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GLYCEROLIPID BIOSYNTHESIS

IN PEA ROOT PLASTIDS

by

Lingru Xue

A thesis submitted to the Faculty of Graduate
Studies and Research in partial fulfilment
of the requirements for the degree of
Master of Science

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Abstract

Pea root plastids were isolated by differential centrifugation and resulting crude plastid fraction was purified by centrifugation through 10%(v/v) Percoll. Marker enzymes indicated that greater than 50% of the plastids were recovered essentially free from mitochondrial and endoplasmic reticulum contamination. The optimum *in vitro* conditions for glycerolipid biosynthesis from [U-^{14}C] glycerol-3-phosphate have been determined. Total glycerolipid biosynthesis was approximately 15 nmole/hr/mg protein in the presence of 200 μM glycerol-3-phosphate, 0.5 mM each of NADH and NADPH, 15 mM KH_2CO_3, 0.05 mM CoA, and 2 mM each of ATP and $MgCl_2$, 100 mM Bis Tris Propane (pH 7.5) and incubated at the standard temperature of 25°C. ATP, Coenzyme A and a divalent cation are absolutely required for glycerolipid biosynthesis, whereas reduced nucleotides and bicarbonate improve the synthesis to varying degrees. Dihydroxyacetone phosphate had little effect, while dithiothreitol, detergent and Mn^{2+} inhibited activity. Under the optimum conditions, isolated pea root plastids mainly synthesized approximately 15% phosphatidic acid, 16% phosphatidylcholine, 13% phosphatidylglycerol, 32% triacylglycerol. Galactolipid synthesis occurred only when UDP-galactose was supplied. Different concentrations of some cofactors resulted in alterations of glycerolipid distribution. Phospholipase A_2 and *Rhizopus* lipase digestions of phospholipids and neutral lipids revealed that radioactive fatty acids were preferentially esterified to position *sn 2* of each glycerolipid with generally 2-4 times as much radioactivity as position *sn 1*. Pea root plastids are composed of approximately 62% phospholipid, 24% neutral lipid and 14% glycolipid. Within these classes PG, TAG, and the

galactolipids are the major components representing 24, 12, and 12% of the total plastid lipids.

Résumé

Des plastes de racine de pois ont été isolés par centrifugation différentielle et purifiés par centrifugation sur 10% (v/v) de Percoll. Un rendement de plus de 50% et une absence de contamination par des mitochondries et du réticulum endoplasmique ont été déduits de la mesure des activités enzymatiques de marqeurs. Les conditions optimales de biosynthèse *in vitro* des glycérolipides à partir d'[U-^{14}C] glycérol-3-phosphate ont été déterminées. La biosynthèse totale de glycérolipides est de l'ordre de 15 nmoles/h/mg de protéines, en présence de 200 uM de glycérol-3-phosphate, de 0,5 mM de NADH et de NADPH, de 15 mM de $KHCO_3$, de 0,05 mM de Coenzyme A, de 2 mM d'ATP et de $MgCl_2$ et de 100 mM de Bis Tris propane à pH 7,5 pour une incubation à la température standard de 25°C. Le Coenzyme A et un cation divalent sont absolument nécessaires pour cette biosynthèse alors que les nucléotides réduits et le bicarbonate l'augmente dans des proportions variables. Le dihydroxyacétone phosphate a peu d'effet tandis que le dithiothréitol, un détergent et le Mn^{2+}inhibent l'activité. Dans les conditions optimales, des plastes de racine de pois isolés synthétisent principalement environ 15% d'acide phosphatidique, 16% de phosphatidylcholine, 13% de phosphatidylglycérol et 32% de triacylglycérol. La synthèse des galactolipides survient uniquement en présence d'UDP-galactose. Des concentrations variables de certains cofacteurs aboutissent à des modifications dans la répartition des glycérolipides. Des digestions de phospholipides et de lipides neutres avec la phopholipase A_2 ou avec la lipase de *Rhizopus* ont révélé que les acides gras sont préférentiellement estérifiés en position *sn*2 des glycérolipides, avec un rapport de 2 à 4 par rapport à ceux estérifiés en position *sn*1. Les plastes de racine de pois

sont composés d'approximativement 62% de phopholipides, de 24% de lipides neutres et de 14% glycolipide. Dans ces classes PG, TAG et les galactolipides sont les composantes majeures réprésentant 24, 12 et 12% du total des lipides des plastes.

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I wish to acknowledge my sincere appreciation to my supervisor, Dr. Salvatore A. Sparace for his guidance, support, understanding and invaluable help throughout the course of this research and during the preparation of this thesis. I would also like to thank Dr. Kathryn F. Kleppinger-Sparace for her advice and assistance.

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ACP	acyl carrier protein
BCCP	biotin carboxyl carrier protein
BSA	bovine serum albumin
βME	beta mercapto ethanol
B.T.P.	bis tris propane
CHAPS	3-[(3-cholamidopropyl)-dimethylamonio]-1-propane-sulfonate
DAG	diacylglycerol
DHAP	dihydroxyacetonephosphate
DGDG	digalactosyldiacylglycerol
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetic acid
FFA	free fatty acids
GLC	gas liquid chromatography
MAG	monoacylglycerol
MGDG	monogalatosyldiacylglycerol
OAA	oxaloacetic acid
PA	phosphatidic acid
PC	phosphatidylcholine
PE	phosphatidylethanolamine
PEP	phosphoenolpyruvate
PG	phosphatidylglycerol
PI	phosphatidylinositol
SQDG	sulfoquinovosyldiacylglycerol
TAG	triacylglycerol
TLC	thin layer chromatography

1 Introduction

Lipids are a diverse group of molecules which have long been recognized as major constituents of all biological membranes and perform several major functions in plants. Phospholipids, galactolipids and sterol esters form the central hydrophobic barrier of membranes. Cuticular lipids and wax esters form a coating on the aerial surface of plants which serves to prevent water loss and as a protection from environmental and biological stress. An analogous material, suberin, is formed by underground organs or in response to wounding. In most seeds, triacylglycerol is a major form of carbon storage. Plant triacylglycerols from seeds of soybean, sunflower, maize, etc. are a major source of calories for human consumption. Lipids also may serve a variety of less well defined functions such as hormones, second messengers, insect attractants, and defense chemicals (phytoalexins) (Moore, 1982).

Membrane glycerolipids contain two long chain fatty acids esterified to the *sn*1 and 2 positions of glycerol. These fatty acids are almost always 16 or 18 carbons in length and contain from 0 to 3 *cis* double bonds. Attached to the third position on the glycerol backbone is a polar head group. The combination of the non-polar fatty acyl chains and the polar head group leads to the amphipathic properties of membrane lipids. Most of such lipids spontaneously form bilayer or micellar structures when mixed with water, and in biological systems they are organized into the classic fluid bilayer which allows membranes to act as barriers, with selective permeabilities to materials and thereby maintain the separation between the internal and external environment of cells. Within cells, membranes form and enclose many of the morphologically distinguishable organelles.

Fatty acid biosynthesis and glycerolipid metabolism are

essential processes in membrane biogenesis. They have been extensively studied in a variety of plant tissues and organelles, such as plastids, particularly in leaves and developing or germinating oilseeds. The structural feature and physiological role shared by all plastids (proplastids, leucoplasts, amyloplasts, chromoplasts, etioplasts, and chloroplasts) are that they are bound by a pair of outer membranes, known as the envelope and that all are capable of synthesising fatty acids and glycerolipids. Unfortunately, studies on nongreen plastids have lagged far behind those conducted on their chloroplast counterpart. Moreover, roots, have largely been ignored as ┐ tissue for such studies mostly because it is very difficult to prepare large amounts of intact nongreen plastids free from contaminating membranes and mitochondria. Furthermore, the presence of starch grains in plastids makes the preparation of intact plastids relatively more difficult. Among nongreen plastids studied, chromoplasts and etioplasts have been the main organelles for investigations of the metabolic capabilities of the plastids, and the structure, chemical composition, and functions of envelope membranes (Douce *et al.*, 1984). Mazliak *et al* (1972) investigated fatty acid biosynthesis in organelle preparations from a variety of nonphotosynthetic tissues including the roots of broad bean and wolf bean. They reported that plant mitochondria were capable of fatty acid biosynthesis and that such activity was dependent on the presence of suitable respiratory substrates and the usual cofactors for fatty acid biosynthesis. However, no mention was made regarding the role of the plastids in the biosynthesis of fatty acids in roots. Sparace *et al.* (1988) determined that fatty acid synthesis in germinating pea root tissues occurred in the plastids. Subsequently, Stahl *et al.* (1991) characterized the optimum *in vitro* cofactor

requirements for fatty acid biosynthesis in pea root plastids.

The study of lipid metabolism in non photosynthetic plastids has been continued here by characterizing glycerolipid biosynthesis in highly purified pea root plastids.

The objectives of this research were the following:

1. Develop a method to separate and purify the plastids from pea root.
2. Determine the purity of the plastid fraction using selected marker enzymes.
3. Determine the profile of glycerolipid biosynthesis from ^{14}C-acetate and ^{14}C-G3P pea in root plastids.
4. Optimize the *in vitro* incubation conditions and individual cofactor requirements for maximum glycerolipid biosynthesis from ^{14}C-G3P in pea root plastids.
5. Determine the effects of different incubation conditions and cofactor concentrations on the total and proportions of glycerolipids synthesized.
6. Characterize the distribution of radioactivity from ^{14}C-acetate and ^{14}C-glycerol-3-phosphate within the newly-synthesized glycerolipids.
7. Determine the cold endogenous fatty acid and glycerolipid composition of pea root plastids.

2 Literature Review

2.1 Fatty Acid Biosynthesis

In 1953, several investigators observed the capacity of isolated leaf chloroplasts to convert acetate to long chain fatty acids (Newcomb & Stumpf, 1953). The process of *de novo* fatty acid biosynthesis takes place almost exclusively in the

stroma of all kinds of plastids in plant cells (Yamada *et al.*, 1975; Weaire & Kekwick, 1975; Ohlrogge *et al.*, 1979; Mudd *et al.*, 1985; Sparace *et al.*, 1988). These involve plastids from photosynthetic tissues such as leaves and stems, and the nonphotosynthetic plastids such as chromoplasts, leucoplasts and amyloplasts present in flowers, fruits, seeds, roots and tubers. The pathway for fatty acid biosynthesis has been clearly defined and closely resembles that of the prokaryotic system in *E. coli* (Goodwin and Mercer, 1983). The general biosynthetic route to long chain, saturated and unsaturated fatty acids is shown in figure 2.1.

The biosynthesis of fatty acids may be divided into three distinct phases. Firstly, acetyl-CoA is carboxylated to yield malonyl-CoA; this reaction is catalysed by acetyl-CoA carboxylase. Secondly, C_2 units derived from malonyl-CoA sequentially condense together to form a fatty acid of intermediate chain length which is usually palmitic acid (16:0). This second phase is a multistep process, which is catalysed by a multienzyme complex called fatty acid synthase. For the condensation process to begin, acetyl-CoA is required as a primer. The methyl and carbonyl carbons of the acetyl residue become the terminal(ω) and the penultimate carbons, respectively, of the final fatty acid product of the synthase. All the other carbons are derived from malonyl residues. The third phase of fatty acid biosynthesis involves the elongation and subsequent desaturation of palmitic acid through the concerted action of fatty acid elongation and desaturation systems. Desaturation involves two pathways, in which palmitate (16:0), stearate (18:0) and oleate (18:1) fatty acids are synthesized *de novo* in the plastids (Roughan *et al.*, 1979). Oleate thus formed may either be desaturated directly in the plastids via the prokaryotic pathway (Browse, 1991) or be exported from plastids as CoA esters to enter the

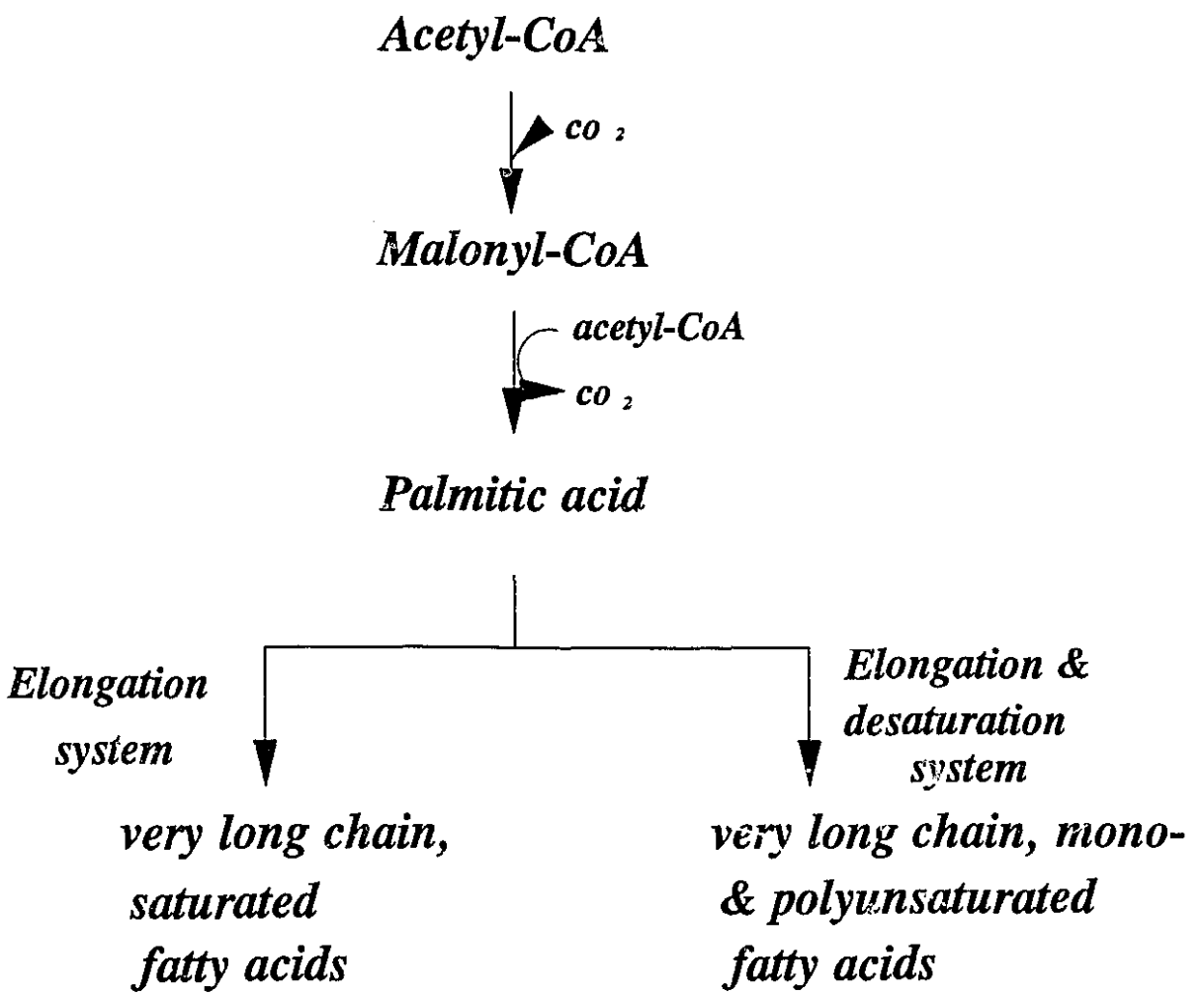

Figure 2.1 Outline of the biosynthetic route to very long chain, saturated and unsaturated fatty acids

eukaryotic pathway outside the plastids, particularly in the endoplasmic reticulum (Moore, 1982). This latter organelle is responsible for most of the production of linoleate (18:2), linolenate (18:3) and long chain fatty acids of 20 carbons and more (Moore, 1982).

The reactions of fatty acid biosynthesis are summarized in table 2.1, and are discussed individually as follows:

2.1.1 Acetyl-CoA Synthesis

The acetate is free to move to the chloroplast, where it is rapidly converted to acetyl-CoA in the stroma (Jacobson & Stumpf, 1972). This reaction (Eq.1) is catalysed by acetyl-CoA synthetase (EC 6.2.1.1.). The enzyme has a requirement for ATP and Mg^{++}.

There is an alternative mechanism for acetyl-CoA synthesis, in which acetyl-CoA can be supplied by the action of pyruvate dehydrogenase which requires NAD^{+} (Eq.2). Although this enzyme occurs both in the mitochondria and in plastids, it is now believed that the plastid isozyme has sufficient activity to account for *in vivo* rates of fatty acid synthesis (Dennis *et al.*, 1990). Plastids also contain a very active acetyl-CoA synthase, since isolated chloroplasts or developing seed plastids can rapidly incorporate radiolabelled acetate into fatty acids. Therefore, it appears that acetyl-CoA for fatty acid biosynthesis can be derived either from the pyruvate dehydrogenase reaction inside the plastid or extraplastidial production of acetate followed by its activation inside the plastid by acetyl-CoA synthase (Dennis *et al.*, 1990).

2.1.2 Formation of Malonyl-CoA

Malonyl-CoA is believed to be the key intermediate in fatty acid biosynthesis. In plants it is formed by the

Table 2.1 The reactions of fatty acid biosynthesis

(Eq.1)	$CH_3COOH + CoA.SH + ATP \longrightarrow CH_3CO.S.CoA + AMP + PPi + H_2O$
(Eq.2)	$CH_3COCOOH + CoA.SH + NAD^+ \longrightarrow CH_3.CO.S.CoA + CO_2 + NADH + H^+$
(Eq.3)	$CO_2 + CH_3CO.S.CoA + ATP + H_2O \longrightarrow COOHCH_2CO.S.CoA + ADP + Pi$
(Eq.4)	$CH_3CO.S.CoA + HS.ACP \longrightarrow CH_3CO.S.ACP + CoA.SH$
(Eq.5)	$HOOCCH_2CO.S.CoA + HS.ACP \longrightarrow HOOCCH_2CO.S.ACP + CoA.SH$
(Eq.6)	$CH_3CO.S.ACP + HOOCCH_2CO.S.ACP \longrightarrow CH_3COCH_2CO.S.ACP + ACP + CO_2$
(Eq.7)	$CH_3COCH_2CO.S.ACP + NADPH + H^+ \longrightarrow CH_3CH(OH)CH_2CO.S.ACP + NADP^+$
(Eq.8)	$CH_3CH(OH)CH_2CO.S.ACP \longrightarrow CH_2CH=CHCHO.S.ACP + H_2O$
(Eq.9)	$CH_3CH=CHCO.S.ACP + NADH + H^+ \longrightarrow CH_3CH_2CH_2CO.S.ACP + NAD^+$
(Eq.10)	$Acetyl\text{-}CoA + 7Malonyl\text{-}CoA + 14NADPH + 14H^+ \longrightarrow Palmitate + 7CO_2 + 14NADP^+ + 8CoA.SH + 6H_2O$
(Eq.11)	$CH_3(CH_2)_{14}CO.S.ACP + H_2O \longrightarrow palmitate + HS.ACP$
(Eq.12)	$palmityl\text{-}ACP + G3P \longrightarrow lyso\text{-}PA + HS.ACP$

(Eq.1) is catalysed by acetyl-CoA synthetase
(Eq.2) pyruvate dehydrogenase
(Eq.3) acetyl-CoA carboxylase
(Eq.4) acetyl-CoA:ACP transacylase
(Eq.5) malonyl-CoA:ACP transacylases
(Eq.6) acetyl-ACP:malonyl-ACP condensing enzyme
(Eq.7) β-Ketoacyl-ACP Reductase
(Eq.8) D(-)-β-Hydroxyacyl-ACP Dehydratase
(Eq.9) enoyl-ACP reductase
(Eq.11) palmityl-ACP thioesterase
(Eq.12) G3P-acyltransferase

carboxylation of acetyl-CoA (Eq.3). Acetyl-CoA carboxylase (EC 6.4.1.2), the enzyme catalysing equation 3, is a multienzyme complex. This complex readily dissociates into three different proteins: biotin carboxyl carrier protein (BCCP), biotin carboxylase and a carboxyltransferase. In higher plants, there appear to be at least two different acetyl-CoA carboxylases (Goodwin & Mercer, 1983). Like that of *E. coli,* the enzyme can be separated into BCCP and the two enzymes; however, while unlike that of *E. coli*, the three components tend to reaggregate readily and the two enzymes have much higher molecular weights. The enzyme from spinach chloroplasts is peculiar in that the BCCP component is tightly bound to the thylakoid membranes while the two enzymes are present in the stroma. The biotin carboxylase component is activated by Mn^{+} and is quite stable while the carboxyltransferase is activated by Mg^{2+} and is labile (Jacoboson & Stumpf, 1972).

In leaves, acetyl-CoA carboxylase has been shown to be localized in chloroplasts (Kannangara & Jensen, 1975; Mohan & Kekwick, 1980; Slack *et al.*, 1981; Thomson & Zalik, 1981). Its activity, which is low in the dark, is increased significantly upon illumination (Slabas *et al.*, 1986). The enzyme from maize was shown to be sensitive to changes in pH, ATP, ADP and Mg^{2+} that were likely to occur in chloroplasts during illumination (Nikolau & Hawke, 1984). The combined effects could bring about a 24-fold increase of acetyl-CoA carboxylase activity. Similar alterations are also known to affect acetate activation and fatty acid synthesis in spinach chloroplasts (Slabas *et al.*, 1986; Sauer & Heise, 1983). Moreover, the wheat germ carboxylase was also shown to be tightly controlled *in vitro* through its requirement for ATP and its inhibition by ADP and AMP (Eastwell & Stumpf, 1983).

2.1.3 Utilization of Malonyl-CoA to Form Palmitic Acid

De novo fatty acid synthesis is the sequence of repeating reactions utilising acetyl-CoA and malonyl-CoA to produce fatty acids. The overall reaction (Eq.10) is catalysed by a multienzyme complex called the fatty acid synthase which includes seven enzymes and acyl carrier protein (ACP). The process is subdivided into 3 phases, which are initiation phase, elongation phase and termination phase resulting in the formation of acyl-ACP thioesters for export from the plastids (Stumpf, 1980). All the enzymes involved are located in the stroma (Stumpf, 1984).

2.1.3.1 Acetyl-CoA:ACP Transacylase

The reaction catalysed by Acetyl-CoA:ACP Transacylase (EC 2.3.1.38) is shown in equation 4, table 2.1. The process is initiated by acetyl-CoA which acts as the primer for fatty acid synthesis. In the first cycle, the acetyl moiety of acetyl-CoA is the acyl unit which is elongated. This reaction is thought to be a rate-limiting step for the overall fatty acid synthetase activity (Murphy and Stumpf 1981), since the specific activity of this enzyme is very low in many preparations. The pH optimum is 8.1 and the enzyme is completely inhibited by 5 mM arsenite.

2.1.3.2 Malonyl-CoA:ACP Transacylase

This enzyme (EC 2.3.1.39) catalyses the transfer of the malonyl moiety of malonyl-CoA to ACP (Eq.5). As acetate is converted to acetyl-CoA by acetyl-CoA synthetase , it is further metabolized to malonyl-CoA by the action of acetyl-CoA carboxylase. These enzymes have been purified from a number of plant tissues including avocado (Caughey and Kekwick, 1982), barley (Hoj and Mikkelsen, 1982), spinach (Stapleton and Jaworski, 1984), soybean (Guerra and Ohlrogge,

1986), and leek (Lessire *et al.*, 1983), as well as from the cyanobacterium (Stapleton and Jaworski, 1984). In soybean two isoforms of the enzyme were found in leaf tissue (Guerra and Ohlrogge, 1986). Both the biotin carboxylase and the transcarboxylase occur exclusively in the stroma phase of plastids, with Mn^{+} being the specific metal activator for the former enzyme while Mg^{+} is the activator for the latter transcarboxylase (Stumpf, 1976). Once malonyl-CoA is formed, it is rapidly transferred to ACP to form malonyl-ACP, which continues in the process of fatty acid biosynthesis as discussed below.

2.1.3.3 Acyl-ACP:Malonyl-ACP Condensing Enzyme

The reaction catalysed by acyl-ACP:malonyl-ACP condensing enzyme (EC 2.3.1.41; Eq.6) results in the formation of a new C-C bond between the methylene carbon of malonyl-ACP and the carboxyl carbon of the acyl unit. In the process, the free carboxyl of malonyl is released while original acyl-enzyme thioester bond is cleaved. The enzyme is also called β-ketoacyl-ACP synthase, fatty acid synthetase I, or 3-oxoacyl-ACP synthase.

2.1.3.4 β-Ketoacyl-ACP Reductase

In this reaction (Eq.7) the β-keto moiety is reduced by β-ketoacyl-ACP reductase (EC 1.1.1.100) with the production of D(-)-β-hydroxybutyryl-ACP. The reductant utilized by the enzyme is NADPH. This enzyme has been reported in two forms and, in the case of avocado, the NADPH form has been resolved from that using NADH (Caughey and Kekwick, 1982). In other tissues, such as safflower and spinach, NADPH is a more effective substrate than NADH (Shimakata and Stumpf, 1982b; Shimakata and Stumpf, 1982c). The NADPH-specific reductase has been purified from avocado fruit and rapeseed (Schuz *et*

al., 1982). The reaction has its optimal activity at pH 6.5 (Stumpf, 1987).

2.1.3.5 D(-)-β-Hydroxyacyl-ACP Dehydratase

In the reaction catalyzed by this enzyme (EC 4.2.1.58; Eq.8) the elements of water are removed from the D(-)-β-hydroxybutyryl-ACP, producing a 4C *cis*-$\Delta^{2,3}$-dehydroacyl-ACP (crotenyl-ACP). The enzyme optimum pH is 7.8, and its best substrate is 2-crotenoyl-ACP.

2.1.3.6 Enoyl-ACP Reductase

Enoyl-ACP reductase (EC 1.3.1.9) catalyses the reaction shown in Eq.9. Two forms of the enzyme have also been detected. Type I utilizes crotenyl-ACP and is NADH-specific (Shimakata and Stumpf, 1982a). Type II uses 2-decenyl-ACP as substrate and NADPH in preference to NADH. Both types are present in safflower, castor bean, and rape seeds (Slabas *et al.*, 1984), although type I seems to be the form present in leaf tissue (Shimakata and Stumpf, 1982b). It has optimal activity at pH 6.5 (Stumpf, 1987).

As a result of the reactions described in section 2.1.3. to this point (Eq.'s 4-9) the first elongation cycle is thus completed. The product is the saturated four carbon fatty acyl-ACP, namely butyryl-ACP. This moiety re-enters the elongation pathway beginning with the reaction of condensing enzyme (Eq.6) where it replaces acetyl-ACP. After six more cycles of two carbon unit additions, the final product is palmityl-ACP.

2.1.4 Termination Reactions

Two different reactions, are involved in effectively terminating of the process of fatty acid biosynthesis. In the first, palmityl-ACP thioesterase (EC 3.1.2.14), localized in

the inner envelope, catalyses the reaction that releases free palmitic acid (Eq.11) which is thus regarded as the end product of *de novo* fatty acid synthesis (Harwood, 1975). The palmitate can then be metabolized by outer envelope acyl-CoA synthetase (EC 6.2.1.3) to become palmityl-CoA which can move to the endoplasmic reticulum or mitochondria where it is processed by the "eukaryotic" pathway (Roughan and Slack, 1982, 1984). The enzyme exhibits strong preference for oleoyl-ACP derivatives, and, therefore, this termination reaction does not usually represent the metabolically preferred fate of palmityl-ACP (Ohlrogge, 1987). In the second termination mechanism, glycerol-3-phosphate acyltransferase (EC 2.3.1.15) catalyses the transfer of the palmityl residue from ACP to the *sn*-1-hydroxyl group of *sn*-glycerol-3-phosphate (Eq.12) to form lysophosphatidic acid (LPA). This LPA is the precursor of phospholipid synthesis in plastids.

2.1.5 Acyl Carrier Protein

The discovery of isoforms of acyl carrier protein (ACP) raised the question of what role they play in plant lipid formation. The possibility that the different isoforms were present in different parts of the cell was discounted by experiments showing that the spinach chloroplast contained both isoforms and in the same ratio as whole leaf extracts (Ohlrogge and Kuo, 1985). Moreover, barley ACP-I and ACP-II were both capable of supporting *in vitro* fatty acid synthesis, with identical products being accumulated (Hoj and Svendsen, 1984). This excluded the possibility that only one form of ACP was used by fatty acid synthetase. However, these workers suggested that the two isoforms might interact differently with different ACP-dependent enzymes. In particular they theorized that one form could be used for *de*

novo synthesis of fatty acids while the other participated in glycerolipid formation (Hoj and Svendsen, 1984).

The above suggestions were tested directly with the spinach ACP isoforms. In keeping with the results with barley, both isoforms of spinach ACP caused identical rates and patterns of products in a fatty acid synthesizing system (Guerra *et al.*, 1986). Moreover, detailed examination of the malonyl-CoA:ACP transacylase failed to reveal any difference in ACP isoform reactivity. In marked contrast, oleoyl-ACP thioesterase and glycerol-3-phosphate acyltransferase were both strongly affected by ACP isoforms (Guerra *et al.*, 1986). These two enzymes determine the fate of oleate produced by the plastid. Thus, when oleoyl-ACP thioesterase activity is predominant, oleate is then released for reesterification to oleoyl-CoA and exported to the endoplasmic reticulum for possible further modification. In contrast, direct acylation of glycerol-3-phosphate keeps the acyl chain within the plastid and would be characteristic of the so-called "prokaryotic pathway" (Heinz and Roughan, 1983; Roughan and Slack, 1984). The different forms have different activities in the oleoyl-ACP thioesterase and oleoyl-ACP:glycerol-3-phosphate acyltransferase reactions. Therefore, this difference may afford a means for the cell to use ACP isoform expression to control the proportions of oleate exported or retained within the plastid. The apportionment of acyl chains between these two pathways may be controlled by a number of factors including glycerol-3-phosphate concentration and the relative activity of thioesterase versus acyltransferase. The experiments with ACP isoforms suggest that the relative abundance of these may also help to control the "prokaryotic" and "eukaryotic" pathways (Guerra *et al.*, 1986). However, since triacylglycerol synthesis in seeds occurs predominantly outside the plastid, high acylthioesterase activity would be

needed, and the occurrence of ACP-II as the major isoform in spinach seeds is not in keeping with such a requirement.

2.1.6 Fatty Acid Elongation Systems

Palmitic acid, produced by the plant fatty acid synthetase, can be elongated by the successive addition of 2C units to the carboxyl end to produce a range of straight-chain, saturated fatty acids from C_{18}-C_{30}. Higher plants appear to have two elongation systems. One called fatty acid synthetase II system or the C_{16}-->C_{18} system that elongates palmitic acid to stearic acid within the plastid. The other is usually called the fatty acid elongase system III or C_{18} elongation system. This system elongates stearic acid and unsaturated C_{18+} fatty acids to the very long chain fatty acids (Goodwin and Mercer, 1983). These systems are multienzyme complexes resembling the fatty acid synthase I in that they involve a multistep process. The latter system is located on the endoplasmic reticulum and utilizes acyl-CoA derivatives as substrates (Harwood, 1989). The collaboration between plastids and the extraplastidic part of the cell also has implications for fatty acid desaturation and complex lipid synthesis (Harwood, 1989).

2.1.6.1 Fatty Acid Synthetase II System

(Eq.13) palmityl-ACP + malonyl-ACP + NADPH + H^+ ----> stearyl-ACP + CO_2 + $NADP^+$ + H_2O + ACP

The C_{16}-->C_{18} elongation system (shown in Eq.13) has been found in the soluble stroma phase of chloroplasts (Harwood, 1975). Its substrates are palmityl-ACP and malonyl-ACP, for which the enzyme shows absolute specificity. It requires NADPH and its products are stearyl-ACP and CO_2. This process is roughly equivalent to one cycle of the elongation phase of

the process catalysed by fatty acid synthase I system discussed earlier. Work on spinach chloroplasts has revealed an *in vitro* pH optimum range of 7.8-8.6 and an inactivation temperature of 37°C for fatty acid synthetase II (Stumpf, 1975).

2.1.6.2 Fatty Acid Elongase System III

The C_{18} elongation system is bound to endoplasmic reticular membranes. It utilizes both saturated and unsaturated C_{18} and very long chain fatty acids up to 30 carbons in length. This system is required mainly for the formation of unusual storage lipids in seeds, suberin in roots and cutin and waxes in the surface layers of plant tissues. Active fractions from germinating peas were shown to use malonyl-CoA as the sources of the C_2 addition unit and to require NADPH as reductant (Harwood, 1988). Stearyl-CoA is the most probable substrate (Harwood and Russel, 1984). In addition to the products of fatty acid biosynthesis, phosphatidylcholine may also serve as a source of fatty acids for elongation. Lipids, especially various phosphatidylcholines, were capable of providing acyl chains for elongation in germinating peas (Jordan and Harwood, 1980). Although acyl carrier protein stimulated total synthesis of fatty acids (Palmitate and stearate accumulating as acyl-ACPs), the very-long-chain products were found esterified exclusively to acyl lipids in germinating peas (Sanchez and Harwood, 1981).

2.1.7 Fatty Acid Desaturations

The major unsaturated fatty acids in plants are three related C_{18} molecules: oleic (18:1), linoleic (18:2), and α-linolenic acids (18:3) (Harwood, 1980). These compounds make up to 80% or more of the total fatty acids of most higher

plant tissues. Their *cis* double bonds are introduced by an aerobic mechanism. The desaturation enzymes are generally soluble and concentrated in the stroma of proplastids or chloroplasts (Harwood, 1988). They require an input of electrons from NADPH or from water via photosystems I and II. Ferredoxin can act as an intermediate electron carrier (Stumpf, 1984).

Most eukaryotic organisms including plants possess a Δ^9 desaturase. This is an enzyme system which introduces a *cis*-Δ^9 double bond into a saturated fatty acid regardless of the length of its carbon chain; however, stearic acid is the most common substrate resulting in the formation oleic acid (18:1, 9c). The enzyme requires NADPH and molecular oxygen for activity. In higher plants, the system is soluble in the stroma of the chloroplast and utilizes stearoyl-ACP as a substrate (Stumpf, *et al.*, 1984; Goodwin and Mercer, 1983). The overall reaction is represented in equation 14:

(Eq.14) $18{:}0\text{-ACP} + NADPH + H^+ + 1/2O_2 \longrightarrow 18{:}1\text{-ACP} + NADP^+ + H_2O$

The desaturase from developing safflower seeds is specific for stearoyl-ACP. Stearoyl-CoA and palmitoyl-ACP give only 5% and 1%, respectively, of the activity observed with stearyol-ACP (McKeon and Stumpf, 1982).

Further desaturations use complex lipid substrates. The first of these, catalysed by a Δ^{12}-desaturase, seems to use phosphatidylcholine as the major substrate in most plant tissues. In order for such a reaction to be followed, oleate has to be hydrolysed from oleoy-ACP (Shine *et al.*, 1976), activated to oleoyl-CoA in the plastid envelope (Joyard and Stumpf, 1981) and transferred to the *sn*-2 position of phosphatidylcholine. Such a sequence of reactions allows

oleate to be modified by the "eukaryotic pathway" that takes place on the endoplasmic reticulum (Roughan, 1987). This desaturase system has a complete dependence on O_2 and either NADH or NADPH (Stymne and Stobart, 1987).

The final (Δ15) desaturase has been studied with whole tissues or subcellular fractions. In leaf tissues it is clear that linoleic acid esterified to monogalactosyldiacylglycerol is the substrate (Heinz and Harwood, 1977; Wharfe and Harwood, 1978; Roughan *et al.*, 1979; Jones and Harwood, 1980). However, nongreen seedlings that accumulate significant amounts of α-linolenate may use phosphatidylcholine as substrate (Harwood, 1988). The leaves of some plant species (16:3 plants) contain *cis*-Δ7,10,13-hexadecatrienoate in their monogalactosyldiacylglycerol. In these species the desaturation of palmitate to hexadecatrienoate also appears to occur on the galactolipid (Thompson *et al.*, 1986). Genetic studies suggest that the same desaturases as those forming α-linolenate are involved (Somerville, 1987).

2.1.8 Metabolic Source of Carbon for Fatty Acid Biosynthesis

Acetyl-CoA is generally regarded as the precursor for fatty acid synthesis. Acetyl-CoA can be generated by the action of the pyruvate dehydrogenase/decarboxylase complex. However, until recently it was debated whether plastids contained sufficient enzyme to provide enough acetyl-CoA for fatty acid synthesis. In the past it was thought that mitochondrial generation of acetyl-CoA was involved. This acetyl-CoA was thought to be cleaved to free acetate that could then move to the plastid and reconverted to acetyl-CoA. This indirect pathway was proposed from experiments with spinach leaf fractions (Murphy & Stumpf, 1981), but its application to plant tissues in general has been questioned

by Givan and Hodgson (1983). Moreover, chloroplastic pyruvate dehydrogenase of high activity has now been demonstrated in chloroplasts from a variety of plants such as in pea (Camp and Randell, 1985). Unfortunately, the physiological source of pyruvate for fatty acid biosynthesis has not been resolved. Either cytoplasmic or plastidic glycolysis is involved. However, there are reports that the uninterrupted pathway from 3-phosphoglycerate is missing from chloroplasts of mustard cotyledon tissues (Liedvogel *et al.*, 1986). Although chloroplasts from mustard cotyledons have been shown to be capable of generating enough pyruvate for pyruvate dehydrogenase activity, other sources of pyruvate, such as from the cytosol, may also be used (Liedvogel *et al.*, 1986).

In contrast to the controversy that has surrounded the sources of acetyl-CoA for fatty acid synthesis in chloroplasts, there seems to be general agreement that nongreen plastids contain not only an active fatty acid synthesizing system, but also the necessary glycolytic enzymes and pyruvate dehydrogenase. Evidence comes from a variety of plastids, including the leucoplasts of developing castor oilseed (Miernyk & Dennis, 1983) and of cauliflower (Journet & Douce, 1985), the chromoplasts of daffodil flowers (Klening & Liedvogel, 1980), and plastids from safflower and linseed cotyledons (Browse & Slack, 1985) and pea root plastids (Qi and Sparace, unpublished observations). 2C units can be provided from pyruvate by the pyruvate dehydrogenase complex in nonphotosynthetic plastids. The demonstration that isolated developing castor oilseed endosperm plastids incorporate ^{14}C-pyruvate into fatty acids indicated that a pyruvate dehydrogenase complex must be present and the separation of endosperm plastids free from mitochondrial contamination demonstrated the presence of this complex in these plastids (Reid *et al.*, 1977).

Beevers and coworkers (Fritsch & Beevers, 1979) have reported that the pyruvate dehydrogenase complex is also present in the plastids of germinating castor beans, so that the glycolytic pathway in these organelles could provide pyruvate for the generation of acetyl-CoA for fatty acid synthesis. However, the presence of pyruvate dehydrogenase complex in plastids of germinating castor bean seeds has been disputed by Rapp and Randall (Rapp and Randall, 1980), who found no such complex in the plastid fraction of this seed. Weaire and Kekwick (1975), in a study of fatty acid synthesis in avocado mesocarp and cauliflower bud tissues, demonstrated that glucose was almost as effective a precursor of fatty acids as acetate, but succinate and citrate were not incorporated, indicating that in these tissues plastids most likely synthesize acetyl CoA from glucose via glycolytic metabolism. In general, acetyl-CoA for fatty acid synthesis in leaf tissues appears to be generated primarily by the action of a plastidic pyruvate dehydrogenase. However, the use of mitochondrial acetyl-CoA is still a possibility, at least in spinach tissues (Heinz *et al.* 1987).

2.1.9 Precursors Used for Studies of Fatty Acid Synthesis

In 1952, Newcomb and Stumpf fed several radioactive substrates to slices of cotyledons of both maturing and germinating peanut seeds. In both systems, acetate was the most effective of those substrates tested for incorporation into long-chain fatty acids (Newcomb and Stumpf, 1953). Acetate is immediately activated to [^{14}C]-acetyl-CoA by plastid acetyl-CoA synthetase. Since a pyruvate dehydrogenase complex and acetyl-CoA synthetase coexist in plastids of several plant species as described earlier, they have the ability to convert both [2-^{14}C]pyruvate and free acetate to acetyl-CoA and subsequently into fatty acids. Radiolabelled

hexoses and trioses from glycolysis and the pentose phosphate pathway have also been used as precursors for fatty acid synthesis (Yamada and Usami, 1975; Miernyk and Dennis, 1983), but these yield low rates of activity probably due to poor uptake across the plastid inner membrane.

The radiolabelled intermediates of fatty acid synthesis, [^{14}C]acetyl-CoA and [^{14}]malonyl-CoA, have been shown in some instances to enter the plastid and become incorporated into fatty acids. However, the plastid envelope is generally impermeable to these acyl-CoA derivatives (Liedvogel and Bauerle, 1986). In any case, the use of these compounds does not allow for the study of strictly *de novo* fatty acid synthesis as they bypass the initial steps of the pathway. Depending on the purposes of different studies, other precursors are also used, such as [^{14}C]CO_2 with bean leaves (Eberhardt and Kates, 1957), [^{14}C]bicarbonate by spinach chloroplasts (Murphy and Leech, 1977) and [^{3}H]H_2O by germinating seeds (Harwood and Stumpf, 1970).

2.1.10 Source of ATP and Reducing Power for Fatty Acid Biosynthesis

ATP and reduced nucleotides are essential for fatty acid biosynthesis. ATP is required for the synthesis of acetyl-CoA, and malonyl-CoA by acetyl-CoA synthetase and acetyl-CoA carboxylase, respectively. These cofactors are provided by different mechanisms in plastids from different tissues. There are many advantages for biosynthetic pathways in chloroplasts since chloroplasts are the primary source of carbon precursors, reducing power and ATP in the green plant cell. Plastids isolated from pea roots contain all the enzymes of the pentose phosphate pathway as well as most of those involved in glycolysis (Emes and Folwer, 1979; Borchert *et al.*, 1993). Nonphotosynthetic plastids can thus obtain

carbon precursors from these pathways. Chloroplasts can also obtain carbon precursors from the Calvin cycle, in addition to the biosynthetic pathways for fatty acids synthesis (Stumpf, 1975).

The ATP required for fatty acid biosynthesis can be derived from the glycolytic pathway which occurs both in plastids and the cytosol of developing oil seeds (Dennis and Miernyk, 1982). Within this pathway, phosphoglycerate kinase and pyruvate kinase are thought to provide ATP through substrate level phosphorylation. Kleppinger-Sparace *et al.*, (1992) demonstrated that pea root plastids can independently generate sufficient ATP to support high rates of fatty acid biosynthesis when provided the components of the triose-phosphate shuttle mechanism. They concluded that phosphoglycerate kinase was an important enzyme for ATP production in these plastids. However, adenylate kinase may also be involved. Similarly, fatty acid biosynthesis in chromoplasts from daffodil flower petals also requires ATP (Kleinig and Liedovogel, 1980). However, ATP can be replaced by glycolytic intermediates which increase the endogenous level of ATP by substrate level phosphorylation of ADP. Phosphoenolpyruvate (PEP) is the most effective glycolytic intermediate in stimulating fatty acid synthesis, but the other intermediates, such as dihydroxyacetone phosphate (DHAP), can also be used. As in other systems, ATP is required for maximal activity for fatty acid synthesis in developing castor plastids (Boyle *et al.*, 1990). P[illegible] simulates fatty acid synthesis and is approximately [illegible] times as effective as ATP through the generation of ATP within the organelle by the action of the plastid pyruvate kinase.

In plastids with a complete glycolytic pathway, some of the reducing power (in the form of NADH) for fatty acid synthesis could be supplied by the glyceraldehyde 3-phosphate

dehydrogenase reaction. NADH can also be generated in the reaction catalyzed by pyruvate dehydrogenase in those plastids containing this enzyme (Camp and Randall, 1985). NADPH is probably supplied through glucose-6-phosphate dehydrogenase or 6-phosphogluconate dehydrogenase in the oxidative pentose phosphate pathway in this organelle (Dennis and Miernyk). Alternatively, in chloroplasts the NADPH and ATP required is derived from the light phase of photosynthesis (Stumpf, 1984; Goodwin and Mercier, 1983).

2.2 Glycerolipid Biosynthesis

As mentioned earlier, glycerolipids are found mainly in membranes or oil droplets, where they function as structural membrane components or food stores, respectively. The composition of plant membrane lipids depends on a number of factors including the tissue type, subcellular localization and environmental influences (Harwood, 1980). In order of abundance, most plant membranes are composed of phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylinositol (PI), and phosphatidylglycerol (PG) (Harwood, 1980). Minor amounts of phosphatidylserine (PS) and the plasmalgen derivatives of phosphatidylcholine and phosphatidylethanolamine also occur (Harwood, 1980). Diphosphatidylglycerol is found exclusively in mitochondrial membranes (Bligny and Douce, 1980). Plasma membranes tend to have a larger neutral lipid content than other plant cell membranes (Hardwood, 1989). Additionally, they may have a rather high content of phosphatidylethanolamine and are enriched in phosphatidylinositol compared to other membranes (Harwood, 1985). In other nonphotosynthetic membranes of plants, such as mitochondrial, peroxisome and microsomal membranes, PC and PE together generally comprise over 70% of the total phospholipids substitutes (Mazliak *et al.*, 1975).

In contrast, in photosynthetic chloroplast membranes phosphatidylglycerol is the major, sometimes only, phospholipid (Harwood, 1980). Chloroplast thylakoids have a uniquely different composition. In addition to phospholipid, these membranes contain three glycosylglycerides that make up over 85% of the total acyl lipid. These lipids are monogalactosyldiacylglycerol (MGDG), digalactosyldiacylglycerol (DGDG) and sulfoquinovosyldiacylglycerol (SQDG). Finally, neutral lipids, including sterols, are generally present but only in very small amounts in chloroplast membranes. The detection of large quantities of phosphatidic acid (PA), monoacyl glycerides and free fatty acids is usually the result of degradation during isolation (Harwood, 1989).

In contrast to the process of fatty acid biosynthesis, glycerolipid synthesis can be carried out by many organelles of plant cells, particularly the endoplasmic reticulum, mitochondria and plastids (Joyard and Douce, 1987). The general pathway for glycerolipid biosynthesis is outlined in figure 2.2.

The assembly of membrane glycerolipids may be considered to occur in two fairly distinct stages. The first is the sequential transfer of fatty acids onto positions 1 and 2 of glycerol-3-phosphate to form phosphatidic acid. This provides the diacylglycerol portion of the lipid. The second stage is the addition of the head group to position 3 of the glycerol moiety. The head group thus defines the class or type to which the lipid belongs (Barron and Stumpf, 1962). The pathway of phospholipid metabolism in plants appears to closely follow that for animals originally established by Kennedy (1961). The central metabolite, PA, may be utilized to form either DAG or CDP-diacylglycerol (CDP-DAG). From DAG, PC, PE or TAG may be formed, while from CDP-DAG, PI, PGP or

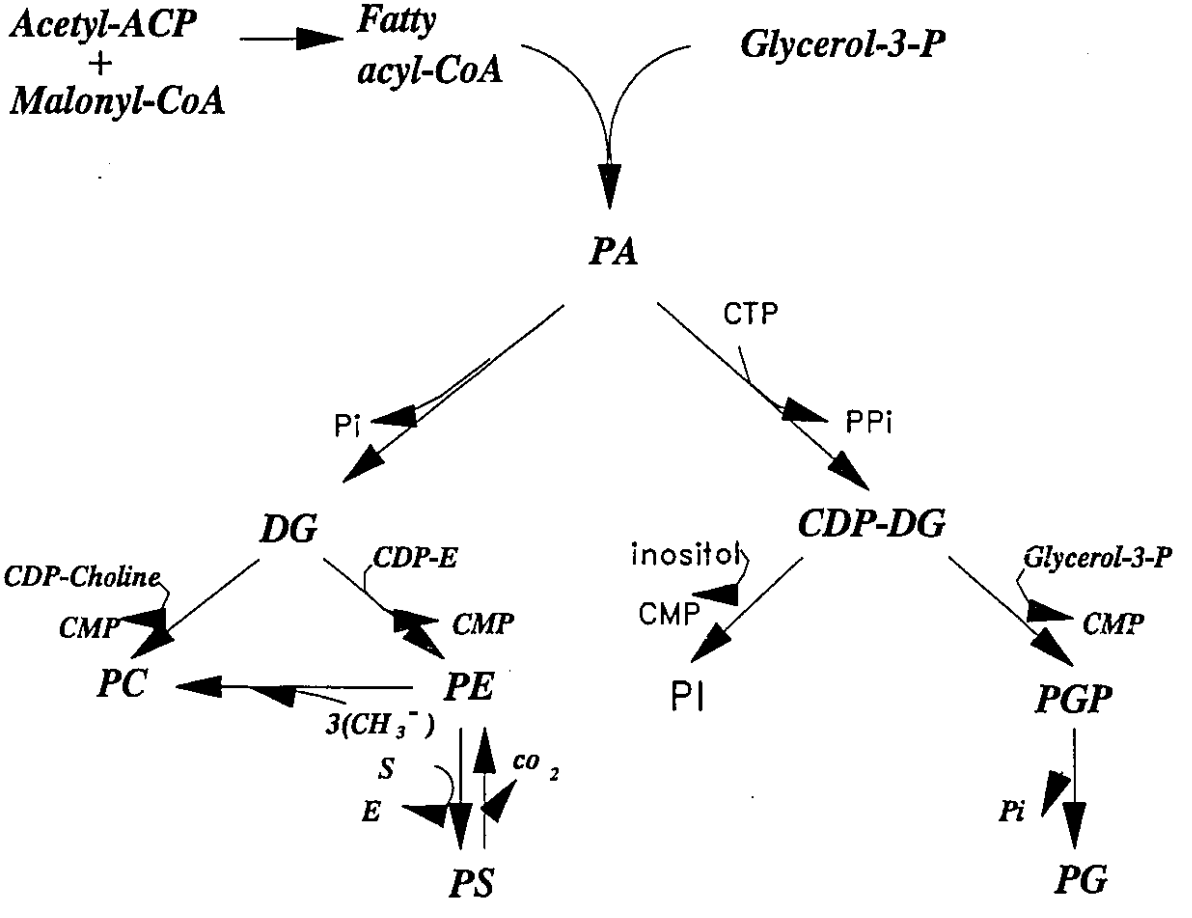

Figure 2.2 The glycerol phosphate pathway (Kennedy, 1961)

PS may be formed. Some interconversions among phospholipid types, for example, exchange of serine for ethanolamine of PE to form PS or methylation of PE to form PC, can also occur (Moore, 1982).

2.2.1 Precursors for Glycerolipid Synthesis

^{14}C labelled glycerol-3-phosphate (G3P) is most frequently used as a precursor to study glycerolipid biosynthesis. This is because it is the most immediate precursor and represents the backbone of the lipid structure to which the fatty acid chains and headgroups become esterified. G3P also can potentially label all or many of the intermediates and products of glycerolipid biosynthesis in various organelles. G3P generally exits in the cytoplasm and freely diffuses or is readily translocated across cellular membranes making it available for lipid assembly at cellular membranes, such as the plastid, mitochondrial or endoplasmic reticular membranes (Roughan *et al.*, 1980). In plant cells, G3P is synthesized from dihydroxyacetonephosphate (DHAP) or 3-phosphoglyceraldehyde by a cytoplasmic triose phosphate dehydrogenase (Harwood, 1989). In photosynthetic tissue, DHAP from the Calvin cycle is the principle carbohydrate exported from the chloroplast (Stumpf, 1987). In the dark or in nonphotosynthetic tissue, DHAP can be derived from starch by glycolysis (Dennis and Miernyk, 1982). Alternately, radiolabelled free glycerol was readily incorporated into lipids by maize leaves (Slack, *et al.*, 1977) and by avocado mesocarp microsomes (Barron and Stumpf, 1962). But in pea root plastids it yielded limited capacity for incorporation into lipid (Stahl, 1990). It is no longer believed that free glycerol is a naturally occurring contributor to the G3P pool since this requires the action of a glycerokinase which consumes ATP.

Similarly, acetate has also been extensively used for the study of glycerolipid biosynthesis because it is readily incorporated into fatty acids which will be used for glycerolipid biosynthesis. For *in vitro* studies, however, acetate is only useful for such purposes with plastids, since they are the only organelle capable of fatty acid biosynthesis as mentioned earlier.

Finally, labelled fatty acid moieties, and various head group donors, such as CDP-choline, UDP-galactose as discussed later, are also employed to study more specific aspects or reactions in lipid synthesis (Moore, 1982). It has been shown that acyl-CoA derivatives are the preferred substrates for most glycerolipid synthesis.

2.2.2 PA Biosynthesis

PA is synthesized through the action of two successive acyltransferase reactions shown in eq.15 and eq.16.

(Eq.15) G3P + Acyl-ACP/CoA ----> 1-Acyl-G3P + ACP/CoA

(Eq.16) 1-Acyl-G3P + Acyl-ACP/CoA ----> PA + ACP/CoA

As indicated, fatty acids as either the ACP or CoA forms, are used for acylation of G3P, depending on the subcellular compartment. Plastids utilize acyl-ACP's while the endoplasmic reticulum and mitochondria use the CoA derivatives. Plastidic enzymes seem heavily studied. The first, *sn*-G3P acyltransferase (EC 2.3.1.15) transfers fatty acyl groups from acyl carrier protein in plastids to position 1 of *sn*-G3P (Eq.15). The second, enzyme, 1-acyl-*sn*-G3P acyltransferase (EC 2.3.1.51), similarly catalyses the transfer of fatty acyl groups to position 2 of *sn*-G3P

(Eq.16).

Glycerol-3-phosphate acyltransferase was purified from pea and spinach leaves. The enzyme is soluble in the stroma, and in peas occurs in two isomeric forms (Bertrams and Heinz, 1979). It has a pH optimum of 7.0-7.4 which is significantly more acidic than the pH of chloroplast stroma during illumination (Joyard and Douce, 1987), and it utilizes both C_{18} and C_{16} fatty acids. *In vitro* the enzyme will utilize acyl-CoAs as well as acyl-ACPs (Bertrams and Heinz, 1979). However, when mixtures of oleoyl-CoA and oleoyl-ACP are used, a strong preference for the acyl-ACP substrate is seen (Frentzen *et al.*, 1983). A marked preference of the enzyme for the *sn1* position is characteristic for the enzyme. The second transferase (1-acyl-*sn*-glycerol-3-phosphate acyltransferase), in contrast to the first, is firmly bound to the chloroplast envelope membrane (Joyard and Douce, 1977). It may be rate limiting for phosphatidate formation in some species such as pea but apparently not in spinach (Gardiner *et al.*, 1984). This enzyme can also use acyl-CoAs as well as acyl-ACPs but the latter are considered to be the physiological donors. In plastids, this enzyme prefers C_{16} fatty acids as the ACP derivative. When [^{14}C]palmitoyl-ACP and [^{3}H]palmitoyl-CoA were used together, over 85% of the label incorporated was always [^{14}C]palmitate, regardless of the concentration of palmitoyl-CoA used (Frentzen *et al.*, 1983). In contrast to the plastid enzymes, the corresponding enzymes from both microsomes and mitochondria preferentially utilize acyl-CoA substrates. As with chloroplasts, the first of this acyltransferase uses either C_{16} or C_{18} fatty acids. However, the second acyltransferase preferentially directs C_{18} fatty acids to the *sn*-2 position (Sauer and Heise, 1982). The combined specificities of the two acyltransferases in these compartments therefore ensure that, for most plants, a *sn*-1-

oleoyl, *sn*-2-palmitoyl species of phosphatidate is produced by chloroplasts. PA synthesis is of particular interest because this phospholipid serves as precursor to all other glycerolipids. As indicated in figure 2.2, phosphatidic acid can be metabolized in two directions to form either CDP-DAG or DAG.

2.2.3 CDP-diacylglycerol Biosynthesis

(Eq.17) PA + CTP ----> CDP-diacylglycerol + PPi

In phospholipid biosynthesis, nucleoside triphosphates of either cytidine or deoxycytidine activate the phosphate group of PA for subsequent transfer of a potential polar head group. The reaction between CTP and PA yields cytidine diphosphate-DAG (CDP-DAG) (Eq.17) and pyrophosphate, which is catalysed by phosphatidyltransferase (EC 2.7.7.41). The enzyme activity has been detected in isolates of plastids (Andrews and Mudd, 1985), mitochondria (Douce *et al.*, 1972) and endoplasmic reticulum (Kleppinger-Sparace and Moore, 1985) from various plant tissues.

2.2.4 PGP and PG Biosynthesis

(Eq.18) *sn*-G3P + CDP-DAG ----> PGP + CMP

(Eq.19) PGP + H_2O ----> PG + Pi

CDP-DG is an important intermediate for the biosynthesis of PI, PS, PG. The synthesis of PG involves two specific reactions. In the first, (Eq.18) phosphatidylglycerophosphate (PGP) is synthesized by glycerophosphate:CDP diacylglycerol phosphatidyltransferase (EC 2.7.8.5). PG is then produced by phosphatidylglycerophosphate phosphohydrolase (EC 3.1.3.27) as shown in Eq.19 where the terminal phosphate is removed.

Evidence for PG synthesis by this pathway was first presented by Sastry and Kates (1966) using spinach leaf extracts. In both the endoplasmic reticulum and mitochondria of castor bean endosperm, Moore (1974) observed a pH optimum of 7.3. Addition of Mn^{2+} leads to maximum stimulation of both enzymes and is optimal at 5 mM. Triton X-100 stimulates the reaction and a variety of sulfhydryl reagents inhibit. Despite the fact that PG is an important component of chloroplast membranes and can be synthesized by chloroplasts, other plastids such as pea root plastids, are also capable of making their own PG (Stahl and Sparace, 1991). The reactions involved have demonstrated a strong requirement for divalent cations, which can be satisfied by either Mg^{++} or Mn^{++}, but in some cases, Mn^{++} inhibits the PG synthesis (Andrews and Mudd, 1985; Xue and Sparace, unpublished observations).

2.2.5 PI Biosynthesis

(Eq.20) myo-Inositol + CDP-DAG ----> PI + CMP

CDP-DAG is also a precursor for PI formation. CDP-diacylglycerol:myo-inositol phosphatidyltransferase (EC 2.7.8.11) catalyses the reaction shown in Equation 20 to generate PI. Phosphatidylinositol synthase has been demonstrated in a mitochondrial preparation from cauliflower inflorescence by Mudd (1980) and in a spinach leaf particulate preparation by Kates (Kates and Marshall, 1975). This enzyme catalyses the transfer of the phosphatidic acid residue of CDP-DAG to myo-inositol to form phosphatidylinositol and CMP. This reaction also requires Mn^{2+} and has a pH optimum of 8.0-8.5 in castor bean endosperm while Triton X-100 at all concentrations tested inhibits the reaction (Sexton and Moore, 1981). On the other hand, the spinach (Kates *et al.*, 1975) and soybean (Carman *et al.*,

1980) enzymes are stimulated by Triton X-100.

2.2.6 PS Biosynthesis

(Eq.21) L-Serine + CDP-DAG ----> PS + CMP

(Eq.22) PE + ethanolamine <===> PS + L-serine

The synthesis of PS is catalysed primarily by serine phosphatidyltransferase (EC 2.7.8.8; Eq. 21). Kates has demonstrated the presence of this enzyme in a particulate fraction from spinach leaves. However, there is another mechanism for the formation of PS. It is catalysed by PE:L-serine phosphatidyltranferase which exchanges L-Serine for the ethanolamine of PE (Eq. 22). The reaction has been reported for both castor bean (Moore, 1975) and pea seedlings (Vandor and Richard, 1968) with the pH optimum of 7.8, but was not detected in spinach leaves (Marshall and Kates, 1974).

2.2.7 DAG Biosynthesis

(Eq. 23) PA + H_2O ----> DAG + Pi

DAG is synthesized from PA by phosphatidate phosphatase, also called phosphatidate phosphohydrolase (EC 3.1.3.4). The first demonstration of phosphatidate phosphohydrolase activity in plant subcellular fractions was by Kates *et al.*, (1955). It has been confirmed that this phosphatase in plants is located on the endoplasmic reticulum (Harwood and Price-Jones, 1987) and has a pH optimum of 9.0 and apparently does not require any divalent cations. In contrast, there are 3 acid pH optimum peaks of phosphatidate phosphatase in the soluble fraction from castor bean endosperm (Moore and Sexton, 1978) and the reaction requires Mg^{++} (Journet and Douce, 1987). An important additional location of this

enzyme in plants is the plastid envelope. This is of particular relevance for glycerolipid synthesis in plastids. The reaction has been shown to be located on the inner envelope membrane (Block *et al.*, 1983). Once formed, DAG is the precursor for PC, PE, TAG, SQDG and galactolipid biosynthesis.

2.2.8 PC Biosynthesis

(Eq.24) Choline + ATP ----> Phosphocholine

(Eq.25) Phosphocholine + CTP ----> CDP-Choline +PPi

(Eq.26) DAG + CDP-Choline ----> PC + CMP

(Eq.27) PE + 3(CH_3) ----> PC

Choline phosphotransferase (EC 2.7.8.2) synthesizes most of the PC in plant cells. The major route to produce PC is the nucleotide pathway which involves the direct transfer of choline from cytidine diphosphate choline to DAG by this enzyme (Moore, 1982). CDP-choline is derived from free choline by a rate limiting two step reaction. Choline is activated by the Mg^{++}-dependent choline kinase (EC 2.7.1.32)(Eq.24) using ATP. Then cholinephosphate cytidyltransferase (EC 2.7.7.15) transfers the phosphocholine moiety to CTP releasing PPi (Eq.25). In addition to the above mechanism, there is an alternative route for the formation of PC. This involves the methylation of phosphatidylethanolamine. The methylation reactions (Eq.27) have been studied in potato tuber, spinach leaf and castor bean (Harwood 1989), in which the formation of PC from PE by sequential methylation of the ethanolamine moiety was catalysed by S-adenosyl-L-methionine:phosphatidylethanolamine N-methyl-transferase (EC. 2.1.1.17). The optimal pH for the

reaction is 9.0 in castor bean and 8.0 in spinach leaf.

2.2.9 PE Biosynthesis

(Eq.28) DAG + CDP-Ethanolamine ----> PE + CMP

The nucleotide pathway for phosphatidylethanolamine synthesis occurs by a series of reactions analogous to those for PC (i.e. ethanolamine kinase, EC 2.7.1.82; ethanolaminephosphate cytidyltransferase, EC 2.7.7.14; and ethanolamine phosphotransferase, EC 2.7.8.1) (Macher and Mudd, 1974). They reported a pH optimum of 7.5 and an optimal requirement of either 8 mM Mg^{2+} or 2 mM Mn^{2+} for spinach leaf microsomal ethanolaminephosphotransferase activity. The final enzyme of the nucleotide pathway has been examined in subcellular fractions from several plants. The enzymes from both spinach leaves and castor bean endosperm show similar properties to the cholinephosphotransferase. Moreover, CDP-choline inhibits formation of PE in both systems. Therefore, it has been suggested that the same enzyme catalyses formation of both phospholipids (Harwood, 1989). However, different conclusions have been made by various workers.

Although the nucleotide pathway is by far the most important pathway for PE synthesis in plants, the phospholipid can also be formed by the decarboxylation of PS or by base exchange (Mudd, 1980). Phosphatidylserine decarboxylase (EC 4.1.1.65) activity has been clearly demonstrated in spinach leaf homogenate fractions by Kates (1975).

2.2.10 TAG Biosynthesis

(Eq.29) DAG + Acyl-ACP/CoA ----> TAG + ACP/CoA

The major pathway for triacylglycerol (TAG) formation in

plants is the glycerol-3-phosphate or Kennedy pathway. The reaction is performed by diacylglycerol acyltransferase (EC 2.3.1.20)(Eq.29) after phosphate of PA is hydrolysed by phosphatidate phosphohydrolase. Phosphatidate phosphohydrolase activity may be rate limiting for TAG formation in many situations (Harwood, 1989). The third acylation of the glycerol moiety occurs at the *sn*-3 position and is non-specific for fatty acid precursors. Selectivity depends mainly on their availability (Roughan and Slack, 1982; Stymne and Stobart, 1987). The endoplasmic reticulum is the primary site for TAG synthesis, and hence the cytoplasmic acyl-CoA pool is the source of the third fatty acid.

An alternative mechanism for TAG synthesis also exists. In this mechanism, DAG is synthesized directly from PC through a reversal reaction by CDP-choline: diacylglycerol cholinephosphatransferase; the resulting DAG is used for TAG synthesis. In this fashion, fatty acids that have been desaturated on the DAG of PC can be directly incorporated into TAG. However, acyl exchange is also involved as discussed earlier (Roughan and Slack, 1982). In any case it thus appears that the acyltransferases involved in TAG synthesis are not as 'restricted' as those involved in membrane glycerolipid synthesis, as many fatty acids other than 16:0 and 18:1 may be used as acyl donors in TAG synthesis.

2.2.11 Glycolipid Biosynthesis

2.2.11.1 Galactolipid Synthesis

(Eq.30) DAG + UDP-Galactose ----> MGDG + UDP

(Eq.31) 2MGDG ----> DGDG + DAG

Chloroplast thylakoids contain glycosylglycerides as their major lipid constituents. These are primarily MGDG and

DGDG and lesser amounts of SQDG. The biosynthesis of the galactolipids involves the transfer of galactose from UDP-galactose by UDP-galactose:DAG galactosyltransferase (EC 2.4.1.46; Eq.30). The enzyme has been localized in the inner envelope membrane of spinach chloroplasts (Joyard and Douce, 1987), but in outer envelope membrane of pea chloroplasts (Cline and Keegstra, 1983). The activity of this enzyme is stimulated by Mg^{2+} or Mn^{2+} (Harwood, 1989).

Digalactosyldiacylglycerol (DGDG) can be synthesized directly from two molecules of MGDG in the absence of UDP-galactose (Eq.31)(Joyard and Dource, 1987). In a recent study, no evidence could be found for the participation of a postulated UDP-galactose:MGDG galactosyltransferase (Heemskerk *et al.*, 1990). The MGDG:MGDG galactosyltransferase of intact chloroplasts from both 16:3 and 18:3 plants is sensitive to thermolysin treatment, which suggests that it is in the outer plastid envelope in both types of plants (Heemskerk *et al.*, 1990).

2.2.11.2 Sulfoquinovosyldiacylglycerol Synthesis

The pathway first outlined by Benson has been tested in a number of systems of higher plants (Harwood, 1975). It involves a reaction (Eq. 32) analogous to that shown for MGDG biosynthesis in equation 30.

(Eq.32) DAG + UDP-SQ ----> SQDG + UDP

Sulfoquinovosyldiacylglycerol (SQDG) is the predominant plant sulfolipid. The biosynthetic route for sulfolipid formation is not very well understood, but the proposed reaction is thought to occur at the chloroplast inner envelope membrane where SQDG is believed to obtain its DAG moiety from the same pool that is used for galactolipid synthesis (Mudd and Kleppinger-Sparace 1987; Joyard *et al.*,

1987). It is now fairly certain that the source of sulfate is adenosylphosphatosulfate (APS), rather than PAPS (Shiff and Hodson, 1973).

2.2.12 Prokaryotic and Eukaryotic Pathways

As mentioned earlier, the fatty acids synthesized by the plastid may be used for glycerolipid synthesis either within plastid itself, or exported for use by other cell membranes. Although both the plastid and the extraplastidial membranes, such as endoplasmic reticulum, are able to synthesize glycerolipids, the natures of the DAG moieties synthesized in these two compartments are dissimilar. The difference lies in the type of fatty acid esterified to position 2 of the glycerol backbone (Joyard and Douce, 1977). In glycerolipid synthesis in both nongreen plastids as well as chloroplasts, position 2 of glycerol backbone is occupied almost exclusively by 16 carbon fatty acids. Both 16 and 18 carbon fatty acids are found at position 1. This fatty acid distribution is characteristic of the lipids synthesized by prokaryotic pathway.

In most higher plants, the first enzyme of the prokaryotic pathway, the stromal acyl-ACP:glycerol-3-P acyltransferase, is highly specific for 18:1-ACP (Frentzen *et al.*, 1983), while the second acylation is catalysed by a membrane-bound acyl-ACP-1:lysoPA acyltransferase, which specifically utilizes 16:0-ACP to form 1-18:1, 2-16:0-PA. Like chloroplasts in which PG is synthesized from prokaryotic PA in all higher plants (Roughan and Slack, 1982), PG in nongreen plastids of pea root is also synthesized from prokaryotic pathway (Xue and Sparace, unpublished observations). In 16:3 plants, a prokaryotic DAG pool is formed from PA by the action of PA phosphatase (Joyard and Douce, 1977). In 18:3 plants, such a DAG pool is not formed.

Thus, chloroplastic PG is the only prokaryotic lipid in these plants, while a DAG pool derived exclusively from the eukaryotic pathway is used for the synthesis of other chloroplast lipids in these (18:3) Plants (Heinz *et al.*, 1983).

On the other hand, the lipids synthesized by the endoplasmic reticulum are so-called 'eukaryotic' in nature. Position 2 of these lipids is occupied primarily by 18 carbon fatty acids. In these lipids as well, position 1 may contain either 18 carbon or 16 carbon fatty acids. The positional distribution of the fatty acids is established by the activities of the enzymes which transfer the fatty acids onto the glycerol backbone of the lipid, and this specificity thus varies in the different compartments.

The results of many experiments suggest that PC which is synthesized in the endoplasmic reticulum is the precursor for the eukaryotic glycolipids. After its assembly in the endoplasmic reticulum, at least the DAG portion from PC is transported to the plastid (Frentzen, 1986). PC is the major structural lipid of almost all the extrachloroplast membranes of the cell, and it is also a substrate for fatty acid desaturation. It is rapidly turned over to provide DG for synthesis of chloroplast lipids. The results of *in vivo* labelling (Slack and Roughan, 1975) and the description of a microsomal 18:1-PC desaturase from leaves (Slack and Roughan, 1976) provided evidence that PC is the predominant substrate for 18:1 desaturation on the eukaryotic pathway. In leaf mesophyll cells, a major proportion of the PC synthesized in the endoplasmic reticulum is further metabolized to provide DAG for the synthesis of galactolipids and sulfolipid for chloroplast membrane biogenesis. The mechanism of lipid transfer between the endoplasmic reticulum and the chloroplast is not precisely known.

2.2.13 Compartmentation and Regulation of Fatty Acid and Glycerolipid Synthesis

Compartmentation is a major mechanism for regulating metabolism. This compartmentation is achieved by delimitation of certain regions of the cell by a membrane whose permeability can be regulated. A major component of these membranes, one which determines much of their basic behaviour, is phospholipids. The proper phospholipid environment is also important for the functioning of membrane-bound enzymes. Thus, the synthesis of phospholipids, and its coordination with the appearance of specific metabolic pathways, is of considerable interest (Moore, 1982; Dennis and Miernyk, 1982).

As mentioned earlier, in plant tissues fatty acid synthesis exclusively occurs in the plastids with subsequent modification elsewhere. The elongation reactions leading to fatty acids above 16 carbons in length appear to be numerous and multiply compartmented (Harwood, 1979). Palmitate elongases have been described from plastids, microsomes, and soluble fractions. Stearoyl elongase activity resides in microsomes of a variety of plant tissues. The initial desaturation step appears to occur in the plastids resulting in the formation of oleate (Roughan *et al.*,1980). Further desaturation to linoleate and linoleneate occurs in microsomal fractions apparently with phosphatidylcholine as an intermediate (Harwood, 1980; Roughan *et al.*, 1980; Stumpf, 1980).

Unlike fatty acid synthesis, the compartmentation of phospholipid synthesis is complex, apparently involving at least three compartmentations to produce the final products. However, the endoplasmic reticulum plays the major role in phospholipid synthesis. The synthesis of PC and PE in the

mitochondria and Golgi apparatus, and PG in chloroplasts are considered relatively minor exceptions. Phospholipid synthesis is absent in other organelles, specifically microbodies (Moore *et al.*, 1973) and plasma membrane (Demandre *et al.*, 1978). PC is primarily synthesized on endoplasmic reticulum with configuration of *sn*-2 C_{18} fatty acids. Although mitochondria and chloroplast do not synthesize large amounts of PC, they do contain considerable amount of PC. Since some organelles apparently can not synthesize the lipids for their membranes, they are thought to be transferred from the sites of the synthesis to those organelles. Two major mechanisms for the transfer have been proposed. The first requires membrane to membrane contact, which is an integral part of the membrane-flow hypothesis (Moore, 1975). The second mechanism relies on carriers for individual molecules, the so-called phospholipid transfer proteins (Kader, 1977).

Phospholipid transfer proteins have been reported by several workers (Douady *et al.*, 1978; Kader, 1977; Mazliak *et al.*, 1980; Yamada, 1980). Concern with the origin of PC in the chloroplast has led to tests for phospholipid transfer proteins in photosynthetic tissue and their ability to transfer PC to chloroplasts. Tanaka *et al.*(1980) and Julienne & Kader (1981) have reported such activities from spinach, oat, and pea leaves, with the capacity to transfer radioactive PC from liposomes to plastids. Dubacq *et al.* (1984) demonstrated that microsomal PC is transferred to chloroplasts by phospholipid transfer proteins in spinach and pea leaves. Murphy & Kuhn (1981) also attempted to assay such activity from spinach, but concluded the only stimulation obtained was an artifact, probably resulting from high salt concentrations in the extract leading to increased membrane fusion.

3 Materials and Methods

All materials and methods are based on those developed by S. A. Sparace and co-workers (Sparace *et al.*, 1988; Stahl and Sparace, 1991; Kleppinger-Sparace *et al.*, 1992).

3.1 Materials

Pea seeds (*Pisum sativum*, cv Improved Laxton's Progress) were purchased from Stokes Seed Co., St. Catharines, Ontario. 200g of the seeds was soaked overnight in running tap water. Imbibed seeds were surface-sterilized for 5 minutes in 5% (v/v) commercially obtained hypochlorite solution ('Javex'), and thoroughly rinsed three times with sterile water. Operating in a laminar flow hood, 20 to 25 pea seeds were placed in each petri dishes (10cm x 2cm) containing filter paper moistened with 7ml sterile water. The petri dishes with the peas were wrapped in foil and the seeds were allowed to germinate at 25°C in complete darkness for 5 days. During this time, roots developed to about 2-4 cm in length and the total yield of root tissue ranged between 10 and 15 grams.

All chemicals, such as cofactors, buffers, lipid standards and lipases, were purchased from the Sigma Chemical Co. (Saint Louis, Missouri). Organic solvents were of analytical grade and were supplied by Caledon or Fisher Laboratories Ltd. and the J. T. Baker Chemical Co. Concentrated acids and bases were from Anachemia, and radiolabelled precursors were purchased from ICN Radiochemicals and Amersham Co. Polygram SIL G/UV_{254} thin-layer-chromatography plates were purchased from Brinkmann Instruments (Rexdale, Ontario).

3.2 Plastid Isolation

All procedures for the isolation of plastids were

carried out at 4°C or on ice. The root tips were excised just below the hypocotyl and the total mass was recorded. Plastids are isolated by a method modified from Stahl and Sparace (1991) and Emes and England (1986) as summarized in figure 3.1. Roots were homogenized in 2 ml homogenization medium (buffer A) containing 50 mM Tricine buffer (pH 7.5), 0.33 M sorbitol, 1 mM ethylenediaminetetraacetic acid (EDTA), 1 mM $MgCl_2$ and 0.1% bovine serum albumin (BSA). The homogenate was successively filtered through two layers each of 250- and 20-μm nylon mesh to remove intact cells, nuclei and cell debris. The filtrate was centrifuged at 2000rpm (500g) for 8 minutes to yield the plastid-enriched fraction. These plastids were then resuspended in 0.5-1.0 mL of buffer A and overlaid on 5 mL of centrifugation buffer (pH 7.5), containing 50 mM Tricine, 0.33 M sorbitol, 0.1% (w/v) BSA and 10% (v/v) Percoll, and purified by centrifugation at 6000rpm (4000g) for 5 minutes. Plastids pelleted through Percoll were washed by resuspending them in 5.0 mL of 1.0 mM bis-tris-propane (B.T.P.) buffer (pH 7.5, buffer B) containing 0.33 M sorbitol and centrifuged at 3000rpm (1000g) for 5 minutes to remove residual Percoll and BSA. All centrifugations were performed in a Beckman type 28 fixed angle rotor. Washed plastids were finally resuspended in sufficient volume of buffer B to yield a suspension of plastid protein concentration of approximately 10 mg/mL. The compositions of all buffers are summarized in appendix 1.

3.3 Marker Enzymes

Marker enzymes used for the identification of subcellular fractions were particulate fatty acid synthetase for the plastids (Sparace, 1988), fumarase for mitochondria (Racker, 1950), and NADPH-Cytochrome c reductase for the endoplasmic reticulum (Lord *et al.*, 1973). Marker enzymes for

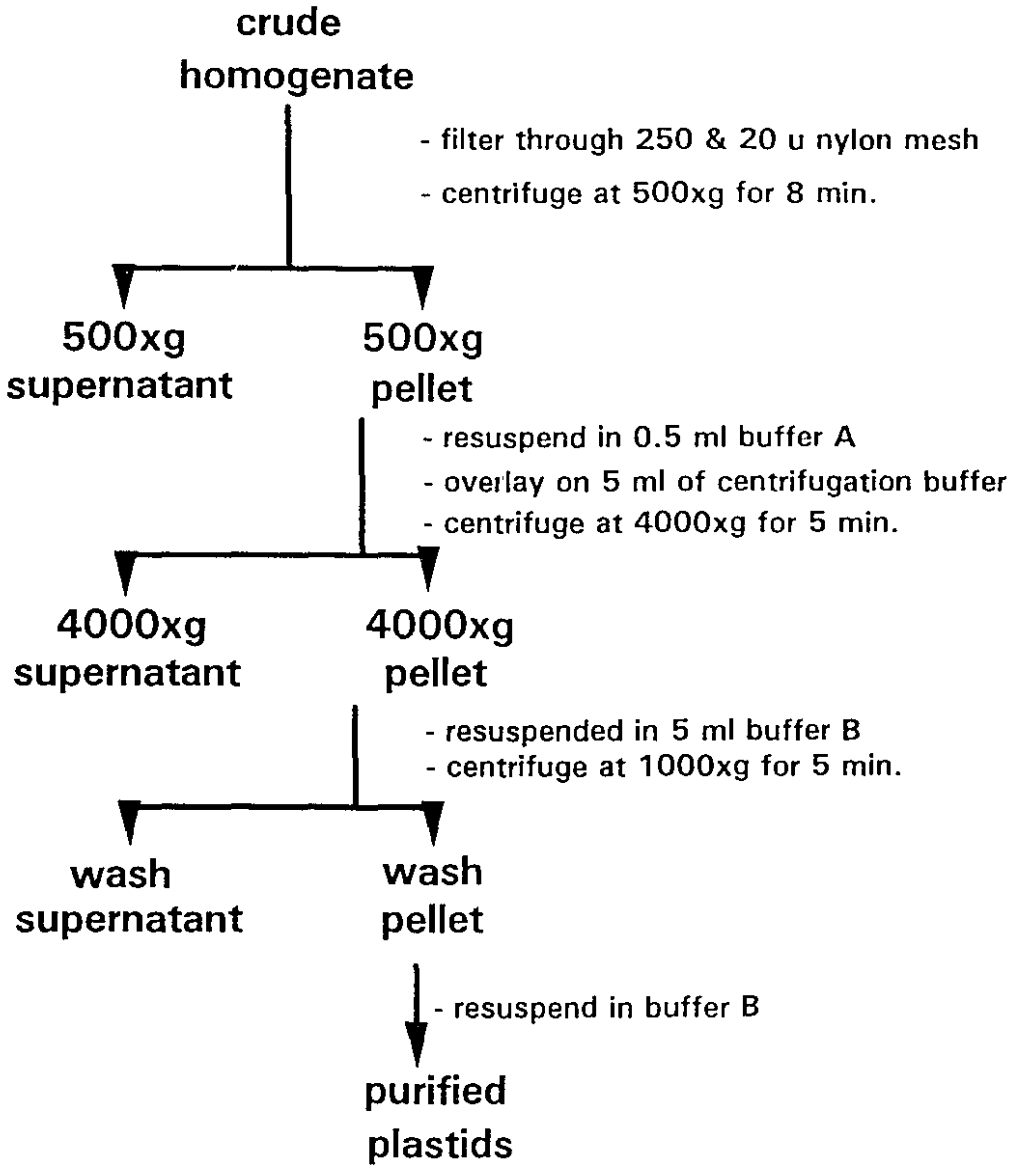

Figure 3.1 The flow diagram of protocol for plastid isolation

other subcellular organelles, such as the plasma membrane, tonoplast, golgi bodies and microbodies, were not performed since these organelles are not commonly associated with fatty acid and glycerolipid metabolism. The activities of marker enzymes among the subcellular fractions from the procedure of the isolation were determinated in duplicate and the results averaged. Spectrophotometric assays were performed with a Beckman model DU-40 spectrophotometer.

3.4 Plastid Incubation Conditions for Glycerolipid Biosynthesis

Initially, the optimum conditions established for fatty acid biosynthesis in pea root plastids (Stahl and Sparace, 1991) were used except that [U-^{14}C]glycerol-3-phosphate was used as a tracer instead of [1-^{14}C]acetate. 40 μL of isolated plastids (40-60 μg protein) were incubated in a 0.5 mL total reaction mixture containing 1.2 μCi [U-^{14}C]glycerol-3-phosphate, 0.1 M bis-tris-propane buffer (pH 8.0), 0.31 M sucrose, 0.2 mM acetate, 0.5 mM each of NADH and NADPH and CoA, 1 mM $MnCl_2$, 6 mM each of ATP and $MgCl_2$, 15 mM $KHCO_3$ and 0.16 mM ^{14}C-G3P and G3P. The total incubation time was 1 hour at 25°C. The concentrations of each cofactor were individually varied to determine optimum concentrations for glycerolipid biosynthesis. Once the optimum cofactor concentrations and buffers were determined, all experiments were repeated under the new re-adjusted optimum conditions to verify the optimum conditions for glycerolipid biosynthesis.

The final incubation conditions for maximum glycerolipid biosynthesis consisted of 0.1 M bis-tris-propane buffer (pH 7.5), 0.31 M sorbitol, 0.2 mM acetate, 15 mM $KHCO_3$, 0.5 mM each of NADH and NADPH, 2 mM each ATP and $MgCl_2$, 0.05 mM CoA and 0.16 mM G3P in the total volume of 0.5 mL. The concentrations of all stock solutions, final concentrations

and the standard volumes used for each reaction tube are summarized in appendix 2.

The effects of other possible cofactors or reagents on glycerolipid biosynthesis were also tested. These included dithiothreitol, dihydroxyacetone phosphate (DHAP), Triton X-100, CHAPS, ADP, UDP-galactose, LysoPC, βME, $KHPO_3$, $CaCl_2$, KCl, KNO_3.

3.5 Fatty Acid and Glycerolipid Extraction

Total fatty acid synthesis from 1 μCi of [1-^{14}C]acetate (11-14 μCi/μmole) was measured under the optimum conditions for fatty acid biosynthesis (Stahl and Sparace, 1991). Incubation reactions were terminated after 1 hour by the addition of 0.1 mL 8 N NaOH and then mixed, capped tightly and heated at 80°C for 1 hour. This process denatures the fatty acid synthetase enzymes and at the same time hydrolyses the acyl chains from the glycerol backbone, CoA and ACP moieties. The mixture was cooled, 0.1 mL 12 N HCl was added, and the free fatty acids were extracted as described by Mudd and DeZacks (1981). For the analysis of glycerolipids synthesized from either radioactive fatty acids via acetate incorporation or radioactive glycerol-3-phosphate, reactions were terminated by the addition of 3 mL chloroform/ methanol/ acetic acid (1:2:0.1, v/v) followed by vortexing. One mL each of 1 M KCl and chloroform were added followed by a further vortexing to produce an even emulsion. Phase separation was facilitated by a 1 minute spin at maximum velocity (approximately 500g) in an IEC table top centrifuge using a #221 swinging bucket rotor. The chloroform phase containing all the lipids was washed with 1.5 mL chloroform/methanol/water (3:48:47, v/v) followed by vortexing, centrifugation and removal of aqueous phase as described above. The chloroform extract of fatty acids or

glycerolipids was dried down under nitrogen with gentle warming to approximately 35°C using a Reacti-Therm heating unit and finally redissolved in 1 mL chloroform per tube. Two 25 μl aliquots of each sample were dispensed into scintillation vials for radioactivity measurement (section 3.8). The remainder of each sample was capped under nitrogen and stored at -25°C for lipid analysis.

3.6 Lipid Separation by Thin Layer Chromatography

Glycerolipids were separated by one-dimensional thin-layer-chromatography on plastic-coated Brinkman polygram SIL G/UV_{254} TLC plates.

All plates were prerun to the top in a solvent solution consisting of acetone/acetic acid/water (100:2:1, v/v). Lipid samples were dried down under nitrogen, and then 50 μL chloroform was added and spotted (using a drawn out pasteur pipette) on 1.5 cm bands 1 cm apart and 1.5 cm from the bottom of the plate. Lipid standards were applied separately to the sides of the plate and between sample lanes in the amount of 15-20 μg for each standard. The solvent system routinely employed to separate the different lipids was that devised by Sparace and Mudd (1982) as follows:

1. Hexane/ether/acetic acid (80:20:1, v/v). To separate the neutral lipids, such as TAG, DAG, MAG, sterol, sterol esters, free fatty acids, by using one solvent system without prerun.

2. Double solvent system. After samples were applied, prerun plates were first developed in acetone/acetic acid/water (100:2:1, v/v) which separated and moved the neutral lipids to the top of the plate. After drying the plate under slow flowing nitrogen for at least two hours, the polar lipids remaining at the origin were developed in the same direction to 13 cm from the bottom by a second solvent

solution consisting of either chloroform/methanol/acetone/acetic acid/ water (50:10:20:15:5, v/v) or chloroform/methanol/ammonium hydroxide/water (65:35:2:2:, v/v).

The lipids were located by slightly staining with iodine vapours and identified by co-chromatography with standards. Radioactive lipid products were localized by autoradiography using Kodak X-Omat AR film and an exposure time of approximately 14 days, depending on levels of radioactivity. Zones on the plate corresponding to radioactive lipids were scraped off directly into vials and measured by liquid scintillation counting or saved for further analyses. Sample lanes were maintained under nitrogen until the individual lipids were scraped into screw-cap test tubes for direct transesterification (section 3.7) and subsequent fatty acid analysis by gas liquid chromatography (section 3.7).

3.7 Determination of Fatty Acid Composition

Fatty acid methyl esters were prepared with BF_3 as described by Beare-Rogers (1969). Two mL of 14% (w/v) boron trifluoride in methanol were added to samples previously dried under nitrogen or samples on silica gel from TLC plates. Tubes were then sealed tightly with a teflon-lined screw-caps and heated in a 65°C water bath for 30 minutes. After cooling on ice, 3 mL of water were added and the sample was extracted three times with 2.5 mL hexane. Emulsions were formed by vortexing and phase separation occurred after a one minute spin at maximum velocity in a table top centrifuge as described earlier. The upper hexane phase was drawn off with a pasteur pipette and collected into a new screw-cap centrifuge tube. Hexane extracts were dried completely under nitrogen and redissolved in 1 mL hexane. Two 25 μL aliquots

of radioactive fatty acid preparations were counted by liquid scintillation counting (section 3.8) to give an estimate of the recovery of fatty acid methyl esters. The rest of the sample was dried down and finally redissolved in 5-10 μL hexane which was used for gas liquid chromatographic analysis.

The methyl esters of radioactive fatty acids were analyzed by GLC using a Varian model 3400 gas chromatography equipped with a 1.8 m x 4 mm glass column packed with 10% CSP-509 on Chromosorb W operated isothermally at 185°C. Injector and detector temperatures were 275 and 300°C, respectively. A stream splitter diverted approximately 80-90% of the effluent prior to a flame ionization detector to a Packard model 894 gas proportional counter radioactivity detector. Radioactivity and mass were quantified with a Varian model 4290 dual channel integrator. Samples were run for 15 minutes each, and the fatty acid peaks identified by co-chromatography with known standards of palmitate (16:0), stearate (18:0), oleate (18:1), linoleate (18:2) and linolenate (18:3). Duplicate injections were performed except when radioactivity levels were insufficient.

The analysis of unlabelled fatty acids was made by capillary column gas liquid chromatography as described above except that a fused silica column (0.25mm x 30 m) with 0.25u of dura bond 225 was used. The column temperature was programmed starting with 175°C. After holding 5 minutes the column was raised to 215°C at the rate of 2°C/min. Samples were run for a total of 26 minutes each, and fatty acids were identified as described earlier and quantified with methylpentadecanoate as an internal standard.

3.8 Liquid Scintillation Counting of Radioactive Products

All radioactive lipids and fatty acids were quantified

with a Beckman model LS 5801 Liquid Scintillation counter optimized for ^{14}C measurement. Samples were dispensed into disposable poly-Q vials, and were dried (if added in solvent). 5 mL of ICN Universol cocktail was added and samples were vortexed. Samples were counted for 5 minutes and measurements were automatically corrected for background radiation and quench as determined by 'H number'.

3.9 Protein Determination

Protein of isolated plastids was determined according to Lowry *et al.* (1951). Bovine serum albumin at a concentration range of 0 to 150 μg/ml was used as a standard. Twenty-five μl aliquots of protein samples were precipitated with 2 ml of 10% (w/v) trichloroacetic acid at 4°C for 20 minutes in order to remove any interfering substances. The protein was pelleted by centrifugation in a table top centrifuge at maximum velocity for 10 minutes and the supernatant was removed with a pasteur pipette. The protein residue was then redissolved in 1 ml of 0.1 N NaOH. One ml of a mixture of one volume 1% (w/v) $CuSO_4 \cdot 5H_2O$, one volume 2% (w/v) sodium potassium tartrate and 20 volumes 10% (w/v) sodium carbonate in 0.5 N NaOH was added to each sample and to 1 ml of each standard, mixed and allowed to stand for 5 minutes. Finally, 3 ml of Folin phenol reagent (diluted 1:10 in water) was added and mixed immediately, and the color was allowed to develop at room temperature for 30 minutes. Both reagents were prepared just before use. Absorbances were measured at 540 nm in polystyrene cuvettes with a Beckman model DU-40 spectrophotometer and sample concentration values were automatically calculated by the Quant-II Linear soft-pac module. All samples and standards were analyzed in duplicate and the results averaged with standard deviations of less than 5% of means.

3.10 Glycerolipid Composition Analysis

The glycerolipid samples, which involved the lipids synthesized from ^{14}C-acetate and cold acyl lipid from intact plastids, were separated and recovered from TLC plates. The labelled glycerolipids (between 50,000 and 250,000 DPM) were digested with either *Rhizopus arrhizus* lipase (for neutral lipids), or phospholipase A_2 from cobra venom (for phospholipids) (all from Sigma Chemical Co.) (Fischer *et al.*, 1973; Sparace and Mudd, 1982). Aliquots of radioactive lipid were spiked with approximately 500 μg of the corresponding lipid standard. Seventy-five μl of Triton X-100 in chloroform (30 μg/ml) was added and the mixture was dried down under nitrogen. One ml of 0.2 mM Tris, pH 7.5 containing 20 mM $CaCl_2$ was added and the mixture sonicated thoroughly. All digestions were initiated by the addition of 1.0 ml of enzyme solutions (about 100 units of phospholipase A_2, 1000 units for *Rhizopus* lipase), carried out at room temperature for 3 hours with vigorous shaking and finally terminated and also extracted by the addition of solution (chloroform/methanol/acetic acid, 1:2:0.5, v/v) as described earlier. The chloroform-soluble products released from the *Rhizopus arrhizus* and phospholipase A_2 digestions were separated by TLC using the double solvent system discussed earlier. All radioactive products of the lipase digestions were localized, identified, and analyzed as described earlier.

4 Results and Discussion

Optimum incubation condition for glycerolipid biosynthesis from ^{14}C-G3P were determined in isolated pea root plastids. First, marker enzymes were used to determine the purity of the plastid fraction. Second, a preliminary experiment was performed under conditions previously

developed for fatty acid biosynthesis to provide an indication of the relative cofactor requirements for glycerolipid biosynthesis. Third, the effects of varying substrate concentration, cofactor concentration and other parameters on glycerolipid biosynthesis and distribution were studied. Finally, the positional analysis of radioactive fatty acids on glycerol molecule was carried out by the digestions with different lipases and the total cold glycerolipid and fatty acid compositions of each lipid were determined. Each plotted data point represents the average of at least two replicate treatments.

4.1 Marker Enzymes

Subcellular fractions from pea (*Pisum sativum L.*) roots have been prepared by differential and percoll gradient centrifugation techniques. After centrifugation of crude pea root plastids (500xg fraction) through Percoll gradient the recovery of fatty acid synthetase, a marker enzyme for plastids, in washed 4000xg pellet was 53% of total activity applied (table 4.1). The activity of this enzyme had been clearly concentrated into this particulate fraction, whereas the activities of marker enzymes for mitochondria and the endoplasmic reticulum, fumarase and NADPH:Cytc reductase respectively, could not be detected. This indicates that the purified plastid preparations obtained are essentially free of contamination by other subcellular organelles which are commonly associated with glycerolipid biosynthesis and metabolism. With similar plastid preparations latency of glutamate synthase and electron microscopy indicated that the plastids are generally 90-95% intact (Emes and England, 1986).

The stability of the isolated plastids on ice, in terms of their capacity for fatty acid biosynthesis, was tested by

Table 4.1 Recovery of total activities of marker enzymes among subcellular fractions isolated from pea roots

	NADPH:Cyt c reductase (μmol/min)	Fumarase (μmol/min)	Fatty acids synthetase (μmol/hr)
	Total Crude Activity		
Crude Homogenate	0.711 ±0.024	2.123 ±0.346	0.111 ±0.002
	% of Crude Activity		
500xg Supernatant	88.9 ± 3.1	54.4 ± 5.9	37.2 ± 2.3
500xg Pellet	10.8 ± 0.9	16.7 ± 3.5	45.5 ± 4.3
4000xg Supernatant	13.1 ± 0.9	6.3 ± 0.9	0
4000xg Pellet	0	0	53.3 ± 1.5
4000xg Percoll	0	0	0

Stahl (1990). They found that the total activity was reduced gradually over time with only 41% of the initial activity remaining after 2 hours on ice. Also, the proportions of 16:0 and 18:1 synthesized were affected significantly. Lengthening the delay from 0 to 2 hours resulted in an increase in 18:1 from 39% to 60% and decrease in 16:0 from 49% to 28%. Therefore, the time of plastid for preparation and initiation of reactions was kept as short as possible (generally 30-40 minutes).

4.2 Optimization of Glycerolipid Biosynthesis

4.2.1 The Effects of Cofactor Deletion

Prior to this study, very little was known regarding the optimum cofactor requirements for glycerolipid biosynthesis in pea root plastids. However, in a previous study Stahl (1990) defined the co-factor requirements for fatty acid biosynthesis in these plastids, and that the products of *de novo* fatty acid biosynthesis (palmitate, stearate and oleate) were readily incorporated into glycerolipids. Thus, under the conditions established for fatty acid synthesis, a preliminary experiment was conducted to determine the relative dependency of glycerolipid biosynthesis from G3P on the cofactors required for fatty acid biosynthesis. The results of this experiment are presented in table 4.2. Glycerolipid biosynthesis is completely dependent on exogenously supplied ATP and Co enzyme A with less than 5% of the control activity observed when they are omitted. A divalent cation is also required. Mg^{2+} is preferred, while Mn^{2+} can partly substitute but is inhibitory when in the presence of Mg^{2+}. Similarly, reduced nucleotides are required. NADH is preferred over NADPH. The patterns of cofactor requirements for glycerolipid biosynthesis from G3P

Table 4.2 The Effects of cofactor deletion on total glycerolipid synthesis and product Distribution

Treatment	Relative Activity % of Ctrl	Lipid Distribution, % of the Total Radioactivity					
		Origin & Lyso-PA	PA	PC	PG	DAG	TAG
Control*	100.0	2	31	9	13	21	25
- ATP	5.3	12	10	13	60	3	2
- Mg^{2+}	24.9	2	25	11	8	29	26
- ATP & Mg^{2+}	5.8	28	10	10	37	7	9
- Mn^{2+}	125.0	8	18	22	12	28	12
- Mg^{2+} & Mn^{2+}	18.5	6	11	11	3	40	28
- CoA	4.9	6	14	12	58	6	3
- NADH	74.1	2	34	13	11	21	19
- NADPH	92.3	2	39	10	17	19	14
- NADH,NADPH	67.1	1	37	9	10	24	19
- $KHCO_3$	54.6	2	32	13	4	17	32
- Acetate	77.0	2	30	10	14	18	25

* (Rate of complete control activity was 8.6 nanomoles G3P/hr/mg)

observed with pea root plastids are similar to those observed for fatty acid biosynthesis in pea root plastids (Stahl,1990) and other non-photosynthetic plastids (Kleinig and Liedvogel, 1978,). These observations indicated that the processes of fatty acid and glycerolipid biosynthesis, particularly in pea root plastids, are tightly coupled, and that glycerolipid biosynthesis in these plastids likely has fatty acid biosynthesis as a corequisite. Finally, it is interesting to note that glycerolipid biosynthesis in pea root plastids is not greatly dependent on exogenously supplied acetate. This suggests that these plastids, at the time of isolation and bioassay, contain sufficient carbon (perhaps via glycolytic metabolism) to sustain much of the fatty acid biosynthesis required for glycerolipid synthesis. Based on the findings summarized above, further studies of glycerolipid biosynthesis were carried out. These include determining the optimum cofactor concentrations for glycerolipid biosynthesis and the effects of each on lipid product distribution.

4.2.2 Effects of Incubation Time with ^{14}C-acetate or ^{14}C-G3P

Studies of the effects of incubation time were carried out in two separate experiments with labelled ^{14}C-acetate or ^{14}C-G3P (figure 4.1 and 4.2, respectively). Incubation time was ranged from 5 minutes to 6 hours. In both cases the total activity of glycerolipid biosynthesis from either ^{14}C-acetate or ^{14}C-G3P was linear for up to six hours. These data suggest that in the presence of the optimal amounts of required cofactors, pea root plastids are relatively stable and that incubation conditions were still sufficient for glycerolipid biosynthesis for up to six hours. In contrast, the rate of glycerolipid synthesis from ^{14}C-acetate by pea chloroplasts began to decline after 30 minutes incubation time (Andrews and Mudd, 1985).

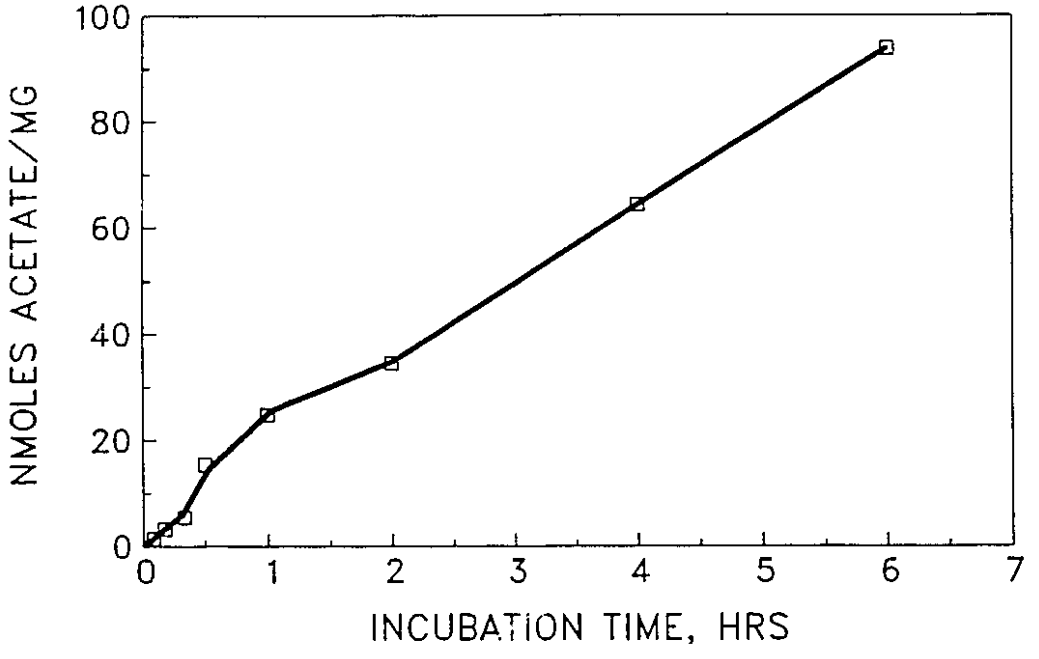

Figure 4.1 The effects of incubation time on glycerolipid biosynthesis from [1-^{14}C]acetate in pea root plastids. Plastids were incubated under the standard conditions of 160μM acetate and 1mM G3P and $MnCl_2$, 6mM ATP and $MgCl_2$, 0.5mM CoA, NADH and NADPH, 15mM $KHCO_3$, 0.31M sorbitol, in 0.1M Bis tris propane (pH 8) and at 25°C. Total activity represents nmoles acetate incorporated per mg plastid protein. Data points represent the mean of two replicates.

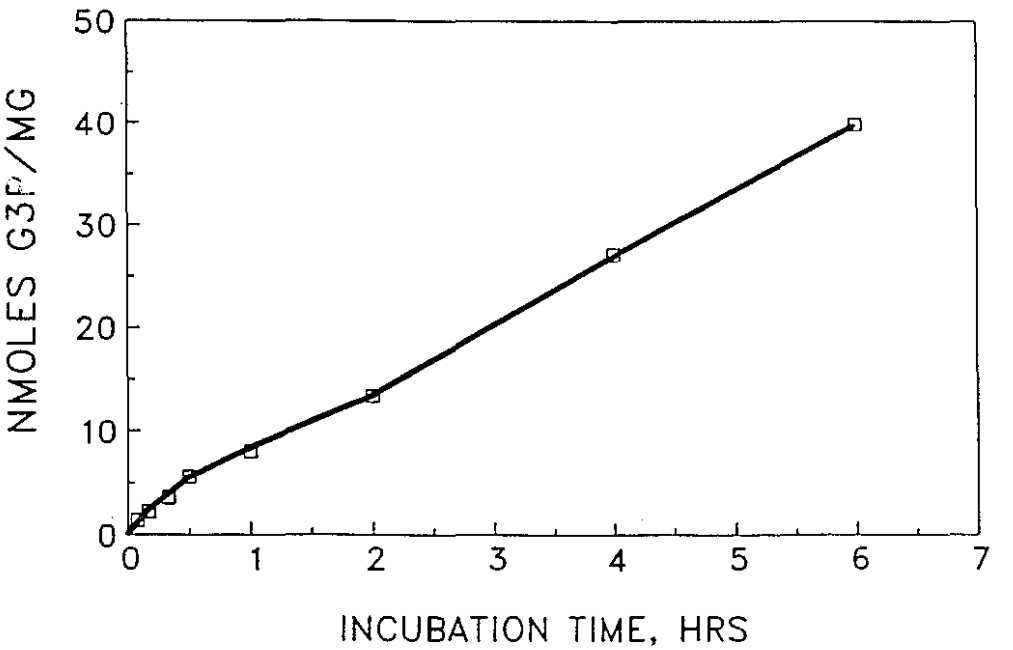

Figure 4.2 The effects of incubation time on glycerolipid biosynthesis from [U-^{14}C]G3P in pea root plastids. Plastids were incubated under the standard conditions of 160μM G3P and acetate, 2mM ATP and Mg^{2+}, 0.05mM CoA, 0.5mM NADH and NADPH, 15mM $KHCO_3$, 0.31M sorbitol, in 0.1M Bis tris propane (pH 7.5) and at 25°C. Total activity represents nmoles G3P incorporated per mg plastid protein. Data points represent the mean of two replicates.

The effects of incubation time on the proportions of each glycerolipid synthesized from ^{14}C-acetate and ^{14}C-G3P were also determined. The results of these investigations are summarized in table 4.3. Both precursors were incorporated into essentially the same glycerolipid products and intermediates, except that less radioactivity from ^{14}C-G3P was detected in PI. Similarly, at the shortest time periods, label from both precursors accumulated preferentially in PA (40-50%) while at the longest time periods, radioactivity accumulated in PG, PC, DAG. These observations are consistent with the known patterns and pathways of glycerolipid metabolism in chloroplasts of spinach, wheat and barley (Roughan and Slack, 1982) and other organelles, such as chromoplasts of daffodil (Liedvogel and Kleinig, 1979), amyloplasts of sycamore (Alban *et al.*, 1989). However, the results presented here are generally in agreement with those in an earlier study, except in one respect. Using the same precursors, Stahl (1990) showed that the decrease in PA was countered mainly by a increase in the accumulation of up to 27 and 40% TAG at 6 hours incubation with acetate and G3P, respectively.

4.2.3 The Effects of Plastid Protein Concentration

Glycerolipid biosynthesis was measured in the presence of different concentrations of plastids. The results (figure 4.3) show that under standard reaction conditions, total lipid synthesis increased linearly as plastid protein concentration increased to 0.125 mg/ml. Concentrations of plastid protein from 0.125 mg/ml to 0.225 mg/ml gave proportionately less for glycerolipid biosynthesis indicating that one or more cofactors became limiting. Therefore, the amount of plastid protein normally used was between 0.05 mg/ml to 0.125 mg/ml.

Table 4.3 The effects of incubation time on the distribution of radioactivity among glycerolipids synthesized from acetate and glycerol-3-phosphate by pea root plastids

Treatment	Lipid Distribution, % of the Total Radioactivity						
	Origin & Lyso-PA	PA	PI	PC	PG	MAG	DAG	TAG
^{14}C-acetate								
5 Min.	6	46	2	5	11	23	5	1
10	6	52	1	3	7	21	8	1
20	5	49	1	4	9	17	14	2
30	3	47	1	4	9	16	17	2
60	3	38	3	8	12	14	18	3
120	3	25	3	15	17	14	16	7
240	2	26	2	17	15	15	17	7
360	1	26	2	19	15	14	15	9
^{14}C-G3P								
5 Min.	23	41	0	4	13	0	13	5
10	16	42	0	5	16	1	14	6
20	10	39	0	7	23	1	15	6
30	5	41	0	6	22	2	16	7
60	2	33	0	7	26	3	18	10
120	2	31	0	6	26	5	19	11
240	1	23	0	7	28	8	17	15
360	1	17	0	5	23	16	15	17

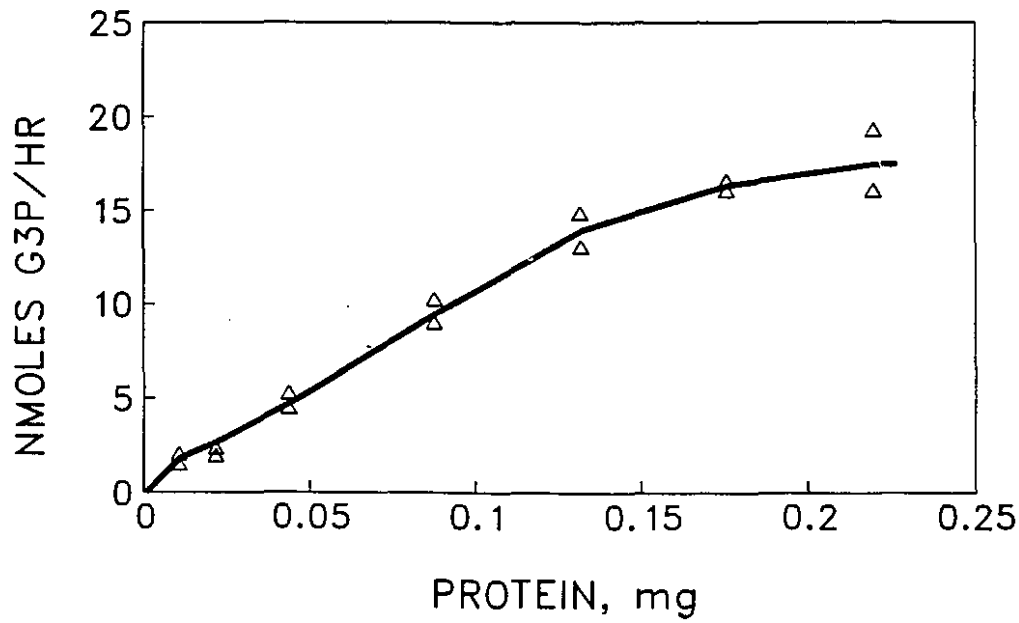

Figure 4.3 The effects of plastid protein concentration on glycerolipid biosynthesis from [U-^{14}C]G3P in pea root plastids. All other incubation conditions were as summarized in figure 4.2. The total activity represents nmoles G3P incorporated per hour.

The concentration of plastid protein had essentially no effect on the proportions of radiolabelled glycerolipid produced (data not shown).

4.2.4 Effects of Glycerol-3-Phosphate

Glycerol-3-phosphate is absolutely required for glycerolipid biosynthesis, since it is essential as a substrate for acylation. Glycerol-3-phosphate was supplied at concentrations of up to 5 mM to determine the saturation concentration for the *de novo* biosynthesis. As shown in figure 4.4, the specific activity increased sharply to 6.6 nmole/mg. However, 0.16 mM was routinely used in order to maintain a sufficiently high specific radioactivity and permit a more economical use of the tracer.

G3P concentration had relatively small effects on overall distribution of radioactivity among glycerolipids (table 4.4). The greatest effects were observed with PG and PA where the levels of these radioactive lipids decreased by roughly 10% and increased 2 fold, respectively, over the range of G3P tested. These observations suggest that the rate of PG synthesis from PA (equations 17-19, sections 2.2.3 and 2.2.4) was saturated and thus resulted in a greater accumulation of PA.

In an earlier study, Stahl and Sparace (1991) found that G3P was not a requirement for fatty acid biosynthesis in pea root plastids. However, activity was stimulated by approximately 42% by the addition of up to 0.5 mM G3P. Further, fatty acid distributions were shifted to favour the accumulation of palmitate within this range of G3P.

4.2.5 Effects of pH and Buffers

Stahl and Sparace (1991) tested five different physiological buffers with a similar pK_a and at equal

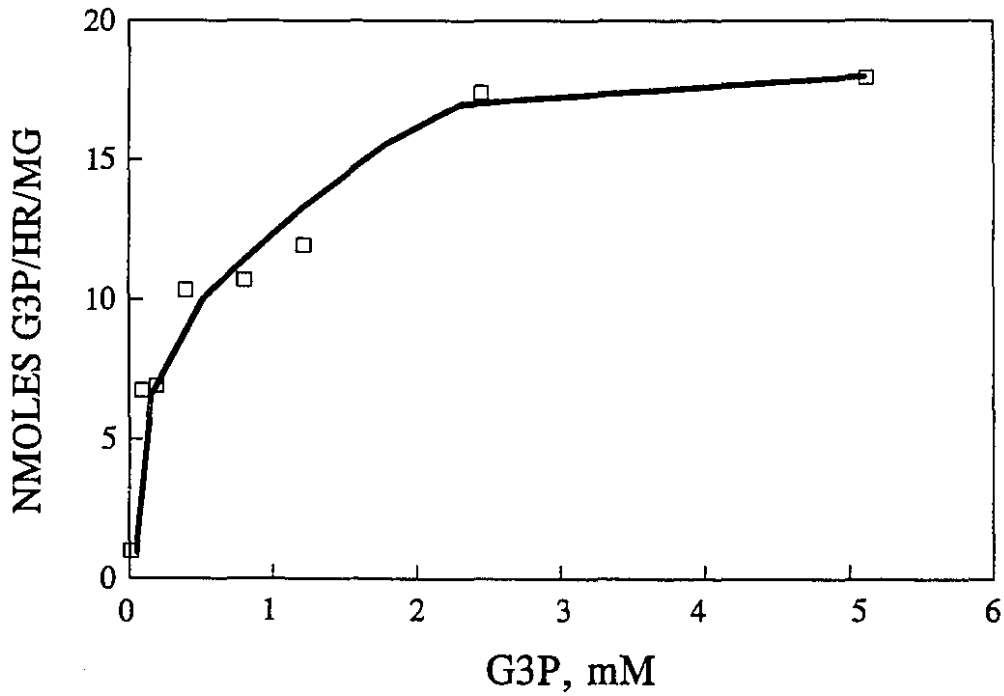

Figure 4.4 The effects of G3P concentration on glycerolipid biosynthesis from [U-^{14}C]G3P in pea root plastids. All other incubation conditions were as summarized in figure 4.2. The total activity represents nmoles G3P incorporated per hour per mg protein. Data points represent the mean of two replicates.

Table 4.4 The Effects of Glycerol-3-Phosphate Concentration on the Distribution of Radioactivity among Glycerolipids Synthesized by Pea Root Plastids

Treatment (mM G3P)	Origin & Lyso-PA	PA	PI	PC	PG	MAG	DAG	TAG
			% Distribution					
Control*	3	13	14	11	28	1	20	10
0.1	2	13	5	11	32	1	22	14
0.2	2	18	6	9	25	1	20	18
0.4	2	21	8	8	21	1	21	19
0.8	3	20	7	9	19	2	21	19
1.2	4	24	8	9	20	2	20	14
2.4	6	20	7	9	20	2	22	15
5.0	6	22	8	9	23	2	17	12

(Control = carrier free w/ concentration of 0.0148 mM)

concentrations over a pH range between pH 6 and pH 10. They found that Bis-tris-propane and Tricine generally gave the greatest activities for the fatty acid biosynthesis in pea root plastids. Based on their results, only the effects of Bis-tris-propane and Tricine on glycerolipid biosynthesis were examined. These buffers both gave virtually identical results (figure 4.5). The maximum activity for glycerolipid biosynthesis was obtained at pH 7.5. In contrast, for fatty acid biosynthesis, Stahl and Sparace (1991) showed that Bis-tris-propane gave approximately 40% greater activity than Tricine and their optimum pH was 8.

As before for total glycerolipid biosynthesis, Bis-Tris-Propane and Tricine buffers had virtually identical effects on the proportions of radiolabelled glycerolipids synthesized. The changes observed with varying pH's seemed to largely reflect rather complex changes in activities of individual enzymes of glycerolipid metabolism. It is tempting, but rather difficult, to speculate which enzyme may have the greatest overall influence on glycerolipid biosynthesis. Phosphatidic acid phosphatase (Eq.23, section 2.2.7) and the enzymes involved in PG biosynthesis (Eq. 18 and 19, section 2.2.4) seem to have the greatest influence. At relatively neutral pH's (7, 7.5), PG synthesis is greatest, and causes the greatest depletion of PA. At higher pH's (8-9), PG synthesis is apparently inhibited by approximately 60%, leading to an accumulation of PA and DAG, the latter via PA phosphatase.

4.2.6 The Effects of ATP Concentrations

As mentioned earlier, non-photosynthetic plastids, including pea root plastids, are generally dependent on exogenously supplied ATP to support fatty acid and glycerolipid biosynthesis. ATP is required for the synthesis

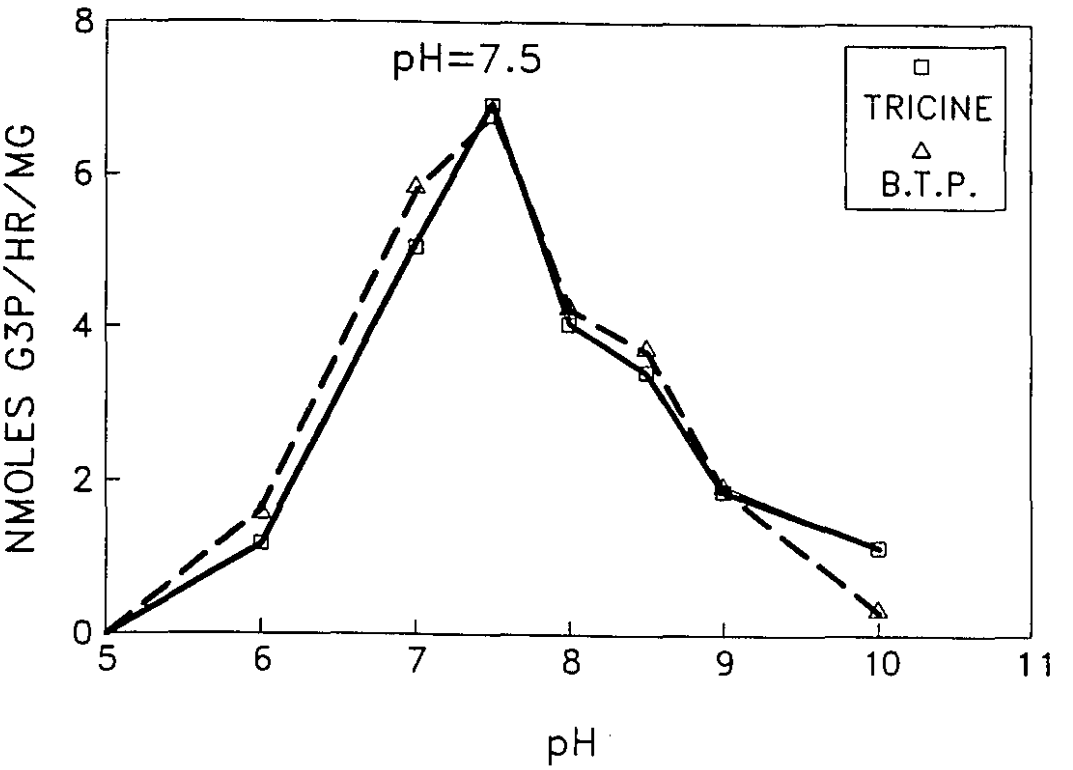

Figure 4.5 The effects of incubation pH and buffer on glycerolipid biosynthesis from [U-^{14}C]G3P in pea root plastids. All other incubation conditions were as summarized in figure 4.2. The total activity represents nmoles G3P incorporated per hour per mg protein. Data points represents the mean of two replicates.

Table 4.5 The effects of incubation pH on lipid distribution

	Lipid Distribution, % of the Total Radioactivity							
pH	Origin & Lyso-PA	PA	PI	PC	PG	MAG	DAG	TAG
6.0	9.5	37.5	9.0	10.0	6.5	7.5	8.5	12.0
7.0	5.0	17.5	4.5	13.5	22.0	3.5	16.5	18.0
7.5	4.0	17.5	3.0	12.5	23.0	2.5	19.5	18.0
8.0	4.5	24.5	5.0	14.0	10.0	3.0	24.0	15.0
8.5	2.0	24.0	4.5	17.0	8.5	2.5	25.0	18.0
9.0	4.5	22.0	3.5	19.0	7.5	2.0	27.0	14.5
10.0	11.0	20.5	4.5	13.0	18.0	2.5	20.5	10.0

No significant differences were observed between the effects of Bis Tris Propane vs. Tricine on glycerolipid distributions. Thus, data shown are the averages obtained for each buffer.

of both acetyl-CoA and malonyl-CoA by acetyl-CoA synthetase and acetyl-CoA carboxylase, respectively (Stumpf, 1984). In a preliminary experiment, the effects of ATP concentration ranging from 0 to 12 mM were tested in the presence of equimolar amounts of Mg^{2+}. This was because of the fact that ATP and Mg^{2+} form a complex in an 1 to 1 molar ratio at physiological pH (Goodwin and Mercer, 1983). The result of this preliminary experiment indicated that ATP was optimal at 2 mM (data not shown). This was confirmed in a subsequent experiment where Mg^{2+} was held constant at 2 mM ATP concentration was varied (figure 4.6). Studies of plastids from other tissues report that glycerolipid synthesis is ATP dependent, but with different optimum ATP concentrations such as 4 mM (Journet and Douce 1985) and 8 mM (Browse and Slack, 1985).

ATP also had effects on the distribution of glycerolipid synthesized. Table 4.6 shows that as ATP concentration increased from 0.25 to 10 mM, the proportion of PA decreased from 49 to 17%, while TAG increased from 17 to 30%. The proportion of PC and DAG also increased to different levels. These observations suggest that ATP may have a regulatory effect on phosphatidic acid phosphatase and glycerolipid biosynthesis. The results presented here are in agreement with those obtained with pea chloroplasts studied by Andrews and Mudd (1985). However a significant difference was that in pea chloroplasts PG was increased but in pea root plastids DAG and TAG were increased. This is reasonable since PG is a significant component of chloroplasts while TAG is significant in both pea roots and root plastids (see section 4.3.2). However, ATP had no effect on the proportions of fatty acids synthesized (Stahl, 1990), since ATP is utilized in the reactions which are the initiation steps committing acetate to the synthesis of fatty acids.

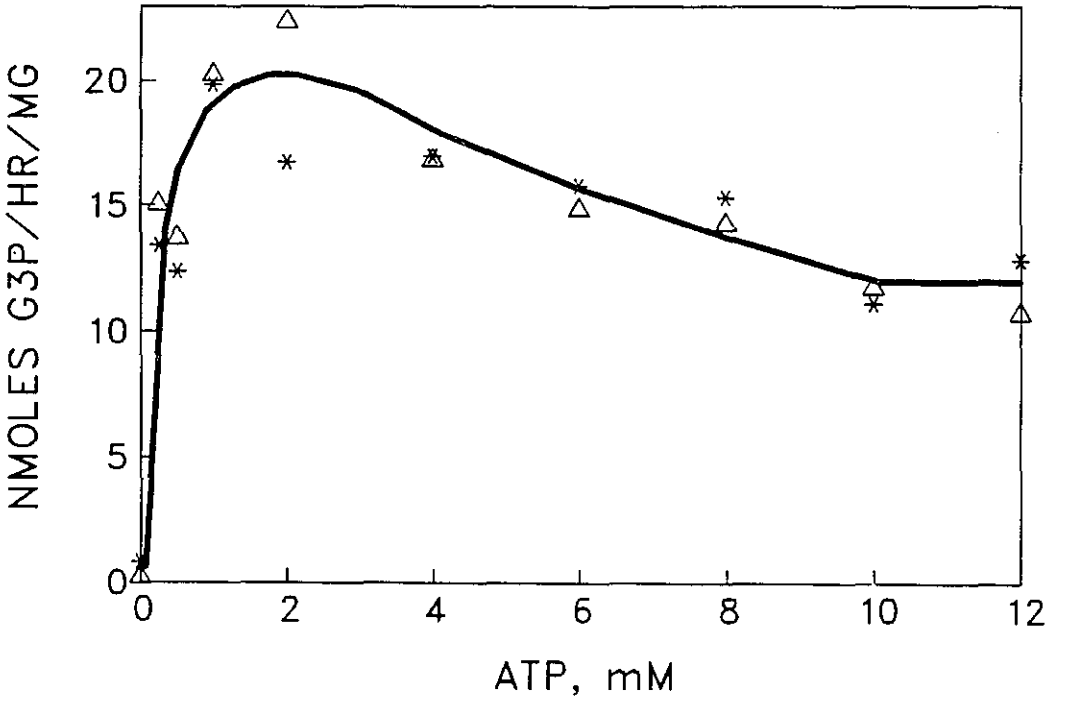

Figure 4.6 The effects of ATP concentration on glycerolipid biosynthesis from [U-^{14}C]G3P in pea root plastids. All ATP concentrations were with 2mM Mg^{2+} and all other incubation conditions were as summarized in figure 4.2. Specific activity represents nmoles G3P incorporated per hour per mg protein.

Table 4.6 The Effects of ATP Concentrations on Lipid Distribution

Treatment (mM ATP)	Lipid Distribution, % of the Total Radioactivity					
	Origin & Lyso-PA	PA	PC	PG	MAG	DAG	TAG
0 *	30	21	16	9	12	6	6
0.25	1	49	16	6	3	8	17
0.5	3	49	11	12	3	7	16
1.0	1	50	14	11	2	6	15
2.0	2	39	18	12	2	9	18
4.0	1	24	22	10	3	15	25
6.0	1	18	25	9	3	17	27
8.0	1	18	27	9	3	14	28
10.0	4	17	24	9	3	13	30
12.0 *	2	13	9	3	3	17	51

(* denotes samples that had very low radioactivity and therefore data presented may not be accurate)

4.2.7 The Effects of Mg^{2+} and Mn^{2+} Ions

As mentioned earlier (section 4.2.1), a divalent cation is essential for maximum rates of glycerolipid biosynthesis in pea root plastids. The requirements for Mg^{2+} and Mn^{2+} were determined in a series of experiments. As mentioned earlier, Mg^{2+} is the preferred cation, with equivalent concentrations of Mn^{2+} giving only 5 to 10% of the Mg^{2+} stimulated activity (data not shown). In the absence of Mn^{2+}, Mg^{2+} gave maximum rates of glycerolipid biosynthesis, when it was 0.5 to 5 mM (figure 4.7). In the presence of 2 mM Mg^{2+}, all concentrations of Mn^{2+} tested inhibited glycerolipid biosynthesis up to 25% with 5 mM Mn^{2+} (figure 4.8) . The results presented in figure 4.7 show that the optimum concentration of Mg^{2+} is the same as that of ATP, which is at 2 mM.

With the exception of PG, Mg^{2+} and Mn^{2+} had little effect on the overall pattern of radiolabelled glycerolipid accumulation (table 4.7). Mg^{2+} caused in increase in the amount of PG synthesized from about 20 to 30%, which is in agreement with Andrews and Mudd (1985) who showed that Mg^{2+} was essential for PG synthesis from PA in pea chloroplasts. In contrast to Sparace and Mudd (1982) who showed that Mn^{2+} caused a four-fold stimulation of PG synthesis in spinach chloroplasts. Mn^{2+} had essentially no effect on the distribution of radiolabelled lipids.

4.2.8 The Effects of Coenzyme A

As mentioned earlier, CoA was an absolute requirement for glycerolipid synthesis. The maximal stimulation of glycerolipid synthesis occurred between 0.01 and 0.05 mM with the specific activity increasing dramatically from only 4.8% of the maximum at 0 mM CoA to nearly 80% of the maximum at

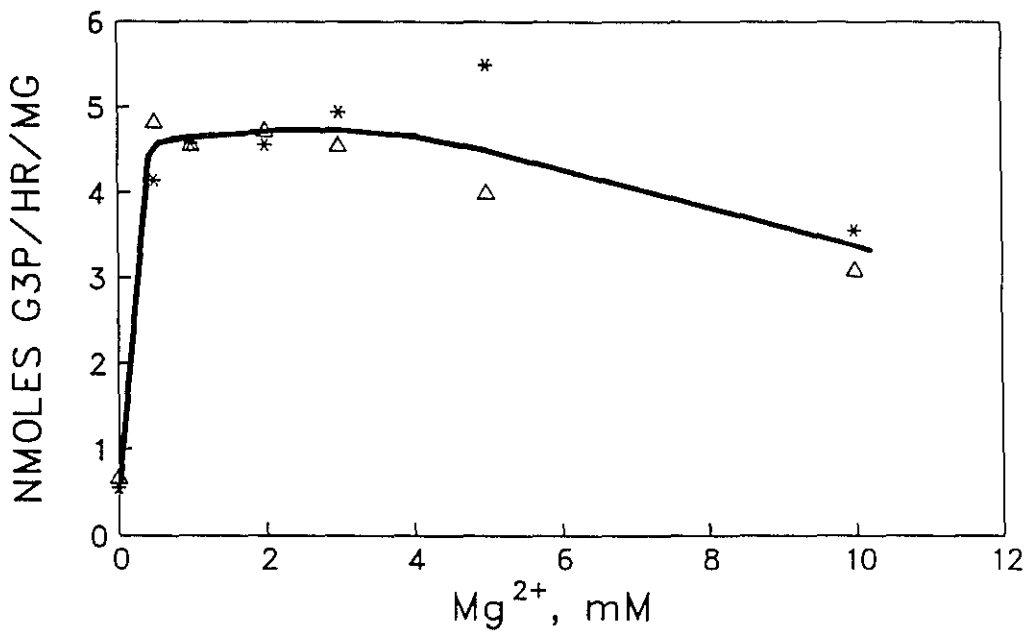

Figure 4.7 The effects of Mg^{2+} ion concentration on glycerolipid biosynthesis by [U-^{14}C]G3P in pea root plastids. All incubation conditions were as summarized in figure 4.2. Specific activity represents nmoles G3P incorporated per hour per mg protein.

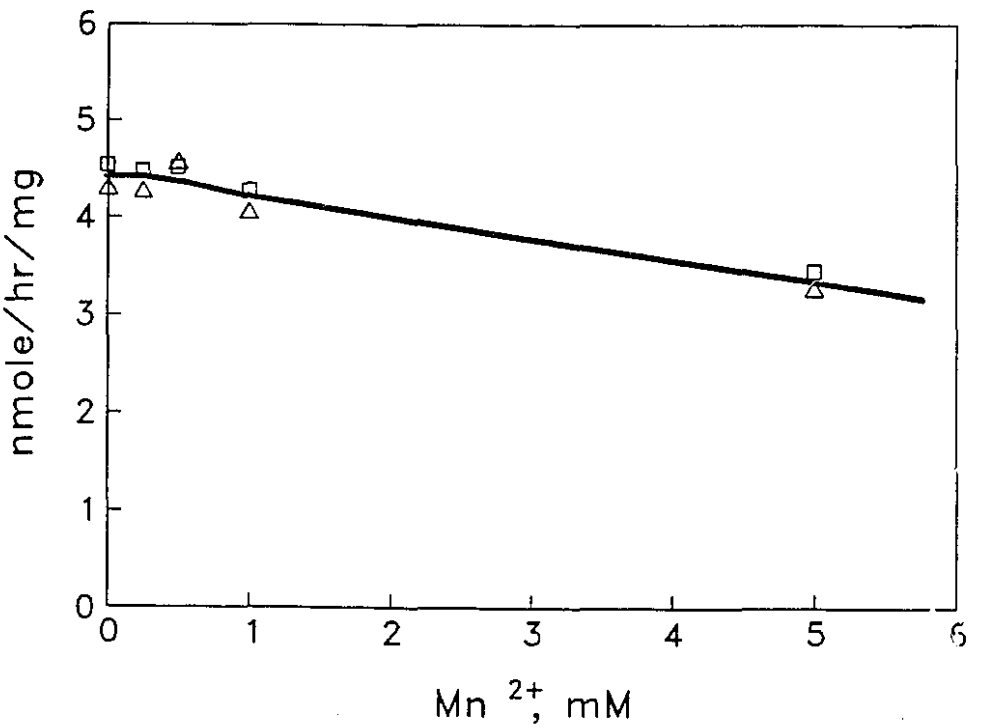

Figure 4.8 The effects of Mn^{2+} ion concentration on glycerolipid biosynthesis by [U-^{14}C]G3P in pea root plastids. All incubation conditions were as summarized in figure 4.2. Specific activity represents nmoles G3P incorporated per hour per mg protein.

Table 4.7 The Effects of Mg^{2+} and Mn^{2+} Concentrations on Lipid Distribution

Treatment	Lipid Distribution, % of the Total Radioactivity					
	Origin & Lyso-PA	PA	PC	PG	MAG	DAG	TAG
Mg^{2+} (mM)							
0 *	20	27	11	9	6	18	9
0.5	9	32	4	23	4	21	8
1.0	5	28	6	29	3	22	6
2.0	4	30	6	31	3	20	6
3.0	7	28	6	28	2	22	7
5.0	6	26	8	31	2	21	5
10.0	13	36	8	18	3	18	4
Mn^{2+} (mM) **							
0	1	19	8	32	2	22	6
0.25	/	21	9	32	2	23	6
0.5	7	22	8	32	1	21	7
1.0	5	18	10	41	1	18	7
5.0	5	30	6	33	1	18	7

(* denotes samples that had very low radioactivity and therefore data presented may not be accurate; **: in the presence of 2 mM Mg^{2+})

the lowest concentration (0.005 mM) tested (figure 4.9). CoA concentrations greater than 0.1 mM became inhibitory. These results are in agreement with Stahl's for optimum rates of fatty acid biosynthesis (1990). The rapid response to CoA and saturation of its effects are somewhat expected. CoA serves as the recyclable activator and carrier of the substrates for fatty acid biosynthesis (acetate and malonate), and therefore very low concentrations of it would be predicted to be sufficient. CoA concentrations had essentially no effect on lipid distribution (appendix 3).

4.2.9 The Effects of NADH and NADPH

The reduced nucleotides NADH and NADPH are required in the β-ketoacyl-ACP reductase and 2-enoyl-ACP reductase steps of *de novo* fatty acid biosynthesis, respectively, as well as the desaturation of stearoyl-ACP (Stumpf, 1984). These cofactors are only indirectly required for plastidic glycerolipid biosynthesis, since the latter process is dependent on active fatty acid biosynthesis. As shown in figure 4.10, the addition NADH and NADPH individually resulted in a 20-35% stimulation in the rates of glycerolipid biosynthetic activity. At all concentrations tested NADH was preferred slightly over NADPH. However, the greatest stimulation of glycerolipid biosynthesis was observed when both nucleotides were used simultaneously at equal molar concentrations. This resulted in a 2-fold or greater increase in lipid biosynthesis that was slightly reduced at concentrations above 0.5 mM. These results are in agreement with Stahl (1990) and Stumpf *et al.*, (1982). Generally the concentrations of reduced nucleotides had no significant effects on the distribution of the lipids synthesized (appendix 4).

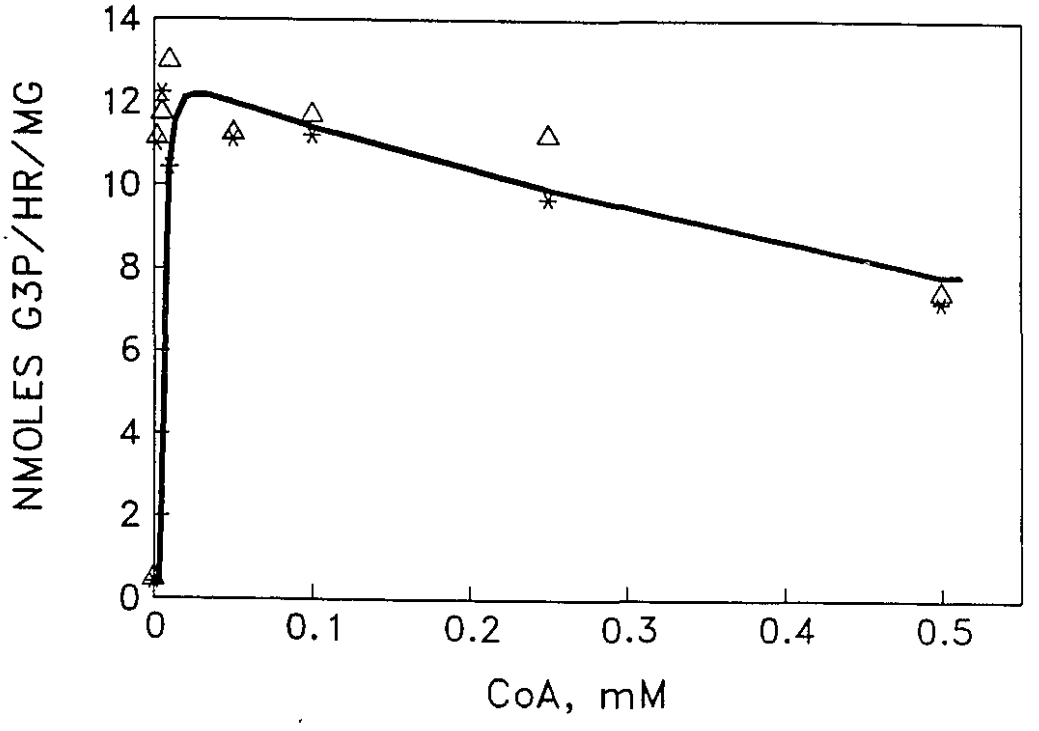

Figure 4.9 The effects of CoA concentration on glycerolipid biosynthesis from [U-^{14}C]G3P in pea root plastids. All other incubation conditions were as summarized in figure 4.2. Specific activity represents nmoles G3P incorporated per hour per mg protein.

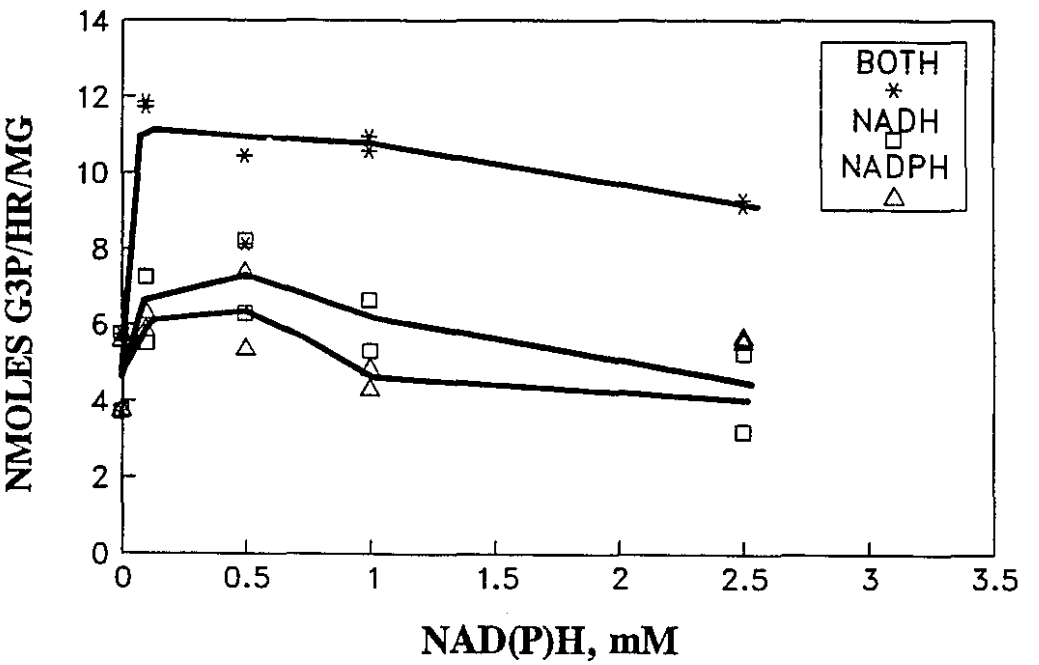

Figure 4.10 The effects of NADH and NADPH concentrations on glycerolipid biosynthesis from [U-^{14}C]G3P in pea root plastids. All other incubation conditions were as summarized in figure 4.2. Specific activity represents nmoles G3P incorporated per hour per mg protein.

4.2.10 The Effects of Bicarbonate

Potassium bicarbonate up to 25 mM caused approximately 15-20% stimulation of total glycerolipid synthesis from G3P (figure 4.11). This relatively small stimulation is somewhat expected since bicarbonate functions as a substrate of fatty acid synthesis from acetate, and that acetate is not essential for glycerolipid biosynthesis. In contrast, however, for the fatty acid biosynthesis, a 3.6 fold increase in specific activity was observed at the saturating level of 15 mM compared to that when no bicarbonate was supplied (Stahl, 1990). These results suggest that the endogenous levels of fatty acid intermediates, and more basal rates of synthesis are largely sufficient for *in vitro* rates of glycerolipids biosynthesis in pea root plastids.

There were no effects of HCO_3^- concentrations on lipid distribution (appendix 5).

4.2.11 The Effects of Other Additions to the Standard Reaction Mixture

The effects of additional possible cofactors or metabolites on glycerolipid synthesis were tested. The concentration of each cofactor tested are shown in table 4.8. Of all the substances tested, βME and DTT had the greatest effects. At 1 mM, these thiols were essentially completely inhibitory to glycerolipid biosynthesis by pea root plastids. This was somewhat surprising since DTT and other reducing reagents are frequently added to *in vitro* incubation mixtures for maximum rates of fatty acid and lipid biosynthesis in other systems. For example, 1 mM DTT stimulates fatty acid biosynthesis in cauliflower bud plastids (Journet and Douce, 1985) and pea chloroplasts (Andrews and Mudd, 1985). Fatty acid synthesis in spinach chloroplasts, however, was inhibited by the addition of DTT (Sauer and Heize, 1983).

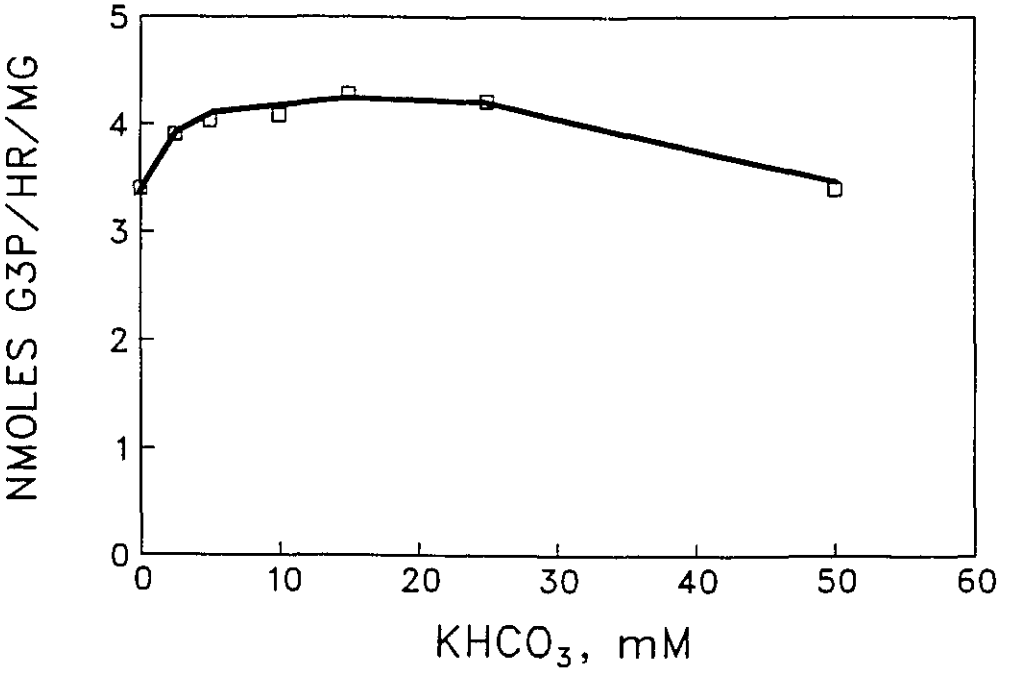

Figure 4.11 The effects of $KHCO_3$ concentrations on glycerolipid biosynthesis from [U-^{14}C]G3P in pea root plastids. All other incubation conditions were as summarized in figure 4.2. Specific activity represents nmoles G3P incorporated per hour per mg protein. Data points represent the mean of two replicates.

Table 4.8 The effects of other cofactor addition on lipid synthesis and product distribution

		Distribution, % of the Total Radioactivity						
Treatment	Relative Activity % of Ctrl.	Origin & Lyso-PA	PA	PC	PG	DAG	TAG	MGDG
Control*	100	2	25	19	20	20	14	-
ADP(2mM)	108	2	26	19	26	14	13	-
DHAP(0.25mM)	112	2	25	14	28	13	12	-
UDP-Galactose (0.5mM)	120	3	21	17	26	9	11	15
Lyso-PC(0.01mM)	48	4	24	20	18	24	11	-
βME(1mM)*	4	1	15	28	39	10	7	-
DTT(1mM)*	2	3	36	13	26	14	8	-
Triton(0.1mM)	121	4	34	11	12	26	10	-
CHAPS(0.1mM)	103	2	33	14	24	19	8	-
KH_3PO_4(3mM)	93	2	28	13	22	18	16	-
KCl(1mM)	104	1	31	18	23	14	13	-
KNO_3(1mM)	106	1	26	16	29	13	15	-

(Control:Rate of complete control activities was 8.6 nanomoles G3P/hr/mg; *: very low activities)

These workers suggested that this inhibition was due two possible reasons: first, the formation of acetyl-DTT reduced the available acetate for fatty acid biosynthesis, and second that DTT promoted dark Calvin cycle CO2 fixation which reduced the availability of ATP. The former possibility may be operating with pea root plastids, but the latter cannot since these plastids are non-photosynthetic.

Lyso-PC (0.01 mM) had the next greatest effect on glycerolipid biosynthesis, inhibiting activity by greater than 50%. This is likely due to disruption of the plastid, with intact plastids required for lipid biosynthesis. However 0.1 mM Triton X-100 improved total lipid synthesis by about 20% (presumablely by increasing the permeability of the plastid envelope for metabolite uptake) while the same concentration of CHAPS had essentially no effect on total lipid synthesis. Both of these detergents resulted in an 8-9% increase in the amount of PA accumulated. While CHAPS slightly stimulated PG synthesis. With spinach chloroplast, however, Triton gave a 2.4 fold stimulation of acetate incorporation into glycerolipids (Roughan *et al.*, 1976) while it completely inhibited PG synthesis (Mudd and DeZacks, 1981).

UDP-galactose (0.5 mM) caused a 20% increase in total lipid biosynthesis which for the first time in this study was accompanied by an accumulation of 15% MGDG. This latter observation was expected since UDP-galactose is an essential cofactor for galactolipid biosynthesis that originates in the cytosol (Joyard and Douce, 1987).

DHAP (0.25 mM) caused a 12% increase in total glycerolipid synthesis which was accompanied by an 8% increase in the amount of PG. This was likely due to the ease of DHAP uptake and its conversion to G3P for PG synthesis. Finally, the effects of other substances on total

glycerolipid biosynthesis and product distribution were relatively small.

4.3 Analyses of Fatty Acid And Glycerolipid Compositions

4.3.1 Analyses of Newly Synthesized Fatty Acids in Glycerolipids Synthesized from Acetate by Pea Root Plastids

Acetate-labelled glycerolipids were purified and analyzed for their total radioactive fatty acid composition (table 4.9). In general, these lipids contained only labelled palmitate, stearate, and oleate with less than 0.1 to 8.7% of short chain fatty acids tentatively identified as myristate and laurate. These fatty acids were not uniformly distributed among the various glycerolipids. Neutral lipids (TAG and DAG) contained roughly equal amounts (43-49%) of palmitate and stearate. PA, PG and PI all contain primarily (76-85%) palmitate, suggesting that these lipids are most likely of the "prokaryotic" configuration described by Browse and Somerville (1991). However, PC, containing primarily (63%) C_{18} Fatty acids is most likely of the "eukaryotic" configuration.

When acetate-labelled phospholipids and neutral lipids were digested with the appropriate lipases, with the exception of PA which contained essentially equal amounts of total ^{14}C-fatty acids in positions *sn*-1 and *sn*-2 of the lipid, an asymmetric distribution of radioactive fatty acids was observed (table 4.10). The remaining phospholipids (PI, PC, PG) contained roughly 2 to 4 times as much radioactivity in position *sn*-2 as compared to *sn*-1. However, this pattern was reversed and even more extreme for the neutral lipids. In view of the known pathways of glycerolipid biosynthesis, the asymmetric distributions of fatty acids in these lipids are somewhat difficult to explain. There may be two pools of PA involved, one for phospholipid biosynthesis and one for

Table 4.9 Radioactive fatty acid compositions of acetate-labelled glycerolipids synthesized by pea root plastids

Lipids	12:0	14:0	16:0	18:0	18:1
	% of radioactivity				
PA	3.2	0.1	72.3	3.7	20.1
PI	-	-	84.8	-	15.2
PC	8.7	7.7	27.5	9.2	55.1
PG	0.7	-	76.4	0.8	31.1
DAG	0.1	2.2	48.5	0.1	47.9
TAG	3.6	1.6	43.6	2.1	49.0

(- Denotes not detected; 12:0, 14:0, 16:0, 18:0 and 18:1 correspond to lauric , myristic, palmitic, stearic and oleic acids, respectively.)

Table 4.10 Distribution of radioactivity among products of phospholipase A_2 and Rhizopus lipase digestions of ^{14}C-acetate labeled glycerolipids

Lipids		% of 14C-Lipids	12:0	14:0	16:0	18:0	18:1
			% Radioactivity of each positions				
PA	*sn2*	46.0±1.8	-	-	80.0	-	20.0
	sn1	54.0±2.8	2.5	-	55.9	8.7	32.9
PI	*sn2*	80.1±10.9	4.2	-	9.7	-	86.1
	sn1	19.9±3.8	-	-	55.9	-	44.1
PC	*sn2*	78.2±5.2	-	-	19.1	-	80.9
	sn1	21.8±2.9	*	*	*	*	*
PG	*sn2*	70.7±1.8	-	-	88.1	-	11.9
	sn1	29.3±0.9	-	-	44.8	-	55.2
DAG	*sn2*	13.7±3.8	-	-	73.3	-	26.7
	sn1	86.3±6.4	2.6	1.4	56.8	2.4	36.8
TAG	*sn2*	10.8±1.5	3.3	-	32.7	-	64.0
	sn1,3	89.2±1.0	-	1.1	41.8	1.8	54.2

(- denotes not detected, * = data not available; 12:0, 14:0, 16:0, 18:0, 18:1 correspond to lauric, myristic, palmitic, stearic and oleic acids, respectively.)

neutral lipid biosynthesis. The former might be derived from pre-existing pools of cold LysoPA. Alternatively, considerable deacylation and reacylation of pre-existing lipids may also be occurring during the process of *de novo* fatty acid biosynthesis. The compositions of radioactive fatty acids in each of the *sn*-1 and *sn*-2 positions generally confirmed that PA and PG are of the prokaryotic configuration, while PC is largely eukaryotic and the neutral lipids are likely a mixture of both prokaryotic and eukaryotic configuration. However, in disagreement with the total fatty acid data, the positional analyses of PI suggest that this lipid may be of the eukaryotic configuration.

4.3.2 Analyses of Cold Endogenous Fatty acids and Glycerolipids of Pea Root Plastids

All previous studies in this work have emphasized the *in vitro* capacity of pea root plastids to synthesize glycerolipids from labelled acetate and G3P. However, in order to fully understand the lipid biosynthetic capacities of those plastids, it is essential to know the native or endogenous glycerolipid and fatty acid compositions of these plastids. The glycerolipid composition of pea root plastids was determined as summarized in table 4.11. On a mole percent basis, the membranes of pea root plastids consist of approximately 62% phospholipids, 14% glycolipids and 24% neutral lipids (including free fatty acids and MAG). This is in marked contrast to chloroplasts which contain only minor amounts of phospholipids and up to 80-90% galactolipid (Gounaris, 1986). However, lipid distributions within these classes are generally in agreement with chloroplasts. Among the phospholipids, PG predominates in both pea root plastids and chloroplasts (Stahl and Sparace, 1990; Andrews and Mudd, 1985), while the galactolipids also predominate among the

Table 4.11 Mole % composition of the total acyl lipids in intact pea root plastids before incubating with the precursors

Lipids	% of Total
PA	11.2 ± 0.1
PI	7.3 ± 0.4
PC	19.8 ± 1.1
PG	24.2 ± 6.5
MGDG	5.9 ± 0.9
DGDG	6.7 ± 1.0
SQDG	1.2 ± 0.1
FFA	4.7 ± 1.1
MAG	3.9 ± 0.2
DAG	2.9 ± 1.4
TAG	11.9 ± 0.1

glycolipids in these plastids. These difference reflect the fact that the root plastids are essentially plastid envelopes devoid of any thylakoids that normally contain the galactolipids of chloroplasts. However, it is interesting to note that the root plastids also contain unusually high amounts of TAG, the significance of which is not presently known.

Fatty acid analysis of cold acyl lipids from root plastids revealed that these lipids contained relatively typical fatty acids (table 4.12). However, the glycolipids and neutral lipids contained small amounts of eicosenoic acid (20:1, 15 and 18% in FFA and MAG, respectively). Based on their fatty acid compositions, lipids of pea root plastids (except for SQDG) fell into 3 groups. First, were the phospholipids which contained primarily palmitate and linoleate. Within this group, however, PG stood out with the greatest amounts of linolenate (34%). Second, in addition to these fatty acids, the galactolipids contained approximately 60% linolenate which is consistent with the fact that pea is an 18:3 plant (Frentzen *et al.*, 1983; Heinz and Roughan, 1982). Lastly, the neutral lipids (DAG and TAG) contained greater than 30% oleate. SQDG was somewhat unique in that it contained over 50% palmitate with the remaining fatty acids each representing 3 to 15% of the total.

5 Conclusions

Pea root plastids are an excellent system for the study of lipid metabolism in a nonphotosynthetic plastid. The plastids described here are free from contamination by other organelles (endoplasmic reticulum and mitochondria) which could potentially influence the results obtained in this study. Pea root plastids readily incorporate G3P or acetate (the latter via *de novo* fatty acid biosynthesis and acylation

Table 4.12 Mass % fatty acid composition of each lipid in pea root plastids before incubating with the precursors

Lipids	16:0	18:0	18:1	18:2	18:3	20:1
PA	46.8	5.5	5.3	38.2	4.2	-
PI	25.2	4.8	7.5	57.9	4.6	-
PC	13.6	3.5	5.8	70.8	6.3	-
PG	24.1	4.0	5.5	32.1	34.2	-
MGDG	4.8	3.8	2.7	22.5	65.1	1.2
DGDG	12.9	5.4	5.2	16.1	58.7	1.7
SQDG	53.9	15.9	6.5	14.1	3.3	6.3
FFA	33.0	17.2	18.0	12.9	3.3	15.6
MAG	25.4	18.9	15.2	17.3	5.1	18.1
DAG	29.6	9.3	33.8	18.2	4.3	4.8
TAG	7.1	3.0	31.8	45.8	11.7	0.6

(- denotes not detected)

of G3P) into the intermediates and products of glycerolipid metabolism. These plastids are completely dependent on an exogenous supply of ATP, Coenzyme A, and Mg^{2+} while reduced nucleotides, acetate, bicarbonate and detergents each give up to a two fold stimulation of total glycerolipid synthesis. Under the optimum incubation conditions, rates of glycerolipid synthesis are generally 5 to 10 nanomoles G3P incorporated/hr/mg plastid protein. Reduced sulfhydryl reagents and lyso-PC are potent inhibitors of glycerolipid biosynthesis, while Mn^{2+} inhibits Mg^{2+}-stimulated activity. Radioactivity from G3P and acetate is recovered in essentially in all constituent glycerolipids including TAG, but not the glycolipids. The proportions of these lipids synthesized was greatly dependent on incubation pH, ATP and Mg^{2+} concentrations. Phosphatidic acid phosphatase and the enzymes associated with PG synthesis from PA were apparently most affected. Labelled MGDG accumulated only in the presence of UDP-galactose, while no labelled DGDG or SQDG were ever detected. Lipids labelled with acetate contained radioactivity in both the *sn*1 and 2 positions of G3P (primarily palmitate and oleate) and were of both the eukaryotic and prokaryotic configuration.

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Appendices

Appendix 1 Compositions of buffers used for pea root plastid isolations

Buffers		Concentration (mM)
Buffer A	Tricine, pH=7.5	50
	Sorbitol	330
	EDTA(Na_2)	1
	$MgCl_2$	1
	BSA	0.1% (w/v)
Buffer B	B.T.P.	1
	Sorbitol	330
Centrifugation buffer	Tricine	50
	Sorbitol	330
	BSA	0.1% (w/v)
	Percoll	10.0% (v/v)
Incubation buffer	B.T.P.	250
	Sorbitol	625

Appendix 2 Compositions and Volumes of Stock Reagents Used to Make the Standard Incubation Mixture for Glycerolipid Biosynthesis

Solutions	Stock solution-concentraton ((00(mM)	Volume (μl)	Final concentraton (mM)
ATP	20	50	2
$MgCl_2$	20	50	2
CoA	2	12.5	0.05
NADH*	20	12.5	0.5
NADPH*	20	12.5	0.5
$KHCO_3$	330	25	15
Acetate	4	20	0.16
G3P	4	20	0.16
^{14}C-G3P		25	
H_2O		3	
Total reaction mixture		260	
Incubation buffer		200	
Purified plastids		40	
Total volume of each reaction tube		500	

(* in buffer B)
(all solutions were maintained at -20°C prior to use)

Appendix 3 The effects of coenzyme A concentrations on lipid distribution

Treatment (mM CoA)	Lipid Distribution, % of the Total Radioactivity						
	Origin & Lyso-PA	PA	PC	PG	MAG	DAG	TAG
0 *	19	21	5	18	10	17	10
0.002	4	41	3	8	3	20	21
0.005	4	40	4	9	3	22	19
0.01	4	39	5	9	2	25	17
0.05	4	39	4	10	2	22	19
0.1	3	36	4	12	2	22	21
0.25	3	36	12	13	2	16	17
0.5	6	45	4	16	2	13	15

(* = The total radioactivities are very low)

Appendix 4 The Effects of NADH and NADPH Concentrations on Lipid Distribution

Treatment	Lipid Distribution, % of the Total Radioactivity						
	Origin & Lyso-PA	PA	PC	PG	MAG	DAG	TAG
NADPH(mM)							
0	8	36	6	15	4	19	13
0.1	16	32	4	11	4	19	13
0.5	9	34	3	15	3	21	15
1.0	7	34	1	8	4	26	20
2.5	8	38	3	14	3	19	14
NADH(mM)							
0	5	35	12	14	2	17	15
0.1	2	29	10	20	2	15	20
0.5	4	30	10	12	3	23	18
1.0	4	31	11	19	2	17	15
2.5	6	30	10	19	2	19	15
NADPH&NADH(mM)							
0	6	34	12	15	1	18	16
0.1	7	44	4	9	3	23	10
0.5	5	26	8	20	3	24	14
1.0	9	28	7	16	21	15	3
2.5	6	34	4	9	4	25	18

Appendix 5 The Effects of HCO_3^- Concentrations on Lipid Distribution

Treatment (mM HCO_3^-)	Lipid Distribution, % of the Total Radioactivity					
	Origin & Lyso-PA	PA+PC	PG	MAG	DAG	TAG
0 *	2	64	4	6	20	4
2.5	2	54	7	2	21	14
5.0	2	51	7	9	22	9
10.0	2	58	6	3	21	10
25.0	2	51	11	2	22	13
50.0	2	46	11	2	27	13

(* = The total radioactivities are very low. Because of the TLC solvent system PA and PC were not separated.)