Effect of Exogenous Dolichyl Monophosphate on a Developmental Change in Mannosylphosphoryldolichol Biosynthesis

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SUMMARY: The initial rate of mannosylphosphoryldolichol formation by pig brain white matter is 2.9 to 3.3-fold higher in membranes from actively myelinating animals as compared to similar preparations from adults. Exogenous dolichyl monophosphate stimulated mannolipid synthesis in both preparations indicating that the level of the acceptor lipid was rate-limiting. The relative enhancement, however, was higher in membranes from adult animals reducing the ratio of initial rates for young/adult. Exogenous dolichyl monophosphate also stimulated the labeling of a mannosylated oligosaccharide lipid and mannoproteins, including a polypeptide (apparent molecular weight of 100,000) not labeled by gray matter membranes.

One stage in the biosynthesis of some glycoproteins involves the formation of a mannosylated N,N'-diacetylchitobiose core while linked to dolichol by a pyrophosphate bridge. In this assembly process mannosylphosphoryldolichol (MPD) plays an important role as a mannosyl donor (for reviews of the lipid intermediate pathway see refs. 1 and 2). While many of the reactions in this biosynthetic scheme are well documented, there is virtually no information available regarding regulatory controls that might be exerted on this pathway.

Earlier work in this laboratory has shown that white matter membranes from calf (3) and pig brain (4) catalyze the synthesis of MPD, a mannosylated oligosaccharide lipid (MOL) and several mannoproteins. In this paper we show that the rate of MPD synthesis is clearly higher in membrane preparations from the actively myelinating animals than in similar preparations from adults. Enzymatic studies utilizing exogenously supplied dolichyl monophosphate (dol-P) indicate that the apparent developmental difference is due partially to a lower amount of acceptor lipid in the membranes prepared from adult animals. Moreover, the stimulation of MPD labeling afforded by exogenous dol-P also resulted in enhanced incorporation of mannose into oligosaccharide lipid and membrane-associated mannoproteins. One of the mannose-labeled glycoproteins has essentially the same molecular weight (100,000) as the myelin glycoprotein that contains an oligosaccharide unit consisting primarily of mannose and N-acetylglucosamine (5). These results provide

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experimental support for the proposal that the cellular level of dol-P could play a role in regulating the rate of glycoprotein assembly during the period of active myelination. Portions of this work have been presented in preliminary form (6).

Materials: GDP-[U-^{14}C]mannose (221 mCi/mmol) was purchased from New England Nuclear Corp. Dolichyl monophosphate was obtained from Sigma Chem. Co. or prepared by chemical phosphorylation (7) of pig liver dolichols from Serdary Research Laboratories. Bio-Gel P-6 was obtained from Bio-rad Laboratories. Synthetic β-mannosyphosphoryldolichol used as a chromatographic standard was a generous gift from Dr. C.D. Warren, Harvard Medical School.
Animals: The pigs used in age-related experiments and their maintenance have been previously described (8). For actively myelinating subjects (19-22 days old) white matter was pooled from the brains of 5-8 litter-mates. The dam (11-54 mo. old) of each litter was used as the source of adult tissue. Pig brains used in other experiments were obtained from a local slaughterhouse.
Enzyme preparation: Brains were placed on ice immediately upon removal and membrane preparations were begun within 2 h after death of the animals. Membranes from either white or gray matter were prepared as described earlier (9) and suspended in 0.1 M Tris-HCl, pH 7.1, 1.0 mM EDTA, 250 mM sucrose at concentrations of 10-20 mg of protein/ml. Protein was measured by the method of Lowry et al. (10). Freshly prepared enzyme was used in assays of initial rates of [^{14}C]MPD synthesis in age-related studies. For other experiments enzyme was stored at -17° C until used.
Assay procedure: The transfer of [^{14}C]mannose from GDP-[^{14}C]mannose into [^{14}C]MPD, [^{14}C]MOL and [^{14}C]mannoprotein was assayed by the method described previously (3). The identification of the [^{14}C]mannolipid as [^{14}C]MPD was based on the same hydrolytic and chromatographic criteria used for the [^{14}C]mannolipid from calf brain including cochromatography with synthetic β-mannosylphosphoryl-dolichol on SG-81 paper in acidic, basic and neutral solvent systems (3).
Preparation of [Man-^{14}C]oligosaccharide and [Man-^{14}C]glycopeptides: Reaction mixtures containing enzyme (7 mg of protein), 70 mM Tris-HCl, pH 7.1, 180 mM sucrose, 0.7 mM EDTA, 20 mM $MnCl_2$, 2.5 mM AMP, 0.04% Triton X-100, 8.0 μM GDP-[^{14}C]mannose (440 cpm/pmole) and where indicated 200 nmoles dol-P in a total volume of 0.5 ml were incubated for 60 min at 37° C. The labeled oligosaccharide lipid and glycoprotein fractions were recovered as described elsewhere (3). [Man-^{14}C]oligosaccharide was released from the lipid carrier by treatment with 0.1 N HCl in 80% tetrahydrofuran at 50° C for 60 min. [Man-^{14}C]glycopeptides were prepared from labeled glycoproteins with Pronase as described in an earlier paper (9).

RESULTS AND DISCUSSION: Based on the rate of accumulation of cholesterol (11) or cerebroside (12), the period of most rapid myelination in pig brain is known to be between the first and fifth week after birth. The rate of MPD synthesis has been compared for white matter membranes from actively myelinating pigs and adult animals in the absence and presence of exogenous dol-P, the acceptor lipid. The results in Table I show that in the absence of exogenous dol-P the initial rate of MPD synthesis was from 2.9 to 3.3-fold higher in membranes from the younger animals in three separate comparisons. The concentration of labeled sugar nucleotide in all assays was saturating for membrane preparations from young and adult animals, and the apparent Km values for GDP-mannose were 2.3×10^{-7} M and 2.0×10^{-7} M, respectively. Thus, the higher level of MPD syn-

TABLE I

THE EFFECT OF EXOGENOUS ACCEPTOR LIPID ON THE DEVELOPMENTAL DIFFERENCES IN THE INITIAL RATE OF MANNOSYLPHOSPHORYLDOLICHOL SYNTHESIS

	Dol-P Added (nmoles)	[^{14}C]MPD Formed (cpm/mg Protein) Myelinating	Adult	Ratio of Initial Rates Myelinating/Adult
Exp. A	None	10,896	3,784	2.9
Exp. B	None	11,208	3,358	3.3
	8.8	21,669	7,903	2.7
	17.5	30,685	12,035	2.6
	35.0	49,683	20,084	2.5
	70.0	76,173	31,355	2.4
Exp. C	None	12,572	4,245	3.0
	12.8	33,142	18,321	1.8
	25.5	44,494	27,742	1.6
	51.0	66,441	40,468	1.6
	102.0	93,347	55,467	1.7

Each reaction mixture contained enzyme (1.0 to 1.2 mg protein), 50 mM Tris-HCl, pH 7.1, 125 mM sucrose, 0.5 mM EDTA, 20 mM $MnCl_2$, 0.04% (v/v) Triton X-100, 2.5 M GDP[^{14}C]mannose (440 cpm/pmole) and the indicated amounts of dolichyl monophosphate in a total volume of 0.2 ml. Exogenous dol-P was dispersed in 2.0% Triton X-100 by ultrasonication and the amount added was measured by the Bartlett procedure for phosphorus (17). The reaction mixtures were incubated at 37° C for 2 minutes and formation of [^{14}C]MPD assayed as described under MATERIALS AND METHODS. Myelinating pigs were 19-22 days old in all experiments. Adult animals were 11 months, 54 months, and 15 months old in Experiments A,B and C, respectively.

thesis in membrane preparations from actively myelinating animals is not due to an alteration in the reactive site for the nucleotide sugar substrate occurring during maturation. The labeling of mannolipid was linear with respect to the amount of membrane protein in these assay mixtures.

The possibility that the lower specific activity in membrane preparations from adult animals was due to a lower level of endogenous dol-P was examined by comparing the labeling of MPD in the presence of varying amounts of exogenous dol-P. The data in Table I (exps. B and C) show that the labeling of MPD is stimulated in preparations from young and adult animals by the addition of increasing amounts of exogenous dol-P indicating that the level of acceptor lipid in both preparations is rate-limiting. However, the relative enhancement of MPD synthesis was proportionally higher for membranes from adult animals. This significantly reduced the ratio of initial rates of MPD synthesis for young/adult

TABLE II

STIMULATION OF [^{14}C]MANNOSE INCORPORATION INTO OLIGOSACCHARIDE LIPID AND MANNOPROTEINS BY EXOGENOUS DOLICHYL MONOPHOSPHATE

Dol-P Added (nmoles)	[^{14}C]Mannose (cpm) incorporated into MPD	MOL	MANNOPROTEIN
None	6,751	80	557
56	173,604	468	1,278

Each reaction mixture contained enzyme (1.0 mg of membrane protein), 2.5 mM AMP, 0.04% (v/v) Triton X-100, 3.2 μM GDP-[^{14}C]mannose (440 cpm/pmole) and, where indicated 56 nmoles dolichyl monophosphate in a total volume of 0.2 ml. The reaction mixtures were incubated at 37° C for 30 minutes and the incorporation of radioactivity into the labeled products was assayed as described under MATERIALS AND METHODS.

suggesting that at least one subfraction of the adult white matter membranes has lower activity due to a reduction in the content of endogenous dol-P. If the apparent developmental change were due solely to a difference in the amount of endogenous acceptor lipid, the addition of the highest levels of exogenous dol-P, that are approaching saturation, would result in equivalent amounts of activity in both preparations. However, the data for exp. C in Table I show that as the level of exogenous dol-P approaches saturation, the ratio of young/adult does not become 1.0, but clearly reaches a plateau at a ratio of approximately 1.6. This result suggests that at least one subfraction of the white matter membrane preparation from actively myelinating animals is higher in activity than adults because it contains more MPD synthase.

In a related study, Breckenridge and Wolfe (13) found that mannolipid biosynthesis by postnuclear pellets from embryonic chick brains was 2.4 times higher at 14 days than in enzyme preparations from 8 day embryos. Based on neurochemical criteria from another report (14), the higher level of mannolipid synthesis corresponds to the onset of myelination. In preliminary studies (15), membranes from pre-myelinating, fetal pig brains synthesize less MPD than membranes from brains of actively myelinating pigs.

The effect of exogenous dol-P on the incorporation of [^{14}C]mannose from GDP-[^{14}C]mannose into the [^{14}C]MOL and the membrane-bound [^{14}C]mannoproteins was also measured. The data in Table II show that stimulation of MPD labeling also resulted in enhanced incorporation of [^{14}C]mannose into MOL and manno-

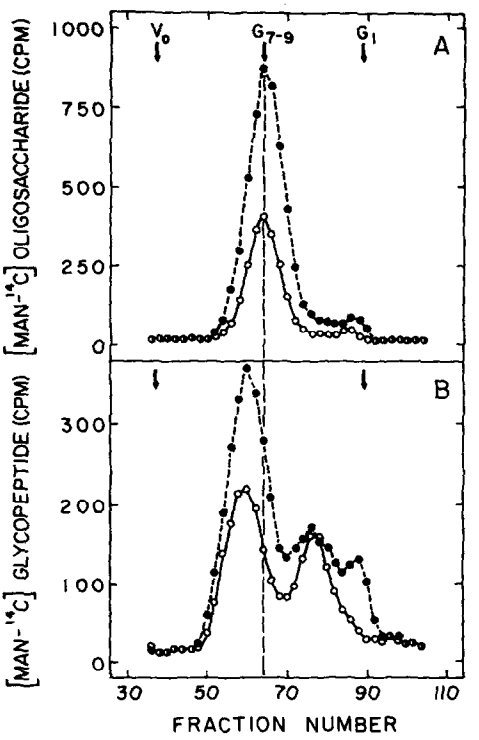

Fig. 1 Chromatographic comparison on Bio-Gel P-6 of [^{14}C]oligosaccharide and [Man-^{14}C]glycopeptides labeled in the absence or presence of exogenous dolichyl monophosphate. [Man-^{14}C]oligosaccharide and [Man-^{14}C]glycopeptides were prepared as described under MATERIALS AND METHODS. [Man-^{14}C]oligosaccharide (Panel A) or [Man-^{14}C]glycopeptides (Panel B) labeled in the absence (o—o) or presence (•---•) of exogenous dol-P were applied to a Bio-Gel P-6 column (1.5 x 75 cm) and eluted with 0.1 M NaCl. Fractions (1.0 ml) were collected and counted. Calibration markers were: V_o, blue dextran; G_{7-9}, [Man-^{14}C]oligosaccharide prepared as previously outlined (16); G_1, mannose.

proteins. This result indicates that at least one labeled glycoprotein was glycosylated via MPD and/or the mannosylated oligosaccharide lipid intermediate.

The lipid-linked [Man-^{14}C]oligosaccharide units formed in the presence or absence of exogenous dol-P are chromatographically identical when analyzed on a Bio-Gel P-6 column (Fig. 1, panel A). After the labeled mannoproteins were converted to glycopeptides by Pronase treatment, chromatographic analysis revealed that labeling of a glycopeptide, appearing to be slightly larger than the lipid-linked oligosaccharide, was stimulated by the addition of dol-P (Fig. 1, panel B). A smaller glycopeptide (fractions 70-80) was unaffected by exogenous dol-P. All of these data are compatible with the conclusion that the level of dol-P can regulate the synthesis of MPD and MOL, and by this mechanism modulate the rate of assembly of some white matter glycoproteins.

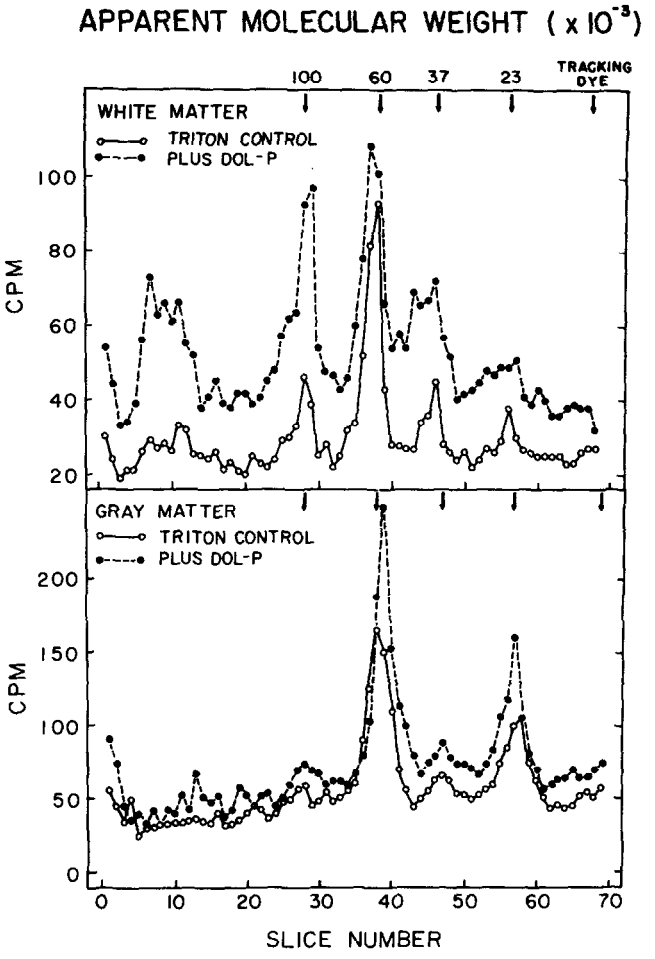

Fig. 2 SDS-polyacrylamide gel electrophoresis of [^{14}C]mannoprotein from pig white matter (Panel A) and gray matter (Panel B) in the absence (o—o) or presence (•---•) of exogenous dolichyl monophosphate. Reaction mixtures containing enzyme (15-20 mg of protein) 60 mM Tris-HCl, pH 7.1, 150 mM sucrose, 0.6 mM EDTA, 15 mM $MnCl_2$, 0.03% Triton X-100, 4.7 μM GDP[^{14}C]mannose (440 cpm/pmole) and 240 nmoles dol-P where indicated, in a total volume of 1.7 ml were incubated for 2 h at 37° C to prepare labeled mannoprotein. Labeled mannoprotein (2000-5000 cpm, 500 μg protein) was analyzed by the procedure described earlier (3).

To estimate the size and number of the glycoproteins labeled *in vitro* with GDP-[^{14}C]mannose in the absence or presence of exogenous dol-P, the [^{14}C]-mannoproteins were analyzed by SDS-polyacrylamide gel electrophoresis. A mannose-containing glycoprotein with an apparent molecular weight of 60,000 is the major labeled polypeptide for both white and gray matter (Fig. 2). Of considerable interest is the finding that a polypeptide having an apparent molecular weight of 100,000 was labeled only by membranes from white matter, the portion

of the brain enriched in oligodendroglial cells. The observation that labeling of this glycoprotein is markedly stimulated by exogenous dol-P (Fig. 2, upper panel) suggests that the glycosylation of this polypeptide proceeds via [^{14}C]-MPD and/or the [^{14}C]mannosylated oligosaccharide lipid. The possibility that this glycoprotein is related to the mannose- and N-acetylglucosamine-containing glycoprotein associated with myelin (5) is now being studied. Labeling of a large molecular weight product found as a broad zone between slices 5-13 is also dramatically stimulated by the addition of exogenous dol-P to white matter membranes. It is not yet known if this peak represents several unresolved components or is an aggregate of lower molecular weight glycoproteins. The labeling of a smaller polypeptide in gray matter was also stimulated by the presence of exogenous dol-P (Fig. 2, lower panel). It has an apparent molecular weight (23,000) similar to the mannose- and N-acetylglucosamine-containing surface antigen reported for rat brain (18).

In summary, the initial rate of MPD formation is higher in white matter membrane preparations from actively myelinating animals than in similar preparations from adult animals. While supplying exogenous dol-P augments MPD labeling for both ages, the ratio of myelinating/adult is reduced as would be expected if the difference in MPD synthesis were partially due to a lower level of dol-P in the membranes from adult pigs. The experimental finding that the addition of exogenous dol-P also enhanced [^{14}C]mannose incorporation into an oligosaccharide lipid and glycoprotein provides experimental support for the idea that alteration to the cellular level of dol-P could play a role in regulating the lipid-mediated assembly process for glycoproteins.

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