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Polyprenyl Phosphates as Coenzymes in Protein and Oligosaccharide Glycosylation [and Discussion]

F. W. Hemming and G. W. Gooday

Phil. Trans. R. Soc. Lond. B 1978 **284**, 559-568
doi: 10.1098/rstb.1978.0089

Phil. Trans. R. Soc. Lond. B. **284**, 559–568 (1978)
Printed in Great Britain

Polyprenyl phosphates as coenzymes in protein and oligosaccharide glycosylation

By F. W. Hemming
Department of Biochemistry, Queen's Medical Centre, The University, Nottingham, NG7 2UH, U.K.

Partly saturated polyprenols of the dolichol type occur in all branches of the plant kingdom although in green plants they are quantitatively minor compared with the shorter polyprenols of the ficaprenol and betulaprenol type. Nevertheless, in all members of the plant kingdom so far studied it is the dolichyl phosphates that function in glycosyl transfer. In yeasts, dolichyl phosphate mannose is primarily an intermediate in the O-mannosylation of some membrane-bound proteins. Further mannosylation of the mannoprotein does not involve dolichyl phosphate. Yeast membrane preparations also catalyse the sequential transfer from the appropriate nucleoside diphosphate sugar of *N*-acetylglucosaminyl phosphate, *N*-acetylglucosamine, several mannose residues and several glucose residues to dolichyl monophosphate to form a dolichyl diphosphate oligosaccharide. The oligosaccharide is then transferred *en bloc* to protein to form an N-glycosidically linked glycoprotein. The transfer of mannose and glucose to the dolichyl diphosphate oligosaccharide is probably via dolichyl monophosphate sugar derivatives. Tunicamycin inhibits specifically the transfer of *N*-acetylglucosamine phosphate which in turn blocks protein N-glycosylation. Evidence for corresponding processes in O- and N-glycosylation of proteins of hyphal fungi is also available but much less well established. The N-glycosylation of proteins by membrane preparations of green plants appears to occur by a process very similar to that found in yeast. In addition, the biosynthesis of β1–3 and β1–4 linked oligoglucans and also of an algal cellulose primer (a glucoprotein) via dolichyl phosphate glucose has been reported. Possible consequences of these phenomena are discussed.

Introduction

Polyprenyl phosphates function as coenzymes in several glycosylation reactions. This function is shown at its simplest in the general scheme in figure 1. Two glycosyl transferases (*a* and *b*) and the polyprenyl phosphate are located in a membrane. The last mentioned acts as an intermediary carrier in the glycosylation of the acceptor. The polyprenyl phosphate is in effect a membrane-bound coenzyme. The primary donor is a nucleoside diphosphate sugar. During the reaction catalysed by enzyme *a* one sugar-1-phosphate linkage (in the NDP sugar) is replaced by another (in the polyprenyl P sugar), i.e. the transfer potential is retained. This process has been described in all types of living organisms. The acceptor may be a protein, to produce a glycoprotein, or a polysaccharide (Waechter & Lennarz 1976). In several cases where the scheme has been described the coenzymic involvement of polyprenyl phosphate has been shown to be obligatory.

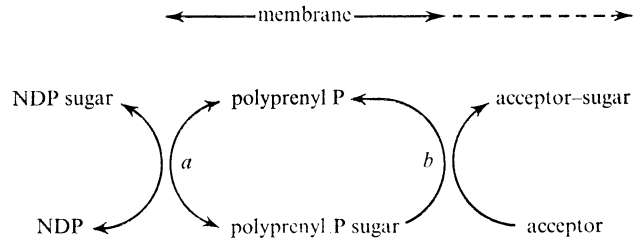
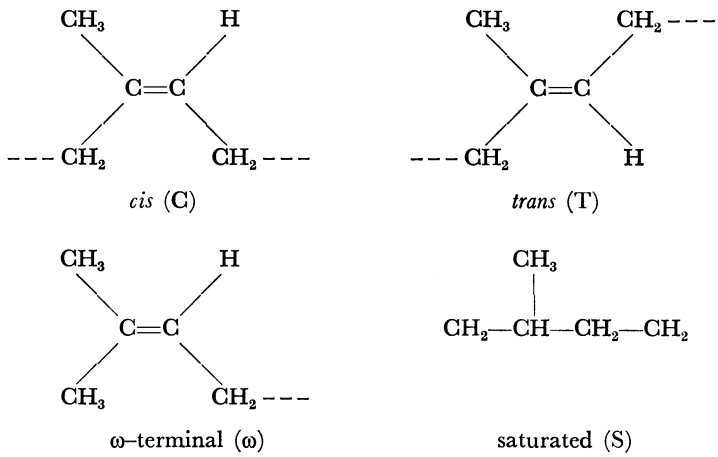


FIGURE 1. A generalized scheme showing the simplest type of involvement of a polyprenyl phosphate in glycosyl transfer.

PLANT POLYPRENOLS: A SUMMARY

Polyprenols are made up of long chains of isoprene residues linked head to tail with a terminal hydroxyl group (Hemming 1974). The isoprene residues may be in the *cis* or *trans* configuration or alternatively be a terminal ω-residue or a saturated residue:



The abbreviations shown can be used as shorthand to describe the essential features of plant polyprenols (table 1). In higher green plants, photosynthetic tissue contains polyprenols of the ficaprenol type which may accumulate to reach relatively large concentrations (e.g. 1 mg/g tissue, wet mass). However, in all plants the only polyprenols so far shown to be involved in glycosylation processes are those of the dolichol type. Dolichols are predominantly very long poly-*cis* prenols with a saturated α-residue. Some fungal versions may also be modified slightly at the ω-terminus (Hemming 1974).

TABLE 1. POLYPRENOLS FOUND IN PLANTS

higher green plants:		
common	ω T_3 C_{6-9} – OH	ficaprenol type
rare	ω T_2 C_{3-6} – OH	betulaprenol type
trace	ω T_x C_y S – OH ($x+y \approx 17$)	dolichol type
yeast	ω T_2 C_{9-13} S – OH	dolichol type
Aspergillus	S_2 T_2 C_{14-17} S – OH	dolichol type

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Experimental approach

Studies of the involvement of these compounds in glycosylation reactions are usually carried out with relatively crude cell-free membrane preparations comparable with mammalian microsomal preparations. These are incubated under suitable conditions with a nucleoside diphosphate [^{14}C or ^{3}H]sugar for a few minutes (table 2). The lipid-soluble components are then recovered and assayed for radioactivity (table 3). A mixture of chloroform and methanol (2:1 by volume) is usually adequate for extraction of polyprenyl monophosphate sugars whereas a mixture of chloroform, methanol and water (1:1:0.3 by volume) is preferred for extraction of polyprenyl disphosphate oligosaccharides present. Butanol will extract both types of compounds. These can be identified by their position of elution from an anion exchanger (e.g. DEAE-cellulose) and by thin layer and paper chromatography. In addition they are both characteristically resistant to mild treatment with alkali (unlike acyl esters) but they readily release the sugar portion upon mild treatment with acid (unlike O-glycosides, e.g. steryl glucosides).

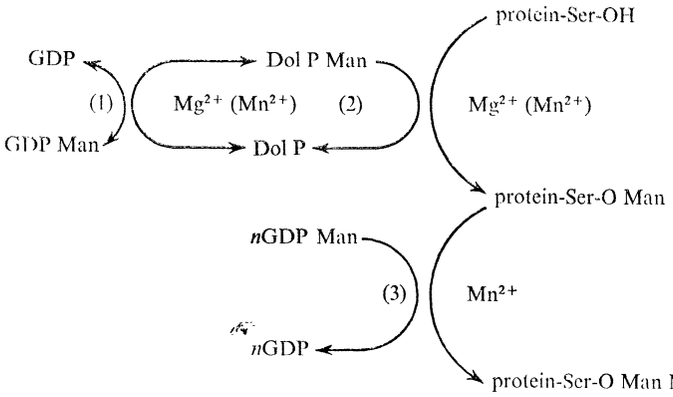


Figure 2. O-mannosylation of yeast protein (after Tanner). Steps 1, 2 and 3 are catalysed by mannosyl transferases, that catalysing step 3 having an absolute requirement for Mn^{2+} whereas the others can use either Mn^{2+} or Mg^{2+}. Man, mannose; Dol–P, dolichyl phosphate.

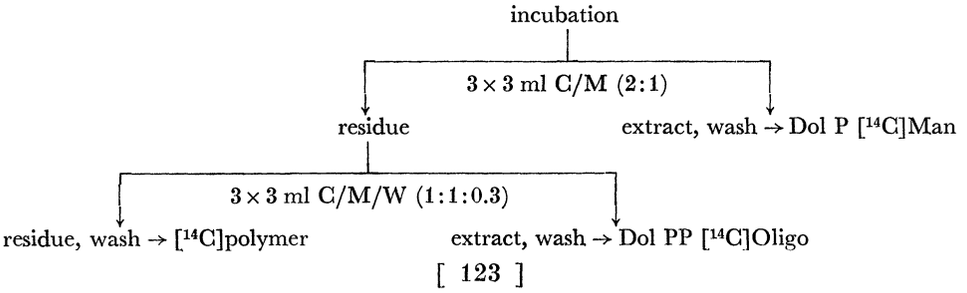
Table 2. Typical incubation conditions

membrane preparation	0.2–1.0 mg protein
Tris–HCl	22 mM, pH 7.4
$MgCl_2$	8 mM
GDP [^{14}C]mannose	3 μM (0.05 μCi)
total volume	200 μl

Incubate for 30 min at 30 °C, with shaking.

Table 3. A typical programme of extraction after incubation

(C, $CHCl_3$; M, MeOH; W, H_2O)



The glycosylated acceptor is usually recovered as the insoluble residue left after organic solvent extraction. This is assayed for radioactivity after extensive washing. Generally, putative exogenous acceptors do not function in this membrane system and often the small amount of endogenous acceptor in the membrane preparation limits the amount of radioisotope transferred.

Protein glycosylation in yeasts

O-*mannosylation*

Using these approaches, Tanner's group (Sharma, Babczinski, Lehle & Tanner 1974) demonstrated that the scheme in figure 2 operates in *Saccharomyces cerevisiae*. A feature that aided the elucidation was the discovery that when Mg^{2+} was the sole metal ion present only the mono-mannosylated protein was recovered. The polyprenol was identified as dolichol by treatment of the polyprenyl phosphate with phosphatase and mass spectrometric investigation of the polyprenol liberated (dolichyl phosphate, unlike an allylic polyprenyl phosphate, is stable to mild treatment with acid). Enzymic proteolysis characterized the final product as glycoprotein and β-elimination in an alkaline solution confirmed the O-glycosidic linkage.

Figure 3. Complex oligosaccharides of yeast glycoproteins. GlcNAc, *N*-acetylglucosamine; Man, mannose; A and B, other sugars.

A similar involvement of dolichyl phosphate in protein mannosylation by another yeast, *Hansenula holstii*, has been described by Bretthauer & Wu (1975).

Several yeast glycoproteins carry oligosaccharide groups of this type but they have not been found in any animals or green plants. The scheme in figure 2 is unique to yeasts and other fungi.

N-*glycosylation with oligosaccharides*

More complex oligosaccharide chains may also be present on yeast glycoproteins (e.g. invertase and acid phosphatase). These contain a core of two *N*-acetylglucosamine residues linked to several mannose residues (figure 3). The proximal *N*-acetylglucosamine is linked via an N-glycosidic bond with an asparagine residue in the protein chain. The mannose residues may carry other sugars. The work of Lehle & Tanner (1975), Nakayama, Arahi & Ito (1976), Palamarczyk (1976), Reuvers, Habets-Willems, Reinking & Boer (1977) and Parodi (1977) has led to a proposal (Parodi 1977) that the proximal core of these oligosaccharides is assembled and transferred to protein in *S. cerevisiae* in a manner very similar to that operating in animal cells (figure 4). In fact, steps 3 and 7 have not yet been demonstrated in yeast cells. The enzyme (or possibly enzymes in view of the multiplicity of protein acceptors) catalysing step 6 has a wide specificity *in vitro* (and possibly *in vivo*) to the oligosaccharide being transferred. Thus the sugar portions of the products of steps 2, 3, 4 and 5 can be transferred to protein. The activity of this cycle (figure 4) is much less than that of the scheme in figure 2. One result of this is that much less of the intermediates of figure 4 is available for characterization. Despite this the lipid-soluble

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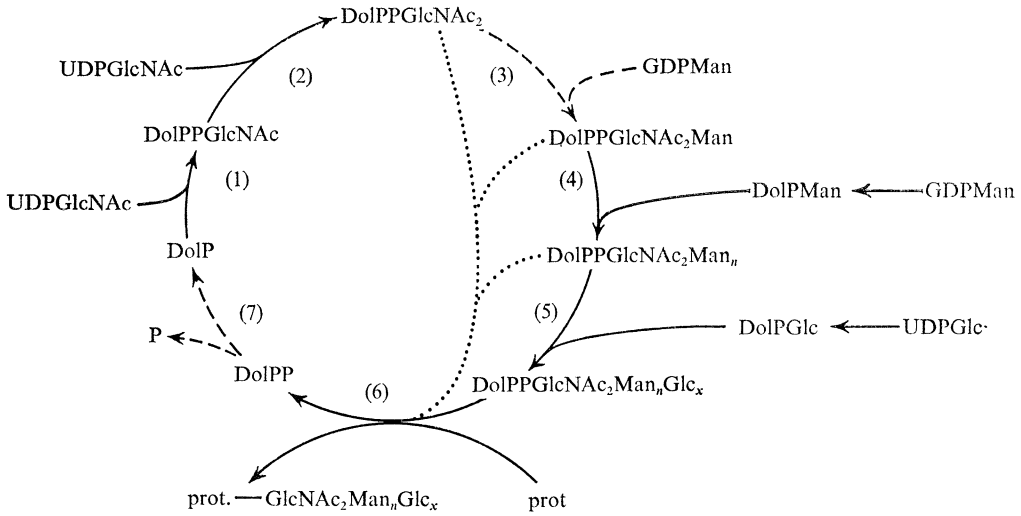
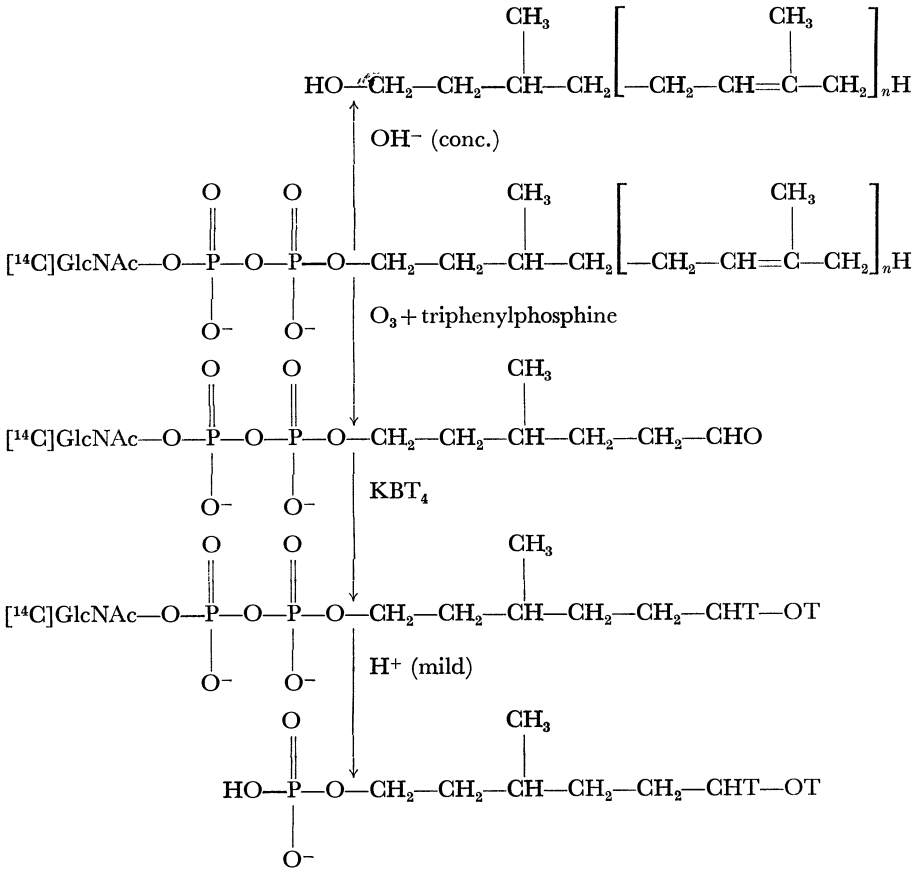


FIGURE 4. *N*-glycosylation of yeast protein (after Parodi). The broken line indicates steps not yet demonstrated directly. The dotted line indicates alternative steps that have been demonstrated with membrane fractions ($n \approx 12$ and $x \approx 4$).

TABLE 4. OZONOLYTIC DEGRADATION AND TRITIATION OF THE PRODUCTS AS A SENSITIVE METHOD OF DETECTING DOLICHOL AS PART OF A LIPID DIPHOSPHATE SUGAR DERIVATIVE

(Also shown is the effect of strong alkali treatment.)



portion of the product of reaction 2 has been identified as dolichol by (*a*) a combination of ozonolytic degradation followed by treatment with borotritiide (table 4) and partly by (*b*) high pressure liquid chromatography analysis of the product of strong treatment with alkali (Reuvers, Boer & Hemming 1978). The results of studies with synthetic polyprenyl monophosphates by Pless & Palamarcyzk (1977) show that the enzyme catalysing step 2 (figure 4) prefers markedly the polyprenyl phosphate to have an α-isoprene (saturated) residue and a poly-*cis* rather than an all *trans* isoprenoid chain; the actual chain length is less important. The same series of experiments demonstrated a much lower specificity in step 1 of figure 2 for the length or stereochemistry of the chain but a slight preference for an α-saturated residue. The enzyme catalysing step 2 (figure 2) demonstrated a more marked but similar preference with regard to donor polyprenyl phosphate sugar.

It has been demonstrated that the antibiotic tunicamycin specifically inhibits step 1 of figure 5 (Takatsuki, Khono & Tamura 1975; Tkacz & Lampen 1975). The earlier observation that tunicamycin interrupts the appearance of invertase and acid phosphatase at the yeast cell surface confirms that these two enzymes are among the proteins glycosylated by the scheme in figure 5.

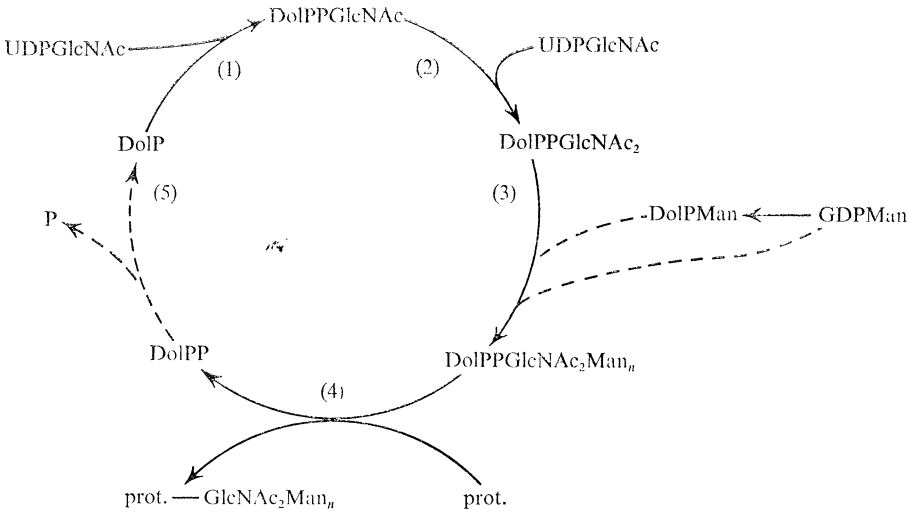


FIGURE 5. *N*-glycosylation of protein of higher plants. Whether mannose is donated to step 3 directly from GDP Man or Dol P Man is uncertain. Step 5 has not been demonstrated.

PROTEIN GLYCOSYLATION IN HYPHAL FUNGI

O-*mannosylation*

The situation in hyphal fungi will probably turn out to be similar to that in yeasts. The mannosylation of dolichyl phosphate by membrane preparations of *Aspergillus niger* was described several years ago (Barr & Hemming 1972; Letoublon, Comte & Got 1973). The further transfer of the mannose to proteins to form an *O*-glycoside has been established both in *Aspergillus niger* (Letoublon & Got 1974) and in *Neurospora crassa* (Gold & Hahn 1976). In both of these organisms the activity of this scheme (steps 1 and 2, figure 2) is much lower than in yeasts.

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N-*glycosylation with oligosaccharide*

It has proved very difficult to demonstrate in hyphal fungi a scheme similar to that in figure 4 (Harrison & Hemming 1977, unpublished work). However, there is no doubt that *Aspergillus niger* forms this type of glycoprotein (Rudick & Elbein 1975). Also it has been reported that at sublethal concentrations, tunicamycin inhibits cell membrane synthesis in *Aspergillus* and in *Penicillium* spp. (Katoh *et al.* 1976). This results in the normal hyphal growth of these fungi being replaced by the formation of giant, multinucleate (up to 50 nuclei), spherical cells. The process is reversible and if the giant cells are placed in medium not containing tunicamycin they revert to normal hyphal growth.

Differentiation and aggregation of cells of the slime mould *Dictyostelium discoideum* have been related to the presence of glycoproteins in the plasma membrane. Recently, Crean & Rossomando (1977) reported the formation of a polyprenyl monophosphate mannose by a plasma membrane-enriched membrane preparation of these cells. The relevance of the presence of this enzymic activity to glycoprotein biosynthesis and to differentiation in *Dictyostelium* remains to be established.

Protein glycosylation in green plants

It has been known for several years that cell-free preparations of mung bean (*Phaseolus aureus*) hypocotyls will catalyse the transfer of mannose from GDP mannose to protein via a lipid phosphate (Alam & Hemming 1973; Kauss 1969; Villemez & Clark 1969). More recent studies with this plant (Forsee, Valkovich & Elbain 1976; Lehle, Fataczek, Tanner & Kauss 1976; Roberts & Pollard 1975) support the scheme shown in figure 5. It is not yet determined if the immediate source of mannose is dolichyl phosphate mannose or GDP mannose. However, it is known (*a*) that mannose is transferred from GDP mannose to dolichyl phosphate mannose; (*b*) that mannose is transferred from GDP mannose to the oligosaccharide of the lipid and of the glycoprotein; and (*c*) that mannose is transferred from dolichyl phosphate mannose to glycoprotein. Reaction 5 is also hypothetical.

Forsee & Elbein (1975, 1977) have also shown that membrane fractions of cotton bolls are capable of catalysing most of the steps in the scheme (figure 5). Whether or not mannose can be transferred directly from dolichyl phosphate mannose to form an O-glycosidic linkage as in yeasts and fungi is not yet clear. However, in studies with membrane preparations of *Phaseolus aureus*, Ericson & Delmer (1977) have reported that [^{14}C]mannose transferred from GDP [^{14}C]mannose to protein was not bound by O-glycosidic linkage. The results of their studies are also in keeping with the scheme in figure 5. It should also be mentioned that the inhibition in green plants of step 1 in figure 5 by tunicamycin has been described very recently (Ericson, Gafford & Elbein 1977).

The nature of the glycoprotein(s) so formed is not yet known. It is suggested that it is an integral membrane component (Forsee *et al.* 1976). Many plant glycoproteins contain the trisaccharide $GlcNAc_2Man$ as a subunit of the oligosaccharide chain proximal to the protein (Sharon 1974).

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Glucan formation in green plants

The glucosylation of polyprenyl phosphate occurs in the golgi of *Phaseolus aureus* (Bowles, Lehle & Kauss 1977) and in *Pisum sativum* (Pont Lezica, Brett, Martinez & Dankert 1975; Pont Lezica, Romero & Dankert 1976; Brett & Leloir 1977). The process is quantitatively minor compared with sterol glycosylation. It is not yet clear if dolichyl phosphate glucose is involved in a scheme such as that described in yeast (figure 4). There is, however, evidence accumulating in favour of a rôle in the biosynthesis of oligoglucans and polyglucans.

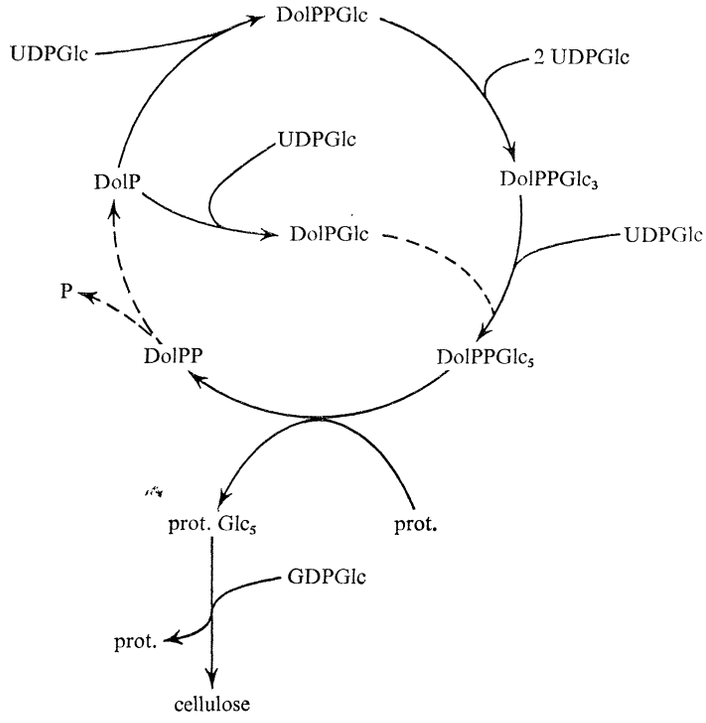

Figure 6. A possible scheme of cellulose biosynthesis in *Prototheca zopfii* (after Pont Lezica, unpublished work).

The formation of glucans by preparations of roots of *Pisum sativum* via a lipid-linked intermediate was suggested by Brett & Northcote (1975). The synthesis of both β1–3 and β1–4 linked glucans was described. More recently, work with *Prototheca zopfii* has provided a convincing case for the scheme shown in figure 6 (Hopp, Romero, Daleo & Pont Lezica 1978). In this scheme a primer oligoglucoprotein is formed from UDP glucose using dolichyl phosphate as a coenzyme. This oligoglucan portion then acts as an acceptor of glucose from GDP glucose in the production of cellulose.

Evidence for the lipid involved in green plants being dolichol is strong (Pont Lezica *et al.* 1975, 1976; Daleo & Pont Lezica 1977; Brett & Leloir 1977). In fact Daleo & Pont Lezica (1977) have described a cell-free system prepared from etiolated pea seedlings capable of biosynthesizing dolichol phosphate from isopentenyl pyrophosphate which will then function as an acceptor of glucose from UDP glucose.

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Consequences of the involvement of dolichyl phosphate

In many instances, though not all (Dallner 1977, private communication), the final product of the glycosylation process is on the opposite side of a membrane from the initial sugar donors, the nucleoside diphosphate sugars. It is possible to interpret this situation as an argument for the hydrophobic dolichyl phosphate carrying hydrophilic sugars across the hydrophobic membrane. However, there is no evidence for movement of dolichyl phosphate sugars across membranes and a flip-flop mechanism from one lipid leaflet of a membrane to the other is likely to be very slow. In addition it is clear that sugar residues of several nucleoside diphosphate sugars (e.g. fucose, galactose and sialic acid in mammalian systems) can traverse membranes during the glycosylation of glycoproteins without the intermediacy of dolichyl phosphate. Possibly the location of dolichyl phosphate-requiring enzymes is a critical factor. For example, they may function in a hydrophobic environment in which hydrophilic nucleoside disphosphate sugars are not suitable substrates. As in the case of the glycosylations not requiring dolichyl phosphate, it is probable that the movement of sugar residues from one side of the membrane to the other is a consequence of the glycosylation process overall and not of the involvement of dolichyl phosphate *per se*.

Nevertheless, the involvement of dolichyl phosphate does have important consequences. Its concentration appears to be very low and generally rate limiting. Thus the rate of glycosylation may be controlled by effecting a change in the concentration of dolichyl phosphate. Bearing in mind the current view that membrane bound glycoproteins are important in cell–cell interactions during differentiation and development and that the formation of cell wall polymers is also important, any factor that may affect the rate of their formation is potentially important. Many of the exohydrolases produced by yeasts and fungi are glycoproteins. The production of these enzymes in yeasts can be turned off by tunicamycin owing to inhibition of the first step in the dolichyl phosphate cycle. There is an obvious potential here for controlling the invasiveness of fungi by controlling the rate of production of these hydrolases through the dolichyl phosphate cycle.

Finally, a further consequence of uncertain significance concerns the configuration of glycosidic bonds in the final product. It seems likely that the enantiomeric configuration of a sugar is inverted each time a sugar-1-phosphate link is broken. The intermediate formation of a dolichyl monophosphate sugar involves an extra inversion resulting in a different configuration in the final product from that that may have been formed if dolichyl phosphate had not been involved. On the other hand involvement of dolichyl diphosphate mono- or oligosaccharides causes no further change in enantiomeric configuration.

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Discussion

G. W. Gooday (*Department of Microbiology, University of Aberdeen, U.K.*). Does Professor Hemming think that there is any significance in the observations that some of the transferase enzymes are activated *in vitro* by Mn^{2+} much more strongly than by Mg^{2+}? Is it possible that *in vivo* there is a compartmentation of Mn^{2+} that could have a regulatory rôle?

F. W. Hemming. The significance of the different requirements *in vitro* of the two mannosyl transferases for Mn^{2+} and Mg^{2+} was that it enabled the experimenter to study the two enzymes separately. The *in vivo* relevance of the metal ion requirements is still an open question with little or no direct experimental evidence.

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