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Pathway of protein glycosylation in the trypanosomatid *Crithidia fasciculata*

(dolichol/glycoprotein/protozoa/oligosaccharides)

ARMANDO J. PARODI*, LUIS A. QUESADA ALLUE*, AND JUAN J. CAZZULO†

*Instituto de Investigaciones Bioquímicas "Fundación Campomar," Obligado 2490, 1428 Buenos Aires, Argentina; and †Instituto Nacional de Diagnóstico e Investigación de la Enfermedad de Chagas "Dr. Mario Fatala Chabén," Avenida Paseo Colón 568, 1063 Buenos Aires, Argentina

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ABSTRACT **Cells of the insect parasite *Crithidia fasciculata* incubated with [^{14}C]glucose were found to possess only one lipid-bound oligosaccharide with solubility in chloroform/methanol/water mixtures and net charge similar to the charges of dolichol pyrophosphate derivatives. The saccharide moiety could be released from lipid by mild acid hydrolysis. Several enzymatic and chemical treatments of the oligosaccharide indicated that the latter had the structure Manα→Manα→Manα→[Manα→Manα→Man(α1→6)]Man→GlcNAc(β1→4)GlcNAc. Two labeled oligosaccharides were liberated from proteins by a sequential treatment with a protease and endo-β-*N*-acetylglucosaminidase H. One of the protein-bound oligosaccharides had the same structure as the lipid-linked compound, whereas in the second oligosaccharide some mannose residues had been replaced by galactose units, but both compounds migrated as did a Man$_7$GlcNAc standard. These were the largest oligosaccharides obtained even after short labeling periods. It is suggested that glycosylation of proteins in the protozoan *Crithidia fasciculata* does not involve glucosylated lipid-bound oligosaccharides as intermediates.**

It has become evident in recent years that glycosylation of asparagine residues in eukaryotic cell proteins involves dolichol pyrophosphate (DolPP)-bound oligosaccharides as intermediates (1). It has been reported that in animal tissues (2, 3), yeasts (4–6), and probably also in insects (7) and plants (8), the oligosaccharide transferred from the lipid derivative to proteins is composed of two *N*-acetylglucosamine, nine mannose, and three glucose residues. A report by Lehle suggests that in yeast the oligosaccharides containing two or three glucose residues are equally transferred to protein (9). However, no evidence was presented indicating that the oligosaccharides used in the assay had the same specific activity.

The protein-bound oligosaccharides are then processed by loss of some of their monosaccharide constituents and addition of other residues directly from the respective sugar nucleotides. We here report evidence suggesting that in the protozoan *Crithidia fasciculata*, an insect obligate parasite, the oligosaccharide transferred to protein contains two *N*-acetylglucosamine and seven mannose residues and that the oligosaccharide may be processed once bound to protein.

MATERIALS AND METHODS

Materials. [^{14}C]Glucose (284 Ci/mol; 1 Ci = 3.7 × 10^{10} becquerels) was from New England Nuclear. Jack bean α-mannosidase type III and *Streptomyces griseus* protease type VI were purchased from Sigma and endo-β-N-acetylglucosaminidase H (endo H), from Miles.

Isolation of Lipid-Bound Oligosaccharides. *C. fasciculata* cells (*Anopheles* isolate ATCC 11745) were grown in the medium described by Bacchi *et al.* (10) without agar at 28°C. Six hundred milliliters of culture containing about 2 g of cells (logarithmic phase) were cooled on ice and centrifuged at 2500 × *g* for 10 min at 4°C. The cell pellet was resuspended in 30 ml of ice-cold minimal Eagle's medium without glucose but containing 5 mM sodium pyruvate (11). The suspension was centrifuged and the cells were washed twice more with 30 ml of the same solution. The cells were finally resuspended in 10 ml of the same solution containing 600 μCi of [^{14}C]glucose and incubated for the time periods and at the temperatures mentioned in the text. Aliquots (2 ml) were withdrawn and mixed with 10 ml of chloroform/methanol (3:2, vol/vol). The protein interphases were washed twice more with 12 ml of chloroform/methanol/water (3:2:1, vol/vol) and once with 6 ml of water and extracted four times with 1 ml of chloroform/methanol/water (1:1:0.3, vol/vol). The extracts were pooled, dried, dissolved in 1 ml of the last mixture, and passed through 0.6 × 6 cm columns of DEAE-cellulose (acetate form) equilibrated with the same solvent. The columns were washed with chloroform/methanol/water (1:1:0.3) (8 ml), with the same solvent containing 10 mM ammonium formate (5 ml), and finally with the same solvent containing 30 mM ammonium formate (6 ml). The substances that eluted at the last ammonium formate concentration were the lipid-bound oligosaccharides.

The glucose-labeled Dol*PP*-bound oligosaccharides from rat liver and the glucose- and mannose-labeled Dol*PP*-bound oligosaccharide from dog thyroid were obtained as described (4).

Isolation of Oligosaccharides from Glycoproteins. The denatured protein pellets that remained after extraction of lipid-linked oligosaccharides were extensively washed with water and incubated for 10 days at 37°C under a toluene atmosphere in 1 ml of a solution containing 0.05 M Tris-maleate buffer (pH 7.6), 5 mM $CaCl_2$, and 0.5 mg of *S. griseus* protease. Similar amounts of the enzyme were added every 2 days.

Trichloroacetic acid was then added up to 10% final concentration and the supernatant fluids were extracted six times with 5 ml of diethyl ether. The aqueous phases were passed through a 95 × 1.2 cm Bio-Gel P-6 column equilibrated with 0.1 M pyridine/acetate buffer (pH 5.0). Elution was performed with the same solvent. A radioactive peak appeared in the fractions where oligosaccharides containing between 4 and 14 monosaccharide residues were eluted. The labeled substances were spotted on paper and subjected to electrophoresis in 5% (wt/vol) formic acid. A positively charged peak containing about 80% of the label appeared 9 cm from a neutral substance. The charged compounds were eluted and incubated for 48 hr at 37°C in 0.25 ml of 0.05 M triethylamine/acetate buffer (pH 5.5) containing 0.01 unit of endo H. One unit is the amount of enzyme

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Abbreviations: Dol, dolichol; endo H, endo-β-*N*-acetylglucosaminidase H; R_{Man}, ratio between the migration of a fraction and that of mannose.

required to hydrolyze 1 μmol of $Man_5GlcNAc_2Asn[^{14}C]$acetyl per min at the saturated substrate concentration, at pH 5.0 and 37°C. The solution was then spotted on paper and subjected again to paper electrophoresis in 5% formic acid. About 45% of the labeled material was neutral, thus indicating that it had been released from glycopeptides by the last enzymatic treatment.

Standards. Tritiated oligosaccharides ($Man_6GlcNAc$ to $Man_9GlcNAc$) from an adenovirus glycoprotein were a generous gift from Rosalind and Stuart Kornfeld. ^{14}C-Labeled oligosaccharides from hen oviduct were obtained as described above for the isolation of oligosaccharides from *C. fasciculata* glycoproteins. Oviduct slices had been incubated with [^{14}C]glucose as described (5). The size of the oviduct oligosaccharides was assessed by paper chromatography with solvent A and the viral oligosaccharides as internal standards. Manooligosaccharide standards were obtained as described (12).

Methods. Conditions for mild and total acid hydrolysis, reduction with $NaBH_4$, treatment with KOH, acetolysis, and degradation with α-mannosidase have been described (4). Paper electrophoresis was performed for 180 min at 17 V/cm. The solvents for paper chromatography were: A, 1-propanol/nitromethane/water (5:2:4, vol/vol); B, 1-butanol/pyridine/water (1:0.3:0.3, vol/vol); and C, 1-butanol/pyridine/water (4:3:4, vol/vol). Incubation of oligosaccharides with endo H was as described above for the glycopeptides.

RESULTS

Isolation of Lipid-Bound Oligosaccharides. The procedure employed for the isolation of putative Dol*PP* derivatives from *C. fasciculata* cells incubated with [^{14}C]glucose was based on the properties of similar compounds isolated from animal tissues and yeasts (1, 5). The lipid-bound oligosaccharides remain with the protein interphase in chloroform/methanol/water (3:2:1) and are insoluble in water, but they can be extracted with chloroform/methanol/water (1:1:0.3). Labeled compounds with the solubility properties mentioned above were extracted from the protozoan cells. In addition, part of those compounds were retained by a DEAE-cellulose column equilibrated with chloroform/methanol/water (1:1:0.3), from which they were eluted at the same salt concentration required for elution of a glucose-labeled Dol*PP* derivative from rat liver. Dol-*P*-Glc, also from rat liver, was not retained by the column.

Size of the Oligosaccharide Moiety. The protozoan compound that was eluted from the DEAE-cellulose column at the same salt concentration as Dol*PP* derivatives was subjected to mild acid hydrolysis (pH 2, 10 min, 100°C) followed by a chloroform/methanol/water (3:2:1) partition. The water-soluble compounds present in the upper phase were spotted on paper and developed with solvent A. As can be seen in Fig. 1*A*, a single substance appeared. It migrated farther than the glucosylated oligosaccharide standards ($Glc_3Man_9GlcNAc_2$, $Glc_2Man_9GlcNAc_2$, and $Glc_1Man_9GlcNAc_2$) from rat liver. The *Crithidia* compound, previously treated with endo H, migrated the same as an internal tritiated octasaccharide standard ($Man_7GlcNAc$) isolated from an adenovirus glycoprotein and as a ^{14}C-labeled oligosaccharide of the same composition obtained from hen oviduct glycoproteins and spotted on a parallel lane (Fig. 1*B*). The same result was obtained whether the cells were incubated with [^{14}C]glucose for 15 min at 5°C, for 10 min at 20°C, or for 3, 5, 10, 15, 20, 40, or 60 min at 28°C.

Presence of *N*-Acetylglucosamine Residues. The compound liberated from the lipid by the mild acid treatment was neutral when run on paper electrophoresis with 5% formic acid as solvent (Fig. 2*A*). However, two positively charged peaks appeared after the compound was reduced with $NaBH_4$ and

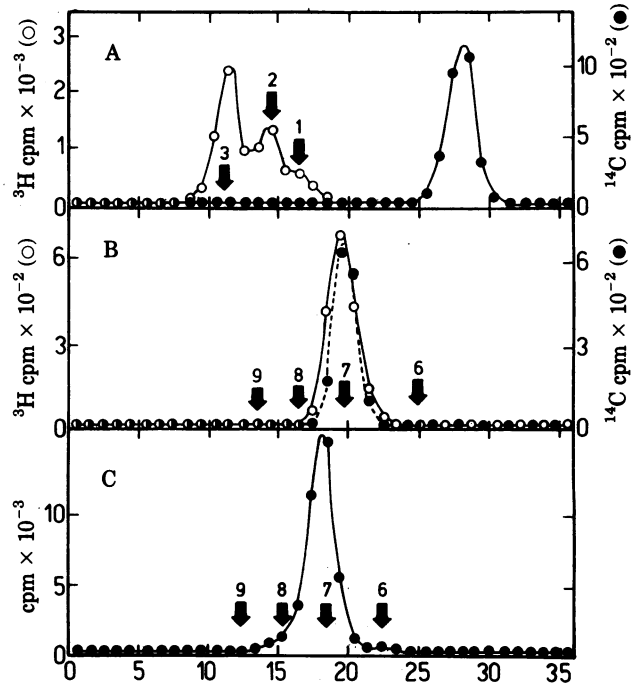


FIG. 1. Paper chromatography of oligosaccharides. (*A*) The ^{14}C-labeled lipid-bound oligosaccharide isolated from *C. fasciculata* was subjected to mild acid hydrolysis followed by a chloroform/methanol/water (3:2:1) partition. The upper phase was spotted on paper together with the following [^3H]glucose-labeled standards: 1, $Glc_1Man_9GlcNAc_2$; 2, $Glc_2Man_9GlcNAc_2$; and 3, $Glc_3Man_9GlcNAc_2$. (*B*) The ^{14}C-labeled oligosaccharide was obtained as described above, treated with endo H, and spotted on paper together with a [^3H]mannose-labeled oligosaccharide ($Man_7GlcNAc$). The following oligosaccharides from oviduct were run on a parallel lane: 6, $Man_6GlcNAc$; 7, $Man_7GlcNAc$; 8, $Man_8GlcNAc$; and 9, $Man_9GlcNAc$. (*C*) The oligosaccharides liberated from *C. fasciculata* glycoproteins were spotted on paper. The cells had been incubated for 10 min at 28°C. The same standards mentioned for *B* were run on a parallel lane. Solvent A was used in all cases.

treated with 2 M KOH at 100°C for 30 min (Fig. 2*B*). Apparently the slow-migrating substance was the precursor of the fast one, because a longer alkaline treatment resulted in a decrease of the slow- and in an increase of the fast-migrating compound (Fig. 2*C*). The fact that an incubation of the compound with endo H previous to the reduction and treatment with alkali abolished the appearance of the fast peak confirmed that the presence of charged substances was due to the deacetylation of first one and then a second *N*-acetylglucosamine residue (Fig. 2*B*). The enzyme cleaves the bond between the two *N*-acetylglucosamine residues of the high-mannose type oligosaccharides (13).

Total Acid Hydrolysis. Total acid hydrolysis of the oligosaccharide followed by paper chromatography with solvent B revealed the presence of labeled mannose residues (Fig. 3*A*). Apparently the *N*-acetylglucosamine residues were not labeled even after 60 min of incubation of the cells with [^{14}C]glucose.

α-Mannosidase Treatment. The oligosaccharide was sequentially treated with endo H and α-mannosidase. Paper chromatography with solvent C revealed the presence of two reaction products (Fig. 4*A*). One of them migrated as did mannose, whereas the second one ran with a $R_{Man} = 0.890$ between that monosaccharide and a mannobiose standard. Because all the label was present in mannose residues and *N*-acetylglucosamine migrates farther than mannose in this solvent, it was speculated that the compound that migrated with R_{Man}

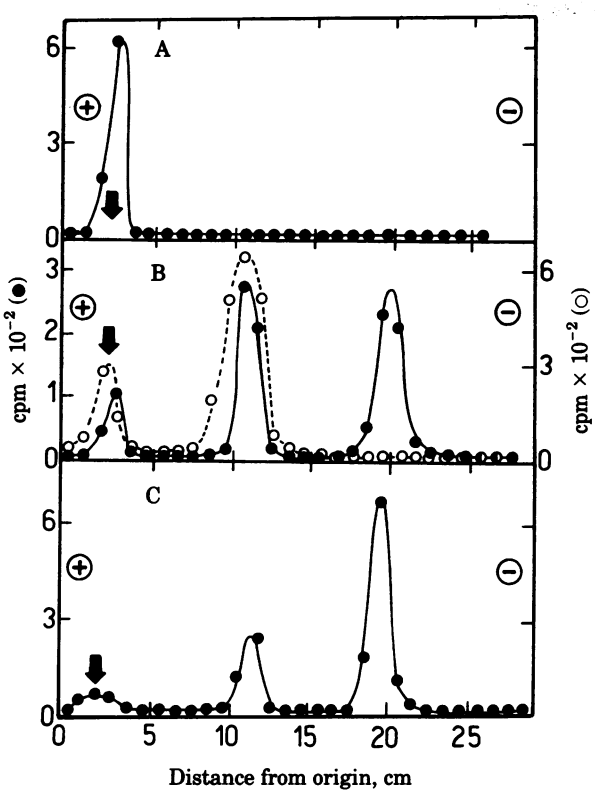


FIG. 2. Presence of *N*-acetylglucosamine residues. The oligosaccharide released from lipid as described in Fig. 1*A* was reduced with $NaBH_4$, treated with 2 M KOH at 100°C for 0 min (*A*), 30 min (*B*, ●), and 120 min (*C*) and run on paper electrophoresis in 5% formic acid. An aliquot was treated with endo H before reduction and a 30-min alkaline treatment (*B*, ○). The arrows indicate the position of unlabeled glucose standards.

= 0.890 could be the disaccharide Man-GlcNAc. This was confirmed when it was found that reduction of the compound with $NaBH_4$ followed by treatment with 2 M KOH at 100°C for 60 min and paper electrophoresis in 5% formic acid revealed the presence of a positively charged peak. This result indicated that the oligosaccharide could be almost completely degraded by α-mannosidase. Similar enzymatic treatments of a glucose- and mannose-labeled oligosaccharide ($Glc_3Man_9GlcNAc_2$) isolated from dog thyroid resulted in the appearance of mannose and a substance that migrated with a R_{Man} = 0.145, thus showing that neither of the glycosidases was contaminated with glucosidases (Fig. 4*C*).

Acetolysis. The endo H-treated oligosaccharide was subjected to acetolysis under conditions in which mainly α1→6 bonds between mannose residues are cleaved. Paper chromatography of the degradation product with solvent C showed the presence of a compound that migrated like mannotriose as well as a larger oligosaccharide (Fig. 5*A*). When the latter was rerun with solvent A together with appropriate standards, it migrated in the position of a pentasaccharide ($Man_4GlcNAc$). The endo H-treated oligosaccharide from dog thyroid ($Glc_3Man_9GlcNAc$) gave three main products when subjected to acetolysis, namely mannobiose, mannotriose, and a larger compound that ran with a R_{Man} = 0.195 (Fig. 5*C*). This substance probably had the following structure: $Glc_3Man_4GlcNAc$ (14).

Isolation of Oligosaccharides from Glycoproteins. The procedure employed for the liberation of the endo H-sensitive oligosaccharides from the glycoproteins present in the pellets that remained after extraction of the lipid-bound oligosaccharides is described in *Materials and Methods*. The amount of labeled

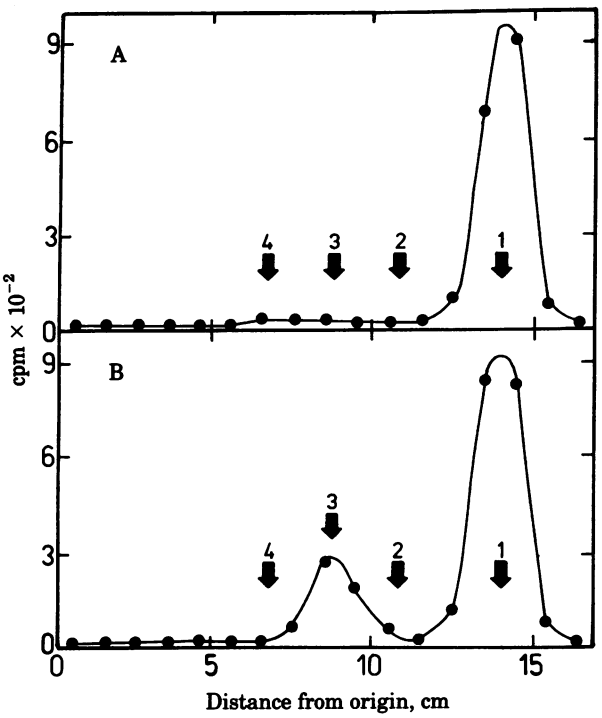


FIG. 3. Total acid hydrolysis. The oligosaccharides liberated from lipid (*A*) or glycoprotein (*B*) were subjected to total acid hydrolysis and paper chromatography with solvent B. The standards used were: 1, mannose; 2, glucose; 3, galactose; and 4, glucosamine. The oligosaccharides used in the experiments depicted in Figs. 3*B*, 4*B*, and 5*B* were a pool of the substances that migrated as a $Man_7GlcNAc$ standard, isolated from cells incubated for 10, 20, 40, and 60 min at 28°C.

oligosaccharides obtained increased linearly until at least 60 min of incubation of cells with [^{14}C]glucose at 28°C.

Size of Oligosaccharides Isolated from Glycoproteins. The compounds isolated from cells incubated for 10, 20, 40, or 60 min at 28°C migrated as an octasaccharide standard ($Man_7GlcNAc$) when run on paper chromatography with solvent A (Fig. 1*C*).

The results reported below were obtained with a pool of the saccharides that migrated as did the $Man_7GlcNAc$ standard.

Sugar Composition. Besides the *N*-acetylglucosamine residues that accounted for the sensitivity of the glycopeptides to cleavage by endo H, the oligosaccharides released from the proteins contained labeled mannose and galactose residues as revealed by total acid hydrolysis and paper chromatography with solvent B (Fig. 3*B*).

α-Mannosidase Degradation. α-Mannosidase treatment of the oligosaccharides liberated from proteins followed by paper chromatography with solvent C showed that at least two oligosaccharides were present in the sample that migrated as the $Man_7GlcNAc$ standard (Fig. 4*B*).

One of them gave mannose and the disaccharide Man-GlcNAc as degradation products, the same as the lipid-bound compound, whereas the other produced mannose and a larger oligosaccharide that migrated as a pentasaccharide (R_{Man} = 0.480). Further incubation of this compound with α-mannosidase did not modify its migration on paper chromotography. Labeled mannose and galactose residues were present in the α-mannosidase-resistant substance as judged by total acid hydrolysis and paper chromatography.

Acetolysis. The acetolysis pattern of the oligosaccharides liberated from proteins was very similar to that of the lipid-bound compound, but in this case a slightly larger proportion of a compound migrating as a mannobiose standard was obtained (Fig. 5*B*).

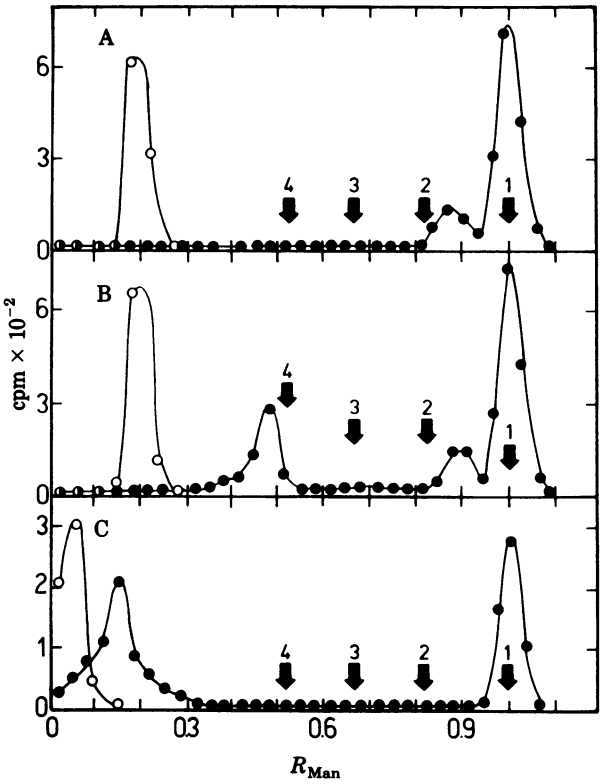


FIG. 4. α-Mannosidase degradation. (*A*) The oligosaccharide obtained as described in Fig. 1*A* was sequentially treated with endo H and α-mannosidase and subjected to paper chromatography. (*B*) The same as *A* but the oligosaccharide was that released from proteins. (*C*) The glucose- and mannose-labeled oligosaccharide ($Glc_3Man_9GlcNAc$) from dog thyroid was used in this case. In all cases ○ and ● correspond to α-mannosidase-untreated and treated samples, respectively; 1, 2, 3, and 4 correspond to the positions where mannose, mannobiose, mannotriose, and mannotetraose standards migrated. Solvent C was used. See legend to Fig. 3.

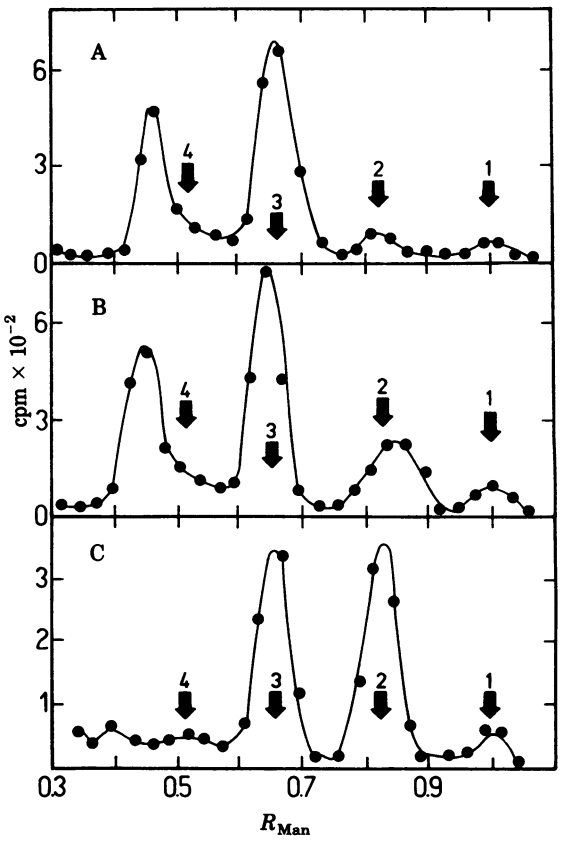
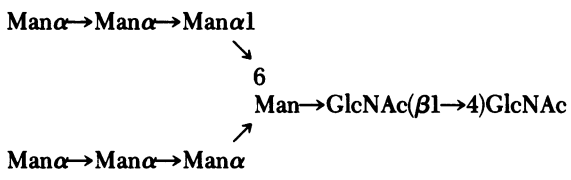


FIG. 5. Acetolysis of oligosaccharides. The endo H-treated oligosaccharides were subjected to acetolysis for 10 hr at 37°C followed by paper chromatography. The same standards and solvent as in Fig. 4 were used. The samples were: (*A*) *C. fasciculata* oligosaccharide liberated from lipid; (*B*) *C. fasciculata* oligosaccharide released from proteins, and (*C*) dog thyroid oligosaccharide ($Glc_3Man_9GlcNAc$). In this case a reaction product that migrated with a $R_{Man} = 0.195$ was omitted from the figure. See legend to Fig. 3.

DISCUSSION

A single lipid-bound oligosaccharide with solubility in chloroform/methanol/water mixtures and net charge similar to the charges of Dol*PP* derivatives was isolated from *C. fasciculata* cells that actively synthesized endo H-sensitive glycoproteins. It is tentatively suggested that, similar to what occurs in higher eukaryotic cells, the lipid residue in the lipid-bound oligosaccharide is a polyprenol and that the lipid and saccharide moieties are joined by a pyrophosphate bridge. The last suggestion is based on the elution profile of the compound from DEAE-cellulose columns and by the fact that the oligosaccharide could be liberated by mild acid treatment. No evidence for the presence of a glucosylated compound was obtained. The results reported suggest the following structure for the saccharide moiety:



The anomeric configurations of the linkages between mannose and between *N*-acetylglucosamine residues appeared to be similar to those of known Dol*PP*-bound oligosaccharides (15). There is an uncertainty concerning the bond between the mannose and *N*-acetylglucosamine residues that was not cleaved by α-mannosidase. In the Dol*PP*-bound oligosaccharides isolated from higher organisms that bond has a β configuration (15). It should be noted that a Dol*PP* derivative with a saccharide moiety similar to that depicted above has been found to be an intermediate in the synthesis of the saccharide donor of protein glycosylation in animal cells, namely $Glc_3Man_9GlcNAc_2$-*P*-*P*-Dol (15). A single block, affecting the enzyme responsible for the addition of the eighth mannosyl residue, could account, therefore, for the accumulation in *C. fasciculata* of the lipid-bound oligosaccharide depicted above.

When animal, plant, or fungal cells were labeled and the Dol*PP* derivatives were isolated by using procedures comparable to those employed here, the main compound found was $Glc_3Man_9GlcNAc_2$-*P*-*P*-Dol (2, 5, 8). This was one of the principal arguments for concluding that this compound is the saccharide donor of protein glycosylation in those organisms. In a similar way it may be assumed that the lipid-bound oligosaccharide isolated from *C. fasciculata* cells is the saccharide donor of protein glycosylation in this protozoan. A very strong indication that this assumption is correct is the fact that no endo H-sensitive oligosaccharide larger than that bound to lipid was released from glycoproteins even after short labeling periods. It should be mentioned that oligosaccharides containing two *N*-acetylglucosamine and between six and nine mannose residues or two *N*-acetylglucosamine, nine mannose, and between one and three glucose residues isolated from animal or yeast gly-

colipids or glycoproteins are sensitive to endo H (5, 13, 16, 17).

Two different oligosaccharides that migrated as a $Man_7GlcNAc$ standard were released from glycoproteins.

One of them had the same sugar composition and the same anomeric configuration of the linkages as the lipid-bound oligosaccharide, whereas the second compound was partially resistant to α-mannosidase degradation. This was due to the replacement of some mannose residues by galactose units, thus indicating that a processing of the protein-bound oligosaccharides had occurred.

The close similitude between the acetolysis patterns of the lipid-bound and the protein-bound oligosaccharides also reinforces the idea of a precursor–product relationship between them. It is of interest that Hunt has described a phytohemagglutinin- and concanavalin A-resistant Chinese hamster ovary cell line that accumulates a lipid-linked oligosaccharide containing seven mannose and two *N*-acetylglucosamine residues (18). However, evidence was presented indicating that a minor component containing two or three glucose units in addition to the nine residues mentioned above was the real precursor in protein glycosylation.

Preliminary experiments performed with epimastigote cells of *Trypanosoma cruzi*, the agent of Chagas disease, have shown that the oligosaccharide released by mild acid hydrolysis from a lipid derivative isolated as described here migrated on paper chromatography as did a $Man_9GlcNAc_2$ standard. Moreover, the largest oligosaccharide isolated from glycoproteins had the same mobility on paper as the lipid-bound compound, even after short labeling periods. It seems therefore, that protein glycosylation in *T. cruzi*, as well as in *C. fasciculata*, which is also a trypanosomatid, does not involve the saccharide $Glc_3Man_9GlcNAc_2$.

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