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Ravi Kothapalli

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LA THÈSE A ÉTÉ MICROFILMÉE TELLE QUE NOUS L'AVONS RECEUE
DISTRIBUTION AND METABOLISM
OF
POLYISOPRENOID ALCOHOLS IN PLANTS

By

Kothapalli Ravi
Department of Biochemistry

Submitted in partial fulfilment
of the requirements for the degree of
Doctor of Philosophy

Faculty of Graduate studies
The University of Western Ontario
London, Ontario
September, 1985.

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ABSTRACT

The phosphorylated derivatives of $\alpha$-unsaturated polyisoprenoid alcohols (polyisoprenols) are obligatory intermediates in the biosynthesis of cell wall glycans in bacteria, whereas $\alpha$-saturated polyisoprenoid alcohols (dolichols) are essential intermediates in the biosynthesis of asparagine-linked glycoproteins in eucaryotic organisms. Although a number of polyisoprenoid alcohols from plants have been characterized, there is much more to learn about the distribution, metabolism and function of polyisoprenoid alcohols in plants.

Polyisoprenoid alcohols were isolated from seeds of different plant species and purified by high pressure liquid chromatography. The presence of dolichols in dicotyledonous seeds, and both dolichols and polyisoprenols in monocotyledonous seeds was established by nuclear magnetic resonance and infrared spectral analysis. The amounts of polyisoprenoid alcohol present in various seeds ranged from 1 to 16 mg/100 g of seeds.

The formation of dolichyl pyrophosphate linked oligosaccharides and three new proteins were demonstrated in soybeans during germination. These observations support the existence of dolichol dependent glycosylation in plants.
The fate of dolichols during germination was investigated using the soybean as a model. The dolichol content of germinating soybeans gradually decreased over the first three days of germination, whereas the concentration of dolichyl phosphate rose from undetectable levels to 1.4 μg/seedling. This suggests that during germination endogenous dolichol may be phosphorylated by a kinase.

Dolichol kinase and dolichyl phosphate phosphatase (Dol-P phosphatase) activities were identified in soybean microsomes and both enzymes were characterized. The activities of dolichol kinase and Dol-P phosphatase were measured throughout germination. An increase in dolichol kinase activity was observed up to three days of germination and the activity then gradually decreased. Dol-P phosphatase activity gradually increased up to day three of germination and remained at high levels.

The submicrosomal distribution of Dol-P phosphatase and dolichol kinase activities was investigated. The high activities of Dol-P phosphatase and dolichol kinase found in rough endoplasmic reticulum could provide a mechanism for regulation of Dol-P concentration by a phosphorylation and dephosphorylation mechanism. The controlled Dol-P levels in turn may regulate the biosynthesis of asparagine-linked glycoproteins during germination.
This investigation was supported by a National Overseas Scholarship from the Government of India to the author, and a Medical Research Council grant to Dr. K. K. Carroll. The author expresses his thanks to the Govt. of India, and MRC of Canada.
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Most importantly I thank my wife, Devi and daughters Pallavi and Prathima for their patience and understanding during the course of this work. I finally thank my parents for their encouragement throughout my studies.
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</tr>
<tr>
<td>Asn</td>
<td>asparagine</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine-5' triphosphate</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
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<td>CAPS</td>
<td>3-(Cyclohexylamino) propane sulfonic acid.</td>
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<td>HEPS</td>
<td>N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid</td>
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TCA  trichloroacetic acid
Thr  threonine
TLC  Thin layer chromatography
UDP  uridine-5' diphosphate
UMP  uridine-5' monophosphate
UTP  uridine-5' triphosphate
CHAPTER I: INTRODUCTION

The long chain polyisoprenoid alcohols are of interest at present because of participation of their derivatives in cell wall biosynthesis, asparagine-linked glycoprotein biosynthesis and other possible functions such as membrane fluidity. Polyisoprenoid alcohol consists of six or more isoprene units linked head to tail and have a terminal hydroxyl group. The term polyrenol has been used collectively for all polyisoprenoid alcohols, regardless of whether the α-isoprene unit is saturated or unsaturated. No convenient term is available to refer specifically to α-unsaturated polyisoprenoid alcohols. Therefore in this thesis the term polyrenol is used for α-unsaturated compounds to distinguish it from the α-saturated polyisoprenoid alcohols (2,3 dihydroderivatives) are called dolichols. The term polyisoprenoid alcohol refers to both polyrenols and dolichols (Fig. 1.1). The total number of isoprene units in the molecule is shown following a dash. For example the polyrenol containing 16 isoprene units is referred to polyrenol-16.

Depending on the configuration of isoprene units (cis or trans) the plant isoprenoid alcohols fall into different types (Hemming; 1974, 1978). They are solanesol, ficaprenol, betulaprenol and dolichol types (Table 1.1).
STRUCTURES OF POLYISOPRENOID ALCOHOLS

(a) $H\left[ \begin{array}{c} \text{CH}_3 \\ \text{CH}_2 - \text{C} = \text{CH} - \text{CH}_2 \\ \text{CH}_2 - \text{C} = \text{CH} - \text{CH}_2 \text{OH} \end{array} \right]$  

POLYPRENOL

(b) $H\left[ \begin{array}{c} \text{CH}_3 \\ \text{CH}_2 - \text{C} = \text{CH} - \text{CH}_2 \\ \text{CH}_2 - \text{CH} - \text{CH}_2 - \text{CH}_2 \text{OH} \end{array} \right]$  

DOLICHOL

Figure 1.1
TABLE 1.1

Types of polyisoprenoid alcohols

<table>
<thead>
<tr>
<th>TYPE</th>
<th>STRUCTURE</th>
</tr>
</thead>
<tbody>
<tr>
<td>SOLANESOL</td>
<td>$\text{wT}_9\text{-}10$ (all trans)</td>
</tr>
<tr>
<td>FICAPRENOL</td>
<td>$\text{wT}_3\text{C}_6\text{-}9\text{-OH}$ (tri trans polycis)</td>
</tr>
<tr>
<td>BETULAPRENOL</td>
<td>$\text{wT}_2\text{C}_3\text{-}6\text{-OH}$ (di trans polycis)</td>
</tr>
<tr>
<td>DOLICHOL</td>
<td>$\text{wT}_2\text{C}<em>3\text{C}</em>{10\text{-}20}\text{-S-OH}$ ($\alpha$-saturated)</td>
</tr>
</tbody>
</table>

w = w end of polyisoprenoid alcohol
T = trans
C = cis
S = saturated
1.1 Polyisoprenoid alcohols - earlier studies

Solanesol-9, a long chain polyisoprenoid alcohol, was first isolated in 1956 (Rowland et al.) from tobacco leaves (Nicotiana tabacum). While searching for solanesol in animal tissue, Pennock et al., in 1960 isolated an isoprenoid alcohol of greater chain length which they named "dolichol". In Greek "dolichos" means long. In 1963 Hemming et al. isolated spadicol (all trans decaprenol) and dolichol from the spadix of skunk cabbage (Arum maculatum). Later Burgos et al. (1963) isolated dolichol from pig liver and studied its properties. Short chain polyisoprenoid alcohols (containing 6 to 9 isoprene units) were isolated from silver birch wood (Betula verrucosa) and designated as betulaprenols (Lindgren, 1955). After their discovery of the polyisoprenoid alcohols from the spadix of Arum maculatum, Hemming, Pennock and their workers started a systematic search for polyisoprenoid alcohols. Castaprenols (tri-trans polycis) 10, 11, 12, and 13 were isolated (Wellburn et al., 1967) from horse chestnut (Aesculus hippocastanum), and castaprenol like materials were obtained from a number of angiosperms (Wellburn and Hemming, 1966). Picaprenols 10, 11, 12 and 13 were isolated from the rubber plant (Ficus elasticus) (Stone et al., 1967).
1.2 Isolation and characterization of polyisoprenoid alcohols

Polyisoprenoid alcohols are stable to strong alkali treatment and readily extractable into non-polar solvents such as diethyl ether, hexane or petroleum ether. Most procedures used to isolate polyisoprenoid alcohols are based on the procedure described by Burgos et al. (1963). Polyisoprenoid alcohols are extracted into non-polar solvents after saponification of the tissue and the lipid classes are then separated by various column techniques including chromatography on Florisil (Carroll et al., 1973), Lipidex (Mankowski et al., 1976), alumina (Keller and Alair, 1977) and Sephadex LH-20 (Keenan and Krusczek, 1975) and TLC. Recently HPLC has proved to be a versatile tool for purification and quantitation of polyisoprenoid alcohols by using normal phase (Keller et al., 1982b; Palmer et al., 1984) and reverse phase columns (Keenan et al., 1977; Tavares et al., 1977; Freeman et al., 1980). Polyisoprenoid alcohols can be detected at 254 nm as p-nitrobenzyl derivatives (Keenan et al., 1977) or directly at 210 nm (Freeman et al., 1980). Polyisoprenoid alcohols were characterized by their mobility on TLC plates and by IR, NMR and MS spectral data. The amount of pure polyisoprenoid alcohols can be estimated by direct gravimetric estimation (Burgos and Morton, 1962; Butterworth and Hemming, 1968; Rupar, 1978),
or by HPLC (Freeman et al., 1980). Spectrophotometry, using the Chugaev color reaction (Hilderson et al., 1984) and radioisotope dilution (Keller and Adair, 1977) have been used for quantitation of polyisoprenoid alcohols.

In the 1970's a different approach led to the discovery of phosphate derivatives of dolichols in a number of plant species. Pont Lezica et al. (1975) extracted an acidic glucose acceptor into organic solvent from wheat germ (Triticum vulgare), pea (Pisum sativum) and soybeans (Glycine max). The possibility of this material being a sterol or fatty acid ester was ruled out because of its acidic nature and stability in mild alkali. The lipid sugar phosphate isolated was compared with standard ficiprenyl phosphate and Dol-P. It behaved like Dol-P in a number of ways such as chromatographic behavior, stability for phenolic treatment and catalytic hydrogenation. Brett and Leloir (1977) did similar experiments with soybeans and reported the presence of Dol-P. Later the mannolipid was isolated and purified from cotyledons of mung bean (Phaseolus aureus) and the structure was determined by MS (Delmer et al., 1978). However doubts were expressed about the methodology of the above experiments. Characterization of dolichol by catalytic hydrogenation was questioned (Kean, 1977), because instability of Dol-P was observed in different solvent systems. Ericson et al (1978) reported that the
lipid carrier for GlcNAc in mung bean and cotton fibres is different from that of mannose.

Very recently long chain betulaprenol type polyisoprenols were isolated from the leaves of Ginkgo biloba (14 to 22 isoprene units. Ibata et al., 1983). Similar types of polyisoprenols were isolated from leaves of twelve different species of conifers and they contained 12 to 28 isoprene residues (Ibata et al., 1984).

1.3 Biosynthesis of polyisoprenoid alcohols

Polyisoprenoid alcohols are formed from acetate via mevalonate (Fig. 1.2). Biosynthesis follows the standard terpenoid route (Hemming, 1974). By using doubly labelled mevalonate [(4R)[2-¹⁴C, 4-³H] and (4S)[2-¹⁴C, 4-³H]], Hemming (1970) observed the addition of isoprene units of polyisoprenoid alcohols in a stereochemically specific manner. Addition of isoprene units by cis-prenyl transferase to farnesyl pyrophosphate gives ditrans, polycis-isoprenoid alcohols. Addition to geranylgeranyl pyrophosphate gives tritrans polycis isoprenoid alcohols. Addition of isoprene residues to either farnesyl pyrophosphate or geranylgeranyl pyrophosphate by trans prenyl transferases leads to all trans polyisoprenoid alcohols. Occurrence of these transferases in plants is yet to be reported. Very little is known about the biosynthesis of polyisoprenoid alcohols in plants.
Figure 1.2 The pathway of polyisoprenoid alcohol biosynthesis

MVA = mevalonic acid, MPP = 5-pyrophosphate-mevalonic acid, DMAPP = dimethylallyl pyrophosphate, GPP = geranyl pyrophosphate, FPP = farnesyl pyrophosphate
Incorporation of mevalonate into betulaprenol by wood tissue of silver birch (Gough and Hemming, 1970), algae Prototheca zopfi (Hopp et al., 1978a) and incorporation of IPP into Dol-P by cell-free preparation of pea epicotyls have been reported (Daleo and Pont Lezica, 1977). Mitochondria and Golgi apparatus were reported as sites of Dol-P synthesis in algae. However these studies did not establish how much radioactivity from mevalonate or isopentenyl pyrophosphate enters into polyisoprenoid alcohols or their ester forms. In peas and algae only 2% and less than 8% of radioactivity was reported to be incorporated from mevalonate and isopentenyl pyrophosphate into the Dol-P fraction respectively.

The biosynthetic end product of this pathway is still not known either in plants or in animals. The end product may be Pol-P-P, Pol-P, polyprenol, Dol-P-P, Dol-P or free dolichol. The stage at which saturation of the α-isoprene unit occurs in dolichol biosynthesis is not known. There are some reports regarding α-saturation of polyprenols in rat liver but none of them are conclusive (Vigo and Adair, 1982; Chojnacki and Dallner, 1983; Ekstrom et al., 1984). Another interesting problem is whether the biologically active monophosphate form of dolichol is formed by dephosphorylation of the pyrophosphate form or by the phosphorylation of free dolichol.
Regulation of polyisoprenoid alcohol biosynthesis appears much more complex in plants than in animals, since there are a number of compounds branching from mevalonate (Loomis and Croteau, 1980). Hemming (1983) reported the possibility of regulation at the HMG CoA reductase level. More work will be needed to understand this complex regulation.

1.4 Polyisoprenoid alcohols and bacterial cell wall glycan biosynthesis.

Important concepts were developed from studies with bacteria in understanding the role of polyisoprenoid alcohols in biological systems. The idea of participation of lipid intermediates in the transfer of sugars in biosynthesis of glycans originally emerged from studies with procaryotes. The lipid moiety in several bacteria was identified by direct chemical analysis as "undecaprenol" (a fully unsaturated polyisoprenoid alcohol containing 11 isoprene units) (Thorne and Kodicek, 1966; Higashi et al., 1967). Undecaprenyl phosphate has been recognized as an essential intermediate in the biosynthesis of different bacterial wall glycans such as polymannans, peptidoglycans, O' antigen and capsular polysaccharides. The detailed mechanisms have been described in several reviews (Osborn, 1969; Strominger et al 1972; Lennarz and Scher., 1972; and Hemming, 1974). The general mechanism of glycan synthesis is as
follows; The sugar or sugar phosphate is transferred to the undecaprenyl phosphate by glycosyl transferases. Then the sugar moiety is transferred from undecaprenyl phosphate to the growing polymer.

It was suggested that the amount of undecaprenyl phosphate is regulated by Pol-P phosphatase and polyrenol phosphokinase (Higashi et al., 1970) and then, this in turn regulates cell wall glycan biosynthesis in procaryotes. Similar regulation has been postulated for glycoprotein biosynthesis in eucaryotes.

1.5 Polyisoprenoid alcohols and glycoprotein biosynthesis

1.5.1 Structure and function of glycoproteins

Glycoproteins can be classified into two major groups according to the nature of the carbohydrate-peptide linkage. They are 1). O-glycosidic (if the carbohydrate is linked to serine, threonine, hydroxylysine or hydroxyproline) and 2). N-glycosidic (if the carbohydrate is linked to asparagine) (Fig. 1.3). Hybrid types of glycoproteins containing both N- and O-linked chains in the same protein molecule have been reported in animals but not in plants. However two types of O-glycosidic linkages in the same protein have been observed in both animals and plants (Sharon and Lis, 1979). Asparagine-linked glycoproteins can be classified further into 3 types. (Fig. 1.4). A common feature of all the
GLYCOSIDIC LINKAGE

1. O-GLYCOSIDIC

2. N-GLYCOSIDIC

Figure 1.3
Figure 1.4 Types of asparagine-linked glycoproteins
classes is that they contain a common pentasaccharide. More detailed information is available about the isolation, characterization, biosynthesis and functions of N-linked glycoproteins in animals in several reviews (Sharon and Lis, 1979; Parodi and Leloir, 1979; Mills and Adamany, 1981; Hubbard and Ivatt, 1981; Staneloni and Leloir, 1982).

The number of well characterized asparagine-linked, plant glycoproteins is small. This class includes compounds such as enzymes, lectins, toxins, and storage proteins. Soybean agglutinin (Lis and Sharon, 1978), bromelain from pineapple (Ishihara, 1979), are some of the well characterized asparagine linked glycoproteins (Fig. 1.5). Storage proteins like legumin from pea (Browder and Beevers, 1978) phaseolin and phytohemagglutinin from kidney bean (Phaseolus vulgaris) (Davis and Delmer, 1979), and lima bean lectin (Phaseolus lunatus) (Misaki and Goldstein, 1977) are also N-linked glycoproteins. In addition to these, 12 proteins of the glyoxysomal membranes of castor beans (Ricinus communis) formed during germination (Bergner and Tanner, 1981) and α-amylase from rice seedlings (Miyata and Akazawa, 1982) have been identified as asparagine linked glycoproteins.

The role of the sugar moiety in glycoproteins is not clear. In addition to providing stability and protection against proteolytic breakdown, the carbohydrate moiety may
(a) Soybean agglutinin

\[
\text{Asn} \xrightarrow{\beta} \text{GlcNAc} \xrightarrow{\beta_{1,4}} \text{GlcNAc} \xrightarrow{\beta_{1,4}} \text{Man} \xrightarrow{\alpha_{1,6}} \text{Man} \xrightarrow{\alpha_{1,2}} \text{Man} \xrightarrow{\alpha_{1,3}} \text{Man} \xrightarrow{\alpha_{1,2}} \text{Man}
\]

(b) Bromelain from pineapple

\[
\text{Asn} \xrightarrow{\beta_{1,4}} \text{GlcNAc} \xrightarrow{\alpha_{1,3}} \text{Fuc} \xrightarrow{\beta_{1,4}} \text{GlcNAc} \xrightarrow{\beta_{1,2}} \text{Xyl} \xrightarrow{\alpha_{1,6}} \text{Man} \xrightarrow{\alpha_{1,2}} \text{Man}
\]

Figure 1.5 Structures of some well characterized asparagine-linked glycoproteins from plants.
participate in other functions such as cell-cell interaction, and signals for recognition. More work remains to be done to understand the role of carbohydrates in glycoproteins.

1.5.2 Participation of polyisoprenoid alcohols in glycoprotein biosynthesis.

The involvement of Dol-P as an essential intermediate in biosynthesis of asparagine-linked glycoproteins is well established in the animal kingdom. Several reviews have appeared on this aspect (Hemming, 1974; Mills and Adamamy, 1981; Parodi and Leloir, 1979; Staneloni and Leloir, 1982. I will summarize the literature concerning Dol-P dependent glycosylation in animal systems and then discuss the same process in plants in detail.

1.5.3 Biosynthesis of asparagine-linked glycoproteins in animals

Synthesis of asparagine-linked glycoproteins through Dol-P has been well established (Fig. 1.6). First, GlcNAc-1-P is transferred to Dol-P from UDP-GlcNAc by an enzyme GlcNAc-1-P transferase. This reaction is inhibited by the antibiotic tunicamycin. Another GlcNAc is transferred from UDP-GlcNAc. Then five mannose residues are transferred to the Dol-P-P-GlcNAc₂. The first Man added is in the β-configuration, and the remaining four are in the α-configuration. Four more mannose residues are added from Dol-P-Man.
Figure 1.6 Dol-P dependent glycosylation of proteins
Mannosyltransferase transfers mannose from GDP-Man to Dol-P. After the formation of Dol-P-P-GlcNAc₂-Man₉, three glucose residues are added (structure shown in Fig. 1.7). All enzymes responsible for transfer of sugars to Dol-P are located in the rough endoplasmic reticulum (RER). The oligosaccharide chain is then transferred en bloc from the lipid to an asparagine moiety of the nascent protein. The oligosaccharide is transferred to a specific asparagine residue of the protein which is found in the amino acid tripeptide sequence, -Asn-X-Thr (or) Ser, where X can be any of the 20 amino acids except aspartic acid. The glucose residues on the oligosaccharides facilitate faster transfer of the oligosaccharide from the lipid to the asparagine residue of the polypeptide (Turco et al., 1977; Murphy and Spiro, 1981; Sharma et al., 1981). The oligosaccharide chain is processed immediately after transfer to protein. The three glucose residues are removed after transfer. In the case of high mannose types of glycoprotein, additional mannose residues are added, whereas in complex types all mannose residues except three are removed and other sugars like GlcNAc, Gal, and SA residues are added. Secretory proteins and proteins destined to various organelles within the cell are capped sequentially with other sugars. Most of the processing reactions take place in the Golgi.
Figure 1.7 Structure of Dol-P-P-linked oligosaccharide
1.5.4 Glycoprotein biosynthesis in plants

The formation of glycolipids (believed to be intermediates in glycolipid synthesis in animals, plants and in yeast) from nucleoside diphosphate sugars has been discovered simultaneously in animals, plants and yeast during the 1970's. (Kauss, 1969; Villemez, 1969; Caccam et al., 1969; Tanner, 1969; Behrens and Leloir, 1970). Progress in animal systems was very rapid compared to plants. A number of experiments in animal and yeast systems revealed the involvement of Dol-P in asparagine linked glycoprotein synthesis (discussed in section 1.5.3). Now it is known that plants also synthesize asparagine linked glycoproteins through a similar mechanism. Excellent reviews have appeared on this aspect (Elbein, 1979; Lehle and Tanner, 1983).

Various transferases, which transfer sugar units such as Man, Glc, and GlcNAc from their nucleoside diphosphates to polyisoprenoid alcohols have been identified in mung beans and cotton fibres (Kauss, 1969; Clark and Villemez, 1973; Alan and Hemming, 1973; Forsee and Elbein, 1975; 1977; Roberts and Pollard, 1975 Forsee et al., 1976; Lehle et al). The formation of glucose containing Dol-P-P-oligosaccharides was observed in alfalfa (Medicago sativa) roots (Staneloni et al., 1980), soybean roots and developing pea cotyledons (Staneloni et al., 1981). Synthesis of Dol-P-P-GlcNAc-Man₉.
Dol-PP-GlcNAc$_2$-Man$_9$-Glc$_3$ was identified in vitro in membrane preparations from tobacco and spinach (Spinach oleracea, Lehle, 1981). Recently five major lipid-linked oligosaccharides from mung bean seedlings were isolated and characterized (Hori et al., 1982). Transfer of oligosaccharides from the lipid moiety to endogenous protein has been demonstrated in peas and soybeans. It was also found that the lipid bound oligosaccharide with three glucose residues is transferred faster than oligosaccharides without glucose (Staneloni et al., 1981). Moreover evidence has also been presented that glycosylation of $\alpha$-amylose takes place co-translationally (Miyata and Akazawa, 1982). Processing reactions which are established in the animal system have not yet been confirmed in plants. However contradictory results have been reported by two groups of workers. One group of workers detected glucose on protein bound oligosaccharide (Staneloni et al., 1981) and others failed to detect any glucose (Browder et al., 1980). Even though the processing reactions have not so far been confirmed it is probable that some processing reactions take place in plants also.

In the animal system (as mentioned in the previous section) the first 5 mannose residues are transferred from the GDP-Man, while the remainder come from Dol-P-Man. This pattern is not yet established in plants even though
formation of Dol-P-Man and Dol-P-Glc have been identified. Inhibition of oligosaccharide formation by tunicamycin was also observed in plants (Ericson et al., 1977; Hori and Elbein, 1981).

1.5.5 Site of N-linked glycoprotein biosynthesis

According to the signal hypothesis (Blobel and Dobberstein, 1975) biosynthesis of membrane or secretory protein (mostly glycoproteins) starts in the RER. The nascent hydrophobic "signal peptide" binds to the RER and then transfers newly synthesized protein into the cisternal space of the RER. Then proteolytic cleavage of the signal peptide and glycosylation take place. Subsequently the newly formed proteins are transported into Golgi where various modifications or processing reactions takes place before the proteins reach their destinations. Most of these steps have been demonstrated in the biosynthesis of asparagine-linked glycoproteins in animal systems (Fig. 1.8).

Even though not much is known about the subcellular site of asparagine-linked glycoprotein biosynthesis in plants, a similar mechanism has been proposed for the synthesis of α-amylase, an N-linked glycoprotein in barley. The topology of the glycosyl transferases and the lipid-linked saccharides within the membranes of animal origin has been reviewed (Hanover and Lennarz, 1981; Snider and Rogers, 1984). Such topological distribution.
Figure 1.8 Proposed pathway of glycoprotein synthesis and secretion
remains to be elucidated in plants.

A number of glycosyl transferases involved in the dolichol pathway were found in endoplasmic reticulum of castor bean endosperm and pea cotyledons and stems. Very little activity was observed in glyoxysomes and mitochondria (Marriot and Tanner., 1979; Nagahashi and Beevers, 1978, Nagahashi et al., 1978; Mellor and Lord., 1979; Burr et al., 1979). In mung bean a different distribution or compartmentalization of enzymes was observed. Mannosyl transferases, which transfer mannose from GDP-Man to Dol-P, were observed in ER, whereas the other enzymes which transfer mannose to endogenous acceptor were in Golgi (Lehle et al., 1978).

Little information is available regarding the modification or processing of the oligosaccharide moiety which were extensively studied in animal systems. Fucosyl transferase was found in ER of castor bean. In the animal system, as described in the previous section, sugars like fucose and SA are added by enzymes in Golgi. Chadwick and Northcote (1980) observed the formation of Dol-P-Glc in plasma membrane. The significance of this reaction is not known.

1.5.6 Regulation of asparagine-linked glycoprotein synthesis in plants.

Although much progress has been made in understanding the mechanism of asparagine-linked glycoprotein
biosynthesis, there is considerably less information available about the regulation of their biosynthesis. Activities of any one of the enzymes of the pathway or the availability of their substrates could exert control on glycoprotein biosynthesis. Experimental evidence indicates that asparagine-linked glycoprotein biosynthesis is regulated by the concentration of Dol-P (Carson et al., 1981; Lucas and Levin., 1977; Harford et al., 1977). The Dol-P concentration is in turn regulated by the relative activities of Dol-P phosphatase and dolichol kinase. Hence characterization and distribution of these enzymes was of considerable interest in the recent past in the animal system. Virtually no information is available regarding the enzymes of Dol-P metabolism in plants. Identification and characterization of Dol-P phosphatase and dolichol kinase and distribution of their activities will be helpful in understanding the regulation of glycoprotein biosynthesis in plants.

1.6 Polyisoprenoid alcohols and cellulose biosynthesis.

Involvement of polyisoprenoid alcohols in cellulose biosynthesis is not yet clearly established. Hopp et al., (1978b) reported the involvement of Dol-P and ficaprenyl phosphate in cellulose biosynthesis by particulate preparations from the Chlorophyta prototheca zopfi. (Fig. 1.9). It was observed that coumarin, an inhibitor of cellulose biosynthesis, inhibits
Figure 1.9 Proposed role of dolichol in cell wall biosynthesis
the transfer of the oligosaccharide chain from the lipid to the protein acceptor which is a primer in the biosynthesis of cellulose (Hopp et al., 1978c). These results are not confirmed by other workers. More ever there is no evidence to support a similar pathway in higher plants.

1.7 Other possible functions for polyisoprenoid alcohols

Since polyisoprenoid alcohols are present in all tissues so far tested. Other functions such as membrane fluidity for polyisoprenoid alcohols are slowly emerging. Experiments along this line have already begun in our laboratory.

Increasing amounts of polyisoprenoids were observed with increasing age of leaves. (Wellburn and Hemming., 1966; Ibata et al., 1983). In animal systems accumulation of dolichol was observed with normal aging (Pullarkat and Reha, 1984), Alzheimer's disease (Wolfe et al., 1982) and neuronal ceroid lipofuscinosis (Palo et al., 1982; Ng Ying Kin et al., 1982). It seems that catabolism of dolichol is very slow or negligible. Recent experiments with rats injected with [1-14C] dolichol showed little catabolism after even four months (Rip and Carroll., unpublished results). This also may be true in other organisms. However the catabolism of polyisoprenoid alcohols in animals and plants remain obscure.
1.8 The aim and scope of the thesis.

The aim of the thesis is to investigate initially the nature of polyisoprenoid alcohols in seeds. After establishing that seeds contain sufficient amounts of dolichols, experiments were designed to learn more about the role and metabolism of polyisoprenoid alcohols during germination and early development of seedlings.

Experiments described in Chapter 2 delineate the isolation, characterization and quantitation of polyisoprenoid alcohols from different types of seeds. Enzyme activities like Dol-P phosphatase and dolichol kinase which are not so far reported in plants were identified in microsomes of soybean seedlings. Identification and properties of these enzymes are described in Chapters 3 and 4.

During germination the changes in the levels of dolichol, Dol-P, and activities of Dol-P phosphatase and dolichol kinase were measured. Formation of Dol-P from dolichol was observed. These results and the possible regulation of Dol-P concentration by Dol-P phosphatase and dolichol kinase are described in Chapter 5.

In order to gain more information regarding the regulation of Dol-P levels, the submicrosomal distribution of both Dol-P phosphatase and dolichol kinase was studied. Isolation and characterization of submicrosomal fractions and localization of Dol-P phosphatase and dolichol kinase
activity is described in Chapter 6.

The results reported in this thesis together with the published literature by other workers strongly supports the presence of dolichols and their participation in glycoprotein biosynthesis. In addition to this the later parts of the work described in this thesis will be helpful in understanding the regulation of N-linked glycoprotein biosynthesis in plants.
CHAPTER 2: ISOLATION AND IDENTIFICATION OF POLYISOPRENOID ALCOHOLS FROM SEEDS

2.1 Introduction

Many plant glycoproteins are known to contain an N-glycosidic linkage between GlcNAc and the amide group of asparagine residues (Sharon and Lis, 1979). The participation of specific lipid sugar carriers in the biosynthesis of these types of glycoproteins is also well known in animals, yeast and higher plants (Hemming, 1974; Elbein, 1979; Staneloni and Leloir, 1982). In animals and yeast, the lipid portion has been characterized and found to be an α-saturated polyisoprenoid alcohol containing 14-21 isoprene units, i.e. dolichol (Parodi and Leloir, 1979). However, the occurrence of polyisoprenols in bovine pituitary gland and hen oviduct has also been reported. (Radominska-Pyrek et al., 1979; Hayes and Lucas, 1980). The distribution of dolichol in human tissues was extensively studied in our laboratory (Carroll et al., 1973; Rupar and Carroll, 1978) and its occurrence in various animals was summarized by Dallner and Hemming (1981).

The lipid portion, which participates in bacterial cell wall biosynthesis (very similar to that of dolichol in animals which is involved in glycoprotein synthesis), was identified as bactoprenol, an unsaturated
polyisoprenol containing eleven isoprene units (Hemming, 1974). There are several reports in the literature that suggest by indirect evidence (described in Chapter 1) that the lipid carrier in plants is also dolichol. However, doubt has been expressed regarding the characterization of plant polyisoprenoid alcohols by catalytic hydrogenation (Kean, 1977). In addition to this, a number of polyisoprenoid alcohols isolated from plants have been characterized as polypropens (Chapter 1), which have no known biological function.

In order to learn more about the nature of the polyisoprenoid alcohols in plants, a systematic study of their occurrence was undertaken in a variety of seeds. The results are presented in this Chapter.

2.2 Materials and Methods

2.2.1 Chemicals

Pig liver [1-\(^{3}\text{H}\)]dolichol (12.5 Ci/m mol) was purchased from New England Nuclear, Boston, MA. Silica Gel 60 H was from E.Merck, Darmstadt, Germany. Florisil and chloroform-d were from Merck, Sharp and Dohme, Montreal, PQ. Chromium (VI) oxide pyridine complex was obtained from Eastman Kodak Co., Rochester, NY. Dichloromethane was from Aldrich Chemicals, Milwaukee, WI. Polypropenol-\(^{18}\) from Prunus avium was obtained from Calbiochem, San Diego, CA. Human liver dolichol and the individual homologues were isolated and purified by HPLC.
Methanol, 2-propanol (HPLC grades) and all other chemicals used were either from Fisher Scientific Co., or Canlab., Toronto, Ont.

2.2.2. Seeds

Soybeans (Glycine Max, var-Maple Arrow) were from United Co-operatives of Ontario, Ilderton, Ont. All other seeds were purchased from local seed stores.

2.2.3 Extraction of polyisoprenoid alcohols from seeds

Finely ground seeds (10 grams) were digested by refluxing for 10 hr in a mixture of 150 ml of 30% (w/v) KOH, 30 ml ethanol and 0.5 g pyrogallol. The solution was then extracted with diethyl ether and the ether extracts were washed with water (Burgos et al., 1963). Ether was removed by rotary evaporation and the lipids dissolved in a small amount of hexane.

2.2.4 Isolation of polyisoprenoid alcohols from soybean and wheat germ

Polyisoprenoid alcohols were isolated from soybeans and wheat germ as described above, except that large amounts of materials were used.

Florisil chromatography: The nonsaponifiable lipids were chromatographed on a Florisil column. Columns of different sizes and corresponding volumes of eluting solvents were used depending upon the amount of lipid to be loaded (Table 2.1). The Florisil was first
TABLE 2.1

Separation of nonsaponifiable lipids on various sizes of Florisil columns

<table>
<thead>
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<th>Weight of adsorbent</th>
<th>12g</th>
<th>30g</th>
<th>150g</th>
</tr>
</thead>
<tbody>
<tr>
<td>Column dimensions</td>
<td>1.2x20cm</td>
<td>1.5x30cm</td>
<td>2.5x50cm</td>
</tr>
<tr>
<td>Approximate lipid load</td>
<td>&lt;0.3g</td>
<td>0.3-1.0g</td>
<td>1.0-5.0g</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Eluting solvent</th>
<th>Lipid classes eluted</th>
<th>ml of solvent</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hexane</td>
<td>Hydrocarbons</td>
<td>25</td>
</tr>
<tr>
<td>Diethylether-hexane (5:95)</td>
<td>Hydrocarbons</td>
<td>50</td>
</tr>
<tr>
<td>Diethylether-hexane (15:95)</td>
<td>Fatty alcohols</td>
<td>75</td>
</tr>
<tr>
<td>Diethylether-hexane (25:75)</td>
<td>Sterols</td>
<td>75</td>
</tr>
</tbody>
</table>

(From Carroll, K.K. 1976).
deactivated with 7% water at least 12 hours before use as described by Carroll (1976). After loading the columns with nonsaponifiable lipids, they were eluted with hexane, hexane-5% diethyl ether, and hexane-15% diethyl ether. The polyisoprenoid alcohols appear in the 15% ether in hexane fraction (Fig. 2.1).

2.2.5 Purification of polyisoprenoid alcohols by HPLC

A Hewlett Packard 1084-B instrument with dual pumps was used. For purification of polyisoprenoid alcohols from the 15% ether in hexane fraction collected from the Florisil column, a semi-preparative (Whatman) C$_{18}$-reverse phase column was used. The column temperature was maintained at 45°C and the eluates were monitored at 210 nm with a Hewlett Packard 1030 B variable wave length detector. Sequential gradients of HPLC grade methanol and isopropanol at a flow rate of 4 ml/min were used as mobile phase. Over the first 10 min the concentration of isopropanol was increased from 10 to 35% and then from 35 to 80% over the next 40 min. The areas with retention times corresponding to standard human liver dolichol were collected.

2.2.6 Quantitation of polyisoprenoid alcohols

For quantitation of polyisoprenoid alcohols a 10 μm C$_{18}$-reverse phase column (0.45 cm x 25 cm) was used. Solvent and column temperatures were maintained at 40°C,
EXTRACTION AND PURIFICATION OF POLYISOPRENOID ALCOHOLS

SEEDS

HYDROLYSIS

KOH ETHANOL PYROGALLOL

EXTRACTION OF THE NONSAPONIFIABLE LIPIDS

ETHER

CHROMATOGRAPHY

FLORISIL COLUMN

HEXANE 5% ETHER

HEXANE 15% ETHER

HEXANE 25% ETHER

HYDROCARBONS POLYISOPRENOID ALCOHOLS STEROLS

Figure 2.1
and 45 °C respectively. Eluates were monitored at 210 nm with a Hewlett Packard 1030B variable wavelength detector. The HPLC grade methanol and isopropanol at a flow rate of 2 ml/min were used in a sequential gradient as a mobile phase. The isopropanol concentration was increased from 10 to 35% over the first 5 min and from 35 to 80% over the next 15 min. The amount of polyisoprenoid alcohols was calculated as follows: HPLC was standardized by injecting a known amount of dolichol and then calculating the total area under the peak. From this a concentration of \(9.5 \times 10^{-6}\) ug per unit area was calculated and used for calculating all unknown samples. By this method 40 ng of polyisoprenoid alcohols can be detected (Freeman et al., 1980).

2.2.7 Estimation of size of polyisoprenoid alcohols

The size of the polyisoprenoid alcohols was calculated by comparison with the retention times of dolichols or polyprenols of known chain length.

2.2.8 Oxidation of soybean polyisoprenoid alcohols

Soybean polyisoprenoid alcohols were oxidized to aldehyde forms as described by Collins and Hess (1972). About 30 mg of soybean polyisoprenoid alcohol was dissolved in 1 ml of dichloromethane. Dipyridine chromium (VI) oxide (50 mg) dissolved in 1 ml of dichloromethane was added to the polyisoprenoid alcohol solution and then
stirred vigorously. A brown reduced chromate was formed. It was dried under a stream of nitrogen. The residue was dissolved in 2 ml of hexane. The solution was applied on a Florisil column. The Florisil column was first washed with 20 ml of hexane and then polyisoprenal was eluted with 50 ml of 5% diethyl ether in hexane.

2.2.9 Oxidation of polyisoprenoid alcohols from wheat germ

Preparation of active manganese dioxide: Manganese dioxide was prepared essentially as described by Attenburrow et al., 1952. A solution of manganese sulfate (111 g in 150 ml water) and a 117 ml of 40% sodium hydroxide were added simultaneously during one hour to a hot stirred solution of 16% potassium permanganate (600 ml). Manganese dioxide precipitated soon after the start of the reaction as a fine brown solid. Stirring was continued for another hour and the solid was collected by centrifugation and then washed with water until the washings were colorless. The solid was dried in an oven at 100-200°C and ground to a fine powder and stored in tightly closed bottles.

Polyisoprenoid alcohols (100 mg) purified from wheat germ by HPLC were dissolved in 10 ml of chloroform and then treated with 1 gram of MnO₂ and kept at 4°C for 16 hours. The unoxidized free alcohols and the oxidized forms were separated by Silica Gel 60 H TLC plates. The
plates were developed in hexane:diethyl ether:acetic acid (65:35:1). Two spots were obtained, one corresponding to the aldehyde form of polyrenol and the other having the Rf of polyrenol and dolichol (Rf=0.34).

2.2.10 NMR and IR spectra of the compounds

**Soybean polyisoprenoid alcohol**: NMR Spectra of soybean polyrenol were recorded in CDCl₃ (as solvent) using a Varian T-60 spectrometer. Tetramethyilsilane was used as internal reference standard. IR spectra for the oxidation product were recorded in a Beckman spectrophotometer.

**Wheat germ polyrenol**: NMR Spectra were recorded in CDCl₃ using a Varian XL-100 spectrometer operating at 100.1 MHz. Tetramethyilsilane was used as the internal reference standard. IR spectra of oxidation product of wheat germ polyisoprenoid alcohols were recorded in a Beckman spectrophotometer (Component 11).

2.3 Results

2.3.1 HPLC of polyisoprenoid alcohols from different seeds

The non-saponifiable lipid of different seeds obtained after Florisil chromatography was injected into an HPLC equipped with a C₁₈-reverse phase column. The HPLC profiles are presented in Fig. 2.2 and 2.3. Four to
Figure 2.2 HPLC profiles of (15% ether in hexane fraction from Florisil column) dicotyledonous seeds

(a) Rapeseed; (b) Peanut; (c) Soybean;
(d) Navy bean; (e) Mung bean (f) Peas.
The numbers shown above the peaks indicate the numbers of isoprene units present in those peaks.
Figure 2.3  HPLC profiles of (15% ether in hexane fraction from the Florisil column) monocotyledonous seeds and wheat germ.

(a) Rye; (b) Maize; (c) Barley; (d) Wheat;
(e) Rice; (f) Wheat germ.

The numbers shown above the peaks indicate the numbers of isoprene units in those peaks.
ten homologues of polyisoprenoid alcohols with chain length 13 to 22 were observed. Two different patterns were observed; one typical of dicotyledonous seeds and the other of monocotyledonous seeds (split peaks). In order to characterize the polyisoprenoid alcohols from soybeans, they were purified by HPLC (Fig. 2.4). For comparison retention times of human liver dolichols are shown in the Figure 2.4 (a).

2:3:2 Identification of polyisoprenoid alcohols from soybean and wheat germ

The NMR spectrum was recorded for purified soybean polyisoprenoid alcohols (Fig. 2.5). It was identical to the spectrum of human liver dolichol (Rupar, 1979). To confirm the \( \alpha \)-saturation, a portion of the purified polyisoprenoid alcohol was oxidized to the corresponding aldehyde and IR spectrum was recorded. A C=O stretch bond was observed at 5.78 \( \mu \)m (Fig. 2.6), indicating the absence of a double bond in the position to the aldehyde (Burgos et al., 1963).

A split peak observed on HPLC for monocotyledonous seeds could be reproduced by mixing equal amounts of dolichol-18 and polypropenol-18, and injecting the mixture into the HPLC. This suggests that the peaks obtained with monocotyledonous represented a mixture of dolichols and polypropenols. Wheat germ proved to be a rich source of such material (18 mg/100g) and was used as the source of the
Figure 2.4 HPLC profiles of dolichol and soybean polyisoprenoid alcohols.

(a) Human liver dolichol. The numbers above the major peaks represent, from left to right, the retention times for dolichols containing 18, 19, 20 and 21 isoprene units respectively.

(b) Shows the fraction from soybeans eluted from the Florisil column with 15% diethyl ether in hexane.

(c) Shows the four major peaks in (b) separated by HPLC with retention times similar to those of human dolichols 16–19.
Figure 2.5 Nuclear magnetic resonance spectrum of polyisoprenoid alcohol isolated from soybeans.
Figure 2.6 Infrared spectrum of soybean polyisoprenal (aldehyde)
fairly large amounts (100mg) of polyisoprenoid alcohols required for chemical characterization.

By taking advantage of the known sensitivity of the allylic alcohols (polyrenols) to oxidation by MnO₂ (Attenburrow et al., 1952) wheat germ polyisoprenoid alcohols could be shown to contain both dolichols and polyrenols. Experiments with authentic dolichol and polyrenols indicated that only about 5% of dolichol is oxidized under conditions that result in complete oxidation of polyrenols to the corresponding aldehydes (Fig. 2.7 Lane A, B, C and D). Before oxidation, the polyisoprenoid alcohol fraction from wheat germ ran as a single spot on TLC plates (Fig. 2.7 Lane E). After oxidation two spots were obtained, one of which ran with the same Rf as dolichol (component I) and the other with the Rf of polyrenol (component II, Fig. 2.7 Lane F). Figure 2.8 (a) shows the HPLC profile of purified wheat germ polyisoprenoid alcohols, whereas Figure 2.8 (b) shows the HPLC profile after oxidation, i.e. a mixture of both oxidized and unoxidized compounds. The two components observed on TLC, were scraped and extracted into chloroform:methanol (2:1). HPLC profiles for component I and component II are shown in Figure 2.8 (c) and (d).

It is interesting to note that the retention times of component I remained as they were and those of component II were altered. The NMR spectrum recorded for component i
Figure 2.7 TLC of (A) human liver dolichol; (B) human liver dolichol after MnO₂ oxidation; (C) polyisoprenol-18; (D) polyisoprenol-18 after MnO₂ oxidation; (E) wheat germ polyisoprenoid alcohols; (F) wheat germ polyisoprenoid alcohols after MnO₂ oxidation. Small aliquots of standards or test samples were spotted on Silica Gel 60 H plates and run in a solvent system of hexane/diethyl ether/acetic acid (65:35:1, v/v/v). Compounds were visualized by exposure to iodine vapours.
Figure 2.8 HPLC profiles of:

(a) Wheat germ polyisoprenoid alcohols
(b) Wheat germ polyisoprenoid alcohols after MnO₂ oxidation
(c) Component I from oxidation mixture (b) after TLC
(d) Component II from oxidation mixture (b) after TLC

The figures above individual peaks indicate the retention times for the particular homologue(s) involved. For example, the figures 11.10, 12.34 and 13.55 indicated in panel (c) correspond to retention times for dolichols containing 15, 16, and 17 isoprene units respectively.
confirms the dolichol nature (Fig. 2.9). When examined by IR spectroscopy (Fig. 2.10) component II was shown to possess a C=O stretch at 5.97 μm, which is indicative of a double bond αβ to the aldehyde group. These results indicate that all the monocotyledonous seeds contain both polyisoprenols and dolichols and that the two forms encompass the same size range. All dicotyledonous seeds so far examined contain only dolichols.

2.3.4 Quantitation of Polyisoprenoid Alcohols by HPLC.

The concentration of polyisoprenoid alcohols present in seeds of various plants varied from 1-16 mg per 100 grams (Fig. 2.11). [1-3H]Dolichol was used as a tracer in the isolation procedure to correct for losses incurred during extraction and isolation.

2.4 Discussion

This is the first comprehensive study on the distribution of polyisoprenoid alcohols in plant seeds. They were found to contain considerable amounts of polyisoprenoid alcohols. Highly purified polyisoprenoid alcohols were isolated and their structures were confirmed by NMR and IR. The presence of dolichols in soybeans and other dicotyledonous seeds was clearly established. In soybeans dolichols 17 and 18 together make up about 60% of the total material. Table 2.2 summarizes the major homologues present in different species of seeds. An interesting finding is that the wheat germ and
Figure 2.9 Nuclear magnetic resonance spectrum of component I from wheat germ.
Figure 2.10 Infra red spectrum of component II from wheat germ
Figure 2.11 Amounts of dolichols and polyisoprenoid alcohols present in:

a) Dicotyledonous seeds
b) Monocotyledonous seeds
<table>
<thead>
<tr>
<th>SOURCE</th>
<th>DICOTYLEDONS</th>
<th>MONOCOTYLEDONS</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
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</tr>
<tr>
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<td>MAJOR HOMOLOGUES</td>
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<tr>
<td>14 - 18</td>
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<td>15, 16</td>
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</tr>
</tbody>
</table>

<table>
<thead>
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<th>SOURCE</th>
<th>DICOTYLEDONS</th>
<th>MONOCOTYLEDONS</th>
</tr>
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<tbody>
<tr>
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</tr>
<tr>
<td>Soybean</td>
<td>Navy bean</td>
<td></td>
</tr>
<tr>
<td>Mung bean</td>
<td>Peas</td>
<td></td>
</tr>
<tr>
<td>Rye</td>
<td>Maize</td>
<td></td>
</tr>
<tr>
<td>Barley</td>
<td>Wheat</td>
<td>Rice</td>
</tr>
</tbody>
</table>

TABLE 2.2
monocotyledonous seeds contain both dolichols and polyrenols. The reason for the presence of both dolichol and polyrenol in these seeds is not known. The possibility of the occurrence of short chain polyrenols in dicotyledonous seeds could not be ruled out, because a number of peaks shorter than 13 isoprene units were observed on HPLC, which are not yet characterized (Fig. 2.3 and 2.4).

The biosynthesis of dolichols is thought to proceed by a pathway leading first to Pol-P-P, followed by saturation of the α-residue to give Dol-P which is sequentially dephosphorylated to free dolichol (Vigo and Adair, 1982). The presence of free polyrenols in monocotyledonous seeds may mean that dephosphorylation without saturation of the α-isoprene unit occurs more readily in these species. On the basis of indirect evidence, two reports (Brett and Leloir, 1977; Delmer et al., 1978) have suggested that dolichol intermediates are involved in the biosynthesis of asparagine-linked glycoproteins in germinating seeds. These intermediates are normally phosphorylated forms of dolichol. Dolichols, present in seeds may participate in the biosynthesis of asparagine-linked glycoproteins which in turn may be required for germination and further development of seedlings. It is conceivable that the polyrenols in monocotyledonous seeds serve a different function from
that of dolichols. Plants have both N-linked glycoproteins and cell walls where participation of both dolichols and polyisoprenoids could be possible in their biosynthesis.

The method developed for separating dolichols and polyisoprenoids from wheat germ is very simple, straightforward and in fact better than the one described by Keller et al (1982a; 1982b). They used normal phase HPLC, where retention times of dolichols and polyisoprenoids are very close and the separation of individual homologues is not possible. Reverse phase HPLC is required to identify the individual homologues.

It should be mentioned that the procedure developed here may be useful to develop an assay for the α-isoprene reductase (not reported so far), since this assay requires a method for the estimation of dolichols and polyisoprenoids from a reaction mixture.
Chapter 3: CHARACTERIZATION OF DOLICHYL PHOSPHATE PHOSPHATASE FROM SOYBEANS

3.1 Introduction

Although Dol-P phosphatase activity was detected and its properties were studied in animal systems (Idoyaga-Vargas et al., 1980; Wedgwood and Strominger, 1980; Adrian and Keenan, 1981; Burton et al., 1981; Rip et al., 1981) no information is available for plants. The free dolichols and their fatty acyl ester forms are identified as the major forms of dolichols present in mammalian tissues (Butterworth and Hemming, 1968). Only 10% or less is present in Dol-P form (Rip et al., 1983). It is apparent that a similar type of distribution is present in plants, because high concentrations of dolichols plus dolichyl acyl esters and only trace amounts of Dol-P have been observed in plants (Pont-Lezica et al., 1975; Brett and Leloir, 1977; Ravi et al., 1983, 1984). Dol-P phosphatase is considered to be a biosynthetic enzyme, since the end products in biosynthesis are often free isoprenoid alcohols rather than pyrophosphate or phosphate forms. During biosynthesis, Dol-P phosphatase and dolichol kinase together may regulate the concentration of Dol-P.

Since this is an important enzyme in dolichol metabolism, attempts have been made to identify it in germinating soybeans. An assay for Dol-P phosphatase was
developed and activity was identified in microsomes isolated from germinating soybeans. The properties of this Dol-P phosphatase are the subject of this chapter.

3.2 Materials and Methods

3.2.1 Chemicals

[1-\(^3\)H]Dolichol (12.5 Ci/m mol) was purchased from New England Nuclear, Boston, MA. Silica Gel H was obtained from E. Merck, Darmstadt. Tris-base, BSA and DTT were from Sigma, St. Louis, MO., PA, PE, PC, lyso PA, were from Serdary Research Laboratories, London, Ont. AMP, CMP, UMP, GMP, ATP, CTP, and G-6-P from Boehringer, Dorval, PQ, Triton X-100 and maleic acid were from Eastman Kodak Co., Rochester, NY. All other chemicals used were from Fisher Scientific Co., Toronto, Ont.

3.2.2 Preparation of microsomes from germinating soybeans

Soybeans were soaked overnight in water and then germinated in moist vermiculite in the dark at room temperature. On the fifth day of germination, the cotyledons were removed and used for the isolation of microsomes. Microsomes were isolated essentially as described by Cherry (1974). Cotyledons were homogenized in 0.5 M sucrose containing 10 mM HEPES; pH 7.4. The homogenate was filtered through Miracloth and centrifuged for 20 min in Sorval SS-34 rotor at 18,500 rpm (40,000g).
The resulting supernatant was centrifuged at 42,000 rpm for 1 hr in Beckman Ti 60 rotor (100,000g) and then the pellet was washed and suspended in 0.5 mM sucrose-10 mM HEPES (pH 7.4). The protein content of the microsomes was estimated on aliquots washed with 5% cold perchloric acid, using the method of Lowry et al (1951), and BSA was used as standard.

3.2.3 Phosphorylation of [1-\(^{3}\)H]dolichol

[1-\(^{3}\)H]Dolichol was chemically phosphorylated by the method developed by Danilov and Chojnacki (1981). Dolichol (about 30 mg) dissolved in organic solvent was placed in a 5 ml tube (Reservoir I) and the solvent was evaporated completely under nitrogen. The residue was dissolved in 2 ml of hexane. When [1-\(^{3}\)H]dolichol was phosphorylated, 2 ul of 1-propanol was added and the tube was capped tightly with several layers of parafilm. In another identical tube (Reservoir II) 1 ml hexane, 20 ul POCl\(_3\) (210 \(\mu\)mol) and 30 \(\mu\)l triethylamine (210 \(\mu\)mol) were added and a micro stirring bar was inserted. The tube was capped with several layers of parafilm. Both tubes were connected using 10 cm of polypropylene tubing (1 mm internal diameter). The solution from the reservoir I was transferred slowly (15 to 30 min) to the reservoir II. During the transfer the contents in the reservoir II were mixed gently by magnetic stirrer and stirring was continued for 15 min after completion of the transfer.
The mixture was then added to 10 ml of a mixture of acetone:water:triethylamine (8:1:0.2 by vol) and left for 18 h to convert dolichyl phosphate dichloride into Dol-P. Then the mixture was concentrated on a rotary evaporator to 1 ml aqueous residue. The residue was dissolved in 3 ml of l-propanol and evaporated. The residue was again dissolved in 5 ml of benzene and evaporated. Evaporations with benzene were repeated several times. After formation of a crystalline precipitate, it was left for 1 hour and then the precipitate was removed by filtration. The clear benzene solution was dried and dissolved in 2 ml of chloroform and applied to a 12 g silicic acid column (1.2 x 20 cm). The column was initially eluted with 75 ml chloroform followed by chloroform:methanol (9:1). Dol-P formed during the reaction will enter into the chloroform:methanol (9:1) fraction. The Dol-P containing fraction was evaporated to dryness under nitrogen and dissolved in 5 ml of chloroform:methanol (2:1). The mixture was washed with 0.6 ml water and 1.6 ml PUP. The chloroform layer was dried under nitrogen and the residue was dissolved in 5 ml hexane.

3.2.4 Dolichyl phosphate phosphatase assay

The activity of Dol-P phosphatase was measured by following the conversion of [1-\(^3\)H]Dol-P to [1-\(^3\)H]dolichol. The standard 200 \( \mu \)l Dol-P phosphatase assay contained a final concentration of 100 mM
Tris-maleate, pH 7.4; 1 µM [1-³H]Dol-P (200,000 dpm); 0.05% Triton X-100 (w/v); 10 mM EDTA and 50 µg/ml microsomal protein. Mixtures were incubated in duplicate at 37°C for 30 min and the reaction was terminated by addition of 4 ml of chloroform:methanol (2:1). Reaction mixtures were washed sequentially with 0.6 ml water and 1.6 ml FUP and the solution was dried under a stream of nitrogen. The residue was dissolved in a small volume of chloroform:methanol (2:1). This solution was then applied on Silica Gel H TLC plates and developed in a solvent system of hexane:ether:acetic acid (65:35:1). The bands corresponding to the dolichol standard were located by exposing plates to iodine vapour. Areas corresponding to standard dolichol were scraped into vials and counted in 10 ml acidified Scinti Verse.

3.3 Results
3.3.1 Kinetics

An enzyme that dephosphorylates Dol-P was detected in microsomes of soybean cotyledons. The enzyme activity was linear with respect to time, up to 60 min (Fig. 3.1a), and protein concentration up to 100 µg/ml reaction mixture (Fig. 3.1b). An apparent $K_m$ of 5 µM was calculated for Dol-P, using a double reciprocal plot (Fig. 3.1c).

3.3.2 Effect of pH, detergent, divalent metal ions, DTT and fluoride

A broad pH optimum, ranging from pH 7 to 9 was
Figure 3.1 (a) Dolichol formed as a function of time in Dol-P phosphatase assay. The assay solution and experimental conditions were described in Section 3.2.4 except that the reaction was terminated at different time intervals. (b) Dolichol formed as a function of protein concentration in Dol-P phosphatase assay. Conditions were described in Section 3.2.4 except for variations in microsomal protein. (c) Lineweaver-Burk plot of Dol-P phosphatase. Assay and conditions were described under section 3.2.4 with Dol-P at concentrations of 1 to 10 μM.
observed (Fig. 3.2). Detergent was necessary for maximal
enzyme activity. In the absence of detergent, the enzyme
activity was very low (less than 10% of optimal activity).
The optimum concentration of Triton X-100 required for
maximal activity was determined as 0.05% for 50 µg of
protein by measuring the enzyme activity with different
detergent concentrations at different protein
concentrations (Fig. 3.3). Divalent metal ions do not
appear to be required for enzyme activity. Added Ca\(^{++}\)
and Mn\(^{++}\) were inhibitory and Mg\(^{++}\) had no effect.
Effects of different metal ions are reported in Table 3.1.
EDTA activates the enzyme. Similar activation was observed
in rat liver by Bełocopitow and Bocoboinik (1982). Slight
inhibition was observed with fluoride. DTT had no effect
on the enzyme (Table 3.1).

3.3.2 Substrate specificity

Water-soluble phosphates: In order to test the
substrate specificity, a number of phosphate-containing
compounds were tested. None of the compounds competed with
Dol-P as substrates. The list of the compounds is given
in Table 3.2.

Phospholipids: A number of phospholipids were tested
for their effects on Dol-P phosphatase activity. All the
phospholipids tested at 1 mM concentration inhibited the
enzyme activity by at least 90%. However at 0.1 mM
concentration PE and PC activated the enzyme while PA and
Figure 3.2  Effect of pH on Dol-P phosphatase activity.
The standard assay mixture was used except
that the pH of the buffer was varied as
indicated. O, Na acetate-acetic acid;
▲, Tris-maleate; 0, Tris-HCl
Figure 3.3 Effect of different detergent concentrations on Dol-P phosphatase activity. Assays contained various detergent concentrations as indicated. 
0 - 0%, • - 0.025%, ▲ - 0.05%, □ - 0.15%, △ - 0.5%.
All other experimental conditions were as described under standard assay.
TABLE 3.1

The effect of divalent cations, EDTA, DTT, and NaF on soybean microsomal Dol-P phosphatase activity.

<table>
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<th>Addition</th>
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<tr>
<td>EDTA (10 mM)</td>
<td>128</td>
</tr>
<tr>
<td>MgCl₂ (10 mM)</td>
<td>102</td>
</tr>
<tr>
<td>MnCl₂ (10 mM)</td>
<td>60</td>
</tr>
<tr>
<td>CaCl₂ (10 mM)</td>
<td>86</td>
</tr>
<tr>
<td>DTT (10 mM)</td>
<td>86</td>
</tr>
<tr>
<td>NaF (20 mM)</td>
<td>85</td>
</tr>
</tbody>
</table>

The composition of control assay was as indicated in Section 3.2.4 with the exception that the assays did not contain EDTA.
TABLE 3.2

The effect of water soluble phosphorus-containing compounds on Dol-P phosphatase activity.

<table>
<thead>
<tr>
<th>Addition</th>
<th>Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>None (Control)</td>
<td>100</td>
</tr>
<tr>
<td>Na₂P₄O₇</td>
<td>98</td>
</tr>
<tr>
<td>Na-β-glycerophosphate</td>
<td>104</td>
</tr>
<tr>
<td>G-6-P</td>
<td>98</td>
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<tr>
<td>AMP</td>
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<tr>
<td>ATP</td>
<td>104</td>
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<tr>
<td>CTP</td>
<td>84发明</td>
</tr>
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</table>

Control assays were carried out as indicated in section 3.2.4.

Water soluble phosphates used at 5 mM concentration.
lyso PA were still inhibitory (Table 3.3). Belocopitow and Boscoboinik (1982) have reported similar effects of phospholipids on rat liver enzyme. Soybean Dol-P (chemically phosphorylated) competed effectively with pig liver [1-\textsuperscript{3}H]Dol-P. This shows that endogenous Dol-P could be the natural substrate for the enzyme. Competitive type of inhibition was observed with PA (Fig. 3.4).

3.4 Discussion

These studies clearly indicate the presence of an enzyme in soybean microsomes which dephosphorylates [1-\textsuperscript{3}H]Dol-P. This dephosphorylation was not due to the action of non-specific phosphatases, since a number of phosphate-containing compounds including glucose-6 phosphate, sodium pyrophosphate, and sodium-\(\beta\)-glycerophosphate, did not compete with Dol-P. Fluoride, which has been used as a general inhibitor of phosphatase activity, did not inhibit the Dol-P phosphatase activity significantly even at 20 mM concentration. Dol-P appears to be the natural substrate for the soybean Dol-P phosphatase. The effects of phospholipids on Dol-P phosphatase are of considerable interest. The possibility that PA phosphatase acts primarily on Dol-P appears unlikely, since the reported PA phosphatase activity had pH optima of about pH 5 and it was inactive at pH 7 (Mudd, 1980). Various effects of phospholipids on Dol-P phosphatase activity are not
### TABLE 3.3

The effect of various phospholipids on Dol-P phosphatase activity.

<table>
<thead>
<tr>
<th>Addition</th>
<th>Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>None (control)</td>
<td>100</td>
</tr>
<tr>
<td>PA</td>
<td>2</td>
</tr>
<tr>
<td>PC</td>
<td>152</td>
</tr>
<tr>
<td>PE</td>
<td>145</td>
</tr>
<tr>
<td>Lyso-PA</td>
<td>15</td>
</tr>
</tbody>
</table>

Control assay was carried out as indicated in section 3.2.4. Phospholipids were used at 0.1 mM.
Figure 3.4 Competitive inhibition of Dol-P phosphatase activity by PA (Lineweaver-Burk plot). Dol-P concentration was held constant while PA was used at several different concentrations. 

0--- no PA; Δ---1.6 μM PA; □---2.4 μM PA; 

●--- 3.2 M PA.
clearly understood. Concentrations of some phospholipids (especially PA) are known to increase during germination (Harwood, 1980). Increased concentration of PA may inhibit the Dol-P phosphatase activity and thereby maintain Dol-P concentration at a higher level. This suggests that the phospholipids may play an important, but indirect role in the regulation of glycoprotein synthesis during germination. The importance of this enzyme in regulation of Dol-P will be discussed in Chapter 5.
Chapter 4: CHARACTERIZATION OF DOLICHOL KINASE FROM SOYBEANS

4.1 Introduction

Dol-P serves as an essential intermediate in the biosynthesis of asparagine-linked glycoproteins (Waechter and Lennarz, 1976). There is considerable evidence to suggest that glycoprotein synthesis in mammalian cells is regulated by the availability of Dol-P (Lucas and Levin, 1977; Harford et al., 1977; Hubbard and Robbins, 1980; Coolbear and Hookerjea, 1981). As mentioned in Chapter 3, dolichol and dolichyl acyl esters are the major forms of dolichol in various tissues. Dolichyl fatty acyl esters may serve as a source of free alcohols. The free alcohols in turn may be phosphorylated by the action of dolichol kinase. Dolichol kinase activity has been observed in mammalian tissues (Allen et al., 1978; Burton et al., 1979; Rip and Carroll, 1980), sea urchin (Rossignol et al., 1981), and the proteoan, Tetrahymena pyriformis (Gandhi and Keenan, 1983b). A similar enzyme, which phosphorylates polyprenol-11, has been identified in bacteria (Higashi et al., 1970; Kalin and Allen, 1979).

The concentration of Dol-P may be regulated by the relative activities of Dol-P phosphatase and dolichol kinase. Bacterial undecaprenyl phosphate, an obligatory intermediate in cell wall biosynthesis, is, regulated by means of undecaprenol kinase (Higashi et al., 1970) and
Pol-P phosphatase (Goldman and Strominger, 1972; Sandermann and Strominger, 1972).

Although the Dol-P dependent glycosylation of proteins is reasonably established in plants not much is known about the dolichol phosphorylating enzyme. Dolichol kinase activity has been identified in the microsomes of soybean seedlings and its properties are described in this Chapter.

4.2 Materials and Methods
4.2.1 Chemicals

[1-14C]Dolichol (50 mCi/m mol) was a gift from the Kuraray Chemical Co., Okayama, Japan. [1-14C]Dol-P was prepared according to the method of Danilov and Chojnacki as described in Chapter 3.2.3. Silica Gel 60 H was obtained from E. Merck, Darmstadt, Germany. Soybeans (Glycine max var. Maple Arrow) were obtained from the United Co-operative of Ontario, Ilderton, Ont.

4.2.2 Germination of soybeans

Soybeans were soaked overnight in water and germinated in moist vermiculite. They were kept in the dark at room temperature. On the third day of germination the whole seedlings were used for isolation of microsomes.

4.2.3 Isolation of microsomes

Microsomes from soybeans were isolated as described in Chapter 3, section 3.2.2. Protein was estimated on
portions of microsomes washed with cold 5% perchloric acid using the method of Lowry et al, 1951.

4.2.4. Assay of dolichol kinase

Dolichol kinase activity was measured by monitoring the conversion of [1,14C]dolichol to [1-14C]Dol-P. The standard 200 μl assay contained final concentrations of 100 mM Tris-maleate; pH 7.4; 10 μM [1-14C]dolichol (222,000 dpm), 20mM Mg++, 10mM DTT, 10 mM NaF, 0.38% Triton X-100 and 5 mg/ml of microsomal protein per ml of reaction mixture. Reaction mixtures were incubated in triplicate at 37°C for 30 min. The reaction was terminated with 4 ml of chloroform:methanol (2:1). For control assays, first 4 ml of chloroform:methanol (2:1) was added followed by enzyme. Each reaction mixture was washed with 0.6 ml of water followed by 1.6 ml of FUP and dried under nitrogen. The residue was dissolved in a small amount of chloroform:methanol (2:1) and separated by TLC on Silica Gel H developed in chloroform:methanol:ammonium hydroxide:water (65:35:4:4). Bands corresponding to Dol-P were located with iodine vapour, scraped into scintillation vials and radioactivity counted.

4.2.5 HPLC of Dol-P

For HPLC, bands corresponding to Dol-P were scraped from the TLC plates, before exposing to iodine vapour. The lipids were extracted with chloroform:methanol:water
(2:1:0.2) and washed with water (Chaudhary et al., 1982). The organic phase was dried under nitrogen. The residue was dissolved in a small amount of chloroform:methanol (2:1) and a small amount of Dol-P was added to serve as carrier. This solution was injected into a Hewlett Packard 1084-B HPLC equipped with an analytical C_{18}-reverse phase column. The column temperature was maintained at 45 °C and the eluates were monitored at 210 nm. Methanol:isopropanol (50:50) containing 10 mM H_3PO_4 was used as a mobile phase at a flow rate of 2 ml/min (Chaudhary et al., 1982).

4.3 Results
4.3.1 Identification of [1-^{14}C]Dol-P as reaction product

The reaction product from several kinase assays was pooled and then applied to TLC plates alongside authentic [1-^{14}C]Dol-P, [1-^{14}C]dolichol and control (0, time) assay. The plates were developed in chloroform:methanol: ammonium hydroxide:water (65:35:4:4). They were then exposed to Kodak X Omat AR film for a period of two days. The product of the kinase assay clearly has the same chromatographic mobility as Dol-P. (Fig 4.1).

Corroborative evidence that [1-^{14}C]Dol-P is formed during the in vitro assay was obtained using HPLC. Reaction products from several assays were pooled and analyzed by HPLC (Conditions were described in section...
Figure 4.1 Autoradiogram of TLC plate (shows the formation of $[1^{-14}C]$Dol-P from $[1^{-14}C]$dolichol). TLC plates was developed in chloroform:methanol:water:ammonium hydroxide (65:35:4:4). (A); control assay (0, time), (B); standard assay, (C); $[1^{-14}C]$Dol-P, (D); $[1^{-14}C]$ dolichol.
4.2.4). The radioactivity eluted coincident with Dol-P homologues, and the two major peaks of radioactivity correspond in size to the two major homologues of the dolichol used as substrate. (Fig. 4.2). The reaction product formed was identical regardless of whether it was produced in the presence or absence of CTP and/or metal ions.

4.3.2 Control of Dol-P phosphatase activity in kinase assay

In order to control Dol-P phosphatase activity in the kinase assay, both fluoride and a CTP-sparing triphosphate have been used successfully in mammalian systems (Rip and Carroll, 1980; Allen et al., 1978; Gandhi and Keenan, 1983b; Burton et al., 1979). Therefore, in initial studies both CTP and ATP were added to the assay medium together with 10mM NaF. However, in subsequent studies it became evident that soybean Dol-P phosphatase was not particularly sensitive to fluoride, and that added ATP and CTP were actually inhibitory (Table 4.1). PA, a competitive inhibitor of soybean Dol-P phosphatase (Ravi et al., 1983) was used in the kinase assay to protect newly formed [1-14C]Dol-P. Unfortunately, the 0.5 mM concentration required for inhibition of Dol-P phosphatase also inhibited kinase activity by about 50%. (Table 4.1). The kinase reaction was therefore carried out without PA, ATP and CTP, By substituting [1-14C] Dol-P
Figure 4.2  HPLC of product formed in kinase assay.

--------- Absorbance at 210 nm. (the two major peaks correspond to Dol-P containing 19 and 20 isoprene units respectively).

- •••• Distribution of radioactivity in the product of kinase assay. (HPLC conditions are described in Materials and Methods).
TABLE 4.1

Effect of different nucleoside triphosphates (at 10 mM) on dolichol kinase activity in the absence or presence of 0.5 mM PA,

<table>
<thead>
<tr>
<th>NTP</th>
<th>dpm / assay -PA</th>
<th>dpm / assay +PA</th>
</tr>
</thead>
<tbody>
<tr>
<td>---</td>
<td>5972</td>
<td>2378</td>
</tr>
<tr>
<td>ATP</td>
<td>3502</td>
<td>1395</td>
</tr>
<tr>
<td>CTP</td>
<td>3835</td>
<td>1595</td>
</tr>
<tr>
<td>GTP</td>
<td>4028</td>
<td>1885</td>
</tr>
<tr>
<td>UTP</td>
<td>4965</td>
<td>2112</td>
</tr>
</tbody>
</table>

Standard assay mixture was used.
for [1-\(^{14}\)C] dolichol in the kinase assay, it was calculated that about 20% of the Dol-P formed during the reaction was lost due to phosphatase reaction.

### 4.3.3 Phosphate donor

Bacterial polyprenol kinases have a nearly absolute requirement for ATP as phosphate donor, while eucaryotic cells have a similar requirement for CTP in order to phosphorylate dolichol (Hiqashi et al., 1970; Allen et al., 1978; Kalin and Allen, 1979; Rip and Carroll, 1980). However in soybean kinase assays addition of CTP between 50 \(\mu\)M and 5 mM resulted in a level of conversion no higher than obtained in the complete absence of triphosphates (Table 4.2). Other possible phosphate donors such as ATP, UTP, and GTP likewise gave no increase in the basal level of dolichol kinase either when assayed alone or in the presence of PA.

### 4.3.4 Kinetics

Dol-P was formed in the kinase assay was in a nearly linear fashion for 45 min (Fig. 4.3a) and product formation was directly proportional to protein concentration to 6.25 mg/ml (Fig. 4.3b). The Dol-P formation was linear up to 75 \(\mu\)M dolichol, the substrate (Fig. 4.3c). The apparent \(K_m\) of 55 \(\mu\)M dolichol was calculated by Lineweaver-Burk plot (Fig. 4.3c inset).
TABLE 4.2

Effect of different concentrations of CTP on dolichol kinase activity

<table>
<thead>
<tr>
<th>CTP (μM)</th>
<th>dpm/assay</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>5882</td>
</tr>
<tr>
<td>50</td>
<td>5934</td>
</tr>
<tr>
<td>250</td>
<td>5391</td>
</tr>
<tr>
<td>500</td>
<td>5394</td>
</tr>
<tr>
<td>1000</td>
<td>4937</td>
</tr>
<tr>
<td>5000</td>
<td>4318</td>
</tr>
</tbody>
</table>

Standard reaction mixture was used with different CTP concentrations indicated above.
Figure 4.3  Dolichol kinase activity with (a) time  
(b) protein concentration and (c) substrate.  
(a) The reaction was stopped at different time  
intervals as indicated. Standard reaction  
mixture described in materials and methods was  
used (b) Different protein concentrations  
were used in the assay. (c) Different  
substrate concentrations were used in the  
assay. The apparent $K_m$ was calculated by a  
Lineweaver-Burk plot (inset).
4.3.5 Effect of pH

Various buffers (sodium acetate-acetic acid pH 4-5, Tris-maleate pH 6-7.5, Tris-HCl pH 8-10, CAPS 9.5 -11) were used at 100 mM concentration. The kinase has two pH optima, the first at about 7.5 (Tris-maleate buffer) and the second at about 9.5 with CAPS buffer (Fig. 4.4). The second optimum was not observed with Tris- HCl, since activity increased fairly linearly between pH 8 and 10. The physiological significance of the second pH optimum is not known, although similar pH behavior has been observed for CTP-dependent dolichol kinases from Tetrahymena pyriformis (Gandhi and Keenan, 1983b) and proteins solubilized from bovine liver microsomes (Allen et al., 1982).

4.3.6 Requirement of metal ions and detergents

The soybean dolichol kinase was maximally activated with 20 mM Mg²⁺. However, exogenous divalent metal ions are not essential since over 80% of maximum activity was obtained without any addition. Other metal ions were inhibitory. Maximum inhibition was observed with Zn²⁺ (Table 4.3a). In addition assays without both Mg²⁺ (20mM) and EDTA (10mM) gave 71% of the activity of the standard assay which contains 20 mM Mg²⁺. The assays lacking 20 mM Mg²⁺ but including EDTA (10 mM) gave 90% of the activity of the standard assay (Table 4.3b).
Figure 4.4 Effect of pH on dolichol kinase activity.
Assays contained 100 mM buffer and all other conditions were the same as the standard assay described in Materials and Methods. Sodium acetate-acetic acid (○); Tris-maleate (○); Tris-HCl (x); CAPS (■).
TABLE 4.3(a)

Effect of divalent ions on dolichol kinase activity.

<table>
<thead>
<tr>
<th>Metal ion</th>
<th>mM</th>
<th>dpm/assay</th>
<th>% Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>--</td>
<td>5568</td>
<td>100</td>
</tr>
<tr>
<td>Mg$^{++}$</td>
<td>20</td>
<td>6792</td>
<td>121</td>
</tr>
<tr>
<td>Mn$^{++}$</td>
<td>20</td>
<td>4156</td>
<td>75</td>
</tr>
<tr>
<td>Ca$^{++}$</td>
<td>10</td>
<td>4081</td>
<td>73</td>
</tr>
<tr>
<td>Zn$^{++}$</td>
<td>10</td>
<td>457</td>
<td>8</td>
</tr>
</tbody>
</table>

*All assays contain 5 mM EDTA

$^{a}$DTT was not added.
TABLE 4.3(b)

Effect of EDTA and Mg\(^{++}\) on dolichol kinase activity.

<table>
<thead>
<tr>
<th>Assay</th>
<th>dpm/assay</th>
<th>% Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Without metal +</td>
<td></td>
<td></td>
</tr>
<tr>
<td>With out EDTA</td>
<td>4660</td>
<td>71</td>
</tr>
<tr>
<td>With Mg(^{++}) (2 mM)</td>
<td>6560</td>
<td>100</td>
</tr>
<tr>
<td>Without Mg(^{++}) 10 mM EDTA</td>
<td>5910</td>
<td>90</td>
</tr>
</tbody>
</table>
Dolichol kinases are generally membrane-bound and their polyisoprenoid substrate is water-insoluble. Detergents are therefore a prerequisite for maximum enzyme activity. Triton X-100 was used in this enzyme assay. In the absence of Triton X-100, only 14% of activity observed at the optimum detergent concentration (0.38%) was obtained. The amount of \([1^\text{14}}\text{C}]\text{Dol-P}\) formed decreased with higher detergent concentration so that 50% inhibition was observed at 1.5% Triton (Fig. 4.5).

4.3.7 Reaction end products

As indicated above, only ATP and CTP can serve as substrates for polyisoprenoid kinases so far reported. But addition of ATP or CTP or both ATP and CTP was inhibitory (Table 4.1). Significant activity was observed in the absence of nucleoside triphosphates. That means that an endogenous substance must be acting as phosphate donor. It was hoped that product inhibition data might reveal the identity of the endogenous phosphate donor for the soybean dolichol kinase. Initially, addition of 10 mM ADP, CDP, GDP and UDP to the reaction mixture gave activities of 87, 70, 89, and 84% of control respectively (Table 4.4). These experiments suggested that CDP was more inhibitory than the other three nucleoside diphosphates and the possibility was tested over a series of concentrations with CDP and ADP (Fig. 4.6). The greater inhibition observed with CDP suggests that it is the end product of the
Figure 4.5 Effect of detergent on dolichol kinase activity. Different concentrations of Triton X-100 were used in the assay mixture.
**TABLE 4.4**

Effect of different nucleoside diphosphates (10 mM) on dolichol kinase assay.

<table>
<thead>
<tr>
<th>NDP</th>
<th>dpm/assay</th>
<th>% Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>---</td>
<td>6809</td>
<td>100</td>
</tr>
<tr>
<td>ADP</td>
<td>5939</td>
<td>87</td>
</tr>
<tr>
<td>CDP</td>
<td>4757</td>
<td>70</td>
</tr>
<tr>
<td>GDP</td>
<td>6057</td>
<td>89</td>
</tr>
<tr>
<td>UDP</td>
<td>5735</td>
<td>84</td>
</tr>
</tbody>
</table>

The standard assay was used with various nucleoside diphosphates.
Figure 4.6 Effect of nucleoside diphosphates on dolichol kinase activity. Nucleoside diphosphates were added to the standard assay mixture: ADP (●); CDP (○).
The graph shows the relationship between dolichol kinase activity (% control) and concentration (mM) for two different treatments. The dotted line represents one treatment, and the solid line represents another. As the concentration increases, the dolichol kinase activity decreases, indicating a negative correlation between the two variables.
kinase reaction, and that endogenous CTP is the source of
the phosphate group in [1-\(^{14}\)C]Dol-P. Dol-P, the second
reaction product of the dolichol kinase was not inhibitory
under the conditions of our assay even at concentrations
of 100–150 \(\mu\)M (Fig. 4.7). Formation of [1-\(^{14}\)C]Dol-P was
greatly enhanced by addition of 25 –75 \(\mu\)M unlabeled Dol-P
to the assay medium. The kinase activity obtained at 75 \(\mu\)M
Dol-P was 215\% of that in its absence. This stimulatory
effect can be explained at least partially on the basis of
a [1-\(^{14}\)C]Dol-P sparing effect of unlabeled Dol-P.
Non-labeled dolichol added to the kinase assay competes
effectively with [1-\(^{14}\)C] dolichol as substrate (Table
4.5).

4.4 Discussion

Dolichol kinase activity was observed for the first
time in a higher plant. The soybean microsomal enzyme has
some unique and interesting properties that have not been
observed with dolichol and polyrenol kinases of animal
and bacterial origins.

All polyrenol kinases of procaryotes are ATP-
dependent and all dolichol kinases so far characterized
are CTP-dependent (Higashi et al., 1970; Rip and
Carroll, 1980; Allen et al., 1978; Kalin and Allen.,
1979). The soybean kinase needs neither ATP nor CTP for
maximal activity. All the nucleoside triphosphates
including ATP and CTP in fact interfered with the rate of
[1-\(^{14}\)C]Dol-P formation.
Figure 4.7 Effect of Dol-P on dolichol kinase activity.
Cold Dol-P was added to the standard reaction mixture at the indicated concentrations.
TABLE 4.5

Effect of unlabeled dolichol on dolichol kinase activity

<table>
<thead>
<tr>
<th>Dolichol added (M)</th>
<th>[1-(^{14})C]Dol-P formed dpm/assay</th>
<th>% Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>6771</td>
<td>100</td>
</tr>
<tr>
<td>50</td>
<td>6458</td>
<td>95</td>
</tr>
<tr>
<td>100</td>
<td>4367</td>
<td>65</td>
</tr>
<tr>
<td>150</td>
<td>3284</td>
<td>49</td>
</tr>
<tr>
<td>200</td>
<td>2984</td>
<td>44</td>
</tr>
</tbody>
</table>

To the standard assay mixture different concentrations of dolichol were added.
A second unique property of the soybean enzyme is that 80% of maximal activity is observed without addition of any divalent metal ions. Previously reported dolichol kinases are activated primarily by Mg\(^{++}\) (Rip and Carroll, 1980; Allen et al., 1978) or by Ca\(^{++}\) (Gandhi and Keenan, 1983a; Burton et al., 1979; Rossignol et al., 1981). Calcium activation involves calmodulin (Gandhi and Keenan, 1983a, 1983b) while Mg\(^{++}\) activates by a different mechanism. The other properties, including pH optimum and inhibition by CDP, are similar to those of CTP-dependent kinases activated by Mg\(^{++}\) or Ca\(^{++}\). The kinase reaction observed here is more sensitive to inhibition by CDP than by other nucleoside diphosphates. This suggests that endogenous CTP may be the phosphate donor for \([1-^{14}C]\)Dol-P formation. The 20% stimulation of activity observed in the presence of Mg\(^{++}\) ion suggests that endogenous Mg\(^{++}\) may also be involved. If a Mg-CTP salt represents the endogenous substrate for the endogenous substrate for the kinase reaction then this complex or Mg\(^{++}\) and CTP separately must be compartmentalized within the RER in such a manner that they are not lost during isolation of microsomes. It would appear that exogenous metals and CTP either do not enter this compartment or the endogenous concentrations are already saturating.

Although dolichol kinase plays an important role in glycoprotein biosynthesis in animals (Burton et al., 1979) as do polyprenil kinases in bacterial cell wall
biosynthesis (Higashi et al., 1970), there is little information concerning the function of dolichol kinase in plants. It is well established that the end product of de novo biosynthesis in some organisms is dolichol rather than Dol-P (Rossignol et al., 1983). In this situation dolichol kinase represents a very important enzyme since it in fact catalyzes the terminal step in the de novo synthesis of Dol-P. This monophosphorylated form is an essential intermediate in the formation of asparagine-linked glycoproteins and its concentration in many situations limits the rate of glycoprotein synthesis (Hemmings, 1982). The amount of dolichol decreased whereas the Dol-P concentration increased during germination (Chapter 5). This suggests phosphorylation of free dolichol during germination by dolichol kinase rather than de novo synthesis. In developing sea urchin embryos a similar conversion of preformed dolichol to the activated form has been observed (Rossignol et al., 1983).

The Dol-P formed during germination may be used for the biosynthesis of glycoprotein enzymes for the hydrolysis of the food reserves in the cotyledons. Miyata and Akazawa (1982) recently reported that the enzyme \( \alpha \)-amylase, which is involved in the hydrolysis of starch, is an asparagine-linked glycoprotein (Bergner and Tanner (1981) observed the synthesis of 12 asparagine-linked glycoproteins in the germinating castor bean endosperm.
Studies on rat liver (Rip and Carroll, 1982) and calf brain (Scher et al., 1984) indicated that dolichol kinase is localized primarily in the RER of the cell. This compartment in rat liver also contains Dol-P phosphatase (Rip et al., 1983) although at a relatively low specific activity compared to plasma membrane (Rip et al., 1981) and lysosomes (Appelkvist et al., 1981). The simultaneous presence of dolichol kinase, Dol-P phosphatase and their respective substrates in the RER of rat liver makes a possible mechanism for the modulation of Dol-P concentration by cycles of phosphorylation and dephosphorylation. A similar mechanism may also regulate Dol-P concentration during germination. Changes in the concentration of dolichol and Dol-P and activities of both Dol-P phosphatase and dolichol kinase will be discussed in the next chapter.
CHAPTER 5: DOLICHOL AND DOLICHYL PHOSPHATE LEVELS
DURING GERMINATION AND EARLY DEVELOPMENT
OF SEEDLINGS.

5.1 Introduction
Many details of Dol-P dependent protein glycosylation are well known in mammalian systems (Waechter and Lennarz, 1976; Parodi and Leloir, 1979). Recent studies on various plant tissues have also provided more information about Dol-P mediated protein glycosylation in plants (Elbein, 1979; Lehle, 1983). However considerably less information is available regarding the regulation of biosynthesis of N-linked glycoproteins. There are some reports suggesting that the regulation of asparagine-linked glycoprotein biosynthesis is at the level of Dol-P in animal systems (Lucas and Levin, 1977; Carson et al., 1981). It was also shown that Dol-P levels are developmentally regulated (Lucas and Levin, 1977; Lennarz, 1983).

In order to understand the regulation of asparagine-linked glycoprotein biosynthesis in plants, the amounts of dolichol and Dol-P were estimated throughout germination and early development of soybeans. Changes in activities of dolichol kinase and Dol-P phosphatase were also measured, since these enzymes may be involved in regulating the amounts of Dol-P that are available for glycoprotein biosynthesis.
5.2 Materials and Methods

5.2.1 Chemicals

[1-^{14}C]Dolichol (specific activity 50 mCi/mmol) was a gift from the Kuraray Chemical Co, Okayama, Japan. [1-^{3}H]Dolichol (specific activity 12.5 Ci/mmol) was obtained from New England Nuclear, Boxborough, MA. Dolichol was isolated from human liver obtained at autopsy (University Hospital, London) and purified by HPLC. [1-^{14}C]Dol-P was prepared from [1-^{14}C]dolichol by the method of Danilov and Chojnacki (1981) as described in Chapter 3 section, 3.2.3. [2-^{3}H]Man (specific activity 27.2 Ci/m mol), GDP-[1-^{3}H]Man of specific activity 10 Ci/mm mol, and UDP-[6-^{3}H]GlcNAc of specific activity 6.6 Ci/mol were purchased from New England Nuclear, Boston, MA. Soybean embryonic axes were isolated manually.

5.2.2 Germination of soybeans and isolation of microsomes

Soybeans were germinated and microsomes were isolated from soybean seedlings as described in Chapter 4 (sections 4.2.2 and 4.2.3).

5.2.3 Extraction of dolichol from germinating soybeans and seedlings

Five seeds or seedlings were finely ground and digested by refluxing for 12 h in a mixture of 35 ml of 30% KOH, 10 ml ethanol and 0.1 g pyrogallol.
Nonsaponifiable lipids were extracted with diethyl ether, washed three times with water, and concentrated by rotary evaporation. Dolichol was extracted and estimated as described previously (Chapter 2, section 2.2.3 and 2.2.6). The dolichol content of different parts of soybean seedlings was also estimated.

5.2.4 Extraction of total Dol-P

Groups of ten seeds or seedlings were used for extraction of total Dol-P. Lipids were extracted with chloroform:methanol (2:1), using 20 ml/g tissue. [1-3H]Dol-P was added at this stage as a tracer to allow correction for losses incurred during the isolation process. The lipids extracted in chloroform:methanol (2:1) were dried by evaporation in a vacuum rotary evaporator and a stream of nitrogen. The lipids were then redissolved in chloroform:methanol (2:1) containing 0.1 N HCl. This mixture was incubated in sealed tubes at 55°C for 90 min to convert Dol-P-P and Dol-P-P-saccharides into free Dol-P (Bayes et al., 1973). The lipids were washed with water and then with FUP. The washed lipids were dried under nitrogen and dissolved in a small amount of chloroform:methanol (2:1).

The residue remaining after chloroform:methanol extraction was reextracted into chloroform:methanol:water (10:10:3), dried under nitrogen, dissolved in a small amount of chloroform:methanol: 0.8 N HCl (10:10:3) and
incubated at 90 °C for 60 min, to convert Dol-P-P oligosaccharides into Dol-P (Baynes et al., 1973).
After acid hydrolysis, the ratio of chloroform:methanol was adjusted to 2:1 and the lipids were washed with water followed by FUP and finally dried under nitrogen.

The lipids from the chloroform:methanol (2:1) and chloroform:methanol:water (10:10:3) extract were combined and dissolved in a small amount of chloroform:methanol (2:1) and run on thin layer plates coated with Silica Gel 60 H which were developed in chloroform:methanol:ammonium hydroxide:water (65:35:4:4). The areas corresponding to authentic Dol-P were scraped and extracted with chloroform:methanol:water (2:1:0.2) and washed with water (Chaudhary et al., 1982).

5.2.5 Quantitation of dolichol and Dol-P

A Hewlett-Packard 1084 B HPLC equipped with a C18 reverse phase column was used to quantitate dolichol and Dol-P. The operating conditions for estimation of dolichol were the same as described in Chapter 2 (section 2.2.6).

For quantitation of Dol-P, the conditions were the same as for dolichol except that the isopropanol contained 10 mM phosphoric acid. Dolichol and Dol-P content of the samples were estimated by summing the areas of individual peaks and comparing them to the areas obtained for standard dolichol and Dol-P. Losses incurred during extraction were corrected for by using [1-3H]dolichol or [1-3H]Dol-P.
5.2.6 Dolichol kinase assay

Dolichol kinase activity was measured by following the conversion of [1-14C]dolichol to [1-14C]Dol-P. The assay was the same as described in Chapter 4 (4.2.4).

5.2.7 Dol-P phosphatase assay

The activity of Dol-P phosphatase was measured by following the conversion of [1-14C]Dol-P to [1-14C]dolichol. The assay mixture contained 10 μM Dol-P (232,000 dpm). The remaining reaction mixture and assay conditions were the same as described in Chapter 3 (Section 3.2.4).

5.2.8 In vivo radiolabelling experiments with soybeans and soybean embryos

Groups of 5 seeds were soaked in 5 ml of distilled water containing [2-3H]Man at 10 μCi/ml for 15 hr. After imbition of water, they were germinated. After different time intervals the lipids were extracted as described for embryos. The radioactivity incorporated into various fractions was counted.

Lots of 30 soybean embryos were incubated separately in 0.8 ml of water in the presence of GDP-[1-3H]Man (2.5 x 10^6 dpm) or UDP-[6-3H]GlcNac (1.6 x 10^6 dpm).

5.2.9 Extraction of lipids into chloroform:methanol (2:1)

After incubation of embryos in water containing
UDP-[6-\(^3\)H]GlcNAc or GDP-[1-\(^3\)H]Man or seeds in water
containing [2-\(^3\)H]Man, for different time intervals, they
were washed thoroughly with water and then extracted into
5 ml chloroform:methanol (2:1). The chloroform:methanol
fraction was washed with 0.6 ml water and 1.6 ml FUP and
dried under a stream of nitrogen. The residue was
dissolved in 10 ml of ScintiVerse then acidified and
counted.

5.2.10 Extraction of lipids with chloroform:methanol:
water (10:10:3)

The residue after chloroform:methanol (2:1)
extraction was re-extracted with 4 ml of
chloroform:methanol:water (10:10:3). The supernatant was
dried and dissolved in ScintiVerse and counted. The
residue left after these extractions was counted.

5.2.11 Extraction of radioactive long-chain
oligosaccharides

Embryos (30) were incubated in 0.8 ml of water
containing UDP-[6-\(^3\)H]GlcNAc (1.6 X 10^6 dpm) for about
18 hours. Then they were thoroughly washed and extracted
with 4 ml chloroform:methanol (2:1). The remaining residue
was washed twice with 2.5 ml of 50% methanol. After the
washings the residue was extracted twice with 4 ml
chloroform:methanol:water (10:10:3) and pooled and dried
under a stream of nitrogen.

Acid hydrolysis: The above residue was dissolved in
5.1 ml of 20% methanol containing 0.02 N HCl. This solution was heated to 100 C for 25 min. After acid hydrolysis, 5ml of chloroform was added and then centrifuged. The upper water layer contained oligosaccharides (Hori and Elbein, 1982).

5.2.12 Size of oligosaccharides

The layer containing oligosaccharides was loaded on to a Biogel-P6 column and eluted with 0.1 M ammonium formate pH 7.0 (containing 0.02% sodium azide). The size of oligosaccharides was calculated by comparing with known standards (column was previously standardized with known molecular weight substances i.e. Blue Dextran (6000), large oligosaccharide from fetuin (2978), desialated large oligosaccharide from fetuin (2005), stachyose (667), Man (180). This part of the work was carried out at CPRI with the help of Dr. Rupar.

5.2.13 Extraction of proteins

Proteins from one gram of tissue (seeds or seedlings) were extracted into 20 ml of 2% SDS (containing 0.1% 2-mercaptoethanol). The extract was heated at 100'C for 10 min and centrifuged. The supernatant was used for identification of proteins by electrophoresis (Pusztar et al., 1983).

5.2.14 SDS-Polyacrylamide gel electrophoresis

Protein samples were run on 1.5 mm thick, 10%
polyacrylamide gels as described by Laemmli (1970). After electrophoresis proteins were fixed in glacial acetic acid 10% (v/v); methanol, 30% (v/v). Proteins and glycoproteins were visualized using Coomassie brilliant blue and glycoproteins by PAS as described by Fairbanks et al. (1971).

5.3 Results
5.3.1 Changes in dolichol and Dol-P concentrations during germination

The dolichol content of germinating soybeans increased about 39% during imbibition, then decreased gradually over the next 2-4 days so that the concentration on day 3 was 21% less than that in dry seeds (Fig. 5.1a). On the third day of germination when the dolichol concentration was low, Dol-P concentration was 1.4 ug/seedling (Fig. 5.1b). The Dol-P content of dry seeds was so low that it could not be detected. The dolichol content of seedlings again increased after the third day of germination and this continued over the next 10 days in seedlings exposed to a 14 h light cycle after 3 days of germination in dark. The dolichol content of seedlings kept continually in the dark increased up to day 5 and then remained constant until day 10 (Fig. 5.1a). After day 3, Dol-P concentration decreased so that nothing was observed by day 5 of germination.
Figure 5.1 (a) Levels of dolichol in soybean seedling during germination

- Seedlings kept in dark
- Seedlings exposed to a 14 h light cycle.

(b) Levels of Dol-P in soybean seedlings during germination
5.3.2 Dolichol content of different parts of soybean seedlings

Dolichol was isolated from the roots, stems, leaves and cotyledons of 15 day old seedlings grown in light after day 3. Cotyledons contained the highest concentration of dolichol at this time (Fig. 5.2).

5.3.3 Changes in dolichol kinase and Dol-P phosphatase during germination

Dolichol kinase activity was detected after 24 h of germination and the level of activity increased about 5-fold between day 1 and 3. After day 4, kinase activity decreased quite significantly, suggesting that the major requirement for Dol-P for glycosylation events related to germination occurs before day 5 under present experimental conditions (Fig. 5.3). Dol-P phosphatase activity also increased up to day 3 of germination in parallel with the increase in kinase activity. However, the increase in Dol-P phosphatase appeared to be more rapid at first and activity remained high up to day 5 (Fig. 5.4).

5.3.4 Incorporation of [2-^3^H]Man into seeds

[2-^3^H]Man was taken up by the imbibing seeds. A very small amount of incorporated Man was extracted into the chloroform:methanol (2:1) fraction whereas a relatively large amount was extracted into the chloroform:methanol:water (10:10:3) fraction (Fig. 5.5a).
Figure 5.2 Distribution of dolichol within soybean seedlings. The dolichol content of different parts was measured as described in the methods.
Figure 5.3 Dolichol kinase activity during germination.
   Microsomes isolated from soybean seedlings at different days after germination were used as enzyme source. Assays were as described under methods.
DOL-P FORMED
(dpm x 10^-3/assay/30 min.)

GERMINATION TIME (days)

[Graph showing the relationship between DOL-P formed and germination time.]
Figure 5.4. Dol-P phosphatase activity during germination. Soybean microsomes obtained at different days after germination were used as source of enzyme. Assay conditions were described under Methods.
5.3.5 **Incorporation of \([1^{-3}H]Man\) and \([6^{-3}H]GlcNAc\) into soybean embryos**

In order to demonstrate the formation of Dol-P-P-oligosaccharides initially soybean seeds were used. It was found difficult to carry out these studies with whole seedlings. Similar results were obtained when isolated soybean embryos were incubated in the presence of GDP-[\(^{1-3}\)H]Man. Incorporation of \([1^{-3}H]Man\) from GDP-[\(^{1-3}\)H]Man into chloroform:methanol (2:1), chloroform:methanol:water (10:10:3) soluble lipids and into remaining residue is shown in Figure 5.5b. Similarly \([6^{-3}H]GlcNAc\) incorporation is shown in Figure 5.6. Incorporation of \([1^{-3}H]Man\) and \([6^{-3}H]GlcNAc\) into chloroform:methanol (2:1) soluble lipids was nearly constant throughout the experimental periods, whereas incorporation of \([1^{-3}H]Man\) and \([6^{-3}H]GlcNAc\) into chloroform:methanol:water (10:10:3) soluble lipids was proportional to the time of the experiment.

5.3.6 **Size of oligosaccharides extracted from soybean embryos**

The size of oligosaccharides linked to Dol-P-P was estimated by using a Biogel P6 column. The length of the oligosaccharides was estimated to be 5 to 9 sugars (Fig. 5.7).

5.3.7 **Glycoprotein synthesis**

Total proteins (100μg) extracted from the
Figure 5.5 (a) $[2^{-3}H]$Man incorporation into
(0) Chloroform:methanol (2:1) fraction and
(0) Chloroform:methanol:water (10:10:3) of
seeds.

(b) $[1^{-3}H]$Man incorporation from
GDP-$[1^{-3}H]$Man into
(0) Chloroform:methanol (2:1) fraction,
(0) Chloroform:methanol:water (10:10:3)
fraction and
(0) Residue.
Figure 5.6 Incorporation of [6-^3^H]GlcNAc from UDP-[6-^3^H]GlcNAc into

(●) chloroform:metanol (2:1) fraction and
(0) chloroform: methanol: water (10:10:3)

fraction of soybean embryos
Figure 5.7 Radioactivity eluted from Bio-gel P6 column. Peaks (1), (2) and (3) correspond to 9, 5 and 1 sugars respectively.
seedlings were applied on lanes of a slab gel. After electrophoresis the protein bands were stained with PAS for glycoproteins. Three new protein bands of mol. weight about 18 K D were observed from the third day of germination onwards (Fig. 5.8). At the same time the Dol-P concentration was also high.

5.4 Discussion

Virtually nothing is known about the metabolism of dolichol. After identification of dolichol in seeds, we decided to follow the changes in concentration of dolichol during germination, in order to investigate the fate of dolichol.

The dolichol content of 5 seeds was followed from dry seeds to 10 days of germination. During the first 12 h of imbibition, the dolichol content increased which appeared to be the real value since extraction of imbibed seeds may be more complete than that of dry seeds.

The simultaneous decrease in dolichol and increase in Dol-P content over the first 3 days of germination suggested that Dol-P was being formed by phosphorylation of existing endogenous dolichol by the dolichol kinase described in Chapter 4. The Dol-P phosphatase activity obtained with soybean microsomes appears to be quite high relative to the level of kinase activity present. The developmental changes observed for Dol-P phosphatase were more difficult to rationalize although high levels of this
Figure 5.8 SDS-Polyacrylamide gel electrophoresis of proteins extracted at different stages of germination.

(1) Stained with Coomassie brilliant blue
(2) Stained with PAB
A. 15 hours after germination; B. day 1
C. day 2; D. day 3; E. day 4; F. day 5;
G. standards. (phosphorylase b, 94,000;
albumin, 67,000; ovalbumin, 43,000;
carbonic anhydrase, 30,000; trypsin
inhibitor 20,100; and α-lactalbumin,
14,000).
enzyme are consistent with a biosynthetic pathway, where the end product is dolichol rather than Dol-P. Similar developmental changes in Dol-P content and in activities of dolichol kinase and Dol-P phosphatase have been reported for early stages of sea urchin development (Lennarz 1983). In these studies it was observed that gastrulation was blocked by the presence of compactin (a competitive inhibitor of HMG-CoA reductase) indicating that the de novo synthesis of dolichol or Dol-P is required for development up to the gastrula stage. Based on these observations of increased levels of kinase activity in these studies, it would appear that dolichol is also the main biosynthetic end product in the soybeans. However, this does not necessarily mean that hydrolysis of Dol-P will always be the favoured direction of reaction because experiments with whole microsomes measure the contribution of RER, SER and Golgi compartments while only RER is involved in transfer of oligosaccharide to protein. Previous experiments in our laboratory (Rip and Carroll., 1982) established that dolichol kinase is localized primarily in the RER of rat liver, while most of the Dol-P phosphatase activity appeared in the Golgi fraction. In this system also, studies on submicrosomal distribution of these enzymes are important and this will be discussed in the next chapter.
In order to establish the involvement of Dol-P in asparagine-linked glycoprotein synthesis in soybeans, some experiments were done with soybeans and \([2^{-3}H]Man\). Even though \([2^{-3}H]Man\) was taken up by seeds during germination, it was found difficult to germinate seeds continuously in the \([2^{-3}H]Man\) environment. However, similar results were obtained when isolated soybean embryos were incubated in the presence of GDP-[1^{-3}H]Man. In this system extensive incorporation of \([1^{-3}H]Man\) and \([6^{-3}H]GlcNAc\) into Dol-P-P-oligosaccharides and glycoproteins was observed over the 16 h duration of experiments. Formation of penta to nona saccharides was observed in soybean embryos which were analyzed by Biogel P6 chromatography. Appearance of 3 new glycoproteins was also observed from the third day of germination onwards.

Previous studies (Marriot and Tanner, 1979) indicated that incorporation of mannose from GDP-Man into glycoprotein in castor bean was maximum between 3 and 4 days after germination. These observations, together with the results presented here, provide reasonable evidence for Dol-P participation in glycoprotein biosynthesis during soybean germination. The activities of Dol-P phosphatase and dolichol kinase and changes in the levels of dolichol and Dol-P concentrations provide a possible means of regulation of glycoprotein synthesis during germination.
CHAPTER 6: SUBMICROSOMAL DISTRIBUTION OF DOLICHOL KINASE AND DOLICHYL PHOSPHATE PHOSPHATASE

6.1 Introduction

The presence of dolichol kinase and Dol-P phosphatase activity in soybean microsomes has been established (Chapters 3 and 4). Activities of these enzymes have been measured throughout germination and early development (Chapter 5). The highest specific activity of Dol-P phosphatase was observed in microsomes. Although a biosynthetic role has been proposed for Dol-P phosphatase activity in the animal system, the highest specific activity was observed in Golgi and plasma membranes, (Rip et al, 1981; Appelkist et al, 1981; Scher et al, 1984) which do not synthesize dolichol. Dol-P phosphatase and dolichol kinase in RER and Golgi may regulate Dol-P levels by phosphorylation and dephosphorylation cycles.

To understand the possible regulation of Dol-P by a phosphorylation and dephosphorylation mechanism in soybeans, the distribution of dolichol kinase and Dol-P phosphatase activities was determined in submicrosomal fractions.

6.2 Materials and Methods

6.2.1 Chemicals

[1-14C]Dolichol was a gift from Japan as mentioned
in Chapter 4: NADH and NADPH are from Calbiochem, Behring Diagnostics, La Jolla, CA. Cytochrome C type IV was from the Sigma Chemical Co. St. Louis, MO. All other chemicals are from either Can Lab or Fisher Chem Co. Toronto, Ont. Soybean seeds were obtained from the same suppliers as in Chapter 4.

6.2.2 Isolation of microsomes

Soybeans were germinated up to 3 days and microsomes were isolated as described in chapter 5 with some modifications. Sucrose 0.25 M containing 10 mM HEPES of pH 7.5 was used. The first centrifugation was done at 14,000 rpm for 20 min in a JA 20 Beckman rotor (23,000g).

6.2.3 Submicrosomal fractionation

All sucrose solutions used were buffered to pH 7.5, using 10 mM HEPES and KOH and were kept at 4°C.

Isolation of Golgi: Microsomes isolated from 200 grams of 3-day old soybean seedlings, were washed with 0.25 M sucrose. After the washing, the microsomes were dissolved in 27 ml of 0.25 M sucrose and then homogenized using a Dounce homogenizer. An equal volume of 2.05 M sucrose was added and the mixture was homogenized again to bring the sucrose concentration to 1.15 M. Two ml of 2.05 M sucrose solution was placed as a cushion in each of the six tubes of a Beckman SW 28 rotor. An aliquot of 9 ml microsomal suspension (1.15 M) was layered with 15 ml of 1.10 M sucrose and 10 ml of 0.4 M sucrose and then
centrifuged at 25,000 rpm for 4 hours at 4 °C. The material banding at the interface between 0.4 and 1.10 M sucrose was taken as the Golgi fraction (Rip and Carroll, 1982). Golgi membranes were aspirated, then diluted with two volumes of 0.25 M sucrose and sedimented into a pellet by centrifuging in a Ti 60 rotor at 42,000 rpm for 1 hour. Golgi pellets were taken up into a small volume of 0.25 M sucrose using a Dounce homogenizer and were frozen at -20 °C until further use.

Isolation of SER and RER:

After separation of Golgi, the remaining microsomes were recovered from the bottom layers of the SW 28 tubes, pooled, diluted two-fold with 0.25 M sucrose and pelleted by centrifugation in a Ti 60 rotor for 1 hour at 42,000 rpm. The resulting microsomal pellets were combined and suspended in 84 ml of 0.25 M sucrose solution. The solution was then made 0.015 M with respect to CsCl, layered into six tubes each containing 15 ml of 1.3 M sucrose-0.015 M CsCl and centrifuged in a Ti 60 rotor at 58,000 rpm for 110 min. Smooth microsomes were recovered from the interface and rough microsomes from the pellet. The rough microsomal fraction was suspended in 0.25 M sucrose and the smooth microsomes were aspirated and diluted two-fold with 0.25 M sucrose. These fractions were pelleted by centrifuging in a Ti 60 rotor for 1 hour at 42,000. Each fraction was suspended in a small volume of 0.25 M sucrose and stored at -20 °C.
6.2.4. Marker Enzymes

NADH and NADPH cytochrome C reductase assays

NADH and NADPH Cytochrome C reductase activities were measured as described by Bowles and Kauass (1976). NADH and NADPH solutions were prepared in 15mM Tris HCl pH 7.4, whereas cytochrome C was in phosphate buffer pH 7.4. The NADH cytochrome C reductase assay contained 100 μl of 200 mM sodium phosphate buffer pH 7.4, 50 μl of 0.5% cytochrome C, 5 μl of 0.5% NaCN, 140 μl of water and 10 μl of membrane fraction. The reaction was started by addition of 50 μl of 0.3% NADH, followed by measuring at 550 nm in a DU-8B spectrophotometer. To investigate the effect of antimycin on enzyme activity 5 μl of 0.2% antimycin (in ethanol) was added.

The assay mixture for NADPH cytochrome C reductase activity contained 100 μl of 200 mM sodium phosphate buffer pH 7.4, 50 μl of 0.5% cytochrome C, 5 μl of 0.5% NaCN, 50 μl of H₂O and 100 μl of membrane fraction. The reaction was started by the addition of 50 μl of 0.3% NADPH, followed by measuring at 550 nm in a DU-8B spectrophotometer.

IDPase activity

IDPase activity was measured 2 days after isolation of membranes. The assay was the same as described by Bowles and Kauass (1976). The assay mixture contained 600 μl of 100 mM Tris-HCl, pH 7.5; 250 μl of 10 mM IDP and 50 μl of 20 mM MgCl₂ and 100 μl of H₂O. Reaction was
started by the addition of 100 μl of membranes. Reaction mixtures were incubated in triplicate at 25°C for 1 hr. Reaction was terminated by addition of 1.4 ml of 12.5% TCA. For control assays, enzyme was added after incubation of the reaction mixture and immediately followed by TCA. Phosphate was determined by the method of Allen (1940).

RNA estimation

RNA was estimated as described by Cherry (1973). All centrifugations were done in a Beckman model J2-21 using a JA-20.1 rotor. Fractions (about 0.5 mg of protein) were precipitated with 5 ml of cold 5% PCA and centrifuged at 10,000 rpm for 10 min. The residue was washed twice with 1.5% PCA each time the precipitate was pelleted by centrifugation at 10,000 rpm for 10 min. The residue was then dissolved in 5 ml of ethanol:ether (2:1) and incubated at 50°C for 30 min. After incubation, it was again centrifuged at 10,000 rpm for 10 min and the pellet was dissolved in 5 ml of 5% PCA and heated in a water bath at 70°C for 40 min. The supernatant after centrifugation at 10,000 rpm for 10 min was taken and measured at 260 nm against a 5% PCA blank in the spectrophotometer.

6.2.5 Electron microscopy

The fractions were fixed overnight at 4°C using Millonig's fixative (2% paraformaldehyde; 3% glutaraldehyde; 1% acrolein; 2.5% DMSO; 0.001 M CaCl₂ in
0.1 M cacodylate buffer, pH 7.6). They were washed several times with cold cacodylate buffer. Then they were enrobed in 2% Noble agar in the same buffer and spun down. Pellets were cut into small blocks (0.5 mm square) and kept in osmium tetroxide at room temperature for 1 hour followed by 0.5% uranyl acetate and water for 1 hour. Sections were dehydrated in acetone, Vestopal and acetone. Then the sections were encapsulated into gelatin and polymerised at 60°C for 24 hours. Thin sections were cut using glass knives and a Reichert ultra microtome. Sections were picked up on 200 mesh Formvar and carbon coated grids and post-stained with 1% uranyl acetate in water followed by Reynolds lead stain. Sections were viewed and photographed on a Phillip's 300 Electron Microscope at KV 60, using Kodak fine grain positive film.

6.2.6 Dolichol kinase and Dol-P phosphatase assay

Dolichol kinase activity was measured as described previously (Chapter 4) with some modifications. Low protein concentrations (0.2 mg of protein/ml reaction mixture) were used. Hence low Triton X-100 (0.2 %) was used in these assays. Dol-P phosphatase activity was measured as described in Chapter 3).

6.3 Results

6.3.1 Distribution of marker enzymes in submicrosomal fractions

In order to prepare bulk fractions, discontinuous
sucrose gradient centrifugation was used. Since there is no reliable method to separate submicrosomal fractions in bulk quantities available in plants, (Bowles and Kauss., 1976) a method which has been used successfully for separation of rat liver submicrosomal fractions has been used (Rip and Carroll., 1982). Antimycin insensitive NADH and NADPH cytochrome C reductase activities (considered as markers for endoplasmic reticulum) were measured in all fractions. In our analysis SER, contained the highest specific activity of NADH cytochrome C reductase activity, whereas the highest NADPH cytochrome C reductase activity was observed in Golgi (Fig. 6.1).

IDPase activity is known as a specific marker for Golgi (Ray et al., 1969). The highest activity of IDPase was observed in Golgi (Figure 6.1), followed by SER and RER. RNA was estimated in all fractions. RER has the highest RNA content (Fig. 6.2). However the enzyme markers are not totally reliable in assaying the purity of the fractions (Bowles and Kauss, 1976), since it is believed that the intracellular membranes of endoplasmic reticulum, Golgi and plasma membranes are in a dynamic state in germinating seeds. Hence electron micrographs of fractions are indispensable for characterization.

6.3.2 Electron microscopy of submicrosomal fractions

Electron micrographs of different fractions are shown in Figure 6.2. All these fractions appeared as vesicles.
Figure 6.1  Distribution of markers in different fractions.

(a) RNA content of different fractions
(b) NADH Cytochrome C reductase activity
(c) NADPH Cytochrome C reductase activity
(d) IDPase activity
(The values represent an average of six measurements).
Figure 6.2 Electron micrographs of (a) microsomes; 
(b) RER; (C) SER; (d) Golgi.

(Magnification. X 69,300)
It is very difficult to isolate these organelles with intact membranes, because rigorous homogenization was required to break the cell walls. However, the presence of ribosomes on the membranes confirms that the fraction was enriched in RER. The smooth nature of the vesicles in Figure 6.2 suggests that they are SER, and Golgi apparatus formed into larger vesicles.

6.3.3 Distribution of Dol-P phosphatase and dolichol kinase activities in submicrosomal fractions

The distribution of Dol-P phosphatase and dolichol kinase was shown in the Figure 6.3. The highest dolichol kinase activity was observed in RER and SER and comparatively low activity in Golgi. However, the highest Dol-P phosphatase activity was observed in RER followed by Golgi and SER.

6.4 Discussion

Dolichol kinase and Dol-P-phosphatase activities were measured in soybean microsomes isolated during various stages of germination (Chapter 5). It is interesting to note that high Dol-P phosphatase activity was observed when compared to dolichol kinase activity throughout germination and early stages of development. Similarly, high Dol-P phosphatase activity was observed in brain tissue (Scher et al., 1984). In rat liver and calf brain Dol-P phosphatase activity was observed in RER (Rip et al., 1882; Scher et al., 1984), where most of the
Figure 6.3 Distribution of (a) dolichol kinase and (b) Dol-P phosphatase in different submicrosomal fractions. (The values represent an average of four measurements).
Dol-P dependent glycosylation events take place. It was suggested that in RER, Dol-P phosphatase may participate in regulation of Dol-P levels together with dolichol kinase activity. In addition to that it may participate in \textit{in vivo} synthesis of dolichol, since RER is considered to be the site of dolichol synthesis.

In soybean seedlings the highest specific activity of Dol-P phosphatase was identified in RER, where most of the glycosyl transferases which transfer sugars to dolichol were observed (Nagahashi and Beevers, 1978; Nagahashi et al., 1978; Marriott and Tanner, 1979). Considerably high activity of Dol-P phosphatase activity was observed in Golgi. It is reasonable to assume that Dol-P phosphatase activity found in RER and Golgi may participate in the biosynthesis of dolichol. In plants, both RER and Golgi have been reported as sites of dolichol synthesis. The significance of the presence of Dol-P phosphatase and dolichol kinase in SER is not known.

The presence of dolichol kinase and Dol-P phosphatase in RER could provide a regulation of Dol-P by phosphorylation and dephosphorylation cycles. It should be mentioned at this point that all studies were done \textit{in vitro}. We still do not know how these activities are regulated in cells. These enzymes may be localized on different sides of the membrane or different pools may exist for Dol-P phosphatase and dolichol kinase.
activities. More work in these lines will be helpful in understanding the regulation of glycoprotein biosynthesis in plants.
Chapter 7: OVER-ALL SUMMARY AND CONCLUSIONS

Recently many basic reactions involved in Dol-P dependent glycosylation in plants have been identified (Elbein, 1979; Lehle, 1984). However the presence of dolichols in plants was not unambiguously identified. Indirect evidence supports the presence of Dol-P in germinating seeds, whereas direct isolation, and characterization by NMR and IR revealed the occurrence of only polyisoprenols in the leaves of many plants (described in Chapter 1). In order to investigate the precise nature of polyisoprenols in plants with special reference to seeds, a systematic study was undertaken.

A lipid fraction enriched in polyisoprenoid alcohols was isolated from a variety of seeds, using Florisil chromatography. The material obtained from dicotyledonous seeds gave a family of symmetrical peaks containing 13 to 22 isoprene units on HPLC. A similar fraction from monocotyledonous seeds and wheat germ yielded on HPLC a family of peaks which were non-symmetrical (split-peaks) and appeared to be made up of nearly equal amounts of two components. Polyisoprenoid alcohols ranging from 13 to 22 isoprene units were purified from mono- and dicotyledonous seeds on HPLC by using a semipreparative column.

Polyisoprenoid alcohols isolated from the dicotyledonous seeds were unequivocally identified as
dolichols by NMR and IR spectral analysis. An asymmetrical peak of the monocotyledonous type could be produced by mixing equal amounts of dolichol-18 and polyisoprenol-18 and injecting the mixture into HPLC. This suggested that the peaks obtained with monocotyledonous seeds and wheat germ might be a mixture of dolichols and polyisoprenols. Polyisoprenols (but not dolichols) can be oxidized to their aldehyde forms by MnO₂. Two components (component I and component II) formed after MnO₂ oxidation, were separated by TLC. Analysis by NMR and IR of the components I and II, confirmed the presence of both dolichols and polyisoprenols in the asymmetrical peaks, i.e. in monocotyledonous seeds.

Dolichols after phosphorylation may participate in asparagine-linked glycoprotein biosynthesis. The significance of the presence of both dolichols and polyisoprenols in monocotyledonous seeds is not known. Polyisoprenols of short chain length (less than 12 isoprene units) which are not yet characterized may be also present in dicotyledonous seeds. These are several possible explanations for the presence of polyisoprenols in monocotyledonous seeds. Polyisoprenols may be immediate precursors for dolichols, or they may participate in cell wall biosynthesis during germination of seeds. A similar function may be performed by dolichols or polyisoprenols of short chain length (if present) in dicotyledonous seeds.
Dolichol was also isolated from the roots, stems, leaves and cotyledons of 15 day old seedlings. Cotyledons contain the highest concentration of dolichol at this time.

In order to investigate the fate of dolichol during germination, the amounts of dolichol and Dol-P were estimated in a fixed number of seeds or seedlings at different time intervals during germination. The dolichol content of germinating soybeans gradually decreased over the first three days, so that on the third day it was 25% lower than in dry seeds. After the third day the amount of dolichol in seedlings (exposed to a 14 h light cycle after the third day of germination in the dark) increased and on the tenth day it was 45% higher than in dry seeds.

Only trace amounts of Dol-P were found in dry seeds, but Dol-P concentration increased and reached a peak of 14 μg/10 seedlings on third day and declined to undetectable levels in later stages of development. The simultaneous decrease in dolichol and increase in Dol-P content over the first 3 days of germination suggests that Dol-P is being formed by phosphorylation of existing endogenous free dolichol. In addition to this, it also suggests that dolichol is the end product of biosynthesis from mevalonate. The above mentioned changes of dolichol and Dol-P, prompted the search for Dol-P phosphatase and dolichol kinase activity in soybeans. Assays were
developed to measure Dol-P phosphatase and dolichol kinase activities. Using soybean microsomes, activities of both enzymes were identified. This is the first demonstration of the occurrence of Dol-P phosphatase and dolichol kinase in plants.

Dol-P phosphatase activity was linear with respect to time and protein concentration. It had a broad pH optimum (pH 7-9). Triton X-100 was necessary for significant enzyme activity. Enzyme activity was slightly enhanced by EDTA. Divalent metal ions were not required for enzyme activity. A number of phosphorylated compounds were tested as enzyme substrates. None of them competed with Dol-P as substrate. At very low concentrations, phospholipids like PC and PE slightly activated the enzyme. However, at higher concentration they each inhibited the enzyme activity. PA showed competitive inhibition.

The dolichol kinase had some unique and interesting properties. Kinase activity was linear with time, protein concentration and the concentration of one of the substrates, dolichol. The second substrate for all known dolichol kinases, CTP, was not required for maximal activity. CTP and other nucleoside tri- and diphosphates particularly GDP, were inhibitory. Inhibition observed with CDP is believed to be end product inhibition. In addition, 80% of maximal activity was obtained in the absence of divalent cations although a low level of
stimulation was noted with Mg++. The inhibitory and slight stimulatory effects of CDP and Mg++ suggest that endogenous CTP and Mg++ are preferentially utilized in the dolichol kinase reaction. An apparent $K_m$ of 55 $\mu$M was determined for dolichol.

The relative activities of dolichol kinase and Dol-P phosphatase are believed to be involved in maintaining the Dol-P concentration during asparagine-linked glycoprotein synthesis. In order to get information about the role of these enzymes in the regulation of Dol-P concentration during germination, activities of both enzymes were measured during germination and early development.

Dolichol kinase activities increased over the first 3-4 days of germination and then became less active. The activity of Dol-P phosphatase increased over the first five days after germination. The specific activity of Dol-P phosphatase obtained with soybean microsomes appears to be quite high relative to the level of kinase activity. The significance of this is not known. It should be mentioned that the maximum amount of Dol-P was observed when dolichol kinase activity was also high. This suggests that hydrolysis of Dol-P is not always the favoured direction in vivo. However, it is not clear why Dol-P concentration was decreased to undetectable levels after the fifth day of germination. More work remains to be done to understand this aspect.
The submicrosomal distribution of Dol-P phosphatase and dolichol kinase activities was investigated. Unlike animal systems, the highest specific activity of both Dol-P phosphatase and dolichol kinase is present in the RER. Higher levels of Dol-P phosphatase activity and low kinase activity was observed in Golgi, which is very similar to the animal system. Dol-P phosphatase and dolichol kinase present in RER and Golgi may regulate the Dol-P concentration by phosphorylation and dephosphorylation cycles. In addition, Dol-P phosphatase present in RER and Golgi may be involved in de novo synthesis of dolichol in those compartments. More work needs to be done to understand Dol-P regulation. The experiments described in this thesis will be helpful in understanding the regulation of Dol-P and N-linked glycoprotein biosynthesis during seed germination.

Formation of Dol-P-P-oligosaccharides was demonstrated by using soybeans and soybean embryos. The appearance of 3 new glycoprotein bands was also observed from the third day of germination onwards. Characterization of glycoproteins formed during germination may shed more light on the regulation of Dol-P dependent glycosylation during germination.
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