Gitel, S. N., Medina, V. M., & Wessler, S. (1984) *J. Biol. Chem. 259*, 6890–6895.
Gonias, S. L., & Pizzo, S. V. (1983a) *Biochemistry 22*, 536–546.
Gonias, S. L., & Pizzo, S. V. (1983b) *Ann. N.Y. Acad. Sci. 421*, 457–471.
Grassetti, D. R., & Murray, J. F. (1967) *Arch. Biochem. Biophys. 119*, 41–49.
Harpel, P. C. (1973) *J. Exp. Med. 138*, 508–521.
Howard, J. B. (1981) *Proc. Natl. Acad. Sci. U.S.A. 78*, 2235–2239.
Howell, J. B., Beek, T., Bates, B., & Hunter, M. J. (1983) *Arch. Biochem. Biophys. 221*, 261–270.
Jesty, J. (1986a) *Anal. Biochem. 152*, 402–411.
Jesty, J. (1986b) *J. Biol. Chem. 261*, 8695–8702.
Krebs, G., Guinand, S., & Breda, C. (1978) *C. R. Seances Acad. Sci., Ser. D 286*, 1219–1222.
Laemmli, U. K. (1970) *Nature (London) 227*, 680–685.
Lowry, O. H., Rosenbrough, N. J., Farr, A. L., & Randall, R. (1951) *J. Biol. Chem. 193*, 265–275.
Miletich, J. P., Jackson, C. M., & Majerus, P. W. (1978) *J. Biol. Chem. 253*, 6908–6916.
Rinderknecht, H., Fleming, R. M., & Geokas, M. C. (1975) *Biochim. Biophys. Acta 377*, 158–165.
Salvesen, G. S., Sayers, C. A., & Barrett, A. J. (1981) *Biochem. J. 195*, 453–461.
Sixma, J. J., Sakariassen, K. S., Beeser-Visser, N. H., Ottenhof-Rovers, M., & Bolhuis, P. A. (1984) *Blood 63*, 128–139.
Sottrup-Jensen, L., Petersen, T. E., & Magnusson, S. (1980) *FEBS Lett. 121*, 275–279.
Sottrup-Jensen, L., Hansen, H. F., & Christensen, U. (1983) *Ann. N.Y. Acad. Sci. 421*, 188–208.
Starkey, P. M. (1979) in *The Physiological Inhibitors of Coagulation and Fibrinolysis* (Collen, D., Wiman, B., & Verstraete, M., Eds.) pp 221–230, Elsevier/North-Holland, Amsterdam.
Swenson, R. P., & Howard, J. B. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 4313–4316.
Towbin, H., Staehelin, T., & Gordon, J. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 4350–4354.
Van der Graaf, F., Rietveld, A., Keus, F. J. A., & Bouma, B. N. (1984) *Biochemistry 23*, 1760–1766.
Van Leuven, F., Cassiman, J. J., & Van den Berghe, H. (1981) *J. Biol. Chem. 256*, 9023–9027.
Wang, D., Wu, K., & Feinman, R. D. (1981) *J. Biol. Chem. 256*, 10934–10940.
Wang, D., Yuan, A. I., & Feinman, R. D. (1983) *Ann. N.Y. Acad. Sci. 421*, 90–97.
Wang, D., Yuan, A. I., & Feinman, R. D. (1984) *Biochemistry 23*, 2807–2811.

Tetrahymena pyriformis Cells Are Deficient in All Mannose-P-dolichol-Dependent Mannosyltransferases but Not in Mannose-P-dolichol Synthesis†

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ABSTRACT: Cells of the ciliated protozoan *Tetrahymena pyriformis* incubated with [^{14}C]glucose were found to synthesize Man-P-dolichol and Glc-P-dolichol, as well as $Glc_3Man_5GlcNAc_2$-P-P-dolichol, the latter being the main and largest lipid derivative formed. The missing mannose residues were those known to be transferred from Man-P-dolichol in other systems. Formation of Man-P-dolichol and of dolichol-P-P-oligosaccharides containing up to five mannose units was detected in cell-free assays containing protozoan membranes, rat liver dolichol-P, unlabeled $Man_{4-9}GlcNAc_2$-P-P-dolichol from pig liver, and GDP-[^{14}C]Man. Under exactly the same conditions but with UDP-[^{14}C]Glc instead of GDP-[^{14}C]Man, Glc-P-dolichol and dolichol-P-P-oligosaccharides containing five mannose and one to three glucose residues were formed in the absence of the pig liver compounds. In the presence of the latter, dolichol-P-P derivatives containing nine mannose and one to three glucose units were also synthesized. It is concluded that *T. pyriformis* cells are deficient in all Man-P-dolichol-dependent mannosyltransferases but not in Man-P-dolichol synthesis. The role of the latter compound in this microorganism is unknown.

N-Glycosylation in wild-type mammalian, plant, insect, and fungal cells is initiated by the transfer of an oligosaccharide ($Glc_3Man_9GlcNAc_2$, Figure 1) from a dolichol-P-P derivative to asparagine residues in proteins (Hubbard & Ivatt, 1981; Kornfeld & Kornfeld, 1985). GDP-Man appeared to be the donor of the first five mannose residues (c, d, e, f, and g, Figure 1) added in the assembly of the oligosaccharide, whereas the other four units (h, i, j, and k, Figure 1) were found to be transferred from Man-P-dolichol (Rearick et al., 1980b). On the other hand, Glc-P-dolichol appeared to be the donor of the three glucose residues (Parodi, 1979; Staneloni et al., 1980). Assays performed with yeast and mammalian membranes have shown that removal of the glucose residues from the lipid-linked oligosaccharide drastically reduces the rate of transfer of the latter to protein (Parodi, 1981; Turco et al., 1977; Spiro et al., 1979). On the other hand, removal of mannose residues h–k did not affect the rate of transfer of oligosaccharides containing three glucose residues (Spiro et al., 1979; Staneloni et al., 1981). The successive addition of

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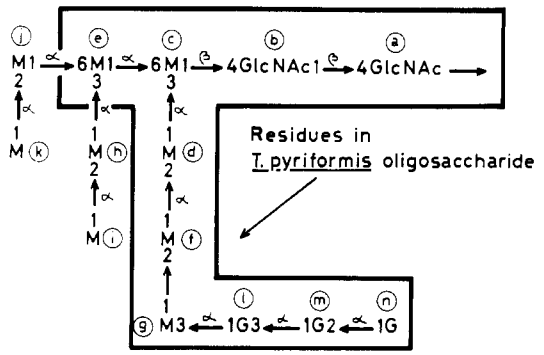

FIGURE 1: Structure of oligosaccharides. The structure is that of the oligosaccharide transferred to protein in wild-type mammalian and yeast cells (Hubbard & Ivatt, 1981; Kornfeld & Kornfeld, 1985). The lettering used for the identification of the individual monosaccharide residues (a, b, c, ...) follows the same order as the addition of the monosaccharides in the assembly of the oligosaccharide (Chapman et al., 1979). In *T. pyriformis* the oligosaccharide is devoid of residues h, i, j, and k. In mammalian cells those four residues are transferred from Man-P-dolichol, whereas the five other mannose units are transferred from GDP-Man (Rearick et al., 1981b). M and G stand for mannose and glucose, respectively.

monosaccharides to the dolichol-linked oligosaccharide follows in vivo a unique order that is depicted in Figure 1 (Chapman et al., 1979).

Certain wild-type flagellated protozoa belonging to the Trypanosomatidae family appeared to be rather unique in nature as they synthesized and transferred to protein in vivo unglucosylated oligosaccharides that contained six, seven, or nine mannose units, depending on the species (Parodi et al., 1981, 1984; Parodi & Quesada-Allue, 1982; Previato et al., 1986). The structures of the oligosaccharides were similar to those of the intermediates in the synthesis of $Glc_3Man_9GlcNAc_2$ in mammalian cells. We have recently conducted a cell-free characterization of the enzymatic defects responsible for the phenotypes observed in trypanosomatids: membranes isolated from them were able to synthesize Man-P-dolichol but not Glc-P-dolichol. In addition, species forming $Man_6GlcNAc_2$ or $Man_7GlcNAc_2$ in vivo were defective in vitro in the transfer of the seventh, eighth, and ninth residues or in that of the eighth and ninth residues, respectively, to the lipid-linked oligosaccharide (unpublished results). This provided the first evidence that at least three different Man-P-dolichol-dependent enzymatic activities are involved in the synthesis of the complete oligosaccharide.

Several years ago, Keenan et al. (1973, 1975) described the synthesis of Glc-P-dolichol as well as the transfer of glucose residues from the latter compound to a lipid-linked oligosaccharide in cell-free assays containing membranes from a ciliated protozoan, *Tetrahymena pyriformis*. The structure of the oligosaccharide formed, however, was not characterized. In this paper we are reporting that the main and largest dolichol-P-P-linked oligosaccharide synthesized in vivo in *T. pyriformis* has the composition $Glc_3Man_5GlcNAc_2$. This is in accordance with the known structures of protein-linked oligosaccharides present in two lysosomal enzymes secreted by this protozoan (Taniguchi et al., 1985). The microorganism appeared to be defective in all Man-P-dolichol-dependent mannosyltransferases but not in Man-P-dolichol synthesis. This is the first description of an eucaryotic cell having these characteristics.

EXPERIMENTAL PROCEDURES

Materials. Rat liver dolichol-P, grade III, Jack bean α-mannosidase, and endo-β-*N*-acetylglucosaminidase H were from Sigma. [^{14}C]Glucose (325 Ci/mol) was purchased from American Radiolabeled Chemicals. UDP-[^{14}C]Glc (285 Ci/mol) and GDP-[^{14}C]Man (216.5 Ci/mol) were prepared according to the method of Wright and Robbins (1965) with slight modifications. Proteose peptone and yeast extract were from Difco. Amphomycin was kindly provided by Dr. Miklos Bodanszky, Case Western Reserve University, Cleveland, OH.

Standards. [^{14}C]$Man_{5-9}GlcNAc$ standards were obtained from hen oviduct glycoproteins as described before (Parodi et al., 1981). Mannooligosacchardes were prepared by acetolysis of [^{14}C]$Man_{5-9}GlcNAc$. [*glucose*-^{14}C]-$Glc_{1-3}Man_5GlcNAc_2$ and [*glucose*-^{14}C]$Glc_3Man_4GlcNAc$ were prepared by α-mannosidase digestion of the corresponding oligosaccharides containing nine mannose residues. These were obtained by mild acid hydrolysis of the dolichol-P-P derivatives synthesized by rat liver microsomes incubated with UDP-[^{14}C]Glc (Parodi et al., 1973). The oligosaccharides were separated by paper chromatography with solvent A prior to the enzymatic treatment. Each oligosaccharide yielded two products, one containing four and the other five mannose units.

Preparations. Rat liver microsomes and the pig liver extract were prepared as described before (Parodi et al., 1972; Parodi, 1979). *Tetrahymena pyriformis* cells (an inoculum of wild-type strain PA was kindly provided by Dr. Mark Coggiano, Department of Zoology, University of Maryland) were grown in 2% proteose peptone and 0.2% yeast extract if labeling of intact cells was envisaged or in 2% glucose, 1% proteose peptone, and 0.2% yeast extract if membranes were to be prepared. The latter were obtained as follows: cells were harvested in the exponential phase (2 L of medium) and resuspended in solution A (15% glycerol, 10 mM Tris–maleate[1] buffer, pH 7.6, 5 mM NaEDTA, and 5 mM 2-mercaptoethanol) and centrifuged for 5 min at 3000*g*. Cells were again resuspended in 40 mL of the same solution and disintegrated in an Omnimixer blender (1.5 min at maximum speed). The suspension was centrifuged at 20000*g* for 15 min and the whole pellet resuspended in solution A at protein concentrations of 40–45 mg/mL. Usually 0.5–1 mL was obtained from each preparation.

Labeling of Cells. Harvested cells (1–2 g wet weight) were resuspended in 15 mL of Eagle's solution devoid of glucose but containing 5 mM sodium pyruvate and centrifuged for 5 min at 3000*g* (Engel & Parodi, 1985). Cells were then resuspended in an equal volume (wet weight) of the same solution containing 300–500 μCi of [^{14}C]glucose and incubated at room temperature for 8 min.

Isolation of in Vivo Labeled Dolichol Derivatives. The cell suspension was diluted to 5 mL with water and poured into 25 mL of chloroform/methanol (3:2). Dolichol-P derivatives were isolated from the lower phase as described before (Parodi & Quesada-Alue, 1982). Briefly, the lower phase was dried and subjected to mild saponification. The unsaponified material was applied to a DEAE-cellulose (acetate form) column equilibrated with chloroform/methanol (2:1). The dolichol-P derivatives were then eluted with an ammonium formate gradient in the same solvent. Dolichol-P-P oligosaccharides were isolated from the proteinaceous interphase of the chloroform/methanol/water (3:2:1) partition as described previously (Parodi et al., 1981). Briefly, the denatured proteins were washed twice with chloroform/methanol/water (3:2:1) and once with water. They were then extracted 4 times with chloroform/methanol/water (1:1:0.3). The extracts were

[1] Abbreviations: AMP, adenosine 5′-monophosphate; EDTA, ethylenediaminetetraacetic acid; GDP, guanosine diphosphate; Tris, tris(hydroxymethyl)aminomethane.

pooled and applied to a DEAE-cellulose (acetate form) column equilibrated with the same solvent. Dolichol-P-P-oligosaccharides were eluted with an ammonium formate gradient in chloroform/methanol/water (1:1:0.3).

In Vitro Synthesis of Dolichol-P Derivatives. The incubation mixtures contained, in a total volume of 100 μL, 50 mM Tris–maleate buffer, pH 7.6, 10 mM $MgCl_2$, 10 mM AMP, 0.6% Triton X-100, the indicated amounts of dolichol-P, 0.05 μCi of UDP-[^{14}C]Glc or GDP-[^{14}C]Man, and 1–1.5 mg of *T. pyriformis* membrane proteins. After 10 min at 30 °C, 0.4 mL of methanol, 0.6 mL of chloroform, and 0.15 mL of water were added. The lower phases were washed twice with 0.3 mL of chloroform/methanol/water (1:16:16) and counted.

In Vitro Synthesis of Dolichol-P-P Derivatives. The incubation mixtures contained, in a total volume of 100 μL, 50 mM Tris–maleate buffer, pH 7.6, 10 mM $MgCl_2$, 10 μg of dolichol-P, 10 mM AMP, 0.6% Triton X-100, pig liver extract (1 μmol of total phosphate), 0.5 μCi of GDP-[^{14}C]Man or 0.8 μCi of UDP-[^{14}C]Glc, and 4–5 mg of rat liver or 2–3 mg of *T. pyriformis* membrane proteins. After 10 min at 37 °C (rat liver membranes) or at 30 °C (protozoan membranes), the reactions were stopped by the addition of 0.4 mL of methanol, 0.6 mL of chloroform, and 0.15 mL of water. The lower phases were washed twice with 0.3 mL of chloroform/methanol/water (1:16:16). The interphases were washed once with 1.5 mL of the latter solution and once with 1 mL of water and extracted 3 times with 1 mL of chloroform/methanol/water (1:1:0.3). The extracts were pooled and mixed with the lower phases.

Characterization of Acceptors in the Pig Liver Extract. The extract contained material soluble in the lower phase of a chloroform/methanol/water (3:2:1) partition of the whole liver as well as material remaining with the proteinaceous interphase that was soluble in chloroform/methanol/water (1:1:0.3). Both materials were mixed, dried, solubilized in the latter solvent, and subjected to DEAE-cellulose column chromatography. Substances eluting at the same ammonium formate concentration as dolichol-P-P derivatives were pooled. The presence in the extracts of compounds having the structure of the in vivo precursors in the formation of $Glc_3Man_9GlcNAc_2$-P-P-dolichol in mammals was ascertained by using an incubation mixture containing rat liver membranes, GDP-[^{14}C]Man, and rat liver dolichol-P.

As depicted in Figure 2A, labeled $Man_{5-9}GlcNAc_2$-P-P-dolichol was formed almost exclusively upon addition of the extract to the incubation mixture. The oligosaccharides formed were submitted to acetolysis, a procedure that preferentially cleaves α(1,6) bonds between mannose residues. Upon such treatment $Man_9GlcNAc_2$ produced only part of the expected products, mannotriose and mannobiose (Figure 2B). The relative proportion of label in them was 5% and 95%, respectively. Reduction of the latter compound followed by strong acid hydrolysis and paper chromatography with solvent B revealed that 12% of the total label was in residue j and 83% in residue k (Figure 1). This, together with the absence of labeled acetolysis products migrating as Man_4GlcNA_2, indicated that unlabeled dolichol-P-P-linked $Man_8GlcNAc_2$, $Man_7GlcNAc_2$, $Man_6GlcNAc_2$, and/or $Man_5GlcNAc_2$ with the correct precursor structures were present in the pig liver extract.

Acetolysis of the other oligosaccharides shown in Figure 2A did not produce the fragments expected for compounds having exclusively the structures of the intermediates formed in vivo. For instance, acetolysis of $Man_6GlcNAc_2$ yielded mannotriose, mannobiose, and mannose (Figure 2B). In addition to

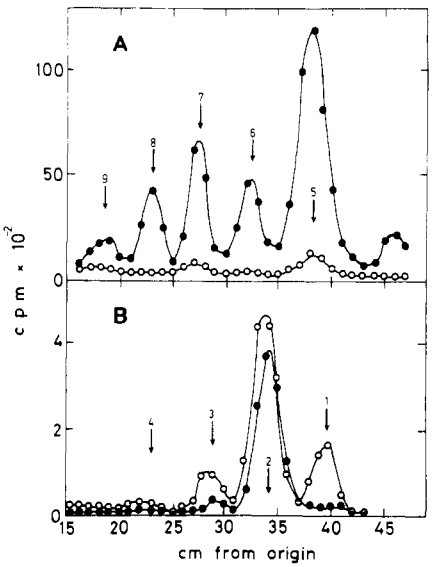

FIGURE 2: In vitro synthesis of mannose-labeled dolichol-P-P-oligosaccharides by rat liver membranes. (A) Derivatives synthesized in the presence (●) or absence (○) of the pig liver extract with GDP-[^{14}C]Man in the incubation mixture were submitted to a mild acid hydrolysis followed by paper chromatography with solvent A. (B) Substances migrating as standard 9 (●) or 6 (○) in part A were subjected to acetolysis followed by paper chromatography in solvent A. Standards: (1) mannose; (2) mannobiose; (3) mannotriose; (4) $Man_4GlcNAc$; (5) $Man_5GlcNAc$; (6) $Man_6GlcNAc$; (7) $Man_7GlcNAc$; (8) $Man_8GlcNAc$; (9) $Man_9GlcNAc$.

$Man_4GlcNAc_2$ the only expected product for the in vivo formed $Man_6GlcNAc_2$ is mannobiose (Figure 1). Reduction of the disaccharide followed by paper electrophoresis in 0.1 M sodium molybdate buffer, pH 5.0, revealed that mannosyl-α(1,3)mannose was among the acetolysis products, because part of the label behaved as a neutral compound under such conditions (Parodi et al., 1983). This, together with the absence of label migrating as $Man_4GlcNAc_2$ in Figure 2B, indicated that unlabeled $Man_5GlcNAc_2$-P-P-dolichol with the structure of the precursor formed in vivo was present in the extract. Similar analysis of labeled $Man_8GlcNAc_2$ and $Man_7GlcNAc_2$ depicted in Figure 2A confirmed the presence in the pig liver extract of unlabeled dolichol-P-P-linked $Man_{5-8}GlcNAc_2$ having the structures of the intermediates in the formation of $Glc_3Man_9GlcNAc_2$. Rearick et al. (1981b) have reported before that upon incubation of mammalian membranes with GDP-Man and dolichol-P several isomers of the smaller dolichol-P-P-oligosaccharides were formed. The larger compounds had a unique structure similar to that of the in vivo formed intermediates. The most plausible interpretation of this result is that the mannosyltransferases may partially act in vitro not in the same order as in vivo, thus producing "wrong" intermediates that are probably poor substrates for the following reactions. According to this interpretation the largest compound formed in vivo and in vitro should have, as observed, the same structure.

Methods. Strong and mild acid hydrolysis, α-mannosidase and endo-β-*N*-acetylglucosaminidase H treatments, acetolysis, reduction with $NaBH_4$ and treatment with 2 N KOH, and paper electrophoresis in 5% formic acid and in 0.1 M sodium molybdate (pH 5.0) were as described before (Parodi et al., 1973, 1981, 1983; Engel & Parodi, 1985). Total phosphate was measured according to the method of Ames (1966). Chromatography was performed on Schleicher & Schuell 2043-a papers or on silica gel 60 Merck glass plates with solvents A [1-propanol/nitromethane/water (5:2:4)], B [1-butanol/pyridine/water (6:4:3)], C [chloroform/2-

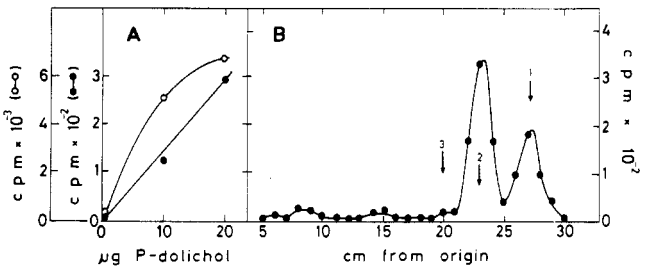

FIGURE 3: Synthesis of dolichol-P derivatives. (A) In vitro synthesis of [^{14}C]Man-P-dolichol (O) or [^{14}C]Glc-P-dolichol (●). (B) In vivo synthesis. The derivatives were purified as indicated under Experimental Procedures and subjected to a mild acid hydrolysis. The hydrophilic moieties thus liberated were run on paper chromatography with solvent B. Standards: (1) mannose; (2) glucose; (3) galactose.

propanol/95% ethanol/glacial acetic acid (2:2:3:1)], or D [1-propanol/water (7:3)].

RESULTS

In Vitro and in Vivo Synthesis of Glc-P-dolichol and Man-P-dolichol. Incubation of *T. pyriformis* membranes with rat liver dolichol-P and GDP-[^{14}C]Man or UDP-[^{14}C]Glc led to the dolichol-P-dependent formation of liposoluble compounds with both sugar donors (Figure 3A). In both cases the reaction products were degraded under mild acid conditions, yielding the same labeled monosaccharide residue present in the respective sugar nucleotide used in the incubation. This was ascertained on paper chromatography of the hydrolysis products with solvent B. Moreover, migration of the dolichol-P derivatives on thin-layer chromatography with two solvent systems was the same as that of the compounds formed by liver microsomes (solvent C R_f = 0.53 for Man-P-dolichol and R_f = 0.63 for Glc-P-dolichol; solvent D R_f = 0.66 for both derivatives). Depending on the membrane preparation, the amount of Man-P-dolichol synthesized was 3–20-fold higher than that of Glc-P-dolichol. Both derivatives were also formed when intact cells were incubated with [^{14}C]glucose; chromatography of the hydrophilic moieties liberated by mild acid hydrolysis of the dolichol-P derivatives synthesized revealed the presence of both labeled glucose and mannose residues (Figure 3B). The migration values of both derivatives on thin-layer chromatography with solvent D were the same as that of Man-P-dolichol synthesized by rat liver microsomes.

In Vivo Synthesis of Dolichol-P-P-oligosaccharides. T. pyriformis cells incubated with [^{14}C]glucose synthesized dolichol-P-P derivatives whose main hydrophilic moiety migrated on paper chromatography as a $Glc_3Man_5GlcNAc_2$ standard. A much smaller amount of label migrating as $Glc_2Man_5GlcNAc_2$ was also observed (Figure 4). Strong acid hydrolysis of the main oligosaccharide yielded labeled glucose and mannose units (Figure 5A). Although no labeled glucosamine residues appeared in the pattern, the oligosaccharide contained two *N*-acetylhexosamine residues: the compound was neutral in an acidic medium but two positively charged substances were produced after reduction and treatment with alkali. This result was obtained even when the oligosaccharide was treated with endo-β-*N*-acetylglucosaminidase H prior to reduction and treatment with alkali, thus indicating that it was resistant to the enzyme (Figure 5B). $Glc_3Man_9GlcNAc_2$ from rat liver also yielded two charged compounds upon treatment with KOH, but only one of such compounds was produced when the saccharide was previously treated with endo-β-*N*-acetylglucosaminidase H (Figure 5C,D).

Treatment of the *T. pyriformis* oligosaccharide with α-mannosidase produced only a partial degradation of the compound. The reaction product migrated as a $Glc_3Man_4GlcNAc_2$ standard (Figure 5E). The same pattern was obtained when $Glc_3Man_9GlcNAc_2$ from rat liver was similarly treated (Figure 5F). Further incubation of the undegraded protozoan compound with the enzyme reproduced the pattern shown in Figure 5E. It is known that the presence of the glucose residues in dolichol-P-P-linked oligosaccharides partially hinders removal of mannose residue e by α-mannosidase (Figure 1) (Parodi & Cazzulo, 1982).

Although the oligosaccharide was not fully characterized, all data reported support the structure indicated in Figure 1 for the main dolichol-P-P-linked oligosaccharide formed in vivo

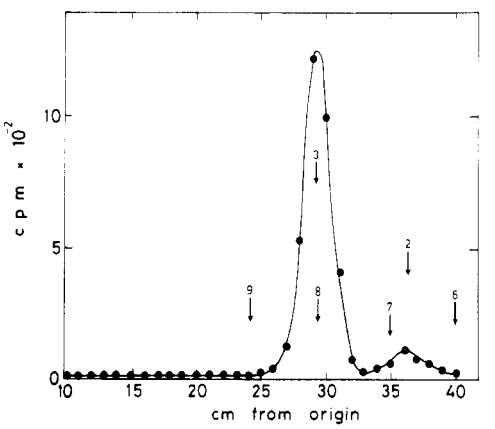

FIGURE 4: In vivo synthesis of dolichol-P-P-oligosaccharides. The labeled dolichol-P-P-oligosaccharides were subjected to a mild acid hydrolysis followed by paper chromatography with solvent A. Standards: (2) $Glc_2Man_5GlcNAc_2$; (3) $Glc_3Man_5GlcNAc_2$; (6) $Man_6GlcNAc$; (7) $Man_7GlcNAc$; (8) $Man_8GlcNAc$; (9) $Man_9GlcNAc$.

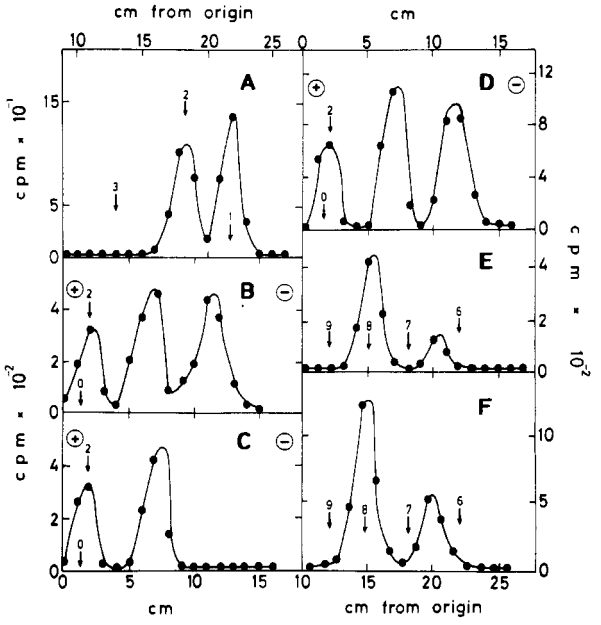

FIGURE 5: Characterization of dolichol-P-P-oligosaccharide synthesized in vivo. (A) The main oligosaccharide in Figure 4 was subjected to strong acid hydrolysis followed by paper chromatography with solvent B. (B) The oligosaccharide was treated with endo-β-*N*-acetylglucosaminidase H, reduced with 0.1 M $NaBH_4$, treated for 30 min at 100 °C with 2 N KOH, and neutralized with concentrated perchloric acid, and the supernatant was subjected to paper electrophoresis in 5% formic acid. (C, D) The same as (B) but with [*glucose*-^{14}C]-$Glc_3Man_9GlcNAc_2$ from rat liver. In (D) the enzymatic treatment was omitted. (E, F) The oligosaccharide from *T. pyriformis* (E) or [*glucose*-^{14}C]$Glc_3Man_9GlcNAc_2$ from rat liver (F) was treated with α-mannosidase and run on paper chromatography with solvent A. Standards: (1) mannose; (2) glucose; (3) glucosamine; (6) $Man_6GlcNAc$; (7) $Man_7GlcNAc$; (8) $Man_8GlcNAc$; (9) $Man_9GlcNAc$. O stands for origin.

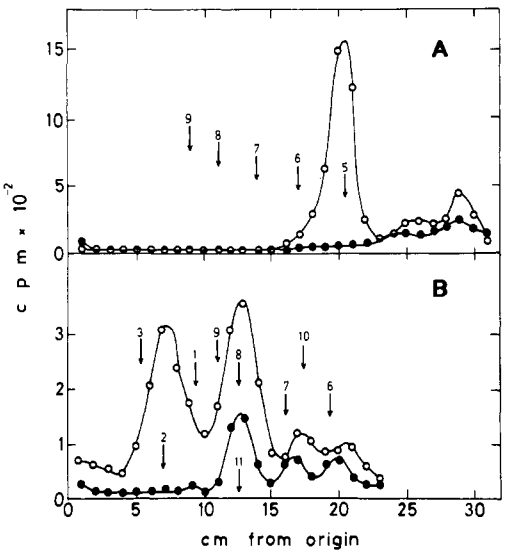

FIGURE 6: In vitro synthesis of dolichol-P-P-oligosaccharides by *T. pyriformis* membranes. (A) The derivatives were synthesized with (○) or without (●) pig liver extract in the presence of GDP-[^{14}C]Man. The reaction products were subjected to a mild acid hydrolysis followed by paper chromatography with solvent A. (B) The same as in (A) but with UDP-[^{14}C]Glc instead of GDP-[^{14}C]Man. Standards: (1) $Glc_1Man_9GlcNAc_2$; (2) $Glc_2Man_9GlcNAc_2$; (3) $Glc_3Man_9GlcNAc_2$; (5) $Man_5GlcNAc_2$; (6) $Man_6GlcNAc$; (7) $Man_7GlcNAc$; (8) $Man_8GlcNAc$; (9) $Man_9GlcNAc$; (10) $Glc_2Man_5GlcNAc_2$; (11) $Glc_3Man_5GlcNAc_2$.

in this microorganism (please note that the structure proposed is that devoid of residues h–k in Figure 1). As will be discussed below, the known structures of protein-lined oligosaccharides present in *T. pyriformis* support the conclusion that this oligosaccharide is the one transferred to protein in this microorganism. The proposed lipid-linked compound (Figure 1) is known to be resistant to endo-β-*N*-acetylglucosaminidase H due to the absence of substitution in residue e (Maley et al., 1978). In order to check the possibility that lipid-linked oligosaccharides with compositions $Glc_3Man_{6-9}GlcNAc_2$ were not detected in cells incubated with [^{14}C]glucose for 8 min due to their rapid transfer to protein, the presence of endo-β-*N*-acetylglucosaminidase H sensitive oligosaccharides in the denatured protein pellet that remained after extracting the dolichol-P-P-linked oligosaccharides with organic solvents was investigated. The experimental procedure employed was that currently used in our laboratory for the isolation of protein-linked, endo-β-*N*-acetylglucosaminidase H sensitive oligosaccharides from protozoan cells incubated with the same labeled precursor (Parodi et al., 1981, 1983; de la Canal & Parodi, 1985). No labeled protein-linked, endo-β-*N*-acetylglucosaminidase H sensitive oligosaccharide was detected in *T. pyriformis*. As naturally occurring oligosaccharides with compositions $Glc_{0-3}Man_{6-9}GlcNAc_2$ are sensitive to endo-β-*N*-acetylglucosaminidase H due to the fact that they all present a substitution in residue e, the absence of oligosaccharides sensitive to the enzyme indicates that no oligosaccharides with compositions $Glc_3Man_{6-9}GlcNAc_2$ had been transferred to protein.

In Vitro Synthesis of Mannose-Labeled Dolichol-P-P-oligosaccharides. Formation of the title compounds was assayed in incubation mixtures containing protozoan membranes, rat liver dolichol-P, GDP-[^{14}C]Man, and an extract from pig liver containing unlabeled $Man_{4-8}GlcNAc_2$-P-P-dolichol.

As depicted in Figure 6A, addition of the pig liver extract only stimulated the formation of dolichol-P-P-linked oligosaccharides having five or less mannose units. It should be emphasized that, as shown under Experimental Procedures, the extract contained the suitable substrates for the addition of the sixth, seventh, eighth, and ninth mannose residues. As will be seen below, it also contained the substrates for the addition of glucose units.

In Vitro Synthesis of Glucose-Labeled Dolichol-P-P-oligosaccharides. A different result was obtained when the incubation was performed under exactly the same conditions used for the formation of mannose-labeled dolichol-P-P derivatives (Figure 6A) but with UDP-[^{14}C]Glc instead of GDP-[^{14}C]Man. In the absence of the pig liver extract in the incubation mixture, substances behaving as lipid-linked $Glc_{1-3}Man_5GlcNAc_2$ were formed (Figure 6B). Addition of the extract to the incubation mixture not only increased the labeling of the above-mentioned compounds but also promoted the formation of lipid-linked oligosaccharides migrating as $Glc_{1-3}Man_9GlcNAc_2$ standards (Figure 6B). In the assays performed in the presence of the pig liver extract with GDP-[^{14}C]Man or UDP-[^{14}C]Glc (Figure 6), the amount of [^{14}C]Man-P-dolichol formed was 2.8-fold higher than that of [^{14}C]Glc-P-dolichol.

The results reported indicated, therefore, that *T. pyriformis* cells are deficient in all Man-P-dolichol-dependent mannosyltransferases.

Fate of Man-P-dolichol in T. pyriformis. The fact that, as shown above, Man-P-dolichol was synthesized both in vivo and in vitro in this protozoan but that the mannose residues known to be transferred from that derivative in mammalian as well as in other eucaryotic cells were absent from the dolichol-P-P-oligosaccharides posed the problem of the fate of Man-P-dolichol in *T. pyriformis*. Omission of dolichol-P and addition of amphomycin (a known inhibitor of Man-P-dolichol synthesis) to an incubation mixture otherwise similar to that used for the synthesis of mannose-labeled dolichol-P-P derivatives (Figure 6A, presence of pig liver extract) drastically reduced [^{14}C]Man-P-dolichol formation without affecting synthesis of oligosaccharides containing five or less mannose units. This indicated that in this microorganism, the same as in mammals and protozoa, the first five mannose residues are transferred from GDP-Man (Rearick et al., 1981b; Muller et al., 1984).

In another set of experiments *T. pyriformis* membranes were incubated for 15 min at 30 °C with [^{14}C]Man-P-dolichol and 10 mM $MgCl_2$ or 10 mM $MnCl_2$ or 10 mM NaEDTA in the presence of 0.1%, 0.6%, or 1.2% Triton X-100. The bulk of the radioactivity remained in a mild acid labile compound (presumably the substrate used) soluble in the lower phases of chloroform/methanol/water (3:2:1) partitions of the reaction mixtures. The fate of Man-P-dolichol in this microorganism remains, therefore, unknown.

DISCUSSION

Results reported here show that the main and largest dolichol-P-P derivative synthesized in vivo in *T. pyriformis* has the composition $Glc_3Man_5GlcNAc_2$. This agrees with the structures of the carbohydrate moieties of two lysosomal enzymes secreted by this microorganism that have been reported by Taniguchi et al. (1985). The oligosaccharides appeared to have the compositions $Glc_{0-3}Man_{2-5}GlcNAc_2$, and the structure of the largest compound ($Glc_3Man_5GlcNAc_2$) was similar to that proposed for the lipid-linked saccharide (Figure 1). No saccharides with compositions $Glc_{0-3}Man_{6-9}GlcNAc_2$ were found in the lysosomal enzymes, this result being in accordance to our inability to detect those oligosaccharides in whole cell proteins. The fact that $Glc_3Man_5GlcNA_2$ was the main and largest dolichol-P-P-linked oligosaccharide formed in intact cells and that protein-linked oligosaccharides

with compositions $Glc_{0-3}Man_{2-5}GlcNAc_2$ but not with $Glc_{0-3}Man_{6-9}GlcNAc_2$ were found indicates that $Glc_3Man_5GlcNAc_2$ was the oligosaccharide transferred in protein N-glycosylation in *T. pyriformis*.

It has been reported that certain mammalian cells synthesized $Glc_3Man_5GlcNAc_2$-P-P-dolichol instead of the usual oligosaccharide containing nine mannose residues under energy-depleted conditions such as glucose starvation (Gershman & Robbins, 1981; Rearick et al., 1981a). Energy depletion, however, appeared not to be responsible for results obtained with *T. pyriformis* as both Taniguchi et al. (1985) and ourselves have used wild-type cells grown under nutrient-rich conditions in well-aerated flasks.

Two mutant mammalian cell lines, one of them derived from a mouse lymphoma and the other from Chinese hamster ovary, have also been reported to synthesize $Glc_3Man_5GlcNAc_2$ as the main and largest dolichol-P-P-linked compound, even under energy-rich conditions (Chapman et al., 1980; Stoll et al., 1982). In both cases the defect was found to be the absence of synthesis of Man-P-dolichol, the known donor of the missing residues. The mutant mouse lymphoma cells were reported to have all Man-P-dolichol-dependent mannosyltransferases as incubation of microsomes, with $Man_5GlcNAc_2$-P-P-dolichol and Man-P-dolichol, led to formation of lipid-linked $Man_{6-9}GlcNAc_2$.

T. pyriformis appeared to synthesize [^{14}C]Glc-P-dolichol and [^{14}C]Man-P-dolichol both in vivo and in vitro. The relative amounts of the derivatives formed in vivo are unknown, as it was not possible to determine the specific activities of the dolichol-P-linked glucose and mannose residues. In vitro, however, under similar experimental conditions [^{14}C]Man-P-dolichol synthesis was 3–20-fold higher (depending on the membrane preparation) than that of [^{14}C]Glc-P-dolichol. This microorganism appeared to be defective not only in the enzymatic activity responsible for the transfer of the sixth mannose residue, a defect that would per se hinder further in vivo elongation of the oligosaccharide, but also in the activities involved in the addition of the seventh, eighth, and ninth residues. The function of Man-P-dolichol in *T. pyriformis* remains to be found. The other known role for Man-P-dolichol in eucaryotic cells, besides its involvement in the synthesis of dolichol-P-P-oligosaccharides, is as intermediate in the mannosylation of serine/threonine residues in proteins, but so far this reaction has been found to occur only in fungi (Sharma et al., 1974; Bretthauer & Wu, 1975; Gold & Hahn, 1976). We were unable to detect transfer of mannose units from [^{14}C]Man-P-dolichol to endogenous *T. pyriformis* membrane proteins under conditions similar to those employed in the fungal systems. Due to the evolutionary pressure, protozoan cells generally do not express unnecessary genetic information. In this repsect they differ, therefore, from laboratory-made mutant cells. The fact that Man-P-dolichol is synthesized in *T. pyriformis* strongly suggests that this lipid derivative might have another, as yet unknown, role in this protozoan.

As mentioned above, in addition to *T. pyriformis*, other protozoa such as the trypanosomatids were found to have modified pathways of protein N-glycosylation. In these parasitic protozoa the defective steps appeared to be the synthesis of Glc-P-dolichol and in some species also the addition of certain mannose residues (unpublished results). Muller et al. (1984) have reported that membranes from the photosynthetic protozoan *Volvox carteri* formed $Glc_1Man_5GlcNAc_2$ as the largest dolichol-P-P derivative. No experiments performed in vivo were presented, however, to corroborate this result. The failure to obtain further elongation of the oligosaccharide could have been due to possible improper experimental conditions. On the other hand, another photosynthetic protozoan, *Euglena gracilis*, was shown to form in vivo $Glc_3Man_9GlcNAc_2$-P-P-dolichol, thus indicating that some protozoa are able to synthesize the complete lipid-linked compound (de la Canal & Parodi, 1985).

Nothing is known about the structure of genes coding for the transferase activities that have been found to be defective in certain protozoa. Once gene probes become available, it would be interesting to know if the observed alterations in the pathway leading to protein N-glycosylation are a consequence of mutations or deletions in the genome or if, alternatively, these microrganisms have never acquired the full genetic information for the pathway.

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REFERENCES

Ames, B. N. (1966) *Methods Enzymol. 8*, 115–118.
Bretthauer, R. K., & Wu, S. (1975) *Arch. Biochem. Biophys. 167*, 151–160.
Chapman, A., Li, E., & Kornfeld, S. (1979) *J. Biol. Chem. 254*, 10243–10249.
Chapman, A., Fujimoto, K., & Kornfeld, S. (1980) *J. Biol. Chem. 255*, 4441–4446.
de la Canal, L., & Parodi, A. J. (1985) *Comp. Biochem. Physiol., B: Comp. Biochem. 81B*, 803–805.
Engel, J. C., & Parodi, A. J. (1985) *J. Biol. Chem. 260*, 10105–10110.
Gershman, H., & Robbins, P. W. (1981) *J. Biol. Chem. 256*, 7774–7780.
Gold, M. H., & Hahn, H. J. (1976) *Biochemistry 15*, 1808–1814.
Hubbard, S. C., & Ivatt, R. J. (1981) *Annu. Rev. Biochem. 50*, 555–583.
Keenan, R. W., Matula, J. M., & Holloman, L. (1973) *Biochim. Biophys. Acta 326*, 84–92.
Keenan, R. W., Kruczek, M., & Fusinato, L. (1975) *Arch. Biochem. Biophys. 167*, 697–705.
Kornfeld, R., & Kornfeld, S. (1985) *Annu. Rev. Biochem. 54*, 631–664.
Maley, F., Tarentino, A. L., & Trimble, R. B. (1978) in *Glycoproteins and Glycolipids in Disease Processes* (Walborg, E. F., Jr., Ed.) pp 86–103, ACS Symposium Series 80, American Chemical Society, Washington, DC.
Muller, T., Bause, E., & Jaenicke, L. (1984) *Eur. J. Biochem. 138*, 153–159.
Parodi, A. J. (1979) *J. Biol. Chem. 254*, 8343–8352.
Parodi, A. J. (1981) *Arch. Biochem. Biophys. 210*, 372–382.
Parodi, A. J., & Cazzulo, J. J. (1982) *J. Biol. Chem. 257*, 7641–7645.
Parodi, A. J., & Quesada-Allue, L. A. (1982) *J. Biol. Chem. 257*, 7637–7640.
Parodi, A. J., Behrens, N. H., Leloir, L. F., & Carminatti, H. (1972) *Proc. Natl. Acad. Sci. U.S.A. 69*, 3268–3272.
Parodi, A. J., Staneloni, R. J., Cantarella, A. I., Leloir, L. F., Behrens, N. H., Carminatti, H., & Levy, J. (1973) *Carbohydr. Res. 26*, 393–400.
Parodi, A. J., Quesada-Allue, L. A., & Cazzulo, J. J. (1981) *Proc. Natl. Acad. Sci. U.S.A. 78*, 6201–6205.
Parodi, A. J., Lederkremer, G. Z., & Mendelzon, D. H. (1983) *J. Biol. Chem. 258*, 5589–5595.

Parodi, A. J., Martin-Barrientos, J., & Engel, J. C. (1984) *Biochem. Biophys. Res. Commun. 118*, 1–7.
Previato, J. O., Mendelzon, D. H., & Parodi, A. J. (1986) *Mol. Biochem. Parasitol. 18*, 343–353.
Rearick, J. I., Chapman, A., & Kornfeld, S. (1981a) *J. Biol. Chem. 256*, 6255–6261.
Rearick, J. I., Fujimoto, K., & Kornfeld, S. (1981b) *J. Biol. Chem. 256*, 3762–3769.
Sharma, C. B., Babczinski, P., Lehle, L., & Tanner, W. (1974) *Eur. J. Biochem. 46*, 35–41.
Spiro, M. J., Spiro, R. G., & Bhoyroo, V. D. (1979) *J. Biol. Chem. 254*, 7668–7674.
Staneloni, R. J., Ugalde, R. A., & Leloir, L. F. (1980) *Eur. J. Biochem. 105*, 275–278.
Staneloni, R. J., Tolmasky, M. E., Petriella, C., & Leloir, L. F. (1981) *Plant Physiol. 68*, 1175–1179.
Stoll, J., Robbins, A. R., & Krag, S. S. (1982) *Proc. Natl. Acad. Sci. U.S.A. 79*, 2296–2300.
Taniguchi, T., Mizuochi, T., Banno, Y., Nozawa, Y., & Kobata, A. (1985) *J. Biol. Chem. 260*, 13941–13946.
Turco, S. J., Stetson, B., & Robbins, P. W. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 4411–4414.
Wright, A., & Robbins, P. W. (1965) *Biochim. Biophys. Acta 104*, 594–596.

Pyrene-Labeled Gangliosides: Micelle Formation in Aqueous Solution, Lateral Diffusion, and Thermotropic Behavior in Phosphatidylcholine Bilayers†

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ABSTRACT: By use of the excimer technique, the formation in aqueous solution of pyrene-labeled ganglioside micelles and their lateral diffusion and distribution in phosphatidylcholine membranes were investigated. For these studies 12-(1-pyrenyl)dodecanoic acid was covalently attached to the ceramide part of lysogangliosides G_{M1}, G_{M2}, G_{M3}, G_{D1a}, and G_{D1b}. The 12-(1-pyrenyl)dodecanoic acid substitute of phosphatidylcholine was used for comparison. All pyrene-labeled gangliosides were present in aqueous solution in a predominantly micellar form down to 2×10^{-8} M, which is the technical limit of this method. The tendency to aggregate is highest for PyG_{D1a} and PyG_{D1b}. In fluid dipalmitoylphosphatidylcholine bilayers the excimer-to-monomer fluorescence intensity ratio of pyrene-labeled gangliosides PyC_{M1}, PyG_{M2}, PyG_{M3}, PyG_{D1a}, and PyG_{D1b} increases linearly with ganglioside concentration. The calculated diffusion coefficients for gangliosides are comparable to 1.6×10^{-7} cm^2/s, which is the diffusion coefficient of pyrene-labeled phosphatidylcholine [Galla, H.-J., & Hartmann, W. (1980) *Chem. Phys. Lipids 27*, 199–219]. In comparison to phosphatidylcholine, the diffusion of monosialogangliosides is slightly increased, with that diffusion of disialogangliosides being slightly decreased. Ca^{2+} ions up to 200 mM do not affect ganglioside diffusion significantly. The shape of the lipid phase transition curves obtained by the excimer technique yields information on the lateral distribution of the tested probe molecules. Pyrene-labeled phosphatidylcholine was taken as reference for a system with complete miscibility but nonideal mixing. 1-Acyl-2-[10-(1-pyrenyl)decanoyl]-*sn*-glycero-3-phosphocholine (PyPC) is known to be randomly distributed in the gel and in the fluid-crystalline lipid phase of dipalmitoylphosphatidylcholine bilayer membranes. It distributes preferentially into the fluid phase in the phase-transition region. In comparison, PyPC in dimyristoylphosphatidylcholine membranes is an example of a system with nearly ideal mixing [Hresko, R. C., Sugar, J. P., Barenholz, Y., & Thompson, T. E. (1986) *Biochemistry 25*, 3813–3828]. Phase-transition curves of pyrene-labeled gangliosides exemplify a nearly ideal mixing system with PyG_{D1a} or PyG_{D1b} producing best effects. The monosialogangliosides, however, exhibit less ideality of mixing, the deviation from an ideal mixing behavior increasing with decreasing number of both neutral sugar residues and sialic acid groups. Addition of Ca^{2+} triggers a tightening of the phosphatidylcholine bilayer and thus induces a change in the lateral distribution of the gangliosides at the phase transition. The system passes into a nonideal mixture, which holds true for PyG_{M3} and PyG_{M2} though less so for PyG_{M1}. PyG_{D1a} and PyG_{D1b} exhibit almost ideal mixing even in the presence of 200 mM Ca^{2+}. This clearly demonstrates that the favorable interaction of G_{D1a} or G_{D1b} with phosphatidylcholine is due to a better fit of the head group dipoles, as was postulated by Maggio et al. [Maggio, B., Cumar, F. A., & Caputto, R. (1980) *Biochem. J. 189*, 435–440]. This Ca^{2+} effect slightly below the phase-transition temperature of the host lipid is time dependent. The increase in excimer formation of pyrene-labeled gangliosides is immediate with concentrations up to 100 mM. However, above this Ca^{2+} concentration the immediate response is followed by a prolonged increase.

Gangliosides are sialic acid containing glycosphingolipids which are minor but essential components of the vertebrate plasma membrane. In extraneural tissue gangliosides are implicated in various surface recognition and physiological processes. For example, G_{M1} and G_{M3} have been shown to regulate cell proliferation. Moreover, G_{M1} and G_{M3} control ganglioside biosynthesis and prevent oncogenic transformation (Bremer et al., 1984; Hakomori, 1981). Gangliosides are

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