that may affect both the conformation and antigenicity of the protein. This review intends to discuss why glycosylation of vaccine candidate proteins could be relevant to vaccine design and understanding of *P. falciparum* biology in its human host.

Asexual blood-stage glycoproteins of P. falciparum exhibit unusual glycosylation patterns

Protein glycosylation is now viewed as a highly dynamic and versatile set of post-translational modifications that regulate intermolecular and intercellular interactions as well as adaptive functions in cellular metabolism (Hart, 1992). Also, evidence is accumulating to indicate that glycosylation-deficient cells may survive *in vitro*, whereas the same glycosylation defects introduced in the mouse blastocyst by homologous recombination result in embryonic death at mid-gestation (Ioffe and Stanley, 1994; Metzler *et al.*, 1994).

The presence of sugar residues in human malarial parasite proteins has been shown by metabolic labeling (Fenton *et al.*, 1989), lectin binding (Ramasamy, 1987) and enzymatic digestion studies (Ramasamy and Reese, 1986; Jakobsen *et al.*, 1987). A significant finding was the observation that malarial proteins, particularly MSP1 of *P. falciparum* contain α-linked galactose residues. It was further shown that removal α-linked galactose residues significantly reduced the binding of the MSP1 to its antibody (Ramasamy and Reese, 1986; Jakobsen *et al.*, 1987). The presence of naturally-occurring antibodies directed against the α-galactosyl epitope in humans and Old World monkeys (Galili, 1993), however, does not appear to perturb the blood-stage maturation of *P. falciparum*.

The lack of *N*-glycosylation in *P. falciparum* was suggested by Ramasamy and Reese (1986) and confirmed recently (Dayal-Drager *et al.*, 1991; Nasir-ud-Din *et al.*, 1992; Nasir-ud-Din *et al.*, 1990; Dieckmann-Schuppert *et al.*, 1992; Dieckmann-Schuppert *et al.*, 1993). The major glycosylation type of immunoprecipitated MSP1 and its processed products was shown instead to be *O*-glycosyl with GlcNAc linked directly to serine or threonine (Dayal-Drager *et al.*, 1991; Nasir-ud-Din *et al.*, 1992; Nasir-ud-Din *et al.*, 1990). The presence of GlcNAc, *O*-linked to serine were also shown to be the predominant mode of hexosamine to protein linkage in *P. falciparum* (Dieckmann-Schuppert *et al.*, 1992; Dieckmann-Schuppert *et al.* 1993), as in *S. mansoni* (Nyame *et al.*, 1987). The presence and activity of an *O*-GlcNAc transferase was also demonstrated in *P. falciparum* (Dieckmann-Schuppert *et al.* 1993). The available data indicate the existence of protein-bound sugar moieties consisting of GlcNAc residue in β-configuration linked to the hydroxyl group of serine or threonine residues of the protein, and additional sugars may be linked to the first sugar. *O*-GlcNAc

modifications have been suggested to be regulatory (Hayes and Hart. 1994), in that they may (i) alter the conformation of a protein, (ii) regulate its level of serine/threonine phosphorylation, (iii) determine its involvement in multimeric complexes and (iv) modulate its half-life and proteolytic processing in host macrophages. Most *O*-GlcNAc modified-proteins described to date are cytoplasmic or nuclear (Hart, 1992). However, lymphocyte activation has been shown to be accompanied by the appearance on cell-surface proteins of *O*-GlcNAc residues accessible to galactosyltransferase (Torres and Hart, 1984). In *P. falciparum*, it appears that MSP1 and MSP2 proteins bearing *O*-linked GlcNAc or GalNAc are both intra- and extracellular. The extracellular, *O*-GlcNAc-modified MSP glycoproteins (Nasir-ud-Din *et al.*, 1992) that interact with erythrocyte plasma membranes are likely to do so in specific conformations and successful inhibition of their interactions may be preferentially brought about by antibodies that recognize such conformations and the carbohydrates involved in their maintenance.

MSP1 and MSP2 glycoproteins are anchored to the parasite membrane through glycosyl-phosphatidyl-inositol (GPI) structures (Haldar *et al.*, 1985) which have been shown to induce inflammatory cytokine production by host macrophages (Schofield and Hackett, 1993). Among the malarial GPIs, two major structures have been identified (Gerold *et al.*, 1994). One consists of an ethanolamine-phosphate-trimannose-glucosamine glycan linked to phosphatidylinositol (Gerold *et al.*, 1994), quite similar to the basic design of GPI anchors found in protozoan and metazoan glycoproteins and glycolipids (McConville and Ferguson, 1993). The other contains four instead of three mannoses in its glycan moiety and represents the GPI structure that links glycoproteins to membranes in *P. falciparum* (Gerold et al., 1994). A modification of the inositol ring, making the GPI structure resistant to a phosphatidyl-inositol specific phospholipase C (PiPLC) was observed, but not yet characterized (Gerold et al., 1994).

The 19 kDa, carboxy-terminal fragment of MSP1 bears the GPI anchor (Blackman *et al.*, 1990) and is involved in complex formation at the surface of the merozoite. Upon erythrocyte invasion, only the 19 kDa component of the complex is carried into the infected erythrocyte (Blackman *et al.*, 1990). This specific behavior may be related to the presence of a GPI-anchor on this fragment, as GPI anchors are known to transfer from one membrane to another (Rifkin and Landsberger, 1990; Ilangumaran *et al.*, 1995). The

anchor glycans and phosphatidylinositol termini also contribute to the pathogenic properties of the malaria toxins and represent antigenic targets *in vivo*. The concept of an "anti-disease" vaccine was elaborated (Playfair *et al.*, 1990) to emphasize this aspect and develop immunological tools to inactivate these toxins. Finally, a protease activity also appears to be released from merozoites by phosphatidylinositol-specific phospholipase C, suggesting the presence of a GPI-anchor on the p76 serine protease of *P.falciparum* (Braun-Breton *et al.*, 1988).

Comprehensive investigations on the structure and functions of the different carbohydrate moieties in these asexual blood stage antigens are essential to understand the pathogenic properties of these molecules and to define their contribution to protective immunity. The proteins obtained by chemical synthesis or recombinant DNA technology that lack carbohydrate moieties on the protein surface or in the anchor are therefore devoid of important structural determinants of their *in vivo* toxicity, pathogenicity and immunogenicity.

Concluding remarks

In asexual stages of the parasite, there are between 10 and 15 proteins that are glycosylated. The glycosylation pattern determined for such proteins may vary depending upon the time of the asexual cycle during which metabolic labeling is carried out. Thus, the observed glycosylation products are a complex mixture and reflect the multistage asexual cycle of the parasite. The *O*-linked GlcNAc modifications at the surface of MSP1 and MSP2 are established (Dayal-Drager *et al.*, 1991; Nasir-ud-Din *et al.*, 1992; Nasir-ud-Din *et al.*, 1990; Dieckmann-Schuppert *et al.*, 1992; Dieckmann-Schuppert *et al.*, 1993) but the site of glycosylation and complete structural information on the oligosaccharide chains or carbohydrate moieties of these glycoproteins are currently lacking.

MSP1 and MSP2 glycoproteins belong to an ever increasing category of *O*-GlcNAc glycosylated proteins. Several possible functions of these proteins have been considered and recognized (Hayer and Hart, 1994). It is likely that the addition of *O*-linked GlcNAc in MSPs may regulate the proteolytic processing and complex formation of MSP1 that occurs upon erythrocyte invasion by merozoites (Blackman et al., 1990). Furthermore, the control of serine/threonine phosphorylations expected of *O*-GlcNAc

additions is also of importance during the intraerythrocytic maturation of the parasite, and it would be interesting to evaluate the therapeutic potential of drugs that interfere with this process.

The GPI-anchor of MSP1, MSP2 and the p76 protease is also likely to confer specific properties regarding their behaviour in merozoite and erythrocyte plasma membranes during invasion. The lateral mobility of GPI-anchored glycoproteins differs from that of transmembrane glycoproteins (Zhang et al., 1991) and GPI-anchored surface molecules are also capable of transferring to other cells (Rifkin and Landsberger, 1990; Ilangumaran *et al.*, 1995). The leishmanial GPI-molecules are known, for instance, to cause profound perturbations in the intracellular membrane traffic of their macrophage host (Winter *et al.*, 1994), and it is conceivable that malarial GPI-molecules are in part responsible for the important plasma membrane remodeling that takes place during erythrocyte invasion (Simoes *et al.*, 1992).

The blood-stage merozoite surface glycoproteins are modified by numerous post-translational glycosylation modifications which endow them with a wide spectrum of biological properties at their surface (*O*-GlcNAc addition) and carboxy-termini (GPI anchors). The glycobiology of *P. falciparum* therefore deserves careful attention in considering vaccine designs and therapeutic measures to alleviate the pathological consequences of parasite multiplication in the human host.

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