THE SYNTHESIS OF TRIGLYCERIDE AND OTHER NON-POLAR ACYL LIPIDS BY PROTOPLASTS AND CHLOROPLASTS FROM BARLEY LEAF.

A thesis presented in partial fulfilment of the requirements for the degree of Master of Science with Honours in Biochemistry at Massey University.

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ABSTRACT

Protoplasts, isolated from barley leaf, were incubated with $H^{14}CO_3$ and (1-^{14}C) acetate. The radioactivity was found incorporated into the lipids sulpholipid, phosphatidyl choline, phosphatidyl ethanolamine, phosphatidyl glycerol, and monogalactosyl diglyceride as identified by thin layer chromatography. Large quantities were found in an unidentified region referred to in this thesis as U_{SF} (Unknowns near the solvent front). This U_{SF} was much less polar than the phospholipids and galactolipids and ran in the same region as plant pigments. U_{SF}, by using TLC with several solvent systems, was identified as consisting of acyl lipids; the major proportion was triglyceride (approximately 55%) with diglyceride, free fatty acid and monoglyceride also present. The radioactivity in the triglyceride was found, by GLC of methylated esters, to be contained in the fatty acids; mainly in linoleate (18:2) and palmitate (16:0) and in lesser concentrations in stearate (18:0) and oleate (18:1).

Endogenous levels of triglyceride were found to be very low - only 14.8 nmoles /g of fresh tissue.

The results of experiments with sections of tissue of different maturity suggested that the high level of incorporation into acyl lipids was related to tissue immaturity.

Chloroplasts were isolated by a traditional blending method and by bursting prepared protoplasts. The latter technique produced chloroplasts of a higher quality. Examination of the incubation

products when chloroplasts were incubated with (1-^{14}C) acetate showed once more the appearance of acyl lipids. Triglyceride made up 35% of the U_{SF} from chloroplasts incubations. The label accumulated mainly in palmitate (16:0) on the triglyceride.

These results suggest that at least part of the triglyceride synthesis in the plant cell is occurring within the chloroplast, contrary to the findings represented in the literature.

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ACP	acyl carrier protein
ADP	adenosine 5-diphosphate
ATP	adenosine 5-triphosphate
BSA	bovine serum albumin
CDP	cytosine 5-diphospate
CTP	cytosine 5-triphosphate
Chl	chlorophyll
CoA	coenzyme A
DHAP	dihydroxy acetone phosphate
DG	diglyceride
DGAT	diacyl glycerol acyl transferase
DGDG	digalactosyl diglyceride
DTT	dithiothreitol
EDTA	ethylenediamine tetraacetic acid
f.a.	fatty acid
f.a.s.	fatty acid synthetase
f.f.a	free fatty acid
GLC	gas-liquid chromatography
G3P	glycerol-3-phosphate
HEPES	N-2-hydroxyethylpiperazine-N -2-ethane sulphonic acid
MES	2[N-morpholino]ethane sulphonic acid
Mg	magnesium ion
MG	monoglyceride
MGDG	monogalactosyl diglyceride
MW	molecular weight

NADH	nicotinamide adenine dinucleotide, reduced form
NADPH	nicotinamide adenine dinucleotide phosphate, reduced form
PA	phosphatidic acid
PC	phosphatidyl choline
PE	phosphatidyl ethanolamine
PEP	phosphoenol pyrurate
PGA	phosphoglycerate
Pi	phosphate
PL	phospholipid
POPOP	1,4 bis [2-(5-phenyloxazolyl)]-benzene
PPO	2,5-diphenyl oxazole
PPi	pyrophosphate
sn	sterospecific number
SL	sphingolipids
TG	triglyceride
TLC	thin layer chromatography
Tricine	N-Tris (hydroxymethyl) methylglycine
UDP-gal	uridine 5-diphosphate D-galactose

Lipid nomenclature

Fatty acids are designated by the short hand notation of the number of carbon atom: number of double bonds eg: 16:0 stands for hexadecanoic acid (palmitic acid). The trivial names of the fatty acids and the complex lipids have often been used for convenience.

CHAPTER 1. INTRODUCTION

Plants possess a variety of lipids:- lipids that compose the outer epidermal layers between the plant and the environment (eg; waxes and wax esters), seed storage lipids (eg; triglycerides that often contain unusual fatty acids), lipids in the plant cell membrane and the membranes of the organelles (eg; phospholipids, the glycolipids in the chloroplast). The lipid composition of each plant organelle varies probably reflecting the differences in the function of each membrane.

1.1 Chloroplast Lipids

The chloroplast envelope is comprised of two outer membranes which have high concentrations of phospholipid, particularly phosphatidyl choline. The envelope surrounds a group of membranes known as the thylakoids, the site of the photosynthetic process. These membranes have a high protein: lipid ratio. The same lipids are present in both the envelope and the thylakoids (see table 1.1), with the exception of chlorophyll that is absent in the envelope, but the proportions differ in the two membrane systems.

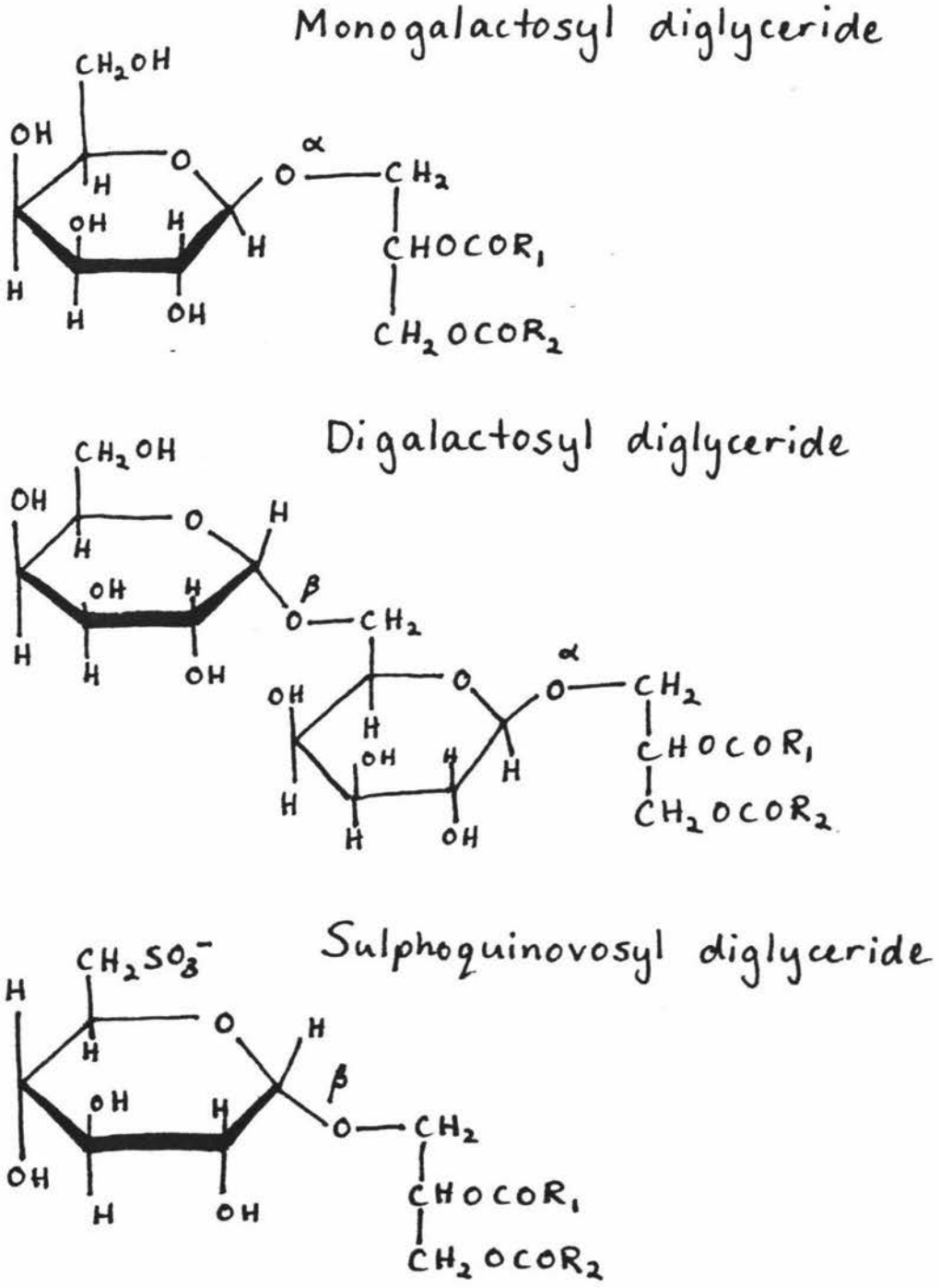
Lipids distinct to the chloroplasts are the glycolipids:- monogalactosyl diglyceride and digalactosyl diglyceride making up the largest proportion of the lipids present, as well as approximately 5% sulphoquinovosyl diglyceride. All of these molecules have a polar region, the sugar moiety and a non-polar region, the fatty acid chain, (see figure 1.1). There are some phospholipids present but a notable absence of phosphatidyl ethanolamine. It is the glycolipids that form

Table 1.1 The Lipid Composition of the Chloroplast Envelope and Thylakoids from Vicia faba.

Chloroplast Fraction	Lipid Composition (% of total lipids)			
	MGDG	DGDG	PC	PG
Envelope	29	32	29	9
Thylakoids	65	26	3	6

(Leech & Murphy, 1976)

Figure 1.1 The Structure of the Chloroplast Glycolipids



the basic layer of the thylakoid membrane. Most of the fatty acids present are 16 or 18 carbon atoms in length and are highly unsaturated, more so in the thylakoid membrane than in the envelope. In most plant species the ∝ linolenic acid content is more than 40% and can, in some plants, account for over 90% of the fatty acids. This probably explains the high fluidity seen in the thylakoid membrane when compared to other biological systems. Also present is a fatty acid unique to photosynthetic tissues, trans-3-hexadecanac acid (16:1), not present in the envelope (MacKender and Leech, 1974) and only found acylated to phosphatidyl glycerol (Bartels et al., 1976).

The concentration of the glycolipids and phosphatidyl glycerol (the typical chloroplast lipids) can be regarded as indicators of the number of chloroplasts within the tissue. A marked rise in monogalactosyl diglyceride, linolenic acid (the chief fatty acid it contains), digalactosyl diglyceride, and to an extent phosphatidyl glycerol occurs in instances where the chloroplast numbers are higher. This has been shown to occur in matured maize and wheat plants, with an associated increase in chlorophyll concentration (Gray, et al., 1967) and when illuminating etiolated plants (Appelqvist, et al., 1968; Murphy & Stumpf, 1979).

1.2 The Role of Galactolipids

Galactolipids are only found as components in the membrane system of chloroplasts and cyanobacteria. No evidence supports the proposal that

galactolipids are involved directly in the photosynthetic chain as electron donors or acceptors. Large amounts of galactolipid can be removed from photosynthetic membranes by treatment with lipase without any marked effect on electron transport (Anderson et al., 1974). The current theory is that their role instead is to provide a framework in which an electron transport chain can operate.

The galactolipid lacks electrical charge and has a small head group in comparison with highly unsaturated fatty acid chains. This provides a fluid environment in which electron transfer, can occur, thereby promoting photosynthesis. Monogalactosyl diglyceride is more prominent in appressed granal membranes, the regions in between thylakoid stacks, than in stromal membranes. These are regions of high curvature and where the protein:lipid ratio is high. The galactolipids may also have a role in allowing optimal packing of large proteins in the thylakoid membranes, and of stabilizing high curvature. This is possibly due to the monogalactosyl diglyceride's physical structure of a liquid crystalline hexagon, with water cylinders in a lipid matrix. The hydrocarbon chains are orientated outwards. If the structure is hydrogenated it turns into a 'gel', and is unable to operate in the same way (Kleoniki and Barber, 1983). For this reason the fatty acids in monogalactosyl diglyceride rarely vary, they are almost exclusively 16:3 and/or 18:3. The structure is highly sensitive to the degree of unsaturation and any slight variation would cause alteration in its structure and phase properties in the membrane.

1.3 Regulation of Fluidity

Monogalactosyl diglyceride is thought to be the most important lipid in regulating the fluidity of the chloroplast membrane at different temperatures. Anabaena variabilis, a blue-green alga with a similar lipid composition to the chloroplast, has been used as a model system in studying fluidity regulation. Experiments showed that the principle process used to change fluidity over a temperature range was by altering desaturation of palmitic acid (16:0) and oleic acid (18:1) in monogalactosyl diglyceride (Sato and Murata, 1980). In higher plants the fatty acid composition changes with a change in temperature. This may occur by induction or modification of desaturase activity (Harwood, 1979).

1.4 Changes in Chloroplast Lipids during Development and the Effect of Light

Differentiation of etioplasts into chloroplasts is light stimulated. A change in the lipid composition of the chloroplast during the stages of development was noticed as early as 1968 (Appelqvist, et al., 1968). Experiments to investigate this difference in membrane composition are generally done in one of two ways. Light can be shone on dark grown plants to stimulate chloroplast development and samples taken at respective times. Or alternately transverse sections of the tissue are cut in distal to basal regions of the leaves of monocotyledons, such as maize and barley, to obtain tissue at different stages of maturity. These experiments showed that the basal regions were high in phosphatidyl choline and phosphatidyl ethanolamine. In the distal

sections, which were more mature with fully developed chloroplasts, the concentrations of phosphatidyl choline and phosphatidyl ethanolamine drop and most of the other lipids increase, Δ^{3t} hexadecenoic acid in phosphatidyl glycerol was only detected in the more mature sections. Fully developed chloroplasts in the distal regions of the leaf were less active in fatty acid biosynthesis (Hawke et al., 1976). The basal regions were found to contain saturated fatty acids with chain lengths of 20 or more carbon atoms. In the distal regions the fatty acids were of shorter chain length and unsaturated (Leech et al., 1973; Hawke et al., 1974).

This suggests that the desaturase is associated with an increase in chloroplast numbers. However there is conflicting evidence. Desaturase activity has been found to be greater in younger tissues (Cherif, et al., 1975; Kannangara et al., 1972; Jacobson, 1974), not induced by light, (Browse et al., 1981), and not dependent on chlorophyll content (Hawke and Stumpf, 1980).

1.5 Synthesis of Plant Lipids

1.5.1. Saturated Fatty Acid Biosynthesis

(a) Location

The synthesis of saturated fatty acids occurs in the plant by a de novo system, using acetyl CoA, acyl carrier protein and several enzymes. The proplastid is the site of synthesis in the seed and developing tissue (Harwood and Stumpf, 1971; Vick and Beevers, 1978; Weavie and Kekwick, 1975; Simcox et al., 1977), although synthesis has also been found to occur in the chromoplasts of the daffodil (Kleinig and Liedvogel, 1980). In leaf the site of de novo fatty acid synthesis is the chloroplast.

(b) Carbon Source

The source of acetyl CoA for fatty acid biosynthesis in photosynthetic tissue is not clear. In the seed the pathway is better understood. Sucrose, made by the leaf, is transported to the developing seed where it is converted to fatty acids. The first step is conversion of sucrose to glucose-1-phosphate, by a series of reactions (Yamada et al., 1974; Simcox, et al., 1977), and then further degradation to pyruvate by glycolytic enzymes. Pyruvate is converted to acetyl CoA by pyruvate dehydrogenase. The ATP requirement is self-supporting, being formed in the glycolytic path and consumed in the acetyl CoA carboxylation step. There is a generation of reducing equivalents by glycolysis and consumption by fatty acid synthetase so the reducing equivalents are cycled. Complete conversion of sucrose to acetyl CoA requires a combination of cytosolic and proplastid enzymes.

Early experiments by Yamada and Nakamuru using ^3H_2O incorporation into

spinach chloroplasts suggested that the path also operated in the leaf (Yamada et al., 1975).

Oxaloacetate, malate and citrate are poor substrates suggesting that the malate synthetase and citrate lyase reactions were not involved in the formation of acetyl CoA for fatty acid synthesis (Yamada, 1975). Incorporation studies of $H^{14}CO_3$ into lipids also supported the Yamada scheme occurring in the leaf (Murphy and Leech, 1981).

Incorporation followed

$CO_2 \rightarrow$ PGA $\rightarrow$ PEP $\rightarrow$ Pyruvate $\rightarrow$ Acetyl CoA $\rightarrow$ fatty acid

so pyruvate could also be used as an effective substrate (Murphy and Leech, 1977). This implied that pyruvate dehydrogenase was present in the chloroplast. Other workers however have found pyruvate to be a poorer substrate then other compounds such as acetate (Roughan et al., 1978; Roughan et al., 1976). Pyruvate dehydrogenase is found to be present in the plastids of seeds and in mitochondria but either absent or with very low activity in chloroplasts (Roughan et al., 1979; Murphy and Stumpf, 1981). So in photosynthesising tissue there is no evidence of the Yamada scheme occurring.

In 1981 it was demonstrated that an acetyl CoA synthetase was present in leaf and localized totally within the chloroplast compartment. The synthetase has a mechanism identical with the enzyme in yeast and animals (Kuhn et al., 1981).

$$\text{Acetate} + \text{ATP} + \text{CoA} \xrightarrow{Mg^{2+}} \text{acetyl CoA} + \text{AMP} + \text{PPi}$$

The acetate probably comes from the mitochondria as they have been shown to contain acetyl CoA hydrolase (Murphy and Stumpf, 1981).

So the supply of acetyl CoA for the chloroplast is thought to occur by the following route: pyruvate dehydrogenase decarboxylates pyruvate in the mitochondria producing acetyl CoA. This acetyl CoA is acted on by a hydrolase forming acetate. The acetate is metabolically inert in the leaf cell until it is transported to the chloroplast stroma. This is the only compartment containing the acetyl CoA synthetase. Acetate is converted to acetyl CoA which is unable to permeate the chloroplast envelope so is available for fatty acid synthesis (Roughan and Slack, 1977; Murphy and Leech, 1981).

Uptake of acetate by the chloroplast is rapid; particularly when a divalent cation is present and in an acid medium.

$CH_3COO^- + H^+ \rightleftharpoons CH_3COOH.$

An acid medium pushes the above equilibrium to the right. The non-ionic form is more readily assimilated by the chloroplast. Experimentally it has been shown that 1mM passes across the chloroplast envelope in less than 5 seconds at a physiological pH of 7.9 (Jacobson and Stumpf 1972), demonstrating how rapid the uptake is <u>in vivo</u>.

(c) De Novo Synthesis of Palmitic Acid

The first step of fatty acid synthesis, using acetyl CoA as the precursor, is catalysed by acetyl CoA carboxylase.

$$\text{acetyl CoA} + HCO_3 + \text{ATP} \xrightarrow{Mg^{2+}} \text{malonyl CoA} + \text{ADP} + Pi$$

This protein has been purified from various plant sources: parsley cell cultures, wheat germ and barley embryo, avocado plastids and spinach chloroplasts. Early work suggested that a biotinyl protein was associated with the thylakoids and that the other components were soluble, being located in the stroma, similar to the acetyl CoA carboxylase in E Coli (Kannangara and Stumpf, 1973; Kannangara and Stumpf ,1972). However, recent work shows that the components occur as a single protein, of molecular weight of approximately 500,000 daltons that is linked to the biotin Mohan and Kekwick, 1980; Nikolau and Hawke, 1973).

In fatty acid biosynthesis in animals and micro-organisms the carboxylation of acetyl CoA is the key regulatory step. This has not been established in plant systems and the data is conflicting. In oil palm embryos it appears to be a rate limiting step (Turnham and Northcote, 1982), while in maize leaves it is not (Nikolau et al., 1981). The system may operate differently in non-photosynthetic tissue. However it has been observed that the responses of purified acetyl CoA Carboxylase are stimulated by the environment of an illuminated chloroplast - a high ATP concentration, a low ADP concentration, a pH of 8.0 and a magnesium ion concentration of 3.0mM (Nikolau and Hawke, 1983). This may be the mechanism of control and may explain the differences in research findings.

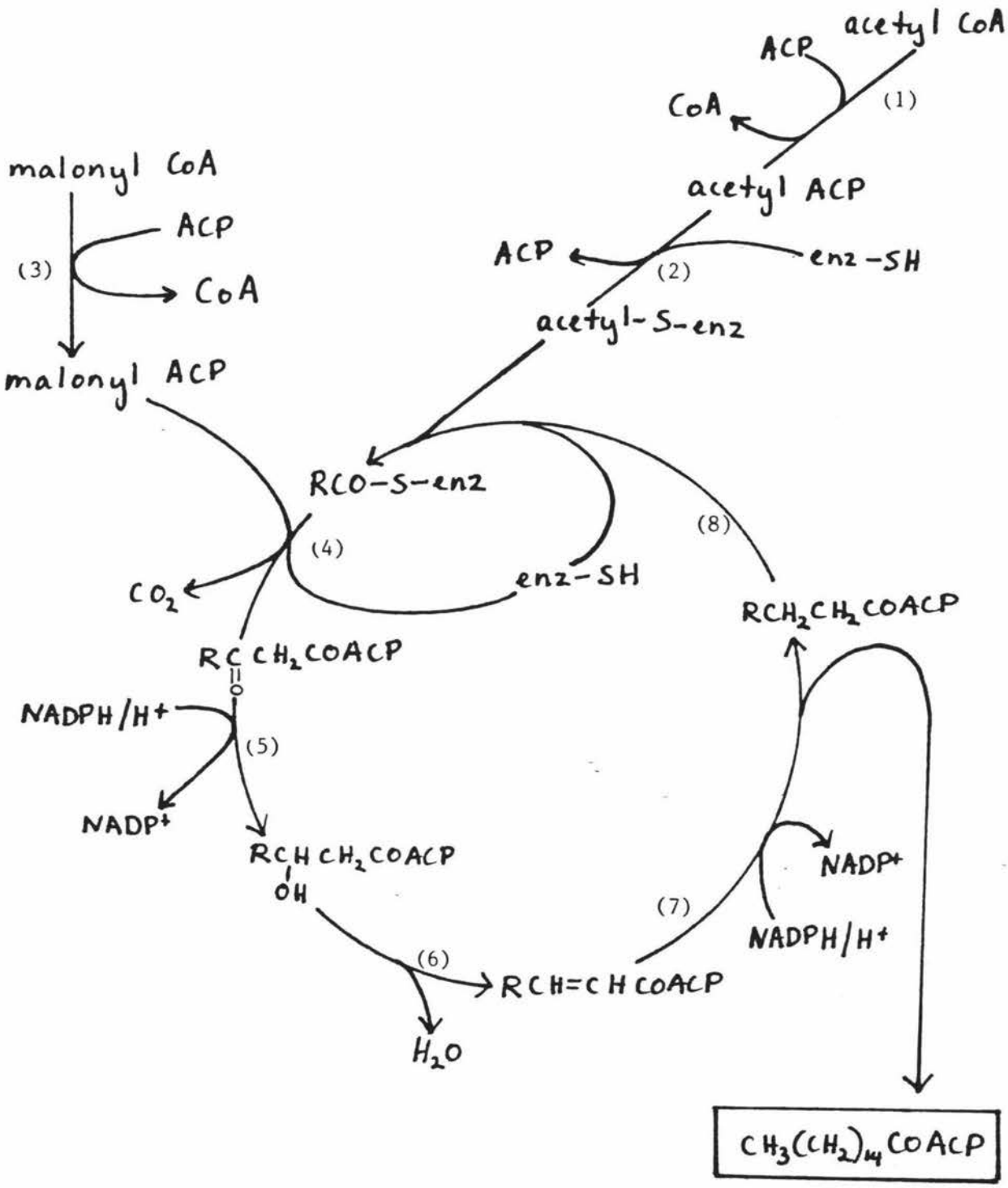
Elongation takes place by the sequential addition of two carbon units and CO_2 release from a malonyl-ACP that is added onto the growing acyl chain (see figure 1.2). Acyl carrier protein is an essential requirement of fatty acid biosynthesis. All of the acyl carrier protein in spinach is found to be located within the chloroplast (Cohlrogge et al., 1979). The ACP derivatives are formed by the action of transacylases (see numbers 1 and 3 in figure 1.2) on acetyl and malonyl CoA. A malonyl CoA - ACP transacylase has been characterized from spinach which has similar properties to the enzyme in cyanobacteria; nearly identical in MW, pH optimal, thermal stability and the random sequential mechanism (Stapleton and Jaworski, 1974). Indeed the whole of the plant synthetase seems to be more like the bacterial than the animal complexes; they are soluble but can be resolved into a number of separate protein fractions (Brooks and Stumpf, 1966).

The NADPH required is supplied from the photosynthetic transport chain, which may explain the light-dependence of fatty acid synthesis. The final product of the fatty acid synthetase is palmitoyl ACP which can be converted to palmitic acid by a hydrolase or can be utilized for the synthesis of longer chain fatty acids.

(d) Elongation of Palmitoyl-ACP to Stearoyl-ACP

In higher plants stearic acid is an elongation product rather than a product of de novo synthesis (Harwood and Stumpf, 1971).

Figure 1.2 Mechanism of the Soluble Fatty Acid Synthetase in Plant Chloroplasts.



(1) ACP acyl transacylase

(2) acetyl group binds to a cysteine residue of the β keto acyl ACP synthetase enzyme

(3) ACP malonyl transacylase

(4) β keto acyl ACP synthetase

(5) β keto acyl ACP reductase

(6) dehydratase

(7) enoyl ACP reductase

(8) acyl group is transferred to the cysteine residue of β keto acyl ACP synthetase and the cycle repeats 6 more times.

In safflower seed it has been shown to be distinct from the fatty acid synthesising system. The palmitoyl-ACP elongase requires palmitoyl ACP, malonyl CoA, and NADPH or NADH. Acetyl CoA can not operate as a carbon donor. The system differs in that its pH optima is higher than for palmitoyl ACP synthesis, ie. pH 7.8 - 8.6 compared with 7.0 for the fatty acid synthetase reaction (Jaworski, et al., 1974).

1.5.2 Oleic Acid Biosynthesis (Desaturation of Stearoyl - ACP).

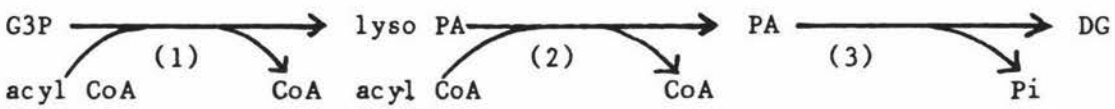
The desaturation of stearoyl-ACP was first demonstrated in 1968 in Euglena gracilis and spinach chloroplasts (Nagai and Bloch, 1968). Since then it has been found to be widespread. The reaction is carried out by a soluble protein, stearoyl-ACP desaturase, found in proplastids and chloroplast stroma. The enzyme is specific for its ACP thioester and has only negligible activity with stearoyl-CoA (Jaworski and Stumpf, 1974; Stumpf and Porra, 1976).

Two electrons are required, from either NADPH or reduced ferredoxin. There is a highly active acyl ACP thioesterase, producing oleic acid (Shine et al, 1976). In addition there are several acyl-CoA synthetases, in particular a long chain acyl CoA synthetase associated with the envelope that has a high affinity for oleate (Roughan and Slack, 1977). A switching system is thought to operate using the thioesterase and the synthetase, that serves to regulate the length of the fatty acid chains (Ohlrogge et al., 1978). Oleoyl ACP is acted on by the two enzymes that have a high specificity for the 18:1 fatty acid. This causes the synthesis of oleoyl CoA from the ACP derivative. CoA esters are only available for the action of acyl transferases attaching fatty acids

into glycerolipids, and are not substrates for further desaturations in the chloroplast (Kannangara and Stumpf, 1971), so the oleoyl CoA is either shipped out of the chloroplast or used for galactolipid synthesis.

1.5.3 Formation of a Diglyceride Intermediate

The conversion of fatty acids into chloroplast lipids firstly involves the formation of a diglyceride intermediate. This can occur in either the endoplasmic reticulum or the chloroplast and follows the scheme.



(1) glycerol-3P acyl transferase

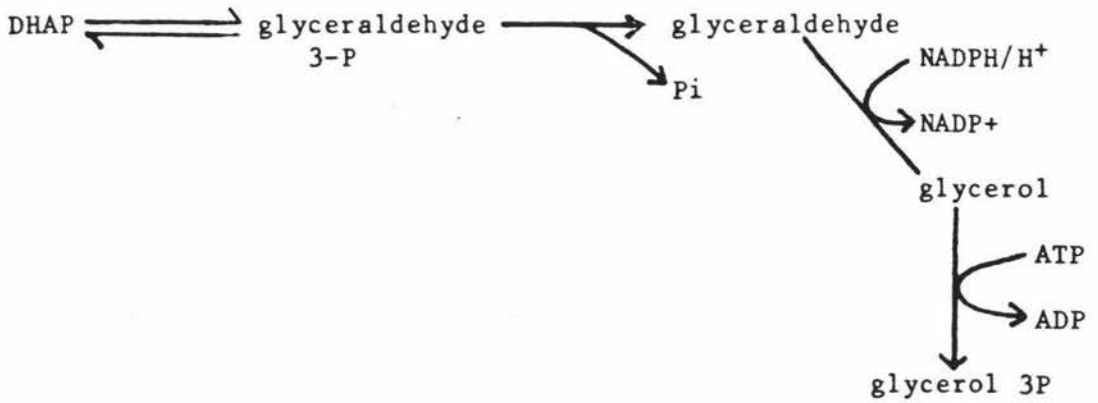
(2) lyso-PA acyl transferase

(3) phosphatidate phosphatidohydrolase

In the chloroplast the first esterification occurs in the stroma by a soluble enzyme that has a preference for oleoyl-CoA as the acyl donor (Bertrams and Heinz, 1978). The next two enzymes in the pathway are in the chloroplast envelope (Joyard and Douce, 1977; Joyard and Douce, 1979).

The enzymes for the synthesis of the precursor glycerol-3-phosphate are located in the cytoplasm, and the compound is brought into the chloroplast by an exchange for dihydroxyacetone-phosphate which is a

product naturally exported by the illuminated chloroplast. The synthesis of glycerol-3-phosphate is thought to occur by the route:-



Diglyceride is an intermediate in the formation of leaf lipids. It occupies a branching point which has three possible fates:- it can either remain in the envelope to be utilized for galactolipid synthesis, be exported out of the chloroplast for synthesis of phosphatidyl ethanolamine and phosphatidyl glycerol, or be used for sulphonovysl diglyceride synthesis that is thought to occur in the chloroplast stroma.

1.5.4 Formation of Phosphatidyl Choline and Oleate to Linoleote Desaturation.

Most fatty acids in the chloroplast are polyunsaturated. There are high concentrations of linolenic acid. Whole leaf labelling studies support the theory that in the biosynthesis pathway of polyunsaturated fatty acids desaturation occurs in the following manner:

oleate ($9\text{-}C_{18:1}$) ⟶ linoleate ($9,12\text{-}C_{18:2}$) ⟶ linolenate ($9,12,15\text{-}C_{18:3}$)

This was first proposed by Harris and James in 1965, and since confirmed in maize leaves (Hawke and Stumpf, 1980), in cucumber cotyledons (Murphy and Stumpf, 1980), and in a wide variety of angiosperms and lower plants (Cherif et al., 1975).

When illuminated chloroplasts instead of whole leaves are incubated with ^{14}C-acetate, with the exception of oleic acid, only negligible concentrations of unsaturated fatty acids are labelled. This is explained by the proposal that a desaturation occurs outside the chloroplast while the fatty acid is esterified to phosphatidyl choline.

Diglyceride is a precursor for the synthesis of phospholipids which are formed in the endoplasmic reticulum. When whole leaf is fed $^{14}CO_2$ major amounts of label are found to be incorporated into phosphatidyl choline and phosphatidyl glycerol (Simpson and Williams, 1979; Roughan, 1970) in a shorter time then into galactolipids (Slack and Roughan, 1975).

Chloroplasts do not contain the enzymes for phosphatidyl choline synthesis and have only recently been found to be capable of synthesising phosphatidyl glycerol. In Euglena gracilis it was found that isolated chloroplasts could synthesize CDP-diglyceride and then phosphatidyl glycerol, reactions that were formerly thought to only occur in microsomal fractions (Mudd and Dezacks, 1981). The phosphatidyl glycerol in the chloroplast is distinct in that it contains 16:1Δ^{3t} at sn-2 position. This fatty acid is formed by desaturation of palmitic acid (Roughan, 1975). The diglyceride for phosphatidyl choline synthesis does not come from the chloroplast but is synthesised in the endoplamic reticulum.

Oleoyl CoA from the chloroplast is transferred almost exclusively onto phosphatidyl choline at sn-2 position (Roughan, 1975; Drapier et al.,

1982). The incorporation of oleate into phosphatidyl choline can occur by two processes, either by transfer directly from oleoyl CoA or by an exchange reaction for pre-existing more unsaturated fatty acids in phosphatidyl choline (Stymne and Glad, 1981). It is after esterification that the second desaturation occurs in both leaves and developing tissue (Stymne and Appelqvist, 1980; Stymne and Appelqvist, 1978; Slack, et al., 1973; Bloch, and Roughan, 1975). This is quantitatively the most important reaction to provide linoleate (Gurr and Brawn, 1970). The acyl transferase and oleate desaturase are tightly coupled in the endoplasmic reticulum, of pea leaf, to cause a "metabolite chanelling". It is thought that it is the amount of 18:1 phosphatidyl choline, in particular phosphatidyl choline with 18:1 at both sn-carbon positions; that determines the activity of the desaturase (Murphy et al., 1984).

The desaturation step on phosphatidyl choline has been reported by several workers using pulse labelling studies (Roughan, 1975; Hawke and Stumpf, 1980). Only one desaturation occurs as no 18:3 - phosphatidyl choline has been detected (Gurr et al., 1969; Heinz and Harwood, 1977). The situation appears to be different in other non-photosynthetic sites, from studies of phospholipid linked linoleate desaturation in linseed cotelydons (Browse and Slack, 1981). Phosphatidyl ethanolamine is also capable of being desaturated (Pugh and Kates, 1973). The desaturase requires O_2 and NADPH. An oleoyl desaturase protein has been found in microsomal (10,000g) fractions in pea leaves but is absent in chloroplasts (Dubacq, et al., 1976). For desaturation of oleic acid a co-operation between the endoplasmic

reticulum and chloroplasts is necessary (Tremolieres and Mazliak, 1974; Tremolieres et al., 1980; Roughan et al., 1980; Hawke et al., 1974).

An alternative desaturase system has been suggested that desaturates oleoyl CoA directly. It has been found in: Candida lipolytica (Pugh and Kates, 1975; Vijay and Stumpf, 1971; Abdelkader, et al., 1973), and Chlorella vulgaris (Gurr, 1971).

In Chlorella vulgaris the oleoyl desaturase is unusual in that it recognises the site for the double bond from the carboxyl group. The oleoyl CoA desaturase has a less extensive role and has a higher activity at low temperatures than the phosphatidyl choline oleoyl desaturase. It has been suggested that it may have a role in temperature regulation of membrane fatty acid composition. There has however been some disagreement about whether this alternative desaturase exists (Slack et al., 1979).

The phosphatidyl choline synthesised is transported to the chloroplast envelope, to be incorporated into the membrane, by an exchange protein. This has been shown with the use of double labelled phosphatidyl choline in Avena (Ohnishi and Yamada, 1982). The protein has recently been purified and characterized from spinach by Kader, Julienne and Vergnolle in 1984, based on their earlier work (Julienne et al., 1981). It is active in transporting phosphatidyl choline and phosphatidyl glycerol from the endoplasmic reticulum to the mitochondria as well as to the chloroplasts. The protein is small (MW

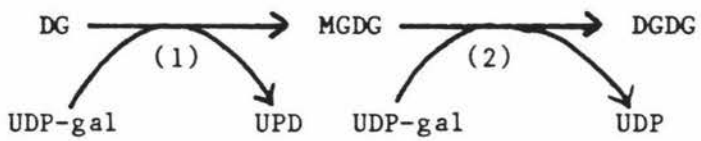
9000), stable at low temperatures and very non-specific for phospholipids.

The phosphatidyl choline can either be included in the membranes of the chloroplasts or the endoplasmic reticulum or it can be converted back to diglyceride and used for galactolipid synthesis. Labelling studies have shown that fatty acids move from phosphatidyl choline onto galactolipids (Hawke and Stumpf, 1980; Stobart et al., 1980).

1.5.5 Galactolipid synthesis and linoleoyl desaturation

(a) Pathway from Diglyceride

Galactolipid synthesis takes place in the chloroplast envelope (Douce, 1974). The scheme for galactolipid synthesis, that is now accepted, is by a stepwise addition of galactose units onto a diglyceride molecule.



(1) galactose:diglyceride galactosyl transferase

(2) this enzyme has yet to be identified

This has been supported by ^{14}C-UDP-gal incorporation studies (Ongun and Mudd, 1968). Addition of glycerol-3-phosphate a precursor for DG; UDP-gal and Triton X-100, believed to release the transferase from the envelope; are found to increase the monogalactosyl diglyceride concentration (McKee and Hawke, 1979), at the expense of unesterified fatty acid accumulation (Roughan and Slack, 1981).

The galactosylation occurs in two separate steps by 2 different enzymes to form monogalactosyl diglyceride and digalactosyl diglyceride respectively. The first is responsible for formation of the

glycosidic bond and has been purified. It is tightly bound to the membrane. The second enzyme is less well characterized. It is also associated with the chloroplast envelope but is less tightly bound than the galactose:diglyceride galactosyl transferase. The enzyme can cause the exchange of galactosyl groups between galactolipid molecules. It does not require UDP-galactose. This in part may explain why large amounts of diglyceride glycerol are found in chloroplast envelopes (Douce and Joyard, 1980).

There is also a third enzyme located on the outer surface of the outer chloroplast envelope that catalyses synthesis of tri and tetra galactosyl diglycerides (Dorne et al., 1982). The significance of this enzyme is not known.

In blue-green alga there is an alternative synthesis route. A glucose unit is often added onto the diglyceride followed by epimerization to form the galactolipid (Sato and Murata, 1982). This does not appear to happen in higher plants.

During thylakoid biogenesis massive transport of galactolipids from the chloroplast envelope occurs, possibly by lateral diffusion (Siebertz et al., 1979).

(b) Source of UDP-gal

Chloroplasts are unable to synthesise UDP-galactose (Bertrams et al., 1981). The enzymes required are found as soluble proteins in the cytosol (Konigs and Heinz, 1974).

glucose-1-P $\xrightarrow[(1)]{}$ UDP glucose $\xrightarrow[(2)]{}$ UDP-gal

(1) UDP-glucose pyrophosphorylase

(2) UDP-glucose 4 epimerase.

The UDP-galactose is unable to cross the chloroplast envelope. To enable it to be utilized the enzymes involved in galactolipid synthesis are organized on the external sides of the envelope.

(c) Diglyceride source

The diglyceride for galactolipid synthesis can arrive from two different sources. The diglyceride may come directly from the chloroplast envelope made from chloroplast fatty acids. Generally this diglyceride is sn-1 oleate, sn-2 palmitate. Or alternatively the diglyceride can be from the endoplasmic reticulum made by breakdown of phosphatidyl choline. This diglyceride usually has linoleate at both sn positions (Roughan et al., 1980).

Labelling studies have shown that high concentrations of labelled linoleate will transfer from phosphatidyl choline onto monogalactosyl diglycerate via a diglyceride intermediate (Ohnishi and Yamada, 1982; Slack et al., 1977; Williams et al., 1976). The fatty acid on the monogalactosyl diglyceride can then undergo further desaturation.

The distinction of which diglyceride pool is used results in two different types of monogalactosyl diglyceride being produced:- a sn-1 18:3, sn-2 16:3 monogalactosyl diglyceride made from chloroplastic diglyceride and termed "prokaryotic" and an sn-1 18:3, sn-2 18:3,

monogalactosyl diglyceride made from diglyceride from the phosphatidyl choline in the endoplasmic reticulum termed "eukaryotic" (Roughan and Slack, 1982).

The prokaryotic monogalactosyl diglyceride recognised by the 16 carbon fatty acid at sn-2 position, found in "16:3 plants", is made completely by the chloroplast (Siebertz, 1979). It is found in highest concentrations in the lower plants, in particular the blue green algae and Chlorella vulgaris (Stafford and Nichols, 1970). But it is present in degrees in other plants in a 'spotted' distribution. In spinach approximately 50% of the monogalactosyl diglyceride is prokaryotic and 50% is eukaryote. The eukaryotic monogalactosyl diglyceride with the 18 carbon fatty acid at both positions are more predominant in plants of more advanced genera and are thought to be of physiological advantage. These findings may support the theory that chloroplasts first arose as bacteria living symbiotically in a cell. The bacteria first produced their own prokaryotic galactolipids using raw materials from the cell and later adapted to utilize the cell-made diglyceride. The 16:3 plants are cases where the prokaryotic pathway has persisted.

Small amounts of monogalactosyl diglyceride other than 18:3 at both positions are found to exist in higher, non - 16:3, plants. This is probably caused by pool mixing; as the chloroplast produces and then exports a prokaryotic diglyceride and is at the same time importing a eukaryotic diglyceride. This could explain the confusion in research findings when the fatty acid composition of monogalactosyl diglyceride has been studied (Roughan and Slack, 1982).

(d) Desaturation of linoleoyl to α-linolenate

In 1969 Mudd et al. suggested that highly unsaturated galactolipids predominated because of the specificity of the enzyme catalysing the transfer of galactose from UDP galactose for polyunsaturated diglyceride acceptors (Mudd et al., 1969). These findings were disproved in 1971 and it was suggested that polyunsaturated monogalactosyl diglyceride arose either by desaturation after monogalactolipid synthesis or by transacylation (Eccleshall and Hawke, 1971). It is now widely accepted that the desaturation of linoleate occurs while it is esterified to monogalactosyl diglyceride. This has been demonstrated by labelling studies (Murphy and Stumpf, 1980; Hawke and Stumpf, 1980; Ohnishi and Yamada, 1980; Ohnishi and Yamada, 1982) and by the use of herbicides that inhibit the linolease desaturase (Lem and Williams, 1981). 18:3 phosphatidyl choline is found only in negligible concentrations and time labeling studies show that as 18:2 monogalactosyl diglyceride levels decrease with time 18:3 monogalactosyl diglyceride levels increase simultaneously (Wharfe and Harwood, 1978).

It was earlier proposed that the synthesis of α-linolenate was not from linoleic acid desaturation but that the synthesis occurred by an elongation of a shorter chain desaturated fatty acid (Kannangara, et al., 1973; Jacobson et al., 1973; Jacobson et al., 1973). Later work has not supported these findings. The scheme was disproved by Murphy & Stumpf in 1979.

Apart from the production of linolenate there are other desaturation reactions that occur while the fatty acids are esterified to monogalactosyl diglyceride. Some palmitate desaturation occurs (Roughan et al., 1979; Jamieson and Reid, 1971).

In some algae groups linoleate can also be synthesised on monogalactosyl diglyceride as well as by the normal route on phosphatidyl choline (Appleby et al., 1971). Desaturation does not appear to occur on digalactosyl diglyceride (Sato and Murata, 1982).

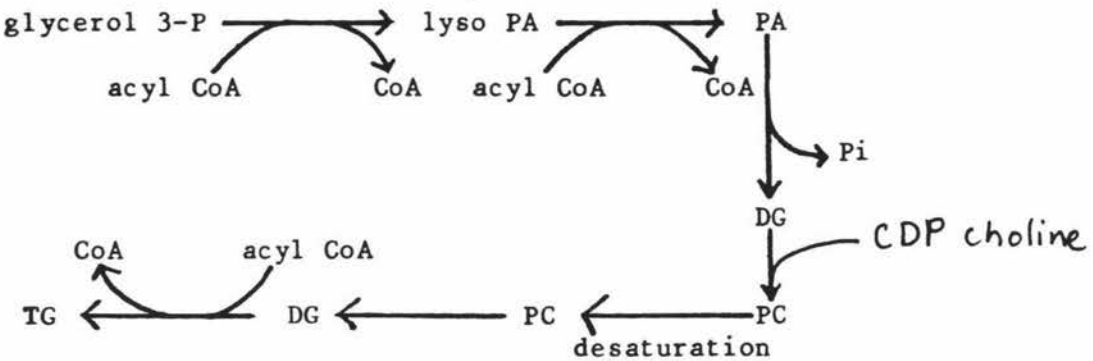
1.5.6 Triglyceride Synthesis

Triglyceride synthesis is thought to be fundamentally the same in plants and animals proceeding by the Kennedy pathway already characterized in animals. This pathway is most active in the seed tissue of plants at certain stages of development. The pathway for synthesis occurs in the endoplasmic reticulum. Acyl CoA esters are required, these are supplied by the chloroplast (Roughan and Slack, 1982).

There appears to be two different groups of triglyceride. One is predominately composed of fatty acids of 20 and 22 carbon chains and appears to be synthesised in the animal manner. The other group is comprised of mainly polyunsaturated fatty acids and the fatty acids appear to come from phosphatidyl choline. A loss of radioactivity from phosphatidyl choline and an equivalent increase in triglyceride occurs (Wilson et al., 1978).

In experiments with soybean cotyledons triglycerides are the main acceptor molecules of fatty acids from phosphatidyl choline (Stymne and Appelqvist, 1980).

The synthesis of these triglycerides occurs by a modified Kennedy pathway which attaches a diglyceride onto phosphatidyl choline, where desaturation occurs, before the last synthesis step to form the triglyceride.



The enzymes have been shown to be present in avacado mesocarp microsomes (Barron and Stumpf, 1962).

There have been few studies of triglycerides in photosynthetic tissues. Recently diacyl glycerol acyl transferase (DGAT), the only enzyme unique to the triglyceride biosynthetic pathway,has been found in spinach leaves (Martin and Wilson, 1983). It has a pH optimum and Mg^{2+} requirement similar to the other enzymes of the glycerolipid pathway. It has a specificity for palmitoyl CoA so this could be a factor in determining triglyceride fatty acid composition.

The function of triglyceride in leaves is unknown. It is stored in intraplastid oil bodies and may be for use as an energy reserve.

1.5.7 Summary

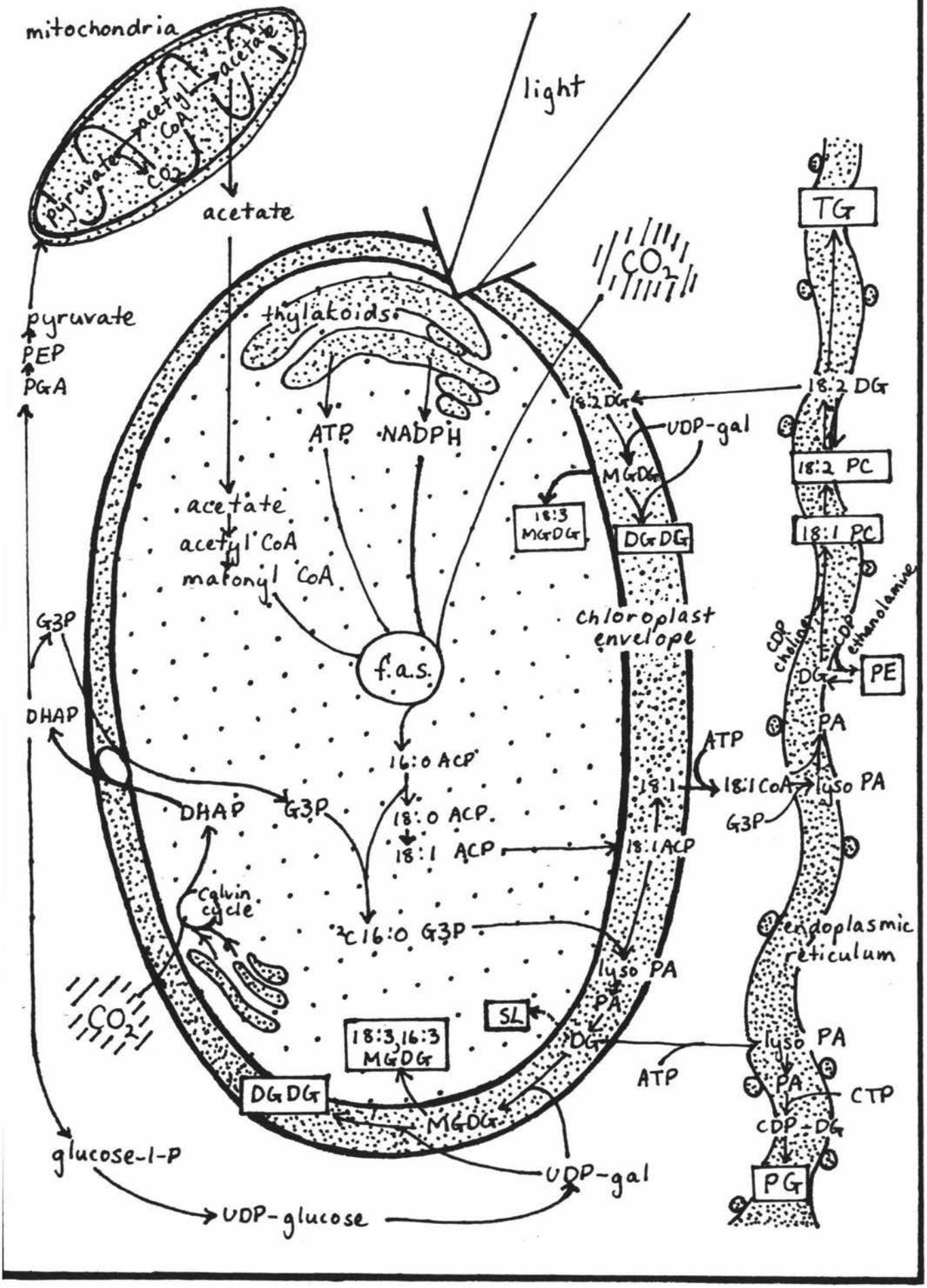
The synthesis of plant lipids in photosynthetic tissue requires not just the chloroplast but involves the interaction of several organelles and the cytosol in the plant cell (see figure 1.4).

1.6 The Use of Protoplasts in Experiments

In studies of lipid metabolism in plants, protoplasts are frequently used as they have several advantages over other tissues. Protoplasts are cells from plants or fungi that have had their cell wall removed. Initially the technique was developed by Cocking (Cocking, 1960) in the isolation of protoplasts from tomato root tip cells. It is now used on many plant types.

Working with whole leaf tissue has certain disadvantages. There are difficulties in transporting radioactive substrates across the cell wall, and there are often problems of translocation by the phloem and xylem. Protoplasts avoid these problems and still contain all the plant cell organelles and their interacting pathways. Removal of the cell wall does not markedly change the cells lipid composition or its ability to synthesis lipids (Kuhn and Stumpf, 1981). The other major advantage protoplasts have is for use as a convenient starting material for the preparation of intact plant organelles. Protoplasts are easily lysed and the organelles can then be separated out without interferring

Figure 1.3 The Organelle Interaction Involved in the Lipid Biosynthesis of a Photosynthetic Plant Cell.



substances from the cell wall, eg; phenolics, coming in contact with them.

Protoplasts are prepared from tissue harvested after a dark period, when there will be little or no starch. The starch grains can cause lysis of the protoplasts during centrifugation, as they have a high sedimentation co-efficient and may spin either through the chloroplast envelope or the plasmalemma. The tissue is usually sliced although some workers have just floated the whole leaf in solution (Ohlrogge et al., 1978) after the epidermis has been peeled off or abraded in some manner. Often cutting of the tissue is done in solution to prevent the entry of air or, alternatively, it is put under a vacuum after cutting. The tissue is digested in a solution of pectinase and cellulase, and in some instances macerase, for 2-5 hours. Often the enzyme solution is changed during the digestion as the enzymes may bind irreversibly to the cell walls (Kanai and Edwards, 1973).

The protoplasts are purified by a step-gradient of sucrose and either sorbitol or mannitol, although a metrizamide/sorbitol gradient has been used (Wirtz et al., 1980). In some instances the density of the sucrose medium has been increased by the addition of dextran (Spalding et al., 1979; Edwards et al., 1979).

Normal functioning of the protoplasts can be checked by; simple observation under light microscope, $^{14}CO_2$ fixation (Sicher, 1982), or by CO_2 or light dependent oxygen evolution rates using an oxygen electrode. Oxygen electrode medis for protoplasts usually contain

$CaCl_2$ which prevents aggregation and inhibits photosynthesis by contaminating chloroplasts produced from ruptured protoplasts (Leegood and Walker, 1983). Rates of oxygen evolution by protoplasts are favourable, indicating that they are as active as the intact plant leaf tissues in their photosynthetic activities (Nishimura and Akazawa, 1975). In spinach rates of over 100 μmoles O_2/mg chlorophyll/hr have been suggested to indicate "normal" metabolic activity (Kuhn and Stumpf, 1981). Crop plants have given rates of: maize 120-182 μ moles O_2/mg chlorophyll/hr (Day et al., 1981); wheat 100-150 or greater (Edwards et al., 1978; Leegood and Walker, 1979); and barley of approximately 180 (Bell, thesis 1983).

1.7 Chloroplast Isolation

Until recently intact chloroplasts have only been prepared from spinach, as this plant has a low phenol content. Chloroplasts have been prepared by mechanical disruption of the leaves in a blender. It is only recently that class A chloroplasts, ie; whole intact chloroplasts (Halliwell, 1981), have been prepared from non-spinach sources. Mechanically this has been achieved by fast grinding using Polytron blenders in semi-solid ice solutions followed by short centrifugations (Leegood and Walker, 1979). But a more gentle method is by osmotic rupture of protoplasts.

Chloroplasts are prepared from protoplasts by placing the newly made protoplasts into a medium of lower osmolarity and then passing them rapidly through nylon mesh, causing breakage of the cells. The protoplasts should be lysed in solutions free of salts to reduce

aggregation of organelles. A cation free media has been shown to increase the percent intactness and enhance separation of intact and broken chloroplasts (Nakatani and Barber, 1979).

After lysing, the chloroplasts are washed. A sucrose gradient can be used to separate them from remaining protoplasts but it has the disadvantage of dehydrating the chloroplast. Separation of the chloroplast from the protoplasts can also be achieved by placing a silicone oil layer between sorbitol and sucrose layers and spinning. Only the chloroplasts pass through the silicone oil layer, the protoplasts remain above, so can be effectively separated off.

The chloroplast media often contains small quantities of EDTA, $MgCl_2$ or $MnCl_2$, and phosphate. The chloroplasts are particularly sensitive to the phosphate level, because the major flux regulation across the chloroplast envelope is mediated by a phosphate translocator. This means if there is excessive phosphate the forced export of phosphorylated intermediates leads to a breakdown of the Calvin cycle and consequent lowering of the rate of photosynthesis. However, too little phosphate will cause the secondary effect of a decrease in the ATP:ADP ratio (Leegood and Walker, 1983).

Microscope examination of chloroplasts shows them to be intact by the appearance of a halo and no visible thylakoids. Intactness can also be tested by the ferricyanide test which is used inversely. Ferricyanide reacts with the thylakoid membrane but is unable to cross the chloroplast envelope. However the percentage intactness that the test

gives tends to be an overestimate, as some resealing; class A_2 chloroplasts; occurs (McC Lilley et al., 1975).

Oxygen electrode experiments have shown rates of greater than 100 μ moles O_2/mg chl/hr from wheat flag leaf (Leegood and Walker, 1979), and 100-150 from wheat (Edwards et al., 1978).

1.8 The Aim of This Study

In the experiments described in this thesis protoplasts and chloroplasts have been used to study the synthesis of non-polar lipids. High amounts of label from (1-^{14}C) acetate were found to be incorporated into a compound identified as triglyceride, by protoplasts. The significance of the label into triglyceride in seven day old barley plants is not understood but experiments suggest that it may be a function of immature tissue. Chloroplasts were also isolated from barley leaf, both by the traditional blending method and by lysing pre-made protoplasts. Incorporation studies with the barley chloroplast suggested that the triglyceride synthesis was, at least in part, occurring inside the chloroplast organelle, contrary to studies recorded in the literature.