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Biosynthesis of Glycoconjugates in Mitochondrial Outer Membranes.

I. Evidence for a Direct *N*-Glycosylation of Endogenous Protein Acceptors from Nucleotide Sugars

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The results reported in this paper show two distinct ways for the incorporation of *N*-acetylglucosamine into mitochondrial outer membranes. The first one is the glycosylation of dolichol acceptors, which is indicated by the inhibition of the synthesis of these products by the inhibitors of the dolichol intermediates (tunicamycin and GDP). The second one is the incorporation of *N*-acetylglucosamine into protein acceptors directly from UDP-*N*-acetylglucosamine. This second way of glycosylation is only localized in mitochondria outer membranes.

The existence of a direct route for *N*-glycoprotein biosynthesis has been based on the following evidence. First, the synthesis of the *N*-acetylglucosaminylated protein acceptors was not inhibited by tunicamycin or GDP. Second, the addition of exogenous dolichol-phosphate did not change the rate of biosynthesis of glycosylated protein material. Third, the sequential incorporation of *N*-acetylglucosamine and mannose from their nucleotide derivatives in the presence of GDP and tunicamycin led to the synthesis of glycosylated protein material which entirely bound to Concanavalin A-Sepharose. The oligosaccharide moiety of the glycosylated protein material resulting from the direct transfer of sugars from their nucleotide derivatives to the protein acceptor is of the *N*-glycan type. On sodium dodecylsulphate polyacrylamide gel electrophoresis, this glycosylated material migrated as a marker protein with a molecular weight between 45 000 and 63 000. HPLC chromatofocusing analysis revealed that the fraction studied was anionic. The oligosaccharide moiety of the glycoprotein material can only be elongated by the incorporation of *N*-acetylglucosamine and galactose from their nucleotide derivatives.

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An examination of general reviews [1-3] on *N*-glycoprotein biosynthesis in animal cells and tissues shows that it is now well established that the biosynthesis of the asparagine-linked oligosaccharide units of glycoproteins is initiated by the "en bloc" transfer of a preformed oligosaccharide from an oligosaccharide-pyrophosphoryl-dolichol intermediate to an asparagine residue of the polypeptide chain. Moreover, the use of chemically synthesized dolichol-oligosaccharides as exogenous substrates [4-7] confirms the role of dolichol as a carrier of the growing *N*-oligosaccharide chain. Some years ago, our laboratory localized glycosyltransferase activities in mitochondrial outer membranes that transfer sugars from their nucleotide precursors into polyprenic acceptors [8-10]. From these studies, dolichol-phosphoryl-mannose, dolichol-diphosphoryl-*N*-acetylglucosamine, dolichol-pyrophosphoryl-*N,N'*-diacetylchitobiose and dolichol-phosphoryl-glucose have been well characterized.

Dolichol-pyrophosphoryl-oligosaccharides and glycosylated protein products have been found, but not investigated in depth. Moreover, lipid-linked oligosaccharides are synthesized to a limited extent while *N*-acetylglucosamine units are incorporated into proteins to a much greater extent. From these last two results, it was of interest to find out if in the mitochondrial outer membrane the polyprenic acceptors and proteins are involved in the same *N*-glycoprotein biosynthesis pathway or if they belong to two different ways of *N*-glycosylation.

The results reported in this paper demonstrate the existence of two different *N*-glycosylation pathways in the mitochondrial outer membranes: the pathway of dolichol intermediates involved in the *N*-glycosylation of proteins, and a second pathway for a direct incorporation of *N*-acetylglucosamine and mannose from their nucleotide precursors into protein acceptors.

Experimental Procedures

Materials

Mitochondria were isolated from adult female OF_1 mice (25 g). The following compounds were obtained from New England Nuclear, Boston, MA, USA: UDP-*N*-acetyl-D-[^{14}C]glucosamine (284 mCi/mmol), GDP-D-[^{14}C]mannose (282 mCi/mmol), UDP-D-[^{14}C]galactose (337 mCi/mmol), GDP-L-[^{14}C]fucose (255 mCi/mmol), CMP-[^{14}C]sialic acid (254 mCi/mmol). Concanavalin A-Sepharose 4B was from Pharmacia Fine Chemicals (Piscataway, NJ, USA). *Streptomyces griseus* protease, guanosine-diphosphate, tunicamycin and Triton-X-100 were from Sigma, St. Louis, MO, USA; Bio-Gel P-30 (200-400 mesh) was from Bio-Rad Laboratories, Richmond, CA, USA; and silica gel thin layer plates from E. Merck, Darmstadt, W. Germany.

Preparation of Mitochondria and Outer Membranes

Mitochondria were isolated according to the procedure described by Weinbach [11] and modified by Bustamente *et al.* [12]. Mouse livers were homogenized in a Dounce Homogenizer and the homogenate was fractionated by differential centrifugation in 250 mM sucrose, 10 mM Tris-HCl pH 7.4 as described previously [8]. Washed mitochondria were suspended in the same buffer and purified by mild ultrasonic treatment [8].

Outer membranes were broken up by swelling of purified mitochondria in 10 mM potassium phosphate pH 7.4.

Preparation of Subcellular Fractions

Fraction C 30 000 g: The post-mitochondrial supernatant was centrifuged at 30 000 × *g* for 30 min. The pellet was homogenized in 10 mM Tris-HCl pH 7.4 containing 250 mM sucrose and centrifuged at 10 000 × *g* for 15 min. The supernatant was centrifuged at 30 000 × *g* for 30 min to give the pellet, C 30 000 g (Golgi apparatus rich fraction). This second 30 000 × *g* supernatant was discarded.

Fraction C 100 000 g: The first 30 000 × *g* supernatant was centrifuged at 100 000 × *g* for 60 min to give the pellet, C 100 000 g (endoplasmic reticulum-rich fraction).

Standard Assay

a) Incorporation of *N*-acetyl-[^{14}C]glucosamine from UDP-*N*-acetyl-[^{14}C]glucosamine.
Protein (5-20 mg) of mitochondrial outer membrane was suspended in 20 mM MES buffer pH 6.1 (4 mg of protein/ml) containing 5 mM $MnCl_2$ and 1.9 μM UDP-*N*-acetyl-[^{14}C]glucosamine. After 30 min at 37°C the reaction was stopped.

b) Incorporation of [^{14}C]mannose from GDP-[^{14}C]mannose.
Protein (5-20 mg) of mitochondrial outer membrane was suspended in 20 mM MES buffer pH 6.8 (4 mg of protein/ml) containing 5 mM $MnCl_2$ and 6.1 μM GDP-[^{14}C]mannose. After 30 min at 37°C the reaction was stopped.

c) Sequential incorporation of *N*-acetyl-[^{14}C]glucosamine and [^{14}C]mannose from their nucleotide precursors.
Protein (5-20 mg) of mitochondrial outer membrane was suspended in 20 mM MES buffer pH 6.1 (4 mg of protein/ml) containing 5 mM $MnCl_2$, 50-200 μg tunicamycin and 1.9 μM UDP-*N*-acetyl-[^{14}C]glucosamine. After 30 min at 37°C, the medium was centrifuged. The supernatant was discarded and the pellet was suspended in 20 mM MES buffer pH 6.8 containing 5 mM $MnCl_2$ GDP and 6.1 μM GDP-[^{14}C]mannose. After 30 min at 37°C the reaction was stopped.

d) Sequential incorporation of *N*-acetyl-[^{14}C]glucosamine, [^{14}C]mannose, [^{14}C]galactose, [^{14}C]sialic acid and [^{14}C]fucose.
The sequential incorporation of the two first sugars (*N*-acetyl-[^{14}C]glucosamine and [^{14}C]mannose) was done as described in c) above. The concentration of each labelled nucleotide sugar was as follows: 1.9 μM UDP-*N*-acetyl-[^{14}C]glucosamine and 6.1 μM UDP-[^{14}C]-galactose, CMP-[^{14}C]sialic acid and GDP-[^{14}C]fucose. The incorporation of these latter sugars is described in the Results section. The medium was incubated at 37°C for 30 min.

In each case, the reaction was stopped by addition of 20 vol of chloroform/methanol, 2/1 by vol. The protein pellet was washed with water three times more and extracted with chloroform/methanol/water, 10/10/3 by vol, twice more.

Gel Permeation Chromatography

Bio-Gel P-30 (200-400 mesh) chromatography was performed on a column (2 × 150 cm) and run in 100 mM pyridine/acetic acid pH 5.4 containing 0.1% sodium azide. Bio-Gel P-6

(100-200 mesh) chromatography was performed on a column (1 × 100 cm) and run in 100 mM pyridine/acetic acid pH 5.4 containing 0.1% sodium azide.

Lectin Affinity Chromatography

The radiolabelled sample was applied to a column (Pasteur pipette) containing Concanavalin A-Sepharose in Tris buffered saline (TBS; 150 mM NaCl, 10 mM Tris, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 0.02% sodium azide, pH 7.2). The material was eluted from the column with TBS, then α-methyl mannoside in TBS. The fractions collected were counted by liquid scintillation.

Thin Layer Chromatography

TLC of the glycosylated polyprenic products was performed on a silica gel 60 plate which was developed three times in chloroform/methanol/ammonium hydroxide/water, 70/44/5/5, by vol. The radioactivity on the plate was located by radioscanning (Scanner 2022 Packard).

HPLC Technique

HPLC was carried out on a Waters (Waters Associates, Milford, MA, USA) equipment. Chromatography was performed on a MonoP HR 5/20 (Pharmacia) column which had been previously equilibrated with 75 mM Tris-acetic acid buffer, pH 9.3. Elution was achieved with an internal linear pH gradient (pH 9.0 to pH 6.0) prepared with Polybuffer 96 (Pharmacia), followed by an external non-linear pH gradient: 4 - 3.5 - 3 - 2.5 (25 mM formic acid adjusted with concentrated ammonium hydroxide) at a flow rate of 1 ml/min.

SDS-Polyacrylamide Gel Electrophoresis

Analytical polyacrylamide gel electrophoresis was performed in the presence of sodium dodecylsulphate (SDS) on 10% acrylamide slab gels according to the procedure of Laemmli [14]. The gels were stained and destained as described [15]. Gels were sliced and allowed to dry according to the method of Goodman and Matzura [16]. Radioactivity was monitored by liquid scintillation counting.

Preparation of Glycopeptides from the Standard Assay

The labelled delipidated protein pellet was suspended in 50 mM Tris buffer pH 8 containing 2 mM $CaCl_2$ and protease (1 mg/5 mg labelled delipidated protein). The mixture was incubated at 40°C under toluene for 48 h. Two additional protease solutions were added during incubation. The digest was chromatographied on a Bio-Gel P-30 column (2 × 150 cm).

Hydrazinolysis of the Labelled Glycopeptides

The hydrazinolysis procedure was carried out as described by Takasaki *et al.* [17]. Under these conditions there is no degradation at the reducing end of the released oligosaccharides.

Table 1. Incorporation of *N*-acetyl-[^{14}C]glucosamine into polyprenic acceptors and protein material in the presence of inhibitors of the glycosylation of polyprenic compounds.

Each incubation mixture contained 6 mg of outer membrane protein suspended in 20 mM MES buffer pH 6.1 containing 5 mM $MnCl_2$ and 1.9 μM UDP-*N*-acetyl-[^{14}C]glucosamine. Assay 1: control + 50 μg of tunicamycin. Assay 2: control + 2 mM GDP. Assay 3: control + 50 μg of tunicamycin and 2 mM GDP. Assay 4: control + 0.1 mM dolichol-monophosphate.

Incubation mixtures	Incorporation (cpm)		
	2:1 Extract	10:10:3 Extract	Protein pellet
Control	2030 (2%)	400 (0.4%)	96070 (97.6%)
Assay 1	930	244	92320
R 1[a]	0.46	—	0.96
Assay 2	2492	320	128136
R 2[a]	1.23	—	1.30
Assay 3	936	280	115927
R 3[a]	0.46	—	1.20
Assay 4	28025	2225	95225

[a] R 1, etc = ratio Assay 1, etc : Control.

Acid Hydrolysis of Reduced Oligosaccharides

Reduced oligosaccharides were hydrolyzed in 1 N HCl at 100°C for 2 h. The hydrolysates were evaporated to dryness and then reacetylated with acetic anhydride.

Paper Chromatography of N-Acetylhexosamines and Acetylhexosaminitols

For descending paper chromatography, Whatman No. 1 paper was moistened with 0.05 M sodium borate containing 0.01 M NaCl, pH 8.4, and dried overnight at room temperature. The development was carried out for 20-36 h with ethyl acetate/2-propanol/pyridine/water, 7/3/2/2 by vol, which is a solvent for separation of *N*-acetylglucosamine and *N*-acetylglucosaminitol. The unlabelled monosaccharides were detected with alkaline silver nitrate. The radioactive monosaccharides were detected by liquid scintillation.

Results

Two Glycosylation Pathways in Mitochondrial Outer Membranes

Mitochondrial outer membranes were incubated with UDP-*N*-acetyl-[^{14}C]glucosamine as described in Table 1. The results summarized in Table 1 show three labelled fractions.

Fraction "2:1 extract" (control) contained 2% of the radioactivity; TLC revealed two compounds respectively identified as dolichol-pyrophosphoryl-di-*N*-acetylchitobiose (Dol-P-P-GlcNAc$_2$) and dolichol-pyrophosphoryl-*N*-acetylglucosamine (Dol-P-P-GlcNAc) plus another labelled compound which was located at the origin of the chromatogram, and which probably is a dolichol-pyrophosphoryl-oligosaccharide resulting from the

Table 2. Comparative study of the incorporation of *N*-acetyl-[^{14}C]glucosamine into polyprenic acceptors and protein material in three subcellular fractions in the presence of inhibitors of the glycosylation of polyprenic compounds.

Incubation mixtures[a]		Incorporation (cpm)		
		2:1 Extract	10:10:3 Extract	Protein pellet
OM[b]	control[e]	2028	400	96070
	assay[f]	936	260	115927
		54%	35%	
C 30000 g[c]	control	10852	732	4048
	assay	344	168	2656
		97%	77%	35.5%
C 100000 g[d]	control	2428	596	8042
	assay	316	308	3312
		87%	48%	59%

[a] The conditions of incubation were identical to those described in Table 1. The inhibitors (tunicamycin and GDP) were only used together.
[b] OM: outer membrane.
[c] C 30000 g: Golgi apparatus rich fraction.
[c] C 100000 g: endoplasmic reticulum rich fraction.
[e] control: subcellular fraction (6 mg of protein) + UDP-[^{14}C]GlcNAc.
[f] assay: subcellular fraction (6 mg of protein) + UDP-[^{14}C]GlcNAc + tunicamycin + GDP.
[g] The percent values represent the % inhibition by tunicamycin and GDP calculated as follows: % = (1 — assay/control) × 100.

elongation of Dol-P-P-[^{14}C]GlcNAc$_2$ by endogenous guanosine-diphosphate-mannose (GDP-Man). The addition of tunicamycin, an inhibitor of the biosynthesis of Dol-P-P-GlcNAc and Dol-P-P-GlcNAc$_2$ decreased the synthesis of these compounds (Assay 1). The addition of guanosine diphosphate (GDP), an inhibitor of the biosynthesis of Dol-P-Man, slightly increased the synthesis of the "2:1 extract" compounds (Assay 2). The addition of both inhibitors (tunicamycin plus GDP, Assay 3) did not modify the inhibition caused by tunicamycin alone (Assay 1).

The fraction "10:10:3 extract" (control) contained 0.4% of the radioactivity, which was insufficient for analysis by TLC. The effect of inhibitors (Assay 1, 2, 3) could not be studied due to the low incorporation.

The fraction "protein pellet" (control) contained 97.6% of the radioactivity. The addition of tunicamycin did not alter the labelling of the protein pellet (Assay 1). The addition of GDP slightly increased the rate of the radioactivity incorporated in the protein material. The addition of both (GDP plus tunicamycin) did not result in any further modifications.

The addition of exogenous dolichol-phosphate (0.1 mM) to the incubation mixture only increased the biosynthesis of the dolichol-linked products (Table 1, Assay 4). Under these conditions, the synthesis of Dol-P-P-[^{14}C]GlcNAc and Dol-P-P-[^{14}C]GlcNAc$_2$ (2:1 extract) was increased much more than the synthesis of Dol-P-P-[^{14}C]oligosaccharides (10:10:3 extract) while the rate of radioactivity incorporated into the protein material was unmodified.

These data suggest two different ways for the incorporation of *N*-acetyl-[^{14}C]glucosamine into mitochondrial outer membranes: 1) glycosylation of dolichol-acceptors, which is supported by the inhibition of the synthesis of these products by the inhibitors of the dolichol intermediates; 2) glycosylation of protein acceptors directly from nucleotide-sugars, which is confirmed by the absence of inhibition of the glycosylated products synthesis by GDP and tunicamycin.

In order to show that the second way of glycosylation only occurs in the mitochondrial outer membrane (OM), other subcellular fractions such as the Golgi apparatus rich fraction (C 30 000 g) and the endoplasmic reticulum rich fraction (C 100 000 g) were incubated with UDP-*N*-acetyl-[^{14}C]glucosamine as described in Table 1. The results are summarized in Table 2. The incorporation of the *N*-acetyl-[^{14}C]glucosamine into the polyprenic acceptors (extracts 2:1 and extracts 10:10:3) was similar in the three subcellular fractions (OM, C 30 000 g and C 100 000 g) except for the 2:1 extract (control) resulting from the C 30 000 g fraction which contained five times more radioactivity. The incorporation of *N*-acetyl-[^{14}C]glucosamine into protein acceptors of the OM fraction was 24 and 12 times higher, respectively, than the incorporation into protein acceptors of the C 30 000 g fraction and the C 100 000 g fraction. The addition of both inhibitors (tunicamycin and GDP) to the incubation mixtures (assay) dramatically decreased the incorporation of *N*-acetyl-[^{14}C]glucosamine into polyprenic and protein acceptors of the C 30 000 g fraction and the C 100 000 g fraction. Under these conditions the incorporation of the *N*-acetyl-[^{14}C]glucosamine into polyprenic acceptors of the OM fraction decreased too, while the incorporation into protein acceptors increased.

All these data show two important points:
1) the synthesis of *N*-acetylglucosaminylated protein compounds is much higher in the mitochondrial outer membrane than in the Golgi apparatus-rich fraction and the endoplasmic reticulum-rich fraction;
2) in these two latter subcellular fractions the inhibition of the synthesis of the *N*-acetylglucosaminylated dolichol compounds involves the decrease of the incorporation of *N*-acetylglucosamine into the protein acceptors. In the mitochondrial outer membrane the inhibition of the synthesis of these compounds does not lead to the decrease of the incorporation of *N*-acetylglucosamine into the protein acceptor. Based on these data the second way for the incorporation of *N*-acetylglucosamine into protein acceptors from a nucleotide-donor does not arise from any membrane contaminants, especially the C 30 000 g and C 100 000 g fractions.

In order to obtain further evidence for the existence of a second route of glycosylation, mitochondrial outer membranes were incubated with GDP-[^{14}C]mannose. The results summarized in Table 3 show three labelled fractions:
1) The fraction "2:1 extract" (control) contained 77% of the radioactivity. TLC revealed two compounds, one of which was Dol-P-Man (major product) and the other labelled compound was located at the origin of the chromatogram, and is probably a dolichol-pyrophosphoryl-oligosaccharide (minor product) resulting from the elongation of endogenous Dol-P-P-GlcNAc$_2$ by GDP-[^{14}C]mannose. The addition of tunicamycin did not change the rate of the radioactivity incorporated in the "2:1 extract" (Assay 1'). The addition of GDP dramatically decreased the labelling of the "2:1 extract", especially the synthesis of Dol-P-[^{14}C]Man (Assay 2', Assay 3').
2) The fraction "10:10:3 extract" (control) contained 9.6% of the radioactivity; TLC revealed the presence of a small amount of Dol-P-Man and two products which migrated as

Table 3. Incorporation of [^{14}C]mannose into polyprenic acceptors and protein material in the presence of inhibitors of the glycosylation of polyprenic compounds.

Each incubation mixture contained 6 mg of outer membrane protein suspended in 20 mM MES buffer pH 6.8 containing 5 mM $MnCl_2$ and 6.1 μM GDP-[^{14}C]mannose. Assay 1': Control + 50 μg of tunicamycin. Assay 2': Control + 2 mM GDP. Assay 3': Control + 50 μg of tunicamycin and 2 mM GDP. Assay 4': Control + 0.1 mM dolichol-monophosphate.

Incubation mixtures	Incorporation (cpm)		
	2:1 Extract	10:10:3 Extract	Protein pellet
Control	128 144 (77%)	15 910 (9.6%)	22 410 (13.4%)
Assay 1'	122 610	19 600	24 140
R 1'[a]	0.96	1.2	1.07
Assay 2'	2 830	1 430	8 140
R 2'[a]	0.02	0.09	0.36
Assay 3'	4 140	2 100	9 260
R 3'[a]	0.03	0.13	0.40
Assay 4'	294 900	49 900	19 000

[a] R 1' etc = ratio Assay 1', etc : Control.

dolichol-pyrophosphoryl-oligosaccharides. The addition of tunicamycin slightly increased the rate of incorporation of the radioactivity (Assay 1'). The addition of GDP greatly decreased the synthesis of the labelled products (Assay 2', Assay 3').
3) The fraction "protein pellet" (control) contained 13.4% of the radioactivity incorporated in protein acceptors. The addition of tunicamycin did not change this percentage of radioactivity (Assay 1'). On the other hand, the addition of GDP significantly decreased the incorporation of radioactivity into the protein material (Assay 2', Assay 3').

The addition of exogenous dolichol-phosphate (0.1 mM) to the incubation mixture increased the incorporation of the [^{14}C]mannose in the lipid fractions (2:1 extract and 10:10:3 extract; the latter contained a significant amount of labelled Dol-P-Man) and slightly decreased the incorporation of [^{14}C]mannose in the protein fraction (Table 3, Assay 4'). The latter data, especially the inhibition of the synthesis of lipid-linked oligosaccharides and glycosylated protein material by GDP, emphasize the existence of a dolichol intermediates pathway.

In order to confirm and to investigate the existence of these two ways of glycosylation, we studied the sequential incorporation of *N*-acetyl-[^{14}C]glucosamine and [^{14}C]mannose into mitochondrial outer membranes. The results summarized in Table 4 show three labelled fractions (control) as described previously. The addition of tunicamycin and GDP (Assay) inhibited the synthesis of "2:1 extract" products, but did not alter the synthesis of "10:10:3 extract" products. This latter result may be explained by the elongation of the large lipid-linked oligosaccharides from endogenous lipid-linked oligosaccharides and exogenous GDP-[^{14}C]mannose. The glycosylation of protein material was increased by the addition of tunicamycin and GDP. These results show that the sequential incorporation of *N*-acetyl-[^{14}C]glucosamine and [^{14}C]mannose does not change all the results described above.

Table 4. Sequential incorporation of *N*-acetyl-[^{14}C]glucosamine and [^{14}C]mannose into polyprenic acceptors and protein material in the presence of inhibitors of glycosylation of polyprenic compounds.

Each incubation mixture contained 10 mg of outer membrane protein. Mitochondrial outer membranes were incubated as described under "Experimental Procedures". The control did not contain any inhibitors such as tunicamycin or GDP.

Incubation mixtures	Incorporation (cpm)		
	2:1 Extract	10:10:3 Extract	Protein pellet
Control	119808 (36%)	7662 (2%)	205560 (62%)
Assay[a]	31941	7119	271620
R[b]	0.26	0.93	1.3

[a] Assay = control + tunicamycin and GDP.
[b] R = ratio Assay:Control.

In order to determine if mannose units were incorporated in an *N*-acetylglucosaminylated protein pellet, it (Table 4, Assay) was digested by pronase and a ^{14}C-glycopeptide fraction was isolated on Bio-Gel P-30, which was analyzed on Concanavalin A-Sepharose (Con A-Sepharose). All the radioactivity was bound to Con A-Sepharose and eluted with 10 mM α-methyl-mannoside (Fig. 1). From this result, the glycosylated protein material (Table 4, Assay) was shown to contain at least two α-linked mannose residues. These data show that the [^{14}C]mannose units incorporated in *N*-acetylglucosaminylated protein material arise from GDP-mannose only; under these experimental conditions the synthesis of dolichol intermediates is greatly inhibited (Table 1, Assay 3; Table 3, Assay 3').

All these data provide good evidence for the existence of two distinct glycosylation pathways in mitochondrial outer membranes: 1) the dolichol intermediates pathway; 2) the glycosylation (*N*-acetylglucosamine, mannose) of protein acceptors directly from nucleotide sugars (UDP-*N*-acetylglucosamine, GDP-mannose).

Nature of the Oligosaccharide Linkage

The "protein pellet" fractions resulting from the incubation, Assay 1 (Table 1), and from the incubation, Assay (Table 4), were digested by pronase and fractionated on Bio-Gel P-30. The two pools of ^{14}C-glycopeptides were subjected to hydrazinolysis under the conditions described in the "Experimental Procedure" section, in order to check that the oligosaccharide moiety is of the *N*-glycan type. After hydrazinolysis, the reducing extremity of the released [^{14}C]-oligosaccharide chain was reduced with tritiated sodium borohydride. The tritiated [^{14}C]-oligosaccharide fractions were subjected to paper chromatography using 1-butanol/ethanol/water, 4/1/1 by vol, in order to eliminate all tritiated impurities. The labelled oligosaccharide fractions thus obtained were analyzed by Bio-Gel P-6 chromatography. Fig. 2 shows the elution profile of the reduced radioactive oligosaccharide fraction arising from the [^{14}C]-glycopeptide pool labelled with *N*-acetyl-[^{14}C]glucosamine (a) and the elution profile of the reduced radioactive

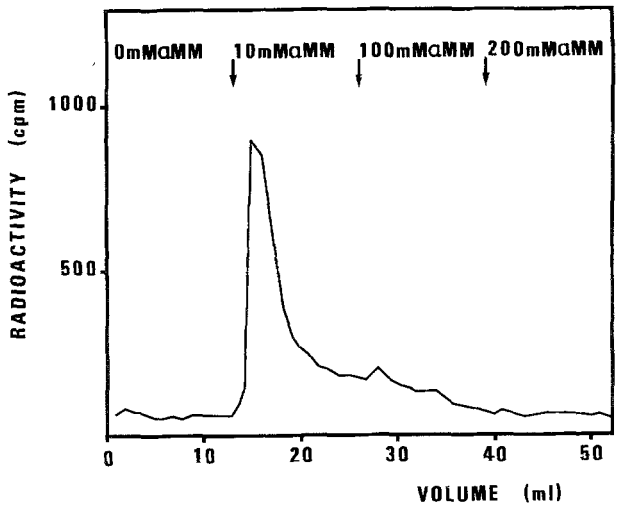

Figure 1. Concanavalin A-Sepharose chromatography of the ^{14}C-glycopeptides. A concanavalin A-Sepharose column (0.5 × 4 cm) was eluted with TBS, then 10 mM, 100 mM and 200 mM α-methyl mannoside (αMM) in TBS. Fractions (1 ml) were collected and counted for radioactivity.

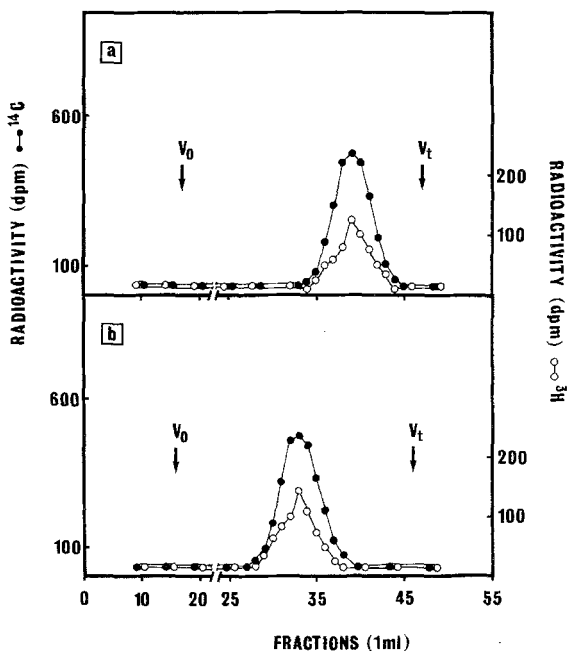

Figure 2. Gel filtration of the double labelled oligosaccharide fractions released by hydrazinolysis. The labelled material was analyzed on a Bio-Gel P-6 column eluted with 100 mM pyridinium acetate pH 5 containing 0.5 mM sodium azide.

a) elution profile of the reduced radioactive oligosaccharide fraction arising from the ^{14}C-glycopeptide pool labelled with *N*-acetyl-[^{14}C]glucosamine.

b) elution profile of the reduced radioactive oligosaccharide fraction arising from the ^{14}C-glycopeptide pool labelled with *N*-acetyl-[^{14}C]glucosamine and [^{14}C]mannose.

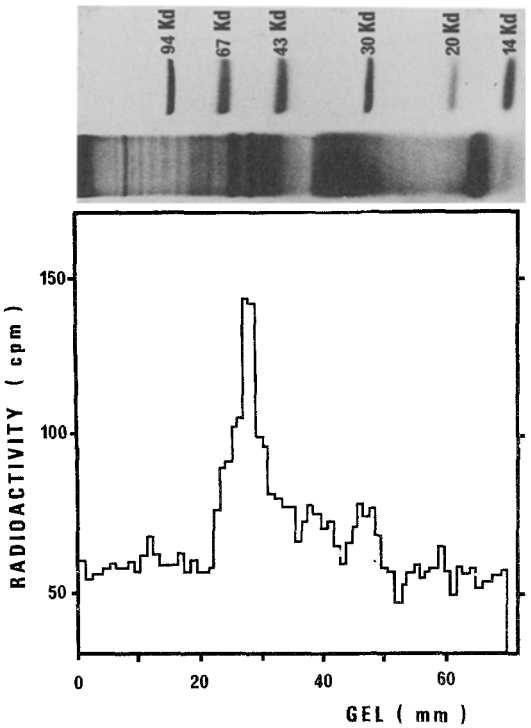

Figure 3. SDS 10% polyacrylamide gel electrophoresis of the ^{14}C-glycosylated protein fraction. Triton-X 100 supernatant containing ^{14}C-glycosylated protein material was applied to the SDS polyacrylamide gel. The figure shows the protein pattern and the radioactive profile of the ^{14}C-glycosylated protein material.

oligosaccharide fraction arising from the [^{14}C]-glycopeptide pool labelled with *N*-acetyl-[^{14}C]glucosamine and [^{14}C]mannose (b). In both cases, each radioactive oligosaccharide fraction was eluted as a single peak, double labelled with ^{3}H and ^{14}C. The reduced oligosaccharides were hydrolyzed in 1 N HCl at 100°C for 2 h and the resulting hydrolyzates were subjected to paper chromatography as described in the "Experimental Procedures" section. In both cases, the double labelling was only identified in the *N*-acetylglucosaminitol area. These results show the existence of *N*-glycosidic linkages characterized by the presence of *N*-acetylglucosamine in the studied glycoprotein. Both ^{14}C-glycopeptide pools were subjected to mild β-elimination under Carlson's conditions which only cleaves *O*-glycosidic linkages. No double labelled oligosaccharides were released by this treatment. This result rules out the presence of *O*-glycosidic linkages in the studied glycoprotein.

Analysis of the Labelled Protein Fraction

In order to inhibit the biosynthesis of the glycosylated dolichol compounds, tunicamycin and GDP were added to the incubation mixture described under "Experimental Procedures". At the end of the incubation time, the reaction mixture was centrifuged, the supernatant was discarded and the protein pellet was washed three times with MES buffer pH 6.8. The washed pellet was solubilized with 1.5% Triton X-100.

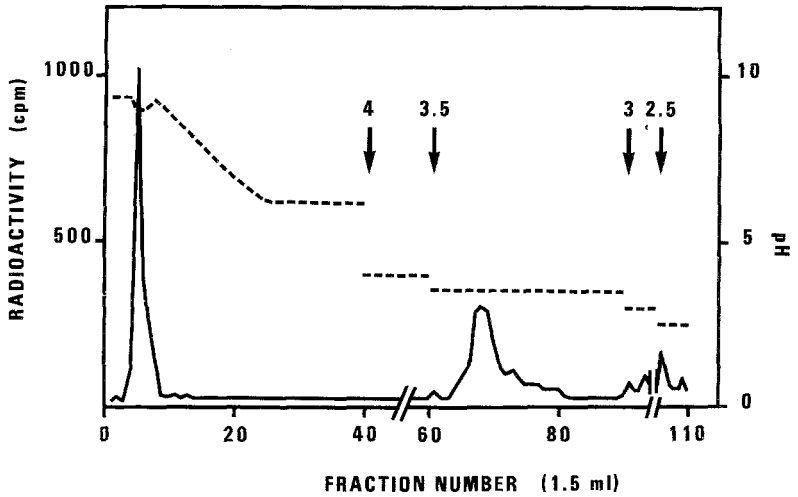

Figure 4. Chromatofocusing of ^{14}C-glycosylated protein fraction. The ^{14}C-glycosylated protein material was applied to the Polybuffer 96 exchanger pre-equilibrated with 75 mM Tris-acetic acid buffer pH 9.3. Elution was carried out with an internal linear pH gradient from 9 to 6, then with an external non-linear pH gradient: 4 - 3.5 - 3 - 2.5. The figure shows the radioactive profile of the solubilized ^{14}C-glycosylated protein fraction.

After centrifuging, 80-90% of the labelled material was recovered in the Triton supernatant. This supernatant was analyzed by SDS polyacrylamide gel electrophoresis and HPLC-chromatofocusing.

The gel electrophoresis analysis of the solubilized ^{14}C-material is shown in Fig. 3. The gel displayed several protein bands. The radioactive pattern showed a single labelled peak which migrated between 67 000 and 43 000 Da, estimated by use of marker proteins.

HPLC-chromatofocusing on the ion exchange PBE 96 combines internal pH gradient and discontinuous gradient in a pH range from 4 to 2. The representative profile is shown in Fig. 4; 35% of the radioactive material was recovered in a single sharp peak eluted at the dead volume of the column. Most of the remainder was eluted in a broad peak at around pH 3.5, followed by some trails of radioactive material. The last fraction eluted at low pH is very anionic.

All these results show that the mitochondrial outer membrane synthesizes a glycoprotein composed of a neutral subunit and an anionic subunit.

Nature of the Oligosaccharide Chain of the Studied Glycoprotein

The asparagine-linked oligosaccharides of *N*-glycoproteins have heterogenous structures which generally fall into two categories, "high mannose" and "complex". Both classes have an inner core of Man_3-$GlcNAc_2$ at the reducing terminus; they differ in that high-mannose glycans contain additional linked mannose residues, while complex oligosaccharides carry other external sugars, such as *N*-acetylglucosamine, galactose, fucose and sialic acid. In order to determine whether the oligosaccharide chain of the

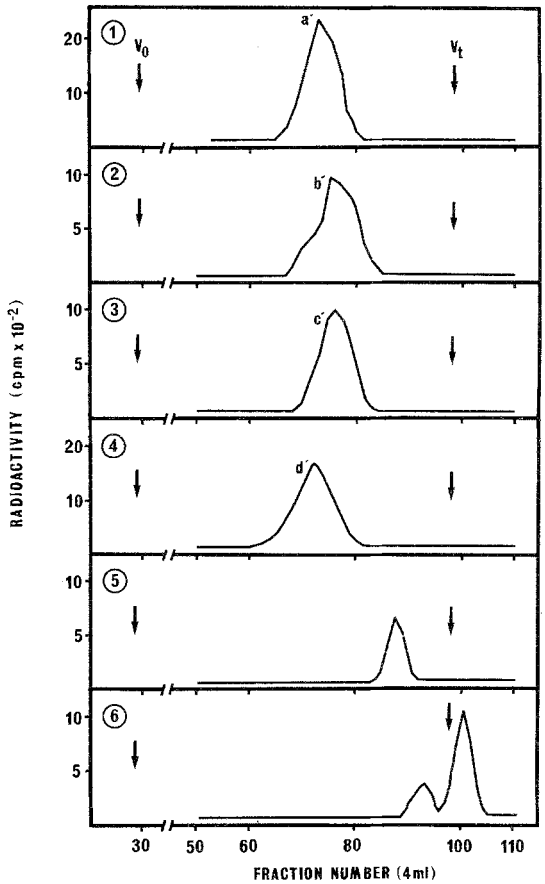

Figure 5. Gel filtration of the ^{14}C-glycopeptides from ^{14}C-glycosylated protein fractions. The labelled protein material resulting from the different incubation mixtures described in the section "Nature of the Oligosaccharide Chain of the Studied Glycoprotein" were digested with pronase. The released ^{14}C-glycopeptides were applied to a Bio-Gel P-30 column (2 × 150 cm) eluted with 100 mM pyridinium acetate pH 5 containing 0.5 mM sodium azide. The figure shows the profiles as follows: 1) the incubation mixture contained UDP-[^{14}C]GlcNAc only; the other incubation mixtures each contained unlabelled UDP-GlcNAc plus the following additional nucleotide sugars: 2) GDP-[^{14}C]Man, 3) GDP-Man + UDP-[^{14}C]GlcNAc, 4) GDP-Man + UDP-GlcNAc + UDP-[^{14}C]Gal.

studied glycoprotein belongs to the complex type, we studied the sequential addition of the external sugars to the growing oligosaccharide chain *in vitro*. The following nucleotide sugars: UDP-*N*-acetylglucosamine (UDP-GlcNAc), GDP-mannose (GDP-Man), UDP-galactose (UDP-Gal), CMP-*N*-acatylneuraminic acid (CMP-NeuAc) and GDP-fucose (GDP-Fuc) were added one after the other to the incubation mixture. The labelled protein material resulting from the different incubations was digested with pronase and the resulting digests were analyzed on Bio-Gel P-30 chromatography.

In the first experiment only the nucleotide sugars participating in the incorporation of each external sugar were labelled, all the others nucleotide sugars were unlabelled. Bio-Gel P-30 profiles (Fig. 5) represent the analysis of each digested incubation mixture.

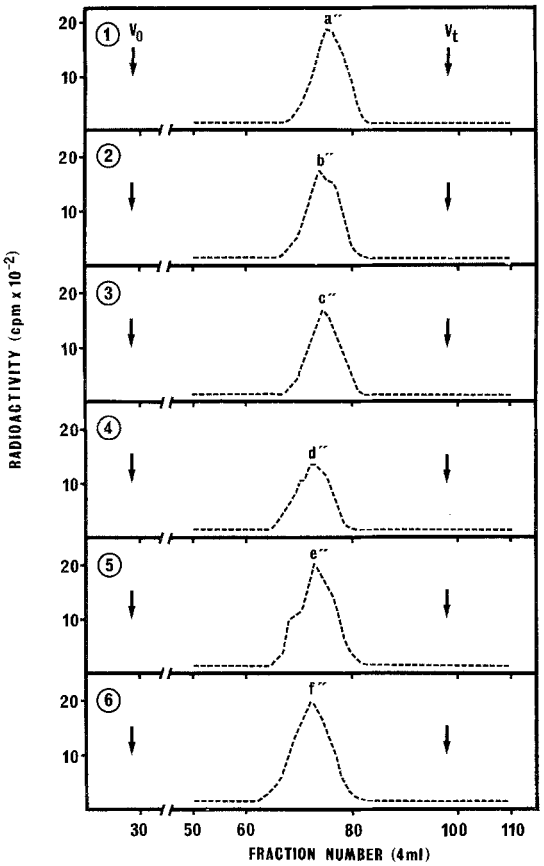

Figure 6. Gel filtration of the ^{14}C-glycopeptides from ^{14}C-glycosylated protein fractions. The labelled protein material resulting from the different incubation mixtures described in the paragraph "Nature of the Oligosaccharide Chain of the Studied Glycoprotein" was digested with pronase. The released ^{14}C-glycopeptides were applied to a Bio-Gel P-30 column (2 × 150 cm) eluted with 100 mM pyridinium acetate pH 5 containing 0.5 mM sodium azide. The figure shows the profiles as follows: 1) the incubation mixture contained UDP-[^{14}C]GlcNAc only; the other incubation mixtures each contained UDP-[^{14}C]GlcNAc plus the following additional unlabelled nucleotide sugars: 2) GDP-Man, 3) GDP-Man + UDP-GlcNAc, 4) GDP-Man + UDP-GlcNAc + UDP-Gal.

When the outer membrane was incubated with UDP-[^{14}C]GlcNAc only, (1); the corresponding digested ^{14}C-material was eluted as a single peak **a'**. Further addition of GDP-[^{14}C]Man, (2); GDP-Man plus UDP-[^{14}C]GlcNAc, (3) and GDP-Man plus UDP-GlcNAc plus UDP-[^{14}C]Gal, (4); respectively, gave the peaks **b',c'** and **d'**. The peaks **a', b',c'** and **d'** were eluted in the same area. Further addition of CMP-[^{14}C]NeuAc, (5); or GDP-[^{14}C]Fuc, (6), gave one and two labelled fractions, respectively. In each case, these latter fractions were not eluted in the area of the peaks **a', b', c',** and **d'**.

In the second experiment only the nucleotide sugar (UDP-[^{14}C]GlcNAc) involved in the incorporation of the most internal sugar was labelled, the other nucleotide sugars (GDP-Man, UDP-GlcNAc, UDP-Gal, CMP-NeuAc and GDP-Fuc) were unlabelled. The labelled protein material obtained from the different incubations was digested with pronase and the resulting digests were analyzed on a Bio-Gel P-30 column. Fig. 6 shows

the Bio-Gel P-30 profiles resulting from the analysis of each digested incubation mixture. The use of unlabelled GDP-Man, UDP-GlcNAc and UDP-Gal in the different incubation media, (2)-(4), instead of their labelled counterparts did not change the elution position of each resulting glycosylated fraction (cf. Fig. 5). The addition of unlabelled GDP-Fuc or CMP-NeuAc gave one fraction each (Fig. 6, lanes 5 and 6). These latter fractions (peaks **e''** and **f''** in Fig. 6) were eluted in the same position as the other fractions corresponding to the peaks **a'',b'', c''** and **d''** (Fig. 6).

All these results show that mannose, *N*-acetylglucosamine and galactose participate in the sequential elongation of the same nascent oligosaccharide chain of the studied glycoprotein. In addition, they suggest that under the experimental conditions used neither GDP-fucose nor CMP-*N*-acetylneuraminic acid participate in the elongation of the oligosaccharide chain of the studied glycoprotein or that mitochondrial outer membrane contains an endogenous pool of both nucleotide sugars. We therefore conclude that the oligosaccharide chain of the studied glycoprotein is a complex type chain.

Discussion

In this paper we have demonstrated that mitochondrial outer membranes catalyze the incorporation of *N*-acetylglucosamine from UDP-*N*-acetylglucosamine, and mannose from GDP-mannose into endogenous polyprenic acceptors as well as into membrane-associated protein acceptors. The glycosylated products are Dol-P-Man, Dol-P-P-GlcNAc, Dol-P-P-$GlcNAc_2$, lipid-linked oligosaccharides and mannosylated protein material. A large amount of the *N*-acetylglucosaminylated protein material is synthesized. Several critical experimental results provide evidence that nucleotide sugars are the direct donors for the glycosylation of polyprenic acceptors and protein material. In the first experiment, tunicamycin was used to block the incorporation of *N*-acetylglucosamine from UDP-*N*-acetylglucosamine in polyprenic acceptors. Tunicamycin has been shown to inhibit specifically the transfer of *N*-acetylglucosamine-1-phosphate from sugar nucleotide to dolichol monophosphate by brain membrane preparations [18] and in other eucaryotic systems [19-22]. In our conditions of incubation, only the synthesis of *N*-acetylglucosaminylated polyprenic products was decreased, the synthesis of *N*-acetylglucosaminylated protein material was not altered. The additon of exogenous dolichol monophosphate only increased the synthesis of glycosylated products resulting from the dolichol intermediates pathway, the rate of formation of *N*-acetylglucosaminylated protein products was unchanged.

The comparative study of the incorporation of *N*-acetylglucosamine into polyprenic acceptors and protein acceptors in the mitochondrial outer membrane fraction, Golgi apparatus-rich fraction and endoplasmic reticulum-rich fraction shows that this second way for the incorporation of *N*-acetylglucosamine from its nucleotide donor into protein acceptors is only localized in the mitochondrial outer membrane and does not result from subcellular contamination. In the second experiment, GDP was used to inhibit the biosynthesis of Dol-P-Man [23] which is the donor of the last four mannose residues for the elongation of the heptasaccharide-lipid [3], and on the other hand, indirectly to inhibit the transfer of oligosaccharide moiety to the protein acceptor. In this case, the incorporation of mannose alone into polyprenic acceptors and protein accep-

tors was dramatically decreased. In the presence of the inhibitors of the dolichol intermediates biosynthesis, the sequential incorporation of *N*-acetylglucosamine and mannose into protein acceptors was increased, only the incorporation of these sugars into polyprenic products was inhibited. Consequently, the carbohydrate moiety of the glycoprotein products synthesized under these conditions does not result from any transfer from the dolichol derivatives. The affinity of the oligosaccharide moiety of these products to Concanavalin A-Sepharose shows that the oligosaccharide chain must contain two α-linked mannose residues. The oligosaccharide linkage is of the *N*-glycan type. The SDS polyacrylamide gel electrophoresis analysis of this glycosylated protein material shows an *N*-glycoprotein having a molecular weight estimated to be between 45 000 and 63 000, and composed of a neutral subunit and an anionic subunit. The sequential elongation of the oligosaccharide moiety in the presence of external sugars shows that the studied *N*-glycoprotein is a complex glycoprotein. All the data reported here present a convincing argument for the existence of two different glycosylation pathways in mitochondrial outer membranes: 1) the dolichol intermediate pathway; 2) a direct pathway for *N*-glycoprotein biosynthesis using nucleotide sugars as unique donors of carbohydrate units for the *N*-glycosylation of protein acceptors.

Among the extensive literature on the different ways for *N*-acetylglucosamine incorporation in the biosynthesis of *N*-glycoprotein, it is of interest to consider some studies reporting the existence of other pathways that do not follow the established rule. Lehle and Tanner [24] reported that yeast membranes catalyze the transfer of both di-*N*-acetylchitobiose and mannosyl-di-*N*-acetylchitobiose from their dolichol derivatives to endogenous glycoprotein; they also demonstrated the participation of Dol-P-Man in the formation of *O*-glycosidic linkages in yeast cell wall mannoprotein. Harford and Waechter [25] demonstrated the transfer of di-*N*-acetylchitobiose from dolichol diphosphate to grey matter glycoprotein. In this case, tunicamycin prevented the enzymatic labelling of the grey matter glycoprotein having an apparent molecular weight of 24 000. Hoflack *et al.* [26, 27] found two pathways for *N*-glycosylation of proteins in rat-spleen lymphocytes: the classical way involving the dolichol pathway, and another way in which *N*-acetylglucosamine and chitobiosyl residues were transferred to proteins from their dolichol intermediates; the glycoprotein biosynthesized can be further elongated in the presence of GDP-mannose resulting in an oligomannosyl moiety. The latter two research groups demonstrated the direct transfer of the disaccharide from the carrier lipid to protein by using tunicamycin to block glycolipid biosynthesis. Chen and Lennarz [28] provided good evidence for the direct transfer of a trisaccharide from its lipid-linked form to the oviduct membrane-associated protein, a fact which did not exclude the participation of an oligosaccharide-lipid in glycosylation of hen oviduct membrane protein [29-31]. These reports, using tunicamycin, show that these second pathways for *N*-glycosylation are dolichol-dependent and they are alternate pathways for *N*-glycoprotein biosynthesis through dolichol intermediates.

Two crucial papers reported the transfer of *N*-acetylglucosamine from its nucleotide form to an exogenous protein, ribonuclease A. Khalkhali and Marshall [32] characterized an *N*-acetyl-β-D-glucosaminyltransferase activity in human serum. This transferase was able to catalyze the glycosylation *in vitro* of the asparagine residue present in the sequence Asn-Leu-Thr in bovine pancreatic ribonuclease. Rat liver microsomes have been shown to catalyze the formation of Dol-P-P-GlcNAc [33] and further the incorpora-

tion of *N*-acetylglucosamine from UDP-GlcNAc into bovine pancreatic ribonuclease A [34]. These two *N*-acetylglucosaminyltransferases were characterized and partially purified by Arakawa and Mookerjea [35]. They showed that the addition of exogenous dolichol-monophosphate resulted in a stimulation of the purified "dolichol" enzyme, but did not affect the purified "RNase A" enzyme. Furthermore, tunicamycin could inhibit only the "dolichol" enzyme. our results are in accord with these two latter reports which suggest the existence of two independent pathways for the incorporation of *N*-acetylglucosamine. The characterization of the oligosaccharide moiety of the *N*-glycoprotein material synthesized without dolichol intermediates in the mitochondrial outer membrane will give more information about the existence of two distinct pathways for *N*-glycoprotein biosynthesis in mitochondria.

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