A Developmental Change in Dolichyl Phosphate Mannose Synthase Activity in Pig Brain

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The initial rate of dolichyl phosphate mannose biosynthesis was measured in white-matter membranes from pig brain at various ages from before birth throughout the period of most rapid brain development. Dolichyl phosphate mannose synthase activity increased from prenatal values to a maximum in 3 week-old animals, and gradually decreased to adult values after 8 weeks of age. The nature of the developmental change was investigated by enzymic and biochemical comparisons of the membrane preparations from the most active age (3 weeks) and adult controls. The specific activity of dolichyl phosphate mannose synthase in preparations from actively myelinating animals was approx. 3-fold higher than adults when mannosyl lipid formation was assayed with saturating concentrations of GDP-[¹⁴C]mannose and utilizing only endogenous acceptor lipid. No major variations were found in the apparent K_m values for GDP-mannose or exogenous dolichyl monophosphate. However, the ratio of dolichyl phosphate mannose synthase activity for myelinating animals/adult animals decreased significantly when large amounts of exogenous dolichyl monophosphate were added to the incubation mixtures. Dolichyl phosphate mannose synthase activity was also compared in white-matter membranes depleted of endogenous dolichyl monophosphate by enzymic mannosylation or treatment with butanol. When these preparations were assayed with identical amounts of exogenous dolichyl monophosphate, the dolichyl monophosphate-depleted membranes from actively myelinating animals contained only 20–30% more dolichyl phosphate mannose synthase activity. Overall, these studies strongly suggest that the developmental change in dolichyl phosphate mannose synthase activity is due primarily to the presence of a relatively lower amount of endogenous dolichyl monophosphate being accessible to the mannosyltransferase in the white-matter membranes from adult animals.

The function of dolichyl monophosphate as an 'activated' glycosyl-carrier lipid in the biosynthesis of asparagine-linked oligosaccharides in animal tissues and yeast is now firmly established (for reviews see Lucas & Waechter, 1976; Waechter & Lennarz, 1976; Hemming, 1977; Parodi & Leloir, 1979). Enzymic studies performed in this laboratory have been directed at understanding the role of polyisoprenoid glycolipid intermediates in the assembly of *N*-glycosidically linked carbohydrate chains in central nervous tissue (Waechter & Scher, 1979).

The presence of a myelin-associated glycoprotein in the central nervous system has been demonstrated,

and structural analyses indicate that myelin-associated glycoprotein contains asparagine-linked saccharide chains (Quarles, 1979). In related studies, indirect evidence was obtained suggesting that a membrane glycoprotein having an apparent molecular weight similar to the major myelin-associated glycoprotein was glycosylated via lipid intermediates in membrane preparations from white matter, but not grey matter (Harford *et al.*, 1977). Considering the possibility that myelin-associated glycoproteins are glycosylated by dolichol derivatives, it was of interest to see if the rate of synthesis of any glycolipid intermediates was increased concurrently with active myelination in white-matter membranes. White matter was chosen for these studies because it is enriched in oligodendrocytes, the cells responsible for myelin biogenesis in the central nervous system (Bunge, 1968). Recent immunocytochemical

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studies on rat brain have provided evidence that the myelin-associated glycoprotein is present in oligodendrocytes and myelin of developing central nervous system (Sternberger *et al.*, 1979).

In this investigation we have focused on the initial rate of dolichyl phosphate mannose biosynthesis. Dolichyl phosphate mannose has been shown to serve as a mannosyl donor in the elongation of a dolichol-bound oligosaccharide in brain (Waechter *et al.*, 1976; Harford & Waechter, 1979), as reported for other animal tissues (Waechter & Lennarz, 1976). In the present paper we report that the maximal initial rate of dolichyl phosphate mannose synthase activity corresponds closely to the most active period of myelination. Various enzymic approaches have yielded data consistent with the conclusion that the age-related change in mannosyl synthesis is due principally to differences in the amount of dolichyl monophosphate accessible to the mannosyltransferase. The implications for modulation of the amounts of the carrier lipid, dolichyl monophosphate, as one type of regulatory control on the lipid intermediate pathway are discussed. Portions of this work have appeared in a preliminary communication (Harford *et al.*, 1977).

Materials and Methods

Materials

GDP-[U- 14 C]mannose (sp. radioactivity 246 Ci/mol) was purchased from New England Nuclear Corp. (Boston, MA, U.S.A.). Dolichyl monophosphate and cholesterol were obtained from the Sigma Chemical Co. (St. Louis, MO, U.S.A.). Triton X-100 was from Packard Instrument Co. (Downers Grove, IL, U.S.A.). All other chemicals and materials were purchased from standard commercial sources.

Preparation of enzyme

The pigs used in these developmental studies and their maintenance have been described by Earl *et al.* (1971). Brains were placed on ice immediately after removal, and membranes were prepared by the procedure described previously (Waechter & Harford, 1977). This method included the centrifugation through 0.9M-sucrose to remove contaminating myelin by flotation. Protein was determined by the method of Lowry *et al.* (1951), with bovine serum albumin as standard.

The data for young animals were obtained with white-matter membranes prepared from five to eight littermates at each age. The white-matter membranes from the dam (12–15 months) of each litter were used for comparison with adult tissue.

Preparation of phospholipid-depleted membranes

Phospholipid-depleted membranes were prepared

by soaking the white-matter membranes in butanol at low temperature. In this procedure the standard membrane preparation was diluted with water to a suspension of 2mg of membrane protein/ml and centrifuged at 39000g for 20min. The membranous pellet was resuspended in cold water at a protein concentration of 5mg/ml and freeze-dried. The freeze-dried membrane powder was then homogenized in 10ml of ice-cold butanol by 10 strokes with pestle B of a Dounce homogenizer (Kontes). The suspension in butanol was stirred at 0–4°C for 1h, and the delipidated membrane suspension was sedimented by centrifugation at 39000g for 5min. The butanol extract was discarded, and the pellet was dried under vacuum. Phospholipid-depleted membranes were suspended in 0.1M-Tris/HCl (pH 7.1)/0.25M-sucrose/1mM-EDTA and stored at –17°C for use as enzyme. A determination of lipid phosphorus (Bartlett, 1959) indicated that 80% of the total membrane phospholipid was removed by the treatment with butanol.

Assay for the enzymic transfer of [14 C]mannose from GDP-[14 C]mannose into pig brain endogenous acceptors

All assays of the initial rate of dolichyl phosphate mannose synthesis were carried out in the presence of an optimal concentration of MnCl_2 and saturating amounts of GDP-[14 C]mannose. The incorporation of [14 C]mannose into dolichyl phosphate [14 C]mannose, a [14 C]mannosylated oligosaccharide phospholipid and [14 C]mannoprotein during incubation of white-matter membranes with GDP-[14 C]mannose was measured as described previously (Waechter *et al.*, 1976).

Determination of radioactivity

Radioactive samples were counted in scintillation vials with ACS (Amersham Corp.). All of the results reported in the present paper are average values obtained by counting each sample for radioactivity for 10min.

Results

Development of porcine brain

Brain development in mammals is characterized by a period of a rapid increase in brain size, termed the 'growth spurt' (Davison & Dobbing, 1968). This rapid growth period is the result of cell proliferation and myelin accumulation. As indices of brain development and myelination, we have measured the average brain weight and cholesterol content of the miniature swine. The same animals were used for the studies on developmental changes in dolichyl phosphate mannose synthase activity described below.

The results in Fig. 1 show that brain growth is

Fig. 1. Brain growth and cholesterol accumulation during development in pig brain

The mean brain weight for four to seven littermates is shown. The standard deviation within each litter was within $\pm 10\%$. Cholesterol was extracted from whole-brain homogenates and measured by the method of Searcy & Bergquist (1960). For this determination one randomly chosen brain from each litter was used. Values for adults (12 months) were 67.5 g for brain weight and 30.2 mg/g wet wt. for brain cholesterol content.

in progress from the day 90 of gestation through a 5–6 week postnatal period. Since myelin contains substantial amounts of cholesterol, the amount of sterol was determined as a measure of the myelination process (Fig. 1, ●). The greatest increase in cholesterol content occurred during the period between 2 and 6 weeks of age. Although the average brain weights of adult animals used in these experiments were lower than in a larger breed of swine used by Dickerson *et al.* (1967), the data for cholesterol content are in close agreement. On the basis of the developmental change in cholesterol content seen here, it appears that rapid myelination occurs when these animals are approx. 2–3 weeks old. Similar studies on the basis of the rate of accumulation of cholesterol (Dickerson & Dobbing, 1967) and cerebroside (Sweasy & Patterson, 1975) indicate that there is active myelination occurring in porcine central nervous tissue between 2 and 5 weeks.

Developmental change in dolichyl phosphate mannose synthase activity

When the initial rate of dolichyl phosphate

Fig. 2. Initial rates of dolichyl phosphate mannose synthase activity in white-matter membranes during development

Reaction mixtures containing white-matter membranes (0.4–0.8 mg of protein), 50 mM-Tris/HCl (pH 7.1), 125 mM-sucrose, 0.5 mM-EDTA, 20 mM-MnCl₂ and 5 μ M-GDP-[¹⁴C]mannose (440 c.p.m./pmol) in a total volume of 0.1 ml were incubated for 2 min at 37°C. Formation of [¹⁴C]-mannolipid was assayed as described in the Materials and Methods section.

mannose synthesis was measured in white-matter membranes prepared from pigs of various ages during the 'growth-spurt' period, mannolipid synthesis began to increase at birth and maximal activity was observed in membranes from 21 day-old pigs (Fig. 2). The initial rate of dolichyl phosphate mannose synthesis decreased thereafter, declining to adult values in 8 week-old animals. The initial rate of transfer of [¹⁴C]mannose from GDP-[¹⁴C]-mannose into dolichyl phosphate [¹⁴C]mannose, catalysed by white-matter membranes from 21 day-old pigs, was approx. 3-fold higher relative to adult animals. This peak of dolichyl phosphate mannose synthase activity correlates closely with the period of active myelination.

To examine the developmental change in dolichyl phosphate mannose synthesis in more depth, extensive enzymic studies were made on the mannosyltransferase activity in white-matter membranes from actively myelinating pigs (3 weeks) and adults. From Fig. 3 it can be seen that the amount of mannolipid synthesized was linearly dependent on the amount of white-matter membranes added, and the ratio in myelinating animals to that in adults remained approx. 3.0 at all concentrations of membrane protein. Dolichyl phosphate mannose synthase activity has also been shown to be 3-fold

Fig. 3. Dependence of dolichyl phosphate mannose synthase activity on the amount of white-matter membranes added

White-matter membranes (amount of protein indicated) from actively myelinating (●) and adult (○) pigs were incubated with 0.05 M-Tris/HCl (pH 7.1)/0.125 M-sucrose/0.5 mM-EDTA/20 mM-MnCl₂/5 μM-GDP-[¹⁴C]mannose (440 c.p.m./pmol) for 2 min at 37°C. The formation of manno-lipid was measured as described in the Materials and Methods section.

higher in three other litters (19–22 days) compared with the corresponding adult controls (Harford *et al.*, 1977).

To see if the reactive sites for the sugar nucleotide and acceptor lipid substrates were developmentally altered, the apparent K_m values for GDP-mannose and exogenous dolichyl monophosphate were compared for the actively myelinating and adult animals. The apparent K_m value for GDP-mannose was found to be 0.2 μM for both myelinating and adult animals (Fig. 4a). Only a minor variation was seen in the apparent K_m values for dolichyl monophosphate in the myelinating (0.5 mM) and adult animals (0.8 mM) (results not shown). In the light of the similarity of these kinetic parameters, it seems unlikely that the developmental change in dolichyl phosphate mannose synthase activity is due to modification of the reactive sites for these substrates.

Biochemical comparison of white-matter membranes prepared from adults and actively myelinating pigs

Recent studies have shown that dolichyl phosphate mannose synthase activity is extremely low in myelin preparations from bovine white matter (Harford *et al.*, 1979). Since the myelin content in brains of adult animals is higher than in 21 day-old pigs, it was necessary to determine whether the observed developmental difference in manno-lipid biosynthesis was due simply to larger amounts of myelin present in the membrane preparations from adults. Therefore, we have made a comparison of

Fig. 4. Double-reciprocal (Lineweaver-Burk) plots for dependence of manno-lipid synthesis on the concentration of GDP-mannose by untreated (a) and phospholipid-depleted (b) membrane preparations

All incubation mixtures contained enzyme (1.6–1.8 mg of membrane protein) from young (○) or adult (●) white matter in 0.05 M-Tris/HCl (pH 7.1)/0.125 M-sucrose/0.5 mM-EDTA/20 mM-MnCl₂ and the indicated concentration of GDP-[¹⁴C]mannose (440 c.p.m./pmol). Incubations of the phospholipid-depleted membranes (b) also contained 0.15 mM-dolichyl monophosphate and 0.04% Triton X-100. After a 2 min incubation at 37°C, [¹⁴C]mannose incorporation into dolichyl phosphate mannose was measured as described in the Materials and Methods section.

the white-matter membrane preparations from adults and actively myelinating pig brains on the basis of several important biochemical criteria.

Since myelin is a relatively lipid-rich membrane, increased amounts of myelin in the white-matter membrane preparations from adults would be reflected in the ratios of phospholipid and cholesterol to membrane protein. A comparison of these ratios in the membranes from 21 day-old animals and adults revealed that there are only minor variations in the two enzyme preparations (Table 1). Moreover, a specific radioimmunoassay for myelin basic protein (Cohen *et al.*, 1975), a major myelin component, indicated that basic protein accounts for less than 2% of the membrane protein in both enzyme preparations (Table 1). This result presents

Table 1. Lipid and myelin basic protein content in white-matter preparations from myelinating and adult pigs
Membranous enzyme preparations were obtained from white matter as described previously (Waechter & Harford, 1977). Cholesterol was extracted with acetone/ethanol (1:1, v/v) and quantitatively determined by the method of Searcy & Bergquist (1960). Phospholipids were extracted by the procedure of Folch *et al.* (1957) and lipid phosphorus was determined by the method of Bartlett (1959). Myelin basic protein was determined by radioimmunoassay (Cohen *et al.*, 1975). Membrane protein was measured by the procedure of Lowry *et al.* (1951).

Membrane preparation	Cholesterol ($\mu\text{g}/\text{mg}$ of protein)	Phospholipid (nmol/mg of protein)	Myelin basic protein ($\mu\text{g}/\text{mg}$ of protein)
Myelinating	440 ± 8	422 ± 21	11
Adult	460 ± 13	377 ± 16	13

a strong argument against either preparation containing significant amounts of myelin. It is quite likely that most of the myelin recovered in the crude microsomal fraction was removed by the flotation step on 0.9M-sucrose in the enzyme preparation.

Effect of exogenous dolichyl monophosphate on the initial rate of mannolipid biosynthesis by untreated white-matter membranes

To test if the lower specific activity of dolichyl phosphate mannose synthase associated with white-matter membranes prepared from adult pigs was due to less mannosyltransferase or endogenous acceptor lipid, assays were performed *in vitro* in the presence and absence of exogenously supplied dolichyl monophosphate. The Triton X-100 concentration (0.03–0.04%) that provided the optimal stimulation was determined for these studies (Fig. 5). It can be seen from Fig. 5 that this detergent concentration had no effect on the mannosyltransferase activity in the absence of exogenous dolichyl monophosphate. As shown above, when the synthesis of mannolipid was dependent on endogenous acceptor lipid, the initial rate of dolichyl phosphate mannose synthesis was 3-fold faster in preparations from actively myelinating pigs compared with the older controls (Fig. 6a). From the data in Fig. 6(a) it is also clear that the enzymic transfer of [^{14}C]mannose from GDP-[^{14}C]mannose into dolichyl phosphate [^{14}C]mannose is stimulated by exogenous dolichyl monophosphate in both preparations. This finding suggests that the amount of endogenous dolichyl monophosphate is rate-limiting in mannolipid formation. However, although the addition of increasing amounts of exogenous dolichyl monophosphate markedly stimulates mannosyltransferase activity in both white-matter preparations, the relatively higher enhancement in membranes from adults results in a decrease in the ratio for myelinating animals to that in adults. At a concentration of acceptor lipid approaching saturation the mannosyltransferase activity in the membranes from young animals is only 1.5-fold

Fig. 5. Effect of Triton X-100 concentration on the stimulation of dolichyl phosphate mannose synthase activity by exogenous dolichyl monophosphate
Reaction mixtures containing pig white-matter membranes (2.0mg of protein), 0.05 M-Tris/HCl (pH 7.1), 0.125 M-sucrose, 0.5 mM-EDTA, 20 mM-MnCl₂, 5 μM -GDP-[^{14}C]mannose (440 c.p.m./pmol), 42 nmol of exogenous dolichyl monophosphate and the indicated amount of Triton X-100 in a total volume of 0.2 ml were incubated for 2 min at 37°C. Symbols: ●, plus dolichyl monophosphate; ○, control (dolichyl monophosphate omitted). Formation of dolichyl phosphate [^{14}C]mannose was measured as described in the Materials and Methods section.

greater than the activity in the membranes of the adults (Fig. 6a, x). These data are compatible with the hypothesis that lower amounts of accessible dolichyl monophosphate in the white-matter membranes of adults are partially responsible for the developmental change in dolichyl phosphate mannose synthase activity. Since the ratio of activity in young animals compared with adults is 1.5 at the highest dolichyl monophosphate concentration, it is

Fig. 6. Effect of exogenous dolichyl monophosphate on the initial rate of dolichyl phosphate mannose synthesis in untreated (a) and phospholipid-depleted (b) membranes

Pig white-matter enzyme (1.0–1.5 mg of protein), 0.05 M-Tris/HCl (pH 7.1), 0.125 M-sucrose; 0.5 mM-EDTA, 20 mM-MnCl₂, 5 μM -GDP-[¹⁴C]-mannose (440 c.p.m./pmol), 0.04% (w/w) Triton X-100 and the indicated amounts of exogenous dolichyl monophosphate in a total volume of 0.2 ml were incubated for 2 min at 37°C. Formation of dolichyl phosphate [¹⁴C]mannose was measured as described in the Materials and Methods section. Myelinating pigs (3 weeks) were compared with their adult (12–15 months) dams. Phospholipid-depleted membranes were prepared with butanol as described in the Materials and Methods section.

quite possible that at least one subfraction of the white-matter membranes from actively myelinating pigs contains a slightly increased level of the mannosyltransferase.

To further investigate the possibility that white-matter membranes from the animals of different ages contained similar amounts of dolichyl phosphate mannose synthase, but different amounts of

Fig. 7. Time course for the synthesis of dolichyl phosphate mannose by white-matter membranes from actively myelinating (O) and adult (●) pigs and reinitiation on the addition of exogenous dolichyl monophosphate

Reaction mixtures contained white-matter membranes (0.5–0.7 mg of protein), 50 mM-Tris/HCl (pH 7.1), 125 mM-sucrose, 0.5 mM-EDTA, 20 mM-MnCl₂ and 5 μM -GDP-[¹⁴C]mannose (440 c.p.m./pmol) in a total volume of 0.1 ml. After 42 min dolichyl monophosphate (40 nmol) dispersed in Triton X-100 (final concentration, 0.04%) was added to membranes from actively myelinating (□) and adult animals (■). At this time point, control incubations from young (O) and old (●) animals received 0.04% Triton X-100 without dolichyl monophosphate and the transfer of [¹⁴C]-mannose into mannosyl lipid was assayed as described in the Materials and Methods section. The reinitiated rate of mannosyl lipid synthesis is shown in the inset with an expanded time scale.

dolichyl monophosphate available to the mannosyltransferase, another experimental approach was followed. The strategy in this study was to deplete the pool of endogenous acceptor lipid by enzymic mannosylation producing a membrane preparation that was totally dependent on the addition of exogenous dolichyl monophosphate. In Fig. 7 it can be seen that when white-matter membranes from young and old pigs are incubated with GDP-[¹⁴C]mannose, the incorporation of [¹⁴C]mannose into [¹⁴C]mannosyl lipid reaches a plateau after 20 min. After 20 min the incorporation of label into oligosaccharide lipid and glycoprotein is extremely slow. There is also very little [¹⁴C]mannose and [¹⁴C]mannose 1-phosphate appearing in the incubation mixture. Therefore, it is very unlikely that the plateau value represents a steady state in a rapid-turnover process in which dolichyl phosphate

[^{14}C]mannose is actively functioning as a mannosyl donor in the synthesis of the oligosaccharide lipid intermediate (Harford & Waechter, 1979) or being extensively degraded. In fact, after a 20 min incubation GDP-[^{14}C]mannose was still present at a concentration in 10-fold excess of the apparent K_m . The higher plateau value for actively myelinating animals suggests that in this '*in vitro*' system the amount of available dolichyl monophosphate can influence the extent as well as the initial rate of dolichyl phosphate mannose synthesis. In any case, on the addition of equivalent amounts of exogenous dolichyl monophosphate the synthesis of dolichyl phosphate mannose is resumed in both preparations, and the reinitiated rate of mannosyl lipid formation in the membranes from myelinating animals is only 1.2-fold higher than in membranes from adults (Fig. 7, inset). These results also indicate that the age-related change in dolichyl phosphate mannose synthase activity is probably not due to a decrease in the amount of mannosyltransferase, but to a lower amount of available dolichyl monophosphate.

Comparison of dolichyl phosphate mannose synthase activity in phospholipid-depleted white-matter membranes

Evidence for lower amounts of dolichyl monophosphate in white-matter membranes from the adult animals was also adduced with phospholipid-depleted enzyme preparations obtained by treating membranes with butanol as described in the Materials and Methods section. Before comparing the dolichyl phosphate mannose synthase activity in the two preparations, several enzymic parameters were examined. Although virtually no dolichyl phosphate [^{14}C]mannose was formed from GDP-[^{14}C]mannose in the absence of exogenous dolichyl monophosphate, the addition of exogenous acceptor lipid resulted in a concentration-dependent synthesis of [^{14}C]mannolipid (Fig. 8). As seen with the untreated membranes (Fig. 5), the optimal Triton X-100 concentration for dolichyl monophosphate stimulation in phospholipid-depleted membranes was 0.03–0.04%. At a dolichyl monophosphate concentration of 0.3 mM, the initial rate of mannosyl lipid synthesis in butanol-treated membranes was approx. 50% of the rate observed in untreated membranes. Thus a substantial amount of the dolichyl phosphate mannose synthase activity survived the treatment with butanol. Moreover, the apparent K_m values for GDP-mannose in the preparations from actively myelinating and adult pigs were both $0.4\ \mu\text{M}$ (Fig. 4b). From double-reciprocal plots an apparent K_m of $0.04\ \text{mM}$ was calculated for dolichyl monophosphate in membrane preparations from both young and adult animals (results not shown).

When the dependence of the initial rate of dolichyl

Fig. 8. Dependence of dolichyl phosphate mannose synthesis by phospholipid-depleted membranes on exogenous dolichyl monophosphate

White-matter membranes (1.5 mg of protein) depleted of endogenous phospholipid by treatment with butanol were incubated with 0.05 M-Tris/HCl (pH 7.1), 0.125 M-sucrose, 0.5 mM-EDTA, 20 mM-MnCl₂, 0.04% Triton X-100, $5\ \mu\text{M}$ -GDP-[^{14}C]mannose (440 c.p.m./pmol) and the indicated amount of exogenous dolichyl monophosphate at 37°C. At each time point mannosyl lipid synthesis was measured by the assay procedure described in the Materials and Methods section.

phosphate mannose formation on the concentration of dolichyl monophosphate was compared for white-matter preparations obtained from young and old subjects in the presence of saturating amounts of GDP-[^{14}C]mannose, marked stimulation was seen in both cases (Fig. 6b). However, in contrast with untreated membranes the ratio of mannosyltransferase activity in myelinating animals to that in adult animals was approx. 1.3 at all acceptor lipid concentrations (Fig. 6b, x). Our interpretation of these results is that the amounts of dolichyl phosphate mannose synthase in the two preparations are nearly equal. These data reinforce the evidence that the developmental difference observed in untreated membranes is due primarily to a diminished amount of accessible dolichyl monophosphate in adult animals. The finding of a ratio of dolichyl phosphate mannose synthase activity in this experiment of 1.3 is also in accord with the conclusion described above that at least one subfraction of the white-matter membranes from actively myelin-

ating animals may possess a slightly higher amount of the mannosyltransferase.

Effect of exogenous dolichyl monophosphate on the incorporation of [14 C]mannose into oligosaccharide lipid and glycoprotein during incubation of white-matter membranes with GDP-[14 C]mannose

The enzymic studies in which dolichyl monophosphate was added exogenously suggest that the initial rate of synthesis of dolichyl phosphate mannose by white-matter membranes is limited by the amount of available dolichyl monophosphate. If modulation of dolichyl monophosphate availability plays a regulatory role in the lipid intermediate pathway, it would be expected that when mannosylation was stimulated by augmentation of the amount of accessible dolichyl monophosphate, there would be a consequent enhancement in the mannosylation of the dolichol-bound oligosac-

charides and the glycosylation of polypeptide acceptors.

The experiment depicted in Fig. 9 demonstrates that when white-matter membranes are incubated with GDP-[14 C]mannose and increasing amounts of exogenous dolichyl monophosphate, under conditions that stimulate dolichyl phosphate mannose formation, there is a concentration-dependent enhancement of [14 C]mannose incorporation into oligosaccharide lipid and glycoprotein. These results show that in this 'in vitro' system the lipid-mediated mannosylation of dolichol-bound oligosaccharides and the addition of mannosylated oligosaccharides to polypeptide acceptors are responsive to changes in the rate of dolichyl phosphate mannose synthesis.

Discussion

The information on the regulatory aspects of the lipid intermediate pathway for asparagine glycosylation has not expanded as rapidly as the knowledge of the individual lipid-mediated glycosyl transfer reactions. Now that the biosynthesis of *N*-glycosidically linked oligosaccharides is understood in substantial detail, it should be possible to investigate the various potential control sites in this complex assembly process. The present paper reports evidence for a developmental change in the dolichyl phosphate mannose synthase activity associated with white-matter membranes. The differences in mannosyltransferase activity between myelinating and adult animals appear to be a result of changes in the amount of available acceptor lipid. The prenatal samples were not sufficient for a detailed comparison with actively myelinating animals. The highest activity is found at an age closely corresponding to the period of active myelination. During this period oligodendroglial cells would be actively synthesizing myelin components, including myelin-associated glycoprotein. Since myelin-associated glycoproteins contain asparagine-linked oligosaccharides, it is reasonable to expect that the formation of glycolipid intermediates might be accelerated at this stage of development.

To better understand the nature of the modulation in dolichyl phosphate mannose synthase activity, extensive biochemical and enzymic comparisons were made with white-matter preparations from actively myelinating (21 days) and adult animals. When the 'in vitro' assays were dependent on the amount of endogenous acceptor lipid, the young animals catalysed the transfer of [14 C]mannose from GDP-[14 C]mannose into mannosylated lipid at an initial rate 3-fold higher than the same preparations from adults. No significant changes in the apparent K_m values for GDP-mannose or dolichyl monophosphate were detected, indicating that there

Fig. 9. *Effect of exogenous dolichyl monophosphate on the incorporation of [14 C]mannose into oligosaccharide lipid and glycoprotein*

White-matter membranes (1.7 mg of protein) were incubated with 0.05 M-Tris/HCl (pH 7.1), 0.125 M-sucrose, 0.5 mM-EDTA, 20 mM-MnCl₂, 5 μ M-GDP-[14 C]mannose (440 c.p.m./pmol) and the indicated amount of exogenous dolichyl monophosphate dispersed in Triton X-100 (final concentration, 0.04%). After incubation for 20 min at 37°C, the amount of [14 C]mannose incorporated into oligosaccharide lipid and glycoprotein was measured as described in the Materials and Methods section. Abbreviation used: MOCL, [14 C]mannosylated oligosaccharide carrier lipid.

were no developmentally related alterations in the reactive sites for these substrates. However, the age-related differences in dolichyl phosphate mannose synthase activity were partially abolished when the enzymic comparison was made in the presence of nearly saturating concentrations of exogenous dolichyl monophosphate. The compensatory effect of exogenous dolichyl monophosphate suggested that the higher activity in the young animals is probably due to a relatively higher amount of endogenous dolichyl monophosphate accessible to the mannosyltransferase, or obversely a deficit of dolichyl monophosphate in the adults. Since the ratio of dolichyl phosphate mannose synthase activity for myelinating animals to that for adults approached, but did not reach, 1.0, it appears that the white-matter membranes from the actively myelinating animals also possess a slightly higher level of mannosyltransferase. This conclusion was corroborated by assays performed *in vitro* with membranes depleted of endogenous dolichyl monophosphate by either enzymic mannosylation or treatment with butanol. Under these conditions dolichyl phosphate mannose synthase activity was dependent on the addition of exogenous dolichyl monophosphate. When assayed in the presence of identical amounts of exogenous acceptor lipid, the phospholipid-depleted membranes from actively myelinating pigs exhibited 20–30% more mannosyltransferase activity than similar preparations from the appropriate adult controls. Considering all the available data, it seems reasonable to conclude that the developmental change in dolichyl phosphate mannose synthase activity observed in these white-matter membranes is due primarily to a higher level of available dolichyl monophosphate in the preparations from actively myelinating animals. Only 20–50% of the increased activity can be accounted for by a higher amount of the mannosyltransferase.

Additional studies reported here with microsomal preparations from white matter also demonstrate that mannose incorporation into an oligosaccharide lipid intermediate and glycoprotein is accelerated as a consequence of enhanced dolichyl phosphate mannose synthesis produced by the addition of exogenously supplied dolichyl monophosphate (Fig. 9). It is plausible that the later stages of the lipid intermediate pathway are similarly responsive to increased concentrations of dolichyl monophosphate availability *in vivo*. It is noteworthy that when white-matter membranes were incubated with GDP-[¹⁴C]mannose, the addition of exogenous dolichyl monophosphate markedly stimulated the labelling of a polypeptide having an apparent mol.wt. of 100000 (Harford *et al.*, 1977), quite similar to the glycoprotein associated with myelin in the central nervous system (Quarles, 1979). The

addition of exogenous dolichyl monophosphate during incubation with GDP-mannose has also been found to stimulate the labelling of oligosaccharide lipid and mannoprotein in retinal preparations (Kean, 1977).

Another example of age-related differences in dolichyl phosphate mannose synthase activity was previously reported for embryonic chick brain (Breckenridge & Wolfe, 1973). In those studies mannosyl lipid formation was 2.4-fold higher in 14 day embryos than in 8 day embryos when post-nuclear pellets were incubated with GDP-[¹⁴C]-mannose in the absence of exogenous acceptor lipid. However, when the activities were compared in the presence of exogenous dolichyl monophosphate, mannosyl lipid synthesis was stimulated in both preparations, but in this case the 8 day embryos exhibited a 4-fold higher dolichyl phosphate mannose synthase activity. These results suggest that the 8 day embryos actually contained more mannosyltransferase, but it was not expressed because of a relatively lower level of accessible dolichyl monophosphate. In similar experiments Harford *et al.* (1979) found that an axolemma-enriched membrane fraction from bovine corpus callosum had only 14% as much dolichyl phosphate mannose synthase activity compared with the microsomal fraction when assays are totally dependent on endogenous acceptor lipid. However, when compared in the presence of high amounts of exogenous dolichyl monophosphate, the axolemma-enriched preparation contains 42% of the activity observed with microsomal membranes. The regulatory significance of membranes containing the mannosyltransferase, but being relatively deficient in dolichyl monophosphate, is not yet clear.

Evidence for developmental changes in dolichyl monophosphate content and/or accessibility have also been reported for other systems. Studies with chick oviduct membranes indicate that an increased amount of dolichyl phosphate mannose synthase activity found during hormone-induced differentiation is caused by an increased concentration of dolichyl monophosphate (Lucas & Levin, 1977). In a later report, the loss of dolichyl phosphate mannose synthase activity during erythrocyte maturation was attributed to the lack of available endogenous acceptor lipid (Lucas & Nevar, 1978).

Although there is now good evidence for modulation of dolichyl monophosphate concentrations in several systems, the factors governing the biosynthesis of the carrier lipid remain to be elucidated. It is known that a substantial percentage of dolichol in animal tissues is present as the esterified form (Butterworth & Hemming, 1968; Rupar & Carroll, 1978). We have previously proposed that the coupled action of a specific lipase, capable of deacylating dolichyl esters, and dolichol kinase could provide

an effective mechanism for influencing the cellular concentration of dolichyl monophosphate. CTP-mediated phosphorylation of dolichol in calf brain (Burton *et al.*, 1979) and other animal tissues (Allen *et al.*, 1978) has been documented. If the substrate for the reductase converting dehydrololichol into dolichol is the free polyprenol, dolichol kinase would be responsible for completing the synthesis of dolichyl monophosphate *de novo*. It will also be of great interest to learn more about the pathway for dolichol biosynthesis *de novo* involving isopentenyl pyrophosphate (Grange & Adair, 1977; Daleo *et al.*, 1977). Studies on cultured aorta smooth-muscle cells suggested that hydroxymethylglutaryl-CoA reductase may function as the rate-controlling enzyme in dolichyl monophosphate biosynthesis (Mills & Adamany, 1978). Other work with L-cell cultures (James & Kandutsch, 1979) and rat liver (Keller *et al.*, 1979) indicates that hydroxymethylglutaryl-CoA reductase is probably not the major control point in these systems. If modulation of dolichyl monophosphate levels is indeed an important regulatory control mechanism for protein glycosylation *in vivo*, investigations on the rate-controlling steps in polyisoprenol metabolism will be extremely beneficial to the understanding of the regulation of glycoprotein assembly.

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