# LIPID BIOSYNTHESIS IN ISOLATED BARLEY PROTOPLASTS

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# ABSTRACT

In most studies of fatty acid and lipid synthesis in plants there has been poor incorporation of radioactive label from acetate into linoleic (18:2) and linolenic (18:3) acids. Consequently the amounts of these fatty acids found in the galactolipids in such studies are much less than their observed endogenous levels.

In the present study incorporation of $H^{14}CO_3^-$ and (1-$^{14}$C) acetate into lipids of barley protoplasts was examined. $CO_2$-dependent $O_2$ evolution rates of the protoplasts were around 180 µmol $O_2$/h/mg Chl and intactness was also ascertained by phase contrast microscopy. Incubating protoplasts with 1mM $H^{14}CO_3^-$ or 50 µM (1-$^{14}$C) acetate resulted in 146.2 and 17 nmol/mg Chl being incorporated into lipids respectively after 1 hour. A concentration of 10 mM was optimal for $HCO_3^-$ incorporation and up to 580 nmol/mg Chl was incorporated into lipids at the end of 1 hour. $Mg^{++}$ and $P_i$ ions used at 2 mM had little effect on $HCO_3^-$ incorporation while $PP_i$ appeared to be slightly inhibitory. Acetate assimilation and its incorporation into lipids was markedly affected by pH and pH 5.8 was chosen for the assay medium. In 20 hour incubations 162 nmol acetate/mg Chl was incorporated. About 33% of label from acetate was found in each of palmitic (16:0) and oleic (18:1) acids with less than 9% in each of stearic (18:0), linoleic and linolenic acids. There was little or no incorporation of acetate into DGDG and less than 10% into each of PG, MGDG, PE and U (unknown lipid). Incorporation into PC after 2½ hours was 36.8% then decreased to 8.9%. Acetate incorporation was most significant into $U_{SF}$ (another unknown

lipid), being 73.4%. Although acetate was incorporated into a range of glycerolipids, incorporation into constituent 18:2 and 18:3 of these lipids was not significant.

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# LIST OF ABBREVIATIONS

| | |
|---|---|
| ACP | acyl-carrier protein |
| ATP | adenosine 5'-triphosphate |
| BSA | bovine serum albumin |
| Chl | chlorophyll |
| CoA | coenzyme A |
| DAG (or DG) | diacylglycerol (or diglyceride) |
| DGDG (or DDG) | digalactosyldiacylglycerol (or digalactosyldiglyceride) |
| EDTA | ethylenediamine tetraacetic acid |
| FA | fatty acid |
| FFA | free fatty acid |
| fr. wt | fresh weight |
| g.l.c. | gas-liquid chromatography |
| HEPES | N-2-hydroxyethylpiperazine-N'-2-ethane sulphonic acid |
| MES | 2[N-morpholino] ethane sulphonic acid |
| MG | monoacylglycerol (or monoglyceride) |
| MGDG (or DGD) | monogalactosyldiacylglycerol (or monogalactosyldiglyceride) |
| NADH | nicotinamide adenine dinucleotide, reduced form |
| NADPH | nicotinamide adenine dinucleotide phosphate, reduced form |
| PA | phoshatidic acid |
| PC | phosphatidylcholine |
| PE | phosphatidylethanolamine |
| PEP | phosphoenolpyruvate |
| PG | phosphatidyglycerol |
| PGA | phosphoglycerate |
| PI | phosphatidylinositol |
| POPOP | 1,4-bis[2(5-phenyloxazolyl)] benzene |
| $PP_i$ | pyrophosphate |
| PPO | 2,5-diphenyloxazole |
| TG | triacylglycerol (or triglyceride) |
| TLC | thin-layer chromatography |
| U, $U_{SF}$ | unknown compounds (see Section 3.6) |
| UDP -gal | uridine 5'-diphosphate D-galactose |

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# CHAPTER 1

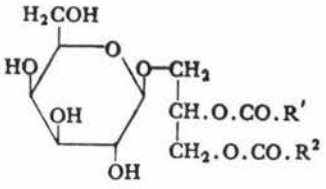
# INTRODUCTION

## 1.1 GENERAL INTRODUCTION

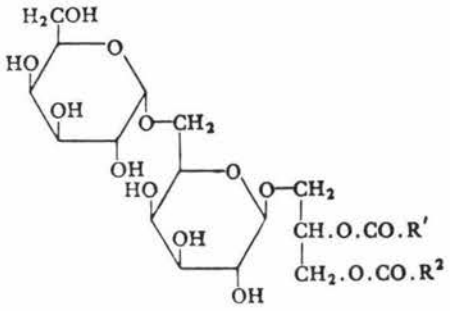
The lipids of plants, principally phospholipids and galactolipids, comprise up to 10% of the dry weight of the photosynthetic tissue. The chloroplasts contain a large proportion of the total lipid (Kates, 1970) and nearly all the galactolipid found in beet and tobacco leaves is located in the chloroplasts (Wintermans, 1960; Ongun *et al*, 1968).

Monogalactosyldiglyceride (MGDG) and digalactosyldiglyceride (DGDG), originally discovered in wheat flour by Carter *et al* (1956), are the two major galactolipids found in plants (Sastry, 1974). The structures of the two galactolipids were established by Carter *et al* (1961) in wheat flour (Figure 1-1) and Sastry and Kates (1964) subsequently isolated pure MGDG and DGDG from runner bean leaves and found them to be identical to those from wheat flour. In photosynthetic tissues (leaves) MGDG consistently exceeds the amount of DGDG (Roughan & Batt, 1969). They contain MGDG and DGDG in concentrations ranging from about 0.6-15 µmol/g and 0.5-7 µmol/g fresh weight, respectively (Sastry, 1974).

In general, the lipids of photosynthetic tissues, particularly the galactolipids, contain a high amount of polyunsaturated lipids (Sastry, 1974). MGDG appears to be more unsaturated than DGDG (Stumpf, 1980). $C_{16}$ fatty acids, when present, are mostly located at the C-2 position and $C_{18}$ acids at the C-1 position of galactolipids (Safford & Nichols, 1970; Siebertz 1977; Rulkötter *et al*, 1975). However saturated and monoenoic fatty acids, found as




MGDG




DGDG

FIGURE 1-1: The structures of monogalactosyldiacylglycerol (MGDG) and digalactosyldiacylglycerol (DGDG).

($R^1$ and $R^2$ are long chain fatty acyl residues (Hawke, 1973))

minor constituents of galactolipids, are esterified in position-1 of glycerol with 18:2 or 18:3 in position-2 (Noda and Fujiwara, 1967). 18:1 and 18:2 together usually account for less than 10% of the fatty acids in galactolipids.

Two types of photosynthetic tissue are distinguishable on the basis of their fatty acid content - the galactolipids of "18:3 plants" contain almost all 18:3 while the "16:3 plants" have 16:3 as well as 18:3 in their galactolipids. Major molecular species of galactolipids in 18:3 plants are mono- and digalactosyl linolenins with some DGDG molecules having 18:3 at C-1 and 16:0 at C-2 positions (Douce and Joyard, 1980; see Figure 1-2). In 16:3 plants, as well as the 18:3-18:3 combinations in MGDG and DGDG, MGDG containing 16:3 at C-1 and 1:3 at C-2 and 18:3-16:0 combinations in DGDG are common (see Figure 1-2).

The exact biological function of galactolipids is uncertain. Anderson *et al* (1974) ascertained that their direct involvement in the photosynthetic process was unlikely since large amounts of galactolipid could be removed from photosynthetic membranes, by treatment with lipases, without marked effects on electron transport. It is clear that the galactolipids which represent 80% of the polar lipids in chloroplasts are the principal constituents of the fluid bilayer acting both as a permeability barrier to polar molecules and as a flexible framework capable of accommodating a variety of proteins (Stumpf, 1980).

Plants are the principal sources of 18:2 and 18:3 and provide the primary source of these polyunsaturated fatty acids for animals. Animals use linoleic and linolenic acids in the synthesis of long chain polyunsaturated fatty acids such as $\gamma$-homolenic and arachidonic acids which

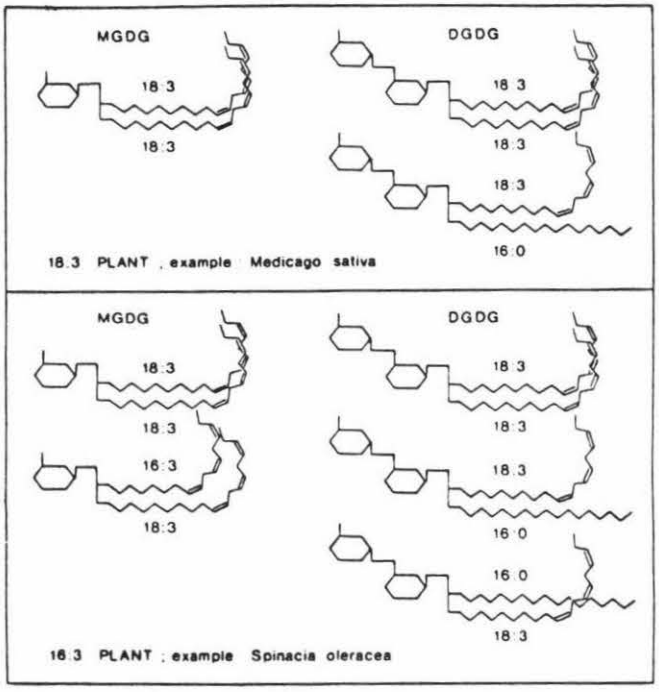



FIGURE 1-2: Structure of the major molecular species of galactolipids (MGDG and DGDG) found in 18:3 and 16:3 plants. (Douce and Joyard, 1980)

are essential for normal growth and development (Guarniere and Johnson, 1970) and are precursors of the prostaglandins and thromboxanes (Samuelsson *et al*, 1978).

The inability of isolated chloroplasts to synthesise poly-unsaturated fatty acids at the high rates that would be expected from the quantities found in chloroplast lipids has been attributed by a number of workers (Harris *et al*, 1967, Hawke *et al* , 1974; Kannangara & Stumpf, 1972a; Nakamura & Yamada, 1975) to the removal of other cellular components and to the loss of soluble cofactors during chloroplast preparation. However Roughan and Slack (1982) have claimed that isolated chloroplasts capable of *in vivo* photosynthetic rates would not have lost any cofactors normally required. They consider such chloroplasts would be self-sufficient for *de novo* fatty acid synthesis and that the effects of any additions would be representative of cytoplasmic influences on internal chloroplast function.

In this present study, protoplasts isolated from barley leaf tissue were used to investigate the synthesis of fatty acids and their distribution in lipids, in particular in the galactolipids MGDG and DGDG. Protoplasts produce advantages over isolated chloroplasts in that possibilities for interplay between chloroplasts and other cellular components are retained. At the same time the disadvantages of using tissue to investigate precursor-product relationships are overcome.

## 1.2 BIOSYNTHESIS OF SATURATED FATTY ACIDS

### 1.2.1 Initial Steps

Although $CO_2$ is the primary carbon precursor of fatty acids, acetyl-CoA is the immediate precursor of fatty acids in plant tissues. Sucrose, formed from $CO_2$, is the principal transport photosynthetate in the plant cell, and it is converted to glucose-1-phosphate (glucose-1-P) by a series of reactions documented by Yamada *et al.* (1974) and Simcox *et al*. (1977). Glucose-1-P is degraded in the cytoplasm by glycolytic enzymes to phosphoenol pyruvate (PEP) which is then further converted either to malic acid or pyruvic acid. Strong evidence suggests these reactions occur in proplastids which are large organelles found in developing (Simcox *et al*, 1977; Weaire and Kekwick, 1975; Yamada *et al*, 1974) as well as germinating seed tissue (Vick and Beevers, 1978), and also to a limited extent in isolated chloroplasts (Murphy and Leech, 1977). A portion of the pyruvate formed by the above reactions is channelled away from the TCA cycle system to provide acetyl-CoA for fatty acid biosynthesis.

Yamada and Nakamura (1975) demonstrated the incorporation of $^3H_2O$ into fatty acids of spinach chloroplasts in the presence of unlabelled PGA, PEP and pyruvate and hence proposed the operation of the following pathway in illuminated chloroplasts: PGA → PEP → pyruvate → acetyl-CoA → fatty acids. The PEP or pyruvate carboxylation, citrate lyase reaction and malate synthetase reaction were shown by them not to be involved in the formation of acetyl-CoA and fatty acids.

From the incorporation of ($^{14}$C) bicarbonate, (2-$^{14}$C) pyruvate, (1-$^{14}$C) acetate and (U-$^{14}$C) C phosphoglycerate into fatty acids, and from isotope competition experiments, Murphy and Leech (1977, 1978) concluded the source of acetyl-CoA in isolated spinach chloroplasts was by the

pathway proposed by Yamada and Nakamura (1975). Murphy and Leech (1977) concluded that fatty acids were synthesised via acetyl-CoA from $CO_2$ supplied to chloroplasts and leaves as ($^{14}C$) bicarbonate and $^{14}CO_2$ respectively. Chloroplasts synthesised acetyl-CoA at 36% of the rate of intact leaves (Murphy & Leech, 1978).

Roughan *et al* (1979c) found that exogenouse (1-$^{14}C$) acetate was superior to (2-$^{14}C$) pyruvate, (2-$^{14}C$) malonate and $NaH^{14}CO_3$ for fatty acid biosynthesis by spinach chloroplasts by factors of 3-4, 140 and 7-8 respectively. Both commercially available (2-$^{14}C$) pyruvate and (2-$^{14}C$) malonate were contaminated with 3-6% (w/w) acetate thus casting some doubt on experiments using these substrates (Yamada & Nakamura, 1975). It was concluded that products of $CO_2$ fixation were probably not significant carbon sources for fatty acid synthesis in the presence of exogenous acetate. Furthermore, Roughan *et al* (1979a) found that pyruvate dehydrogenase activity was absent in spinach chloroplasts, although pyruvate decarboxylation was observed. Results obtained by Murphy & Stumpf (1981) imply that the decarboxylation activity is related to acetolactate synthetase which utilises pyruvate as a substrate to form acetolactate together with $CO_2$. In the spinach leaf cell Murphy & Stumpf (1981) found appreciable pyruvate dehydrogenase activity in the mitochondrion but only low activity in isolated chloroplasts. Spinach leaf mitochondria were also found to accumulate (1-$^{14}C$) acetate following incubation of mitochondria with (1-$^{14}C$) acetyl-CoA thus verifying an earlier report of Stumpf (1972) that acetyl-CoA hydrolase is present in this organelle. Kuhn *et al* (1981) determined that acetyl-CoA synthetase is localised solely in the chloroplast, and also that 1 mM free acetate was present in the spinach leaf cell. A highly active acetyl-CoA synthetase localised in spinach chloroplasts is capable of converting acetate to acetyl-

CoA at a rate of 8 µmol/mgChl/h (Kuhn & Stumpf, 1981). Thus Murphy & Stumpf (1981) propose that acetyl-CoA generated from pyruvate by pyruvate dehydrogenase in the mitochondria is hydrolysed to acetate which then moves to the chloroplast. The acetate would be converted to acetyl-CoA in the chloroplast by the action of acetyl-CoA synthetase and be available for fatty acid synthesis. Studies by Murphy & Leech (1981) support this proposal.

Chloroplastic acetyl-CoA carboxylase (ACC) converts acetyl-CoA to malonyl-CoA utilising bicarbonate and ATP (Kannangara & Stumpf, 1972b). It was thought that ACC activity operated through chloroplastic protein components - biotin carboxyl carrier protein (BCCP) localised in the thylakoids and stromal biotin carboxylase and transcarboxylase (Kannangara & Stumpf, 1972b). However it is now recognised that ACC is a single biotin protein with a molecular weight of approximately 50,000 daltons (Mohan & Kekwick, 1980; Nikolau & Hawke, 1982 (unpublished)). Acetyl-CoA and malonyl-CoA are converted to their respective ACP derivatives by transacylases and subsequently utilised for fatty acid biosynthesis.

There is good evidence in plant tissues that the synthesis of palmitic acid (16:0), the most abundant and important saturated fatty acid in higher plants, involves a *de novo* system; so called because it utilises acetyl-CoA, malonyl-CoA, acyl carrier protein (ACP), and the coordinated activity of soluble, nonassociated enzymes (palmitoyl-ACP synthetase) to form, as its terminal product, palmitoyl-ACP (Stumpf, 1980).

Using antibodies raised against purified spinach ACP, Ohlrogge *et al* (1979) documented the essential requirement of ACP for all *de novo* fatty acid biosynthesis in the spinach leaf cell. All of the ACP in the spinach

leaf cell was found to be localised in the chloroplast, and so it followed that the chloroplast is the specific site for fatty acid synthesis.

### 1.2.2 Formation of Palmitate and Stearate

Although the usual products of acetate incorporation by chloroplasts are palmitate, oleate, some stearate, and small amounts of linoleate and linolenate (James, 1962), the initial product of *de novo* fatty acid synthesis in plants (eg Stumpf, 1977) as in animals (Vance and Bloch, 1977) is palmitoyl-ACP. Requirements for its synthesis include ACP, NADPH, NADH, acetyl-CoA and malonyl-CoA (Jaworski *et al*, 1974). It was demonstrated by Jaworski *et al* (1974) that palmitoyl-ACP may be elongated to stearoyl-ACP by a system which requires palmitoyl-ACP, malonyl-CoA and NADPH. The elongation system was present in extracts from safflower seeds, avocado mesocarp, and stroma of spinach chloroplasts. It was shown also by Jaworski *et al* (1974) that the elongation system differed from the *de novo* fatty acid synthetase system in requiring only NADPH for full activity whereas the synthetase required both NADPH and NADH.

## 1.3 BIOSYNTHESIS OF UNSATURATED FATTY ACIDS

### 1.3.1 Biosynthesis of Oleic Acid

Stearoyl-ACP desaturase was first demonstrated in *Euglena gracilis* and in spinach chloroplasts by Nagai and Bloch (1968). The enzyme has since been found in a wide variety of plant tissues - in developing safflower seeds, spinach chloroplasts, avocado mesocarp (Jacobson *et al*, 1974), soybean cotyledons (Stumpf and Porra, 1976), jojoba

cotyledons (Pollard *et al* , 1979) and coconut endosperm (Oo and Stumpf, 1979). In all plants examined, stearoyl-ACP desaturase is a soluble protein found either in the proplastid of the cell or in the stroma of the chloroplast (Stumpf, 1980). An input of two electrons either from NADPH or $H_2O$ via photosystems I and II is required and the intermediate electron carrier protein between the electron pair and molecular oxygen in plants is ferredoxin (Stumpf, 1980).

Free oleic acid, rather than oleoyl-ACP, is the product of the desaturase activity (Jacobson *et al*, 1974) because of the presence of an acyl-ACP thioesterase which rapidly hydrolyses oleoyl-ACP to free oleic acid (Sine *et al* , 1976).

### 1.3.2 Biosynthesis of Linoleic and Linolenic Acids

Harris & James (1965a) and Harris *et al* (1965) were the first to investigate the biosynthesis of linoleic and linolenic acids in plant leaves and the algae *Chlorella vulgaris* . Intact or chopped leaf tissue of castor bean and lettuce, and whole cells of *Chlorella vulgaris* , were shown to desaturate (1- $^{14}C$) oleic acid to linoleic and linolenic acids (Harris & James, 1965a). Fractionation studies suggested that the desaturase activity resided in the chloroplast (Harris & James, 1965a). Sequential desaturation of oleic acid to linoleic and linolenic acids was implied from labelling patterns (Harris & James, 1965a,b; Harris *et al* , 1965). James (1963) had earlier concluded that oleic acid was sequentially desaturated to linoleic and linolenic acids from studies of acetate incorporation in *Ricinus communis* leaves.

Evidence for the sequential desaturation of stearate to linolenate, via oleate and linoleate, has gathered steadily. Cherif *et al* (1975) from labelling studies in intact leaves, seeds, flowers, seedlings and roots from byrophytes, gymnosperms and algae, concluded that such a system was operating. Further support for the sequential desaturation pathway has been obtained from $^{14}CO_2$ and (1-$^{14}$C) acetate feeding experiments in pumpkin (Roughan, 1970), spinach and sorghum (Roughan, 1975), developing maize leaf (Slack and Roughan, 1975), broad bean (Williams *et al*, 1976, Heinz and Harwood, 1977) and *Anthriscus*, *Chenopodium*, and spinach (Siebertz & Heinz, 1977; Murphy and Stumpf, 1979).

An alternative pathway to the sequential desaturation of stearate to linolenate via oleoyl and linoleoyl intermediates was proposed by a number of workers. It was thought that trienoic acids such as 12:3 (Kannangara *et al*, 1973) or 16:3 (Jacobson *et al*, 1973a,b) could serve as precursors for elongation to linolenate.

## 1.4 Substrates of Oleate and Linoleate Desaturation

Some controversy has existed as to whether oleate and linoleate are desaturated as the free acid, the CoA or ACP derivative, or as the acyl moiety of lipids such as phosphatidylcholine (PC) and MGDG. A new concept was introduced into the study of long-chain fatty acid synthesis in plants by Nichols *et al* (1967). The incorporation of (2-$^{14}$C) acetate into individual glycerolipids of greening *Chlorella vulgaris* cells was monitored and it was found that one group of lipids, including phosphatidylglycerol (PG), PC and MGDG, had higher turnover rates for certain fatty acids than did another group

of lipids consisting of DGDG, sulpholipid (SL), phosphatidylethanolamine (PE) and phosphatidylinositol (PI). Nichols *et al* (1967) suggested that the lipids with the rapid fatty acid turnover might be involved as carriers or intermediates in the desaturation process.

Prior to this Harris & James (1965a), using whole cell homogenates and subcellular preparations of *Chlorella vulgaris* had found that oleoyl-CoA was the preferred substrate for desaturation to linoleate and linolenate. Vijay and Stumpf (1971, 1972) found an oleoyl-desaturase in microsomal preparations of developing safflower seeds which was specific for oleoyl-CoA and inactive with oleoyl-ACP. Although it was observed that both oleoyl and linoleoyl groups were transferred to position 2 of PC by a very active acyl transferase, they concluded that the oxygen ester did not serve as a substrate for desaturation. Abdelkader *et al* (1973), using microsomes from aged potato tuber slices, and Dubacq *et al* (1976), using a microsomal and mitochondrial preparation from pea leaves, showed that oleoyl-CoA could be desaturated to linoleoyl-CoA before being incorporated into PC.

It had been shown by Harris *et al* (1967) that (1-$^{14}$C) oleoyl-CoA could be desaturated by *Chlorella vulgaris* chloroplasts and that high levels of $^{14}$C from (1-$^{14}$C) oleoyl-CoA were in PC. It was concluded that PC might be involved in the desaturation reaction (Harris *et al*, 1967). Both phospholipids and glycolipids were implicated in fatty acid desaturation by Nichols *et al* (1967) and Nichols (1968) from labelling studies also with *Chlorella*.

Gurr *et al* (1969) and Roughan (1970, 1975) found that PC might be involved in desaturation as a carrier molecule. It was shown in *Chlorella vulgaris* that oleic acid, in

the presence of ATP and CoA, and oleoyl-CoA were rapidly incorporated into PC and newly synthesised linoleate was esterified to PC (Gurr *et al*, 1969; Gurr & Brawn, 1970; Gurr, 1971). Using pumpkin leaves Roughan (1970, 1975) observed that (1-$^{14}$C) acetate was incorporated into oleic acid of PC and desaturation to linoleate and linolenate of oleic acid followed. The failure of linolenate to accumulate in PC coupled with the rising levels of both linoleate and linolenate in other glycerolipids, in particular MGDG and DGDG, led Roughan (1970, 1975) to suggest that PC, as well as being the site for oleate desaturation, was also a donor of polyunsaturated fatty acids for the synthesis of other polar lipids. Further support for PC being the substrate for oleate desaturation in leaves has been obtained by Hawke & Stumpf (1980a) using maize leaves, Browse *et al* (1981) using spinach leaf discs and Slack *et al*, (1976) using microsomal preparations from immature maize leaf.

It has been shown by Dubacq *et al* (1976) and Slack *et al* (1976, 1979) that the 18:1 PC desaturase in plants is membrane-bound and probably in the endoplasmic reticulum. Kader (1977) and Stymne & Appelqvist (1978) have demonstrated that the enzyme requires $NADH_2$ and $O_2$ and that it is inhibited by cyanide.

Evidence for the co-operation between chloroplasts and other cellular organelles for the synthesis of polyunsaturated fatty acids has been suggested by studies of Hawke *et al* (1974) and Tremolieres & Mazliak (1974). Slack, Roughan and co-workers proposed that oleic acid synthesised in the chloroplasts is transferred to PC of the endoplasmic reticulum and further desaturated to linoleic acid (Slack and Roughan, 1975; Slack *et al*, 1977). Linoleate-PC thus formed is converted to linoleate-DG in the endoplasmic reticulum and then transferred to

the chloroplast where it is metabolised to linolenate-MGDG via linoleate-MGDG (Roughan *et al*, 1979b). Spinach chloroplast envelopes have been shown to contain a long-chain acyl-CoA synthetase which would presumably facilitate the movement of oleic acid out of the chloroplast by formation of oleoyl-CoA at the plastid/cytosol interface and its subsequent release into the cytoplasm (Roughan and Slack, 1977). Drapier *et al* (1982) have demonstrated that ($^{14}$C) oleate is specifically exported towards the external medium from isolated pea chloroplasts. Addition of microsomes resulted in a stimulated exportation and incorporation of the exported oleate into PC.

Hawke and Stumpf (1980a) have shown that 18:1-PC is a precursor of 18:2-PC in maize tissue. This has been implied also in studies of (1-$^{14}$C) acetate incorporation into the glycerolipids of soybean cell suspensions (Wilson *et al*, 1978), ($^{14}$C) oleic and ($^{14}$C) linoleic incorporation into glycerolipids of greening cucumber (Murphy & Stumpf, 1980) and in (1-$^{14}$C) acetate, (2-$^{3}$H) glycerol and ($^{14}$C) 18:1 feeding experiments of *Avena* leaves (Ohnishi & Yamada, 1980, 1982). This desaturation reaction occurs in the endoplasmic reticulum (Stymne & Appelqvist, 1978; Slack *et al*, 1979).

From the kinetics of incorporation of 18:2 and 18:3 into MGDG, a transfer of linoleate from PC to MGDG was suggested (Hawke & Stumpf, 1980a). Results of $^{14}CO_2$ assimilation experiments using *V. faba* leaf slices are in accordance with $C_{18}$ fatty acids being transferred from PC to MGDG as a diglyceride (Williams *et al*, 1976; Simpson & Williams, 1979; Lem & Williams, 1981). Slack *et al* (1977) also concluded that diglycerides derived from PC were utilised for MGDG synthesis and studies of Hawke & Stumpf (1980a) using maize seedlings implied that linoleate-diglyceride was a precursor of linolenate-MGDG. From labelling studies

of glycerolipids in *Avena* leaves Ohnishi & Yamada (1980, 1982) in addition have hypothesised that linoleate-PC in the endoplasmic reticulum is transferred to the chloroplast by a phosphatidylcholine-exchange protein (PCEP) and then converted to linoleate-MGDG via linoleate-diglyceride in the chloroplast. The existence of a PCEP has been demonstrated in *Avena* and spinach leaves (Tanaka *et al*, 1980; Julienne *et al*, 1981) and its presence implied by studies of Jones & Harwood (1980) in lettuce chloroplasts.

The desaturation of 18:2 while associated with MGDG, to form 18:3-MGDG, has been suggested by a number of studies (Hawke & Stumpf, 1980a, 1980b; Ohnishi & Yamada, 1980, 1982; Wilson *et al*, 1978; Roughan *et al*, 1979b). However Siebertz & Heinz (1977) deduced from the labelling pattern of $C_{16}$-fatty acids of MGDG from *Anthriscus cerefolium* and spinach that palmitate was sequentially desaturated to 16:3 and, for the desaturation of $C_{18}$-fatty acids of MGDG, a similar labelling pattern was also observed.

The desaturation of ($^{14}C$) oleoyl-CoA by homogenates from soya bean cotyledons, and the resultant distribution of the ($^{14}C$) linoleate and ($^{14}C$) linolenate products in glycerolipids, led Stymne & Appelqvist (1980) to suggest that there is an intimate coupling between PC and linoleate desaturation. Earlier, Heinz & Harwood (1977) and Wharfe and Harwood (1978) concluded that PC was not involved in 18:2 desaturation. Similarly Stymne and Appelqvist (1980) concluded that MGDG did not play a role in 18:2 desaturation since very little 18:2 was transferred to that lipid. Their results however indicated that considerable desaturation of the linoleate moiety took place after it had been transferred from the CoA molecule to complex lipids, in particular PC. They claimed that PC acts as a donor of both linoleate and linolenate to other complex lipids.

Based on other studies, for instance Stobart *et al* (1980) looking at ($^{14}$C) oleate desaturation in etiolated barley leaves, $^{14}CO_2$ feeding experiments of *V. faba* leaf discs by Williams (1980), and the examination of the effects of two substituted pyridazinone herbicides on the incorporation of $^{14}CO_2$ into the fatty acids of *V. faba* leaf discs by Lem & Williams (1981), is the proposal that 18:2 and 18:3 may be formed from 18:1-PC by sequential desaturation and then transferred from PC as a diglyceride to the chloroplast envelope for galactosylation to form MGDG. Desaturation of $C_{18}$ fatty acids is believed to occur in both PC and MGDG, and also in the diglyceride intermediate (Lem & Williams, 1981). Lem & Williams (1981) observed in herbicide treated tissue that radioactivity accumulated in MGDG, DGDG and sulpholipid 18:2 at the expense of 18:3, but the specific activity of MGDG 18:3 continued to increase throughout the experiment indicating only partial inhibition of MGDG 18:3 synthesis. It has been concluded that there are at least two sites for 18:2 desaturation to form 18:3 - one associated with MGDG in the chloroplast, which was inhibited by herbicide, and one or more extra-chloroplastic sites which were not inhibited by herbicide (Lem & Williams, 1981). The specific activity data was not compatible with free acyl exchange of fatty acids between PC in the cytoplasm and MGDG in the chloroplast, as suggested earlier by Wharfe & Harwood (1978) and Harwood (1979).

## 1.5 GALACTOLIPID SYNTHESIS

It is clear from the previous section that galactolipid and polyunsaturated fatty acid syntheses are interrelated, with the alternative possibilities of the final desaturation step in the biosynthesis of trienoic fatty acids occurring in diglyceride, a precursor of galactolipids, or

MGDG (Lem & Williams, 1981). Early studies (Ferrari & Benson, 1961; Neufeld & Hall, 1964; Ongun & Mudd, 1968) were consistent with the biosynthesis of galactolipids in plants occurring by a stepwise addition of galactose residues to diacylglycerol from UDP-galactose (UDP-gal):

$$\text{1,2-Diacyl-sn-glycerol} + \text{UDP-Gal} \rightarrow \text{MGDG} + \text{UDP}$$
$$\text{MGDG} + \text{UDP-Gal} \rightarrow \text{DGDG} + \text{UDP}$$

Douce (1974) and Douce & Benson (1974) subsquently clearly demonstrated that the chloroplast envelope is the major site of UDP-Gal incorporation into both MGDG and DGDG in leaf cells.

UDP-Gal:diacylglycerol galactosyltransferase catalyses the synthesis of MGDG from UDP-Gal and endogenous diacylglycerol (Joyard & Douce, 1976b) and the enzyme has been demonstrated to be associated with chloroplast envelope membranes (Joyard & Douce, 1976a). The formation of DGDG may be catalysed either by a UDP-Gal:MGDG galactosyltransferase which utilises UDP-Gal for the galactosylation of MGDG (Ongun & Mudd, 1968; Williams *et al*, 1975; Heinz, 1977) or by a galactolipid:galactolipid galactosyltransferase in which direct exchange of galactosyl groups between two MGDG molecules occurs to generate diacylglycerol (DAG) as well as DGDG (van Besouw & Wintermans, 1978; Wintermans *et al*, 1981; van Besouw *et al*, 1981). The results of pulse-chase experiments, using *V. faba* leaves, by Williams *et al* (1975) support the view that DGDG is formed by galactosylation of MGDG. However Dorne *et al* (1982) have demonstrated that galactolipid:galactolipid galactosyltransferase is located on the outer face of the outer chloroplast envelope and that it catalyses the synthesis of unnatural galactolipids (tri-DGDG and tetra-galactosyldiacylglycerol). They suggest that the enzyme is probably not involved directly in DGDG synthesis

and that "it is clear therefore that the enzyme responsible for DGDG synthesis still remains to be identified".

The role of diacylglycerol in the biosynthesis of galactolipids is firmly established in the above studies, but the origin of the diacylglycerol moiety in galactolipids is not so sure. Renkonen and Bloch (1969) found that cell-free extracts of *E. gracilis* catalysed the transfer of acyl groups from thioesters of ACP or CoA to MGDG and was stimulated by *sn*-glycerol 3-phosphate. This experiment strongly suggested that DAG was formed by the acylation of *sn* -glycerol 3-phosphate followed by the dephosphorylation of phosphatidic acid. The observation by Douce and Guillot-Salomon (1970) that intact plastids isolated from spinach and maize leaves incorporated label from *sn* -($^{14}$C) glycerol 3-phosphate into MGDG provided direct support for this pathway. Two acyltransferases (Joyard and Douce, 1977, 1979; Bertrams and Heinz, 1976, 1979) and a specific alkaline phosphatidic acid phosphatase (Joyard & Douce, 1979) have since been demonstrated in chloroplast envelope membranes. Douce & Joyard (1980) have claimed that the chloroplast envelope, and probably the inner membrane, contains the complete array of galactolipid biosynthetic machinery. Saturated and monounsaturated fatty acids, synthesised in the chloroplast stroma by the multienzyme complex fatty acid synthetase, along with galactose and glycerol are believed to be assembled in the chloroplast envelope to form the galactolipid molecule (see Figure 1-3).

Roughan and Slack (1982) on the other hand have proposed a model in which there are two pathways leading to galactolipid synthesis - one which is entirely chloroplastic and the other which involves the endoplasmic reticulum as well (see Figure 1-4). In the former pathway (1-18:1-2-16:0) DAG, produced in the chloroplast from 18:1-ACP

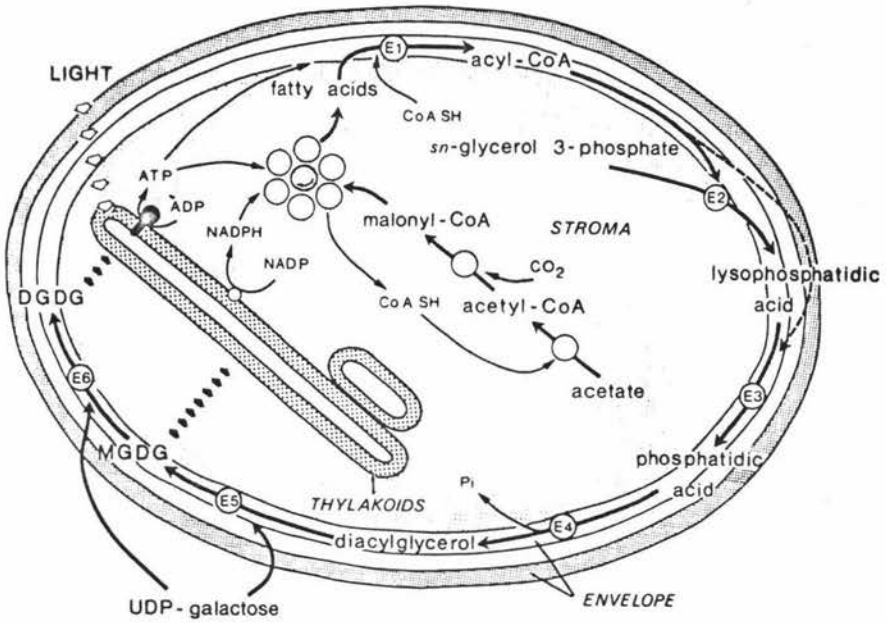



FIGURE 1-3: Model of galactolipid synthesis in the chloroplast as proposed by Douce and Joyard (1980).

and 2-16:0-G3P, gives rise to (1-18:1-2-16:0) MGDG which then undergoes desaturation to form (1-18:3-2-16:3) MGDG. The formation of MGDG upon the addition of UDP-Gal to DAG synthesised by isolated chloroplasts has been demonstrated by McKee and Hawke (1979) and Roughan *et al* (1980). Roughan *et al* (1979b) have shown that the fatty acids of the resultant (1-18:1-2-16:0) MGDG are desaturated *in situ* to yield molecular species with increasingly greater degrees of desaturation. Siebertz *et al* (1980) have detected the same sequence of molecular species of MGDG resulting from this desaturation in spinach leaves labelled in the galactose moiety *in vivo* with $^{14}CO_2$.

In the pathway involving the chloroplast and extrachloroplastic sites, 18:1-ACP generated by the chloroplast becomes 18:1-CoA in the cytoplasm and (18:1, 18:1) PC is consequently formed via the acylation by 18:1-CoA of G3P in the endoplasmic reticulum (see Figure 1-4). Desaturation of (18:1, 18:1) PC occurs and (18:2, 18:2) PC (Slack and Roughan, 1975; Slack *et al*, 1977) and perhaps small amounts of (18:3, 18:3) PC (Stobart *et al*, 1980) give rise to DAG in the endoplasmic reticulum which is subsequently incorporated into MGDG in the chloroplast envelope. Further desaturation and galactosylation of MGDG, to form DGDG, may occur in the chloroplast envelope.

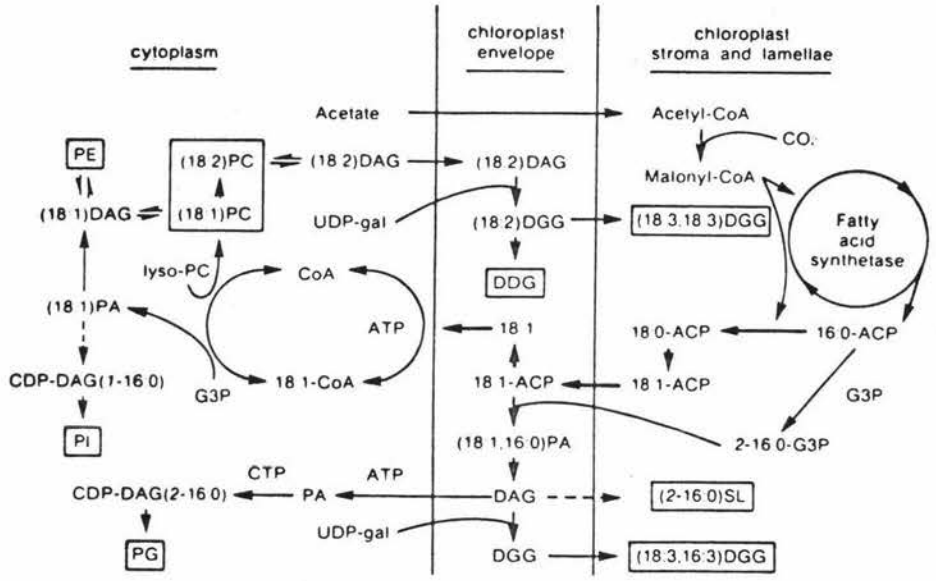



FIGURE 1-4: Model of galactolipid synthesis in the leaf cell as proposed by Roughan and Slack (1982).

(where DGG ≡ MGDG and DDG ≡ DGDG)

# CHAPTER 2

# MATERIALS

## 2.1 PLANT MATERIALS

Barley (*Hordeum vulgare*) and maize (*Zea mays* var. XL45) seeds were purchased from Arthur Yates and Co. Ltd N.Z. Seeds were soaked overnight in water and sown in trays of peat/pumice (1:4, v/v) potting mixture supplied with Hoagland's solution A (Hoagland & Arnon, 1938). Plants were grown in a controlled environment with day/night temperatures, vapor pressure deficits, and equivalent relative humidities at 25/20°C, 10/5 mbar and 68/78%, respectively. Day-length was 12 h and photosynthetically active radiation of 400-700 nm range was 170 w/m². After 6-7 days growth plants were harvested by cutting the shoots at the base. Protoplasts were isolated from whole seedling tissue of barley. When maize was used as a source of protoplasts, the coleoptile and first leaf were removed.

## 2.2 REAGENTS

The following chemicals were obtained from Sigma Chemical Co. St Louis: D-sorbitol, pectinase, MES, BSA Fraction V, POPOP and PPO. Cellulase (Cellulysin$^{TM}$) was from Calbiochem, La Jolla. HEPES was from Hopkins and Williams, Essex, England. Silica Gel G (type 60) was from E. Merck, Darmstadt, Germany. Sodium (1-$^{14}$C) acetate and sodium ($^{14}$C) bicarbonate were obtained from the Radiochemical Centre Ltd, Amersham, England. Ethylenesuccinate-methylsiliconecopolymer (EGSS-X) was from Applied Science Laboratories Inc., California, USA and Chromosorb Q 100/120 from Alltech Associates, Deerfield, U.S.A. All other chemicals were of an appropriate grade obtained from normal chemical suppliers. All solvents were redistilled before use.

# CHAPTER 3

# METHODS

## 3.1 PREPARATION OF MESOPHYLL PROTOPLASTS

The method used was based on that described by Day *et al* (1981) for *Zea mays* mesophyll protoplasts. Leaves were first abraded with 160 grit carborundum to break the epidermal layer, rinsed with water and blotted dry (Kojima *et al* (1979)). Using a razor blade the leaves were cut transversely into segments approximately 1 mm wide. 4g of sliced leaves were placed in 40 ml of incubation medium consisting of 0.5 M sorbitol, 0.2 mM $CaCl_2$, 0.2 mM $KH_2PO_4$, 1 mM $MgCl_2$, 10 mM MES buffer, 0.2% (w/v) BSA, 0.2% (w/v) pectinase and 2% (w/v) cellulase adjusted to pH 5.5 with NaOH. The tissue was evacuated on a water pump and then digested for 2 hours, with very gentle shaking, at 28°C and a light intensity of 64 $W/m^2$ ($6.4 \times 10^8$ $ergs/m^2/s$) (Edwards *et al* (1979). At the end of digestion the leaf tissue was filtered through a 124 µm nylon mesh and washed with solution A (0.6 M sorbitol, 0.2 mM $CaCl_2$, 0.2 mM $KH_2PO_4$, 1 mM $MgCl_2$, 0.2% BSA, 5 mM HEPES buffer adjusted to pH 7.8 with NaOH). The combined filtrate and washings were centrifuged at 300g for 3 min and the supernatant discarded. The pellet was gently resuspended in 7.5 ml of solution B (identical to solution A except that sucrose replaced sorbitol), overlaid with 3 ml of solution A, and centrifuged at 350g for 5 min. The band of protoplasts which aggregated at the sucrose-sorbitol interface was carefully removed with a Pasteur pipette. The protoplasts were concentrated by recentrifuging at 350g for 3 min in solution A and then resuspended and stored on ice in about 2 ml of solution A.

## 3.2 DETERMINATION OF INTACTNESS OF PROTOPLASTS

### 3.2.1 Phase Contrast Microscopy

Protoplasts were examined using phase contrast microscopy at magnifications of 300x and 600x to get a visual impression of their intactness.

### 3.2.2 Oxygen Evolution

$CO_2$ - dependent oxygen evolution was measured polarographically at 30°C with a Rank electrode system (Rank Bros, Cambridge, U.K.). Protoplasts containing approximately 50 µg of chlorophyll were added to $O_2$ electrode buffer [0.6 M sorbitol, 2 mM $KH_2PO_4$, 2 mM $MgCl_2$, 1 mM EDTA, and 25 mM HEPES adjusted to pH 7.5 with NaOH (or 25 mM MES adjusted to pH 5.8)] and 5 mM $PP_i$ (adjusted to pH 7.5 with HCl). After the baseline had stabilised $NaHCO_3$ was added to a concentration of 10 mM bringing the total volume of the reaction mixture to 2 ml. Illumination was provided by a Hanimex Rondette 1200 RF projector with an irradiance of 85 W/m² ($8.5 \times 10^8$ ergs/m²/s) at the surface of the electrode vessel.

## 3.3 INCUBATION OF PROTOPLASTS WITH $^{14}C$-BICARBONATE

Protoplasts were incubated in a volume of 0.5 ml containing 0.25 ml of oxygen electrode buffer [1.2 M sorbitol, 4 mM $KH_2PO_4$, 4 mM $MgCl_2$, 2 mM EDTA, and 50 mM HEPES adjusted to pH 7.5 with NaOH (Day *et al* (1981)), 5 mM $PP_i$ (this was later omitted as it was found to be inhibitory), 10 mM $H^{14}CO_3^-$ (5 µmoles, 1.84 µCi) and protoplasts equivalent to 50 - 55 µg of chlorophyll. The time of incubation was usually one hour. Reactions were stopped by the addition of sufficient chloroform: methanol (2:1) to form a single phase. Water was

then added to give two phases. The top water phase was removed and the remaining $CHCl_3$ layer washed successively with 1% $NaHCO_3$, 0.1 M NaCl and three times with water. The final $CHCl_3$ solution containing total lipid was dried under a stream of $N_2$ and then redissolved in 1 ml chloroform. A suitable aliquot was dried in a scintillation vial, 5 ml of toluence scintillant added, and radioactivity determined using a Beckman L5 8000 liquid scintillation system. When the total $HCO_3^-$ assimilated by protoplasts was examined, the reaction was stopped by adding 0.1 ml 6 M HCl. 0.1 ml was removed from each tube and dried on small squares of 3 mm paper and then radioactivity determined. Total lipid was then extracted and determined as before.

## 3.4 INCUBATION OF PROTOPLASTS WITH (1-$^{14}$C) ACETATE

Protoplasts were incubated in a volume of 0.5 ml containing 0.25 ml of oxygen electrode buffer (1.2 M sorbitol, 4 mM $KH_2PO_4$, 4 mM $MgCl_2$, 2 mM EDTA and 50 mM MES adjusted to pH 5.8 with NaOH), 50 µM (1-$^{14}$C) acetate (25 nmoles, 0.25 - 10.0 µCi) and protoplasts equivalent to 50 - 55 µg of chlorophyll. Incubation time varied. Reactions were stopped by the addition of sufficient chloroform : methanol (2:1) to form a single phase. Water was then added to give two phases. The top water phase was removed and the remaining $CHCl_3$ layer washed successively with 1% acetic acid, 0.1 M NaCl and three times with water. The final $CHCl_3$ solution containing total lipid was dried under a stream of $N_2$ and then redissolved in 1 ml chloroform. A suitable aliquot was dried in a scintillation vial and radioactivity determined.

## 3.5 INCUBATION CONDITIONS

Incubations were carried out at 25°C in 15 ml round-bottom tubes in a photosynthetic Warburg apparatus. The tubes were agitated at 78 cycles/min and sixteen 40 watt tungsten lamps provided a light intensity of about 25,000 lux (or $8 \times 10^8$ ergs/m²/s) at the tube surface.

## 3.6 SEPARATION OF LIPIDS BY THIN-LAYER CHROMATOGRAPHY

Lipids were separated by thin-layer chromatography (t.l.c.) on silica gel G layers. Glass plates (5 cm x 20 cm) were spread with a slurry of adsorbant in distilled water (1:2, w/v) to a thickness of 0.25 mm with a commercial spreader (Desaga, Heidelberg, Germany). Plates were allowed to air dry for 15 to 20 min at room temperature before activation for 4 hours at 110°C.

The lipid material was applied as a narrow band 2 cm long and markers were placed either side as single spots. In most separations plates were developed in $CHCl_3$ : MeOH : acetic acid : $H_2O$ (85:15:10:3.5, v/v). After drying the plates were strip-scanned using a Packard Radiochromatogram Scanner (for conditions see 3.9) to give an indication of radioactivity distribution. Plates were then placed in a tank containing a few iodine crystals and after a few moments the following bands could be clearly seen: PC, DGDG plus PG, MGDG and to a lessor extent PE (see Figure 3-1). The bands "U" (immediately above MGDG and stained a bright yellow in iodine vapor) and "$U_{SF}$" (everything between U and the solvent front (SF)) contained a large proportion of radioactivity as evidenced by each strip scan.

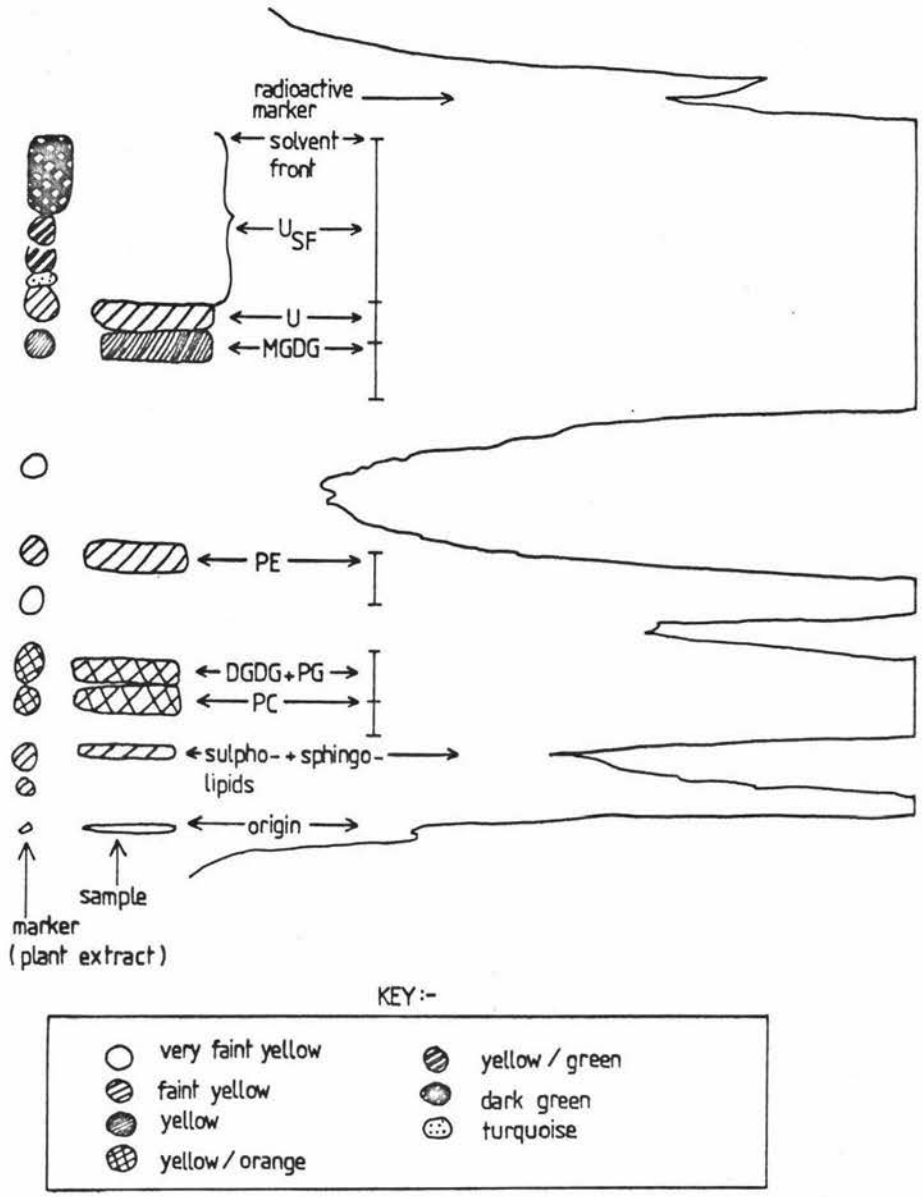



FIGURE 3-1: Thin-layer chromatogram (TLC) and scan of sample developed in $CHCl_3$ : MeOH : acetic acid : $H_2O$ (85:15:10:3.5, v/v)

Bands showed up as indicated upon placing TLC plate in iodine vapour.

Hence these unidentified bands (U and $U_{SF}$) were extracted as well as PC, DGDG plus PG, PE and MGDG from the silica gel using $CHCl_3$ : MeOH : HCOOH : $H_2O$ (97:97:4:2, v/v). Total lipid and U were developed in toluene : ethyl acetate : 95% EtOH (2:1:1, v/v) and $U_{SF}$ was developed in hexane : diethyl ether : acetic acid (70:30:1, v/v). Methyl esters obtained by methylating U and $U_{SF}$ with 0.5 M NaOH/methanol were developed in hexane : diethyl ether (9:1, v/v).

## 3.7 PREPARATION OF METHYL ESTERS OF FATTY ACIDS

Methyl esters of fatty acids were prepared by the method of van Wyngaarden (1967). An aliquot of lipid extract to be analysed was placed in a 20 ml tube with a ground glass neck. The solvent was removed by a stream of $N_2$ and 2 ml of 0.5 M methanolic NaOH added. The tube was attached to a water condenser and refluxed on a sand bath for 3 min. 2 ml of 14% boron trifluoride in methanol (w/v) was added through the condenser and refluxing continued for a further 3 min. 5 ml of hexane was added through the condenser and then the tube was removed from the sand bath and allowed to cool. After adding sufficient water to bring the hexane layer to the top of the tube, the hexane layer upon clearing (aided by centrifugation) was transferred to a glass-stoppered centrifuge tube using a Pasteur pipette. The bottom layer was further washed twice with hexane, vortexing in between washings and the washings added to the tube. Hexane was removed with a stream of $N_2$ and the methyl esters redissolved in 1 ml of hexane. A suitable aliquot was removed for the determination of radioactivity. Recovery of radioactivity in methyl esters prepared from lipids was usually in the range of 70-100% of the initial radioactivity.

## 3.8 GAS-LIQUID CHROMATOGRAPHY OF METHYL ESTERS OF FATTY ACIDS

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

The column was fitted with an effluent stream splitter diverting three quarters of the sample to the collector jet and the remainder to the flame ionisation detector. Samples along with a standard methyl ester mixture containing 16:0, 18:0, 18:1, 18:2 and 18:3 were injected on to the column in a volume of no more than 10 µl. Levels of fatty acids in the samples themselves were too low to be detected as peaks on the chart recorder.

The column was held at 164°C with the injector and detector temperatures at 210°C and 190°C respectively. The carrier gas was oxygen-free $N_2$ flowing at 32 ml/min with hydrogen and air supplied to the detector at 17 ml/min and 250 ml/min respectively. Radioactive effluent of each fatty acid, as monitored from the chart recorder, were collected in a pyrex tube loosely packed with 0.1 - 0.2 g of glass wool moistened with scintillation solution. The methyl esters were eluted from the collection tubes by one 5 ml aliquot of scintillation fluid followed by a further 1 ml into scintillation vials. A small hand operated air pump was used to flush the solvent through the glass wool and aid draining.

## 3.9 DETERMINATION OF RADIOACTIVITY

Samples were counted in Triton X-100/toluene (1:2, v/v) containing 0.4% PPO and 0.01% POPOP. Radioactivity was determined with a Beckman Model LS8000 scintillation counter. Radioactivity on chromatograms was detected by scanning on a Packard Model 7200 Radiochromatogram Scanner. Counting conditions were 1.26% iso-butane in helium (v/v) flowing at 120 ml/min, voltage: 1.15 Kv, slit width: 2.5 mm, time constant: 30s or 10s, linear range: 300 or 3000 and scanning rate: 0.5 cm/min.

Autoradiographs of t.l.c. plates were obtained by exposure to Agfa-Gevaert Osray M3 X-ray film for almost 5 weeks in a light-proof box. Films were developed and fixed with Kodak Liquid X-ray developer and fixer respectively.

## 3.10 CHLOROPHYLL DETERMINATION

Chlorophyll was determined by the method of Arnon (1949). 25 µl of protoplast suspension in solution A was made up to 5 ml with 80% acetone, mixed well and filtered. Absorbances were read at 645 nm and 663 nm. Chlorophyll concentration was given by the following relationship: -

$$\text{Concentration (mg/ml)} = (20.2 \times A_{645} + 8.02 \times A_{663}) \times \frac{5}{0.025} \times \frac{1}{1000}$$

where $A_{645}$ and $A_{663}$ are the absorbances at 645 nm and 663 nm respectively.

# CHAPTER 4

# RESULTS

## 4.1 COMPARISON OF YIELD OF PROTOPLASTS USING (i) CUT AND (ii) ABRADED MAIZE LEAVES

The procedure of Day *et al* (1981) was followed to isolate protoplasts. Two petri dishes were set up, one containing 1 g of abraded leaves in 10 ml of incubation medium and the other 1 g of leaves, chopped into segments approximately 1 mm in width, in 10 ml of incubation medium. After digestion the suspended protoplasts were transferred to a centrifuge tube and the tissue rinsed exhaustively with further digestion buffer in order to harvest the protoplasts. The protoplasts obtained were made up to a volume of 1.1 ml with solution A. 0.5 ml of each 1.1 ml suspension of protoplasts was made up to 5 ml with 80% acetone for chlorophyll determination as described in section 3.10. The chlorophyll content of 1 g of untreated plant material was also determined. 966 µg Chl/ml was released from the untreated plant tissue, while 506 µg Chl/ml and 220 µg Chl/ml was released from the cut and abraded tissue respectively. It can be seen that abrading the leaves enabled some digestion to occur resulting in the release of protoplasts, but to a lesser extent than when segments of plant leaves were used. To obtain the maximum yield of protoplasts therefore, it was decided to firstly abrade the leaves before cutting them into segments for digestion.

Examination of the maize protoplasts by phase microscopy after digestion revealed that about 75% of them were intact.

## 4.2 QUALITY OF PROTOPLASTS OBTAINED FROM BARLEY LEAVES

Barley leaves were abraded and then sliced into sections 1 mm in width before digestion according to the procedure of Day *et al* (1981). The protoplasts thus obtained were examined by phase contrast microscopy and found to be superior to those obtained from maize. 90-95% of the protoplasts were intact and their appearance was better than maize protoplasts. Their membranes were more clearly outlined and there was hardly any clumping of chloroplasts to one side of the protoplast as was seen in some maize protoplasts.

Since the appearance and degree of intactness of protoplasts isolated from barley were better than from maize, it was decided to use barley tissue as a source of protoplasts in future experiments.

## 4.3 MEASUREMENT OF INTACTNESS OF PROTOPLASTS ISOLATED FROM BARLEY

### 4.3.1 Phase Contrast Microscopy

Protoplasts were examined at 300x and 600x magnification at the end of each preparation using phase contrast microscopy. The leaves of 6-7 day old barley seedlings yielded protoplasts which had an appearance of intactness as seen in Figure 4-1. During each set of incubations of protoplasts with substrates, a control incubation was also set up containing protoplasts and all the materials except the radioactive substrate. A portion of this was removed for examination periodically over the incubation time. At the beginning of each incubation 90-95% of the protoplasts were intact. The protoplasts were typically bright

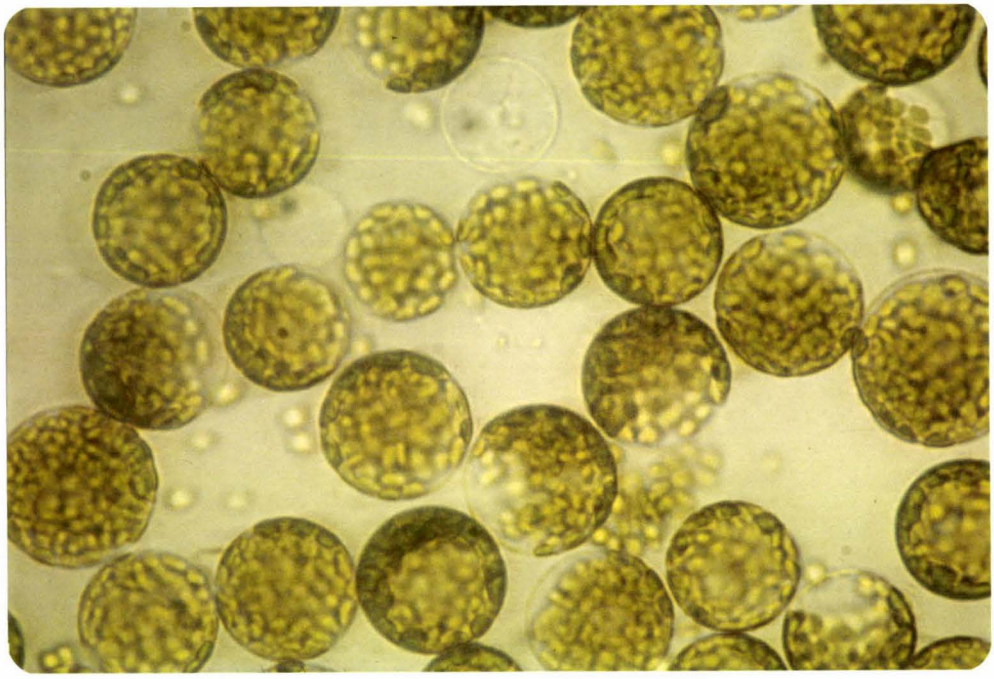


FIGURE 4-1: A representative field from a preparation of protoplasts

800 x magnification

green with clearly outlined membranes and few had spilt their chloroplasts. After $2\frac{1}{2}$ hours the protoplasts looked similar but with a little more spillage and a degree of intactness of about 80%. At 5 hours also the protoplasts still looked quite good, but the spillage was more noticeable and there were quite a number of half empty protoplasts. About 65% were intact. After 10 hours incubation the protoplasts were not as green and the outlines of the membranes had become indistinct. At least half of them were spilt. By the end of 20 hours the suspending solution was a yellowy-green and there were very few intact protoplasts remaining.

### 4.3.2 Oxygen Evolution

The metabolic activity of protoplasts can be readily determined by measuring $CO_2$-dependent $O_2$ evolution with an oxygen electrode (Nishimura and Akazawa, 1975). Rates greater than 100 µmol $O_2$ /hr/mg Chl indicate that the protoplasts have retained "normal" metabolic activity (Kuhn and Stumpf, 1981). The same buffer was used in the $O_2$ evolution assay mixture as was used in the corresponding incubation mixtures of protoplasts for lipid synthesis. Namely, HEPES at pH 7.5 when $H^{14}CO_3^-$ was the substrate and MES at pH 5.8 when (1-$^{14}$C) acetate was the substrate for lipid synthesis. The rates of $CO_2$-dependent $O_2$ evolution obtained when using HEPES buffer ranged from 102.02 - 246.96 µmol $O_2$/hr/mg Chl in 9 observations with the median at 198.34 µmol $O_2$/hr/mg Chl. Using MES buffer the rates ranged from 110.61 - 171.55 µ mol $O_2$ /hr/mg Chl in 4 observations with the median at 148.41 µmol $O_2$/hr/mg Chl. It was noticeable that the rates of $CO_2$ dependent $O_2$ evolution using MES buffer were not as high as when the HEPES buffer was used.

## 4.4 UTILISATION OF $H^{14}CO_3^-$ AND (1-$^{14}$C) ACETATE BY BARLEY PROTOPLASTS FOR LIPID SYNTHESIS

### 4.4.1 Rate of Total $H^{14}CO_3^-$ Assimilation by Barley Protoplasts

Protoplasts equivalent to 56 µg Chl were incubated in the assay mixture as described in Section 3.3. 5 mM $PP_i$ was included, according to Lilley *et al* (1975), and 1 mM $HCO_3^-$ (500 nmoles) was used along with 1.84 µCi $H^{14}CO_3^-$.

The rate of total $H^{14}CO_3^-$ assimilation by the protoplasts was rapid over the first 10 minutes and then began to plateau (see Figure 4-2). At the end of 1 hour 78.2% of the added label had been assimilated.

### 4.4.2 Rate of $H^{14}CO_3^-$ Incorporation into Total Lipids of Barley Protoplasts

Incubation conditions were the same as those in section 4.4.1.

$H^{14}CO_3^-$ incorporation into total lipids of the protoplasts increased steadily over the hour (see Figure 4-3). It did not slow down after 10 minutes as the $H^{14}CO_3^-$ assimilation into protoplasts did. After 1 hour the incorporation of $H^{14}CO_3^-$ into lipids was 146.22 nmol/mg Chl which was 2.09% of the total $H^{14}CO_3^-$ assimilated into protoplasts at this time.

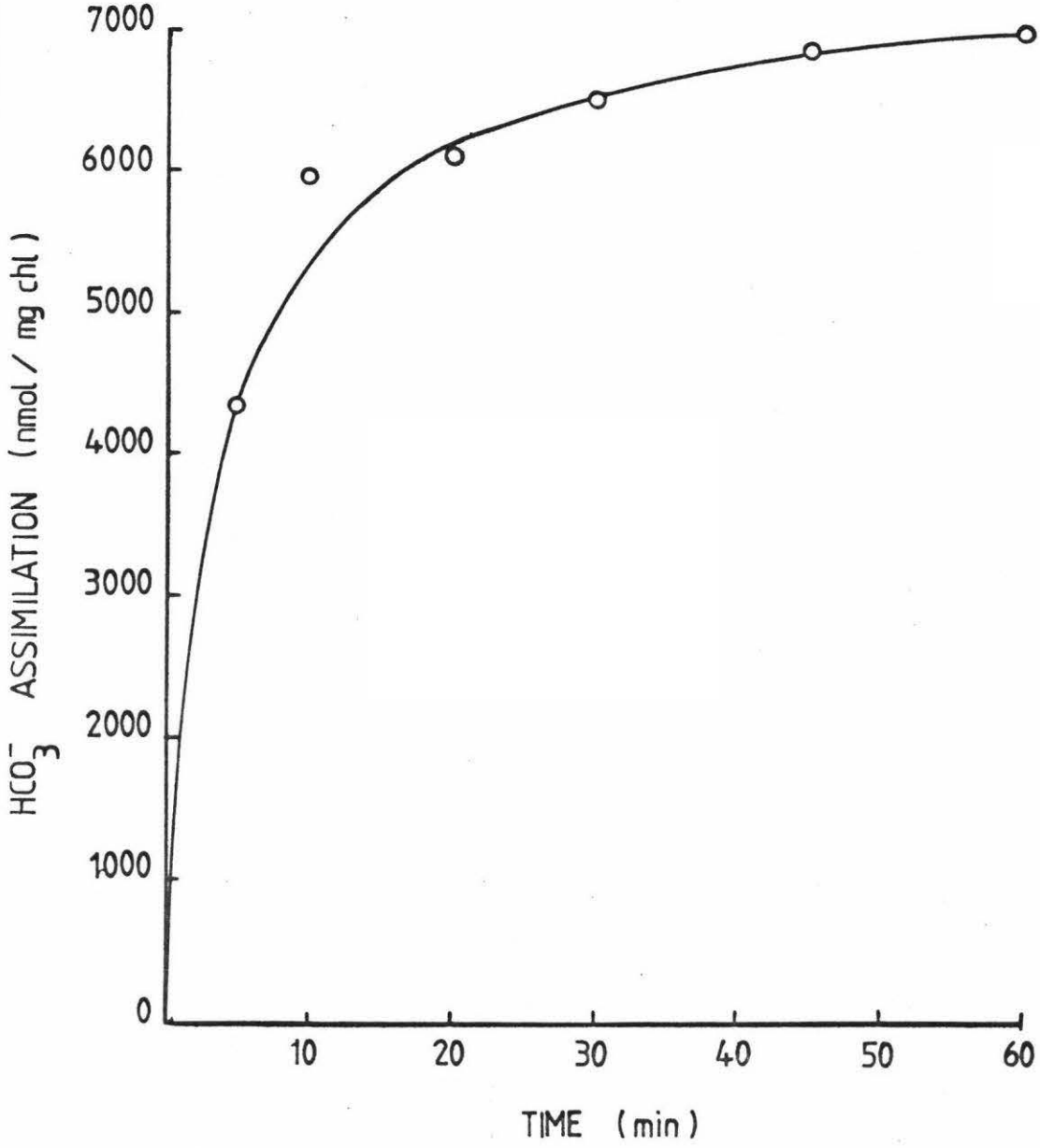



FIGURE 4-2: The rate of total $H^{14}CO_3^-$ assimilation by barley protoplasts.

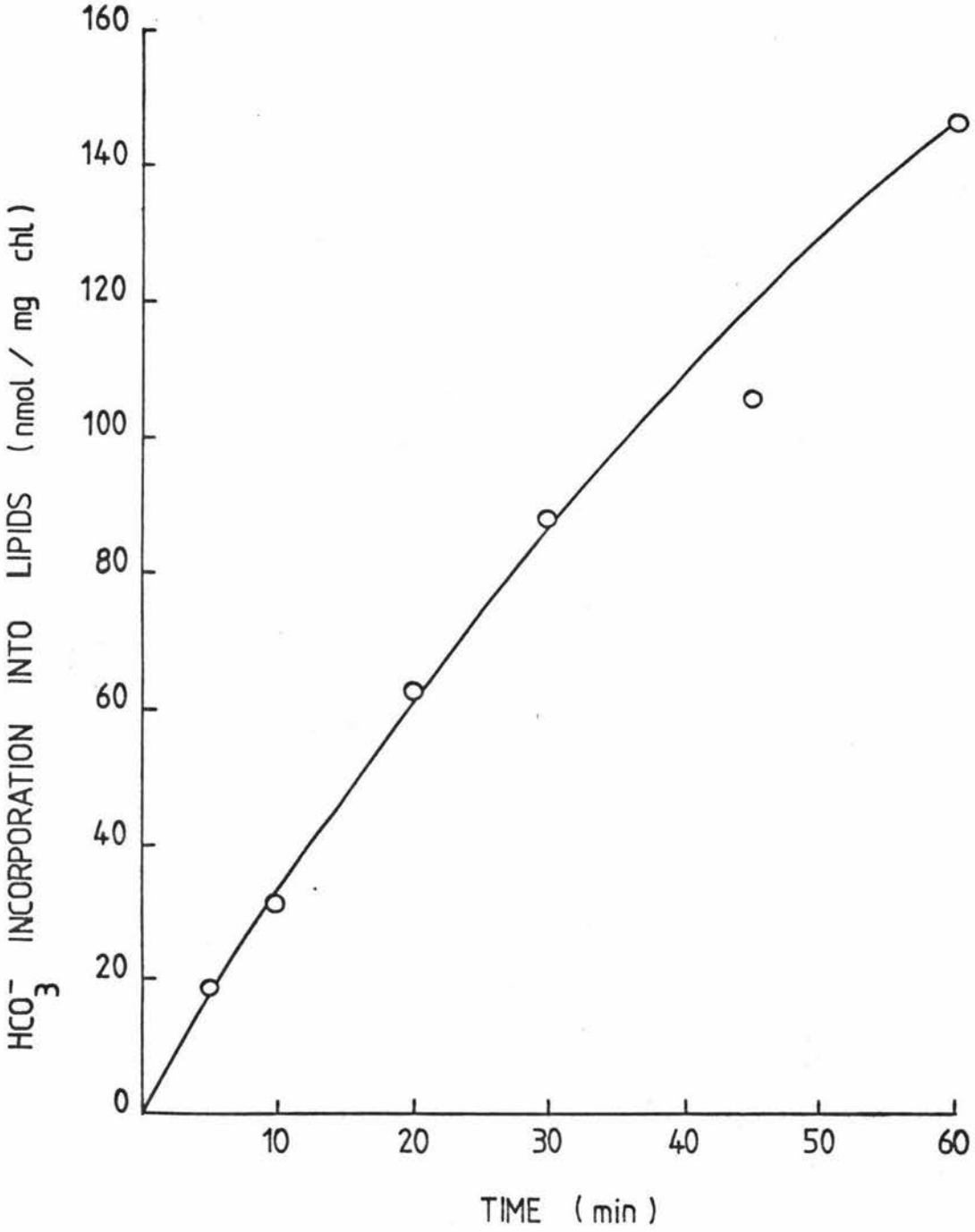



FIGURE 4-3: The rate of $H^{14}CO_3^-$ incorporation into the total lipids of barley protoplasts.

### 4.4.3 Rate of (1-$^{14}$C) Acetate Incorporation into Total Lipids of Barley Protoplasts

Protoplasts equivalent to 56 µg Chl were incubated in the assay mixture as when examining $HCO_3^-$ incorporation (see Section 3.3) except that 50 µM acetate (25 nmoles) was used along with 0.25 µCi (1-$^{14}$C) acetate.

Incorporation of (1-$^{14}$C) acetate into total lipids of barley protoplasts increased gradually (see Figure 4-4). After 1 hour 3 nmol acetate/mg Chl had been incorporated which was 0.46% of the added substrate. In a parallel experiment (Section 4.4.2) $H^{14}CO_3^-$ incorporation into total lipids after 1 hour was 1.46% of substrate added.

## 4.5 VARIATIONS OF REACTION CONDITIONS FOR THE INCORPORATION OF $H^{14}CO_3^-$ INTO LIPIDS OF BARLEY PROTOPLASTS

### 4.5.1 Effect of $HCO_3^-$ Concentration on $H^{14}CO_3^-$ Incorporation into Lipids of Barley Protoplasts

Protoplasts equivalent to 50 µg Chl were incubated in the assay mixture, as described in Section 3.3, for 1 hour. 5 mM $PP_i$ was included and 1.84 µCi $H^{14}CO_3^-$ was used. The unlabelled $HCO_3^-$ concentration was varied from 1 mM to 30 mM. The amount of $H^{14}CO_3^-$ incorporated into lipids of barley protoplasts increased steadily up to 10 mM $HCO_3^-$ concentration and then began to plateau (see Figure 4-5). It was decided to use a concentration of 10 mM $HCo_3^-$ in future studies of $H^{14}CO_3^-$ incorporation into lipids.

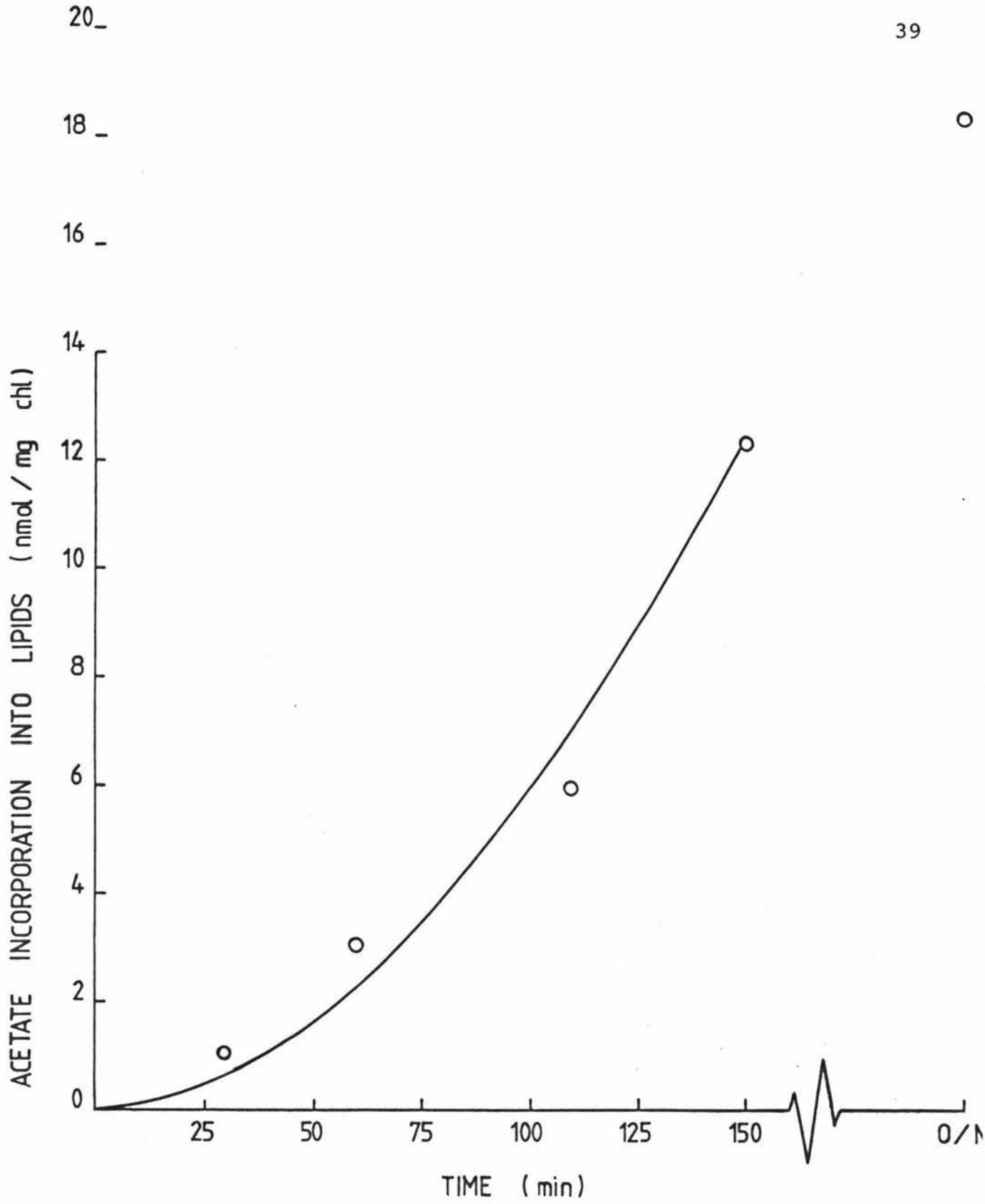



FIGURE 4-4: The rate of (1-$^{14}$C) acetate incorporation into total lipids of barley protoplasts.

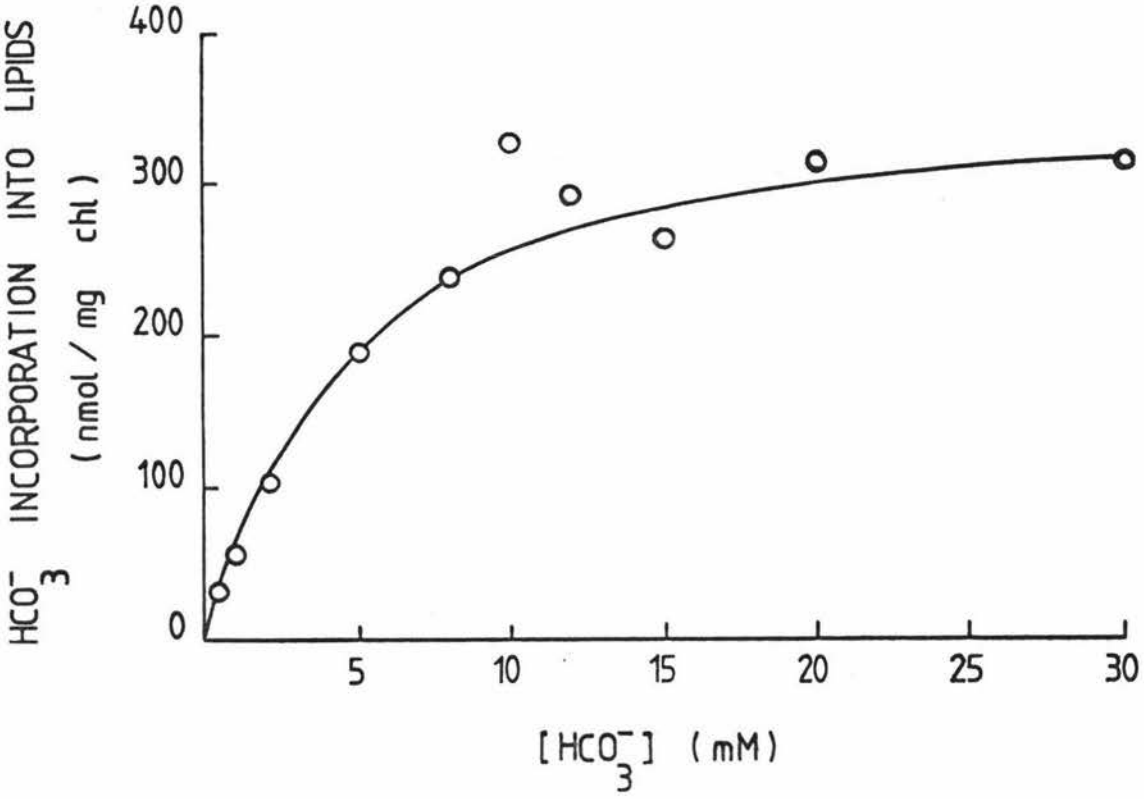



FIGURE 4-5: The effect of $HCO_3^-$ concentration on the incorporation of $H^{14}CO_3^-$ into lipids of barley protoplasts.

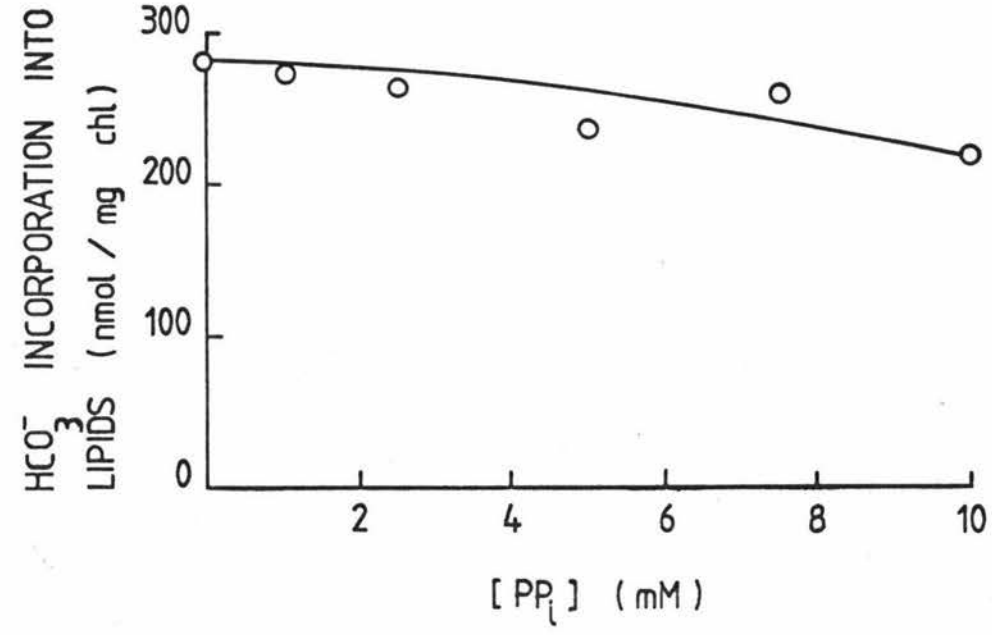



FIGURE 4-6: The effect of $PP_i$ concentration on the incorporation of $H^{14}CO_3^-$ into lipids of barley protoplasts.

### 4.5.2 Effect of $PP_i$ Concentration on $H^{14}CO_3^-$ Incorporation into Lipids of Barley Protoplasts

Protoplasts equivalent to 50 µg Chl were incubated in the assay mixture, as described in Section 3.3, for 1 hour. 10 mM $HCO_3^-$ and 1.84 µCi $H^{14}CO_3^-$ were used while $PP_i$ concentration was varied from 0-10 mM.

Maximum $H^{14}CO_3^-$ incorporation into lipids of barley protoplasts (281.4 nmol/mg Chl) was obtained at 0 mM $PP_i$ and increasing the $PP_i$ concentration appeared to be slightly inhibitory to $H^{14}CO_3^-$ incorporation (see Figure 4-6). It was therefore decided to omit $PP_i$ in later incubations of protoplasts with $H^{14}CO_3^-$.

### 4.5.3 Effect of $P_i$ Concentration on $H^{14}CO_3^-$ Incorporation into Lipids of Barley Protoplasts

Protoplasts equivalent to 50 µg Chl were incubated in the assay mixture, as described in Section 3.3, for 1 hour. $PP_i$ was omitted. 10 mM $HCO_3^-$ and 1.84 µCi $H^{14}CO_3^-$ were used while $P_i$ concentration was varied from 0-10 mM.

The incorporation of $H^{14}CO_3^-$ into the lipids of barley protoplasts increased slightly from 0 - 3 mM $P_i$ and tapered off a little at concentrations greater than this (see Figure 4-7). It was decided to continue to keep $P_i$ in the oxygen electrode buffer at 2 mM.

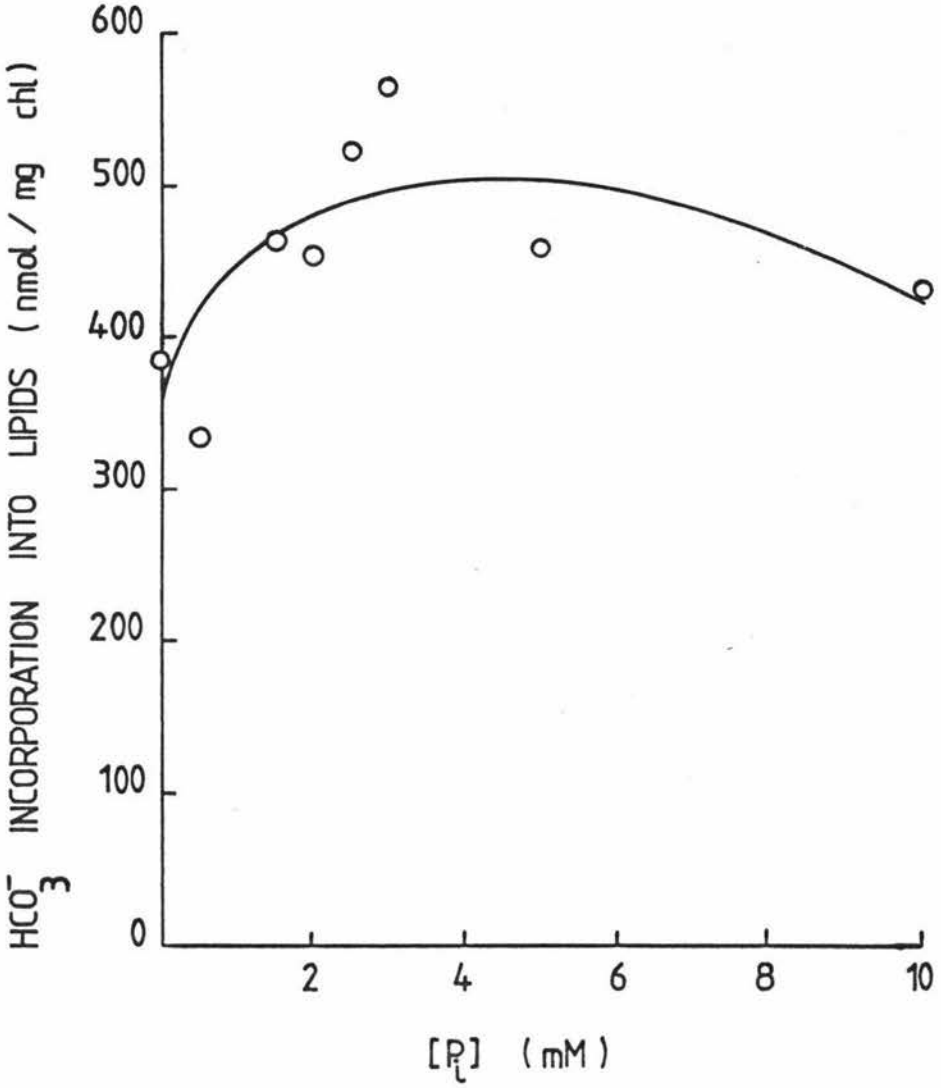



FIGURE 4-7: The effect of $P_i$ concentration on the incorporation of $H^{14}CO_3^-$ into lipids of barley protoplasts.

### 4.5.4 Effect of $Mg^{++}$ Concentration on $H^{14}CO_3^-$ Incorporation into Lipids of Barley Protoplasts

Protoplasts equivalent to 50 µg Chl were incubated in the assay mixture, as described in section 3.3, for 1 hour. 10 mM $HCO_3^-$ and 1.84 µCi $H^{14}CO_3^-$ were used and $PP_i$ was omitted. The concentration of $Mg^{++}$ was varied from 0-10 mM. The incorporation of $H^{14}CO_3^-$ into lipids of barley protoplasts was much the same as when $P_i$ concentration was varied (see Figure 4-8). It was therefore also decided to keep $Mg^{++}$ concentration in the oxygen electrode buffer at 2 mM.

### 4.5.5 Effect of pH on $H^{14}CO_3^-$ Incorporation into Lipids of Barley Protoplasts

Protoplasts were incubated in the assay mixture as described in Section 3.3 for 1 hour. The pH of the oxygen electrode buffer was varied between 6.8 and 8.4. The incorporation of $H^{14}CO_3$- into lipids of barley protoplasts increased rapidly up to pH 7.5, plateaued between pH 7.5 and 8.0 and then started to decrease at the more alkaline pH (see Figure 4-9). The neutral pH of 7.5 was obviously the most favourable for $H^{14}CO_3^-$ incorporation into lipids, acidic and alkaline pHs being inhibitory, and the pH of the oxygen electrode buffer was continued to be used at 7.5.

### 4.5.6 Effect of Time on $H^{14}CO_3^-$ Incorporation into Lipids of Barley Protoplasts

Protoplasts were incubated in the assay mixture as described in Section 3.3. $H^{14}CO_3^-$ incorporation into lipids of barley protoplasts increased steadily up to 60 minutes (see Figure 4-10). After this the increase was not quite as great and

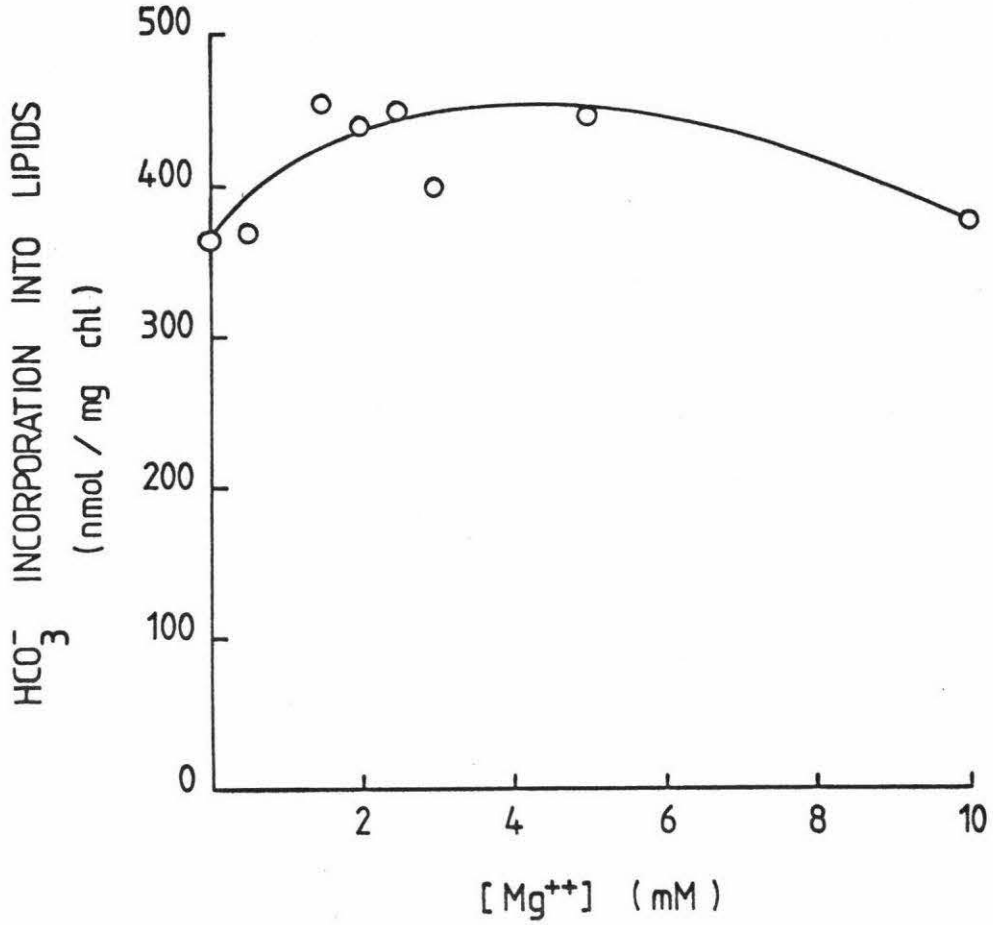



FIGURE 4-8: The effect of $Mg^{++}$ concentration on the incorporation of $H^{14}CO_3^-$ into lipids of barley protoplasts.

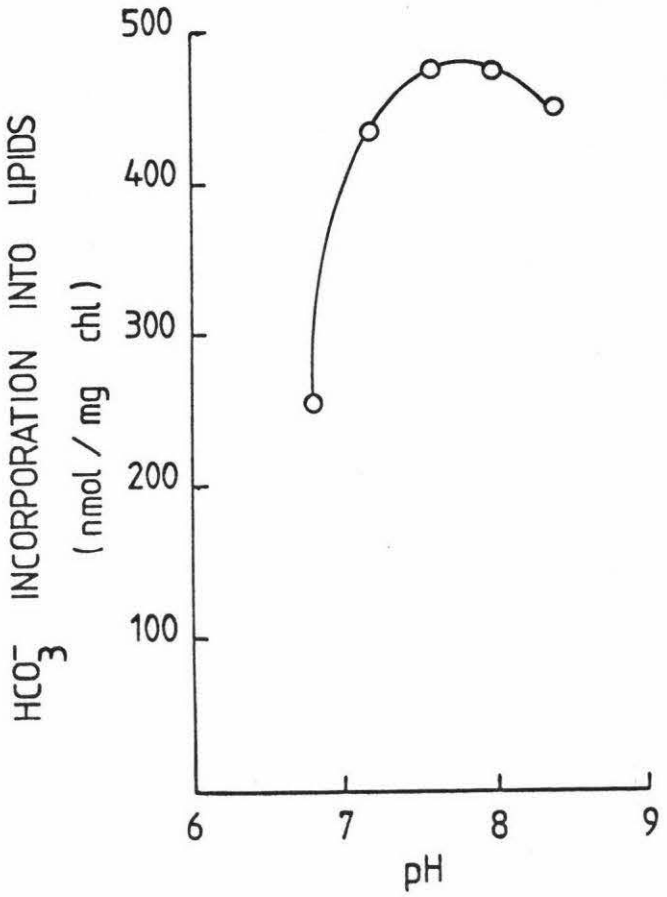



FIGURE 4-9: The effect of pH on $H^{14}CO_3^-$ incorporation into lipids of barley protoplasts.

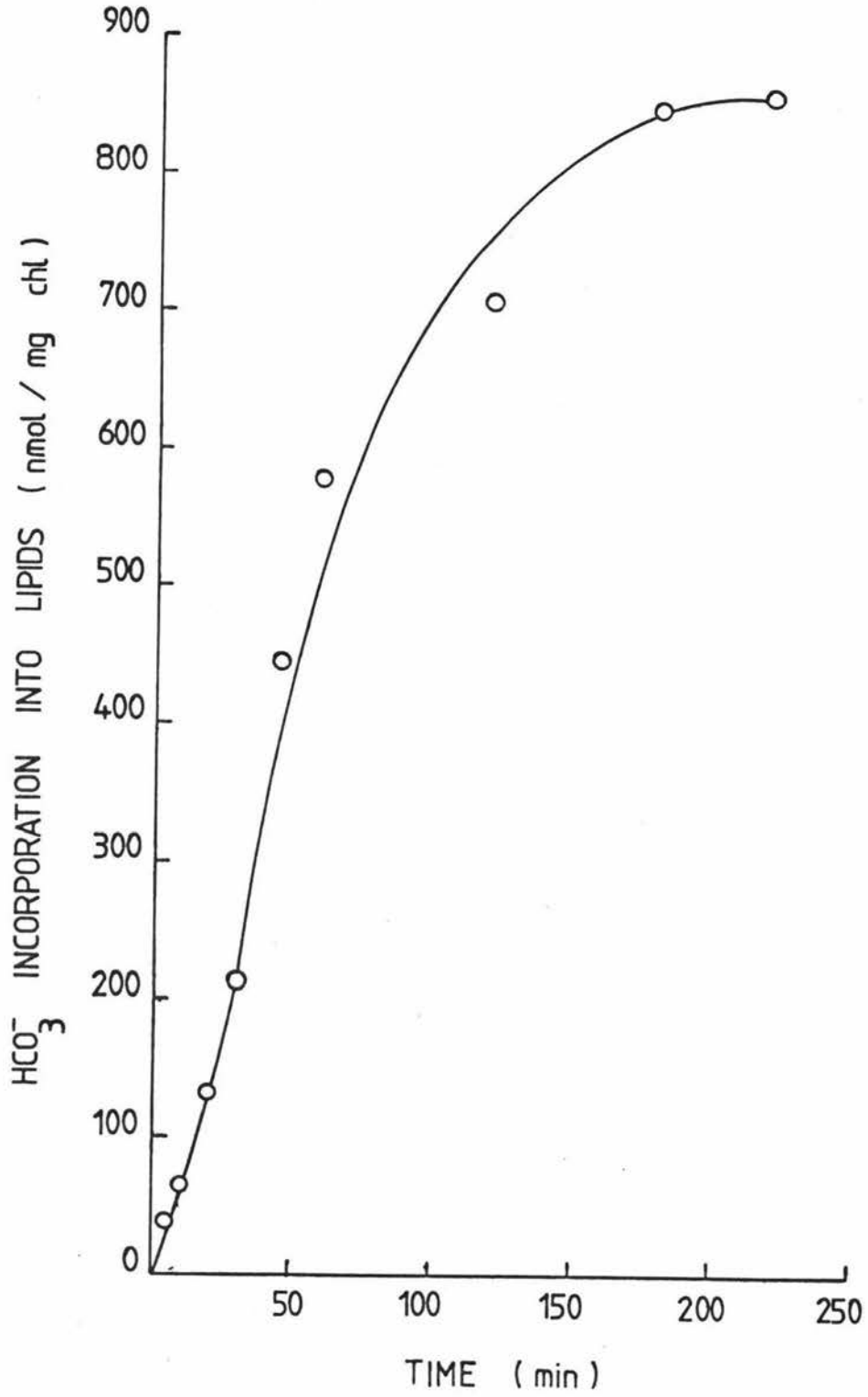



FIGURE 4-10: The effect of time on $H^{14}CO_3^-$ incorporation into lipids of barley protoplasts.

between 180 and 220 minutes was very small. From this it appeared that 60 minutes was the optimum time to leave the assay for $H^{14}CO_3^-$ incorporation into lipids.

### 4.5.7 Effect of Acetate on the Incorporation of $H^{14}CO_3^-$ into Lipids of Barley Protoplasts

Protoplasts were incubated in the assay mixture as described in Section 3.3 for 1 hour. Acetate concentration was varied from between 0 and 1 mM.

The incorporation of $HCO_3^-$ into lipids of barley protoplasts did not appear to be affected much by the presence of acetate (see Figure 4-11).

## 4.6 INCORPORATION OF (1-$^{14}$C) ACETATE INTO LIPIDS BY BARLEY PROTOPLASTS

### 4.6.1 Influence of pH on the Assimilation of (1-$^{14}$C) Acetate by Barley Protoplasts

Protoplasts were incubated for one hour in the assay mixture, as described in Section 3.4, with 50 µM (1-$^{14}$C) acetate (25 nmoles, 0.243 µCi) to measure assimilation at different pHs. The pH was varied between 5 and 7 using MES buffer and between 6.8 and 8.4 using HEPES buffer. Additional assays containing no radioactive acetate were set up at pH 5, 7 and 8.4 in order to examine the appearance of the protoplasts under phase contrast microscopy at the end of the incubation. At each pH the protoplasts appeared

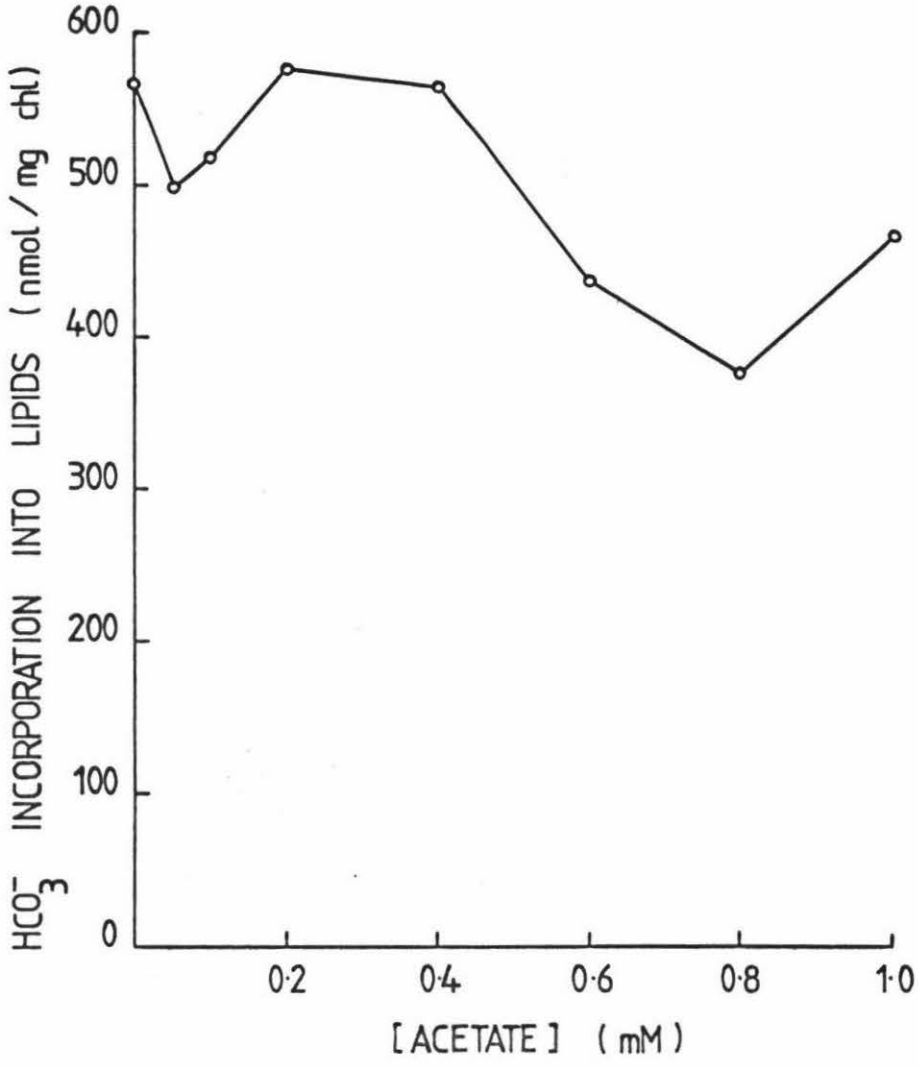



FIGURE 4-11: The effect of acetate concentration on $H^{14}CO_3^-$ incorporation into lipids by barley protoplasts.

to be intact after one hour. A further three assays were set up, again containing no radioactive acetate to measure the recovery of protoplasts, based on chlorophyll determination, after incubation.

After incubation the tubes containing the assay mixtures were centrifuged at 300g for 3 minutes and the supernatant discarded. The pelleted protoplasts were resuspended in solution A containing 50 µM acetate to wash out any labelled acetate which had not been assimilated by the protoplasts. The resulting suspension of protoplasts was again centrifuged at 300g for 3 minutes. The supernatant was discarded and the pellet of protoplasts resuspended in solution A containing no sorbitol to release protoplast contents. The volumes were determined and then 10 µl removed from each for radioactivity determination. In separate incubations chlorophyll measurements were made in order to determine the recovery of protoplasts in the extraction procedure. This was found to be 75% ± 1%. This correction factor was used in the calculation of acetate assimilation.

Acetate assimilation by barley protoplasts was greatest at the acidic pH of 5, being 160.77 nmol/mg Chl, which is 32.2% of the added label. Acetate assimilation was much lower at the higher pHs examined (see Figure 4-12 and Table 1).

It was suspected that the unassimilated acetate may not have been completely removed with the single acetate wash. Therefore the experiment was repeated using a second wash containing 50 µM acetate and the level of radioactivity monitored in the supernatants. Assays were set up as before so that protoplasts could be examined at the end of incubation and their recovery determined.

The average recovery of protoplasts, as measured by chlorophyll levels, was 74.5% which is approximately the same as found earlier when one acetate wash was used. The

TABLE 1: THE EFFECT OF pH ON (1-$^{14}$C) ACETATE ASSIMILATION BY BARLEY PROTOPLASTS

| pH | Acetate assimilation | |
|---|---|---|
| | nmol/mg Chl | % of added label |
| 5.0 | 160.8 | 32.2 |
| 5.4 | 151.7 | 30.4 |
| 5.8 | 100.7 | 20.2 |
| 6.2 | 61.4 | 12.3 |
| 6.6 | 38.5 | 7.7 |
| 7.0 | 25.3 | 5.1 |
| 6.8 | 39.1 | 7.8 |
| 7.2 | 26.7 | 5.4 |
| 7.6 | 23.6 | 4.7 |
| 8.0 | 17.4 | 3.5 |
| 8.4 | 20.1 | 4.0 |

Protoplasts were incubated with buffers MES (pH 5.0 - 7.0) and HEPES (pH 6.8 - 8.4) for 1 hour and then subjected to a single wash with acetate (see Section 4.6.1 for details).

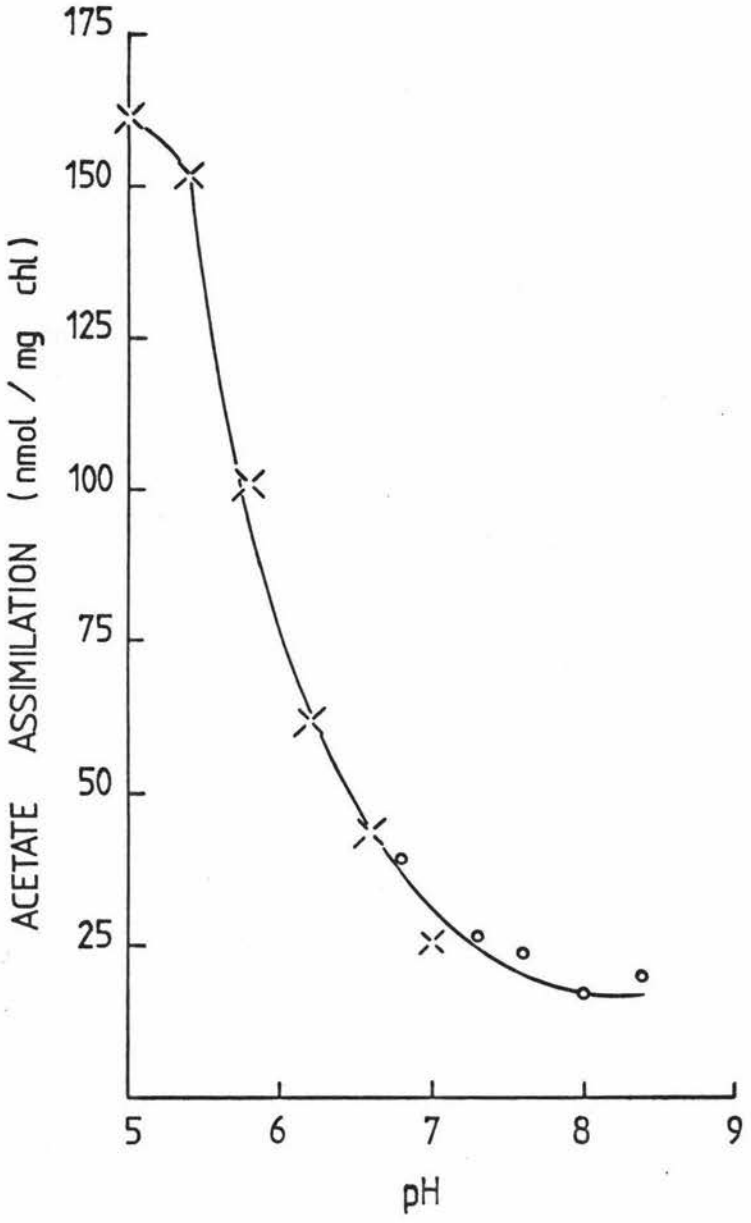



FIGURE 4-12: The effect of pH on (1-$^{14}$C) acetate assimilation by barley protoplasts.

Protoplasts were incubated with buffers MES (X) and HEPES (O) for 1 hour and then subjected to 1 acetate wash. (see Section 4.5.1 for details).

protoplasts incubated at pH 5 this time did not look quite as good as those at pHs 7 and 8.4. It was found that the second washing ("supern. 3") of the protoplasts during their recovery removed an additional small amount of label which was greatest at low pHs (see Table 2). This may reflect lower stability of protoplasts under mild acid conditions. This was confirmed by observations under phase contrast microscopy. It will be noted that incubations were for $1\frac{1}{2}$ hours in this experiment rather than for 1 hour as in the earlier experiment. Although the levels of acetate assimilation were not quite as great as in the first experiment, the more favourable acetate assimilation under slight acidic conditions was confirmed (see Figure 4-13).

TABLE 2: THE EFFECT OF pH ON (1-$^{14}$C) ACETATE ASSIMILATION BY BARLEY PROTOPLASTS

| pH | Acetate content nmol/mg Chl | | | Acetate assimilation | |
|---|---|---|---|---|---|
| | Supern.1 | Supern.2 | Supern.3 | nmol/mg Chl | % of added label |
| 5.0 | 197.1 | 84.4 | 29.4 | 137.2 | 27.3 |
| 5.4 | 193.7 | 92.8 | 23.3 | 122.4 | 24.3 |
| 5.8 | 184.9 | 85.8 | 22.9 | 105.0 | 20.9 |
| 6.2 | 239.3 | 67.7 | 13.6 | 82.9 | 17.3 |
| 6.6 | 209.1 | 57.9 | 9.4 | 63.5 | 12.7 |
| 7.0 | 304.6 | 61.8 | 6.2 | 49.7 | 10.1 |
| 6.8 | 257.1 | 70.4 | 12.2 | 52.5 | 10.5 |
| 7.2 | 261.1 | 58.3 | 7.0 | 33.1 | 6.6 |
| 7.6 | 285.5 | 68.3 | 8.3 | 19.3 | 3.9 |
| 8.0 | 276.6 | 43.4 | 3.2 | 22.1 | 4.2 |
| 8.4 | 269.4 | 42.0 | 2.2 | 16.6 | 3.5 |

Protoplasts were incubated with buffers MES (pH 5.0 - 7.0) and HEPES (6.8 - 8.4) for $1\frac{1}{2}$ hours and subjected to two washes with acetate (see Section 4.6.1 for details).

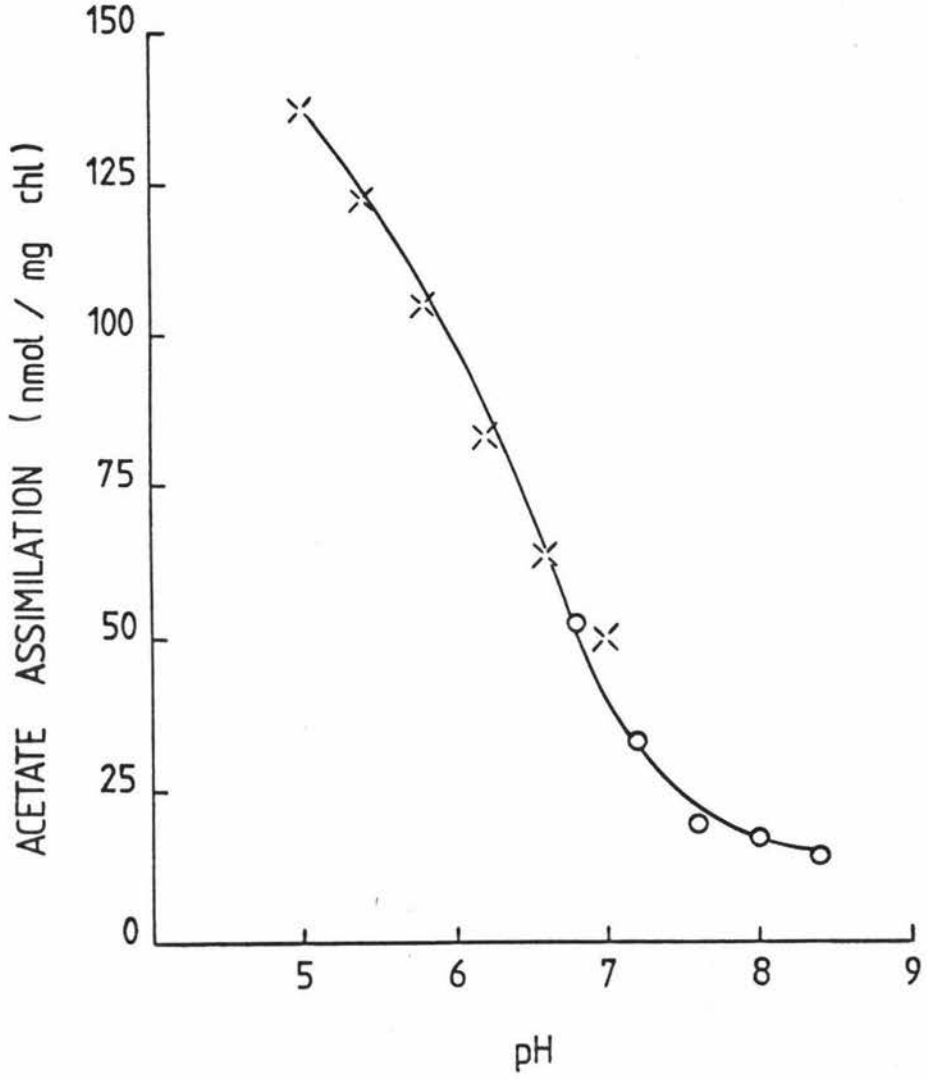



FIGURE 4-13: The effect of pH on (1-$^{14}$C) acetate assimilation by barley protoplasts.

Protoplasts were incubated with buffers MES (X) and HEPES (O) for 1½ hours and then subjected to 2 acetate washes. (see Section 4.5.1 for details).

### 4.6.2 (1-$^{14}$C) Acetate Incorporation into Lipids of Barley Protoplasts over a pH Range

Acetate incorporation into the lipids of barley protoplasts after one hour was 6.9 nmol/mg Chl at pH 5, which was about 5% of the acetate assimilated. With increasing pH, acetate incorporation decreased (see Figure 4-14).

MES buffer at pH 5.8 was chosen for later incubations of protoplasts with acetate rather than pH 5 because the protoplasts were less stable in the more acidic environment.

### 4.6.3 Rate of (1-$^{14}$C) Acetate Incorporation into Lipids of Barley Protoplasts

Protoplasts were incubated in the assay mixture, as described in Section 3.4, with 50 µM (1-$^{14}$C) acetate (25 nmoles; 0.243 µCi). Acetate incorporation into lipids of barley protoplasts increased in a sigmoidal fashion up to a value of 87.4 nmol/mg Chl at 4 hours (see Figure 4-15). Leaving the incubation overnight (approximately 18 hours) increased the level of incorporation only by a small amount to 93 nmol/mg Chl. The incorporation of acetate into lipids after 60 minutes was 17 nmol/mg Chl which was 3.4% of added substrate.

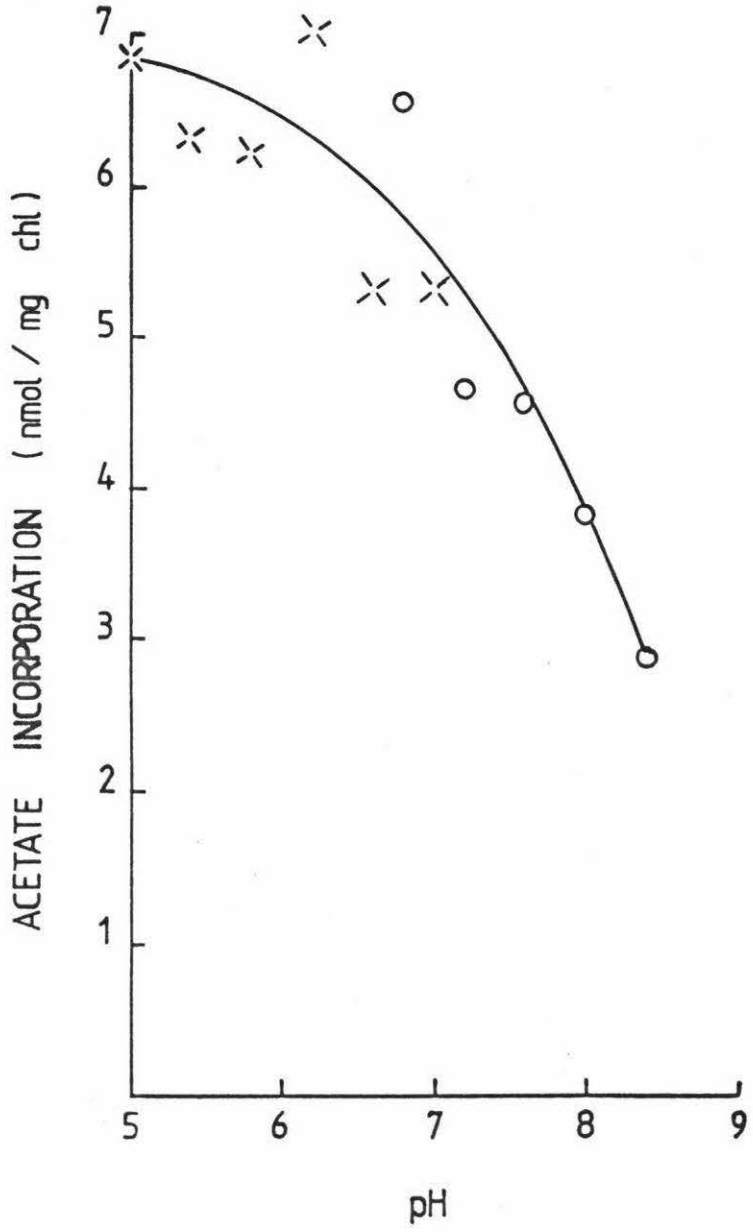



FIGURE 4-14: The effect of pH on (1-$^{14}$C) acetate incorporation into the lipids of barley protoplasts.

Protoplasts were incubated with buffers MES (X) and HEPES (O) for 1 hour.

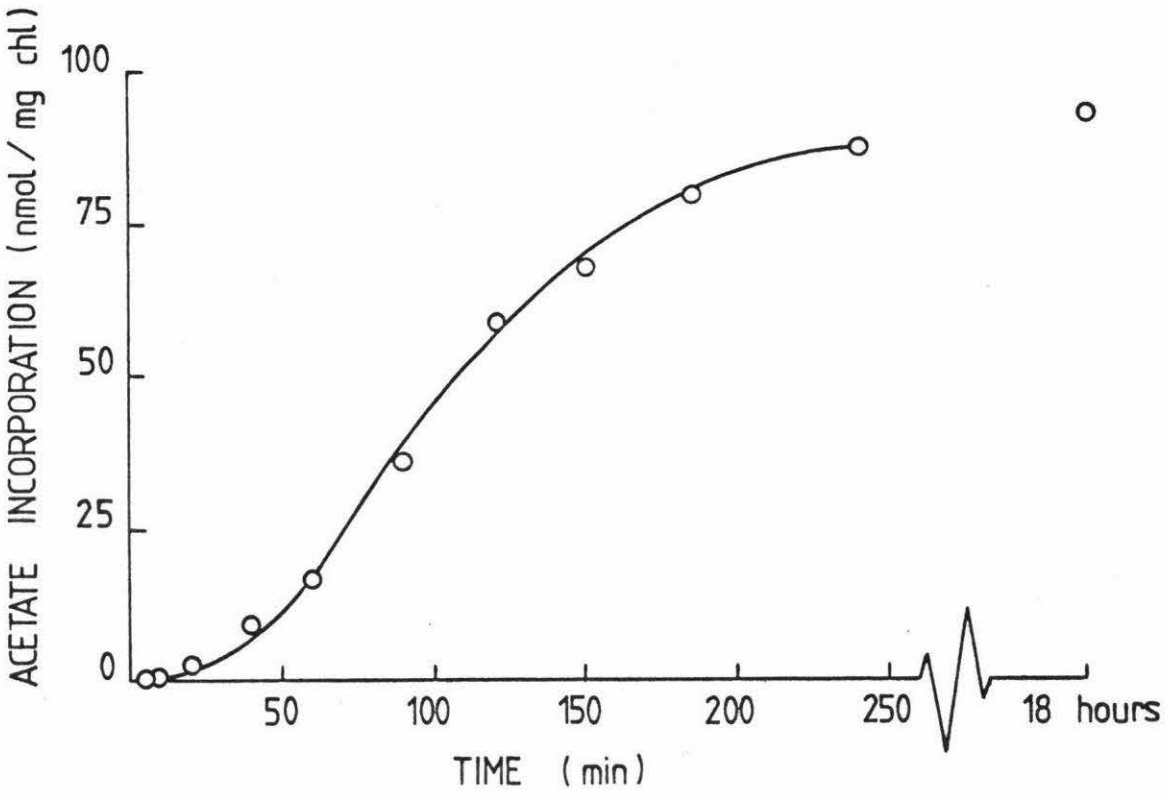



FIGURE 4-15: The rate of (1-$^{14}$C) acetate incorporation into the lipids of barley protoplasts.

### 4.6.4 Rate of (1-$^{14}$C) Acetate Incorporation into Constituent Fatty Acids of Total Lipids

Protoplasts were incubated in the assay mixture with 50 µM (1-$^{14}$C) acetate (25 nmoles, 10 µCi), as described in Section 3.4, for 2½, 5, 10 and 20 hours. Total lipids were recovered at the end of each incubation time as described in Section 3.4. A known portion (approximately 50,000 dpm) of the total lipid material obtained at each time was removed for methylation of fatty acids as described in Section 3.7. Methyl esters of fatty acids were analysed for the distribution of label as described in Section 3.8.

The rate of acetate incorporation into the lipids of barley protoplasts was greatest up to 5 hours and then the rate of incorporation was more gradual (see Figure 4-16 and Table 3). At the end of 20 hours 62 nmoles of acetate had been incorporated into lipids which was 12.4% of the added label.

Palmitic and oleic acids were the major fatty acids synthesised and to a lessor extent linoleic, stearic and linolenic acids in decreasing order (see Figure 4-17 and Table 3).

TABLE 3: (1-$^{14}$C) ACETATE INCORPORATION INTO THE CONSTITUENT FATTY ACIDS OF TOTAL LIPIDS

| Time Hours | Proportion of label in fatty acids % of total | | | | |
|---|---|---|---|---|---|
| | 16:0 | 18:0 | 18:1 | 18:2 | 18:3 |
| 2½ | 22.5 ( 7.3) | 6.2 (2.0) | 50.0 (16.2) | 16.5 (5.3) | 2.9 (0.9) |
| 5 | 29.3 (12.7) | 6.3 (2.7) | 41.5 (18.1) | 18.2 (7.9) | 2.7 (1.2) |
| 10 | 30.6 (16.2) | 7.6 (4.0) | 40.8 (21.5) | 15.2 (8.0) | 3.6 (1.9) |
| 20 | 32.0 (21.4) | 9.6 (5.9) | 33.7 (21.1) | 14.0 (8.7) | 6.1 (2.7) |

nmol/mg Chl ( )

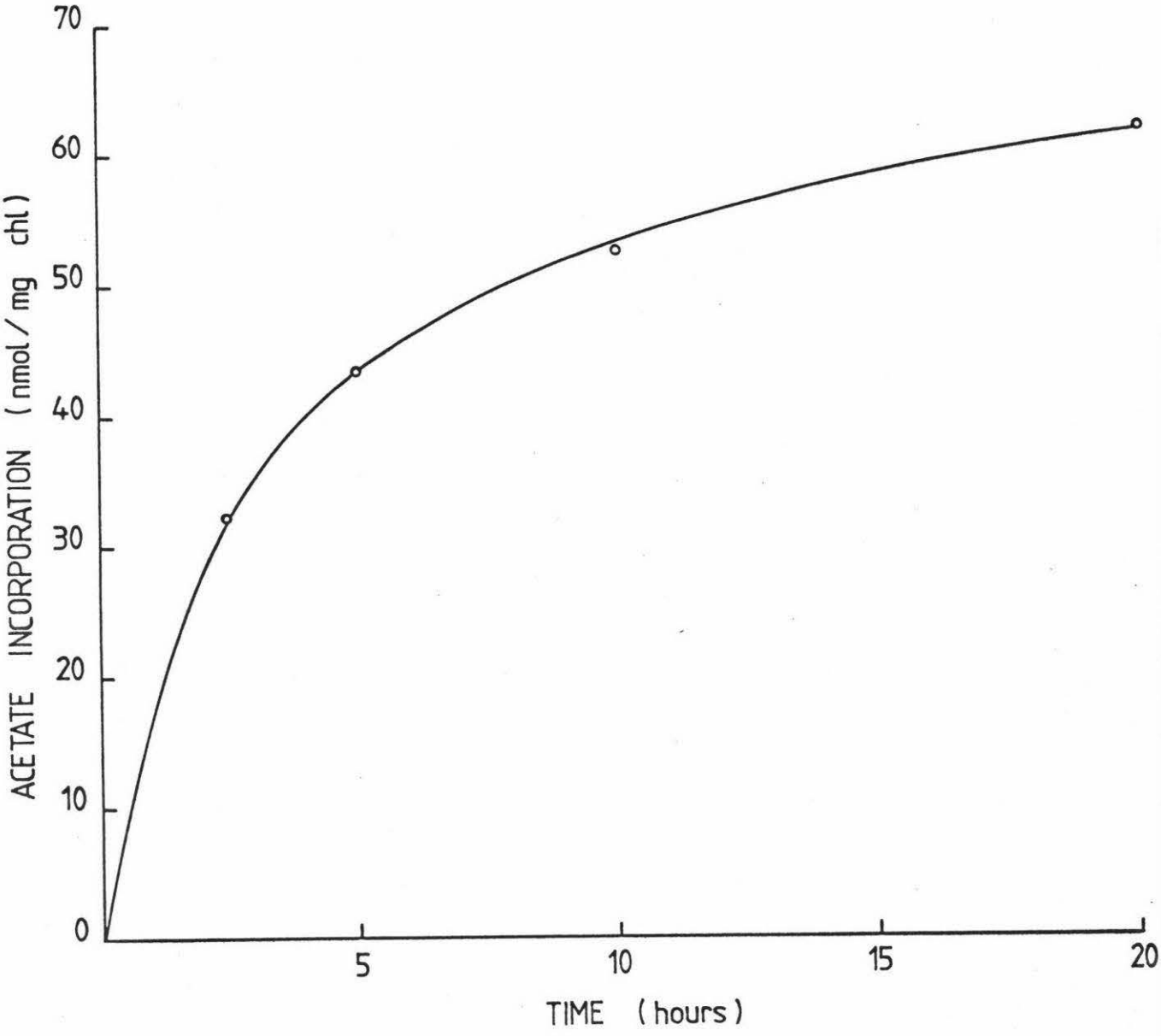



FIGURE 4-16: The rate of (1-$^{14}$C) acetate incorporation into the total lipids of barley protoplasts.

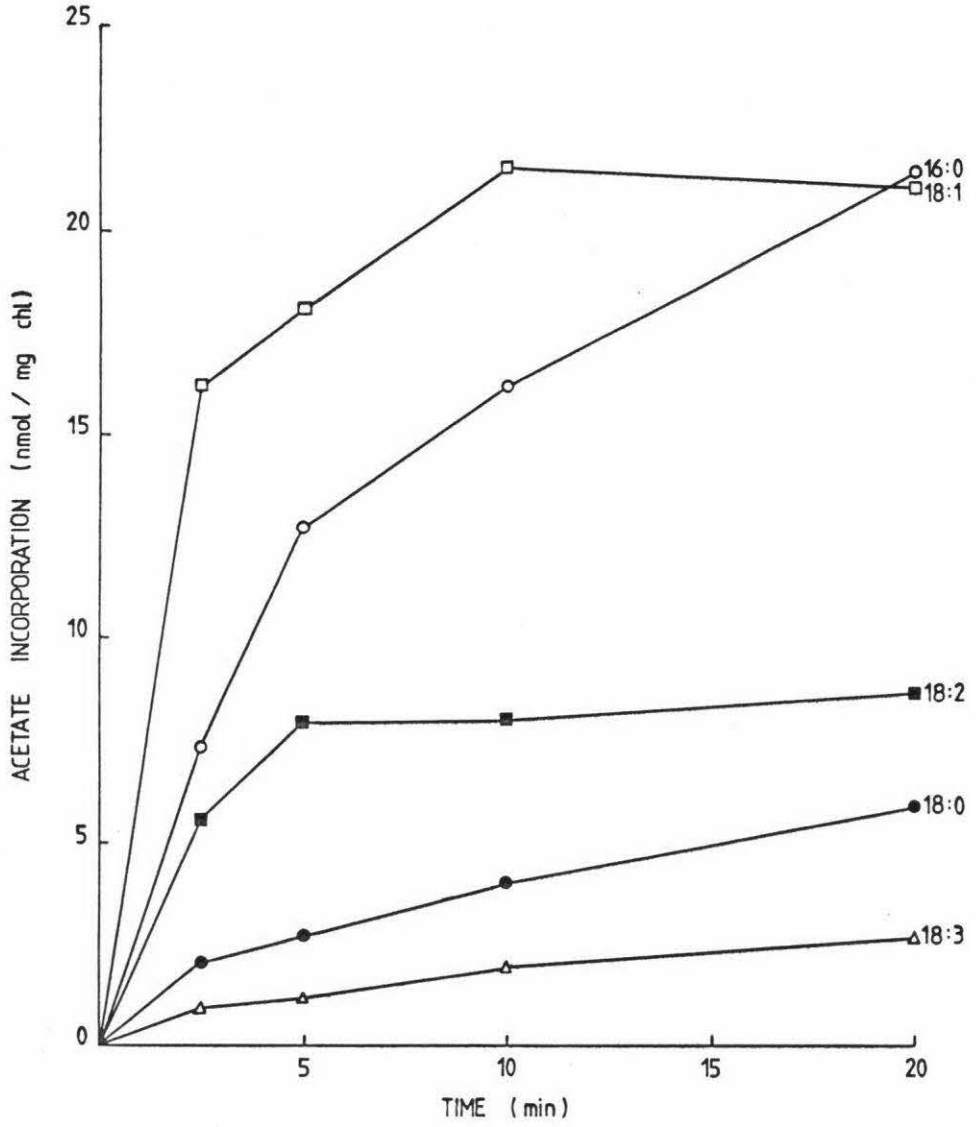



FIGURE 4-17: The rate of (1-$^{14}$C) acetate incorporation into the constituent fatty acids of total lipids.

### 4.6.5 Rate of (1-$^{14}$C) Acetate Incorporation into Constituent Fatty Acids of PC, DGDG plus PG, PE, MGDG, U and $U_{SF}$

The total lipids extracted from protoplasts after incubation with acetate were separated by thin-layer chromatography using $CHCl_3$ : MeOH : acetic acid : $H_2O$ (85:15:10:3.5, v/v) as described in Section 3.6. A small portion of total lipids material was also developed in toluene : ethyl acetate : 95% ethanol (2:1:1) along with MGDG and DGDG markers to confirm the presence of these galactolipids. Furthermore, known portions of each lipid corresponding to approximately 50,000 dpm were developed in the above solvents and autoradiograms obtained.

PC, PE, and MGDG were separated in the $CHCl_3$ : MeOH : $CH_3COOH$ : $H_2O$ solvent and DGDG plus PG ran together just above PC (see Figures 3-1, 4-21, 4-22). A high level of radioactivity was located between the solvent front and MGDG after TLC in the $CHCl_3$/MeOH solvent. The nature of the radioactive compounds have not been identified and have been designated U and $U_{SF}$. U ran immediately above MGDG and $U_{SF}$ was taken as the region between U and the solvent front. Radioactivity determinations of each of these showed that most of the label appeared in $U_{SF}$ (see Figure 4-18 and Table 4). Up to $2\frac{1}{2}$ hours acetate incorporation into PC was nearly as great as into $U_{SF}$, and then it decreased with time while acetate incorporation into $U_{SF}$ increased. The incorporation of label into U, PE, MGDG and DGDG plus PG did not increase significantly. Analysis of the distribution of label in the fatty acids (as described in Section 3) of these lipids followed a similar pattern. Initially most of the label appeared in the palmitic and oleic acids of PC. With time the amount of label in these two fatty acids of PC decreased dramatically. Label in linoleic

TABLE 4: INCORPORATION OF (1-$^{14}$C) ACETATE INTO LIPIDS AND FATTY ACIDS BY BARLEY PROTOPLASTS

| Time | Incorporation of acetate into lipids | | Distribution of label between lipids % | | | | | | Distribution of label between fatty acids % | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Hours | nmol/mg Chl | % of label | PC | DGDG&PG | PE | MGDG | U | $U_{SF}$ | 16:0 | 18:0 | 18:1 | 18:2 | 18:3 |
| 2½ | 32.3 | 6.5 | 36.8 | 4.3 | 3.1 | 8.0 | 3.2 | 44.6 | 22.5 | 6.2 | 50.0 | 16.5 | 2.9 |
| 5 | 43.5 | 8.7 | 30.4 | 3.0 | 3.3 | 6.5 | 3.2 | 53.7 | 29.3 | 6.3 | 41.5 | 18.2 | 2.7 |
| 10 | 52.7 | 10.6 | 18.7 | 2.7 | 2.5 | 4.7 | 4.3 | 67.2 | 30.6 | 7.6 | 40.8 | 15.2 | 3.6 |
| 20 | 62.0 | 12.4 | 8.9 | 7.5 | 2.0 | 4.4 | 3.8 | 73.4 | 32.0 | 9.6 | 33.7 | 14.0 | 6.1 |

U and $U_{SF}$ are unidentified lipids

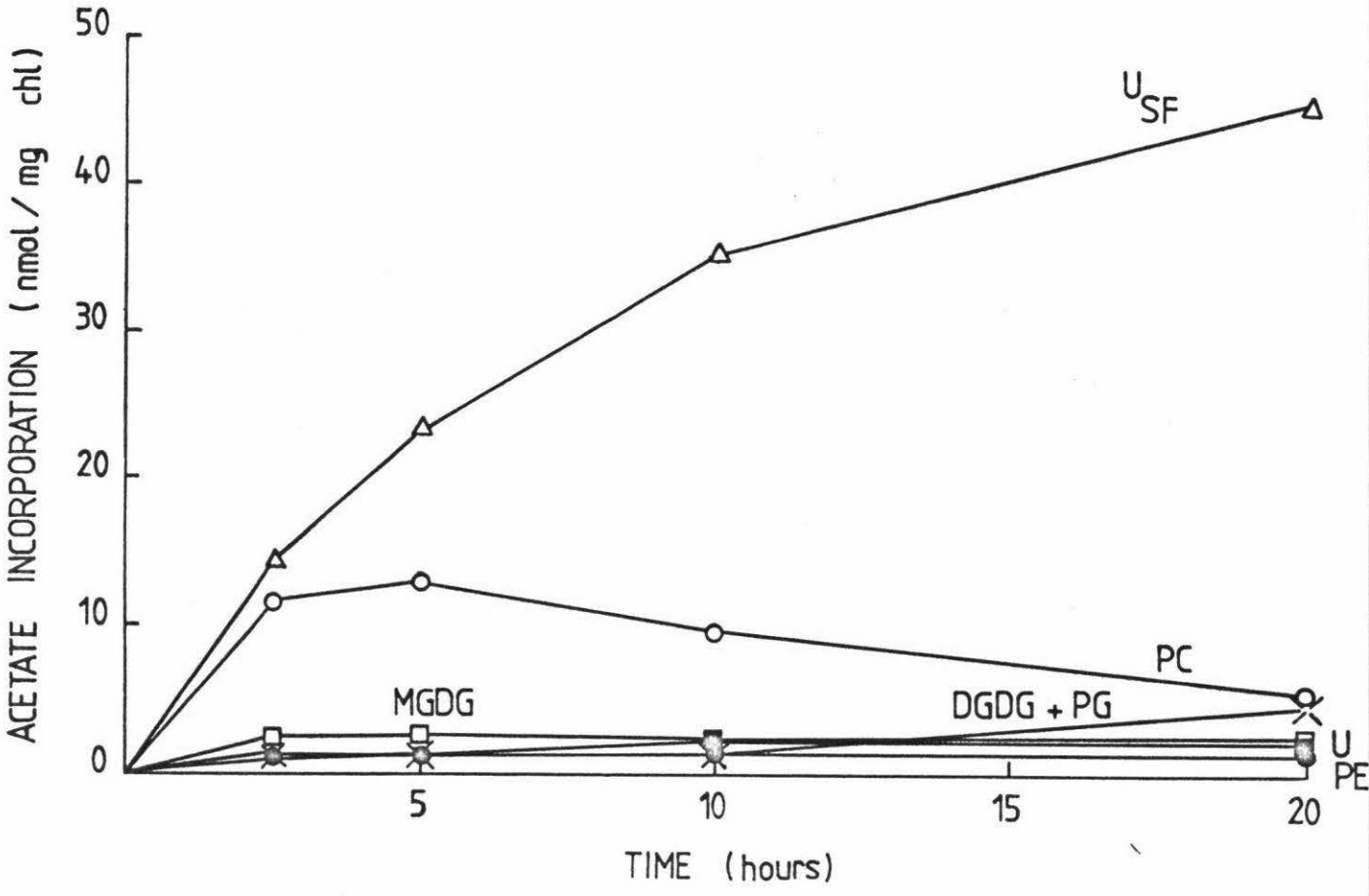



FIGURE 4-18: The rate of (1-$^{14}$C) acetate incorporation into the lipids of barley protoplasts.

U and $U_{SF}$ are unidentified lipids.

and stearic acids decreased to a lesser extent in PC and linolenic acid levels remained about the same (see Figure 4-19). There was not much increase of label into the fatty acids of PE, MGDG, and DGDG plus PG, apart from an increase into palmitic acid in the latter. However the rate of incorporation of acetate into palmitic and oleic acids, and to a lesser extent into stearic and linoleic acids, of $U_{SF}$ increased significantly (see Figure 4-20).

A scan for radioactivity of the plate of total lipids developed in toluene : ethyl acetate : 95% ethanol (2:1:1, v/v) confirmed the presence of MGDG. The presence of DGDG was not so certain. However the autoradiograms of the plates developed in this solvent showed radioactivity corresponding to MGDG, but little or none corresponding to DGDG (see Figures 4-21 and 4-22). A radioactive band corresponded to PG which ran just below the DGDG marker and above the other phospholipids which remained at the origin. Autoradiograms of the plates developed in $CHCl_3$ : MeOH : $CH_3COOH$ : $H_2O$ (85:15:10:3.5, v/v) confirmed the presence of radioactivity which had been detected by the scanning technique. From the above it would appear that radioactivity in the combined PG/DGDG band is due mainly to PG. The region termed "$U_{SF}$" consisted of several radioactive bands.

### 4.6.6 Attempt to Determine the Identity of Unknown Compounds U and $U_{SF}$

The unknown bands U and $U_{SF}$ were eluted from the TLC chromatograms obtained from development of the total lipids in $CHCl_3$ : MeOH : $CH_3COOH$ : $H_2O$ (85:15:10:3.5, v/v).

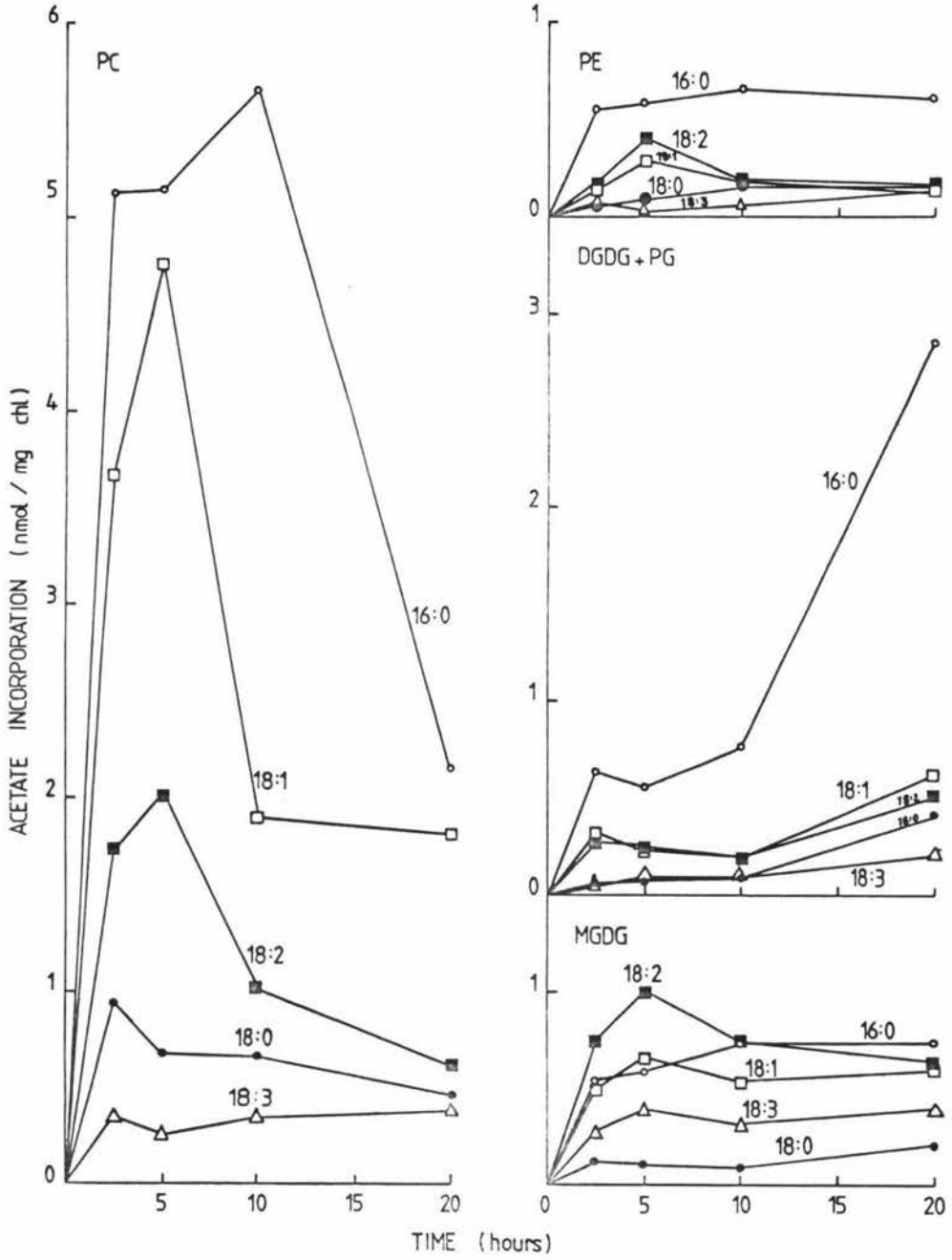



FIGURE 4-19: Rates of (1-$^{14}$C) acetate incorporation into the fatty acids of lipids.

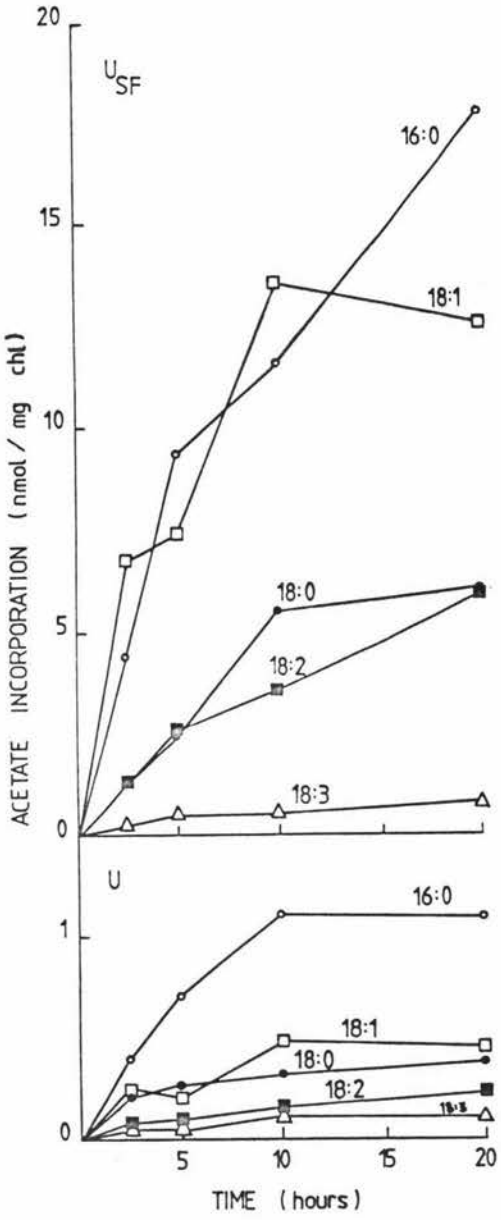



FIGURE 4-20: Rates of (1-$^{14}$C) acetate incorporation into the fatty acids of unidentified lipids.

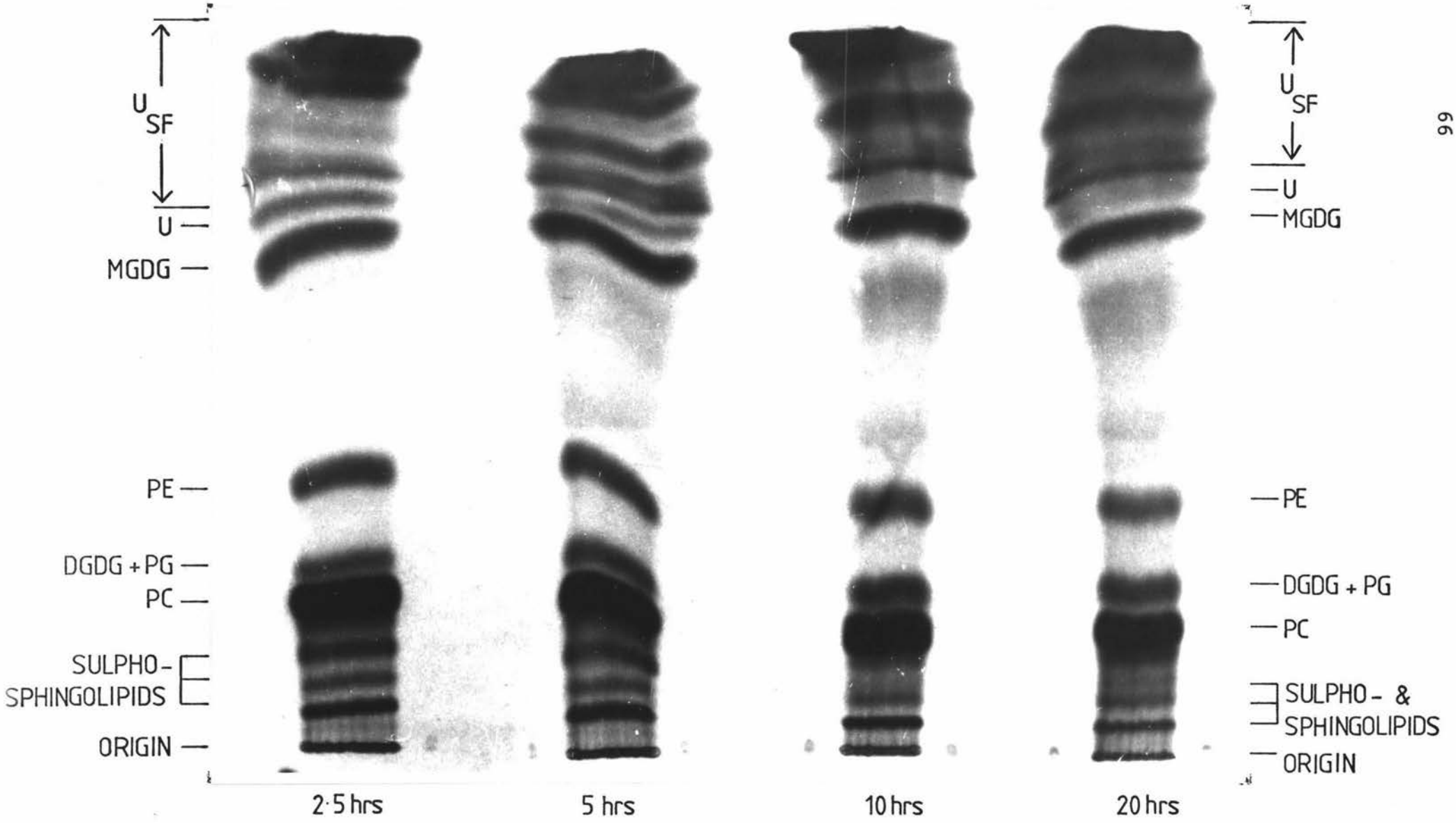

U
SF
U
MGDG
PE
DGDG + PG
PC
SULPHO-
SPHINGOLIPIDS
ORIGIN
2·5 hrs
5 hrs
10 hrs
20 hrs
U
SF
U
MGDG
PE
DGDG + PG
PC
SULPHO- &
SPHINGOLIPIDS
ORIGIN

FIGURE 4-21: Autoradiograms of TLC of total lipid developed in $CHCl_3$ : MeOH : $CH_3COOH$ : $H_2O$ (85:15:10:3.5, v/v)

Protoplasts were incubated with 50 µM (1-$^{14}$C) acetate (25 nmoles, 10 µCi) and approximately 50,000 dpm of extracted lipid chromatographed and autoradiograms obtained (see Sections 4.6.5 and 3.9 for details).

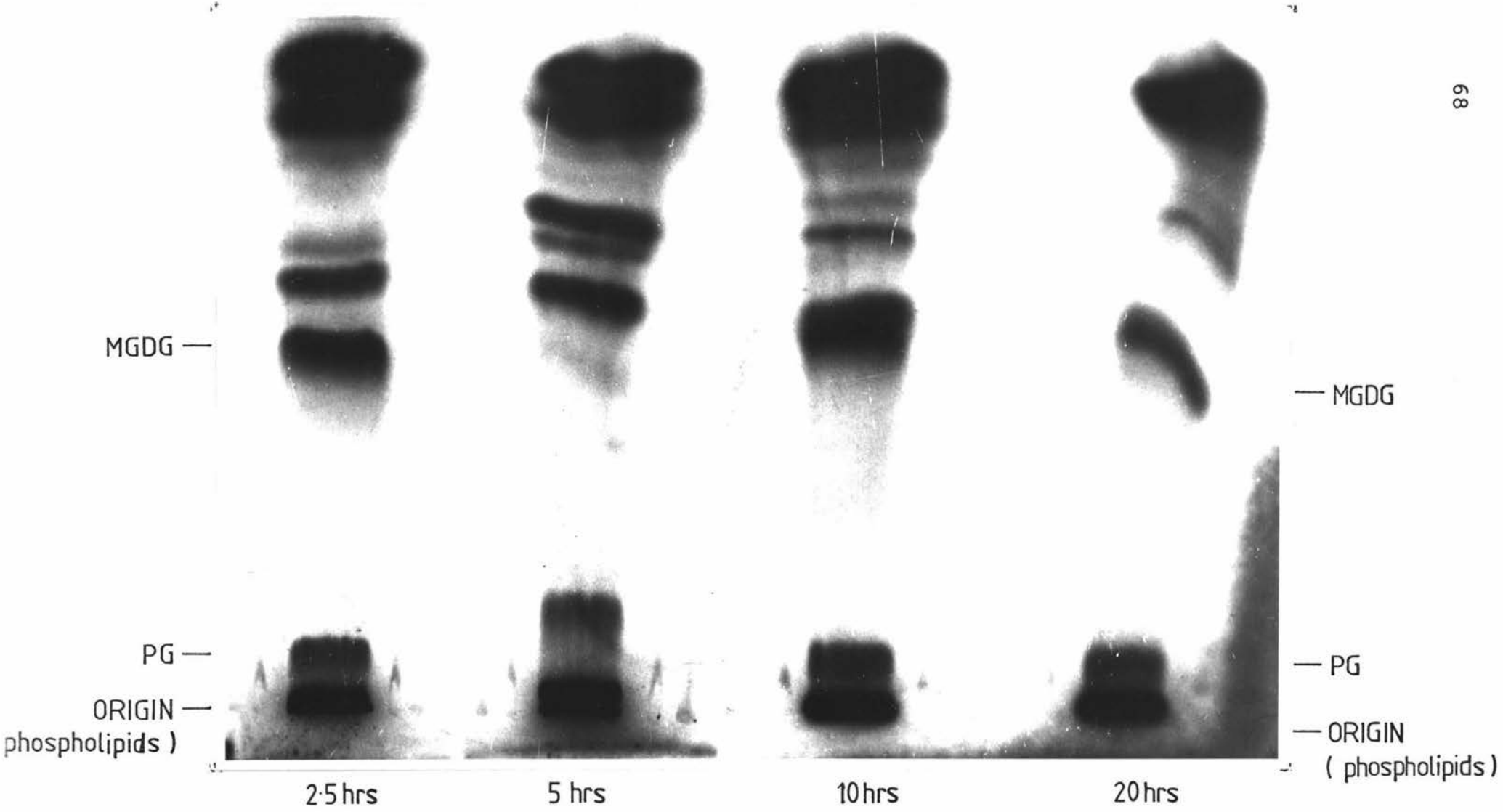

MGDG
PG
ORIGIN
phospholipids )
2·5 hrs
5 hrs
10 hrs
20 hrs
MGDG
PG
ORIGIN
( phospholipids )

FIGURE 4-22: Autoradiograms of TLC of total lipid developed in toluene: ethyl acetate: 95% ethanol (2:1:1, v/v)

Protoplasts were incubated with 50 μM (1-$^{14}$C) acetate (25 nmoles, 10 μCi) and approximately 50,000 dpm of extracted lipid chromatographed and autoradiograms obtained (see Sections 4.6.5 and 3.9 for details).

U and $U_{SF}$ were chromatographed in solvents toluene : ethyl acetate : 95% ethanol (2:1:1, v/v) and hexane : diethyl ether : acetic acid (70:30:1, v/v) respectively and the chromatograms scanned for radioactivity. Furthermore U and $U_{SF}$ eluted from thin layers were hydrolysed and reacted with $BF_3$/HCl as described for the preparation of methyl esters from acyl lipids. The methylated material of each was chromatographed in hexane : diethyl ether (9:1, v/v) and scanned for radioactivity.

The distribution of the label in the chromatogram of U in toluene : ethyl acetate: 95% ethanol (2:1:1, v/v) was mostly over the upper half of the plate while a small amount remained at the origin (see Figure 4-23 and Table 5). Upon methylation of U, most of the label appeared in band 3 which corresponded to the fatty acid methyl ester used as marker (see Figure 4-25 and Table 7), and approximately 1/5 of the material remained at the origin. This implies that most of U consists of acyl lipid.

The strip scan and subsequent radioactivity determination of bands of the chromatogram of $U_{SF}$ implied that some of the label was in MG (band 1) and TG (band 4) (see Figure 4-24 and Table 6). Upon methylating $U_{SF}$ most of the label appeared in band 3 which corresponded to the methyl ester fatty acid marker (see Figure 4-25 and Table 8). It appears also that most of $U_{SF}$ consists of acyl lipid.

TABLE 5: DISTRIBUTION OF LABEL IN BANDS OBTAINED FROM CHROMATOGRAM OF U IN TOLUENE : ETHYL ACETATE : 95% ETHANOL (2:1:1, v/v)
(see Figure 4-23 opposite)

| Band | % of total label |
|---|---|
| 1 | 4.3 |
| 2 | 12.2 |
| 3 | 29.5 |
| 4 | 7.6 |

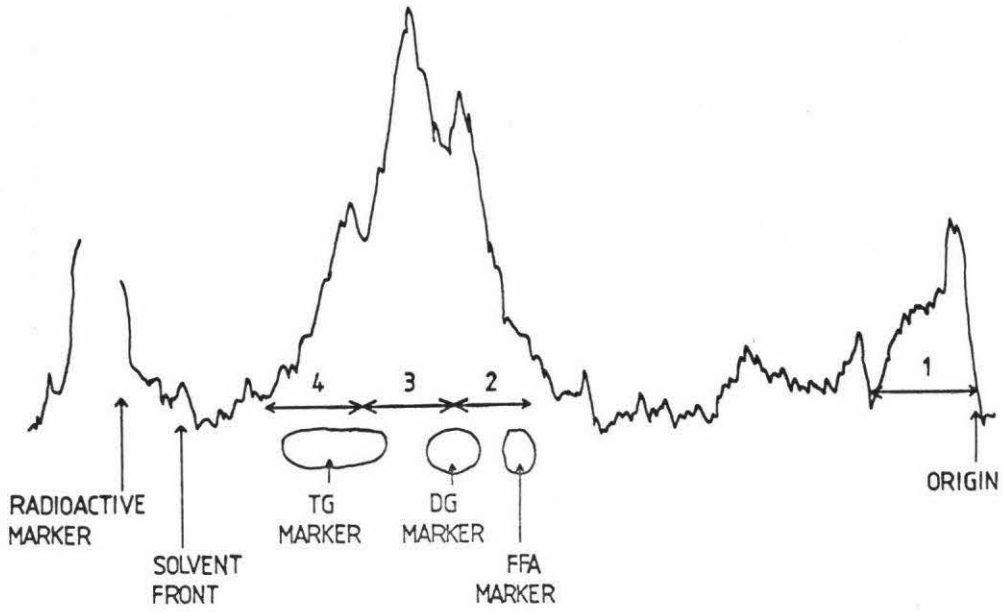



FIGURE 4-23: Radioactive scan of chromatogram of U in toluene : ethyl acetate : 95% ethanol (2:1:1 v/v).

Approximately 13,150 dpm were applied to the t.l.c. plate in a narrow band 1.5 cm in length. Conditions of scanning were: slit width 2.5 mm, time constant 30s, linear range 300, and the rest as in Section 3.9.

TABLE 6: DISTRIBUTION OF LABEL IN BANDS OBTAINED FROM CHROMATOGRAM OF $U_{SF}$ IN HEXANE : DIETHYL ETHER : ACETIC ACID (70:30:1, v/v)
(see Figure 4-24 opposite)

| Band | % of total label |
|---|---|
| 1 | 10.7 |
| 2 | 6.9 |
| 3 | 2.3 |
| 4 | 18.9 |
| 5 | 9.5 |

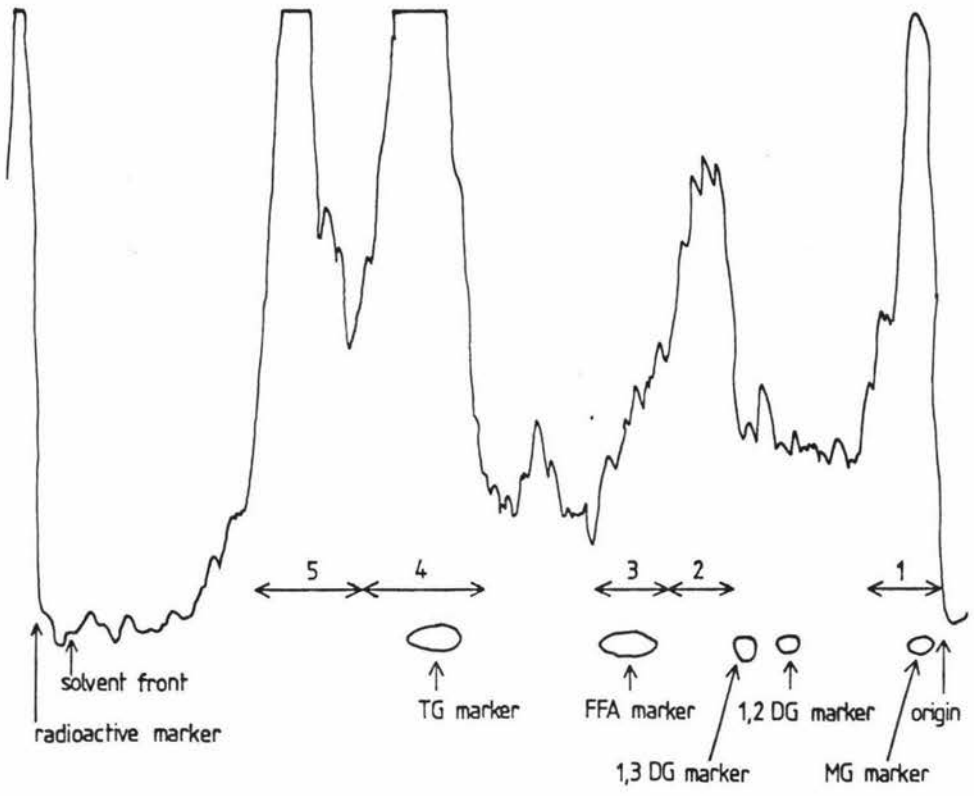



FIGURE 4-24: Radioactive scan of chromatogram of $U_{SF}$ in hexane : diethyl ether : acetic acid (70:30:1, v/v)

Approximately 49,000 dpm were applied to the t.l.c. plate in a narrow band 1.5 cm in length. Conditions of strip scanning were: slit width 2.5 mm, time constant 30s, linear range 300, and the rest as in Section 3.9.

TABLE 7: DISTRIBUTION OF LABEL IN BANDS OBTAINED FROM CHROMATOGRAMS OF METHYLATED U IN HEXANE : DIETHYL ETHER (9:1, v/v)
(see Figure 4-25 opposite)

| Time Hours | % of total eluted counts | | |
|---|---|---|---|
| | Band 1 | Band 2 | Band 3 |
| $2\frac{1}{2}$ | 11.5 | 0 | 86.0 |
| 5 | 20.9 | 2.0 | 77.2 |
| 10 | 20.4 | 10.9 | 67.4 |
| 20 | 17.0 | 6.2 | 76.8 |

TABLE 8: DISTRIBUTION OF LABEL IN BANDS OBTAINED FROM CHROMATOGRAMS OF METHYLATED $U_{SF}$ IN HEXANE : DIETHYL ETHER (9:1, v/v)
(see Figure 4-25 opposite)

| Time Hours | % of total eluted counts | | |
|---|---|---|---|
| | Band 1 | Band 2 | Band 3 |
| $2\frac{1}{2}$ | 17.6 | 2.6 | 80.0 |
| 5 | 24.8 | 1.6 | 74.0 |
| 10 | 3.3 | 0 | 97.0 |
| 20 | 9.0 | 3.2 | 87.8 |

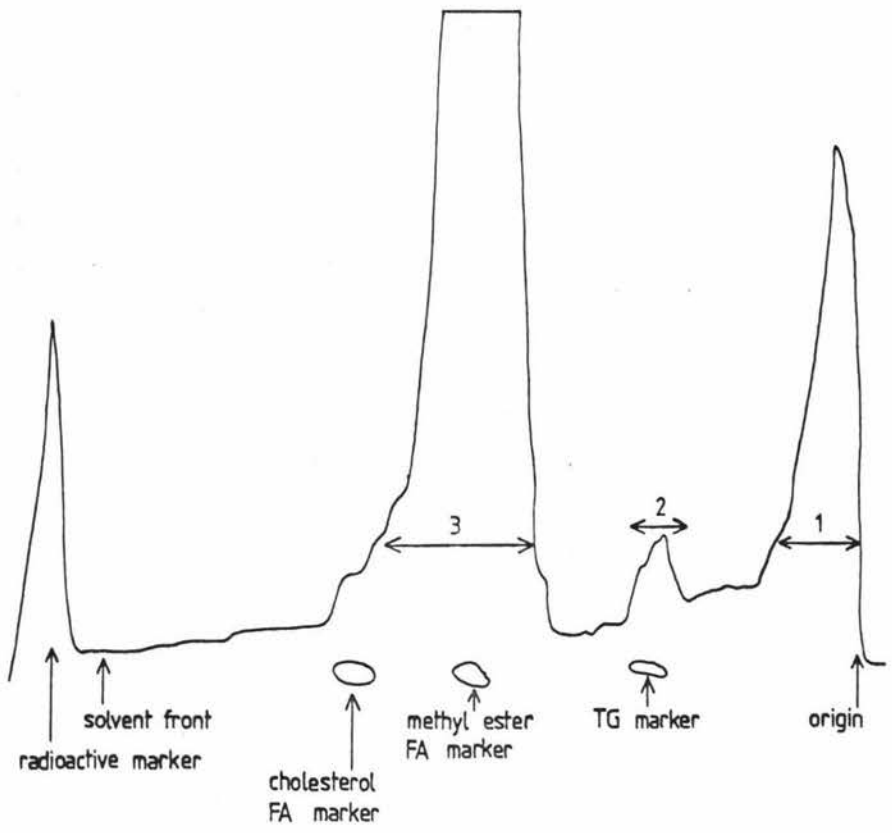



FIGURE 4-25: A typical scan of a chromatogram obtained from development of methylated U or $U_{SF}$ in hexane : diethyl ether (9:1, v/v)

The amount of radioactive material applied in a narrow band 1.5 cm in length to the t.l.c. plate ranged from 20,000 - 80,000 dpm.

Conditions of strip scanning were: slit width 2.5 mm, time constant 30s, linear range 300, and the rest as in Section 3.9.

# CHAPTER 5

# DISCUSSION

## 5.1 PROTOPLAST PREPARATION

Protoplasts for metabolic studies have been isolated from plant tissues in recent years by the use of various enzyme solutions and conditions. Kanai and Edwards (1973) used an incubation time of 3-5 hours to release protoplasts from maize leaf segments in a simpler enzyme solution compared to the one employed in this study (Day *et al*, 1981) and in particular it lacked pectinase and BSA. Sicher (1982) however digested barley tissue in the dark at 30°C for only $1\frac{1}{2}$ hours in a medium containing macerase as well as cellulase but no BSA. With other plant tissues longer incubation times have been used to release protoplasts with and without pectinase or macerase and BSA. For example Kuhn & Stumpf (1981) incubated spinach leaves for 4 hours at 30°C and Edwards *et al* (1979) *Panicum miliaceum* for $4\frac{1}{2}$ hours at 30°C. Wheat (Edwards *et al*, 1978) and oat (Briggs *et al*, 1982) tissue were incubated for 3 hours at 25°C and 28°C respectively for the release of protoplasts and Bhojwani (1982) plasmolysed white clover for 3 hours before incubating the tissue for 16 hours in the dark at 25°C in an enzyme solution containing driselase as well as both cellulysin and macerase.

The sucrose-sorbitol density gradient (Day *et al*, 1981) yielded highly purified barley protoplasts, as seen by phase contrast microscopy, which were intact, as evidenced by $CO_2$-dependent $O_2$ evolution rates. Edwards *et al* (1978) and Sicher (1982) used a similar density gradient for purification of wheat and barley protoplasts respectively. A density gradient formed from sucrose and sorbitol

solutions containing different amounts of dextran $T_{40}$ was used by Edwards *et al* (1979) to achieve the separation of both bundle sheath and mesophyll protoplasts at about 95% purity. In other studies initial filtration after digestion of leaf tissue and subsequent centrifugation of protoplast suspensions has alone been used (Kanai and Edwards, 1973; Briggs *et al*, 1982).

## 5.2 INTACTNESS OF PROTOPLASTS AND CHLOROPLASTS ISOLATED FROM OTHER TISSUE

Usual values for $CO_2$-dependent $O_2$ evolution were around 180 µmol $O_2$/hr/mg Chl indicating that the barley protoplasts had retained "normal" metabolic activity (Kuhn & Stumpf, 1981). Kuhn (1980) obtained values of around 60 µmol/hr/mg Chl for spinach protoplasts and Leegood and Walker (1981) obtained values of 96 and 136 µmol/hr/mg Chl for wheat protoplasts. Robinson *et al* (1979) however obtained a rate of 167 µmol/hr/mg Chl by wheat protoplasts for $CO_2$-dependent $O_2$ evolution. Rates for $CO_2$-dependent $O_2$ evolution by chloroplasts have been found to be about 120 µmol/hr/mg Chl in spinach (Lilley *et al*, 1975) and wheat (Robinson *et al*, 1979; Leegood and Walker (1981). Roughan *et al* (1979a) on the other hand have obtained rates of up to 369 µmol/hr/mg Chl for spinach chloroplasts and attributed such high rates to using low-ionic-strength buffers (Nakatani & Barber, 1977) during the isolation of chloroplasts from spinach leaves. Also it was found that addition of catalase (Kaiser, 1976) was required for maximum rates of $O_2$ evolution, rates being 20-30% lower in its absence (Roughan *et al*, 1979a). However in the present study addition of catalase did not affect $CO_2$-dependent $O_2$ evolution rates by barley protoplasts.

## 5.3 FACTORS AFFECTING INCORPORATION OF SUBSTRATE INTO LIPIDS

Both $Mg^{++}$ and $P_i$ were found not to have much effect on the incorporation of $HCO_3^-$ into barley protoplast lipids, probably because the protoplasts were already saturated with these ions from the isolation medium. Since $PP_i$ was included in the $CO_2$-dependent $O_2$ evolution assay (Lilley *et al*, 1975), it was also added when looking at $HCO_3^-$ incorporation into lipids. However it was found to be slightly inhibitory to $HCO_3^-$ incorporation and was consequently omitted. 10 mM proved to be the optimum concentration for $HCO_3^-$ as was used by Lilley *et al* (1975) in $CO_2$-dependent $O_2$ evolution assays and by workers following $HCO_3^-$ incorporation into chloroplastic lipids (McKee & Hawke, 1978; Murphy & Leech, 1978; Roughan *et al*, 1979a). Acetate was not found to be inhibitory to $HCO_3^-$ incorporation into barley protoplast lipids. On the other hand, Roughan *et al* (1979a) found in isolated spinach chloroplasts that fatty acid synthesis from $HCO_3^-$ was decreased in the presence of 0.16 mM acetate and even more so when acetate was increased to 0.4 mM.

50 µM acetate was used in all assays examining the incorporation of acetate into lipids of barley protoplasts. Roughan *et al* (1976) showed in isolated spinach chloroplasts that between 100 and 150 µM acetate was optimal for acetate incorporation into lipids, therefore greater incorporation of acetate into barley protoplast lipids would probably have been achieved using these higher concentrations.

The pH profile for acetate assimilation by, and incorporation into lipids of barley protoplasts was similar to that obtained by Kuhn & Stumpf (1981) for spinach protoplasts. Incorporation was greatest at an acidic pH of around 5

and its decrease paralleled the increase in pH. The inverse is seen for spinach chloroplasts (Nakamura & Yamada, 1975) - at the acidic pHs acetate incorporation is low, but with increasing pH incorporation increases to become optimal around pH 8.0.

## 5.4 INCORPORATION OF SUBSTRATE INTO POLYUNSATURATED FATTY ACIDS AND LIPIDS OF PLANTS

Studies to ascertain the intracellular location and mechanism of desaturation of oleic and linoleic acids to linolenic acids have not so far been conclusive. *De novo* syntheses of a range of glycerolipids and 18:2 and 18:3 acids as constituents of mainly MGDG is observed in studies using leaf tissue, such as intact spinach leaves (McKee & Hawke, 1978; Murphy & Leech, 1981), *V. faba* leaf discs (Williams *et al*, 1976) and excised leaves (Slack & Roughan, 1975). Until recently significant synthesis of 18:2 and 18:3 has not been observed in isolated chloroplasts, even when the range of lipids synthesised by chloroplasts was enhanced by the addition of UDP-galactose and glycerol 3-P (McKee & Hawke, 1978, 1979). However Roughan *et al* (1979b) found in incubations containing both UDP-galactose and spinach chloroplasts that there was appreciable synthesis of both di- and trienoic acids and that these were specifically located within the polar-lipid fraction of chloroplast extracts. Yet the 18:2 and 18:3 levels still were not near the high amounts of these acids found in intact leaf tissue.

Isolated barley protoplasts were able to synthesise a range of glycerolipids, but as in most other investigations, most of the label was incorporated into palmitic and oleic acids of lipid material with insignificant labelling of linoleic and linolenic acids. At optimal conditions,

$HCO_3^-$ incorporation into barley protoplast lipids over the linear part of the graph was about 500 nmol/mg Chl/h and acetate incorporation was about 8.7 nmol/mg Chl/h. Murphy & Leech (1981) have obtained a rate of 610 nmol/mg Chl/h for $HCO_3^-$ incorporation into spinach chloroplasts. In the only other study of incorporation of a labelled precursor into lipids of protoplasts, Kuhn & Stumpf (1981) showed that acetate incorporation into lipids of spinach protoplasts was around 1150 nmol/mg Chl/h. However despite the much greater incorporation, synthesis of 18:2 and 18:3 acids was still low. Roughan *et al* (1979a) have achieved rates of up to 1500 nmol/mg Chl/h for acetate incorporation into lipids of isolated spinach chloroplasts.

Intact spinach leaves exposed to $^{14}CO_2$ have been found to incorporate $CO_2$ into lipids at a rate of 7600 nmol/mg Chl/h (Murphy & Leech, 1981). Label entered into all parts of the lipid molecule and the incorporation rate of $CO_2$ into fatty acids alone was 2160 nmol/mg Chl/h. In the same study, isolated spinach chloroplasts incubated with $H^{14}CO_3^-$ incorporated all of the label into fatty acids at 610 nmol/mg Chl/h and the range of lipids synthesised was very limited compared with the intact leaf. Murphy & Leech (1981) have proposed that acetate derived from the leaf cell mitochondria is the physiological fatty acid precursor in chloroplasts since studies of Roughan *et al* (1979a, 1979c) and Murphy (1981, unpublished results) have shown that isolated chloroplasts are able to synthesise fatty acids at rates approaching those in whole leaves (Murphy & Leech, 1981) only if (1-$^{14}$C) acetate rather than $H^{14}CO_3^-$ is used as the substrate.

A comparison of the lipids synthesised from $H^{14}CO_3^-$ and (1-$^{14}$C) acetate by barley protoplasts was not made in this study. It would have been interesting to see whether the protoplasts were capable of synthesising the same range

of lipids from both precursors. Both barley protoplasts and spinach protoplasts (Kuhn, 1980) were able to synthesise from acetate a range of glycerolipids as in leaf studies, but as in chloroplasts significant synthesis of 18:2 and 18:3 was not observed.

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