THE BIOSYNTHESIS OF GALACTOLIPIDS IN RED CLOVER (*Trifolium pratense*)

A thesis presented in partial fulfilment of
the requirements for the degree of
Master of Science
in Biochemistry

By

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ABBREVIATIONS

Acyl-MGDG	-	6-O-acyl derivative of MGDG.
ACP	-	acyl carrier protein
AES	-	automatic external standardisation
ATP	-	adenosine 5'-triphosphate
C	-	Curie
C/M	-	Chloroform-methanol solution
CoA	-	coenzyme A
cpm	-	counts per minute
DEGS	-	diethylene glycol succinate
DGDG	-	digalactosyl diglyceride
DGG	-	digalactosyl glyceride
dpm	-	disintergrations per minute
DSC	-	Differential Scanning Calorimetry
DTA	-	Differential Thermal Analysis
Gal	-	Galactose
gm	-	gram
FFA	-	free fatty acid
l	-	litre
M	-	molar
MGDG	-	monogalactosyl diglyceride
MGDGH	-	hydrogenated MGDG
MGG	-	monogalactosyl glycerol
mgm	-	milligram
m.u.	-	mass units
NaDEDTC	-	sodium diethyl dithiocarbamate
PC	-	phosphatidyl choline
PE	-	phosphatidyl ethanolamine
PG	-	phosphatidyl glycerol
PI	-	phosphatidyl inositol
POPOP	-	1,4 bis (2-(5-phenyloxazolyl))-benzene
PPO	-	2,5-diphenyl oxazole
PVP	-	polyvinyl pyrrolidone
rac	-	racemic
sn	-	stereospecific numbering
SL	-	sulfo lipid
S.P.	-	sintering point

TGDG - trigalactosyl diglyceride

tol/EtOH/EtAc - toluene-ethanol-ethyl acetate solvent

Tris - Tri-(hydroxymethyl) methylamine

UDPgal - uridine 5'-diphosphate D-galactose

v - volume

Lipid nomenclature:

For the specific structural designation of complex lipids containing a glyceryl moiety, the nomenclature suggested by the IUPAC-IUB Commission on Biochemical Nomenclature (European J. Biochem. (1967) 2, 127) has been followed. The trivial names of complex lipids have been used when it is more appropriate to do so.

Fatty acids are designated by the shorthand notation of number of carbon atoms:number of double bonds, e.g. 16:3 refers to hexadecatrienoic acid.

Chapter One

REVIEW OF LITERATURE

1.1 Introduction:

Galactolipids occur in all cells capable of the photosynthetic evolution of oxygen. The main galactolipids are monogalactosyl diglyceride (MGDG), digalactosyldiglyceride (DGDG) and sulfolipid (SL), MGDG being the most abundant. These three together form the major nonpigment lipids in both leaves and green algae, accounting for about 40% of the total lipids. Nearly all of the cellular MGDG of plants is present in the chloroplasts and is concentrated in the lamellae.

Galactolipids contain unusually high percentages of polyunsaturated acids (MGDG contains greater than 90% in some plants), the major one being α-linolenic acid (9,12,15-octadecatrienoic acid; 18:3). Thus in higher plants most of the MGDG is monogalactosyl dilinolenoyl glycerol. MGDG may be the actual substrate of the desaturation reactions of α-linolenic biosynthesis.

Since the chemical characterisation of the galactolipids, many of the enzymatic reactions of their biosynthesis and degradation have been elucidated. An enzymatic transacylation which forms acylated galactolipids not normally present in leaves, has been discovered in plant homogenates. This complicates galactolipid metabolic studies using chloroplast preparations.

As the principal non-pigment lipid component of chloroplasts, galactolipids fulfil the usual structural role of lipids, being essential components of the lamellae. Their specific association with the photosystem suggests that they may have other specialised functions. They are thought to be necessary for the special arrangement of chlorophyll mole-

cules and associated enzymes in the photosystem to give the optimum geometry for light trapping and election transfer and the high content of 18:3 may be associated with this special role. The fatty acid portions of galactolipids in the lamellae probably provide the hydrophobic environment necessary for election transfer.

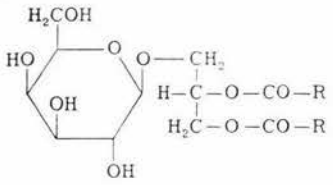
Recently galactolipids have been reviewed by Eccleshall (1970), Hawke (1971), Kates (1970) and Tremolieres (1970).

1.2 Occurrence:

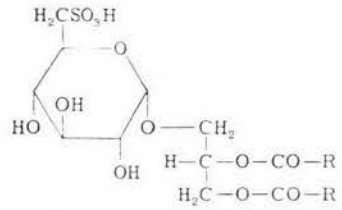
In addition to the main galactolipids MGDG, DGDG and SL (Benson, 1963), minor amounts of sterol- and acyl-sterol glycosides (Nichols, 1963; Eichenberger and Newman, 1968), polygalactosyl diglycerides (Webster and Chang, 1969; Galliard, 1969) glucocerebrosides (Carter and Koob, 1969) and a complex glycolipid called phytoglycolipid (Carter et al., 1969) are present in plant tissue. The structures of these are given in Figure 1.

The 6-O-acyl derivatives of MGDG and DGDG have been found as artifacts in aqueous spinach leaf homogenates (Heinz, 1967a; Heinz and Tulloch, 1969; Wintermans et al., 1969) but are not detected in total lipid extracts of leaves. These have also been shown to be lipid components of the bacterium Bifidobacterium bifidum (Exterkate and Veerkamp, 1969). Heinz demonstrated that 6-O-acyl MGDG is formed by an enzymatic transacylation to MGDG as acceptor in leaf homogenates (Heinz, 1967b). 6-O-acyl MGDG has been isolated and characterised from wheat flour of which it seems to be a normal constituent (Myhre, 1968; MacMurray and Morrison, 1970). However this does not necessarily mean that it is present as a normal constituent of wheat seed, as a 6-O-acylating enzyme may be released during the milling.

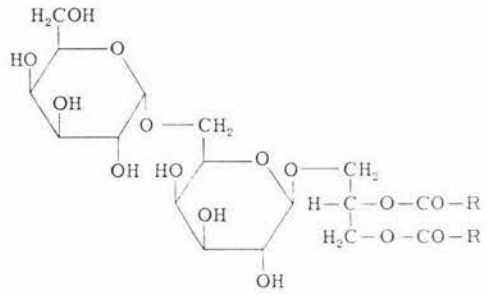
Detailed composition data of lipids in photosynthetic tissue



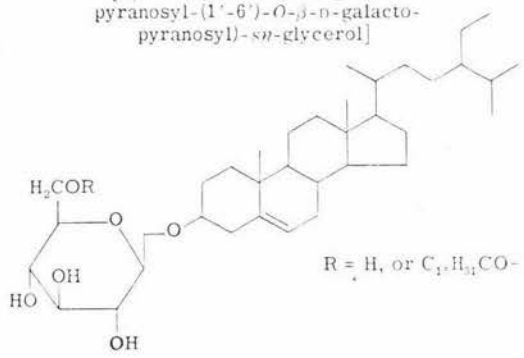
Monogalactosyl diglyceride
(1, 2-diacyl-3-*O*-β-D-galacto-pyranosyl-*sn*-glycerol)



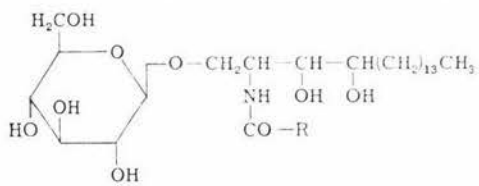
Sulfoquinovosyl diglyceride
[1, 2-diacyl-3-*O*-(6′-sulfo-α-D-quinovopyranosyl)-*sn*-glycerol]



Digalactosyl diglyceride
[1, 2-diacyl-3-*O*-(α-D-galacto-pyranosyl-(1′-6′)-*O*-β-D-galacto-pyranosyl)-*sn*-glycerol]



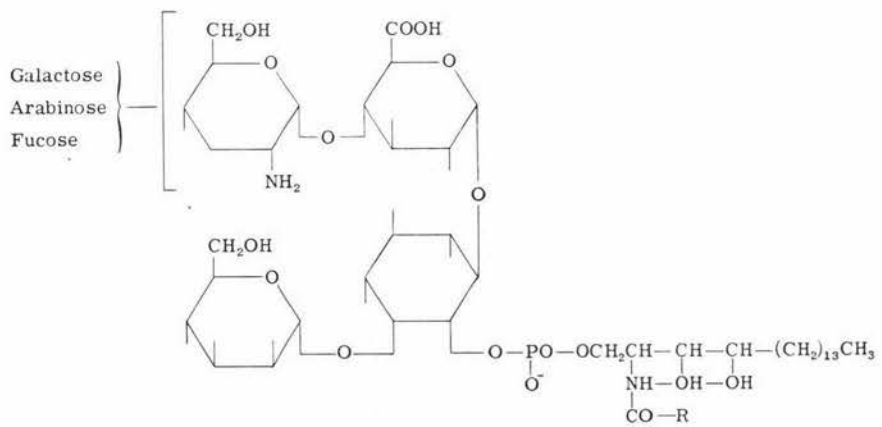
β-Sitosterol glucoside or (6-acyl) glucoside
[β-D-glucopyranosyl-(1-3′)-β-sitosterol
or
6-acyl-β-D-glucopyranosyl-(1-3′)-β-sitosterol]



Glucocerebroside
[*N*-acyl-β-D-glucopyranosyl-(1-1′)-phytosphingosine]

Figure 1.

Structures of Plant Glycolipids



Phytoglycolipid
N-Acyl-phytosphingosyl-1-phosphoryl-1′-*O*-{2′-*O*-mannosido-6′-*O*-[(fucosyl-arabinosyl-galactosyl)-D-glucosamido-(1→4)-D-glucuronido]}-inositol

are known for very few plants even though MGDG and DGDG have been identified in numerous species including higher plants, algae, some flagellates, and the blue green algae. The known data are summarised in Kates (1970), Hawke (1971) and Eccleshall (1970). Spinach leaf is the most completely analysed tissue and Table 1 gives the data for this species and some pasture species.

The molar ratio of MGDG to DGDG for the leaf tissues of various species examined by Roughan and Batt (1969) varied from 1.0 to 2.4. Webster and Chang (1969) found that the molar ratio of galactolipids in Spinach was MGDG:DGDG:trigalactosyl diglyceride (TUDG): tetragalactosyl diglyceride = 60:30:5:1.

Kates (1970) makes the observation that there appears to be a clear-cut distinction between the lipid composition of the photosynthetic apparatus of plants that evolve oxygen during photosynthesis (Hill reaction) and those that do not. Leaf chloroplasts and green and blue-green algae contain in common MGDG, DGDG, SL, and phosphatidyl glycerol, whereas photosynthetic bacteria which are incapable of the Hill reaction have only phosphatidyl glycerol.

From the data for spinach in Table 1, it can be seen that much of the lipid of whole leaf is concentrated in the chloroplast (33-36% of its dry weight) and about half the dry weight of the chloroplast lamellae is lipid. Data on the cellular distribution of galactolipids are limited, as relatively few studies have been made with cell fractions. Those that have been carried out, show that chloroplast lamellae contain most of the cellular galactolipid. (Table 2).

Table 1.

Overall quantitative lipid composition of photosynthetic tissues.[1]

	Spinach leaves[2]			Red Clover[2] leaves	Perrenial[3] Ryegrass leaves	Alfalfa leaves[4] (Vernal, 20°C)
	Total	Chloroplasts	Lamellae			
Phospholipids	23	10	12	21		40.4
MGDG	20	30	22	16	20.9	26.5
DGDG	13	15	15	8	12.8	24.0
Sulpholipids	4	5	7	+		9.1
Sterol and sterol esters	1	0.2	0	1.4		
Sterol glycosides	0.5	traces	0			
Cerebrosides	+	0	0			
Chlorophylls	13	23	21			
Carotenoids	2	4	3			
Quinones	2	1	3			
Waxes and hydrocarbons	9	0	0	0.4		
Ceryl alcohol	9	0	0			
Glycerides	+	0	-	2.1		
Total lipid (% of tissue dry weight)	9	33-36	49	7		

(1) Expressed as % by wgt of total lipids. Gaps indicate values not determined.
(2) Kates (1970).
(3) Gray et al. (1967).
(4) Kuiper (1970).

Table 2

Lipid composition of fractionated tissue.

		Phospholipids				Galactolipids		
		PI	PG	PE	PC	SL	DGDG	MGDG
sugar beet[2]	total leaf[1]	5.2	9.4	11.6	24.2	9.7	14	25.9
	chloroplasts[1]	2.4	9.6	3.9	9.6	5.5	24.8	44.2
	cytoplasm[1]	9.0	9.0	22.3	44.3	15.4	0	0
	chloroplast (% of cellular lipid).	27	60	20	23	33	100	100
tobacco chloroplasts[3] (% of cellular lipid).		nd	74	nd	nd	76	88	83

nd not determined
(1) % of total lipid.
(2) Wintermans (1960).
(3) Ongun, Thomson and Mudd (1968).

Recently, Bishop et al. (1971) have examined the distribution of galactolipids in mesophyll and bundle sheath chloroplasts because of the different photosynthetic capabilities of these two types of chloroplasts (Slack, 1970). Their analyses show that the galactolipid content of bundle sheath chloroplasts is significantly higher on a chlorophyll basis than that of mesophyll chloroplasts. They suggest that the molar ratios of galactolipid to chlorophyll reflect the degree of grana formation in a chloroplast, as bundle sheath chloroplasts show good grana formation in contrast to the agranal mesophyll chloroplasts.

MGDG is present in nonchlorophyllous plant tissue such as dark-grown Euglena, etiolated leaves, potato tubers, wheat flour etc., and also in mammalian nervous tissue (Eccleshall, 1970).

1.3 Structure and Properties:

(a) Characterisation of Galactolipids:

Carter and coworkers (1956, 1961a, b) elucidated the structures of DGDG and MGDG obtained from wheat flour. They isolated a mixed glycolipid fraction which gave two distinct carbohydrates on mild alkaline hydrolysis (Carter et al., 1956). Both of these were shown to consist of galactose and glycerol only and the proportions agreed with those of monogalactosyl glycerol and digalactosyl glycerol. The first (MGG) consumed 3 moles of periodate to give 1 mole of formic acid and 1 mole of formaldehyde; the second (DGG) consumed 5 moles of periodate to give 2 moles of formic and 1 of formaldehyde. These results, and the action of α and β galactosidases are consistent with the structures:

β-D-galactopyranosyl-1-glycerol

and β-D-galactopyranosyl-(1→6)-O-α-D galactopyranosyl-1-glycerol.

Later work (1961a and b) gave saponification equivalents consistent with 2 acyl groups per molecule of both MGDG and DGDG. Complete methylation and alkaline deacylation gave products which in both cases consumed 1 mole of periodate and released formaldehyde. Acid hydrolysis of these deacylated compounds yielded 2,3,4,6-tri-O-Me-D-galactose and free glycerol for MGDG, and 2,3,4-tri-O-Me-D-galactose, 2,3,4,6-tetra-O-Me-galactose and free glycerol for DGDG. This meant that the two acyl groups were located on the glycerol for both MGDG and DGDG.

Wickberg (1958a, b) synthesized a series of α and β galactosides of 1 and 3-O-sn-glycerol which were found to have characteristic and different infra red (IR) spectra. Collaboration between

Wickberg, and Carter's group established the absolute configuration of the glycerol in the wheat flour MGDG and DGDG as 3-O-sn-glycerol. Supporting evidence comes from the radiochemical experiments of Miyano and Benson (1962). The ^{14}C- labelled galactosyl glycerols formed on deacylation of the lipid products of Chlorella when photosynthesis occurs in the presence of $^{14}CO_2$ were oxidised with nitrogen dioxide. The oxidation products were hydrolysed to yield glyceric acid. Cocrystallisation of this with salts of glyceric acid of known configuration confirmed the above assignment for the glycerol configuration of MGDG and DGDG.

Sastry and Kates (1964a) showed that the galactosyl diglycerides from runner bean leaves have structures identical with those from wheat flour. Analysis gave the required molar ratios of galactose, glycerol and fatty acid. Alkaline hydrolysis gave 1 mole of MGG and DGG from 1 mole of MGDG and DGDG respectively. These galactosyl glycerols had melting points and infra red spectra identical with those of Carter and Wickberg, and reacted with periodate to yield formaldehyde in molar ratios close to the theoretical values of 3:1 for MGG and 5:1 for DGG.

Hence the structures of the plant galactolipids are:

MGDG: 1,2-di-O-acyl, 3-O-(β-D galactopyranosyl)-sn-glycerol.

DGDG: 1,2-di-O-acyl, 3-O-(β-D galactopyranosyl-(1→6)-O-α-D--galactopyranosyl)-sn-glycerol. (See Figure 1.)

Galliard (1969) isolated trigalactosyl diglyceride (TGDG) from potato tubers. This had a fatty acid ester to galactose ratio of 1.53:1 and a galactose to glycerol ratio of 2.9:1. Its infra red

spectrum was similar to those of MGDG and DGDG, the OH peaks (about 3400 cm^{-1}) decreasing in size TGDG > DGDG > MGDG. The deacylation products of these three when run in two solvents, had R_f's characteristic of an homologous series. The molar ratio of periodate consumed to formaldehyde liberated was 7:1 for the deacylated TGDG and it was not hydrolysed by galactosidase. Thus the structure of TGDG was shown to be:

1,2-di-O-acyl, 3-O-(β-D-galactopyranosyl-(1→6)-O-α-D-
-galactopyranosyl-(1→6)-O-α-D-galactopyranosyl)-sn glycerol.

Webster and Chang (1969) identified TGDG in spinach chloroplasts. It had galactose:glycerol:ester ratios, periodate reaction, and hydrolysis products from exhaustive methylation consistent with the above structure. Another galactolipid was also isolated. It behaved with MGDG, DGDG and TGDG as an homologous series on TLC, both as the intact and deacylated lipids. It was concluded that the fourth galactolipid was tetragalactosyl diglyceride.

(b) Characterisation of acylated galactolipids:

Heinz (1967) isolated an acyl galactosyl diglyceride from a spinach leaf homogenate which was held at 4°C for 6-8 hrs. It was not found when the leaves were placed into boiling water before homogenation, indicating enzymic synthesis in the homogenate. The pure lipid was more hydrophobic than MGDG and was composed of glycerol, galactose and fatty acids in a molar ratio of 1:1:3. After deacylation crystalline galactosyl glycerol was obtained and identified as 3-O-(β-D-galactopyranosyl)-sn-glycerol by comparison of its melting point, optical rotation, and infra red spectrum with those of the

series of galactosyl glycerols synthesized by Wickberg (1958b). The stoichiometry of this MGG was consistent with attachment of the galactose at the 3 position of the glycerol.

In order to determine the position of the three acyl groups, the glycolipid was methylated and saponified to yield trimethyl galactosyl glycerol. On hydrolysis this substance gave a mixture of 65% 2,3,4-, 30% 2,3,6- and a small amount of 2,4,6-tri-O-methyl D galactose. This suggested that two acyl groups were attached to the glycerol and the third to the 6 position of the galactose. Heinz and Tulloch (1969) showed by nuclear magnetic resonance (NMR) spectroscopy that the third acyl group is wholly at the 6 position. Presumably the two extra trimethyl galactoses obtained previously in the methylation study were artifacts produced by acyl migration. The NMR spectra of the acyl MGDG and 6-O-acyl MGDG synthesized from spinach MGDG were indistinguishable. They differed from the MGDG spectrum in that the signal due to the proton at galactose C6 was displaced downfield about 0.5 ppm, which is characteristic of acylated primary alcohols. Corroborating evidence was obtained from the spectra of acetylated MGDG and acetylated acyl MGDG, the latter having no acetate signal corresponding to C6.

Thus the structure of this acylated MGDG is:

1', 2'-di-O-acyl-3'-O-(6-O-acyl-β-D galactopyranosyl)-sn-glycerol.

Myher (1968) isolated and characterised this lipid from soft wheat flour. It contained fatty acids and galactosyl glycerol in the molar ratio 3:1, and methylation studies suggested that the third acyl group was at C6. Periodate oxidation studies confirmed this as the product was tri-O-acyl-dialdehyde and free fatty acids were not

released. Reduction of the dialdehyde by $NaBH_4$ followed by saponification, acid hydrolysis and a further reduction gave glyceritol and ethylene glycol as expected for the tri-O-acyl dialdehyde derived from 6-O-acylated MGDG.

Wintermans et al. (1969) found three new galactolipids formed by spinach chloroplasts. On deacylation, two of these were shown to be derived from MGDG and one from DGDG. The ester:galactose ratios obtained were variable. However, it was suggested that the digalactolipid was monoacylated (ratio of 1.6 or higher) and that the most hydrophobic MGDG derivative may be the acylated MGDG of Heinz (ratio usually well above 3).

Exterkate and Veerkamp (1969) identified monoacyl MGDG, diacyl MGDG and monoacyl DGDG in lipid extracts of the cell walls of Bifidobacterium bifidum. The identification was made on the basis of their deacylation products and their fatty acid:galactose:glycerol ratios.

(c) Fatty acid composition:

The fatty acid composition of leaf tissues and galactolipids from various sources have been reviewed by Kates (1970), Hawke (1971) and Eccleshall (1970). Table 3 gives data for spinach and some pasture species. Whilst data for fractionated plant tissue are limited, most MGDG is present in the chloroplast and thus the composition of MGDG isolated from whole leaf should reflect the chloroplast composition.

Plant MGDG is generally characterised by the fatty acids 18:3, 18:2, 18:1, 18:0 and 16:0. However the proportions of these

Table 3.

Fatty acid composition of lipids of leaf tissue.

Source	Lipid	Fatty acids (%) 12:0	14:0	16:0	Δ^{3t}16:1	16:3	18:0	18:1	18:2	18:3
Spinach[1]	MGDG	-	-	tr	tr	30	-	1	1	67
	DGDG	-	-	6	-	3	1	4	3	84
	SL	-	-	27	-	-	-	6	39	28
Alfalfa[2] (grown at 30°C)	MGDG	1.1	2.3	7.0	-	-	2.2		10.9	76.5
	DGDG	2.6	3.2	21.2	-	-	9.0		29.8	34.2
	SL	0.6	2.0	30.4	-	-	7.1		24.7	35.2
Perennial Ryegrass[3]	MGDG	-	0.2	3.4	-	-	0.9	1.9	3.2	90.4
Barley seedlings[3]	MGDG	-	tr	4.8	-	-	1.3	1.6	1.0	85.6
Fescue[4]	MGDG	-	-	0.6	0.1*	-	0.3	0.3	1.5	97.2
	DGDG	-	-	14.6	3.4*	-	1.1	1.3	4.6	75.0

(1) Kates (1970)
(2) Kuiper (1970)
(3) Gray et al. (1967)
(4) Hawke (unpublished)
* Δ^{9}16:1.

are dependent on such variables as light environment, age of the tissue, and temperature, with genetic differences superimposed on these. The galactolipids are extremely rich in triene fatty acids, usually α-linolenic acid, and are the most unsaturated of the leaf lipids. MGDG and DGDG may possess up to 97% and 93% respectively of their

fatty acids as α-linolenic, with MGDG generally being more unsaturated than DGDG.

Spinach MGDG has a characteristically high proportion (about 30%) of a second trienoic acid, 7,10,13-hexadecatrienoic acid (16:3), whereas DGDG contains little of this. 16:3 has also been found in some green algae and rape leaves.

α-linolenic acid (cis 6,9,12 octadecatrienoic acid) which is usually associated with animal type metabolism, is abundant in the Boraginaceae family of herbaceous plants and present at lower levels (less than 3%) in some members of the Carophyllaceae (Jamieson and Reid, 1971). The latter also contain significant amounts of 18:4.

The fatty acid composition of blue green algae can be quite variable compared with that of plants. Fatty acids such as α-linolenic acid, 18:4, and C_{20} and C_{22} acids have been found in various species. Anacystis nidulans MGDG is entirely lacking in polyunsaturated fatty acids and this fact may be relevant to a discussion of the role of polyunsaturated fatty acids in the chloroplast.

Galactolipids of nonchlorophyllous tissues of higher plants contain mainly linoleic and linolenic acids, but the levels of 18:3 are lower than those for green leaf MGDG.

Heinz (1967b) obtained a crude lipid free preparation of the spinach enzyme that synthesizes 6-O-acyl MGDG. He showed that the third fatty acid attached to C_6 of galactose of acyl MGDG was derived from which ever lipid was added to the enzyme in the presence of MGDG. The third fatty acid of acyl MGDG formed by a spinach homogenate was found to come from DGDG (Table 4). The acyl MGDG fatty acid compo-

sition is intermediate between the MGDG and DGDG compositions. The 0.2% 16:1 present in the acyl MGDG probably came from phosphatidyl glycerol which is the main phospholipid of spinach chloroplasts and contains 30-40% 16:1.

Table 4.

Fatty acid composition of galactolipids isolated from spinach leaf homogenate+.

Fatty acid	MGDG	DGDG	AMGDG	AMGDG*
16:0	-	8.2	2.4	2.7
16:1	-	-	0.2	-
16:2	0.4	-	0.5	0.3
16:3	22.8	3.1	17.0	16.3
18:0	-	0.4	-	0.1
18:1	0.9	2.6	1.4	1.5
18:2	1.5	4.2	2.0	2.4
18:3	74.4	81.5	76.5	76.7

\+ Heinz (1967b). Fatty acid composition in %.
* Acyl MGDG formed during incubation of crude enzyme preparation with MGDG and DGDG.

(d) Specific composition of galactolipids:

Nichols and Moorhouse (1969) used argentation TLC to separate Chlorella MGDG into five molecular fractions containing one to five double bonds per molecule. Fatty acid analysis of these fractions demonstrated the significant feature that in each case the major component acids did not differ in degree of unsaturation by more than one double bond. For instance, the fraction with two double bonds per molecule was almost entirely composed of species with two monoenoic

acids per molecule and the only fraction which contained trienoic acids was the one with five double bonds per molecule.

Pancreatic lipase specifically removes the fatty acids from the glycerol 1-position of galactosyl diglycerides and has been used to determine the positional arrangement of galactolipids obtained from algae and plants. For algal MGDG's which generally have a more complex fatty acid composition than plant MGDG's, the major factor controlling the arrangement of fatty acids is chain length (Safford and Nichols, 1970). Thus C_{18} acids are concentrated at the 1-position and C_{16} acids at the 2-position.

In plant leaf MGDG's with the characteristically high 18:3 content, it is obvious that the dilinolenoyl molecular species is predominant and consequently positional tendencies are not pronounced. Noda and Fujiwara (1967) concluded that for MGDG from Artemisia princeps leaves which contains 94% 18:3, the fatty acids at the 2-position are more unsaturated than those at the 1-position. This is the type of specificity obtained in animal, plant (Sastry and Kates, 1964a), and algal (Safford and Nichols, 1970) phospholipids which are more saturated than MGDG's. However for MGDG's from the plant species which contain significant levels of 16:3 besides 18:3 (e.g. Spinach), the algal type of specificity applies with the shorter chain 16:3 concentrated at the 2-position and 18:3 at the 1-position (Safford and Nichols, 1970; Auling et al., 1971). These apparently contradictory results could possibly be due to the differences in the overall fatty acid compositions of the MGDG's studied. For instance, in Anchusa MGDG which contains about 40% 18:4 besides 18:3, there is no possibility of chain length specificity as nearly

all of the fatty acids are C_{18}. Safford and Nichols found that in this case the 18:4 is concentrated at the 2-position in accordance with the unsaturation specificity noted by Noda and Fujiwara.

Auling et al. also used gas-liquid chromatography of TMS ethers of hydrogenated galactolipids to examine the molecular species. Galactolipids with two C_{18} residues (C_{18}/C_{18}) and C_{16}/C_{18} were present in plants but C_{16}/C_{16} was absent as would be expected for positional specificity determined by chain length. In spinach MGDG there was about 50% C_{16}/C_{18}; 50% C_{18}/C_{18} and in the DGDG 27% C_{16}/C_{18}; 73% C_{18}/C_{18} in accord with the much lower levels of 16:3 in DGDG compared with MGDG. 10% C_{16}/C_{16} was present in the alga Anabaena which is to be expected as its MGDG contained more than 50% C_{16} acids. Galactolipids from plants with mostly 18:3 were not examined in this study.

(e) Chemical synthesis and Physical properties:

Wehrli and Pomeranz (1969) have recently developed a method for synthesizing optically active MGDG. It involves acylation of the primary hydroxyl groups of 2,5-methylene-D-mannitol, cleavage of the mannitol moiety between C_3 and C_4 using lead tetra-acetate, reduction of the resulting aldehyde, attachment of galactose, hydrolysis of the acetal, acylation of the OH group and hydrazinolysis of the acetylated glycolipids. A simpler procedure in which the optical activity at C_2 is lost, is also described. The acyl groups used were 8:0, 10:0, 12:0, 16:0, 18:0 and 18:2.

Heinz (1971) prepared a series of semisynthetic MGDG's and DGDG's having identical or different acyl groups attached to C_1 and

C_2 of the glycerol. He used natural galactolipids as starting material, blocked the galactosyl hydroxyls with O-1-methoxyethyl groups, removed the acyl groups with sodium methoxide, reesterified with new fatty acids and hydrolysed the protecting groups with boric acid. The acyl groups used were acetyl, 16:0, 16:1, 18:0 and 18:1.

Heinz and Tulloch (1969), have described a method for synthesizing 6-O-acyl MGDG involving trimethylsilylation of the galactosyl hydroxyl's of hydrogenated MGDG from spinach, specific hydrolysis of the 6-O-methyl silyl group with potassium carbonate in methanol, acylation of the 6-hydroxyl with stearoyl chloride and hydrolysis of the rest of the blocking groups with water.

Synthetic galactolipids with known fatty acids in definite positions will be useful substrates for studying galactolipid transformations such as the acyl MGDG forming activity, especially if the fatty acids are ^{14}C labelled. Highly purified synthetic galactolipids are also useful as standards in studying their physical properties. Table 5 gives a summary of melting point and optical data for galactolipids from various sources.

The sintering point is due to a crystal structure transition and can be observed in a microscopic melting point apparatus, especially when polaroid optics are used. Useful techniques for studying thermal behaviour are differential thermal analysis (DTA) and differential scanning calorimetry (DSC) which were reviewed by Ladbrooke and Chapman (1969). In DTA the sample and an inert reference material are heated or cooled at the same rate and the difference in temperature between them is recorded. If the sample undergoes a thermal reaction, a peak is obtained, its direction indicating whether the transition is

Table 5

Melting points and optical rotations for some galactolipids.

Lipid	SP (°C)	MP (°C)	$[\alpha]_D^{21}$	conc.[5]	Reference
Hydrogenated MGDG	91-92.5	151.5-153.5	-2.28°	5.6	1
from plants	122	149			2
	100-102	162-163	-1.97°[6]		3
Synthetic MGDG					
$R_1 = R_2$ = acetyl		142-143	-3.19°	5.3	1
$R_1 = R_2$ = 16:0	91-93	152-154	-2.04°	4.7	
$R_1 = R_2$ = 18:1			-2.27°	5.6	
R_1 = 18:1, R_2 = 16:0			-2.04		
R_1 = 18:0, R_2 = 16:0	93-94	152-154	-2.24°	3.8	
R_1 = 16:0, R_2 = acetyl			-4.17°	4.5	
rac $R_1 = R_2$ = 16:0	54.5				4
rac $R_1 = R_2$ = 18:0	60-65				
$R_1 = R_2$ = 16:0	56				
6-O-acyl MGDG	67-69	79	-1.30°[6]	5.23[7]	3

1. Heinz (1971)
2. Sastry and Kates (1964a)
3. Heinz and Tulloch (1969)
4. Wehrli and Pomeranz (1969)
5. Concentration in pyridine for $[\alpha]$ determination
6. $[\alpha]_D^{25}$
7. In chloroform. SP. - sintering point.

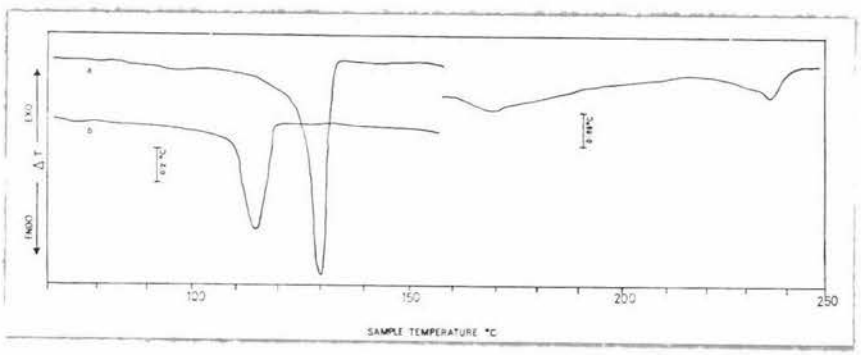
endothermic or exothermic. In DSC the temperatures of a sample and a reference are maintained at an equal level or a fixed differential throughout the analysis and the variation in heat flow to the sample required to maintain this level during a transition is measured. Peak area gives a measure of the heat of the transition. Very small amounts of impurities (0.5 mole %) affect the peak shape. This can be allowed for to give a correct heat of fusion and an estimation of purity based on an application of van't Hoff's equation.

Although polymorphic behaviour is common with lipids, no thermal analyses of galactolipids have been found in the literature. Triglycerides and phospholipids have been studied extensively. As the polar phospholipids are closer in structure to galactolipids, the thermal behaviour of phosphatidyl cholines (PC) will be described. PC's are hygroscopic and are normally obtained from organic solvents in hydrated form. Anhydrous 1,2-dibehenoyl PC (two 22:0 acyl groups) can be obtained by slow crystallisation from dry diethyl ether/chloroform mixtures. Figure 2 gives the DTA data for this lipid. A large endothermic transition, many degrees below the melting point (236°C), occurs on heating, corresponding to transition to a liquid-crystalline phase (curve a). If the lipid is heated above this transition, cooled to room temperature and reheated a different transition occurs at a lower temperature with a smaller heat change due to transition to the liquid-crystalline phase from a second polymorphic form. This form is extremely hygroscopic and forms the monohydrate on exposure to the atmosphere with a further reduction in transition temperature (not shown). Several forms of the monohydrate have been obtained by crystallisation from different solvents.

IR spectra for MGDG and DGDG from both plant and synthetic sources have been published (Allen et al., 1966; Heinz, 1967a; Wehrli and Pomeranz, 1969). These spectra are similar with strong bonds for: OH (3400 cm^{-1}, broad), CH_2 (2940, 2860, 1460 and 710 cm^{-1}), ester C = O (1730 cm^{-1}) and ester C-O- (1165 cm^{-1}), alcohol C-O- (1070 cm^{-1}) and cis double bonds for unsaturated material (3000, 1650, 690 (shoulder)) (Sastry and Kates 1964a).

The IR spectrum of acyl MGDG is similar to these although

Heinz does claim that the OH peak is relatively smaller than that of MGDG as expected. However this is only a reliable difference if all traces of water are removed from the samples.



DTA curves for anhydrous 1,2-dibehenoyl-PC crystallised from $CHCl_3$/ether.
(a) Crystalline material heated from room temperature.
(b) Material previously heated to 160°C, cooled to room temperature and reheated. The origin of the small transition near 170°C is not known (Ladbrooke and Chapman, 1969).

Figure 2.

Mass spectroscopic analysis of MGDG has not been described in the literature. Trimethyl silyl (TMS) ethers of polar and nonpolar-lipids have been used to increase their volatility and to give more characteristic fragmentation modes in mass spectrometry. For example, the molecular species of sterol glycosides were separated by GLC as the TMS ethers and identified by mass spectrometry (Laine and Elbein, 1971). However mass spectrometry of lipids is especially useful if the

unblocked lipids give characteristic and identifiable spectra as they can be extracted and identified after TLC.

Triglycerides have been studied (Barber et al., 1964) including the molecular weight range tripalmitin through to tribehenin. In all cases a parent molecular ion was obtained and it was possible to distinguish the acyl group attached at position 2 from those at positions 1 and 3 of the glycerol moiety.

Recently Klein (1971a) has described fragmentation patterns for unblocked dipalmityl-, dioleoyl- and 1-stearoyl, 2 oleoyl-glycerophosphorylcholines. Significant differences between these were observed, and fragment ions were identified by high resolution mass measurements. Ions associated with the hydrocarbon chains, glycerol esters and phosphorylcholine were identified. For dioleoyl PC a small peak corresponding to the molecular ion was obtained. Subsequently a more detailed description of the fragmentation sequence has been published (Klein, 1971b). By observing metastable transitions in the first field free region of a double focussing spectrometer it was shown that the major ions in the spectrum were produced by election impact processes and not by pyrolysis. Many of the ions were related to each other by metastable processes.

NMR spectra of MGDG and DGDG in pyridine or $CDCl_3$ have been described (Heinz, 1971; Heinz and Tulloch, 1969; Wehrli and Pomeranz, 1969). MGDG in pyridine gives the following signals downfield from the proton signal of tetramethylsilane internal standard. signals from acyl groups: terminal $-CH_3$, triplet at 0.88 ppm; bulk of internal $-CH_2-$ at 1.28 ppm. and $-CH_2-$ triplet at 2.33 ppm.

other signals: galactose H_1, doublet at 4.73 ppm; glycerol H_2', multiplet at 5.53 ppm.

6-O-acyl MGDG in pyridine has the same acyl signals as MGDG (Heinz and Tulloch, 1969) but the signal from galactose H_1 is a doublet superimposed on the galactose H_6 multiplet at 4.70 ppm. The use of NMR in determining the position of attachment of the third acyl group of acyl-MGDG was described in Section 1.2(b).

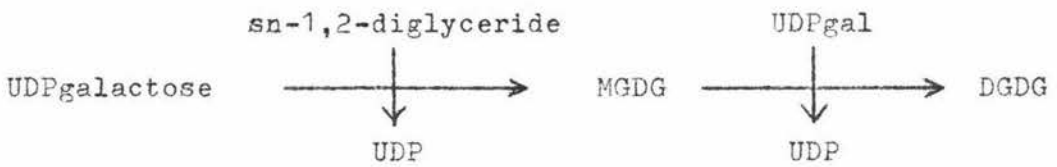
1.4 Biosynthesis:

(a) The overall synthetic pathway:

Most of our present knowledge of galactolipid biosynthesis has come from studies on Chlorella, spinach or Euglena using radioactive substrates.

Studies on the incorporation of CO_2 into the lipids of Chlorella (Ferrari and Benson, 1961) showed that MGDG was the most rapidly labelled, followed by DGDG, suggesting that the latter was synthesized by galactosylation of the MGDG. They assumed that UDP galactose (UDPgal) was the immediate galactose donor and proposed the following reaction scheme:

$CO_2 \rightarrow$ phosphoglyceric $\rightarrow \rightarrow \rightarrow$ UDPglucose $\rightarrow$ acid



Incorporation of Galactose:

Neufield and Hall (1964) tested this scheme by incubating a chloroplast preparation from spinach leaves with UDPgal ^{14}C. This resulted in the incorporation of ^{14}C into mono-, di-, tri- and possibly tetra- galactosyl diglycerides. Incubation with UDP glucose ^{14}C also gave incorporation into galactolipids but as it was at a lower level it was attributed to the UDP galactose epimerase present in chloroplasts.

Ongun and Mudd (1968) and Mudd et al. (1969) confirmed these studies. They also showed that MGDG isolated from chloroplast was the acceptor for the second galactosylation to give DGDG and that

DGDG possibly gives TGDG. Some evidence was obtained for the presence of two separate enzymes for the synthesis of MGDG DGDG which would be likely because of the different nature of the two linkages involved. Optimum conditions for incorporation into galactolipids were pH 7.2 in Tris or phosphate buffers at 37°C. Higher pH favoured MGDG rather than DGDG or TGDG synthesis.

The incorporation of ^{14}C from UDPgal^{14}C into galactolipids has been demonstrated in several other species suggesting the generality of this reaction for MGDG-containing organisms. Euglena gracilis has some significant differences from the spinach system (Lin and Chang, 1971). About 70% of the galactose incorporated in one hour was actually transferred in two minutes. The MGDG:DGDG ratio was 1:2, compared with about 3:1 for spinach (Chang and Kulkarni, 1970). Photoauxotrophic and photoheterotrophic Euglena incorporate UDPgal^{14}C but strict heterotrophs do not (Matson et al., 1970). The Gramineae; fescue, ryegrass, fog and phalaris also synthesize galactolipids using UDP galactose (Eccleshall and Hawke, 1971).

An important step towards a more complete understanding of galactolipid biosynthesis was the preparation of a soluble sub-chloroplast fraction from spinach leaves which could incorporate UDPgal^{14}C into galactolipids - mainly MGDG and DGDG (Chang and Kulkarni, 1970). This had pH and temperature optima similar to the intact chloroplast enzyme of Mudd. Different K_M values for MGDG and DGDG were obtained and this could indicate separate enzymes for the two galactosylations. Some stimulation of the reaction by light was observed.

Recently Chang (1970) published data from experiments in which sulfhydryl inhibitors were added to incubations of spinach

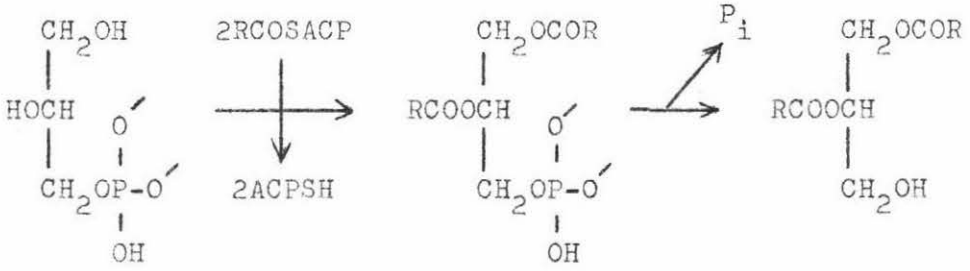
chloroplasts with UDPgal ^{14}C. The results suggest that the galactosyl transfer enzymes contain one or more sulfhydryl groups. Parachloromercuribenzoate, iodoacetate and low levels (0.5 µmole) of Hg^{2+} inhibited the incorporation of ^{14}C into galactolipids almost completely. The reducing agent, mercaptoethanol, restored more than 60% of the activity present before the addition of Hg^{2+}. Similar results have been obtained with Euglena chloroplasts (Lin and Chang, 1971).

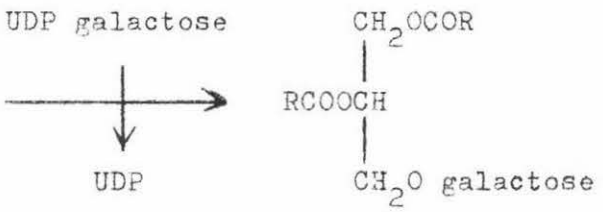
It is generally accepted that DGDG's are synthesized from MGDG's by galactose addition via UDP galactose. Ongun and Mudd (1968) were able to demonstrate such a precursor-product relationship using radioactive MGDG and chloroplasts, the endogenous lipids of which were removed by acetone extraction. However, in a similar experiment with Euglena no such relationship was observed (Lin and Chang, 1971). These workers suggested that a separate biosynthetic pathway involving a digalactosyl-acceptor intermediate may exist for DGDG. A further complication is the consistent difference in the fatty acid composition of MGDG and DGDG isolated from the same tissue. If DGDG is formed from MGDG, then the galactosylation must be very specific for MGDG of a particular fatty acid composition, or some sort of deacylation-reacylation must occur.

Incorporation of fatty acids and identification of diglyceride as galactose acceptor:

Knowledge of the pathway of fatty acid incorporation into galactolipids has come from the experiments with photoauxotrophic Euglena of Renkonen and Bloch (1969). They showed that cell-free extracts catalyse the transfer of oleoyl and stearoyl (but not palmi-

toyl) groups from acyl carrier protein (ACP) thioesters into MGDG, but not phospholipids or DGDG. Fatty acids from coenzyme A (CoA) thioesters are incorporated into both MGDG and phospholipids. The incorporation of the ACP esters was stimulated by α-glycerophosphate and UDP galactose to a lesser extent. These results suggested the following reactions in which α-glycerophosphate accepts fatty acid groups from ACP and possibly CoA, to form phosphatidic acids, followed by dephosphorylation to 1,2 diglycerides before reaction with UDP galactose:





Support for such a system has come from experiments with isolated chloroplasts and etioplasts of spinach and maize (Douce and Guillot-Salomon, 1970). These incorporated label from sn-glycerol-3--phosphate-^{14}C into MGDG in the presence of CoASH, ATP and UDP galactose. Exogenous fatty acids were not required. There was no incorporation into DGDG and these workers consider that the enzymes responsible for galactosylation of diglycerides and MGDG's are spatially separated.

Evidence for diglyceride being the initial galactose acceptor, has come from work with acetone extracted chloroplasts.

These acetone powder preparations require the readdition of the acetone extract before they will incorporate UDPgal^{14}C into MGDG. Ongun and Mudd (1968) were the first to show that the addition of diglyceride could give the same stimulatory action as the acetone extract. They also made a crude fractionation of the acetone extract. Of the three fractions obtained, only one, containing all the MGDG, most of the DGDG and a small amount of the phosphatidyl glycerol of the extract, stimulated the incorporation into MGDG. Either the galactolipids were degalactosylated by galactosidase in the acetone powder, or the incorporation obtained with this fraction was due to exchange reactions. Very little free diglyceride was in fact present in the total acetone extract.

Active acetone powders could not be prepared from Gramineae chloroplasts (Eccleshall and Hawke, 1971). The acetone powder of Euglena chloroplasts (Lin and Chang, 1971) was active when the extract was added. No attempt to add diglyceride to the powder was reported.

(b) Fatty acid desaturation and the polyunsaturated fatty acids of MGDG:

It has been shown that the chloroplast is the major site of fatty acid synthesis in leaf tissue (Harris et al., 1967) and that isolated chloroplasts can synthesize long chain fatty acids up to and including stearic acid. This synthesis is stimulated by ACP and probably involves a sequence similar to that worked out in detail for bacteria (Nagai and Bloch, 1967). However, while leaves or tissue slices will efficiently incorporate ^{14}C-acetate into monoenoic and polyenoic acids, and can convert oleic acid directly to linoleic and linolenic acids by oxygen and light dependent reactions, repeated attempts to demonstrate formation of polyunsaturated fatty acids by

isolated chloroplasts have been unsuccessful (Harris et al., 1967). This is a major gap in our knowledge of fatty acid biosynthesis in the plant leaf. As the chloroplast contains most of the polyunsaturated fatty acids of the leaf, it seems unlikely that the site of desaturation is outside of it. Thus the desaturase must be very sensitive to the procedures used for disrupting leaf tissue.

The actual form of the acyl group in the substrate required by the desaturase is also unknown. Furthermore, in the particular case of the fatty acids of MGDG, it is still uncertain whether their characteristically high unsaturation is determined before diglyceride galactosylation, or whether desaturation occurs within the MGDG molecule following its synthesis.

Acetone powder experiments:

Studies in which diglycerides of known structure were added to spinach acetone powders to determine whether or not the galactosylating enzyme is specific for more unsaturated diglycerides, have given conflicting results.

Ongun and Mudd (1968) showed that diolein stimulates incorporation from UDPgal ^{14}C into MGDG, whereas dipalmitin does not. Mudd et al. (1969) used diglyceride fractions of varying degree of unsaturation prepared from spinach and egg phospholipids. It was found that the more unsaturated diglycerides gave the greater stimulation of incorporation, dilinolenin being the best. These results suggest that the galactosyl transferase does exhibit selectivity for unsaturated diglyceride acceptors. In contrast, a separate investigation using synthetic diglycerides, failed to show any selectivity, although this

conclusion may be dependent on the weight ratio of diglyceride to acetone powder (Eccleshall and Hawke, 1971). At present, the local concentration of diglyceride at cellular enzyme sites needed for comparison, is unknown.

It is difficult to compare these experiments. The synthetic diglycerides contained both the 1,2- and 2,3- isomers, whereas those prepared enzymatically from natural sources would be predominantly the 1,2- isomers. Moreover, the triunsaturated fatty acid used in the synthetic diglycerides was α-linolenic acid, whereas the natural one is α-linolenic acid. However the most significant difference is probably in the method of adding the diglyceride to the incubation. While Mudd et al. added the diglyceride as a suspension in Tris buffer containing detergent, Eccleshall and Hawke added it to the acetone powder dissolved in acetone, which was then removed under nitrogen. Eccleshall (1970) suggests that the organic solvent addition is preferable as it should allow maximum interaction between enzyme and substrate, thus minimizing the influence of differences in diglyceride water solubility on the incorporation.

Comparative experiments using acetone preparations of spinach chloroplasts, both methods of diglyceride addition and natural and synthetic diglycerides are required to explain these discrepancies. If it is active with diglyceride as galactose acceptor, then similar studies using the acetone powder of Euglena would be useful. If the soluble subchloroplast preparation of spinach (Chang and Kulkarni, 1970) could be further purified so that diglyceride had to be added for activity, this system could be investigated as an alternative to the acetone powder.

Other experimental approaches:

Eccleshall and Hawke (1971) found that when MGDG synthesized from UDPgal ^{14}C by whole chloroplasts was fractionated according to the nature of the constituent fatty acids, the radioactivity was distributed widely among the MGDG's of different fatty acid composition, suggesting that the galactosylation is nonspecific with respect to the diglyceride.

An earlier study of MGDG synthesis by Chlorella (Nichols and Moorhouse, 1969) indicated significant changes occurring in the fatty acid composition following de novo synthesis. They incubated cells with 2-C^{14} sodium acetate and extracted the MGDG which was fractionated into species of varying unsaturation using argentation TLC. When the fatty acids of these fractions were subjected toradiochemical analysis, it was found that the specific activity of a single fatty acid varied considerably among the various species in which it occurred. There was also a time dependent increase in the specific activity of any one fatty acid in the more unsaturated species. In later experiments (Safford and Nichols, 1970), the positional composition of these MGDG species was determined using pancreatic lipase. These workers suggested that the compositional and metabolic data for Chlorella are consistent with the theory that sequential desaturations occur following de novo synthesis of MGDG. They envisage a series of events such as:

$$\begin{bmatrix} 18{:}0 \\ 16{:}0 \\ \text{gal} \end{bmatrix} \rightarrow \begin{bmatrix} 18{:}1 \\ 16{:}0 \\ \text{gal} \end{bmatrix} \rightarrow \begin{bmatrix} 18{:}1 \\ 16{:}1 \\ \text{gal} \end{bmatrix} \rightarrow \begin{bmatrix} 18{:}2 \\ 16{:}1 \\ \text{gal} \end{bmatrix} \rightarrow \begin{bmatrix} 18{:}2 \\ 16{:}2 \\ \text{gal} \end{bmatrix} \rightarrow \begin{bmatrix} 18{:}3 \\ 18{:}2 \\ \text{gal.} \end{bmatrix}$$

Even if desaturation does occur after MGDG synthesis, MGDG may not be the actual substrate form of the acyl groups acted on by the

desaturase. Phosphatidyl choline is considered by some to be the true substrate for desaturation (Roughan, 1970; Gurr, 1971). Vijay and Stumpf (1971) have shown that oleoyl CoA is the substrate for a microsomal desaturase in castor seeds. If acyl groups are desaturated either attached to phosphatidyl choline or to CoA, then there must be an acyl transfer to MGDG. No evidence for such a transfer exists at present.

(c) Control of Galactolipid biosynthesis:

It is well documented that the action of light on etiolated leaves or dark-grown heterotrophic algal cells results in rapid synthesis of chlorophyll, carotenoids, galactolipids, sulfolipids and phosphatidyl glycerol associated with the formation of functional chloroplasts (Kates, 1970). Such light dependence of chloroplast formation results in a positive correlation between galactolipid and chlorophyll concentrations in plants. This is true even during greening under abnormal conditions such as with Euglena in the presence of protein inhibitors (Bishop and Smillie, 1970) or manganese deficient Euglena (Constantopoulos, 1970). Bishop et al. (1971) commented that the molar ratio of galactolipid to chlorophyll for chloroplasts of a particular type, appears to reflect the degree of grana formation. An increase in the 18:3 content of galactolipids also occurs during greening (Appelquist et al., 1968).

Unser and Mohr (1970) obtained evidence for phytochrome mediated galactolipid synthesis in mustard seedlings under far-red light, which appeared to be independent of the normal light stimulated membrane synthesis. Newman (1971) studied the effects of red and far-red light on fatty acid desaturation in barley leaves and concluded that desaturation as such was not phytochrome mediated.

The effect of temperature on alfalfa leaves from cold-sensitive and cold-hardy varieties was examined by Kuiper (1970). The MGDG and DGDG content of the leaves was found to be inversely related to the temperature in the range 15 to 30°C. Cold-hardy plants had generally higher galactolipid contents than cold sensitive plants. Changes in fatty acid composition with temperature were small, the main differences occurring in the levels of 18:3 in the MGDG, being higher at lower temperatures in both varieties.

Seasonal variation in galactolipids has not been studied in detail. Jamieson and Reid (1969) observed a variation in the content of C_{18} polyenoic acids of Myosotis scorpioides with the time of year. A seasonal variation which was independent of plant maturity, occurred in the level of lipid-bound sugar clover leaves (Bailey, 1964).

1.5 Galactolipid transformations:

In the living plant cell the galactolipids exist in a dynamic state of equilibrium as has been shown with studies using radioisotopes (Ferrari and Benson, 1961; Sastry and Kates, 1965). To maintain the required level of galactolipids, the steady state cycle of degradative and synthetic reactions must be under sensitive control. Enzymic compartmentalisation probably gives this control (e.g. synthetic enzymes located in the chloroplast; hydrolytic enzymes located in cytoplasmic particles analagous to the lysosomes of animal cells).

Homogenisation of leaf tissues disrupts this compartmentalisation and a number of highly active enzymes are released which rapidly degrade and transform the principal lipid components. Consequently in the isolation and determination of leaf lipids, these enzymes must be

initially inactivated by heat or solvent treatment. However, for galactolipid studies using metabolically active chloroplast preparations, this is not possible and incubation conditions must be found to eliminate or minimise these reactions so that the reaction of interest can be studied.

The main enzymes effecting galactolipid transformations are galactolipases, galactosidases and acyl transferases.

(a) Galactolipases:

Although galactolipases are present in a wide range of plant species they have not been studied extensively in species other than Phaseolus where they are particularly active (Sastry and Kates, 1964b; Helmsing, 1969) and in spinach (Helmsing, 1967; Wintermans et al., 1969). Partially purified galactolipase preparations behave differently towards MGDG and DGDG, e.g. pH optima are 7.0 and 5.6 respectively, their affinity for DGDG is higher than that for MGDG and the two activities have different stabilities on storage at 4°C (Sastry and Kates, 1964b). However Helmsing (1969) obtained a single protein fraction containing both activities whose specific activities were in the ratio 2:1 (MGDG: DGDG). Cysteine completely inhibited the enzyme.

Mono- and di- galactosyl glycerols accumulate during galactolipid breakdown but lyso galactolipids have not been detected. It is assumed that these are transient intermediates and the second acyl group must be removed much faster than the first. Sastry and Kates found that the runner bean enzyme was active towards the unsaturated plant galactolipids but not towards saturated galactolipids. This could reflect solubility differences in these substrates rather than

substrate specificity.

The potato tuber contains a general lipolytic acyl hydrolase which acts on several classes of lipids (Galliard, 1970 and 1971). Its galactolipase activity is similar to that of spinach in several respects but for this enzyme the lyso galactolipid is detected. An acyl transfer activity which transfers acyl moieties from lipid to alcohols present in the mixture, is associated with the potato tuber lipase.

Galactolipases impair the Hill reaction of isolated chloroplasts (Bamberger and Park, 1966). There is also a parallel uncoupling of photophosphorylation (Wintermans et al., 1969) which appears to be an indirect effect of linolenic acid released by the lipases.

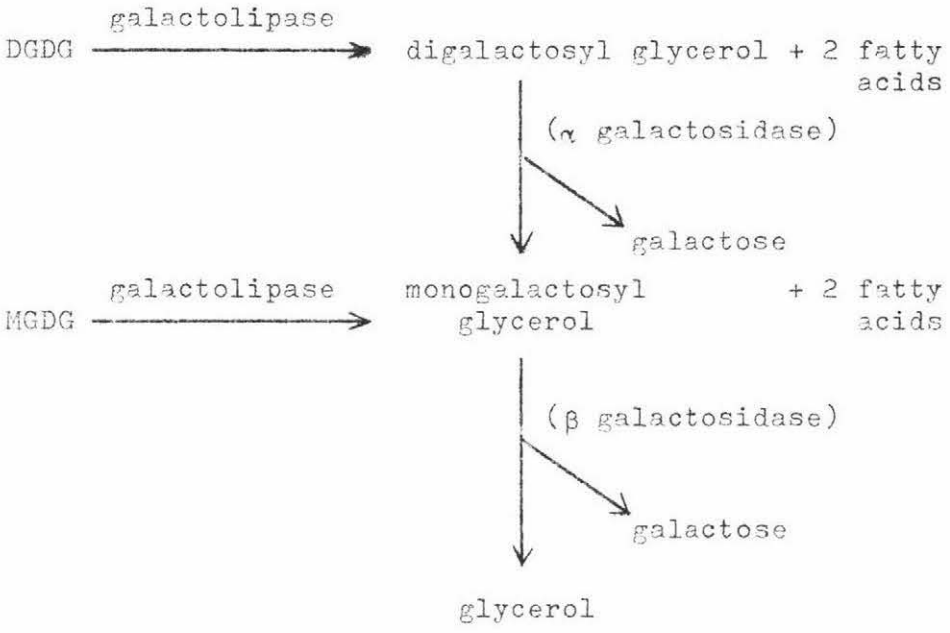
(b) Galactosidases:

α - and β - galactosidase activities have been isolated from spinach leaves (Gatt and Baker, 1970). The two enzymes were separated from each other and the α galactosidase purified 30 times; β 1000 times. p-nitrophenyl galactosidases were used as the assay substrates and acidic pH optima (α 5.3; β 4.2) were obtained. Hydrolysis of MGDG by β galactosidase was much slower than the p-nitrophenyl substrate although the rate was similar to that for the action of galactolipase on MGDG. Neither enzyme hydrolysed DGDG. This could be because the DGDG used was more saturated than spinach DGDG. Another possibility is that the enzyme does not hydrolyse the native lipid, but will split off galactose from the deacylated lipid.

The acidic pH optima obtained are similar to those of the corresponding animal enzymes which are located in lysosomes. Although most of the galactosidase activity of spinach was in the supernatant

after blending the leaf tissue, grinding did give a higher proportion in particulate fractions. If plant galactosidase is present in 'lysosome-like' particles, membranes of these must be very fragile and the enzymes easily released even on gentle grinding in isotonic buffered medium.

The galactolipases and galactosidases of plants are the enzymes necessary for complete hydrolysis of galactolipids. These reactions can be summarised as follows:



(c) Transacylation:

In experiments involving incubation of chloroplast preparations with UDPgal ^{14}C, it is commonly noted that besides the usual incorporation into MGDG, DGDG and polygalactolipids, smaller amounts of several other radioactive lipids are formed (Webster and Chang, 1969; Ongun and Mudd, 1968; Chang and Kulkarni, 1970). Most of these are probably acylated derivatives of galactolipids.

Heinz (1967b) partially purified the enzyme responsible for the formation of acyl-MGDG in spinach leaf homogenates. He showed that acyl-MGDG is formed mainly by acyl transfer from DGDG to MGDG. The pH optimum was pH 4.6 and acyl-MGDG accounted for about 20% of the leaf lipids after a 3 hour incubation at this pH. The acid pH optimum is in common with those of the galactolipases and galactosidases. No attempt to determine the cellular distribution of the enzyme was made and the crude enzyme was isolated from the 20,000 xg supernatant of the homogenate. This does not preclude the possibility of its location within a membrane bound organelle similar to the lysosome of animals as it may be readily released during homogenisation.

In view of the formation of other acyl derivatives of galactolipids (Wintermans et al., 1969) and acylated sterol glucoside (Ongun and Mudd, 1970) by spinach leaf homogenates it is interesting to speculate on the possible existence of a general transacylating activity in plant leaf cells. Whilst these acylated derivatives have not been found as normal constituents of leaves, their formation could be important in plant cell decomposition. It is more likely that they arise as artifacts of homogenisation such as an alteration in specificity of a normal acylating enzyme caused by the changed environment (pH, polarity etc.) in the locality of the enzyme. In this regard the transacylating activity associated with the lipolytic acyl hydrolase of potato tuber (Galliard, 1970) may be of significance.

1.6 Possible functions of the galactolipids:

The chloroplast contains a highly organised membranous system in which are localised the light trapping systems responsible

for the photochemical reactions of photosynthesis. In electron micrographs these membranes resemble double plasma membranes and are known as thylakoids. In the chloroplasts of most higher plants the thylakoids are arranged in stacks to form grana which are inter-connected through the stroma by single thylakoids (lamellae). The chlorophylls and other pigments associated with the photosystem are confined to the thylakoids and are particularly concentrated in the grana (Weier and Benson, 1966). Studies with chloroplast fragments indicate that about 5% of the chlorophyll a is strongly oriented at particular sites and the rest is relatively unoriented. Apart from the pigments, the galactolipids are the major lipids present in the thylakoids and as such must be fundamental structural components of these membranes. This is emphasised by the morphological changes in thylakoids observed after prolonged treatment with galactolipase (Bamberger and Park, 1966). The surfactant nature of the galactolipids with their neutral hydrophilic region and highly unsaturated hydrophobic region, makes them ideal for stabilising the sheet-like structure of membranes in an aqueous environment; a role fulfilled by phospholipids in other membranes.

Various models of the detailed molecular structure of the thylakoids have been proposed, and like all membrane models they are mainly speculative at this stage of our knowledge. Weier and Benson (1966) have reviewed these models and other data relevant to the molecular nature of the chloroplast membrane. They conclude that the evidence of studies using polarised light, low angle Xray diffraction and the electron microscope, favours a subunit structure rather than a leaflet structure. They propose their own model based on the surfactant nature of the galactolipids and the structure of the branched paraffin chains of the chlorophylls and plastoquinones.

Subunits of lipoprotein are arranged in layers and stabilised by the hydrophobic association of branched and unsaturated hydrocarbon chains with the hydrophobic internal structure of the lamellar protein. It is postulated that the surfaces of the membrane exposed to the stroma are lined with the hydrophilic groups of the galactolipids with the linolenic acid chains embedded within the subunits. The regions of contact between thylakoids (e.g. in the grana) are thought to be highly hydrophobic and capable of binding chlorophyll, carotenoids and quinones. Hydrophobic association between the linolenoyl chains of galactolipids and the proteins is considered to be strong as the 3 double bonds give a twisted configuration to the chain conducive to close association with a single protein chain and the double bonds may associate strongly with the π orbitals of the aromatic amino acids of the membrane protein. An entropic stabilisation would arise from the disruption of hydration shells surrounding the groups concerned. Some support for such hydrophobic association has come from experiments in which lamellar proteins and chloroplast lipids were combined (Ji and Benson, 1968). Recombination of galactolipids and chlorophyll with protein in the presence of lipid competitors was dependent on the nature of the hydrophobic groups and not the hydrophilic groups of the competitor. Galactolipids may give the optimum orientation of the enzymes involved in electron transfer and photophosphorylation by such interaction.

The interdependence between chlorophyll levels and the degree of unsaturation and level of galactolipids has already been established (section 1.4 (c)). This suggests that a close physical association between galactolipids and chlorophyll may exist. Trosper and Sauer (1968) demonstrated that purified MGDG and DGDG form relatively strong one-to-one complexes with chlorophyll even in the presence of water.

Also chlorophyll is randomly dispersed by MGDG in monolayers. These workers suggested that the bulk fraction of the randomly oriented pigment in the lamellae may be associated with MGDG. Rosenberg (1967) proposed a more specific association between galactolipids and chlorophyll. The 16 carbon phytol chain of chlorophyll bears four methyl groups at positions 3, 7, 10 and 15 which introduce twists in the chain similar to the pockets or twists in the linolenic acid chains. Space filling molecular models show that the two chains are complementary and fit together closely in a space saving arrangement. London - Van der Waals dispersion/attraction forces would stabilise such a close fit. Furthermore the fit of the phytyl methyl groups into the linolenyl pockets would allow stabilisation of the order of a hydrogen bond because of induced polar interaction between the double bonds and the methyl groups. It has been shown that a film of chlorophyll is much more stable superimposed on a film of oleoyl alcohol than on a film of stearoyl alcohol. The small group of highly oriented chlorophyll molecules in the photosystem is thought to provide an 'electron sink' for the 'light trap' and to be the ultimate electron donor for the electron transfer chain. It is possible that the galactolipids are important in arranging these chlorophyll molecules in order to maximise the electron transfer.

The occurrence of galactolipids in all photosynthetic organisms capable of the Hill reaction has been mentioned (section 1.2). Galactolipids give a stimulatory effect on the rate of cytochrome c photoreduction by spinach chloroplasts (Chang and Lundin, 1964). A Scenedesmus mutant that was defective in the Hill reaction had morphologically normal chloroplasts but its α - linolenic acid content was very much less than in the parent wild type; otherwise it had a

normal fatty acid spectrum. This suggested that the degree of unsaturation of galactolipids may be important in electron transport; especially the Hill reaction. However Anacystis nidulans does not contain polyunsaturated fatty acids and yet is capable of the Hill reaction. It seems likely that the hydrophophic portion of galactolipids provides the medium of low dielectric constant required for efficient electron transport rather than being directly involved as a redox compound in electron transport.

An early proposal for a function for the galactose moiety of galactolipids was as a metabolically active carbohydrate reservoir exchangeable with photosynthetic intermediates (Benson et al., 1959). The galactolipids of plants and algae readily incorporate ^{14}C when grown in the presence of $^{14}CO_2$. However turnover experiments with fully expanded pumpkin leaves show that the specific activity of the phospholipids is greater than that of galactolipids which would not be expected if $^{14}CO_2$ fixed in photosynthesis passed through the galactose moiety.

The possible involvement of galactolipids in α-linolenic acid biosynthesis has been discussed (section 1.4 (b)). This would not be a primary role for galactolipids as most of the linolenic acid synthesised by the chloroplast is located in the galactolipids.

Chapter 2

THE AIM OF THE PRESENT WORK

In studies of galactolipid metabolism using isolated chloroplast preparations from plants, a number of lipids are formed besides MGDG and DGDG. The work reported in this thesis was undertaken to find the conditions for maximum formation of MGDG and DGDG by Red Clover (*Trifolium pratense*) chloroplast preparations. The structure and significance of galactolipids other than MGDG and DGDG was also investigated.

Chapter Three

MATERIALS AND METHODS

Materials and Analytical Techniques:

3.1 Plant Tissues:

Spinach (Spinacea oleracea) leaves were obtained from commercial sources.

Ryegrass (Lolium perenne) and Fescue (Festica elation) leaf tissue was harvested from clones in a field.

The first samples of Red Clover (Trifolium pratense) leaves were cut from clones grown in the open and later ones were harvested from pure Red clover pasture.

3.2 Reagents and Solvents:

All chemicals used were analytical, Laboratory or Technical reagents.

Solvents were distilled before use.

3.3 Radioactive Materials:

The following were obtained from the Radiochemical Centre (Amersham, England):

D-Galactose-1-^{14}C, specific activity of 55.7 mC/mM.

n-Hexadecane-1-^{14}C, specific activity of 0.781 μC/gm.

Uridine diphospho-(D-galactose-^{14}C(U)) ammonium salt, specific activity of 240 mC/mM. (UDPgal^{14}C).

3.4 Chromatography:

(a) Thin-layer chromatography (TLC):

Glass plates (5 cm x 20 cm, 10 cm x 20 cm or 20 cm x 20 cm) were spread with a slurry of Silica gel G (Merck) with water (2:1, v/w) at a thickness of 0.25 to 0.5 mm using a spreader made by Desaga (Heidelberg, Germany). The plates were allowed to settle at room temperature and then activated by heating at 110°C for at least one hour.

Solvents used to separate galacto-lipids were:

Toluene / ethyl acetate / 95% ethanol (2:1:1; v/v).

Chloroform / methanol (C/M) (10:1; v/v).

Water saturated diethyl ether / isopropanol / methanol (100:4.5:3; v/v).

Hexane / diethyl ether / glacial acetic acid (70:30:1; v/v) and Hexane / diethyl ether (80:20; v/v) were used for free fatty acids and fatty acid methyl esters respectively.

Lipids were detected by spraying.the plate with 2,7 - dichloro (R) fluorescein (0.2%, w/v in methanol) and viewing under ultraviolet light, wavelength 360 mm, or by spraying with H_2SO_4 / dichromate (50% conc H_2SO_4, 0.5% potassium dichromate) and charring in an oven at 120°C.

(b) Column chromatography:

Silicic acid columns were used in the purification of MGDG and unknown galactolipids.

Fine particles were removed from the silicic acid

(Malinkrodt), by suspending about 120 gms in distilled water in a tall measuring cylinder and allowing it to settle. The water and fines were decanted and the residual slurry activated in an oven at 110°C for at least twenty four hours. The activated silicic acid was suspended in hexane and held under vacuum in a dessicator for several minutes to remove air bubbles. To pack the column (2.5 mm diameter), it was filled with hexane and the silicic acid slurry spooned in carefully until a packed height of 35 to 40 cm was reached.

The mixed lipid sample (1.0 to 1.5 gms) was applied to the top of the column in a minimum (4 to 5 mls) of hexane / chloroform; 1:1 v/v). Eluting solvents were passed through the column via a reservoir consisting of a one litre separating funnel fitted to the top of the column. Fractions (10 or 15 ml) were collected automatically in test-tubes and assayed using TLC with the total mixed lipid extract as standard.

(c) Gas-liquid chromatography (GLC):

Methyl esters of the fatty acids from lipids were prepared for GLC analysis by hydrolysis and reaction with boron trifluoride-methanol reagent (BF_3 14% by weight in methanol obtained from Applied Science Laboratories). The solvent was removed from the lipid sample in a stoppered testtube under a nitrogen stream, and 0.5 ml of 0.5N methanolic NaOH added. After several minutes refluxing until the lipid had dissolved, 0.7 ml of BF_3 reagent were added through the condenser and reflux continued for another 3 minutes. Hexane (approx. 2 mls) was added and the reflux tube removed from heat. After about a minute of further reflux off the heat, to facilitate extraction of the methyl esters, the tube was cooled and the hexane floated to the top on 0.1M NaCl. This hexane layer was removed with a pasteur pipette and

injected straight into the GLC column after concentration to about 50 l under a stream of dry nitrogen.

The methyl esters were analysed on a Packard Gas Chromatograph using a 2 mm x 6 ft column of 12% diethylene glycol succinate (DEGS) on Chromosorb W, at an oven temperature of 165°C and on a Varian Aerograph (model 1520) chromatograph using a ¼ inch x 5 ft column of 12% DEGS at a temperature of 165°C. Both these chromatographs were fitted with flame ionisation detectors and used nitrogen as carrier gas.

Radioactive fatty acid methyl esters were analysed on the Varian Aerograph with a stream splitter attached prior to the detector so that 4/5 of the gas flow was diverted into a Nuclear Chicago radioactive flow counter. Helium carrier gas was used. Mass peaks and radioactive peaks were traced onto a single chart output.

Peaks were identified by comparing retention times with mixed fatty acid methyl ester standards obtained from the Hormel Institute and British Drug Houses.

3.5 Radioisotope scanning and counting:

Duplicate aliquots of radioactive extract in chloroform were evaporated to dryness under a stream of nitrogen and 10 mls of toluene scintillation fluid (2,5 - diphenyloxazole (PPO, 0.6%) and 1,4 bis /2-(5-phenyloxazolyl)/ - benzene (POPOP, 0.05%) in toluene) added to the counting vial.

For aqueous radioactive samples 10 mls of Bray's scintillation fluid (naphthalene (60 gm), PPO (4 gms), POPOP (200 mgm), methanol (100 ml), ethylene glycol (20 ml) and p-dioxane to make

1 litre) were used.

The vials were counted using the preset ^{14}C channel in a Packard Tri-carb Liquid Scintillation Spectrometer model 3375. The counting rates had a percentage standard error which was less than 2%. Quenching of scintillation was corrected for by dividing the counts per minute by the Automatic External Standardisation (AES) value. A plot of AES against counting efficiency, constructed by counting a measured weight of n-hexadecane-1-^{14}C in the presence of increasing amounts of total lipid extract, showed that AES and efficiency were equivalent in the AES range 0.2 to 0.65. All lipid samples counted fell in this range.

TLC plates (5 cm x 20 cm) were scanned for radioactivity using a Packard Radio-chromatogram scanner, model 7200, with a mixture of 1.3% isobutane - 98.7% helium as carrier gas. Optimum conditions for scanning were: gas flow 110 cc/min, high voltage 1.15 kv, time constant 30 sec., slit width 2.5 mm. Speed of counting was in the range 5 to 20 cm/hr, and the scale usually 0-300 cpm. Relative peak areas were measured on the scans using a planimeter and expressed as percentages of the total radioactivity present.

The resolution of radioactive peaks by the scanner was checked by making radio autographs of the TLC plates. Xray films were held over the plates in light tight boxes for two to three weeks and then developed.

Experimental Procedures:

3.6 Leaf-slice incubations and lipid extraction:

Whole fescue and clover leaves were harvested, washed with

water and blotted dry. Fescue leaves were cut into one mm. slices with a razor blade. Clover leaves were sliced longitudinally to give approx. 5 mm wide lengths which were then cut into 1 mm slices.

The incubation mixture was composed of:

0.5 gm sliced leaf tissue

0.2M phosphate buffer, pH7.4 (1.0 ml for fescue, 1.5 ml for clover)

50 µmoles $KHCO_3$

100 µmoles Sodium acetate

1 µmole Sodium diethyl dithiocarbamate (NaDEDTC).

2.5 µmoles cysteine hydrochloride (cys-HCl).

The volume was made up with water to the minimum required to wet all the tissue (about 2.2 ml for fescue and 3 ml for clover).

Radioactive galactose-1-^{14}C was added and the incubation performed at 30°C with shaking, in room light, for four hours.

The reaction was stopped by addition of sufficient C/M(1:2) to give one phase and the solution refluxed for one hour. The solution was transferred to a separating funnel through a wad of glass wool in a filter funnel to retain the leaf residues which were washed with C/M (2:1). The chloroform extract was washed 3 times with 0.1M NaCl (1% in galactose). Galactose was added to the wash to dilute out any radioactive galactose which may have remained in the chloroform phase. The chloroform layer was taken to dryness on a rotary evaporator and the total lipids redissolved in 1 ml of chloroform. Aliquots were taken for radioactive counting and scanning.

3.7 Chloroplast isolation, incubation and lipid extraction:

Chloroplast suspensions were prepared by two methods. All operations were carried out at 0-4°C. Chlorophyll content was estimated using 80% acetone solutions, according to Arron (1949).

Method I (after Spencer and Wildman, 1964).

This method was used in preliminary experiments to compare three methods of disruption of the plant tissue for release of chloroplasts.

Leaf tissue was chilled in a cold room for 1 to 2 hours, then 7 gms net weight were homogenised by one of the three methods in a medium (3 volumes v/w) consisting of:

25 mM Tris-HCl buffer pH 7.4

250 mM sucrose

1 mM magnesium acetate

4 mM cysteine-hydrochloride

2.5% Ficoll

5% Dextran 40T

The three homogenising techniques were:

(1) Slicing - the tissue was minced as finely as possible with dewaxed razor blades.

(2) Grinding - the tissue was ground with acid-washed sand in a mortar.

(3) Blending - the tissue was homogenised in a small blendor.

The resulting brei was strained through one layer of Miracloth and centrifuged for 2 min. at 30xg and 2x10 min. at 3020xg. The crude chloroplast pellet was suspended in 0.1M Tris-HCl buffer pH 7.4, containing 1 mM cysteine -HCl.

Method II:

This method was developed for preparation of clover chloroplasts which would give maximum incorporation of radioactive UDPgal ^{14}C into the galactolipids.

It involved grinding or slicing prechilled and washed leaf tissue in a medium (2 to 3 volumes, v/w) consisting of:

0.01 M Na_2HPO_4 - KH_2PO_4 buffer, pH 7.4.

0.2 M sucrose

4 mM cysteine - HCl.

0.1 mM sodium diethyl dithiocarbamate (NaDEDTC)

1% polyvinyl pyrrolidine (PVP).

The homogenate was strained through one layer of Miracloth or two layers of cheese cloth and centrifuged for 2 min. at 120 xg and 10 min. at 3020 xg. The crude pellet was suspended in 0.1 M Tris-HCl buffer (1 mM in cysteine -HCl) pH 7.4.

For radioactive experiments, aliquots of the chloroplast preparation in Tris -HCl were incubated with microlitre aliquots of the UDPgal ^{14}C solution, at 38°C with shaking in room light.

The reaction was stopped by adding sufficient C/M (1:2) to give one phase and by boiling for 5 minutes. After low speed centrifugation, the supernatant was decanted, and the residue washed twice more with C/M (2:1) using agitation on a vortex mixer. The bulked extracts were washed 2 or 3 times with 0.1 M NaCl and the chloroform layer evaporated to dryness. The total lipids were taken up in chloroform. Suitable aliquots were taken for radioisotope counting and scanning.

3.8 Galactolipid purification and hydrogenation:

Silicic acid columns were used in the preparation of MGDG and the unknown galactolipids by a modification of the method of Heinz and Tulloch (1969). The polarity of the eluting solvents was increased at a much slower rate than that used by Heinz to prevent band mixing on the column which was loaded with lipid close to its absorbing capacity.

(a) Preparation of hydrogenated MGDG:

Hydrogenated MGDG was prepared as a control and guide for the identification of the two unknown galactolipids.

144 gms of washed clover leaves were boiled for ten minutes in ethanol to denature enzymes (galactolipases and galactosidases). Water was added and the leaves homogenised. C/M (1:2) was added to the homogenate to give one phase and the mixture refluxed for 1 hour to extract the lipids. The leaf residue was filtered on a Buchner filter and washed with C/M (2:1) and ether. The combined extracts were washed twice with 0.1 M NaCl and taken to dryness in a rotary evaporator (yield 3.1 gms). Approximately 1.2 gms of the lipid was dissolved in 6 ml of chloroform / hexane (C/H, 1:1) for application to the silicic acid column. Column fractions were assayed by TLC using the toluene / ethyl acetate / ethanol (2:1:1 v/v) solvent. MGDG was identified by comparison with the total extract as TLC standard. MGDG appears as the major clover lipid component with a characteristic R_f when sprayed with dichlorofluorescein and viewed under U.V. light.

The following solvent elution scheme was used.

H/C/DE	(10:10:5)	500 ml	Pigments and neutral lipids removed
H/C/DE	(10:10:9)	500 ml	
C/A	(9:1)	400 ml	
C/A	(9:2)	440 ml	
C/A	(9:3)	480 ml	MGDG removed.
C/M	(9:3)	400 ml	Other galactolipids (DGDG and TGDG) and some MGDG removed.
C/M	(2:1)	300 ml	

H/C/DE - hexane / chloroform / diethyl ether.

C/A - chloroform / acetone.

C/M - chloroform / methanol.

The diethyl ether was distilled, dried over calcium hydride and redistilled before use.

MGDG was obtained in fractions 205 to 224 (10 ml each) free of DGDG or other lipids but with a small proportion of one pigment. These fractions were bulked (yield 167 mgms) and 139 mgms immediately hydrogenated.

For the hydrogenation, the lipid was dissolved in about 20 mls of methanol. 400 mgms of catalyst (10% palladium on charcoal) were added and the tube shaken under 40 psi of hydrogen on an hydrogenator (Parr Instrument Co.) for 18 hours. The tube was centrifuged and the supernatant decanted from the charcoal which was extracted twice more with warm chloroform. The combined extracts were taken to dryness on a rotary evaporator (yield 82.5 mgms). The product consisted of a white powder which gave a single spot on TLC corresponding to both the MGDG in the column fractions and the MGDG in the total clover lipid extract. The pigment was probably absorbed onto the charcoal. 35 mgms of the hydrogenated MGDG were twice crystallised from C/M (1:9) (Final yield

28.9 mgms).

(b) Preparation of hydrogenated, unknown galactolipids:

A crude chloroplast preparation was obtained from 150 gms wet weight of clover leaves using method II. The leaves were homogenised with a Waring Blendor in 500 mls of suspension medium without the PVP. The lipids from a small portion of the chloroplast suspension were extracted for a zero time control, whilst the rest of the suspension was held six hours at room temperature in the dark. The lipids were then extracted with chloroform / methanol (1:1 v/v), the extract washed twice with NaCl and evaporated to dryness on a rotary evaporator (yield 1.1 gms). 1 gm of this total lipid was dissolved in 5 ml of chloroform / hexane (1:1) and applied to a silicic acid column. Column fractions were collected automatically and assayed by TLC using C/M (10:1) as solvent, and the zero time and six hour lipid samples as standards. The following solvent elution scheme was used:

H/C/DE	515 ml	(50:50:3)	Removed neutral lipids (free fatty acids) and pigments.
H/C/DE	208 ml	(50:50:4)	
H/C/DE	440 ml	(50:50:10)	
H/C/DE	460 ml	(50:50:15)	Removed mixture of galactolipids and pigments.

Fractions 25 to 48 (15 ml each) were composed of free fatty acids (FFA) and a small amount of pigment. The FFA was identified by (a) methylation using diazomethane which gave long chain methyl esters as shown by TLC with hexane / diethyl ether (80:20) as solvent, (b) by infra red spectroscopy and comparison with the IR spectrum of palmitic acid and (c) by its behaviour on TLC in chloroform / methanol / NH_4OH

(85:15:2) solvent. In this solvent FFA runs very close to the origin as it is in a predominantly ionized form due to the alkaline ammonia. Lipid esters, especially MGDG, have a much higher R_f.

The mixed galactolipid fraction (approx. 100 ml; fraction 122 collected in a flask) contained a considerable amount of pigments, MGDG, and smaller quantities of at least two other lipids. Two lipids ran ahead of MGDG in TLC with C/M (10:1), and their R_f's corresponded to those of radioactive lipids running ahead of MGDG in UDPgal ^{14}C incorporation experiments.

Most of fraction 122 was hydrogenated by the method used with MGDG. This removed the pigments. The hydrogenated lipid fractions corresponding to the unknown radioactive lipids were then purified by preparative TLC with C/M (10:1) as solvent. These purified lipids samples were used for identification by mass spectrometry.

3.9 Chemical analysis of MGDG:

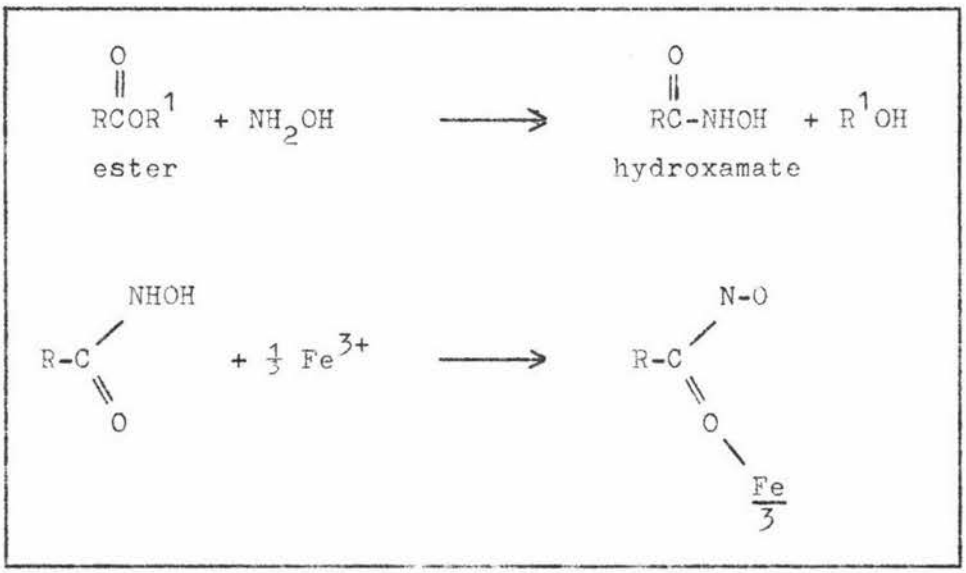
The following procedure was used to analyse the galactose, glycerol and fatty acid ester content of the hydrogenated MGDG. Fatty acid esters were estimated on the whole lipid and as free fatty acids (methyl esters determined by GLC) liberated by alkaline hydrolysis.

To keep weighing errors to a minimum approximately 5 mgms of MGDG were weighed out on a six figure balance, made up to a known volume with ethanol / diethyl ether (3:1) and suitable aliquots taken for triplicate acyl ester determinations and for triplicate hydrolyses.

(a) Fatty acyl ester determination:

The reagents and procedure of Morgan and Kingsbury (1959) were used:

The MGDG in 4 ml of ethanol / diethyl ether (3:1) was estimated along with a series of standards of palmitoyl-distearoyl glycerol in the range 0 to 4 μequivalents of fatty acid ester. In this method, reaction with hydroxylamine forms the hydroxamate of the acid part of the ester. The hydroxamate is then chelated with trivalent Fe^{3+} to form a brown coloured complex with an absorption maximum at 515 mμ.



The absorbance at 515 mμ was read on an Hitashi 101 Spectrophotometer. Visible spectra of the reacted standards and MGDG showed that their wavelength maxima were coincident at 515 mμ.

(b) Hydrolysis of MGDG:

(1) Mild alkaline hydrolysis:

After addition of a suitable known weight of methyl heptadecanoate (Me 17:0) as an internal fatty acid standard, the

solvent was removed from the MGDG aliquots by a stream of nitrogen.

5 mls of 0.5 M NaOH in methanol were added and the lipid refluxed for one hour.

5.5 mls of 0.5 M HCl were added at the completion of reflux.

The liberated free fatty acids were removed from the acidified hydrolysate with hexane washes. It was found that one wash was sufficient as the presence of the internal standard obviates the need for 100% extraction of free fatty acids. Residual free fatty acid in the aqueous methanolic phase did not interfere with the galactose and glycerol determinations.

The hexane was saved for fatty acid estimation. The methanolic phase was taken to dryness on a rotary evaporator for acid hydrolysis.

(2) Acid hydrolysis:

5 mls of 2N HCl were added to the residue of the alkaline hydrolysis and refluxed for 4 hours.

The hydrolysate was made up to 10 ml with water and 2 ml aliquots taken for glycerol and galactose determination.

(c) Galactose determination:

The reagents and procedure of Dubois et al. (1956) were used.

To the 2 ml duplicate acid hydrolysis samples and a series of standards (0 to 100 µgms of galactose) were added 30 µl of 80% phenol and 5 ml of conc. sulphuric acid (rapid). The tubes were stood 10 min. at room temperature, shaken, and held at 27°C for 15 min. The absorbance of the light brown colour was read at 487 mµ.

Visible spectra of standards and sample showed that the wavelength maxima at 487 mμ were coincident.

Preliminary work also showed that the presence of equimolar amounts of glycerol in the standards did not interfer with the estimation.

(d) Glycerol determination:

The reagents and procedure of Hanahan and Olley (1958) were used.

Glycerol is too volatile to use as the standard for this estimation. As the recommended standard, monomethyl dimethyl hydantoin was not available, mannitol was used.

The method depends upon the release of formaldehyde from glycerol on addition of periodate. Subsequently the formaldehyde is reacted with chromotropic acid. Mannitol releases the same weight of formaldehyde per mole as does glycerol.

4 ml aliquots of standards in the range 0-50 μgms equivalent of glycerol were estimated along with the acid hydrolysate samples made up to 4 ml.

The purple colour was read at 570 mμ and spectra showed that the wavelength maximum is 570 mμ for both sample and standard.

Hanahan and Olley found that 1 mgm of glucose gives the equivalent reaction of 19.0 μgms of glycerol. This is less than 2% interference. Hence the effect of the presence of galactose in the acid hydrolysate was negligible.

(e) Estimation of fatty acids as methyl esters using GLC:

The hexane was removed from the total fatty acid sample under a stream of nitrogen. The fatty acids were methylated using 0.1 ml BF_3 -MeOH reagent in a centrifuge tube fitted with a teflon lined screw cap by heating for about 5 min in an oven at 110°C. On cooling approximately 2 mls of hexane were added. The hexane was floated up to the top of the tube by addition of 0.1 M NaCl. After shaking and settling, the hexane layer was removed using a Pasteur pipette and injected straight into the GLC column after concentration to about 50 µl under a stream of nitrogen. The total fatty acids in the MGDG sample were calculated from the ratio of hexadecanoate plus octadecanoade methyl esters to Me 17:0.

Physical Techniques:

3.10 Melting point determination:

The melting points of galactolipids were determined on a Reichert hot stage microscopic melting point apparatus fitted with polaroid optics.

3.11 Differential Scanning Calorimetry (DSC):

This sensitive technique for examining the melting behaviour of compounds was used with the hydrogenated MGDG as a check on purity.

The sample was placed in a crimped aluminium pan of 20 µl capacity (approx $\frac{1}{4}$ inch in diameter) designed for use with volatile samples and fitted with a lid which was hermetically sealed to the pan under pressure. The instrument was a Perkin Elmer Model DSC 1B,

temperature calibrated with high purity melting point standards. The nitrogen gas flow rate was 30 ml/min.

3.12 Infra red spectroscopy:

Two instruments were used, a Beckman IR20 and a Perkin Elmer 137.

Lipid samples were run as potassium bromide disks. About 2 mgms were ground to a fine powder with about 250 mgms of KBr (British Drug Houses Laboratory Reagent). The disks (1 cm in diameter) were formed in a piston-cylinder device under a pressure of 20 to 25 tons provided by an hydraulic press.

3.13 Mass Spectrometry:

An AEI model MS 9 instrument was used. The galactolipid samples were inserted into the source chamber on a solid sample probe.

3.14 Electron Microscopy:

Chloroplast preparations were prepared for examination by members of DSIR staff. The micrographs were taken with a Philips EM 200 electron microscope.

Chapter Four

RESULTS

4.1 Comparison of grinding, slicing and blending techniques of chloroplast preparation:

The preparation of chloroplasts for incubation with UDPgal^{14}C to show incorporation of radioactivity into the galactolipids requires disruption of the plant leaf tissue. To examine the effect of the disruption technique on the ability of chloroplasts to form radioactive galactolipids, chloroplasts were prepared from spinach and ryegrass leaves using the three methods described in Section 3.7, method I and incubated as follows:

(a) Spinach:

The final volume of the chloroplast suspensions was 2 ml. Duplicate 0.2 ml aliquots were incubated with 4 l of UDPgal^{14}C (59,000 dpm) made up to a final volume of 0.5 ml with 0.1M Tris-MCl buffer, pH 7.4 for 1 hour at 30°C. The amount of radioactivity that was incorporated into the total lipid extracts of the chloroplasts is given in Table 6. When the total lipids were separated by TLC in toluene/ethanol/ethyl acetate (2:1:1) (tol/EtOH/EtAc) and scanned for radioactivity, several peaks in addition to MGDG and DGDG were obtained (Figure 3). The percent contribution of each peak was calculated from the peak areas and these data are shown in Table 7.

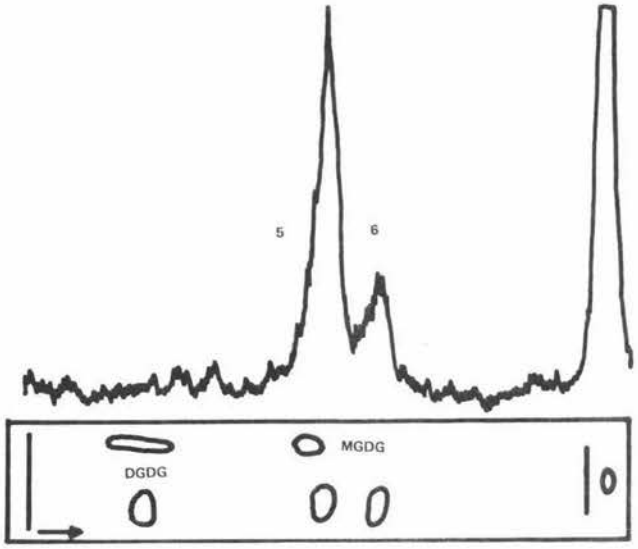


Figure 3

Radiochromatogram scans of lipids from UDPGal--14- C incubations of Spinach chloroplasts prepared by three different homogenising techniques. (Materials and Methods Section 3.7 Method I). Solvent Tol-EtOH-Etac.

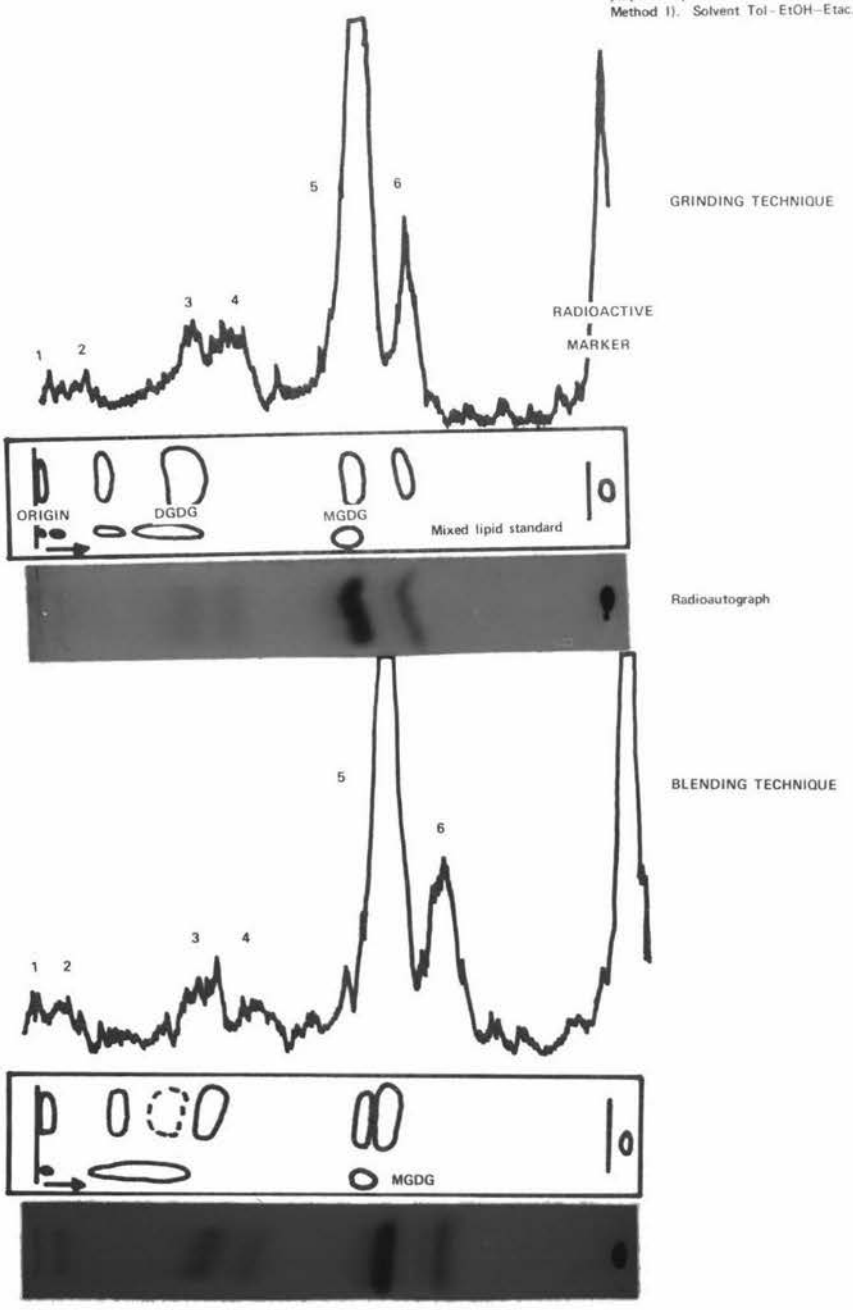


Table 6

Incorporation of radioactivity from UDPgal ^{14}C into the total lipids of spinach chloroplasts prepared using 3 homogenising techniques.

Technique	Incorporation			Chlorophyll* (µgms)	dpm^{t} per 50 µgm	Average
	Total (dpm)	%	Average			
Slicing	10,000	17.0	16.6	31	16,100	15,800
	9,600	16.3		31	15,500	
Grinding	13,500	22.9	18.9	49	13,800	11,400
	8,700	14.8		49	8,900	
Blending	13,900	23.5	20.2	39	17,800	14,700
	9,400	15.9		39	12,000	

* total chlorophyll in incubation mixture.
t incorporation on chlorophyll basis.

Table 7

Incorporation of radioactivity from UDPgal ^{14}C into lipid components of spinach chloroplasts prepared using three homogenising techniques. Results expressed as percentage of total.

Technique	Peak number (See Fig. 3) and Identity					
	1 TGDG	2	3 DGDG	4 X	5 MGDG	6 Y
Slicing	t	-	3	3	70	24
Grinding	t	t	9	11	62	16
Blending	1	4	9	4	60	21

t - trace.

(b) Ryegrass:

15 gm samples of leaf tissue were used and the final volume of the chloroplast suspensions was 6 ml. Duplicate 1 ml samples were incubated with 5 µl UDPgal^{14}C (56,200 dpm) for 1 hour at 30°C. The radioactivity incorporated into the total lipids extracted from the chloroplasts is given in Table 8. Table 9 records the percentage incorporation into the lipid fractions separated by TLC using tol/EtoH/EtAc solvent.

For both spinach and ryegrass chloroplasts the % incorporation into total lipids was about the same for the grinding and blending techniques and somewhat higher than the incorporation for slicing. However the chlorophyll levels were also much lower for slicing. Chlorophyll levels give an indication of the number of chloroplasts or chloroplast fragments present. Thus slicing disrupted the tissue the least of the three techniques and so gave the lowest yield of chloroplasts. On this basis the order of decreasing harshness of the three techniques was: grinding, blending, slicing. The best comparison of the incorporation figures is on a chlorophyll basis as given in the last two columns of Tables 6 and 8. Note that these figures are based on 50 µgms of chlorophyll for spinach and 500 µgms for ryegrass as the latter had a much lower galactolipid synthesising activity. On a chlorophyll basis slicing and blending gave similar incorporation figures and grinding incorporation was somewhat lower. Presumably the harsher techniques removed more of the galactolipid synthesising enzyme from the chloroplasts and chloroplast fragments so that more was lost in the supernatant during the centrifugation steps of the preparation of the chloroplast suspension.

Table 8

Incorporation of radioactivity from UDPgal ^{14}C into total lipids of ryegrass chloroplasts prepared using 3 homogenising techniques.

Technique	Incorporation			Chlorophyll (µgms)	dpm per 500 µgms	Average
	Total (dpm)	%	Average			
Slicing	9,600	17.0	16.7	210	23,200	22,700
	9,200	16.4		210	22,100	
Grinding	29,800	53.1	41.5	760	19,700	15,400
	16,800	29.8		760	11,100	
Blending	21,300	37.9	37.7	500	21,200	21,000
	21,000	37.5		500	20,900	

Table 9

% incorporation of radioactivity from UDPgal ^{14}C into lipid components of ryegrass chloroplasts prepared using 3 homogenising techniques.

Technique	Lipid Component				
	TGDG	DGDG	X	MGDG	Y
Slicing	t	15	13	61	7
Grinding	6	16	17	42	19
Blending	3	22	18	41	6

Lipids X and Y are referred to later in the thesis. Incorporation into X was resolved from the MGDG incorporation by the scanner only for the ryegrass chloroplasts, although the radioautographs for the spinach chloroplasts showed that low levels of X were formed. TGDG is thought to be trigalactosyl diglyceride because of its low R_f and by comparison with the incorporation pattern of Webster and Chang (1969) for spinach chloroplasts.

For both plants the patterns of incorporation into specific lipid components were similar for the blending and grinding techniques and these techniques gave more complex patterns than slicing. This difference probably arose because the harsher techniques made the enzymes responsible for synthesis of DGDG, TGDG, and Y more accessible to substrates such as UDPgal ^{14}C and MGDG than did slicing.

Although these results suggest that slicing gives the most intact and active chloroplasts, the levels of incorporation obtained with the other methods are sufficient for radioactive experiments. Grinding was found to be the most convenient technically and was used for preparation of clover chloroplasts. Preliminary experiments showed that for clover little difference in the pattern of incorporation occurred between chloroplasts prepared by grinding and by slicing.

4.2 Experiments with Red Clover:

(a) The effect of PVP and NaDEDTC on synthesis of radioactive galactolipids by chloroplasts:

First attempts to demonstrate incorporation of radioactivity from UDPgal ^{14}C into the lipids of Red Clover chloroplasts were

unsuccessful. These chloroplasts were prepared by grinding or slicing leaf tissue in a suspension medium of phosphate buffer (pH 7.4) and 0.1M sucrose and straining, centrifuging, resuspending in 0.1M Tris-MCl as in Method II, (Section 3.7). It was found that the addition of 0.1mM NaDEDTC and 1% P.V.P. gave significant levels (greater than 5%) of incorporation into the total lipids. An experiment was designed to examine the effects of these two additives on the incorporation.

Chloroplasts were prepared from 5 gm samples of leaf tissue using Method II (Section 3.7) according to the protocol shown in Table 10. The final volume of each chloroplast suspension was 1 ml in Tris-HCl buffer pH 7.4. 1 µl of UDPgal^{14}C (92,000 dpm) was added to each for incubation for one hour at 30°C. The incorporation into the total lipids extracted from the chloroplasts is given in Table 10.

Table 10

The effect of PVP and NaDEDTC on incorporation from UDPgal^{14}C into the total lipids of Red Clover chloroplasts.

Protocol		Incorporation		Chlorophyll (mgm)	dpm per mgm	%
1% PVP	0.1mM NaDEDTC	Total (dpm)	%			
-	-	9,900	10.7	0.92	10,800	12.7
-	+	10,000	10.8	0.61	16,400	17.8
+	-	17,300	18.8	0.81	21,400	23.2
+	+	19,300	20.9	0.92	20,900	22.7

In this particular experiment an incorporation of about 11% into the total lipids was obtained with neither PVP nor NaDEDTC present, whereas the first attempts gave less than 2% incorporation. Although

similar quantities of plant material and grinding were used for preparation of the chloroplasts for each incubation of the protocol, there was significant variation in the levels of chlorophyll. This arose because of variations in the extent of grinding (e.g. differences in quantities of sand, varying pressure on the pestle), a process which is difficult to standardise. The last column of Table 10 gives the % incorporation per mgm of chlorophyll. The best incorporation figures for comparison would be somewhere between the values corrected and uncorrected for chlorophyll as the extent of grinding introduces two opposing effects. Harsh grinding releases more chloroplasts from the leaf tissue but also gives greater disruption of the chloroplasts and loss of the galactolipid synthesising enzymes in the supernatant during the centrifugation than does mild grinding.

Table 10 shows that the presence of PVP and NaDEDTC approximately doubles incorporation, the effect of PVP being greatest. Both NaDEDTC and PVP were added in subsequent chloroplast preparations as in Method II (Section 3.7).

Radiochromatograms of the total lipids, using C/M (10:1) as solvent, showed that incorporation into MGDG was very low (less than 4%) and there was no incorporation into DGDG. Nearly all of the radioactivity was present in two peaks; one with an R_f slightly greater than MGDG (lipid Y) and one with an R_f slightly less (lipid X). These lipids are not normally detectable in Red Clover chloroplasts as there were no fluorescent spots corresponding to the X and Y peaks after spraying the TLC plate with dichlorofluorescein. The proportions of these two peaks in the four parts of the protocol were approximately the same, i.e. 70-75% Y and 20-25% X.

(b) Fractionation of chloroplast radiolipids to check the pattern of incorporation from UDPgal ^{14}C:

In the first experiments with Red Clover chloroplasts the levels of incorporation were so low that the TLC plates had to be overloaded with the total lipid in order to give measurable peaks on the radiochromatogram. The tol/EtOH/EtAc solvent gave insufficient separation of the radiolipids and only one peak corresponding to a MGDG standard was obtained. Examination of the autoradiograph showed that in fact there were three radiolipids X, MGDG and Y.

Figure 4 gives the radiochromatogram scans of lipid from Red Clover chloroplasts prepared by Method II and incubated with UDPgal ^{14}C. This particular experiment gave 5.6% incorporation into the total lipids. The separation obtained with the tol/EtOH/EtAc solvent is shown, as is the separation obtained with C/M (10:1). This second solvent was found to be the best for resolving X, MGDG, Y and for separating these from the pigments. The four fractions a, b, c and d, were scraped off the TLC plate run in C/M (10:1), the lipids extracted and re-chromatographed with a galactolipid standard using tol/EtOH/EtAc to confirm that incorporation into MGDG was minimal. Fraction b contained all the MGDG from the total lipid and yet no significant radioactivity was detected in the radiochromatogram. Fractions a and c contained the two radioactive peaks X and Y respectively. Fraction d contained all the DGDG which was not radioactive.

Figure 4 demonstrates a problem encountered with chromatographing standards on the 5 x 20 cm TLC plates needed to fit the scanner. The standards are spotted close to the edges of the plate where differences in the solvent due to evaporation, and differences in the thickness of the silica gel sometimes occur. These cause unpredictable

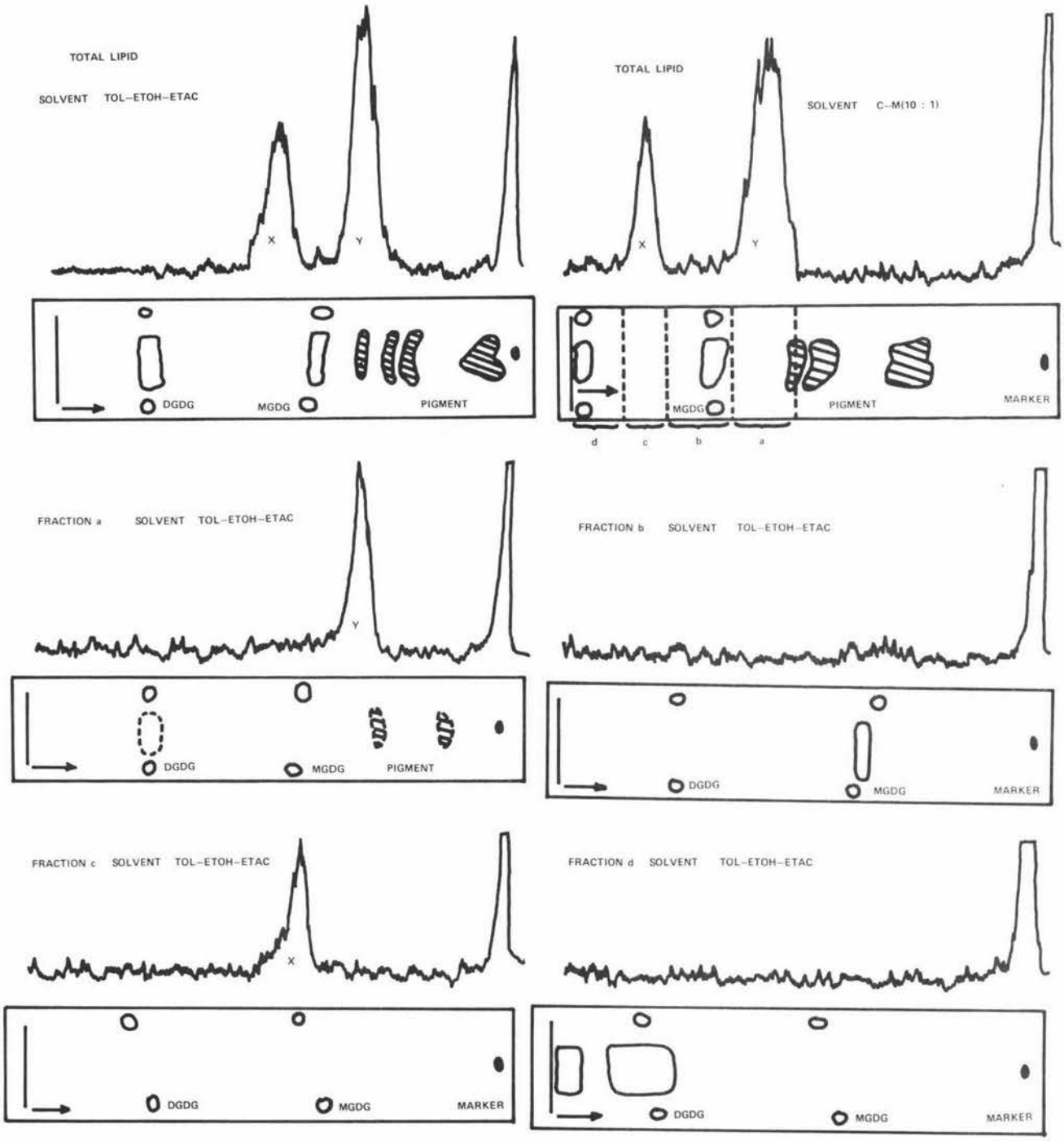


Figure 4

Radiochromatogram scans of lipid from clover chloroplasts incubated with UDPGal–14–C

variations in the R_f's of the standards as illustrated by the MGDG sample and standards rechromatographed in fraction b.

This experiment also shows that the 2 major radioactive peaks do not correspond to any major lipid components of the Red Clover sample that could be detected with dichlorofluorescein.

(c) Comparison of radioactive incorporation into galactolipids of Fescue and Red Clover chloroplasts incubated with UDPgal ^{14}C:

As the pattern of incorporation observed with Red Clover chloroplasts was unusual, it was compared with the pattern of Fescue chloroplasts.

Chloroplasts were prepared from 20 gms of each of the two plant tissues using Method II. The final volume of the suspensions was 1.5 mls and 0.5 ml samples were incubated with UDPgal ^{14}C (121,000 dpm) for 1 hour at 30°C. Incorporation into the total lipid extracts is given in Table 11, and radiochromatogram scans of the component lipids separated by TLC in C/M (10:1) are shown in Figure 5.

Table 11.

Incorporation into the total lipids of Clover and Fescue chloroplasts after incubation with UDPgal ^{14}C.

Tissue	Chlorophyll (mgms)	Incorporation	
		Total (dpm)	%
Fescue	0.210	43,200	36
Clover	0.220	8,300	7

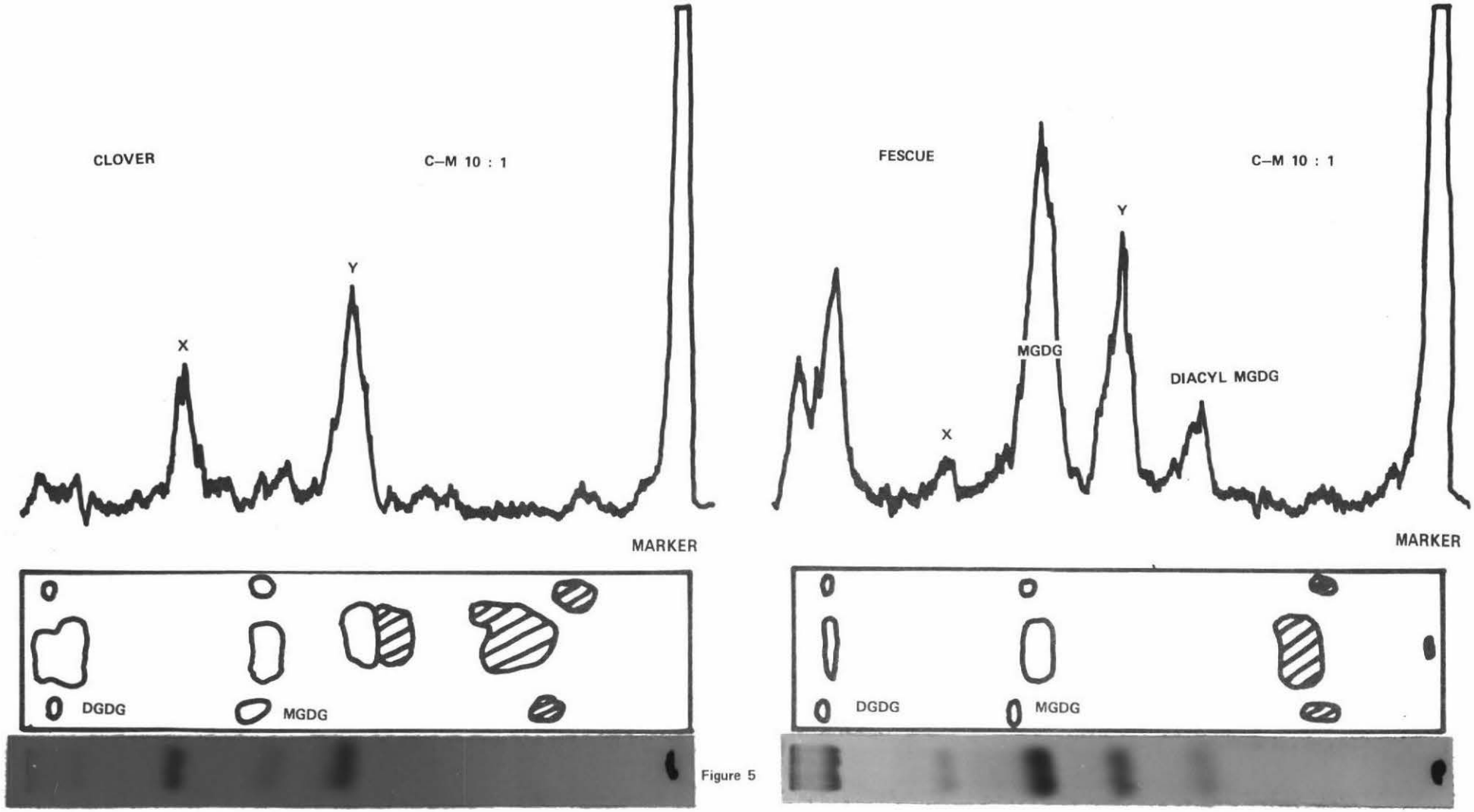


Figure 5

Radiochromatogram scans of lipid extracts of Fescue and Red Clover chloroplasts after incubation with UDPGal–14–C

Considerable differences occurred, both in total incorporation (36% for Fescue; 7% for Red Clover) and in the pattern of incorporation. For Fescue MGDG was the main radiolipid and in addition there was incorporation into Y, DGDG, diacyl MGDG (identified later in Section 4 a lipid at the origin and a small amount into X. In contrast Y was the principal radiolipid for Red Clover with a smaller peak for X and a very small amount of incorporation into MGDG.

(d) Comparison of radioactive incorporation into galactolipids of Fescue and Red Clover leaf slices incubated with Gal ^{14}C:

The method outlined in Section 3.6 was used. The radiolipids were extracted, counted and separated on TLC using tol/EtOH/EtAc solvent. Table 12 presents the data for the incorporation into the total lipids and the radiochromatogram scans are shown in Figure 6.

Table 12

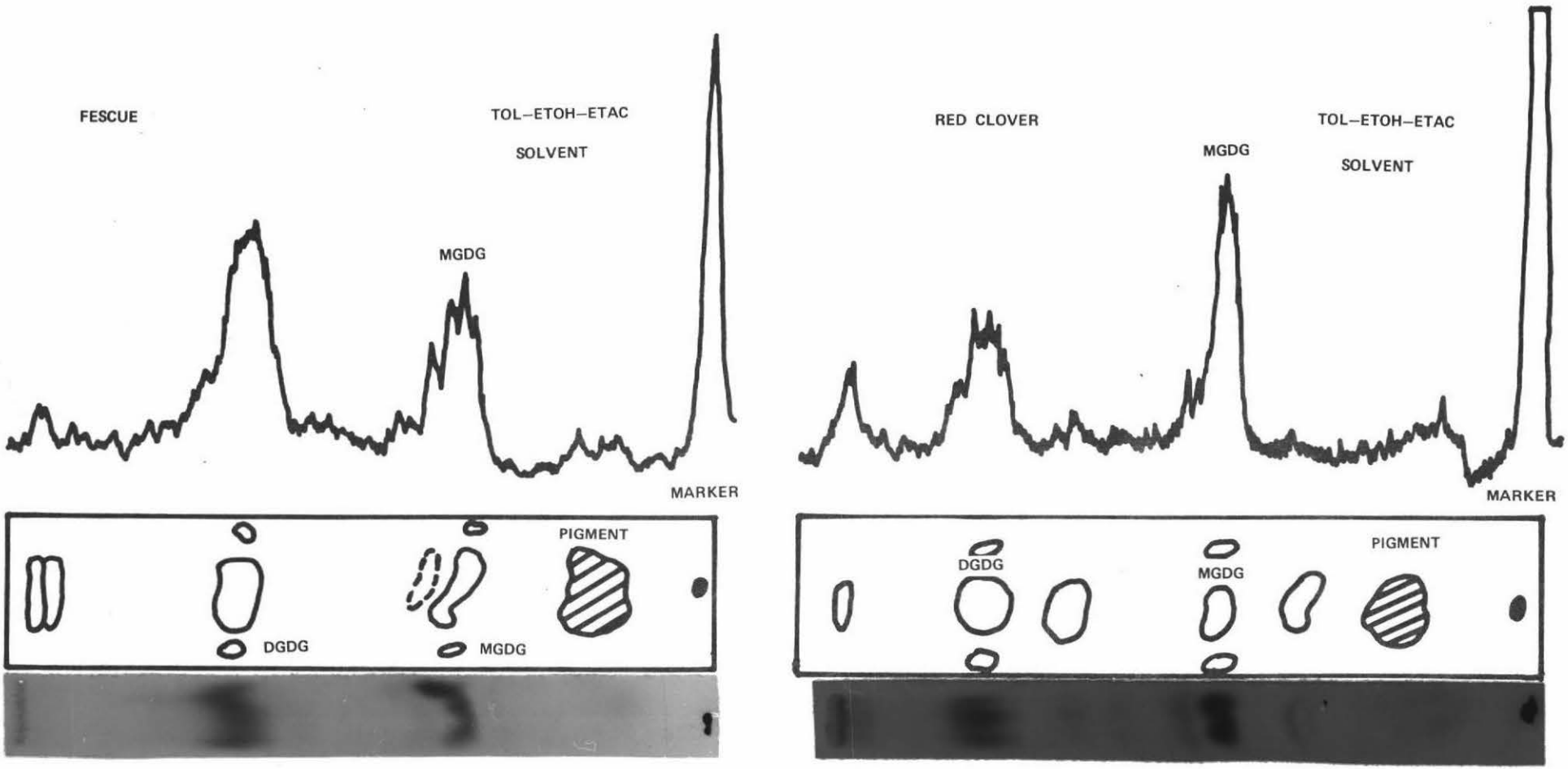
Incorporation of radioactivity into total lipids of Red Clover and Fescue leaf slices incubated with Gal ^{14}C.

Tissue	Radioactivity added (dpm)	Incorporation	
		Total (dpm)	%
Fescue	890,000	24,000	2.7
Red Clover	4,400,000	45,000	1

The levels of incorporation for this type of incubation were much lower than those for incubations of chloroplasts with UDPgal ^{14}C. This is to be expected as the gal ^{14}C must be transported through the leaf tissue to the chloroplasts and the UDP derivative formed. In

Figure 6

Radiochromatogram scans of lipid extracts of Fescue and Red Clover leaf slices after incubation with [illegible]Gal–14–C



contrast UDPgal ^{14}C is readily available to the enzyme for galactolipid synthesis in chloroplast incubations. Table 12 shows that Fescue leaf slices gave greater incorporation than Red Clover leaf slices which is consistent with the chloroplast incubations.

The radiochromatograms show that the patterns of incorporation for Red Clover and Fescue leaf slices were similar and rather different from the patterns obtained with the UDPgal ^{14}C incubations (Section 4.2 (c)). Incorporation was mainly into MGDG and DGDG with slightly more into DGDG. There was more incorporation into a lipid at the origin for Red Clover compared with Fescue. The radioautographs show some further fine differences between the two plants. For Red Clover there was slight incorporation into lipids corresponding to X and Y. However this incorporation was insufficient for detection by the scanner.

(e) Comparison of sodium chloride and sucrose based suspension media for chloroplast preparation:

Chloroplasts were prepared by Method II using two different suspension media; one was the normal 0.2 M sucrose based medium of this method and in the other the sucrose was replaced by 0.35 M NaCl so that the effects of the two media on galactolipid synthesis could be compared.

20 gms of leaf tissue were used for each chloroplast preparation. The final volume of the chloroplast suspensions for incubation was 0.5 ml containing 0.25 mgms of chlorophyll. 5 µl of UDPgal ^{14}C (42,400 dpm) were added and the incubation performed for one hour at 30°C.

Table 13 gives the data for incorporation into the lipid

extracts of the chloroplasts.

Table 13

Incorporation of radioactivity into Red Clover chloroplasts prepared using NaCl and sucrose based suspension media and incubated with UDPgal ^{14}C.

Suspension Medium	Incorporation into total lipids		% Incorporation into lipid components		
	dpm	%	X	MGDG	Y
NaCl	3,200	7.5	41.8	4.5	53.7
Sucrose	3,100	7.4	26.5	4.5	69.0

Within the limits of experimental measurement the incorporations into the total lipids and MGDG were the same for both suspension media. Most of the radioactivity was in lipids X and Y and the relative proportion of these was slightly different for the two media.

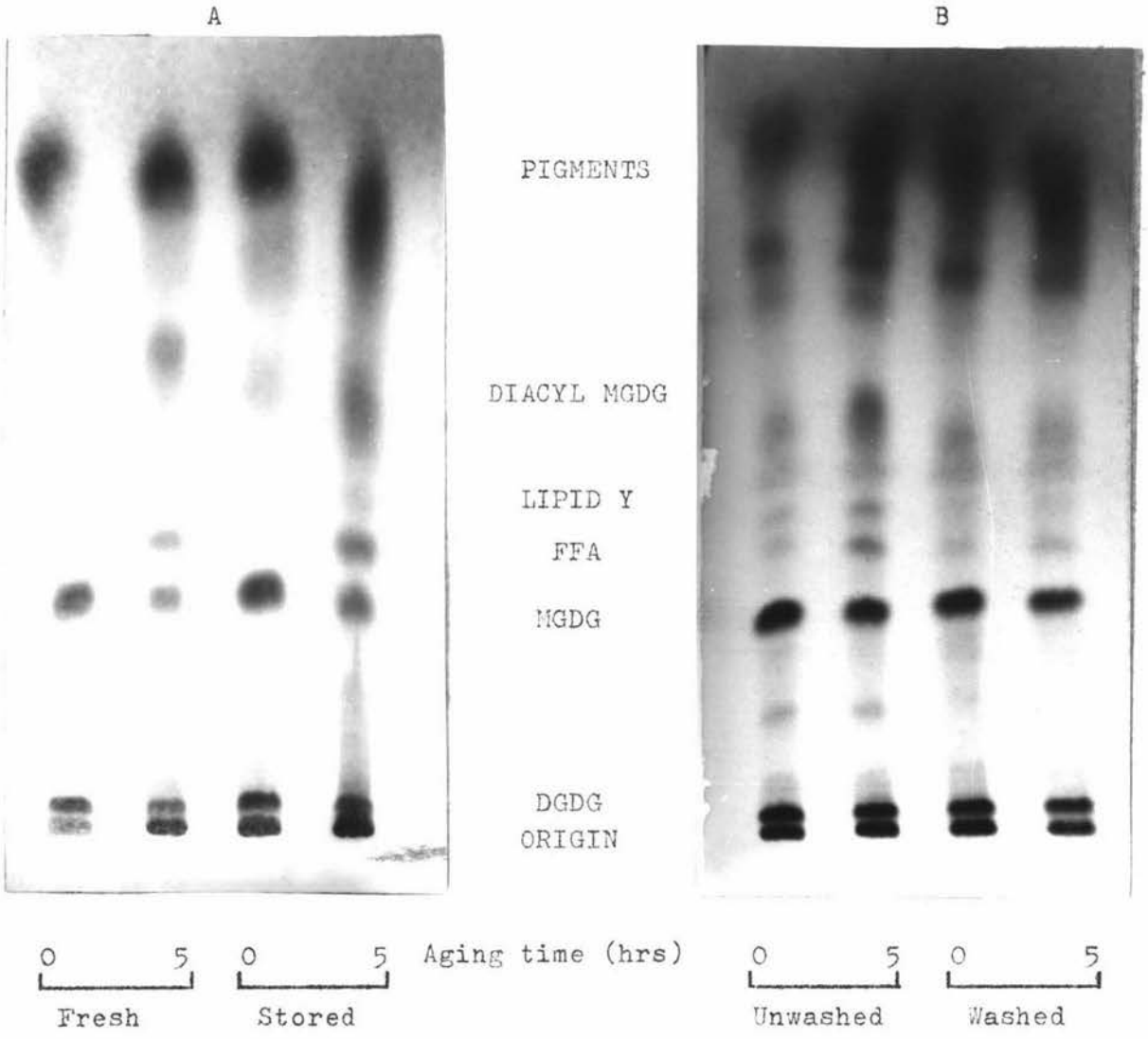
(f) Galactolipid transformations in aged Red Clover chloroplasts:

In the radiochemical experiments with Red Clover, fluorescent spots corresponding to the radioactive peaks X and Y were not usually detected after spraying the TLC plates with dichlorofluorescein. Chloroplasts were aged for 5 hours without the addition of UDPgal ^{14}C so that the non-radioactive galactolipid transformations could be detected. Two separate experiments were performed; one to compare chloroplasts prepared from fresh leaf tissue with chloroplasts from tissue stored overnight at $4^{o}C$ (A), and the other to compare washed and unwashed chloroplasts (B). Zero time controls were included for each chloroplast preparation.

Chloroplasts for part A were prepared by grinding 5 gms of tissue in the suspension medium of Method II. Chloroplasts for part B were prepared similarly with P.V.P. excluded from the suspension medium. The homogenates were strained and centrifuged at 120 x g for 2 min. Washed chloroplasts were prepared from the 120 x g pellet by two further centrifugations at 3020 x g for 10 mins. The four chloroplast pellets from centrifugation were each resuspended in 30 ml of the suspension media and two 10 ml aliquots taken: one for the zero time control from which the lipids were extracted immediately, and the other for the aged chloroplasts which were stored 5 hrs in the dark at room temperature before lipid extraction. Equal quantities of the total lipid extracts were applied to TLC plates, developed in C/M (10:1) and charred. Photographs of the TLC plates are shown in Figure 7.

Figure 7A shows that overnight storage of leaf tissue before chloroplast preparation did not affect the galactolipid transformations. Three lipids were formed at the expense of MGDG and DGDG during the 5 hour aging period for chloroplasts from both fresh and stored tissue, as shown by the relative intensities of the lipid spots. The positions of two of these relative to MGDG corresponded to the positions of the radioactive peaks diacyl MGDG and Y observed in the radiochemical experiments. The quantity of Y was much greater than X, which is the reverse of the radioactive peaks. The third lipid formed did not correspond to any of the radioactive peaks and was later identified as free fatty acid.

As P.V.P. is partly soluble in chloroform and was extracted with the lipids (appearing as a streaking effect from the origin in Figure 7B), it was excluded in the preparation of chloroplasts for part B. Comparison of the lipids from the unwashed chloroplasts in Figure 7B with the lipids of Figure 7A shows that the absence of P.V.P.



Thin layer chromatograms of lipids from Red Clover chloroplasts extracted immediately and after aging for 5 hrs at room temperature in the dark. Solvent: C/M (10:1)

Detection: charring. A. Chloroplasts from fresh leaf tissue and tissue stored overnight at $4^{\circ}C$.

B. Unwashed and washed chloroplasts (See text for washing method).

FFA - free fatty acids.

Figure 7

slightly reduced the amount of the three lipids formed during aging. Washing of the chloroplasts stopped the formation of these three lipids.

(g) The pH dependence and the effect of washed chloroplasts on the formation of radioactive galactolipids by Red Clover chloroplasts incubated with UDPgal ^{14}C:

In an attempt to increase incorporation into MGDG, the pH dependence of the incorporation of radioactivity from UDPgal ^{14}C into Red Clover chloroplasts was examined using incubations at 4 pH's (6.2, 6.8, 7.4 and 7.8; the effective range of the phosphate buffer used for chloroplast preparation). At each pH washed and unwashed chloroplasts were compared to see if the washing decreased formation of the lipids X and Y as observed in the previous section (4.2 (f)). A further sample of washed chloroplasts was incubated with UDPgal ^{14}C in parallel with these and at completion, excess nonradioactive UDP galactose was added to dilute the UDPgal ^{14}C for a further incubation.

10 gms of leaf tissue were used to prepare chloroplasts at the different pH's by grinding in the medium of Method II with the phosphate buffer at the appropriate pH. After straining and centrifugation at 120 x g for 2 min the supernatants were divided into two parts. One part was centrifuged at 3020 x g for 10 min ('unwashed' chloroplasts) and the other centrifuged twice at 3020 x g for 10 min ('washed' chloroplasts). The pellets were resuspended in 1 ml of 0.1 M Tris-HCl buffer containing 1 mM cysteine at the correct pH's. They were incubated with 15 µl UDPgal ^{14}C (35,000 dpm) for 1 hr at 38°C after 0.1 ml samples were taken for chlorophyll determination.

At the completion of the incubation the lipids were extracted. The second sample at pH 7.4 was not extracted after the

incubation; instead it was incubated with 25 µl of a stock solution of non radioactive UDPgal (2.5 mgm/ml) for a further hour and the lipids extracted. The incorporation into the total lipids of all samples is presented in Table 14. The % incorporation into specific lipid components calculated from radiochromatogram scans (samples of which are shown in Figure 8) is given in Table 15.

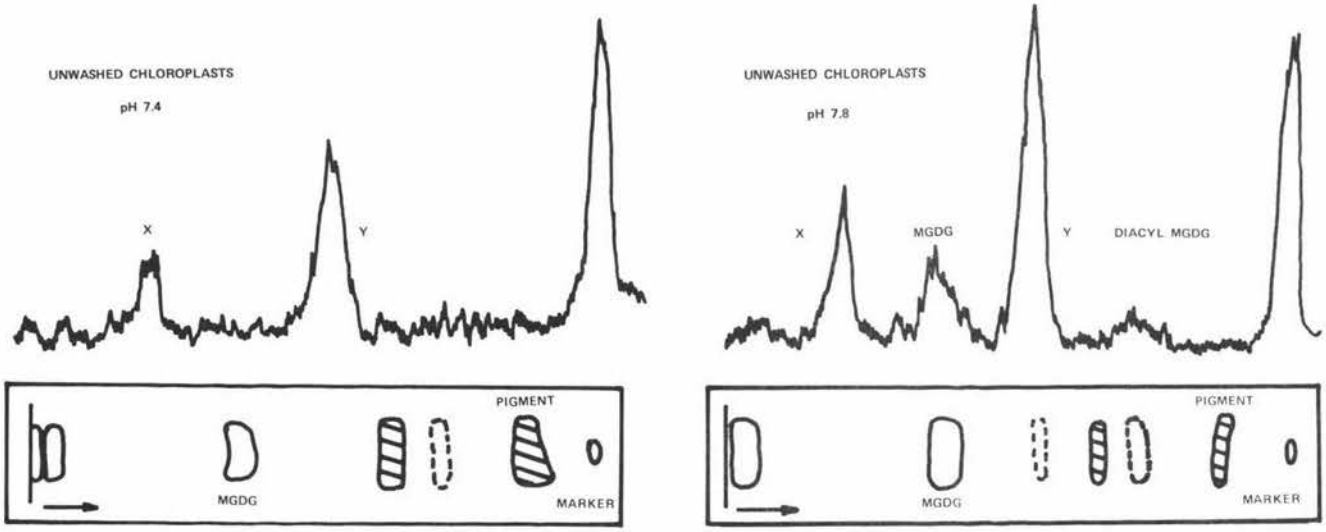
The results of a similar, separate experiment in which McIlvaine buffer (0.1 M citric acid/0.2 M Na_2HPO_4) at pH 7.4 was used to prepare and suspend washed and unwashed chloroplasts are also included in Tables 14 and 15. The radioactivity of the UDPgal ^{14}C added for this part was not determined.

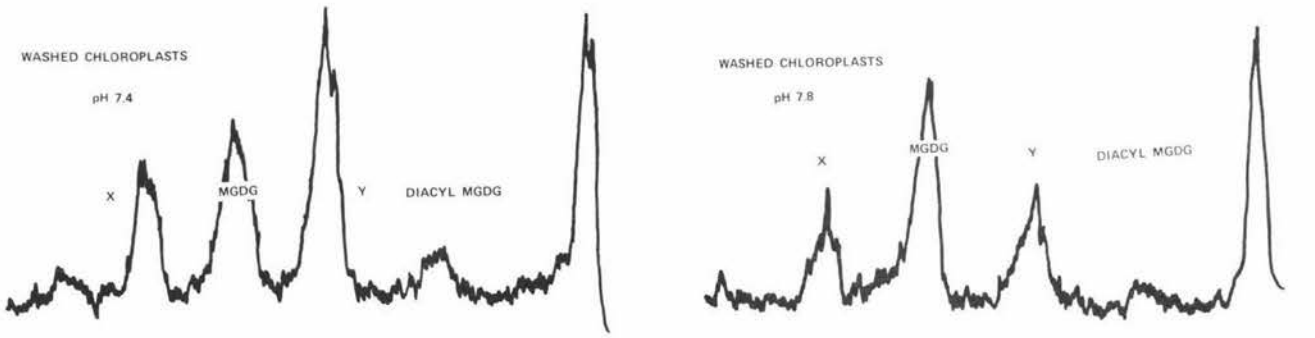
Table 14

Incorporation of radioactivity into total lipids of washed and unwashed chloroplasts incubated at various pH's with UDPgal ^{14}C.

pH	Unwashed Chloroplasts			Washed Chloroplasts		
	Chlorophyll (mgms)	Incorporation Total (dpm)	%	Chlorophyll (mgm)	Incorporation Total (dpm)	%
Phosphate Buffer						
6.2	1.4	1,500	4	1.2	1,800	5
6.8	1.3	8,000	23	1.0	10,800	31
7.4	1.1	4,100	12	0.7	8,000	23
7.8	0.8	7,300	21	0.6	9,400	27
7.4*					8,400	24
McIlvaine Buffer						
7.4	0.7	12,400		0.6	11,500	

* Lipids from washed chloroplasts incubated with UDPgal ^{14}C for 1 hour, then diluted with nonradioactive UDPgal and reincubated 1 hour.





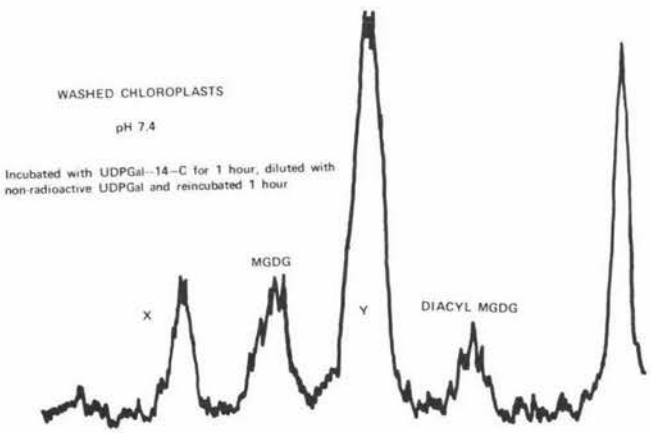


Figure 8

Examples of radiochromatogram scans of lipid extracts from Red Clover chloroplast incubations with UDPGal--14--C at various pH's (Solvent C--M (10 : 1))

Table 14 shows that for both the washed and unwashed chloroplasts, the lowest pH (pH 6.2) gave low incorporation (4%) whilst the other pH's gave much higher incorporation (20 to 30%). The chlorophyll concentrations decreased with increasing pH so that on a chlorophyll basis the highest pH's gave the highest incorporation into the total lipids although the variation between pH 6.8-7.8 was not large compared with the variation between pH 6.2 and pH 6.8. Incorporation into the total washed chloroplast lipids was slightly higher than that for unwashed chloroplasts at each pH using the phosphate buffer.

Table 15

% incorporation of radioactivity into component lipids of washed and unwashed chloroplasts from Red Clover incubated with UDPgal ^{14}C at various pH's.

pH	Unwashed Chloroplasts					Washed Chloroplasts				
	DGDG	X	MGDG	Y	DIACYL MGDG	DGDG	X	MGDG	Y	DIACYL MGDG
Phosphate Buffer										
6.2	-	-	-	100	-	-	-	-	74	26
6.8	-	16	2	75	7	-	11	39	34	16
7.4	-	28	-	72	-	3	18	30	40	9
7.8	3	21	19	56	6	-	16	48	27	9
7.4*						2	13	17	59	9
McIlvaine Buffer										
7.4	13	21	29	29	8	9	8	58	5	20

* see Table 14 for details.

Table 15 shows that for both washed and unwashed chloroplasts the % incorporation into MGDG and X increased with pH and that there was a corresponding decrease in incorporation into X and diacyl MGDG.

At pH 7.4 the results were slightly inconsistent with this trend, probably because of variation in the grinding of the tissue. McIlvaine buffer at pH 7.4 gave the highest % incorporation into MGDG both for the washed (29%) and unwashed (58%) chloroplasts. There was little incorporation into DGDG (less than 3%) for any of the phosphate buffer samples. However the McIlvaine buffer gave about 10% incorporation into DGDG. Washed chloroplasts prepared using both buffers at pH's greater than pH 6.2 gave much higher % incorporation into MGDG than did unwashed chloroplasts. This is well illustrated by comparing the scans in Figure 8.

From these results it can be concluded that the conditions for optimum incorporation into MGDG were: washed chloroplasts prepared using McIlvaine buffer at pH 7.4 to pH 7.8.

A comparison of the incorporation patterns for the washed chloroplasts at pH 7.4 before and after reincubation with nonradioactive UDP galactose shows that the radioactivity in Y increased from 40% to 59% during the second incubation (demonstrated qualitatively in Figure 8 and quantitatively in Table 15). There was also a decrease in the radioactivity in MGDG from 30% to 17% and a much smaller decrease for X from 18% to 13%. Incorporation into the total lipids during the second incubation was negligible (Table 14) because the specific activity of the UDPgal ^{14}C was decreased at least 200-fold on addition of the nonradioactive UDP galactose. Thus the change in the pattern of radioactivity which occurred during the second incubation must be due to synthesis of $Y^{14}C$ from MGDG-^{14}C.

(h) The incorporation of radioactivity into the galactolipids of washed chloroplasts incubated with UDPgal ^{14}C in the presence of a particulate fraction obtained from the washings:

This experiment was designed to test whether the changed incorporation pattern caused by washing chloroplasts was due to the removal of one (or more) enzymes in a particulate fraction of the washings.

15 gms of Red Clover leaf tissue were ground in the medium of Method II with the phosphate buffer at pH 7.4. After straining and centrifuging at 120 x g for 2 min the chloroplast suspension was divided into three equal parts and each part centrifuged at 3020 x g for 10 minutes. Two of these pellets were resuspended in suspension medium and again centrifuged at 3020 x g for 10 min. All three pellets (i.e. one unwashed, two washed) were then resuspended in 0.1 M Tris-HCl pH 7.8 for incubation. The supernatant from the final centrifugation of one of the washed samples was centrifuged at 10,000 x g for 10 min, the pellet suspended in 0.5 ml 0.1 M Tris-HCl pH 7.8, and added back to the chloroplasts. The final volume of all three chloroplast suspensions was 1 ml. UDPgal ^{14}C was added to each and then incubated for 1 hour at 38°C with shaking.

The lipids were extracted at the end of the incubation. The incorporation data are presented in Table 16 and the radiochromatograms are reproduced in Figure 9. Hydrogenated diacyl MGDG prepared by the method described in Section 3.8(b) was developed on the TLC plate together with the radioactive lipids in two of the radiochromatograms using two solvents: C/M (10:1) and water saturated diethyl ether/ isopropanol/methanol (100:4.5:3). Comparison of C with D in Figure 9 shows that this second solvent was not as effective as C/M (10:1) in separating neither MGDG from X nor Y from diacyl MGDG, but it did give

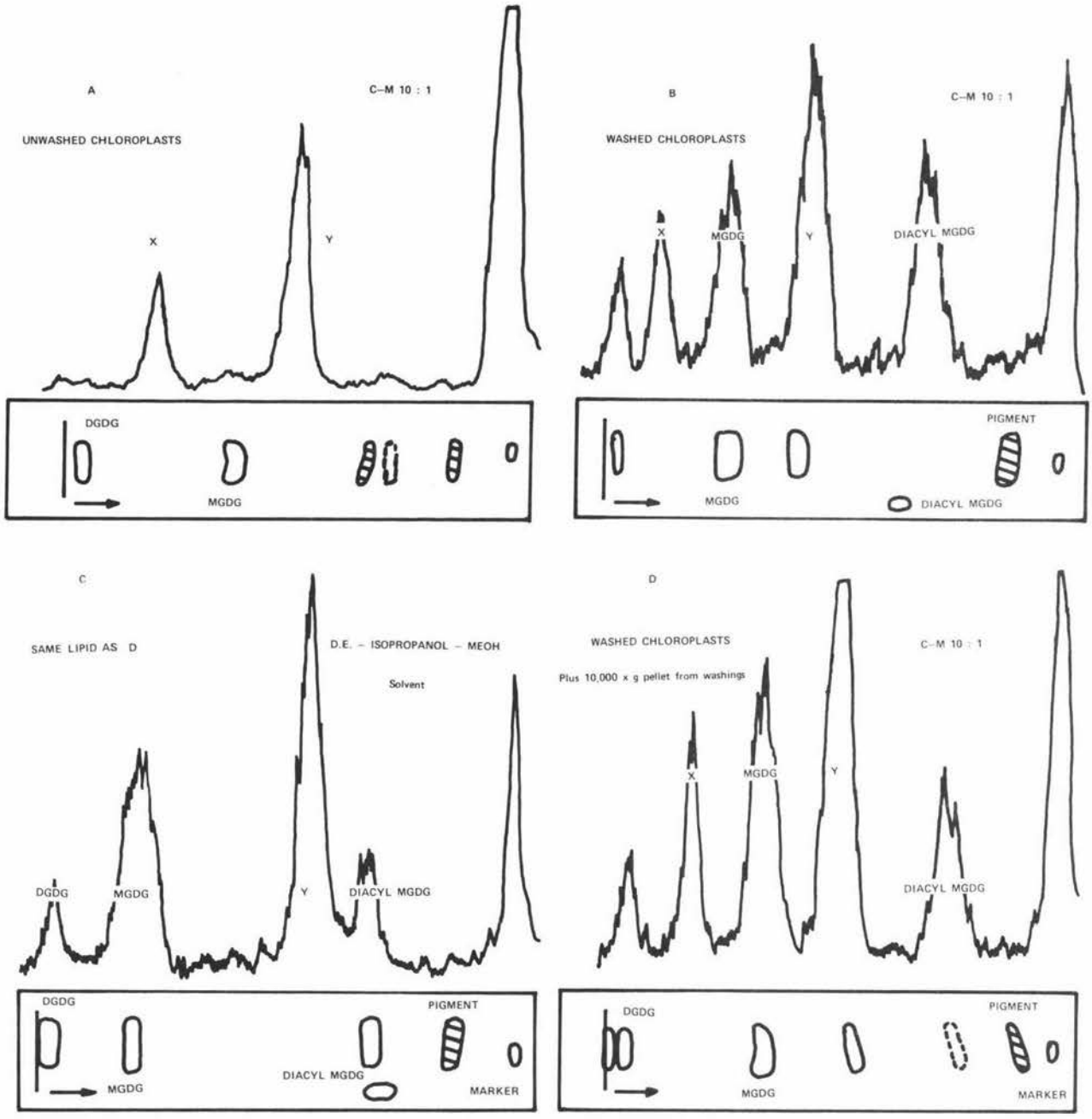


Figure 9

Radiochromatogram scans of lipid extracts from Red Clover chloroplasts incubated with UDPGal–14–C at pH 7.8. Comparison of incorporation into unwashed and washed chloroplasts with washed chloroplasts plus 10,000 x g pellet from the washings

Table 16

Incorporation of radioactivity from UDPgal ^{14}C into Red Clover galactolipids by: (1) unwashed chloroplasts,
(2) washed chloroplasts and
(3) washed chloroplasts plus a particulate fraction from the washings.

Type of Chloroplasts	14C Added to sample (dpm)	Incorporation into total lipids		% Incorporation into component lipids				
		(dpm)	%	DGDG	X	MGDG	Y	DIACYL MGDG
Unwashed	438,000	76,000	17	-	24	6	67	3
Washed	146,000	63,000	43	7	11	20	35	27
Washed + 10,000 x g pellet from washings	146,000	101,000	70	5	15	23	41	16

a good separation between MGDG and Y.

If enzymes removed in washing were contained in the particulate fraction, the pattern of incorporation for washed chloroplasts in the presence of this fraction should have been similar to that for unwashed chloroplasts. (e.g. incorporation into MGDG would be closer to 6% than to 20%). In fact the difference between the patterns for washed chloroplasts in the presence and absence of the particulate fraction was very small and the incorporation into MGDG actually increased slightly. Hence the changed pattern of incorporation into the galactolipids of Red Clover chloroplasts caused by washing is not due to the removal of enzymes in this particulate fraction.

(i) Electron micrographs of Red Clover chloroplast preparations:

Unwashed chloroplasts, washed chloroplasts and the 10,000 x g pellet from the washings were prepared for electron microscopy by D.S.I.R. staff. Difficulty was experienced in cutting sections of the unwashed chloroplasts because fine sand particles were present. There was much less sand in the other two samples and sections of these were examined under the electron microscope for (1) the extent of chloroplast disruption (2) the presence of organelles other than chloroplasts and (3) the extent of removal of such organelles by washing. The following selected electron micrographs are reproduced in Figure 10:

A. Intact chloroplast.

B. Chloroplast stripped of its outer membrane and stroma.

C. Chloroplast fragments and other organelles.

] Washed chloroplast preparation.

D. Chloroplast fragments and other organelles from the particulate fraction.

Key to labelling:

CF	Chloroplast fragment
G	Granum
L	Lamellae
M	Mitochondrion (osmotically distended)
P	Peroxisome
S	Stroma
St	Starch grain.

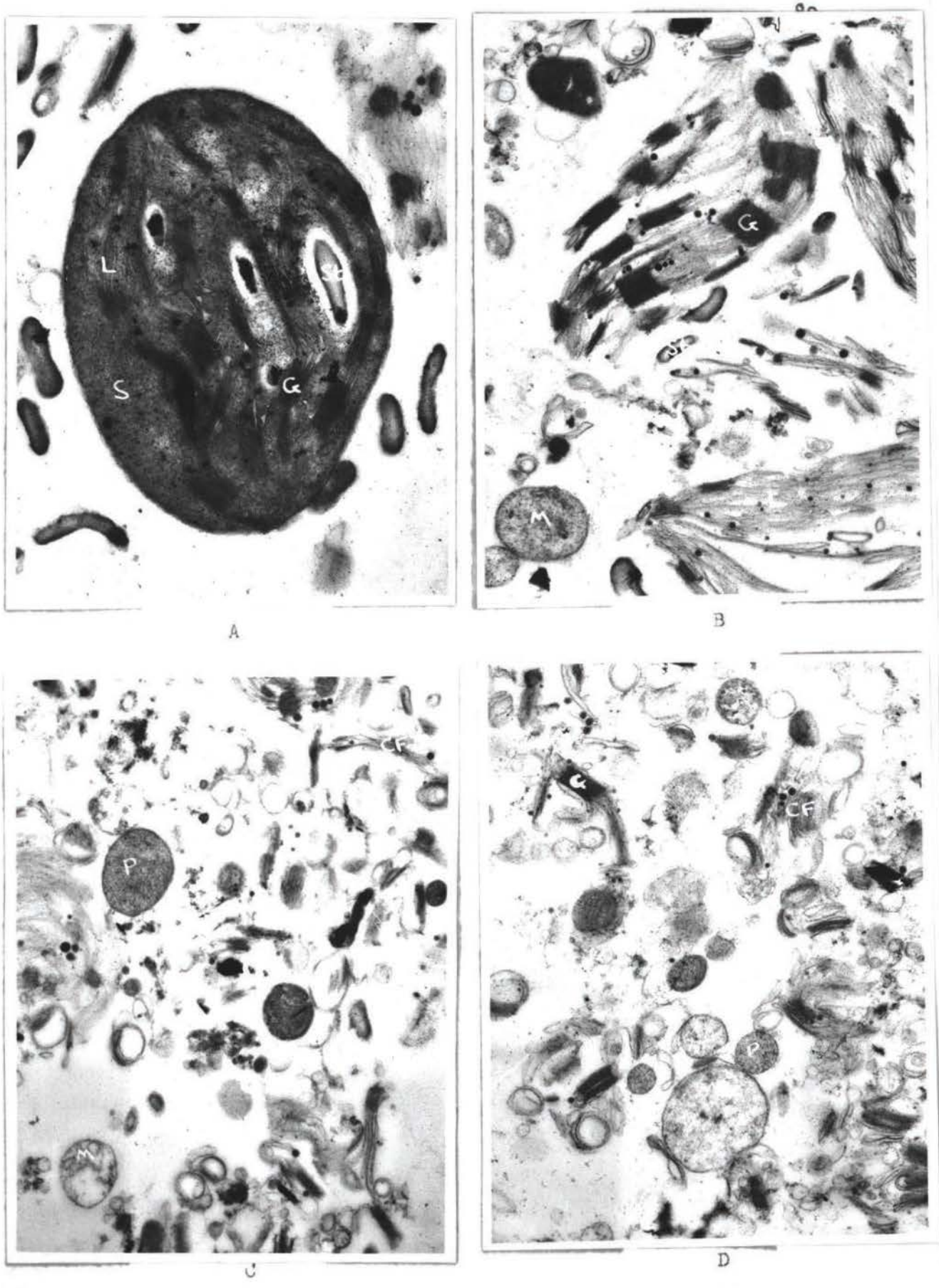


Figure 10 Electron micrographs of chloroplast preparations
Magnifications: A X18,000. B X12,000. C X12,000
D X14,000. See text for Key.

The washed chloroplast preparation contained many intact chloroplasts (A) but most were stripped of their outer membrane and much of their stroma (B). As well as chloroplasts there were many chloroplast fragments, peroxisomes and mitochondria. The particulate fraction from the washings consisted of chloroplast fragments, (grana with associated lamellae) peroxisomes and mitochondria. It can be concluded that the washing process removed many of these contaminants. However 'washing' required resuspension of the chloroplasts in the suspension medium aided by dispersal of the pellet using a glass rod. It is likely that this disrupted many of the intact chloroplasts to give a higher proportion of stripped chloroplasts in the washed preparation.

4.3 Analyses of Red Clover galactolipids:

(a) Fatty acid composition:

(i) Red Clover lipids:

The fatty acid composition of Red Clover galactolipids and phospholipids determined using GLC of the methyl esters is given in Table 17. MGDG, DGDG and total phospholipids were extracted from chloroplast lipids by preparative TLC using the tol/EtOH/EtAc solvent. Phosphatidylcholine (PC) and phosphatidyl glycerol (PG) were separated from the total phospholipid by preparative TLC using C/M/H_2O (65/35/4,v/v/v) solvent. Diacyl MGDG was extracted from an aged chloroplast preparation using silicic acid column chromatography and preparative TLC as described in Section 3.8(b). The GLC chromatographs of the fatty acyl methyl esters of the galactolipids are reproduced in Figure 11.

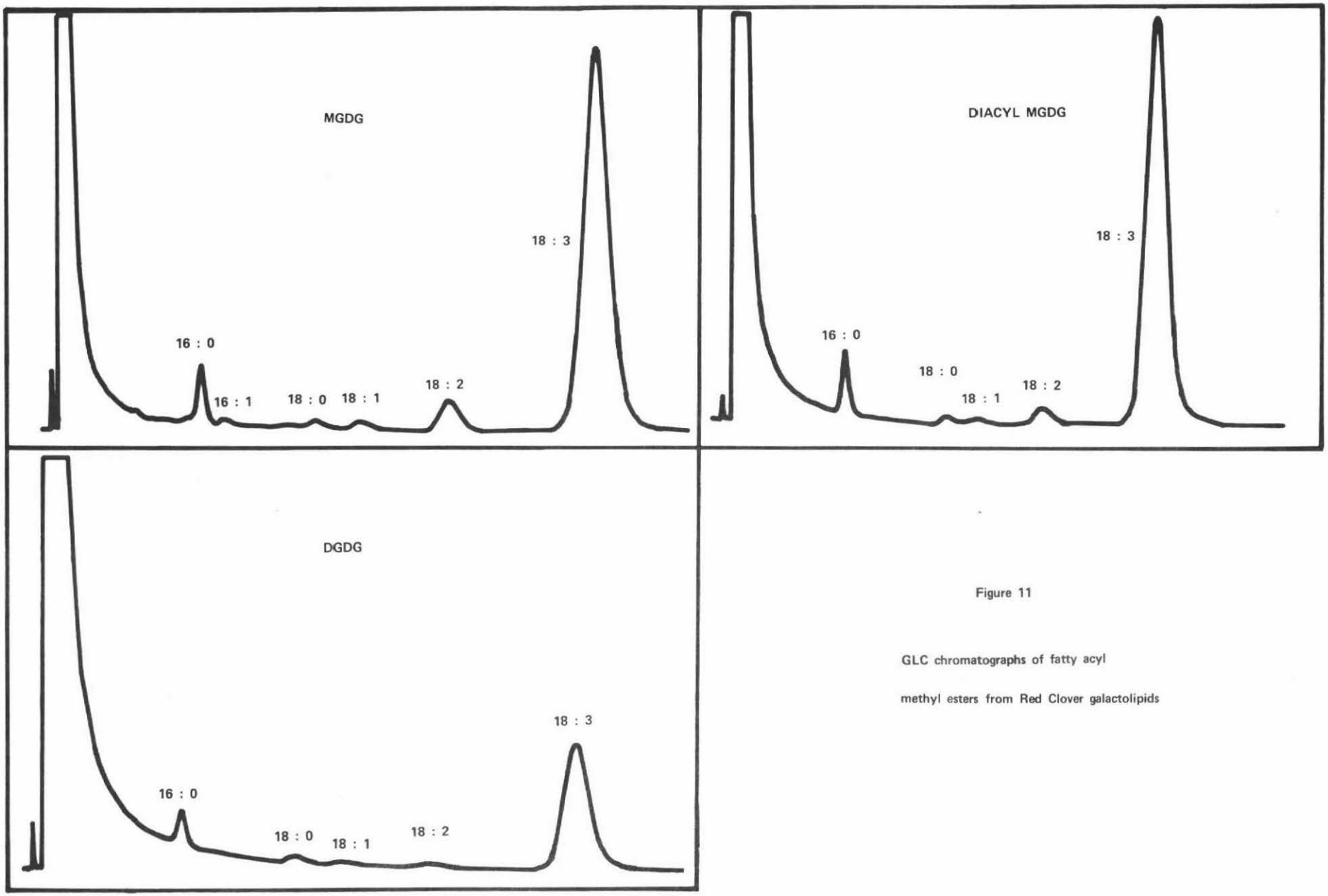


Figure 11

GLC chromatographs of fatty acyl methyl esters from Red Clover galactolipids

Table 17

The fatty acid composition of Red Clover galactolipids and phospholipids. Results expressed as area percentage of the total fatty acids.

Lipid	12:0	14:0	16:0	16:1$_{\triangle}$	^{3t}16:1	16:3	18:0	18:1	18:2	18:3
MGDG	t	t	4.2	t	-	-	1.0	1.2	5.7	87.6
DIACYL MGDG	t	t	6.9	-	-	-	1.2	0.9	3.7	87.4
DGDG	t	t	8.0	-	-	-	2.1	1.3	1.5	87.3
HYDROGENATED MGDG	t	t	3.0	-	-	-	97	-	-	-
PG	t	t	14.2	-	22.9	2.1	3.4	5.0	8.2	44.2
PC	t	t	23.8	t	3.5	t	3.9	3.7	21.7	40.9

t - trace.

Table 17 shows that the three galactolipids MGDG, DGDG and diacyl MGDG have high contents (87 to 88%) of linolenic acid. The fatty acid composition of diacyl MGDG is intermediate between the compositions of MGDG and DGDG. The hydrogenated MGDG was prepared by column chromatography as described in Section 3.8 (a) and contains 97% stearic acid and 3% palmitic acid.

The two phospholipids contained much lower proportions (40-44%) of linolenic acid than did the galactolipids. Compared with the galactolipids, PC had a high content of 16:0 and 18:2. PG contained 22.9% of the fatty acid $\triangle^{3t}$16:1, which is characteristic of this lipid. The fatty acid compositions of the freee fatty acids formed during aging of chloroplasts prepared at two different times of the year were determined. These two compositions were different although both were intermediate between the galactolipid and PC fatty acid compositions. It is likely that both galactolipases and phospholipases were present in

the chloroplasts and the differences in the free fatty acid compositions were due to different proportions of these two enzymes.

(ii) Radioactive Spinach galactolipids:

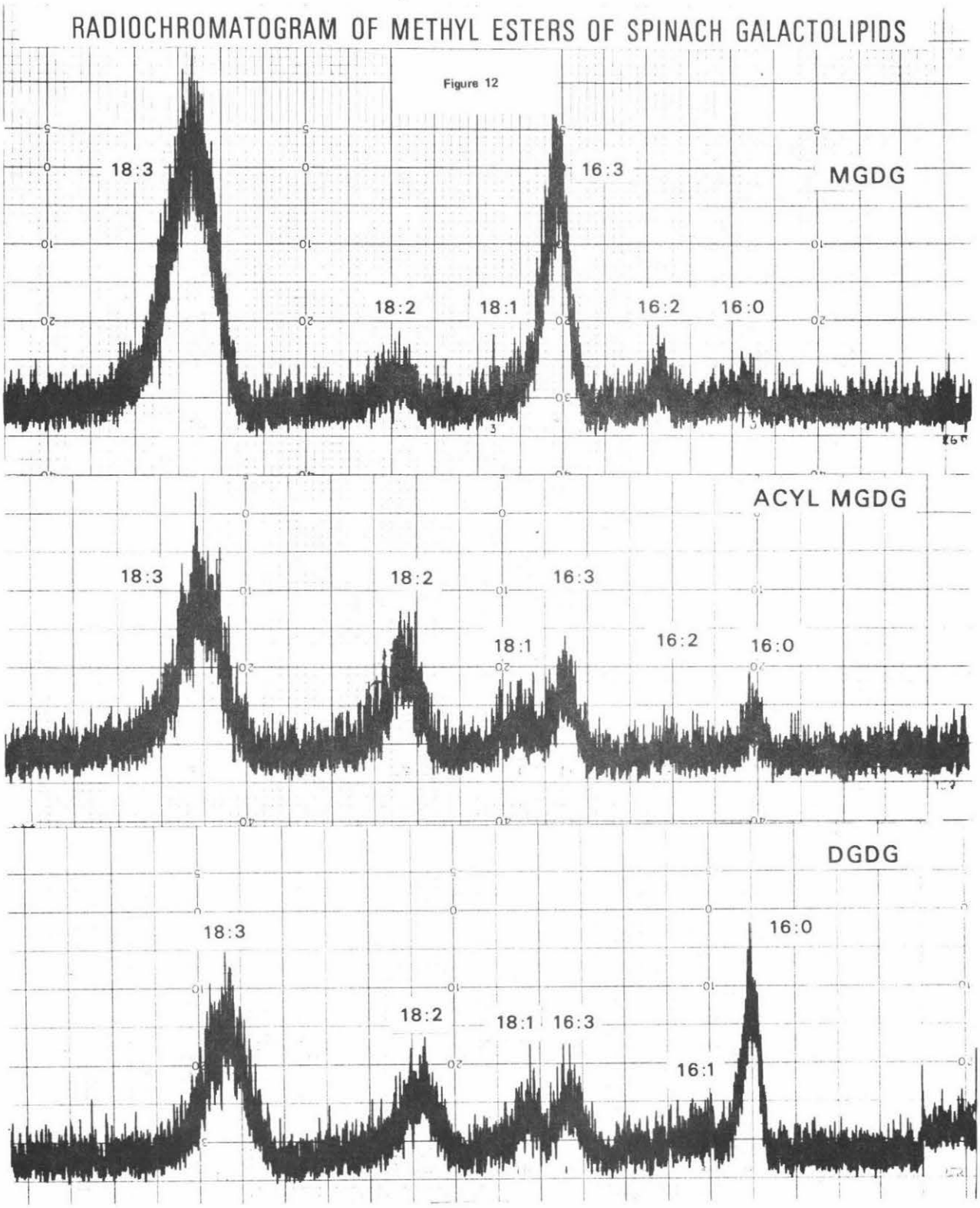
A radioactive total lipid extract from spinach was obtained as a gift from Dr. Tapper of the D.S.I.R. The potted spinach from which it was extracted had been exposed to $^{14}CO_2$ for 24 hours to form radioactive plant protein. There was considerable radioactivity in both the galactosyl glycerol moiety and the fatty acyl groups of the galactolipids. A radiochromatogram of the TLC of the total lipid using C/M (10:1) showed that much radioactive lipid Y had been formed during the initial extraction of the radioactive plant protein. MGDG, DGDG and lipid Y were obtained from the total lipid by preparative TLC and their fatty acids analysed by a GLC fitted with a radioactivity detector. The radiochromatograms of the fatty acids are reproduced in Figure 12. Table 18 gives the % radioactive composition of the fatty acids.

Table 18

The % radioactivity in fatty acids of Spinach galactolipids extracted from leaves after they had been exposed to $^{14}CO_2$ for 24 hrs.

Lipid	16:0	16:1	16:2	16:3	18:1	18:2	18:3
MGDG	3.8	-	4.2	26.2	1.2	5.4	58.5
LIPID Y	8.3	-	4.5	10.2	7.7	23.6	45.9
DGDG	20.4	5.9	-	9.9	8.6	14.5	40.8

The % radioactivity in each fatty acid from lipid Y (labelled acyl MGDG in Figure 12) is intermediate between the % radioactivity in the same fatty acid from MGDG and DGDG for all fatty acids except 18:2. The % radioactivity in 18:2 for lipid Y is higher than that for both MGDG and DGDG.


RADIOCHROMATOGRAM OF METHYL ESTERS OF SPINACH GALACTOLIPIDS
Figure 12
MGDG
18:3
18:2
18:1
16:3
16:2
16:0
ACYL MGDG
18:3
18:2
18:1
16:3
16:2
16:0
DGDG
18:3
18:2
18:1
16:3
16:1
16:0

(b) Chemical analysis of hydrogenated MGDG:

MGDG was isolated from Red Clover chloroplasts by silicic acid column chromatography and hydrogenated as described in the Methods Section 3.8 (a). The purpose of preparing this hydrogenated MGDG (MGDGH) was to use it as a control for the identification of the unknown lipids formed by Red Clover chloroplasts. Relatively large quantities (approximately 80 mgms) of MGDG were isolated and as its content of C_{18} fatty acids was high, hydrogenation yielded a lipid with 97% stearic acid present. Hence MGDGH was readily purified by repeated crystalisation from C/M (1:9). Its purity, checked by a melting point determination and DSC analysis was found to be high. It was used to test the feasibility of applying mass spectroscopic analysis to the identification of diacyl MGDG. Its IR spectrum was compared with that of diacyl MGDG. A chemical analysis for the galactose, glycerol and fatty acid composition was also made using the method described in section 3.9 to check that MGDG had indeed been isolated and to test the feasibility of using the same method for identifying diacyl MGDG.

The results of the chemical analysis are shown in Table 19.

If the molar ratios are corrected to the nearest whole number it can be concluded that the composition of the hydrogenated MGDG is the theoretical value of 2:1:1:1 for the ratio FA:galactose:glycerol:MGDG. However the variation in the determinations is large (greater than 10%) despite the purity of the galactolipid. Hence this method was not applied to the analysis of diacyl MGDG which was obtained in very small quantities (5 to 10 mgms) and was impure.

Table 19

Analytical data for hydrogenated MGDG.
Two separate analyses were made.

Constituent	Determination 1 (μmoles)	Determination 2 (μmoles)
MGDG analysed	1.0	1.0
Fatty acyl esters[t]	2.0	1.8
Fatty acids*	2.4	2.1
Galactose	1.2	0.9
Glycerol	1.2	0.8
Ratios**		
FA:gal:glycerol	1.8:1.0:1.0	2.2:1.0:0.9
FA:gal:glycerol:MGDG		
experimental	2.2:1.2:1.2:1.0	2.0:0.9:0.8:1.0
theoretical	2:1:1:1	2:1:1:1

t colorimetric technique.

* determined from GLC of methyl esters with internal standard.

** Fatty acid value used was the average of the 2 for each determination.

(c) Infra-red spectra of diacyl MGDG and hydrogenated MGDG:

The IR spectra of hydrogenated MGDG and diacyl MGDG in KBr disks are reproduced in Figure 13. A standard polystyrene peak at 1600 cm^{-1} is shown on the top spectrum. Similar peaks were obtained for hydrogenated MGDG as those reported in the literature (see Section 1.2 (e)).

The diacyl MGDG sample was the nonhydrogenated lipid separated by preparative TLC in C/M (10:1) from the mixed galactolipid fraction prepared by column chromatography as described in section 3.8 (b). Its R_f in C/M (10:1) corresponded to that of radioactive diacyl MGDG

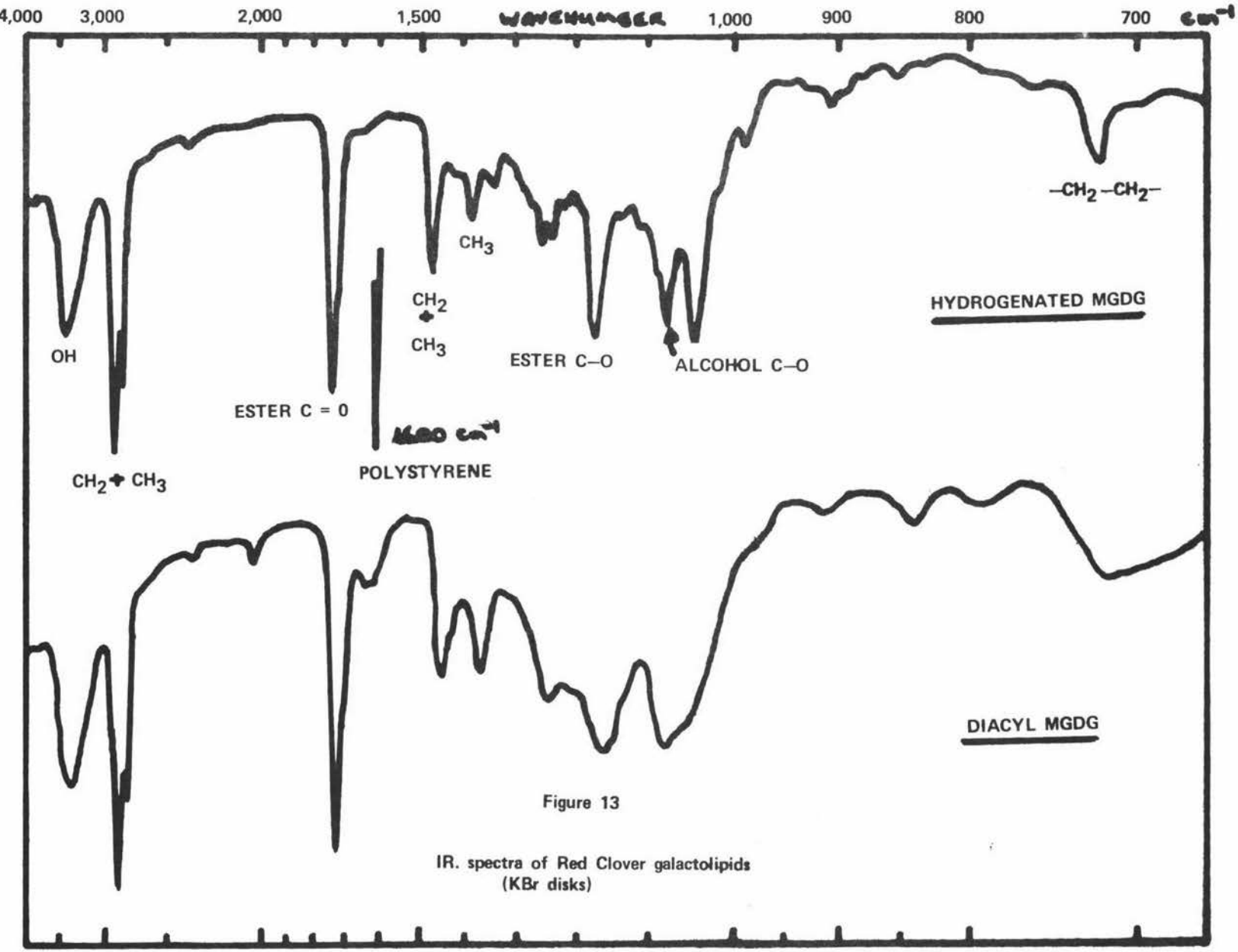


Figure 13

IR. spectra of Red Clover galactolipids
(KBr disks)

obtained in the radiochemical experiments. The spectrum of diacyl MGDG is very similar to that of the hydrogenated MGDG, with an additional peak at 1650 cm^{-1} and a slight shoulder at 3000 cm^{-1} due to the cis double bonds.

(d) Melting point determination and differential scanning calorimetric analysis of hydrogenated MGDG:

As a measurement of purity, the melting point of the hydrogenated MGDG was determined using a hot stage microscope. Viewed through the microscope, the dried MGDGH consisted of small broken crystals. On heating, this sample underwent a transition to larger, more regular facetted crystals at 100°C (sintering point). Further heating resulted in melting at 158°C. Cooling of the melt to 40°C gave large irregular multi-facetted crystals and on reheating a new sintering point at 75°C was observed. There was no transition at 100°C on this second determination and the melting point was a little lower at 153°C. Both determinations gave sharp melting points with melting occurring over a range of about 0.5°C indicating high purity.

A further sample of MGDGH was analysed by DSC as a more accurate check on purity and melting point and to study the polymorphic behaviour in more detail. The melting curves and a cooling curve are reproduced in Figure 14. The same sample was used throughout and the conditions for the analyses were:

scan speed: 8.34 degrees/min
chart speed: 60 inches/hour
range: 4.53 (curves 2-5) and 2.35 (curve 1).

Curve 1 is the first melt after introduction of the sample (crystallised from $C/M/H_2O$) into the sealed pan. There were two

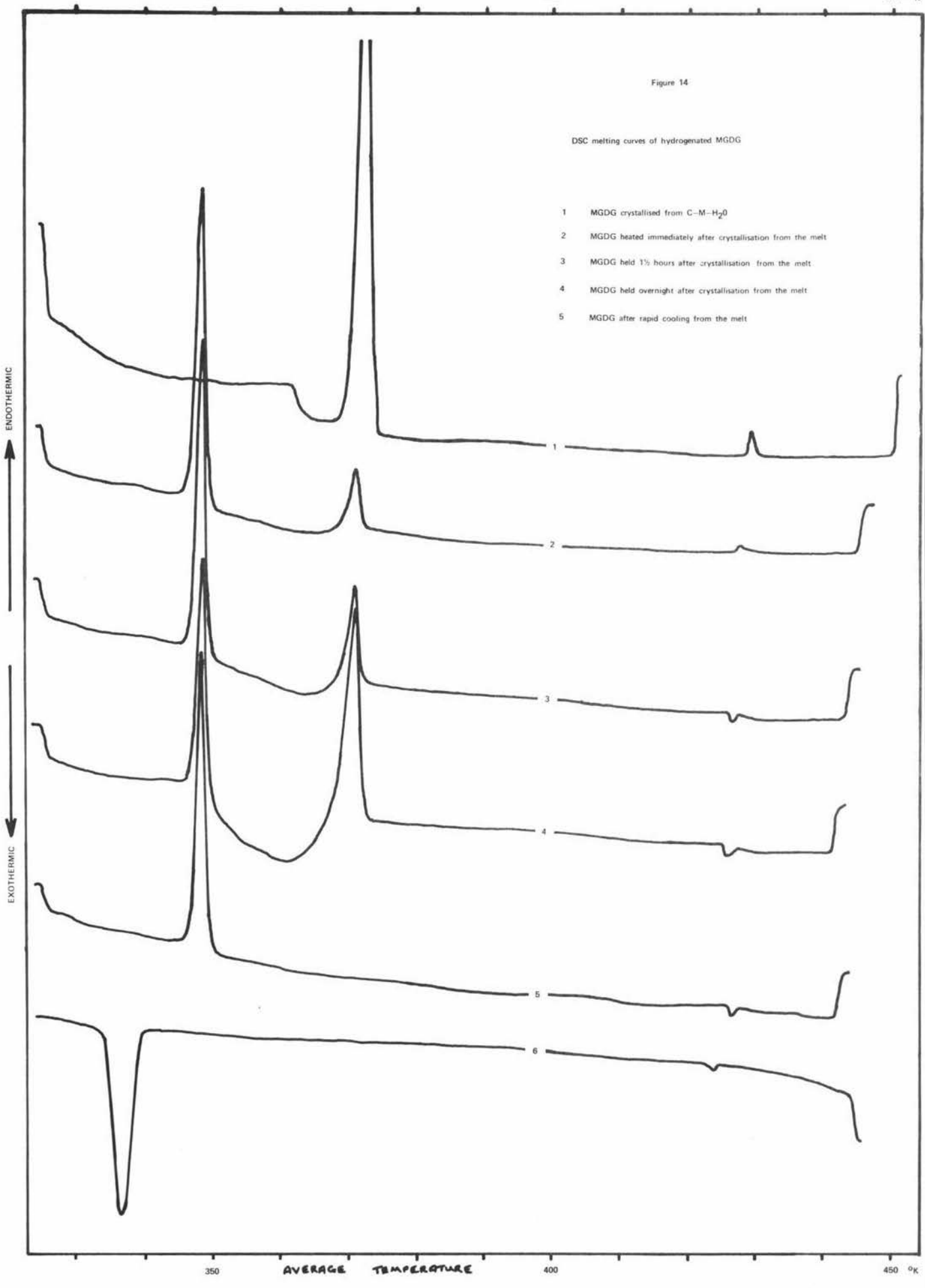


Figure 14

DSC melting curves of hydrogenated MGDG

1 MGDG crystallised from C–M–H_2O

2 MGDG heated immediately after crystallisation from the melt

3 MGDG held 1½ hours after crystallisation from the melt

4 MGDG held overnight after crystallisation from the melt

5 MGDG after rapid cooling from the melt

endothermic transitions: a large one at 95°C (368°K) and a much smaller one at the melting point of 156°C (429°K).

Curves 2 to 4 were obtained by cooling the melt to 53°C (326°K) and reheating after holding at this temperature for increasing periods. There were 3 endothermic transitions: the two observed in curve 1 plus another at 72.5°C (345.5°K). The size of the peak at 95°C increased with holding time and the size of the peak at 72.5°C decreased.

Curve 5 was obtained by cooling the melt as rapidly as the apparatus would allow and reheating immediately. The peak at 95°C was completely absent.

Curve 6 was a typical cooling curve. Note that the peaks were shifted downscale as is usual with cooling curves. The transition equivalent to that obtained at 95°C in the melting curves was absent.

The three transitions observed with DSC occur at temperatures which are in good agreement with the temperatures observed with the hot stage microscope, and the narrow, sharp peaks obtained indicate high purity (greater than 99%).

The polymorphic behaviour displayed by the MGDGH is similar to that of phosphatidyl choline described in Section 1.2 (e) and illustrated by Figure 2. It is most simply explained by postulating two crystalline forms of the solid MGDGH . The β form which transforms to a liquid-crystalline phase at 95°C, is the most stable form and is obtained by crystallisation from C/M/H_2O (curve 1). The α form which transforms to the liquid-crystalline phase at 72.5°C is the form obtained by rapid crystallization of the melt (curves 5 and 6). The melting point at 156°C corresponds to a low energy (small peak area) transition from the

liquid-crystalline phase to the liquid. A slow α to β transformation takes place at room temperature so that β accumulates when α is held at room temperature as shown by curves 2, 3 and 4. Hence on heating solid crystallised from the melt and held for $1\frac{1}{2}$ hours as in curve 3, the α transforms to the liquid-crystalline phase at 72.5°C and the β which was formed during the holding period transforms to the liquid crystalline phase at 95°C.

An α to β transition also occurs just above the melting point of α as shown by the exothermic trough between the α and β peaks in curves 3 and 4. This was further demonstrated by 'tempering' the sample. α was heated to just above the temperature of its transformation to the liquid crystalline phase and by holding at this temperature for sufficient time, (approximately 1 hour) all the sample was transformed into β. On cooling and reheating, the melting curve resembled curve 1. The [illegible] of this transition appeared to be dependent on the amount of β present to seed it, as the size of the exothermic trough increased from curve 2 through to curve 4.

Little can be deduced about the structure of the two crystalline forms, although considering the hydrophillic nature of some of the phosphatidyl choline crystalline forms (see Section 1.2 (e)), it is likely that water of crystallisation is involved in these phase changes.

(e) Mass spectroscopic analysis of hydrogenated MGDG and hydrogenated diacyl MGDG:

Mass spectroscopy was used to identify the structure of the lipid diacyl MGDG which was formed in radiochemical and aging experiments with Red Clover chloroplasts. Hydrogenated diacyl MGDG was prepared from a total lipid extract of aged chloroplasts as described

in Section 3.8 (b). Figure 9 parts B and C shows radiochromatograms in which diacyl MGDG^{14}C was chromatographed with the hydrogenated diacyl MGDG standard in two solvents; C/M (10:1) and water saturated diethyl ether/isopropanol/methanol (100:4.5:3.0). It can be seen that the radioactive peaks correspond with the hydrogenated lipid.

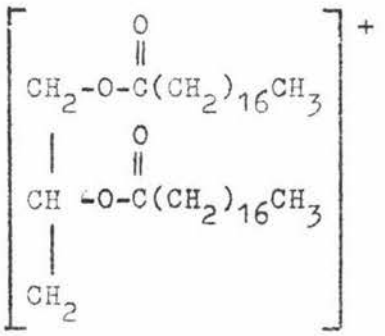
Mass spectroscopic analysis of galactolipids has not been previously reported in the literature. As its purity and chemical composition had been established, hydrogenated MGDG (97% 18:0 - see Section 4.3 (a)) was analysed first to test the feasibility of using unblocked galactolipids and to examine their general fragmentation pattern. A detailed description of the fragmentation sequence was not required and only intense peaks relevant to the identification of the diacyl MGDG were examined in the spectra.

A stable spectrum with high molecular mass peaks of measurable intensity was obtained for MGDGH at high temperatures (250°C). No molecular ion was detected and the ion of highest mass was at 768 m.u. which corresponds to the molecular weight of MGDGH containing two stearoyl groups minus water. At this high mass, the spectrum was too weak for high resolution mass measurement. There were intense peaks for fragment ions at 606, 607, 284 and 163 m.u. The results of high resolution mass determinations made on three of these and calculated masses and formulae were as follows:

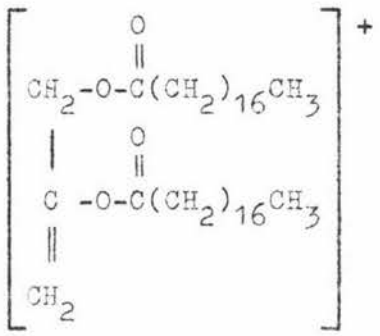
peak	measured mass	calculated mass	formula
606 m.u.	606.5564	606.5581	$C_{39}H_{74}O_4$
607 m.u.	607.5632	607.5659	$C_{39}H_{75}O_4$
163 m.u.	163.0606	163.0606	$C_6H_{11}O_5$

The most likely structures of these are:

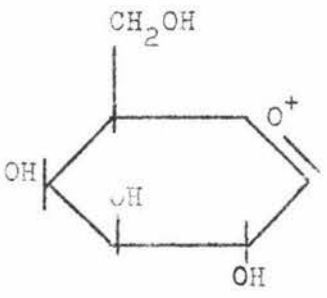
$C_{39}H_{75}O_4$



$C_{39}H_{74}O_4$

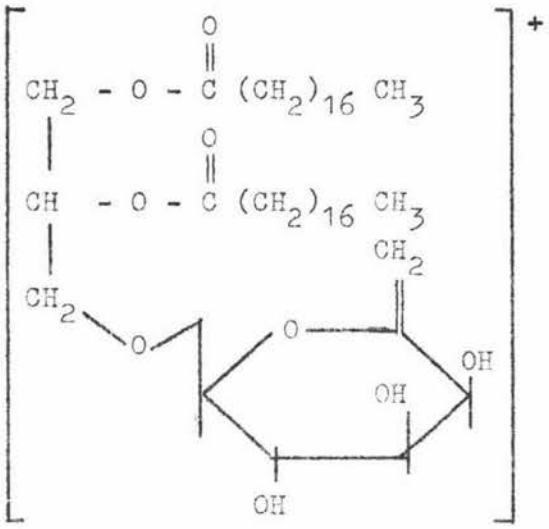


$C_6H_{11}O_5$



The peak at 284 m.u. is at the molecular weight of stearic acid which made up 97% of the total fatty acids of the MGDGH. There was also a series of peaks spaced at 14 m.u. at masses lower than 284 m.u. which are characteristic of stearic acid fragmentation.

Hence the most abundant ions in the spectrum were associated with the hydrocarbon chains, glycerol esters and galactose of MGDGH. The absence of a molecular ion and the presence of the peak at 768 m.u. suggests that water was immediately lost from the molecule; probably from the galactose moiety to give an ion of the type:



An important fragmentation occurred at the ether bond between the galactose and glycerol moieties to give the ions with masses 606, 607 and 163 m.u. Fragmentation of the glycerol ester bonds must yield intact stearic acid.

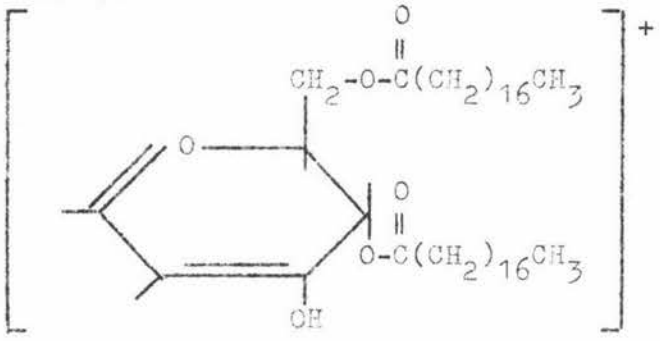
The most intense high molecular weight peaks obtained with diacyl MGDG heated to 250°C, were at 606, 607, 676, and 677 m.u. The stearic acid fragmentation peaks noted for MGDGH were also present and a peak at 163 m.u. was of very low intensity relative to the stearic acid peaks compared with the 163 m.u. peak for MGDGH. As the sample temperature increased to 250°C the intensities of the peaks at 606 and 607 m.u. decreased in proportion to the peaks at 676 and 677 m.u. Further analyses on recrystallised lipid would be required to determine whether this effect is due to temperature dependent pyrolysis or due to the presence of two or more slightly different compounds of varying volatility.

The peaks at 606, 607 and 284 m.u. have been described for the MGDGH spectrum. High resolution mass measurements were made on 676 m.u. and 677 m.u.:

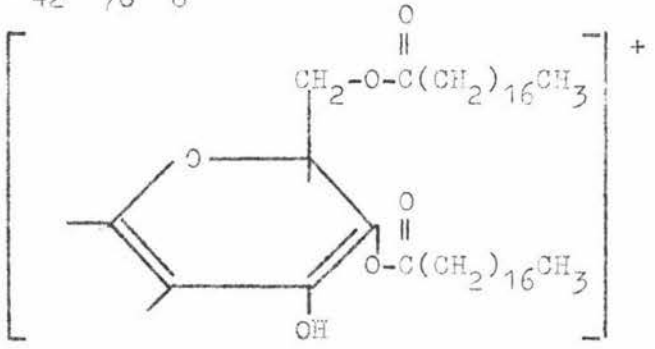
peak	measured mass	calculated mass	formula
676 m.u.	676.5596	676.5638	$C_{42}H_{76}O_6$
677 m.u.	677.5665	677.5719	$C_{42}H_{77}O_6$

The most acceptable structures for these two ions are:

$C_{42}H_{77}O_6$



$C_{42}H_{76}O_6$



The positions of the 2 stearoyl groups on the ring can not be deduced from the mass spectra.

On the basis of these results, the following structure for diacyl MGDG is proposed:

1,2-di-O-acyl-3-O (diacyl-β-D-galactopyranosyl)-sn-glycerol.

The fragmentation of this molecule is similar to that of MGDGH except that cleavage of the ether linkage between the galactose and glycerol yields the fragment ions at 676 and 677 m.u., instead of 163 m.u., besides the ion at 284 m.u.

Chapter 5

DISCUSSION

5.1 Incorporation of radioactivity into the galactolipids of Red Clover chloroplasts:

P.V.P. and NaDEDTC were required in the suspension medium used to prepare Red Clover chloroplasts for incorporation of radioactivity from UDPgal^{14}C into the chloroplast galactolipids. P.V.P., which had the greater effect on incorporation, binds strongly with phenolic compounds (tannins). Tannins are widespread in plants and often occur in high concentrations. They have been shown to inhibit the activities of many plant enzymes and this effect has been reviewed by Loomis and Battaile (1966). Phenols form very strong hydrogen bonds with N-substituted amines and hence with protein peptide bonds. Furthermore, tannins are readily oxidised by phenol oxidases which are common in plant tissues, to form quinones. These compounds can oxidise essential protein groups and also react to form covalent bonds with SH groups, terminal amino groups and N-terminal prolines of proteins. P.V.P. forms hydrogen bonds with tannins to give stable insoluble complexes.

The effect of P.V.P. on Red clover chloroplast incorporation suggests that Red Clover has a high content of tannins. NaDEDTC forms a strong complex with copper and its effect on incorporation was probably due to inhibition of copper containing polyphenol oxidase.

Red Clover chloroplasts were much less active in incorporating radioactivity from UDPgal^{14}C than were Fescue chloroplasts. Furthermore, the principal radiolipids formed were lipids X and Y and sometimes smaller

amounts of diacyl MGDG while the main component for Fescue was MGDG with smaller amounts of X, Y, diacyl MGDG and DGDG. Incorporation into X and Y was also observed for spinach and ryegrass chloroplasts in the experiments designed to compare different homogenising techniques. Synthesis of radioactive galactolipids by Red Clover chloroplasts has not been reported previously, but incorporation into lipids other than MGDG and DGDG (including lipids with similar R_f's to X and Y) by chloroplasts isolated from various plants has been observed (Eccleshall, 1970; Webster and Chang, 1969; Ongun and Mudd, 1968; Chang and Kulkarni, 1970). However in all these instances incorporation into MGDG was the highest, whereas Red Clover chloroplasts consistently gave relatively low % incorporation into MGDG.

Incubation of Red Clover leaf slices with Gal ^{14}C gave low incorporation into the main lipid components MGDG and DGDG with only traces (less than 1%) into X and Y. This was a similar pattern to that of Fescue and agreed with the patterns obtained for Fescue and Barley leaf slices by Eccleshall (1970). P.V.P. was not required in the leaf slice incubations. As the leaf tissue was largely intact the gal ^{14}C must have diffused or may have been transported through the tissue to the chloroplasts for formation of the UDPgal. derivative and galactolipid synthesis. The incorporation pattern observed this way should reflect the true proportions of the final products more than the chloroplast incubations. Thus the observance of the formation of X, Y and diacyl MGDG is a function of the chloroplast preparation.

5.2 Identification of galactolipids formed by Red Clover chloroplasts:

In the chloroplast incubations with UDPgal ^{14}C, little or no lipid was detected on the TLC plates at positions corresponding to the

radioactive peaks X, Y and diacyl MGDG. Wintermans et al. (1969) studied the formation of similar lipids in nonradioactive aging experiments with spinach chloroplasts. Red Clover chloroplasts gave a similar pattern of lipid formation in aging experiments, the principal lipid formed being diacyl MGDG with smaller amounts of Y and X. Free fatty acids were also formed in quite large amounts despite the addition of cysteine-HCl which Helmsing (1969) found inhibited the galactolipase from runnerbean.

Diacyl MGDG was identified using mass spectroscopy and the structure was established as 1,2-di-O-acyl-3-O (di-O-acyl-β-D--galactopyranosyl)-sn-glycerol. The position of attachment of the galactosyl acyl groups was not determined. Hydrogenated MGDG was used as a control comparison for the diacyl MGDG identification and fragmentation between the galactose and glycerol moieties occurred for both lipids. Their IR spectra were very similar, with some small differences in the fingerprint region. This is the first reported identification of diacyl MGDG from plant material. It has been shown to be present in the lipids of Bifidobacterium bifidum (Exterkate and Veerkamp, 1964). Mass spectra of galactolipids have not been reported previously and the spectrum obtained with MGDGH shows that the galactolipids are sufficiently volatile to yield useful information. Hydrogenated lipids were used because hydrogenation gave a more homogenous structure, and also pigments which were not removed by column chromatography were removed during the hydrogenation. Details of fatty acid composition are lost by hydrogenation but the simpler spectra enable identification of the basic structure of the lipid. The principle of isolating hydrogenated lipids of unknown structure for identification by mass spectrometry is a useful one, especially as this work with galactolipids and the studies

of Klein (1971a and b) with phospholipids show that polar lipids can be analysed without the blocking of active groups.

The MGDGH prepared for use as a standard for the identification of diacyl MGDG was shown to be pure by its sharp melting point and the sharp peaks in its DSC analyses. Polymorphic behaviour was also observed and explained by postulating two crystalline forms for MGDGH which transform to a liquid crystalline phase at different temperatures (95°C and 72.5°C). The transition temperature (95°C) and melting point (156°C) obtained with the DSC agree quite well with a sintering point of 91-92.5°C and a melting point of 151.5-153.5 reported by Heinz (1971). Although reported melting point data do not include a transition at 72.5°C, Wehrli and Pomeranz (1969) reported a M.P. of 60-65°C for chemically synthesized monogalactosyl distearoyl glyceride and they had probably observed this transition rather than the true melting point.

Insufficient Y was formed in 5-6 hour aged chloroplast preparations to enable its isolation and identification by mass spectrascopy. However its position relative to MGDG in TLC using the water saturated diethyl ether/isopropanol/methanol (100:4.5:3.0) solvent, is similar to that of acyl MGDG identified by Heinz (1967a). Also, Y was shown to be formed from MGDG in radiochemical experiments with chloroplasts. More diacyl MGDG was formed in the 5 hour aging experiments relative to Y than was the case in the shorter 1 hour incubations with UDPgal ^{14}C. This also suggests that Y was a precursor for diacyl MGDG. It is postulated that Y is monoacyl MGDG and thus MGDG, Y and diacyl MGDG form an homologous series as the equal spacings between these observed in TLC using C/M (10:1) would suggest is the case.

Lipid X was not identified, although it could be monoacyl DGDG as it had a similar position relative to MGDG after TLC in C/M (10:1) as did monoacyl DGDG identified by Wintermans (1969) in spinach chloroplast preparations.

The free fatty acids were identified as such by several methods outlined in Section 3.8(b).

5.3 Improved incorporation into MGDG of Red Clover chloroplasts:

In order to study the details of MGDG synthesis by Red Clover chloroplasts using UDPgal ^{14}C, higher levels of incorporation into MGDG and DGDG were required. Hence various conditions for the preparation and incubation of these chloroplasts were studied.

It was found that the highest incorporation into MGDG was obtained if the phosphate and Tris-HCl buffers used for preparation and incubation of the chloroplasts were in the range pH 7.4-7.8. The use of McIlvaine buffer for both preparation and incubation was even better, although it was tested at only one pH value (pH 7.4). This pH effect could have been due to the pH dependence of the transacylating enzymes which form X, Y and diacyl MGDG or it could have been due to the decreased binding of proteins by tannins with increased pH (Loomis and Battaile, 1966). Heinz (1967b) found that the enzyme which synthesizes acyl MGDG by transacylation from DGDG to MGDG in spinach homogenates has a pH optimum of 4.5 and its activity rapidly decreases with increasing pH. Probably both effects were operating.

Washing of chloroplasts removed peroxisomes, mitochondria and chloroplast fragments which were present in the unwashed chloroplast preparation. Washing also gave increased incorporation into the total

lipids and into MGDG. If the change in incorporation pattern obtained by washing the chloroplasts was due to the removal of transacylating enzymes in the particulate fraction of the washings, then the incubation of washed chloroplasts with the particulate fraction would have given an incorporation pattern similar to that of unwashed chloroplasts. This did not occur, although incorporation into the total lipids was increased. It appears that the enzymes were removed in the supernatant fraction of the washings and were not constituents of contaminating organelles in the unwashed chloroplasts. The most likely explanation of the effects of washing is that the resuspension of the chloroplast pellet disrupted more chloroplasts allowing increased contact between UDPgal^{14}C and the enzyme which synthesises the galactolipids. The transacylating enzymes which are removed by the washing must be relatively soluble and easily accessible to solvent (e.g. located in the stroma).

Wintermans et al. (1969) found that chloroplasts isolated in sucrose-containing media had high galactolipid transacylating activity whereas chloroplasts isolated in sodium chloride containing media did not. NaCl removes stromal protein and so the transacylating enzymes were thought to be located in the stroma. In the present study no large differences between these two media were observed for incubations of Red Clover chloroplasts with UDPgal^{14}C.

It can be concluded that the best conditions for incorporation from UDPgal^{14}C into Red Clover chloroplasts are to use washed chloroplasts prepared with McIlvaine buffer in the pH range 7.4 to 7.8.

5.4 The nature of the galactolipid transacylating enzymes:

Monoacyl MGDG, diacyl MGDG and a small amount of monoacyl DGDG were formed in the aging experiments at the expense of MGDG and DGDG. It appears that the synthesis of these acylated derivatives by Red Clover chloroplasts is similar to the synthesis of monoacyl MGDG in spinach leaf homogenates (Heinz, 1967b). That is, monoacyl MGDG is formed by transfer of an acyl group from DGDG to MGDG and diacyl MGDG is formed by a further transacylation from DGDG to monoacyl MGDG. The fatty acid composition of the diacyl MGDG was consistent with such a synthesis, as it was intermediate between the fatty acid compositions of MGDG and DGDG. Further experiments are needed to conclusively establish this point. As insufficient monoacyl MGDG was formed in the aging experiments to purify and make a fatty acid analysis, a similar comparison for this lipid could not be made. However the radioactive monoacyl MGDG from spinach did have a radioactive fatty acid composition intermediate between MGDG and DGDG.

The formation of free fatty acids occurred at the same time as these transacylations in the aged chloroplast preparations. The fatty acid composition of this fraction was intermediate between the fatty acid compositions of PC and MGDG. It is probable that both phospholipases and galactolipases were active during the aging. The lipase activity was removed by washing of the chloroplasts.

Wintermans *et al.* (1969) observed that galactolipase activity occurred at the same time as the transacylations. They assayed for free fatty acids using a colorimetric technique. However, in their published TLC of the lipids from chloroplasts before and after aging, there was no lipid labelled as free fatty acid. The TLC spot

corresponding to free fatty acid was shown to contain an MGDG derivative by paper chromatography of the deacylated lipid. However the authors did not exclude the possibility that free fatty acid was also present. In Heinz's (1967a) study of acyl MGDG formation in spinach homogenates, no mention of free fatty acid formation was made. However in his published TLC using the water-saturated diethyl ether/isopropanol/ methanol solvent, there is a major lipid with an R_f slightly less than that of acyl MGDG, which was probably free fatty acid.

Acylated galactolipids have not been detected in the normal lipids of plant leaf tissue. Thus the enzyme or enzymes responsible for their formation in leaf homogenates or chloroplast preparations are more likely to be modified enzymes produced by the disruption of the plant material, than they are to be normal enzymes of galactolipid metabolism in the intact plant leaf cells. There are two ways in which this could occur. Firstly the enzymes could have a function in the cell different from galactolipid synthesis, but capable of transacylating galactolipids when the tight compartmentalisation of the cell is disrupted so that they are made accessible to this abnormal substrate. Secondly, the enzymes may have a normal role in galactolipid metabolism, but disruption of the cell changes their specificity through such phenomena as local pH or polarity changes or binding reactions with tannins or quinones. The lipase of general specificity isolated from potato tubers (Galliard, 1970) was able to transfer acyl moieties from lipid to alcohols present in the incubation mixture. This provides a good example of the type of enzyme that could act on galactolipids as described above.

SUMMARY

1. Leaf slices prepared from Red Clover (Trifolium pratense) and Fescue (Festica elation) incorporated galactose ^{14}C into MGDG and DGDG but acylated MGDG and DGDG were not formed.

2. Chloroplast preparations from fescue incorporated ^{14}C galactose from UDP-^{14}C galactose into MGDG with only small amounts present in acylated MGDG. Red Clover chloroplast preparations required the presence of polyvinyl pyrrolidone a tannin complexing reagent or sodium diethyldithiocarbamate before incorporation of ^{14}C into galactolipids could be measured. There was little incorporation into MGDG and DGDG most of the radioactivity being present in three acylated galactolipids.

3. With chloroplasts from Red Clover maximum incorporation of ^{14}C from UDP^{14}C galactose into MGDG was obtained using a citrate phosphate buffer pH 7.4 during preparation, washing, and incubation of the chloroplasts.

4. The mass spectrum of pure hydrogenated MGDG isolated from Red Clover leaves is described. Intense fragment ions derived from the hydrocarbon, glycerol and galactose moieties were present. The usefulness of mass spectroscopy for identifying lipids after hydrogenation is discussed.

5. One of the acylated galactolipids formed by the chloroplasts was hydrogenated and identified using mass spectroscopy. It was found to be consistent with the structure:

   1,2-di-O-acyl-3-O (di-O-acyl-β-D-galactopyranosyl)-
   -sn-glycerol.

   This lipid has not been previously identified in plant homogenates.

6. The other two acylated derivatives were tentatively identified as monoacyl MGDG and monoacyl DGDG. The significance of the

formation of the three acylated galactolipids by transacylation reactions is discussed.

7. DSC analysis of hydrogenated MGDG showed that two crystalline forms of this lipid exist, each having different temperatures of transition to a liquid crystalline phase. This polymorphic behaviour can explain some differing melting point data reported in the literature.

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