LIPID BIOSYNTHESIS IN ISOLATED CHLOROPLASTS

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ABSTRACT

Two notable features of previous work on lipid biosynthesis by isolated chloroplasts have been:- (a) The inability of chloroplasts to incorporate more than small amounts of acetate into the main constituent fatty acids of the chloroplast lipids, namely linoleic (18:2) and linolenic (18:3) acids. (b) The poor incorporation of fatty acids synthesized into galactolipids, which are the main chloroplast lipids. Both of these aspects of lipid biosynthesis were investigated using chloroplasts isolated from spinach, maize and sweetcorn. Initial attempts to improve the synthesis of polyunsaturated fatty acids from [1-^{14}C]acetate were not successful. Consequently the main object of the investigation was directed towards increasing the incorporation of long chain fatty acids into galactolipids in the hope that increased galactolipid synthesis might also lead to increased desaturation of oleate to linoleate and linolenate.

Factors affecting the rates of acetate incorporation into lipids by spinach, maize and sweetcorn chloroplasts were investigated. Optimum concentrations of acetate, ATP and CoA were found to be about 0.5mM-acetate (spinach somewhat higher at 0.75mM-acetate), 0.5mM-ATP and 0.25mM-CoA under the incubation conditions used in the present study. Acetate concentration had a major effect on the rate of incorporation; optimisation of ATP and CoA concentrations gave only small enhancements of acetate incorporation. The effect of divalent cations was also investigated for spinach chloroplasts. Optimum Mg^{++} was 3.0mM; addition of 1mM-Mn^{++} in the presence of 1mM-Mg^{++} gave a comparable stimulation of acetate incorporation. Acetate incorporation by spinach chloroplasts was also enhanced by the addition of Triton X-100, sn-glycerol-3-phosphate and UDP-galactose.

Maximum incorporation rates obtained for maize and sweetcorn chloroplasts were 20-30nmol of acetate/mg chlorophyll/h which are up to 10-fold higher than previously reported rates for maize. Rates of up to 500nmol of acetate/mg chlorophyll/h were obtained for spinach chloroplasts which compare favourably with the rates obtained by other workers using chloroplasts isolated from younger leaf tissue.

Oleic and palmitic acids with small amounts of stearic

acid were the main fatty acids synthesized from acetate by isolated chloroplasts from all three sources. Little synthesis of linoleic and linolenic acids was achieved and changes in acetate, ATP and CoA concentrations had no significant effect on the synthesis of polyunsaturated fatty acids from acetate. Triton X-100 and divalent metal ion concentrations also had little effect on the synthesis of polyunsaturated fatty acids by spinach chloroplasts.

The synthesis of diglycerides (DG) by isolated chloroplasts from spinach, maize and sweetcorn was enhanced by the addition of sn-glycerol-3-phosphate (G-3-P). Synthesis of monogalactosyldiglyceride (MGDG) was enhanced by the addition of UDP-galactose particularly if G-3-P was also present. Triton X-100 greatly enhanced the synthesis of DG and also (in the presence of UDP-galactose) MGDG by spinach chloroplasts. Spinach chloroplasts gave higher rates of DG and MGDG synthesis than either maize or sweetcorn chloroplasts.

The synthesis of MGDG from DG by spinach chloroplasts was investigated by double-labelling experiments, using [1(3)-^{3}H]sn-glycerol-3-phosphate and [1-^{14}C]acetate, fatty acid analysis and positional distribution of the incorporated fatty acids. The synthesis of MGDG was shown to occur without prior modification of the fatty acid composition of the DG.

It was evident from the incorporation of oleate and palmitate into DG (and subsequently into MGDG) and from the positional distribution of these two fatty acids that a specific acylation of G-3-P occurred synthesizing mainly 1-oleoyl, 2-palmitoyl-sn-glycerol. The effects of altering the proportions of oleate and palmitate synthesized on the relative amounts of these fatty acids incorporated into DG (and MGDG) were investigated. The results suggested that palmitate was incorporated into position 2 first followed by oleate into position 1. If there was more palmitate than oleate synthesized some palmitate could be also incorporated into position 1.

The rates of DG synthesis calculated from [1(3)-^{3}H]-sn-glycerol-3-phosphate incorporation were considerably greater than those calculated from [1-^{14}C]acetate incorporation indicating that a considerable dilution of the label from [1-^{14}C]acetate had occurred and that a major proportion of the fatty acid carbon had come from an alternative source.

Bicarbonate, present in the reaction medium, was found to be utilized by spinach chloroplasts for the synthesis of fatty acids and lipids. Thus bicarbonate was probably the alternative source of fatty acid carbon. The fatty acids and lipids synthesized by spinach chloroplasts from exogenous acetate and bicarbonate were very similar.

Although high rates of DG and MGDG synthesis have been achieved in the course of the present study by the addition of appropriate metabolites, stimulation of synthesis of these lipids did not alter the rates of synthesis of linoleic and linolenic acids from acetate. Other attempts to increase polyunsaturated fatty acid synthesis from acetate by isolated chloroplasts were also unsuccessful. The use of chloroplasts isolated from developing maize leaf sections had little effect on the rates of linoleic and linolenic acids synthesized from acetate. The addition of a 100,000 X g particulate preparation from leaf homogenate to isolated maize and spinach chloroplasts though stimulating overall incorporation of acetate, gave only minor increases in the proportion of linoleic and linolenic acids synthesized. The stimulation of phosphatidylcholine synthesis by the particulate fraction, in the presence of isolated chloroplasts, failed to result in any dramatic increases in the proportions of polyunsaturated fatty acids synthesized.

These findings are discussed in relation to the current understanding of fatty acid and lipid synthesis and recent *in vivo* and *in vitro* studies of plant lipid synthesis.

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To my Parents

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ABBREVIATIONS

ACP	acyl carrier protein
ATP	adenosine 5'-triphosphate
A_{645}	absorbance at 645nm
BCCP	biotin carboxyl carrier protein
BSA	bovine serum albumin
^{o}C	degrees Celsius
CDP-choline	cytidine 5'-diphosphate choline
Ci	Curie
cm	centimetre
cm^3	cubic centimetre
CoA	coenzyme A
DCCD	N,N'-dicyclohexylcarbodiimide
DCMU	3-(3,4-dichlorophenyl)-1,1-dimethylurea
DEGS	diethylene glycol succinate
DG	diglyceride (or diacylglycerol)
DGDG	digalactosyldiglyceride (or digalactosyl-glycerol)
d.p.m.	disintegrations per minute
DTT	dithiothreitol
E. coli	*Escherichia coli*
FCCP	4-trifluoromethoxyphenylhydrazone
FFA	free fatty acids
ft-candle	foot-candle (1 ft-candle = 10.7639 lx)
g	gram
g	force of gravity
G-3-P	*sn*-glycerol-3-phosphate
g.l.c.	gas-liquid chromatography
h	hour
i.d.	internal diameter
kv	kilovolt
l	litre
lyso-PA	lyso-phosphatidic acid (or monoacyl-*sn*-glycerol-3-phosphate)
lx	lux
M	molar
mg	milligram
MG	monoglyceride (or monoacylglycerol)
MGDG	monogalactosyldiglyceride (or monogalactosyl-diacylglycerol)

MGMG	monogalactosylmonoglyceride (or monogalactosyl-monoacylglycerol)
min	minute
mM	millimolar
mmol	millimole
mol	mole
NADH	nicotinamide adenine dinucleotide, reduced
NADPH	nicotinamide adenine dinucleotide phosphate, reduced
nm	nanometre
nmol	nanomole
p., pp.	page, pages
PA	phosphatidic acid
PC	phosphatidylcholine
PE	phosphatidylethanolamine
PEP	phosphoenolpyruvate
PG	phosphatidylglycerol
2-PGA	2-phosphoglyceric acid
3-PGA	3-phosphoglyceric acid
POPOP	1,4 bis[2-(5-phenyloxazolyl)]-benzene
PPO	2,5-diphenyloxazole
s	second
sn	stereospecific numbering
SQDG	sulphoquinovosyldiglyceride (or sulphoquinovosyl-diacylglycerol)
TG	triglyceride (or triacylglycerol)
t.l.c.	thin-layer chromatography
Tricine	N-Tris(hydroxymethyl) methylglycine
Tris	Tris(hydroxymethyl) aminomethane
UDP-galactose (UDP-gal)	uridine 5'-diphosphate D-galactose
UK	unknown compound (see Methods, p. 36)
v	volume
wt	weight
µ	micro

NOMENCLATURE

For the specific structural designation of complex lipids containing a glycerol moiety, the nomenclature suggested by the IUPAC-IUB Commission on Biochemical Nomenclature (Eur. J. Biochem. (1967) 2, 127-131) has been followed. However, the trivial names of complex lipids are used when it is more appropriate. Widely used abbreviations, e.g. MGDG for monogalactosyldiglyceride, have also been used for the sake of brevity. These are defined on pp. xxi-xxii.

Fatty acids are designated by the shorthand notation of number of carbon atoms:number of double bonds, e.g. 18:3 refers to linolenic acid.

Other abbreviations and the format for the figures and tables in this thesis followed the guide lines set down by the Biochemical Journal (Biochem. J. (1975) 145, 1-20).

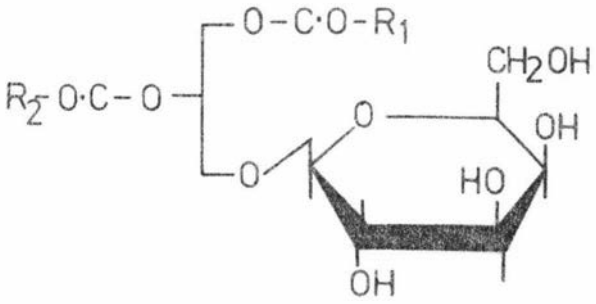
Chapter 1
INTRODUCTION

1.1 General Introduction

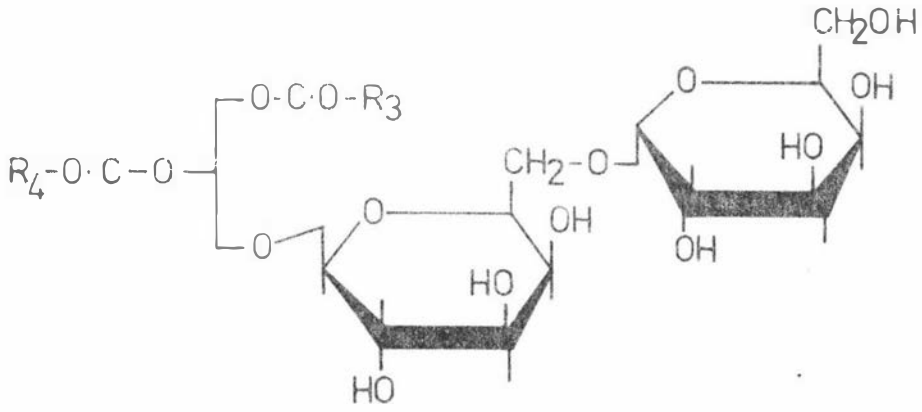
Lipids, principally phospholipids and galactolipids comprise up to 10% of the dry weight in photosynthetic tissue. A large proportion of the total lipid is located in the chloroplasts (Kates, 1970) which contain nearly all of the galactolipid found in the leaf tissue from beet and tobacco (Wintermans, 1960; Ongun et al., 1968).

Monogalactosyldiglyceride (MGDG) and digalactosydiglyceride (DGDG) are the two major galactolipids found in plants (Sastry, 1974) and their structures were determined by Carter et al. (1961) on MGDG and DGDG isolated from wheat flour (Fig. 1-1, p. 2). Sastry and Kates (1964a) confirmed that the MGDG and DGDG from runner bean leaves had identical structures to those from wheat flour. The distinctive feature of lipids in photosynthetic tissues, particularily the galactolipids, is their high content of polyunsaturated fatty acids, mainly dienoic (linoleic (18:2) and hexadecadienoic (16:2)) and trienoic (linolenic (18:3) and hexadecatrienoic (16:3)) fatty acids with very small amounts of the fatty acids commonly found in animal lipids (palmitic (16:0), stearic (18:0) and oleic (18:1)) (Sastry, 1974). Linolenic acid can constitute up to 90% of the fatty acids of MGDG. There is little opportunity for fatty acid positional specificity, but in plants, such as spinach, which contain a considerable amount of C_{16} fatty acids (mainly 16:3), these show a preference for position 2 of the MGDG molecules (Safford and Nichols, 1970; Auling et al., 1971).

The presence of galactolipids in all photosynthetic tissue capable of O_2-evolution (James and Nichols, 1966), the observation of increases in galactolipids containing polyunsaturated fatty acids with increasing levels of chlorophyll either in developing leaf tissue (Appelqvist et al., 1968a; 1968b; Leech et al., 1971) or in the greening of etiolated tissue (Bishop and Smillie, 1970) and the high galactolipid content of the chloroplast lamella (Mackender and Leech, 1974) led to the suggestion that they may be involved in the photosynthetic process (Constantopoulos and Bloch, 1967). The direct involvement of galactolipids in the light reaction or electron transport



1,2-diacyl-[β-D-galactopyranosyl(1' → 3)]-sn-glycerol



1,2-diacyl[α-D-galactopyranosyl-(1 → 6')-β-D-
galactopyranosyl(1' → 3)]-sn-glycerol

Fig. 1-1. The structures of MGDG and DGDG
(R_1, R_2, R_3 and R_4 are long chain fatty acyl residues (Carter et al., 1961))

reactions is unlikely since large amounts of galactolipid can be removed from photosynthetic membranes, by treatment with lipases without marked effects on electron transport (Anderson et al., 1974). It has been proposed that the role of galactolipids is to provide the correct environment for the assembly of the photosynthetic pigments and protein complexes allowing only limited mobility of pigment molecules necessary for photosynthetic activity (Nichols and James, 1968).

Plants provide animals with their only source of linoleic and linolenic acids, since they can not be synthesized by animals. They are required by animals for the synthesis of long chain polyunsaturated fatty acids such as arachidonic acid which are essential for normal growth and development (Guarniere and Johnson, 1970). Arachidonic acid and the other long chain polyunsaturated fatty acids are precursors for the synthesis of prostaglandins (van Dorp et al., 1964; Bergstrom et al., 1964).

1.2 Fatty Acid Biosynthesis

1.2.(a) De novo Biosynthesis

Much of our detailed knowledge of fatty acid biosynthesis has come from studies of bacterial and animal systems (Volpe and Vagelos, 1973; Katiyar and Porter, 1977). Two distinct fatty acid synthetases are recognizable. One type is a multienzyme protein complex found in yeast and animal tissues. In yeast, the fatty acid synthetase can be resolved into two polyfunctional components, each a polyfunctional polypeptide (Schweizer et al., 1973). The second type is that found in Escherichia coli (E. coli) where the enzymes of the fatty acid synthetase are obtained as individual protein components from ruptured cells. The individual enzymes and reactions of the E. coli synthetase have been extensively studied and the pathway determined (Fig. 1-2, p. 4). A feature of the E. coli synthetase is the involvement of a free carrier protein for the growing fatty acid chain called acyl carrier protein (ACP) (Majerus and Vagelos, 1967). In contrast, the yeast "ACP" like protein represents a region of one of the multi-functional polypeptide chains of fatty acid synthetase (Schweizer et al., 1973).

The E. coli synthetase utilizes the ACP thioesters of acetate and malonate as substrates for the synthesis of fatty acids. These are synthesized from their respective coenzyme A (CoA) derivatives by the action of transacylases (Fig. 1-2, p. 4).

(1.) $CH_3CO-S-CoA + HS-ACP \rightarrow CH_3CO-S-ACP + CoA-SH$

acetyl CoA-ACP transacylase

(2.) $CH_3CO-S-ACP + HS-E_{cond} \rightarrow CH_3CO-S-E_{cond} + ACP-SH$

acetyl transferase

(3.) $HOOC-CH_2CO-S-CoA + HS-ACP \rightarrow HOOC-CH_2CO-S-ACP + CoA-SH$

malonyl CoA-ACP transacylase

(4.) $HOOC-CH_2CO-S-ACP + CH_3CO-S-E_{cond} \rightarrow CO_2 + HS-E_{cond} + CH_3COCH_2CO-S-ACP$

condensing enzyme*

(5.) $CH_3COCH_2CO-S-ACP + NADPH + H^+ \rightarrow NADP^+ + CH_3CHOHCH_2CO-S-ACP$

β-ketoacyl-ACP reductase

(6.) $CH_3CHOHCH_2CO-S-ACP \rightarrow H_2O + CH_3CH=CHCO-S-ACP$

β-hydroxyacyl-ACP dehydrase

(7.) $CH_3CH=CHCO-S-ACP + NADPH + H^+ \rightarrow CH_3CH_2CH_2CO-S-ACP + NADP^+$

enoyl-ACP reductase

Fig. 1-2 The reaction sequence catalyzed by the fatty acid synthetase system of E. coli

Steps 4 to 7 are repeated, further malonyl-ACP being incorporated, until palmityl-ACP is synthesized and relaesed by the action of palmityl-ACP thioesterase (Majerus and Vagelos, 1967).

*β-ketoacyl-ACP synthetase

Unlike the multienzyme complex system, which has a specific requirement for NADPH, the *E. coli* system can utilize both NADH and NADPH for the reduction steps in fatty acid biosynthesis (Vagelos *et al.*, 1966; Weeks and Wakil, 1968).

Smirnov (1960) was the first to demonstrate the capacity of isolated spinach chloroplasts to incorporate [1-^{14}C] acetate into long chain fatty acids. He found that ATP, CoA and Mn^{++} were required and light was shown to stimulate several-fold the incorporation of acetate. These observations were confirmed and extended by other workers, all of whom suggested that the chloroplast was the major site of fatty acid biosynthesis in the leaf (Smirnov, 1962; Stumpf and James, 1963; Mudd and McManus, 1962). Yamada and Nakamura (1975) deduced from $^{3}H_2O$ incorporation studies that 83% of the fatty acid biosynthetic capacity of the spinach leaf resided in the chloroplast.

Mudd and McManus (1962) showed that, in broken chloroplast preparations, ATP, CoA, NADH and NADPH are essential components for fatty acid biosynthesis from acetate. They also noted that bicarbonate enhanced the incorporation of acetate. Using lettuce chloroplasts, Brooks and Stumpf (1965, 1966) found that the fatty acid synthetase was located in the stroma, thus indicating a soluble synthetase. Fractionation procedures demonstrated that the synthetase required a heat stable fraction (crude ACP) and that this could be replaced by *E. coli* ACP, as found earlier by Overath and Stumpf (1964) with a soluble synthetase from avocado mesocarp. The acyl carrier proteins isolated from avocado mesocarp and spinach chloroplasts were found to be very similar in amino acid composition to that of *E. coli* ACP (Simoni *et al.*, 1967; Simoni and Stumpf, 1969). This finding together with the observed stimulation of chloroplast fatty acid biosynthesis by ACP and the soluble nature of the chloroplast synthetase, has led to the conclusion that the plant synthetase is of the *E. coli* type (Stumpf, 1975, 1976).

1.2.(b) The Source of Acetyl-Coenzyme A in Plant Leaves

The primary source of carbon for fatty acid synthesis in plants is from CO_2-fixation, but early attempts to obtain significant fatty acid synthesis from CO_2 (as HCO_3^-) by isolated chloroplasts were unsuccessful and lead to the proposal

that chloroplasts did not possess a self-contained pathway for the synthesis of acetyl-CoA from CO_2 (Everson and Gibbs, 1967; Sherratt and Givan, 1973).

Yamada and Nakamura (1975) found that label from ^3H_2O was incorporated into the fatty acid chain in the presence of unlabelled 3-phosphoglycerate (3-PGA), phosphoenolpyruvate (PEP) and pyruvate. This was indirect evidence that a pathway for the utilization of 3-PGA for the biosynthesis of fatty acids exists in isolated spinach chloroplasts. They also showed that PEP or pyruvate carboxylation, the citrate lyase reaction and the malate synthetase reaction were not involved in the formation of acetyl-CoA used for fatty acid biosynthesis. Yamada and Nakamura (1975) proposed that 3-PGA was converted to acetyl-CoA in the chloroplast by the following pathway:

3-PGA ⟶ PEP ⟶ pyruvate ⟶ acetyl-CoA, before utilization for fatty acid biosynthesis.

Murphy and Leech (1977, 1978) demonstrated conclusively that isolated spinach chloroplasts could incorporate $H^{14}CO_3^-$ into fatty acids. They attributed their success to using chloroplasts capable of high rates of photosynthetic carbon reduction comparable to those found in the intact leaf. They deduced from the incorporation of [U-^{14}C]phosphoglycerate, [2-^{14}C]pyruvate, [^{14}C]bicarbonate and [1-^{14}C]acetate as well as from isotope competition experiments that the pathway involved was that proposed by Yamada and Nakamura (1975).

The enzymes required for the pathway: phosphoglyceromutase (3-PGA ⟶ 2-PGA) and phosphopyruvate hydratase (enolase, 2-PGA ⟶ PEP) have been found in chloroplasts from Vica faba and Zea mays (Leese, 1972). Pyruvate kinase has been found in tobacco chloroplasts (Bird et al., 1973) and pyruvate dehydrogenase in spinach chloroplasts (Yamada and Nakamura, 1975; Murphy and Leech, 1977). The rates of [^{14}C]bicarbonate incorporation intolipids by isolated spinach chloroplasts, observed by Murphy and Leech (1978), were approximately 36% of the rates of $^{14}CO_2$ incorporation into lipids observed with intact leaves.

Most of the studies of fatty acid biosynthesis in chloroplasts and indeed in leaf, leaf sections, seeds and other

plant preparations have utilized acetate as the source of carbon (Hitchcock and Nichols, 1972; Stumpf, 1975; Harwood, 1975). Acetate is rapidly taken up by isolated chloroplasts (Jacobson and Stumpf, 1972) and converted to acetyl-CoA by acetyl-CoA synthetase which is located in the stroma (Roughan and Slack, 1977).

Acetyl-CoA carboxylase, which is associated with both the stroma and the lamellar membranes of spinach chloroplasts, converts acetyl-CoA to malonyl-CoA utilizing bicarbonate and ATP (Kannangara and Stumpf, 1972b, 1973). Kannangara and Stumpf (1972b) have shown that the biotin carboxyl carrier protein (BCCP) component of the enzyme is located in the lamellar membrane, whereas the carboxylase and transcarboxylase activities are associated with the stroma. Stumpf (1975) has suggested that the location of BCCP in the lamellar membrane ensures its proximity to the site of synthesis of ATP which is required for the formation of carboxy-biotin. Acetyl-CoA and malonyl-CoA are then converted to their respective ACP derivatives by transacylases before utilization for fatty acid biosynthesis (Fig. 1-2, p. 4).

1.2.(c) The Effect of Light on Fatty Acid Biosynthesis

The special feature of fatty acid biosynthesis in the chloroplast is the stimulation by light found by Smirnov (1960), Stumpf and James (1962, 1963), Mudd and McManus (1962) and Stumpf *et al*. (1967). The stimulatory effect of light was attributed to the production of ATP and NADPH from photosynthesis (Stumpf and James, 1962, 1963). Later studies by Stumpf *et al*. (1963) using inhibitors of photophosphorylation, such as *p*-chlorophenyldimethylurea and NH_4^+, led them to the conclusion that acetate incorporation into fatty acids by chloroplasts was dependent on non-cyclic photophosphorylation. Non-cyclic photophosphorylation, which results in ATP. NADPH and O_2 production, could not be replaced in fatty acid biosynthesis by cyclic photophosphorylation, which produces only ATP, However, the addition of ATP, NADPH and O_2 to chloroplasts in the dark did not promote acetate incorporation to the same level as observed in the light (Stumpf *et al*., 1967).

Nakamura and Yamada (1975b) reinvestigated the role of light in fatty acid biosynthesis and found that 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) an electron

transport inhibitor and carbonylcyanide 4-trifluoromethoxy-phenylhydrazone (FCCP) a photophosphorylation uncoupler, both inhibited fatty acid synthesis. However, they found that NH_4Cl and phlorizin, at concentrations sufficient to specifically inhibit ATP formation, only partially inhibited fatty acid synthesis. Since FCCP acts directly on the high energy intermediate (or state) involved in driving ATP synthesis while NH_4Cl and phlorizin prevent ATP synthesis without dissipating the high energy state (Gross and San Pietro, 1969), it would appear that fatty acid synthesis may be directly linked to the high energy intermediate (or state). Nakamura and Yamada (1975b) also found that the addition of N,N'-dicyclohexylcarbodiimide (DCCD), which has been found to stabilize the high energy intermediate (McCarthy and Racker, 1967), in the presence of NH_4Cl and phlorizin, restored fatty acid biosynthesis to full activity which supports the proposed linking of fatty acid synthesis to the high energy intermediate.

1.2.(d) Elongation of Palmitate by Chloroplasts

The final products of acetate incorporation by isolated chloroplasts are palmitate, oleate and some stearate with only small amounts of linoleate and linolenate (James, 1962). However, early studies by Hawke and Stumpf (1965a) using barley seedlings, Macey and Stumpf (1968) with pea seedlings, arsenite inhibition of C_{18} fatty acid synthesis in germinating pea seeds and avocado mesocarp (Harwood and Stumpf, 1971, 1972) have established that palmitate was the end-product of *de novo* fatty acid biosynthesis. Kannangara and Stumpf (1972c) found that in the presence of arsenite there was substantial inhibition of C_{18} fatty acid synthesis coupled with a 3-fold rise in palmitate synthesized by isolated chloroplasts further indicating that palmitate was the end-product of *de novo* synthesis.

Although the presence of an elongation system has been recognized in avocado mesocarp (Harwood and Stumpf, 1972) and safflower seed preparations (Harwood and Stumpf, 1971), few investigations on the capacity of chloroplasts to elongate acyl-ACP derivatives have been reported (Nagai and Bloch, 1967; Jaworski *et al.*, 1974). Using spinach chloroplast preparations, Nagai and Bloch (1967) found that octanoyl-ACP, decanoyl-ACP and lauryl-ACP were elongated in the presence of malonyl-CoA

to C_{16} and C_{18} fatty acids. Similar results were obtained with a preparation from Euglena gracilis, in addition palmitoyl-ACP was elongated to C_{18} fatty acids (Nagai and Bloch, 1967). However, it was not until the studies of Jaworski et al. (1974) that the capacity of chloroplasts to elongate palmitoyl-ACP was demonstrated. Jaworski et al. (1974) found that the elongation system was located in the stroma of spinach chloroplasts, indicating a soluble enzyme system like that found in safflower seeds and avocado mesocarp (Harwood and Stumpf, 1971, 1972).

The location of both fatty acid biosynthesis and the elongation system in the chloroplast stroma (Brooks and Stumpf, 1965; Jaworski et al., 1974), the utilization of ACP derivatives by both systems, the presence of thioesterases capable of hydrolysing ACP derivatives (Shine et al., 1976a) and the inability of plants to synthesize ACP derivatives from long chain fatty acids or their CoA esters (Jaworski et al., 1974) all indicate that there must be some close association between the two enzyme systems.

1.3 The Biosynthesis of Unsaturated Fatty Acids

Oleic acid is the only unsaturated fatty acid synthesized to any extent from acetate by isolated chloroplasts from maize (Hawke et al., 1974a), spinach (Stumpf and James, 1963; Kannangara and Stumpf, 1972a; Nakamura and Yamada, 1975a; Murphy and Leech, 1977, 1978) and lettuce (Brooks and Stumpf, 1966). Very little ^{14}C is incorporated from [1-^{14}C]acetate into polyunsaturated fatty acids which are the major fatty acids present in chloroplast lipids (Sastry, 1974).

Attempts to improve the biosynthesis of polyunsaturated fatty acids by isolated chloroplasts have been made using chloroplasts isolated from the developing maize leaf (Hawke et al., 1974a), greening etiolated pea leaf tissue (Panther and Boardman, 1974) and immature spinach leaf tissue (Kannangara and Stumpf, 1972a; Kannangara et al., 1973a). Spinach chloroplasts which had high photosynthetic O_2-evolving capacities coupled with high rates of acetate incorporation into fatty acids (Roughan et al., 1976) or high rates of photosynthetic carbon reduction (Murphy and Leech, 1977, 1978) failed to give any improvement of polyunsaturated fatty acid biosynthesis.

The inability of isolated chloroplasts to synthesize polyunsaturated fatty acids at the high rates that would be

expected from the quantities found in chloroplast lipids has been described as 'an outstanding problem of lipid biochemistry in higher plants' (Hawke et al., 1974a). This consistent inability to synthesize polyunsaturated fatty acids has encouraged workers to examine the possible involvement of other cellular organelles, particularly the microsomes, at some stage of the biosynthetic process (Hawke et al., 1974a; Tremolieres and Mazliak, 1974; Slack and Roughan, 1975; Slack et al., 1976).

1.3.(a) Biosynthesis of Oleic Acid

Nagai and Bloch (1965, 1966, 1968) demonstrated the presence of a soluble stearyl acyl carrier protein desaturase system from photoauxatrophic Euglena gracilis. They were able to show that the desaturase consisted of three components: a ferredoxin: $NADP^+$ reductase, the desaturase and a ferredoxin. All three components, together with O_2 and NADPH, were required for the desaturation of stearyl-ACP. However, the purified reconstituted enzyme system could desaturate stearyl-CoA as rapidly as stearyl-ACP, thus the nature of the actual substrate for the desaturation of stearate to oleate was not entirely clear (Nagai and Bloch, 1968). Chloroplast preparations from spinach leaves could also desaturate stearyl-ACP to oleic acid (Nagai and Bloch, 1968).

Jacobson et al. (1974) demonstrated that the stearyl ACP desaturase was located in the stroma of spinach chloroplasts. The desaturase was specific for stearyl-ACP and, as found by Nagai and Bloch (1968), required O_2, ferredoxin and an electron donor. Various electron donors could be used by the chloroplast system: NADPH and ferredoxin-NADP reductase or illuminated photosystem 1 in the presence of ascorbate and dichlorophenol-indophenol (Jacobson et al., 1974). The desaturase preparation was more active when prepared from chloroplasts obtained from immature spinach leaf than from mature leaves. (Jacobson et al., 1974).

Stumpf and co-workers have found stearyl-ACP desaturase not only in spinach chloroplasts (Jacobson et. al., 1974) but also in soluble extracts from avocado mesocarp (Jacobson et al., 1974), developing safflower seeds (Jaworski and Stumpf, 1974a) and developing and germinating soybean cotyledons (Stumpf and Porra, 1976).

Stumpf and co-workers used enzymatically prepared stearyl-ACP (Jacobson et al., 1974; Jaworski and Stumpf, 1974a; Stumpf and Porra, 1976) which gave higher rates of desaturation than were observed using chemically prepared stearyl-ACP (Jaworski and Stumpf, 1974a; Nagai and Bloch, 1965, 1966, 1968). Though stearyl-ACP was the substrate for the desaturase, free oleic acid rather than oleoyl-ACP was the product (Jacobson et al., 1974). This was resolved by Shine et al. (1976a) to be a result of the action of acyl-ACP thioesterases which rapidly hydrolyse oleoyl-ACP to free oleic acid.

1.3.(b) The biosynthesis of Linoleic and Linolenic Acids

Initial studies on the biosynthesis of linoleic and linolenic acids were conducted by Harris, James and co-workers in the early 1960's using a variety of leaf tissues and Chlorella vulgaris cells (Harris and James, 1965a; Harris et al., 1965). They demonstrated that [1-^{14}C]oleic acid was desaturated to linoleic and linolenic acids by intact or chopped leaves from castor bean, lettuce and whole cells of Chlorella vulgaris.

Harris and James (1965a) found that oleoyl-CoA was a better substrate for desaturation, but oleoyl-CoA could be replaced by free oleic acid in the presence of CoA, ATP and Mg^{++}. As observed with the desaturation of stearate to oleate the desaturation of oleoyl-CoA required NADPH or NADH and O_2 suggesting a similar desaturation mechanism (Harris and James, 1965a). In later studies with Anabaena variabilis, [1-^{14}C]oleic and [1-^{14}C]stearic acids were desaturated to linoleic and linolenic acids without any apparent degradation and resynthesis of the substrate (Harris and James, 1965b). However, one feature of these studies was the very long incubation time (36-72h) required for any appreciable synthesis of linoleic and linolenic acids (Harris and James, 1965a, 1965b).

From the labelling pattern it was suggested that oleic acid was converted to linoleic and linolenic acids by sequential desaturation (Harris and James, 1965a, 1965b; Harris et al., 1965). This was consistent with the earlier studies of James (1963), who interpreted from acetate incorporation studies with Ricinus communis leaves that oleic acid was sequentially

desaturated to linoleic and linolenic acids.

A considerable amount of work has been carried out since these early studies on the mechanism of polyunsaturated fatty acid biosynthesis. Some of this later work supports the proposed pathway of sequential desaturation of stearate to linolenate via oleoyl and linoleoyl intermediates. However, an alternative proposal has been put forward by Stumpf and co-workers suggesting that desaturation occurs at the C_{12} stage and the polyunsaturated 12:3 precursor is then elongated to 16:3 and 18:3 (Kannangara et al., 1973b).

Cherif et al. (1975) using microdroplets of [1-^{14}C]-acetate, [1-^{14}C]oleate, [1-^{14}C]linoleate and [U-^{14}C] linolenate (as ammonium salts) on intact leaves, seeds, flowers, seedlings, and roots from bryophytes, gymnosperms and algae, concluded from their results that linoleic and linolenic acids were synthesized by sequential desaturation. Tremolieres and co-workers (Tremolieres, 1972; Cherif et al., 1975) found that tissue undergoing greening, developing pea leaves and young leaves of higher plants were more active in the desaturation of C_{18}-fatty acids than mature tissue. Further support for the sequential desaturation of stearate to linolenate, via oleate and linoleate, has come from $^{14}CO_2$ and [1-^{14}C]acetate feeding experiments with a wide variety of plants: 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 and Heinz, 1977). Siebertz and Heinz (1977) deduced from the labelling of C_{16} fatty acids that hexadeca-trienoic acid (16:3) was also synthesized by sequential desaturation of palmitic acid (via 16:1 and 16:2).

Kannangara and Stumpf (1972a) demonstrated that chloroplasts isolated from young spinach leaves were much more active in synthesizing linolenic acid from [1-^{14}C]-acetate than chloroplasts isolated from mature spinach. The proportions of linoleate and linolenate synthesized by chloroplasts from immature tissues were different from the endogenous proportions of these fatty acids. Kannangara and Stumpf (1972a) found that the synthesis of linoleate was inhibited by CN^- which did not affect the synthesis of oleate

and linolenate and proposed that linolenate was synthesized by a different pathway from the previously proposed sequential desaturation route (James, 1963).

Jacobson et al. (1973a, 1973b) found, using disrupted chloroplasts and the stroma fraction prepared from spinach chloroplasts, that hexadecatrienoic acid could be elongated to linolenic acid. The elongation system in these preparations could use either acetyl-CoA or malonyl-CoA as the donor of the 2-carbon unit. The addition of the 2-carbon unit to the carboxyl end of hexadecatrienoic acid (16:3) was confirmed by reductive ozonolysis of the [^{14}C]linolenic acid which gave on g.l.c. a single radioactive peak coincidental with methyl azelate semialdehyde, thus establishing that the [^{14}C]label was in the portion of the molecule between the carboxyl end and the first double bond (carbons 1-9) (Jacobson et al., 1973a). After reduction of the [^{14}C]linolenic acid to [^{14}C]stearic acid, chemical α-oxidation showed that the [^{14}C]label was located in position 2 of the molecule as expected from the utilization of of [2-^{14}C]acetate (Jacobson et al., 1973a). Kannangara et al. (1973b) found this pathway to be operative in leaf slices from spinach and barley, whole cells of Chlorella pyrenoidosa and Candida bogoriensis. Using spinach leaf slices, Kannangara et al. (1973b) found that [^{14}C]labelled 8:0, 10:0 and 12:0 were utilized for linolenic acid synthesis, but not 14:0 or 18:0 and proposed that dodecatrienoic acid (12:3) was the earlist permissable trienoic acid which is then elongated to linolenic acid.

The sequential desaturation of dodecanoic acid to dodecatrienoic acid has yet to be demonstrated, though the first step has been shown to occur in the cytosol of spinach leaf and avocado mesocarp (Sodja and Stumpf, 1976). However, the synthesis of 16:3 by the elongation of 12:3 via 14:3 is inconsistent with the findings of Siebertz and Heinz (1977) who deduced from their data that 16:3 was synthesized by sequential desaturation of palmitate after incorporation into MGDG. Linseed seeds, unlike many other oil seeds, contains very high levels of 18:3 (>70%) (Oulaghan and Wills, 1974). However, fatty acid analysis at various stages of seed development failed to detect any 12:3, 14:3 or 16:3 acids and from this Oulaghan and Wills (1976) proposed that the desaturation of

elongation pathway did not operate in linseed. Slack and Roughan (1975) carried out degradation studies on oleate, which is actively desaturated after incorporation into phosphatidylcholine, the proposed donor of polyunsaturated fatty acids (Roughan, 1970, 1975) and the linolenate of MGDG. They found that the distribution of label in linolenate and oleate were identical and consistent with the sequential desaturation of oleate to linolenate.

Though there is considerable evidence for the sequential desaturation of stearate to linolenate via oleate and linoleate, Leech and Murphy (1976) have suggested that until reductive ozonolysis has been carried out on the linolenate synthesized, one can not conclusively distinguish between the two alternative pathways.

1.3.(c) Substrates for Desaturation of Oleate and Linoleate

A further problem of oleate and linoleate desaturation is whether fatty acids are desaturated as the free acid, the CoA or ACP derivative, or as the acyl moiety of lipids such as phosphatidylcholine (PC) and monogalactosyl-diglyceride (MGDG).

As mentioned earlier, Harris and James (1965a) found that oleoyl-CoA was the preferred substrate for desaturation to linoleate and linolenate by whole cell homogenates and subcellular preparations of Chorella vulgaris. Vijay and Stumpf (1971, 1972), using a microsomal preparation from developing safflower seeds, found an oleoyl-desaturase which was specific for oleoyl-CoA and was inactive with oleoyl-ACP. Although their microsomal preparation contained a very active acyltransferase which transfered oleoyl and linoleoyl groups to position 2 of PC, it was concluded that the oxygen ester did not serve as a substrate for desaturation. Support for this has come from the work of 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, both these systems could desaturate oleoyl-CoA to linoleoyl-CoA before subsequent incorporation into phosphatidylcholine.

Harris et al. (1967) showed that isolated chloroplasts from Chlorella vulgaris could desaturate [1-^{14}C]oleoyl-CoA.

High levels of ^{14}C from [1-^{14}C]oleoyl-CoA were found in PC, it was concluded that PC may be involved in the desaturation reaction (Harris *et al.*, 1967). Nichols *et al.* (1967) and Nichols (1968) deduced from their labelling studies, also with *Chlorella*, that both phospholipids and glycolipids could be involved in fatty acid desaturation.

Gurr and co-workers, using *Chlorella vulgaris* preparations, established that oleic acid (in the presence of ATP and CoA) and oleoyl-CoA were rapidly incorporated into PC before desaturation to linoleate which remained esterified to PC (Gurr *et al.*, 1969; Gurr and Braun, 1971; Gurr, 1971). These results indicated that oleate desaturation was tightly coupled with PC metabolism, but did not rule out the possibility that the acyl moiety could be transfered to the enzyme for desaturation, followed by reacylation to the PC carrier molecule (Gurr *et al.*, 1969).

Although direct desaturation of fatty acids of PC has been demonstrated in bacterial and mammalian systems (Talamo *et al.*, 1973; Pugh and Kates, 1973, 1975, 1977) it has yet to be demonstrated in plant preparations. Kates and co-workers (Kates and Paradis, 1973; Pugh and Kates, 1973, 1975) found that oleate could be desaturated in *Candida lipolytica*, either as the CoA ester or as PC, indicating that two enzyme systems were operative. Pugh and Kates (1977) extended these observations to rat liver microsomes and found that eicosatrienoyl-PC was directly desaturated to give arachidonoyl-PC. No deacylation or reacylation of the acyl moieties was evident in the *Candida* or the rat liver microsomal systems studied (Pugh and Kates, 1973, 1975, 1977).

In higher plants, Roughan (1970, 1975) found that PC was rapidly labelled from [1-^{14}C]acetate and had the highest specific activity at short time intervals. Oleic acid was rapidly labelled and was incorporated into PC, followed by desaturation to linoleate and linolenate. The low levels of linolenate in PC and the rising levels of both linoleate and linolenate in other lipids, particularly MGDG, was interpreted to indicate that PC was not only the site of oleate desaturation, but a donor of polyunsaturated fatty acids for the synthesis of other polar lipids (Roughan, 1970, 1975).

Slack and Roughan (1975) reported similar results from $^{14}CO_2$ and [1-^{14}C]acetate feeding experiments with developing maize leaf and found that the labelled phosphatidylcholine was associated with the microsomes. After the transfer of the maize leaf from labelled acetate to unlabelled acetate, there was a decrease in labelled oleate and linoleate of PC and a concomitant increase in the amount of radioactivity in linoleate and linolenate of MGDG (Slack and Roughan, 1975). During longterm experiments with spinach leaves the radioactivity from the oleoyl moiety of microsomal PC was lost, while radioactivity accumulated in the linolenate of MGDG of the chloroplast (Slack and Roughan, 1975). The utilization of PC as the substrate for oleate desaturation was further supported by Slack et al. (1976) using microsomal preparations from pea seedlings and immature maize leaf. Oleate from oleoyl-CoA was rapidly incorporated into PC followed by desaturation to linoleate and linolenate (Slack et al., 1976).

Williams et al. (1976) supplied $^{14}CO_2$ to Vicia faba leaf discs, and concluded that the transfer of polyunsaturated acyl groups to MGDG probably occurred as diglycerides derived from PC. Pulse-chase experiments, using [2-^{3}H]glycerol and [1-^{14}C]acetate (Slack et al., 1977), demonstrated that similar amounts of ^{14}C and ^{3}H label were lost from PC (^{14}C mainly as dilinoleate) and were accompanied by an accumulation of ^{14}C and ^{3}H label in MGDG (^{14}C mainly as dilinolenate) indicating that diglycerides derived from PC were utilized for MGDG synthesis.

Co-operation between the chloroplasts and microsomes for the synthesis of polyunsaturated fatty acids has been supported by the investigations of Tremolieres and Mazliak (1974) and Hawke et al. (1974a). Tremolieres and Mazliak (1974), using developing pea leaves and subcellular preparations, found evidence for the desaturation of oleate to linoleate in the microsomes, but the desaturation of linoleate to linolenate was associated with the chloroplast fraction. Slack et al. (1977) observed that linoleate was lost from the microsomal PC, but linolenate appeared in MGDG, suggesting that desaturation of linoleate may occur following transfer from PC. A more direct demonstration

of the co-operation between chloroplasts and other cellular organelles has come from the work of Hawke et al. (1974a) using isolated maize chloroplasts and crude mitochondrial and microsomal preparations. The addition of the mitochondrial and microsomal preparations, together or separately, stimulated the synthesis of polyunsaturated fatty acids, although the levels obtained were still well below that expected from the endogenous levels of these fatty acids (Hawke et al., 1974a).

The possibility that lipids other than PC are involved in fatty acid desaturation was proposed by Nichols and co-workers following [2-^{14}C]acetate studies with Chlorella vulgaris and other algae (Nichols et al., 1967; Nichols, 1968; Nichols and Moorhouse, 1969; Appleby et al., 1971). Their findings were consistent with those of Gurr et al. (1969) that PC was a substrate for desaturation of fatty acids, but they deduced from their results that MGDG and other lipids also served as substrates for desaturation. Nichols and Moorhouse (1969) presented evidence for the desaturation of fatty acids of monogalactosyldiglycerides in Chlorella vulgaris following de novo synthesis of the monogalactosyldiglycerides. It would appear that in Chlorella parallel desaturation pathways, one using PC and the other using MGDG as substrates, may be present (Appleby et al., 1971). Appleby et al. (1971) found that Anabaena variabilis, a blue-green alga which lacked PC, utilized MGDG as the substrate for the desaturation of oleate to linolenate.

Recently, evidence that both PC and MGDG may serve as substrates for desaturation has been obtained for higher plants (Heinz and Harwood, 1977; Siebertz and Heinz, 1977). Heinz and Harwood (1977) following the incorporation of [1-^{14}C]acetate, $^{14}CO_2$ and [^{35}S]sulphate into the glycolipids of Vicia faba leaves interpreted their data to indicate that SQDG (sulphoquinovosyldiglyceride), MGDG and PC could act as substrates for desaturation. However, they found that MGDG contained the highest specific radioactivity of linolenate with smaller levels in DGDG and SQDG. After the same incubation times linolenate was never found in more than trace amounts in PC. Heinz and Harwood (1977) interpreted this to suggest that PC was not involved in the synthesis of linolenate in Vicia faba, incontrast to the work of

Williams *et al.* (1976) and Roughan and co-workers (Roughan, 1970, 1975; Slack and Roughan, 1975). Similar results were found by Siebertz and Heinz (1977) from $^{14}CO_2$ feeding experiments with young leaves from *Anthriscus*, *Spinacia* and *Chenopodium*. Siebertz and Heinz (1977) found that the labelling pattern of C_{16}-fatty acids of MGDG from *Anthriscus cerefolium* and *Spinacia oleracea* were consistent with the sequential desaturation of palmitate to hexadecatrienoate. A similar labelling pattern was also observed for the desaturation of C_{18}-fatty acids of MGDG.

It is apparent that several lipids may be involved in the desaturation of fatty acids (as well as CoA and ACP derivatives) and it has been proposed that each cellular organelle may have its own particular substrate for the desaturation of fatty acids (Appleby *et al.*, 1971; Heinz and Harwood, 1977; Siebertz and Heinz, 1977). According to this proposal a PC mediated desaturation would occur in the microsomes and a MGDG mediated desaturation would occur in the chloroplasts. However, the exchange of fatty acids, either as diglycerides derived from PC or as PC, may occur between subcellular fractions, although this has not been demonstrated with chloroplasts *in vitro* (Mazliak *et al.*, 1975).

1.4 Diglyceride and Monogalactosyldiglyceride Biosynthesis

1.4.1 Diglyceride Biosynthesis

Diglycerides can be synthesized either *de novo* from the acylation of *sn*-glycerol-3-phosphate followed by the action of phosphatidate phosphatase or from the hydrolysis of other lipids such as phospholipids and galactolipids.

Cheniae (1965) and Sastry and Kates (1966) demonstrated the incorporation of [^{32}P] *sn*-glycerol-3-phosphate into phosphatidic acid (PA), lysophosphatidic acid (lyso-PA), monoglycerides (MG) and diglycerides (DG) by subcellular preparations from spinach leaves. The microsomal fraction was the most active preparation with only a low activity present in the chloroplast fraction (Cheniae, 1965; Sastry and Kates, 1966). Douce and Guillot-Salamon (1970) found that isolated spinach chloroplasts, maize chloroplasts and maize etioplasts could incorporate [1,3-^{14}C] *sn*-glycerol-3-phosphate into lyso-PA, PA, and DG as well as into PC and PG (phosphatidylglycerol). Further studies by Joyard and Douce (1977)

demonstrated the presence of two acylating enzymes in spinach chloroplasts: a soluble (or loosely bound) acyl-CoA:sn-glycerol-3-phosphate acyltransferase and an envelope membrane bound acyl-CoA:monoacyl-sn-glycerol-3-phosphate acyltransferase, both of which are required for the synthesis of phosphatidic acid. The chloroplast envelope membrane was also found to contain the phosphatidic acid phosphatase inferred from the studies of Douce and Guillot-Salamon (1970) to be present in the chloroplast (Joyard and Douce, 1977).

The microsomal and chloroplastic acylases utilized acyl-CoAs for the acylation of sn-glycerol-3-phosphate (Cheniae, 1965; Sastry and Kates, 1966; Joyard and Douce, 1977). Bertrams and Heinz (1976) observed that the activity of the acyl-CoA:sn-glycerol-3-phosphate acyltransferase varied with the age of the tissue. In young spinach and pea leaves the maximal activity was associated with the soluble proteins from the chloroplast, whereas in older tissue, the highest activity was associated with the microsomes (Bertrams and Heinz, 1976). The utilization of acyl-CoAs is consistent with the location of acyl-CoA synthetases in the chloroplast envelope (Joyard and Douce, 1977; Roughan and Slack, 1977).

Renkonen and Bloch (1969) using a cell-free extract from green Euglena gracilis found that the addition of sn-glycerol-3-phosphate stimulated the incorporation of acyl-ACPs as well as acyl-CoAs into lipids. Shine et al. (1976b) found that microsomes from avocado mesocarp could transfer acyl moieties from CoA derivatives to position 2 of lyso-phospholipids, but with little transfer of acyl moieties from acyl-ACPs. However, Shine et al. (1976b) found that acyl-ACPs were utilized for triglyceride biosynthesis by avocado mesocarp microsomes, as were the CoA derivatives. A granal preparation from spinach chloroplasts could incorporate acyl moieties from acyl-CoAs, but not from acyl-ACPs, into polar lipids (Shine et al., 1976b). It would appear that in higher plants the chloroplasts utilized acyl-CoAs, but not acyl-ACPs, for the acylation of sn-glycerol-3-phosphate. Shine et al. (1976a) have proposed a pathway in which acyl-ACPs are converted to their acyl-CoAs by the action of thioesterases and thiokinases.

Isolated chloroplasts from maize (Hawke et al., 1974a) and spinach (Kannangara and Stumpf, 1972a; Roughan et al., 1976;

Murphy and Leech, 1977, 1978) can incorporate newly synthesized fatty acids into diglycerides, but a large proportion of the fatty acids synthesized remained as free fatty acids. Kannangara and Stumpf (1972a) found that chloroplasts isolated from young spinach leaves incorporated more newly synthesized fatty acids into diglycerides than did chloroplasts isolated from mature tissue. The addition of sn-glycerol-3-phosphate and or Triton X-100 stimulated the incorporation of newly synthesized fatty acids into diglycerides by spinach chloroplasts (Roughan et al., 1976). However, the diglycerides synthesized by isolated chloroplasts, using [1-^{14}C]acetate or $^{14}CO_2$, were composed mainly of palmitate and oleate with low levels of more unsaturated fatty acids (Kannangara and Stumpf, 1972a; Hawke et al., 1974a; Roughan et al., 1976; Murphy and Leech, 1978). Joyard and Douce (1976a) reported that the endogenous diglycerides in the envelope of spinach chloroplasts were very rich in polyunsaturated fatty acids.

A further source of diglycerides is from the metabolism of other lipids, particularly phosphatidylcholine. This had been suggested by Williams et al.(1976) from $^{14}CO_2$ labelling studies with Vicia faba leaves in which PC was proposed to be the donor of polyunsaturated diglycerides for MGDG biosynthesis. Slack et al. (1977) using double labelling techniques, found evidence in developing maize leaf that PC acts as a donor of diglycerides to other lipids, principally MGDG. Slack et al. (1977) have suggested that the diglycerides could be liberated from PC by the combined action of phospholipase D to give phosphatidic acid (Quarles and Dawson, 1969) which is then converted to diglyceride by the action of phosphatidic acid phosphatase.

1.4.2 Monogalactosyldiglyceride Biosynthesis

Ferrari and Benson (1961) proposed from $^{14}CO_2$ labelling studies with Chlorella pyrenoidosa that MGDG was synthesized by the galactosylation of diglycerides and that MGDG was further galactosylated to DGDG. They also proposed that UDP-galactose was the most likely donor of the galactose moiety for the galactosylation of DG to MGDG and MGDG to DGDG. Neufield and Hall (1964) demonstrated the utilization of UDP-galactose for galactolipid biosynthesis by spinach

chloroplasts and showed that other galactose nucleotides could not replace the requirement for UDP-galactose. Neufield and Hall (1964) also found that UDP-glucose was utilized to a small extent for galactolipid synthesis and suggested that the UDP-glucose was first converted to UDP-galactose before incorporation. Ongun and Mudd (1968) found that the galactosylation activity was greater for chloroplasts isolated from young spinach leaves than those prepared from mature tissue. Two enzymes are involved: one for the galactosylation of DG to MDGD and a second enzyme for the galactosylation of MGDG to DGDG. The existance of these two separate enzymes was first indicated by Ongun and Mudd (1968) who found that the second enzyme was easily extracted from their chloroplast preparations. Further evidence for two separate enzymes has been provided by the findings of Chang and Kulkarni (1970) using sub-chloroplast preparations from spinach, Mudd et al. (1969) and Bowden and Williams (1973) using acetone powder preparations from spinach and maize chloroplasts respectively. These workers found that the two enzymes differed in their pH optimum and responded differently to metal ions and inhibitors.

Initial studies of the sub-cellular distribution of the galactosylation enzymes had indicated their presence in the microsomes, mitochondria, chloroplasts and soluble preparations (Mudd et al., 1969; Chang and Kulkarni, 1970; Lin and Chang, 1971; van Hummel, 1974). This led to considerable confusion as to the site(s) of galactolipid synthesis. However, Douce (1974) clearly demonstrated that the chloroplast envelope was the site of galactolipid biosynthesis and this was later confirmed by van Hummel et al. (1975). Their work emphasized the ease with which the envelope could be released from the chloroplast and suggested that it was envelope contamination which led to the apparent presence of the transferase activity in other cellular fractions. Joyard and Douce (1976b) and van Hummel et al. (1975) demonstrated that purified preparations of microsomes and mitochondria were incapable of synthesizing galactolipids from UDP-galactose.

The location of the galactosyltransferase in the envelope ensures its proximity to a large pool of diglycerides

(Joyard and Douce, 1976a, 1976c) and to the site of diglyceride biosynthesis (Joyard and Douce, 1977). The location of the enzyme would also enable the utilization of diglycerides donated from PC for the synthesis of MGDG (Williams et al., 1976; Slack et al., 1977).

1.4.2.1 Substrate Specificity of the Galactosyltransferase

Diglyceride specificity has been demonstrated by preparations from many species of micro-organisms which synthesize hexosyldiglycerides. For example, the Streptococcus faecalis enzyme is specific for unsaturated diglycerides (Pieringer, 1968) and the Pseudomonas diminuta enzyme for unsaturated and shorter chain fatty acids (Shaw and Pieringer, 1977).

Mudd et al. (1969) demonstrated, using acetone powders of spinach chloroplasts as an enzyme source, that polyunsaturated diglycerides were better acceptors of galactose from UDP-galactose than saturated diglycerides. Similarly, Bowden and Williams (1973), using acetone powders from maize chloroplasts, found that diolein was a better accepter of galactose from UDP-galactose than dipalmitin. However, the preference for unsaturated diglycerides was not found by Eccleshall and Hawke (1971) using a similar acetone preparation to that of Mudd et al. (1969).

The possible role of MGDG as a substrate for desaturation of fatty acids, in algae as proposed by Appleby et al. (1971) and Nichols and Moorhouse (1969) and its extension to higher plants by Heinz and co-workers (Heinz and Harwood, 1977; Siebertz and Heinz, 1977), would give rise to polyunsaturated MGDGs without the necessity for the galactosylation of polyunsaturated diglycerides. It was also apparent that the positional specificity of the newly synthesized fatty acids incorporated into MGDG found by Siebertz and Heinz (1977) for C_{16} and C_{18} fatty acids was consistent with the positional distribution of these fatty acids in the endogenous MGDGs (Auling et al., 1971).

Deacylation and reacylation of MGDG after de novo synthesis could also bring about the modification of the fatty acid composition which also would reduce the necessity for the galactosylation of polyunsaturated diglycerides.

The presence of galactolipases has been reported in runner bean leaves and chloroplasts (Sastry and Kates, 1964b; Helmsing, 1969), spinach leaves (Helmsing, 1967) and spinach chloroplasts (McCarthy and Jagendorf, 1965). However, only limited studies have been carried out on the reacylation capacity of leaf homogenates and chloroplast preparations (Safford et al., 1971; Bajwa and Sastry, 1972, 1973, 1975). Leaf homogenates of Spinacia oleracea (Safford et al., 1971), Tetragonia expansa (Bajwa and Sastry, 1972) and chloroplast preparations from Tetragonia expansa (Bajwa and Sastry, 1972, 1975) could incorporate fatty acids into MGDG in the presence of ATP and CoA. Bajwa and Sastry (1972, 1973, 1975) found that unsaturated fatty acids were incorporated at higher rates than saturated fatty acids and the highest rates were obtained with linolenic acid. Enzymatically prepared monogalactosyl-monoglycerides (MGMG) were acylated to MGDG by leaf homogenates (Safford et al., 1971; Bajwa and Sastry, 1972) and acetone powders of chloroplasts (Bajwa and Sastry, 1973) It is evident from these observations that the chloroplast has the capacity to modify the fatty acid composition of its MGDGs.

The final fatty acid composition of MGDG in the chloroplast would be determined by the type of diglycerides utilized for galactosylation and by subsequent modification, such as: desaturation and deacylation-reacylation, after de novo biosynthesis. The relative importance of these alternatives is not known.

Chapter 2

AIMS OF THE PRESENT STUDY

This study is an investigation of the biosynthesis of polyunsaturated fatty acids and monogalactosyldiglycerides (MGDG) by isolated chloroplasts. In particular the work has been directed towards two outstanding problems associated with lipid biosynthesis in photosynthetic tissues: the low incorporation of acetate into linoleate and linolenate by isolated chloroplasts and also the low incorporation of the newly synthesized fatty acids into MGDG.

The initial studies described in this thesis were carried out with the aim of improving [1-^{14}C]acetate incorporation into fatty acids and lipids by isolated chloroplasts from spinach, maize and sweetcorn. This involved an investigation of chloroplast isolation methods, the effect of cofactors and incubation conditions on acetate incorporation.

Several lines of investigation were used in attempts to improve the incorporation of acetate into polyunsaturated fatty acids by isolated spinach and maize chloroplasts. These included a study of the biosynthetic capacity of chloroplasts obtained from maize tissues at different stages of development. In view of the current uncertainty as to the site(s) of desaturation leading to the biosynthesis of the polyunsaturated fatty acid constituents of MGDG in photosynthetic tissues. (namely, the role of microsomal PC as a substrate for fatty acid desaturation followed by transfer to MGDG (Roughan, 1970, 1975; Slack and Roughan, 1975) and MGDG as a substrate for desaturation, following de novo synthesis, in the chloroplast (Nichols and Moorhouse, 1969; Appleby et al., 1971)) the role of other cellular organells was investigated using a non-chloroplastic cell fraction with chloroplasts in a reconstituted system. The role of MGDG as a substrate for the desaturation of fatty acids was investigated by selecting experimental conditions which varied the nature of the final acyl product synthesized from acetate, namely free fatty acids, diglycerides and monogalactosyldiglycerides. During the course of the latter study, the incorporation of newly synthesized fatty acids into MGDG was also investigated.

Chapter 3

MATERIALS AND METHODS

3.1 Materials

3.1.(a) Reagents

Chemicals used in the present study were obtained from the following sources: ATP, CoA, dithiothreitol (DTT), sn-glycerol-3-phosphate (sodium salt), uridine 5'-diphosphate D-galactose (UDP-galactose), cytidine 5'-diphosphate choline (CDP-choline), glucose 6-phosphate, bovine serum albumin-fraction V (BSA), sodium deoxycholate, Tris(hydroxymethyl)amino-methane (Tris), N-Tris(hydroxy methyl)methyl glycine (Tricine), glycerol kinase (E.C. 2.7.1.30, E. coli), pancreatic lipase Type II (E.C. 3.1.1.3, Hog), p-Terphenyl and 2,5-p-diphenyl-oxazole (PPO) were obtained from Sigma Chemical Co., St. Louis, USA. [1-^{14}C]acetic acid, sodium salt (58.1 mCi/mmol), n-[1-^{14}C]-hexadecane (0.781 Ci/g), n-[1,2(n)-^{3}H] hexadecane (1.8 Ci/g) and [1(3)(n)-^{3}H]glycerol (2.3Ci/mmol) from the Radiochemical Centre Ltd., Amersham, England.

Silica gel G and DG from Riedel-De-Haen AS Seelge-Hannover, Germany and MN300 cellulose from Macherey and Nagel, Duren, Germany.

1,4 bis[2-(5-phenyloxazolyl)]-benzene (POPOP) and sucrose from Koch-light Laboratories Ltd., England. Streptomycin sulphate from Glaxo Laboratories, N.Z. and Miracloth from Calbiochem, California, USA.

All other reagents were obtained from BDH Chemicals Ltd., Poole, England or May and Baker Ltd., Dagenham, England. All solvents were redistilled before use.

3.1.(b) Plant Materials

Maize (Zea mays var. Wisconsin 346) and sweetcorn (Zea mays var. Golden Cross Bantam) seeds were obtained from Arthur Yates and Co., Ltd., N.Z. The seeds were soaked in water at approximately 20°C overnight before sowing in trays of peat/pumice (1:4, v/v) potting mixture. Plants were grown for 8 days after sowing in a controlled environment at the Plant Physiology Division's Climate Laboratory, DSIR, Palmerston North, N.Z. Day/night temperatures, vapour pressure deficits and equivalent relative humidities were 25°C/20°C, 10/5 millibars and 68/78% respectively. Daylength was 12h and the photosynthetically active radiation of 400-700nm range was

170 watt per square metre. Half-strength formulation of Hoaglands' solution A (Hoagland and Arnon, 1938) modified by the use of chelated iron was used as a nutrient solution.

Field-grown spinach (Spinacia oleracea) was purchased locally.

3.2 Methods

3.2.1.(a) Preparation of E. coli Acyl Carrier Protein .

Acyl carrier protein from E. coli was partially purified by the method of Majerus et al. (1969). Frozen E. coli paste was suspended in 0.02M-triethanolamine-HCl buffer, pH7.5, containing 0.01M-β-mercaptoethanol (1l/kg of frozen cells) and thawed at 4^{o}C with stirring. Cells were then ruptured in a French pressure cell (twice through at 7-8,000 p.s.i.) which resulted in greater than 85% rupture of cells (Prestidge, 1978). Intact cells and cell membranes were removed by centrifugation at 30,000 X g in a Sorvall RC2-B centrifuge for 30min. The protein concentration was adjusted to 15mg/cm^3 by the addition of further buffer. Streptomycin sulphate (1g/g of protein) was added with constant stirring to precipitate nucleic acids which were then removed by centrifugation at 30,000 X g.

Sufficient 1.0M-triethanolamine-HCl buffer, pH7.5 and 1M-β-mercaptoethanol were added to the supernatant to give a final buffer concentration of 0.1M-triethanolamine-HCl buffer, pH7.5, containing 0.01M-β-mercaptoethanol. Solid ammonium sulphate was added to give 70% saturation and after standing for 15min at 4^{o}C the precipitated proteins were removed by centrifugation at 30,000 X g for 30min. The supernatant was adjusted to pH1.0 by the addition of 10M-HCl, allowed to stand overnight and the precipitated acyl carrier protein collected by centrifugation at 10,000 X g for 20min.

The partially purified acyl carrier protein was resuspended in a minimum volume of 0.05M-imidozole-HCl buffer, pH7.0, containing 0.01M-β-mercaptoethanol and centrifuged at 15,000 X g for 45min to remove insoluble protein.

The preparation contained 40μgACP/mg of protein as determined by the malonyl-S-pantethein-CO_2 exchange reaction (Majerus et al., 1969).

3.2.1.(b) Preparation of [1(3)-^{3}H]sn-glycerol-3-phosphate

[1(3)-^{3}H]sn-glygerol-3-phosphate was synthesized

enzymatically from [1(3)(n)-^{3}H]glycerol by phosphorylation with ATP in the presence of glycerol kinase (E.C. 2.7.1.30) according to the procedure of Chang and Kennedy (1967). The incubation medium consisted of 0.05M-Tris/HCl buffer, pH8.0, 20mM-ATP, 20mM-$MgCl_2$, 20mM-β-mercaptoethanol, BSA (1mg/cm^3), glycerol kinase (20units) and 1mCi-[1(3)(n)-^{3}H]glycerol (2.3Ci/mmol) in a final volume of 5cm^3. After incubation at 37^{o}C for 3h, the incubation medium was heated for 5min in a boiling water-bath and centrifuged at 1,000 X g for 5min to remove the precipitated protein.

The supernatant was layered on to a column (16x1.3cm) of Biorad-AS 1-X8 (200-400 mesh) ion-exchange resin (formate form). Unreacted glycerol was eluted with 80cm^3 of distilled water. Gradient elution of the [1(3)(n)-^{3}H] sn-glycerol-3-phosphate was begun with the gradient mixer vessels containing 250cm^3 of distilled water and 250cm^3 of 4M-HCOOH respectively. A single peak of radioactivity was obtained from the column between 285cm^3 and 360cm^3 of efluent collected. The 15cm^3 fractions were pooled and freeze-dried. The product was then redissolved in 20cm^3 of distilled water.

The purity of the product was assayed by t.l.c. on MN300 cellulose developed in ethyl acetate/glacial acetic acid/water (3:3:1, by vol.) according to the method of Lueking et al. (1973) as modified by Hippman and Heinz (1976). No radioactive glycerol was detected in the preparation when the t.l.c. plates were scaned or following autoradiography.

3.2.2 Isolation of Chloroplasts

3.2.2.(a) Method 1

Maize and sweetcorn plants were harvested by cutting immediately above the potting mix. The coleoptile and the first outer leaf were removed. Maize, sweetcorn and spinach leaf tissue was washed in cold water before use.

Chloroplasts were isolated according to the method of Leese et al. (1971). The tissue was suspended in the isolating medium consisting of 0.5M-sucrose, 0.2%-BSA, 1mM-$MgCl_2$ in 0.067M-phosphate buffer, pH8.0, at a ratio of 4cm^3 of medium per g of tissue. The tissue was homogenized using a Waring blendor at full speed for 3s, followed by mixing and a further 5s homogenization. The homogenate was filtered through 10 layers of cotton organdie and 10 layers of 25μm nylon bolting cloth.

The filtrate was centrifuged for 1min at 2,000 X g in a Sorvall RC3 centrifuge, fitted with an auto-brake, and the resulting pellet resuspended in a minimum volume of isolating medium. The resuspended chloroplasts were filtered through one layer of Miracloth and the chloroplast suspension layered onto $20cm^3$ of 0.6M-sucrose-0.067M-phosphate buffer, pH8.0, containing 1mM-$MgCl_2$ and 0.2%-BSA. The chloroplasts were centrifuged at 300 X g for 15min in a HL-8 rotor fitted with swing-out buckets in the Sorvall RC3 centrifuge. The purified chloroplasts were resuspended in the isolating medium and used immediately.

3.2.2.(b) Method 2

The plant tissue was treated and washed as described in Method 1. Chloroplasts were isolated according to the method used by Jacobson et al. (1973a). The isolation medium contained 0.6M-sorbitol, 60mM-$NaHCO_3$, 10mM-K_2HPO_4, 1mM-$MgCl_2$, 0.1%-sodium ascorbate and 0.1M-Tricine/NaOH buffer, pH7.9. A ratio of isolating medium to tissue of $4cm^3$ of medium per g of tissue was used as before. The tissue was homogenized using a Waring blendor at full speed for 30s. The homogenate was filtered through 4 layers of Miracloth and the filtrate centrifuged for 45s at 3,600 X g. The chloroplast pellet was resuspended in the isolating medium.

3.2.3 Preparation of the Non-chloroplastic Particulate Fraction from Leaf Homogenate

The non-chloroplastic particulate fraction was prepared according to Hawke et al. (1974a). The supernatant from the chloroplast isolation was centrifuged for 10min at 4,000 X g to remove broken chloroplast fragments. The resulting supernatant was centrifuged for 1h at 100,000 X g in a Beckman Model L Preparative Ultracentrifuge. The pellet was resuspended in half the volume of the chloroplast isolating medium used for the chloroplast resuspension. The particulate fraction was added to freshly prepared chloroplasts from the same batch of tissue in the experiments described.

3.2.4 Preparation of Maize Leaf Sections

Leaf sections were prepared according to the procedure of Leese et al. (1971). Pre-chilled maize leaf tissue, after the removal of the coleoptile and the first leaf, was cut into 5 sections. The four lower sections (A-D) were each of

2cm long while section E comprised the remaining distal tissue.

3.2.5 Microscopy of Chloroplasts

3.2.5.(a) Phase-contrast Microscopy

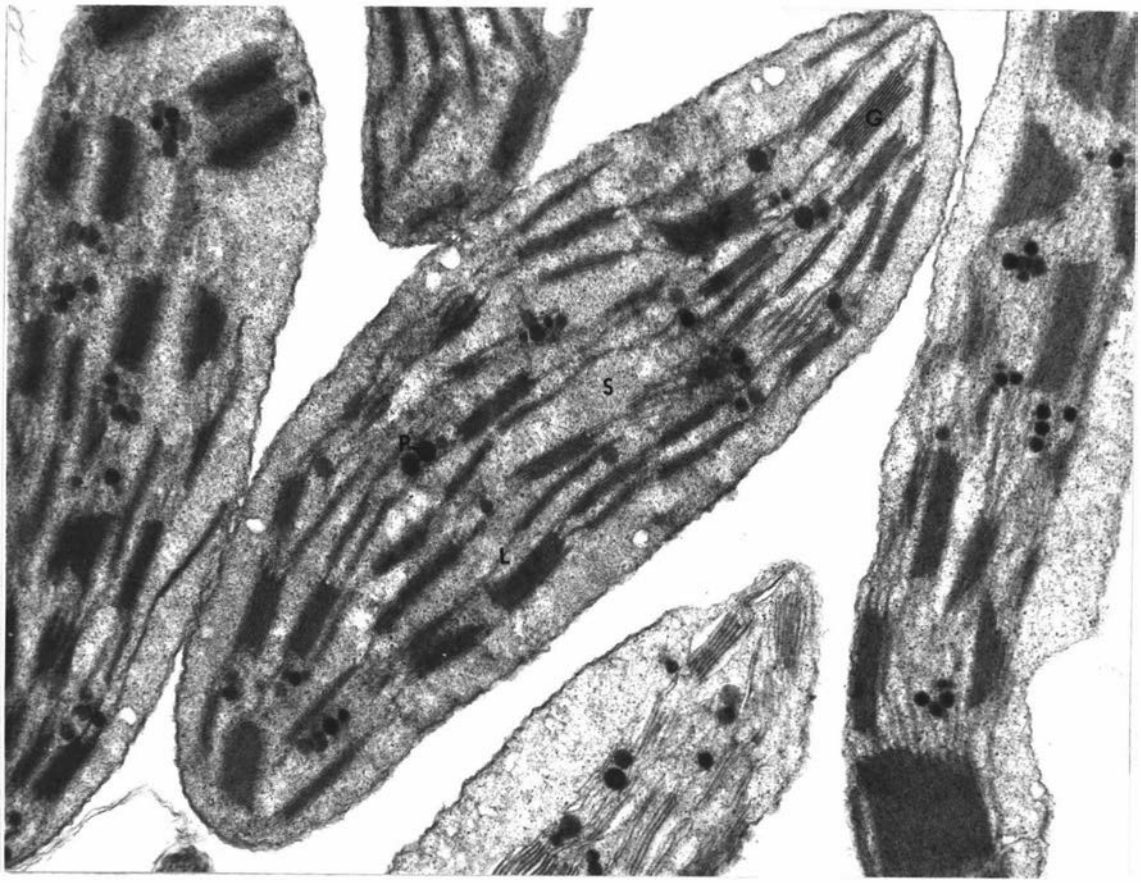
Phase-contrast microscopy was used routinely to assess the degree of intactness of the chloroplast preparations (Ridley and Leech, 1968). At 1,500 X magnification, over 80% of chloroplasts isolated from spinach and maize were intact (ie. Class 1 chloroplasts) while from sweetcorn, generally 50-60% of the chloroplasts were intact.

3.2.5.(b) Electron Microscopy

Isolated chloroplasts were fixed by the method of Karnovsky (1965) using formaldehyde and glutaraldehyde in 0.1M-phosphate buffer, pH7.2. The chloroplasts were centrifuged for 1min at 1,000 X g and left for 3h at 4°C before removing the fixative by washing the pellet three times with 0.1M-phosphate buffer, pH7.2, for 10,10 and 30min intervals. The chloroplast pellet was post-fixed with 1%-OsO_4 in 0.1M-phosphate buffer, pH7.2, for 3-5h at 4°C. The post-fixative was removed and the pellet washed as described above.

Dehydration was carried out by washing the pellet with aqueous ethanol in four steps of increasing ethanol concentration from 25% to 100%, followed by two treatments (10 to 15min) with propylene oxide. The fixed chloroplasts were embedded using a series of solutions of Fluka epoxy resin disolved in propylene oxide: 25%-resin for 1h, 50%-resin for 1h, 75%-resin overnight with a final 100%-resin for 7h.

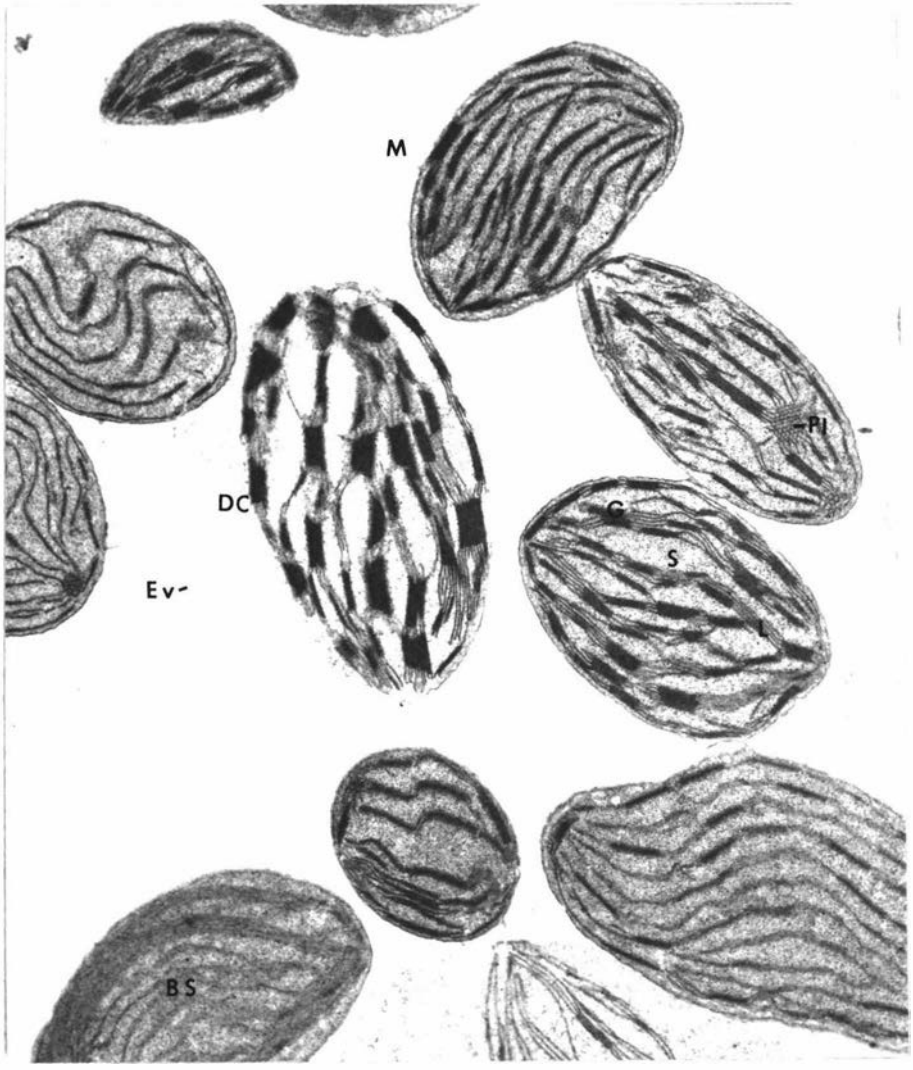
The embedded chloroplasts were then placed in gelatin capsules before curing at 60°C for 2 to 3 days. A LKB-1 microtome was used for thin-sectioning. Sections were placed on 400-mesh copper grids (unsupported). Sections were stained according to Venable and Coggeshall (1965) with uranylacetate followed by lead citrate. Sections were observed in a Philips EM-100 electron microscope and photographed (Plate 1 , p. 30; Plate 2, p. 31; Plate 3, p. 32



EXPLANATION OF PLATE 1

Electron micrograph of spinach (Spinacia oleracea) chloroplasts.

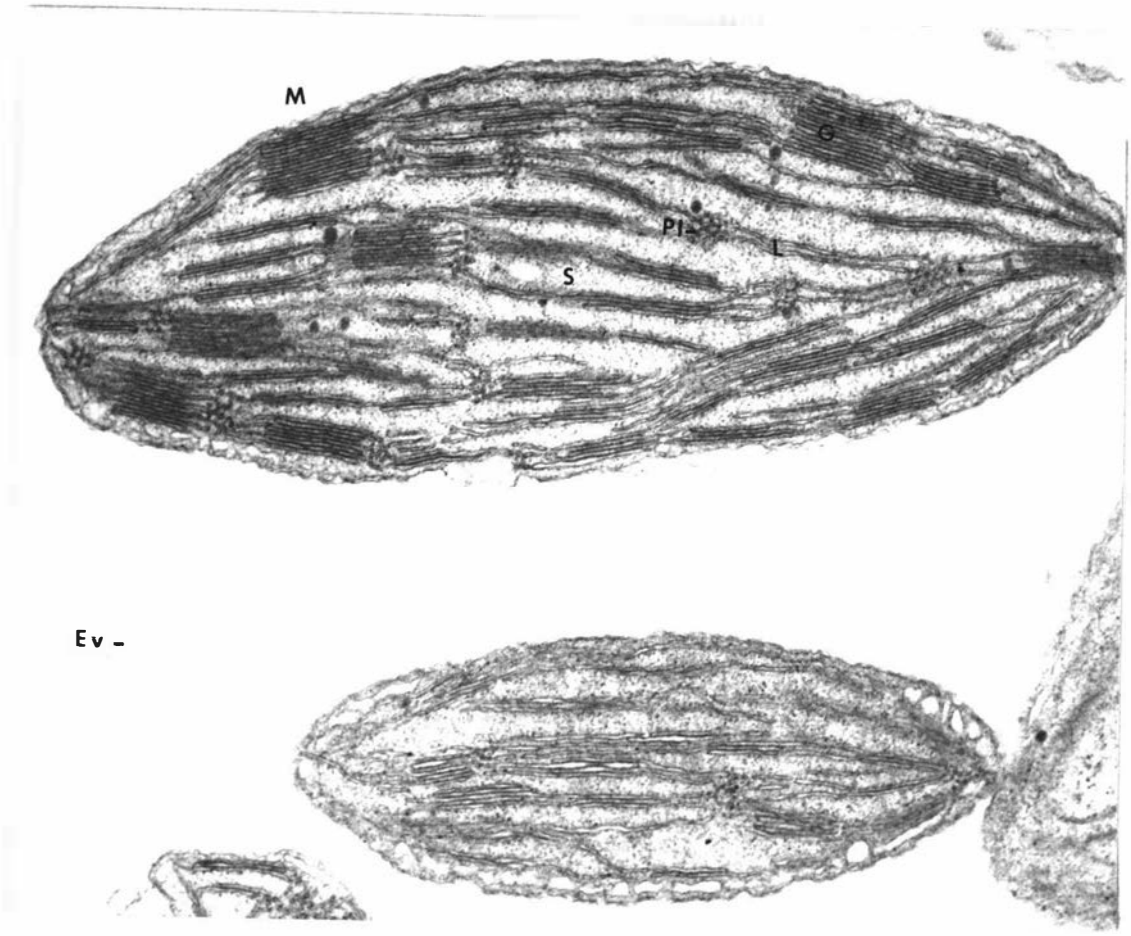
Visible within the chloroplasts are grana (G), stroma (S), stroma lamella (l) and plastoglobuli (P). Spinach chloroplasts were isolated by Method 1 and fixed using formaldehyde/glutaraldehyde. For further details see text. Magnification X 26,600.



EXPLANATION OF PLATE 2

Electron micrograph of maize (Zea mays var. Wisconsin 346) chloroplasts.

Visible are developing mesophyll chloroplasts (M) containing grana (G), stroma (S), stroma lamella (l) and prolamellar bodies (Pl). Also visible are agranal bundle sheath chloroplasts (BS), a disrupted mesophyll chloroplast (DC) and envelope fragments (Ev). Maize chloroplasts were isolated by Method 1 and fixed using formaldehyde/glutaraldehyde. For further details see text. Magnification X 18,000.



EXPLANATION OF PLATE 3

Electron micrograph of sweetcorn (Zea mays var. Golden Cross Bantam) chloroplasts.

Visible within the mesophyll chloroplast (M) are grana (G), stroma (S), stroma lamella (l) and prolamellar bodies (Pl). Also visible is a detached piece of envelope (Ev). Sweetcorn chloroplasts were isolated by Method 1 and fixed using formaldehyde/glutaraldehyde. For further details see text. Magnification X 26,600.

3.2.6 Incubation of Chloroplasts with Substrates

3.2.6.(a) Incubation Medium A

This medium was that used by Hawke et al. (1974a) and contained: 0.3M-sorbitol, 50mM-Tricine/NaOH buffer, pH7.8, 50mM-phosphate buffer, pH7.8, 2.5mM-DTT, 2.0mM-ATP, 0.5mM-CoA, 30mM-$NaHCO_3$, 1.0mM-$MgCl_2$, 0.2mM-NADPH, 0.2mM-NADH, and 0.1cm^3 of chloroplast suspension containing 0.5M-sucrose, 0.067M-phosphate buffer, pH8.0, 1mM-$MgCl_2$ and 0.2%-BSA, in a final volume of 1cm^3 after the addition of substrates and chloroplasts.

3.2.6.(b) Incubation Medium B

This medium was based on the medium used by Jacobson et al. (1973a). The medium contained: 1.0mM-DTT. 2.0mM-ATP, 0.2mM-CoA, 0.5mM-NADPH (replacing the NADPH generating system), 0.5mM-NADH, 20μg-E. coli ACP, and 0.6 cm^3 of chloroplast suspension containing: 0.6M-sorbitol, 0.1M-Tricine/NaOH buffer, pH7.9, 60mM-$NaHCO_3$, 10mM-K_2HPO_4, 1.0mM-$MgCl_2$, and 0.1%-sodium ascorbate in a final volume of 0.8cm^3 after the addition of substrates and chloroplasts. Unlike Medium 1, the sorbitol, buffer, $MgCl_2$, bicarbonate, phosphate and in addition to these some ascorbate were supplied by the chloroplast suspension. Their final concentrations were: 0.4M-sorbitol, 75mM-Tricine/NaOH buffer, pH7.9, 40mM-$NaHCO_3$, 0.75mM-$MgCl_2$, 7.5mM-K_2HPO_4 and 0.075%-sodium ascorbate.

3.2.6.(c) Incubation Conditions

Incubations were carried out in 15cm^3 stoppered tubes placed in a photosynthetic Warburg apparatus (B. Braun, Meisungen, Germany) fitted with sixteen X 40 watt tungsten lamps giving a light intensity of 20,000 to 25,000lx. The temperature of the water-bath was maintained at 20°C ($\pm$ 1.0°C) during the incubation. The reaction tubes were agitated at 78cycles/min.

3.3 Analytical Methods

3.3.1 Chlorophyll Determination

Chlorophyll was determined by the method of Arnon (1949). 100μl of chloroplast suspension in isolation buffer was added to 25cm^3 of 80%-aqueous acetone, mixed well and filtered. The absorbances were read at 645nm and 663nm (Hitachi Model 101 Spectrophotometer). Chlorophyll concentration was given by the following relationship:-

concentration (mg/cm^3) = 0.25 x (20.2 x A_{645} + 8.02 x A_{663})

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

3.3.2 Lipid Extraction and Thin-Layer Chromatography

Lipids were extracted, after the addition of $0.1cm^3$ of 6M-HCl to stop the reaction, according to the procedure of Hawke et al. (1974a). $6cm^3$ of $CHCl_3$/MeOH (2:1, v/v) were added to the reaction mixture and thoroughly dispersing the phases using a vortex mixer. A further $2cm^3$ of $CHCl_3$ and $3cm^3$ of water were added and the two phases thoroughly mixed. After the phases had separated the aqueous layer was removed. The chloroform layer was extracted successively with equal volumes of 1%-acetic acid, 0.1M-NaCl and distilled water (3 extractions). For each extraction the chloroform layer was dispersed in the extractant by means of a vortex mixer. Finally, the chloroform was removed under a stream of nitrogen and the lipids redissolved in $1cm^3$ of $CHCl_3$. Suitable aliquots were removed for radioactivity measurements to determine the incorporation of radioactivity into lipid.

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

Layers impregnated with $AgNO_3$ were prepared by slurrying the adsorbant with a 5%-$AgNO_3$ solution (wt/v). Two cm^3 of $AgNO_3$ solution was used for every g of adsorbant, giving a 10%-$AgNO_3$ in silica gel (wt/wt) (Nichols and Moorhouse, 1969).

MN300 cellulose plates were prepared by homogenizing 15g of MN300 cellulose with $80cm^3$ of distilled water for 30s prior to spreading to a thickness of 0.25mm. The layers were allowed to air dry at room temperature for 2 to 3 days.

The separation of neutral lipids and fatty acids on silica gel layers was achieved by developing with hexane-diethyl ether-glacial acetic acid (50:50:1, by vol.) (hexane solvent). The separation of galactolipids was achieved by

developing with toluene-ethyl acetate-95%-ethanol (2:1:1, by vol) (toluene solvent) and the separation of individual phospholipids by developing with $CHCl_3$-MeOH-glacial acetic acid-water (85:15:10:3, by vol.) (chloroform solvent). Plates (20cm by 20cm) were run in a modified two-dimensional manner (Fig. 3-1, p. 36). The lipid extract was added with markers as a spot at the lower right hand corner and developed in the hexane solvent. After drying the plate was sprayed with 0.05%-2,7-dichlorofluorescein in methanol (wt/v) and viewed under U.V.-light. The areas corresponding to TG, FFA, DG, MG and the region between MG and DG (position of UK, unidentified compound) were transfered to glass centrifuge tubes, or to counting vials as required. The lipids remaining at the origin were then chromatographed in either the toluene or chloroform solvents. The positions of the lipids were determined under U.V.-light after spraying with dichlorofluorescein and the appropriate areas removed as above.

Lipids required for further analysis were extracted from the silica gel using $5cm^3$ of $CHCl_3$-MeOH-diethyl ether (1:1:1, by vol.) following the addition of a few drops of water to deactivate the silica gel.

MGDG and DG were further purified on silica gel layers developed in hexane-diethyl ether-glacial acetic acid (60:40:1, by vol.) and $CHCl_3$-MeOH (9:1, v/v) respectively.

MGDGs of differing degrees of unsaturation were separated on 10%-$AgNO_3$ silica gel layers using $CHCl_3$-MeOH-H_2O (60:22:4, by vol.) as the developing solvent (Nichols and Moorhouse, 1969).

3.3.3 Gas-Liquid Chromatography of Methyl Esters of Fatty Acids

3.3.3.(a) 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 $20cm^3$-tube with a ground glass neck, the solvent was removed by a stream of nitrogen and $2cm^3$ of 0.5M-methanolic NaOH was added. The tube was attached to a water condenser and refluxed on a sand bath for 2min. $2cm^3$ of 14%-boron trifluoride in methanol (wt/v) was added through the condenser and refluxing continued for a

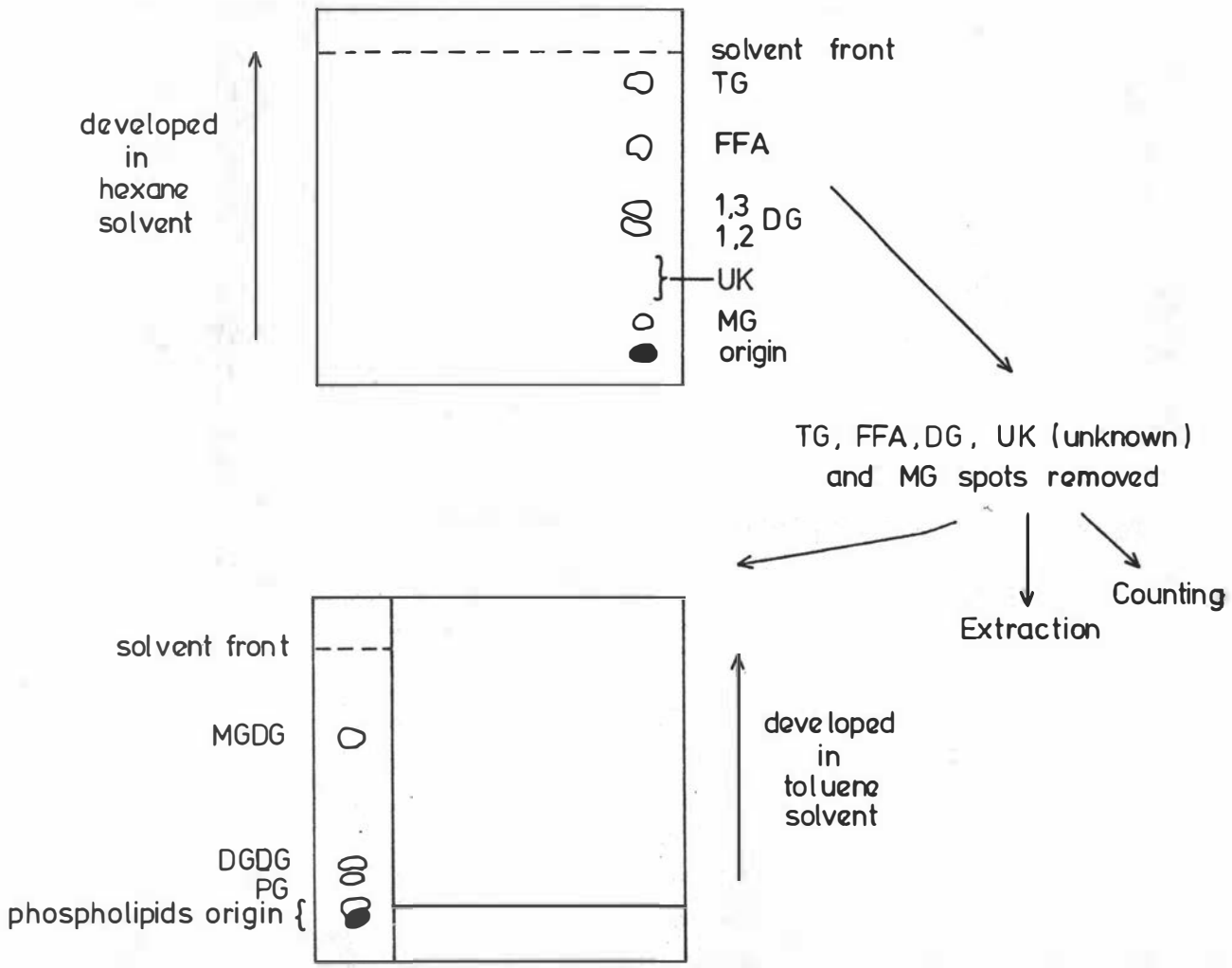


Fig. 3-1. **The modified two-dimensional thin-layer chromatography of lipids**

For further details see text (p. 35).

further 2min. 5cm^3 of hexane was added through the condenser and this mixture allowed to reflux, off heat, for a further 2min . After cooling, sufficient water was added to bring the hexane layer to the top of the tube. The hexane layer was transferredto a glass-stoppered centrifuge tube using a Pasteur pipette. The hexane was removed with a stream of nitrogen and the methyl esters redissolved in an appropriate volume of hexane. A standard methyl ester mixture containing 16:0, 18:0, 18:1 and 18:2 was added for co-chromatography, since little of these fatty acids are present in chloroplast lipids (Sastry, 1974).

3.3.3.(b) Gas-Liquid Chromatography

The methyl esters of fatty acids were analysed using a Varian-Aerograph Model 1520 gas chromatograph fitted with a flame ionisation detector. The glass column (183cm by 0.3cm i.d.) was packed with 9%-diethylene glycol succinate polyester (DEGS) (Annlabs, Connecticut, USA) on Chromosorb Q (60-70 mesh) (Applied Sciences Laboratories, California, USA).

The column was fitted with an effluent stream splitter diverting $\frac{1}{4}$ of the sample to the collector jet and the remainder passed to the flame ionisation detector. Samples were injected on to the column using a 30μl-SGE microlitre syringe (Scientific Glass Engineering Pty. Ltd., Melbourne, Australia).

The column was held at 185^{o}C with the injector and detector temperatures at 205^{o}C and 235^{o}C respectively. The carrier gas was oxygen-free nitrogen, flowing at 47-52cm^3/min, with hydrogen and air supplied to the detector at 22cm^3/min and 250cm^3/min respectively.

3.3.3.(c) Collection of Radioactive Effluent

Samples of the radioactive effluent were collected in a pyrex tube loosely packed with 0.1-0.2g of glasswool moistened with scintillation solution. The methyl esters were eluted from the collection tubes by two 5cm^3 aliquots of scintillation solvent into counting vials. A small hand-operated air-pump was used to flush the solvent through the glasswool and aid draining. Each fatty acid was collected in a separate collection tube as they were eluted of the column as monitored from the chart recorder.

3.3.4 Measurement of Radioactivity

10cm^3 of Triton X-100-toluene scintillation solvent (9g-PPO, 0.3g-POPOP and 1l of Triton X-100 added to 2l of toluene) was used for the counting aqueous solutions and the non-volatile products from $H^{14}CO_3^-$ fixation. 10cm^3 of Toluene scintillation solvent (6g-PPO, 0.24g-POPOP added to 1l of toluene) or 0.5%-ρ-terphenyl in toluene (wt/v) were used for the counting lipid samples after the removal of $CHCl_3$, methyl esters of fatty acids and radioactive areas from t.l.c. -silica gel layers.

Radioactivity was determined using either a Packard Tricarb Model 3375 scintillation counter or a Beckman LS-350 liquid scintillation system. Preset modules were used for counting ^{14}C-isotope. When ^3H-isotope and ^{14}C-isotope were counted together discriminator settings of 0-to 200 for ^3H and 300 to 1,000 for ^{14}C were used on the Beckman LS-350. Counting efficiencies were determined by means of an automatic external standard calibrated against radioactive hexadecane.

Thin-layer chromatography plates were scanned for radioactivity using a Packard Radiochromatogram Scanner Model 7200. The counting conditions were: 1.26%-iso-butane in helium (v/v) at 150cm^3/min; voltage, 1.15kv; slit-width, 2.5mm; time constant, 30s with a scanning rate of 0.5cm/min to 1.0cm/min.

Autoradiographs of t.l.c. layers were obtained by exposing the plates to Kodak RP Royal X-Omat X-ray film for 2 to 4 weeks in a light-proof box. Films were developed and fixed with Kodak Liquid X-ray developer and fixer.

3.3.5 The Determination of the Positional Distribution of Radioactive Fatty Acids in MGDG and DG Synthesized by Spinach Chloroplasts

Hydrolysis of MGDG and DG by pancreatic lipase was carried out essentially by the method of Safford and Nichols (1970). Pancreatic lipase was extracted at 0^{o}C with acetone, diethyl ether-MeOH (2:1, v/v), followed by three washings with diethyl ether to remove endogenous lipids and air-dried (Noda and Fujiwara, 1967).

The incubation medium consisted of: 10cm^3 of 0.2M-Tris/HCl buffer, pH7.6, 0.1cm^3 of 1%-sodium deoxycholate (wt/v)

and 0.3cm^3 of 45%-$CaCl_2$ (wt/v). The MGDG or DG to be hydrolyzed, in 0.1cm^3 of hexane, was added to the incubation medium, the hexane was removed by a stream of nitrogen and the reaction mixture sonicated for 5min using a 100watt ultrasonic disintegrator in the auto-mode at a power output of about 6-microns peak to peak (M.S.E. Ltd., London, England).

The reaction was started by the addition of 50mg-pancreatic lipase and the MGDG and DG hydrolyzed for 3h and 2min respectively at 40°C.

Following the incubation the lipids were extracted into $CHCl_3$ using an equal volume of chloroform to reaction mixture (2 extractions). The pooled chloroform extracts was evaporated to dryness under a stream of nitrogen and redissolved in 1cm^3 of chloroform.

The separation of MGDG, MGMG and FFAs on silica gel layers was achieved by developing with $CHCl_3$-MeOH-Acetone (80:16:4, by vol.). The lipids were located and extracted from the silica gel as described in Section 3.3.2. The methyl esters of the fatty acids were prepared and g.l.c. as described in Section 3.3.3 (pp. 35 to 37).

Chapter 4

RESULTS

4.1 The Incorporation of [1-^{14}C]acetate into Total Lipid and Constituent Fatty Acids by Isolated Chloroplasts

Initially the studies were aimed at improving the incorporation of acetate into lipids and fatty acids. These studies involved a comparison of isolation methods and incubation conditions and an investigation of the effect of varying ATP, CoA and acetate to optimize the concentrations for maximum incorporation of acetate. Incorporation into each of the major constituent fatty acids was examined in the hope of finding conditions that would give increased incorporation into polyunsaturated fatty acids.

4.1.1 A Comparison of Chloroplast Isolation Methods and Incubation Conditions on [1-^{14}C]acetate Incorporation into Lipid

Two procedures of chloroplast isolation, namely the method developed by Leese *et al.* (1971) for *Zea mays* (Method 1) and the isolation method used by Jacobson *et al.* (1973a) for *Spinacia oleracea* (Method 2), were compared to establish which method was the most suitable for the isolation of chloroplasts from both types of tissue.

Isolation Method 1 involved two brief homogenizations of the leaf tissue (for 3s and 5s with mixing between periods) in 0.5M-sucrose-phosphate buffer, pH8.0, containing BSA and Mg^{++}. The chloroplasts were purified using a one-step gradient. Method 2 utilized a single 30s homogenization in 0.5M-sorbitol-Tricine/NaOH buffer, pH7.9, containing bicarbonate, ascorbate, K_2HPO_4 and Mg^{++} (see Methods section 3.2.2, p. 27).

The incubation media used by Hawke *et al.* (1974a) and Jacobson *et al.* (1973a) (Medium A and Medium B respectively) contained the same range of cofactors, but at different concentrations, also *E. coli* acyl carrier protein (ACP) was a component of Medium B but not of Medium A. The bicarbonate, buffer and osmotic requirements for Medium B were supplied by the addition of the chloroplasts resuspended in the isolating medium (see Methods section 3.2.6, p. 33).

Chloroplasts isolated by Method 1 from *Spinacia oleracea* (spinach) and *Zea mays* var. Wisconsin 346 (maize) incorporated more [1-^{14}C]acetate into lipid when incubated in

Medium A than in Medium B (Table 1, p. 42) The much lower incorporation observed when Medium B was used in conjunction with isolation Method 1 could be accounted for by the absence of bicarbonate. Spinach chloroplasts showed comparable rates of acetate incorporation in incubation Medium A when isolated by both methods. However, maize chloroplasts showed poor rates of acetate incorporation when isolated by Method 2 in both incubation media (Table 1, p. 42).

Examination of maize chloroplasts isolated by Method 1 by phase-contrast microscopy showed that approximately 75% were intact whereas maize chloroplasts isolated by Method 2 were only 30 to 40% intact. When maize chloroplasts were isolated by Method 1, but extending the homogenization time to 30s, they possessed only 25% of the biosynthetic activity of chloroplasts isolated by the brief homogenization (3s and 5s with mixing between bursts) procedure (3.5nmol-acetate/mg chlorophyll/30min compared with 15.4nmol-acetate/mg chlorophyll/30min). The longer homogenization time also resulted in a 40 to 50% decrease in the degree of intactness of the maize chloroplasts. In contrast to maize chloroplasts, no obvious alteration in the degree of intactness of spinach chloroplasts or their rates of acetate incorporation arising from the different isolation methods was found when incubated in Medium A (Table 1, p. 42).

On the basis of these results isolation Method 1 (Leese et al., 1971) and the incubation Medium A as used by Hawke et al. (1974a) were routinely used in subsequent experiments.

4.1.2 The Effect of ATP and CoA Concentration on [1-^{14}C]-acetate Incorporation into Lipids and Constituent Fatty Acids by Isolated Chloroplasts

The addition of low concentrations of ATP stimulated acetate incorporation into lipids by isolated spinach, maize and sweetcorn chloroplasts (Fig. 4-1, p. 44). Maximum stimulation of acetate incorporation was achieved at 0.5mM-ATP for spinach and maize and at 1.0mM-ATP for sweetcorn chloroplasts. At concentrations of 2.0mM-ATP and above the incorporation of acetate by isolated chloroplasts from all three tissues decreased as the ATP concentration increased.

Table 1. A comparison of chloroplast isolation methods and incubation conditions on [1-^{14}C]acetate incorporation into lipids

Chloroplasts were isolated from spinach and maize leaf tissue.

Isolation: Method 1. The tissue was homogenized for 3s and 5s with mixing between bursts, in 0.5M-sucrose-0.067M-phosphate buffer, pH8.0, containing 0.2%-BSA and 1mM-$MgCl_2$. Chloroplasts were further purified on a one step gradient using 0.6M-sucrose-0.067M-phosphate buffer, pH8.0 layer.

Method 2. Tissue was homogenized for 30s in 0.6M-sorbitol-0.1M-Tricine/NaOH buffer, pH7.9, containing 1mM-$MgCl_2$, 10mM-K_2HPO_4 and 60mM-$NaHCO_3$. For further details see Methods and Materials (section 3.2.2, pp. 27 to 28). In both methods the chloroplasts were resuspended in the isolating medium and used immediately.

Incubation: Medium A. 300mM-sorbitol, 50mM-Tricine/NaOH buffer, pH7.8, 50mM-phosphate buffer, pH7.8, 2.5mM-DTT, 2.0mM-ATP, 30mM-$NaHCO_3$, 0.5mM-CoA, 1.0mM-$MgCl_2$, 0.2mM-NADPH, 0.2mM-NADH and 100 l(130 to 180μg of chlorophyll) of chloroplast suspension in a final volume of 1cm^3 after the addition of substrates.

Medium B. 1.0mM-DTT, 2.0mM-ATP, 0.2mM-CoA, 0.5mM-NADPH, 0.5mM-NADH, 40μg of *E. coli*-ACP and 0.6cm^3 (700 to 900μg of chlorophyll) of chloroplast suspension in a final volume of 0.8cm^3 after the addition of substrates.

20nmol (1.27μCi)[1-^{14}C]acetate was added to both incubation media and incubated as described in Methods and Materials (section 3.2.6 3.2.6.(c), p. 33).

Isolation medium	Chloroplast source	Incorporation of acetate into lipid (nmol/mg chlorophyll/h) Incubation Medium A	Incubation Medium B
1	*Zea mays* var. Wisconsin 346 (maize)	28.8	18.3
1	*Spinacia oleracea* (spinach)	57.4	4.6
2	*Zea mays* var. Wisconsin 346 (maize)	5.0	6.6
2	*Spinacia oleracea* (spinach)	57.5	31.1

Oleic acid was the major fatty acid synthesized by isolated chloroplasts from all three tissues studied. The amount synthesized from acetate, when ATP concentration was increased, closely followed the variation in acetate incorporation into total lipid (Fig. 4-1, p. 44). At higher ATP concentrations the decrease in oleic acid accounted for a large proportion of the decrease in the total incorporation observed in all chloroplast preparations. The relative changes in the incorporation for the other major fatty acid constituents, when ATP concentration was varied, were much smaller than for oleic acid, but the patterns were generally similar to that of the total lipids (Fig. 4-1, p. 44). The levels of acetate incorporation into linoleic and linolenic acids were low at all ATP concentrations used, although at higher ATP concentrations an increased porportion of the total label was present in these fatty acids since the incorporation rates into these fatty acids did not decrease to the same extent as that observed for oleic acid.

The addition of CoA stimulated acetate incorporation into lipid (Fig. 4-2, p. 46). However, increasing the concentration of CoA beyond 0.25mM-CoA resulted in only minor increases in incorporation.

Increased incorporation into oleic acid accounted for most of the stimulation of acetate incorporation by CoA in the case of maize and sweetcorn chloroplasts. A two-fold increase in the level of labelled linoleic and linolenic acids was also achieved by adding CoA, but the levels were still low (Fig. 4-2, p. 46). However, with spinach chloroplasts, oleic, palmitic and stearic acids all contributed to the increased incorporation of acetate in to total lipid while the levels of label in linoleic and linolenic acids were unchanged by the addition of CoA (Fig. 4-2, p. 46).

4.1.3 The Effect of Acetate Concentration on the Incorporation of [1-^{14}C]acetate into Lipid and Constituent Fatty Acids by Isolated Chloroplasts

Increasing acetate concentration from 0.02mM-acetate to 0.52mM-acetate resulted in a three to four-fold increase in the incorporation of acetate into lipid by spinach chloroplasts and a five to six-fold increase in the case of maize and sweetcorn chloroplasts (Fig. 4-3, p. 48). Maximum

Fig. 4-1. The effect of ATP concentration on the incorporation of $[1-^{14}C]$acetate into lipid and constituent fatty acids by isolated chloroplasts

Chloroplasts were isolated from spinach, maize and sweetcorn leaf tissue by Method 1 and incubated in Medium A (refer Table 1, p. 42) for 30min with 20nmol (1.27μCi)$[1-^{14}C]$acetate.

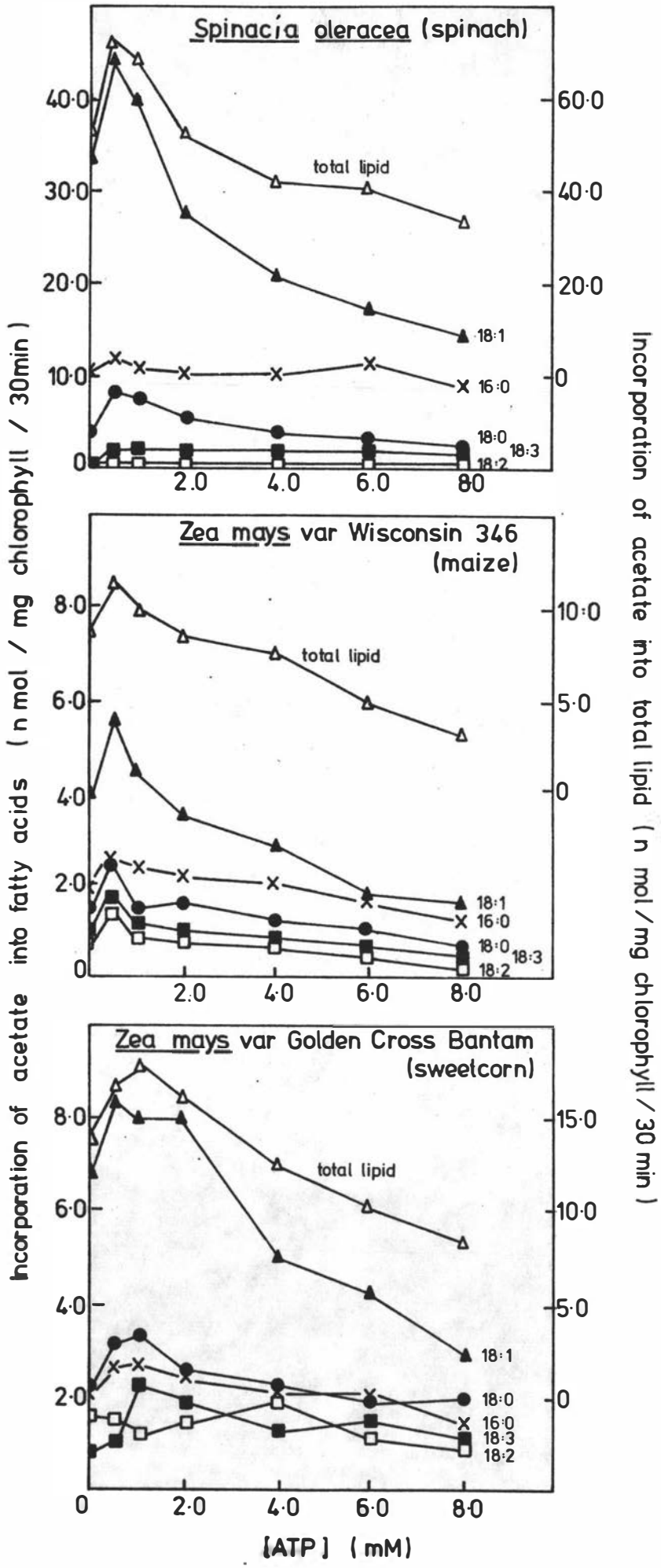
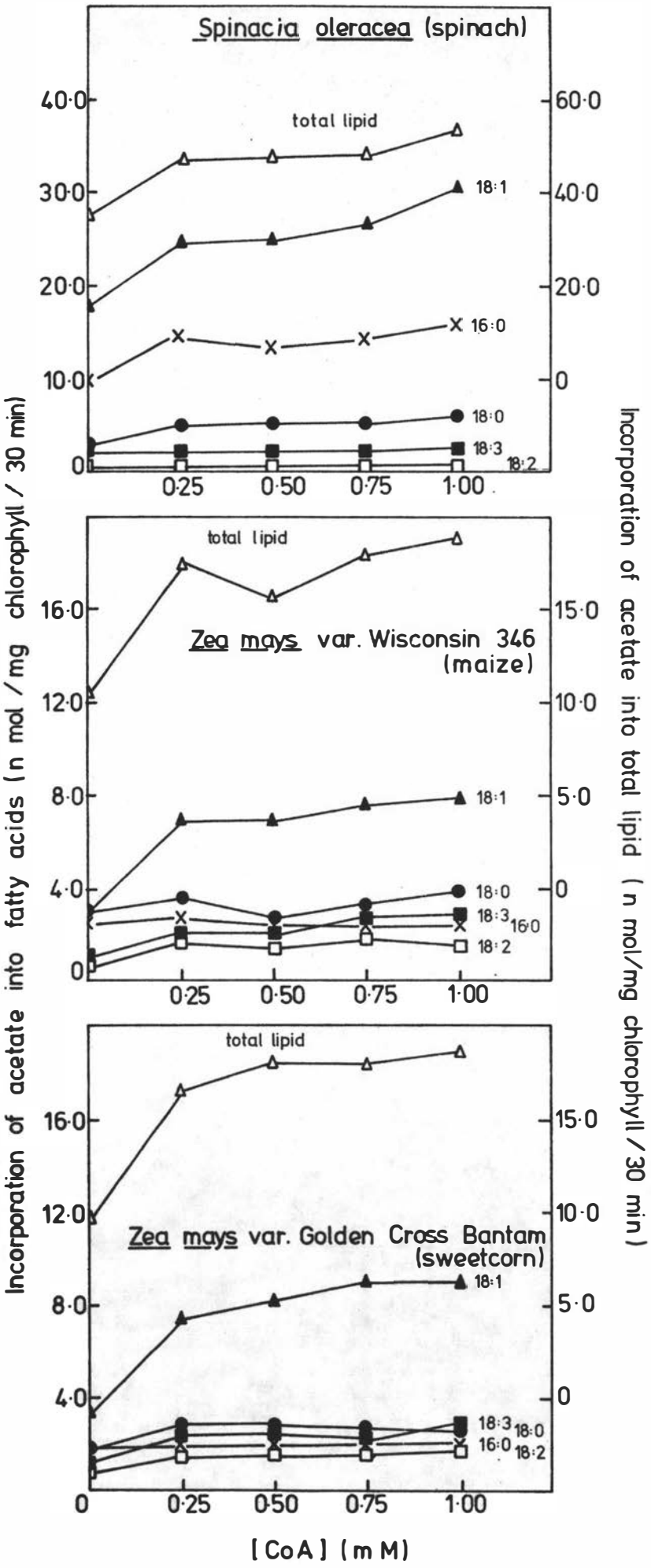

Spinacia oleracea (spinach)
total lipid
18:1
16:0
18:0
18:3
18:2
Zea mays var Wisconsin 346 (maize)
Zea mays var Golden Cross Bantam (sweetcorn)
Incorporation of acetate into fatty acids (n mol / mg chlorophyll / 30min)
Incorporation of acetate into total lipid (n mol / mg chlorophyll / 30 min)
[ATP] (mM)

Fig. 4-2. The effect of Coenzyme A concentration on the incorporation of [1-^{14}C]acetate into lipid and constituent fatty acids by isolated chloroplasts

Chloroplasts were isolated by Method 1 and incubated in Medium A (refer Table 1, p. 42), except that the ATP concentration was 0.5mM. 20nmol-(1.26μCi)[1-^{14}C]acetate was added.


Spinacia oleracea (spinach)
total lipid
18:1
16:0
18:0
18:3
18:2
40·0
30·0
20·0
10·0
0
60·0
40·0
20·0
0
0·25
0·50
0·75
1·00
Zea mays var. Wisconsin 346 (maize)
16·0
12·0
8·0
4·0
15·0
10·0
5·0
Zea mays var. Golden Cross Bantam (sweetcorn)
Incorporation of acetate into fatty acids (n mol / mg chlorophyll / 30 min)
Incorporation of acetate into total lipid (n mol/mg chlorophyll / 30 min)
[CoA] (m M)

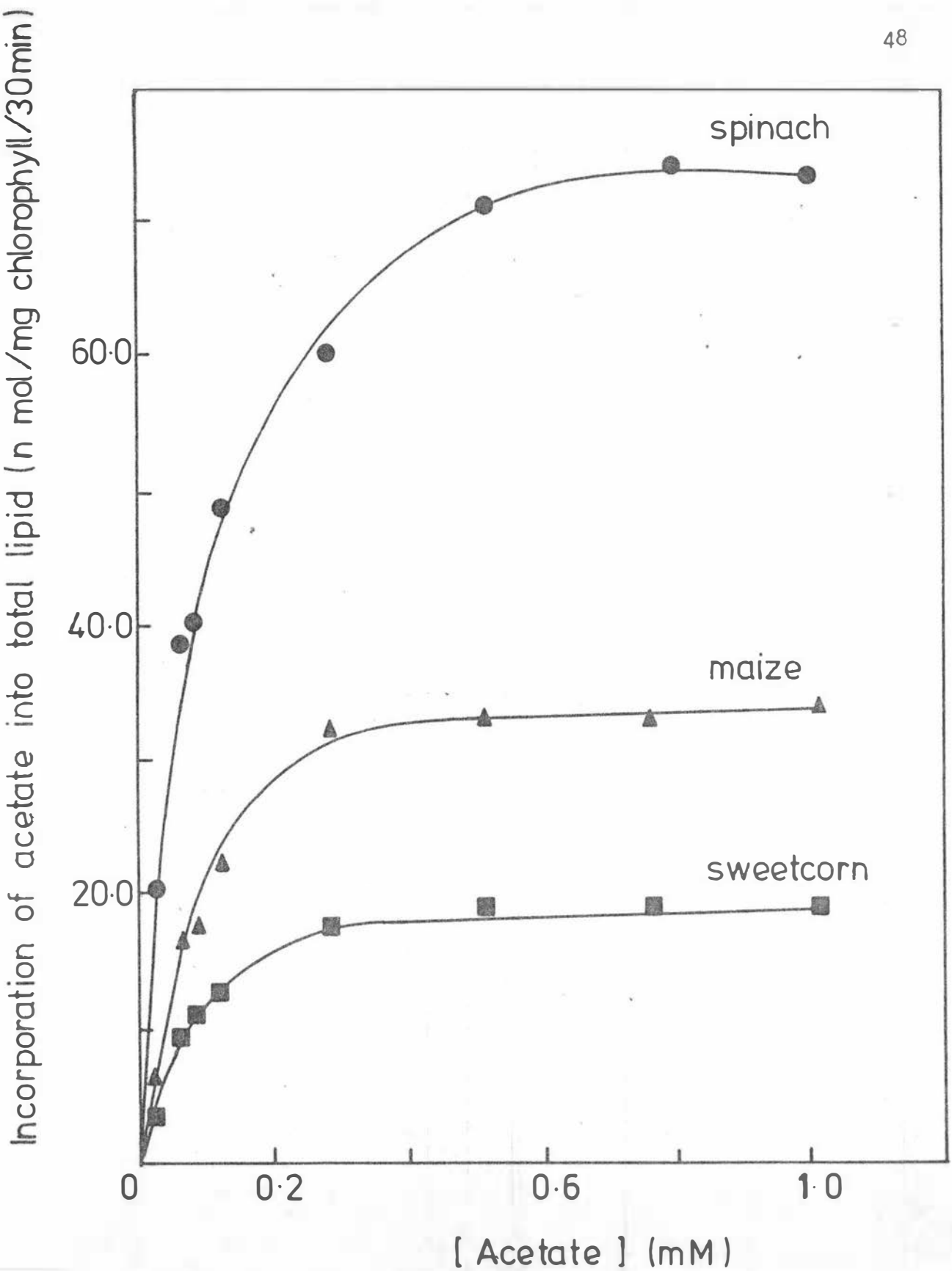


Fig. 4-3. The effect of acetate concentration on the incorporation of $[1-^{14}C]$acetate into lipid by isolated chloroplasts

Chloroplasts were isolated by Method 1 and incubated in Medium A (refer Table 1, p. 42), except that 0.5mM-ATP and 0.25mM-CoA were used. 20nmol-(1,26μCi)$[1-^{14}C]$acetate was added with appropriate amounts of unlabelled acetate to give the required concentration.

incorporation was obtained at about 0.5mM-acetate for maize and sweetcorn chloroplasts and about 0.75 to 1.0mM-acetate for the more active spinach chloroplasts (Fig. 4-3, p. 48).

Varying the concentration of acetate had little effect on the relative proportions of the fatty acids synthesized from acetate by isolated chloroplasts (Table 2, p. 50). Oleic, palmitic and stearic acids constituted 80 to 90% of the label incorporated from [1-^{14}C]acetate while only small amounts of label were incorporated into linoleic and linolenic acids.

4.1.4 Rates of [1-^{14}C]acetate Incorporation into Lipid and Constituent Fatty Acids by Isolated Chloroplasts

The incorporation of acetate into total lipid was approximately linear for the first 20min of incubation for isolated chloroplasts from all three sources (Fig. 4-4, p. 51). The rate of incorporation by maize and sweetcorn chloroplasts decreased considerably after 30 to 40min, but the more active spinach chloroplasts maintained linear incorporation up to 45min.

Oleic, palmitic and stearic acids were the major fatty acids synthesized with only low rates of incorporation into linoleic and linolenic acids (Fig. 4-4, p. 51). The relative importance of each fatty acid varied with the chloroplasts under investigation, with oleic and palmitic acids being the major fatty acids labelled in spinach and maize chloroplasts, whereas in sweetcorn, oleic acid was the predominant fatty acid labelled.

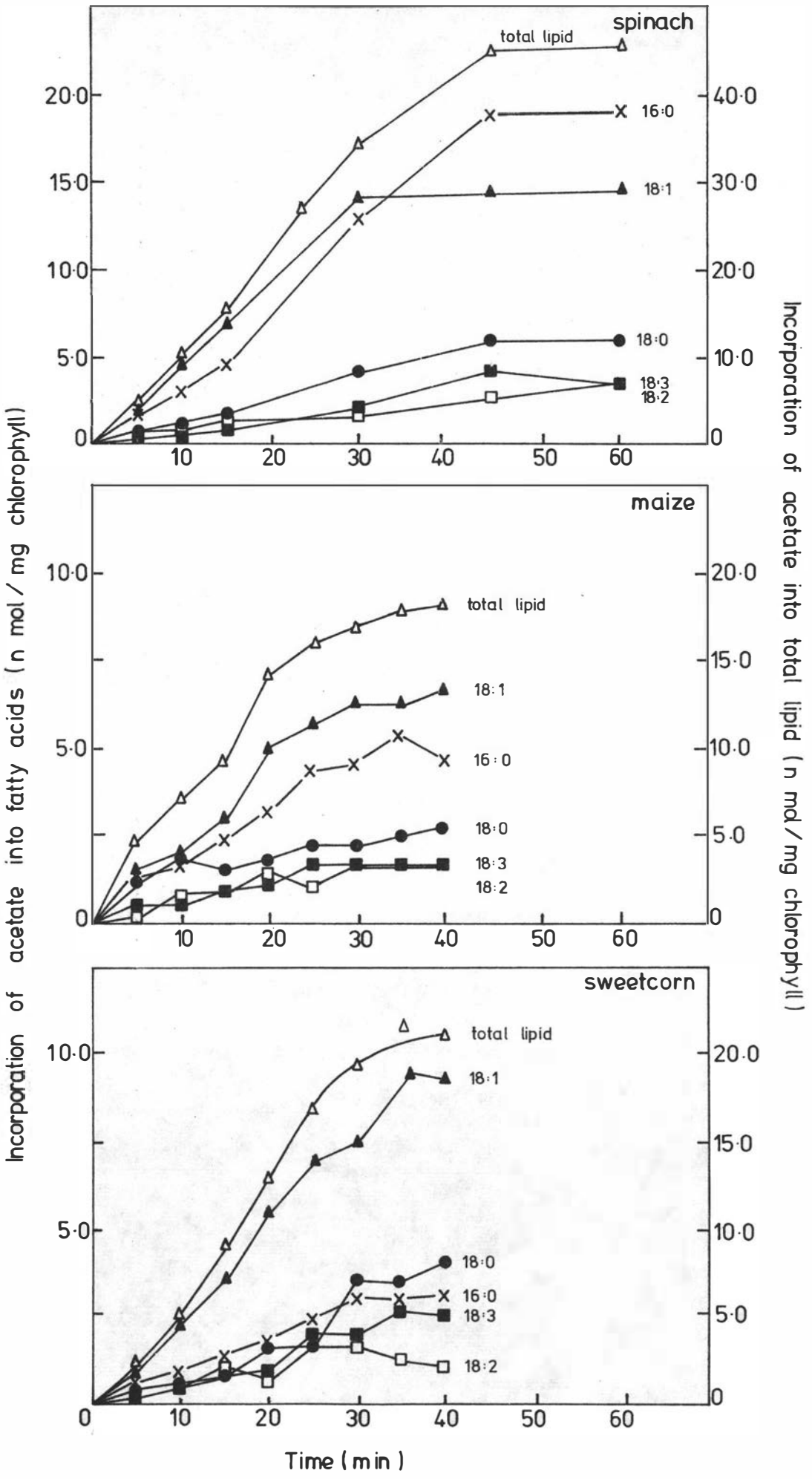
Table 2. The effect of acetate concentration on the incorporation of [1-^{14}C]acetate into fatty acids by isolated chloroplasts

For experimental details see Fig. 4-3 (p. 48).

Acetate concentration mM	Incorporation of acetate into lipid (nmol/mg chlorophyll/30min)	Distribution of label between fatty acids (%)					
		<16:0	16:0	18:0	18:1	18:2	18:3
Spinach chloroplasts							
0.02	20.0	12.7	30.1	10.6	41.1	0.6	4.9
0.12	48.2	7.6	28.0	12.2	45.3	1.6	5.3
0.52	71.0	11.8	22.9	13.5	45.9	2.4	3.5
Maize chloroplasts							
0.02	5.9	2.2	29.2	18.9	47.5	0.8	1.4
0.12	22.4	1.8	29.7	17.2	49.4	0.8	1.1
0.52	32.8	2.4	26.1	17.3	48.5	1.6	4.1
Sweetcorn chloroplasts							
0.02	3.2	7.6	23.6	15.6	41.8	5.4	6.0
0.12	12.5	3.9	28.7	14.8	48.4	1.6	2.6
0.52	19.0	4.7	27.7	15.0	44.8	2.7	5.1

Fig. 4-4. The rates of [1-^{14}C]acetate incorporation into lipid and constituent fatty acids by isolated chloroplasts from spinach, maize and sweetcorn

Chloroplasts were isolated by Method 1 and incubated in Medium A with ATP and CoA concentrations of 0.5mM and 0.25mM respectively (refer Table 1, p. 42). 107nmol (6.73μCi)[1-^{14}C]acetate was used as substrate in a final volume of 1cm^3.


spinach
total lipid
16:0
18:1
18:0
18:3
18:2
maize
total lipid
18:1
16:0
18:0
18:3
18:2
sweetcorn
total lipid
18:1
18:0
16:0
18:3
18:2
Incorporation of acetate into fatty acids (n mol / mg chlorophyll)
Incorporation of acetate into total lipid (n mol / mg chlorophyll)
Time (min)

4.2 Attempts to Improve Polyunsaturated Fatty Acid Biosynthesis by Isolated Chloroplasts

In the experiments reported in section 4.1, only very low rates of acetate incorporation into linoleic and linolenic acids were obtained. Therefore attempts to improve the incorporation of acetate into these fatty acids were made using other approaches. The first approach was to investigate if polyunsaturated fatty acid biosynthesis was affected by the stage of chloroplast development. Developing maize leaf tissue was utilized as a source of chloroplasts at different stages of development. Secondly, the possible involvement of other cell organelles, co-operating with the chloroplasts, in polyunsaturated fatty acid biosynthesis was investigated by the addition of a particulate preparation from leaf tissue containing microsomes and mitochondria to isolated chloroplasts. Finally, the acyl end product was altered by the addition of sn-glycerol-3-phosphate and UDP-galactose to improve diglyceride and monogalactosyldiglyceride biosynthesis respectively, to see if this affected the synthesis of polyunsaturated fatty acids.

4.2.1 The Influence of Chloroplast Development on [1-^{14}C]-acetate Incorporation into Lipid and Constituent Fatty Acids

Leaf sections from developing maize leaf were prepared according to the procedure of Leese et al. (1971) and the chloroplasts isolated by Method 1 and incubated in reaction Medium A, but using 0.5mM-ATP and 0.25mM-CoA which gave the optimum incorporation of acetate. The estimated chlorophyll content of 150 x 10^6-plastids (chloroplasts) is 45, 50, 75, 125, 200μg of chlorophyll, respectively, for plastids isolated from A, B, C, D and E sections of maize leaves (Leese et al., 1971). These values were used to estimate the number of plastids present so that the rates of acetate incorporation per plastid could be compared. On this basis the chloroplasts isolated from section D were twice as active in acetate incorporation as chloroplasts isolated from sections A+B and C and about six-fold greater than the rates observed for chloroplasts isolated from section E (Table 3, p. 54). The expression of the rates of acetate incorporation per mg of chlorophyll gives a completely different result, with the chloroplasts from sections A+B being

Table 3. The incorporation of [1-^{14}C]acetate into lipids and fatty acids by isolated chloroplasts from maize leaf sections

Maize leaf sections were prepared according to the proceedure of Leese et al. (1971) and the chloroplasts isolated by Method 1. The isolated chloroplasts were incubated in Medium A, but with ATP and CoA concentrations of 0.5mM and 0.25mM respectively. 20nmol (1.26μCi)[1-^{14}C]acetate was added in a final volume of 1cm^3 after the addition of substrates and chloroplasts. For definition of leaf sections see Methods section 3.2.4 (p. 28).

leaf section	Incorporation of acetate into lipids (nmol/mg chlorophyll/h)	(nmol/150x 10^6-plastids/h)	Distribution of label between lipids (%) FFA	DG	UK*	MG	MGDG	DGDG	others$^{\alpha}$	Distribution of label between fatty acids (%) <16:0	16:0	18:0	18:1	18:2	18:3
A+B	42.5	2.02	60.4	12.4	5.6	7.4	1.9	0.9	11.4	2.5	34.0	6.0	55.6	0.8	1.1
C	33.3	2.50	61.6	12.3	7.2	3.6	1.1	1.1	13.1	3.7	45.9	7.0	41.5	0.9	1.0
D	30.4	4.13	63.5	11.8	10.3	2.2	1.8	1.4	9.0	1.5	58.0	7.2	31.5	0.5	1.3
E	3.7	0.73	64.4	8.6	5.6	4.3	3.5	3.6	10.0	3.1	52.0	7.0	36.0	1.0	0.9

$^{\alpha}$ more polar lipids (mainly phospholipids). * UK, an unidentified labelled compound

about 45% more active than the chloroplasts from sections C and D and about ten-fold greater than the chloroplasts from section E (Table 3, p. 54).

The distribution of label between the lipid classes did not vary appreciably with the stage of development of the chloroplast. Free fatty acids and diglycerides constituted 60 to 64% and 8 to 12% respectively of the label incorporated into lipid. Monogalactosyldiglyceride and digalactosyldiglyceride were poorly labelled in all chloroplast preparations with only 1 to 4% of the label appearing in these lipids (Table 3, p. 54). The remainder of the label was located in monoglyceride (MG), an unknown (UK) and more polar lipids.

At all stages of development oleic, palmitic and stearic acids constituted most of the fatty acids synthesized from acetate by the chloroplasts (Table 3, p. 54). There was a progressive decrease in the proportions of oleic acid synthesized as the maturity of the chloroplast increased (sections A+B to E) and this was accompanied by an increased proportion of label incorporated into palmitic acid. Only 0.8 to 1.0% and 0.9 to 1.3% of the label incorporated appeared in linoleic and linolenic acids respectively. The stage of chloroplast development did not alter the proportions of linoleic and linolenic acids synthesized from acetate.

4.2.2 The Effect of a Non-chloroplastic Particulate Fraction on the Incorporation of [1-^{14}C]acetate into Lipid and Constituent Fatty Acids by Isolated Maize Chloroplasts

The addition of a non-chloroplastic particulate fraction (sedimented at 100,000 X g), isolated from the maize-leaf homogenate remaining after sedimentation of the chloroplasts, stimulated the incorporation of acetate into lipid (Fig. 4-5, p. 56). The particulate fraction itself showed only a very limited capacity to incorporate acetate into lipid (0.01nmol of acetate/10µl of particulate suspension/30min). The particulate preparation contained chloroplast fragments, but no intact chloroplasts were detected by phase-contrast microscopy.

Increasing the particulate:chloroplast ratio up to 1.0 increased the incorporation of acetate into lipids, but further increases in the ratio had no further effect on incorporation (Fig. 4-5, p. 56).

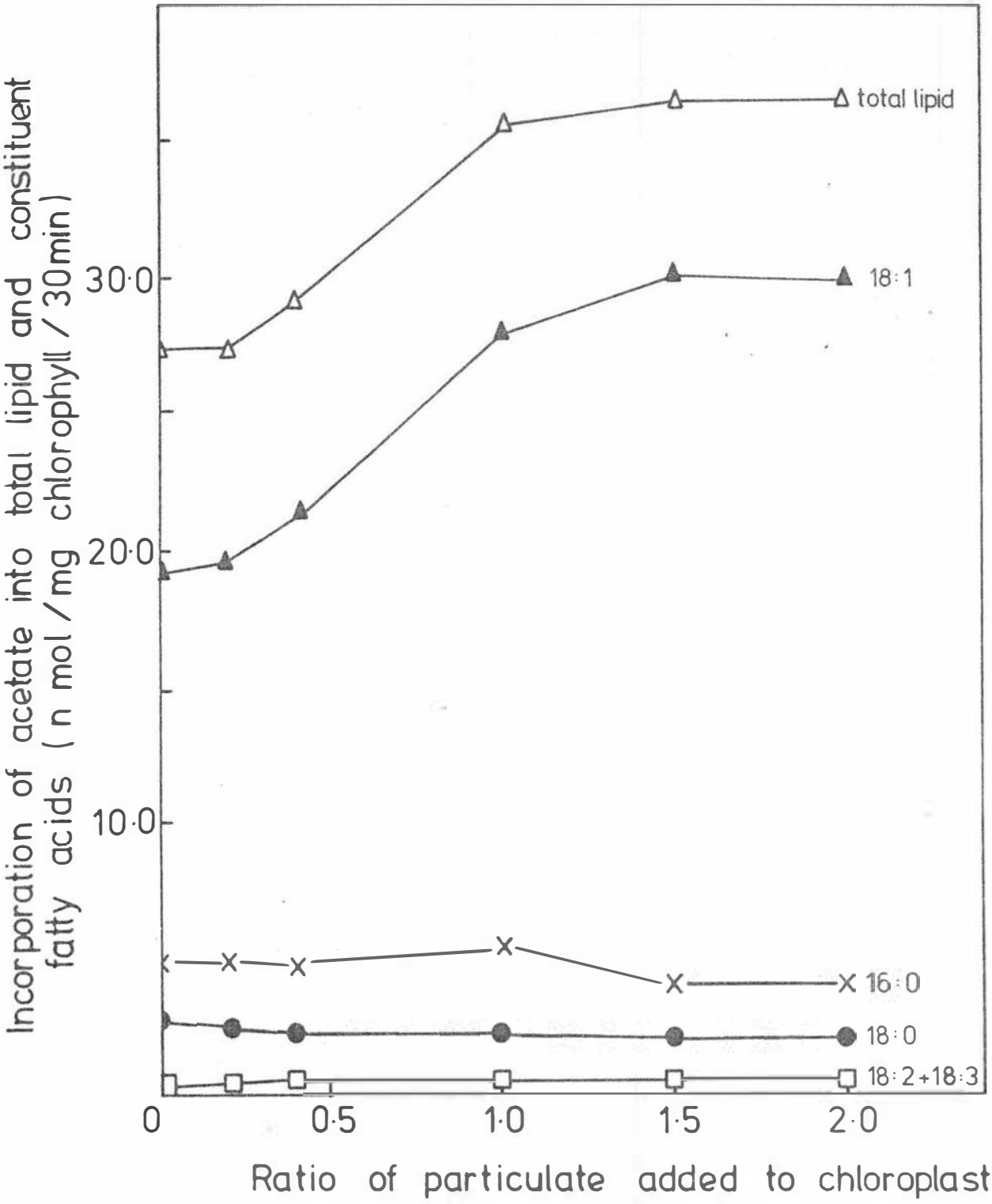


Fig. 4-5. The effect of the particulate fraction:chloroplast ratio on the incorporation of [1-^{14}C]acetate into total lipid and constituent fatty acids by isolated maize chloroplasts

Maize chloroplasts were isolated by Method 1 and incubated in Medium A (refer Table 1, p. 42; Fig. 4-4, p. 51). Various volumes of the particulate fraction suspension, prepared from maize leaf homogenate, were added to the incubation medium giving the required range of particulate fraction:chloroplast ratios (see section 3.2.3, p. 28). 620nmol (6.73μCi) [1-^{14}C]-acetate was added in a final volume of 1cm^3 after the addition substrate, chloroplasts and particulate fraction. The particulate fraction:chloroplast ratio is defined on p. 58.

The increased incorporation was due to the increased synthesis of oleic acid. Only minor changes in the levels of the other major fatty acids were observed. The levels of linoleic and linolenic acids synthesized were low at all particulate:chloroplast ratios (Fig. 4-5, p. 56).

The stimulation of acetate incorporation by the addition of the particulate fraction was confirmed by time-course study of acetate incorporation into lipid by chloroplasts (Fig. 4-6, p. 58). In the absence of the particulate fraction, the proportion of acetate incorporated into oleic acid decreased as time progressed (from 57% at 15min to 40% at 120min, of the incorporated acetate). This was accompanied by an increase in the proportion of acetate incorporated into palmitic acid and to a lesser extent into stearic acid. However, the proportions of linoleic and linolenic acids were unchanged. In the presence of the particulate fraction (particulate:chloroplast ratio = 1) the proportion of oleic acid remained constant after 15min incubation, as did the proportions of palmitic and stearic acids. Again no stimulation of the relative proportions of linoleic and linolenic acids was observed over the 2h incubation (Fig. 4-6, p. 58).

4.2.3 The Effect of sn-glycerol-3-phosphate and UDP-galactose on the Incorporation of [1-^{14}C]acetate into Lipids and Constituent Fatty Acids by Chloroplasts

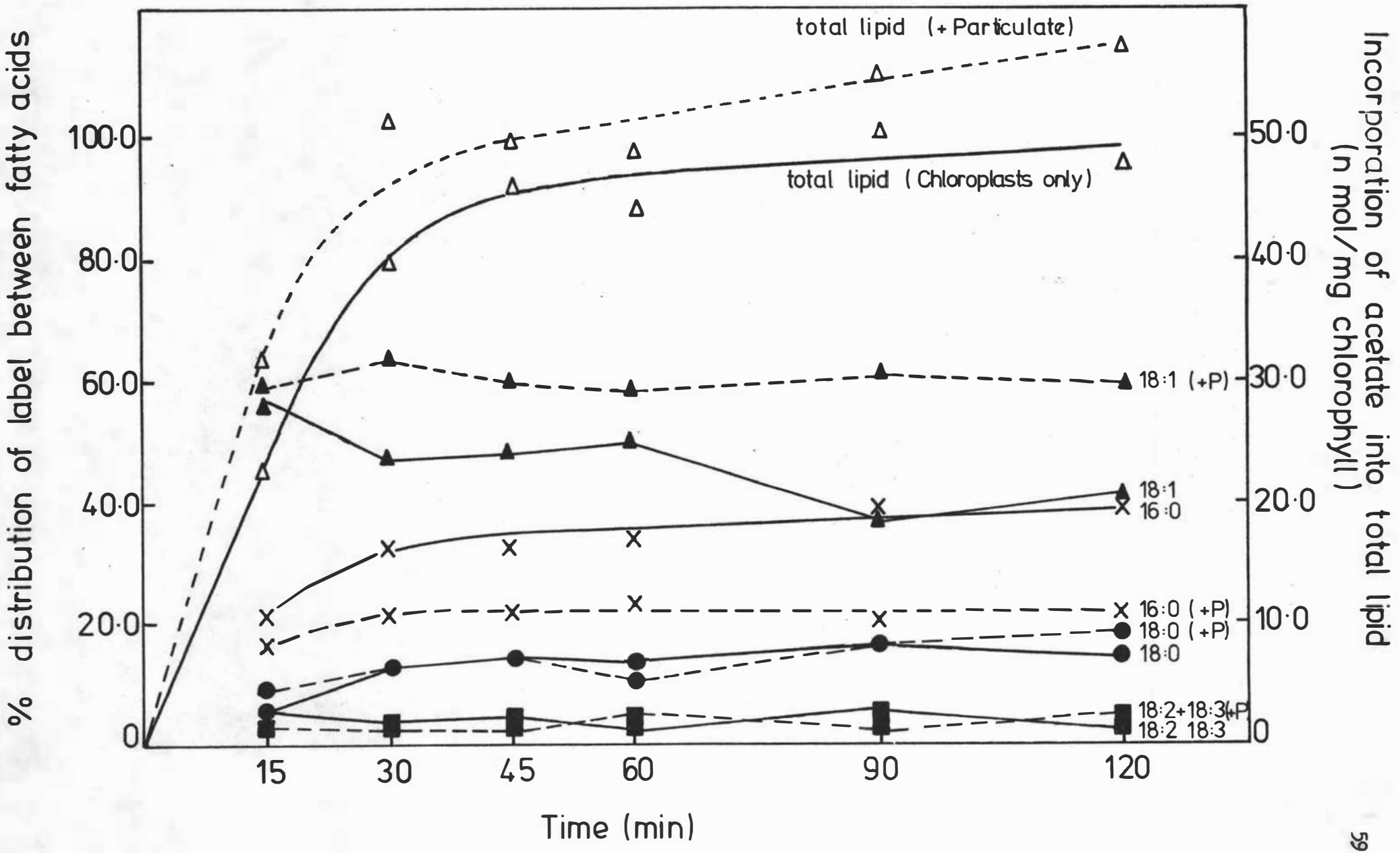
4.2.3.1 Influence of sn-glycerol-3-phosphate and UDP-galactose Concentrations

Free fatty acids (FFA) constituted up to 75% of the ^{14}C incorporated from acetate by isolated chloroplasts from spinach, maize and sweetcorn using incubation Medium A (Fig. 4-7, p. 60; Fig. 4-8, p. 61; Fig. 4-9, p. 62). Diglycerides, monogalactosyldiglycerides and digalactosyldiglycerides constituted 10 to 15%, 1 to 8% and 1to 4% respectively of the incorporated label. The remainder of the label was incorporated into monoglycerides (1 to 6%) and more polar lipids.

Increasing the concentration of sn-glycerol-3-phosphate (G-3-P) up to 5mM, increased the incorporation of acetate into lipid by spinach chloroplasts (Fig. 4-7, p. 60): concentrations above this level resulted no further stimulation of acetate incorporation. However, the incorporation

Fig. 4-6. The effect of the particulate fraction on the rates of [1-^{14}C]acetate incorporation into lipid and constituent fatty acids by maize chloroplasts

The experimental procedure was that described in Fig. 4-5 (p. 56) with a particulate:chloroplast ratio of 1. The final reaction volume was 1cm^3 after the addition of 620nmol (6.73μCi) [1-^{14}C]-acetate, chloroplasts and particulate fraction. The particulate fraction:chloroplast ratio is defined as the ratio of 2 x volume of the particulate fraction suspension added/volume of chloroplast suspension added, where a ratio of 1 is when the amount of particulate fraction added is that amount which was isolated from the same weight of tissue as the amount of chloroplasts.


% distribution of label between fatty acids
Incorporation of acetate into total lipid (n mol/mg chlorophyll)
total lipid (+ Particulate)
total lipid (Chloroplasts only)
18:1 (+P)
18:1
16:0
16:0 (+P)
18:0 (+P)
18:0
18:2+18:3(+P)
18:2 18:3
100·0
80·0
60·0
40·0
20·0
0
50·0
40·0
30·0
20·0
10·0
0
15
30
45
60
90
120
Time (min)

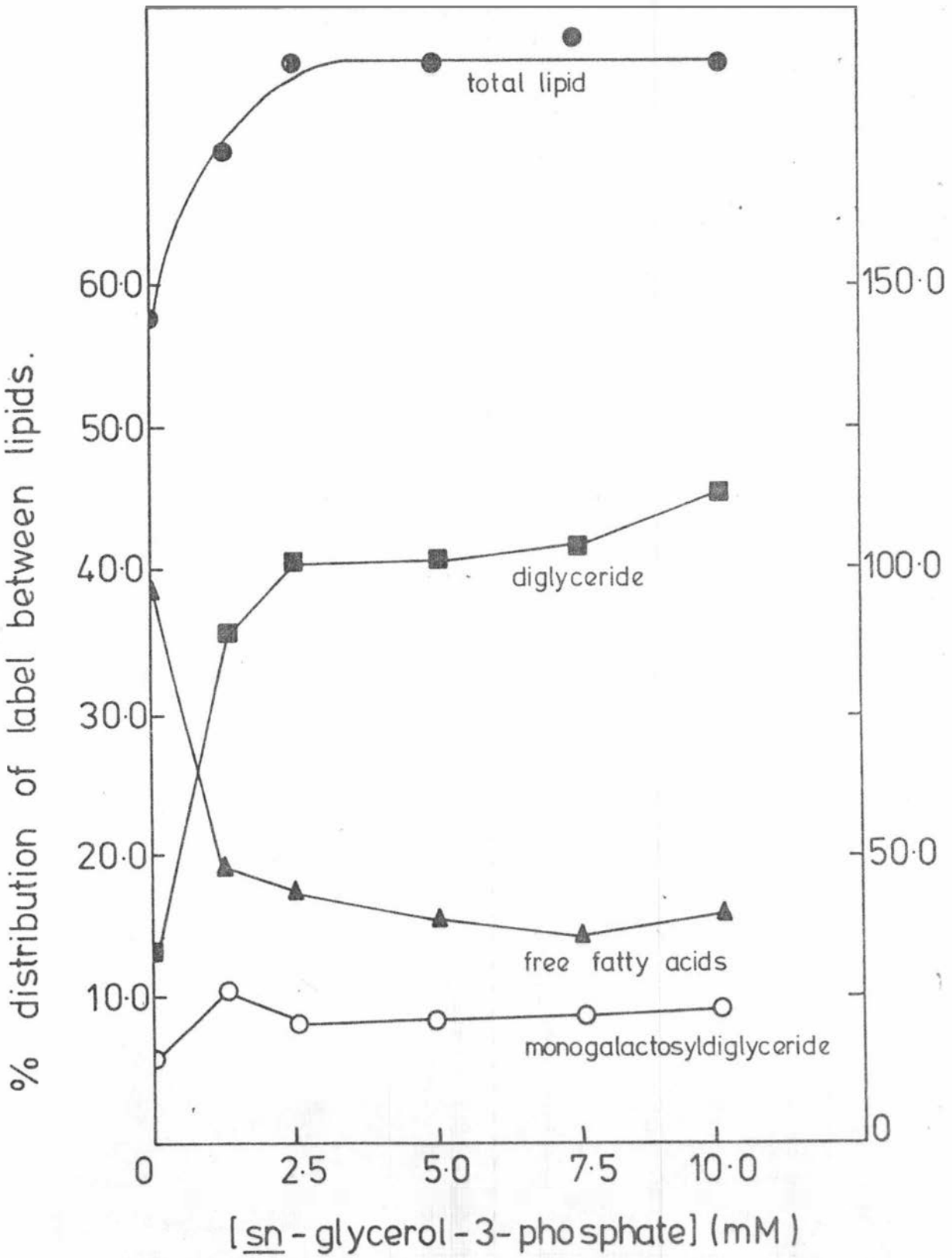


Fig. 4-7. The effect of sn-glycerol-3-phosphate concentration on the incorporation of [1-^{14}C]acetate into lipids by spinach chloroplasts

Chloroplasts were isolated by Method 1 and incubated in Medium A (refer Fig. 4-4, p. 51) containing 109nmol (6.73μCi) [1-^{14}C]acetate and with appropriate amounts of sn-glycerol-3-phosphate to give the required concentration.

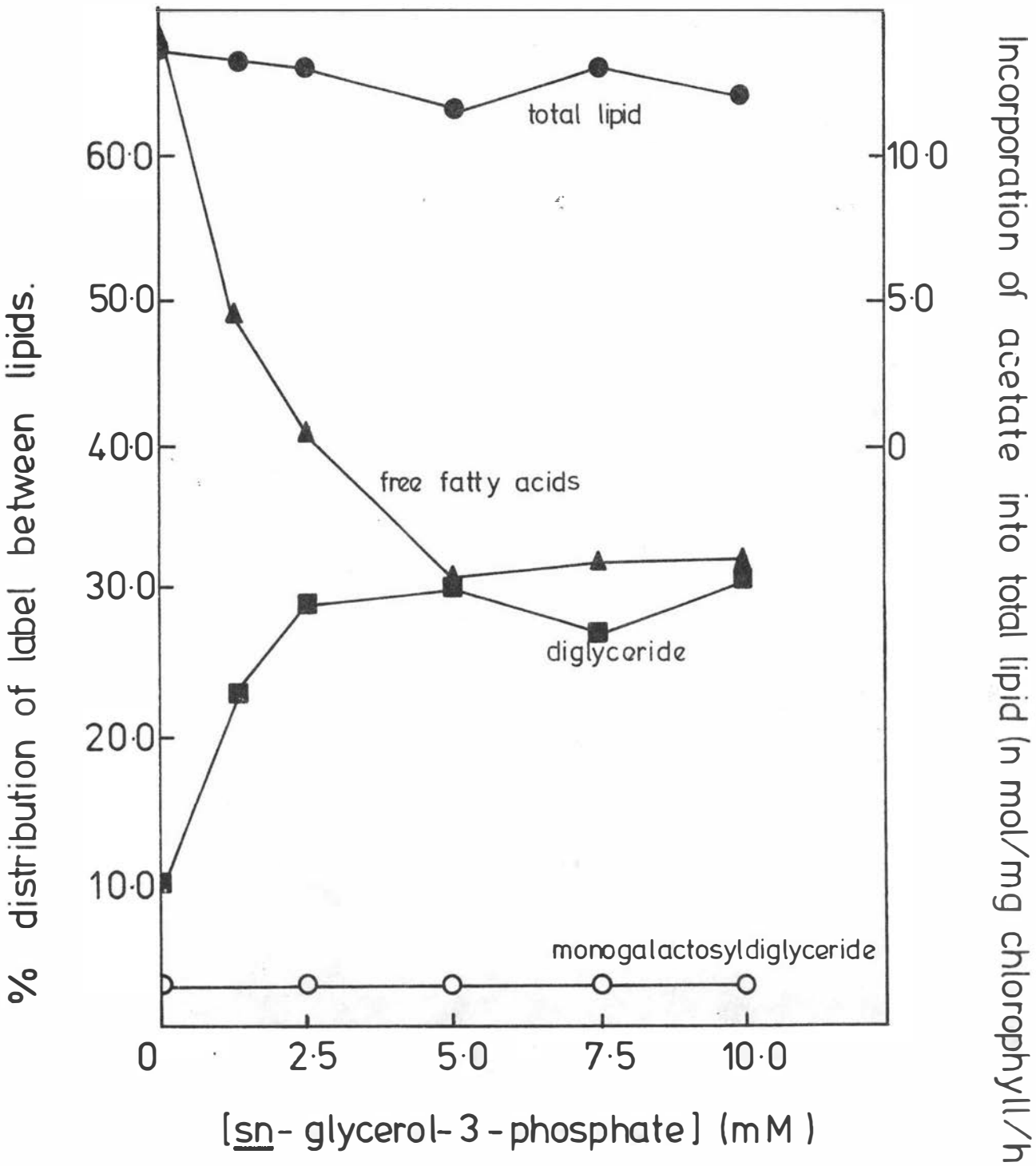


Fig. 4-8. The effect of sn-glycerol-3-phosphate concentration on [1-^{14}C]acetate incorporation into lipids by maize chloroplasts

The experimental procedures were those as described in Fig. 4-7 (p. 60), but using chloroplasts isolated from maize seedling leaf tissue.

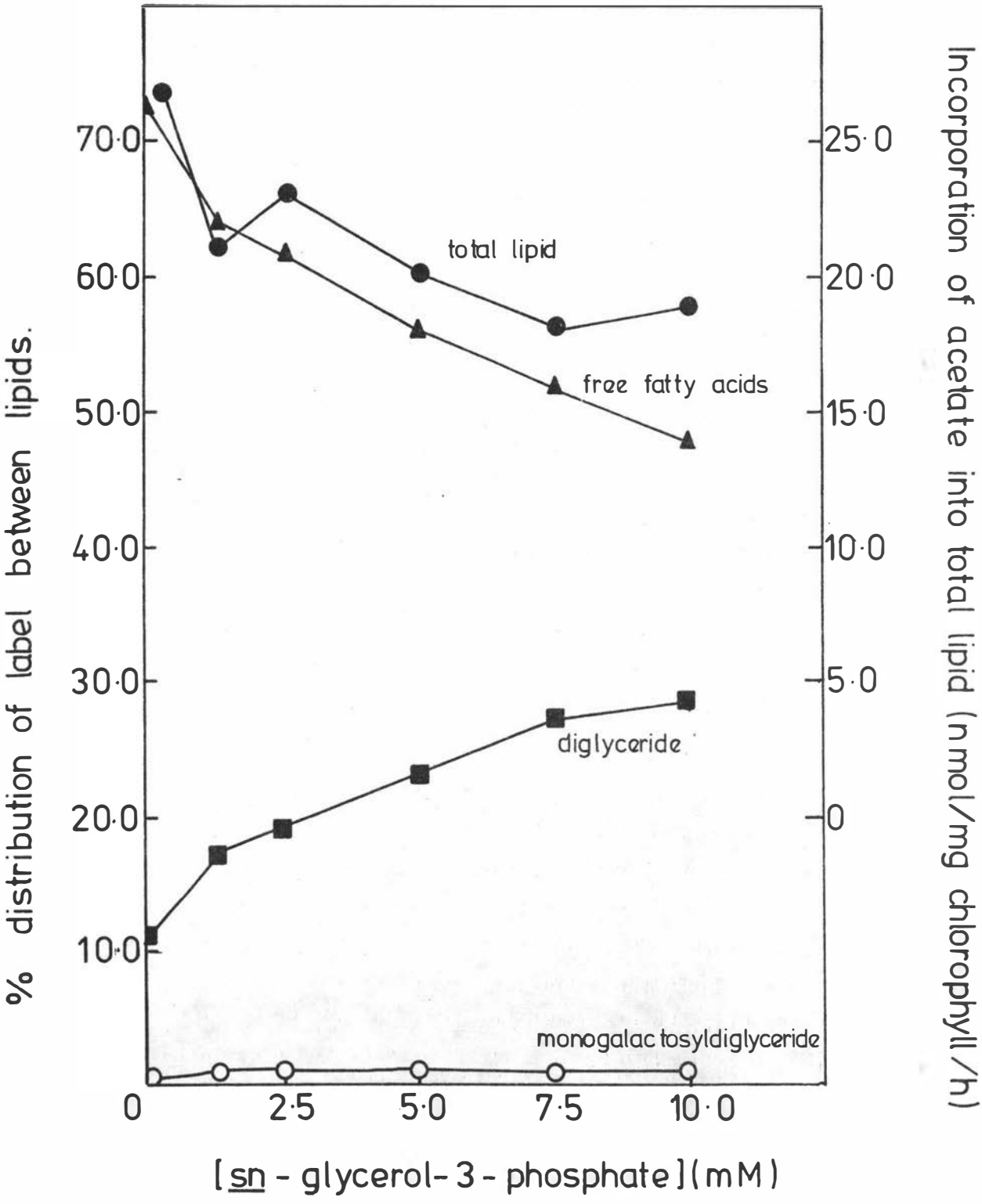


Fig. 4-9. **The effect of sn-glycerol-3-phosphate concentration on [1-^{14}C]acetate incorporation into lipids by sweetcorn chloroplasts**

The experimental procedures were those as described in Fig. 4-7 (p. 60), but using chloroplasts isolated from sweetcorn seedling leaf tissue.

of acetate into lipids by maize chloroplasts was not stimulated by the addition of G-3-P at any of the concentrations used (Fig. 4-8, p. 61). The addition of G-3-P to sweetcorn chloroplasts led to a decrease in the incorporation of acetate into lipids (Fig. 4-9, p. 62).

The addition of G-3-P to chloroplasts prepared from all three tissues increased the incorporation of label into diglycerides and decreased the proportion of label in FFA (Fig. 4-7, p. 60; Fig. 4-8, p. 61; Fig. 4-9, p. 62). The maximum increase in the proportion of diglyceride biosynthesis was obtained at 5mM-G-3-P for spinach and maize chloroplasts and at 7.5mM-G-3-P for sweetcorn chloroplasts. The levels of monogalactosyldiglyceride synthesized from acetate were not significantly altered by the addition of G-3-P to the incubation medium.

Increasing the concentration of UDP-galactose, up to 75μM in the presence of 5.0mM-G-3-P, gave a small increase in acetate incorporation into total lipid by spinach chloroplasts (Fig. 4-10, p. 64), but similar responses were not observed with maize and sweetcorn chloroplasts (Fig. 4-11, p.65).

The addition of UDP-galactose to the incubation medium stimulated the synthesis of monogalactosyldiglyceride from acetate by chloroplasts prepared from each of the three tissues and was accompanied by a decrease in the levels of diglyceride. A small additional decrease in the level of FFA, above that obtained with G-3-P, was also observed with spinach chloroplasts but not with maize or sweetcorn chloroplasts (Fig. 4-10, p. 64; Fig. 4-11, p. 65).

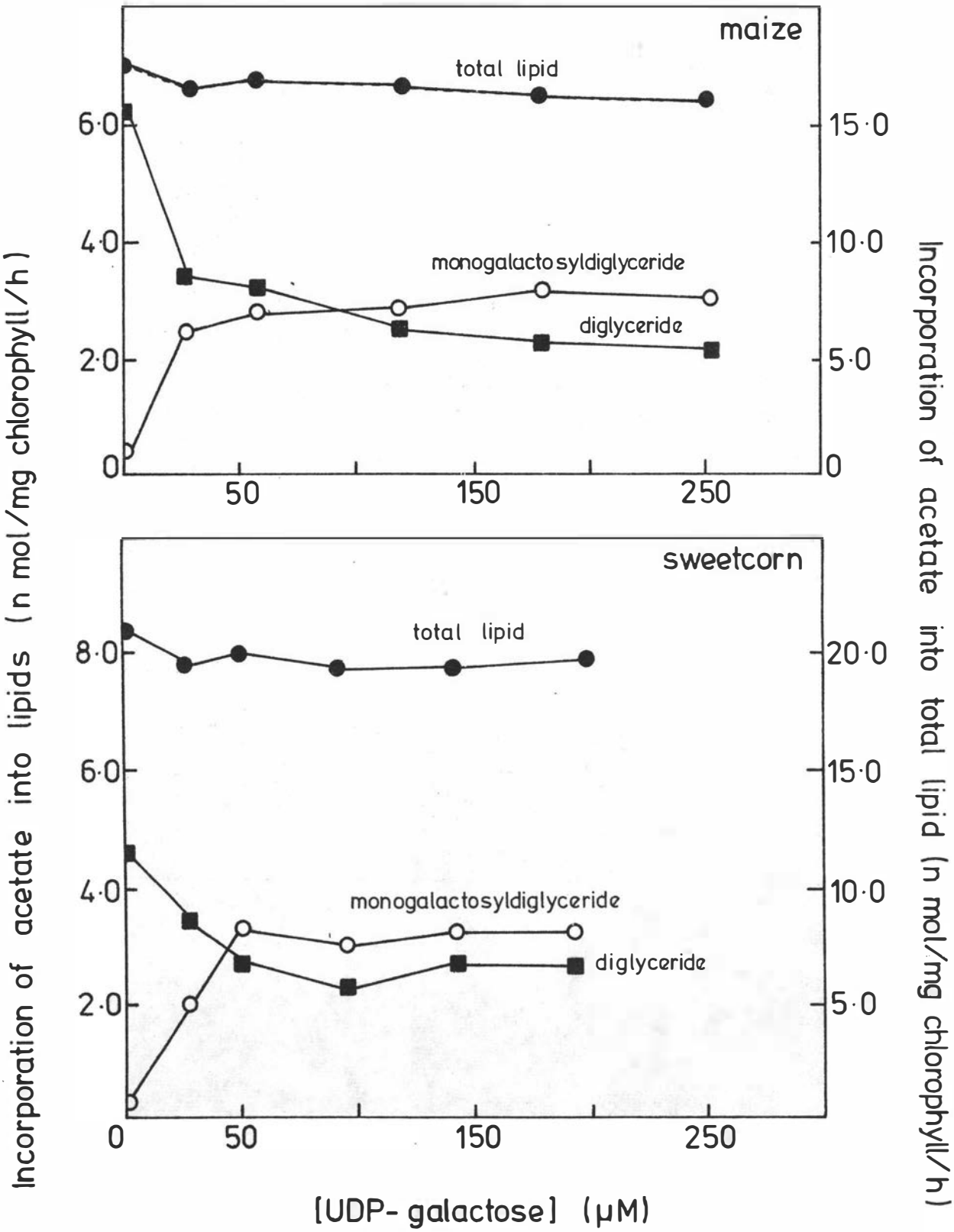


Fig. 4-11. The effect of UDP-galactose concentration on the incorporation of [1-^{14}C]acetate into lipids in the presence of sn-glycerol-3-phosphate by maize and sweetcorn chloroplasts

For experimental procedures see Fig. 4-10 (p. 64).

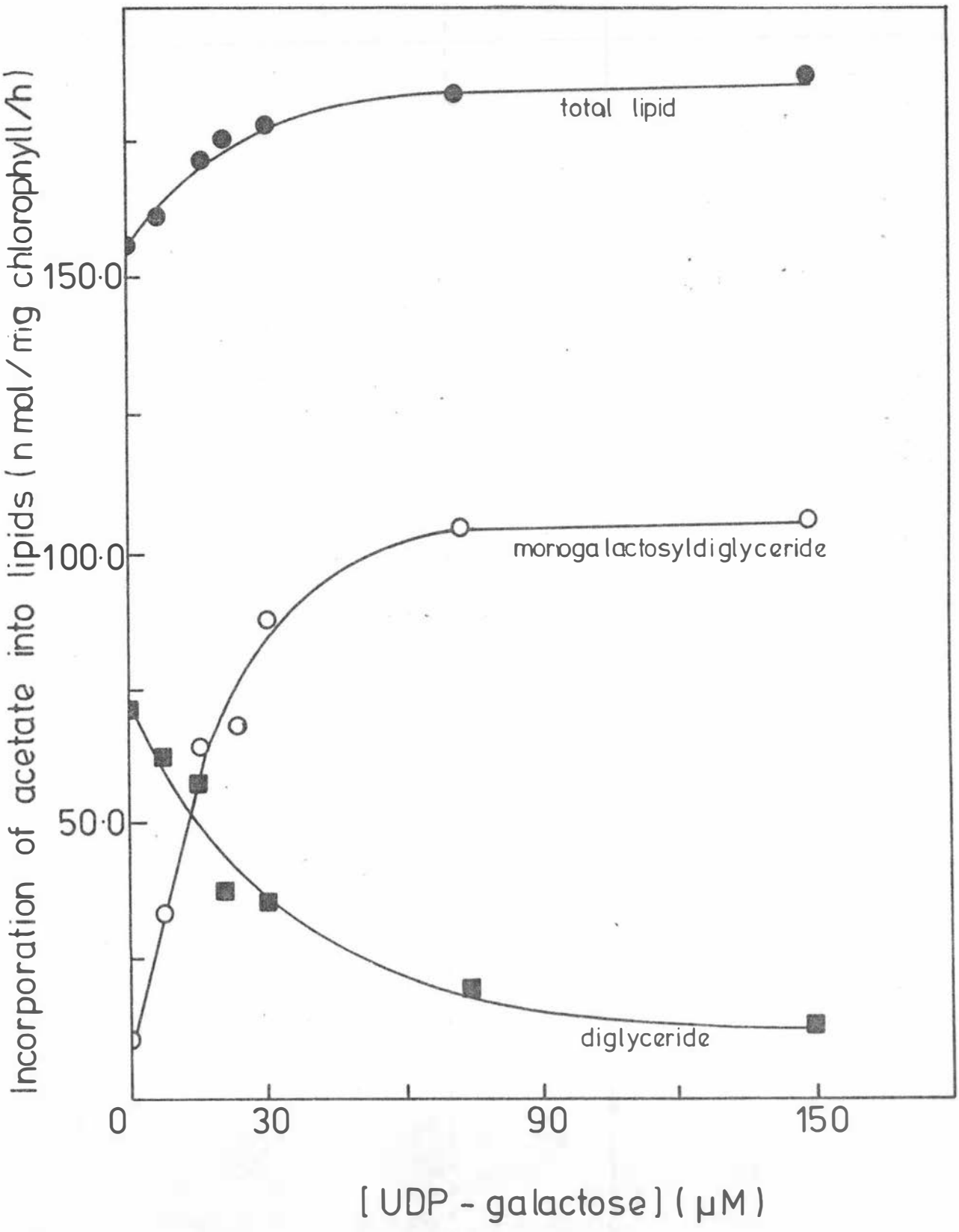


Fig. 4-10. The effect of UDP-galactose concentratino on the incorporation of [1-^{14}C]acetate into lipids in the presence of sn-glycerol-3-phosphate by spinach chloroplasts

Spinach chloroplasts were isolated by Method 1 and incubated in Medium A (refer Fig. 4-4, p. 51) containing 5 mol sn-glycerol-3-phosphate, 104nmol-(6.72μCi)[1-^{14}C]acetate and the required amounts of UDP-galactose in a final volume of 1cm^3 after the addition of substrates and chloroplasts.

4.2.3.2 Incorporation Rates of [1-^{14}C]acetate in the Presence of sn-glycerol-3-phosphate and UDP-galactose

4.2.3.2.(a) Rates of Acetate Incorporation into Lipid

The incorporation of acetate into lipid by chloroplasts at different incubation times, when G-3-P or G-3-P + UDP-galactose were added to the incubation medium, are detailed in Table 4 (pp. 67 to 69). In 60min incubations the addition of G-3-P and G-3-P + UDP-galactose stimulated acetate incorporation by spinach chloroplasts by 40% and 52% respectively. The stimulation by G-3-P was apparent after 20min and the further stimulation by UDP-galactose in the presence of G-3-P was apparent after 30min (Table 4, p. 67). As observed above (Fig. 4.8, p.61), the incorporation of acetate into total lipid by maize chloroplasts was unaffected by the addition of G-3-P or UDP-galactose in the presence of G-3-P (Table 4, p. 68). The inhibition of acetate incorporation into total lipid by G-3-P, with or without added UDP-galactose, was evident early in incubations with sweetcorn chloroplasts (Table 4, p. 69).

4.2.3.2.(b) Influence of sn-glycerol-3-phosphate and UDP-galactose on the Lipids Synthesized by Chloroplasts

The addition of G-3-P stimulated the synthesis of diglycerides with a corresponding decrease in the proportion of free fatty acids (Table 4, pp. 67 to 69). The greatest changes in the proportion of diglycerides arising from G-3-P addition were observed at intermediate incubation times in all chloroplast preparations. The reduction in free fatty acids and increase in diglycerides was greatest for spinach chloroplasts. The addition of UDP-galactose to the incubation medium containing G-3-P increased the proportion of monogalactosyldiglycerides synthesized and reduced the proportion of label in diglycerides. Smaller increases in monogalactosyldiglycerides arose from the addition of UDP-galactose with maize and sweetcorn chloroplasts than with spinach chloroplasts, but the proportion of label in monogalactosyldiglycerides increased with extended incubation times (Table 4, pp. 67 to 69).

Table 4. The effect of sn-glycerol-3-phosphate and UDP-galactose on the rates of [1-^{14}C]acetate incorporation into lipids by isolated chloroplasts

Chloroplasts were isolated by Method 1 and incubated in Medium A (refer Fig. 4-4, p. 52). The final concentrations of sn-glycerol-3-phosphate and UDP-galactose were 5.0mM and 0.15mM respectively, with 104nmol-(6.72μCi)[1-^{14}C]acetate in a final volume of 1cm^3 after the addition of chloroplasts and substrates.

Time min	Additions to incubation medium	Incorporation of acetate into lipids (nmol/mg chlorophyll)	Distribution of label between lipids(%) FFA	DG	MGDG	DGDG	others
Spinach chloroplasts							
10	none	20.5	63.1	10.7	6.9	1.3	18.0
	G-3-P	19.1	29.4	34.1	3.0	1.1	32.4
	G-3-P + UDP-galactose	20.8	27.1	12.0	27.8	1.1	32.0
20	none	49.8	63.9	13.7	2.3	1.2	18.9
	G-3-P	64.2	25.5	44.2	3.8	1.4	25.1
	G-3-P + UDP-galactose	62.8	23.3	10.5	45.4	1.6	19.2
30	none	74.1	55.2	12.4	5.6	2.6	24.2
	G-3-P	83.2	21.4	45.3	6.0	3.0	24.3
	G-3-P + UDP-galactose	95.8	16.9	7.0	54.1	2.8	19.2
45	none	83.7	48.1	15.8	5.9	3.4	26.8
	G-3-P	123.4	17.5	44.8	8.6	3.6	25.5
	G-3-P + UDP-galactose	132.4	12.9	6.2	52.5	4.6	23.8
60	none	108.7	40.6	12.6	6.8	3.8	36.2
	G-3-P	143.7	14.1	36.2	9.3	6.1	34.3
	G-3-P + UDP-galactose	156.7	11.1	5.9	49.8	3.1	30.1

continued on p. 68

Table 4. continued from p. 67

Time min	Additions to incubation medium	Incorporation of acetate into lipids (nmol/mg chlorophyll)	Distribution of label between lipids (%) FFA	DG	MGDG	DGDG	others α
Maize chloroplasts							
10	none	8.2	66.4	9.6	4.7	2.1	17.2
	G-3-P	7.5	54.3	14.8	5.3	1.8	23.8
	G-3-P + UDP-galactose	6.7	52.4	11.8	5.5	2.5	27.8
20	none	10.5	68.6	9.4	2.3	1.7	18.0
	G-3-P	10.2	52.1	18.6	2.8	3.6	22.9
	G-3-P + UDP-galactose	10.8	46.8	13.6	8.4	4.4	26.8
30	none	16.5	67.5	12.0	2.4	1.3	16.8
	G-3-P	15.4	45.9	26.0	2.7	2.1	23.3
	G-3-P + UDP-galactose	15.6	47.7	19.3	11.1	1.4	20.5
45	none	21.0	65.4	13.1	2.5	2.1	16.9
	G-3-P	19.2	47.9	27.6	1.8	2.0	20.7
	G-3-P + UDP-galactose	18.5	43.7	18.9	11.7	1.6	24.1
60	none	21.0	62.1	13.0	2.1	0.7	22.1
	G-3-P	19.4	41.9	32.0	2.9	1.2	22.0
	G-3-P + UDP-galactose	20.5	40.3	16.3	12.1	0.9	30.4

continued on p. 69

Table 4. continued from p. 68

Time min	Additions to incubation medium	Incorporation of acetate into lipids (nmol/mg chlorophyll)	Distribution of label between lipids (%) FFA	DG	MGDG	DGDG	others[α]
Sweetcorn chloroplasts							
10	none	10.0	75.4	5.2	1.4	0.1	17.9
	G-3-P	8.0	61.7	11.6	2.2	2.4	22.1
	G-3-P + UDP-galactose	8.1	58.7	7.3	5.7	3.2	25.1
20	none	20.0	76.8	6.0	1.6	0.8	14.8
	G-3-P	16.0	57.7	14.8	1.8	2.3	23.4
	G-3-P + UDP-galactose	16.0	55.4	10.7	7.6	2.2	24.1
30	none	24.3	75.6	7.6	1.2	1.2	14.4
	G-3-P	16.6	53.5	19.9	3.2	1.9	21.5
	G-3-P + UDP-galactose	18.6	53.5	12.8	10.4	2.5	20.8
45	none	26.6	72.1	8.3	2.7	1.8	15.1
	G-3-P	22.0	51.8	23.5	3.0	2.2	19.5
	G-3-P + UDP-galactose	22.5	50.0	13.5	11.7	1.3	23.5
60	none	28.1	70.0	10.3	1.2	0.7	17.8
	G-3-P	22.2	51.7	21.9	1.6	0.8	24.0
	G-3-P + UDP-galactose	23.1	52.8	14.6	12.2	0.8	19.6

α more polar lipids (mainly phospholipids), MG and an unknown compound (UK).

4.2.3.2.(c) Influence of sn-glycerol-3-phosphate and UDP-galactose on the Fatty Acids Synthesized by Chloroplasts

Details of the rates of synthesis of fatty acids from acetate by spinach, maize and sweetcorn chloroplasts and the effects of G-3-P and UDP-galactose are given in Fig. 4-12 (p. 71), Fig. 4-13 (p. 72) and Fig. 4-14 (p. 73). Oleic, palmitic and stearic acids were the major fatty acids synthesized from acetate by isolated chloroplasts. Only very low rates of synthesis of linoleic and linolenic acids were achieved. In the absence of added G-3-P and G-3-P + UDP-galactose, chloroplasts from all three tissues synthesized 50 to 100% more oleic acid than palmitic acid after 60min (Fig. 4-12, p. 71; Fig. 4-13, p. 72; Fig. 4-14, p. 73). The addition of G-3-P stimulated the synthesis of palmitic acid by spinach and maize chloroplasts and slightly decreased oleic acid synthesis (Fig. 4-12, p. 71; Fig. 4-13, p. 72). Stimulation of palmitic acid synthesis was greatest with spinach chloroplasts. However, with sweetcorn chloroplasts the addition of G-3-P to the incubation medium decreased the synthesis of both oleic and palmitic acids (Fig. 4-14, p. 73).

The addition of both G-3-P and UDP-galactose to spinach chloroplasts stimulated slightly the synthesis of oleic acid above that observed in the presence of G-3-P alone with the rate of palmitic acid synthesis remaining unaltered (Fig. 4-12, p. 71). The synthesis of oleic and palmitic acids by maize and sweetcorn chloroplasts was not altered by the the further addition of UDP-galactose to the incubation medium containing added G-3-P (Fig. 4-13, p. 72; Fig. 4-14, p. 73).

Altering the nature of the acyl end-products synthesized by the addition of G-3-P and UDP-galactose did not alter the synthesis of linoleic and linolenic acids (Fig. 4-12, p. 71; Fig. 4-13, p. 72; Fig. 4-14, p. 73). The synthesis of stearic acid was unaffected by the addition of G-3-P and UDP-galactose.

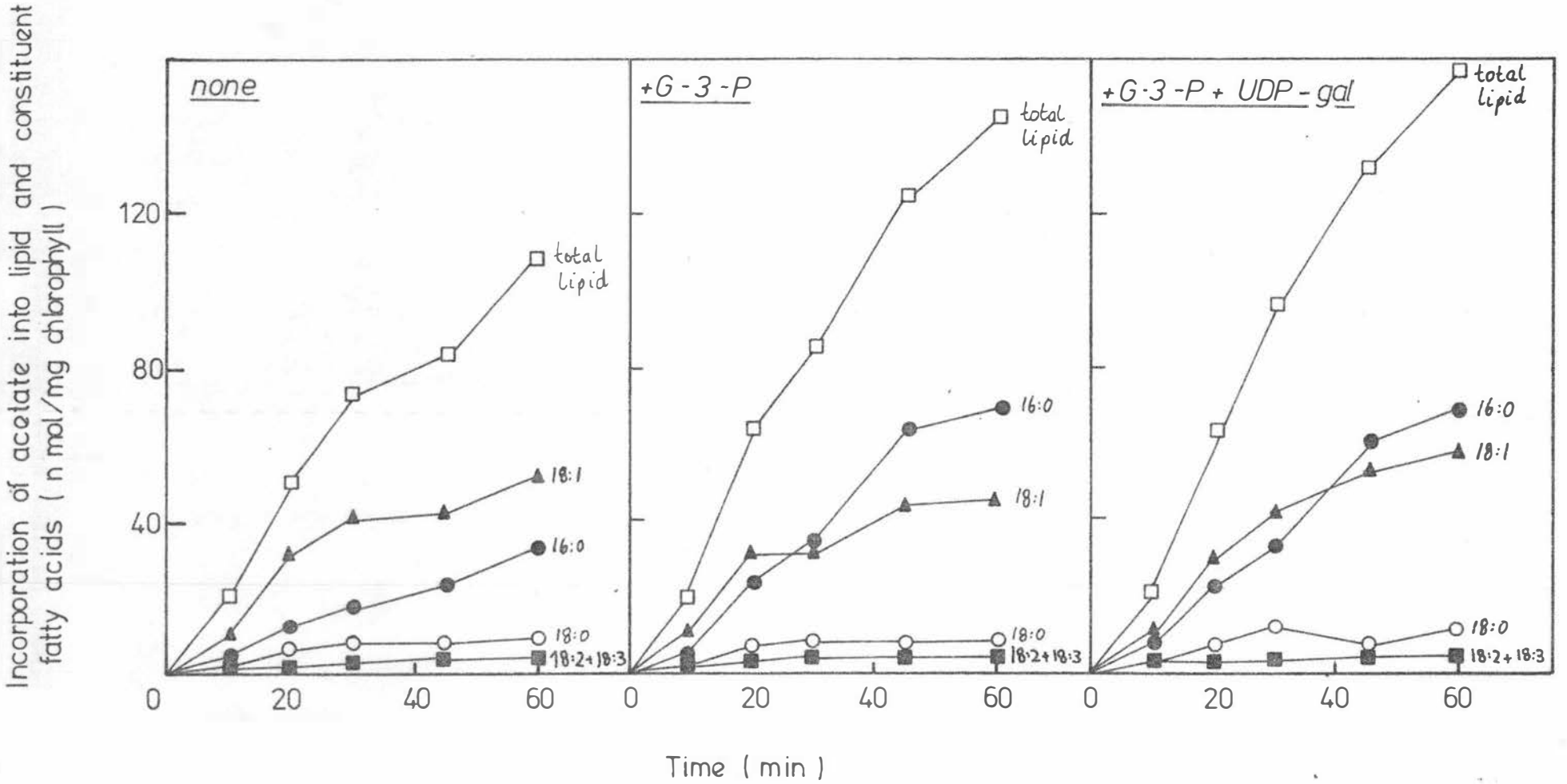


Fig. 4-12. The effect of sn-glycerol-3-phosphate and UDP-galactose on the rates of [1-^{14}C]acetate incorporation into lipid and constituent fatty acids by spinach chloroplasts

The data in this figure represent fatty acid analysis of the total lipids already referred to in Table 4 (p. 67).

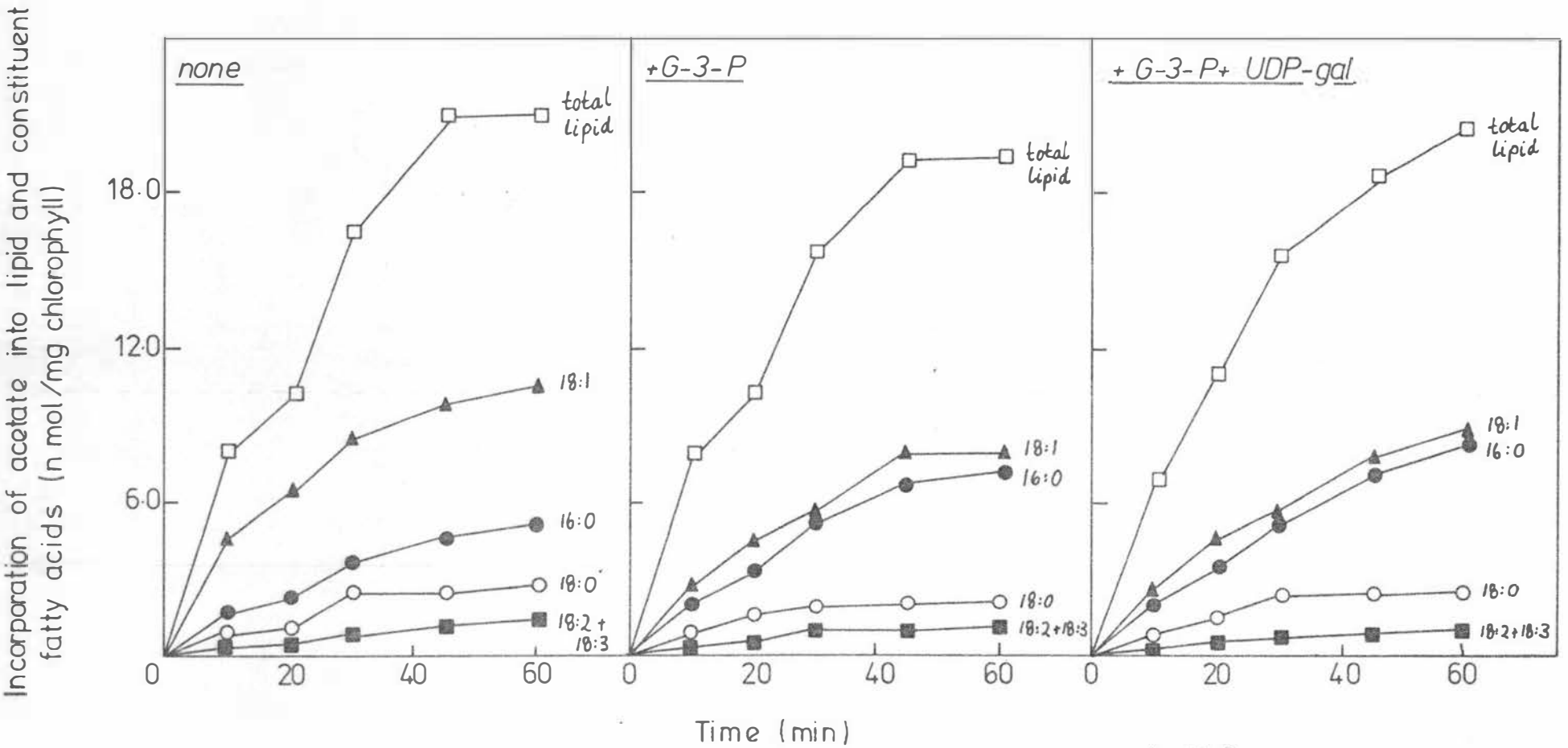


Fig. 4-13. The effect of sn-glycerol-3-phosphate and UDP-galactose on the rates of $[1-^{14}C]$acetate incorporation into lipid and constituent fatty acids by maize chloroplasts

The data in this figure represent fatty acid analysis of the total lipids already referred to in Table 4 (p. 68).

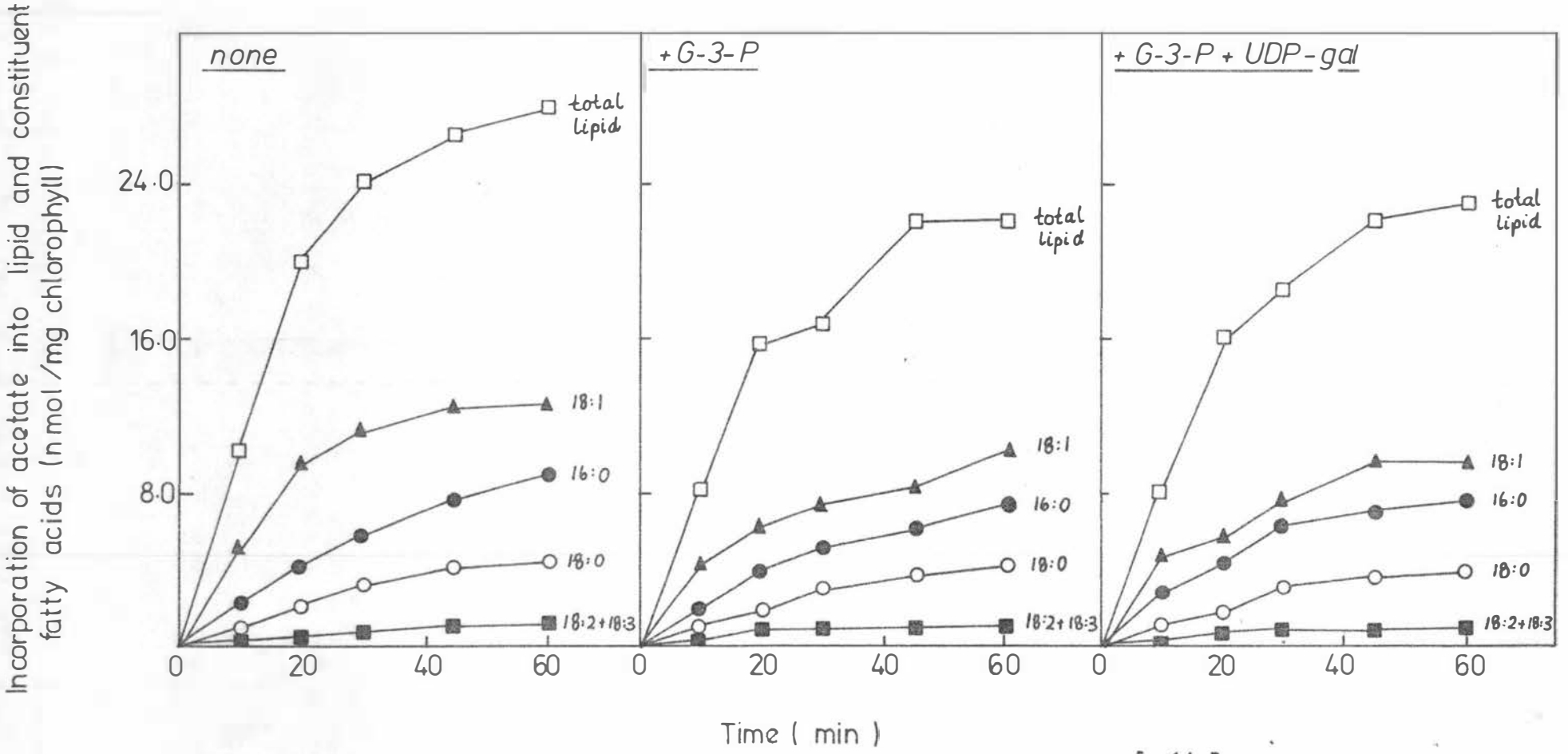


Fig. 4-13. The effect of sn-glycerol-3-phosphate and UDP-galactose on the rates of [1-^{14}C]acetate incorporation into lipid and constituent fatty acids by sweetcorn chloroplasts

The data in this figure represent fatty acid analysis of the total lipids already referred to in Table 4 (p. 69).

4.3 Diglyceride and Monogalactosyldiglyceride Biosynthesis by Spinach Chloroplasts

4.3.1 The Influence of sn-glycerol-3-phosphate and UDP-galactose on the Incorporation of Fatty Acids into Diglycerides and Monogalactosyldiglycerides

In the previous experiments (Fig. 4-12, p. 71; Fig. 4-13, p. 72; Fig. 4-14, p. 73) the addition of G-3-P affected the synthesis of oleic and palmitic acids from acetate, but had little or no effect on the synthesis of other fatty acids. The levels of labelled oleate and palmitate in the MGDG, DG and FFAs synthesized by spinach chloroplasts after 60min, referred to in Table 4 (p. 67), are given in Table 5 (p. 75). In the absence of G-3-P and UDP-galactose equal amounts of oleate and palmitate were incorporated into MGDG while slightly less oleate than palmitate was incorporated into DG (Table 5, p. 75). The free fatty acid fraction was rich in oleic acid giving a 18:1/16:0 ratio of 1.9. It is clear from the oleate:palmitate ratios that the proportions of oleate and palmitate incorporated into MGDG and DG are not necessarily determined by the proportions of these two fatty acids synthesized from acetate.

When G-3-P was added to the incubation medium more palmitate than oleate was synthesized (18:1/16:0 ratio of 0.6, Table 5, p. 75). Similar oleate:palmitate ratios were obtained for MGDG and DG with a slightly lower ratio for the FFAs (Table 5, p. 75). Similar trends were observed when both G-3-P and UDP-galactose were present in the incubation medium. It would appear that in the presence of G-3-P, with or without UDP-galactose, that the proportions of oleate and palmitate incorporated into MGDG and DG reflect the proportions of these two fatty acids synthesized from acetate.

Table 5. The effect of sn-glycerol-3-phosphate and UDP-galactose on the oleate:palmitate ratio of lipids synthesized by spinach chloroplasts

The total lipids synthesized by spinach chloroplasts after 60min incubation with and without added G-3-P or UDP-galactose, were fractionated by t.l.c. (see Methods section 3.3.2 pp. 34 to 35). The levels of radioactivity in oleate and palmitate of total lipids, MGDG, DG and the free fatty acid fraction were determined by g.l.c. of the methyl esters and collection of the radioactive effluent followed by scintillation counting. For further details see Methods sections 3.3.3 (pp. 35 to 38) and 3.3.4 (p. 38). The data in this table represent fatty acid analysis of the 60min samples of spinach chloroplasts already referred to in Table 4 (p. 67).

Additions to incubation medium	Incorporation of acetate into 16:0 and 18:1 (nmol/mg chlorophyll/h)											
	none				G-3-P				G-3-P + UDP-galactose			
	Total lipid	16:0	18:1	18:1 / 16:0	Total lipid	16:0	18:1	18:1 / 16:0	Total lipid	16:0	18:1	18:1 / 16:0
Total lipid	*108.7	32.5	52.0	1.6	143.7	70.0	43.8	0.6	156.7	68.8	56.3	0.8
Monogalactosyldiglyceride	7.4	2.8	2.8	1.0	13.7	8.2	4.8	0.6	78.0	33.5	24.0	0.7
Diglyceride	13.7	6.8	5.2	0.8	50.5	25.0	14.6	0.6	9.2	3.8	2.8	0.7
Free fatty acids	44.2	13.0	25.0	1.9	20.2	10.5	5.0	0.5	17.3	8.8	4.5	0.5

* Total lipid unaccounted for by MGDG, DG and FFA fractions is largely phospholipid (see Table 4, p. 67).

4.3.2 The Influence of Triton X-100 Concentration on the Incorporation of [1-^{14}C]acetate into Lipid

The effect of Triton X-100 on the incorporation of oleate and palmitate into DG and MGDG was investigated since Stumpf and Boardman (1970) had demonstrated that oleic acid synthesis by spinach chloroplasts was stimulated by this detergent. This initial experiment was aimed at finding the Triton X-100 concentration which gave the optimum stimulation of acetate incorporation into lipid and its effect on the lipids synthesized.

Increasing the Triton X-100 concentration up to 160μM doubled acetate incorporation into lipid (Fig. 4-15, p. 77). At higher concentrations (500μM) Triton X-100 was inhibitory.

A five-fold stimulation of diglyceride synthesis was observed at optimal Triton X-100 concentrations, while a small increase in the level of free fatty acids synthesized was obtained using less than optimal Triton X-100 concentrations (Fig. 4-15, p. 77). The increase in DG accounted for over 80% of the increase in total incorporation with the remainder being accounted for by the increase in free fatty acids and phospholipids. The incorporation of long chain fatty acids into MGDG was not significantly increased by the addition of Triton X-100 (Fig. 4-15, p. 77).

4.3.3 The Effect of Triton X-100 on the Rates of [1-^{14}C]-acetate Incorporation into Lipids and Constituent Fatty Acids

4.3.3.(a) The Incorporation of [1-^{14}C]acetate into Lipids

The addition of 0.16mM-Triton X-100 to the incubation medium stimulated the rate of acetate incorporation into lipid (Fig. 4-16, p. 79). The rate of acetate incorporation observed in the presence of Triton X-100 was approximately twice the rate observed when 5mM-G-3-P was added to the incubation medium. The addition of both Triton X-100 and G-3-P resulted in rates of acetate incorporation only slightly higher than those obtained in the presence of Triton X-100 alone (Fig. 4-16, p. 79). The stimulation by Triton X-100 was apparent at shorter incubation times than the effect of G-3-P.

When Triton X-100 was added the rates of DG and FFAs

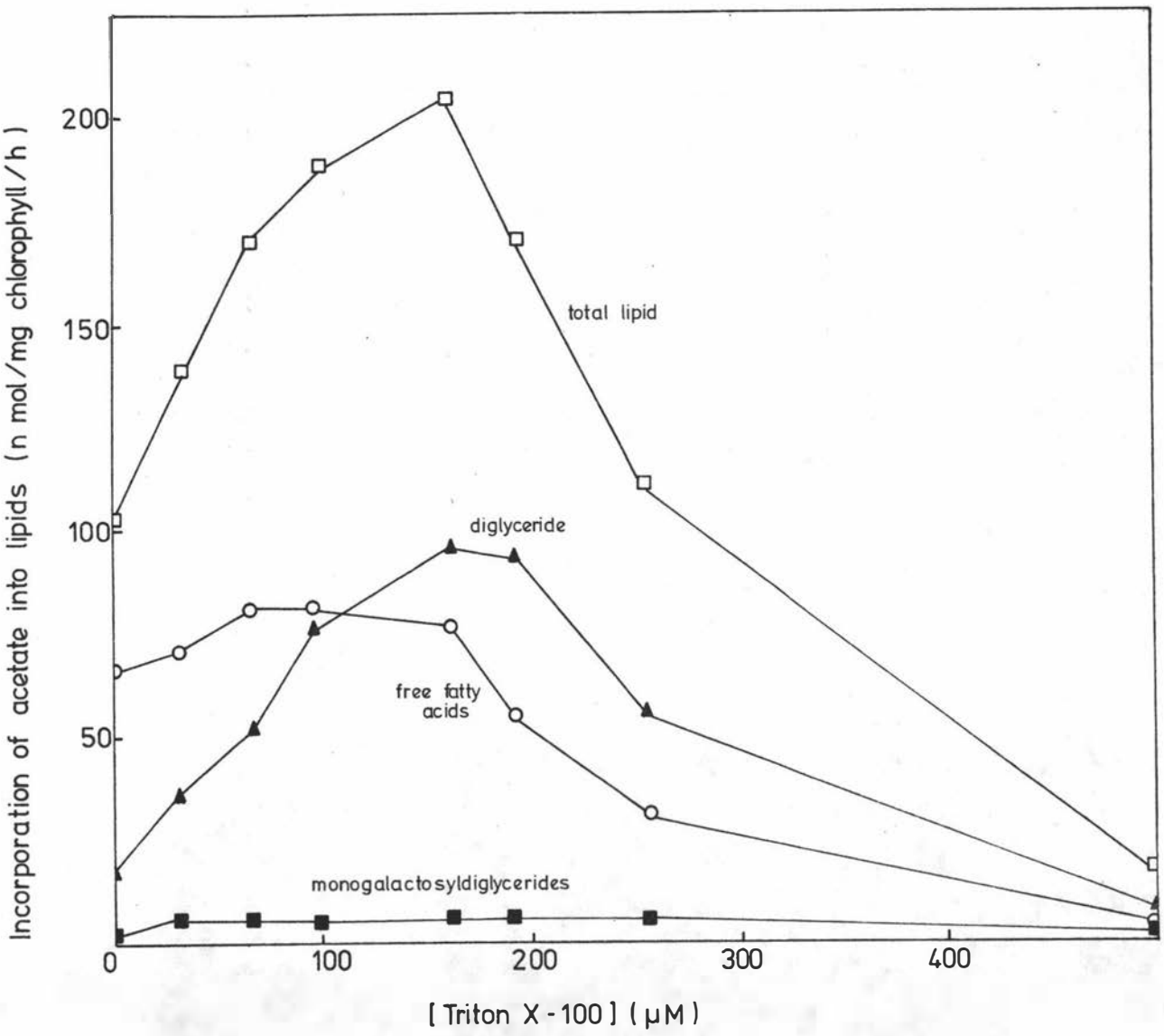


Fig. 4-15. The influence of Triton X-100 concentration on the incorporation of $[1-^{14}C]$acetate into lipids by spinach chloroplasts

Spinach chloroplasts were isolated by Method 1 and incubated for 1h in Medium A (refer Fig. 4-4, p. 51) containing the required amounts of Triton-X-100. 109nmol (6.73μCi) $[1-^{14}C]$acetate was added in a final volume of $1cm^3$ after the addition of substrates and chloroplasts. No G-3-P or UDP-galactose was present in the incubation medium.

synthesis were greater than in the controls or in the presence of G-3-P alone. The addition of both Triton X-100 and G-3-P to the incubation medium gave greater rates of diglyceride synthesis than Triton X-100 alone, while the labelling of the free fatty acid fraction was considerably reduced (Fig. 4-16, p. 79). Though acetate incorporation into other lipids (MG and more polar lipids) increased with the addition of Triton X-100, the proportion of label incorporated into these lipids decreased from about 40% of the total ^{14}C incorporated in the controls to 20% of the total ^{14}C incorporated in the presence of Triton X-100 or Triton X-100 and G-3-P (data not shown).

4.3.3.(b) The Incorporation of [1-^{14}C]acetate into Oleic and Palmitic Acids

The effect of the addition of Triton X-100 on the rates of acetate incorporation into oleic and palmitic acids is shown in Fig. 4-16 (p. 79). There was a six-fold and a four-fold stimulation of palmitic acid and oleic acid synthesis respectively and the rate of oleic acid synthesis was about twice that of palmitic acid, which is in sharp contrast to the effect of G-3-P. When Triton X-100 and G-3-P were both present in the incubating medium, the rates of oleic and palmitic acids synthesis were the same, but the rate of oleic acid synthesis was lower than when Triton X-100 alone was added (Fig. 4-16, p. 79).

Stearic acid accounted for most of the remaining label incorporated (12 to 19% of the total label), with linoleic and linolenic acids contributing together only 4 to 6% of the total label.

4.3.3.(c) The Incorporation of Oleate and Palmitate into Lipids

Fig. 4-16 (p. 79) also shows that Triton X-100 and G-3-P have very different effects on the rates of oleate and palmitate incorporation into diglycerides. With added G-3-P, palmitate incorporation into diglycerides was about twice that of oleate after 60min. The addition of Triton X-100 stimulated the rate of incorporation of both oleate and palmitate into diglyceride, with equal proportions of these two fatty acids being incorporated. The addition of Triton X-100 and G-3-P together, gave the greatest rate of

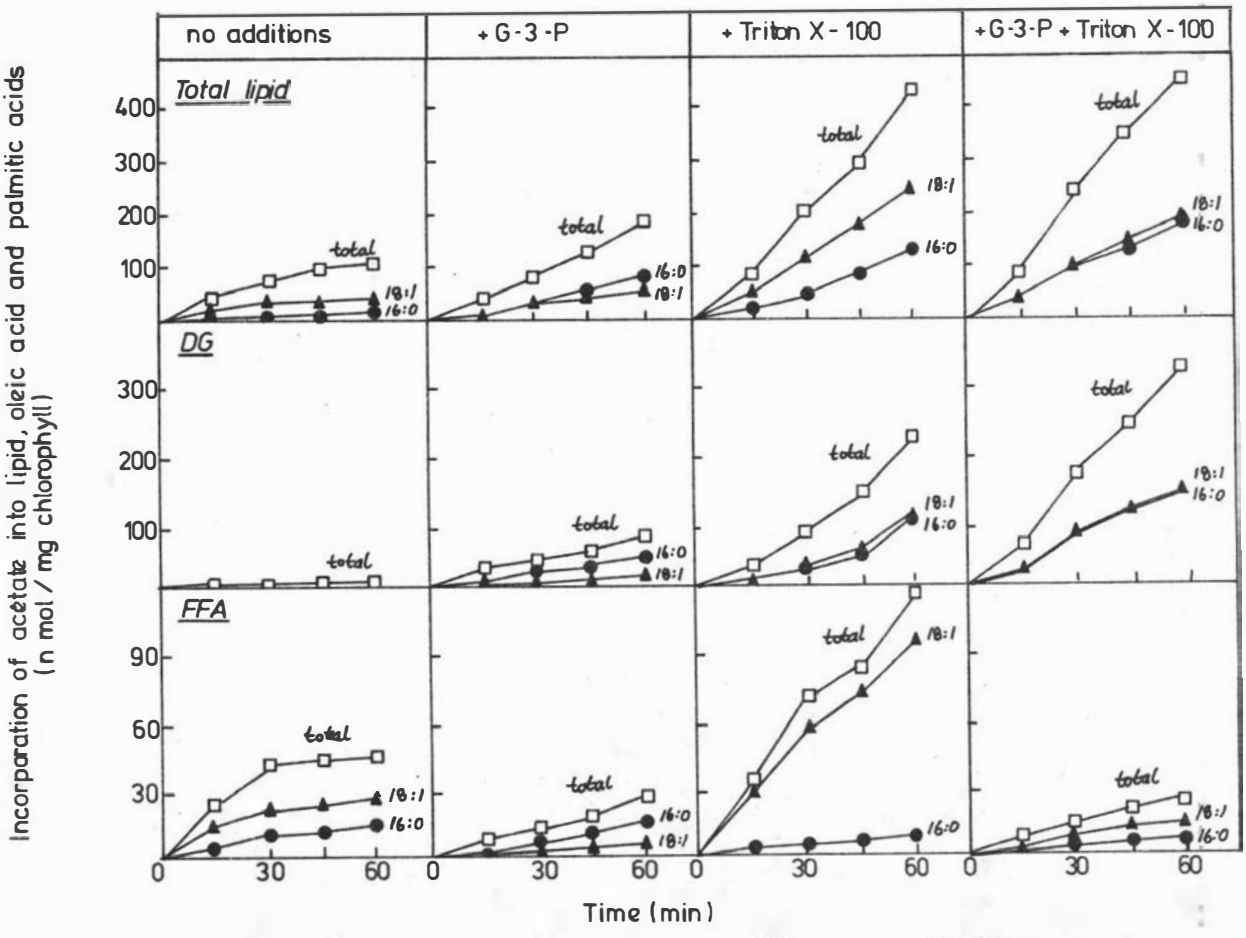


Fig. 4-16. The effect of Triton X-100 on the rates of [1-^{14}C]acetate incorporation into lipids and constituent oleic and palmitic acids by spinach chloroplasts

Chloroplasts were isolated by Method 1 and incubated in Medium A (refer Fig. 4-4, p. 51) containing 109nmol (6.73μCi)[1-^{14}C]acetate in a final volume of 1cm^3 after the addition of substrates and chloroplasts. The final concentrations of sn-glycerol-3-phosphate (G-3-P) and Triton X-100 were 5.0mM and 0.16mM respectively.

both oleate and palmitate into diglycerides and again equal proportions of oleate and palmitate were incorporated (Fig. 4-16, p. 79).

However, in the free fatty acid fraction, Triton X-100 led to much more oleic acid remaining as free fatty acid while having little effect on palmitic acid. In contrast, G-3-P led to less oleic acid remaining as free fatty acids. The presence of both Triton X-100 and G-3-P resulted in less oleic and palmitic acids remaining in the free fatty acids compared with the controls (Fig. 4-16, p. 79).

The effect of Triton X-100, with and without G-3-P, on the oleate:palmitate ratios are summarised in Table 6 (p. 81). It is apparent from the ratios of oleate:palmitate incorporated into diglycerides after 60min (Table 6, p. 81), that the fatty acid composition of the diglycerides synthesized in the control (in the absence of Triton X-100 and G-3-P) or in the presence of Triton X-100 alone, does not reflect the proportions of these two fatty acids synthesized from acetate. However, when G-3-P, with or without Triton X-100, was added to the incubation medium, the proportions of oleate and palmitate incorporated were identical to the proportions of these fatty acids synthesized from acetate (Fig. 4-16, p. 79; Table 6, p. 81).

Table 6. The effect of Triton X-100 on the oleate:palmitate ratio of total lipids, diglycerides and free fatty acids synthesized from [1-^{14}C]acetate by spinach chloroplasts

Oleate:palmitate ratios were calculated from the 60min incubation data from Fig. 4-16 (p. 79).

	Incorporation of acetate into 16:0 and 18:1 (nmol/mg chlorophyll/h)															
	Additions to incubation medium															
	none				G-3-P				Triton X-100				G-3-P + Triton X-100			
Lipid	Total lipid	16:0	18:1	18:1 16:0	Total lipid	16:0	18:1	18:1 16:0	Total lipid	16:0	18:1	18:1 16:0	Total lipid	16:0	18:1	18:1 16:0
Total	110.0	34.5	53.0	1.5	180.9	82.2	52.1	0.6	426.1	126.8	238.8	1.9	435.8	176.8	180.1	1.0
DG	14.8	6.4	7.0	1.1	72.2	46.1	22.0	0.5	221.0	95.2	102.1	1.1	324.7	142.5	140.0	1.0
FFA	44.8	11.9	25.0	2.1	23.7	12.2	6.4	0.5	120.7	8.0	94.1	11.8	23.0	4.0	12.0	3.0

4.3.4 The Effect of UDP-galactose on the Rates of [1-^{14}C]-acetate Incorporation into Lipids and Constituent Fatty Acids

This investigation was essentially the same as that described in the previous section (4.3.3, pp. 76 to 81), except that 0.15mM-UDP-galactose was added to all incubation conditions used, to enhance the synthesis of MGDG and to see what effect this had on the levels of oleic and palmitic acid synthesized.

4.3.4.(a) The Incorporation of [1-^{14}C]acetate into Lipids

The effects of Triton X-100 and G-3-P on acetate incorporation into lipids in this experiment are very similar to those described in the previous experiment. The results show that in the presence of UDP-galactose much of the stimulatory effect of both Triton X-100 and G-3-P is due to increased incorporation into MGDG (Fig. 4-17, p. 83). The addition of Triton X-100 to the incubation medium gave a rate of MGDG biosynthesis twice that obtained when G-3-P was added. The addition of G-3-P and Triton X-100 resulted in the most active synthesis of MGDG at all incubation times with very low rates of DG and FFA synthesis. However, in this experiment the synthesis of free fatty acids in the presence of Triton X-100 was approximately the same as that observed in the control. This was in contrast to the effect of Triton X-100 in the absence of UDP-galactose (Fig. 4-16, p. 79).

4.3.4.(b) The Incorporation of [1-^{14}C]acetate into Oleic and Palmitic Acids

The patterns of oleic and palmitic acids synthesis (Fig. 4-17, p. 83) are very similar to those observed in the absence of added UDP-galactose (Fig. 4-16, p. 79) and indicate that the addition of UDP-galactose had little effect on the proportions of oleic and palmitic acids synthesized from acetate.

4.3.4.(c) The Incorporation of Oleate and Palmitate into Lipids

The effects of Triton X-100 and G-3-P on the proportions of oleate and palmitate in MGDG (Fig. 4-17, p. 83), are much the same as on the incorporation of these fatty acids into diglycerides found in the previous experiment (Fig. 4-16, p. 79). A similar pattern was found in the diglyceride fraction.

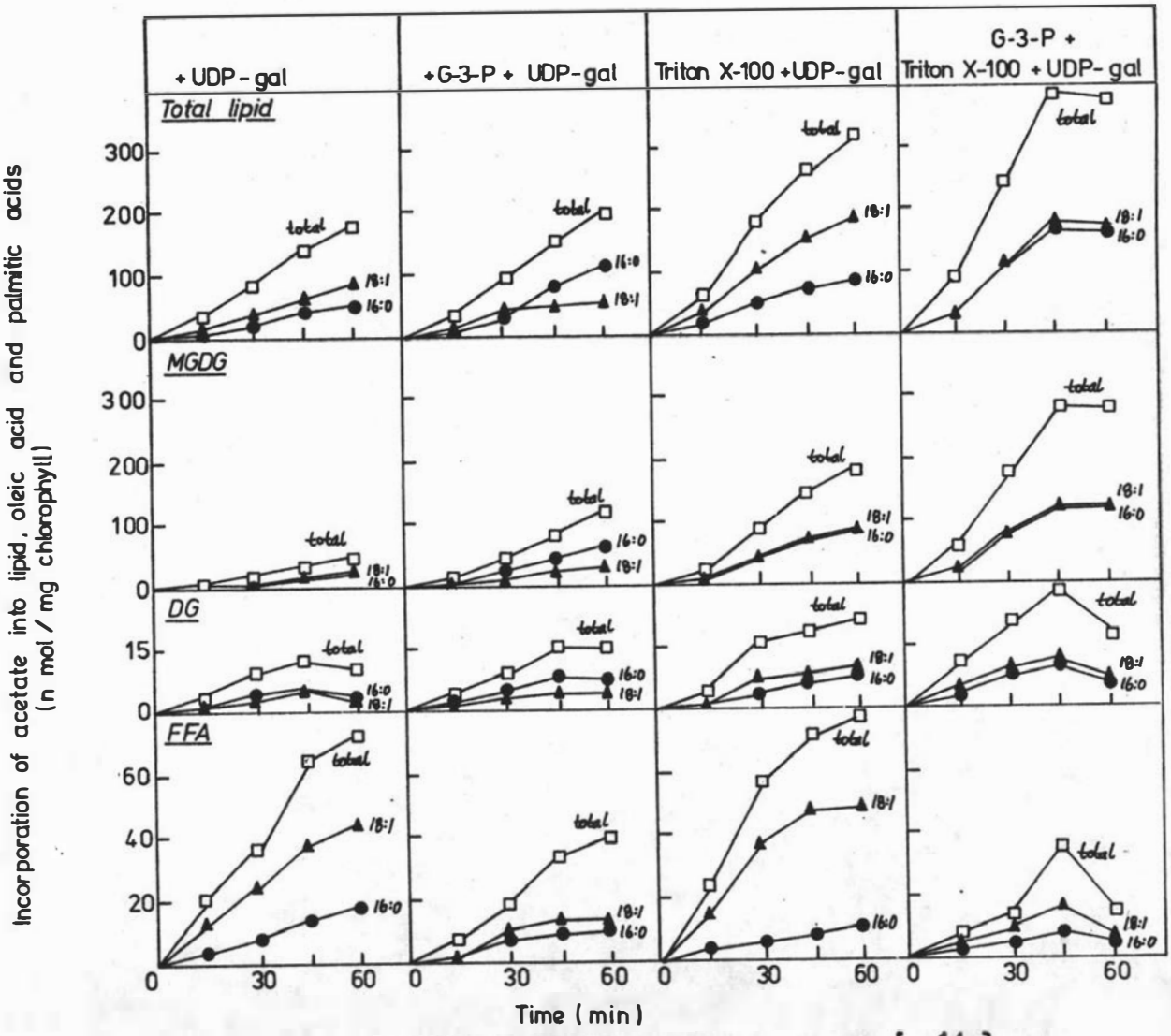


Fig. 4-17. The effect of UDP-galactose on the rates of [1-^{14}C] acetate incorporation into lipids and constituent oleic and palmitic acids by spinach chloroplasts

Experimental procedures were those as described in Fig. 4-16 (p. 79), except that UDP-galactose (UDP-gal), at a final concentration of 0.15mM, was added to all reaction mixtures.

The effects of Triton X-100 and G-3-P separately and together on the ratios of oleate:plamitate (18:1/16:0), in the presence of UDP-galactose, are summarised in Table 7 (p. 85). In the control (no G-3-P or Triton X-100) and in the Triton X-100 treated chloroplasts, the proportions of oleate and palmitate in the DG and MGDG (18:1/16:0 = 1) do not reflect the proportions of these fatty acids synthesized from acetate. However, in the presence of G-3-P (with and without Triton X-100) the proportions of oleate and palmitate in DG and MGDG reflected the proportions of these fatty acids synthesized from acetate (Table 7, p. 85).

In summary, it is apparent from both experiments (sections 4.3.3, pp. 76 to 81, and 4.3.4, pp. 82 to 84) that Triton X-100 and G-3-P have different effects on the synthesis of fatty acids and lipids from acetate. Triton X-100 stimulated the synthesis of fatty acids from acetate, but did not alter the proportions of the fatty acids synthesized, compared with the controls. However, G-3-P also stimulated the incorporation of acetate, but G-3-P enhanced the synthesis of palmitic acid. UDP-galactose had no significant effect on the proportions of the fatty acids synthesized from acetate.

The presence of Triton X-100 and G-3-P separately and together in the incubation medium, enhanced the synthesis of DG and MGDG (in the presence of UDP-galactose). Equal proportions of oleate and palmitate were incorporated into DG and MGDG under all conditions, with the exception being when G-3-P alone (or with UDP-galactose) was added to the incubation medium. In the presence of G-3-P, the proportions of oleate and palmitate incorporated into DG and MGDG, reflected the proportions of these fatty acids synthesized from acetate. In the controls (no G-3-P or Triton X-100) or when Triton X-100 was added to the incubation medium, more oleate than palmitate was synthesized, the free fatty acid fraction contained predominantly oleic acid while equal proportions were incorporated into DG and MGDG (when UDP-galactose was added).

Table 7. The effect of UDP-galactose on the oleate:palmitate ratio of total lipids, monogalactosyldiglycerides, diglycerides and free fatty acids synthesized from [1-^{14}C]acetate by spinach chloroplasts

Oleate:palmitate ratios were calculated from the 60min incubation data from Fig. 4-17 (p. 83).

	Incorporation of acetate into 16:0 and 18:1 (nmol/mg chlorophyll/h)															
	Additions to incubation medium															
	UDP-galactose				G-3-P + UDP-galactose				Triton X-100 + UDP-galactose				G-3-P + Triton X-100 + UDP-galactose			
	Total			18:1	Total			18:1	Total			18:1	Total			18:1
Lipid	lipid	16:0	18:1	16:0	lipid	16:0	18:1	16:0	lipid	16:0	18:1	16:0	lipid	16:0	18:1	16:0
Total	175.3	51.0	85.5	1.7	196.8	105.0	48.5	0.5	318.2	92.9	178.5	1.9	368.3	160.0	168.0	1.1
MGDG	39.4	16.2	17.8	1.1	109.4	61.0	28.8	0.5	177.2	76.1	77.5	1.0	274.0	119.0	121.5	1.0
DG	10.2	4.2	3.8	0.9	14.2	6.4	4.0	0.6	22.6	8.5	9.0	1.1	17.3	7.0	7.5	1.1
FFA	71.5	17.2	43.8	2.5	28.7	10.5	12.0	1.1	69.0	9.0	47.9	5.3	14.4	4.9	7.1	1.5

4.3.5 The Conversion of Diglycerides to Monogalactosyldiglycerides by Spinach Chloroplasts

In the previous experiments (sections 4.3.3, pp. 76 to 81, and 4.3.4, pp. 82 to 84), the monogalactosyldiglycerides synthesized under various conditions contained the same proportions of oleate and palmitate as did the diglycerides synthesized under the same conditions, but in the absence of added UDP-galactose. It would appear from these results that the diglyceride molecule is used as such for MGDG biosynthesis without any modification of the fatty acid content. The following experiments were aimed at comfirming these observations.

4.3.5.(a) The Stimulation of Monogalactosyldiglyceride Biosynthesis by the Addition of UDP-galactose

Diglyceride was the major product of acetate incorporation by spinach chloroplasts in the presence of added G-3-P and Triton X-100, but without UDP-galactose (Fig. 4-18, p. 87). MGDG was poorly labelled under these conditions. Most of the remaining label was present in the FFA fraction (10 to 17% of the total incorporation) with small amounts in MG, DGDG and more polar lipids.

The addition of UDP-galactose, 30min after the commencement of incubation with acetate, stimulated acetate incorporation into total lipid (Fig. 4-18, p. 87) and resulted in a twelve-fold stimulation of MGDG synthesis. The increase in MGDG was accompanied by a correspondingly rapid decrease in the level of diglyceride (Fig. 4-18, p. 87).

The presence of Triton X-100 in the incubation medium enhanced the stimulatory effect of added UDP-galactose, since in other experiments in which no Triton X-100 was present there was little stimulation of acetate incorporation on the addition of UDP-galactose after 30min incubation (Fig. 4-21, p. 95).

4.3.5.(b) The Incorporation of [1(3)-^{3}H]sn-glycerol-3-phosphate and [1-^{14}C]acetate into Lipids by Spinach Chloroplasts

It is evident from Fig. 4-18 (p. 87) that a precursor-product relationship exists between DG and MGDG. Since the fatty acid composition of DG and MGDG were found to be similar (sections 4.3.3, pp. 76 to 81, and 4.3.4, pp. 82 to 84), it is

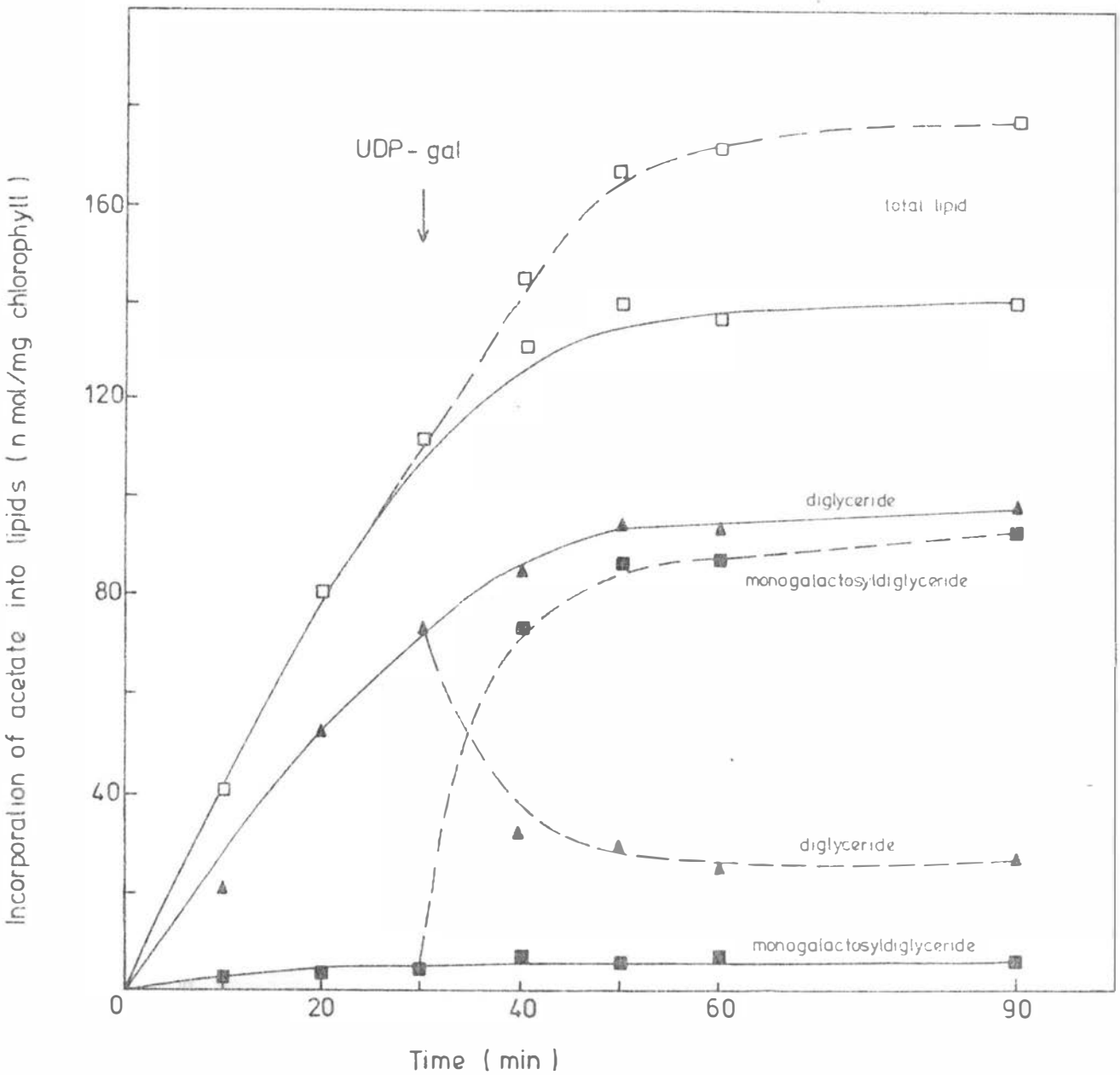


Fig. 4-18. The stimulation of monogalactosyldiglyceride biosynthesis by the addition of UDP-galactose

Spinach chloroplasts were isolated by Method 1 and incubated in Medium A (refer Fig. 4-4, p. 51) containing G-3-P and Triton X-100 at final concentrations of 5.0mM and 0.16mM respectively. 109nmol (6.73μCi)[1-^{14}C]-acetate was added in a final volume of 1cm^3 after the addition of substrates and chloroplasts. UDP-galactose (20μl of solution) was added to one series of tubes, 30min after the commencement of incubation, to give a final concentration of 0.15mM, while the other series of tubes were controls.

apparent that the diglycerides are converted to monogalactosyldiglycerides without deacylation and reacylation of either the diglycerides or the monogalactosyldiglycerides and the exchange of labelled acyl groups with a non-labelled pool. To confirm this the incorporation of [1 (3)-^{3}H]sn-glycerol-3-phosphate and [1-^{14}C]acetate into lipids was investigated. Initally, the effects of G-3-P concentration and UDP-galactose on the incorporation of [1 (3)-^{3}H] sn-glycerol-3-phosphate and [1-^{14}C]acetate into lipids was investigated.

Increasing the concentration of G-3-P increased the incorporation of both acetate and G-3-P into lipid (Table 8, p. 89). FFAs, DG and MGDG constituted 70 to 80% of the label incorporated from [1-^{14}C]acetate, with the remainder of the label distributed between MG, DGDG and more polar lipids. Diglycerides and MGDG contained over 70% of the incorporated label from [1 (3)-^{3}H] sn-glycerol-3-phosphate, with the remainder located in more polar lipids.

Increasing the concentration of G-3-P increased the proportion of ^{14}C label incorporated into diglyceride and was associated with a decrease in the proportion of ^{14}C label in FFAs (Table 8, p. 89). The incorporation of G-3-P into diglycerides decreased slightly with increasing G-3-P concentration and was accompanied by a corresponding increase in the incorporation of G-3-P into MGDG.

The addition of UDP-galactose had little effect on total incorporation, but stimulated the incorporation of label from both [1 (3)-^{3}H] sn-glycerol-3-phosphate and [1-^{14}C]acetate into MGDG (Table 8, p. 89). This was accompanied by a substantial decrease in the proportion of label incorporated from both [1 (3)-^{3}H] sn-glycerol-3-phosphate and [1-^{14}C]acetate into diglyceride. The proportions of both labels in the more polar lipids remained unchanged, though the proportions of label from [1-^{14}C]acetate in FFAs were slightly lower when UDP-galactose was included in the incubation medium.

A similar pattern of results was obtained when this experiment was repeated with maize chloroplasts. The addition of UDP-galactose to the incubation medium stimulated the incorporation of label from both [1 (3)-^{3}H] sn-glycerol-3-phosphate and [1-^{14}C]acetate into MGDG by maize chloroplasts.

Table 8. The influence of sn-glycerol-3-phosphate concentration on the incorporation of [1-^{14}C]acetate and [1(3)-^{3}H]sn-glycerol-3-phosphate into lipids by spinach chloroplasts

Chloroplasts were isolated by Method 1 and incubated in Medium A (refer Fig. 4-4, p. 52) containing 20.9nmol-(1.27μCi)[1-^{14}C]acetate and (7.86μCi)[1(3)-^{3}H]sn-glycerol-3-phosphate, supplemented with unlabelled sn-glycerol-3-phosphate (G-3-P) in a final volume of 1cm^3 after the addition of substrates and chloroplasts. To one series of tubes 0.15μmol-UDP-galactose was also added.

G-3-P concentration mM	Incorporation of acetate into total lipid (nmol/ mg chlorophyll/h)	Distribution of label between lipids (%) FFA	DG	MGDG	others$^{\alpha}$	Incorporation of G-3-P into total lipid (nmol/ mg chlorophyll/h)	Distribution of label between lipids (%) DG	MGDG	others$^{\alpha}$
0.0	42.2	62.5	3.9	3.9	29.7	—	—	—	—
0.5	49.3	31.0	34.2	5.4	29.4	46.8	67.0	6.2	26.8
1.25	57.8	21.8	42.8	6.6	28.8	47.3	69.4	8.0	22.6
2.5	57.5	17.0	50.7	7.5	24.8	65.9	61.2	11.9	26.9
5.0	58.4	15.5	51.4	6.7	26.4	106.2	59.3	14.3	26.4
+ UDP-galactose									
0.0	42.3	47.4	6.3	17.1	29.2	—	—	—	—
0.5	50.0	26.7	10.5	34.2	28.6	45.3	31.0	47.3	21.7
1.25	52.7	18.0	8.2	48.4	25.4	54.6	19.1	55.7	25.2
2.5	60.7	14.2	10.0	51.8	24.0	62.2	15.4	58.8	25.8
5.0	58.1	12.8	10.7	52.3	24.2	109.8	13.8	60.7	25.5

$^{\alpha}$ more polar lipids (mainly phospholipids), MG and an unknown compound (UK).

The increase in label from both substrates into MGDG was accompanied by a corresponding decreases in both ^{14}C and ^{3}H label in diglyceride.

As observed with spinach chloroplasts, the level of G-3-P incorporation into lipid by maize chloroplasts was increased by increasing the G-3-P concentration. However, acetate incorporation by maize chloroplasts was not stimulated by increasing the concentration of G-3-P, in contrast to spinach chloroplasts. The rate of G-3-P incorporation into lipid by maize chloroplasts was comparable to that obtained with spinach chloroplasts (80nmol of G-3-P/mg of chlorophyll/h at 5mM-G-3-P, compared with 106nmol of G-3-P/mg of chlorophyll/h at 5mM-G-3-P for spinach chloroplasts). However, the rate of acetate incorporation into lipid by maize chloroplasts, under the same conditions, was considerably lower (10nmol of acetate/mg of chlorophyll/h. compared with 58.4nmol of acetate/mg of chlorophyll/h for spinach chloroplasts at 0.02mM-acetate. The lower acetate concentration used in these experiments was to aid the detection of both labels together, but was found not to be necessary in later experiments).

4.3.5.(c) Rates of Incorporation of [1(3)-^{3}H]sn-glycerol-3-phosphate and [1-^{14}C]acetate into lipids by Spinach Chloroplasts

The rates of G-3-P and acetate incorporation into lipid were slightly stimulated with the inclusion of UDP-galactose in the incubation medium. The addition of UDP-galactose 30min after the commencement of the incubation, stimulated the rate of acetate incorporation slightly, but the rate of G-3-P incorporation remained unchanged (Table 9, p. 91).

FFAs, DG and MGDG constituted over 70% of the incorporated acetate, while DG and MGDG contained over 50% of the G-3-P incorporated into lipid (Fig. 4-19, p. 92). The addition of UDP-galactose stimulated the incorporation of label from both [1-^{14}C]acetate and [1(3)-^{3}H]sn-glycerol-3-phosphate into MGDG with a corresponding decrease in the levels of both labels in diglyceride (Fig. 4-19, p. 92). However, since no Triton X-100 was present, the levels of acetate incorporation into FFAs were unchanged by the addition of UDP-galactose.

Table 9. Rates of [1-^{14}C]acetate and [1(3)-^{3}H]sn-glycerol-3-phosphate into lipids by spinach chloroplasts

Chloroplasts were isolated by Method 1 and incubated in Medium A (refer Fig. 4-4, p. 52) containing 2.5μmol (7.86μCi)[1(3)-^{3}H]sn-glycerol-3-phosphate and 104.5nmol (6.37μCi)[1-^{14}C]acetate in a final volume of 1cm^{3} after the addition of substrates and chloroplasts. When UDP-galactose addition was required, 20μl of solution containing 0.15μmoles was added after 30min. 2.5μmoles of G-3-P was used instead of the optimum 5.0μmoles because the dilution of the [^{3}H]label at higher concentrations of G-3-P reduced the incorporation of ^{3}H to levels which were time-consuming to count.

	Incorporation of substrate into lipids (nmol/mg chlorophyll)					
	no UDP-galactose added		UDP-galactose added after 30min		UDP-galactose added at zero time	
Time min	acetate	G-3-P	acetate	G-3-P	acetate	G-3-P
10	18.0	42.7	—	—	20.0	57.2
20	46.0	67.2	—	—	49.9	86.0
30	78.5	95.0	—	—	80.1	107.5
35	—	—	90.0	108.8	—	—
40	—	—	99.8	136.8	—	—
45	108.0	145.1	112.0	138.2	116.0	147.5
60	134.5	154.5	147.0	151.0	148.0	157.5

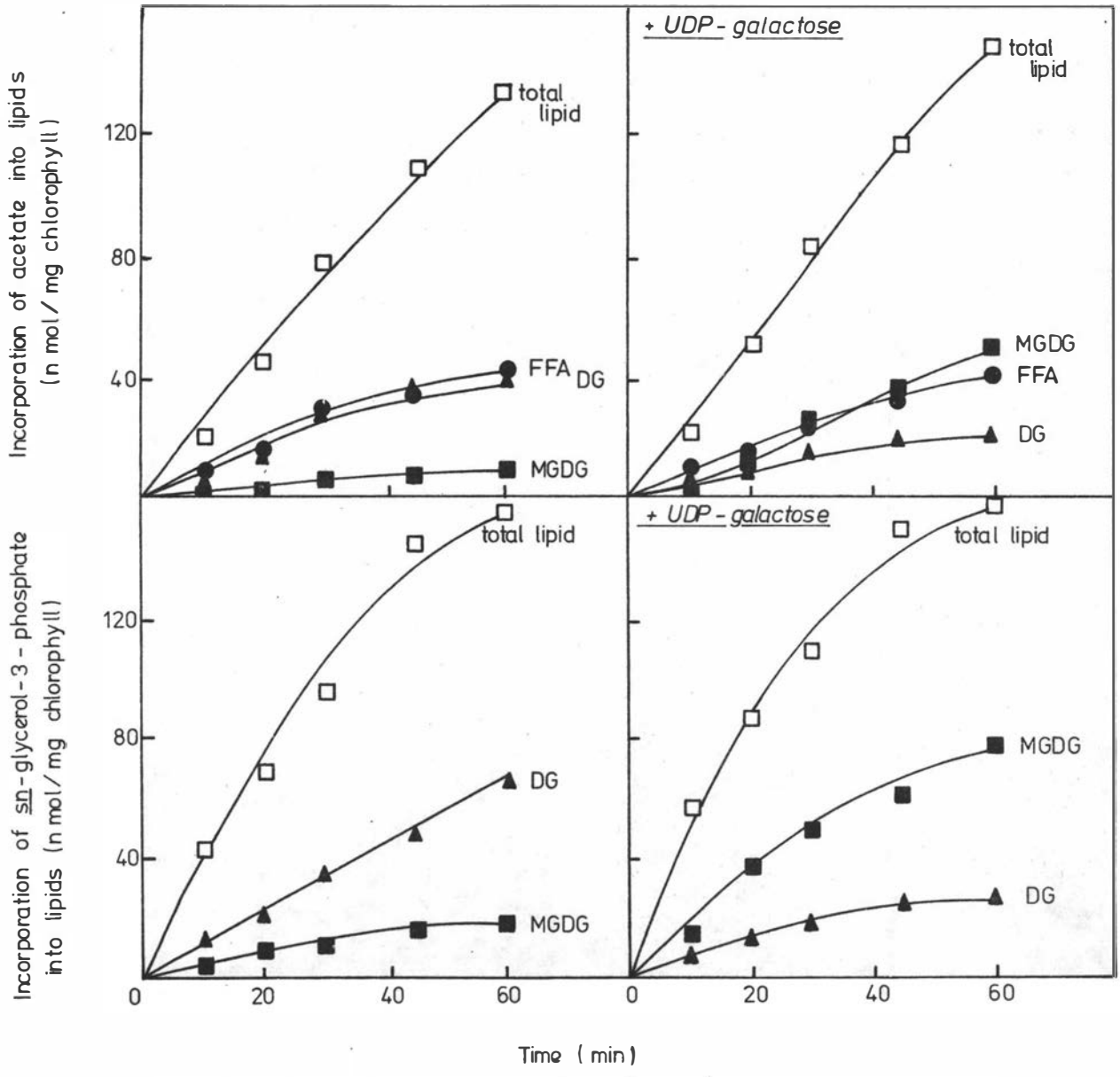


Fig. 4-19. Rates of [1-^{14}C] acetate and [1(3)-^{3}H] sn-glycerol-3-phosphate incorporation into monogalactosyldiglyceride, diglyceride and free fatty acids by spinach chloroplasts

The data in this figure represent lipid analysis of some of the total lipids referred to in Table 9 (p. 91).

In the absence of added UDP-galactose, the molar ratio of G-3-P:acetate incorporated into diglycerides was 2.3 at 10min after the commencement of incubation (Fig. 4-20, p. 94). This value decreased with time to remain at approximately 1.6 from 45 to 60min. The molar ratio of G-3-P:acetate incorporated into MGDG was 4.6 at 10min after the commencement of incubation, but as with diglycerides the ratio decreased to a constant value of a little less than 2 after 60min. When UDP-galactose was included in the incubation medium the same ratios after 10min incubation were appreciably lower for both diglycerides and MGDG than those obtained in the absence of UDP-galactose. However, the difference in the ratios when the incubation was conducted with and without UDP-galactose disappeared after 20min, with similar ratios being maintained throughout the remainder of the incubation period. This pattern applied to both diglycerides and monogalactosyldiglycerides (Fig. 4-20, p. 94). It would appear from these results that during short incubation times the incorporation of G-3-P into DG and MGDG was more rapid than the incorporation of fatty acids synthesized from acetate.

The incorporation of label from both substrates into MGDG was stimulated by the addition of UDP-galactose during the course of the incubation (Fig. 4-21, p. 95). This stimulation of MGDG synthesis was accompanied by a decrease in the amounts of ^{14}C and ^{3}H in diglyceride.

The molar ratio of G-3-P:acetate incorporated into MGDG after the addition of UDP-galactose was similar to the ratio of these substrates incorporated into diglycerides in the absence of UDP-galactose (Fig. 4-22, p. 96). Moreover, the G-3-P:acetate ratios of the diglycerides remaining after the addition of UDP-galactose were very similar, over the time range studied, to those of MGDG and the diglycerides synthesized in the absence of UDP-galactose. The similarity of the ratios for monogalactosyldiglycerides and diglycerides indicate that the diglycerides are converted to monogalactosyl-diglycerides without any detectable exchange of the labelled fatty acids with a non-labelled pool of fatty acids.

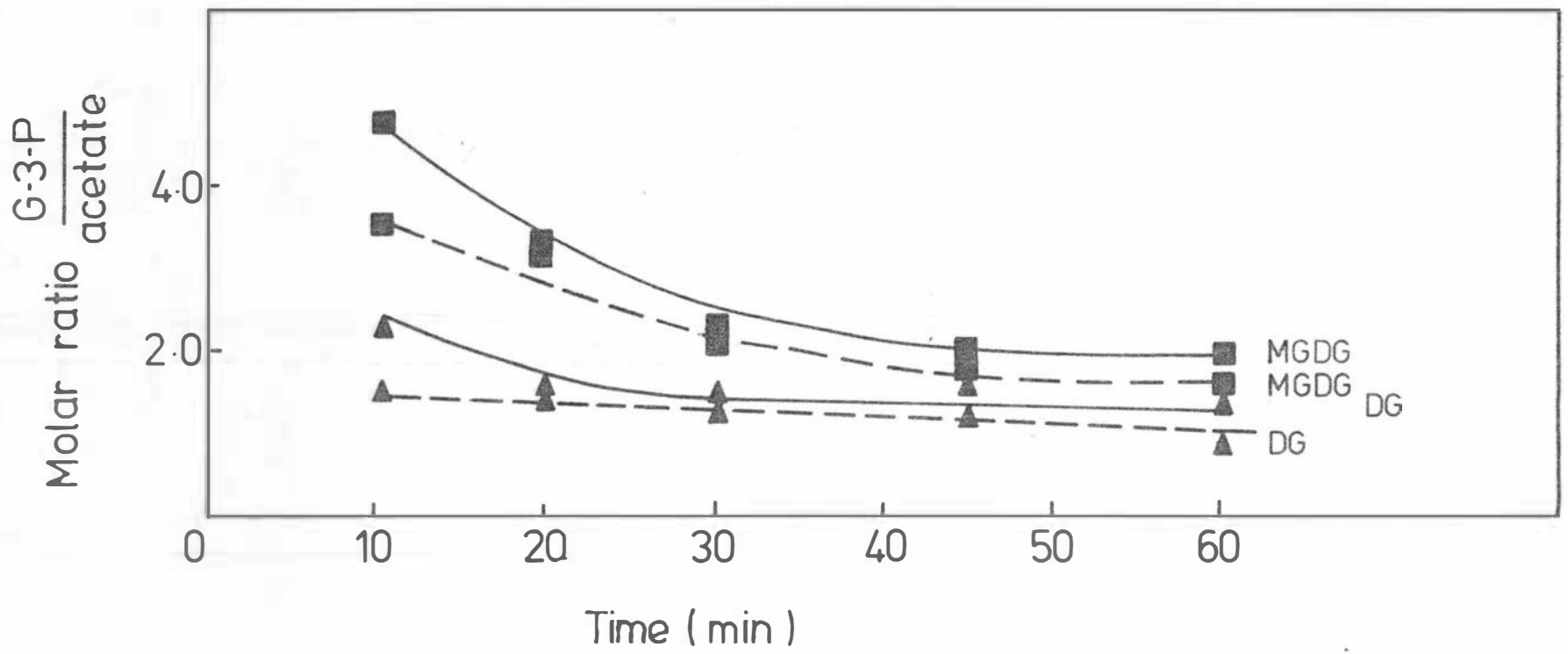


Fig. 4-20. The molar ratio of G-3-P:acetate incorporated into monogalactosyldiglyceride and diglyceride by spinach chloroplasts

Ratios were calculated from the data presented in Fig. 4-19 (p. 92) for monogalactosyldiglyceride (MGDG) and diglyceride (DG) synthesized in the absence and presence of UDP-galactose.

MGDG - UDP-galactose MGDG + UDP-galactose

DG - UDP-galactose DG + UDP-galactose

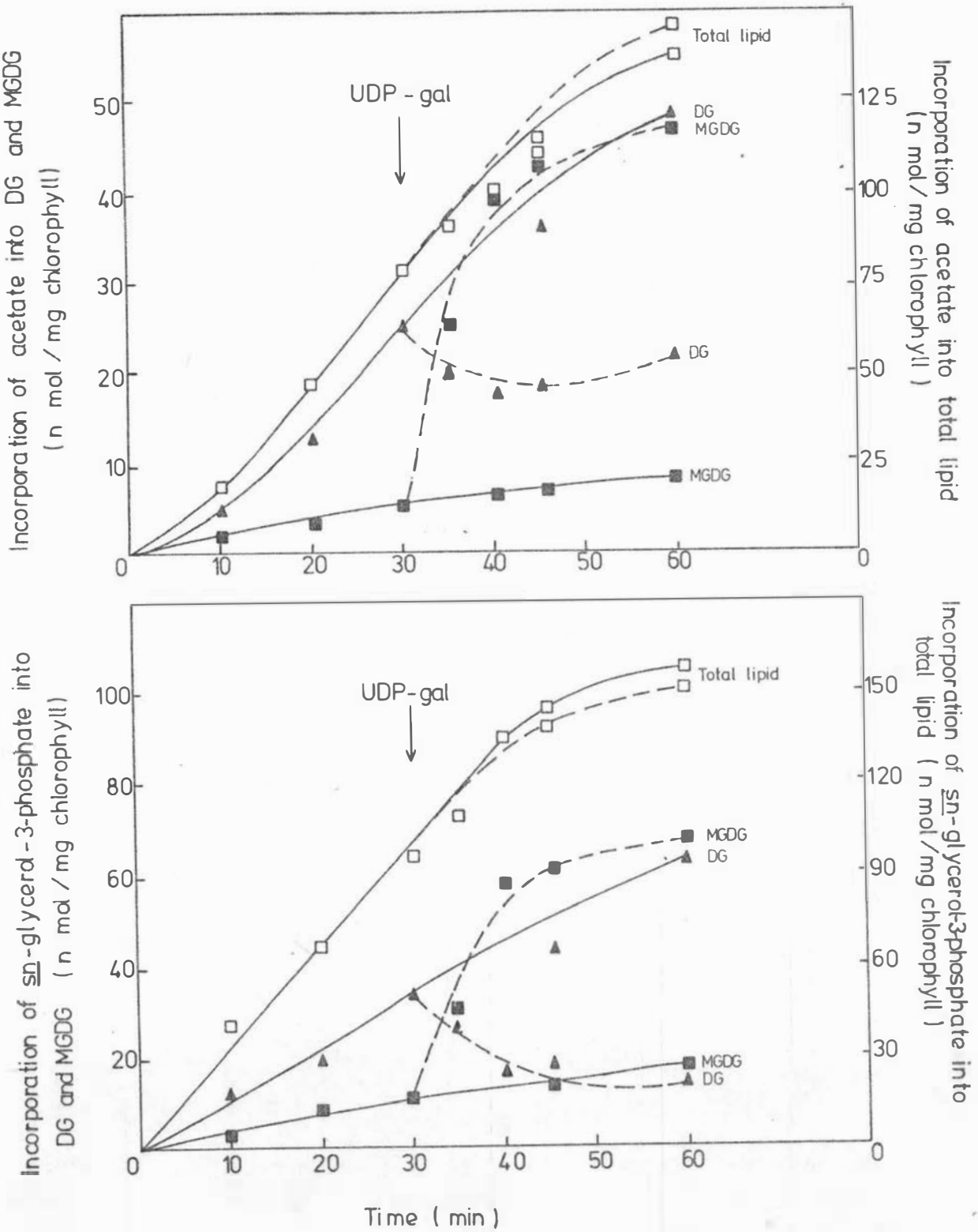


Fig. 4-21. Stimulation of the rates of [1-^{14}C]acetate and [1(3)-^{3}H]sn-glycerol-3-phosphate incorporation into monogalactosyldiglyceride by UDP-galactose

The data in this figure represent lipid analysis of some of the total lipids referred to in Table 9 (p. 91). The unbroken lines represent the lipids synthesized following the addition of UDP-galactose (UDP-gal) 30min after the commencement of the incubation.

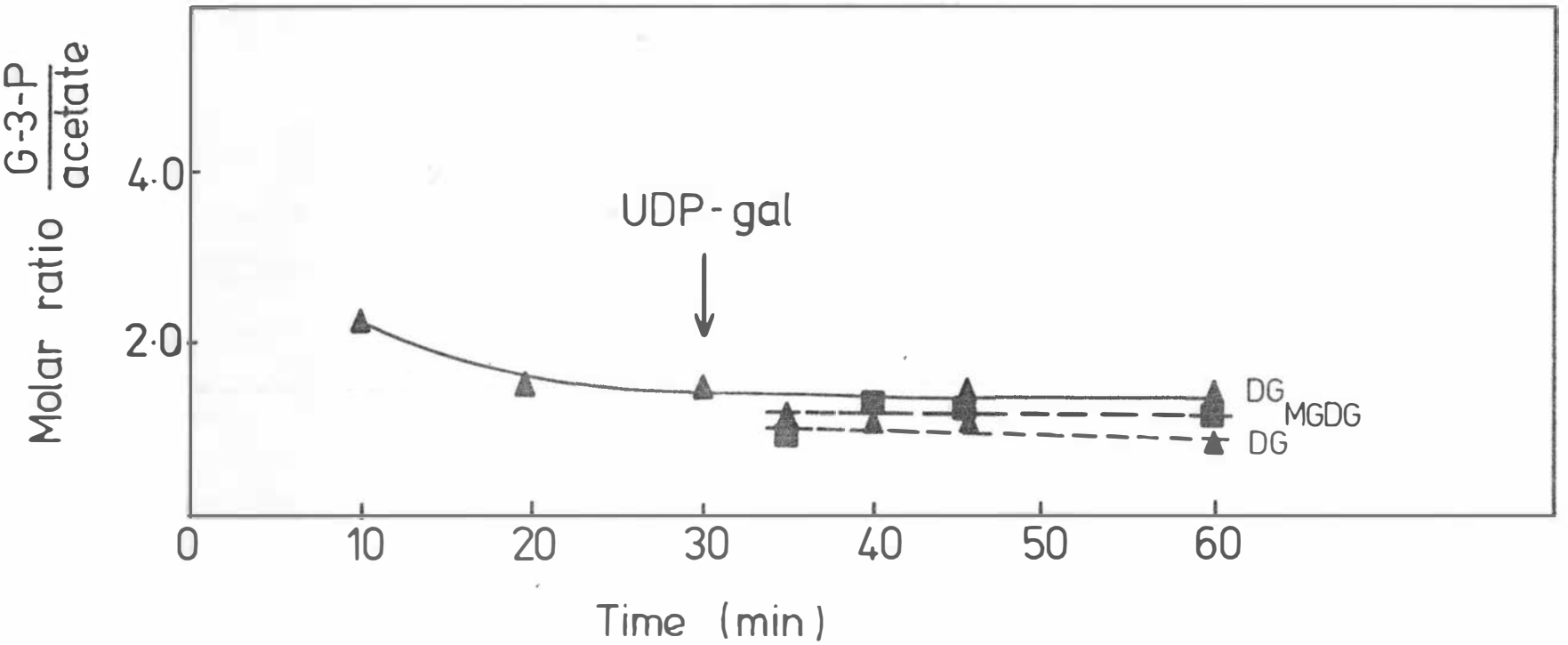


Fig. 4-22. The molar ratio of G-3-P:acetate incorporated by spinach chloroplasts into monogalactosyldiglyceride following UDP-galactose addition 30min after commencement of the incubation

The molar ratios were calculated from the data presented in Fig. 4-21 (p. 95) for diglyceride (DG) synthesized in the absence of UDP-galactose (UDP-gal) or after the addition of UDP-galactose and monogalactosyldiglyceride (MGDG) synthesized after the addition of UDP-galactose.

▲——▲ DG - UDP-galactose

▲– – –▲ DG + UDP-galactose

■– – –■ MGDG + UDP-galactose

4.3.5.(d) The Effect of UDP-galactose on the Incorporation of Oleate and Palmitate into Diglycerides and Monogalactosyldiglycerides by Spinach Chloroplasts

The results of the previous experiment (section 4.3.5.(c), pp. 90 to 96) showed that diglycerides were converted to monogalactosyldiglycerides without any detectable modification. This finding would also be expected to be apparent from the levels of oleate and palmitate in the diglycerides and monogalactosyldiglycerides after the addition of UDP-galactose during the course of the incubation. Thus the levels of oleate and palmitate, synthesized from acetate, were determined for the diglycerides and monogalactosyl-diglycerides synthesized in the previous experiment (Fig. 4-21, p. 95) and are presented in Fig. 4-23 (p. 98).

In the presence of added G-3-P, approximately equal amounts of labelled oleate and palmitate were incorporated into diglycerides during the first 30min of incubation (Fig. 4-23, p. 98). At longer incubation times the palmitate content of diglycerides contained more ^{14}C-label than the oleate constituent. A similar labelling pattern was evident for the constituent oleate and palmitate of MGDG.

The addition of UDP-galactose, 30min after the commencement of the incubation, stimulated the levels of ^{14}C in both oleate and palmitate constituents of MGDG with a corresponding decrease in the levels of ^{14}C in these fatty acid constituents of diglycerides (Fig. 4-23, p. 98). 30min after the addition of UDP-galactose, the proporations of oleate and palmitate in MGDG were identical to the proportions of these fatty acids in diglycerides synthesized in the absence of UDP-galactose.

These results are consistent with the direct conversion of the diglycerides to monogalactosyldiglycerides without any modification of the diglycerides prior to galactosylation.

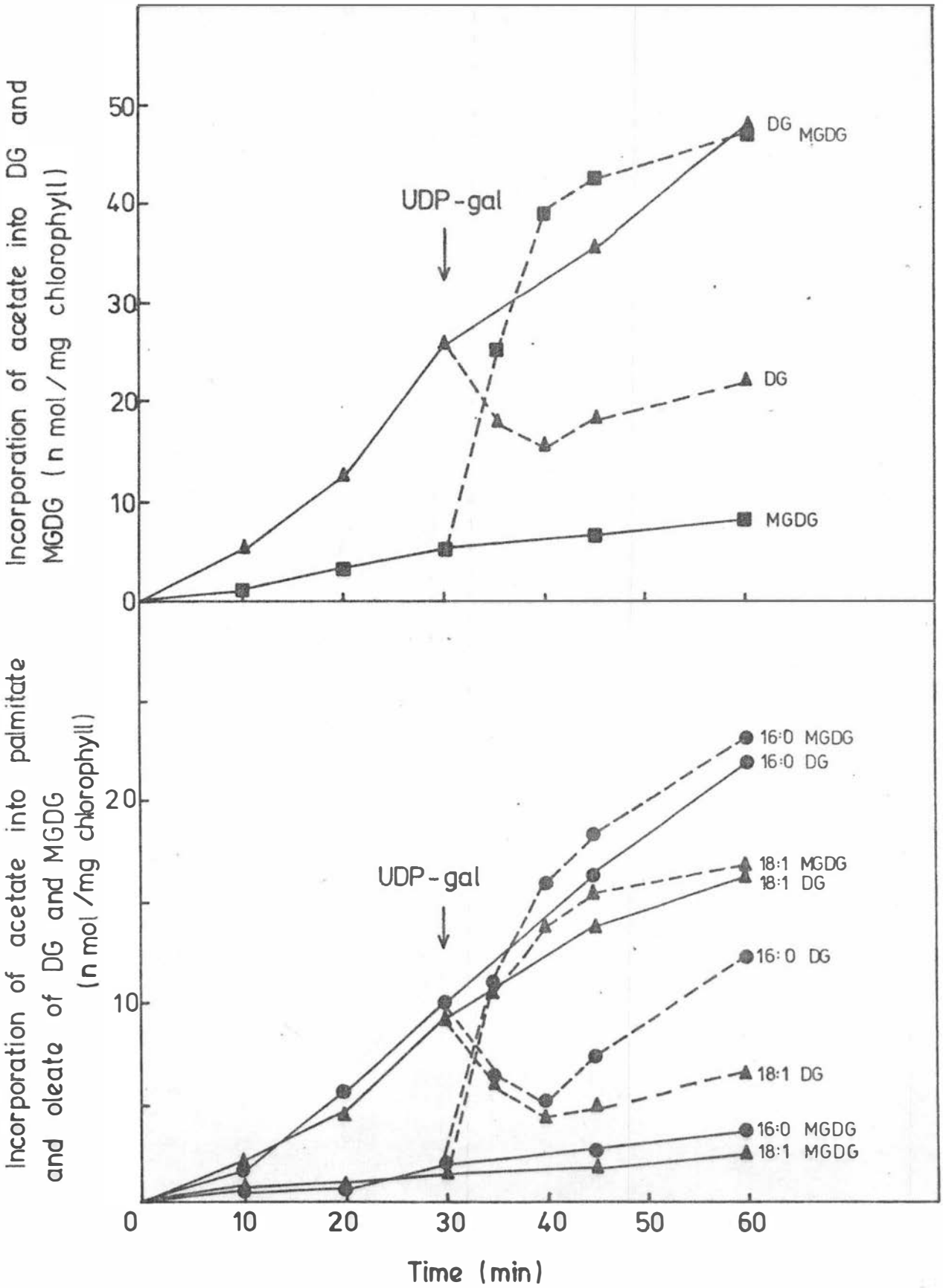


Fig. 4-23. The levels of palmitate and oleate in diglycerides and monogalactosyldiglycerides synthesized by spinach chloroplasts in the absence of UDP-galactose and after the addition of UDP-galactose

The levels of palmitate and oleate were determined for the diglycerides and monogalactosyldiglycerides from the previous experiment (data given in Fig. 4-21, p. 95).

4.3.6 The Positional Distribution of the Constituent Fatty Acids of Diglycerides and Monogalactosyldiglycerides Synthesized by Spinach Chloroplasts

It is apparent from the previous sections (4.3.5.(c), pp. 90 to 96; 4.3.5.(d), pp. 97 to 98) that diglycerides are converted directly to monogalactosyldiglycerides, without any apparent exchange of labelled acyl groups with a non-labelled pool. Since the diglycerides and monogalactosyl-diglycerides synthesized are composed largely of oleate and palmitate, the positional distribution of these two fatty acids within the DG and MGDG molecules will give an insight into the structure of the predominant species synthesized and the positional specificity of the acylating enzymes involved in the acylation of G-3-P. Pancreatic lipase was utilized to remove the fatty acid acylated to position 1 of both DG and MGDG, and the resulting MG and MGMG were isolated and the fatty acid composition determined (see section 3.3.5, pp. 38 to 39).

Palmitate constituted 88 to 95% of the labelled fatty acids in the monoglycerides obtained from diglycerides by hydrolysis with pancreatic lipase. Only small amounts of labelled of oleate, stearate, linoleate and linolenate were present in the monoglycerides (Table 10, p. 100). This shows that palmitate is predominately incorporated into position 2 of diglycerides. Since less than 50% of the total labelled fatty acid in the diglycerides is palmitate (except when the incubation medium is supplemented with G-3-P), the high palmitate content of the monoglycerides indicates that virtually all of the palmitate incorporated is in position 2. Oleate and the small amounts of stearate must therefore be located in position 1 of the diglycerides, while the small amounts of polyunsaturated fatty acids are, presumably, evenly distributed between positions 1 and 2. In the presence of G-3-P, palmitate comprises more than half of the labelled fatty acids. Consequently some palmitate must be located in position 1.

Table 11 (p. 101) shows that palmitate comprised 88 to 91% of the labelled fatty acids in the monogalactosyl-monoglycerides (MGMG) derived from MGDGs by lipase hydrolysis, and as observed with monoglycerides derived from diglycerides, only small proportions of oleate and stearate were present.

Table 10. The distribution of radioactivity in the constituent fatty acids of diglycerides and in the monoglycerides obtained from these diglycerides by hydrolysis with pancreatic lipase

The diglycerides, already referred to in Table 6 (p. 81), containing about 200,000d.p.m. of radioactivity in fatty acids were hydrolysed by pancreatic lipase. The incubation medium condisted of 10cm^3 of 0.2M-Tris/HCl buffer, pH7.6, 0.1cm^3 of 1%-sodium deoxycholate (wt/v), and 45%-$CaCl_2$ (wt/v). The incubation medium containing the diglycerides was sonicated for 5min, 50mg of pancreatic lipase was added and the mixture incubated for 2.5min at 40°C. The lipids were extracted with chloroform and seperated by t.l.c. For further details see Methods section 3.3.5 (pp. 38 to 39).

Experimental treatment (additive to chloroplasts from which DG isolated)		Proportion of label in fatty acids (% of total)			
		16:0	18:0	18:1	18:2 + 18:3
none	DG	41.7	10.0	42.4	5.9
	MG	89.4	3.1	4.2	3.3
G-3-P	DG	56.9	10.0	28.1	5.0
	MG	88.0	2.0	3.5	6.5
Triton X-100	DG	44.4	4.7	46.9	4.0
	MG	94.9	0.7	3.0	1.4
G-3-P + Triton X-100	DG	47.2	4.9	43.1	4.8
	MG	90.6	2.4	3.0	4.0

Table 11. The distribution of radioactivity in the constituent fatty acids of monogalactosyldiglycerides and in the monogalactosylmonoglycerides obtained from monogalactosyldiglycerides by hydrolysis with pancreatic lipase

The monogalactosyldiglycerides, already referred to in Table 7 (p. 85), containing about 200,000d.p.m. of radioactivity in fatty acids were hydrolysed by pancreatic lipase as described in Table 10 (p. 100), but incubated for 3h at 40°C. The lipids were extracted as before and separated by t.l.c. (see Methods section 3.3.5, pp. 38 to 39).

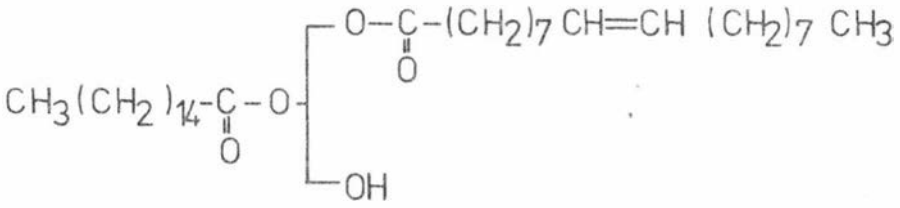
Experimental treatment (additive to chloroplasts from which MGDG isolated)		Proportion of label in fatty acids (% of total)			
		16:0	18:0	18:1	18:2 + 18:3
UDP-galactose	MGDG	40.0	6.4	43.9	9.7
	MGMG$^{\beta}$	87.7	3.6	5.0	3.7
UDP-galactose + G-3-P	MGDG	58.2	4.3	34.0	3.5
	MGMG$^{\beta}$	88.5	1.6	4.9	5.0
UDP-galactose + Triton X-100	MGDG	43.8	6.8	43.0	6.4
	MGMG$^{\beta}$	90.1	2.9	3.9	3.1
UDP-galactose + G-3-P + Triton X-100	MGDG	43.5	8.4	43.3	4.8
	MGMG$^{\beta}$	91.0	3.0	2.3	3.7

$^{\beta}$ monogalactosylmonoglyceride

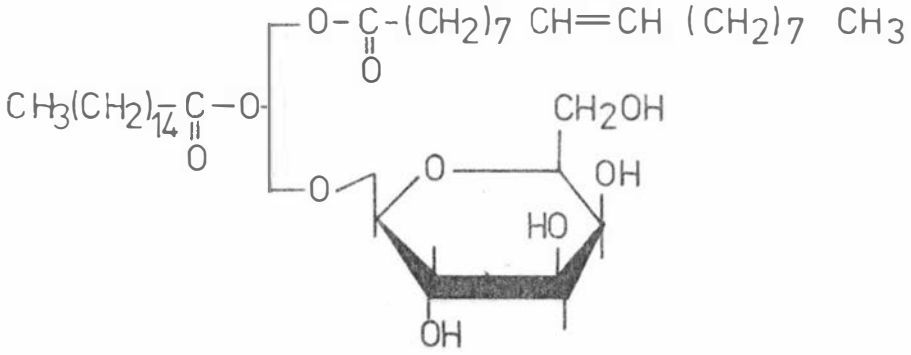
This proportion of palmitate in the monogalactosylmonoglycerides indicates that virtually all of the labelled palmitate in monogalactosyldiglycerides is located in position 2. The exception is, when G-3-P is included in the incubation medium and more than half of the labelled fatty acids synthesized are palmitate, consequently some palmitate is located in position 1 of these monogalactosyldiglycerides. Again, oleate and stearate must therefore be located in position 1 of the monogalactosyl-diglycerides.

Clearly the major diglyceride and monogalactosyl-diglyceride synthesized from acetate by spinach chloroplasts, contains oleate in position 1 and palmitate in position 2 as shown in Fig. 4-24 (p. 103). A small proportion of dipalmitoyl-DG and dipalmitoyl-MGDG would be synthesized when the ratio of oleate:palmitate was less than 1. This was the case when G-3-P was added to the chloroplast incubation medium (Table 6, p. 81; Table 7, p. 85). It is apparent that the diglycerides which accumulate when UDP-galactose is not added to the incubation medium had the same stereospecific distribution of the fatty acids as the monogalactosyldiglycerides synthesized when UDP-galactose was added to the incubation medium.

The incorporation of palmitate and oleate into the same molecule was confirmed by silver nitrate-t.l.c. of the MGDG synthesized in the presence of G-3-P, Triton X-100 and UDP-galactose. Only one band was detected by scanning and subsequent autoradiography.



1-oleoyl, 2-palmitoyl-sn-glycerol
(DG)



1-Oleoyl, 2-palmitoyl-[β-D-galactopyranosyl(1'→ 3)]-sn-glycerol
(MGDG)

Fig. 4-24. The major diglyceride and monogalactosyldiglyceride species synthesized by spinach chloroplasts

4.3.7 The Incorporation of [^{14}C]bicarbonate into Lipids and Constituent Fatty Acids by Spinach Chloroplasts

An outstanding feature of the incorporation of [1-^{14}C]acetate and [1(3)-^{3}H]sn-glycerol-3-phosphate into lipids (sections 4.3.5.(b), pp. 86 to 89; 4.3.5.(c), pp. 90 to 97), is that the amount of label incorporated from acetate into MGDG, DG and total lipid, was considerably less than that expected from the amounts of fatty acids required for the acylation of the G-3-P incorporated into these lipids. For example: the MGDG synthesized 30min after the addition of UDP-galactose in Fig. 4-21 (p. 95), contained 68nmol of G-3-P incorporated/mg of chlorophyll, but only 47nmol of acetate was incorporated/mg of chlorophyll. The acylation of this amount of G-3-P incorporated into MGDG would require 136nmol of fatty acids. Assuming that equal proportions of oleate and palmitate were incorporated, the amount of acetate incorporated would supply approximately 6nmol of these fatty acids. It is apparent that fatty acids were being synthesized from a substrate other than the exogenous acetate. Since Murphy and Leech (1977) had reported the ability of isolated photosynthesizing spinach chloroplasts to incorporate bicarbonate into acyl lipids, the utilization of the bicarbonate present in the incubation medium was investigated as a possible source of fatty acid carbon.

Spinach chloroplasts incorporated ^{14}C from $H^{14}CO_3^-$ into lipid and other non-volatile compounds (Fig. 4-25, p. 105). As the concentration of HCO_3^- increased up to 60mM-HCO_3^-, an approximately linear increase in the incorporation into lipid was observed (Fig. 4-25, p. 105). At 60mM-HCO_3^- the proportion of label in lipid accounted for about 60% of the total incorporation into non-volatile compounds. The incorporation of bicarbonate into either non-volatile compounds or lipids was unaffected by the addition of 100nmol of unlabelled acetate to the incubation medium (Fig. 4-25, p. 105), even though under these conditions 89nmol of acetate/mg of chlorophyll/h was incorporated into lipid.

The effect of adding G-3-P and UDP-galactose on the incorporation of HCO_3^- was investigated in further experiments using a bicarbonate concentration of 10mM. In the absence of added G-3-P and UDP-galactose 173nmol of HCO_3^-/mg of chlorophyll/h

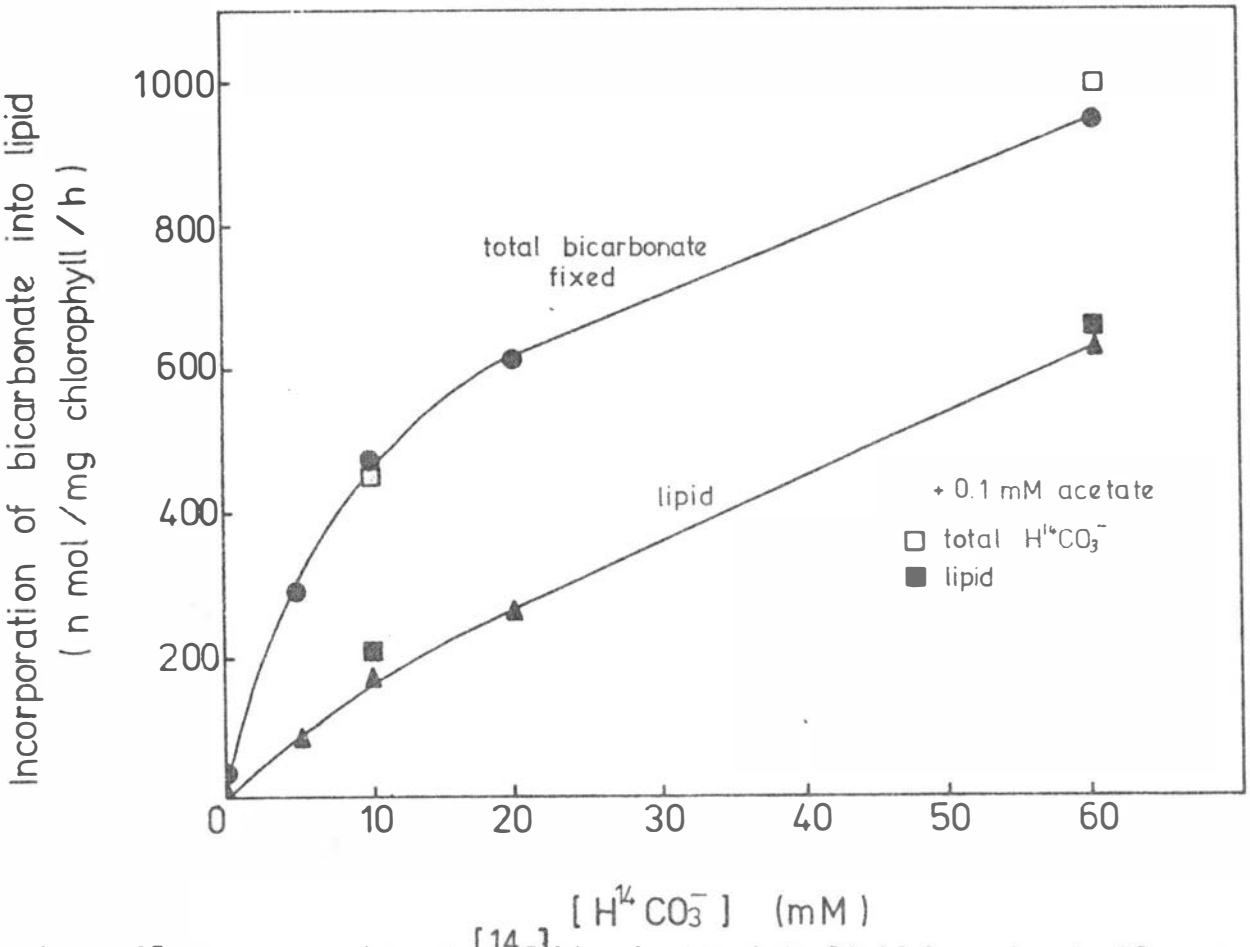


Fig. 4-25. Incorporation of $[^{14}C]$bicarbonate into lipid by spinach chloroplasts

Chloroplasts were isolated by Method 1 and incubated for 1h in Medium A (refer Fig. 4-4, p. 51), except that 0.14μmol (7.5μCi)$[^{14}C]$bicarbonate with various amounts of unlabelled bicarbonate replaced the bicarbonate (30mM) normally used. Total bicarbonate fixed was determined by taking aliquots of the acidified reaction mixture before the addition of $CHCl_3$/MeOH used for lipid extraction (refer Methods section 3.3.2, p. 34). These aliquots were taken to dryness under a stream of nitrogen at 40°C and counted for radioactivity. The lipids were extracted from the remainder of the reaction mixture and aliquots taken for determination of radioactivity.

was incorporated by spinach chloroplasts (Table 12, p. 107). The addition of G-3-P stimulated the incorporation almost two-fold, whereas the addition of UDP-galactose to the incubation medium gave a somewhat smaller stimulation. The addition of G-3-P together with UDP-galactose gave the greatest stimulation of bicarbonate incorporation into lipid.

In the absence of added G-3-P and UDP-galactose, label from bicarbonate was incorporated mainly into free fatty acids and diglycerides, with only minor proportions of the label incorporated into MGDG, PC and more polar lipids (Table 12, p. 107). The addition of G-3-P stimulated the synthesis of diglycerides with a corresponding decrease in the proportion of label in free fatty acids. The addition of UDP-galactose, in the absence of G-3-P, decreased the proportion of label in diglycerides and increased the proportion of label incorporated into MGDG. The addition of both G-3-P and UDP-galactose together increased the proportion of MGDG synthesized to about 50% of the total lipid synthesized. The various additions of G-3-P and UDP-galactose had little effect on the proportions of PC and more polar lipids synthesized.

Oleate, stearate and palmitate were the main fatty acids synthesized from bicarbonate and the proportions of these fatty acids was similar to those synthesized from acetate. The relative proportions of the fatty acids in total lipids synthesized from the two precursors were:- oleate: 52.5 and 68.5%; stearate: 11.8 and 10.2%; and palmitate: 25.2 and 15.7% from bicarbonate and acetate respectively.

Table 12. The incorporation of [^{14}C]bicarbonate into lipids by spinach chloroplasts

Spinach chloroplasts were isolated by Method 1 and incubated in Medium A, except that 10μmol (7.5μCi)[^{14}C]bicarbonate replaced the 30μmol of HCO_3^- normally present in Medium A (the lower concentration of HCO_3^- was used because the dilution of the [^{14}C]label at higher concentrations of bicarbonate reduced the incorporation of ^{14}C to levels which were time-consuming to count). The final volume of the reaction mixture was 1cm^3 after the addition of substrates and chloroplasts. The final concentrations of sn-glycerol-3-phosphate (G-3-P) and UDP-galactose were 5mM and 0.15mM respectively.

Additions to incubation medium	Incorporation of bicarbonate into lipids (nmol/mg chlorophyll/h)	Distribution of label between lipids (%)				
		FFA	DG	MGDG	PC	others[a]
none	173.0	56.6	30.7	3.2	7.6	1.9
G-3-P	303.1	23.4	65.0	3.5	5.0	3.1
UDP-galactose	237.0	54.0	15.5	22.9	5.9	1.7
G-3-P + UDP-galactose	358.1	11.9	29.7	50.7	5.3	2.4

[a] more polar lipids (mainly phospholipids, except phosphatidylcholine (PC)), MG and an unknown compound (UK).

4.3.8 The Effect of Magnesium and Manganese Ion Concentrations on the Incorporation of [1-^{14}C]acetate into Lipids and Constituent Fatty Acids by Spinach Chloroplasts

The investigations described in earlier sections have shown that addition of G-3-P, UDP-galactose and Triton X-100 gives a considerable stimulation of total lipid and fatty acid synthesis. A brief investigation of the effect of divalent metal ion concentration on the rates of acetate incorporation was carried out, since it is important to establish that the concentrations used in incubation Medium A were not limiting either total incorporation or any particular component process in the presence of the various additives. The effect of Mn^{2+} was also investigated, since it had been shown to stimulate acetate incorporation (Nakamura and Yamada, 1975a).

Additional Mg^{2+} was added to the incubation Medium A (which contains 1mM-Mg^{2+}) in the presence of G-3-P, UDP-galactose and Triton X-100. Increasing the Mg^{2+} concentration from 1mM up to 3mM, increased the incorporation of acetate into lipid almost two-fold, but there was little further increases at higher concentrations (Fig. 4-26, p. 109).

Mn^{2+} at concentrations up to 1mM (in addition to the 1mM-Mg^{2+} already present) increased the incorporation of acetate into lipid. At 1mM-Mn^{2+} the incorporation of acetate was stimulated over two-fold, but at higher concentrations the stimulation observed decreased until at 10mM-Mn^{2+} no stimulation of acetate incorporation was observed over the rates obtained in the absence of Mn^{2+} (Fig. 4-27, p. 111).

The increase in lipid synthesized from acetate, obtained either at higher Mg^{2+} concentrations or with Mn^{2+} + Mg^{2+}, was almost entirely due to an increase in the level of MGDG synthesized, as would be expected from the presence of G-3-P, UDP-galactose and Triton X-100 in the reaction medium (Fig. 4-26, p. 109; Fig. 4-27, p. 111).

Approximately equal amounts of oleate and palmitate were synthesized from acetate and the proportions of these two fatty acids was relatively unaltered at all Mg^{2+} and Mn^{2+} concentrations investigated (Fig. 4-26, p. 109; Fig. 4-27, p. 111). These two fatty acids and stearate constituted 88 to 93% of the label incorporated into fatty acids with only minor amounts (2 to 5%) of label present in linoleate and

Fig. 4-26. The effect of Mg^{++} concentration on [1-^{14}C]acetate incorporation into lipids and constituent fatty acids by spinach chloroplasts

Chloroplasts were isolated by Method 1 and incubated for 1h in Medium A (refer Fig. 4-4, p. 51) containing sn-glycerol-3-phosphate, UDP-galactose and Triton X-100 at final concentrations of 5.0mM, 0.15mM and 0.16mM respectively. 104nmol (6.24μCi) [1-^{14}C]acetate was used in a final volume of 1cm^3 after the addition of substrates and chloroplasts. Additional amounts of $MgCl_2$ were added to give the required Mg^{++} concentrations.

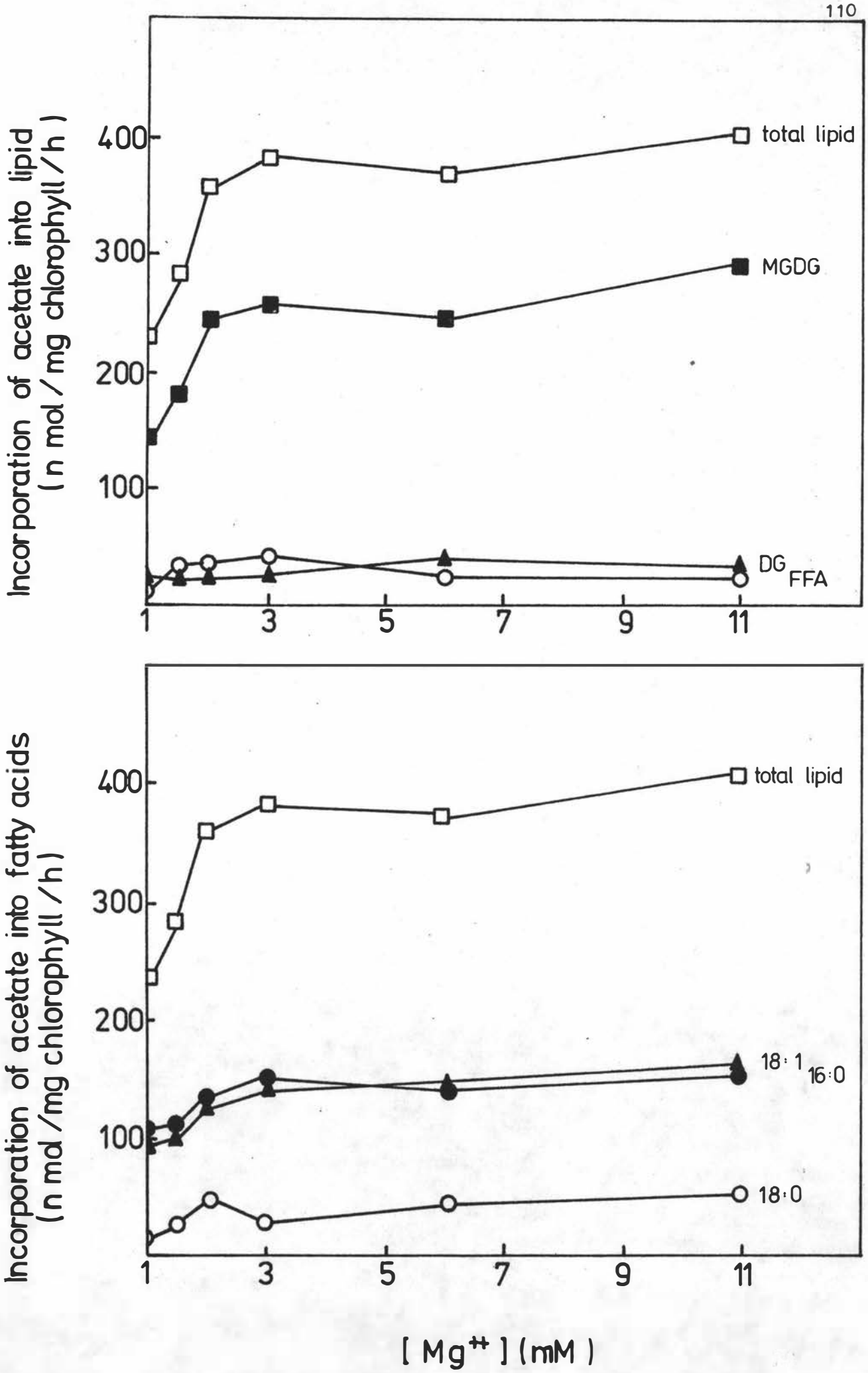
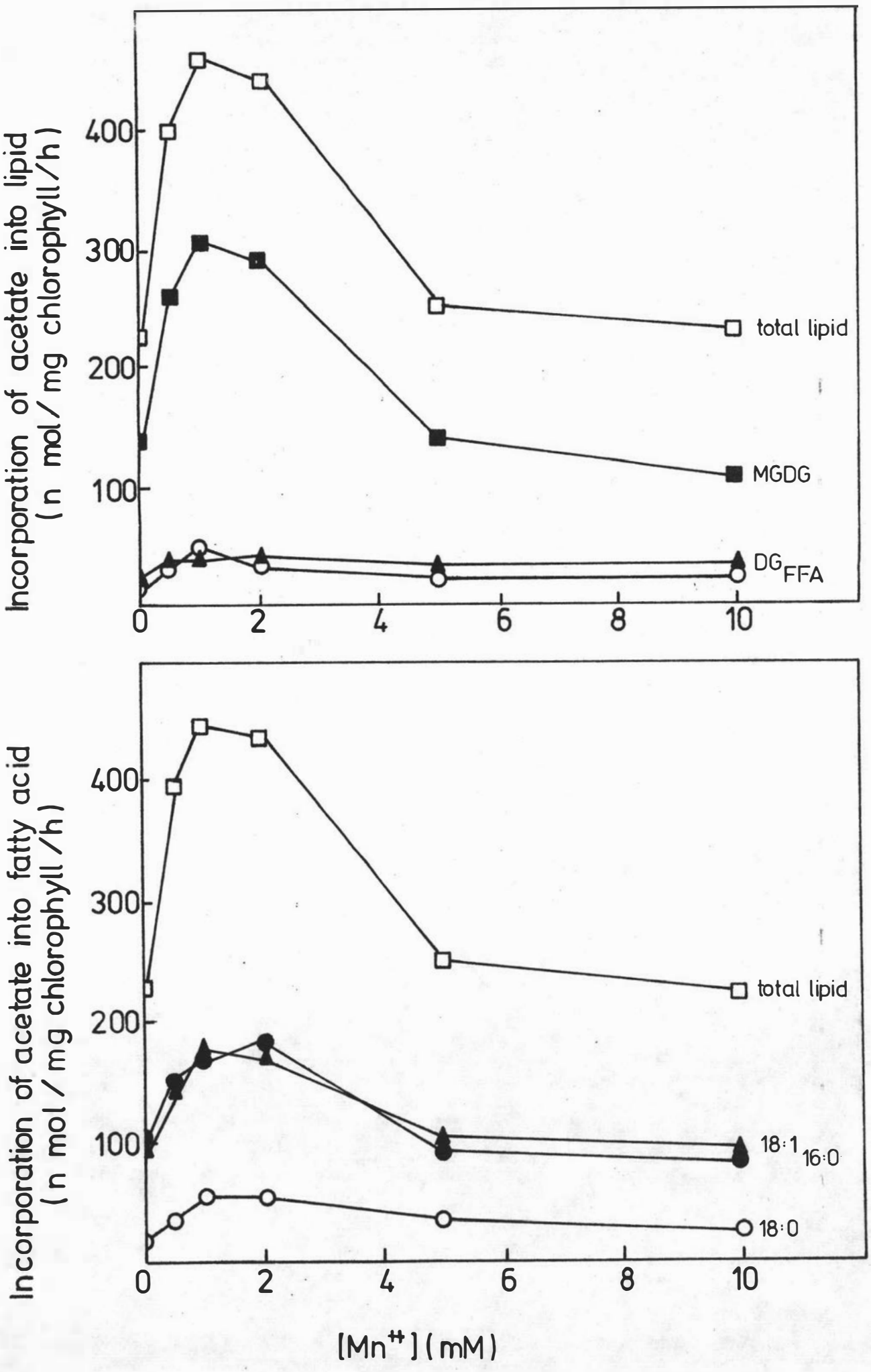

Incorporation of acetate into lipid
(n mol / mg chlorophyll / h)
400
300
200
100
total lipid
MGDG
DG
FFA
1
3
5
7
9
11
Incorporation of acetate into fatty acids
(n mol / mg chlorophyll / h)
400
300
200
100
total lipid
18:1
16:0
18:0
1
3
5
7
9
11
[Mg++] (mM)

Fig. 4-27. The effect of Mn^{++} concentration on [1-^{14}C]acetate incorporation into lipids and constituent fatty acids by spinach chloroplasts

Experimental procedures were those as described in Fig. 4-26 (p. 109), except that various amounts of $MnCl_2$ were added to give the required Mn^{++} concentrations. The Mg^{++} concentration was 1.0mM.


Incorporation of acetate into lipid
(n mol / mg chlorophyll / h)
400
300
200
100
0
2
4
6
8
10
total lipid
MGDG
DG FFA
Incorporation of acetate into fatty acid
(n mol / mg chlorophyll / h)
400
300
200
100
0
2
4
6
8
10
total lipid
18:1 16:0
18:0
[Mn++] (mM)

The remaining label was present in small amounts of medium chain fatty acids (12:0 and 14:0).

4.4 A Possible Role for Phospholipids and Non-chloroplastic Fractions in the Biosynthesis of Polyunsaturated Fatty Acids

Though the synthesis of diglycerides and monogalactosyldiglycerides were stimulated by the addition of G-3-P, Triton X-100 and UDP-galactose, the principal fatty acids synthesized from acetate were oleate and palmitate. Thus, the increased synthesis of DG and MDGD was not accompanied by appreciably enhanced synthesis of linoleate and linolenate, which are the main endogenous fatty acid constituents of MGDG. In view of the postulated roles for phospholipids and microsomal cell fractions in the synthesis of polyunsaturated fatty acids, the effect of the 100,000 X g particulate fraction on lipid and fatty acid synthesis was reinvestigated under conditions of high rates of lipid and fatty acid synthesis. The addition of CDP-choline was used in an attempt to improve the synthesis of phosphatidylcholine (PC). The effect of CDP-choline on the synthesis of lipids by chloroplasts was investigated before its utilization inconjunction with the particulate fraction.

4.4.1 The Effect of CDP-choline on the Incorporation of [1-^{14}C]acetate into Lipids by Spinach Chloroplasts

The addition of CDP-choline in the presence of G-3-P had no effect on the total incorporation of acetate into lipids or on the distribution of the label between the various lipids from that obtained when G-3-P only was added to the incubation medium (Table 4-13, p. 114). The addition of CDP-choline in the presence of G-3-P and UDP-galactose gave a similar distribution of label between lipid classes as that obtained when G-3-P and UDP-galactose were added to the incubation medium. Under all the conditions studied, no stimulation of phosphatidylcholine synthesis was observed.

Table 13. The effect of CDP-choline on the incorporation of [1-^{14}C]-acetate into lipids by spinach chloroplasts

Chloroplasts were isolated by Method 1 and incubated in Medium A (refer Fig. 4-4, p. 52) containing 104nmol (6.24μCi)[1-^{14}C]acetate in a final volume of 1cm^3 after the addition of chloroplasts and substrates. The final concentrations of CDP-choline, UDP-galactose and sn-glycerol-3-phosphate (G-3-P) were 0.18mM, 0.15mM and 5.0mM respectively.

Additions to incubation medium	Incorporation of acetate into lipids (nmol/mg chlorophyll/h)	Distribution of label between lipids (%)				
		FFA	DG	MGDG	PC	others$^{\alpha}$
G-3-P	165.1	25.3	45.8	2.2	2.7	24.0
G-3-P + UDP-galactose	165.7	13.1	6.0	58.5	1.7	20.7
G-3-P + CDP-choline	165.5	21.2	46.7	3.1	2.2	26.8
G-3-P + UDP-galactose + CDP-choline	165.0	12.8	4.6	56.4	1.6	24.6

α more polar lipids (mainly phospholipids, except phosphatidylcholine (PC)), MG and an unknown compound (UK).

4.4.2 The Effect of the Non-chloroplastic Particulate Fraction on the Incorporation of [1-^{14}C]acetate into Lipids and Constituent Fatty Acids by Spinach Chloroplasts

As observed before with maize chloroplasts (section 4.2.2, pp. 55 to 57), the addition of the 100,000 X g particulate fraction stimulated the incorporation of acetate into lipids (Table 14, p. 116). Substituting CDP-choline for UDP-galactose in the incubation medium did not affect the stimulation by the particulate fraction. The increases in acetate incorporation were not due to the incorporation of acetate by the particulate fraction itself, since the particulate fraction incorporated only 0.57nmol of acetate/ 100μl of suspension/h. The values reported in Table 14 (p. 116) have been corrected to take account of the small incorporation of acetate by the particulate fraction.

Addition of the particulate fraction increased the proportion of label in PC (Tabel 14, p. 116). Substitution of UDP-galactose by CDP-choline in the presence of the particulate fraction gave a 16-fold stimulation of PC synthesis over that observed in the control (in the presence of G-3-P, Triton X-100 and UDP-galactose additions to the incubation medium). This was also about 1½ times the proportion of PC synthesized when the particulate fraction was added to the incubation medium containing added G-3-P, Triton X-100 and UDP-galactose. The addition of the particulate fraction also slightly increased the proportion of more polar lipids synthesized from acetate.

The addition of the particulate fraction increased the proportion of label incorporated into oleate and was associated with decreases in the proportions of label in palmitate and stearate (Table 14, p. 116). The addition of the particulate fraction also slightly increased the proportion of label incorporated into linoleate and linolenate. The substitution of CDP-choline for UDP-galactose in the presence of the particulate fraction had no further effect on the fatty acids synthesized.

Table 14. The influence of the particulate fraction on [1-^{14}C]acetate incorporation into lipids and constituent fatty acids by isolated spinach chloroplasts

Chloroplasts were isolated by Method 1 and incubated in Medium A (refer Fig. 4-4, p. 52). The 4,000 to 100,000 x g particulate fraction was prepared from the whole leaf homogenate, prepared from the same batch of tissue, as described in Methods section 3.2.3 (p. 28). The particulate:chloroplast ratio used was 1 (see Fig. 4-5, p. 56). 104nmol (6.24μCi) [1-^{14}C]acetate was used in a final volume of 1cm^3 after the addition of substrates, chloroplasts and particulate fraction. The final concentrations of Triton X-100, sn-glycerol-3-phosphate (G-3-P), UDP-galactose and CDP-choline were 0.16mM, 5.0mM, 0.15mM and 0.17mM respectively.

Additions to incubation medium	Incorporation of acetate into lipids (nmol/mg chlorophyll/h)	Distribution of label between lipids (%)					Distribution of label between fatty acids (%)				
		FFA	DG	MGDG	PC	others[α]	<16:0	16:0	18:0	18:1	18:2 + 18:3
Triton X-100 + G-3-P + UDP-galactose	237.8	19.1	10.3	60.5	0.8	9.3	1.5	39.8	13.6	42.5	2.6
Triton X-100 + G-3-P + UDP-galactose + Particulate fraction	328.6	12.0	10.4	56.7	7.4	13.5	2.0	33.5	9.0	51.4	4.1
Triton X-100 + G-3-P + CDP-choline + Particulate fraction	325.0	14.0	42.8	14.8	12.8	15.6	2.1	33.6	9.4	50.3	4.6

α more polar lipids (mainly phospholipids, except phosphitdylcholine (PC)), MG and an unknown compound (UK).

Chapter 5
DISCUSSION

Several features of lipid biosynthesis by isolated chloroplasts are apparent from the present study:-

1. The rates of acetate incorporation into lipid are dependent on a number of factors, in particular the isolation procedure and the composition of the incubation medium.

2. Palmitic and oleic acids were the predominant fatty acid species synthesized and only very low incorporation of acetate into polyunsaturated fatty acids was achieved.

3. The nature of the lipid products synthesized could be markedly affected by the addition of particular intermediates. This finding has been used to obtain information on the specificity of the acylation of sn-glycerol-3-phosphate and the galactosylation of the diglycerides.

The discussion of these findings to follow will begin with the synthesis of diglycerides and monogalactosyldiglycerides, since the synthesis of these acyl products has a bearing on the rates of acetate incorporation, the fatty acids synthesized and the ultimate synthesis of polyunsaturated fatty acids in the chloroplast. A model of the biosynthetic pathways operating is presented and its relevance to other studies and in vivo lipid metabolism will be discussed.

5.1 The Biosynthesis of Diglycerides and Monogalactosyldiglycerides by Isolated Chloroplasts

5.1.1 Rates of Diglyceride and Monogalactosyldiglyceride Biosynthesis by Isolated Chloroplasts

5.1.1.1 Diglyceride Biosynthesis

5.1.1.1.(a) Effect of sn-glycerol-3-phosphate

In the absence of added G-3-P, free fatty acids were the major product of acetate incorporation by chloroplasts isolated from spinach, maize and sweetcorn (Fig. 4-7, p. 60; Fig. 4-8, p. 61; Fig. 4-8, p. 62; Table 4, pp. 67 to 69). This was in agreement with previous studies by Hawke et al. (1974a) and Roughan et al. (1976). However, Kannangara and Stumpf (1972a) found a high proportion of acetate incorporated by spinach chloroplasts in acyl lipid, especially in the case of chloroplasts isolated from immature spinach leaves. A possible explanation for the high acyl lipid synthesis found

by Kannangara and Stumpf may lie in their inclusion of Triton X-100 in the incubation medium as discussed later (section 5.1.1.1.(b), p. 119).

The present study and the recent work of Roughan et al. (1976) have shown that the addition of sn-glycerol-3-phosphate greatly stimulates the incorporation of newly synthesized fatty acids into diglycerides and other lipids. There have been relatively few previous studies on the effects of G-3-P on lipid synthesis by chloroplasts. Renkonen and Bloch (1969) showed that G-3-P stimulated acyl-ACP and acyl-CoA incorporation into lipids by an Euglena gracilis cell extract, while Douce and Guillot-Salomon (1970) demonstrated [^{14}C]G-3-P incorporation into lipids by maize and spinach chloroplasts. The increased synthesis of DG in the presence of G-3-P was confirmed in the present study for chloroplasts from spinach (Fig. 4-7, p. 60), maize (Fig. 4-8, p. 61) and sweetcorn (Fig. 4-9, p. 62). The stimulatory effect of G-3-P on the proportion of ^{14}C incorporated into DG increased up to a G-3-P concentration around 5.0-10.0mM (Fig. 4-7, p. 60; Fig. 4-8, p. 61; Fig. 4-9, p. 62). The stimulation of diglyceride synthesis by increasing G-3-P concentration was also apparent from the incorporation of ^{3}H labelled G-3-P (Table 8, p. 89).

In contrast to the findings of Roughan et al. (1976), the addition of G-3-P to the incubation medium also stimulated the total incorporation of acetate by spinach chloroplasts (Fig. 4-7, p. 60; Table 4, p. 67). However, this stimulation of total incorporation by G-3-P was only apparent after 30min incubation (Table 4, p. 67). This would account for the failure by Roughan et al. (1976) to observe any stimulation, since they used incubation times up to 20min. Furthermore, the stimulation of acetate incorporation by G-3-P was not observed with maize chloroplasts (Fig. 4-8, p. 61; Table 4, p. 68; Hawke et al., 1974a) while the addition of G-3-P to sweetcorn chloroplasts proved to be inhibitory (Fig. 4-9, p. 62; Table 4, p. 69). The inhibition of acetate incorporation by G-3-P observed with sweetcorn chloroplasts, increased with increasing G-3-P concentration (Fig. 4-9, p. 62).

The stimulation of diglyceride synthesis by the addition of G-3-P would suggest that the endogenous levels of G-3-P were low. This may be due to the loss of G-3-P during the

isolation of the chloroplasts (Walker, 1976). Alternatively, much of the G-3-P required for DG formation may be synthesized outside the chloroplast. The ability of chloroplasts to synthesize sufficent G-3-P to meet their requirements for lipid biosynthesis has not been demonstrated (Leech and Murphy, 1976). The most probable pathway for G-3-P synthesis would be the reduction of dihydroxyacetone phosphate, which is synthesized by the chloroplasts (Heber, 1963). However, the presence of glycerol-3-phosphate dehydrogenase has not been demonstrated in isolated chloroplasts (Leese, 1972) or in leaves (Konigs and Heinz, 1974). An alternative pathway would involve the reduction of glyceraldehyde to glycerol-3-phosphate. A glyceraldehyde-3-phosphatase, for the synthesis of glyceraldehyde, has been found to be associated with chloroplasts from sugar cane (Randall and Tolbert, 1971). However, the glyceraldehyde reductase, for the reduction of glyceraldehyde to glycerol, was located in the 40,000 X g supernatant, but not in the chloroplasts of Vicia faba leaves (Konigs and Heinz, 1974). The ability of leaf tissue to utilize glycerol for lipid synthesis has been demonstrated by Slack et al. (1977), but the site of phosphorylation of the glycerol (glycerol kinase) was not studied. Although $^{14}CO_2$ can be incorporated readily into non-acyl components of leaf lipids (Williams et al., 1976), little label was incorporated from $H^{14}CO_3^-$ by isolated chloroplasts into the glycerol backbone of lipids (Murphy and Leech, 1977). As Leech and Murphy (1976) have suggested, further study on the biosynthetic capacity for G-3-P synthesis by chloroplast is required.

5.1.1.1.(b) Effect of Triton X-100

Triton X-100 stimulated both acetate incorporation and diglyceride synthesis by spinach chloroplasts (Fig. 4-15, p. 77; Fig. 4-16, p. 79) and both were further enhanced when G-3-P was also present (Fig. 4-16, p. 79). The stimulatory effect of Triton X-100 on acetate incorporation was previously noted by Stumpf and Boardman (1970), but these workers did not report any lipid analysis. The stimulation of diglyceride synthesis by Triton X-100 was later demonstrated by Roughan et al. (1976). The presence of Triton X-100 in the reaction medium used by Kannangara and Stumpf (1972a) would probably account for the high proportions of diglyceride and more

polar lipids and the low proportion of free fatty acids found, even though no G-3-P was present.

The mechanism whereby Triton X-100 stimulates the synthesis of DG is unclear. Roughan et al. (1976) have suggested that it may affect the cellular control of DG synthesis or the rate of supply of G-3-P. As discussed earlier, the endogenous level of G-3-P in isolated chloroplasts is low due either to the loss of G-3-P during isolation or the low capacity of the to synthesize G-3-P. It would appear that Triton X-100 may affect the synthesis of G-3-P, but no studies on the effect of Triton X-100 on the incorporation of $^{14}CO_2$ into the glycerol moiety of lipids by isolated chloroplasts have been carried out.

If the only effect of Triton X-100 on the synthesis of diglycerides was the supply of G-3-P, the addition of G-3-P should replace or partially replace the effect of Triton X-100. However, the addition of both Triton X-100 and G-3-P resulted in even higher rates of diglyceride synthesis than with Triton X-100 alone (Fig. 4-16, p. 79). Since the G-3-P concentrations were sufficient to saturate the stimulation of DG synthesis, in the absence of Triton X-100 (Fig. 4-7, p. 60), it is unlikely that further stimulation of DG synthesis by Triton X-100 would have been evident if Triton X-100 simply acted by way of stimulating G-3-P availability. Therefore Triton X-100 may have some effect on the acylating enzymes or their control as inferred by Roughan et al. (1976). Boehler and Ernst-Fonberg (1976) have demonstrated that low concentrations of Triton X-100 release sn-glycerol-3-phosphate transacylase from Euglena gracilis chloroplasts as well as other organelles. Since Joyard and Douce (1977) have found that this enzyme is soluble or loosely bound to the chloroplast envelope membrane, Triton X-100 may well effect the association of this enzyme with the membrane and thereby alter its activity. Since this enzyme catalyses the first step in the acylation of G-3-P (Lands and Hart, 1964), its activity would determine the rate of phosphatidic acid synthesis and subsequently diglyceride synthesis. The determination of the exact nature of the Triton X-100 effect on diglyceride synthesis will require further study.

5.1.1.1.(c) Comparison of the Rates of Diglyceride Synthesis Calculated from [1(3)-^{3}H]sn-glycerol-3-phosphate and [1-^{14}C] acetate Incorporation

From the rate of [1(3)-^{3}H]sn-glycerol-3-phosphate incorporation into DG (Fig. 4-19, p. 92; Fig. 4-21, p. 95) a rate of DG synthesis of 50-70nmol/mg chlorophyll/h is obtained. On the other hand the rate of DG synthesis estimated from the incorporation of acetate was much lower. In the same experiment the rate of acetate incorporation into DG was 45-50nmol of acetate/mg chlorophyll/h. Assuming that 1-oleoyl, 2-palmitoyl-sn-glycerol was the major DG species synthesized, 17nmoles of acetate would be required to synthesize one nmole of the acyl groups which would give a rate of DG synthesis of only 2-4nmol/mg chlorophyll/h. The maximum rate of acetate incorporation into DG was 324.7nmol/mg chlorophyll/h (Table 6, p. 81), this would represent a DG synthesis rate of about 20nmol/mg chlorophyll/h on the basis of the above assumption. This is still considerably lower than the rate calculated from G-3-P incorporation. The difference between the two estimates is probably due to the difference in the extent to which the endogenous precursors are being used simultaneously with the exogenous source. As already discussed, little endogenous G-3-P is available in isolated chloroplasts so that the exogenous precursor constitutes almost the only source for DG synthesis. However, spinach chloroplasts are capable of a considerable rate of endogenous fatty acid biosynthesis fron bicarbonate (see section 5.1.4, pp. 131 to 133) and this endogenous pool will effectively reduce the incorporation of exogenous [1-^{14}C]acetate into DG.

Joyard and Douce (1977) obtained rates of G-3-P incorporation of 12-14nmol/mg protein/h from the incorporation of [^{14}C]G-3-P into lipid by isolated spinach chloroplasts, using the value of 0.112mg chlorophyll/mg protein which they cited for their chloroplasts, this represents a rate of incorporation of 107-126nmol of G-3-P/mg chlorophyll/h. This is in good agreement with the rates of G-3-P incorporation of up to 158nmol of G-3-P/mg chlorophyll/h found in the present study (Table 8, p. 89; Table 9, p. 91). However, DG represents a much larger proportion (50 to 70%) of the total lipid synthesized in the experiments described in this study (Table 8, p. 89) than the proportion (14%) obtained in the work of Joyard and Douce (1977).

The reasons for the different results obtained in this study and those of Joyard and Douce (1977) are unclear. Joyard and Douce (1977) found that MG constituted about 60% of the incorporated label from [^{14}C]G-3-P. MG is noted for its poor and erratic recovery in chloroform/methanol extractions (Bremer, 1963) and may be a factor in the different results obtained. However, Joyard and Douce (1977) also used chloroform/methanol extraction procedures.

5.1.1.2 Monogalactosyldiglyceride Biosynthesis

Neufield and Hall (1964) established that UDP-galactose was the galactosyl donor for galactolipid biosynthesis. Several workers have subsequently studied the effects of added UDP-galactose on the utilization of acyl-ACP (Renkonen and Bloch, 1969), sn-glycerol-3-phosphate (Douce and Guillot-Salomon, 1970; Joyard and Douce, 1977), endogenous diglycerides (Joyard and Douce, 1976a) and diglycerides synthesized from acetate (Kannangara and Stumpf, 1972a).

The finding of the present study that addition of UDP-galactose to isolated chloroplasts incorporating [1-^{14}C]-acetate into lipid stimulates the synthesis of monogalactosyldiglyceride with a corresponding decrease in diglyceride (Fig. 4-10, p. 64; Fig. 4-11, p. 65; Fig. 4-18, p. 87), is in close agreement with the earlier work of Kannangara and Stumpf (1972a). The main difference was the requirement for G-3-P in addition to UDP-galactose to obtain significant MGDG synthesis in the present study since, as discussed earlier, little DG synthesis occurred in the absence of added G-3-P while in the study of Kannangara and Stumpf (1972a) high levels of DG were obtained without added G-3-P. The inclusion of Triton X-100 in the incubation medium, which further stimulated DG synthesis, also gave high rates of MGDG biosynthesis when UDP-galactose was added (Fig. 4-17, p. 83).

The failure of previous workers using [1-^{14}C]acetate (Hawke et al., 1974a; Roughan et al., 1976) and $H^{14}CO_3^-$ (Murphy and Leech, 1977, 1978) to obtain appreciable incorporation of label into MGDG by isolated spinach or maize chloroplasts, was due either to low rates of DG synthesis or, when appreciable rates of DG synthesis were obtained, to the lack of UDP-galactose. The large stimulation of MGDG synthesis again indicates that endogenous levels of this intermediate are

low in isolated chloroplasts. The existing pool of UDP-galactose is probably diluted during isolation of the chloroplasts and further synthesis of UDP-galactose from CO_2 (as HCO_3^-) can not occur since cytosolic enzymes are required for the synthesis of UDP-galactose (Konigs and Heinz, 1974).

Kannangara and Stumpf (1972a) achieved appreciable synthesis of DGDG as well as MGDG upon the addition of UDP-galactose. However, the addition of UDP-galactose to chloroplasts from spinach, maize and sweetcorn (Table 4, pp. 67 to 69), though stimulating the synthesis of MGDG, failed to give any appreciable stimulation of DGDG synthesis. The low rates of DGDG synthesis observed in the present study with spinach, maize and sweetcorn chloroplasts are consistent with previous observations of Mudd et al. (1969), Eccleshall and Hawke (1971), Douce and Guillot-Salomon (1970) and Hawke et al. (1975). The low incorporation of label from [1-^{14}C]acetate into DGDG was probably due to the large pool of unlabelled endogenous MGDG available for galactosylation (Sastry, 1974).

The addition of UDP-galactose also stimulated the incorporation of [1(3)-^{3}H]sn-glycerol-3-phosphate into MGDG, accompanied by a decreased labelling of DG (Table 8, p. 89; Fig. 4-19, p. 92; Fig. 4-21, p. 95). Similar findings were reported by Douce and Guillot-Salomon (1970) and Joyard and Douce (1977).

The rates of MGDG synthesis by spinach chloroplasts, calculated from the data obtained in this study (60-80nmol of MGDG/mg chlorophyll/h; Table 8, p. 89; Fig. 4-19, p. 92; Fig. 4-21, p. 95), were considerably greater than those obtained by other workers. Douce and Guillot-Salomon (1970) calculated a value of 1nmol of MGDG/mg protein/h from the rate of incorporation of [^{14}C]G-3-P into MGDG by spinach chloroplasts in the presence of UDP-galactose. Using the chlorophyll: protein ratio cited by Joyard and Douce (1977) (0.112) to estimate the rate in terms of chlorophyll, this would correspond to a rate of synthesis of 8.9nmol of MGDG/mg chlorophyll/h. Van Hummel et al. (1975) gave a value of 0.436nmol/mg chlorophyll/h from the rate of ^{14}C-UDP-galactose incorporation into galactolipids by spinach chloroplasts. A higher rate of 60nmol/mg chlorophyll/h can be calculated from the data reported by Douce (1974). The low rates observed by

Douce and Guillot-Salomon (1970) were probably due to the low G-3-P concentration used (0.1mM-G-3-P compared with 2.5mM-G-3-P used in this study, Fig. 4-19, p. 92; Fig. 4-21, p.95) and the small amounts of UDP-galactose (2nmoles) used by van Hummel et al. (1975). On the other hand, the concentration of UDP-galactose (45μM) used by Douce (1974) was comparable to those concentrations required for maximum MGDG synthesis found in this study (50-75μM-UDP-galactose, Fig. 4-10, p. 64; Fig. 4-11, p. 65).

The conversion of DG to MGDG was very rapid upon the addition of UDP-galactose (Fig. 4-18, p. 87; Fig. 4-21, p. 95) and was consistent with previous observations of Joyard and Douce (1976a, 1977) with spinach chloroplast envelope preparations.

5.1.2 The Specific Acylation of sn-glycerol-3-phosphate by Isolated Spinach Chloroplasts

Oleic and palmitic acids were the major fatty acids synthesized from exogenous [1-^{14}C]acetate by spinach chloroplasts (Fig. 4-12, p. 71). Only a small proportion of the incorporated label was found in stearic acid, while little labelling of linoleic and linolenic acids was found (Fig. 4-12, p. 71).

In the standard incubation mixture (with no G-3-P) the oleate:palmitate ratio is about 1.5 (Table 5, p. 75; Table 6, p. 81). Addition of G-3-P stimulates the synthesis of palmitate while the synthesis of oleate remains either unchanged or slightly decreased (Table 5, p. 75; Fig. 4-16, p. 79; Table 6, p. 81). The stimulation of palmitate synthesis by G-3-P and of both palmitate and oleate by Triton X-100 will be further discussed in a later section (5.2.(b), pp. 139 to 143). This section will discuss the effect of changing the relative proportions of oleate and palmitate on the fatty acid composition of the diglycerides synthesized.

Addition of G-3-P to the incubation medium markedly affected the relative proportions of oleate and palmitate synthesized (Table 5, p. 75; Fig. 4-16, p. 79; Table 6, p. 81). However, the proportions of oleate and palmitate incorporated into DG did not necessarily reflect the proportions of these two fatty acids synthesized from acetate (Table 5, p. 75; Fig. 4-16, p. 79; Table 6, p. 81).

In the absence of G-3-P the ratio of oleate:palmitate synthesized was 1.5 - 1.7 (Table 5, p. 75; Table 6, p. 81) whereas the ratio of these two fatty acid residues in DG was about 1. The addition of G-3-P increased the proportion of palmitate so that the oleate:palmitate ratio was 0.6 (Table 5, p. 75; Table 6, p. 81) and in this case the ratio of these two fatty acids in DG (0.5 - 0.6) was virtually the same as the ratio of these fatty acids synthesized.

The addition of Triton X-100, though stimulating overall fatty acid synthesis (Fig. 4-16, p. 79), had little effect on the fatty acids synthesized other than a small increase in the proportion of oleate. The ratio of oleate: palmitate (1.9) was similar to that observed in the absence of G-3-P and Triton X-100 (1.5 - 1.6, Table 5, p. 75; Table 6, p. 81). When both Triton X-100 and G-3-P were added together, the ratio of oleate:palmitate was 1 (Table 6, p. 81), largely due to increased palmitate synthesis. However, equal proportions of oleate and palmitate were incorporated into DG under both conditions (Table 6, p. 81). Thus under conditions where oleate was synthesized in excess over palmitate, the amount of oleate incorporated into DG (and subsequently into MGDG) was limited by the amount of palmitate available for the acylation of G-3-P so that a considerable amount of oleic acid remained in the free fatty acid fraction (Table 6, p. 81; Table 7, p. 85). Hawke *et al.* (1974a) and Roughan *et al.* (1976) also found that the free fatty acid fraction contained predominatly oleic acid. These results indicate that the acylation of *sn*-glycerol-3-phosphate is specific in that one palmitate and one oleate moiety are preferentially incorporated into particular positions in the DG molecule. An excess of oleate does not lead to synthesis of the dioleoyl-*sn*-glycerol. This conclusion is confirmed by the finding that the predominant DG synthesized under all conditions was 1-oleoyl, 2-palmitoyl-*sn*-glycerol (Table 10, p. 100; Fig. 4-24, p. 103).

Although oleate rarely becomes incorporated into position 2, even under conditions where a large excess of oleate is synthesized, the positional distribution of the fatty acids in DG and MGDG (Table 10, p. 100; Table 11, p. 101) indicates that palmitate can become incorporated into

position 1 in small quantities. This suggests that the enzyme catalyzing the acylation at position 1 is less specific than that at position 2.

The acylation of G-3-P occurs in a stepwise manner (equations 1 and 2), catalyzed by two separate enzymes in

<u>sn</u>-glycerol-3-phosphate $\xrightarrow[\text{(acyl-ACP)}]{\text{acyl-CoA}}$ monoacyl-<u>sn</u>-glycerol-3-phosphate --(1)

monoacyl-<u>sn</u>-glycerol-3-phosphate $\xrightarrow[\text{(acyl-ACP)}]{\text{acyl-CoA}}$ diacyl-<u>sn</u>-glycerol-3-phosphate --(2)

<u>E. coli</u> (Ray <u>et al.</u>, 1970; Hechemy and Goldfine, 1971), <u>Mycobacterium butyricum</u> (Okuyama <u>et al.</u>, 1977) and spinach chloroplasts (Joyard and Douce, 1977). The acylation can occur by two distinct pathways depending upon the positional specificity of the acyl-CoA:<u>sn</u>-glycerol-3-phosphate acyltransferase (Fig. 5-1, p. 127). The 1-monoacyl-<u>sn</u>-glycerol-3-phosphate pathway predominates in <u>E. coli</u> (Okuyama and Wakil, 1973), yeast (Yamada <u>et al.</u>, 1977) and rat liver (Okuyama <u>et al.</u>, 1971; Tamai and Lands, 1974). However, only recently has there been a report of an organism (<u>Mycobacterium</u>) which utilizes the 2-monoacyl-<u>sn</u>-glycerol-3-phosphate pathway (Okuyama <u>et al.</u>, 1977)

<u>Mycobacterium</u> phospholipids are unusual in their fatty acid pattern: palmitic acid is mainly found in position 2, while oleic acid is found predominantly in position 1 (Okuyama <u>et al.</u>, 1967). However, naturally occurring phospholipids generally contain saturated fatty acids at position 1 and unsaturated fatty acids at position 2 (Hill and Lands, 1970). Okuyama <u>et al.</u> (1977) found that in the presence of G-3-P, oleoyl-CoA and palmitoyl-CoA, a membrane particulate fraction from <u>Mycobacterium</u> synthesized predominatly 1-oleoyl, 2-palmitoyl-sn-glycerol-3-phosphate. The specificity and sequence of synthesis in this organism is clearly similar to that in spinach chloroplasts (Table 10, p. 100; Table 11, p. 101; Fig. 4-24, p. 103). In contrast, <u>E. coli</u> preparations at low concentrations of acceptor (either G-3-P or 1-monoacyl-<u>sn</u>-glycerol-3-phosphate) in the presence of oleoyl-CoA and palmitoyl-CoA synthesize 1-palmitoyl, 2-oleoyl-<u>sn</u>-glycerol-3-phosphate via the 1-monoacyl-<u>sn</u>-glycerol-3-phosphate pathway (Okuyama <u>et al.</u>, 1976).

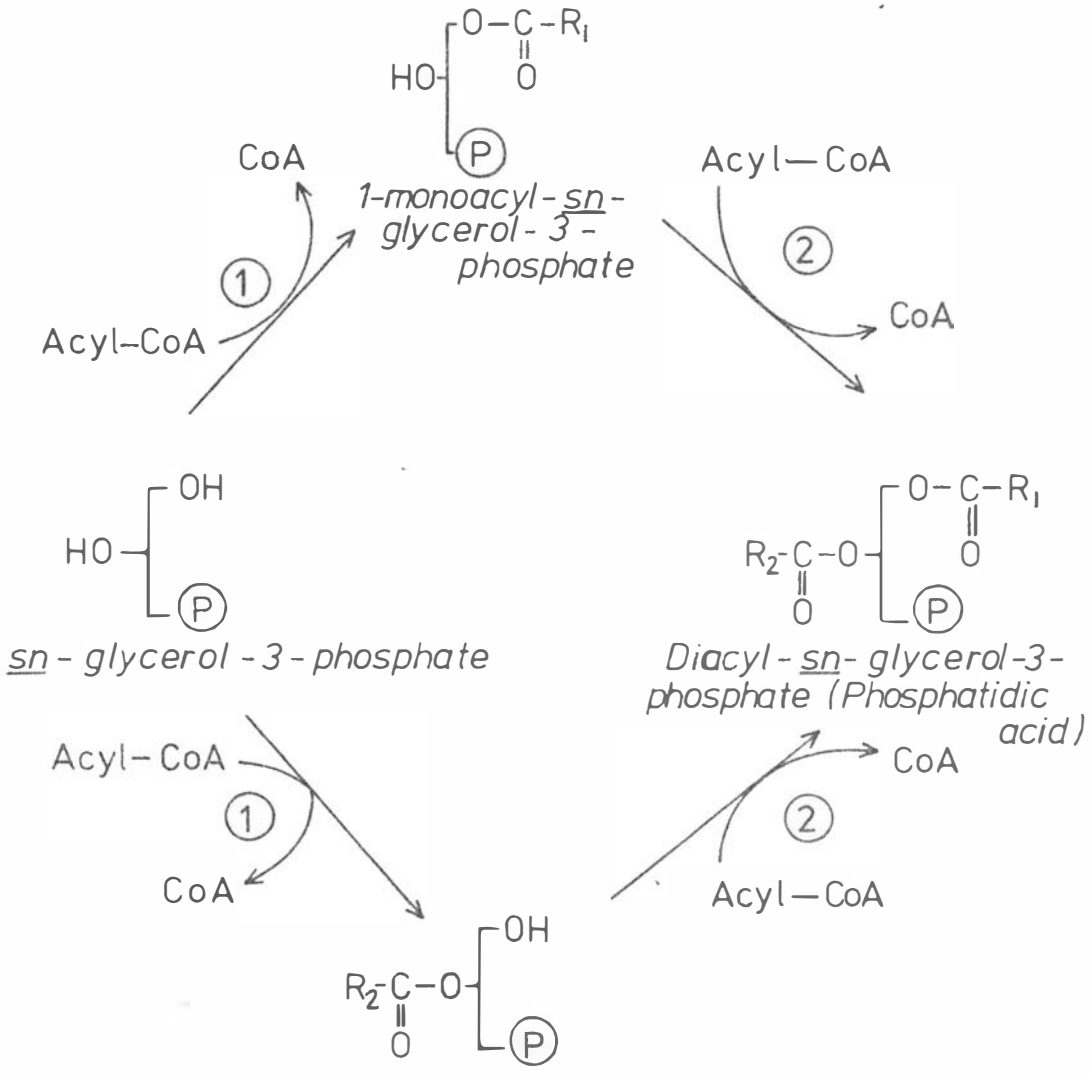


Fig. 5-1. The two pathways for the acylation of sn-glycerol-3-phosphate

1 Acyl-CoA:sn-glycerol-3-phosphate acyltransferase

2 Acyl-CoA:monoacyl-sn-glycerol-3-phosphate acyltransferase

This specificity of the E. coli enzyme system is lost at higher acceptor concentrations above the endogenous in vivo concentrations (Okuyama et al., 1976).

The positional distribution of the fatty acids in the chloroplast DG and MGDG (Table 10, p. 100; Table 11, p. 101) and the effect of G-3-P on the relative proportions of palmitic and oleic acids in the free fatty acids on the one hand and DG and MGDG on the other are clearly consistent with a sequence and specificity of the acylation process identical to that in Mycobacterium (Okuyama et al., 1977).

A lower specificity of the enzyme catalyzing acylation at position 1, so that a second palmitate can be incorporated into this position when there is insufficient oleate, would clearly be advantageous since a high concentration of monoacyl-sn-glycerol-3-phosphate (lyso-PA), with its strong detergent properties would have a deleterious effect on the chloroplast (Rossiter and Strickland, 1960; Hoshina and Nishida, 1975). Alternatively, the lower specificity of this enzyme may be a consequence of the high concentration of the acceptor used. A similar lack of specificity at high acceptor concentrations was found in E. coli (Okuyama et al., 1976) and Mycobacterium (Okuyama et al., 1977). A detailed study of the specificity of the acylating enzymes present in spinach chloroplasts would be required to confirm the above explanation.

The finding that G-3-P stimulates the synthesis of palmitate without increasing the synthesis of oleate, suggests that in the presence of high G-3-P concentrations, palmitate is used preferentially for acylation rather than for elongation and desaturation to oleate. This raises the question of the nature of the acyl substrate which is used in the acylation reactions. In vitro studies have shown that the CoA derivatives can be used by the acylating enzyme systems from E. coli (Pieringer et al., 1967; Ray et al., 1970; Cronan et al., 1970), Mycobacterium (Okuyama et al., 1977) and spinach chloroplast preparations (Bertrams and Heinz, 1976; Joyard and Douce, 1977). However, studies with E. coli (Ray and Cronan, 1975) and Euglena gracilis (Renkonen and Bloch, 1969) have shown that either the ACP derivative or the CoA derivative can be used

for acylation. In E. coli the same enzymes used both the CoA and ACP derivatives (Ray and Cronan, 1975). Shine et al. (1976b) found that a granal preparation from spinach chloroplasts used CoA derivatives rather than the ACP derivatives. It is not known if the envelope acylating enzymes can utilize ACP derivatives. However, Shine et al. (1976a) have proposed a pathway by which ACP derivatives could be converted by the action of thioesterases and thiokinases to their CoA derivatives. If palmitoyl-ACP is a common intermediate for the acylation reaction and the elongation-desaturation pathway to oleate, the increased utilization of the palmitoyl-ACP for acylation in the presence of G-3-P may divert this substrate from elongation to stearyl-ACP (Jaworski et al., 1974) and desaturation to oleoyl-ACP (Jacobson et al., 1974).

5.1.3 The Utilization of Diglycerides for Monogalactosyldiglyceride Biosynthesis

Comparison of the fatty acid composition of the monogalactosyldiglycerides synthesized when UDP-galactose was added to the incubation medium (Fig. 4-17, p. 83) with those of the diglycerides synthesized in the absence of UDP-galactose (Fig. 4-16, p. 79), suggests that the diglycerides were utilized for the synthesis of MGDG without prior modification or selectivity for any particular DG species. Confirmation of this was sought by double labelling experiments in which [1(3)-^{3}H] sn-glycerol-3-phosphate was used to label the glycerol moiety and [1-^{14}C]acetate the acyl moieties of the synthesized lipids.

As discussed earlier the rates of incorporation of ^{3}H labelled G-3-P and ^{14}C labelled acetate into DG are different due to the endogenous synthesis of acetyl units from HCO_3^- which dilutes the incorporation of exogenous acetate. Because of this the molar ratios of G-3-P:acetate incorporated into DG and MGDG will not necessarily be the same, especially after short time intervals (Fig. 4-20, p. 94; Fig. 4-22, p. 96). Thus the G-3-P:acetate ratio in DG was 2.3 while that in MGDG was 4.6 (in the absence of added UDP-galactose) at the 10min sampling time, although the ratios fell to very similar values at longer incubation times (Fig. 4-20, p. 94). Addition of UDP-galactose gave lower ratios in both DG and MGDG (Fig. 4-20, p. 94).

A more conclusive result was obtained when UDP-galactose was added 30min after the commencement of incubation (Fig. 4-21, p. 95), resulting in a rapid conversion of the DG into MGDG. Here the molar ratio of G-3-P:acetate for the newly synthesized MGDG was almost identical to the ratios for the DG synthesized in the absence of UDP-galactose and the DG synthesized after the addition of UDP-galactose (Fig. 4-22, p. 96).

The direct conversion of DG to MGDG, without prior modification, is supported by the similarity of the fatty acid composition of the DG and of the MGDG synthesized prior to UDP-galactose addition and also by that of the MGDG synthesized after the addition of UDP-galactose and the DG synthesized in the absence of UDP-galactose (Fig. 4-23, p. 98). Further support comes from the similarity of the positional distribution of the fatty acids of DG and MGDG (Table 10, p. 100; Table 11, p. 101).

The galactosylation enzyme does not appear to be specific for a particular DG species. Thus when G-3-P is present in the incubation medium, the increased palmitate synthesis leads to the synthesis of both 1-oleoyl, 2-palmitoyl-*sn*-glycerol and 1,2-dipalmitoyl-*sn*-glycerol. The similarity of the oleate:palmitate ratios of the DG synthesized in the presence of G-3-P and the MGDG synthesized in the presence of both G-3-P and UDP-galactose (Table 6, p. 81; Table 7, p. 85), indicate that both DG species have been equally galactosylated to MGDG. This is consistent with earlier findings of Eccleshall and Hawke (1971). However, Mudd *et al.* (1969) and Bowden and Williams (1973) found that the galactosylation enzyme showed a preference for highly unsaturated diglycerides. Furthermore, Kannangara and Stumpf (1972a) found that the MGDG synthesized by young spinach leaf chloroplasts, contained more unsaturated fatty acids than the DG and concluded that this was a result of the preference of the galactosylation enzyme for polyunsaturated diglycerides. While such a selectivity may occur with more unsaturated diglycerides the present study does not suggest any significant discrimination between the fully saturated dipalmitoyl-*sn*-glycerol and the monounsaturated 1-oleoyl, 2-palmitoyl-*sn*-glycerol.

5.1.4 The Incorporation of [^{14}C]bicarbonate into Lipids and Constituent Fatty Acids

It was apparent from the incorporation rates of G-3-P into lipids by spinach (Fig. 4-19, p. 92; Fig. 4-21, p. 95) and maize chloroplasts (see p. 88) that the rates of acetate incorporation into fatty acids could not account for the rates of acyl lipid synthesized (see section 5.1.1.1.(c), pp. 121 to 122). These findings suggested that an alternative source of carbon for fatty acid synthesis was avialable to the chloroplasts.

Non-radioactive bicarbonate was included as a cofactor in the chloroplast incubation medium to provide the CO_2 required for the acetyl-CoA carboxylase reaction (Kannangara and Stumpf, 1972b). This bicarbonate could constitute a carbon source for endogenous synthesis of acetyl units which would be used for fatty acid synthesis. The incorporation of label from [^{14}C]bicarbonate into fatty acids demonstrated that bicarbonate was assimilated by the isolated chloroplasts, metabolized to acetyl-CoA and subsequently incorporated into fatty acids (Fig. 4-25, p. 105). The incorporation of over 600nmol of bicarbonate/mg chlorophyll/h into lipids and constituent fatty acids gives some estimate of the probable contribution that bicarbonate makes to fatty acid biosynthesis (Fig. 4-25, p. 105). The addition of acetate to a final concentration of 0.1mM (the concentration routinely used in this study) had little effect on the rates of bicarbonate incorporation (Fig. 4-25, p. 105), indicating that at this acetate concentration the flow of carbon from acetate did not appreciably affect the rates of carbon flow from bicarbonate.

The rate of bicarbonate incorporation into lipid (173nmol/mg chlorophyll/h, Table 12, p. 107) was similar to that obtained by Murphy and Leech (1978, 161-232nmol/mg chlorophyll/h). The bicarbonate concentrations used was the same in both studies (10mM-HCO_3^-). Though the rate of $H^{14}CO_3^-$ incorporation into lipid were comparable to those of Murphy and Leech (1978), the rates of total $H^{14}CO_3^-$ fixation (0.6μmol/mg chlorophyll/h) were only a small fraction of those rates achieved by them (60-70μmol/mg chlorophyll/h). The reason for the very low $H^{14}CO_3^-$ fixation rates has not

been investigated further in the present study.

The most important finding from this brief study of the incorporation of $H^{14}CO_3^-$ is that the rate of acetate incorporation into lipid is only about 14% of the rate of incorporation of carbon from bicarbonate under comparable conditions (see p. 104). Thus the bicarbonate in the incubation medium is a major source of fatty acid carbon and the rate of exogenous acetate incorporation greatly underestimates the rate of biosynthesis of fatty acids by isolated chloroplasts.

Consistent with the findings of Murphy and Leech (1977, 1978) most of the label from $H^{14}CO_3^-$ incorporated into lipid was associated with free fatty acids and diglycerides (Table 12, p. 107). The addition of G-3-P not only stimulated incorporation of bicarbonate into lipid, but also stimulated the synthesis of diglyceride (Table 12, p. 107). The addition of UDP-galactose, alone or with G-3-P, also stimulated the total incorporation into lipid. The presence of UDP-galactose in the incubation medium stimulated the synthesis of MGDG and was associated with decreased proportion of DG (Table 12, p. 107). Thus the effects of G-3-P and UDP-galactose on the incorporation of ^{14}C from $H^{14}CO_3^-$ into lipids closely resemble those on the incorporation of ^{14}C from [1-^{14}C]acetate (Fig. 4-7, p. 60; Fig. 4-10, p. 64). It is apparent that the failure of Murphy and Leech (1977, 1978) to obtain labelling of MGDG from $H^{14}CO_3^-$ was due to the absence of UDP-galactose in their reaction mixture. In agreement with the findings of Murphy and Leech (1978), little label from $H^{14}CO_3^-$ was incorporated into phosphatidylcholine (Table 12, p. 107). However, incontrast to their findings, the proportion of MG synthesized was relatively small with less than 2% of the label being recovered in MG and other lipids compared with about 25% of the incorporated label in MG reported by Murphy and Leech (1978). However, Murphy and Leech (1978) reported that the recovery of MG from the reaction mixtures using chloroform/methanol gave poor recovery of labelled MG. The recovery of MG from the incubation used in this study was not investigated.

Oleic, palmitic and stearic acids were the main fatty acids synthesized from both [1-^{14}C]acetate and

[^{14}C]bicarbonate (p. 106), with only minor labelling of linoleic and linolenic acids as reported by Murphy and Leech (1977, 1978).

5.1.5 Model of Lipid Biosynthesis in Isolated Spinach Chloroplasts and Its Relevance to In Vivo Biosynthesis

A model to account for the biosynthetic processes observed during the course of this study, particularly with reference to spinach chloroplasts, is presented in Fig. 5-2 (p. 134).

Spinach chloroplasts (and probably all chloroplasts) can utilize both bicarbonate and acetate for the synthesis of fatty acids. The main fatty acids synthesized are oleic and palmitic acids which are utilized, probably as their CoA derivatives, for the specific acylation of G-3-P (Fig. 5-2, p. 134). The synthesis of DG occurs by the 2-monoacyl-sn-glycerol-3-phosphate pathway to give phosphatidic acid (PA) which is dephosphorylated to DG (Fig. 5-2, p. 134). The galactosylation of DG to MGDG occurs without any preference for a particular DG or prior modification of the DG. The major MGDG synthesized is 1-oleoyl, 2-palmitoyl-[β-D-galactopyranosyl(1'$\longrightarrow$3)]-sn-glycerol (Fig. 4-24, p. 103; Fig. 5-2, p. 134).

The composition of the major MGDG species synthesized by isolated chloroplasts is considerably different from the endogenous MGDG of spinach (Allen *et al.*, 1964) and other plants (Sastry, 1974). Spinach MGDG consists almost entirely of hexadecatrienoic acid (30%) and linolenic acid (67%) with only minor amounts of palmitic (trace), stearic (1%) and oleic (1%) acids (Allen *et al.*, 1964).

Auling *et al.* (1971) have shown that hexadecatrienoic acid is restricted to position 2 of MGDG. Therefore the 1-linolenoyl, 2-hexadecatrienoyl-species must constitute 60% of the total MGDG fraction. Thus the positional distribution of the linolenic and hexadecatrienoic fatty acids in the major MGDG species in spinach chloroplasts correspond to the positional distribution of palmitic and oleic acids in the MGDG synthesized by isolated chloroplasts (Table 11, p. 101; Fig. 4-24, p. 103). Siebertz and Heinz (1977) have provided further confirmation, from a study of

Fig. 5-2. Model of lipid biosynthesis in isolated spinach chloroplasts

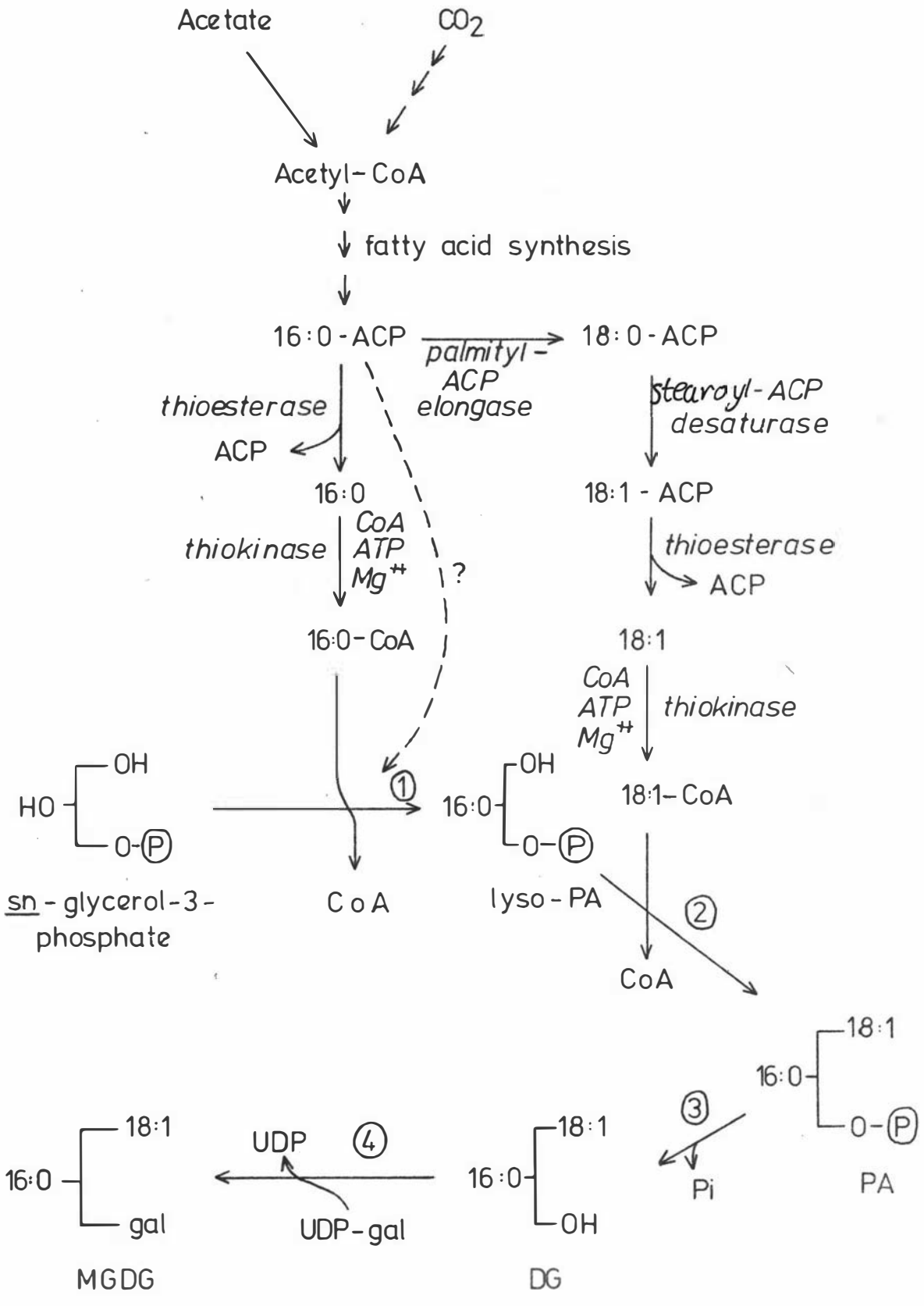
Enzymes:-

1 Acyl-CoA:sn-glycerol-3-phosphate acyltransferase

2 Acyl-CoA:monoacyl-sn-glycerol-3-phosphate acyltransferase

3 Diacyl-sn-glycerol-3-phosphate phosphatase
(Phosphatidic acid phosphatase)

4 UDP-galactose:diacyl-sn-glycerol galactosyltransferase
(Galactosyltransferase)


Acetate
CO_2
Acetyl-CoA
fatty acid synthesis
16:0-ACP
palmityl-ACP elongase
18:0-ACP
thioesterase
ACP
16:0
thiokinase
CoA
ATP
Mg^{++}
?
16:0-CoA
stearoyl-ACP desaturase
18:1-ACP
thioesterase
ACP
18:1
CoA
ATP
Mg^{++}
thiokinase
18:1-CoA
HO
OH
O-P
sn-glycerol-3-phosphate
①
CoA
16:0
OH
O-P
lyso-PA
②
CoA
18:1
16:0
O-P
③
Pi
PA
18:1
16:0
gal
MGDG
UDP
④
UDP-gal
18:1
16:0
OH
DG

the incorporation of $^{14}CO_2$ and [U-^{14}C]acetate into the acyl groups of MGDG *in vivo*, that the newly synthesized MGDG contains ^{14}C labelled C_{18} fatty acids in position 1 and C_{16} fatty acids in position 2. They interperted their findings to indicate that desaturation of palmitic and oleic acids to hexadecatrienoic and linolenic acids respectively occurs after their incorporation into the MGDG molecule (Siebertz and Heinz, 1977). Thus the initially formed MGDG species *in vivo* would be principally 1-oleoyl, 2-palmitoyl-species as found in the present study. The specific incorporation of oleate and palmitate into *sn*-glycerol-3-phosphate (section 5.1.2, pp. 124 to 129) and the absence of any modification of diglycerides prior to their utilization for monogalactosyldiglyceride synthesis, indicates that the stereospecific distribution of C_{16} and C_{18} fatty acids found in spinach MGDG is probably determined at the stage of DG synthesis.

The desaturation of fatty acids while esterified to MGDG was originally proposed by Nichols and co-workers (Nichols *et al.*, 1967; Nichols, 1968; Nichols and Moorhouse, 1969; Appleby *et al.*, 1971) working with green and blue-green algae. This proposal has been extended to include several higher plants such as spinach, *Anthriscus* and *Chenopodium* (Siebertz and Heinz, 1977), rye grass (Bolton and Harwood, 1978), barley, wheat and pea leaves(Wharfe and Harwood, 1978).

Reasons for the failure in the present study to obtain any significant desaturation of either palmitic or oleic acid in the MGDG synthesized by isolated chloroplasts are not understood. This problem will be discussed in a later section (section 5.3.4 pp. 146 to 147).

As discussed above, 60% of the MGDG in spinach chloroplasts contains C_{18} fatty acids in position 1 and C_{16} fatty acids in position 2. This is consistent with the findings of the present study, that the 1-oleoyl, 2-palmitoyl-species is the major MGDG species synthesized by isolated chloroplasts and the proposal that this species is the probable precursor for desaturation of palmitate and oleate of MGDG as observed by Siebertz and Heinz (1977). However, since linolenate constitutes 67% of the chloroplast

MGDG fatty acids, this means that most of the remaining 40% of the MGDG species of spinach chloroplasts is the dilinolenoyl-species. The synthesis of dilinolenoyl-species is not so readily accounted for since the corresponding dioleoyl-*sn*-glycerol, which would be the expected precursor, was not synthesized to any appreciable extent by isolated chloroplasts in the present study.

The synthesis of the dilinoenoyl-MGDG could occur by deacylation and reacylation, replacing hexadecatrienoic acid by linolenic acid. The ability of chloroplast preparations to reacylate fully or partially deacylated MGDG has been demonstrated (Safford *et al.*, 1971; Bajwa and Sastry, 1972, 1973, 1975). The highest rates of acylation being obtained with unsaturated fatty acids, particularly linolenic acid (Bajwa and Sastry, 1972, 1973, 1975). Stumpf and co-workers (Kannangara *et al.*, 1973b; Jacobson *et al.*, 1973a, 1973b) have demonstrated that hexadecatrienoic acid can undergo elongation to linolenic acid, providing a possible source of linolenic acid for the reacylation process.

Another possibility is the existence of a second pathway to MGDG via polyunsaturated diglycerides derived from the metabolism of PC (Williams *et al.*, 1976; Slack *et al.*, 1977). Though Slack *et al.* (1977) demonstrated that diglycerides derived from PC were utilized for MGDG biosynthesis, they noted that it was predominantly the dilinoleoyl-species which was lost from PC, and the dilinolenoyl-species which appeared in MGDG. The actual site of the final desaturation of linoleate to linolenate is not clear. It has been found by many workers that little ^{14}C labelled 18:3 is found in PC during $^{14}CO_2$ or [1-^{14}C]acetate feeding experiments with leaf tissue (Roughan, 1970, 1975; Williams *et al.*, 1976; Heinz and Harwood, 1977; Siebertz and Heinz, 1977; Bolton and Harwood, 1978). Roughan (1970, 1975) has proposed that this is a result of the rapid transfer of 18:3 to other lipids, particularly MGDG. The alternative explanation is that the desaturation of 18:2 moiety of PC to 18:3 does not occur to any great extent and that the desaturation of 18:2 to 18:3 is associated with the chloroplast (Tremolieres and Mazilak, 1974), probably while acylated to MGDG (Siebertz and Heinz, 1977).

It is clearly possible that the diglycerides

for MGDG biosynthesis could be synthesized by two separate pathways; namely, de novo synthesis in the chloroplast (Joyard and Douce, 1977) and synthesis via PC metabolism (Slack et al., 1977). The de novo synthesized 1-oleoyl, 2-palmitoyl-MGDG would be desaturated to 1-linolenoyl, 2-hexadecatrienoyl-MGDG (Siebertz and Heinz, 1977) and the dilinoleoyl-MGDG, synthesized from diglycerides derived from PC, would be desaturated to dilinolenoyl-MGDG. The high proportion (60%) of MGDG containing equal proportions of 16:3 and 18:3 suggests that the de novo synthesis of 1-oleoyl, 2-palmitoyl-MGDG, followed by desaturation, is probably a major pathway for MGDG biosynthesis in spinach. The donation of polyunsaturated diglycerides derived from PC may be happening to a lesser extent. The contribution of these two possible pathways to MGDG biosynthesis may vary from species to species. Thus while both are probably involved in spinach, the donation of polyunsaturated diglycerides from PC may be the major pathway in maize (Slack et al., 1977). Further study is required to elucidate the contribution of these two alternatives.

5.2 Factors Affecting the Rate of Acetate Incorporation into Lipids by Isolated Chloroplasts

Having considered the main features of the process whereby exogenous acetate becomes incorporated into the final acyl products of the chloroplast, a brief discussion of the factors which determine the overall rate of acetate incorporation by isolated chloroplasts will be presented.

A number of factors have been shown to be involved in determining the rate of acetate incorporation into lipids:

5.2.(a) Structural Integrity of the Chloroplasts

It has been clearly established that the ability of chloroplasts to incorporate exogenous acetate into lipids and fatty acids is correlated with their degree of intactness (Nakamura and Yamada, 1975a). The same correlation exists between structural integrity and the rates of CO_2-fixation (Nakamura and Yamada, 1975a) and O_2-evolution (Roughan et al., 1976). Thus the same features of chloroplast structural organization which are required for high rates of photosynthesis are also required for high rates of lipid synthesis (Murphy and Leech, 1977).

The ability of the spinach chloroplasts prepared in the present study to incorporate label from $H^{14}CO_3^-$ and [1-^{14}C]acetate into lipid at rates comparable to those obtained with these substrates by Murphy and Leech (1978) and Roughan et al. (1976) respectively suggests comparable degrees of intactness with these two investigations. Using lipid synthesis as the criteria of chloroplast integrity, the method developed by Leese et al. (1971) for the isolation of maize chloroplasts was suitable for the isolation of spinach chloroplasts as well (Table 1, p. 42). On the other hand, one of the standard methods for the isolation of spinach chloroplasts (Jacobson et al., 1973a) was not satisfactory for isolating maize chloroplasts. The lower proportion of intact maize chloroplasts, as visible by phase-contrast and the low acetate incorporation, probably arose from the greater homogenization time used in the latter procedure. This reflects a greater fragility of maize chloroplasts. An isolation method for spinach chloroplasts using Honda's medium (Stumpf and Boardman, 1970) was also unsatisfactory for the isolation of maize chloroplasts (Hawke et al. 1974a).

5.2.(b) Composition of the Incubation Medium

The presence of bicarbonate in the incubation medium is clearly required for high rates of lipid synthesis. This requirement was first noted by Mudd and McManus (1962) and has become a standard cofactor in reaction mediums when studying acetate incorporation into lipids (Hawke et al., 1974a; Kannangara and Stumpf, 1972a; Nakamura and Yamada, 1975a; Roughan et al., 1976). The lack of bicarbonate in the reaction mixture, when chloroplasts isolated by Method 1 are incubated in Medium B, was largely responsible for the low rates of acetate incorporation (Table 1, p. 42). The stimulatory effect of bicarbonate has been attributed to the CO_2 required in the acetyl-CoA carboxylase reaction (Kannangara and Stumpf, 1972a, 1973).

Low concentrations of ATP enhanced the incorporation of acetate into lipid (Stumpf et al., 1967; Kannangara and Stumpf, 1972a; Nakamura and Yamada, 1975a) with the optimum of 0.5mM (Fig. 4-1, p. 44). This was similar to that used by Nakamura and Yamada (1975a), but lower than the 1-2mM-ATP used by Stumpf and co-workers (Stumpf et al., 1967;

Kannangara and Stumpf, 1972a). In addition, ATP was inhibitory at lower concentrations than previously observed (Stumpf et al., 1967). However, the ATP effect varies according to the maturity of the spinach tissue used as the chloroplast source (Kannangara and Stumpf, 1972a).

Roughan et al. (1976) have demonstrated that when high ATP and CoA concentrations (4mM and 1mM respectively) are used a substantial proportion of the label is lost into the methanol phase as CoA esters during lipid extraction with chloroform/methanol. Such losses are less likely in the present study because the aqueous phase contained lower methanol concentrations.

The reasons for the stimulatory effect of ATP have not been clearly resolved. Stokes and Walker (1971) have reported that the chloroplast is relatively impermeable to ATP. Roughan et al. (1976) suggested a role for exogenous ATP in the formation of oleoyl-CoA, as this occurs in the envelope (Roughan and Slack, 1977) it would preclude the necessity for ATP uptake by the chloroplast. Heldt (1969) has shown that spinach chloroplasts can take up as much as 5μmol of ATP/mg chlorophyll/h which would be adequate to account for the incorporation of 5nmol of acetate/mg chlorophyll/h even if only 0.2% of the ATP was used for fatty acid synthesis. Since the stimulation of acetate incorporation achieved by the addition of 0.5mM-ATP is of this order it seems probable that the limited permeability of the chloroplast envelope is adequate to account for the observed stimulatory effect (Fig. 4-1, p. 44). Heldt (1976) has proposed that this transport mechanism probably functions to provide the chloroplast with ATP generated by glycolysis or respiration in the dark.

The concentration of acetate is another factor determining the overall rate of incorporation. The concentrations used by earlier workers (Stumpf, 1972; Hawke et al., 1974a) were clearly sub-optimal. Optimal incorporation of acetate was achieved at concentrations of 0.3 - 0.5mM-acetate for sweetcorn and maize chloroplasts while the more active spinach chloroplasts required 0.8 - 1.0mM-acetate for maximum incorporation (Fig. 4-3, p. 48). These findings were consistent with the previous findings of Nakamura and

Yamada (1975a) and Roughan et al. (1976) with spinach chloroplasts. However, Roughan et al. (1976) obtained maximum rates of acetate incorporation at lower acetate concentrations (0.1 - 0.15mM) and the maximum rates achieved with spinach chloroplasts were somewhat higher than those obtained in the present study. This difference will be discussed later. The maximum rates of incorporation observed for maize (32.8nmol of acetate/mg chlorophyll/30min) and sweetcorn (19.0nmol of acetate/mg chlorophyll/30min) chloroplasts were ten-fold higher than previously reported for isolated maize chloroplasts (0.4 - 3.0nmol of acetate/mg chlorophyll/30min, Hawke et al., 1974a) at the optimal acetate concentrations (Fig. 4-3, p. 48).

The addition of compounds such as G-3-P, Triton X-100 and UDP-galactose, which promote the synthesis of DG and MGDG, also stimulated acetate incorporation by spinach chloroplasts (Fig. 4-7, p. 60; Fig. 4-10, p. 64; Fig. 4-15, p. 77; Fig. 4-16, p. 79; Fig. 4-17, p. 83) as mentioned earlier in the discussion (section 5.1.1.1.(a), p. 118; section 5.1.1.1.(b), p. 119). For example, the rate of acetate incorporation into total lipids was 435nmol/mg chlorophyll/h in the presence of G-3-P and Triton X-100 compared with 110nmol/mg chlorophyll/h in the absence of both (Fig. 4-16, p. 79). A possible explanation of this effect is that the stimulation of the synthesis of DG and other acyl lipids removes an end-product of fatty acid biosynthesis and so relieves an end-product inhibition of acetate incorporation. It is significant that, while the level of oleic acid in the free fatty acid fraction varies considerably according to the nature of the added compound (G-3-P), the level of palmitic acid in the free fatty acid fraction is very similar under all conditions (Table 5, p. 75; Table 6, p. 81; Table 7, p. 85). Since palmitate is the end-product of de novo fatty acid biosynthesis (Hawke and Stumpf, 1965a; Kannangara and Stumpf, 1972c), the free acid or a derivative would be the most likely compound to exert end-product inhibition. Palmitoyl-CoA has been demonstrated to inhibit acetyl-CoA carboxylase from various sources (see Volpe and Vagelos, 1972; Bloch and Vance, 1977 for references). However, Burton and Stumpf (1966) found that wheat germ acetyl-CoA carboxylase

was unaffected by palmitoyl-CoA. These authors did indeed find an inhibitor of acetyl-CoA carboxylase in lettuce chloroplasts, but its nature was not determined.

The failure of G-3-P to stimulate acetate incorporation by maize chloroplasts was consistent with the previous observations by Hawke et al. (1974a). However, the reasons for the inhibition by G-3-P of acetate incorporation by sweetcorn chloroplasts is unclear and was not investigated further in this study. Though the effect of Triton X-100 on the incorporation of acetate by maize and sweetcorn chloroplasts was not investigated, but Hawke et al. (1974a) has reported that Triton X-100 markedly inhibited incorporation by maize chloroplasts.

At a late stage of the present study a brief investigation of the effect of metal ion concentration on the rates of acetate incorporation was carried out. This study showed that, at least under conditions giving high rates of acyl lipid end-products, the metal ion concentration used would have been sub-optimal. A two-fold stimulation of acetate incorporation by spinach chloroplasts was achieved by increasing the Mg^{++} concentration from 1mM (the concentration normally used in this study) to 3mM which gave maximal rates (Fig. 4-26, p. 109). Alternatively, addition of 1mM-Mn^{++}, in the presence of 1mM-Mg^{++}, gave a comparable stimulation to that obtained with 3mM-Mg^{++} (Fig. 4-27, p. 111). These observations were in accordance with the findings of Nakamura and Yamada (1975a). The relative proportions of the various fatty acids and lipids were unaltered by changing the metal ion concentrations (Fig. 4-26, p. 109; Fig. 4-27, p. 111).

The highest rates of acetate incorporation by spinach chloroplasts reported in the literature are those by Roughan et al. (1976). The rates of acetate incorporation by spinach chloroplasts obtained in this study were somewhat lower than those obtained by Roughan et al. (1976) that is, 100 - 400nmol of acetate/mg chlorophyll/h compared with 300 - 700nmol of acetate/mg chlorophyll/h. However, such comparisons do not warrant undue emphasis. Firstly, the age of the tissue used as a source of chloroplasts influences the number of chloroplasts/unit weight of

chlorophyll (Leese et al., 1971). Hawke et al. (1974a) have pointed out that the apparent difference in the rates of acetate incorporation for chloroplasts isolated from immature and mature spinach tissue (Kannangara and Stumpf, 1972a) was in part a consequence of the difference in chlorophyll content per plastid. Roughan et al. (1976) consistently used young spinach leaf tissue in their work. In the present study more mature tissue was used for much of the work. Secondly, the rates of acetate incorporation were not always linear with time (Fig. 4-16, p. 79). Roughan et al. (1976) based their estimate of incorporation rates on short sampling times up to 15 or 20min whereas in the present study incubation times of 30min or 1h were used since these longer times were required to obtain significant stimulation of DG and MGDG synthesis (Table 4, pp. 67 to 69; Fig. 4-16, p. 79; Fig. 4-17, p. 83). Since incorporation was in some cases non-linear over the period of an hour, the use of the full hour incubation period will clearly give a lower estimate of the rate than a shorter incubation time. Furthermore, in the interests of economy, sub-optimal concentrations of acetate (about 0.1mM) were routinely used in most of the present study, resulting in lower rates of incorporation, but without affecting the fatty acids synthesized (Table 2, p. 50).

5.3 The Biosynthesis of Polyunsaturated Fatty Acids by Isolated Chloroplasts

The failure of isolated chloroplasts to synthesize polyunsaturated fatty acids at the high rates expected from the quantities found in chloroplast lipids has been described as an outstanding problem of lipid biosynthesis in higher plants (Hawke et al., 1974a). One of the original aims of the present study was to extend the observations of previous workers on this problem, especially those representing any progress toward achieving increased rates of polyunsaturated fatty acid biosynthesis. However, the findings of the present study have consistently confirmed the inability of isolated chloroplasts, as prepared by current procedures, to synthesize significant quantities of dienoic or trienoic fatty acids either as free fatty acids or components of acyl lipids.

Oleic and palmitic acids with some stearic acid

were the main fatty acids synthesized from acetate or from bicarbonate under all conditions investigated. The proportions of linoleic and linolenic acids synthesized were never greater than 10-15% of the total fatty acids, Such findings are in agreement with those of the most recent workers (Hawke et al., 1974a; Nakamura and Yamada, 1975a; Roughan et al., 1976; Murphy and Leech, 1977, 1978; Bolton and Harwood, 1978). However, the attempts made to improve polyunsaturated fatty acid biosynthesis merit some discussion.

5.3.1 Effect of Cofactors

Kannangara and Stumpf (1972a) found that ATP and CoA at high concentrations stimulated polyunsaturated fatty acid biosynthesis by spinach chloroplasts. In the present study only very small increases (1-4nmol of acetate/ mg chlorophyll/30min) in the rates of synthesis of linoleic and linolenic acids were obtained and this mainly with maize and sweetcorn chloroplasts (Fig. 4-1, p. 44; Fig. 4-2, p. 46). The difference between the work of Kannangara and Stumpf (1972a) and the present study is likely to be in the maturity of the tissue used to obtain the chloroplasts. Kannangara and Stumpf (1972a) found the stimulation of polyunsaturated fatty acid biosynthesis by ATP and CoA mainly in chloroplasts isolated from young leaves while more mature tissue was used in the present study.

Variation in the concentration of other components of the incubation medium, acetate and divalent metal ions, while affecting total synthesis of fatty acids, as described in the previous section, has had no significant effect on the proportion of polyunsaturated fatty acids synthesized from acetate.

5.3.2 Effect of Chloroplast Maturity

The developing maize leaf provides an ideal tissue from which chloroplasts at different stages of development can be isolated. The basal four cm of the leaf contains largely proplastids while the distal sections contain mature plastids with a transition zone of about 2cm in between (Leese et al., 1971). Various workers have used developing Gramineae species to study the effect of chloroplast maturity on acetate incorporation into lipids (Hawke et al., 1974a, 1974b; Bolton and Harwood, 1978).

Plastids isolated from different maize leaf sections synthesized little linoleic and linolenic acids from acetate (Table 3, p. 54), a finding consistent with the earlier observations of Hawke et al. (1974a) and of Bolton and Harwood (1978). These workers showed that whole leaf sections from maize (Hawke et al., 1974b) and rye grass (Bolton and Harwood, 1978) incorporated a considerable amount of acetate into linoleate (up to 25% of the total acetate in fatty acids in maize and up to 34% in rye grass), particularly in sections from more mature leaf tissue. However, the incorporation of acetate into linolenate was relatively low (up to 4% in maize and up to 6% in rye grass). In isolated chloroplasts from comparable leaf sections, acetate incorporation into linoleic acid does not exceed 5% of the total fatty acids (Table 3, p. 54).

5.3.3 Effect of Addition of a Non-chloroplastic Particulate Fraction

The major difference between the incorporation of acetate into polyunsaturated fatty acids by whole leaf sections and isolated chloroplasts may indicate a requirement for components outside the chloroplast to participate in the desaturation process. The microsomal fraction is the most likely extrachloroplastic site since in non-chloroplastic tissues the desaturases are microsomal (McMahon and Stumpf, 1964; Vijay and Stumpf, 1971, 1972). Slack and Roughan (1975) and Slack et al. (1976) have demonstrated that the desaturation of fatty acids acylated to PC occurs in the microsomal fraction of leaf tissues. Hawke et al. (1974a) found that the synthesis of polyunsaturated fatty acids, especially with chloroplasts isolated from mature maize leaf sections, was increased by the addition of both mitochondrial and microsomal fractions. However, the increased synthesis of polyunsaturated fatty acids was still well below the rates expected from the endogenous levels.

The effect of the non-chloroplastic particulate fraction (100,000 X *g* pellet) from maize, and later from spinach, on acetate incorporation by isolated chloroplasts was investigated. The work with maize was done at an early stage of the present study and showed that the addition of

the particulate fraction enhanced the synthesis of oleic acid, but had no effect on the synthesis of more unsaturated fatty acids (Fig. 4.5, p. 56; Fig. 4-6, p. 58). The failure to observe and stimulation of polyunsaturated fatty acid synthesis is in contrast to the findings of Hawke et al. (1974a). Subsequently, in the light of the results on the stimulatory effects of Triton X-100, G-3-P and UDP-galactose on acyl lipid synthesis, the effect of a similar particulate fraction from spinach was investigated in the presence of the additional components. In this case their was a small enhancement of acetate incorporation into polyunsaturated fatty acids (from 2.6 to 4.2% of the total acetate incorporated) as well as an enhancement of the proportion of acetate incorporated into oleate (Table 14, p. 116). However, an even more striking effect in this experiment was the increased synthesis of PC (from 0.8 to 7.4% of the total acetate incorporated into lipid). This was further enhanced (to 12.8%) by replacing UDP-galactose by CDP-choline (Table 14, p. 116). Since chloroplasts synthesize little PC on their own, even in the presence of CDP-choline (Table 13, p. 114; Table 14, p. 116; Murphy and Leech, 1977, 1978), the PC synthesis must be due to the microsomal component as indicated by the results of other workers (Joyard and Douce, 1976b; Devor and Mudd, 1971). Slack and Roughan (1975) also observed that label incorporated from $^{14}CO_2$ and [1-^{14}C]acetate into PC was associated with the microsomal fraction. The present study supports the proposal of Slack and Roughan (1975) that fatty acids (mainly oleic acid) are transfered to PC of the endoplasmic reticulum probably via synthesis of oleoyl-CoA in the chloroplast envelope (Roughan and Slack, 1977) which is subsequently released into the aqueous medium for incorporation in to PC.

5.3.4 Effect of Stimulating Acyl Lipid Synthesis

Stumpf and co-workers have reported enhanced synthesis of polyunsaturated fatty in the presence of Triton X-100 (Stumpf and Boardman, 1970; Givan and Stumpf, 1971; Kannangara and Stumpf, 1972a). While Triton X-100 was found in the present study to enhance acyl lipid synthesis, but no increase in the proportion of polyunsaturated fatty acids was found. This was consistant with the recent findings

of Roughan et al. (1976).

In view of the suggestion, supported by the results of several previous workers (Nichols and Moorhouse, 1969; Appleby et al., 1971; Siebertz and Heinze, 1977; Bolton and Harwood, 1978), that MGDG is a substrate for desaturation it was possible that stimulation of MGDG synthesis might lead to increased synthesis of polyunsaturated fatty acids. However, in spite of the high rates of MGDG synthesis achieved in the present study by the addition of G-3-P and UDP-galactose enhanced synthesis of polyunsaturated fatty acids was not obtained (Fig. 4-12, p. 71; Fig. 4-13, p. 72; Fig. 4-14, p. 73; Table 11, p. 101). In spite of this finding the results of this study tend to support the view that MGDG is a major substrate for desaturation in vivo (see section 5.1.5, pp. 133 to 138).

5.4 Summary and Suggestions for Further Study

Though several features of lipid biosynthesis in isolated chloroplasts are evident from the present study, most of the main findings point to aspects of lipid metabolism requiring further study. These are discussed below with the purpose of stimulating further investigation.

The principal findings were:-

(a) The rates of acetate incorporation into lipids by spinach, maize and sweetcorn chloroplasts were stimulated by the use of optimal concentrations of acetate, CoA and ATP. Acetate concentration had the major effect on the rates of incorporation while optimisation of ATP and CoA concentrations gave only small enhancements of acetate incorporation. Divalent metal ions, Mg^{++} or Mg^{++} + Mn^{++}, stimulated greatly the incorporation of acetate by spinach chloroplasts. High rates of acetate incorporation by spinach chloroplasts were associated with high rates of acyl lipid synthesis, particularly DG and MGDG synthesis. Triton X-100, sn-glycerol-3-phosphate (G-3-P) and UDP-galactose enhanced the synthesis of acyl lipids (DG and MGDG) with enhanced acetate incorporation.

The maximum rates of acetate incorporation into lipids by maize and sweetcorn chloroplasts (20-30nmol of acetate/mg chlorophyll/h) were up to 10-fold greater than previously reported. Rates of up to 500nmol of acetate/mg chlorophyll/h were obtained for spinach chloroplasts

which compares favourably with the rates obtained by other workers using chloroplasts isolated from younger leaf tissue. However, the rate of acetate incorporation into lipids greatly underestimates the fatty acid biosynthetic capacity of isolated chloroplasts when the rates of acetate incorporation were compared with the rates of acyl lipid synthesis from the incorporation of G-3-P. These results suggested that a large proportion of fatty acid carbon had come from an alternative source than the exogenous acetate. Bicarbonate, present in the incubation medium, was found to be incorporated into fatty acids and acyl lipids by spinach chloroplasts.

(b) The incorporation of newly synthesized fatty acids into lipids, particularly into DG and MGDG, was enhanced by the addition of appropriate metabolites (G-3-P and UDP-galactose). Triton X-100, with and without G-3-P, stimulated the synthesis of DG and in the presence of UDP-galactose the synthesis of MGDG.

Oleate and Palmitate (the main fatty acids synthesized by isolated spinach chloroplasts) were utilized in the specific acylation of sn-glycerol-3-phosphate to give predominately 1-oleoyl, 2-palmitoyl-sn-glycerol and the subsequent MGDG. It was inferred from the proportions of oleate and palmitate incorporated into DG and in the free fatty acid fraction, when different proportions of these two fatty acids were synthesized, that palmitate was probably incorporated into position 2 first followed by oleate into position 1.

From double-labelling experiments using [1-^{14}C]acetate and [1(3)-^{3}H]sn-glycerol-3-phosphate, fatty acid composition and positional distribution of the fatty acids it was evident that the galactosylation of the diglycerides to MGDG occurred without any prior modification of the diglycerides or preference for any particular diglyceride species.

(c) The synthesis of polyunsaturated fatty acids was low with predominantly oleic and palmitic acids synthesized from acetate and bicarbonate. Alteration of cofactor concentrations, the use of chloroplasts isolated from developing maize leaf sections, stimulation of acyl lipid synthesis and the addition of a 100,000 X g particulate preparation from leaf homogenate failed to give any significant stimulation of polyunsaturated fatty acid synthesis. However, the synthesis

of 1-oleoyl, 2-palmitoyl-MGDG species is significant in the light of the recent observation of the desaturation of these fatty acids while acylated to MGDG *in vivo*. The small stimulation of polyunsaturated fatty acid synthesis by the addition of the particulate fraction to isolated chloroplasts, associated with the synthesis of phosphatidylcholine (PC), is also significant since PC is the substrate for the desaturation of oleate in the microsomal fraction.

It is unlikely that the improvement of the rates of acetate incorporation into lipid will stimulate the synthesis of polyunsaturated fatty acids. However, the relative contributions of acetate and bicarbonate to the overall fatty acid synthesis by isolated chloroplasts will need to be determined more closely and may lead to rates of fatty acid synthesis comparable to those *in vivo*.

Though it is inferred that palmitate is acylated to G-3-P at position 2 first and then followed by the incorporation of oleate into position 1, a more direct demonstration of the positional specificity of the acylating enzymes is required. Investigation of the specificity of these enzymes (if any) for particular fatty acids and the effects of acceptor concentrations would greatly extend our understanding acyl lipid synthesis.

The observation that the highest rates of acetate and bicarbonate incorporation into lipids were associated with high rates of DG and MGDG synthesis suggests that the rate of utilization of the newly synthesized fatty acids determines the rate of synthesis. Conditions which promote acetate and acyl lipid formation also lead to an increase in the oleic acid level in the free fatty acid fraction but, significantly, the level of palmitic acid remains constant suggesting that palmitic acid or a derivative may be a regulator of fatty acid synthesis. The observed stimulation of palmitic acid synthesis by G-3-P and the incorporation of palmitate not only into position 2, but also into position 1, without much effect on the synthesis of oleate indicated that palmitoyl-ACP which could have been elongated and desaturated to oleoyl-ACP was diverted for acylation. The ability of the acylating enzymes to utilize ACP derivatives and the effect of these derivatives on fatty acid synthesis requires

investigation.

The mode of action of Triton X-100 on the synthesis of DG is unclear. However, since G-3-P is required to stimulate the synthesis of DG in the absence of Triton X-100 this suggests that either Triton X-100 acts to make available a pool of G-3-P for DG synthesis or stimulates the synthesis of G-3-P by the chloroplast. Investigation of the ability of the chloroplast to synthesize G-3-P may prove fruitful.

The results of the present study suggest two lines of investigation which in the light of *in vivo* studies should lead to the synthesis of polyunsaturated fatty acids. One is the further investigation of the metabolism of the 1-oleoyl, 2-palmitoyl-MGDG species with the aim of stimulating the desaturation of the oleate and palmitate to linolenate and hexadecatrienoate. The reason for the failure to obtain the desaturation of the fatty acids is unclear, but the length of the incubation period may be a factor. The second line of investigation would involve further studies with the addition of the particulate fraction to the isolated chloroplasts. The addition of microsomal fractions, particularly active in the synthesis of PC, may use the oleate synthesized by the chloroplasts for the synthesis of linoleate and linolenate. This reconstituted system could serve as the first step in the study of the transfer of oleate from the chloroplast to the microsomes and subsequently the transfer of the desaturation products to the chloroplast. The relative contributions of these two pathways for the synthesis of polyunsaturated fatty acids, which comprise over 95% of the fatty acids of spinach MGDG, is important to the understanding of MGDG biosynthesis.

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