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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.

Frances Joan McIntosh

1985

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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LIST OF ABBREVIATIONS

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

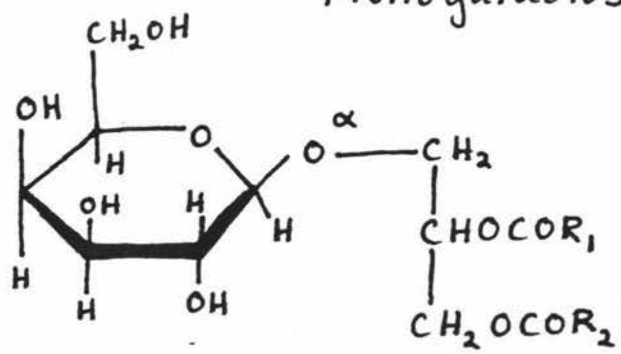
Table 1.1 The Lipid Composition of the Chloroplast Envelope and Thylakoids from *Vicia faba*.

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

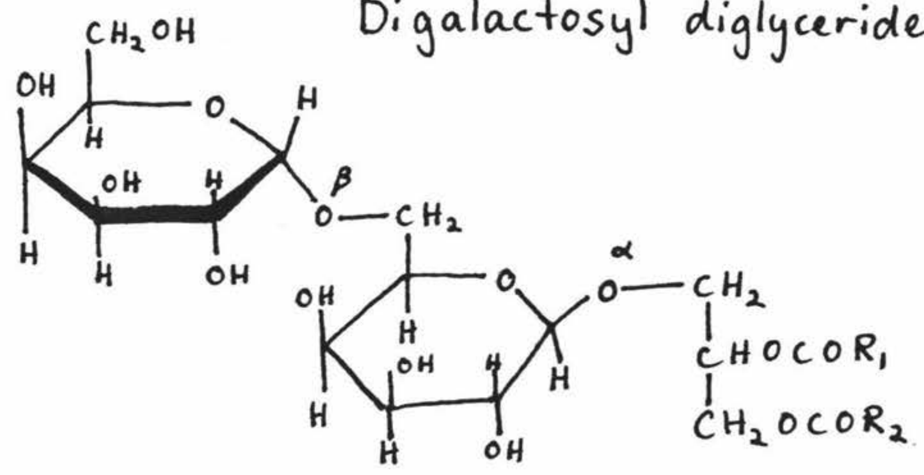
(Leech & Murphy, 1976)

Figure 1.1 The Structure of the Chloroplast Glycolipids

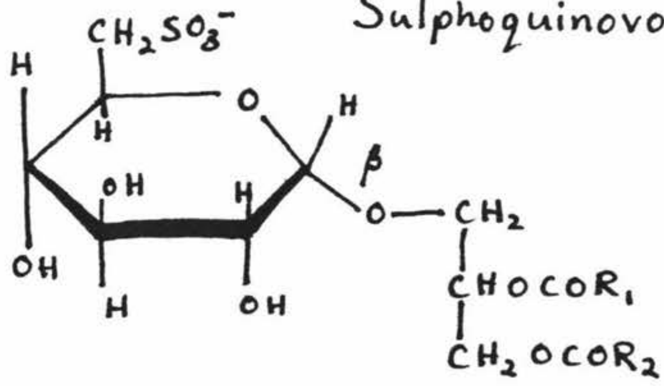
Monogalactosyl diglyceride



Digalactosyl diglyceride



Sulphoquinovosyl diglyceride



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 α 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

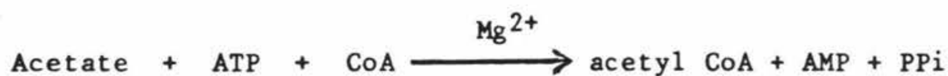
spinach chloroplasts suggested that the path also operated in the leaf (Yamada et al., 1975).

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).

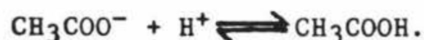


$$\text{Acetate} + \text{ATP} + \text{CoA} \xrightarrow{Mg^{2+}} \text{acetyl CoA} + \text{AMP} + \text{PPi}$$

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.

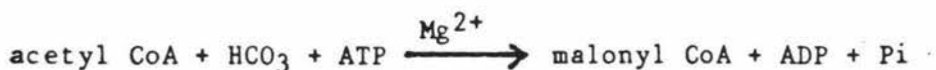


$CH_3COO^- + H^+ \rightleftharpoons CH_3COOH$.

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 *in vivo*.

(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.



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

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.

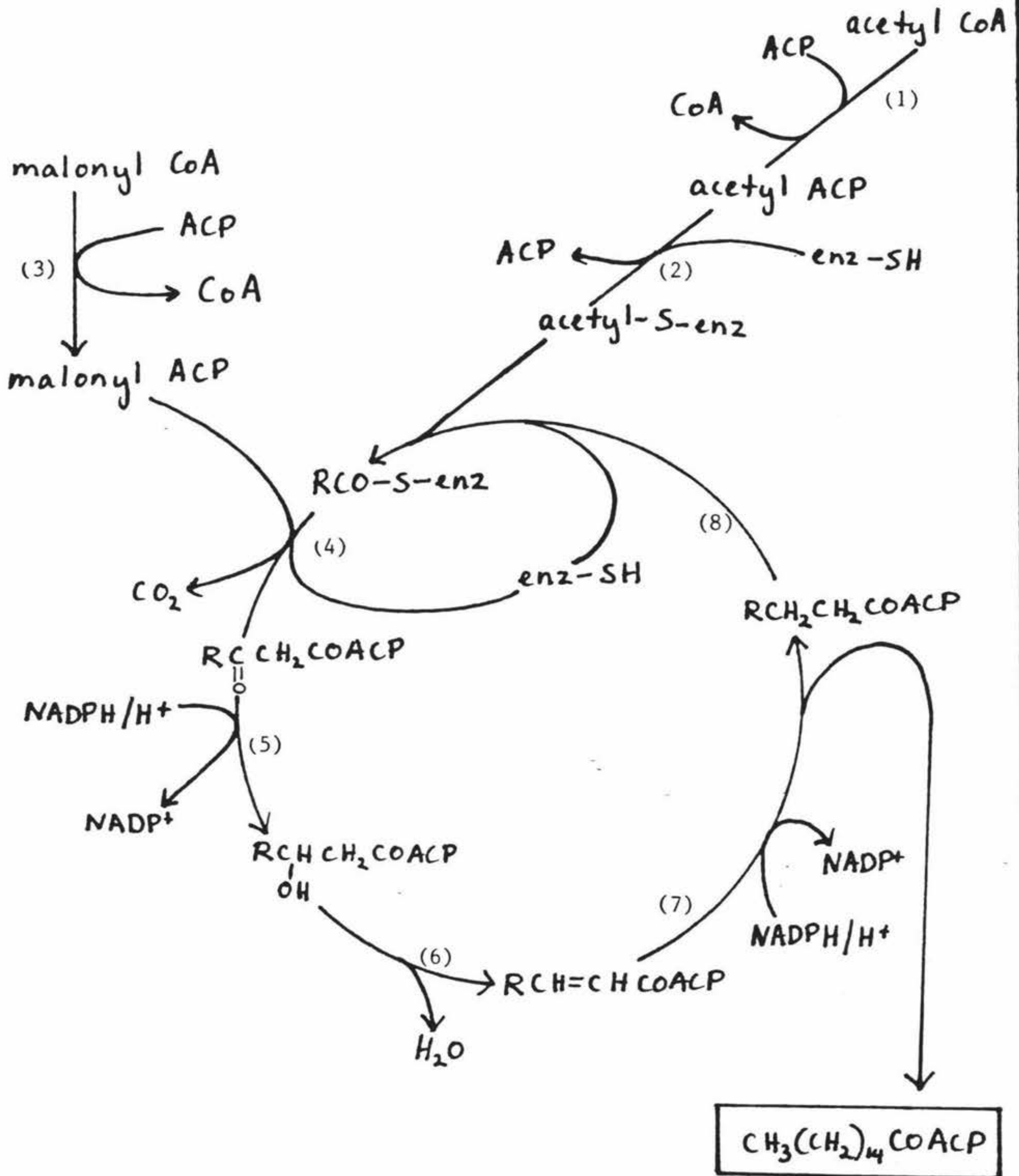
Elongation takes place by the sequential addition of two carbon units and CO_2 release from a malonyl-ACP that is added onto the growing acyl chain (see figure 1.2). Acyl carrier protein is an essential requirement of fatty acid biosynthesis. All of the acyl carrier protein in spinach is found to be 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).

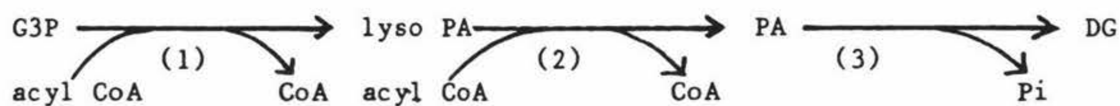
The desaturation of stearoyl-ACP was first demonstrated in 1968 in Euglena gracilis and spinach chloroplasts (Nagai and Bloch, 1968). Since then it has been found to be widespread. The reaction is carried out by a soluble protein, stearoyl-ACP desaturase, found in proplastids and chloroplast stroma. 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:-

$$\text{DHAP} \rightleftharpoons \text{glyceraldehyde 3-P} \longrightarrow \text{glyceraldehyde} + \text{Pi}$$

$$\text{glyceraldehyde} \xrightarrow{NADPH/H^+ \rightarrow NADP^+} \text{glycerol} \xrightarrow{ATP \rightarrow ADP} \text{glycerol 3P}$$

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

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 $16{:}1\Delta^{3t}$ 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

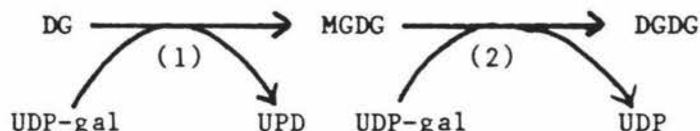
9000), stable at low temperatures and very non-specific for phospholipids.

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).

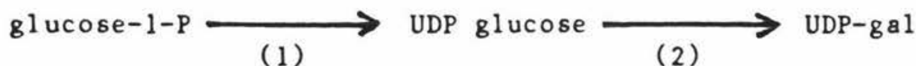
There is also a third enzyme located on the outer surface of the outer chloroplast envelope that catalyses synthesis of tri and tetra galactosyl diglycerides (Dorne et al., 1982). The significance of this enzyme is not known.

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).



(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).

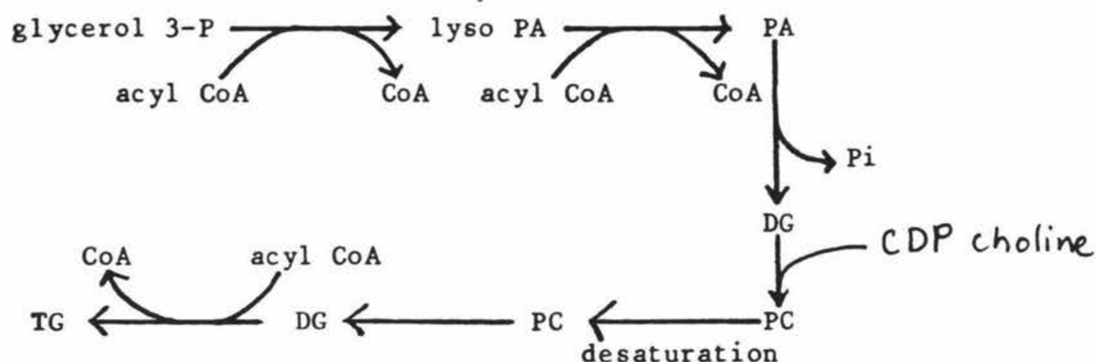
1.5.6 Triglyceride Synthesis

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.

CHAPTER 2. MATERIALS

2.1 Plant Tissues

Maize seeds (Zea mays variety XL45) were purchased from Arthur Yates and Co. Ltd., N.Z. Four varieties of barley (Hordeum vulgare) seeds were used. The varieties Georgie and Fleet were obtained from Arthur Yates Co. Ltd., N.Z. Goldmarker was received from Hodder & Tolley Ltd, NZ, and the variety Triumph was from the Canterbury Malting Co. Ltd., Marton.

The procedure for growing the tissue involved firstly washing the seeds until the pink anti-fungal agent was no longer visible. The seeds were then sown in a peat/pumice (1:4 v/v) mix and watered generously with Hoagland's solution A (Hoagland and Arnon, 1938).

The plants were grown in growth cabinets providing a controlled environment with day/night conditions of: temperature 25/20°C, vapour pressure deficits 10/5 mbar and relative humidities 68/78% respectfully. The day length was fixed at 12hr. The light supplied was in the 400-700nm range at an intensity of 170 w/m^2.

Barley plant tissue was used for experiments after 7 day's growth when approximately 12cm in height.

2.2 Reagents

The following was purchased from Sigma Chemical Co. St. Louis:
D-sorbitol, pectinase, MES, Tricine, BSA Fraction V, POPOP and PPO,

CoA, ATP, NADPH, NADH, DTT. Cellulase (Celluysin ™) was from Calbiochem, La Jolla. HEPES from Hopkins and Williams, Essex, England. Silica Gel G (type 60) was from E Merck, Darmnstadt, Germany. Sodium (1-^{14}C) acetate and sodium ^{14}C-bicarbonate were obtained from the Radio chemical Center Ltd, Amersham, England.

Potassium ferricyanide was from Koch-Light Laboratories Ltd, Colbrook Bucks, England.

Other chemicals used, not listed above, were of an appropriate grade purchased from normal chemical suppliers.

All solvents used were redistilled before use.

CHAPTER 3. METHODS

3.1 Preparation of Protoplasts

The method used to prepare protoplasts was based on the method of Day *et al.* (1981) for *Zea mays*.

Seven-day old plant tissue was cut off at the soil surface. The bottom, approximately $^1/_2$ cm long white stem was cut off and discarded. When maize was used the outer leaf was removed also. Leaves were first abraded with 160 grit carborundum to scratch the epidermal layer, then rinsed and blotted dry. The leaves where cut transversely with a razor blade into approximately 2mm sections. 2.5-5g of sliced leaves were placed in a Petri dish with 40mls of a digestion media containing 0.5M sorbitol, 0.2 mM $CaCl_2$, 0.2 mM KH_2PO_4, 1mM $MgCl_2$, 0.2% (wt/v) BSA, 0.2% (wt/v) pectinase, 2% (wt/v) cellulase and 10mM MES buffer, adjusted to pH 5.5 with NaOH. The tissue as evacuated on a water pump, then left to digest for 3 hrs with gentle shaking at 30 cycles/min, 28°C and a light intensity of 600 $\mu E/m^2/sec$.

After the 3hr digestion the rest of the preparation was done on ice. The leaf tissue was filtered through 124 μm nylon mesh and washed with solution A (0.6M sorbitol, 0.2mM $CaCl_2$, 0.2mM KH_2PO_4, 1mM $MgCl_2$, 0.2% (wt/v) BSA, 5mM HEPES buffer, adjusted to pH 7.8 with NaOH). This filtrate and washings were centrifuged at 300g for 3min. The supernatant was discarded and the pellet resuspended in 7.5cm^3 of Solution B (identical to solution A except that sucrose replaces sorbitol). This was overlaid with 3 mls of solution A forming a step

gradient. After centrifugation at 350g for 5 mins a band of protoplasts was seen to collect at the sucrose - sorbitol interface. This was removed with a Pasteur pipette. The protoplasts were washed with approximately 5 mls of solution A, re-centriguged at 350g for 5 min, supernatant discarded and the protoplast pellet resuspended in 1 - 2 mls of solution A and stored on ice.

3.2 Chloroplast Preparation

3.2.1 Chloroplast Prepartion from Protoplasts

Chloroplasts were prepared from protoplasts by a modified method of Edwards et al. (1978).

The protoplasts prepared as described above were pelleted by spinning at 350 g for 3 min. The pellet was washed in approximately 5 mls of solution C (0.4M sorbitol, 10mM EDTA, 10mM $NaHCO_3$, 0.2 mM KH_2PO_4, 25 mM Tricine, adjusted to pH 8.0), spun at 750 g for 2-3 min and the supernatant discarded. The pellet was resuspended in solution C and allowed to stand for 5 min. The suspension was then passed rapidly through a 22 gauge needle attached to a syringe with a layer of Mira cloth inserted between the needle and the syringe to aid disruption. This was repeated two more times. The suspension was centrifuged at 350 g for 3 min to precipitate whole protoplasts. The supernatant was then centrifuged at 750 g for 2-3 min. The chloroplast pellet was kept on ice as a suspension in 0.5-2 mls of solution C. Chloroplasts were used within 30 minutes of isolation as suggested by Edwards et al, 1978.

3.2.2 Chloroplasts Prepared by a Blending Method

Chloroplasts were prepared by an alternate method as taken from Leegood and Walker (1979) where it was used for wheat.

Tissue was grown and harvested in the normal manner. 20g of leaf tissue was cut into 2 cm sections and homogenized in 150 mls of semi-frozen grinding media (containing 0.33M sorbitol, 10mM $NaHCO_3$, 10mM EDTA, 5mM $MgCl_2$, 0:1 (wt/v) BSA, 0.2 (wt/v) Na D-isoascorbate, 20mM HEPES at pH 7.6) for 25 seconds by an Ultra-turrax polytron blender. The homogenate. was filtered through a Mira-cloth. It was centrigued at 5,500 rpm for 30 s, and the supernatant discarded. The chloroplasts were washed in 5 mls of solution C, re-centrifuged and resuspended in approximately 2 mls of solution C.

Chloroplasts were again used only until 30 minutes after isolation.

3.3 Chlorophyll Determination

The chlorophyll concentration of protoplast and chloroplast suspensions was determined by the method of Arnon (1949).

Between 25-100 1 of the suspension, depending on its concentration, was made up to 5 cm^3 with 80% acetone. It was mixed well, filtered and the absorbance read at 645 nm and 663 nm on a Hitachi Model 101 spectrophotometer with 80% acetone set at nil balance.

The concentration is calculated using the equation:

chlorophyll concentration ($\mu g/cm^3$)

$$= \frac{(20.2 \times A_{645} + 8.02 \times A_{663}) \times 5}{\text{volume of suspension used}}.$$

3.4 Determination of Protoplast Intactness

3.4.1 Phase-Contrast Microscope Examination

Protoplasts were examined by placing some suspension on a 0.1mm counting chamber and observing under magnifications of 300x and 600x on a Zeiss Compact transmitted light microscope KM model 470600, West Germany.

This enabled a visual estimate of protoplast quality and intactness to be made.

3.4.2 Oxygen Evolution

Light-dependent O_2 evolution was checked on every suspension, to see if the protoplasts had normal metabolic activity. Measurements were made at 30°C (the temperature maintained by a circulating water bath) with a YSI (electrode system (Yellow Springs, Ohio, Model 5301). Illumination was provided by a Hanimex Rodette 1200 RF projector focused from 23 cm from the front of the electrode vesel giving an irradiance of 1500 $\mu E/m^2/sec$.

The machine was firstly calibrated using air-saturated distilled water to obtain a maximum O_2 value, and dithionite added for a minimum 0% O_2 value. The vessel was washed out and the assay then performed. 2 mls of a double strength oxygen electrode buffer was added to the electrode vessel with 1.9 mls of H_2O giving a final concentration of

0.6M sorbitol, 2mM KH_2PO_4, 2mM $MgCl_2$, 1mM EDTA and 25mM MES adjusted to pH5.8. (If the incubation to be performed was with bicarbonate instead of acetate the MES buffer was replaced with 25mM HEPES at a pH adjusted to 7.5.) The vessel contents were bubbled with N_2, a base-line established with the electrode, and the protoplasts then added. Protoplast suspension containing approximately 20 μg of chlorophyll was used for most tests, although for experiments concerned with a change in O_2 evolution rates with differing protoplast concentrations different concentrations were used. The rise occurring after protoplast addition was calculated. The light is shielded and the dark rate calculated and deducted.

Values of O_2 evolution are expressed as μmoles O_2/hr/mg chl.

3.5 Determination of Chloroplast Intactness

3.5.1 Phase-Contrast Microscope Examination

The chloroplasts were examined at 600x magnification using a slide with a drop well on a Zeiss Compact transmitted light microscope KM. This allowed a visual estimate of the percentage of Class A chloroplasts to be made.

3.5.2 Oxygen Evolution by Chloroplasts

The oxygen evolution of chloroplasts was checked under the same conditions as for protoplasts (at 30°C, with a YSI oxygen electrode system) except a red filter (Leegood & Walker, 1981) was used in some experiments and the projector was positioned 13 cm from the reaction vessel producing a light intensity of 1000 $\mu E/m^2/sec$.

experiments the red filter was omitted and the light source was placed 23 cm from the reaction vessel producing a light intensity of 1500 $\mu E/m^2/sec$.

The machine was calibrated, 2 mls of a double strength chloroplast oxygen electrode buffer and 1.9 mls of H_2O was added to the electrode vessel (giving a final concentration of 0.4M sorbitol, 10mM EDTA, 10mM NaHCO3, 0.2 KH_2PO_4, 25mm Tricine pH 8.4; the pH suggested by Leegood & Walker, 1981) and the solution was bubbled with N_2 to remove oxygen from the solution. After the baseline was established 100 mls of chloroplast suspension was added, generally containing approximately 20 μg of chlorophyll. The rate was calculated by subtracting the dark value from the rate in light.

Values are again expressed as μmoles O_2/hr/mg chl.

3.5.3 Ferricyanide Test for Chloroplast Intactness

The method used is from Leegood and Walker (1983), based on the original work of McClilley et al (1975). Ferricyanide reduction is catalysed only by naked thylakoids, so the rate of reduction is used inversely to calculate the percent intactness.

The reduction of ferricyanide was followed on a CE 292 Digital Ultra-violet spectrophotometer with recorder at 420nm. In the cuvette was placed 0.89 mls distilled H_2O, 1 ml of a double strength intactness test buffer, 100 mls of chloroplast suspension and lastly 10 mls of a 0.3M potassium ferricyandie solution (giving final concentrations of

0.33M sorbitol, 10mM EDTA, 10mM $NaHCO_3$, 0.2mM KH_2PO_4, 25mM Tricine adjusted to pH 8.4 and 1.5mM Tricine adjusted to pH 8.4 and 1.5mM $K_3Fe(CN)_6$ in a total volume of 2 mls). It was followed on the spectrophotometer until it was possible to accurately calculate the rate of ferricyanide reduction.

This value was compared to the results of an osmotically burst chloroplast sample. The chloroplasts were burst by first mixing 100 mls suspension with 0.89 mls 10mM $MgCl_2$ for one minute. 1 ml of the double strength intactness test buffer was added with the ferricyanide (giving the same final concentrations as before). The rate of ferricyanide reduced was again calculated.

Calculation of the rate of ferricyanide reduction was done by the following method:

ferricyanide reduced /min

$$= \frac{\text{Abs change 1 min}}{\mathcal{E}}$$

$$\mathcal{E} = 1.0 \times 10^3$$

The percent intactness of the chloroplast suspension was calculated by:

$$\text{\% intactness} = \frac{(\text{burst rate} - \text{normal rate})}{\text{burst rate}} \times 100$$

3.6.1 Incubation with Labelled Substrates

3.6.1 Incubation Conditions

Incubations were carried out at 25°C in round bottom 15 ml tubes in a photosynthetic Warburg apparatus. The tubes were shaken at 78 cycles/min at a light intensity of 350 $\mu E/m^2/sec$, an intensity above the irradiance necessary for maximal incorporation values (Sicher, 1982). The light source was provided by sixteen 40 watt tungsten lamps located 4 cm below the tubes.

3.6.2 Incubation of Protoplasts with ^{14}C-Acetate

Protoplasts were incubated in a 0.5 ml volume. The incubation media contained 0.6M sorbitol, 2mM KH_2PO_4, 2mM $MgCl_2$, 1mM EDTA, 25mM MES adjusted to pH 5.8. Acetate concentrations of 300μM was used in later incubations, but in the first incubations lower concentrations of 50μM acetate were used. This generally contained 0.25μCi of ^{14}C-acetate, but in some experiments up to 2.5μCi was used if TLC plates were to be run from samples. A protoplast concentration of approximately 20μg was used. The time of incubation varied in earlier experiments but 3 hours was used in later incubations as this was found to give maximal incorporation while maintaining protoplast integrity. Incubations were stopped by addition of enough chloroform:methanol (2:1, v/v) to form a single phase. Water was then added to give two phases. The top water phase was removed and the remaining chloroform layer was washed successively with 1% acetate acid, 0.1M NaCl and three times with water. Tubes are vortexed and centrifuged in between each washing to mix and separate the phases. The washed chloroform solution was dried under a stream of N_2 on a sand bath and then redissolved in 1 ml

chloroform. A 10 ml aliquot was removed, dried in a scintillation vial, 5 mls of toluene scintillation fluid added and radioactive counts determined, by a liquid scintillation counter.

When the total assimilation of radioactive substrates into plant products was to be examined the reaction was stopped by addition of 0.1 ml 6M HCl. 0.1 mls was removed from the tube, dried on 2 mls circles of 3 mm Whatman paper, 5 mls triton X-100 toluene scintillation solvent added and the radioactivity determined.

3.6.3 Incubation of Protoplasts with ^{14}C-Bicarbonate

Protoplasts were incubated in 0.5 ml volume containing 0.6M sorbitol, 2mM KH_2PO_4, 2 mM $MgCl_2$, 1mM EDTA, 25mM HEPES adjusted to pH 7.5; 10mM ^{14}C-HCO_3 (approximately 1.5 μCi). Approximately 20mg chlorophyll of protoplasts were used. Incubations were, in later experiments, carried out for 1½ hours.

When bicarbonate incorporation into lipid was measured incubations were stopped by chloroform:MeOH (2:1, v/v) addition and then water. The top water phase was removed and the chloroform was washed with 1% $NaHCO_3$, 0.1M NaCl and three times with water. The sample was dried under a stream of N_2, made up to 1 ml and 10 μl removed for counting in toluene scintillation fluid.

When total assimilation was examined, the reaction was stopped by adding 0.1 ml 6 M HCl, 0.1 mls was removed, dried on 3MM Whatman paper

circles, triton X-100 toluene scintillant added and the radioactivity determined.

3.6.4 Incubation of Chloroplasts with ^{14}C-Acetate

Chloroplasts were incubated in a 0.5 ml volume. 100 μl chloroplast suspension (generally containing approximately 20 μg chlorophyll) was incubated in a media that contained 0.4M sorbitol, 10mM EDTA, 10mM $NaHCO_3$, 0.2 mM KH_2PO_4, 25mM Tricine pH 8.4, 10mM ATP, 5mM CoA, 50mM DTT, 4mM NADPH, 4mM NADH, 600mm $NaHCO_3$ (the co-factor concentrations were from McKee, 1979) an acetate concentration of 0.2mm containing 0.25 μCi but in some experiments up to 2.5 μCi of ^{14}C-acetate. Incubations were carried out for 1 hr.

Incubations were stopped and extractions made in the same way as described for protoplast experiments.

3.7 Separation and Identification of Lipids by Thin Layer Chromatography

3.7.1 Prepartion of Plates

The separation of individual lipids after extraction was achieved by thin layer chromatography (TLC) on silica gel type G layers. Glass plates of 5cm x 20cm and 10cm x 20cm were spread with the gel mixed into a slurry with distilled water (1:2 w/v) to a thickness to 0.25mm by a commercial spreader, (Desaga, Heidelberg, Germany). Plates were dried at room temperature for 30 mins and then transferred to a 110°C oven for 3 hrs. They were cooled and stored in a dessicator with silica gel beads to remove air moisture.

3.7.2 Conditions

The lipid was applied to the plate as a narrow band approximately 2 cm long by use of a 25 μl syringe. Markers were placed on either side as single spots. A number of different solvent systems were used in developing the plates. Generally chloroform:MeOH:acetic acid:H_2O (85:15:10:3.5, v/v) was used, with a total lipid extract as marker, for running the total lipid sample. The chromatography tank was lined with blotting paper. After running, the plates were air dried.

3.7.3 Detection of Lipids on Chromatograph

After drying a radioactive marker was applied above the solvent front and the plates strip scanned using a Radiochromatogram scanner. This gave an indication of the distribution of radioactivity. The markers were then either sprayed with a 0.1% (w/v) solution of 2,7 dichlorofluorescein in methanol and viewed under uv light, or the plate was placed in a tank with a few iodine crystals.

The positions of the marker lipids were compared with the trace of the strip scanner to identify the radioactive lipids.

3.7.4 Elution of Lipids from Silica Gel

Lipid bands were extracted by scraping the silica gel off the plate and eluting with chloroform:methanol (2:1,v/v) or for the more polar lipids with chloroform:MeOH:acetic acid:H_2O (97:97:4:2, v/v). The gel is eluted three times and washings dried under a stream of oxygen-free nitrogen. It was made up to 1 ml with chloroform and counts determined on 10 μl in a liquid scintillation system.

Lipid bands were often re-chromatographed in a different solvent system. The solvent system toluene:ethyl acetate:95% ethanol (2:1:1, v/v) (Gray et al., 1967) was used to separate digalactosyl diglyceride and phosphatidyl glycerol but it was also often used for the first chromatographic separation of lipids in a sample. The more non-polar lipids were examined by running in hexane:diethyl ether: acetic acid (70:30:1, v/v) (Mangold and Malins, 1960); (50:50:1, v/v) or hexane:diethyl ether (9:1, v/v) (Marlins et al., 1965).

After lipids were chromatographed for a second time they were eluted off and re-chromatographed in the same solvent system to purify. Radioactive counts were again determined. The percent recovery was calculated after each chromatographic step.

Some plates were used to make autoradiographs to clearly define radioactive bands on the plate.

3.8 Isolation of Lipid from Barley Leaves

Young barley tissue was grown and harvested in the manner previously described. It was cut into approximately 5 cm tranverse sections and immediately blended in a Waring blender with sufficient chloroform:methanol (2:1, v/v) to form a single-phased suspension. It was filtered through Mira-cloth into a separating funnel and left to separate into two phases. The bottom green chloroform phase was transferred into a clean separating funnel, the water phase was

discarded. The Mira-cloth and filtrate were cut into pieces, placed in a round bottom flask and refluxed with chloroform:methanol (2:1, v/v) for 30 mins. The washings were poured off and collected. The filtrate was re-washed with additional chloroform:methanol (2:1, v/v) and refluxed for a further 15 minutes. These washings were also collected. The two washings and the original chloroform mixture were pooled and washed four times with water. The extract was dried down on a rotary evaporator and made up to a known volume with chloroform.

3.9 Determination of the Concentrations of Non-Polar Lipids in Barley Leaves.

1.5 mls of the whole lipid extract was plated onto several silica gel plates. Triglyceride, diglyceride, free fatty acid and monoglyceride markers were placed on the outside zones of the plate. The plates were run in hexane:diethyl ether: acetic acid (70:30:1, v/v) and sprayed with dichloroflorescein and viewed under uv light. The areas corresponding to the marker compounds were scraped off the plate, being careful not to include the markers in with the sample, and eluted from the gel by three sucessive chloroform:methanol (2:1, v/v) additions. They were dried down using N_2 and re-run separately in the same solvent system to purify, except for the monoglyceride sample which was re-run in the more polar hexane:diethyl either: acetic acid (50:50:1, v/v) solvent mixture in which it moves higher up the TLC plate. The samples were again eluted off with chloroform:methanol (2:1, v/v), and dried with N_2.

3.10 Preparation of Methyl Esters

Methyl esters were prepared by the method of Van Wyngarden (1976).

The sample to be used was placed in a round bottom tube and dried under a stream of N_2. 10 mls of a 17 carbon fatty acid solution in chloroform (2 mg/ml) was added to the sample to serve as internal standard for quantification.

2 mls of 0.5M methanolic NaOH was added. The tube was attached to a water condenser, anti-bumping granules added, and refluxed on a sand bath for 2 mins. 2 mls of 14% boron trifluoride in methanol (w/v) was added through the condenser and the refluxing continued for a further 3 mins. 5 mls of hexane was added and the tube was removed from the sand bath and allowed to cool. Water was added to bring the hexane layer to the top of the tube. The tube was centrifuged to clearly define the phases and the hexane layer transferred to a glass stoppered tube using a pasteur pipette.

The bottom layer was washed 2 more times with hexane vortexing between washings and centrifuging. The washings were added to the stoppered tube, and the hexane removed with a stream of N_2. The methyl esters were applied to TLC plates and chromatographed in hexane:diethyl ether (9:1, v/v) along with methyl palmitate as marker. The plate was sprayed with dichloroflorescein and looked at under uv light. The methyl ester spot was identified and removed by scraping the gel from the plate and eluted by three successive washes with diethyl ether. The sample was dried under N_2 and redissolved in approximately 100 μl of hexane.

When lipids with radioactive substrates incorporated were being made into methyl esters the radioactivity was determined from an aliquot of a known volume both before and after methylation and after elution from the TLC plate.

3.11 GLC of Methyl Esters

Methyl esters of fatty acids were analysed using Varian aerograph model 1520B gas chromatograph fitted with a flame ionisation detector. The glass column (183 cm x 0.3 cm) was packed with 9% EGSS-X on chromosorb Q (100/120 mesh).

The column had an effluent stream splitter diverting three quaters of the sample to the collector jet and the remaining quarter to the flame ionisation detector. A standard sample containing 16:0, 18:0, 18:1, 18:2, and 18:3 was injected into the column at the beginning and end of a days use. The samples were injected in a volume of approximately 10 μl.

The column was held at 164°C with injector temperature at 210°C and detector temperature at 190°C. Oxygen-free N_2 was the carrier gas flowing at 32 mls/min with hydrogen and air supplied to the detector at 17 mls/min and 250 mls/min respectively.

When radioactive samples were run before loading onto the gas chromatograph they were spiked with a linseed oil standard to make peaks on the chart recorder visible. The radioactive effluent of each fatty acid was collected in a pyrex tube loosely packed with glass wool and moistened with scintillation solution. The methyl esters were

eluted from the collection tubes by addition of two 5 ml scintillation fluid aliquots, flushed through with a syringe. For each sample the scintillation fluid was collected in a vial and radioactive counts determined.

3.12 Determination of Radioactivity

Non-aqueous samples were counted in a toluene scintillation solvent containing 0.4% PPO and 0.01% POPOP, (w/v). Aqueous samples from experiments examining total assimilation were counted in a Triton X-100:toluene (1:2 v/v) scintillation solvent. Radioactivity was determined with a Beckman Model LS8000 scintillation counter. Radioactivity scans of TLC plates were made by scanning on a Packard Model 7200 Radiochromatogram Scanner. Counting conditions were 1.26% iso-butane in helium (v/v) flowing at 120 mls/min, voltage: 1.15 Kv, slit width: 2.5mm, time constant: 30s or 10s, linear range: 100,300,1000 or 3000 and scanning rate : 0.5cm/min.

Autoradiographs of TLC plates were obtained by exposure to Agfa-Gevaert Osray M3 X-ray film for up to 5 weeks in a light-proof box. Films were processed with kodak liquid X-ray developer and fixer.

CHAPTER 4. RESULTS

4.1 PROTOPLAST ISOLATION AND INCUBATION

4.1.1 Viability of Protoplasts

(a) Visual Examination of Protoplasts

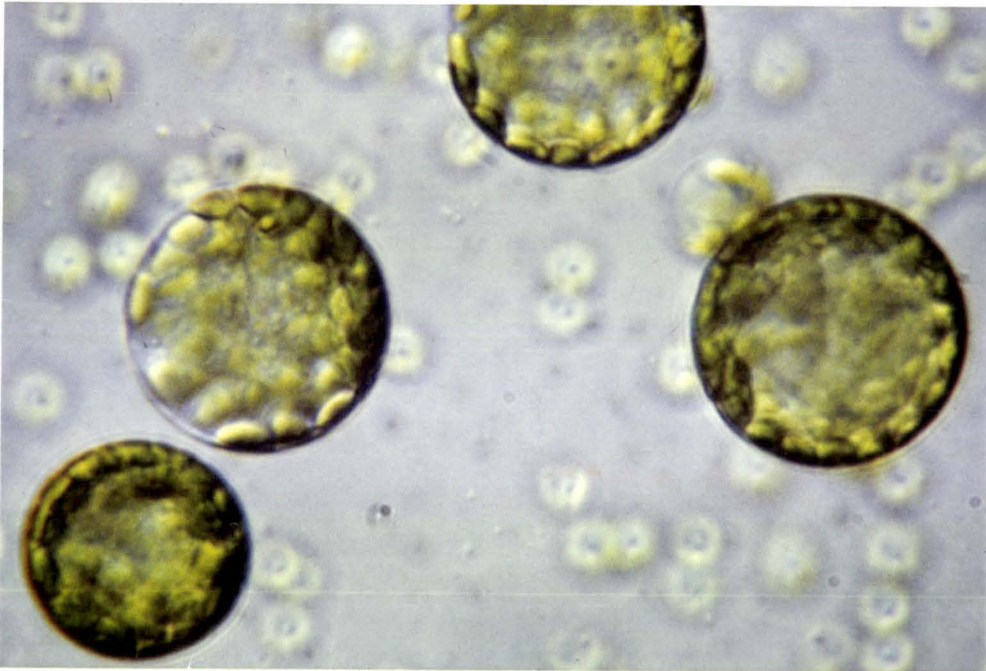
Barley protoplasts, successfully prepared by the method of Day *et al*. (1981) were of higher quality then when maize was used, the tissue suggested in Day's method. Examination of the maize protoplasts directly after isolation under phase contrast microscope (as described in the Methods section 3.4.1) showed intact protoplasts but with the chloroplasts aggregated together on one side of the cell. 'Lop-sided' protoplasts have been obtained by other workers (Kanai and Edwards, 1973). There were many free chloroplasts present in the preparation.

Protoplasts from barley plants were isolated by the same method. Visual examination showed less free chloroplasts with 90-95% of the protoplasts intact (see figure 4.1). The occurrence of protoplasts with chloroplasts clumped on one side, as seen with the maize preparations, was rare. Barley leaf was used as the tissue source in all preparations used in incubations.

(b) Protoplast Oxygen Evolution Values

Light-dependent oxygen evolution values were used as a qualitative determination of protoplast integrity. They were calculated using the O_2 electrode on each protoplast isolation to check whether the suspension was suitable for incubations. Results showed that the quality of the protoplasts isolated seemed partially dependent on the

Figure 4.1 Photograph of Protoplasts from Barley Leaf



Prepared as described in the Methods section 3.1.
Magnification 500x

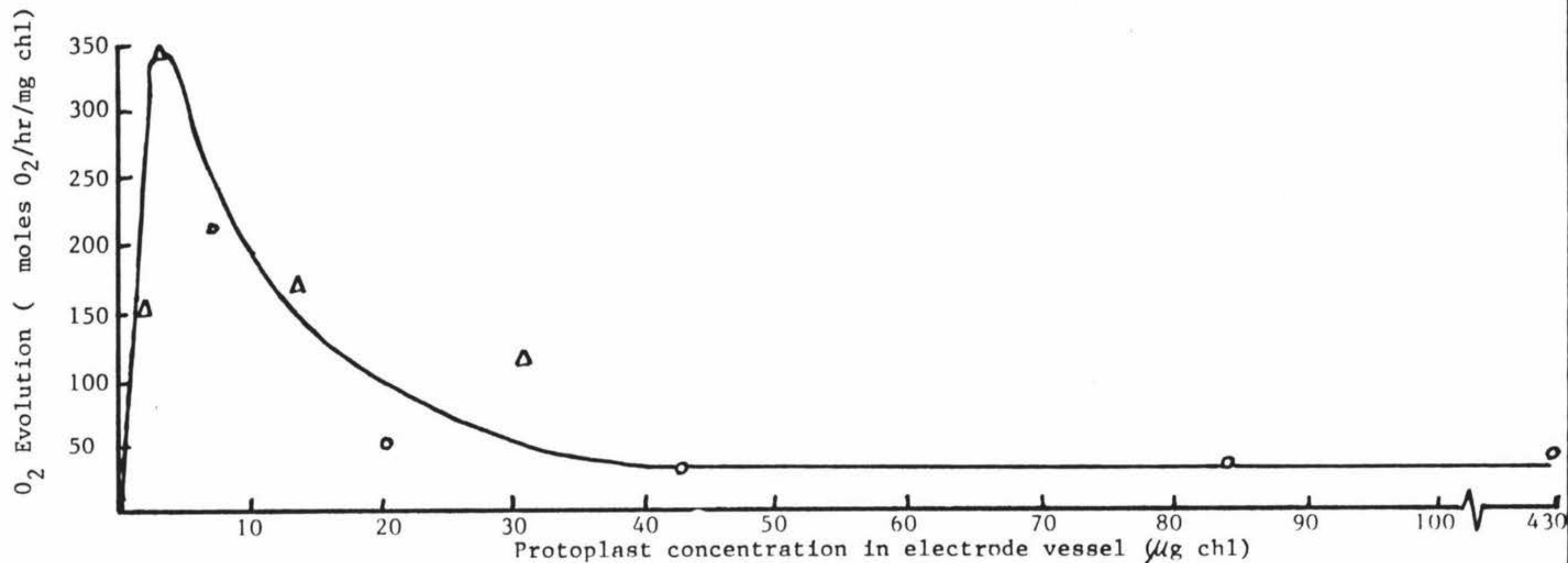
experience of the worker. In the first four months of work the average O_2 evolution value was 90.20 μmoles O_2/hr/mg chl from 14 isolations compared to an average of 152.88 μmoles O_2/hr/mg chl from 9 isolations over the rest of the research period. These results compare favourably with the studies done by other workers; for example spinach gave 33-40 μ moles O_2/mg chl/hr (Nishimura & Akazara, 1975), wheat 86.2 (Leegood & Walker). Kuhn & Stumpf use a value greater than 100 μmoles O_2/hr/mg chl is needed for determining that there is normal metabolic activity.

O_2 evolution values were better if performed at pH 7.5 with HEPES buffer (as when testing integrity for a ^{14}C-bicarbonate incubation) than at pH 5.8 with MES buffer (for a ^{14}C-acetate incubation), by approximately 20%.

A variation is protoplast concentrations in the oxygen electrode was examined to determine whether it had an effect on the O_2 evolution rate. The results (see figure 4.2) showed a peak rate at approximately 2.5 μg chlorophyll concentration, then curving down and leveling at approxmately 50 μg.

An explanation for this observation is that the protoplasts in higher concentrations "shadow" each other, cutting out light supply to cells behind one another.

Figure 4.2 The Effect of Protoplast Concentration on Oxygen Evolution by Barley Protoplasts



Method for measuring oxygen evolution values is as described in Methods section 4.2. Values are from two independent experiments as indicated.

(c) The Variation in Protoplast Yield and Quality Using Different Barley Cultivars.

In the preparation of protoplasts a variation in yield and quality from different seed batches was noticed. This led to an experiment examining the effect of different barley cultivars as the tissue source. There are four types of barley used principally in New Zealand. For each, seeds were obtained, tissue grown and used for isolating protoplasts. Separately 2.5g of tissue was weighed; abraded and cut in the normal manner, digested in 20 mls of digestion media and isolated by the procedure stated in the methods section 3-1. Each were made up to 1 ml of suspension with solution A.

Observation of each suspension under the phase microscope showed no recognizable difference in the appearance of protoplasts from each cultivar. The O_2 evolution rates were determined for use in judging whether there was any appreciable difference in the qualtiy of protoplasts from each cultivar. Yield was calculated from the chlorophyll determination (see table 4.1).

The difference in cultivar for isolating protoplasts appeared to have little effect on the quality of the protoplasts produced. The oxygen evolution values are all within one standard deviation of the mean. The yield values are similar between all cultivars with the exception of Triumph which is lower. This plant breed has a more fibrous appearance than the other three.

Table 4.1. The Variation in the Yield and Quality of Protoplasts Isolated from Different Cultivars of Barley Tissue.

Cultivar	Yield (chlorophyll determination mg/ml)	Quality (O_2 evolution rate moles O_2/hr/mg/ml)
Georgie	308.1	57.7
Goldmarker	310.8	82.2
Fleet	332.5	61.5
Triumph	248.2	93.6

Protoplasts were isolated as described in the Methods section 3.1. Chlorophyll determination was preformed as described in the Methods section 3.3, O_2 evolution rate in Methods section 3.4.2.

All cultivars performed together on the same day under the same conditions. Figures are the average of two results, performed in separate experiments.

4.1.2 Incubations of Barley Protoplasts with Radioactive Substrates

(a) ^{14}C-Acetate Assimilation by Barley Protoplasts.

Incubations were carried out as described in the Methods section 3.6. Several experiments were initially performed varying the reaction conditions to determine maximal incorporation and assimilation of ^{14}C-acetate by the protoplasts. For each a separate non-radioactive incubation was also run allowing a check on protoplast integrity to be made after the incubation. This was determined by visual examination under the phase contrast microscope.

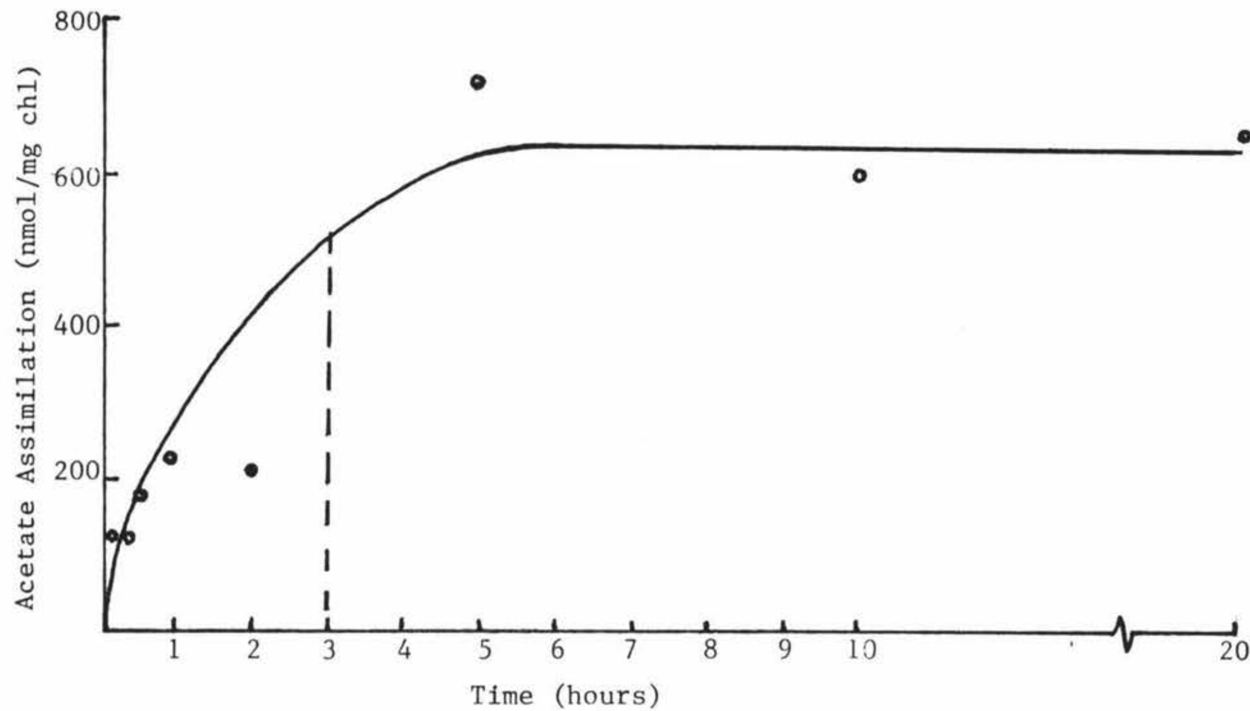
(i) Effect of length of incubation period on ^{14}C-acetate assimilation and incorporation into total lipids by barley protoplasts.

Separate protoplast samples were placed in incubation media for 5 min, 10 min, 20 min, 30 min, 1 hr, 5 hr, 10 hr and 20 hr. Examination of the non-radioactive sample showed that up to 2 hr they were in good condition. The 5 hr sample was partly damaged, the 10 hr had a higher number of broken protoplasts, and in the 20 hr sample the protoplasts were grossly damaged, appearing clumped together and bleached.

Incorporation and assimilation rates (see figures 4.3, and 4.4) start to slow at 3 hrs remaining stationary at 5 hrs. This is attributed to the damage occurring to the protoplasts, at longer incubation times, making them unable to utilise ^{14}C-acetate.

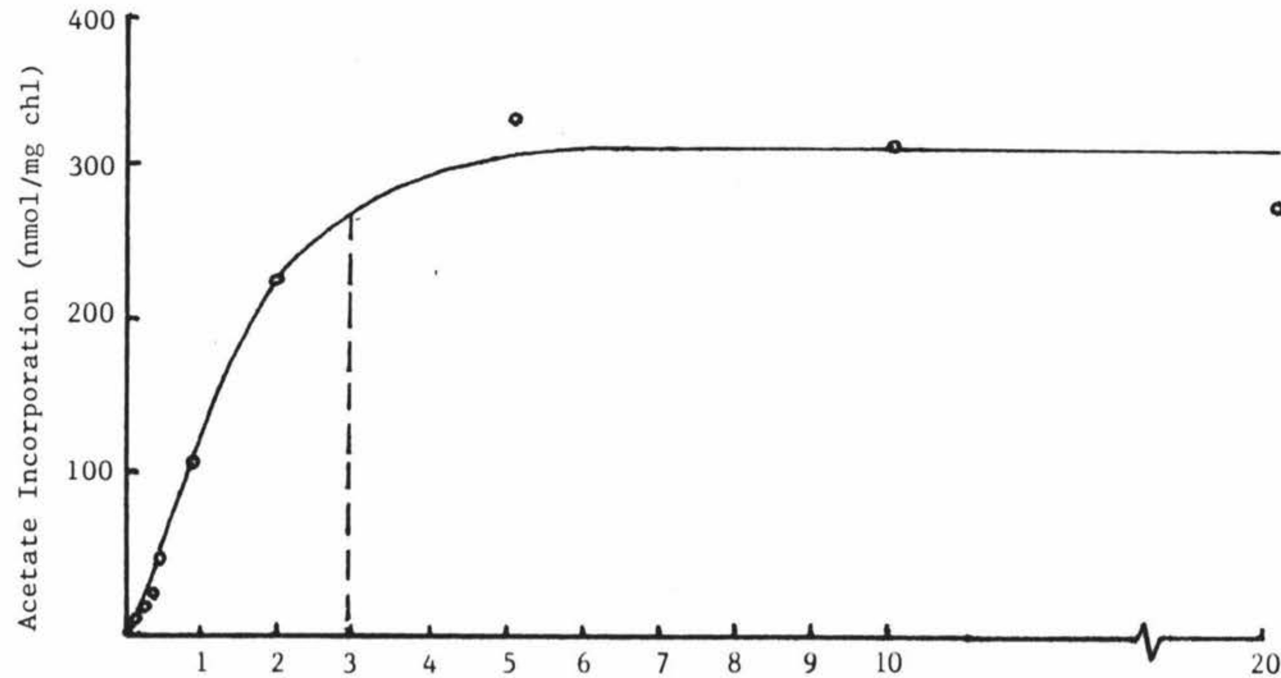
All subsequent incubations were performed for a 3 hr period.

Figure 4.3 The Rate of 1-^{14}C-Acetate Assimilated by Barley Protoplasts



Incubations were carried out as described in the Methods section 3.6.
Length of incubation time was varied. Values are from a single experiment

Figure 4.4 The Rate of 1-^{14}C-Acetate Incorporated into Total Lipid by Barley Protoplasts



Incubations carried out as described in the Methods section 3.6.
Length of incubation time was varied. Values are from a single experiment.

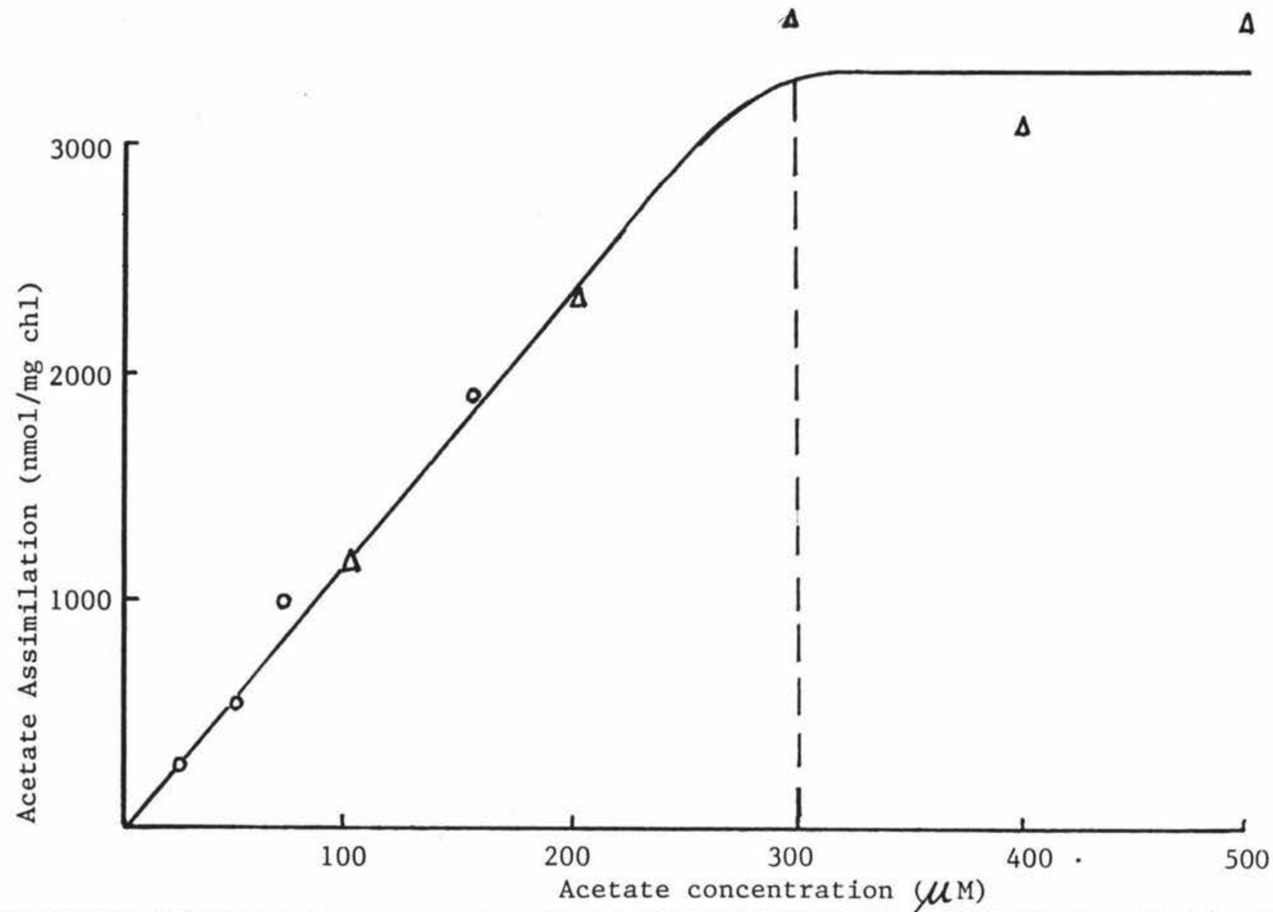
(ii) The effect of acetate concentration on ^{14}C-acetate assimilation and incorporation into total lipids by barley protoplasts.

Protoplasts were incubated in a range of acetate concentrations, from 0-500 μM. All non-radioactive samples showed protoplasts in good condition. The graphs of assimilation (figure 4.5) and total lipid incorporation (figure 4.6) show a steady increase in the range of 0-300 μM followed by a plateau. In all later incubations an acetate concentration of 300 μM was used. In these maximal conditions, 50-65 % of the label assimilated was being used for lipids synthesis. In all later incubations, an acetate concentration of 300 μM was used. Previously concentrations of 50 μM had been used (Bell, 1983; Kuhn & Stumpf, 1981) with incorporation results of approximately 1000 nmoles acetate/mg/chl/hr (Kuhn & Stumpf, 1981).

(iii) The effect of bicarbonate addition on ^{14}C-acetate assimilation and incorporation into total lipids by barley protoplasts.

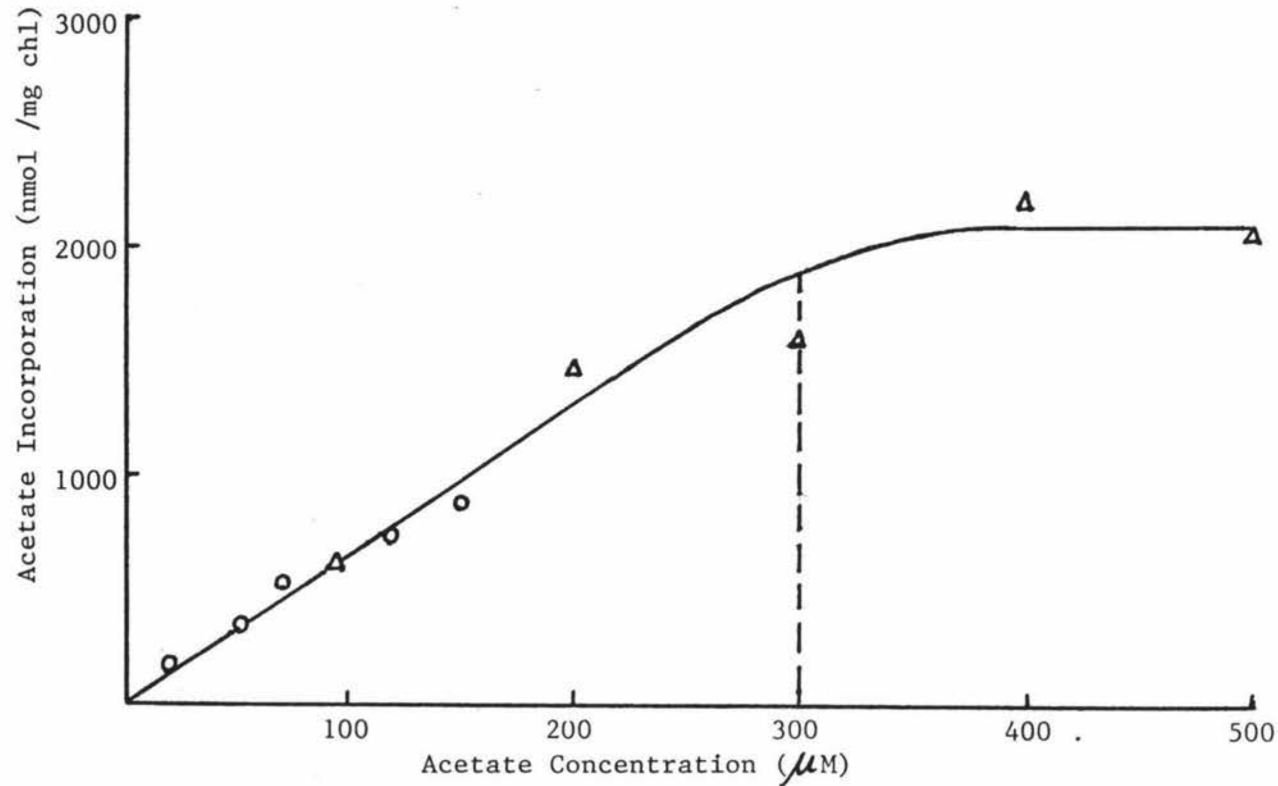
Protoplasts were incubated with bicarbonate in the concentration range of 0-10 mM. The graph of assimilation (figure 4.7) and total lipid incorporation (figure 4.8) showed a steady decrease with increasing bicarbonate. This demonstrated that the bicarbonate enters the same pool as the acetate and that the acetate is not a preferential substrate.

Figure 4.5 Variation in (1-^{14}C) acetate Assimilation by Barley Protoplasts with Differing Acetate Concentrations



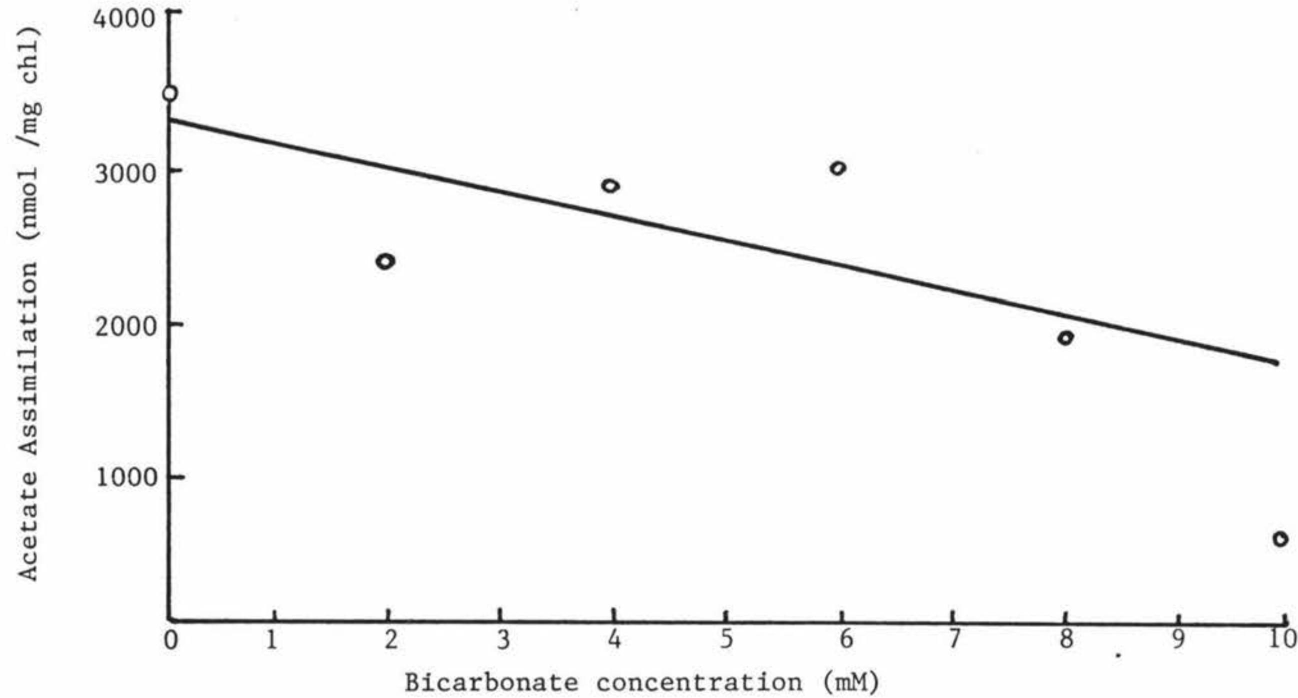
Incubations were carried out as described in the Methods section 3.6. Concentration of acetate was varied. Values are from two separate experiments as indicated.

Figure 4.6 Variation in (1-^{14}C) Acetate Incorporation into Total Lipids by Barley Protoplasts with differing acetate concentrations.



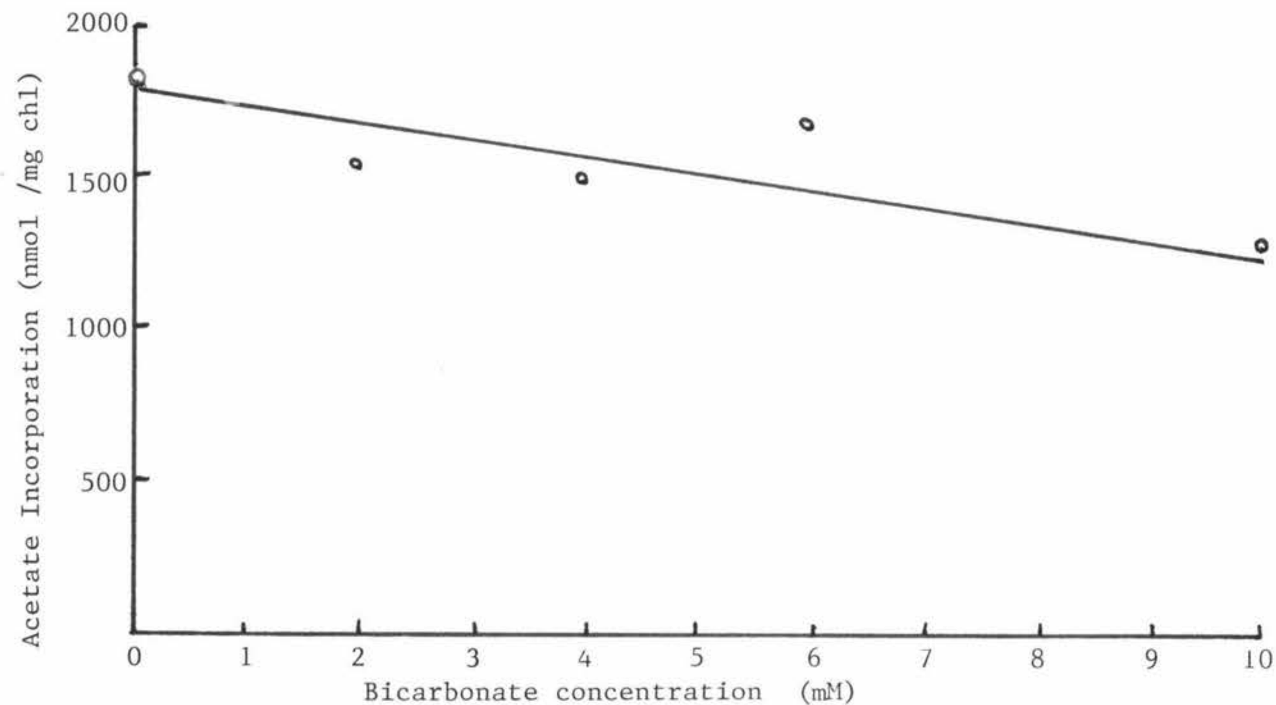
Incubations were carried out as described in the Methods section 3.6 . Concentration of acetate was varied. Values are from two separate experiments as indicated.

Figure 4.7 The Effect of Bicarbonate on (1-^{14}C) Acetate Assimilation by Barley Protoplasts



Incubations were carried out as described in the methods section 3.6, with the addition of a range of bicarbonate concentrations. Values are from a single experiment.

Figure 4.8 The Effect of Bicarbonate on (1-^{14}C) acetate Incorporation into Total Lipids by Barley Protoplasts



Incubations were carried out as described in the Methods section 3.6, with the addition of a range of bicarbonate concentrations. Values are from a single experiment.

(iv) Altering the pH during an incubation of (1-^{14}C)acetate with barley protoplasts to improve assimilation and incorporation into total lipids.

An attempt to improve the incorporation of ^{14}C-acetate into lipids was made by shifting the pH of the incubation media from 5.8 to pH 7.5. pH 5.8 is the maximal pH for transporting acetate into the cell whereas the lipid biosynthetic enzymes have optimal activity at pH 7.5. This was achieved by use of a 100 ml incubation solution, identical in composition to the normal incubation media except the 25mM MES was lowered to 5mM. This "placebo" solution was adjusted first to pH 5.8 with NaOH, left 3 hrs and the pH rechecked, then 10 mls 500mM HEPES solution added and addition of 1.2 mls of 2M NaOH required to change the pH to 7.5. The pH was re-checked after 2 hrs. The volume of HEPES and NaOH required to adjust the pH for the volume of a normal incubation was calculated from these above results. An incubation was carried out in tubes incubated in the normal manner (incubated for 3 hrs with 25mM MES in the media), tubes with NaOH and HEPES addition mid-way in the incubation in a 5mM MES incubation media, and tubes with addition of HEPES only midway. Addition of 25 mls of 500mM HEPES, and 100 μl 1M NaOH (0.01 nmoles) was made after three hours incubation and the incubations carried out for a further 2 hrs.

Values of assimilation and incorporation into total lipid were compared (see table 4.2).

HEPES addition without NaOH (changing the molarity without changing the pH) decreased lipid incorporation. HEPES addition with NaOH changes

Table 4.2 The Effect of Changing the pH from 5.8; to 7.5 during an Incubation on ^{14}C-Acetate Assimilation and Incorporation into Total Lipids.

	HEPES addition	HEPES + NaOH addition
Assimilation	95.46%	162.39%
Total Lipid	50.85%	83.05%

Results are expressed as a % of the results obtained for an incubation with protoplasts peformed in the normal manner (see methods section 3.6) for 3 hrs incubation time. Samples with additions were incubated 3 hrs, in an identical incubation media except for 5mM MES replacing the normal 25mm MES. Additions were made after this time and the samples incubated for a further 2 hrs. All samples were started at pH 5.8. The experiment was done in duplicate, results averaged.

the pH of the medium and unexpectdly caused increased assimilation but did not increase incorporation into lipid. The alteration in pH mid-way through the incubation did not improve the incorporation of radioactive label into lipid products as had been predicted.

(v) Comparison of the assimilation and incorporation into total lipids of ^{14}C-acetate by barley protoplasts under light and dark conditions.

Protoplasts were added to a normal incubation media but in tubes blackened by wrapping in aluminium foil. They were placed in the incubation apparatus and incubated in the normal manner. Samples without aluminium foil wrappings were run at the same time. The experiment was performed 4 times. Results gave an assimilation value 34% of that obtained in light, total lipid incorporation was 9% of the light value. Dark assimilation has previously been found to be $^{1}/_{3}$ of the light value (Kanangara et al., 1971).

(b) ^{14}C-Bicarbonate Assimilation and Incorporation into Total Lipids by Barley Protoplasts.

Incubations were performed by the method described in section 3.6 of the methods. Experiments examining the effect of varying incubation time and bicarbonate concentration were performed to determine maximal conditions for ^{14}C-bicarbonate assimilation and incorporation into lipids. In each experiment non-radioactive incubations were also run to allow a visual check, using the phase-contrast microscope, to be made of protoplast integrity.

(i) Effect of length of incubation period on ^{14}C-bicarbonate assimilation and incorporation into total lipids by barley protoplasts.

Protoplast samples were incubated for differing time periods, the longest being 20 hrs. The non-radioactive samples showed that protoplasts were of good quality up to 10 hr where approximately half the protoplasts were broken. The 20 hr sample on examination had few intact protoplasts.

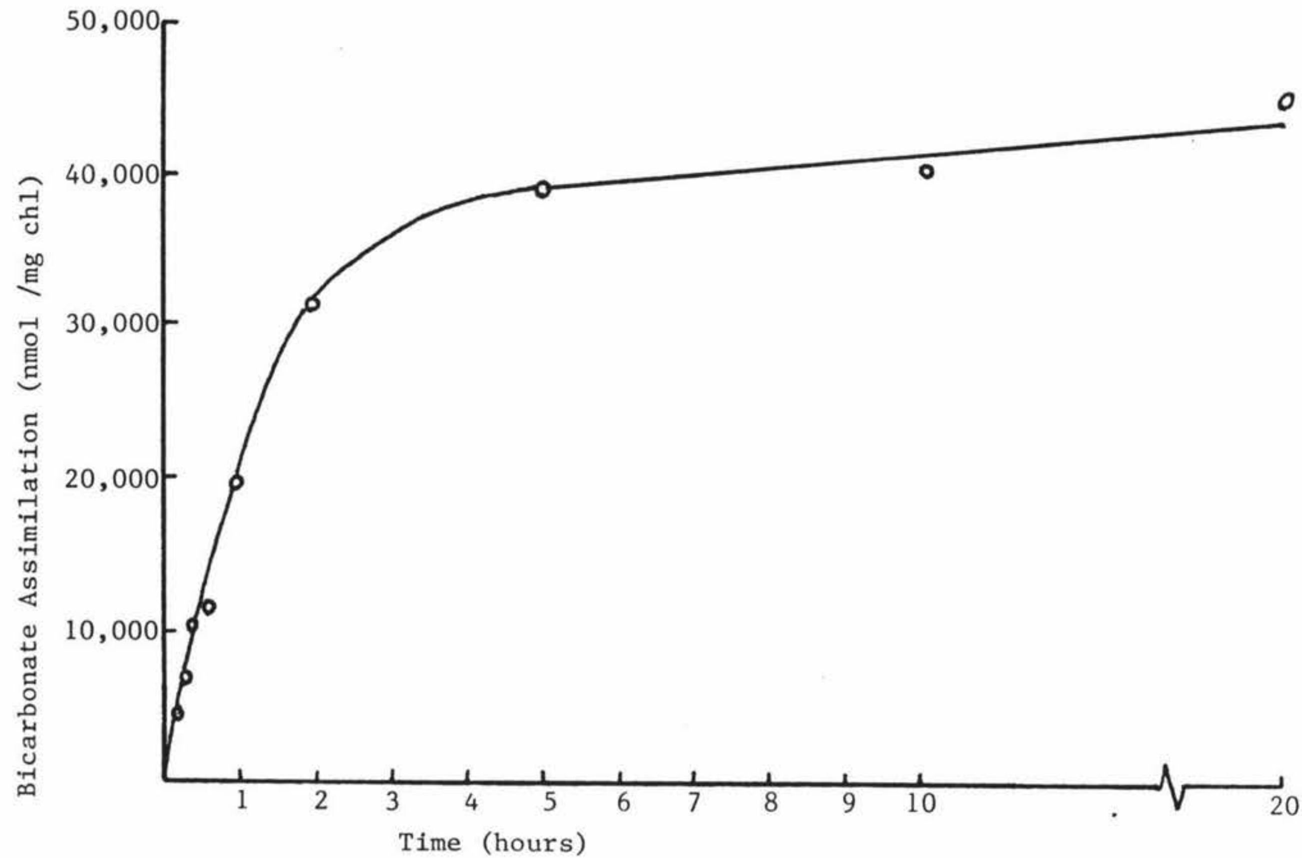
However, incorporation and assimilation values (see figures 4.9 and 4.10) leveled at approximately 1 $^1/_2$ hrs, although the 5 hr sample examined showed protoplasts to be of good integrity. Bicarbonate appears to have a more rapid entry than acetate. After the 1 $^1/_2$ hr point the assimilation still increased while the lipid incorporation decreased suggesting the lipids synthesised were being broken down. 1 $^1/_2$ hrs was used as the incubation time for all later ^{14}C-bicarbonate incubations.

(ii) The effect of bicarbonate concentration on ^{14}C-bicarbonate assimilation and incorporation into total lipids by barley protoplasts.

Protoplasts were incubated in a range of bicarbonate concentrations from 0-30mM. All non-radioactive samples, in each different bicarbonate concentration over the range, had protoplasts intact.

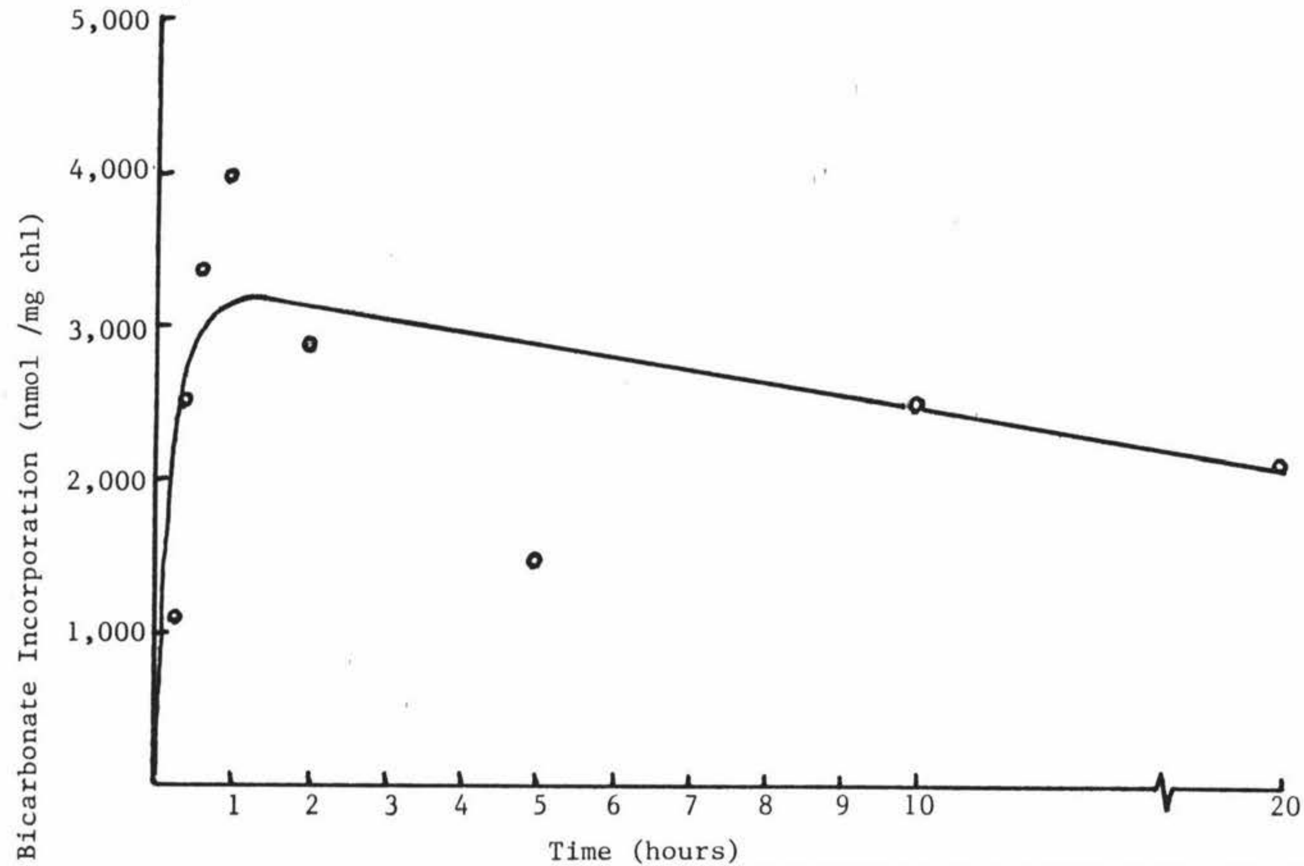
The graph of assimilation (figure 4.11) starts to slow at approximately 10mM bicarbonate, the incorporation into lipid graph (figure 4.12)

Figure 4.9 The Rate of ^{14}C Bicarbonate Assimilation by Barley Protoplasts



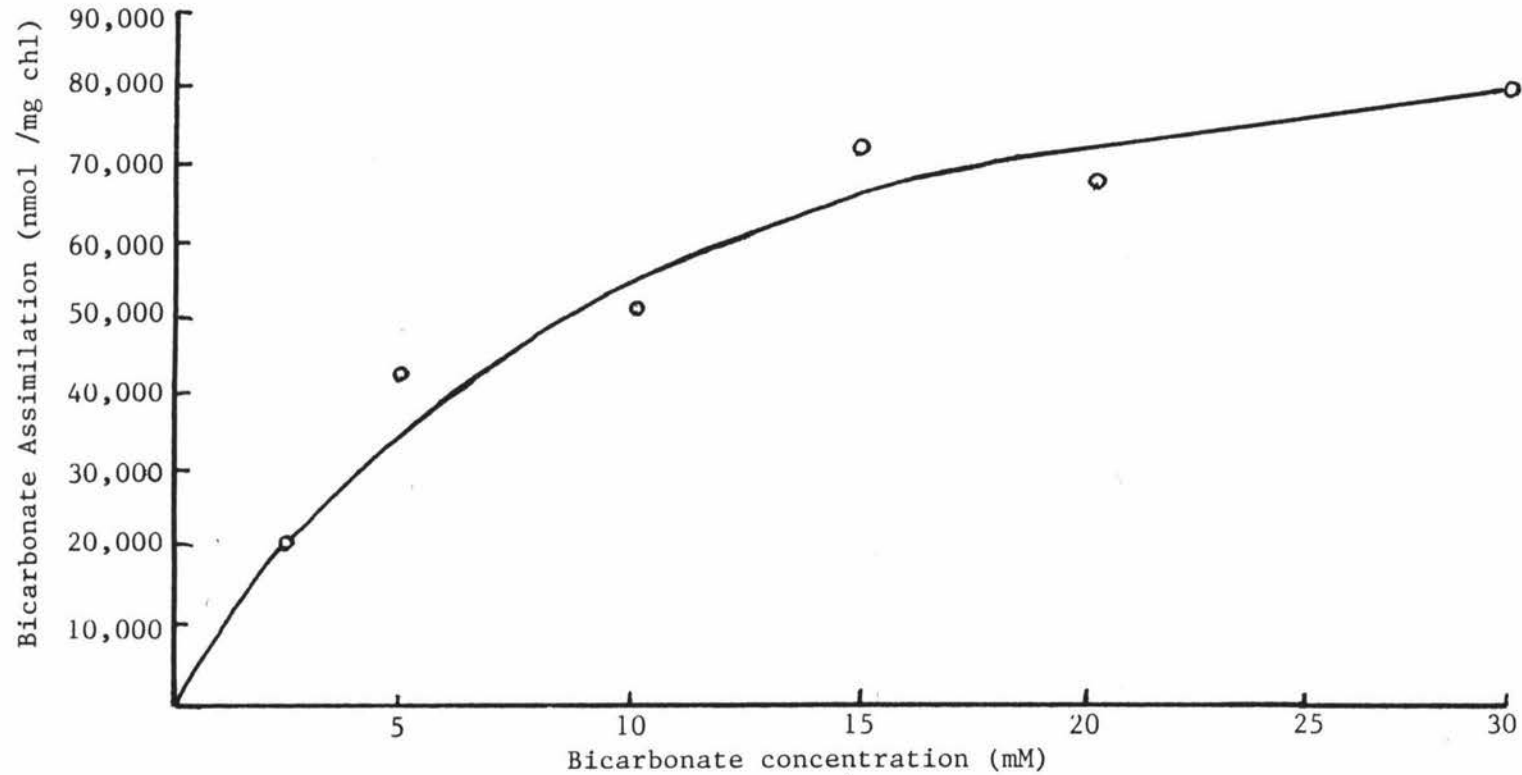
Incubations were carried out as described in the Methods section 3.6. Length of incubation time was varied. Values are from a single experiment.

Figure 4.10 The Rate of ^{14}C-Bicarbonate Incorporation into Total Lipid by Barley Protoplasts



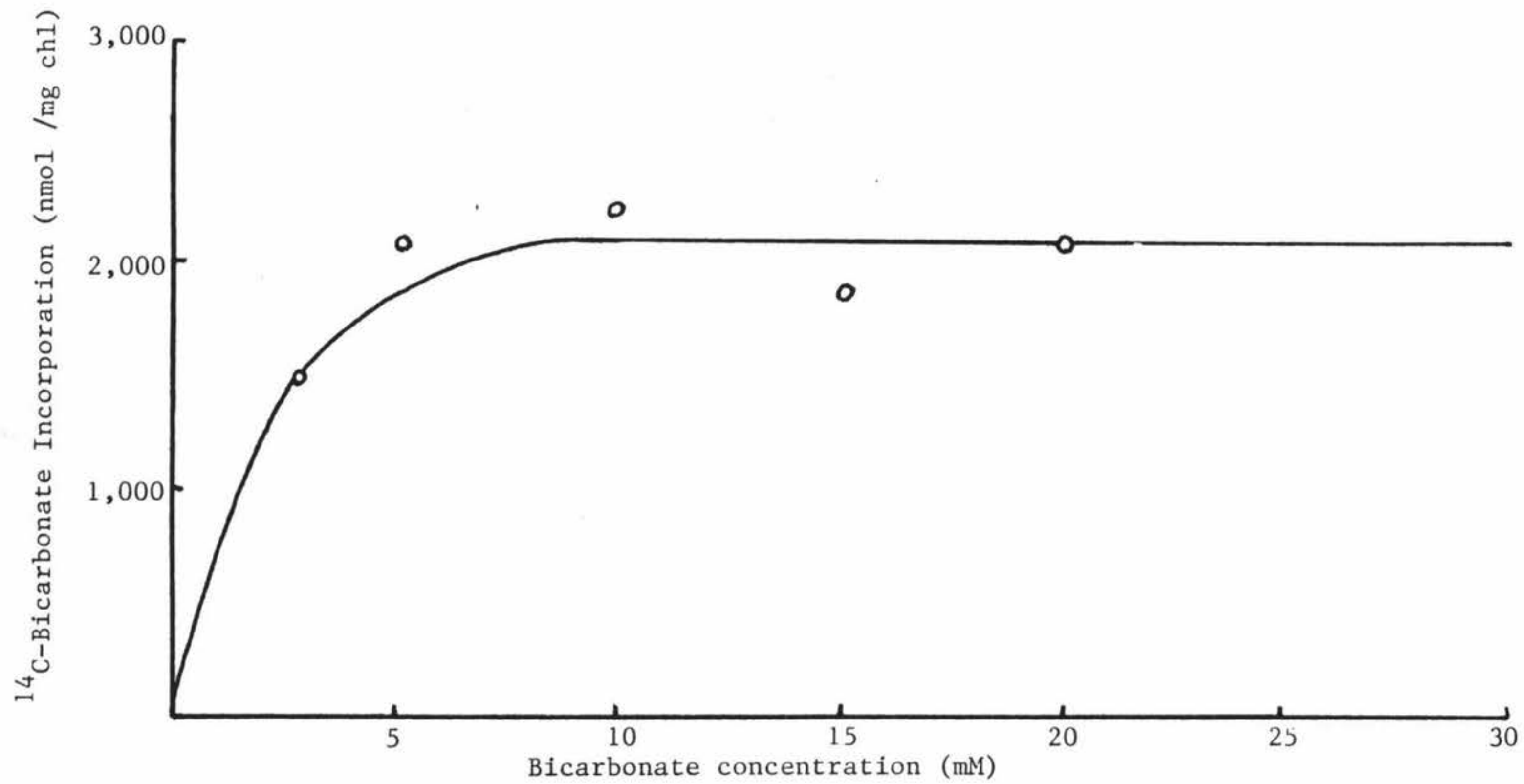
Incubations were carried out as described in the Methods section 3.6 length of incubation time was varied. Values are from a single experiment.

Figure 4.11 Variation in ^{14}C-Bicarbonate Assimilation by Barley Protoplasts with differing Bicarbonate Concentrations



Incubations were carried out as described in the Methods section 3.6. Concentration of bicarbonate was varied. Values are from a single experiment.

Figure 4.12 Variation in ^{14}C-Bicarbonate Incorporation into Total Lipids by Barley Protoplasts with Differing Bicarbonate Concentrations



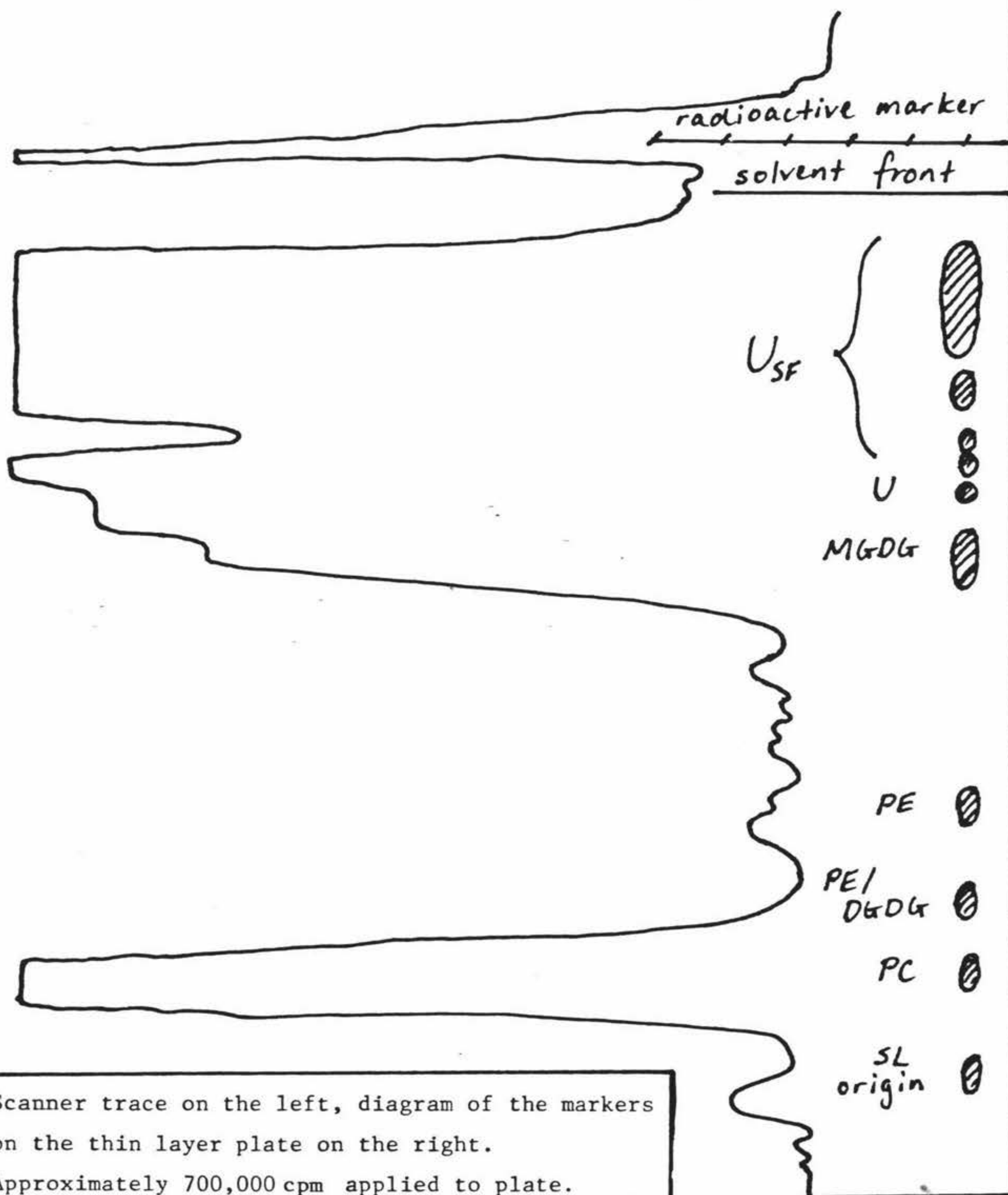
Incubations were carried out as described in the methods section 3.6. Concentration of bicarbonate was varied. Values are from a single experiment.

levels off at a lower bicarbonate level. All subsequent bicarbonate incubations were carried out with 10mM bicarbonate in the incubation medium. The incorporation results obtained by other workers for ^{14}C-bicarbonate and for $^{14}CO_2$ have been in a similar range, eg; spinach leaf protoplasts gave incorporation values of 3300 nmoles/mg chl/hr (Nishimura and Akazawa, 1975).

4.1.3 Thin Layer Chromatography of Total Lipids Extracted from Protoplasts After Incubation with Acetate and Bicarbonate.

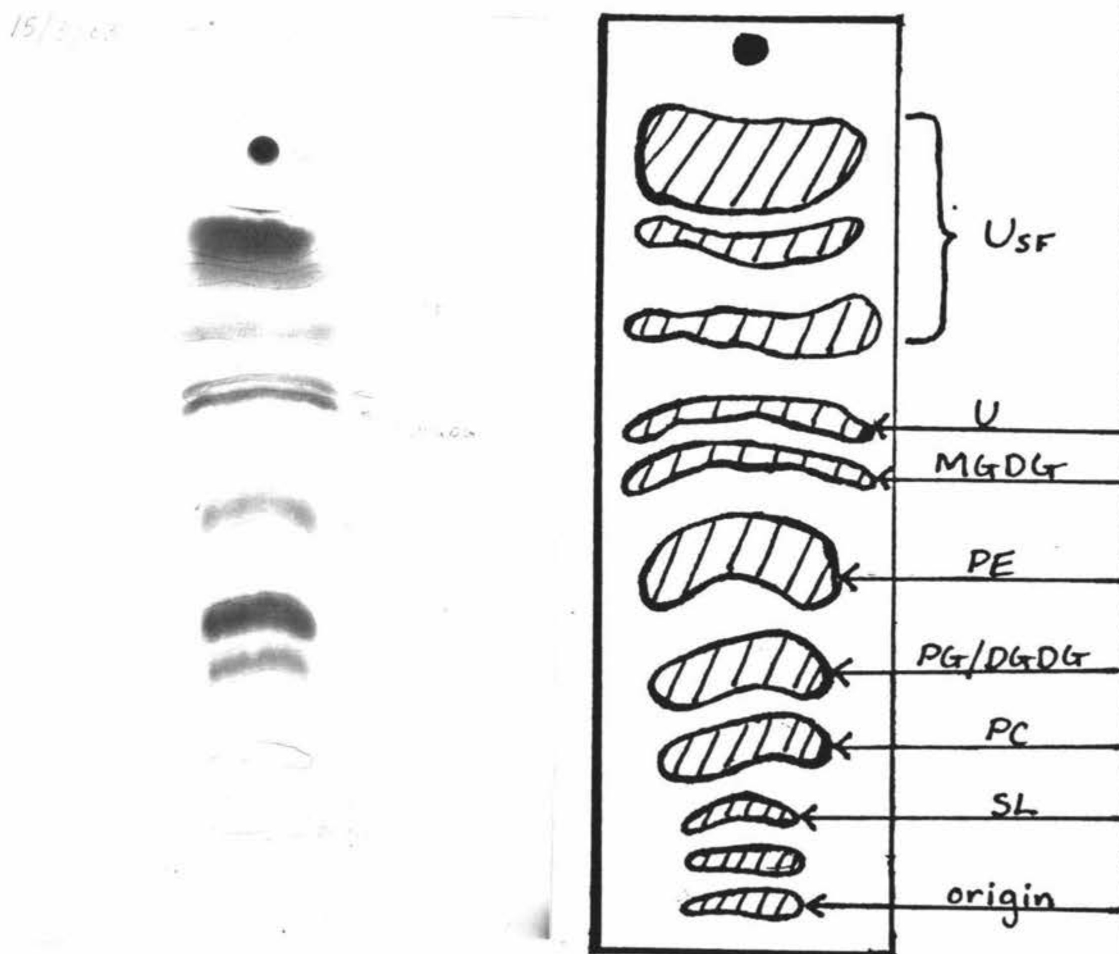
TLC of protoplasts total lipid samples was performed as described in the methods section 3.7. The major sample examined was that produced from (1-^{14}C) acetate incubation. Generally the total lipid sample was first run in chloroform:MeOH:acetic acid:H_2O (85:15:10:3-5, v/v). A scanner trace of the plate and lining up with the markers (see figure 4.13) allowed most of the lipid components to be identified. Incorporation products included SL, PC, a PG/DGDG spot, PE, MGDG and two unknown regions. Autoradiographs were taken of the plates to clearly define regions of high radioactivity (see figure 4.14). Two regions on the plate were unable to be identified. Both of these, titled U and U_{SF} (unknowns to the solvent front) run high up on the plate in the same place as plant pigments. They were highly non-polar. Each component on the TLC plate was eluted off and counts determined on a known aliquot. This allowed the proportions of the components to be calculated (see table 4-3). A large proportion of the radioactivity (68.7%) was found in U_{SF}.

Figure 4.13 Scanner Trace of the Radioactivity of a Chromatogram of the Total Lipids from Protoplast Incubation with ^{14}C-Acetate



Scanner trace on the left, diagram of the markers on the thin layer plate on the right. Approximately 700,000 cpm applied to plate.

Figure 4.14 Autoradiograph of a Chromatogram of a Total Lipid Sample Produced from Incubation of Barley Protoplasts with (1-^{14}C)-Acetate.



Picture of autoradiograph on left, diagram of the autoradiograph on the right. Taken of a plate run in chloroform:meOH:acetic acid:H_2O (85:15:10:3.5, v/v). Approximately 700,000 dpm applied to plate. Left for 2 weeks before developing.

Table 4.3 The Proporations of Lipids Present in a Total Lipid Sample from Incubation of Barley Protoplasts with ^{14}C-Acetate.

Lipid Components	Proportion (% of total dpm)
Sulpholipid	1.7
Phosphatidyl choline	23.9
Phosphatidyl glycerol/ digalactosyl diglyceride	10.5
Phosphatidyl ethanolamine	2.6
Monogalactosyl diglyceride	7.1
U	5.2
U_{SF}	68.7

Total lipid sample from incubation with (1-^{14}C) acetate run in chloroform:MeOH:acetic acid:H_2O (85:15:10:3.5, v/v), components eluted (see methods section 3.7).

Experiment performed 5 times, results averaged.

In order to determine which component of the phosphatidyl glycerol/digalactosyl diglyceride spot contains the radioactivity in the TLC plate run in chloroform:methanol:acetic acid:H_2O (85:15:10:3.5, v/v), the area was eluted off and re-run in toluene:ethyl acetate:95% ethanol (2:2:1, v/v).

This solvent system separates the two compounds; digalactosyl diglyceride runs midway up the plate while phosphatidyl glycerol remains at the bottom near the origin. The plate is run through a scanner after spraying with phospholipid spray to locate the PG marker. The results showed that all of the radioactivity was in PG, none appeared evident in DGDG. This occurrence has been noticed previously (Bell, 1983).

A protoplast total lipid sample extracted after incubation with ^{14}C-bicarbonate was run in chloroform:MeOH:acetic acid:H_2O (85:15:10:3.5, v/v). The plate was scanned, areas identified from this and eluted off. The proportions of each component were calculated (see table 4.4). Comparison of the ^{14}C-acetate incorporation with the ^{14}C-bicarbonate incorporation TLC results showed a different profile of where the radioactivity was being incorporated.

A protoplast total lipid sample extracted after incubation with ^{14}C-acetate in the dark was also run on a TLC plate in chloroform:MeOH:acetic acid:H_2O (85:15:10:3.5, v/v).

Table 4.4 The Proportions of Lipids Present in a Total Lipid Sample from Incubation of Barley Protoplasts with ^{14}C-Bicarbonate.

Lipid Component	Proportion (% to total counts)
Sulpholipid	4.7
Phosphatidyl choline	9.5
Phosphatidyl glycerol/ digalactosyl diglyceride	12.5
Phosphatidyl ethanolamine	0
Monogalactosyl diglyceride	9.7
U	15.3
U_{SF}	48.3

A total lipid sample from incubation with ^{14}C-bicarbonate was run in chloroform:MeOH:acetic acid:H_2O (85:15:10:3.5, v/v), components eluted off (see methods section 3.7). Experiment performed 2 times, results averaged.

The scanner trace of this plate appeared quite different to incubation in light conditions (see figure 4.15) and the proportions of the components were markedly different (see table 4.5). A large proportion of the label was incorporated into monogalactosyl diglyceride. Fatty acid synthesis is more than 94% dependent on light. An increase in oleate has been shown to occur during the day while an increase in desaturated fatty acids occurs at night; the desaturase reactions not requiring light (Browse et al., 1981). The high concentrations of MGDG in the dark are probably due to the enzymes fixing the residual fatty acids on the galactolipid backbone continuing to operate when acetyl CoA carboxylase is inactivated in dark conditions (Nikolau & Hawke, 1983) and no further fatty acids are being synthesised.

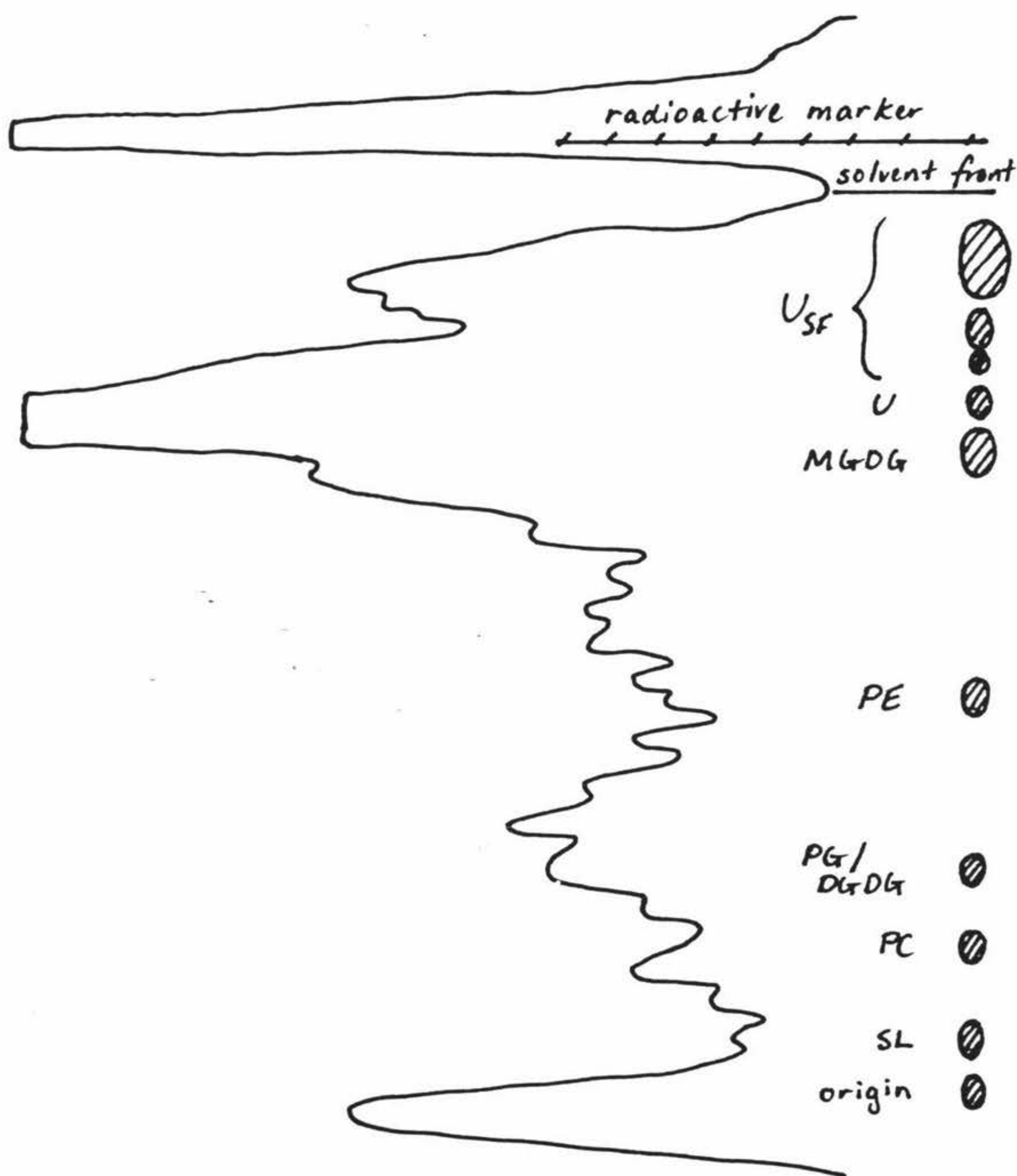
4.1.4 Thin-Layer Chromatography of the Unknowns U and U_{SF} Separated from Total Lipid Samples Extracted from Protoplasts after Incubation with ^{14}C-Acetate

The components U and U_{SF} , purified from TLC of total lipid samples extracted from protoplasts after incubation with (1-^{14}C) acetate, were retained for characterisation.

The unknown U was applied to the TLC plate and chromatographed in the solvent system hexane:diethyl ether (9:1, v/v). The radioactivity remained at the origin.

When U_{SF} was run in hexane:diethyl ether (9:1, v/v) the unknown fraction was separated into products corresponding in mobility to triglyceride, free fatty acid and monoglyceride markers (see figure 4.16). As large

Figure 4.15 Scanner trace of the Radioactivity on a Chromatogram of the Total Lipid from Incubation of Barley Protoplasts with ^{14}C Acetate in the dark.



Scanner trace on the left, diagram of the markers on the thin layer plate on the right. Approximately 27,000 cpm applied to plate. Solvent system was chloroform:MeOH:acetic acid:H_2O (85:15:10:3.5,v/v).

Table 4.5 The Proportions of Lipids Present in a Total Lipid Sample from Incubation of Barley Protoplasts with ^{14}C-Acetate in the Dark.

Lipid Component	Proportion (% to total counts)
Sulpholipid	12.5
Phosphatidyl choline	16.6
Phosphatidyl glycerol/ digalactosyl diglyceride	0
Phosphatidyl ethanolamine	11.1
Monogalactosyl diglyceride	48.3
U	5.0
U_{SF}	6.5

A total lipid sample from incubation with (1-^{14}C) acetate in the dark was run in chloroform:MeOH:acetic acid:H_2O (85:15:10:3.5,v/v), components eluted off (see methods section 3.7). Experiment performed 2 times, results averaged.

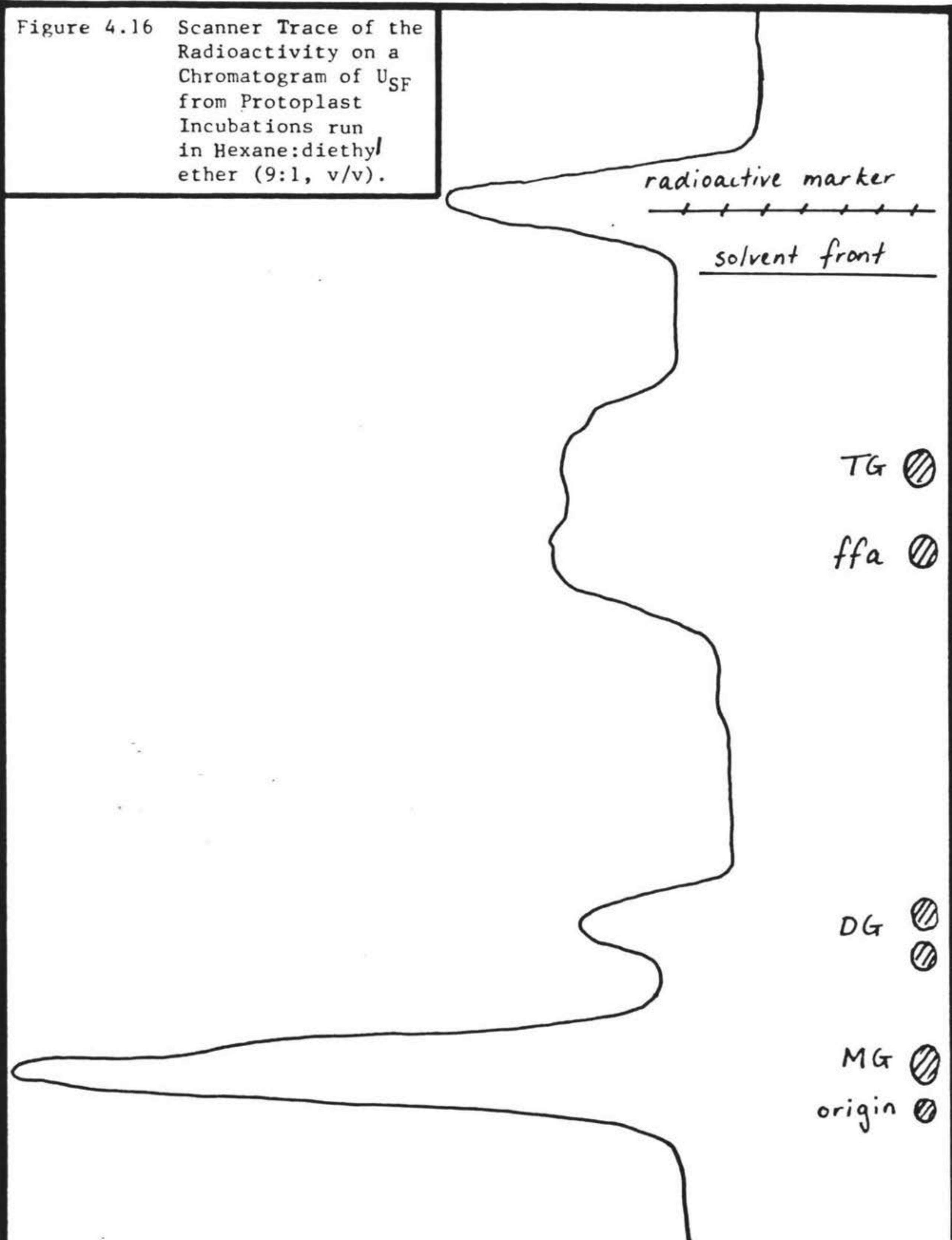


Figure 4.16 Scanner Trace of the Radioactivity on a Chromatogram of U_{SF} from Protoplast Incubations run in Hexane:diethyl ether (9:1, v/v).

Scanner trace on the left, diagram of the markers on the thin layer plate on the right. Approximately 19,000 cpm applied to plate.

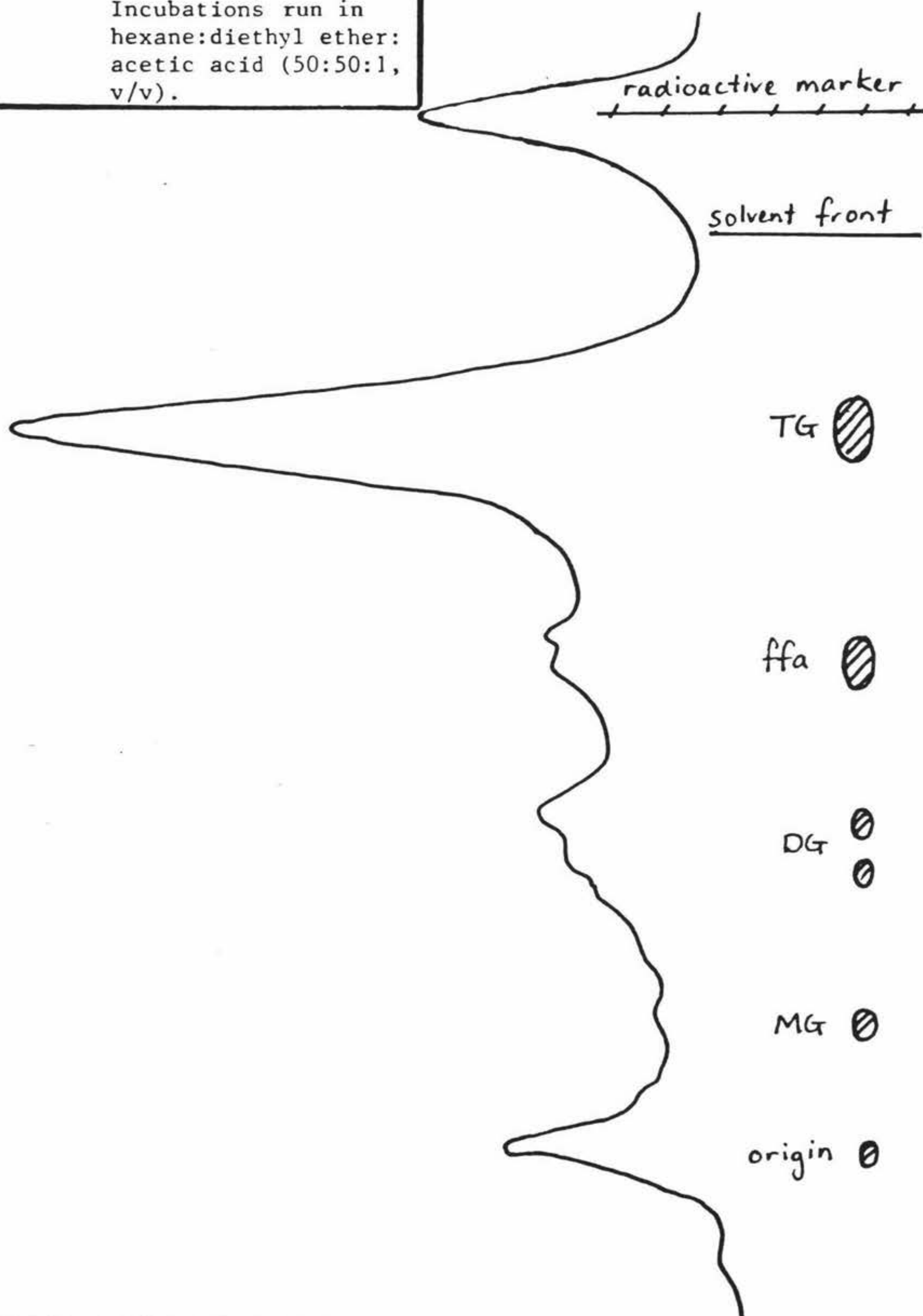


Figure 4.17 Scanner trace of the Radioactivity of a Chromatogram of U_{SF} from Protoplast Incubations run in hexane:diethyl ether: acetic acid (50:50:1, v/v).

Scanner trace on the left, diagram of the markers on the thin layer plate on the right. Approximately 350,000 cpm applied to plate.

amounts of radioactivity failed to migrate from the origin another solvent system was selected.

U_{SF} was chromatographed in the solvent system hexane:diethyl ether:acetic acid (70:30:1, v/v). Again the components corresponded directly with the markers triglyceride, free fatty acid, diglyceride and monoglyceride. In this more polar solvent system monoglyceride migrated far enough away from the origin to allow a clear separation. There was found to be little incorporation into monoglyceride.

The components from this plate were eluted off and the proportions of each component, from the results of radioactive counting, calculated (see table 4.6). U_{SF} was run in one additional solvent system, benzene:diethyl ether:acetic acid (70:4:4:0.2, v/v) to further verify the identity of the components (see figure 4.18).

4.1.5 Gas-Liquid Chromatography of the Components of U_{SF} from Protoplast Incubations with ^{14}C-Acetate.

Diglyceride, free fatty acid, and triglyceride from U_{SF}; obtained from protoplast incubations; were made into methyl esters (as described in the methods section 3.10) and run through the GLC (methods section 3.11). Samples of each fatty acid were collected and counts of radioactivity determined. The percentage of each different fatty acid is calculated (see table 4.7)

The results show that in the triglyceride the label is mainly incorporated into 18:2 and 16:0. For the diglyceride the label is

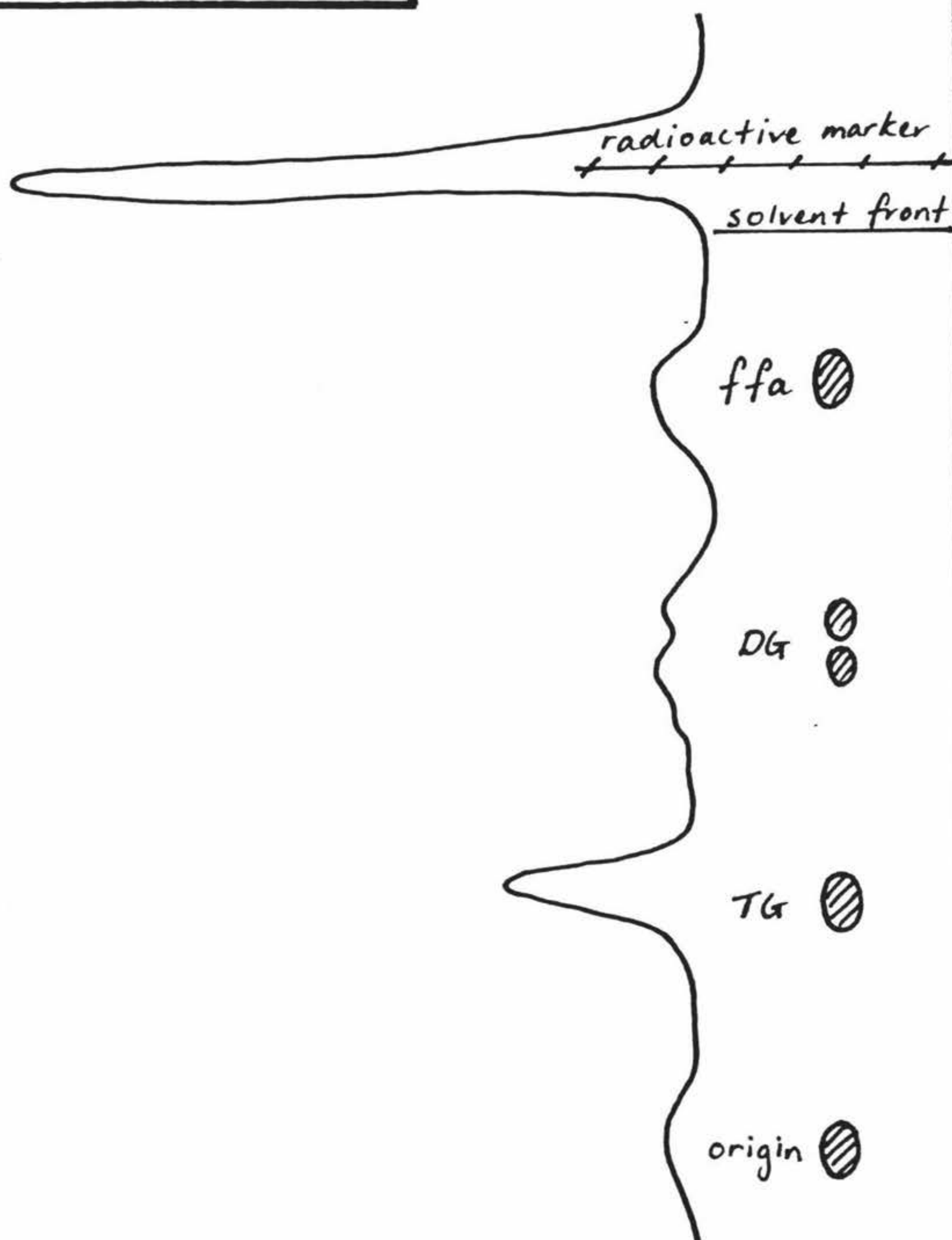
Table 4.6 The Proportions of each Acyl Lipid in U_{SF} from Protoplast Incubations with ^{14}C-Acetate.

Lipid Component	Proportion (% of total counts)
monoglyceride	7.5
diglyceride	16.7
free fatty acid	15.7
triglyceride	56.8

Total lipid sample run in chloroform:MeOH:acetic acid:H_2O (85:15:10:3.5, v/v), U_{SF} eluted off.

U_{SF} run in hexane:diethyl ether:acetic acid (50:50:1, v/v), components eluted off and counts determined. Results are the average of 4 experiments.

Figure 4.18 Scanner Trace of the Radioactivity of a Chromatogram of U_{SF} from Protoplast Incubations run in Benzene:diethyl ether: acetic acid (70:4:4:0.2, v/v)



Scanner trace on the left, diagram of the markers on the thin layer plate on the right. Approximately 15,000 cpm applied to the plate.

Table 4.7 Location of the Label in the Acyl Lipids Incorporated by Barley Protoplasts from Incubation with (1-^{14}C) Acetate.

Fatty acid	Triglyceride	Free fatty acid	Diglyceride
12:0	0.90	4.90	23.90
14:0	1.56	35.04	11.69
16:0	29.67	43.95	29.88
17:0	2.21	4.56	11.25
18:0	6.45	8.45	7.29
18.1	3.97	0.73	1.18
18:2	54.64	1.60	7.41
18:3	1.19	0.85	7.34

Triglyceride, diglyceride and free fatty acids from U_{SF} made into methyl esters (methods section 3.10), then run through the GLC (methods section 3.11). Results expressed as percentage of total counts. Single experiment performed.

present mainly in unsaturated fatty acids, there are high concentrations of 12:0, 14:0 and 16:0. The desaturated fatty acids present are probably formed from the hydrolysis of other lipids, for example, galactolipids (Kaninga and Gemel, 1984). 14:0, 16:0 and smaller quantities of 18:0 and 12:0 are present in the free fatty acid sample.

4.2 The Concentration of Acyl Lipids in Barley Leaf Tissue

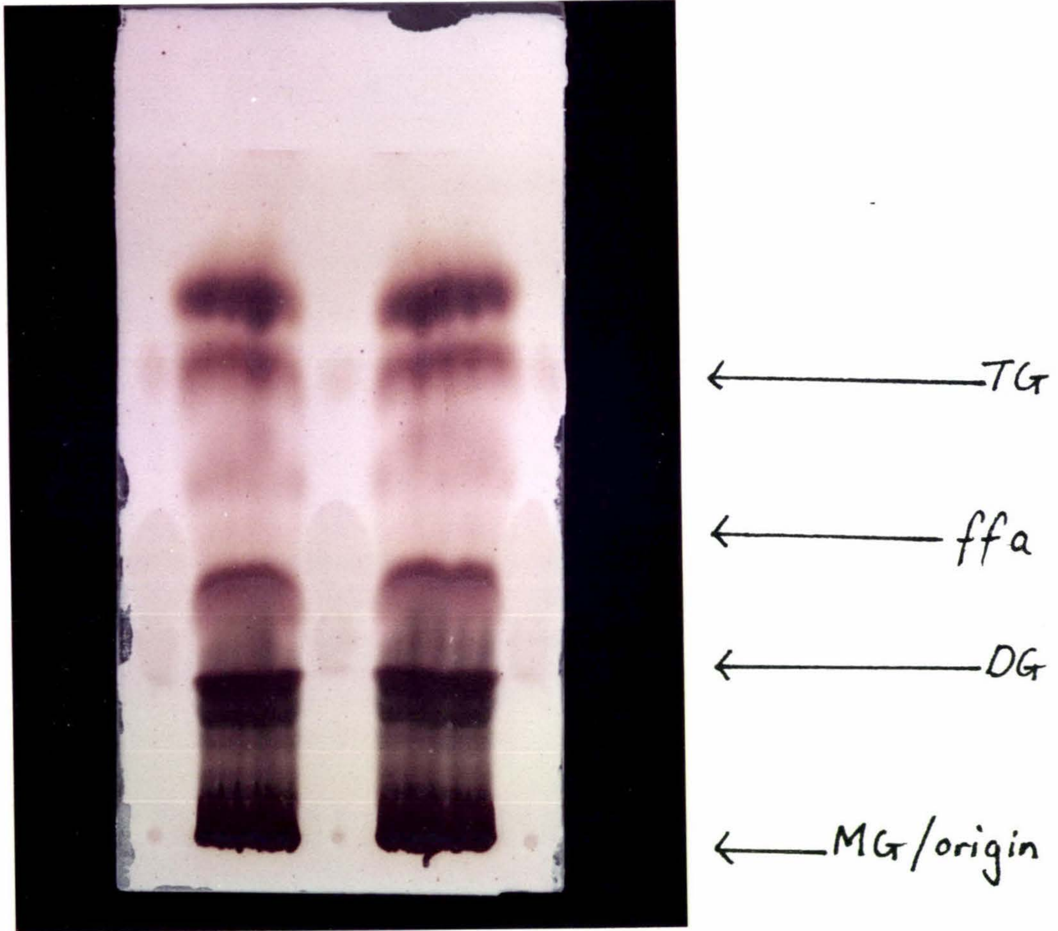
A whole barley leaf extract of known concentration was prepared (see methods section 3.8) and chromatographed twice (see methods section 3.9) to obtain pure fractions of triglyceride, fatty acid, diglyceride and monoglyceride (see figure 4.19). Methyl esters were made up from each fraction (methods section 3.10) and they were examined by GLC (methods section 3.11). The concentration of each acyl lipid was calculated and from this data an estimate of the proportions of each fatty acid was made (see table 4.8 and 4.9).

Acyl lipids were found to be present in only small concentrations. Triglyceride seemed to be composed of mainly 16:0, 18:0 and 18:1 fatty acids, a range of free fatty acids were present, there were high amounts of 18:3 possibly from hydrolysis of other glycerol-lipids in the leaf.

4.3 Incorporation of ^{14}C-Acetate into Acyl Lipids by Barley Leaf Protoplasts from Tissue of Differing Maturity

Barley plants were grown and harvested in the normal manner (as described in the materials section 2.1, and the methods section

Figure 4.19 Photograph of the Chromatogram of a Total Lipid Sample from Whole Barley Leaf Tissue.



Thin layer chromatograph run in hexane:diethyl ether:acetic acid (70:30:1, v/v) as described in the Methods section 3.7.

Table 4-8 Concentrations of Acyl Lipids in Whole Barley Leaf Tissue.

Acyl Lipid	Concentration (nmoles/g of fresh tissue)
triglyceride	14.80
free fatty acid	144.15
diglyceride	8.55
monoglyceride	12.50

Concentrations were calculated from the results of GLC tracings on components obtained from a whole lipid extract from barley leaves. Results are the average of two experiments.

Table 4.9 The Proportions of Fatty Acids Present in Acyl Lipids from Barley Leaf Tissue.

	Acyl Lipid			
f.a.	Triglyceride	Free fatty acid	Diglyceride	Monoglyceride
12:0	trace	0.04	-	-
14:0	0.10	0.04	0.03	-
16:0	1.0	1.0	1.0	1.0
16:1	-	0.22	-	-
18:0	0.50	0.38	0.16	0.31
18:1	0.35	0.53	0.10	0.09
18:2	0.18	0.31	0.14	-
18:3	0.05	2.38	0.25	-
20:0	0.01	0.11	-	-

(Results expressed as a proportion; as compared to 16:0)

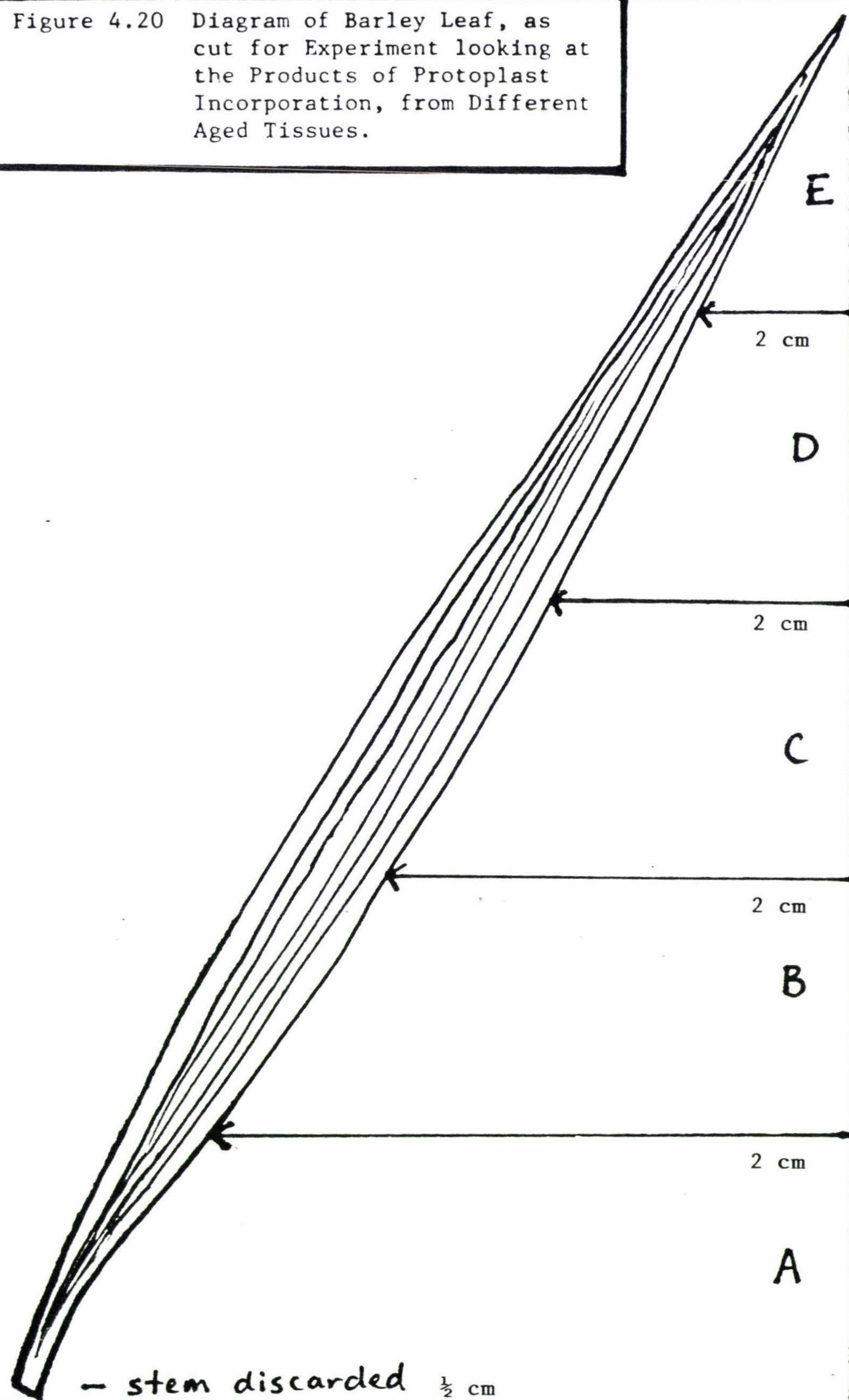
Figures are calculated from the results of GLC tracings on methyl esters of triglyceride, diglyceride, fatty acid,and monoglyceride from barley leaf tissue. Experiment performed twice, results averaged.

3.1). After abrading and washing, the tissue was cut into sucessive 2 cm sections from base to tip and labelled A to E, the more mature sections represented by E, the most immature by A (see figure 4.20). This technique has been used by several workers (Leech et al., 1973; Hawke et al., 1974; Barber & Leech, 1977). Each section was cut into 2mm slices with a razor blade, 2.5g weighed and placed into 20 mls digestion media in a petri dish. Protoplasts were isolated (see methods section 3.1) from each section of leaf. Each was suspended in 1 ml of solution A. Chlorophyll determinations were carried out on each sample (see methods section 3.3). These results (figure 4.21) demonstrated that there was an increased chlorophyll concentration in more mature sections.

Each suspension was examined under the phase-contrast microscope. Protoplasts from basal sections were smaller than normal and lighter in color but were intact. Middle sections had some protoplasts with the chloroplasts collected on one side. Protoplasts were of normal size. In the distal sections protoplasts were abundant but of a lower quality. Some broken protoplasts were evident and there were low concentrations of chloroplasts. An oxygen evolution test was done on each sample to check that they had retained "normal" metabolic activity. Each section had rates greater than 100 μmoles/hr/mg chl, the number recommended by Kuhn & Stumpf for use determining protoplast integrity (Kuhn & Stumpf, 1981) so were considered to be suitable for use in incubations.

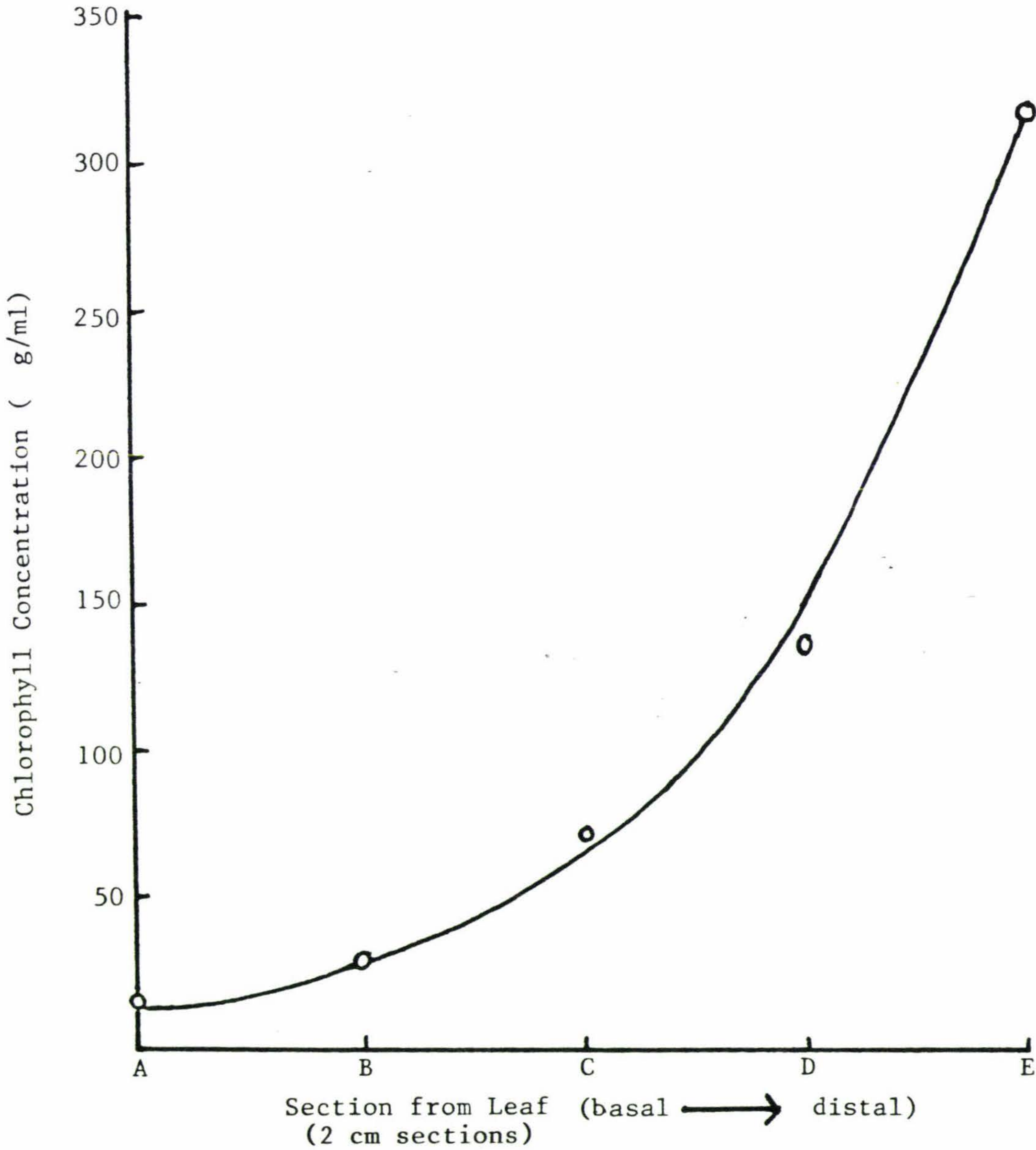
Duplicate sample were incubated for testing total lipid incorporation,

Figure 4.20 Diagram of Barley Leaf, as cut for Experiment looking at the Products of Protoplast Incorporation, from Different Aged Tissues.



Drawing of a Barley leaf, magnified 2x. A to E represent the labelling as described.

Figure 4.21 The Change in Chlorophyll Concentration in Relation to the Maturity of Barley Leaf Tissue



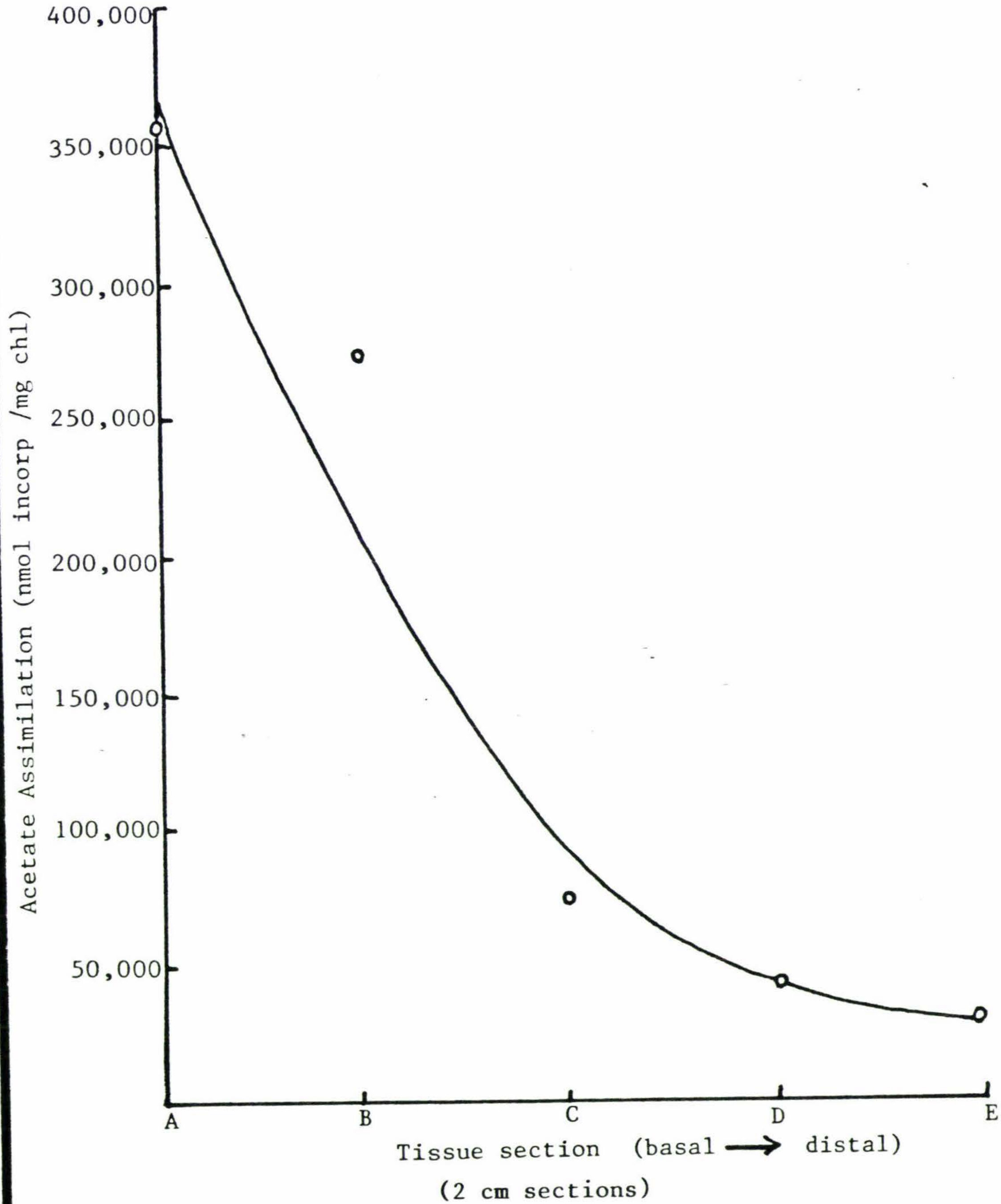
Chlorophyll determination carried out as described in the Methods section 3.3 on samples from different sections of the leaf. Experiment performed once.

and a single sample incubated for testing assimilation of (1-^{14}C) acetate. Incubations were carried out as described in the methods 3.6. After washing, radioactive counts were determined. The results showed (see figures 4.22 and 4.23) that the protoplasts isolated from the basal regions had a greater ability for (1-^{14}C) acetate assimilation and incorporation into lipids.

Each section's total lipid duplicate samples were pooled and the complete sample run in chloroform:MeOH:acetic acid:H_2O (85:15:10:3.5, v/v). The U_{SF} band was identified, eluted off and radioactive counts determined on a known aliquot. Two graphs were drawn. The first, plotted with the leaf section against total U_{SF} counts (figure 4.24), demonstrated the ability of the immature regions to synthesise large amounts of U_{SF} ; nearly nine times greater than mature sections. The second graph (figure 4.25) is of the leaf section against the U_{SF} content as a percentage of the counts in the total lipids. Again there is a higher U_{SF} content in the basal sections (approximately 90% compared with 45%). This graph demonstrates that it is not just the higher quality of protoplasts from the basal sections that are causing the raised U_{SF} values. The U_{SF} makes up a higher proportion of the total lipid.

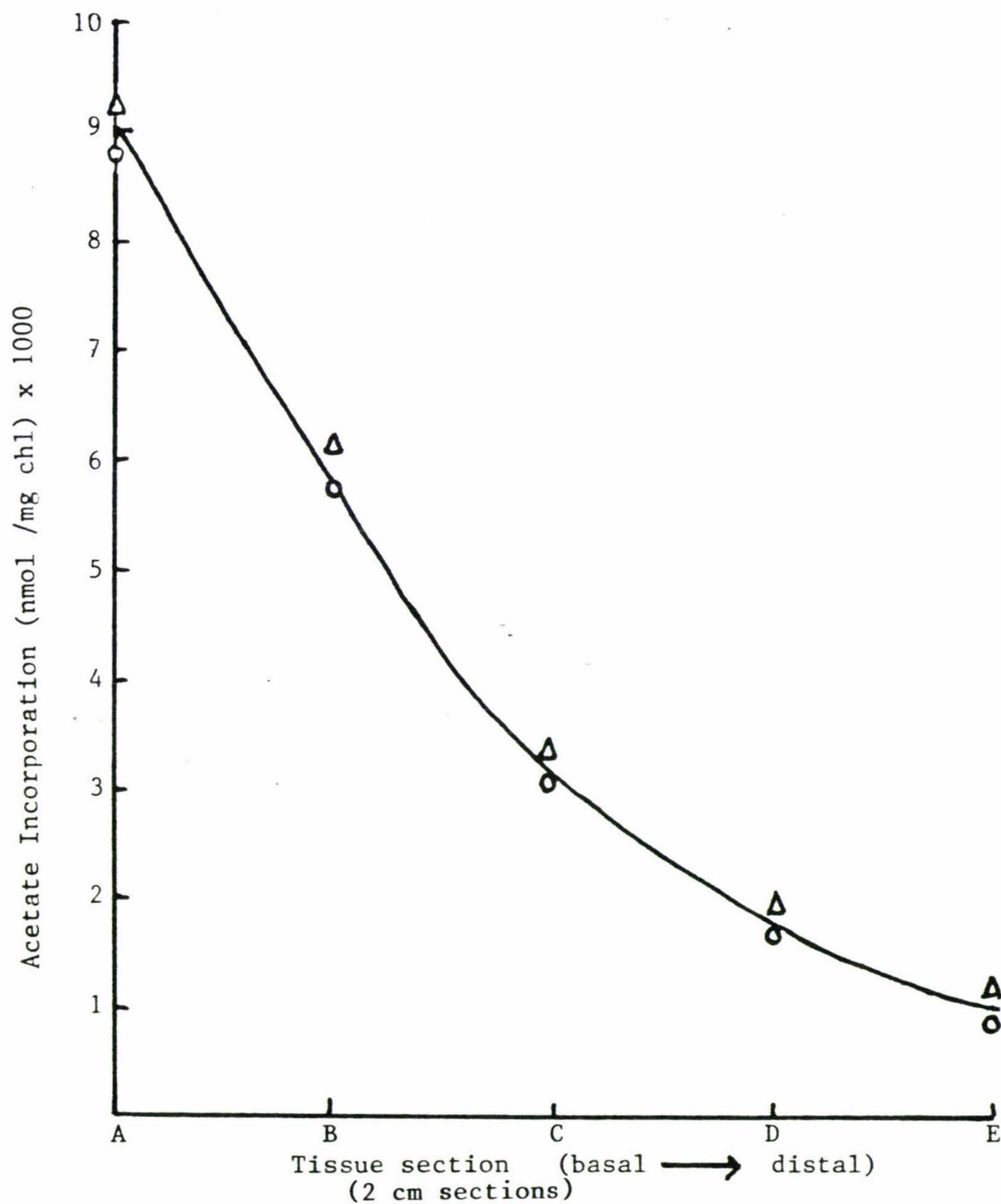
These results suggest that the synthesis of acyl lipids (U_{SF}) is related to tissue immaturity.

Figure 4.22 Assimilation of (1-^{14}C) Acetate by Barley Protoplasts from Tissue of Differing Maturity



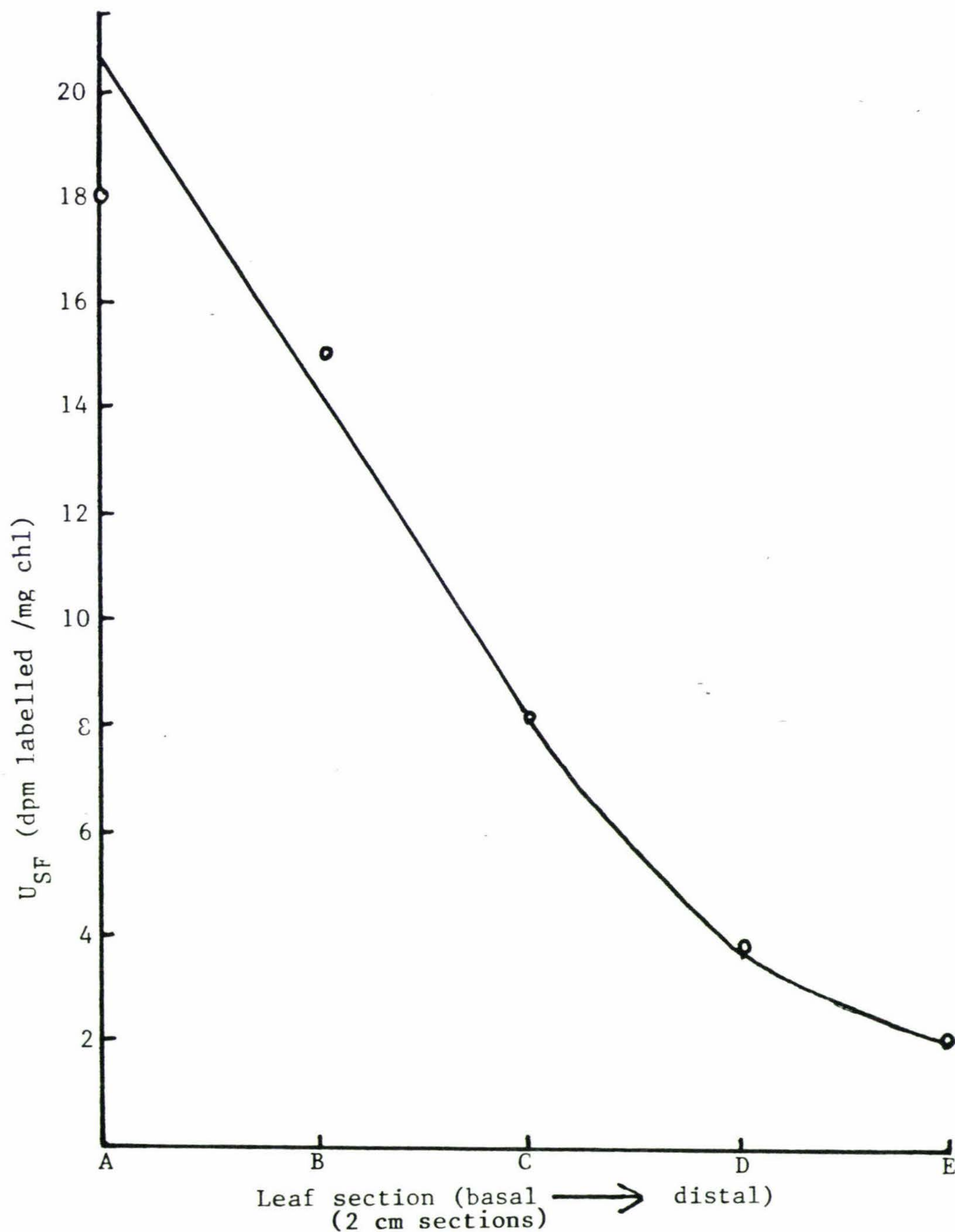
Barley protoplasts were isolated from different sections of the leaf, labelled A to E in a basal to distal direction. Incubations carried out by the method described in Methods section 3.6. Single experiment performed.

Figure 4.23 Incorporation of (1-^{14}C) Acetate into Total Lipids by Barley Protoplasts from Tissue of Differing Maturity



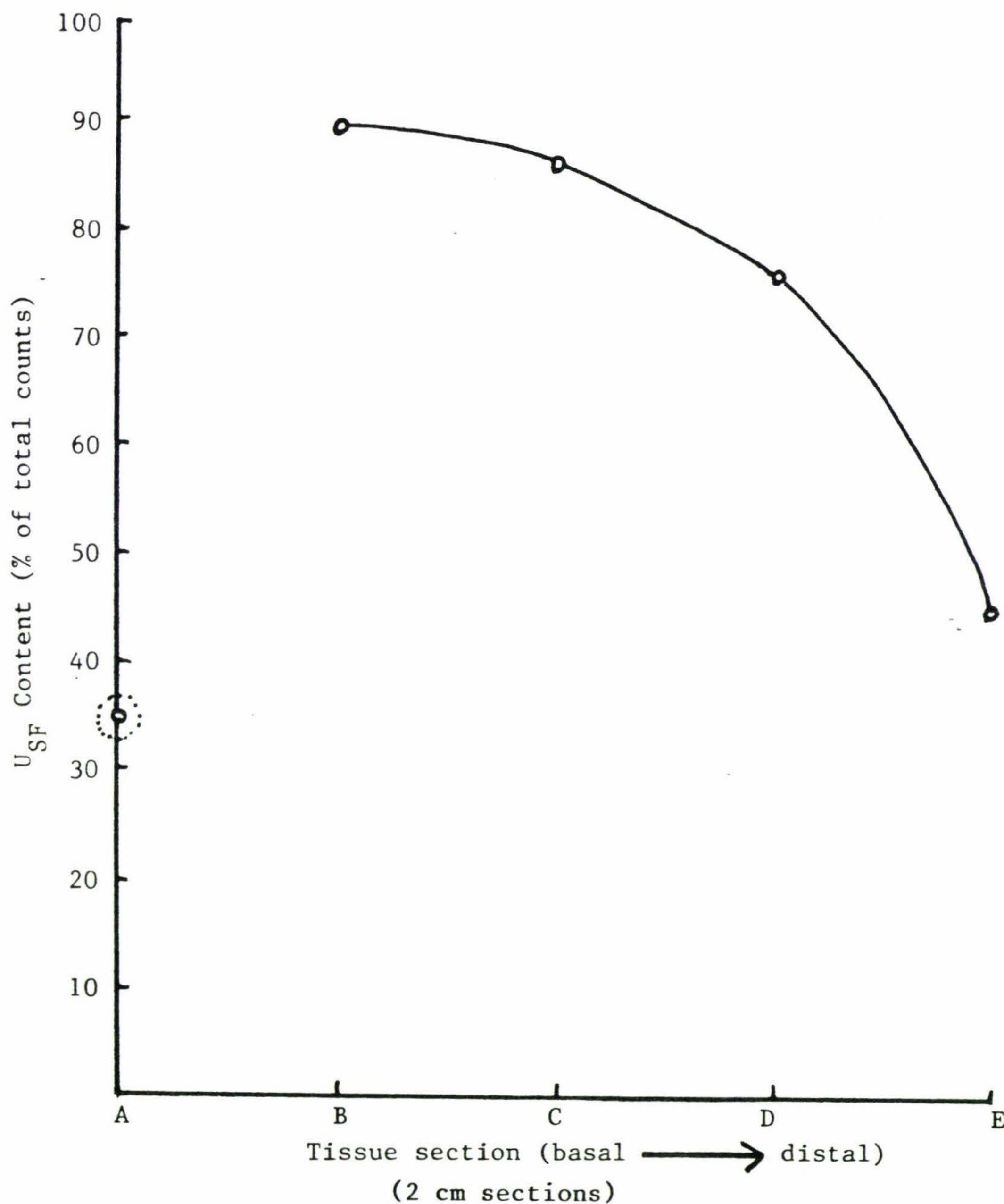
Barley protoplasts were isolated from different sections of the leaf, labelled A to E in a basal to distal direction. Incubations carried out by the method described in Methods section 3.6. Duplicate samples performed in a single experiment.

Figure 4.24 Incorporation of $(1-^{14}C)$ Acetate into U_{SF} by Barley Protoplasts from Tissue of Differing Maturity



Protoplasts isolated from tissue of differing age, and labelled A to E basal to distal incubated as described in the Methods section 3.6. Total lipid sample chromatograph in Chloroform:MeOH:acetic acid:H_2O (85:15:10:3.5, v/v), U_{SF} extracted and counts determined. Single experiment performed.

Figure 4.25 The Proportion of Label from (1-^{14}C) Acetate Incorporated into U_{SF} by Barley Protoplasts from Tissue of differing Maturity



Protoplasts isolated from tissue of differing age, and labelled A to E basal to distal, incubated as described in the Methods section 3.6. Total lipid sample was chromatographed in Chloroform:MeOH:acetic acid:H_2O (85:15:10:3.5, v/v) U_{SF} extracted and counts determined. Single experiment.

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Chloroplast Isolation and Incubation

4.4.1 Viability of Chloroplasts

(a) Visual Examination of Chloroplasts

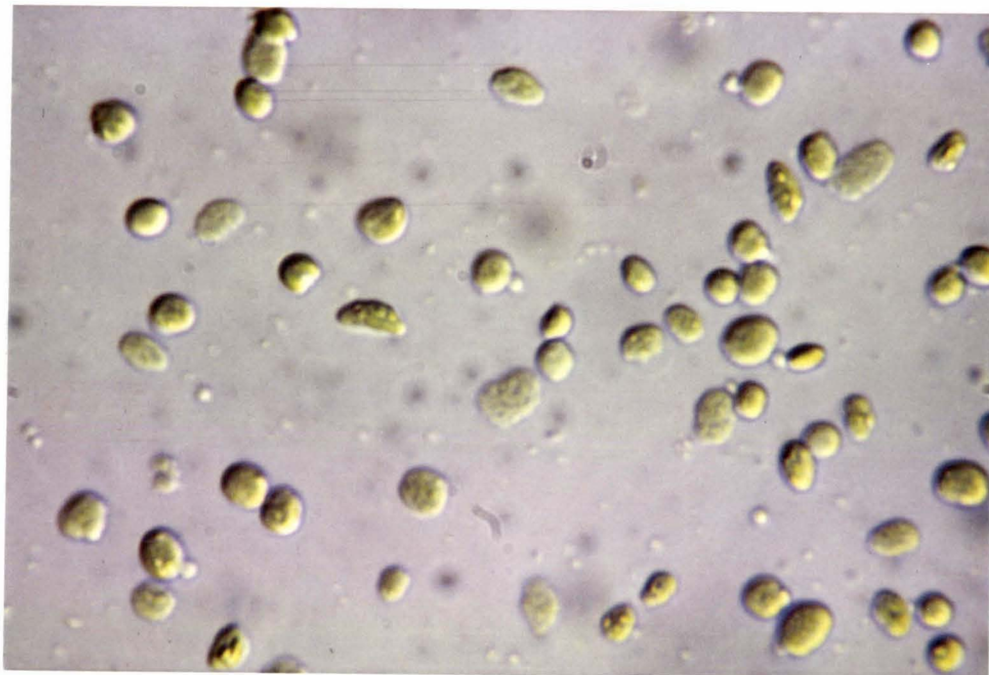
Chloroplasts prepared by both the mechanical method (see methods section 3.2.2) and prepared by rupturing protoplasts (methods section 3.2.1) were examined under phase contrast microscope at 600x magnification. Class A chloroplasts (ie, whole intact chloroplasts) are highly reflective, opaque and distinct in the presence of a "halo". Broken chloroplasts have a granular appearance as the thylakoids are visible.

Chloroplasts prepared from protoplasts appeared to be of high quality. Some broken chloroplasts were evident in most preparations but these were rare and numbered less than 20%. No protoplasts were visible in the suspension.

Chloroplasts prepared by the mechanical method appeared to be of lower quality. More broken chloroplasts were evident and some preparations contained bacteria. The bacteria was assumed to come through from the leaves; this was prevented when isolating by the former method by the two-step gradient used in obtaining protoplasts.

Visual examination showed that rupturing protoplasts provides a better method for isolating chloroplasts. The chloroplasts obtained from this isolation method appear to be of good quality (see figure 4.26).

Figure 4.26 Photograph of Chloroplast from Barley Leaf Tissue



Prepared by rupture of pre-made protoplasts, as described in the Methods section 3.2.1. Magnification 500x.

(b) Chloroplast Oxygen Evolution Rates

As for protoplasts O_2 evolution rates were recorded as a quantitative measure of integrity. Results of chloroplasts prepared from protoplasts averaged 17.5 nmoles O_2 produced /hr/mg chl. However for chloroplasts prepared by mechanical grinding methods results were even lower. Due to the large degree of bacterial contamination in some preparations coming through from the leaves, some experiments showed oxygen uptake.

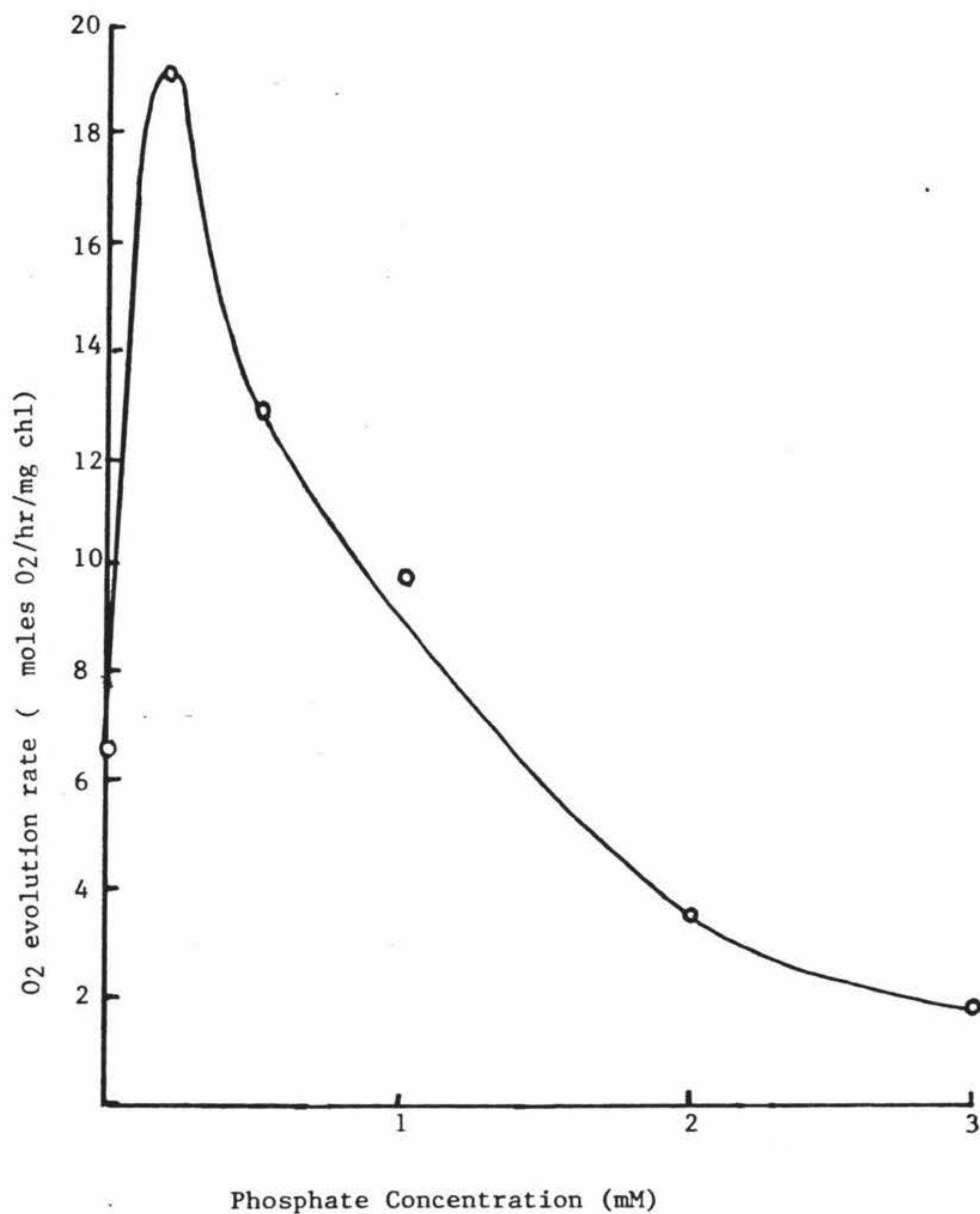
Use of a red filter proved to be needless in experiments with the O_2 electrode, although it has often been used by other workers (Leegood & Walker, 1981). Addition of catalase in the O_2 evolution media increased rates of O_2 evolution by approximately 25%. Catalase has been used previously by other workers (Edwards et al., 1978). It serves as a protective agent against peroxide.

An experiment was performed varying the phosphate concentration between 0-3 mM. The result (see figure 4.27) was a peak evolution rate when the phosphate concentration was 0.25mM. Higher concentrations of phosphate decreased the O_2 evolution rate. Chloroplasts appear to be highly sensitive to the phosphate levels of the media.

(c) Ferricyanide Intactness Test to Estimate the Number of Whole Chloroplasts.

The ferricyanide intactness test gave average results of 75% intactness for chloroplasts preparations isolated by the grinding method, and 95% for chloroplasts prepared by bursting protoplasts.

Figure 4.27 The Effect of Phosphate on the O_2 Evolution by Barley Chloroplasts.



Method for the detection of O_2 evolution as described in section 5.2 of the Methods. Results are from a single experiment.

This test tends to overestimate as some broken chloroplasts reseal (McClilley et al., 1975).

4.4.2 Incubations of Barley Chloroplasts with ^{14}C-Acetate

Incubations of chloroplasts with ^{14}C-acetate, under conditions described in the methods section 3.6 showed rates of assimilation of averaging 1383.02 nmoles/hr/mg chl and incorporation into total lipid averaging 1061.97 nmoles/hr/mg chl. This gives approximately 75% assimilation into lipid (compared with 50-65% for protoplasts). The incubations performed with chloroplasts prepared by mechanical methods gave rates of 721.83 nmoles/hr/mg chl. These results are all high compared with work by other workers, ie. spinach chloroplasts give rates of 100 nmoles acetate/mg chl/hr (Kuhn & Stumpf, 1981) or 175 (McKee & Hawke, 1979), barley - 420 (Kannangara, et al., 1971).

4.4.3 TLC of Total Lipids and Resolution of U_{SF} Components, Extracted from Chloroplasts after Incubation with ^{14}C-Acetate.

The total lipid sample extracted from chloroplasts after incubation with (1- ^{14}C) acetate is run in chloroform:MeOH: acetic acid:H_2O (85:15:10:3.5, v/v) (see methods section 3.7). The components and unknowns are identified from the results of scanning the plate and autoradiographs. The components are eluted, and the proportions of each calculated from radioactive counts (see table 4-10). Results showed incorporation into MGDG, PE and a high portion into U_{SF}. U_{SF} was re-chromatographed in hexane: diethyl ether:acetic acid (50:50:1, v/v). The proportions of the components of U_{SF} were calculated (see table 4-11).

Table 4.10 The Proportions of Lipids Present in a Total Lipid Sample from Incubation of Barley Chloroplasts with ^{14}C-Acetate.

Lipid Component	Proportion (% of total dpm)
Sulpholipid	12.3
Phosphatidyl choline	1.7
Phosphatidyl glycerol/ digalactosyl diglyceride	3.2
Phosphatidyl ethanolamine	8.6
Monogalactosyl diglyceride	28.1
U	9.1
U_{SF}	53.7

Total lipid sample from incubation run in chloroform:MeOH:acetic acid:H_2O (85:15:10:3.5, v/v), components eluted off (see methods section 3.7). Experiments performed 3 times, results averaged.

Table 4-11 The Proporations of Each Acyl Lipid in U_{SF} From Chloroplast Incubations with ^{14}C-Acetate.

Acyl Lipid	Proportion (% of total count)
triglyceride	34.9
free fatty acid	44.3
diglyceride	11.1
monoglyceride	2.3

Total lipid sample run in chloroform:MeOH:acetic acid:H_2O (85:15:10:3.5, v/v), U_{SF} eluted off.

U_{SF} run in hexane:diethyl ether acetic acid (50:50:1, v/v), components eluted and counts determined. Results are the average of four experiments.

To check if the results of the chloroplast incubation, which again showed up large amounts of radioactive label incorporated into U_{SF}, was due to protoplast contamination a separate thin layer plate was chromatographed using the total lipids produced from (1-^{14}C) acetate incubation with chloroplasts isolated by the traditional blending technique, as described in the methods (section 3.2.2). The chromatogram again showed the presence of U_{SF} and when re-chromatographed in the second solvent system, hexane: diethyl ether:acetic acid (50:50:1, v/v), there were again large amounts of label in triglyceride.

4.4.4 Gas-Liquid Chromatography of the Components of U_{SF} from Chloroplast Incubations with ^{14}C-Acetate.

Methyl esters of diglyceride, free fatty acid and triglyceride from U_{SF} were made (see methods section 3.10) and run through the GLC (methods section 3.11). Samples of each fatty acid were collected and radioactivity determined. The percentage of each different fatty acid was determined (see table 4.12).

Results showed that in the chloroplastic triglyceride approximately 70% of the label was incorporated into palmitic acid. For the diglyceride the label accumulated mainly in 12:0 and 14:0. The free fatty acid was almost entirely made up of 18:0. The presence of 18:2 and 18:3 in the preparations (although in relatively small amounts), suggests endoplasmic reticulum contamination as the chloroplast cannot insert these double bonds.

Table 4-12 Location of the Label in the Acyl Lipids Incorporated by Barley Chloroplasts from Incubation with ^{14}C-Acetate

Fatty Acid	Acyl Lipid		
	Triglyceride	Free fatty Acid	Diglyceride
12:0	11.90	0.90	30.90
14:0	3.27	0.86	39.97
16:0	72.19	2.34	6.81
17:0	2.57	0.45	6.81
18:0	1.94	84.97	1.43
18:1	4.36	1.13	3.40
18:2	2.64	7.65	6.44
18:3	1.79	1.72	3.94

Diglyceride, free fatty acid, triglyceride from U_{SF} made into methyl esters (methods seciton 3.10), then run through the GLC (methods section 3.11). Results expressed as % of total counts. Single experiment performed.

CHAPTER 5. DISCUSSION

This thesis was a study of the acyl lipids, in particular triglyceride synthesised by protoplasts and chloroplasts from barley leaf tissue. The concerns were two-fold. One objective was to examine the isolation and incubation with labelled precursors of protoplasts and chloroplasts from 7 day old barley tissue. The second objective was the identification of a lipid product as triglyceride, produced from incubation with labelled precursors. Some further experiments were performed to investigate the appearance of the triglyceride. Some conclusions were made about the role of this triglyceride and its synthesis site in the young barley leaf.

5.1 The Isolation of Protoplasts and Chloroplasts from Young Barley Leaf

A major part of the work in this thesis involved the isolation of protoplasts and chloroplasts from young barley leaf tissue.

The protoplasts, prepared by a method based on Day's (Day, 1981) were of high quality. Their appearance under the phase-contrast microscope, the O_2 evolution rates and the ^{14}C-acetate and ^{14}C-bicarbonate incorporation values were all comparable with the results of other workers.

Chloroplasts caused considerably more difficulty in isolation. When mechanical methods were used there were numerous problems. Studies show that mechanical methods give better results if quick blending

techniques are used and with Polytron blenders, and fast centrifugations (Halliwell, 1981). Even with these considerations there was still a high rate of chloroplast breakage. Often, bacteria present on the barley leaves come through into the final suspension. This has also been noticed by other workers (Appelqvist, 1968). Some groups have found the blending method successful, even with non-spinach sources (Leegood & Walker, 1975).

Another isolation method was used, one which involved using pre-made proplasts, lysing them and separating out the chloroplasts. This method is particularly good for obtaining chloroplasts from grasses and crop plants (barley, wheat, maize) as they have a high fibre content and the mechanical methods cause a significant degree of breakage of the organelles. The method of lysing protoplasts has been used for obtaining other organelles, for example vacuoles from barley protoplasts (Kaiser and Heber, 1984), apart from chloroplasts. The results of my own experiments showed this method to be far better for obtaining Class A chloroplasts (see table 5.1). Other workers doing similar comparisons have had the same conclusions (Leegood and Walker, 1981; Edwards et al., 1979).

O_2 evolution values serve as a direct measure of chloroplast integrity. The results from different plant species often vary to a large degree. Results of 120 μmoles O_2/hr/mg chlorophyll have been obtained for wheat flag leaf (Leegood and Walker, 1979), 150 for Panicum miliaceum (Edwards et al, 1979), 210 for maize (Day et al., 1981). Pea chloroplasts have given rates of 20 μmoles O_2/hr/mg chlorophyll when

Table 5.1 Comparison of the Methods of Isolating Chloroplasts from Barley Leaf Tissue.

	Method	
	Blending Method	Produced via protoplasts
1. O_2 evolution (μmoles O_2/hr/mg chl	not able to be determined due to bacterial contamination	17.5
2. Ferricyanide intactness test (% intact)	75%	95%
3. ^{14}C-Acetate incorporation into total lipids (nmoles incorp./hr/mg chl)	721.83	1061.97

98-100% intactness was shown by the ferricyanide test (Drapier et al., 1982). In this study the rates were low, averaging 17.5 μmoles O_2/hr/mg chlorophyll; but examination under the phase-contrast microscope and the results of the ferricyanide intactness test demonstrated that they had good integrity. These results suggest that the low O_2 evolution rates are peculiar to this plant species and are not a reflection of the quality of the chloroplasts isolated.

The isolation of chloroplasts by lysing protoplasts does however have disadvantages. Compared with blending methods it is far more laborious and time consuming and the yield is much lower. The protoplasts isolated from mature leaves contain only 5% of the chlorophyll present in the starting tissue, those from young leaves contain 10% (Leegood and Walker, 1979; Edwards et al., 1979); there is again a loss on conversion of protoplasts to chloroplasts.

There is the problem of purifying the chloroplasts from the contaminating protoplasts as smaller protoplasts can often get through the lysing procedure without being broken. They can be separated using a sucrose gradient. This however can result in dehydration of the chloroplast (Kuhn and Stumpf, 1981) and subsequent loss of normal function. For this reason the purification step was omitted in the isolation procedure carried out in this study, particularly as phase-contrast microscope examination showed no evidence of contaminating protoplasts. Despite all the problems involved for incorporation studies the difference in the quality of the chloroplasts produced by the new method seems much better then the traditional blending technique.

Once isolated the chloroplasts are suspended in a media of 0.4M sorbitol. This molarity is used for plants such as wheat and barley. Other species, like pea and spinach, seen to function best at 0.33M molarity (Leegood and Walker, 1983).

The concentration of the other ingredients in the media are of importance in maintaining normal operation. Phosphate levels were varied in a preliminary experiment to enable an appropriate level to be determined for use in the medias. The results of this (see results, figure 4.27) showed that it was only in a very narrow concentration range that the chloroplasts had good O_2 evolution rates. The optimal phosphate concentration is 0.25mM. Other workers have obtained identical graphs for chloroplasts from wheat (Leegood and Walker, 1979; Day et al, 1981) Panicum miliaceum (Edwards et al., 1979), spinach and pea (Edwards et al., 1978). The phosphate effect has some temperature dependence, as at a lower temperature a higher concentration is required in the stroma to maintain photosynthesis (Leegood and Walker, 1983). Phosphate concentrations are of particular sensitivity in the chloroplast because the major export of substrates out of the chloroplast is mediated by a phosphate translocator (Heber and Heldt, 1981).

The pH at which incubations are performed is at 8.4. This is the pH where lipid synthesis is maximal. It contrasts with protoplast incubations being performed at pH 5.8, the low pH necessary for uptake of acetate into the cell (Jacobson and Stumpf, 1972). This demonstrates that the limiting point in acetate assimilation is at the cell membrane and not across into the chloroplast.

5.2 The Incorporation of Labelled Precursors into Triglyceride and Other Acyl Lipids by Protoplasts and Chloroplasts from Barley Leaf

When barley protoplasts are incubated with labelled precursors high amounts of the label are found in acyl lipids. When ^{14}C-acetate was used as precursor it accounted for more than 68% of the label in the total lipid products. These acyl lipids were present whether the incubations were carried out in light or dark conditions, with ^{14}C-acetate or ^{14}C-bicarbonate. Approximately 57% of the acyl lipid from U_{SF} was made up of triglyceride (identified by running against markers in several TLC solvent systems). This high concentration of label into triglyceride is unusual. The characteristic lipids of the leaf are the galactolipids. These would be expected to be a major portion of the lipid products.

To try and help in interpreting these results several investigations were made. The first was to test the level of endogenous triglyceride in the whole barley leaf tissue. Triglyceride was found to be present but in very low concentrations, only 14:8 nmoles/g weight of fresh tissue. Results in a similar range were obtained by Martin and Wilson in 1983 with spinach leaf. The fatty acids in the triglyceride from whole barley leaf were the typical plant leaf fatty acids; predominantly 16:0, 18:0, 18:1, 18:2 with smaller amounts of 18:3.

To further investigate the appearance of large amounts of label in the triglyceride an experiment was set up using protoplasts from barley tissue of different maturity. This was achieved by cutting the leaf into graded sections in a basal to distal fashion. The results of this

experiment showed that although the more immature sections were able to incorporate radioactive label more readily, (and so were of higher quality), the proportion of the label incorporated into acyl lipid also increased. This suggests the acyl lipid synthesis is somehow connected with the tissue immaturity.

The significance of the high incorporation into triglyceride is still not understood. Why are there such huge rates of incorporation when the endogenous level of triglyceride in whole leaf is so minute? And how does this involve tissue immaturity? Basically there are three approaches to try and explain this phenomena:-

(a) The increased incorporation into triglyceride that we are seeing is not actually due to a large amount of triglyceride synthesis. One possibility is that in the plant cell two pools of acetate are being formed, one of high specific activity. This high specific activity pool is used preferentially for triglyceride synthesis. This explains the presence of triglyceride with a high specific activity.

(b) The high proportion of label being incorporated into triglyceride by the barley protoplasts, much higher than the leaf de novo triglyceride synthesis, is because the incubation conditions are for some reason favouring this biosynthetic pathway. What these conditions are is uncertain. The optimal conditions for the enzymes of triglyceride synthesis, ie., pH, temperature-dependence and magnesium concentration, are similar to the other enzymes of the glycerolipid pathway. Removal of the cell wall, to make protoplasts, could be causing an abnormal environment for lipid synthesis; although this is refuted in the literature (Kuhn and Stumpf, 1981).

(c) The triglyceride synthesis indicated in these incorporation studies is an accurate account of the rate of synthesis *in vivo*. It is unusually high in immature tissue because it is specific to a certain short developmental phase which involves the fixing of compounds for reserve. A similar event to the maturation of a seed is occurring in the barley leaf.

Barley chloroplast incubations with ^{14}C-acetate again revealed the presence of U_{SF} in large proportions (54%). The largest percentage of this was free fatty acids. This is as expected because the chloroplast is the organelle where fatty acid synthesis occurs in the leaf. However, once again the triglyceride was again present; 35% of the acyl lipids. This triglyceride was found not just in incubations with chloroplasts isolated via protoplasts, suggesting it could be due to protoplast contamination, but also with chloroplasts isolated by mechanical methods. This was not the predicted result as triglyceride synthesis in the leaf is thought to occur around the endoplasmic reticulum in the cytosol (Roughan and Slack, 1982). This may be due to endoplasmic reticulum and other membrane fragments being present even though there are several washings in the isolation procedure. These contaminations have been found to be present even when chloroplasts are isolated from protoplasts and put through density gradients.

The significance of why high concentrations of triglyceride and other acyl lipids are being synthesised from protoplasts and chloroplasts isolated from young barley leaf tissue, is still not understood. It

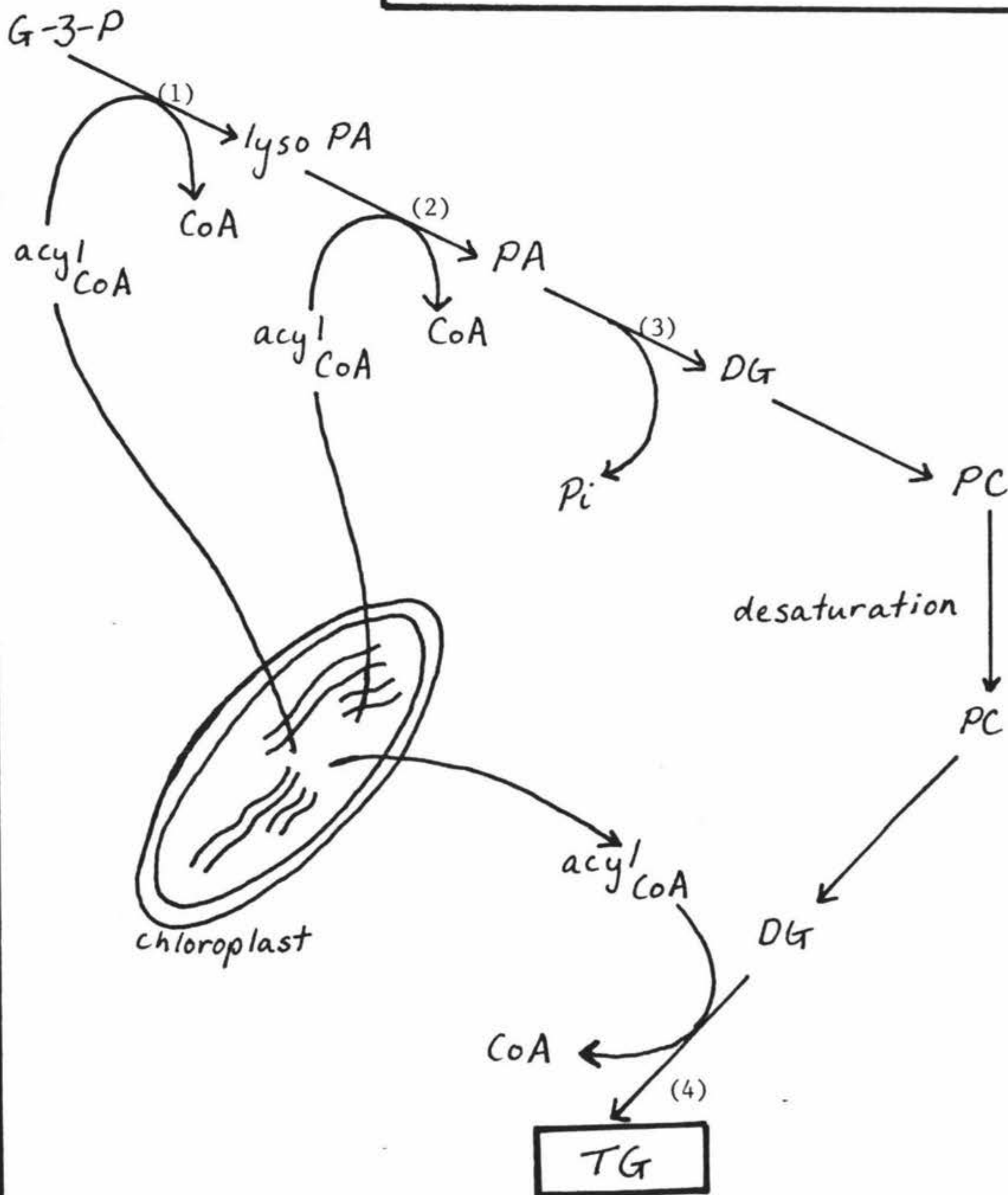
may be a specific developmental stage of the plant for the collection of reserves.

5.3 Triglyceride Synthesis in Leaf Tissue

In the plant triglycerides are classically thought of as the reserve compounds in the seed. It is found in seeds in high concentrations often with characteristic long chain fatty acids of unusual structure. The synthesis of these compounds occurs rapidly in the plastids over a very specific time period (Gurr et al., 1972) coinciding with the maturation of the seed.

Very little research, however, has been done on triglyceride synthesis in the leaf. Triglyceride synthesis in the leaf is via phosphatidyl choline (Roughan and Slack, 1982) and not by the normal Kennedy pathway (see figure 5.1). This was supported in work with soybean cotyledons (Stymne, 1982). This route would allow a second double bond to be included in the fatty acids on the diglyceride, suggesting that the major diglyceride source would be 1,2 dilinoleoyl diglyceride. It also implies that the site for triglyceride synthesis in the leaf is the cytosol as the phosphatidyl choline is unable to be synthesised by the chloroplast itself. The experimental work in this thesis demonstrated that triglyceride made by the protoplasts contained high concentrations of 18:2 (55%) along with 30% 16:0 suggesting that the acyl donor was preferentially 16:0 CoA. This appeared to support the scheme suggested by Roughan and Slack (1982).

Figure 5.1 Operation of the Modified Kennedy Pathway for the Synthesis of TG in the Plant Cell



(1) Glycerophosphate - acyl transferase
(2) 1-acyl glycerol phosphate acyl transferase
(3) phosphatidate phosphatase
(4) Diacyl glycerol acyl transferase (DGAT)

However, when chloroplasts were used in incubations with ^{14}C-acetate surprisingly the triglyceride again appeared. The chloroplastic triglyceride was made up predominately of 16:0 (72%) with some 18:1 present. These results directly conflict with the Roughan and Slack triglyceride synthesis scheme. It suggests that the synthesis of this chloroplastic triglyceride is occurring totally within the chloroplast, without going through phosphatidyl choline and with the enzymes having completely different specificities than predicted.

In 1983 Martin and Wilson analysed DGAT - the only enzyme in the triglyceride biosynthesis pathway that is particular to triglyceride synthesis. This DGAT from spinach leaves is the only one that has been charcterised from plant tissue. It was found to have a pH optima of 8; similar to the other enzymes in the glycerolipid pathway; was temperature dependent, had highest activity with diolein as the diglyceride, and had the lowest K_m for 16:0 CoA substrate. All of these characteristics are similar to the microsomal enzyme from rat adipose tissue (Coleman and Bell, 1976). This suggests that the plant and mammalian enzymes are very similar. Martin and Wilson found that the enzyme was associated with the chloroplast envelopes. How the DGAT enzyme is positioned on the envelope, whether on the outer or inner side, is not clear. This finding still rules out the possibility that triglyceride synthesis is occurring around the phosphatidyl choline synthesis site; in the endoplasmic reticulum in the cytosol.

The problem still remains that the triglyceride synthesised by protoplasts preparations contains different fatty acids to the

chloroplastic triglyceride. The chloroplast's triglyceride contains only 16:0, 18:0 and 18:1. These fatty acids are the main ones produced by the chloroplast; the chloroplast being only able to insert one double bond, that found in oleic acid. The differences in the fatty acids on the triglycerides suggest one of two options. Either the chloroplastic triglyceride is being shipped out of the chloroplast and altered in the cytosol or else the triglycerides are present as two separate pools. When the difficulties of transporting a large triglyceride molecule across the chloroplast envelope are considered, as well as the whole set of enzymes required to elongate and desaturate the fatty acids, the latter suggestion seems more probable.

If there are two separate pools of triglyceride then synthesis must be occurring both inside the chloroplast and in the cytosol. Two different pathways could operate, the cytosolic triglyceride being synthesised by the Roughan and Slack scheme and the chloroplastic triglyceride by the normal Kennedy route that also occurs in the plastids in seeds. This means DGAT must be present in both sites. The fatty acid specificities of the two enzymes would also have to be quite different.

The role of leaf triglyceride in the plant's metabolism is not at present understood. It probably serves as a reserve for the plant cell in a similar, although not so dramatic, way to its usage in the seed. Visually, from electron micrographs, "intraplastic lipid globules" have been found within the chloroplast itself (Appelqvist and Von Wettstein, 1975). These globules may be similar in function to starch grains for

use as a reserve by the chloroplast. This evidence further supports triglyceride synthesis occurring in chloroplasts. If this postulate is correct then enzymes for triglyceride breakdown would be present in the chloroplast, but as yet these enzymes have not been isolated.

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