The Initial Stages of Processing of Protein-bound Oligosaccharides in Vitro*

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Following the rapid enzymatic transfer of an oligosaccharide (GlcNAc2Man3Glc3) from a lipid carrier to endogenous protein acceptors in membrane preparations from NIL fibroblasts, the transferred oligosaccharide chain undergoes processing. Protein-bound oligosaccharides, released from the polypeptide backbone by treatment with endo-β-N-acetylglucosaminidase H, were analyzed by gel filtration and by susceptibility to α-mannosidase digestion. The initial stages of this processing in vitro consist of sequential excision of 3 glucose residues prior to the removal of mannose residues. The array of oligosaccharides generated in vitro by membrane preparations from NIL cells appears to be identical with processed oligosaccharides derived in vivo in intact NIL cells.

In the previous paper in this series (1), we presented the partial structure of the major lipid-linked oligosaccharide (GlcNAc2Man3Glc3) synthesized in NIL fibroblasts. Evidence from our laboratory has shown that this glucose-containing oligosaccharide-lipid is a preferred substrate in the enzymatic transfer of the oligosaccharide to endogenous protein acceptors in vitro (2). Similar observations recently have been reported by Spiro et al. (3). This result and also our other evidence (4), indicating that an oligosaccharide-lipid with a possibly identical structure is the major oligosaccharide-lipid in a variety of cultured cells, led us to propose that this oligosaccharide-lipid is the true intermediate in the synthesis of the core region of asparagine-linked oligosaccharides (4, 5). This proposal includes a "processing" mechanism which excises the 3 glucose residues and mannose residues. Processing is required since the final products of glycosylation, the glycoproteins, do not contain glucose, although there are exceptions (6-9). Several recent reports from other laboratories have provided evidence to support the concept of processing of protein-bound oligosaccharides (10-14).

In this paper, evidence is presented for the molecular events involved in the initial stages of processing in vitro. A mixture of oligosaccharide-lipid labeled with either [3H]glucose or [14C]mannose was prepared and added to fresh preparations of membranes from NIL cells. After a rapid en bloc transfer of the oligosaccharide to endogenous proteins, the 3 glucose residues were sequentially excised, followed by removal of mannose residues from the protein-bound oligosaccharide.

RESULTS

Processing Kinetics of Protein-bound Oligosaccharides

These studies were directed to an evaluation of the fate of the oligosaccharide chain following enzymatic transfer from the lipid carrier to protein in membranes prepared from NIL fibroblasts. Indications that processing of protein-bound oligosaccharides occurs in vitro first became apparent when the transfer of [3H]Glc- and [14C]Man-labeled oligosaccharides from the lipid carrier to endogenous proteins was allowed to proceed longer than 15 min. In this kinetic study, radiolabeled oligosaccharide-lipid was added to membranes. After various periods of time, aliquots were removed and radioactivity incorporated into the residue fraction was measured as shown in Fig. 1. Consistent with our earlier observations (2), labeled oligosaccharide was transferred very rapidly to protein. However, after 4 h of incubation, 82% of the [3H] label was lost from the protein fraction. 14C label was also lost but not as rapidly or as extensively as the [3H] label: 24% of the [14C] label was lost after 4 h.

Radiolabeled material recovered in the combined water extract during the extraction procedure was analyzed by paper chromatography in n-butyl alcohol/pyridine/water (6:4:3, v/v/v) and by gel filtration on Bio-Gel P-2. These results revealed the presence of [3H]glucose and [14C]mannose, demonstrating that only monosaccharides, and not disaccharides or oligosaccharides, are removed from the protein-bound materials (data not shown). Phosphorylated oligosaccharides were also detected in the water extract, presumably originating from pyrophosphatase action on the lipid pyrophosphoryl oligosaccharides.

Gel Filtration of Processed Oligosaccharides

To characterize the protein-bound oligosaccharide, oligosaccharide-lipid labeled with either [3H]glucose or [14C]mannose was added to a fresh membrane preparation. Aliquots were removed at 45 s, 5 min, 12 min, 1 h, and 3 h. The residue fraction was subjected to exhaustive pronase digestion, converting the residue to water-soluble glycopeptides. The mix-

† Portions of this paper (including "Experimental Procedures," part of "Results," Tables I and II, Figs. 7 to 9 and additional Refs. 1 13) are presented in miniprint format immediately following the references. 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. 20014, Request Document No. 78M-1511, cite author(s), and include a check or money order for $1.05 per set of photocopies.

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Initial Stages of Glycoprotein Processing In Vitro

Fig. 1. Kinetics of incorporation of [3H]Glc- and [14C]Man-oligosaccharides by the endogenous acceptors of NIL cell membranes. [3H]Glc-oligosaccharide-lipid (36,000 cpm) and [14C]Man-oligosaccharide-lipid (45,000 cpm) were combined and dried under a stream of nitrogen. MnCl₂ (0.4 ml of 64 mM solution) and 0.4 ml of Tris-buffered saline (0.9% NaCl solution) (0.15 M NaCl, 0.02 M Tris-Cl, pH 7.4) were added and the mixture was sonicated. After addition of 0.4 ml of 3% sodium deoxycholate, the reaction was initiated by the addition of 2 ml of membranes (80 mg) and was stirred at 25°C. At the indicated times, 160-μl aliquots were removed and the reaction was terminated by the addition of 2 ml of chloroform/methanol (3:2) and 300 μl of water. The samples were extracted and radioactivity incorporated into the residue was determined as described under "Experimental Procedures." 0, [3H]Glc-oligosaccharide-lipid; 0, [14C]Man-oligosaccharide-lipid.

Analysis of the oligosaccharides which had been transferred to protein at the earliest time point (45 s) reveals the presence of a single component with a constant ratio of [3H]glucose and [14C]mannose throughout the peak (Fig. 2, Panel I). This oligosaccharide is given the designation of O₀ (G for glycopeptide-derived). O₀ is identical with respect to relative elution coefficient (Table I), [3H]/[14C] ratio (Table II), and susceptibility to α-mannosidase digestion (data not shown), with the product of endo H treatment of the starting lipid-linked oligosaccharide (O₀), demonstrating the en bloc transfer of the oligosaccharide to protein.

After 5 min of incubation (Fig. 2, Panel II) the predominant oligosaccharide is O₀, but a new oligosaccharide, designated A₀, is observed. The ratio of [3H]glucose to [14C]mannose of A₀ is smaller than O₀ (Table II), suggesting the loss of 1 glucose residue. Furthermore, [3H]glucose, but not [14C]mannose, is recovered in the combined water extract during the extraction procedure at this time interval.

At 12 min, the time of maximal incorporation of label into protein, A₀ is still the major oligosaccharide, while A₀ has become more prominent (Fig. 2, Panel III). A third oligosaccharide, B₀, is also observed which has a smaller ratio of [3H] to [14C] than does A₀ (Table II), indicating that it is probably formed by the removal of a glucose residue from A₀.

At 1 h, further processing of the oligosaccharides which are protein-bound yields five distinguishable oligosaccharides, O₀, A₀, B₀, and two new oligosaccharides, C₀ and D₀ (Fig. 2, Panel IV). Since there is no [3H]Glc label coincident with O₀ and D₀, it is tentatively concluded that oligosaccharide C₀ is generated by the removal of the glucose residue from B₀ and that D₀ is formed by the removal of a mannose residue from C₀. There are two features of this profile which are not yet understood. One is the slight increase with time in the [3H]/[14C] ratio of A₀ (Table II). The other is the appearance of small amounts of [3H]glucose-labeled material in a position that is overlapping but not coincident with C₀. It is possible...
that there is a minor pathway of processing of oligosaccharides in which a mannose residue is excised prior to the removal of all 3 glucose residues.

At 3 h, further processing yields two more distinct oligosaccharides, formed presumably by removal of mannose residues from Do (Fig. 2, Panel V). However, by 3 h, it appears that processing has slowed down in the in vitro system since, at this time, there are still significant amounts of Oo, Ao, and Bo. Furthermore, the glycopeptide fraction is completely susceptible to endo H, indicating that the synthesis of complex oligosaccharide units (15) has not been attained in vitro. The addition of UDP-GlcNAc, GDP-Man, and UDP-Gal to the incubation mixture did not affect the oligosaccharide profile. Recent work has shown that the presence of deoxycholate, which is used in the incubation to stimulate the transfer of the oligosaccharide from the lipid carrier to protein acceptors, may inhibit or limit the extent of processing.3 On the basis of these results, it is concluded that we are observing only the initial stages of processing of glycoproteins in vitro.

The \( K_v \) values for the processed oligosaccharides generated at the different time periods in Fig. 2 are listed in Table I. These values are consistent with the following compositions: Oo, Glc3Man3GlcNAc3; Ao, Glc3Man3GlcNAc3; Bo, Glc3Man3GlcNAc3; Co, Man3GlcNAc; Do, Man3GlcNAc; Eg, Man3GlcNAc; Fc, Man3GlcNAc; (16).

**\( \alpha \)-Mannosidase Susceptibility of Processed Oligosaccharides**

Further information concerning the structures of the processed oligosaccharides was obtained by \( \alpha \)-mannosidase digestion. The individual oligosaccharides were purified and treated exhaustively with \( \alpha \)-mannosidase, and the products were analyzed by chromatography on Bio-Gel P-4 (Fig. 3). In Panel I, the \( \alpha \)-mannosidase digestion of \([1^4C]\)Man- and \([3H]Glc\)-labeled Oo reveals the presence of two major radioactive products, \([1^4C]\)mannose in the inclusion volume and an \( \alpha \)-mannosidase-resistant product containing slightly less than half of the \( ^{14}C \) label and all of the \( ^3H \) label.

Similarly, the \( \alpha \)-mannosidase-resistant product of radiolabeled Ao retains slightly less than half of the \( ^{14}C \) label and all of the \( ^3H \) label (Fig. 3, Panel II). Of particular significance is the observation that this product has a smaller \( ^{14}C/^{3H} \) ratio (3.08) and is smaller in size than the \( \alpha \)-mannosidase-resistant product of Oo (\( ^{14}C/^{3H} \) ratio of 4.70). This result is another indication that the first sugar residue that is excised is a glucose and not a mannose residue. If a mannose residue were removed first, then the \( \alpha \)-mannosidase-resistant products of Oo and Ao would have been identical.

As is true for both Oo and Ao, \( \alpha \)-mannosidase digestion of radiolabeled Bo releases greater than half of the total \( ^{14}C \) label as mannose, resulting in the formation of a third \( \alpha \)-mannosidase-resistant product (Fig. 3, Panel III). This product has a smaller ratio of \( ^{14}C/^{3H} \) (1.05) and is smaller in size than the resistant product of Ao. This observation is consistent with the hypothesis that Bo is formed by the excision of a glucose residue from Ao.

As seen in Panel IV of Fig. 3, Co is almost completely susceptible (>95%) to the action of \( \alpha \)-mannosidase. A small peak of \( ^{3}C \)-labeled material slightly larger than mannose is observed. There were sufficient quantities of this material for characterization, but presumably it is the expected \( \beta \)-Man-GlcNAc. The finding that Co is almost completely sensitive to \( \alpha \)-mannosidase supports the earlier conclusion that Co arises by the removal of the last glucose residue from Bo. Consequent exposure of the entire \( \alpha \)-mannosyl oligosaccharide structure leads to almost complete hydrolysis. In Fig. 3, Panel V, the profile of \( \alpha \)-mannosidase-digested Do is similar to that of digested Co since Do is believed to be formed by the removal of a mannose residue from Co.

**Studies on the Reisolated Lipid-linked Oligosaccharide**

**Partial Characterization of Reisolated Oligosaccharide-Lipid**—In order to determine whether the lipid-linked oligosaccharides are susceptible to the processing enzymes, the oligosaccharide-lipid remaining after 12 min of incubation was reisolated by chloroform/methanol/water (1:1:0.3) extraction. An aliquot was subjected to mild acid hydrolysis and treated with endo H, and products were analyzed by gel filtration as shown in Fig. 4, Panel I. When compared to the purified oligosaccharide lipid (Fig. 7), it is clear that considerable modification had occurred. Only 46% of the \([1^4C]Glc\) and 30% of the \([1^4C]\)Man label still bound to lipid is associated with Oo. This result also indicates that at least some of the radio-labeled monosaccharides, recovered in the combined water extract (see above), arose from hydrolysis of lipid linked oligosaccharides.

There are at least two other components labeled with both \([1^4C]Glc\) and \([1^4C]\)Man which remain to be fully characterized. One of the oligosaccharides, Ao, elutes with a \( K_v \) value (0.365) which is identical with that of oligosaccharide Ao (Table II). Treatment of Oo and Ao, with a purified glucosidase from *Penicillium funiculosum* (1) removes a glucose residue from

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5 S. J. Turco and P. W. Robbins, Unpublished observations.
rides will be necessary in order to firmly establish the identity
extensive study of the processing of lipid-bound oligosaccha-
position between AL and the expected BL. It is apparent that
copeptide-derived counterparts, Ao or Bo, since it elutes in a
applied to a column of Bio-Gel P-4 (minus 400 mesh, 1 X 210 cm);
was mild acid-hydrolyzed and treated with endo H, and products were
![Fig. 4. Panel I, Bio-Gel P-4 chromatogram of products of endo H treatment of [3H]Glc- and [3C]Man-oligosaccharides released from lipid by mild acid hydrolysis. (3H]Glc- and (3C]Man-Oligosaccharide-lipids were added to membranes as described under “Experimental Procedures.” After 12 min of incubation, the lipid-linked oligosaccharides were re-extracted. An aliquot (8000 cpm of 3H, 5000 cpm of 3C]Man-oligosaccharides. OL, AL, BL, and CL, lipid-derived oligosaccharides. Panel II, Bio-Gel P-4 chromatogram of products of endo H-treated glycopeptides. Reisolated oligosaccharide-lipid (48,000 cpm of 3H, 30,000 cpm of 3C]Man-oligosaccharides. Oo, A o, B o, and C o, glycopeptide-derived oligosaccharides.]

The "12-min" oligosaccharide lipid mixture was added to a fresh preparation of membranes for 45 s. The nonextractable residue fraction (2500 cpm of 3H, 1500 cpm of 3C]Man-oligosaccharide; 3H to 3C of AL is 2.34 compared to 2.12 for Oo, indicating that Ao has lost the terminal glucose present in Oo (data not shown). However, the ratio of 3H to 3C of Ao is 2.34 compared to 2.12 for Oo. The significance of this is unclear. The other lipid-derived glucose-containing oligosaccharide does not appear to be identical with the glycopeptide-derived counterparts, Ao or Bo, since it elutes in a position between Ao and the expected Bo. It is apparent that extensive study of the processing of lipid-bound oligosaccharides will be necessary in order to firmly establish the identity of the components.

**Preferential Transfer of the Largest Oligosaccharide**—Since the lipid-linked oligosaccharide mixture isolated after 12 min of incubation is a potential donor of oligosaccharides to protein acceptors, the initial products of transfer from the mixture to endogenous membrane acceptors were examined. The "12-min" oligosaccharide lipid mixture was added to a fresh preparation of membranes for 45 s at 25°C and the oligosaccharides transferred to protein were analyzed by gel filtration (Fig. 4, Panel II). Clearly, the unmodified oligosaccharide (Oo) was transferred preferentially during this short incubation. With longer periods of time, the modified oligo-

![Fig. 5. Bio-Gel P-4 chromatograms of products of endo H-treated glycopeptides derived in vivo and in vitro from NIL cells. Processed [3C]Man-oligosaccharides derived in vitro were obtained from incubating [3C]Man-oligosaccharide-lipid with membranes for 12 min, 1 h, and 3 h at 25°C as described in the legend to Fig. 2. Endo H-treated [3H]Man-oligosaccharides, obtained by pulse labeling NIL cells with [3H]mannose for 2, 5, and 10 min (16) were combined with in vitro-generated oligosaccharides and applied to a column of Bio-Gel P-4 (200 to 400 mesh, 1 x 210 cm); 0.7 ml fractions were collected. Oo, [3H]Man-oligosaccharide; Oo, [3C]Man-oligosaccharide. Oo, A o, B o, C o, and D o, glycopeptide-derived oligosaccharides.]

**Comparison of in Vitro and in Vivo Processed Oligosaccharides**

In order to evaluate the physiological relevance of the processing mechanism in vitro, the protein-linked oligosaccharides generated during processing in vitro were compared with those isolated in vivo. Processed oligosaccharides formed in vivo by pulse labeling of intact NIL cells with [3H]mannose for short periods of time (see Ref. 16) were kindly provided by Dr. S. C. Hubbard of this laboratory. These in vitro-derived oligosaccharides were combined with [3C]Man-labeled oligosaccharides processed in vitro for various time intervals and the combined oligosaccharides were applied to a column of Bio-Gel P-4. A comparison of oligosaccharides generated at 12 min in vitro with those generated with a 2-min pulse in vivo is shown in Fig. 5, Panel I. In vitro-derived oligosaccharides Oo, Ao, and Bo co-elute with those isolated in vivo. It appears that processing of oligosaccharides proceeds much faster in the in vivo system. Even with a short in vivo pulse of 2 min, Co is already present. Part of this discrepancy is due to temperature; the in vivo experiments were conducted at 37°C while the in vitro incubations were carried out at 25°C. As shown in Fig. 5, Panel II, a comparison of 5-min in vivo and 1-h in vitro oligosaccharides reveals that the major oligosaccharides are coincident. Similar results are seen in Fig. 5, Panel III, which compares 10-min in vivo- and 3-h in vitro-derived oligosaccharides. Further structural similarities of the
in vivo- and in vitro-derived oligosaccharides were observed following α-mannosidase digestion. The α-mannosidase-resistant products of in vivo- and in vitro-generated O₅, A₅_5, and B₅ were coincident (data not shown).

**DISCUSSION**

The results presented in this paper support and extend our earlier findings (4) concerning processing of asparagine-linked oligosaccharides. The biochemical reactions involved in the initial stage of processing of protein-bound oligosaccharide units in membranes from NIL cells were examined. The major lipid-linked oligosaccharide, which we believe to be the true intermediate in glycoprotein biosynthesis in these cells, was described in the preceding paper (1) as a compound containing 2 N-acetylglucosamine, 9 mannose, and 3 glucose residues. The glucose residues were found to be arranged in a linear sequence occupying the nonreducing end of one of the mannosyl branches. Addition of this lipid-linked oligosaccharide to membranes from NIL cells resulted in a rapid en bloc transfer of the oligosaccharide from the lipid carrier to endogenous protein acceptors. Shortly thereafter, glucose residues were excised from the protein-bound oligosaccharides, followed by removal of mannose residues. This was demonstrated by comparing the $K_d$ values and by the change in the [3H]glucose/[14C]mannose ratio of processed oligosaccharides isolated at various times after transfer. Thus, the $K_d$ values and the change in ratio of $O_5$, $A_5$, $B_5$, and $C_5$ are in agreement with the loss of 3 glucose residues as determined for the conversion of Glc₃Man₉GlcNAc₂ to Man₉GlcNAc (1). Furthermore, structural studies by α-mannosidase digestion of the isolated oligosaccharides are consistent with this conclusion.

On the basis of currently available evidence, the formation of asparaginyl-linked carbohydrate units can be divided into three separate molecular events: synthesis of the oligosaccharide-lipid precursor, transfer of the completed oligosaccharide to protein, and processing of the protein-bound oligosaccharide to form high mannose and complex units (15). The overall glycosylation pathway is depicted in Fig. 6. The various steps in the biosynthesis of the oligosaccharide-lipid precursor have been studied by a number of investigators (reviewed in Refs. 5 and 17). The majority of these studies indicate the oligosaccharide moiety is assembled on the polyisoprenoid lipid, dolicholphosphate, which participates as a carrier lipid, presumably in a cyclic fashion. Following transfer of the oligosaccharide to protein, processing of the oligosaccharide chain begins. It is quite probable that the initial stages of processing described here are common to the formation of both complex and high mannose units. The final stages of processing would include the final excision of mannose residues and the addition of "peripheral" sugars N-acetylglucosamine, galactose, sialic acid, and fucose. In the case of high mannose units, we speculate that a few or no mannose residues would be removed if the ultimate high mannose unit has the same number of, or fewer, residues than the lipid-linked oligosaccharide precursor. If the final unit has more mannose, mannose residues may

![Figure 6: Schematic pathway of biosynthesis of asparagine-linked glycopeptides.](http://www.jbc.org/)

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**FIG. 6.** Schematic pathway of biosynthesis of asparagine-linked glycopeptides.
be added after the oligosaccharide is protein-bound. With regard to the formation of complex units, Kornfeld and co-workers have recently observed that the late stage of processing yields an oligosaccharide (Man₃GlcNAc₆) which is a critical intermediate. They have found that the removal of 2 mannose residues from the heptasaccharide requires the prior attachment of a particular N-acetylgalactosamine residue. Schachter and co-workers (18, 19) have also reported that the addition of a β1,3 N-acetylgalactosamine residue linked to a mannose terminus of a pentasaccharide inner core is a prerequisite for the attachment of a second distinct N-acetylgalactosamine and a fucose residue.

The results demonstrating that the processing sequence in membrane preparations from NIL fibroblasts and liver tissue involves glucose excision prior to mannose removal contrast with recent observations by Staneloni and Leloir (12). They reported that glucose was found in protein-bound oligosaccharides (generated by incubating thyroid slices with radioactive glucose) that seemed to be smaller by about 3 to 5 monosaccharide residues than the lipid-linked oligosaccharide precursor. These results suggest that, while processing of protein-bound oligosaccharides may be ubiquitous, the initial stages of the processing pathway may be common to some cells (removal of all glucose residues before mannose removal) but not to others (premature removal of mannose residues). However, since the experiments of Staneloni and Leloir (12) involve a long incubation period (3 h at 37°C), it is possible that they were not observing the initial stages of processing. For example, in their system, glucose residues may have been added to proteins after processing had been completed. It should be noted that several glycoproteins are known to contain glucose as a constituent of carbohydrate chains (6-9). The origin of these residues is unknown.

The glycosidases responsible for processing of oligosaccharides clearly will possess different properties than known lysosomal glycosidases. We already have evidence that the processing enzymes have activity at neutral pH. In addition, at least one of the glycosidases is associated with the rough endoplasmic reticulum. Other glycosidase(s) are located in both the rough and smooth endoplasmic reticulum, while the mannosidase may be distributed throughout the endoplasmic reticulum and Golgi apparatus. Touster and co-workers have, in fact, characterized an α-mannosidase from Golgi apparatus of rat liver (20, 21).

The factors that will determine whether the protein-bound oligosaccharide is destined to become a high mannose or complex oligosaccharide chain are unknown. It is probable that the structure of the glycosylated protein itself, i.e. primary sequence and tertiary structure, is important in determining the ultimate fate of the oligosaccharide. On the other hand, Ceccarini (22) and Muramatsu et al. (23) have reported that cell density may be a factor in influencing the appearance of the two types of asparaginyl carbohydrate units in cultured cells.

Acknowledgments—We are indebted to L. Grinna, S. C. Hubbard, T. Liu, and A. R. Robbins for helpful critical discussions of this work.

REFERENCES


Additional Refs. 1-13 are found on p. 4566.
Experimental Procedures

Materials. - UDP-[H-3H] (20 Ci/mmol) was purchased from Amersham and [U-14C]GlcNAc was obtained as described previously. Other radioactive and unlabeled radioactive precursors for GlcNAc and mannose, [1-14C]glucosamine and mannose, Type III, from Sigma Chemical Co.; [3H]thymidine from New England Nuclear; and [3H]uridine from Amersham. Labelled glycopeptides and oligosaccharides were purified by Bio-Gel P-2 column chromatography. [3H]GlcNAc and [3H]mannose were purchased from New England Nuclear and [3H]uridine from Amersham. [3H]thymidine was obtained from New England Nuclear. [14C]glucosamine and [14C]mannose were purchased from Amersham.

Enzymes. - Membranes derived from baby hamster kidney cells were prepared as described previously (9). Rat liver membranes were prepared by the method of Ungewickell and Unanue (10) and obtained from Dr. Louis Orten of this laboratory.

Chemicals. - All chemicals were obtained from Sigma Chemical Co. unless otherwise indicated.

Preparation of radioactive glycopeptides. - The preparation of radioactive glycopeptides has been described previously (9). The radioactive glycopeptides were isolated with organic solvents and purified on DEAE cellulose and Sephadex G-25 (11).

In vitro Transfer of Oligosaccharides. - Rat liver membranes were incubated in a medium containing 200 mM NaCl, 50 mM Tris-HCl (pH 7.5) and 2% bovine serum albumin. The reaction mixture was incubated for 2 h at 37°C (13). The reaction was stopped by the addition of a solution containing 0.1 M NaOH and 50 mM EDTA. The reaction mixture was centrifuged to remove the precipitate and the supernatant was used for the assay of NAA-oligosaccharides.

Analysis of NAA-oligosaccharides. - The method of analysis has been described previously (9). The NAA-oligosaccharides were isolated with organic solvents and purified on DEAE cellulose and Sephadex G-25 (11).

Preparation of radioactive reagents. - The preparation of radioactive reagents has been described previously (9).

In vitro Transfer of Oligosaccharides. - Rat liver membranes were incubated in a medium containing 200 mM NaCl, 50 mM Tris-HCl (pH 7.5) and 2% bovine serum albumin. The reaction mixture was incubated for 2 h at 37°C (13). The reaction was stopped by the addition of a solution containing 0.1 M NaOH and 50 mM EDTA. The reaction mixture was centrifuged to remove the precipitate and the supernatant was used for the assay of NAA-oligosaccharides.

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References

Initial Stages of Glycoprotein Processing In Vitro

**Figure 1.** Bio-Gel P-10 chromatogram of products of endo H treatment of [5,6-methyl-2,3-3H]-oligosaccharides released from IgM by mild acid hydrolysis. [5,6-methyl-2,3-3H]-oligosaccharides (600 cpm) and [3H]glucose oligosaccharides, both dimer and monomer, were chromato graphically separated on Bio-Gel P-10 columns. After application of the samples, the column was eluted with 2 M acetic acid. Fractions of 1.2 ml were collected. (a) [3H]deoxyoligosaccharides; (b) [3H]N-acetylglucosamines.

**Figure 2.** Bio-Gel P-10 chromatogram of products of endo H treated liver lysosomes. [5,6-methyl-2,3-3H]-oligosaccharides (600 cpm) and [3H]glucose oligosaccharides, both dimer and monomer, were chromatographed on Bio-Gel P-10 columns. After application of the samples, the column was eluted with 2 M acetic acid. Fractions of 1.2 ml were collected. (a) [3H]deoxyoligosaccharides; (b) [3H]N-acetylglucosamines.

**Figure 3.** Bio-Gel P-10 chromatogram of products of endo H treated lysosomes. [5,6-methyl-2,3-3H]-oligosaccharides (600 cpm) and [3H]glucose oligosaccharides, both dimer and monomer, were chromatographed on Bio-Gel P-10 columns. After application of the samples, the column was eluted with 2 M acetic acid. Fractions of 1.2 ml were collected. (a) [3H]deoxyoligosaccharides; (b) [3H]N-acetylglucosamines.

**Table 1**

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The initial stages of processing of protein-bound oligosaccharides in vitro.
S J Turco and P W Robbins


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