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Structural Characterization of Several Galactofuranose-Containing, High-Mannose-Type Oligosaccharides Present in Glycoproteins of the Trypanosomatid *Leptomonas samueli*†

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ABSTRACT: It was reported before that cells of the trypanosomatid *Leptomonas samueli* incubated with [^{14}C]glucose synthesized dolichol-P-P-linked $Man_9GlcNAc_2$ as the main and largest derivative. It is now reported that this protozoan is deficient in dolichol-P-Glc synthesis as judged from results obtained in a cell-free assay. We have structurally characterized several endo-β-*N*-acetylglucosaminidase H sensitive oligosaccharides present in mature glycoproteins of this parasite. The compounds appeared to have the compositions $Gal_3Man_9GlcNAc_2$, $Gal_2Man_9GlcNAc_2$, $Gal_1Man_9GlcNAc_2$, $Man_9GlcNAc_2$, $Gal_1Man_8GlcNAc_2$, $Man_8GlcNAc_2$, $Gal_1Man_7GlcNAc_2$, and $Man_7GlcNAc_2$. The galactose residues were in all cases in the furanose form and linked to mannoses in nonreducing ends. In the cases of $Gal_1Man_8GlcNAc_2$ and $Gal_1Man_7GlcNAc_2$, the galactose-substituted mannose units were the nonreducing residues originally present in the oligosaccharide transferred from dolichol-P-P ($Man_9GlcNAc_2$) and not the nonreducing termini generated by demannosylation of the latter oligosaccharide. Except for $Gal_3Man_9GlcNAc_2$, the other galactosylated compounds appeared to be mixtures of several isomers.

Protein N-glycosylation in most eucaryotes is initiated by the transfer of an oligosaccharide ($Glc_3Man_9GlcNAc_2$) from a dolichol-P-P derivative to asparagine residues in incompleted or recently completed polypeptide chains. Processing of the oligosaccharide occurring as the glycoproteins migrate through the endoplasmic reticula and the Golgi apparatus leads, in mammalian cells, to the formation of high-mannose-, complex-, or hybrid-type compounds. The high-mannose compounds contain two *N*-acetylglucosamine and five to nine mannose units, and the complex oligosaccharides contain two *N*-acetylglucosamine, three mannose, and additional *N*-acetylglucosamine, galactose, sialic acid, and fucose units, whereas the hybrid compounds have both high-mannose- and complex-type characteristics (Hubbard & Ivatt, 1981; Kornfeld & Kornfeld, 1985).

Trypanosomatids are parasitic protozoa, some of which are the causative agents of endemic diseases in developing countries (Chagas's disease, sleeping sickness, visceral leishmaniasis or kala-azar disease, etc.). Protein N-glycosylation in those microorganisms involves transfer of oligosaccharides devoid of glucose and containing six, seven, or nine mannose units depending on the species (Parodi et al., 1981, 1984a; Parodi & Quesada-Allue, 1982; Previato et al., 1986). *Crithidia fasciculata* and *Crithidia harmosa* were found to transfer $Man_7GlcNAc_2$. Processing of this oligosaccharide involved removal of a single mannose unit from either one of the two nonreducing ends and addition of one galactofuranose residue to the nonreducing mannoses originally present in the transferred oligosaccharide (Figure 1). Thus, two $Gal_1Man_6GlcNAc_2$ isomers were generated (Mendelzon & Parodi, 1986). It should be noted that only galactopyranose units have been found to be present in complex-type, protein-linked oligosaccharides of mammalian cells.

We have also detected galactofuranose residues in high-mannose-type oligosaccharides present in glycoproteins of two species of trypanosomatids transferring $Man_9GlcNAc_2$ from the dolichol-P-P derivative, *Leptomonas samueli* and *Herpetomonas samuelpessoai*. Galactose (and not mannose) residues were liberated from the oligosaccharides under relatively mild acid conditions, and in addition, galactose was converted to arabinose units when the oligosaccharides were first treated with sodium periodate under conditions in which only bonds between exocyclic diols were broken and then reduced with sodium borohydride (Mendelzon et al., 1986).

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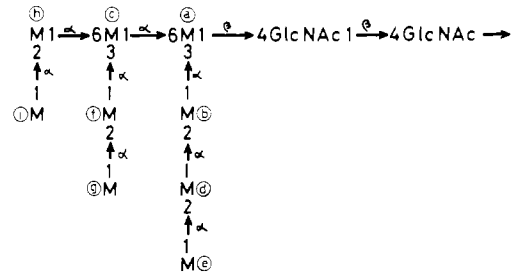

FIGURE 1: Structure of oligosaccharides. The structure depicted is that of the oligosaccharide ($Man_9GlcNAc_2$) transferred to protein in *L. samueli*. The lettering used for the identification of the individual mannose residues (a, b, c, etc.) follows the order of addition of the monosaccharides in the in vivo assembly of $Man_9GlcNAc_2$-P-P-dolichol (Chapman et al., 1979). The compound transferred to protein in *C. fasciculata* is devoid of residues h and i, and one of the protein-linked $Gal_1Man_6GlcNAc_2$ isomers lacks also residue g and has a galactofuranose linked to mannose e whereas the other isomer is devoid of residue e and has a galactofuranose linked to residue g (Mendelzon & Parodi, 1986; Parodi et al., 1981). Proposed structures: compound I ($Gal_3Man_9GlcNAc_2$), one galactofuranose linked to C_2 of residues e, g, and i; compound II ($Gal_2Man_9GlcNAc_2$), one galactofuranose linked to residues e and g, residues e and i, or residues g and i; compound III ($Gal_1Man_9GlcNAc_2$), one galactofuranose linked to residue e, g, or i; compound IV ($Man_9GlcNAc_2$), the depicted structure; compound V ($Gal_1Man_8GlcNAc_2$), the $Man_8GlcNAc_2$ isomer devoid of residue g and having one galactofuranose linked to residue e or i or, alternatively, the $Man_8GlcNAc_2$ isomer devoid of residue e and having one galactofuranose linked to residue g or i; compound VI ($Man_8GlcNAc_2$), devoid of residue e or g; compound VII ($Gal_1Man_7GlcNAc_2$), the $Man_7GlcNAc_2$ isomer devoid of residues g and i and having a galactofuranose residue linked to residue e, the isomer devoid of residues e and g and having a galactofuranose linked to residue i, or, finally, the isomer devoid of residues e and i and having one galactofuranose linked to residue g; compound VII ($Man_7GlcNAc_2$), devoid of residues g and i, of residues e and g, or of residues e and i. M stands for mannose.

In this paper we are reporting the structural characterization of several of those novel oligosaccharides occurring in *L. samueli*.

Experimental Procedures

Materials. Rat liver dolichol-P, grade III, *Streptomyces griseus* protease, type XIV, Jack bean α-mannosidase, and endo-β-*N*-acetylglucosaminidase H were from Sigma. [^{14}C]Glucose (325 Ci/mol) was from American Radiolabeled Chemicals. UDP-[^{14}C]Glc (285 Ci/mol) and GDP-[^{14}C]Man (216.5 Ci/mol) were prepared according to Wright and Robbins (1965) with slight modifications.

Standards. The source of standards employed was as follows: [^{14}C]$Man_{5-9}GlcNAc$, glycoproteins from hen oviduct incubated with [^{14}C]glucose (Parodi et al., 1981); [*glucose*-^{14}C]$Glc_1Man_{7-9}GlcNAc$, glycoproteins from rat liver microsomes incubated with UDP-[^{14}C]Glc (Parodi et al., 1984b); [*glucose*-^{14}C]$Glc_{1-3}Man_9GlcNAc$, dolichol-P-P derivatives from rat liver microsomes incubated with UDP-[^{14}C]Glc (Parodi et al., 1973); [^{14}C]Man_2, [^{14}C]Man_3, [^{14}C]-$Man_3GlcNAc$, and [^{14}C]$Man_4GlcNAc$, acetolysis of [^{14}C]-$Man_{5-9}GlcNAc$; [*glucose*-^{14}C]$Glc_1Man_4GlcNAc$ and [*glucose*-^{14}C]$Glc_1Man_5GlcNAc$, α-mannosidase treatment of [*glucose*-^{14}C]$Glc_1Man_{7-9}GlcNAc$; [^{14}C]ManGlcNAc, α-mannosidase treatment of [^{14}C]$Man_{5-9}GlcNAc$. 2,3,4,6-Tetramethylmannose, 3,4,6-trimethylmannose, 2,4,6-trimethylmannose, and 2,4-dimethylmannose were obtained by permethylation of [^{14}C]$Man_9GlcNAc$ and of ^{14}C-labeled mannan from a wild-type strain of *Saccharomyces cerevisiae* (Parodi, 1981). Permethylation of free galactose followed by strong acid hydrolysis yielded two substances with different migration on thin-layer chromatography in solvent B. The fast migrating compound was identified as 2,3,5,6-tetramethylgalactose as it was the only galactosyl derivative obtained by permethylation of [^{14}C]$Gal_1Man_6GlcNAc_2$ from *C. fasciculata*, a substance in which the galactose is in the furanose form (Mendelzon & Parodi, 1986). The slower compound was identified as 2,3,4,6-tetramethylgalactose as it migrated the same as the tetramethyl derivative obtained upon permethylation of Galα(1→6)Galα(1→6)Glc, a compound produced by mild acid hydrolysis of stachyose in which both galactoses are in the pyranose form. It is known that a high proportion of the furanose form is present in basic anhydrous solutions of galactose, a condition employed in permethylation (Campbell & Bentley, 1972).

Isolation of Labeled Protein-Linked Oligosaccharides. L. samueli cells were grown in the medium described by Bacchi et al. (1974). They were harvested in the late exponential phase, and 0.5–1 g of them was labeled with 300–500 μCi of [^{14}C]glucose as described before for 60 min at room temperature, after which the medium was diluted 10-fold with fresh growth medium containing 10 mM glucose (Parodi et al., 1981). Incubation was stopped after an additional 60-min period. Endo-β-*N*-acetylglucosaminidase H sensitive oligosaccharides were isolated as described previously (Parodi et al., 1981). Briefly, the method involved incubation of lipid-free whole-cell proteins with a protease, isolation of glycopeptides, and treatment of the latter with endo-β-*N*-acetylglucosaminidase H.

Assay for the Synthesis of Dolichol-P Derivatives. Cells were harvested in the exponential phase of growth by centrifugation at 1000*g* for 10 min, washed twice with solution A [0.25 M sucrose, 5 mM sodium ethylenediaminetetraacetate (Na-EDTA), pH 7.2, and 5 mM 2-mercaptoethanol], and ground in a mortar with glass beads until most of the cells were broken. The homogenate was diluted with solution A and centrifuged at 1000*g* for 5 min. The supernatant was then centrifuged at 17300*g* for 15 min. The second supernatant thus obtained was centrifuged for 60 min at 100000*g*. The pellet was resuspended in solution A with a glass–Teflon potter at a protein concentration of 30–60 mg/mL. Reaction mixtures for dolichol-P-[^{14}C]Man and dolichol-P-[^{14}C]Glc synthesis contained, in a final volume of 50 μL, 0.6% Triton X-100, 50 mM tris(hydroxymethyl)aminomethane (Tris)–maleate buffer, pH 7.6, 10 mM $MgCl_2$, 12 mM 5′-AMP, 0–6 μg of dolichol-P, 0.4–0.6 mg of microsomal proteins, and 10 μM GDP-[^{14}C]Man or 9 μM UDP-[^{14}C]Glc. Incubations lasted for 5 min at 28 °C, and reactions were stopped by the addition of 0.5 mL of methanol, 0.75 mL of chloroform, and 0.2 mL of water. The lower phases were washed 3 times with 0.3 mL of chloroform/methanol/water (1:16:16) and counted.

Methods. Treatment with α-mannosidase, acetolysis, and strong acid hydrolysis were as described previously (Engel & Parodi, 1985). Mild acid hydrolysis was performed in HCl, pH 2.2, at 100 °C for 150 min. Permethylation was as described by Hakomori (1964) and Li et al. (1978). Paper chromatography and electrophoresis were performed on Schleicher & Schuell 2043-a papers, and thin-layer chromatography was performed on silica gel 60 Merck glass plates.

The following solvents were employed for paper or thin-layer chromatography: (A) 1-propanol/nitromethane/water (5:2:4); (B) benzene/acetone/water/ammonium hydroxide (50:200:1:1.35); (C) 1-butanol/pyridine/water (4:3:4); (D) 1-butanol/pyridine/water (10:3:3); (E) 1-butanol/pyridine/water (6:4:3). Electrophoresis was performed in 0.1 M sodium molybdate buffer, pH 5.0, for 90 min at 17 V/cm.

Results

Absence of UDP-Glc:Dolichol-P Glucosyltransferase. It

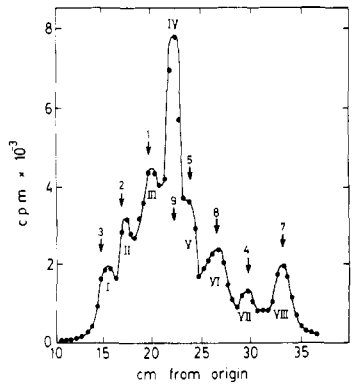

FIGURE 2: Pattern of protein-linked oligosaccharides. Endo-β-*N*-acetylglucosaminidase H sensitive oligosaccharides from cells labeled for 60 min with [^{14}C]glucose and chased for the same period with the unlabeled monosaccharide were isolated as described under Experimental Procedures and run on paper chromatography in solvent A. Standards: (1) $Glc_1Man_9GlcNAc$; (2) $Glc_2Man_9GlcNAc$; (3) $Glc_3Man_9GlcNAc$; (4) $Glc_1Man_7GlcNAc$; (5) $Glc_1Man_8GlcNAc$; (7) $Man_7GlcNAc$; (8) $Man_8GlcNAc$; (9) $Man_9GlcNAc$. I–VIII correspond to different compounds as denominated here.

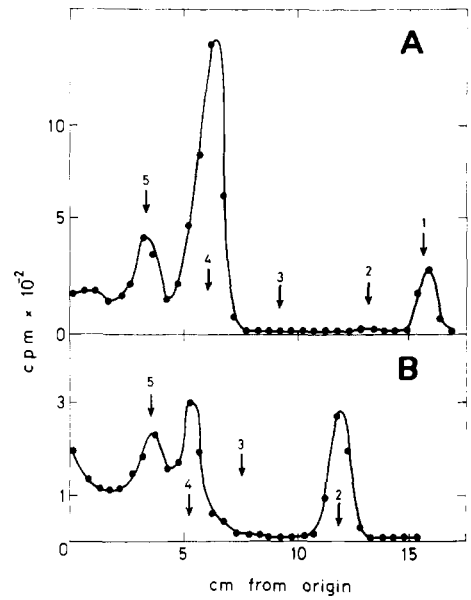

FIGURE 3: Permethylation of oligosaccharides. Compound I (A) or IV (B) was subjected to permethylation followed by strong acid hydrolysis and thin-layer chromatography in solvent B. Standards: (1) 2,3,5,6-tetramethylgalactose; (2) 2,3,4,6-tetramethylmannose; (3) 2,4,6-trimethylmannose; (4) 3,4,6-trimethylmannose; (5) 2,4-dimethylmannose.

was reported previously that *L. samueli* cells incubated with [^{14}C]glucose formed $Man_9GlcNAc_2$-P-P-dolichol as the main and largest derivative (Previato et al., 1986). The structure of the oligosaccharide moiety appeared to be that depicted in Figure 1. We have now characterized the enzymatic defect responsible for the synthesis of the unglucosylated compound. Incubation of rat liver dolichol-P, parasite membranes, and GDP-[^{14}C]Man or UDP-[^{14}C]Glc led to the synthesis of dolichol-P-[^{14}C]Man but not to that of dolichol-P-[^{14}C]Glc. Results were similar to those already obtained for *C. fasciculata*, *Blastocrithidia culicis*, *Leishmania enriettii*, and *Trypanosoma cruzi* membranes (de la Canal & Parodi, 1987). It was determined that failure to form dolichol-P-[^{14}C]Glc was not due to the presence, in the parasite membranes, of an inhibitor of the synthesis or of an enzyme responsible for degradation of dolichol-P-[^{14}C]Glc, nor to a specific and immediate breakdown of UDP-[^{14}C]Glc. It was concluded, therefore, that *L. samueli*, the same as the other parasites, is devoid of the glucosyltransferase involved in dolichol-P-Glc formation. The latter is the known glucosyl donor in the assembly of dolichol-P-P derivatives in mammalian, yeast, and plant cells (Hubbard & Ivatt, 1981; Kornfeld & Kornfeld, 1985). It was previously reported that intact *L. samueli* cells incubated with [^{14}C]glucose synthesized dolichol-P-[^{14}C]Man and not dolichol-P-[^{14}C]Glc (Previato et al., 1986).

Pattern of Protein-Linked Oligosaccharides Present in Mature Glycoproteins. The pattern of endo-β-*N*-acetylglucosaminidase H sensitive oligosaccharides obtained from whole-cell mature glycoproteins is depicted in Figure 2. Substances migrating the same as $Man_9GlcNAc_2$, $Man_8GlcNAc_2$, and $Man_7GlcNAc$ standards (peaks IV, VI, and VIII, respectively) were present as well as five more compounds migrating the same or close to the standards $Glc_3Man_9GlcNAc$, $Glc_2Man_9GlcNAc$, $Glc_1Man_9GlcNAc$, $Glc_1Man_8GlcNAc$, and $Glc_1Man_7GlcNAc$ (peaks I, II, III, V, and VII, respectively). Each of the substances was eluted and rechromatographed at least once in order to eliminate contamination by adjacent compounds.

Structure of Compounds IV, VI, and VIII. These substances had the same composition as the respective standards with which they comigrated as upon an α-mannosidase treatment they were degraded to mannose and ManGlcNAc. This was visualized on paper chromatography of the degradation products in solvent C (not shown but results were similar to those depicted in Figure 6E). Acetolysis under conditions almost specific for breaking α(1→6) bonds between mannose residues produced, in the case of compound IV, fragments migrating on paper chromatography in solvent C as mannobiose, mannotriose, and $Man_4GlcNAc$ standards [results were similar to those shown in Figure 4A in Previato et al. (1986)]. Permethylation of substance IV followed by strong acid hydrolysis and thin-layer chromatography revealed the presence of substances migrating as 2,3,4,6-tetramethylmannose, 3,4,6-trimethylmannose, and 2,4-dimethylmannose standards (Figure 3B). These results indicated that the structure of substance IV was that depicted in Figure 1, identical with that of the dolichol-P-P-linked $Man_9GlcNAc_2$ formed by the parasite.

Fragments obtained upon acetolysis of compound VI were identified as $Man_4GlcNAc$, $Man_3GlcNAc$, mannotriose, and mannobiose (Figure 4A). The small amount of mannose produced may be due to unspecific breakdown of the oligosaccharide. Reduction of mannobiose with $NaBH_4$ followed by paper electrophoresis in sodium molybdate buffer, pH 5.0, revealed that the disaccharide was composed of almost equal amounts of mannoseα(1→2)mannose and mannoseα(1→3)-mannose as nearly half of the label migrated to the anode and the rest behaved as a neutral substance (Parodi et al., 1983a). These results indicated that the main demannosylation pathway of $Man_9GlcNAc_2$ involved removal of residue g (Figure 1) and the minor one removal of residue e.

The same degradation products as those of substance VI (but in different proportions) were produced upon acetolysis of substance VIII (Figure 4B), but in this case the free mannose obtained cannot be accounted for by unspecific breakdown. Reduction and paper electrophoresis of the disaccharide showed that the latter was mainly composed of mannoseα(1→3)mannose as most (but not all) of the label did not migrate. This indicated that $Man_7GlcNAc$ isomers devoid of residues g and i (Figure 1) or of residues e and g or residues e and i were present.

Concerning oligosaccharides containing only mannose and *N*-acetylglucosamine residues, processing of protein-linked

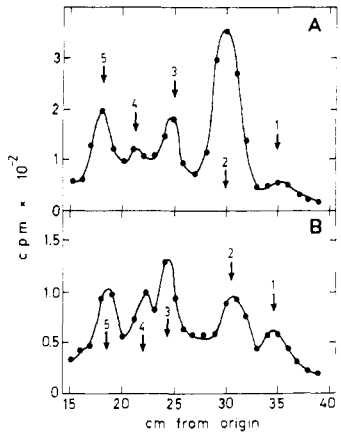

FIGURE 4: Studies on compounds VI and VIII. Compounds VI (A) and VIII (B) were subjected to acetolysis followed by paper chromatography in solvent C. Standards: (1) mannose; (2) Man_2; (3) Man_3; (4) $Man_3GlcNAc$; (5) $Man_4GlcNAc$.

$Man_9GlcNAc_2$ appeared to be, therefore, essentially similar to that described previously for *T. cruzi* epimastigotes (Doyle et al., 1986).

Structure of Compounds I and II. As shown in Figure 2, compound I migrated slightly ahead of a $Glc_3Man_9GlcNAc$ standard. As reported previously, high-mannose-type oligosaccharides containing galactofuranose residues migrate ahead of similar oligosaccharides but with glucopyranose or galactopyranose residues (Mendelzon & Parodi, 1986). Exhaustive treatment of compound I with Jack bean α-mannosidase did not modify the migration of the oligosaccharide on paper chromatography (Figure 5A,B), nor were labeled mannose residues liberated upon such treatment. Mild acid hydrolysis under conditions required for the hydrolysis of hexofuranoses but not of hexopyranoses (150 min at 100 °C in HCl, pH 2.2) resulted in the liberation of galactoses and the concomitant production of an oligosaccharide migrating as a $Man_9GlcNAc$ standard (Figure 5C,D). That the degradation product was actually $Man_9GlcNAc$ was demonstrated by the fact that it was degraded to mannose and ManGlcNAc upon α-mannosidase treatment. The same oligosaccharide ($Man_9GlcNAc$) was also produced if, previous to the mild acid treatment, compound I was treated with α-mannosidase, thus confirming that the latter oligosaccharide was completely resistant to the enzyme. Permethylation of compound I followed by strong acid hydrolysis and thin-layer chromatography revealed the presence of substances migrating as 2,3,5,6-tetramethylgalactose, 3,4,6-trimethylmannose, and 2,4-dimethylmannose (Figure 3A). In other runs it was found that 2,3,4,6-tetramethylgalactose migrated behind 2,3,5,6-tetramethylgalactose (migration ratio 0.78). The result obtained with compound I confirms, therefore, the presence of galactofuranose residues. Comparison of the patterns yielded by compounds I and IV (Figure 3A,B) showed an increase in 3,4,6-trimethylmannose and the disappearance of 2,3,4,6-tetramethylmannose in the former compound. These data indicate that compound I has the composition $Gal_3Man_9GlcNAc$ with one galactofuranose residue linked to each of the nonreducing ends of $Man_9GlcNAc_2$ (residues e, g, and i, Figure 1), most probably to C_2.

On the other hand, compound II migrated slightly ahead of a $Glc_2Man_9GlcNAc$ standard. The same as compound I, it produced $Man_9GlcNAc$ upon a mild acid treatment, simultaneously releasing galactose units. Treatment of compound II with α-mannosidase produced free mannoses and three oligosaccharides migrating ahead of compound II on paper chromatography (Figure 5E). Mild acid treatment of the mixture of the three α-mannosidase degradation products yielded substances that migrated as $Man_8GlcNAc$, $Man_7GlcNAc$, and $Man_6GlcNAc$ standards and that were degraded to mannose and ManGlcNAc upon incubation with α-mannosidase (Figure 5F). Treatment of each of the individual α-mannosidase degradation products shown in Figure 5E with mild acid showed that $Man_8GlcNAc$, $Man_7GlcNAc$, and $Man_6GlcNAc$ in Figure 5F had been respectively produced by the slow, medium, and fast migrating peaks in Figure 5E. The most likely interpretation of these results is that compound II represents $Man_9GlcNAc$ having one galactofuranose residue in two of the nonreducing ends (e and g, g and i, or e and i, Figure 1). It is known that non-mannose residues in high-mannose-type oligosaccharides hinder complete removal of mannoses theoretically susceptible to α-mannosidase degradation. For instance, a single glucose residue linked to residue e in $Glc_1Man_9GlcNAc$ strongly hampers α-mannosidase digestion of residue c, and $Glc_1Man_5GlcNAc$ and $Glc_1Man_4GlcNAc$ are obtained as reaction products (Parodi & Cazzulo, 1982). Therefore, it is expected that if the structure proposed for compound II were correct, galactofuranose residues linked to residues e and g or residues e and i in $Man_9GlcNAc$ would result in the production, upon α-mannosidase and mild acid treatments, of $Man_8GlcNAc$ and $Man_7GlcNAc$. On the other hand, if the galactofuranoses were in residues g and i, both treatments should generate $Man_7GlcNAc$ and $Man_6GlcNAc$. This interpretation of results obtained upon analysis of compound II is strengthened by the fact that compound I appeared to have one galactofuranose residue in each of the three nonreducing ends of $Man_9GlcNAc$ and, as will be seen below, compound III has one galactofuranose in either one of such nonreducing ends.

Structures of Compounds III, V, and VII. These compounds will be dealt with together as they appeared to share certain structural features. The three title compounds migrated the same as or close to $Glc_1Man_9GlcNAc$, $Glc_1Man_8GlcNAc$, and $Glc_1Man_7GlcNAc$ standards, respectively. Treatment of compounds III, V, and VII with mild acid produced respectively $Man_9GlcNAc$, $Man_8GlcNAc$, and $Man_7GlcNAc$, with the simultaneous release of galactose units. Differently to compounds I and II, however, degradation of the title compounds with acid was not complete as part of the label still migrated as the original substances after the treatment (see below). Treatment of compounds III, V, and VII with α-mannosidase resulted in the production of substances migrating the same as or only slightly ahead of $Glc_1Man_4GlcNAc$ and $Glc_1Man_5GlcNAc$ standards. The same result was obtained before upon treatment of $Gal_1Man_6GlcNAc$ and $Glc_1Man_{7-9}GlcNAc$, and as mentioned above, the larger substances were incomplete degradation products (Mendelzon & Parodi, 1986; Parodi & Cazzulo, 1982). The reaction products yielded by compound VII are shown in Figure 6A. Mild acid treatment of substances migrating as $Glc_1Man_4GlcNAc$ produced by α-mannosidase degradation of compounds III, V, and VIII yielded, in all cases, galactose units, together with substances migrating as a $Man_4GlcNAc$ standard. Degradation with mild acid of the three compounds was not complete, however, as part of the label still migrated as $Glc_1Man_4GlcNAc$ after the treatment. Results obtained upon α-mannosidase treatment of compounds III and VII and further mild acid hydrolysis of the respective substances migrating as a $Glc_1Man_4GlcNAc$ standard are shown in Figure 6B,C. Compounds that migrated as $Glc_1Man_4GlcNAc$ after α-mannosidase and mild acid degradations of substances III, V, and VII were composed of

labeled glucose and mannose units as shown by strong acid hydrolysis (1 N HCl at 100 °C for 4 h) followed by paper chromatography (the result obtained with compound VII is shown in Figure 6D). On the other hand, substances migrating as $Man_4GlcNAc$ after α-mannosidase and mild acid treatments of compounds III, V, and VII were degraded to mannose and ManGlcNAc on further treatment with the enzyme, thus showing that they were actually $Man_4GlcNAc$ (Figure 6E). Acetolysis of $Man_4GlcNAc$, generated as mentioned above from compounds III, V, and VII, produced in all cases substances migrating as $Man_4GlcNAc$, manotriose, mannobiose, mannose, and/or ManGlcNAc (Figure 6F; note that in the solvent employed in this experiment ManGlcNAc migrates together with mannose and not between mannose and mannobiose as in the solvent employed in experiment in Figure 6E).

These results indicate that two types of compounds, containing either glucose or galactose residues, were actually present in each of the peaks designated as III, V, and VII. The transient presence of asparagine-linked, $Glc_1Man_9GlcNAc_2$, $Glc_1Man_8GlcNAc_2$, and $Glc_1Man_7GlcNAc_2$ has been previously detected in *T. cruzi* and in other parasites transferring $Man_9GlcNAc_2$ to protein as well as in mammalian, plant, and yeast cells (Lederkremer & Parodi, 1986; Mendelzon et al., 1986; Parodi et al., 1983a,b, 1984b). Except for the latter cells, evidence was obtained indicating that the glucosylated compounds were formed by glucosylation of protein-linked oligosaccharides, UDP-Glc being the monosaccharide donor. Rather unusually, however, in *L. samueli* the glucosylated compounds were still present after a 60-min chase of cells with unlabeled glucose, whereas in other systems they appeared to have much shorter half-lives.

Concerning the galactofuranose-containing compounds, data presented indicate that $Gal_1Man_9GlcNAc$, $Gal_1Man_8GlcNAc$, and $Gal_1Man_7GlcNAc$ were present in peaks III, V, and VII, respectively. In all cases galactofuranoses were linked to nonreducing mannoses and in the case of $Gal_1Man_8GlcNAc$ and $Gal_1Man_7GlcNAc$ to mannoses already in such positions in the oligosaccharide transferred from the dolichol-P-P derivative and not in nonreducing termini generated by demanosylation of $Man_9GlcNAc_2$. Otherwise, substances smaller than $Gal_1Man_4GlcNAc$ or $Man_4GlcNAc$ should have been produced upon α-mannosidase treatment or the same plus mild acid treatments, respectively.

In addition, the products of acetolysis of $Man_4GlcNAc$ indicated that, concerning $Gal_1Man_9GlcNAc$, galactofuranoses were linked to either residue e, g, or i of $Man_9GlcNAc$ (Figure 1). In the first case $Man_4GlcNAc$ generated by succesive treatments with α-mannosidase and mild acid would produce $Man_4GlcNAc$ upon acetolysis. In the second case (substitution in residue g) $Man_4GlcNAc$ would generate mannotriose and ManGlcNAc on acetolysis, and in the third case (substitution in position i) acetolysis would produce mannobiose, mannose, and ManGlcNAc.

Concerning $Gal_1Man_8GlcNAc$, results are compatible with galactofuranose residues being linked to mannose e or i of the main $Man_8GlcNAc$ isomer (devoid of residue g) or to mannose g or i of the minor isomer (devoid of residue e). Concerning $Gal_1Man_7GlcNAc$, data available indicate that galactofuranose residues are linked either to residue e of the $Man_7GlcNAc$ isomer devoid of mannoses g and i or to residue g or i of the isomers devoid of mannoses e and i or of mannoses e and g, respectively.

As in the case of $Gal_1Man_9GlcNAc$, the structures proposed for $Gal_1Man_8GlcNAc$ and $Gal_1Man_7GlcNAc$ explain the production of $Gal_1Man_4GlcNAc$ or $Man_4GlcNAc$ upon α-mannosidase treatment or the same plus mild acid treatments and the products generated by acetolysis of the $Man_4GlcNAc$ thus obtained.

Discussion

Results here presented indicate that galactofuranose-containing, high-mannose-type oligosaccharides with compositions $Gal_{1-3}Man_9GlcNAc_2$ and $Gal_1Man_{7,8}GlcNAc_2$ occur in glycoproteins of the parasitic protozoan *L. samueli*. In all cases the galactose units appeared to be linked to mannoses in nonreducing ends. Demannosylation of the oligosaccharide transferred from the dolichol-P-P derivative ($Man_9GlcNAc_2$) generated nonreducing ends different from those present in the latter compound. Nevertheless, galactofuranose residues in $Gal_1Man_8GlcNAc_2$ and $Gal_1Man_7GlcNAc_2$ were linked to nonreducing termini already present in $Man_9GlcNAc_2$. The same observation concerning the substitution of mannose residues was made before upon analysis of both $Gal_1Man_6GlcNAc_2$ isomers isolated from *C. fasciculata*, a parasite in which $Man_7GlcNAc_2$ is transferred to proteins (Mendelzon & Parodi, 1986).

Galactose-containing, N-linked oligosaccharides in *L. samueli* and *C. fasciculata* appeared to be markedly different from compounds having the same residue present in mammalian glycoproteins as (a) in the protozoan oligosaccharides the galactoses appeared to be in the furanose and not in the pyranose form as in the mammalian compounds and (b) in the latter the galactose residues are in the so-called complex-type oligosaccharides having the general composition $(NeuAc_1Gal_1GlcNAc_1)_{1-5}Man_3GlcNAc_2$ whereas in protozoa the galactose residues are in high-mannose-type oligosaccharides ($Gal_{1-3}Man_{6-9}GlcNAc_2$). Both oligosaccharide types are formed by processing of the oligosaccharides transferred to proteins, $Glc_3Man_9GlcNAc_2$ in mammals and $Man_{7,9}GlcNAc_2$ in the above-mentioned protozoa. It is highly probable that the donor of the galactofuranose residues is UDP-galactofuranose. This sugar nucleotide has been detected in the fungus *Penicillium charlesii*, in which it is involved in the synthesis of a galactomannan (Garcia-Trejo et al., 1971).

It would be expected for the protozoan oligosaccharides to be highly antigenic as no such structures are present in mammalian cells and, in addition, high-mannose-type, N-linked oligosaccharides in intact parasites are readily accessible to the surrounding medium as judged by reaction with appropriate lectins (Mutharia & Pearson, 1987). The antigenicity of these galactofuranose-containing oligosaccharides is yet unknown, however, as it has not been tested experimentally and, in addition, these compounds have been found so far only in species infecting insects and not mammals (*L. samueli*, *H. samuelpessoai*, *C. fasciculata*, and *C. harmosa*, monogenetic species) (Mendelzon & Parodi, 1986; Mendelzon et al., 1986). On the other hand, species infecting both insects and mammals (*T. cruzi*, *Trypanosoma conorhini*, *Trypanosoma dionisii*, and *Leishmania mexicana*, digenetic species) were found not to have galactofuranose units in high-mannose-type oligosaccharides (Parodi et al., 1983a, 1984a; Mendelzon et al., 1986). It is worth mentioning that galactofuranose residues have been detected in digenetic species such as *T. cruzi* and *Trypanosoma brucei*. In these microorganisms, however, galactofuranoses appeared to be present not in high-mannose-type, N-linked oligosaccharides but in compounds of still ill-characterized structure, a lipopeptidephosphoglycan in the former parasite and in the glycan part of the anchoring moiety of the variant surface glycoprotein in the latter (De Lederkremer et al., 1985; Ferguson et al., 1985). It is highly

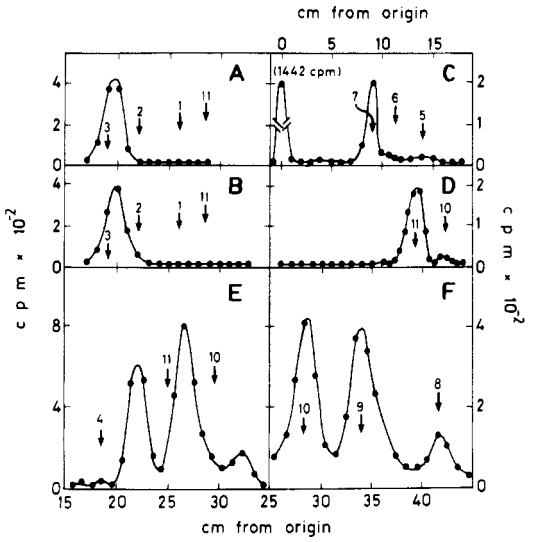

FIGURE 5: Studies on compounds I and II. (A and B) Compound I was run before (A) and after (B) being treated with α-mannosidase. (C) Compound I was subjected to mild acid hydrolysis. (D) The substance in the origin in (C) was eluted and rerun on paper chromatography. (E and F) Compound II was treated with α-mannosidase and run on paper chromatography before (E) and after (F) being subjected to mild acid hydrolysis. Paper chromatography was performed in solvent A (A, B, and D–F) or in solvent D (C). Standards: (1) $Glc_1Man_9GlcNAc$; (2) $Glc_2Man_9GlcNAc$; (3) $Glc_3Man_9GlcNAc$; (4) compound II; (5) mannose; (6) glucose; (7) galactose; (8) $Man_6GlcNAc$; (9) $Man_7GlcNAc$; (10) $Man_8GlcNAc$; (11) $Man_9GlcNAc$.

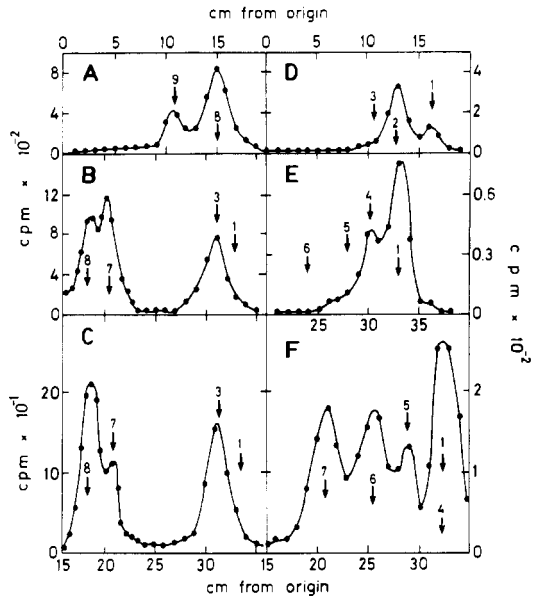

FIGURE 6: Studies on compounds III, V, and VII. (A) Compound VII was treated with α-mannosidase. (B and C) The substance migrating as standard 8 in panel A (C) or the similar substance obtained from α-mannosidase-treated compound III (B) was treated with mild acid. (D) The substance migrating as standard 8 in panel C was subjected to strong acid hydrolysis. (E) The substance migrating as standard 7 in panel B was treated with α-mannosidase. (F) The substance migrating as standard 7 in panel B was subjected to acetolysis. Paper chromatography was performed in solvent A (A–C and F), in solvent E (D), or in solvent C (E). Standards: (1) mannose; (2) glucose; (3) galactose; (4) ManGlcNAc; (5) Man_2; (6) Man_3; (7) $Man_4GlcNAc$; (8) $Glc_1Man_4GlcNAc$; (9) $Glc_1Man_5GlcNAc$.

probable that the *T. cruzi* compound is also part of an anchoring system for membrane proteins as it shares several structural features with the glycan of *T. brucei*. The latter compound is part of the so-called cross-reacting determinant, present in all the different variant surface glycoproteins of *T. brucei*. The cross-reacting determinant is antigenic only when isolated and not in the intact parasite, as it is probably buried in the external surface. It is not known, however, if the galactofuranoses are per se antigenic determinants.

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Registry No. $Man_9GlcNAc_2$, 71246-55-4; galactose, 59-23-4; UDP-Glc:dolichol-P glucosyltransferase, 71061-42-2.

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Trehalose-Containing Lipooligosaccharide Antigens of *Mycobacterium* sp.: Presence of a Mono-*O*-methyltri-*O*-acyltrehalose "Core"†

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ABSTRACT: We have described the surface antigens of *Mycobacterium kansasii* as trehalose-containing lipooligosaccharides (LOS) which at the nonreducing "epitope" end bear a unique amino sugar containing diglycosyl unit, whereas the putative reducing end consists of an acylated α,α-trehalose-containing tetraglucosyl "core" [Hunter, S. W., Jardine, I., Yanagihara, D. L., & Brennan, P. J. (1985) *Biochemistry 24*, 2798–2805]. The presence of a new variation on this core, in *Mycobacterium szulgai*, is now reported, →3)β-D-Glc*p*-(1→6)α-D-Glc*p*(1↔1)3,4,6-tri-*O*-acyl-2-*O*-Me-α-D-Glc*p*, representing the first example of an *O*-methyltrehalose unit in nature. The simplest of the LOS class of glycolipids in *M. szulgai* was defined as α-L-2-*O*-Me-Fuc*p*(1→3)α-L-Rha*p*(1→3)α-L-Rha*p*(1→3)β-D-Glc*p*(1→6)α-D-Glc*p*(1↔1)3,4,6-tri-*O*-acyl-2-*O*-Me-α-D-Glc*p*. Further glycosylation of this nonantigen, by an incompletely defined 6-deoxyhexosyl residue, confers specific antigenicity on the organism. Thus, these extraordinary structures, in a manner analogous to the better known lipopolysaccharides from rough variants of *Enterobactericiae*, are highly amphipathic and display variability not only in the immunogenic, distal region but also in the "invariant" lipophilic core. The contribution of these glycolipids to the hydrophobic barrier, the pseudo outer membrane of mycobacteria, is discussed.

Serological differentiation of most individual species and subspecies within the *Mycobacterium* genus is based on one of several classes of glycolipid antigens, vastly different in elemental structure but each endowed with novel, sometimes exotic, non-reducing-end epitopes (Brennan, 1984). The glycopeptidolipids, notably those from members of the *Mycobacterium avium* complex isolated from patients with acquired immunodeficiency syndrome, have been examined in considerable detail; the structures of the oligosaccharide haptens from the predominant 10 serovariants of the 31-member *M. avium* serocomplex have now been fully elucidated (McNeil et al., 1987; Chatterjee et al., 1987). Another group of surface antigens, the so-called lipooligosaccharides of mycobacteria (Hunter et al., 1983), are examples of a rare principle in carbohydrate chemistry, the existence of glycosidically linked trehalose. Although clearly ubiquitous, only the trehalose-containing, pyruvylated glycolipids of *Mycobacterium smegmatis* (Saadat & Ballou, 1983) and the more glycosylated, antigenic variety in *Mycobacterium kansasii* (Hunter et al., 1983, 1985) have been described in detail. Work on those from other important mycobacterial opportunistic pathogens has been hampered by the difficulty encountered in grasping the structural details of the products from *Mycobacterium szulgai* and others. Although precedent dictated that all such products were anchored on acyltrehalose (Saadat & Ballou, 1983; Hunter et al., 1983; Kamisango et al., 1985), we now report that, while the antigenic lipooligosaccharides of *M. szulgai*, like the others, are endowed with a distinctive non-reducing-end epitope, thereby conferring serological specificity, the other end of the oligosaccharide chain is unique in that it consists of a glycosidically linked mono-*O*-methyltri-*O*-acyltrehalose unit.

Experimental Procedures

Extractions of Antigens. The identity of *Mycobacterium szulgai* (strain Johnson; No. 1878 from the collection maintained at the National Jewish Center) was confirmed by agglutination against rabbit antiserum raised against the homologous strain (Schaefer, 1965). Cultures were grown in Fernbach flasks for 4–5 weeks in 7H11 medium without agar (Hunter et al., 1983) or in a glycerol–alanine–salt medium (Takayama et al., 1975) and then autoclaved, and both cells and medium was evaporated to dryness at 50 °C. The resulting solid was extracted with $CHCl_3/CH_3OH$ (Hunter et al., 1983). Dried extracts were dissolved in the biphasic mixture $CHCl_3/CH_3OH/H_2O$ (8:4:2) (Folch et al., 1957); the contents of the lower organic phase were used. Approximately 6 g of washed lipid were obtained from 100 g of dried cells.

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