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THE BIOSYNTHESIS OF GALACTOLIPIDS

IN

CHLOROPLAST ENVELOPES

A Thesis Presented as Partial Fulfillment for
the Degree of Master of Science in Biochemistry

by

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ABSTRACT

The procedure of Douce _et al_ (1973) was employed for the isolation of envelopes from purified chloroplasts of spinach (_Spinacia oleracea_) and maize (_Zea maize_ var. Wis. 235). Maize chloroplasts gave very low yields of envelope protein and low incorporation of radioactivity from UDP-^{14}C-Galactose into galactolipids. However the use of spinach chloroplasts resulted in higher yields of envelope protein and high levels of a galactosyltransferase that synthesised galactolipids from endogenous lipid substrates and added UDP-^{14}C-Galactose. The products of galactosyltransferase were identified as MGDG and DGDG by comparison with standard lipids on thin layer chromatography. The procedure for the isolation of chloroplast envelopes reported by Poincelot and Day (1973) gave a higher yield of less contaminated envelope membranes and an increased specific activity of galactosyltransferase compared to the results obtained using envelopes isolated by the method of Douce _et al_ (1973)

Total incorporation of radioactivity from 0.3 µM UDP-^{14}C-Galactose by galactosyltransferase was dependent on the time and temperature of incubation and the nature of the incubation buffer. Maximum incorporation (about 72% of the added radioactivity) was obtained upon incubation at 30°C for 30 min, in 50 mM HEPES-NaOH at pH 8.0. MGDG was identified as the major labelled lipid (MGDG:DGDG ratio 1.7:1). Lower pH values gave higher incorporation into DGDG.

A cation dependence of galactosyltransferase was observed and incorporation was stimulated by addition of Ca^{2+}, Mg^{2+} or Ba^{2+}. Maximum incorporation was obtained with 5 mM Ba^{2+}. In contrast 5 mM Cu^{2+} completely inhibited incorporation.

The sulphydryl nature of the chloroplast galactosyltransferase (Chang, 1970; Mudd _et al_ 1971) was confirmed with galactosyltransferase of the chloroplast envelope.

Linoleic acid at 0.72 µM completely inhibited transferase activity. The inhibition by linoleate could be partially removed by addition of about 10 mM Ca^{2+} or Ba^{2+} but 10 mM Mg^{2+} and BSA (30 µg per ml) were without effect.

UMP, UDP and UTP at 1 mM inhibited incorporation by transferase. UDP was the most effective inhibitor and gave 50% inhibition of incorporation at about 5 µM. NADH and PP_i did not significantly affect incorporation.

The addition of exogenous diacylglycerol (1-palmitoyl, 2-oleoyl glycerol or 1, 2-di-linoleoyl glyerol) did not increase the incorporation of radioactive galactose into galactolipids. Incorporation was inhibited by 0.3% Triton X-100 and 6 mM sodium cholate. No radioactivity from added ^{14}C-diacylglycerol was incorporated into MGDG by chloroplast envelopes.

Preincubation of the chloroplast envelopes with phospolipase C or D reduced the total amount of radioactivity incorporated by galactosyltransferase. Transferase activity was detectable after preincubation of the envelopes with trypsin and protease.

The fatty acid composition of MGDG, DGDG and DG from whole tissue, chloroplasts and chloroplast envelopes of spinach is presented. The characteristic highly unsaturated nature of the fatty acids of MGDG and DGDG is in contrast to the relatively saturated fatty acid content of DG isolated from whole tissue and chloroplasts. However, DG isolated from chloroplast envelopes contained predominantly 16:0, 18:1 and 18:3.

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LIST OF ABBREVIATIONS

ATP	adenosine-5'-triphosphate
Tris	tris(hydroxymethyl)aminomethane
BSA	bovine serum albumin
cm	centimetre
CoA and acylCoA	coenzyme A and its acyl derivative
cpm	counts per minute
C_i	curie (3.7×10^{10} s^{-1})
^oC	degrees celsius
DG	1,2-diacylglycerol (diglyceride)
DEGS	di-ethylene glycol succinate
DGDG	digalactosyldiacylglycerol
EDTA	ethylenediaminetetraacetic acid
E	extinction ($\log \frac{I_o}{I}$)
GLC	gas-liquid chromatography
G-3-P	<u>sn</u>-glycerol-3-phosphate
g	gram
x g	x gravitational force
h	hour
l	litre
K_m	Michaelis constant
µg	microgram (10^{-6} g)
p (prefix)	micromicro (10^{-12} x)
µmole	micromole (10^{-6} M)
n (prefix)	millimicro (10^{-9} x)
M	molar (moles per litre)
mM	millimolar (millimoles per litre)
min	minute
MGDG	monogalactosyldiacylglycerol
NADH	nicotinamide-adenine dinucleotide, reduced
P_i	orthophosphate (inorganic)
PP_i	pyrophosphate (inorganic)
%	per cent
PC	phosphatidylcholine
PE	phosphatidylethanolamine
PG	phosphatidylglycerol
PI	phosphatidylinositol
rpm	revolutions per minute
s	second
<u>sn</u>	stereospecific numbering
[illegible]	[illegible]

TLC	thin layer chromatography
PA	phosphatidic acid
lyso-PA	lyso-phosphatidic acid
TTGDG	tetragalactosyldiacylglycerol
TGDG	trigalactosyldiacylglycerol
UMP	uridine-5'-phosphate
UDP	uridine-5'-pyrophosphate
UTP	uridine-5'-triphosphate
UDP-^{14}C-Galactose	uridine-5'-diphosphate-D-U-^{14}C-Galactose
UDP-Galactose	uridine-5'-diphosphate-D-galactose
vol.	volume
wt.	weight
dpm	disintegrations per minute

Chapter 1: REVIEW OF LITERATURE

1.1 Introduction

1.1.1 Structure and occurrence of galactolipids

Galactolipids are abundant in all photosynthetic tissues and photosynthetic organisms. Non-photosynthetic plant tissues, micro-organisms and vertebrate spinal tissue contain smaller amounts of these lipids.

The predominant galactolipids in higher plants are monogalactosyldiacylglycerol (MGDG) (1,2-di-O-acyl, 3-O-(β-D-galactopyranosyl)-sn-glycerol) and digalactosyldiacyldiglycerol (DGDG) (1,2-di-O-acyl, 3--O-(β-D-galactopyranosyl-(1' 6')-O-α-D-galactopyranosyl)-sn-glycerol), (Figure 1). Webster and Chang (1969) reported that spinach chloroplasts had a molar ratio of MGDG:DGDG: Trigalactosyldiglycerol (TGDG):Tetragalactosyldiglycerol (TTGDG) of 60:30:5:1. More recently Joyard and Douce (1976 b) found that in envelopes from spinach chloroplasts the molar ratio of these lipids was 30:38:6:1.

The more complex glycolipids e.g. sterol and acyl sterol glycosides (Eichenberger and Newman, 1968), glucocerebrosides (Carter and Koob, 1969 ; Weber, 1970) and polygalactolipids (Webster and Chang, 1969 ; Galliard, 1969) are found in plant tissue as well, but generally account for less than 10 % of the total galactolipid fraction. (Table 1)

The present discussion will be confined to galactosyldiacylglycerol from higher plants.

TABLE 1

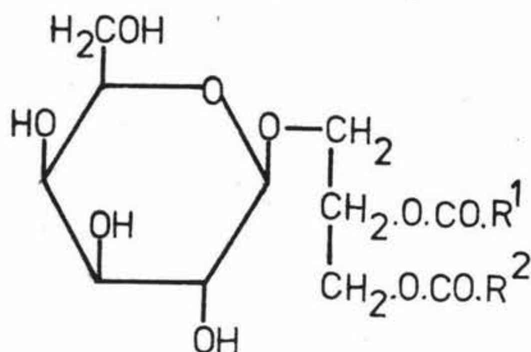
The lipid composition of photosynthetic tissue from spinach (% of total lipids by wt.)

Phospholipids	23	Sterols	1
MGDG	20	Sterol glycosides	0.5
DGDG	13	Chlorophylls	13
Sulpholipid	4	Ceryl alcohol	9
Waxes	9	other lipids	4

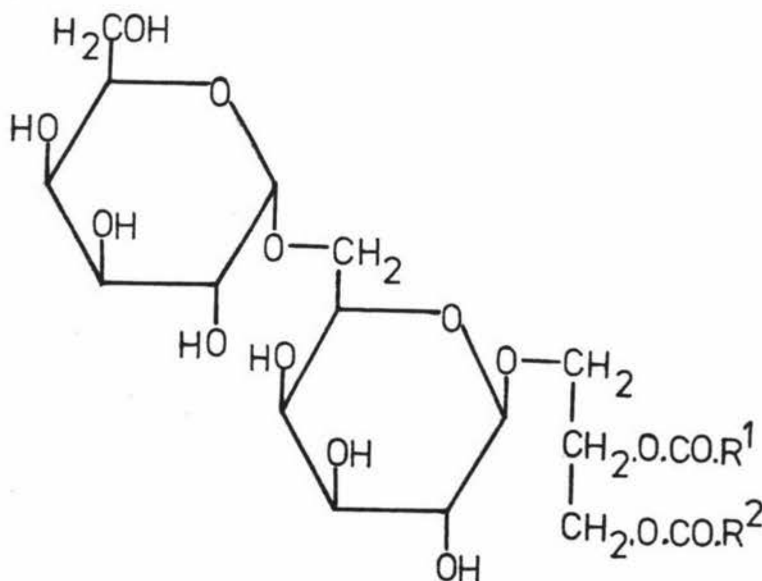
(Data from Kates, 1970)

FIGURE 1

The structure of monogalactosyldiacylglycerol (MGDG) and digalactosyldiacylglycerol (DGDG).



MGDG



DGDG

1.1.2 The cellular distribution of galactolipids

The lipid content of leaf tissue from higher plants ranges between 3 % and 10 % of the dry weight (Hawke, 1971). Galactolipids may account for up to 50 % of the total leaf lipid and up to 90 % of the total galactolipid may be in the chloroplast (Wintermans, 1960; Ongun, Thomson and Mudd, 1968). Mackender and Leech (1974) reported that lamellae and envelopes from *Vicia faba* L. contained 45 % and 30 % respectively of the total chloroplast galactolipid. Similarly Joyard and Douce (1976 b) Hashimoto and Murakami (1975) and Poincelot (1973) found high levels of galactolipid in the lamellae and envelope fractions from spinach chloroplasts. The reported MGDG:DGDG ratio in lamellae ranges from 1.4 - 2:1 and in envelopes from 0.2 - 1:1. The envelope fraction contains a relatively high concentration of DGDG and a much higher level of phospholipid, particularly phosphatidylcholine, than the lamellae fraction (Poincelot, 1973 ; Mackender and Leech, 1974), (Table 2).

The quantitative importance of galactolipid is clear but the specific function of these lipids in photosynthetic tissue is still a subject of controversy. Bishop *et al* (1971) reported that the galactolipid:chlorophyll ratio was about 3 x higher in the granal bundle sheath chloroplasts from maize and sorghum than in the agranal mesophyll chloroplasts from the same tissue. Other workers have noticed a close relationship between the increasing levels of chlorophyll and galactolipid during development of the chloroplasts (Leech *et al* 1973 ; Gray *et al* 1967 ; Fong and Heath, 1977).

The amphipathic nature of galactolipids with both hydrophobic and hydrophilic regions makes them ideal for stabilizing membrane structures. Bamberger and Park (1966) concluded from electron microscopy and galactolipase digestion that much of the lipid in the lamellae is providing a matrix upon which are arranged the proteins of the photosynthetic system.

Table 2

The lipid composition of fractions isolated from chloroplasts of <u>Vicia faba</u> and <u>Spinacia oleracea</u>

(% of total lipid)

	Envelopes[a]	Envelopes[b]	Lamellae[b]	Envelopes[c]	Lamellae[c]	Microsomes[d]
MGDG	29.1	8.4	39.1	22	51	12
DGDG	32.4	29.3	20.1	32	26	8
Sulpholipid	-	5.5	7.3	5	7.1	2
Phosphatidylglycerol	0.9	13.2	16.5	8.4	9.1	3
Phosphatidylcholine	29.6	27.5	10.1	27	3.2	35
Phosphatidylethanolamine	0	+	-	trace	0	30
Phosphatidylinositol	-	-	-	1.3	1.4	7

Data from: (a) Mackender and Leech, (1974) (<u>Vicia faba</u>.)

(b) Hashimoto and Murakami, (1975) (<u>Spinacia oleracea</u>)

(c) Douce <u>et al</u> (1973) (<u>Spinacia oleracea</u>)

(d) Douce (1974) (<u>Spinacia oleracea</u>)

1.1.3 The fatty acid composition of galactolipids

In general, plant lipids and particularly the lipids of photosynthetic tissue contain a high amount of polyunsaturated fatty acids. The major fatty acids of the chloroplast galactolipids are the unsaturated fatty acids linoleic acid (cis-9, cis-12-Octadecadienoic) (18:2), α-Linolenic acid (cis-9, cis-12, cis-15-Octadecatrienoic) (18:3), and cis-7, cis-10, cis-13 -Hexadecatrienoic (16:3); 18:3 in some tissues (e.g. alfalfa, O'Brien and Benson, 1964) accounts for up to 95 % of the acyl groups of MGDG and may account for up to 93 % of the acyl groups of DGDG. The fatty acid composition of MGDG and DGDG from several different plant species is given in Table 3. The more unsaturated or shorter chain fatty acids appear to be concentrated at the 2 position of MGDG. In spinach, 92 % of the 16:3 is found at the 2 position of MGDG with only trace amounts of 16:3 in DGDG. DGDG contains most of the small amounts of saturated fatty acids that are found in galactolipid from spinach.

The fatty acid composition of leaf tissue changes in relation to the age and light environment of the plant. Etiolated tissue contains relatively less unsaturated fatty acid than green tissue. On greening the levels of both unsaturated fatty acid and galactolipid increase (Gray et al 1967 ; Crombie, 1958). The high positional and compositional specificity of the fatty acids has led to proposals that the unsaturated fatty acids would give a high degree of flexibility in a membrane structure and thus contribute to the spacing and orientation required within the membrane for an efficient photosynthetic system and for electron transport (Benson, 1966). However, Nichols et al (1965) have reported that lipids from the Blue-green algae Anacystis nidulans contain only monoenoic fatty acids, therefore 18:3 would appear not to be a prerequisite for an active photochemical system.

Table 3

The fatty acid composition of MGDG and DGDG from whole tissue, chloroplasts and envelopes of several plant species.

(% of total fatty acid)

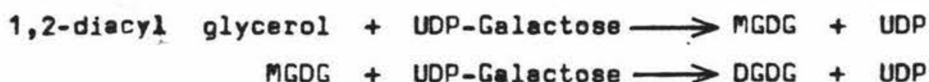
Fatty acid	*Brassica oleracea*[a]		*Medicago sativa* (alfalfa)[b]		*Trifolium pratense*[c]		*Spinacia oleracea*[d]				*Vicia faba*[e]			
							(lamellae)		(envelopes)		(lamellae)		(envelopes)	
	MGDG	DGDG	MGDG	DGDG	MGDG	DGDG	MGDG	DGDG	MGDG	DGDG	MGDG	DGDG	MGDG	DGDG
16:0	-	12	3	14	17	9	trace	4.6	trace	18.8	1.1	4.2	10.3	8.9
16:1	-	-	-	trace	trace	1	-	-	-	-	0	0.4	2.0	0.7
16:2	2	-	-	-	-	-	-	-	-	-	-	-	-	-
16:3	32	1	-	-	-	-	26	4	24.6	4	-	-	-	-
18:0	-	1	trace	0.3	1	2	-	trace	-	trace	0.5	1.8	5.6	3.4
18:1	-	trace	trace	0.4	1	2	0.6	2.5	1	5.1	1.4	2.2	10.9	2.5
18:2	2	11	2	1	6	3	1	0.8	0.6	3.2	2.9	1.7	8.9	3.4
18:3	65	75	95	82	75	83	72	87	73.6	68.6	93.8	89.7	62.1	80.9

Data from: (a) Auling *et al* (1971)
(b) O'Brien and Benson, (1964)
(c) Weenink, (1964)
(d) Douce *et al* (1973)
(e) Mackender and Leech, (1974)

1.2 Biosynthesis of galactolipids

1.2.1 Outline of the biosynthetic pathways

The work of Ferrari and Benson (1961) showed that $^{14}CO_2$ could be incorporated into MGDG and DGDG of Chlorella. MGDG was labelled before DGDG and with increasing time of incubation the percentage of radioactivity in DGDG increased and the percentage in MGDG decreased. Consequently the step-wise synthesis of MGDG and DGDG was proposed.



Neufeld and Hall (1964) tested this scheme by incubating a chloroplast preparation from spinach with UDP-^{14}C-Galactose. The results indicated that ^{14}C-Galactose was incorporated into MGDG and DGDG with significant amounts in the TGDG and TTGDG fractions. Ongun and Mudd (1968) with an acetone powder preparation from spinach chloroplasts, demonstrated that added unsaturated diacylglycerol could stimulate incorporation into MGDG.

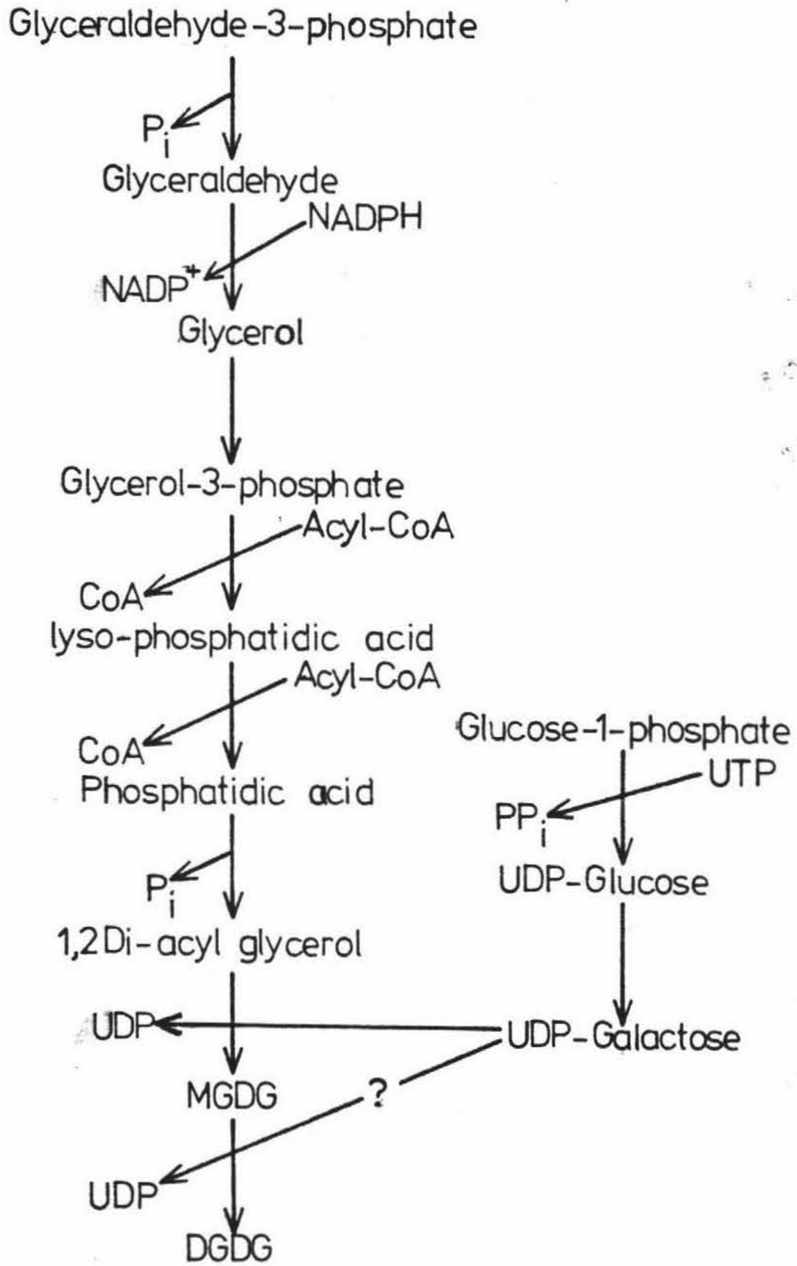
The acyl groups of MGDG appear to be synthesised from acetate in the presence of ATP and CoASH along the established pathways of fatty acid biosynthesis. (Roughan et al 1976)

Recent work by Hippman and Heinz (1976) on the synthesis of glycerol-3-phosphate (G-3-P) and Koings and Heinz (1974) on the precursors for UDP-Galactose has suggested the pathway for galactolipid synthesis given in Figure 2.

Douce and Guillot-Salomon (1970) showed that ^{14}C-G-3-P in the presence of ATP and CoASH was untilised for the synthesis of phosphatidic acid (PA), lyso-phosphatidic acid (lyso-PA) and diacylglycerol (DG). More recently, Joyard and Douce (1977) have identified a membrane bound and a soluble acyltransferase from spinach chloroplasts. These enzymes are active in the synthesis of PA, lyso-PA and DG from G-3-P and the acylCoA esters of palmitic and oleic acid. The lyso-PA product of the soluble acyltransferase is used by the envelope bound transferase to synthesise PA. These authors detected a phosphatidic acid

FIGURE 2

The proposed pathways for the biosynthesis of galactosyldiacylglycerol.



phosphatase in the chloroplasts which could provide endogenous diacylglycerol from PA. In the presence of UDP-Galactose the radioactivity as ^{14}C-glycerol in lyso-PA, PA and DG was found in MGDG.

It would appear that the complete sequence of reactions for the synthesis of MGDG from fatty acids, glycerol-3-phosphate and UDP-Galactose can occur on and in the chloroplast envelope. Free fatty acids from the stroma side are activated by a membrane bound thiokinase (Roughan and Slack, 1977) and are combined with G-3-P by the acyltransferases to form PA. The phosphatase cleaves the ortho-phosphate group of PA and the galactosyltransferase catalyses the transfer of the galactose moiety from UDP-Galactose on to the 3 position of the DG. The specificity of the enzymes in the sequence of reactions as to the fatty acid composition of the lipids is unknown.

Recent work by Van Besouw and Wintermans (1978) has suggested that the synthesis of DGDG requires a galactose carrier other than UDP. The proposed reaction is:

MGDG + MGDG ⟶ DGDG + DG

Evidence for an intermediate carrier molecule in DGDG synthesis had been presented by Lin and Chang (1971) working with Euglena.

1.2.2 Desaturation of the fatty acids

The acyl groups involved in the synthesis of PA by the chloroplast acyltransferase are highly saturated. In contrast the fatty acids in MGDG are highly unsaturated. Therefore the desaturation of these acyl groups must occur after the initial synthesis of PA and before the deposition of the galactolipid in the membrane. The site of desaturation and the nature of the carrier molecule for the fatty acid during desaturation are unknown. Desaturation of the fatty acid as a thiol ester (Vijay and Stumpf, 1971 working with safflower seeds) or as an oxygen ester bound to a lipid carrier (Slack, Roughan and Terpstra, 1976 working with a pea leaf homogenate) has been proposed. The work in this field has proved to be difficult and the variations in

results from different plant species employed by different groups of workers has further complicated this problem.

Williams _et al_ (1976) suggested that the unsaturated diacylglycerol utilised for synthesis of MGDG was derived from a pool of desaturated phosphatidyl choline (PC) by removal of the polar groups at the 3 position. PC has been shown to contain high levels of 18:2 (Roughan, 1975), however, the predominant fatty acid in MGDG is 18:3.

A further desaturation scheme involving the fatty acids on MGDG or phosphatidic acid has been proposed by Appleby _et al_ (1971). Support for this model has come from recent work with _Anthriscus_ (Heinz, 1977). The two schemes for desaturation involving PC in the microscomal fraction and MGDG in the thylakoid and envelope fraction may operate in parallel (Heinz, 1977).

1.2.3 The nature of the diacylglycerol as substrate for the synthesis of MGDG.

Mudd _et al_ (1969) working with an acetone powder of spinach chloroplasts demonstrated that diacylglycerol containing polyunsaturated fatty acids gave the greatest stimulation of incorporation from UDP-^{14}C-Galactose into MGDG. Eccleshall and Hawke (1971) with a similar enzyme preparation showed that a mixture of MGDG species containing different fatty acids were labelled with ^{14}C-Galactose suggesting that galactosyltransferase was not specific for the fatty acid composition of the diacylglycerol.

Bowden and Williams (1973) using an acetone powder of maize chloroplasts showed that exogenous diolein but not dipalmitin or triolein could stimulate incorporation of radioactivity from UDP-^{14}C-Galactose into MGDG. However, incorporation of radioactivity from ^{14}C-diacylglycerol into MGDG has not been demonstrated and consequently it is not clear whether the added diacylglycerol is stimulating incorporation by acting as a substrate for galactosyltransferase although Mudd _et al_ (1969) showed that diacylglycerol methylated at the 3 position could not stimulate incorporation.

Joyard and Douce (1976c) commented that diacylglycerol isolated from envelopes of spinach chloroplasts contained large amounts of the unsaturated fatty acids 18:3 and 16:3 but they did not give the supporting data on the composition of this lipid. They showed that upon addition of UDP-Galactose the large endogenous pool of diacylglycerol in the envelopes (diacylglycerol accounted for about 15 % of the total envelope lipid) was rapidly converted to about equal amounts of MGDG, DGDG, TGDG and a smaller amount of TTGDG. The significance of the high level of incorporation into TGDG, a lipid generally found at very low concentrations in the chloroplast, is not clear.

McKee and Hawke (pers. comm.) found that in the presence of CoASH, ATP, glycerol-3-phosphate and ^{14}C-acetate, spinach chloroplasts synthesised diacylglycerol containing a high proportion of the fatty acids 18:1 and 16:0. The addition of UDP-Galactose resulted in these fatty acids appearing in MGDG. Therefore it would appear that galactosyltransferase does not specifically require highly unsaturated diacylglycerol.

1.2.4 Synthesis of UDP-Galactose

UDP-Galactose is possibly synthesised from glucose-1-phosphate and UTP by the cytoplasmic enzymes UDP-glucose pyrophosphorylase and UDP-glucose epimerase (Konigs and Heinz, 1974). The inner membrane of the chloroplast envelope is impermeable to UDP-Galactose and therefore synthesis of the component outside the chloroplast may pose a problem of transport (Heber, 1974). However, the membrane may not constitute a barrier if the envelope bound galactosyltransferase may accept UDP-Galactose from the intramembranal space. The observation that radioactivity from UDP-^{14}C-Galactose was incorporated into MGDG by a preparation of intact chloroplasts (Douce, 1974) would suggest that exogenous UDP-Galactose is available for synthesis of MGDG by the chloroplast transferase.

1.2.5 Variations in the level of galactolipid in plant tissue.

The light environment (Gray et al 1967), temperature (Kuiper, 1970) and age of tissue (Fong and Heath, 1977) generally have an effect on the amount of galactolipid found in plant tissue.

Hawke et al (1974) found a diversity of fatty acid composition between successive sections from the base to the tip of developing maize plants. The younger, basal tissue synthesised less unsaturated fatty acids than the older tissue. Fong and Heath (1977) observed a close relationship between the increasing levels of chlorophyll, galactolipid and phospholipid in Phaseolus vulgaris during leaf development.

The effect of the environmental temperature on alfalfa leaves from cold hardy and cold sensitive varieties was studied by Kuiper (1970). The MGDG and DGDG content was found to be inversely related to the temperature in the range 15 to 30 °C. Temperature dependent changes were observed in the fatty acid composition of MGDG but generally only in the level of 18:3 (18:3 accounted for 57 % of the fatty acids of MGDG at 30 °C and 88 % at 15 °C). The cold hardy plants had a relatively higher galactolipid content than the cold sensitive plants.

Recently, Tremolieres et al (1978) reported that low temperatures enhanced the synthesis of unsaturated fatty acids from ^{14}C-acetate in a winter variety of rape seed.

The nature of the chemical controls that mediate between the conditions of the environment (light, temperature) and the enzymes of the biosynthesis of galactolipids are unknown.

1.2.6 Galactolipases and galactosidases of plant tissue

Galactolipases have been identified in an extensive variety of plant species including spinach (Wintermans et al 1969) and Phaseolus (Sastry and Kates, 1964). The products of the galactolipases are the mono- and digalactosylglycerols. Galactosidases which remove the galactose moiety from the glycerol have been detected (Marbach et al 1978, in Pisium sativium).

The importance of these enzymes lies in their activity during isolation and analysis of plant tissue and during assay of the enzymes for the synthesis of galactolipids. Galactolipases and galactosidases may provide endogenous compounds that are not normally present in the in vivo system. The effect of these enzymes may be most important during the prolonged time of preparation that is required for isolation of envelopes. However, at the pH of the buffers used in the present study (pH 8.0) these enzymes show a greatly reduced activity.

1.3 The chloroplast envelope

1.3.1 Isolation and composition of the chloroplast envelope

Mackender and Leech (1970) reported the purification of chloroplast envelopes from Vicia faba L. by sucrose gradient centrifugation of disrupted chloroplasts. The isolation of envelopes from spinach chloroplasts (Douce et al 1973 ; Poincelot, 1973) and from bundle sheath chloroplasts of maize (Poincelot, 1977) has been reported.

The purification of envelopes generally involves the osmotic bursting of a preparation of chloroplasts and then centrifugation of the suspension on a discontinuous sucrose gradient to isolate the envelopes from the remaining chloroplast fractions. The techniques for isolation of envelopes used by different workers generally differ in the procedures employed for the osmotic bursting and gradient centrifugation.

Poincelot and Day (1974) reported a method for the isolation of envelopes from spinach chloroplasts that gave a higher yield of less contaminated membranes, a higher proportion of double membrane envelopes and a consequent 10 fold increase in the specific activity of the non-latent Mg^{2+}-dependent ATPase over the values obtained with previous procedures. Sabnis et al (1970) had previously demonstrated the presence of a Mg^{2+}-dependent ATPase between the two envelope membranes of chloroplasts of pea tendril, and consequently this enzyme may be used as a marker for the double membrane envelope. Low levels of the stromal enzyme, ribulose-1,6-diphosphate carboxylase, were detected in envelopes from spinach chloroplasts indicating less than 1 % contamination by stromal material, and the microsomal NAD(P)H:Cyt. C oxidoreductase was not detectable in the envelope preparation. The envelope fraction consisted of two distinct sub-fractions with bouyant densities on a sucrose gradient of 1.08 g cm^{-3} and 1.11 g cm^{-3}. The lighter and the heavier sub-fraction consisted of primarily single and double membrane envelopes respectively. The double membrane fraction had a lipid:protein (mg lipid: mg protein) of 1.74:1.

The lipid composition of the envelope fraction is given in Table 4 The chloroplast envelope has a relatively high DGDG and phospholipid

content when compared with lamellae (Mackender and Leech, 1974 ; Hashimoto and Murakami, 1975 ; Joyard and Douce, 1976b) and is particularly rich in phosphatidylcholine.

TABLE 4

The lipid composition of envelopes and lamellae isolated from spinach chloroplasts.

(% of total lipid)

	Envelope	Lamellae
Monogalactosyldiacylglycerol	32.8	22
Digalactosyldiacylglycerol	33.6	15
Sulpholipid	0.8	7
Trigalactosyldiacylglycerol	1.5	-
Cerebroside	2.7	0
Sterylglucoside	1.1	0
Acylated sterylglucoside	0.8	0
Phosphatidylcholine	9.3	2.9
Phosphatidylglycerol	2.9	7.8
Phosphatidylinositol	1.1	1.3
Phosphatidylethanolamine	1.4	0
lyso-phosphatidylcholine	0.1	-
Sterol	1.9	0
Steryl ester	1.8	0
Chlorophyll	0.8	0
Carotenoid	+	3
Neutral lipids	+	3

(Data from Poincelot, 1973)

The proteins of the chloroplast envelope have been analysed by polyacrylamide gel electrophoresis in sodium dodecylsulphate (Mendiola-Morgenthaler and Morgenthaler, 1974 ; Pineau and Douce, 1974, Joy and Ellis, 1975 ; Sprey and Laetsch, 1975). The polypeptide patterns from the envelope and lamellae fractions are markedly different although several proteins appear to be common to both fractions. The envelope fraction contains about 20 detectable polypeptides with molecular weights ranging between 15,000 and

110,000 daltons. It is possible that these proteins contain lipid and carbohydrate and this may introduce considerable error into estimations of the molecular weight of the polypeptides.

The distinct difference in the protein and lipid composition of the envelopes and the lamellae possibly reflects, to some degree, the different functions required in these fractions, i.e. the involvement of the lamellae in the photosynthetic process and of the envelope as a semipermeable membrane in CO_2 assimilation and metabolite transport.

1.3.2 Properties and possible role of the chloroplast envelope

The envelope, as the outer membrane of the chloroplast may be assumed to have a biochemical and structural importance in maintaining the integrity of the chloroplast. Electronmicroscopy shows the envelope as two membranes separated by an electron translucent space about 30 nm in width (Weier and Thomson, 1962).

Heldt and Sauer (1971) demonstrated the semipermeable nature of the envelope and showed that the inner of the two membranes was the site of specific metabolite transport. Electron microscopy had suggested that the stroma and grana lamellae were formed by invagination of the chloroplast envelope (Menke, 1962) and therefore the envelope was assumed to play a major role in the development and differentiation of the chloroplast.

The procedures for the isolation of contaminant-free membranes developed by Mackender and Leech (1971), Douce et al (1973) and Poincelot (1973), made possible further studies of the enzymes of the envelope. Douce (1974) and Van Hummel et al (1975) reported a highly active galactosyltransferase in the envelope of spinach chloroplasts that utilised added UDP-^{14}C-Galactose and endogenous diacylglycerol for synthesis of MGDG. The envelope has also been shown to be the site of synthesis of DGDG, TGDG and TTGDG (Joyard and Douce, 1976c) but the mechanism for synthesis of these polygalactolipids is still unknown (Lin and Change, 1971 ; Van Besouw and Wintermans, 1978).

Recent work has shown that the envelope contains the necessary enzymes for the synthesis of acylCoA from fatty acid and CoASH

(Roughan and Slack, 1977) and phosphatidic acid and 1,2-diacylglycerol from acylCoA and glycerol-3-phosphate (Joyerd and Douce, 1977 ; Heinz et al 1978). Thus the envelope membrane of the chloroplast is capable of the synthesis of MGDG from fatty acid, CoASH, Glycerol-3-phosphate and UDP-Galactose and as MGDG is the major lipid component of the lamellae this may be consistent with the conclusion that stroma and grana lamellae appear to be derived from the chloroplast envelope (Menke, 1962).

Several groups of workers have studied the permeability and transport properties of the envelope with respect to metabolic and catabolic intermediates such as phosphates and dicarboxylates (Heldt and Rapley, 1970) amino acids (Nobel and Cheung, 1972) and triosephosphates and adenine nucleotides (Fliege et al 1978 ; Heldt, 1969). Poincelot (1976) showed that bicarbonate transport by envelopes isolated from chloroplasts of spinach and sunflower could be directly related to the level of Mg^{2+}-dependent ATPase activity in the envelopes and he suggested that ATPase may function in the transport of metabolites across the envelope membrane.

The nature of the many possible roles of the chloroplast envelope remain to be investigated.

1.4 Membrane-bound enzymes and galactosyltransferase

1.4.1 The biochemistry of membrane-bound enzymes.

The study of membrane-bound enzymes poses several major difficulties that are not evident in the study of soluble enzymes. Possibly the most important difficulty is that of assay of the membrane-bound enzyme. A membrane may contain enzymes other than the enzyme of interest and as further purification without disruption of the membrane may be impossible the assay will not only contain a heterogenous mixture of proteins but some of these proteins may interfere with the assay. The assay may also be affected by changes in the membrane in response to pH, osmotic and temperature alterations (Bramley et al 1971). The spatial arrangement of the enzyme in the membrane may be important particularly since many membranes have limited permeability for many substrates (Lin, 1971). The particulate nature of the enzyme preparation will have an effect on the microenvironment in the assay. The possibility of variations in the level of substrate, product and pH between different regions of the membrane preparation could have a marked effect on the kinetics of the assay. Continuous agitation during assay may contribute to the maintenance of homogeneity, but in the presence of membrane vesicles there are difficulties in ensuring a completely homogenous system.

In general there is a special significance in the binding of an enzyme to a membrane, e.g. to act as a sub-unit of a multienzyme array or for vectorial movement of compounds across the membrane or to satisfy a lipid requirement, and in many cases the enzyme is active only in the presence of the membrane. Therefore any procedure which may disrupt the interaction between the membrane and the enzyme may inhibit activity (Coleman, 1973). It may be possible to remove the enzyme from the membrane without removing all of the lipid (Helenius and Simons, 1975) and thus retain an active enzyme, but the soluble enzyme may have different physical and chemical properties to the membrane-bound form (allotopy) (Racker, 1967).

The solubilization of membrane-bound enzymes is a complex and difficult process. The many techniques employed to remove enzymes

from a membrane include low osmotic strength, sonication, controlled proteolytic digestion, high ionic strength, chaotropic reagents, solvent extraction, phospholipase digestion and detergent solubilization (Coleman, 1973). Detergents have been used most extensively for the solubilization and purification of active enzymes, but a delicate balance normally exists between solubilization and inactivation of the enzyme. In general the inactivation effects are in the order non-ionic (Triton X-100) $<$ bile salts (cholate, deoxycholate) $<$ dodecylsulphate (Coleman, 1973). An inactivated enzyme may be reactivated by the readdition of the appropriate lipid or membrane components, e.g. enzymes of the electron transport system isolated from beef-heart mitochondria by precipitation with 90 % aqueous acetone may be reactivated by addition of cardiolipid, phosphatidylethanolamine or phosphatidylcholine (Brierly et al 1962).

Most of the work on membrane solubilization and reactivation by readdition of lipid has been done with animal and bacterial systems.

1.4.2 Galactosyltransferase of the chloroplast envelope

It has been well established that the envelope of chloroplasts contains galactosyltransferase active in the synthesis of galactolipid from diacylglycerol and UDP-Galactose (Douce, 1974 ; Van Hummel et al 1975 ; Joyard and Douce, 1976b, 1976c). A soluble transferase from spinach chloroplasts was reported by Chang and Kulkarni (1970). The chloroplasts were disrupted by sonication and the supernatant obtained from centrifugation at 30,000 x g for 30 min was reported to contain transferase activity, however, centrifugation at 30,000 x g does not sediment all the chloroplast envelopes from a suspension of disrupted chloroplasts and it is possible that the activity observed by these workers may have been derived from the chloroplast envelope. Nevertheless, Mudd et al (1969) demonstrated that after centrifugation of a spinach leaf homogenate at 100,000 x g for 1 h, as much as 40 % of the transferase activity remained in the supernatant.

The pH and temperature optima and the diacylglycerol specificity

of galactosyltransferase in an acetone powder of spinach chloroplasts was examined by Mudd <u>et al</u> (1969). They showed that the major radioactive product from incubation with UDP-^{14}C-Galactose at pH 7.2 was MGDG. Maximum incorporation (31 % of the added radioactivity) was obtained at an incubation temperature of 45 ^{o}C and incubation at 60 ^{o}C for 20 min gave about 11 % incorporation. The incorporation of radioactivity from UDP-^{14}C-Galactose showed a marked dependence on the incubation buffer and pH of incubation. Lower pH values increased incorporation into DGDG and higher pH favoured the synthesis of MGDG. Incorporation of radioactivity by the acetone powder preparation was dependent on a supply of exogenous diacylglycerol and added polyunsaturated diacylglycerol was shown to be more effective in stimulating incorporation into MGDG than saturated diacylglycerol. (see Section 1.2.3)

The reported K_m values for UDP-Galactose in the synthesis of MGDG range between 2.22 x 10^{-4} and 375 x 10^{-4} mM (Ongun and Mudd, 1968 ; Chang and Kulkarni, 1970 ; Siebertz and Heinz, 1977 ; Van Besouw and Wintermans, 1978). However, the determination of these K_m values was made in the presence of one substrate, UDP-Galactose, and the pool of endogenous diacylglycerol was relied upon for the supply of the other substrate (diacylglycerol). The reported specific activities of the transferase of chloroplast envelopes from several plant species range from 3 - 45,000 pmoles min^{-1} mg $protein^{-1}$ (Heinz, 1977). The inherent problems of the membrane bound nature of galactosyltransferase and the possibilities for wide variations in the level and availability of the endogenous diacylglycerol to transferase may explain the observed range of K_m and specific activity values. Furthermore the wide variations in UDP-Galactose concentration the methods for the isolation of envelopes and the incubation buffer and assay systems with different plant species may have an effect on the observed activity of galactosyltransferase. (see Table 5)

Chang and Kulkarni (1970) observed no cation requirement for the synthesis of MGDG from UDP-^{14}C-Galactose by a relatively crude preparation of transferase from spinach chloroplasts. Van Besouw and Wintermans (1978) found that 10 mM Mg^{2+} was required in the

TABLE 5

Characteristics of galactosyltransferase from spinach chloroplasts

	Major Radioactively labelled Product	K_m UDP-Galactose (mM) MGDG	DGDG	pH optimum	UDP-^{14}C-Galactose concentration	specific activity
spinach chloroplast homogenate a)	MGDG	2.22×10^{-4}	4×10^{-4}	7.4	0.6 µM	242 pmoles h^{-1} mg^{-1}
envelopes of spinach chloroplasts b)	MGDG/ DGDG	3.75×10^{-2}	1.25	MGDG- 7.5 DGDG- 6.5	0.5 mM	MGDG- 2.7 µmoles h^{-1} mg^{-1} DGDG- 1.5 µmoles h^{-1} mg^{-1}
acetone powder of spinach chloroplasts c)	MGDG	-	-	MGDG - 7.5 DGDG - 5.75	0.1 µM	825 pmoles h^{-1} mg^{-1}

Data from: a) Chang and Kulkarni, (1970)

b) Van Besouw and Wintermans, (1978)

c) Mudd et al (1969)

incubation to give maximum incorporation from UDP-^{14}C-Galactose by envelopesof spinach chloroplasts.

A complication in the study of galactosyltransferase of envelopes is the possibility that there may be a number of different enzymes, each catalysing the synthesis of one of the galactolipids, MGDG, DGDG, TGDG, or TTGDG. Evidence for the existence of two separate enzymes in the synthesis of MGDG and DGDG has come from the differences in pH and temperature dependence (Mudd et al 1969 ; Chang and Kulkarni, 1970), values of K_m for UDP-Galactose (Van Besouw and Wintermans, 1978 ; Siebertz and Heinz, 1977 ; Chang and Kulkarni, 1970) and the time of incubation dependent formation of product (Williams et al 1975 ; Joyard and Douce, 1976c ; Bowden and Williams, 1973 ; Ongun and Mudd, 1968). Furthermore, the initial galactosyl transfer reaction in the synthesis of MGDG requires the formation of a β glycosidic bond and the second galactosyl transfer reaction (synthesis of DGDG) requires the formation of an α glycosidic bond.

1.4.3 Galactosyltransferase from animals and bacteria

Membrane-bound galactosyltransferases active in the synthesis of glycosphingolipids in rat kidney (Martensson et al 1974), MGDG of rat brain tissue (Wenger et al 1968), MGDG of a bacterial cell wall (Veerkamp, 1974) and glycoprotein from rat liver membranes (Fraser and Mookerjea, 1977 ; Mookerjea and Yung, 1974) have been studied and in come cases purified from the membrane.

Fraser and Mookerjea (1977) isolated transferase activity from a preparation of rat liver microsomes by treatment of the membranes with Triton X-100 which released the activity into the supernatant on centrifugation at 100,000 x g for 1 h. The enzyme was then purified to apparent homogeneity by affinity chromatography. This enzyme which catalysed the transfer of galactose from UDP-Galactose to a monosaccharide acceptor had a pH optimum of about pH 6.5 and an absolute requirement for Mn^{2+}. The K_m for UDP-Galactose was 10.8 μM.

Incubation of a particulate fraction from Bifidobacterium bifidum var. pennsylvanicus (Veerkamp, 1974) with UDP-^{14}C-Galactose resulted in radioactivity incorporated into 5 major lipids corresponding to

MGDG, DGDG, TGDG and the mono- and diacylgalactosyldiacylglycerol. The addition of dipalmitin to the incubation gave a 7 fold stimulation of incorporation. The enzyme showed a cation dependence and was stimulated by Mn^{2+}, Mg^{2+}, Co^{2+} and Fe^{2+}, but Cu^{2+} strongly inhibited activity. The optimum pH for the synthesis of MGDG was between pH 6.0 - 7.0 and the synthesis of DGDG was stimulated by lower pH (about pH 5.0). A similarity between the chloroplast transferase and the bacterial transferase with respect to the cation requirement, pH dependence and distribution of radioactivity between the galactolipids after incubation with UDP-^{14}C-Galactose is apparent.

Chapter 2: AIM OF THE PRESENT STUDY

Galactosyltransferase active in the synthesis of galactolipids from endogenous substrates and added UDP-^{14}C-Galactose was detected in an acetone powder of spinach chloroplasts (Ongun and Mudd, 1968 ; Mudd _et al_ 1969 ; Eccleshall and Hawke, 1971) and a soluble fraction from a homogenate of spinach chloroplasts (Chang and Kulkarni, 1970). Mudd _et al_ (1969) and Chang and Kulkarni (1970) had examined the pH, temperature, cation and substrate dependence of galactosyltransferase in the relatively crude preparations obtained by these workers from spinach chloroplasts. More recently Douce (1974) and Van Hummel _et al_ (1975) reported a highly active membrane-bound galactosyltransferase associated with the envelope of spinach chloroplasts. The aim of the present study was to examine the characeristics of this envelope-bound transferase with respect to the pH, temperature, cation and substrate dependence of the enzyme and with a view to solubilization of the active enzyme from the membrane.

Chapter 3: Materials and methods

3.1 Materials and analytical techniques

3.1.1 Chemicals and solvents

All chemicals used were analytical laboratory grade obtained from BDH, May and Baker or Sigma. UDP-Galactose, phospholipases, trypsin, protease, bovine serum albumin (Cohn fraction V) and Tricine and HEPES buffers were obtained from Sigma.

Solvents were all distilled and, where appropriate, dried before use.

The buffer used in the isolation of chloroplasts contained sucrose obtained from the N.Z. Sugar Company. All other sucrose solutions were prepared from A.R. grade sucrose (Koch-Light Lab.Ltd.)

Silica gel G for thin layer chromatography was obtained from Riedel-De Hagen A.G. Seelze.

3.1.2 Radioactive compounds

UDP-^{14}C-Galactose lithium or ammonium salt specific activity 322-347 mCi per mmole. U-^{14}C-hexadecane, U-^{14}C-oleic acid and U-^{14}C-linoleic acid were purchased from the Radiochemical Centre, Amersham.

3.1.3 Chromatography

a) Thin layer chromatography (TLC)

i) Preparation of Thin layer.

Glass plates 5 cm x 20 cm or 20 cm x 20 cm were spread with a slurry of silica gel G (according to Stahl) using a commercial spreader (Desaga, Heidelberg, Germany) at 0.5 mm thickness. The plates were air-dried for several minutes and then activated at 100 ^{o}C for 1 h.

ii) Solvent systems.

Solvent system	Compound isolated
Hexane:diethyl ether:glacial acetic acid 70:30:1 (by vol.)	Diacylglycerol
Toluene:ethyl acetate:95% ethanol 50:25:25 (by vol.)	Galactolipids

Chloroform:methanol:glacial acetic acid:water 85:15:10:3 (by vol.)	Phospholipids
Chloroform:methanol:glacial acetic acid:acetone:water 30:6:6:12:3 (by vol.)	Phosphatidylserine and phosphatidylinositol

iii) Detection of compounds on TLC

Lipids were detected by spraying with 2,7 dichlorofluorescein (0.2 % w/v, in ethanol) and viewing under ultraviolet light. Phospholipids were detected using the modified spray of Vaskovsky and Kostestky (1968).

b) Gas-liquid chromatography (GLC)

Methyl esters of long chain fatty acids were prepared with boron trifluoride in methanol and analysed on a Packard gas chromatograph using a colum of 12 % DEGS on Chromosorb 60-70 W at a nitrogen flow rate of 40 ml per min and a column temperature of 162 ^{o}C. The column was fitted with a flame ionization detector. Mass peaks were identified by comparing retention times with methyl esters of known fatty acids.

3.1.4 Incubation procedure

The standard incubation mixture for assay of galactosyltransferase contained: 100 μl enzyme (20-80 μg envelope protein), 50 mM HEPES-NaOH at pH 8.0, 5 mM $MgCl_2$, 0.3 M sucrose and 10 μl UDP-^{14}C-Galactose containing 0.070-0.072 nmoles UDP-Galactose (51,000-55,000 dpm) in a total reaction volume of 260 μl. The final concentration of UDP-Galactose was between 3.0×10^{-7} and 2.7×10^{-7} M. All incubations were carried out in duplicate at 30 ^{o}C and with constant shaking for 30 min unless stated otherwise. Incubations were stopped by adding sufficient chloroform-methanol (2:1 v/v) to give one phase. After standing for 15 min water was added to give two phases, the aqueous layer was removed and the chloroform layer was re-extracted twice further with water. The chloroform layer was then taken to dryness under a stream of nitrogen. The extract was redissolved in 0.5 ml of chloroform and duplicate 0.1 ml aliquots were dried in scintillation vials for determination of the total incorporation into the lipid fraction by liquid scintillation counting. The values for incorporation obtained from the duplicates generally agreed to within

15 %. All values given in all experiments are an average of the duplicates.

3.1.5 Determination of radioactivity

Packard Models 2002 and 3375 Tri-carb liquid scintillation spectrometers and a Beckman LS-350 liquid scintillation system were used for scintillation counting.

The scintillation solvent for counting ^{14}C was a 5 gl^{-1} solution of p-terphenyl in toluene. This solvent gave a counting efficiency of 40 % on a pre-set ^{14}C channel in the Beckman LS-350 scintillation counter.

A quench correction curve of scintillation solvent with a known amount of ^{14}C-hexadecane and variable amounts of a highly pigmented plant extract was prepared and counted on the Beckman LS-350 scintillation counter which was equipped with automatic external standard capability. Disintegrations per min (dpm) were calculated from the counts per min (cpm) recorded by the machine, allowing for background levels of radioactivity, by determining the counting efficiency from the quench correction curve.

The components of the lipid fraction were separated by TLC using toluene:ethyl acetate:95 % ethanol (2:1:1, by vol.) and the distribution of radioactivity was determined on a Packard radiochromatogram scanner Model 7200. Optimum conditions for scanning were: gas flow (1.3 % iso-butane in helium) 120 ml per min, high voltage 1.15 kv, time constant 10 s, slit width 2.5 mm. MGDG and DGDG standard were chromatographed to verify the nature of the labelled compounds. The areas on the thin layer corresponding to the peaks of radioactivity from the scanner were scraped into scintillation vials and counted. The efficiency of counting in the presence of silica gel was determined with lipid of known radioactivity.

3.1.6 Protein determination.

Protein was determined using the method of Lowry *et al* (1951).

Reagent A: 2% Na_2CO_3 in 0.1 M NaOH

Reagent B: 0.5 % $CuSO_4 . 5H_2O$ in 1 % Na-Tartrate

Reagent C: Mix 50 ml A with 1 ml B

Reagent D: Mix one part Folin reagent with 2 parts water.

The sample with 10-200 μg protein in 0.5 ml was added to 5.0 ml of reagent C, and mixed well. After 10 min, 0.5 ml of reagent D was added and the absorbance after 10 min read at 580 nm in a Hitachi spectrophotometer.

The Coomassie Blue method for protein determination (Bradford, 1976) was compared with the Lowry method. The two methods gave similar protein values but due to the possibility of variations in dye-protein interaction (Van Kley and Hale, 1977, Pierce and Suelter, 1977) the Coomassie Blue method was not used for routine analysis. Standard curves for the Lowry and Coomassie Blue methods were prepared with bovine serum albumin (BSA).

3.1.7 Sonication

A MSE (Measuring and Scientific Equipment Ltd.) 100 watt ultrasonic disintegrator was used in the auto mode at a power output of about 6 microns peak-to-peak. The sample was sonicated at 0-4 °C under an atmosphere of nitrogen.

3.1.8 Chlorophyll determination

Chlorophyll was determined using the method of Arnon (1949) in which the absorbance of an aqueous 80 % acetone solution at 645 nm and 663 nm was measured. The equation

$$(20.2 \times E_{645}) + (8.02 \times E_{663}) \times \frac{\text{vol. of 80 \% acetone}}{\text{sample vol.} \times 1000}$$

was used to calculate the mg chlorophyll per ml of sample.

3.1.9 Polyacrylamide gel electrophoresis

Polyacrylamide gel electrophoresis in sodium dodecyl sulphate (SDS) was carried out according to the method of Weber and Osbourn (1969). The envelope sample was incubated for 2 min at 80-100 °C in sodium phosphate buffer containing 5 % (w/v) SDS. To the sample was added 3 μl of tracking dye (0.05 % bromophenol blue in water) 5 % (v/v) β-mercaptoethanol and one crystal of sucrose.

The solution was applied to tube gels containing 7 % acrylamide, 0.33 % N,N, methylene bis-acrylamide, and run at 4 mA per gel for 4-5 h. After running the gels were removed from the tube, the dye front marked and the protein bands stained in Coomassie Blue solution (1.25 g Coomassie Blue in 454 ml 50 % aqueous methanol and 46 ml glacial acetic acid) for 2 h. The gels were destained in water:acetic acid: methanol (18:12:1, by vol.) for several days and then photometrically scanned at 583 nm on a Beckman Acta III spectrophotometer equipped with a gel scanner.

3.1.10 ATPase and FDPase assays.

Mg^{2+}-dependent adenosine triphosphatase and fructose 1,6 diphosphatase were assayed as described by Douce et al (1973).

The ATPase assay contained 0.1 - 2 mg protein and 10 mM ATP with 10 mM $MgCl_2$. After incubation at 37 °C for 20 min, 1 ml of 20 % trichloroacetic acid (TCA) was added, the solution centrifuged and the supernatant assayed for inorganic phosphorus by the method of Taussky and Shorr (1953).

The FDPase assay contained 0.25 - 2.5 mg protein, 0.5 M Tris-HCl pH 8.4, 0.1 M $MgCl_2$, 0.016 M Na_2EDTA and 4 mM fructose 1,6 diphosphate. After incubation at 30 °C for 5 min the reaction was stopped with 2 ml of 5 % TCA and inorganic phosphorus determined as described above.

3.1.11 Preparation of samples for electron microscopy and phase contrast microscopy.

The samples for electron microscopy were prepared as described by Poincelot (1973). The pellets were fixed with 4 % glutaraldehyde in 100 mM phosphate buffer pH 7.0 for 30 min at 4 °C. The glutaraldehyde was decanted and the pellets were washed three times with 5 ml volumes of buffer. The pellets were postfixed with 1 % osmium tetroxide in phosphate buffer for 30 min at 4 °C. The osmium tetroxide was removed and the pellets rewashed with three 5.0 ml changes of water. The fixed samples were dehydrated in a graded acetone series, and embedded in resin (Spurr 1969). Sections were cut, stained with uranyl acetate and lead citrate, and examined

on a Philips EM 200 electron microscope.

The samples for phase contrast microscopy were resuspended in the chloroplast isolation buffer and viewed at 1250 x magnification.

3.1.12 Tryptic, protease and phospholipase digestion

The tryptic, protease and phospholipase digestions were carried out in the standard incubation mixture in the presence of 5 mM Mg^{2+} and 20 mM Ca^{2+} for about 30 min. Transferase activity was determined after incubation with the digestive enzymes by addition of UDP-^{14}C-Galactose and incubation for 30 min at 30 °C.

3.2 Experimental procedures

3.2.1 Isolation of chloroplasts

Chloroplasts were isolated by the method of Leese et al (1971) Whole spinach plants were obtained from the local market on the day or the day after the plants were fresh stock. About 400 g of deribbed leaf tissue was used for each chloroplast preparation. The tissue was homogenized in a Waring blender using 100 g tissue to 400 ml buffer (0.5 M sucrose, 0.067 M KH_2PO_4/Na_2HPO_4 at pH 8.0, 1 mM $MgCl_2$, 0.2 % BSA) for early experiments, but in later experiments 100 g tissue to 200 ml buffer was used with no apparent reduction in the yield of intact chloroplasts. After homogenizing for 3 s and then 5 s the homogenate was filtered through 10 layers of cotton organdie and 10 layers of 25 µ nylon bolting cloth. The filtrate was centrifuged for 90 s at 4,200 rpm (3,000 x g) using a SP-X rotor in a Sorvall RC 3 centrifuge fitted with a fast brake. The resultant crude chloroplast pellet was resuspended in the 0.5 M sucrose buffer described above and layered on 20 ml of a 0.6 M sucrose solution in the same phosphate buffer. This two-zone gradient was centrifuged for 15 min at 1,100 rpm using a HL-8 swing-out bucket head. The purified chloroplast pellet was resuspended in 0.5 M sucrose in 0.067 M KH_2PO_4/Na_2HPO_4 pH 8.0, 1 mM $MgCl_2$, and recentrifuged at 4,200 rpm to remove BSA. The washed chloroplast pellet was used directly for the preparation of envelopes.

Zea mays var. Wisconsin 235 was grown at 23-25 ^{o}C for 7-10 days in constant environment rooms. The plants were harvested and the coleoptile and outer leaf removed. Chloroplasts were isolated by the procedure outlined above of Leese *et al* (1971)

3.2.2 Isolation of chloroplast envelopes

The procedure of Douce *et al* (1973) was used to isolate chloroplast envelopes.

The chloroplast pellet was suspended in 10 mM Tricine-NaOH buffer at pH 8.0 containing 2 mM $MgCl_2$. The suspension was held at 4 ^{o}C for 5 min and then homogenized by making 5 complete passes in a Ten-Broek homogenizer. The homogenized suspension was layered on a discontinuous sucrose gradient consisting of 6 ml each of 1.5 M, 1.2 M, 0.93 and 0.6 M sucrose solutions in 10 mM Tricine-NaOH at pH 8.0, 2 mM $MgCl_2$ and centrifuged for 1 h at 22,500 rpm (51,500 x g) using a SW 25.1 rotor in a Beckman Model L ultracentrifuge. The fractions from the gradient (Results 4.2.1, Figure 6) were diluted with 10 volumes of the 10 mM Tricine NaOH buffer and centrifuged for 30 min at 15,500 rpm (29,000 x g).

The resultant pellets were suspended in the 10 mM Tricine-NaOH buffer and used for protein, chlorophyll and galactosyltransferase determinations.

A modified procedure was employed for the routine preparation of chloroplast envelopes. The crude chloroplast pellet obtained, as described above, after the 90 s centrifugation was resuspended in the 0.5 M sucrose isolation buffer and recentrifuged at 4,200 rpm for 90s. This washed chloroplast pellet was used for preparation of envelopes using a 2-zone gradient consisting of 16 ml 0.88 M sucrose and 8 ml 0.63 M sucrose in 10 mM Tricine-NaOH at pH 8.0, containing 2 mM $MgCl_2$. (Results 4.2.1, Figure 7)

3.2.3 Isolation of phospholipids from spinach leaf tissue

100 g of leaf tissue was homogenized twice with 200 ml volumes of hot chloroform-methanol (2:1 v/v) in a Waring blender. The combined extracts obtained from filtration of the residue were washed three times with water after addition of chloroform to

improve separation of the aqueous and non-polar phases. The chloroform extract was dried under vacuum and redissolved in a minimum volume of chloroform. The phospholipids were isolated by preparative TLC, using chloroform:methanol:glacial acetic acid: water (85:15:10:3, by vol.) and chloroform:methanol:glacial acetic acid:acetone:water (30:6:6:12:3, by vol.)

3.2.4 Preparation and purification of diacylglycerol

1,2-Di-acylglycerides were prepared according to the method of Mattson and Volpenhein (1962). The starting materials for synthesis of 1-palmitoyl, 2-oleoyl glycerol were 1-monopalmitin and oleoyl chloride and for synthesis of 1,2-di-U-^{14}C-linoleoyl glycerol were glycerol and U-^{14}C-linoleoyl chloride. The fatty acid chlorides were prepared from oxalyl chloride and the free fatty acid as described by Mattson and Volpenhein (1962). The U-^{14}C-linoleoyl chloride was prepared by equilibration of unlabelled carrier linoleoyl chloride with U-^{14}C-linoleic acid in hexane for 2 h at room temperature (Borgstrom and Krabisch, 1963). The fatty acid chloride and glycerol or 1-monopalmitin were reacted for 4 h at room temperature.

The 1,2 and 1,3-diacylglycerol were separated from mono-glyceride, triglyceride and the unreacted free fatty acid by column chromatography on 60-100 mesh Florisil in a column 2 cm x 35 cm (Carroll, 1961). Triglyceride eluted from the column with 15 % diethyl ether in hexane and the diacylglycerol eluted with 50 % diethyl ether in hexane. Monoglyceride and free fatty acid were removed from the column with 10 % methanol in diethyl ether. The diacylglycerol fraction consisted of a mixture of 1,2 and 1,3-diacylglycerol with no visible lipid contamination as shown by TLC.

Chapter 4: RESULTS

4.1 Experiments with maize chloroplasts

4.1.1 Isolation of envelopes from maize chloroplasts

Electron micrographs of the purified chloroplast pellet showed a preparation consisting of about 60 % intact, Class I chloroplasts. A representative field of the chloroplast pellet is presented in Figure 3. Examination of the chloroplasts under a light microscope equipped with phase contrast confirmed that about 50 % - 60 % of these organelles were intact. No evidence of non-chloroplastic or bacterial contamination was visible.

The distribution of protein, chlorophyll and galactosyltransferase in the fractions isolated from disrupted chloroplasts by gradient centrifugation is given in Table 6. Fraction 4 contained about 82 % of the chlorophyll and 45 % of the protein. Most of the remaining chlorophyll (16 %) and about 30 % of the protein were concentrated in the pellet. The soluble stromal proteins in the supernatant fraction accounted for about 25 % of the total protein.

Fraction 2 had the highest total incorporation of radioactivity from UDP-^{14}C-Galactose into lipids and the highest specific activity for galactosyltransferase. In each of six separate preparations of envelopes from maize chloroplasts the incorporation by Fraction 2 was less than 2 %. A complete analysis of one preparation is given in Table 6.

The poor yields of the fraction from the discontinuous gradient resulted in insufficient sample for use in electron microcopy.

Table 6

The distribution of protein, chlorophyll and galactosyltransferase in fractions isolated by sucrose gradient centrifugation of disrupted maize chloroplasts.

Procedures: The protein and chlorophyll concentrations were determined as described in 3.1.6 and 3.1.8. The galactosyltransferase was assayed by following the incorporation of radioactivity from UDP-^{14}C-Galactose (55,000 dpm, 0.07 nmoles per incubation) into the lipid fraction (3.1.4).

Fraction	Volume (ml)	Protein (mg)	% of total protein	Chlorophyll (mg)	Protein chlorophyll ratio	Assay of galactosyltransferase Galactose incorporation		
						per fraction (dpm x 10^2)	per 100 µl (% of total)	Specific activity*
1	32.50	7.17	25.02	-	-	715	0.4	25.4
2	1.68	0.07	0.24	trace	-	75.6	0.8	275.6
3	1.20	0.36	1.26	0.11	3.27	13.2	0.2	9.4
4	3.30	12.60	44.96	3.48	3.62	39.6	0.2	0.8
pellet	3.05	8.46	29.52	0.66	12.82	91.5	0.6	2.8
sum	-	28.66	-	4.25	6.74	-	-	-
Disrupted chloroplasts		32.13	-	5.70	5.64	256.8	1.1	2.0

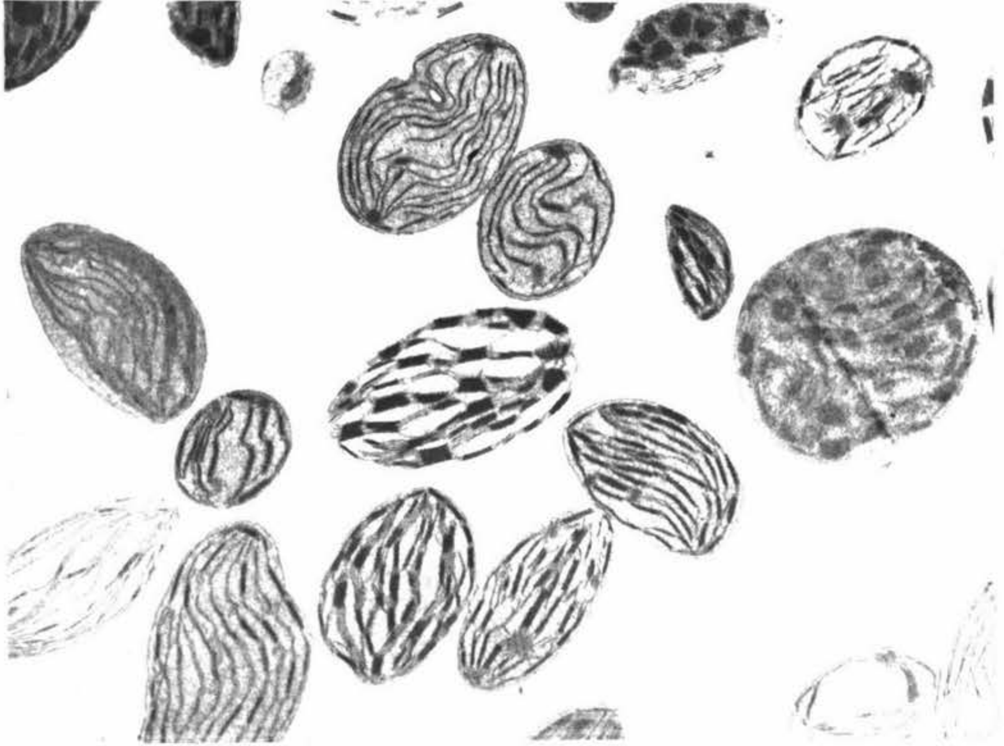
Chloroplasts were isolated from 380 g leaf tissue.

*Specific activity is measured in pmoles ^{14}C-Galactose incorporated h^{-1} mg $protein^{-1}$.

FIGURE 3

Representative field from a preparation of maize chloroplasts.

(a) 12,200 x magnification



4.2 Experiments with spinach chloroplasts

4.2.1 Isolation of envelopes from spinach chloroplasts

The chloroplast preparation consisted of 60 % - 70 % intact, Class I chloroplasts as demonstrated by electron microscopy. A representative field is presented in Figure 4 a. No evidence of non-chloroplastic or bacterial contamination was visible.

The distribution of protein, chlorophyll and galactosyltransferase in the fractions isolated by gradient centrifugation of disrupted chloroplasts is presented in Table 7. The distribution of protein and chlorophyll obtained from spinach chloroplasts is similar to that obtained from maize chloroplasts. However, spinach chloroplasts gave higher yields of protein from all fractions with a 20 fold increase in the yield from Fraction 2. The envelope pellet obtained from Fraction 2 (Douce et al 1973) was generally contaminated with trace amounts of lamellar material. Electron microscopy showed the envelope pellet consisted of membrane vesicles from 0.2 to 1.5 μ in diameter, similar in appearance to the envelope membranes observed by Mackender and Leech (1971), Poincelot (1973), Poincelot and Day (1974), Joyard and Douce (1976 a,b,c) (Figure 4 b).

The envelope pellet showed no detectable fructose 1,6 diphosphatase activity but did have Mg^{2+}-dependent ATPase activity. All fractions assayed incorporated significant amounts of radioactivity from UDP-^{14}C-Galactose into lipids. Fraction 2 had the highest specific activity and incorporated up to 60 % of the added radioactivity.

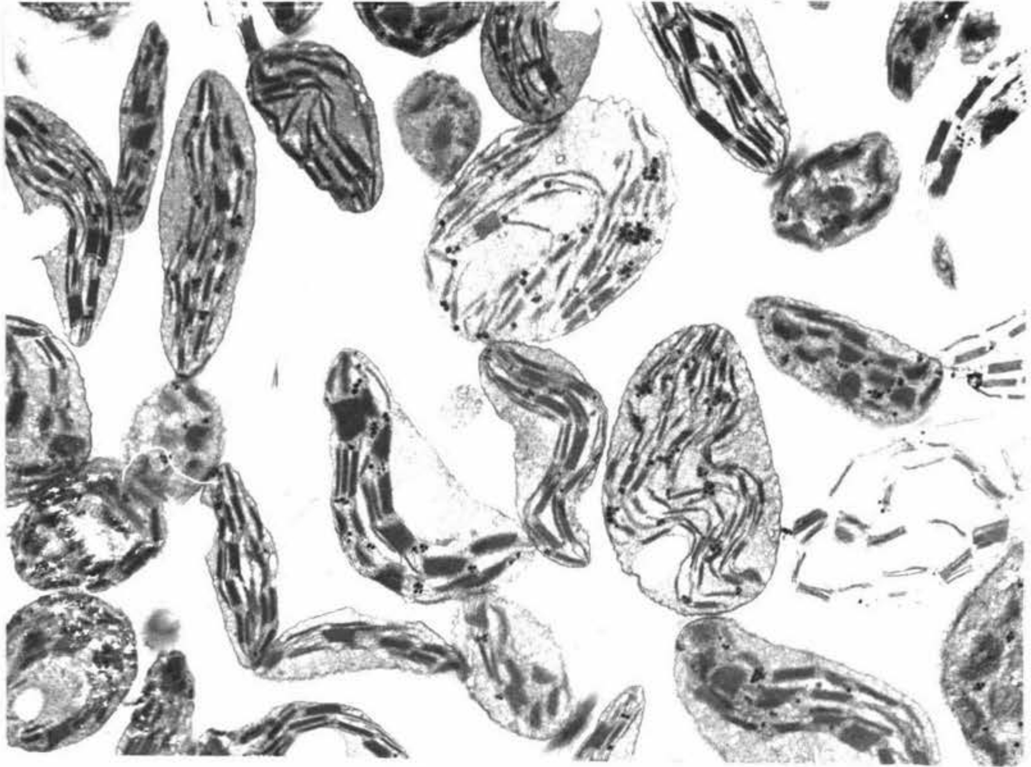
The modified procedure for the isolation of envelopes resulted in higher yields of less contaminated envelope membranes. (Table 8 and Figure 7). Fraction 2 was generally completely free of chlorophyll contamination. Two light-yellow bands were observed at the 0 M - 0.63 M and the 0.63 M - 0.8 M sucrose interphases (Figure 7). Poincelot and Day (1974) reported that these bands were enriched in single and double membrane envelopes respectively. The electron micrographs of the pellets obtained from these fractions are given in Figure 6.

faces

FIGURE 4

A representative field from a preparation of spinach chloroplasts (a) and chloroplast envelopes (b).

(a) 7,000 x magnification



(b) 10,500 x magnification

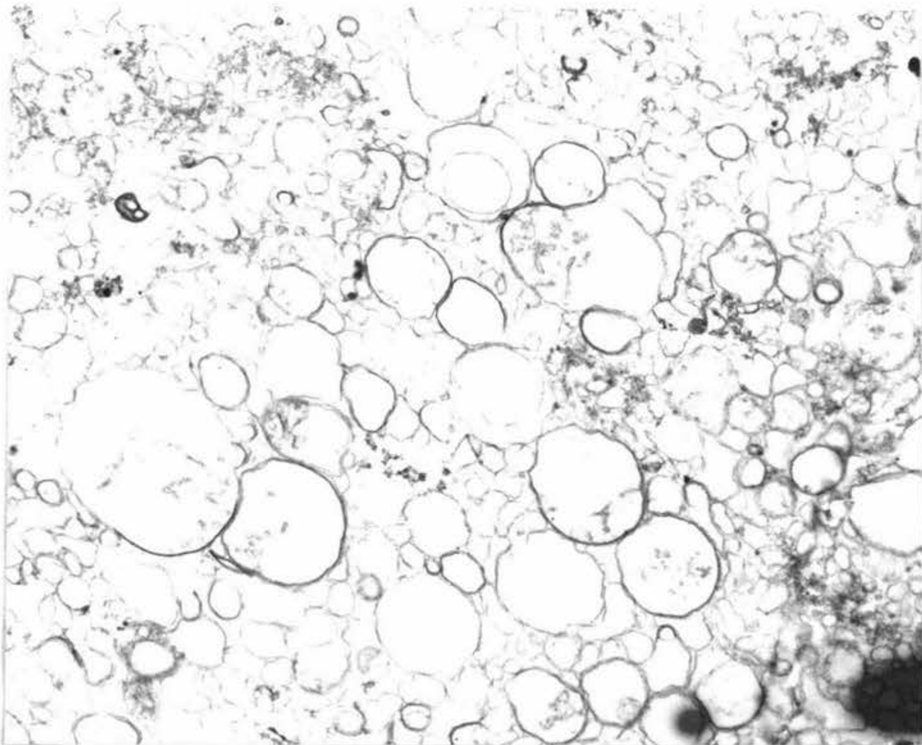


FIGURE 5

The profile of fractions obtained by sucrose gradient centrifugation of disrupted spinach chloroplasts.
(see Table 7)

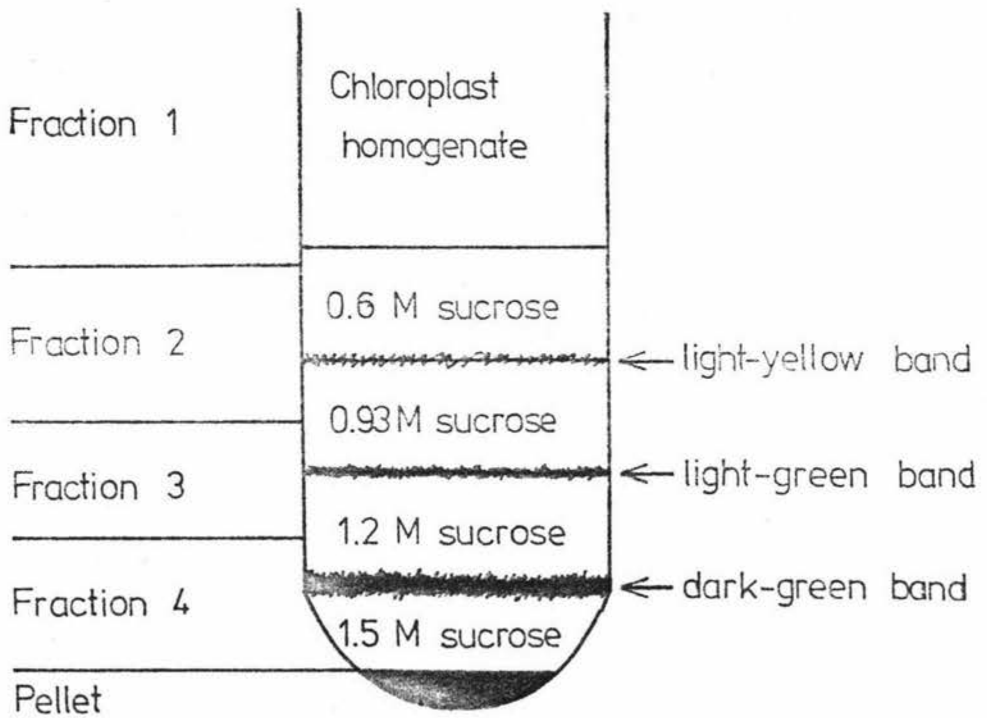


Table 7

The distribution of protein, chlorophyll and galactosyltransferase in fractions isolated by sucrose gradient centrifugation of disrupted spinach chloroplasts.

Procedures: The protein and chlorophyll concentrations were determined as described in 3.1.6 and 3.1.8. The galactosyltransferase was assayed by following the incorporation of radioactivity from UDP-^{14}C-Galactose (55,000 dpm, 0.07 nmoles per incubation) into the lipid fraction (3.1.4) (see figure 5).

Fraction	Volume (ml)	Protein (mg)	% of total protein	Chlorophyll (mg)	Protein / Chlorophyll ratio	Assay of galactosyltransferase Galactose incorporation			Mg^{2+}-dependent ATPase (µmoles P_i, h^{-1}, mg protein^{-1})
						per fraction (dpm x 1000)	per 100 µl (% of total)	Specific activity*	
1	33	49.83	27.8	-	-	421	2.3	21.5	-
2	1.7	1.36	0.8	trace	-	557	59.6	1042	4.61
3	1.5	0.69	0.4	0.15	4.6	117	14.2	430	2.12
4	5.9	55.21	30.8	8.07	6.8	816	25.2	38	1.01
pellet	8.2	72.42	40.3	4.89	14.8	1446	32.1	50	1.26
sum	-	179.51	-	13.10	13.7	-	-	-	-
Disrupted chloroplasts		190.40	-	13.80	13.8	3190	22.7	21	-

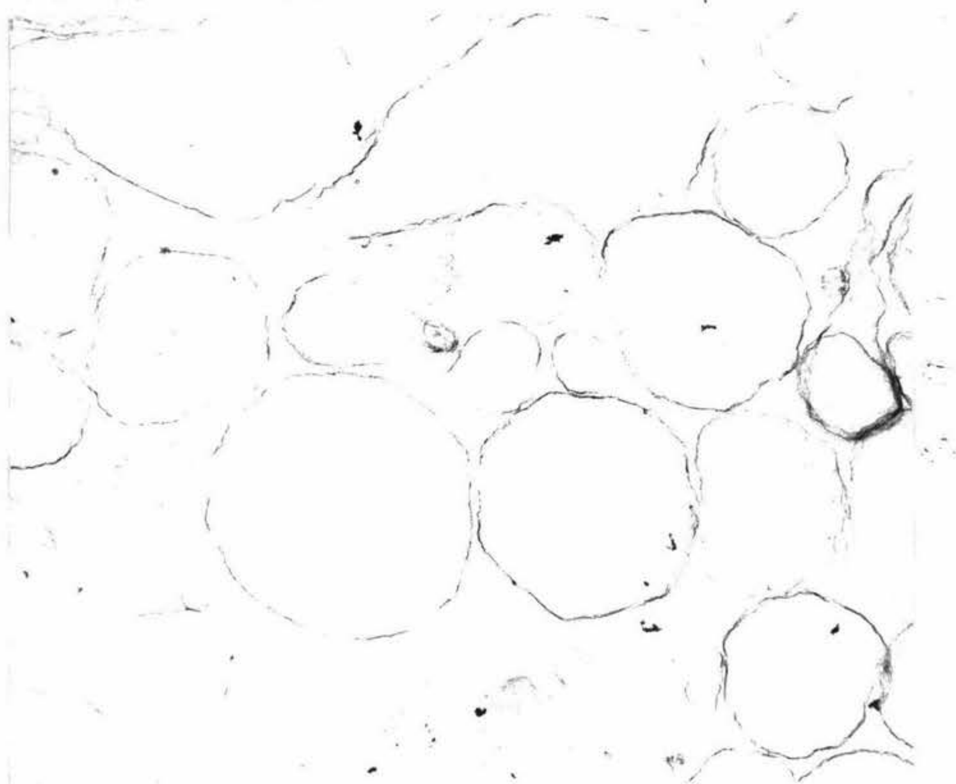
Chloroplasts were isolated from 400 g of leaf tissue

*Specific activity is measured in pmoles ^{14}C-Galactose incorporated h^{-1} mg protein^{-1}.

FIGURE 6

Representative fields of a preparation of envelopes from spinach chloroplasts obtained from fraction 1 (a) and fraction 2 (b) of the modified discontinuous sucrose gradient.

(a) 15,500 x magnification



(b) 15,500 x magnification

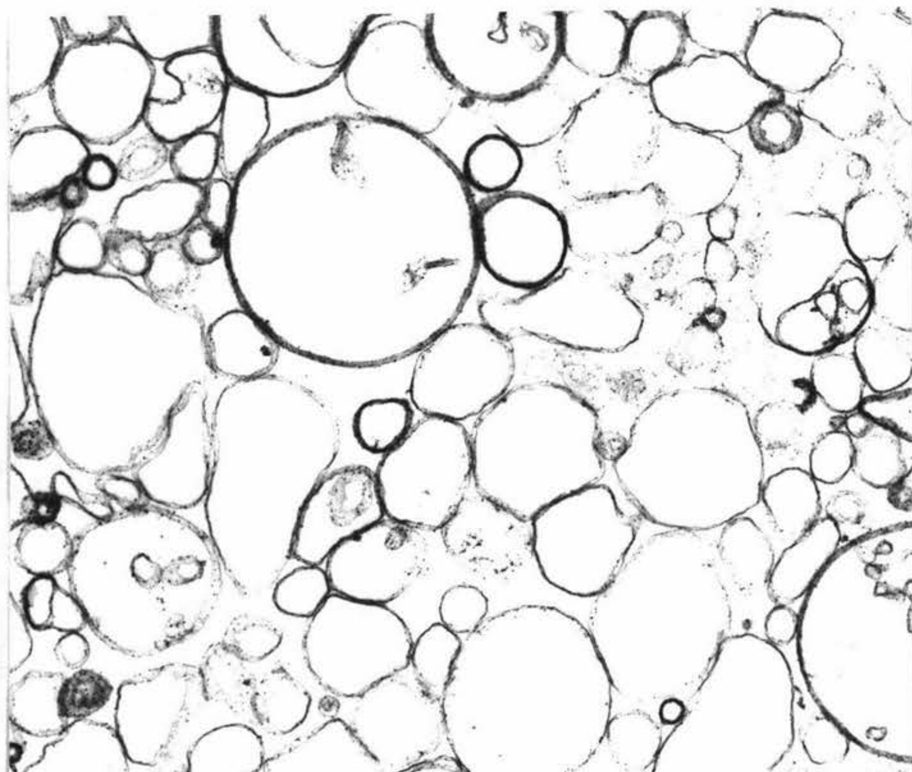
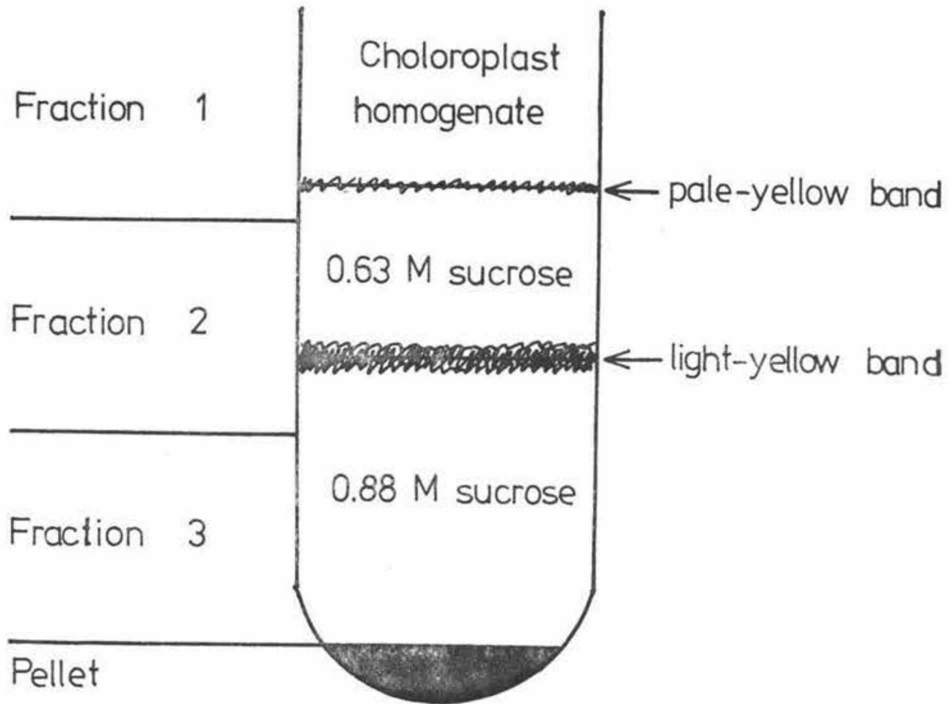


FIGURE 7

The profile of fractions obtained by sucrose gradient centrifugation of disrupted spinach chloroplasts (modified gradient). (see Table 8)



<u>Table 8</u>

The distribution of protein, chlorophyll and galactosyltransferase in fractions isolated by sucrose gradient centrifugation of disrupted spinach chloroplasts. (modified gradient)

Procedures: Protein, chlorophyll and galactosyltransferase were determined as described in Table 7. (see Figure 7).

Fraction	Volume (ml)	Protein (mg)	% of total protein	Chlorophyll (mg)	Protein / Chlorophyll ratio	Assay of galactosyltransferase: Galactose incorporation			Mg^{2+}-dependent ATPase (μmoles P_i, h^{-1}, mg protein^{-1})
						per fraction (dpm x 1000)	per 100 μl (% of total)	Specific activity*	
1	36	334	38.6	-	-	380	1.9	3	0.6
2	5.2	2.08	0.2	-	-	1263	47.7	1691	5.3
3	23	14.03	1.6	trace	-	920	7.8	182	1.0
pellet	23	515.62	59.6	72.9	6.9	5648	48.2	15	-

Chloroplasts were isolated from 420 g leaf tissue

* Specific activity is measured in pmoles ^{14}C-galactose incorporated h^{-1} mg protein^{-1}.

4.2.2 Variations in the level of galactosyltransferase in envelopes isolated from spinach chloroplasts.

Different envelope preparations gave variable levels of incorporation with between 30 % and 70 % of the added radioactive label incorporated into the lipid fraction. The specific activity of the envelope fraction was between 800 - 1900 pmoles ^{14}C-Galactose h $^{-1}$ mg protein^{-1}. The UDP-^{14}C-Galactose concentration in each incubation was between 3.0 x 10^{-7} and 2.7 x 10^{-7} M and was therefore assumed to be at a rate-limiting concentration.

The effect of increasing amounts of envelope protein on the incorporation of added radioactive label into lipids was examined. (Table 9 and Figure 8). The incorporation of radioactivity from UDP-^{14}C-Galactose increases with increasing amounts of envelope protein to a maximum at 100 µg envelope protein per incubation. The distribution of radioactivity in the lipid fraction from the incubation containing 50 µg envelope protein is shown in Figure 9. MGDG is the sole major labelled lipid and less than 3 % of the label was found in the DGDG fraction.

4.2.3 The effect of storage of the envelope preparation on incorporation of radioactivity from UDP-^{14}C-Galactose into lipids.

The effect on transferase activity of storage at 2 °C and -4 °C in 10 mM Tricine-NaOH at pH 8.0 containing 2 mM $MgCl_2$ was examined.

Table 10 shows the enzyme retained activity for up to 10 days at 2 °C but the activity visibally disappeared upon prolonged storage at this temperature. The slightly increased activity of the sample over 10 days at 2 °C may be explained by galactolipase or hydrolysis reactions in the envelope fraction providing an increase in endogenous diglyceride.

The envelope preparation at -4 °C incorporated up to 36 % of the added radioactivity after 44 days storage. Freezing reduced the total incorporation by between 4 % and 10 % but had no effect on the distribution of label in the lipid fraction. The ratio of label incorporated into MGDG and DGDG (MGDG:DGDG) was 20:1 in the frozen preparation and 21:1 in a control experiment.

Table 9

The effect of increasing amounts of envelope protein on incorporation of radioactivity from UDP-^{14}C-Galactose.

Reaction mixture: 10-100 µg envelope protein was incubated in the standard incubation mixture at 30 ^{o}C for 30 min.

Amount of envelope protein added (µg)	Galactose incorporation (dpm)	(% of total)
10	12100	22.0
25	19140	34.8
50	26570	48.3
100	28760	52.3

Figure 8

The effect of increasing amounts of envelope protein on the incorporation of radioactivity from UDP-^{14}C-Galactose into lipids. (see Table 9)

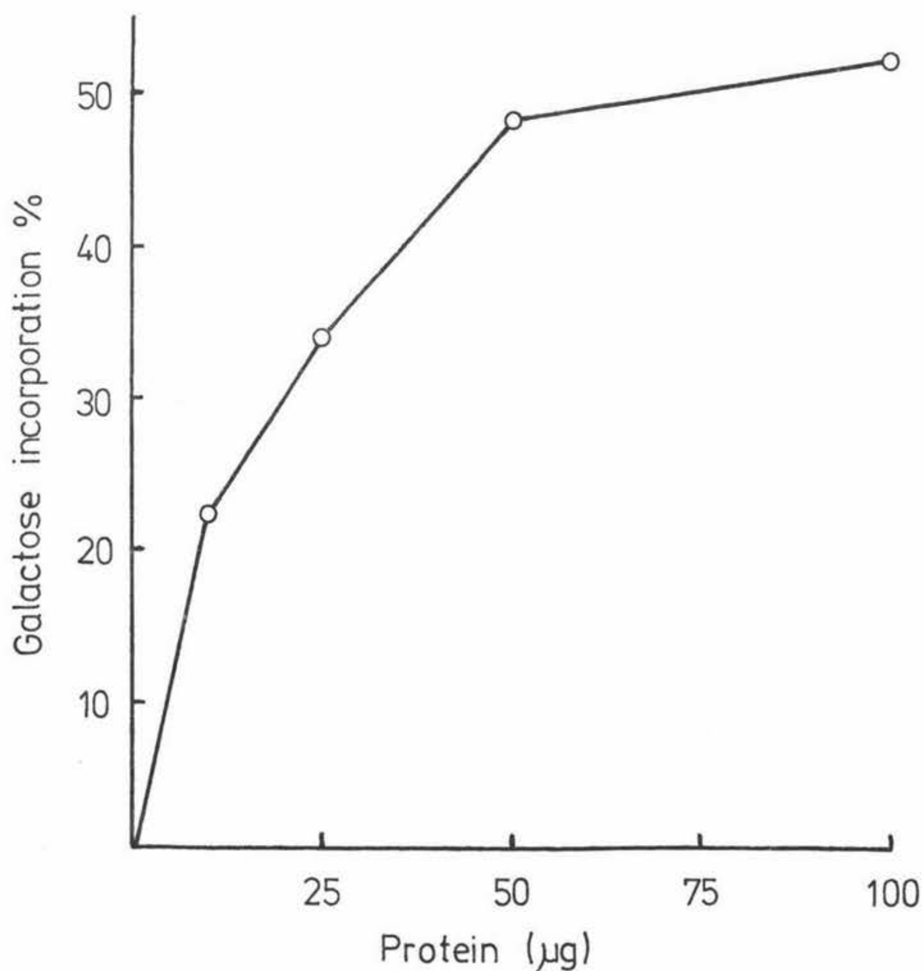


FIGURE 9

Radiochromatogram scan of the lipid extract obtained after incubating 50 μg envelope fraction with UDP-^{14}C-Galactose (55,000 dpm). (see Table 9)

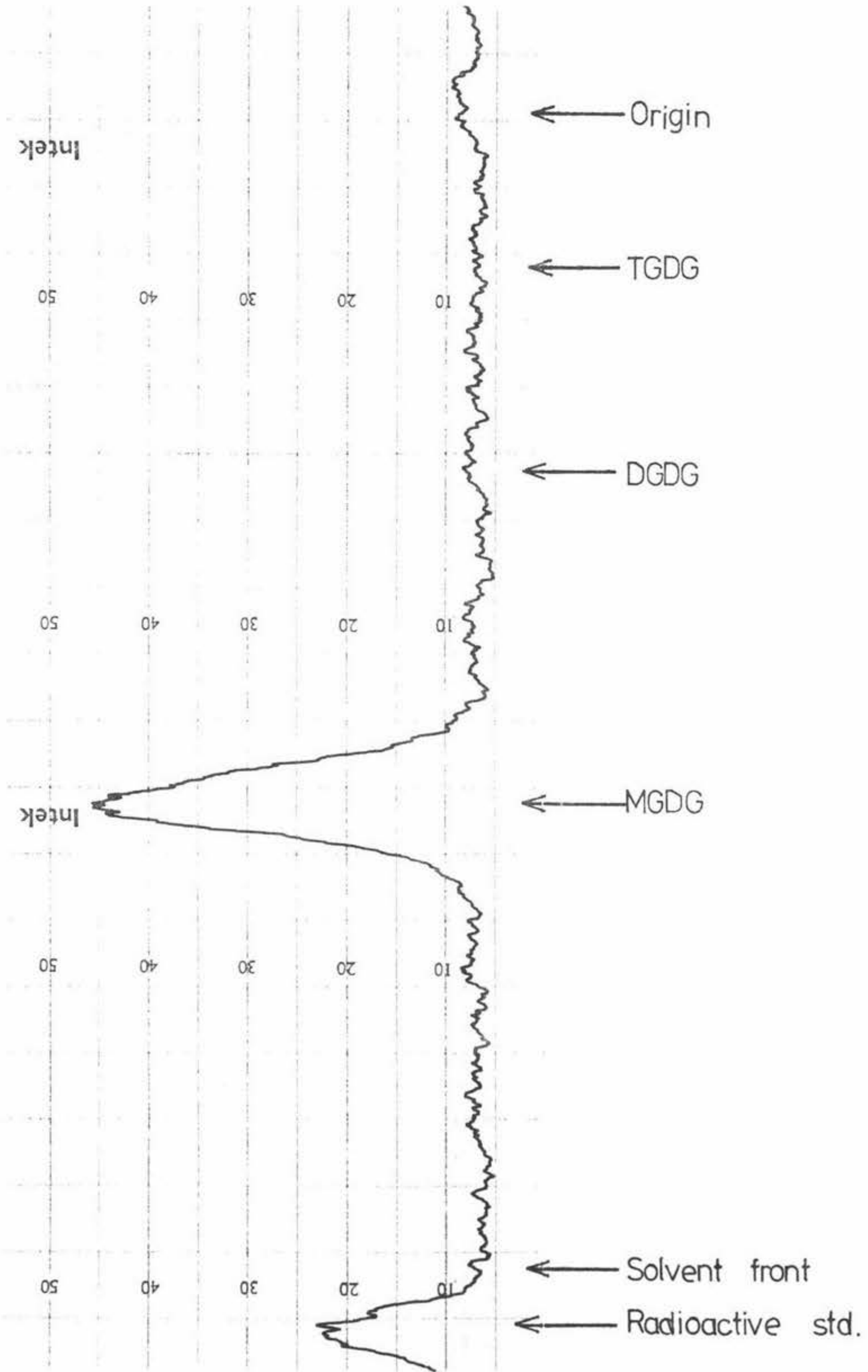


Table 10

The effect of storage of the envelope preparation on incorporation of radioactivity from UDP-^{14}C-Galactose into lipids.
Reaction mixture: 100 μl of envelope fraction (40 μg protein) which had been stored at 2 ^{o}C or at -4 ^{o}C was incubated in the standard incubation mixture for 30 min at 30 ^{o}C.

Days of storage	Galactose incorporation (dpm)	(% of total)
at 2 ^{o}C		
1	22000	40.0
5	25360	46.1
10	28050	51.0
20	715	1.3
at -4 ^{o}C		
0	22230	40.4
5	20120	36.5
44	19000	34.5

4.2.4 The effect of time and temperature of incubation on incorporation of radioactivity from UDP-^{14}C-Galactose into lipids.

The dependence of incorporation of radioactivity on time of incubation at several temperatures was examined to determine the optimum conditions for incubation. The standard incubation mixture was pre-incubated for 10 min at the appropriate temperature prior to addition of UDP-^{14}C-Galactose.

Table 11 and Figure 10 show the maximum level of incorporation was similar for each temperature investigated, but the rate of incorporation was temperature dependent. Lower incubation temperatures required longer incubation times to give maximum incorporation. The MGDG to DGDG ratio remained relatively constant over the range of temperatures that were examined.

The distribution of radioactivity in the lipid fraction over longer times of incubation was examined at 30 ^{o}C (Table 12). After reaching a maximum at about 1 h incubation there was no significant decline in the radioactivity in the lipid fraction. Analysis of the labelled lipids by TLC showed that most of the

label was in MGDG with minor amounts in DGDG and a third component with a lower R_f, tentatively identified as TGDG. The MGDG:DGDG ratio decreased with increasing incubation time from 31:1 after 5 min incubation to 9:1 after 5 h incubation.

TABLE 11

The effect of time and temperature of incubation on incorporation of radioactivity from UDP-^{14}C-Galactose into lipids by chloroplast envelopes.

Reaction mixture: The standard incubation mixture was preincubated for 10 min at the appropriate temperature prior to addition of 10 µl of UDP-^{14}C-Galactose (55,000 dpm, 0.07 nmoles). Incubation was continued for the times stated in the table.

Time of incubation (min)	Galactose incorporation (% of total)		
	at 45 °C	at 30 °C	at 23 °C
5	34.9	19.7	14.5
10	44.5	33.1	22.2
20	50.0	46.0	34.7
30	51.6	53.0	40.3
45	45.0	-	51.4
60	-	54.1	48.0

The distribution of radioactivity between the components of the lipid fraction after incubation at several different temperatures.

Temperature of incubation	Initial rate of incorporation*	Galactose incorporation dpm in MGDG	dpm in DGDG	MGDG/DGDG
45 °C	97.6	22690	1560	14.5
30 °C	55.2	23100	1620	14.3
23 °C	40.6	22600	1720	13

*Determined over the first 5 min of incubation and measured in pmoles galactose incorporated min^{-1} mg protein^{-1}.

Figure 10

The effect of time and temperature of incubation on incorporation of radioactivity from UDP-^{14}C-Galactose into lipids by chloroplast envelopes. (see Table 11)

Key: ○ incubation at 23 °C
□ incubation at 30 °C
● incubation at 45 °C

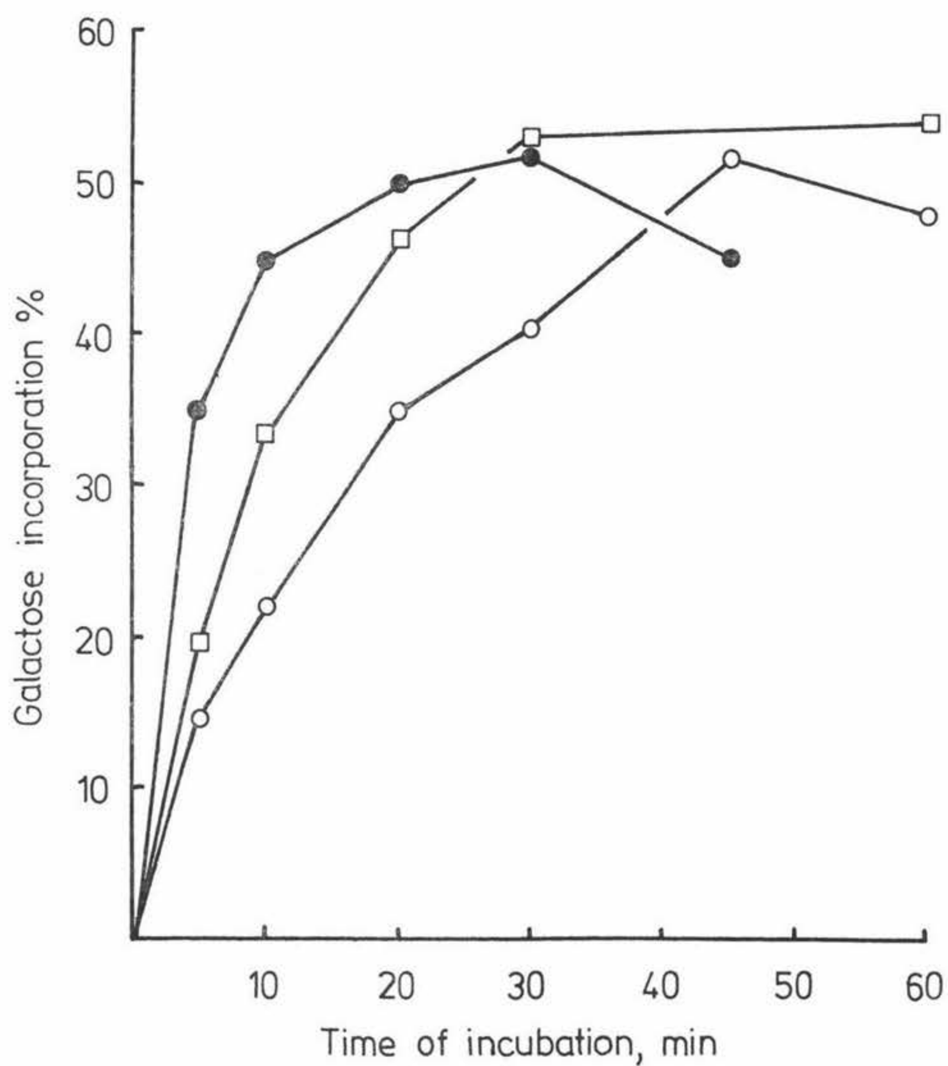


Figure 11

The effect of time of incubation on the incorporation of radioactivity from UDP-^{14}C-Galactose into MGDG, DGDG and TGDG by chloroplast envelopes. (see Table 12)

Key: □ MGDG
■ DGDG
○ TGDG

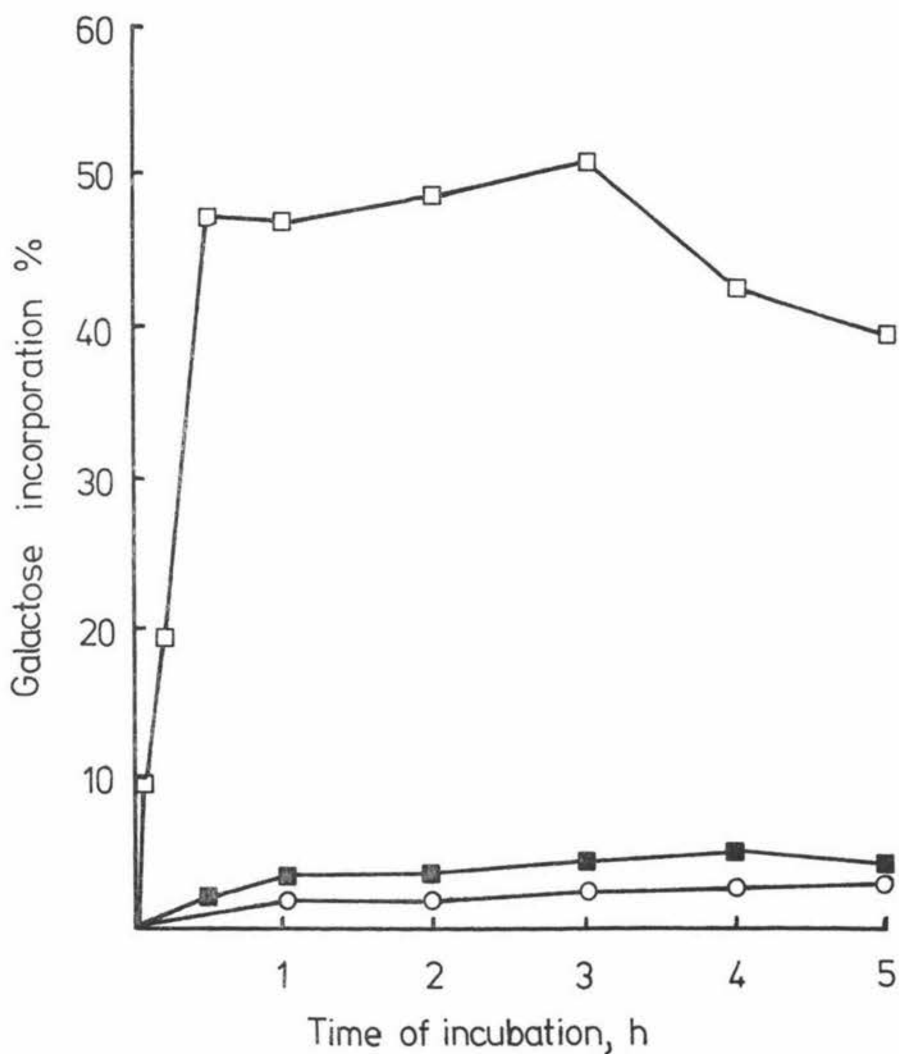


TABLE 12

The effect of time of incubation on the incorporation of radioactivity from UDP-^{14}C-Galactose into MGDG, DGDG and TGDG by chloroplast envelopes.

Reaction mixture: The standard incubation mixture was incubated with 10 μl of UDP-^{14}C-Galactose (55,000 dpm, 0.07 nmoles) for up to 5 h at 30 ^{o}C.

Time of incubation	Galactose incorporation (dpm in,)			(% of total in,)			MGDG/DGDG
	MGDG	DGDG	TGDG	MGDG	DGDG	TGDG	
5 min	4910	160	90	9.6	0.3	0.2	31
10 min	9830	250	120	19.2	0.5	0.2	39
30 min	23870	1200	440	46.8	2.3	0.9	20
1 h	23760	1700	920	46.5	3.3	1.8	14
2 h	24720	1960	1000	48.4	3.8	2.0	13
3 h	25880	2320	1060	50.7	4.5	2.1	11
4 h	21630	2010	1290	42.4	3.9	2.6	11
5 h	19960	2170	1590	39.1	4.2	3.1	9

4.2.5 The nature of the acceptor molecule involved in the synthesis of ^{14}C-MGDG from UDP-^{14}C-Galactose.

The possibility that ^{14}C-Galactose is synthesised by an exchange reaction involving endogenous MGDG and UDP-^{14}C-Galactose was investigated by a pulse-chase experiment. A small amount of UDP-^{14}C-Galactose was incubated with the envelopes to allow the ^{14}C-Galactose to be incorporated into MGDG. This was followed by the addition of a large amount of unlabelled UDP-Galactose which would greatly dilute any unreacted UDP-^{14}C-Galactose and stimulate the synthesis of MGDG. If an exchange reaction was taking place then in the presence of high concentrations of unlabelled UDP-Galactose the incorporation in MGDG could be expected to decrease.

The standard incubation mixture was incubated for 30 min at 30 ^{o}C in the presence of 2.80×10^{-7} M UDP-^{14}C-Galactose to allow radioactivity to be incorporated into envelope MGDG. After 30 min three additions were made to separate sets of incubations:

Table 13

The nature of the acceptor molecule involved in the synthesis of ^{14}C-MGDG from UDP-^{14}C-Galactose.

Reaction mixture: The standard incubation mixture was incubated for 30 min at 30 ^{o}C with 0.07 nmoles UDP-^{14}C-Galactose (55,000 dpm) per incubation. After this time the additions described in the text were made and incubation was continued for up to 3 h at 30 ^{o}C.

Time of incubation (min)	Galactose incorporation (dpm)		
	(A) control	(B) + UDP-^{14}C-Gal	(C) + UDP-Gal
5	17270	-	-
15	26170	-	-
30	25130	-	-
35	23820	42530	24920
45	23020	45640	24730
60	22460	46370	24534
180	23390	46980	23400

A) 10 μl of buffer (control)

B) 10 μl of UDP-^{14}C-Galactose (55,000 dpm, 0.07 nmoles) to give a total amount of added radioactive UDP-^{14}C-Galactose of 0.14 nmoles.

C) 10 μl of unlabelled UDP-Galactose (370 nmoles) to give a final concentration of 1.4 mM.

The incprporation in MGDG remained constant in the presence of high levels of unlabelled UDP-Galactose thus giving no evidence of an exchange reaction (Table 13 and Figure 12). Galactosyltransferase is still active after 30 min incubation as shown by the rapid incorporation of radioactivity by incubation (B) from a further 10 μl aliquot of UDP-^{14}C-Galactose. A radiochromatogram scan of the products of the reaction in the presence of unlabelled UDP-Galactose (incubation (C)) is given in Figure 13. The major radioactive lipid was MGDG with less than 2 % of the label in DGDG. No other radioactive products were detected.

Figure 12

The nature of the acceptor molecule involved in the synthesis of ^{14}C- MGDG from UDP-^{14}C-Galactose. (see Table 13)

Key: ● A (control)
□ B (+ UDP-^{14}C-Galactose)
○ C (+ UDP-Galactose)

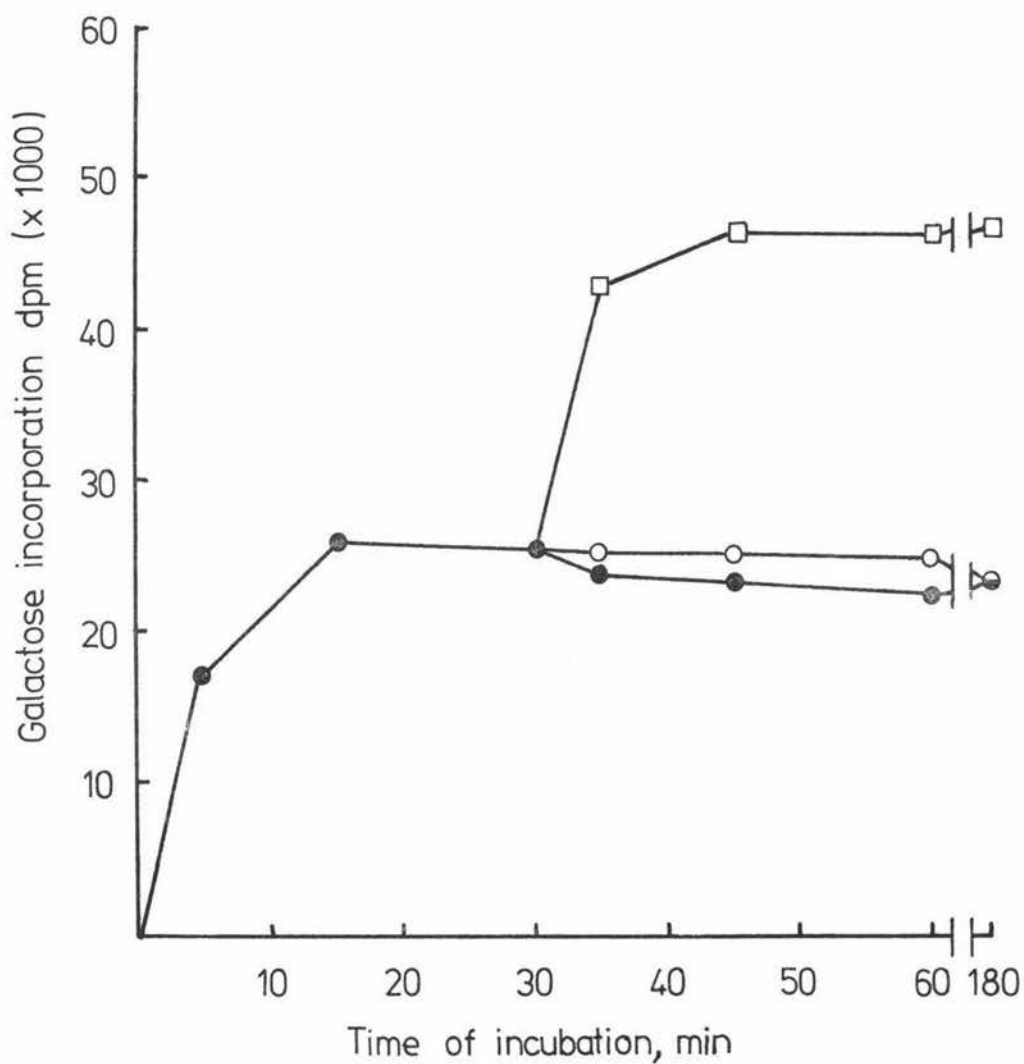
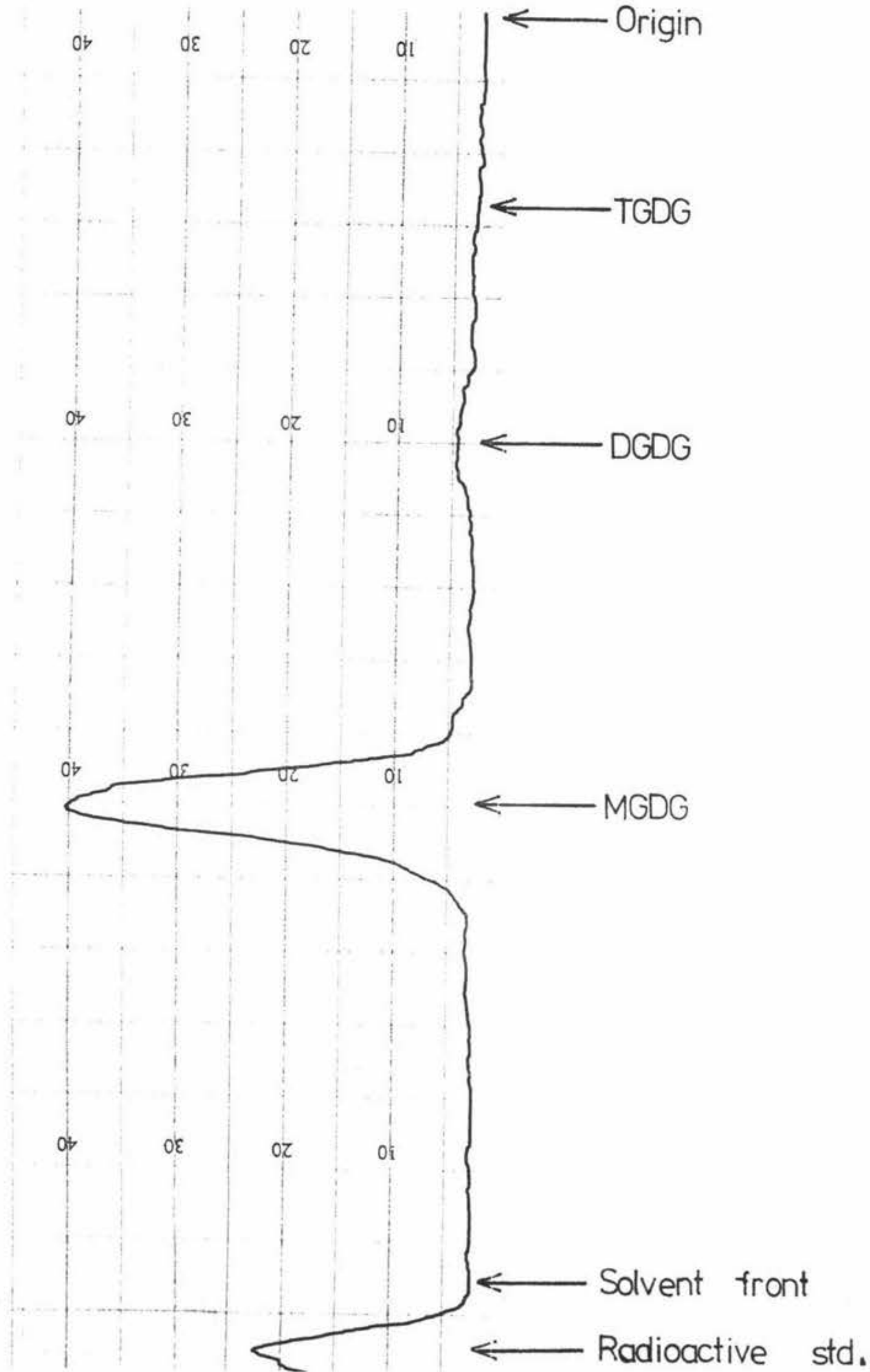


FIGURE 13

Radiochromatogram scan of the products from incubation with 0.28 μM UDP-^{14}C-Galactose for 30 min and then 1.4 mM UDP-Galactose for 30 min (incubation C). (see Table 13)



4.2.6 The effect of the composition of the incubation buffer and pH of incubation on incorporation of radioactivity from UDP-^{14}C-Galactose into lipids by envelopes.

The effect of the composition of the buffer and pH of incubation was investigated to determine the optimum conditions for incorporation. The buffers used were citrate-phosphate, Tris-HCl, Tricine-NaOH, HEPES-NaOH and glycine-NaOH all at 50 mM.

The incubation mixture containing 50 mM buffer at each pH, 5 mM $MgCl_2$, 0.3 M sucrose and 50 μl of the envelope fraction (30 μg protein) in a total volume of 260 μl, was preincubated for 10 min at 30 ^{o}C and then 10 μl of UDP-^{14}C-Galactose (55,000 dpm) was added to start the reaction and incubation was continued for 30 min at 30 ^{o}C.

The maximum incorporation (72 % of the added radioactivity) was obtained with the incubation containing HEPES-NaOH at pH 8.0 (Table 14 and Figure 14). All buffers showed an increase in total incorporation with increasing pH up to pH 8.0. At pH values below pH 6.0 incorporation was reduced to about 10 %. The incorporation over the range pH 7.0 - 9.0 was dependent, in part, on the buffering system. At all pH values examined HEPES-NaOH and Tris-HCl gave a higher incorporation than Tricine-NaOH.

Incorporation into DGDG was highest between pH 6.0 - 7.0 (Table 14 and Figure 15), higher pH values and Tris-HCl and HEPES-NaOH buffers favoured the synthesis of MGDG. HEPES-NaOH at pH 8.5 gave a MGDG:DGDG ratio (dpm in MGDG:dpm in DGDG) of 28:1, Tricine-NaOH at pH 8.0 gave a ratio of 2:1

The results obtained agree with the work of Mudd _et al_ (1969) and Van Besouw and Wintermans (1978) on the general trend of pH dependence, however, the results suggest a pH optimum of about pH 8.0 rather than the optimum of about pH 7.2 - 7.5 obtained by these workers.

TABLE 14

The effect of the composition of the incubation buffer and pH of incubation on incorporation of radioactivity from UDP-^{14}C-Galactose into lipids by chloroplast envelopes.

Reaction mixture: 50 μl envelope fraction (30 μg protein) with 50 mM of each buffer at each pH, 5 mM $MgCl_2$ and 0.3 M sucrose in a total reaction volume of 250 μl was preincubated for 10 min at 30 °C. 10 μl of UDP-^{14}C-Galactose (55,000 dpm, 0.07 nmoles) was added and incubation was continued at 30 °C for a further 30 min.

pH of incubation	Incorporation (%) in incubation containing:				
	citrate	Tricine	Tris	HEPES	glycine
4.0	0	-	-	-	-
5.0	11	-	-	-	-
6.0	51.2	-	-	-	-
7.0	58.9	-	55.7	60.3	-
7.5	-	47.5	60.5	68.4	-
8.0	-	51.7	64.2	71.8	-
8.5	-	-	67.1	61.6	-
9.0	-	60.8	61.9	-	-
9.5	-	-	-	-	61.3

pH of incubation	$\frac{\text{dpm in MGDG}}{\text{dpm in DGDG}}$ (MGDG:DGDG) in incubation containing:		
	Tricine	Tris	HEPES
7.0	-	6.0	9.3
7.5	1.4	-	-
8.0	2.0	8.7	11.7
8.5	-	-	27.9
9.0	11.7	22.0	-

FIGURE 14

The effect of the composition of the incubation buffer and pH of incubation on incorporation of radioactivity from UDP-^{14}C-Galactose into lipids by envelopes. (see Table 14)

Key: ● HEPES-NaOH ■ Tris-HCl □ Tricine-NaOH
○ citrate-phosphate ▲ glycine-NaOH

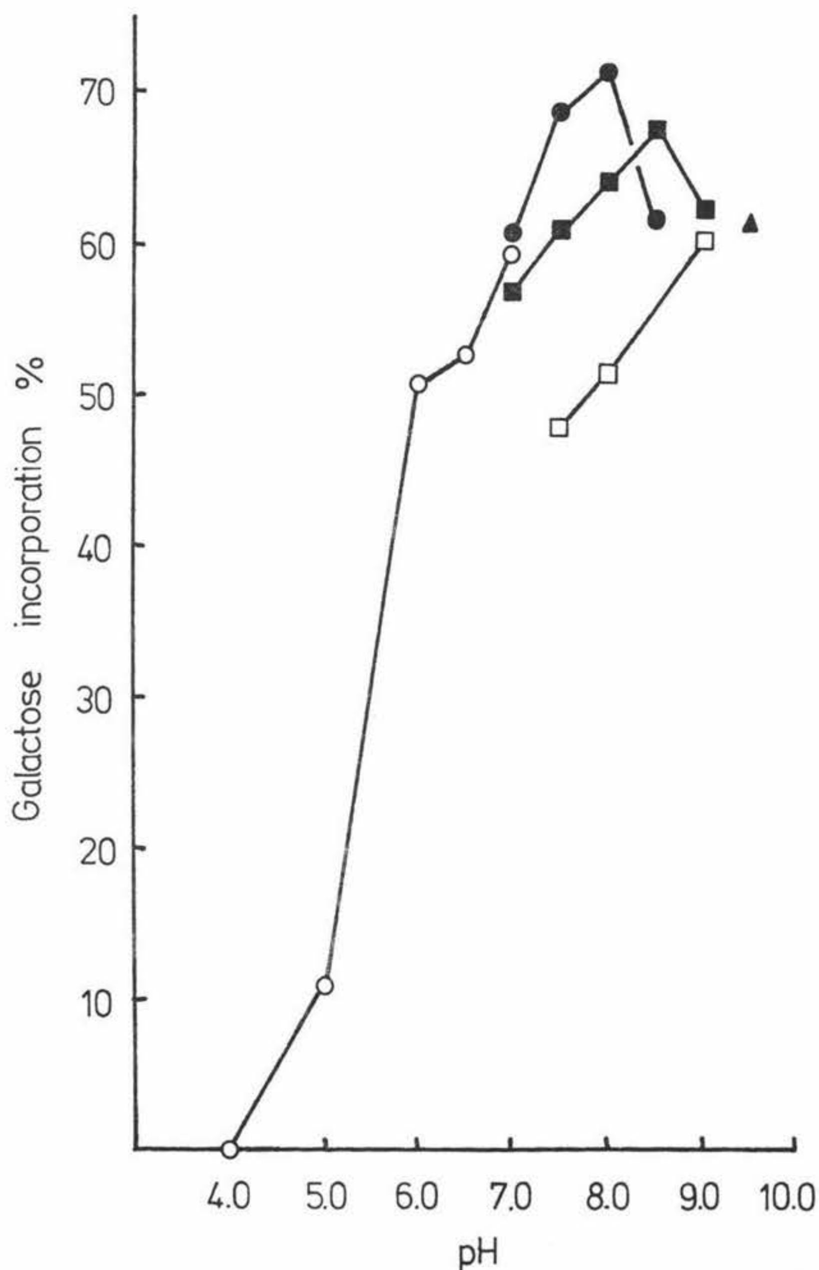
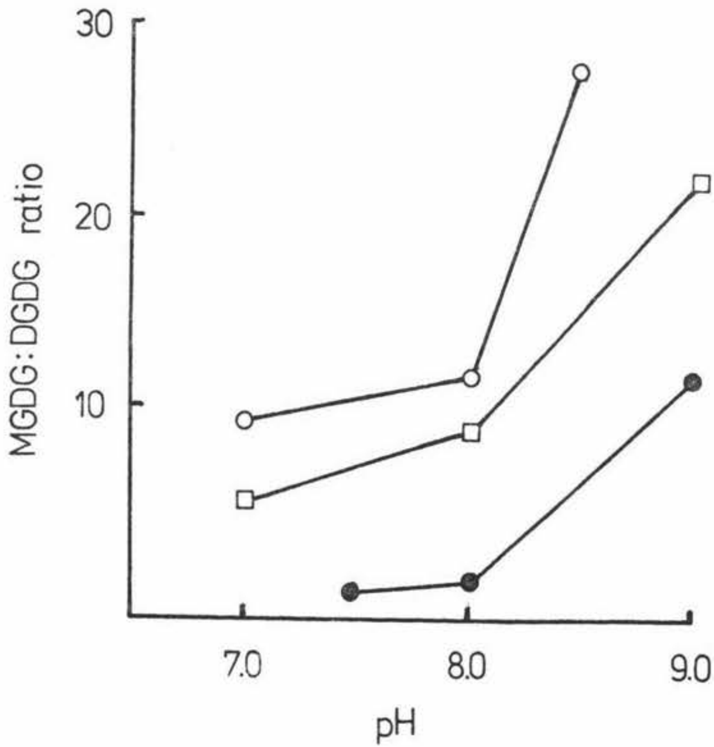


FIGURE 15

The effect of the composition of the incubation buffer and the pH of incubation on the distribution of radioactivity from UDP-^{14}C-Galactose between MGDG and DGDG of chloroplast envelopes. (see Table 14)

Key: ● Tricine-NaOH
□ Tris-HCl
○ HEPES-NaOH



4.2.7 The effect of mono- and divalent cations on incorporation of radioactivity from UDP-^{14}C-Galactose into lipids by a preparation of envelopes.

The effect of the monovalent cations Li^{+}, K^{+} and Na^{+} and the divalent cations Co^{2+}, Ca^{2+}, Cu^{2+}, Cs^{2+}, Mn^{2+}, Ba^{2+} and Mg^{2+} was examined. All of the cations were added as their chloride salts.

Envelopes isolated as described in 3.2.1 and 3.2.2 showed no cation requirement for transferase activity. As the envelope pellet may consist of membrane vesicles the activity could be due to a carry-over of Mg^{2+} from the isolation buffers. Therefore the chloroplast pellet was prepared as described in 3.2.1 but Mg^{2+} was deleted from all the buffers used for the isolation of envelopes. The absence of Mg^{2+} from the modified discontinuous gradient had no visible effect on the yield or contamination of the envelope pellet.

Incorporation of radioactivity from UDP-^{14}C-Galactose by a preparation of envelopes in the absence of any cation was about 16 % (Table 15). Addition of EDTA slightly stimulated incorporation. Of the monovalent cations tested at 5 mM none activated the enzyme and K^{+} gave slight inhibition. The divalent cations Co^{2+} and Cs^{2+} had little effect on incorporation but Cu^{2+} completely inhibited incorporation. In contrast Mn^{2+}, Ca^{2+}, Mg^{2+} and Ba^{2+}, all at 5 mM, stimulated the activity. Ba^{2+} gave the highest incorporation (52 %) and Mn^{2+} the least (27 %).

The cations Ca^{2+}, Mg^{2+} and Ba^{2+} over the concentration range 0.1-50 mM had similar effects on transferase (Table 16 and Figure 16). Incorporation increased with increasing cation concentration to a maximum at about 5 mM, but at concentrations above 5 mM incorporation was increasingly inhibited. At 50 mM Ba^{2+} showed 66 % inhibition of the activity observed at 5 mM and Mg^{2+} and Ca^{2+} showed about 45 % inhibition. Cation concentrations above 20 mM led to coagulation of the envelope preparation and this may account for some of the inhibition observed at high cation concentrations.

TABLE 15

The effect of mono- and divalent cations on incorporation of radioactivity from UDP-^{14}C-Galactose into lipids by a preparation of envelopes.

Reaction mixture: 100 µl envelope fraction (25 µg protein) with 50 mM HEPES-NaOH at pH 8.0, 0.3 M sucrose and 5 mM of the appropriate cation. 10 µl of UDP-^{14}C-Galactose (55,000 dpm) was added and incubation was at 30 ^{o}C for 30 min.

Cation added (5 mM)	Galactose incorporation (% of total added)
none	16.1
Li^{+}	15.6
K^{+}	12.9
Na^{+}	16.3
Co^{2+}	13.1
Cu^{2+}	0.8
Ca^{2+}	46.6
Mn^{2+}	26.8
Mg^{2+}	47.4
Ba^{2+}	52.5
Cs^{2+}	17.2

TABLE 16

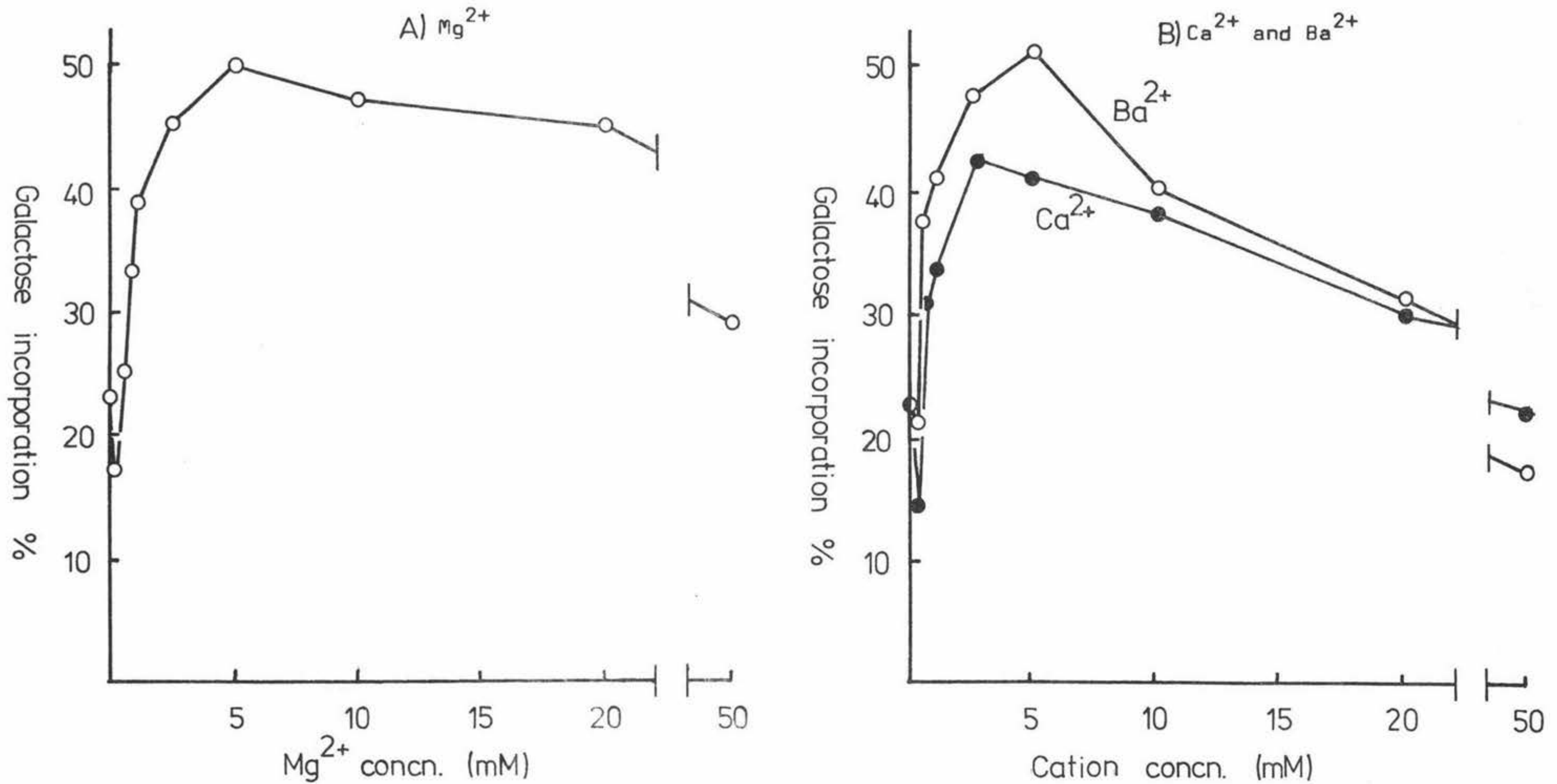
The effect of Ca^{2+}, Mg^{2+} and Ba^{2+} over the concentration range 0.1-50 mM on incorporation of radioactivity from UDP-^{14}C-Galactose into lipids by a preparation of envelopes.

Reaction mixture: As for Table 15 but with 0.1-50 mM of the appropriate cation.

Cation concentration (mM)	Galactose incorporation (% of total added)		
	Mg^{2+}	Ca^{2+}	Ba^{2+}
0	23.3	-	-
0 + 2 mg EDTA	24.0	-	-
0 + 4 mg EDTA	26.4	-	-
0.1	16.7	14.9	22.1
0.5	25.4	31.3	37.9
0.75	33.4	-	-
1.0	38.9	33.9	41.4
2.5	45.1	47.7	42.5
5.0	50.1	40.6	51.3
10.0	47.4	38.1	40.4
20.0	45.2	30.0	31.3
50.0	28.9	21.9	17.2

FIGURE 16

The effect of Ca^{2+}, Mg^{2+} and Ba^{2+} over the concentration range 0.1 - 50 mM on incorporation of radioactivity from UDP-^{14}C-Galactose into lipids by chloroplast envelopes. (see Table 16)



4.2.8 The effect of UMP, UDP, UTP, NADH and pyrophosphate (PP_i) on the incorporation of radioactivity from UDP-^{14}C-Galactose into lipids by chloroplast envelopes.

The effect of the nucleotides UMP, UDP, UTP, NADH AND PP_i as possible effectors in the control of the synthesis of MGDG was examined with a preparation of chloroplast envelopes.

UMP, UDP and UTP inhibited incorporation of radioactivity from UDP-^{14}C-Galactose into MGDG (Table 17 and Figure 17). UDP was the most effective inhibitor and gave 50 % inhibition of incorporation at about 5 μM. UMP and UTP gave 50 % inhibition at about 100 μM and 500 μM respectively. NADH and PP_i showed a slight inhibition of incorporation.

TABLE 17

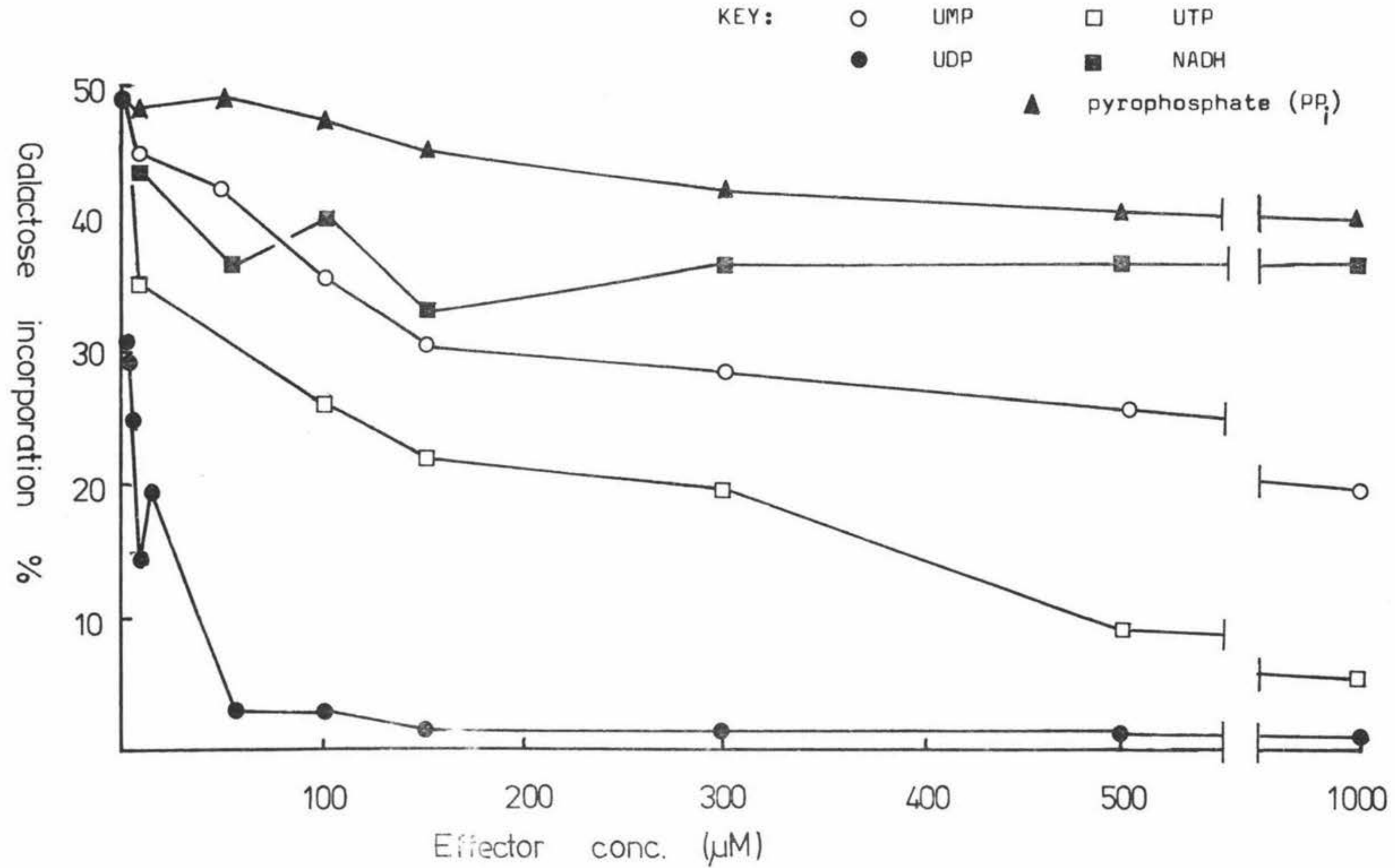
The effect of UMP, UDP, UTP, NADH and PP_i on incorporation of radioactivity from UDP-^{14}C-Galactose into lipids by chloroplast envelopes. Reaction mixture: 100 μl envelope fraction (30 μg protein) was incubated in the standard incubation mixture with 0.1-1.0 mM of each effector and 0.27 μM UDP-^{14}C-Galactose (55,000 dpm) for 30 min at 30 ^oC.

Concentration of possible effector (μM)	Galactose incorporation (% of total added)				
	UMP	UDP	UTP	NADH	PP_i
0	49.6	49.6	49.6	49.6	49.6
0.5	-	30.3	-	-	-
1.0	-	29.1	-	-	-
5.0	-	24.5	-	-	-
10.0	45.2	14.9	35.5	44.0	48.5
15.0	-	19.7	-	-	-
50	43.1	3.0	-	36.7	49.5
100	35.5	3.1	26.4	40.0	47.7
150	30.3	1.8	22.3	33.7	45.1
300	28.1	1.8	19.1	36.0	42.1
500	26.0	1.5	9.8	36.6	40.8
1000	19.7	1.3	5.8	36.2	40.4

It would appear that UDP, a product of the synthesis of MGDG from diacylglycerol and UDP-Galactose by galactosyltransferase may influence transferase activity but the nature of the inhibition was not further investigated.

FIGURE 17

The effect of UMP, UDP, UTP, NADH and PP_i, on incorporation of radioactivity from UDP-^{14}C-Galactose into lipids by chloroplast envelopes. (see Table 17)



4.2.9 The effect of sulphydryl reagents on incorporation of radioactivity from UDP-^{14}C-Galactose into lipids by galactosyltransferase of chloroplast envelopes.

The sulphydryl nature of the chloroplast galactosyltransferase has been reported (Chang, 1970 ; Mudd et al 1971). The effect of the sylphydryl reagents iodoacetate, mercaptoethanol, dithiothreitol and mercury ion on galactosyltransferase of the chloroplast envelope was examined.

The results given in Table 18 show that 10 umoles iodoacetate or Mg^{2+} inhibited incorporation of radioactivity from UDP-^{14}C-Galactose into lipids. In contrast both mercaptoethanol and dithiothreitol showed a slight inhibition of transferase activity. Mudd et al (1971) reported that iodoacetate had no effect on transferase, however, the results of the present study are in agreement with Chang (1970) and show an inhibitory effect of iodoacetate on the enzyme. These results indicate the presence of one or more sulphydryl groups which appear to be necessary for an active galactosyltransferase.

TABLE 18

The effect of sulphydryl reagents on incorporation of radioactivity from UDP-^{14}C-Galactose into lipids by galactosyltransferase of chloroplast envelopes.

Reaction mixture: 100 µl envelopes fraction (25 µg protein) was incubated in the standard incubation mixture with the appropriate sulphydryl reagent and 10 µl UDP-^{14}C-Galactose (55,000, 0.07 nmoles) for 30 min at 30 ^{o}C.

	Galactose incorporation (% of total added)
Control	44.6
Iodoacetate (1 µmole)	1.6
Iodoacetate (10 µmole)	0.8
Hg^{2+} (10 µmole)	5.2
Mercaptoethanol (10 µmole)	42.7
Dithiothreitol (5 µmole)	35.2

4.2.10 Inhibition of the incorporation of radioactivity from UDP-^{14}C-Galactose by linoleic acid and the effect of the cations Ca^{2+}, Mg^{2+}, Ba^{2+} and BSA on the observed inhibition.

Free fatty acids have been shown to inhibit the photosynthetic processes of isolated chloroplasts (Anderson et al 1974 ; Friedlander and Neumann, 1968 ; Friend and Hawcroft, 1967) and the inhibition is thought to be due to disruption of the integrity of the envelope caused by penetration of the hydrophobic fatty acid into the hydrophobic region of the membrane (Okamoto and Katoh, 1977 ; Okamoto et al 1977). The association of galactosyltransferase with membranes of the chloroplast prompted an investigation of the effect of free fatty acids on enzyme activity. Furthermore, transferase was stimulated by cations such as Ca^{2+} and Ba^{2+} which form insoluble salts of fatty acids (experiment 4.2.7). The possibility that this stimulation was caused by removal of an inhibition by endogenous fatty acids and the effect of BSA, a well-known complexing agent with free fatty acids, was investigated.

a) Inhibition of galactosyltransferase by linoleic acid

Addition of linoleic acid inhibited the incorporation of radioactivity from UDP-^{14}C-Galactose by a preparation of chloroplast envelopes (Table 19 and Figure 18). Linoleate at 0.11 mM reduced incorporation by about 50 % and 0.72 mM linoleate completely inhibited incorporation.

b) The effect of Ca^{2+}, Mg^{2+}, Ba^{2+} and BSA on inhibition

The effect of Ca^{2+}, Mg^{2+}, Ba^{2+} and BSA on the inhibition by linoleate was determined by the addition of cations or BSA to the incubation mixture after preincubation of envelopes with linoleate. Incorporation of radioactivity was inhibited about 60 % by 0.18 mM linoleic acid. The inhibition was partially released by preincubation with Ca^{2+} or Ba^{2+} but not by preincubation with Mg^{2+} or BSA (Table 20 and Figure 19). Ca^{2+} at 15 mM and Ba^{2+} at 8 mM respectively gave about 60 % and 50 % stimulation of incorporation of radioactivity from UDP-^{14}C-Galactose into galactolipids in the presence of linoleate. The effect of Mg^{2+} is not unexpected as the salts of this cation are soluble, however, it is not clear why BSA had no effect on inhibition of incorporation by linoleic acid.

TABLE 19

The effect of linoleic acid on the incorporation of radioactivity from UDP-^{14}C-Galactose into lipids by chloroplast envelopes.

Reaction mixture: 100 μl envelope fraction (50 μg protein) was preincubated in the standard incubation mixture with linoleate for 10 min at 30 ^{o}C. After this time, 10 μl UDP-^{14}C-Galactose (55,000 dpm, 0.07 nmoles) was added and incubation was continued for a further 30 min at 30 ^{o}C.

Linoleic acid added (mM)	Galactose incorporation (% of total added)
0	43.5
0.036	39.0
0.071	24.2
0.108	20.1
0.144	19.7
0.180	18.1
0.216	16.8
0.288	6.9
0.360	6.3
0.720	0

FIGURE 18

The effect of linoleic acid on the incorporation of radioactivity from UDP-^{14}C-Galactose into lipids by chloroplast envelopes. (see Table 19)

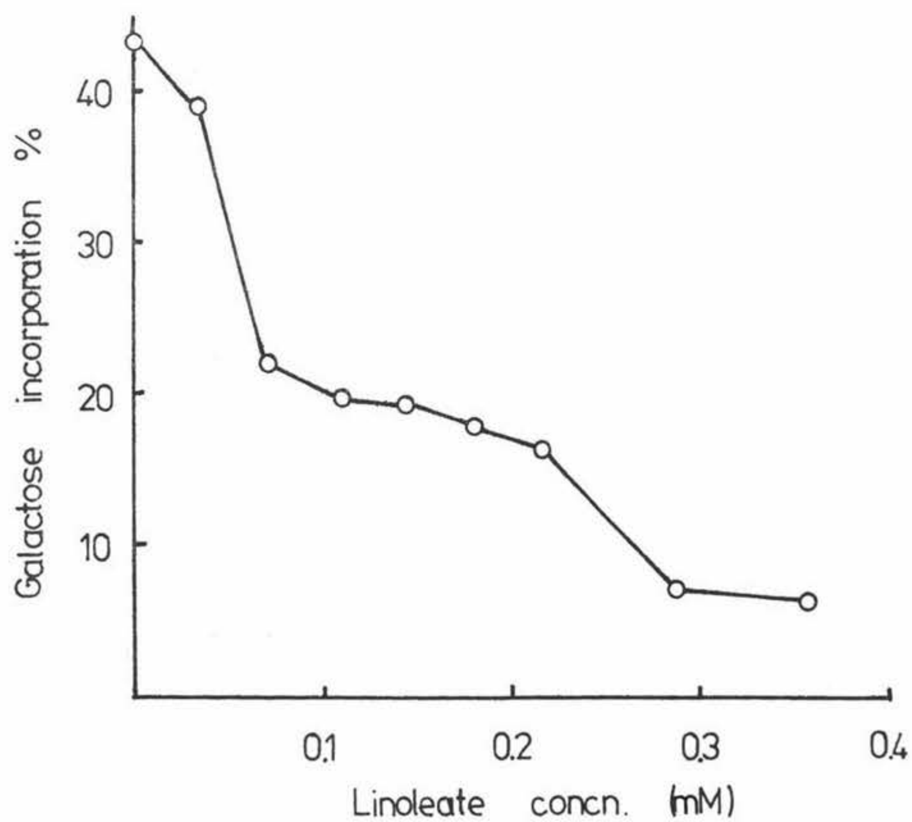


TABLE 20

The effect of Ca^{2+}, Mg^{2+}, Ba^{2+} and BSA on the inhibition of incorporation of radioactivity from UDP-^{14}C-Galactose by linoleic acid.

Reaction mixture: 100 µl envelope fraction (50 µg protein) was preincubated in the standard incubation mixture with 0.18 mM linoleic acid for 10 min at 30 °C. After this time 50 µl of the appropriate cation or BSA solution was added and preincubation was continued for a further 10 min. Transferase activity was determined by the addition of 10 µl UDP-^{14}C-Galactose (55,000 dpm, 0.07 nmoles) and then incubation for 30 min at 30 °C. Values stated in the Table are the means of duplicates from three experiments, i.e. each value is the mean of 6 incubations.

Cation concentration (mM)	Galactose incorporation (% of total added)					
	Ca^{2+}		Mg^{2+}		Ba^{2+}	
	with added linoleate	no added linoleate	with added linoleate	no added linoleate	with added linoleate	no added linoleate
0	17.3	42.1	17.3	42.1	17.3	42.1
2	18.1	31.9	12.6	36.4	19.4	33.6
4	21.3	38.8	20.0	37.2	22.4	37.9
6	24.3	37.6	19.4	38.4	23.4	33.9
8	24.6	35.9	-	-	26.0	30.2
10	25.0	33.9	18.7	37.0	25.9	31.3
12	-	-	15.6	32.3	-	-
15	28.5	30.9	17.1	28.1	19.8	30.8
20	25.2	27.9	16.3	29.0	19.7	27.5
30	18.2	25.8	13.5	21.1	18.1	20.1
50	12.5	11.5	5.3	10.8	15.6	12.9

BSA concentration (µg/ml)	Galactose incorporation (% of total added)
0	17.3
10	12.4
20	17.0
30	16.0

FIGURE 19

The effect of Ca^{2+}, Mg^{2+} and Ba^{2+} on the inhibition of incorporation of radioactivity from UDP-^{14}C-Galactose by linoleic acid. (see Table 20)

(A) Ca^{2+} (B) Mg^{2+} (C) Ba^{2+}

Key: ○ control (no linoleate)

□ with linoleate (50 μg)

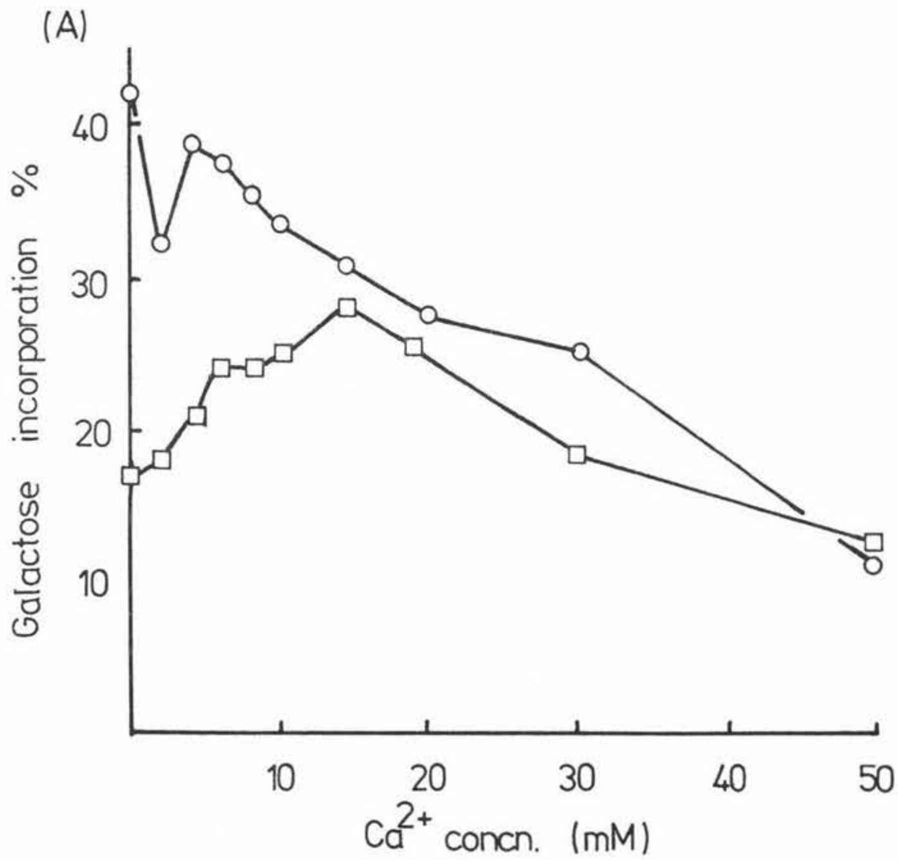
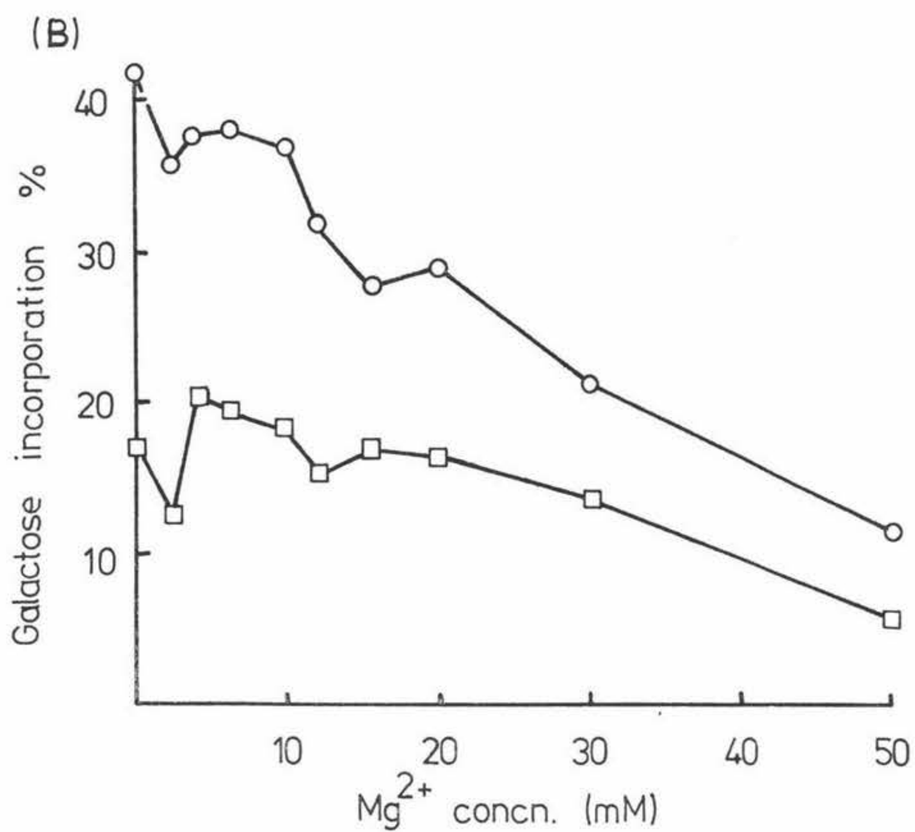
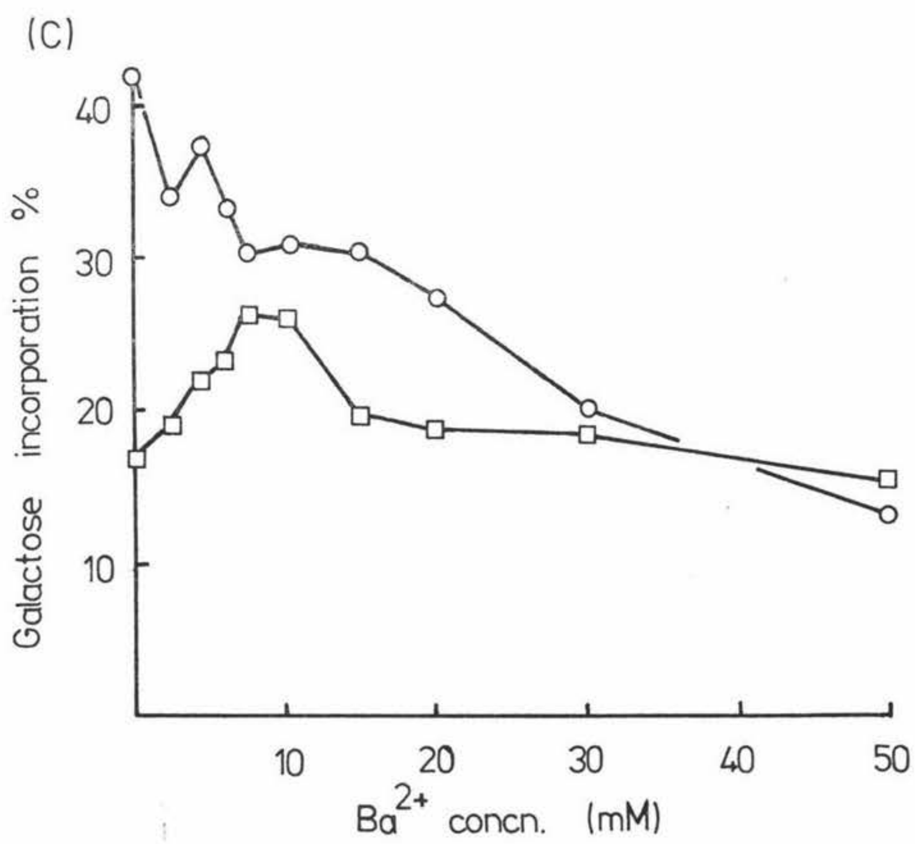


FIGURE 19 continued





4.2.11 The effect of addition of 1,2-diacylglycerol on incorporation of radioactivity from UDP-^{14}C-Galactose into lipids by chloroplast envelopes.

Ongun and Mudd (1968) and Mudd et al (1969) prepared an acetone powder of spinach chloroplasts and showed that the preparation was capable of the synthesis of MGDG from UDP-^{14}C-Galactose provided that exogenous diacylglycerol was supplied to the enzyme. The procedure of Ongun and Mudd (1968) was employed in an attempt to prepare an acetone powder of envelopes of chloroplasts. However, no acetone precipitate was obtained from an envelope preparation containing 3 mg protein. The generally hydrophobic nature of the components of a membrane such as the chloroplast envelope may explain the failure to observe an acetone precipitate since all of the lipid and most of the membrane proteins may be stable in non-polar solvents and therefore may not precipitate from acetone. Consequently, experiments on the effect of addition of diacylglycerol were carried out with a preparation of chloroplast envelopes suspended in buffer.

The effect of an exogenous source of diacylglycerol (DG) on incorporation of galactose from UDP-^{14}C-Galactose by a preparation of chloroplast envelopes was examined.

The addition of 1-palmitoyl, 2 oleoyl glycerol did not stimulate incorporation of radioactivity from UDP-^{14}C-Galactose into galactolipids (Table 21 and Figure 20). Higher concentrations of DG (above 2 mg) inhibited incorporation. The MGDG:DGDG (dpm in MGDG: dpm in DGDG) ratio was increased from 6:1 at 0.75 mg DG to 15:1 at 11 mg DG but the increase was not regular and the significance of this result is unclear.

Since the electron micrographs of the envelope pellet showed the presence of membrane vesicles it was possible that added DG may not be available to the transferase for synthesis of MGDG. Therefore the effect of sonication of the envelope preparation on utilization of DG was examined.

Sonication of chloroplast envelopes in the absence of added DG was inhibitory to the incorporation of galactose from UDP-^{14}C-Galactose (Table 22 and Figure 21). Sonication for 0.5 min reduced incorporation by 40 % but an increase in the time of sonication did not further reduce incorporation. Sonication for 1 min in the presence of 1.5 mg DG reduced incorporation by about 50 % and there was a further reduction

TABLE 21

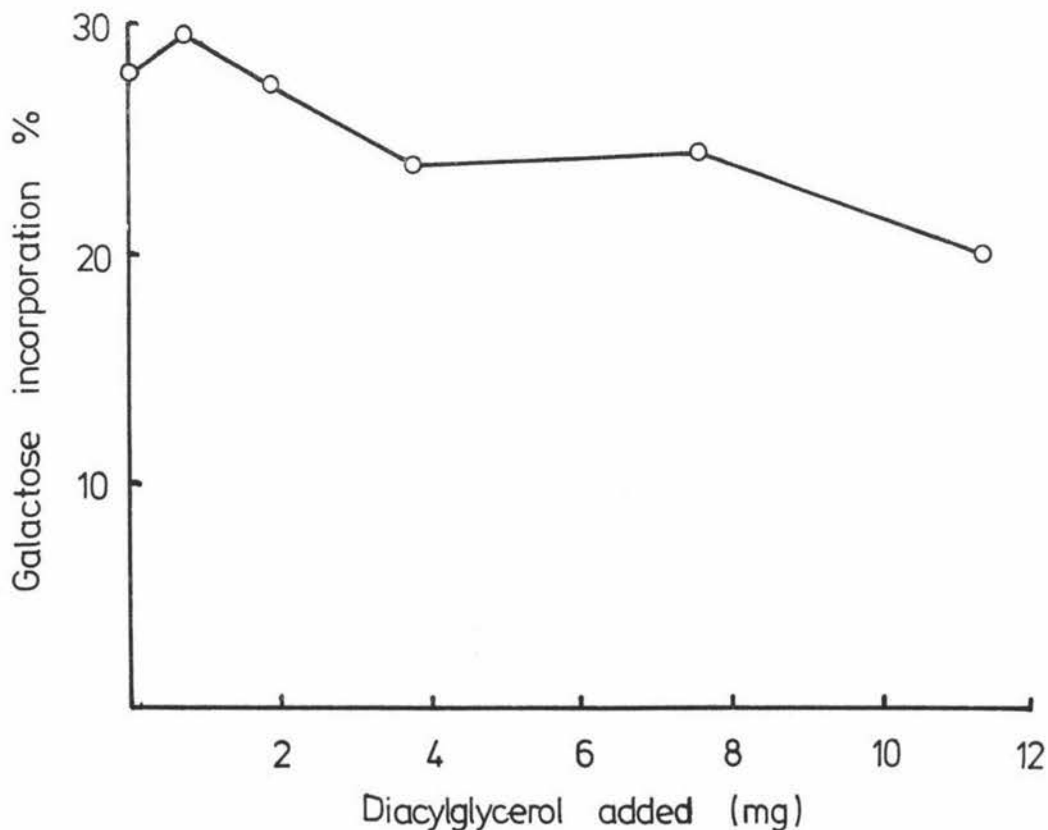
The effect of addition of 1-palmitoyl, 2-oleoyl glycerol on incorporation of radioactivity from UDP-^{14}C-Galactose by chloroplast envelopes.

Reaction mixture: The standard incubation mixture with 40 μg envelope protein, 0 - 11.3 mg DG and 10 μl UDP-^{14}C-Galactose (55,000 dpm) was incubated for 30 min at 30 ^{o}C.

Diacylglycerol added (mg)	Galactose incorporation (% of total added)	dpm in MGDG / dpm in DGDG
0	28.0	11.1
0.75	29.8	6.2
1.88	27.5	8.9
3.77	23.9	8.7
7.54	24.5	14.4
11.31	20.0	15.1

FIGURE 20

The effect of addition of 1-palmitoyl, 2-oleoyl glycerol on incorporation of radioactivity from UDP-^{14}C-Galactose by chloroplast envelopes. (see Table 21)



in incorporation as the time of sonication was increased. Therefore the general effect of sonication of the envelope preparation was to reduce the incorporation of galactose from UDP-^{14}C-Galactose into galactolipids.

TABLE 22

The effect of sonication in the presence and absence of diacylglycerol on incorporation of radioactivity from UDP-^{14}C-Galactose by chloroplast envelopes.

Reaction mixture: The standard incubation mixture with 40 µg envelope protein and with either 1.5 mg DG or with no added DG was sonicated for up to 6 min. After sonication 10 µl UDP-^{14}C-Galactose (55,000 dpm, 0.07 nmoles) was added and incubation was at 30 °C for a further 30 min.

Time of sonication (min)	Galactose incorporation (% of total added) with DG	with no DG
0	36.0	37.4
0.5	-	22.6
1	19.0	25.8
2	18.7	25.8
3	13.7	-
4	12.0	24.2
6	-	23.3

Mudd et al (1969) could not detect radioactivity in MGDG after incubation of an acetone powder of spinach chloroplasts with UDP-Galactose and ^{14}C-diacylglycerol. The experiments of Mudd et al (1969) were repeated with a preparation of chloroplast envelopes and 1-palmitoyl, 2-U-^{14}C-oleoyl glycerol (0.003 µCi per mole) and 1,2-di-U-^{14}C-linoleoyl glycerol (0.005 µCi per mole), and incorporation of radioactivity from ^{14}C-diacylglycerol into MGDG was determined after separation of the lipid components by TLC.

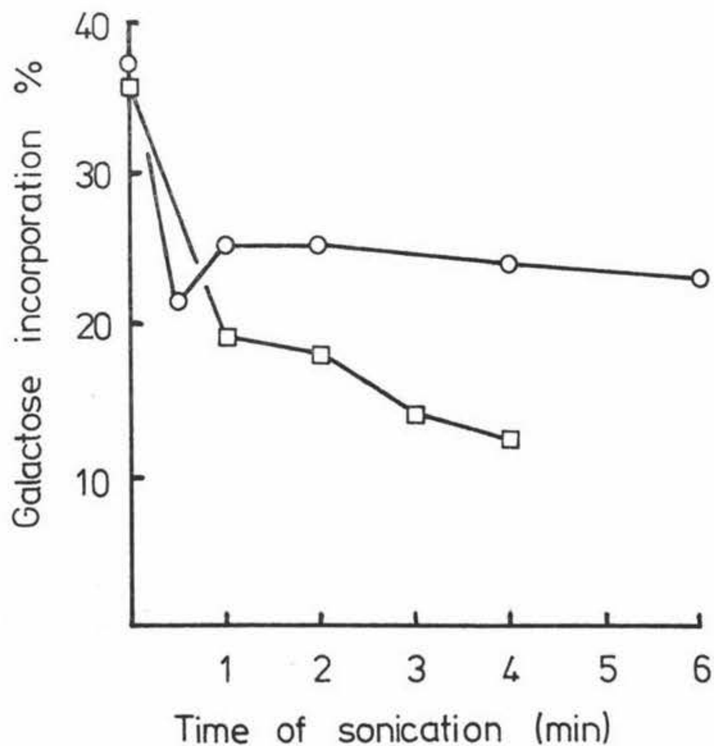
No incorporation of radioactivity from ^{14}C-diacylglycerol into MGDG was observed using 1,2-di-U-^{14}C-linoleoyl glycerol (Table 23). Similar results were obtained with 1-palmitoyl, 2-U-^{14}C-oleoyl glycerol (Data not shown). Increasing the time of incubation (up to 5 h) or sonication of the incubation containing ^{14}C-diacylglycerol did not affect the incorporation of radioactivity from ^{14}C-DG detected in MGDG. Nevertheless, radioactivity was detected in MGDG

FIGURE 21

The effect of sonication in the presence and absence of diacylglycerol on incorporation of radioactivity from UDP-^{14}C-Galactose by chloroplast envelopes. (See Table 22)

Key: □ diacylglycerol added (1.5 mg)

○ no diacylglycerol



when UDP-^{14}C-Galactose (55,000 dpm) and ^{14}C-DG were used as substrates indicating that the transferase was active in the presence of ^{14}C-diacylglycerol.

TABLE 23

The biosynthesis of ^{14}C-MGDG from ^{14}C-diacylglycerol and UDP-^{14}C-Galactose by chloroplast envelopes.

Reaction mixture: The standard incubation mixture containing 40 μg envelope protein and the additions stated in the Table were incubated for 1 h at 30 ^{o}C.

UDP-Galactose concentration	UDP-^{14}C-Galactose concentration	1,2-di-U-^{14}C-linoleoyl glycerol added	Radioactivity in (dpm)	
(mM)	(μM)	(mg)	MGDG	DG
1.4	-	0.88	0	15208
-	0.27	0.88	10501	16071
1.4	-	1.76	24	29372

4.2.12 The effect of Triton X-100 and sodium cholate on incorporation of radioactivity from UDP-^{14}C-Galactose into galactolipids by envelopes of chloroplasts.

Experiment 4.2.11 had shown that added diacylglycerol (DG) did not stimulate incorporation of radioactivity from UDP-^{14}C-Galactose into lipids and no incorporation of radioactivity from ^{14}C-DG was detected in MGDG. However, the added DG may not have been available to galactosyltransferase because of the particulate nature of the envelopes. Therefore, Triton X-100 and sodium cholate were employed as detergents in attempts to disrupt and solubilize the envelope membrane and allow access of exogenous DG to transferase. Varying concentrations of Triton X-100 and sodium cholate were incubated with or without 0.8 mg DG or 0.67 mg triacylglycerol.

Triton X-100 (Table 24 and Figure 22) and sodium cholate (Table 24 and Figure 23) in the absence of DG inhibited incorporation of galactose from UDP-^{14}C-Galactose into galactolipids by chloroplast envelopes. Triton X-100 at 0.083 % (1.2 mM, assuming 648 avg. mwt. for Triton X-100) or sodium cholate at 8 mM completely inhibited incorporation.

When 0.8 mg DG was included in the preincubation mixture both Triton X-100 (Table 24 and Figure 22) and sodium cholate (Table 24 and Figure 23) showed a change in their respective patterns of inhibition of transferase activity and a higher concentration of each detergent was required to inactivate the enzyme. In the case of Triton X-100, but not sodium cholate there was a marked stimulation of incorporation with a maximum at about 0.083 % Triton X-100. This concentration of Triton X-100 in the absence of DG completely inhibited transferase activity. All detectable transferase activity was retained in the envelope membrane. No activity was detected in any supernatant fraction following detergent treatment of chloroplast envelopes.

To determine whether the stimulation of incorporation by Triton X-100 in the presence of DG was due to the DG acting as a substrate for the synthesis of MGDG or simply to an interaction between the detergent and the added DG, 0.67 mg 1-palmitoyl, 2,3-dioleoyl glycerol was substituted for the diacylglycerol in the incubation mixtures containing Triton X-100.

Varying the concentration of Triton X-100 in the presence of triacylglycerol gave a stimulation of incorporation of radioactivity from UDP-^{14}C-Galactose into galactolipids which was similar to that observed with DG. Consequently it is unlikely that the stimulation of incorporation obtained in the presence of DG is resulting from the added DG acting as a substrate for the synthesis of MGDG by transferase.

The effect of sodium cholate on the incorporation of radioactivity from ^{14}C-diacylglycerol into MGDG by chloroplast envelopes was investigated but no incorporation of radioactivity from either 1-palmitoyl, 2-U-^{14}C-oleoyl glycerol or 1,2-di-U-^{14}C-linoleoyl glycerol was detected in MGDG from any of the experiments (Data not shown). Sonication of the incubation mixture and increasing the incubation time up to 5 h were without effect.

TABLE 24

The effect of Triton X-100 and sodium cholate on the incorporation of radioactivity from UDP-^{14}C-Galactose into galactolipids by envelopes of chloroplasts.

Reaction mixture: Lipid was sonicated into buffer containing 0.05 % (w/v) Triton X-100. 100 μl envelope fraction (40 μg protein) was incubated in the standard incubation mixture containing 0 - 0.167 % (w/v) Triton X-100 (about 0 - 2.5 mM) or 1 - 10 mM sodium cholate in the presence and absence of 0.8 mg diacylglycerol or 0.67 mg triacylglycerol for 10 min at 30 ^{o}C. After this preincubation 10 μl UDP-^{14}C-Galactose (51,000 dpm, 0.07 nmoles) was added and incubation was continued for a further 30 min at 30 ^{o}C.

Triton X-100 concentration (%)	Galactose incorporation (% of total)		
	no addition	diacylglycerol added	triacylglycerol added
0	37.0	21.0	15.5
0.0097	28.0	18.1	15.5
0.019	-	12.3	-
0.029	17.9	24.6	26.8
0.039	-	23.8	-
0.048	8.2	32.3	33.6
0.063	-	33.9	31.5
0.083	0.2	34.9	33.0
0.111	-	28.4	22.5
0.139	0	18.5	19.1
0.167	-	8.6	-

Na cholate concentration (mM)	Galactose incorporation (% of total)	
	no addition	diacylglycerol added
0	44.6	32.4
1	34.1	36.3
2	38.0	36.6
3	35.4	38.7
4	25.0	-
5	11.8	32.9
6	1.2	25.8
8	0	13.5
10	0	4.9

FIGURE 22

The effect of Triton X-100 in the presence and absence of diacylglycerol and triacylglycerol on incorporation of radioactivity from UDP-^{14}C-Galactose into galactolipids by envelopes of chloroplasts. (see Table 24)

Key: ○ with 0.8 mg diacylglycerol

● with 0.67 mg triacylglycerol

□ Control (no addition)

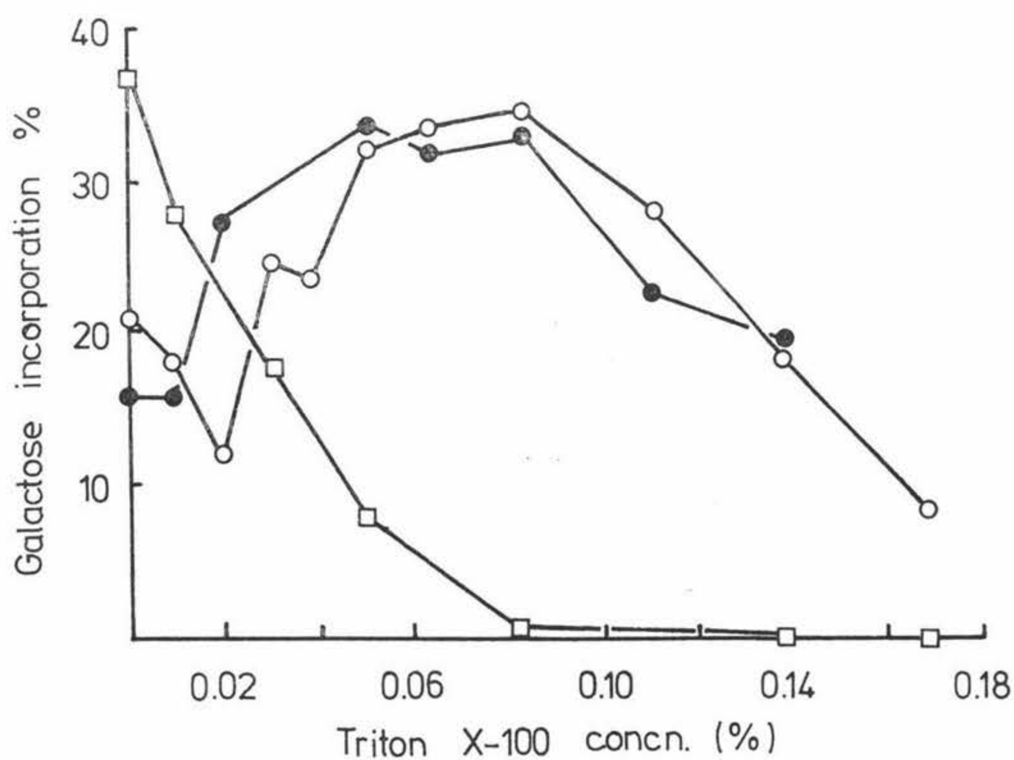
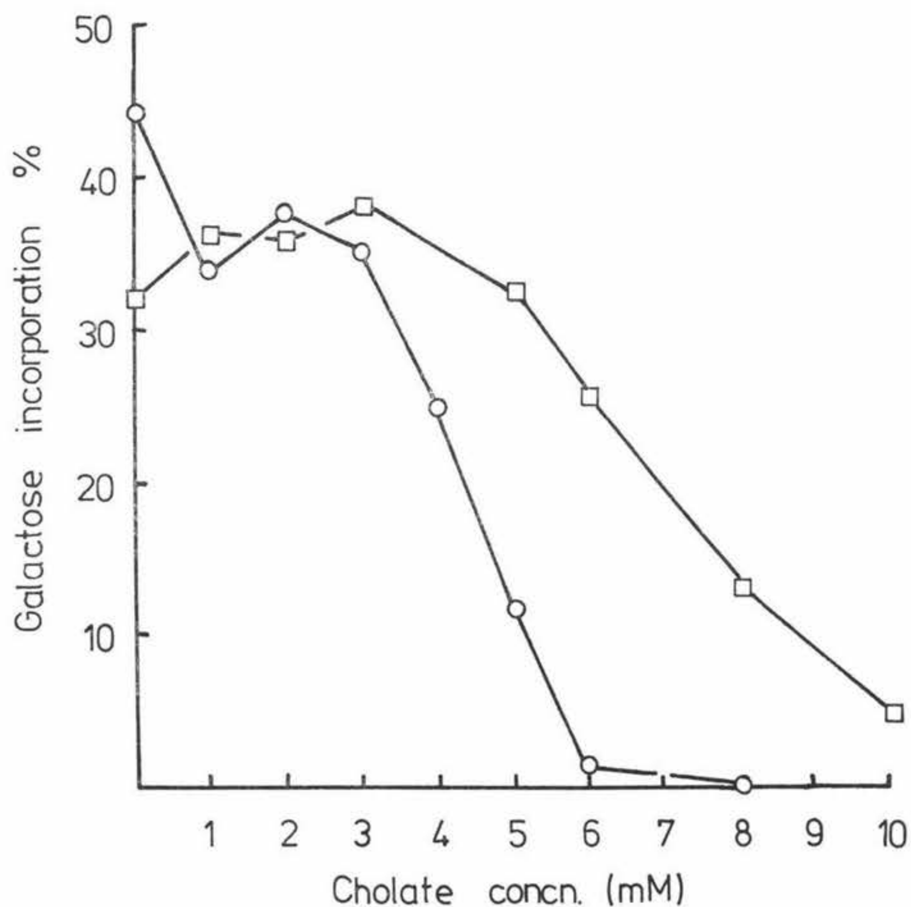


FIGURE 23

The effect of sodium cholate in the presence or absence of diacylglycerol on incorporation of radioactivity from UDP-^{14}C-Galactose into galactolipids by envelopes of chloroplasts. (see Table 24)

Key: □ Control (no addition)

○ with 0.8 mg diacylglycerol



4.2.13 The effect of phospholipase C and D on incorporation of radioactivity from UDP-^{14}C-Galactose into lipids by chloroplast envelopes.

The apparent inactivation of transferase by detergent action on the membrane-bound enzyme (4.2.12) indicated that the enzyme may be dependent for activity on a component of the membrane. The chloroplast envelope is rich in phospholipid (Mackender and Leech, 1974 ; Poincelot, 1973 ; Hashimoto and Murakami, 1975) and consequently it was of interest to examine the effect of phospholipases on the activity of the transferase. The effect of phospholipase C was of particular interest because the hydrolysis product of this enzyme (diacylglycerol) may provide an endogenous source of substrate for the synthesis of MGDG by transferase.

Incubation of chloroplast envelopes with phospholipase C for 10 min had a very slight inhibitory effect on incorporation of radioactivity from UDP-^{14}C-Galactose by galactosyltransferase (Table 25 and Figure 24) but longer periods of incubation led to about 50 % inhibition. Incubation of the envelopes with phospholipase D had a more immediate inhibitory effect and reduced incorporation to a greater extent (80 % inhibition after 3 h incubation) than phospholipase C. Turbidity of the incubation mixture was noted after 30 min of incubation with phospholipase C, but no turbidity was observed during incubation with phospholipase D. The turbidity may be due to accumulation of lipid products (diacylglycerol) of the digestion.

The inhibition of transferase activity may be due to a disruption effect of the phospholipases on the membrane or its components or to an accumulation of the products of phospholipase digestion (phosphatidic acid, diacylglycerol). However, the similar inhibitory response with both phospholipase C and D would suggest that the integrity of the envelope membrane and particularly the phospholipid component of the membrane may have an important role in the activity of the galactosyltransferase.

Phospholipids isolated from spinach and hens egg were sonicated into buffer containing 0.1 % Triton X-100 (w/v) and were added to chloroplast envelope that had been incubated with phospholipase C or D for 30 min. No stimulation of incorporation of radioactivity

from UDP-^{14}C-Galactose into galactolipids was obtained by addition of 1 - 50 μg phosphatidylcholine or phosphatidylglycerol from spinach, or phosphatidylcholine from hens egg. The addition of the phospholipid to the partially digested envelope membrane gave a further inhibition of transferase activity and reduced total incorporation in all incubations to between 2 - 15 %.

TABLE 25

The effect of phospholipase C and D on incorporation of radioactivity from UDP-^{14}D-Galactose into lipids by chloroplast envelopes.

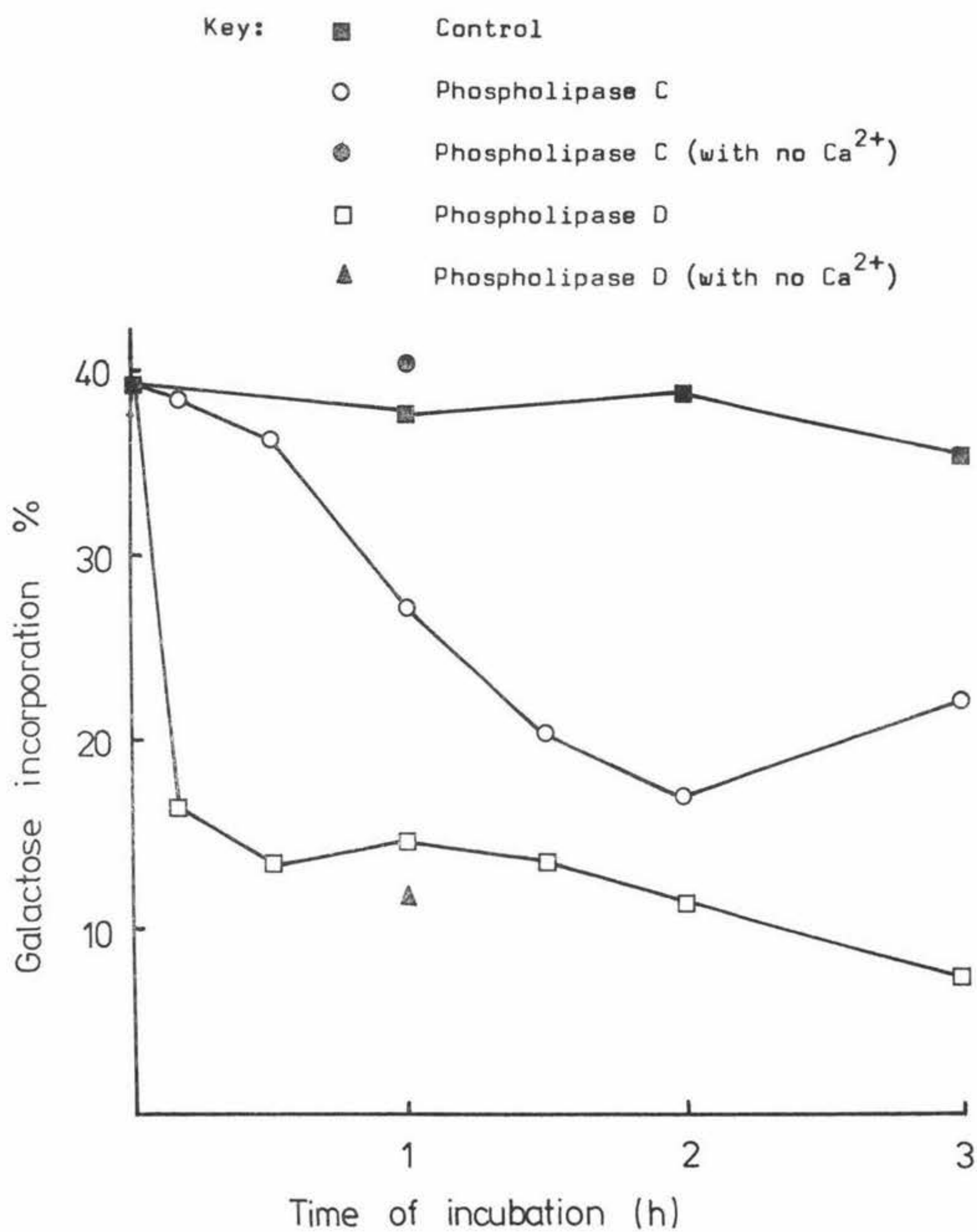
Reaction mixture: The standard incubation mixture containing 40 μg envelope protein, 20 mM Ca^{2+}, and 0.37 units phospholipase C (1 unit releases 1 μmole P_i min^{-1} at pH 7.3) or 0.001 units phospholipase D (1 unit catalyses the cleavage of 1 μmole lecithin h^{-1} at pH 7.0) was incubated for up to 180 min at 30 ^{o}C (Berry et al 1978). After the times of incubation given in the Table, 10 μl UDP-^{14}C-Galactose (51,000 dpm, 0.07 nmoles) was added and incubation was continued for a further 30 min at 30 ^{o}C.

Time of incubation (min)	Galactose incorporation (% of total added)		
	Control	with phospholipase C	with phospholipase D
0	39.0	-	-
10	-	38.7	16.3
30	-	36.3	13.7
60	37.4	27.2 (40.1)*	14.8 (11.4)*
90	-	20.4	13.1
120	39.0	17.2	11.4
180	35.1	22.3	7.8

(* incubation in the absence of Ca^{2+})

FIGURE 24

The effect of phospholipase C and D on incorporation of radioactivity from UDP-^{14}C-Galactose into lipids by chloroplast envelopes.



4.2.14 The effect of proteolytic digestion of chloroplast envelopes with trypsin or protease on incorporation of radioactivity from UDP-^{14}C-Galactose.

Complimentary to the investigation of the effect of phospholipases on the lipid components of the membrane was an examination of the effect of hydrolysis of the protein components of the membrane. Consequently the effect on transferase activity of treatment of the envelopes with trypsin or protease was examined.

The envelope membranes after tryptic digestion and before gel electrophoresis were washed by centrifugation and resuspension in buffer in an attempt to remove the trypsin. Gel electrophoresis of the digested and the undigested envelope pellets was performed as described in methods (3.1.9).

Incorporation of radioactivity from UDP-^{14}C-Galactose into lipids by chloroplast envelopes was reduced by about 60 % after incubation for 30 min with 50 µg protease (Table 26). Protease digestion of the envelope preparation for 30 min gave coagulation of the envelopes and this may account for some of the inhibition of incorporation. Incubation with 100 µg trypsin reduced incorporation by about 30 %. Proteolytic digestion had no effect on the ratio of radioactivity incorporated into MGDG and DGDG.

It appears that galactosyltransferase is relatively resistant to digestion with a 2:1 ratio (w/w) of trypsin to envelope protein. The effect of protease is complicated by the precipitation of the envelopes observed after incubation with this enzyme.

The gel electropherograms obtained from the digested and the undigested envelope pellets are given in Figure 25. The undigested envelope gave a polypeptide pattern similar to that observed by Sprey and Laetsch (1975) with about 9 major, distinguishable protein bands. The digested envelopes gave one broad protein band (as observed by Joy and Ellis, 1975) which ran close behind the dye front on the gel. No other protein bands were observed.

TABLE 26

The effect of proteolytic digestion of chloroplast envelopes with trypsin or protease on incorporation of radioactivity from UDP-^{14}C-Galactose into lipids.

Reaction mixture: The standard incubation mixture at pH 8.0 contained 60 μg envelope protein, 20 mM Ca^{2+} and 100 μg trypsin or 50 μg protease. After preincubation for 30 min at 30 ^{o}C, 10 μl of UDP-^{14}C-Galactose (55,000 dpm, 0.07 nmoles) was added and incubation was continued for a further 30 min at 30 ^{o}C.

	Galactose incorporation (% of total added)	dpm in MGDG / dpm in DGDG
Control	37.7	13
Trypsin (100 μg)	24.9	14
Protease (50 μg)	15.6	14

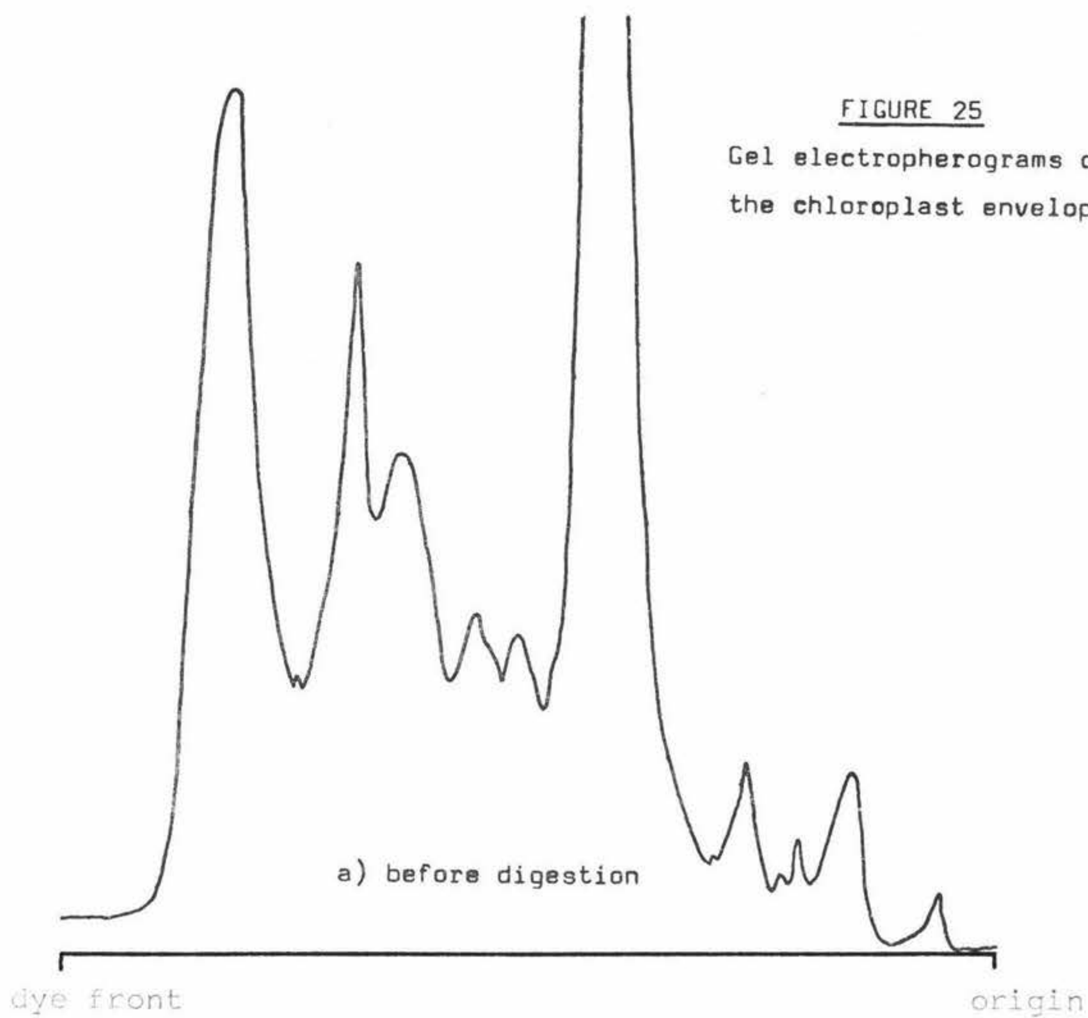
4.2.15 The fatty acid composition of MGDG, DGDG and DG isolated from whole tissue, chloroplasts and chloroplast envelopes of spinach.

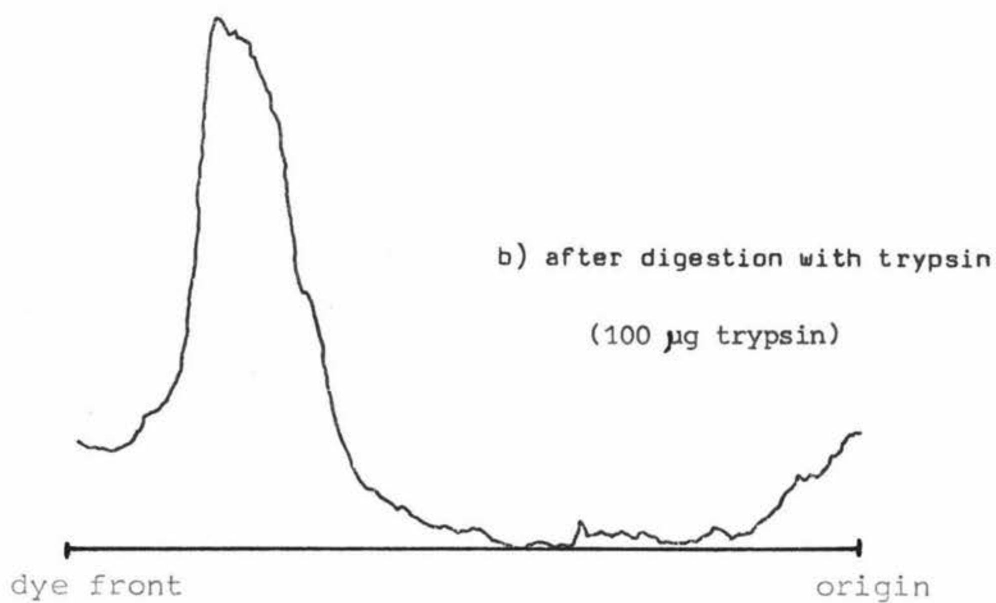
The fatty acid composition of MGDG and DGDG from chloroplasts and chloroplast envelopes of spinach has been extensively studied (Kuiper, 1970 ; Douce et al 1973). Although Joyard and Douce (1976 c) commented that the constituent fatty acids of DG contained high amounts of 18:3 and 16:3,data on the composition was not presented. The fatty acid composition of DG from whole tissue, chloroplasts and chloroplast envelopes is of particular interest in view of the claims that highly unsaturated diacylglycerol is the preferred substrate for MGDG synthesis by transferase (Mudd et al 1969).

The same batch of tissue was used for all of the preparations. Chloroplasts were isolated from 115 g leaf tissue by the method of Leese et al (1971) (methods 3.2) and envelopes were prepared from 300 g tissue (methods 3.2.2). The lipid components were isolated from whole tissue (80 g), chloroplasts and chloroplast envelopes as described in methods (3.2.3) and were separated by TLC. A known amount of heptadecanoic acid (17:0) was added prior to methylation of the fatty acids to serve as an internal standard for calculations from the GLC data.

FIGURE 25

Gel electropherograms of the chloroplast envelope.





The total amounts of MGDG, DGDG and DG from whole tissue, chloroplasts and chloroplast envelopes of spinach, calculated from the GLC data, are given in Table 27.

The fatty acid composition of MGDG, DGDG and DG in each fraction is given in Table 28.

TABLE 27

The MGDG, DGDG and DG content of whole tissue, chloroplasts and chloroplast envelopes of spinach.

Whole tissue (µmoles per mg chlorophyll)			Chloroplasts (µmoles per mg chlorophyll)			Chloroplast envelopes (nmoles per mg envelope protein)		
MGDG	DGDG	DG	MGDG	DGDG	DG	MGDG	DGDG	DG
5.4	4.2	0.4	3.15	1.09	0.05	295	470	58

The MGDG:DGDG ratio of the envelopes is significantly different to the ratio of these lipids obtained from chloroplasts. Assuming a mg lipid per mg protein ratio of 1.74:1 for the envelopes (Poincelot and Day, 1974) DG would account for about 2 % of the total lipid of the chloroplast envelope.

The galactolipids MGDG and DGDG from whole tissue, chloroplasts and chloroplast envelopes are rich in highly unsaturated fatty acids. As observed by previous workers (Kates, 1970 ; Douce et al 1973) MGDG contains high amounts of 18:3 and 16:3 and DGDG is rich in 18:3. In contrast the DG isolated from chloroplasts and whole tissue contains high amounts of the fatty acids 18:1 and 16:0. The DG isolated from chloroplast envelopes has a fatty acid composition intermediate between the highly unsaturated MGDG and the saturated DG of the chloroplast.

TABLE 28

The fatty acid composition of MGDG, DGDG and DG isolated from whole leaf tissue, chloroplasts and chloroplast envelopes of spinach.

Procedures: See text for analytical procedures.

Fatty acid	(% of total fatty acids by weight)								
	Whole tissue			Chloroplasts			Chloroplast Envelopes		
	MGDG	DGDG	DG	MGDG	DGDG	DG	MGDG	DGDG	DG
14:0	0.1	trace	1.1	0.2	0.4	trace	1.92	0.7	4.1
16:0	0.9	14.6	62.4	1.4	11.5	62.8	15.5	25.3	26
16:1	0.3	trace	1.8	0.4	trace	trace	2.0	trace	trace
16:3	25	1.4	-	29.8	1.8	-	18.6	3.3	7.3
18:0	0.6	7.0	3.7	trace	2.3	9.2	4.8	1.9	8.3
18:1	0.7	2.2	19.4	0.4	3.2	21	5.2	4.6	14.2
18:2	1.0	5.6	6.9	0.8	2.8	4.4	2.2	3.4	7.3
18:3	71.4	69.2	4.7	67.0	78	2.6	46.8	60.7	32.7
20:1	-	-	-	trace	-	-	2.9	-	trace

CHAPTER 5 DISCUSSION

Methods for the isolation of chloroplast envelopes have been described for broad bean (Mackender and Leech, 1971) and spinach (Poincelot, 1973 ; Douce et al 1973) which involve disruption of isolated chloroplasts by homogenization after osmotic bursting and then centrifugation of the chloroplast fragments on a discontinuous sucrose gradient. The procedure of Douce et al (1973) was followed in the present study to isolate envelopes from chloroplasts of maize and spinach. It gave low yields of envelopes from purified chloroplasts of maize and the resultant envelope pellet contained very low galactosyltransferase activity. Spinach chloroplasts yielded up to 20 fold more envelope protein and the envelopes gave high incorporation (up to 70 %) of radioactivity from UDP-^{14}C-Galactose into MGDG.

However, as reported by Joyard and Douce (1976b), the procedure of Douce et al (1973) has two major disadvantages which are the requirements for a purified chloroplast preparation and for the inclusion of Mg^{2+} in the buffers of the discontinuous sucrose gradient. In early experiments of the present work the procedure of Leese et al (1971) was used to prepare purified chloroplasts which were suitable for use in the procedure of Douce et al (1973). These procedures resulted in a yield of envelopes of about 2 mg envelope protein per kg starting material. The envelope pellet required up to 5 h preparation time and generally contained small amounts of chlorophyll contamination.

The method for the preparation of envelopes of spinach chloroplasts reported by Poincelot (1973) was further developed by Poincelot and Day (1974) and these workers succeeded in preparing contaminant-free envelopes in higher yields and with a more regular appearance under electronmicroscopy than the membranes isolated by Douce et al (1973) and Joyard and Douce (1976b). The essential feature of the Poincelot and Day (1974) method was a modification of the discontinuous sucrose gradient which led to a more complete separation of the envelopes from the lamellae and other chloroplastic material. This technique resulted in up to 30 % higher yield of less contaminated envelopes from a crude preparation of chloroplasts and a decrease in the preparation time required for the isolation of the envelopes. Consequently the method of Poincelot and Day (1974) was employed in the present study for the routine

preparation of chloroplast envelopes from spinach.

The envelopes of spinach chloroplasts isolated by Douce et al (1973) and Poincelot (1973) were reported to contain a specific Mg^{2+}-dependent ATPase activity. The modified procedure of Poincelot and Day (1974) that gave improved yields of double membraned envelopes also gave up to 10 fold higher Mg^{2+}-dep ATPase activity (80 μmoles P_i h^{-1}mg protein^{-1}). These workers suggested that the ATPase may be associated with the intact double-membraned envelopes and may be lost during isolation of the single-membraned envelopes.

Fractions from the modified discontinuous gradient of the present study, reported to be rich in single-membraned (fraction 1) and double-membraned (fraction 2) envelopes (Poincelot and Day, 1974) did not show significantly different ATPase activities. The low detectable ATPase activity (about 5.3 μM Pi formed , h^{-1}, mg protein^{-1}) in the double-membraned fraction may indicate that this fraction contains a high proportion of single membranes or that the ATPase is easily lost from the envelope during the isolation procedure employed in the present study. However, the double-membraned envelope fraction (fraction 2) appeared, under electron-microscopy, to consist of a high proportion of double-membraned envelopes, which would suggest that the ATPase activity may have been lost during envelope isolation or that the assay system for the ATPase failed to detect this enzyme.

Douce (1974), Van Hummell et al and Joyard and Douce (1976b) reported that a highly specific galactosyltransferase activity was associated with the chloroplast envelope which was concentrated in fraction 2 of the discontinuous sucrose gradient. This was confirmed in the present work. Sub-chloroplast fractions that were prepared by centrifugation on the modified discontinuous gradient showed a distribution of galactosyltransferase similar to that found by Douce et al (1973) with the double-membraned fraction (fraction 2) incorporating about 60 % of the added radioactivity from UDP-^{14}C-Galactose into galactolipids. It is of interest that both the single-membraned fraction (fraction 1) and the double-membraned fraction had a similar specific activity for galacto-syltransferase. If these fractions consist of primarily single and double-membraned envelopes respectively then this would suggest

that the transferase is relatively firmly bound to one or both of the envelope membranes.

Mudd et al (1969) reported that up to 40 % of the detectable chloroplast galactosyltransferase in a spinach leaf homogenate was not sedimented by centrifugation at 100,000 x g for 1 h. However, in the present study all of the galactosyltransferase activity in the supernatant from the modified discontinuous gradient was pelleted by centrifugation at 80,000 x g for 30 min, i.e. no evidence was obtained of a soluble galactosyltransferase, in the supernatant of a homogenate of spinach chloroplasts.

Mudd et al (1969) and Chang and Kulkarni (1970) have examined several characteristics of the galactosyltransferase of spinach chloroplasts and in particular the pH, temperature, cation and substrate dependence of incorporation of radioactivity from UDP-^{14}C-Galactose into galactolipids. Similar aspects of the nature of the galactosyltransferase of the spinach chloroplast were examined in the present study using chloroplast envelopes as the source of the enzyme.

Mudd et al (1969) reported a dependence of transferase activity of an acetone powder of spinach chloroplasts on the temperature of incubation with maximum incorporation (30 % of the added radioactive label) obtained at 40 ^{o}C. Incubation at 60 ^{o}C reduced the total incorporation to about 10 %. Galactosyltransferase activity of the chloroplast envelope showed a similar dependence on the temperature of incubation and increasing the temperature from 23 ^{o}C to 45 ^{o}C increased the total incorporation after 30 min from 40 % to 54 % with a maximum of 53 % at 30 ^{o}C.

It is possible that the membrane-bound nature and consequent lipid association of galactosyltransferase may help to maintain transferase activity at high incubation temperatures such as 45 ^{o}C. Furthermore the high optimum temperature of incubation may be explained by the effect of temperature on the kinetics of interaction between the enzyme and the substrates, particularly the endogenous diacylglycerol. Higher incubation temperatures may promote a

greater freedom of movement for both the protein and the lipid components of the membrane (Singer and Nicholson, 1972).

Mudd et al (1969) and more recently Van Besouw and Wintermans (1978) reported that the optimum pH of incubation for incorporation of radioactivity by transferase of the spinach chloroplast was pH 7.2 - 7.5 and at this pH MGDG appeared to be the major labelled lipid. At lower pH values higher proportions of DGDG were synthesised. A similar trend was noted in the present study on transferase of the chloroplast envelope, however, the optimum pH for incorporation was about pH 8.0. The nature of the incubation buffers used in the present study had a significant effect on the total incorporation of radioactivity into the lipid fraction.

HEPES-NaOH at pH 8.0 gave the highest total incorporation of 72 % of the added label. Maximum incorporation with Tris-HCl was at pH 8.5 (67 %) and Tricine-NaOH at pH 9.0 (61 %). The distribution of radioactivity between MGDG and DGDG was influenced by the nature of the buffer. Tricine-NaOH at all pH values gave higher levels of label in DGDG than Tris or HEPES. HEPES-NaOH at 50 mM and pH 8.0 was employed as the standard incubation buffer because under these conditions a high proportion of the radioactivity was incorporated into MGDG, thus any observed effect on incorporation would, primarily, be an effect on transferase involved in the synthesis of MGDG.

The specific activity of galactosyltransferase in envelopes isolated from spinach chloroplasts was about 55 pmoles min^{-1} mg protein^{-1} when incubated at 30 ^{o}C and pH 8.0. Joyard and Douce (1976c) had reported transferase activity of about 15 nmoles min^{-1} mg protein^{-1} in chloroplast envelopes and more recently Van Besouw and Wintermans (1978) had obtained transferase activity of about 45 nmoles min^{-1} mg protein^{-1}. The concentration of UDP-^{14}C-Galactose in the present study was about 0.3 μM in comparison to 0.5 mM used by Joyard and Douce (1976c) and Van Besouw and Wintermans (1978) which may account for much of the difference in the calculated transferase activity. Nevertheless some of the variation could be due to variations in the age of tissue, method of isolation of chloroplasts and envelopes and to the different incubation conditions (pH, temperature, cation concentration) used by different

workers. The light and temperature environment of the growing tissue have been reported to influence the level of galactolipid in leaf tissue (Gray et al 1967 ; Kuiper, 1970). Large variations in the lipid composition of leaf tissue with respect to the maturity of the tissue have been reported (Fong and Heath, 1977 ; Hawke et al 1974) and the lipid composition may reflect, to some degree, the level of the biosynthetic enzymes in tissue. Variations in transferase activity may also be a consequence of the different techniques used by different workers to isolate chloroplast envelopes. Van Besouw and Wintermans (1978), Joyard and Douce (1976bc) and Douce (1974) used the method of Douce et al (1973) to isolate envelopes. In the present study this method was inferior in the yield of envelope protein and degree of contamination of the envelopes to the method of Poincelot and Day (1974) and consequently the latter method gave an increased specific activity of galactosyltransferase over the method of Douce et al (1973).

The reported K_m for UDP-Galactose for galactosyltransferase is about 40 μM (Van Besouw and Wintermans, 1978). The ideal substrate concentration for the study of enzymatic reactions is about 10-100 x K_m and consequently the UDP-^{14}C-Galactose concentration (0.3 μM) in the present study must be assumed to be rate-limiting. It was impractical to use sufficiently high molar concentrations of UDP-^{14}C-Galactose therefore the addition of unlabelled carrier UDP-Galactose was investigated. However, when unlabelled UDP-Galactose was added to the UDP-^{14}C-Galactose to give a final UDP-Galactose concentration of 30 μM, maximum incorporation was reduced by about 80 % which greatly reduced the amount of radioactive lipid available for analysis. The membrane bound (particulate) nature of galactosyltransferase poses further problems of the rate-limitation as the added substrates and effectors may not be fully accessible to the enzyme and this may lead to heterogeneity of the micro-environment of the assay (Martensson et al 1974).

Chang and Kulkarni (1970) concluded that galactosyltransferase of spinach chloroplasts had no cation requirement for the synthesis of MGDG and none of the cations tested gave a stimulation of incorporation. However these workers used a relatively crude preparation of transferase obtained from a homogenate of spinach chloroplasts and the concentration of Mg^{2+} carried over from the isolation buffers

may have been sufficient to maintain transferase activity. The early studies on transferase of the chloroplast envelope (Douce, 1974 ; Van Hummel et al 1975) were in the presence of Mg^{2+} in the incubation buffer. Van Besouw and Wintermans (1978) reported that 10 mM Mg^{2+} was required in the incubation buffer to give maximum incorporation of radioactivity from UDP-^{14}C-Galactose into galactolipids by the transferase of the chloroplast envelope. Veerkamp (1974) reported a dependence of galactosyltransferase of a bacterial fraction on added Mn^{2+}, Mg^{2+}, Co^{2+} or Fe^{2+} and Fraser and Mookerjea (1977) have described a dependence of galactosyltransferase of rat liver microsomes on added Mn^{2+}.

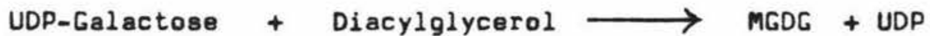
Chloroplast envelopes isolated in buffers containing Mg^{2+} showed no cation dependence. However, when the envelopes were isolated in buffers containing no Mg^{2+} a cation dependence of galactosyltransferase activity was observed. About 16 % of the added radioactive label was incorporated into galactolipids in the absence of added cations and addition of 5 mM Mg^{2+}, Ca^{2+} or Ba^{2+} increased total incorporation to about 47 %, 46 % and 52 % respectively. When the concentrations of the cations were varied over the range 0.1 - 50 mM each cation gave maximum incorporation at about 2.5 mM. Incorporation was increasingly inhibited by cation concentrations above 5 mM. Of the other cations tested (Li^{+}, K^{+}, Na^{+}, Co^{2+}, Cu^{2+}, Mn^{2+}, Cs^{2+}) none significantly stimulated transferase activity and 5 mM Cu^{2+} completely inhibited incorporation.

Ba^{2+} is an unusual cation to stimulate an enzymic reaction and Ca^{2+} with its tendency to form insoluble salts of free fatty acids would appear to be a most unlikely effector for a membrane-bound enzyme involved in the synthesis of lipids.

A similar cation requirement to the chloroplast transferase is shown by a bacterial galactosyltransferase (Veerkamp, 1974). Both are stimulated by Mn^{2+}, Ca^{2+} and Mg^{2+}, and Cu^{2+} was completely inhibitory. The effect of Mg^{2+} up to 50 mM was similar for both enzymes but the data for Ca^{2+} and Ba^{2+}, over this concentration range, was not reported for the bacterial enzyme. Furthermore, the pH dependence of the distribution of radioactivity between MGDG and DGDG and the polygalactolipids was similar for both enzymes; lower

pH values result in an increase incorporation into DGDG and higher pH values favour the synthesis of MGDG.

The influence of substrate, product or cofactor concentrations on the galactosyltransferase reaction has not been reported and some of these aspects were examined in the present work. The most effective nucleotide inhibitor is UDP, a product of the reaction:



UDP gave 50 % inhibition of the reaction at about 5 μM, whereas 500 μM UMP or 100 μM UTP were required to give a similar level of inhibition. NADH and PP_i were without effect. The low concentration of UDP required for inhibition may pose a problem of control for the plant cell.

Kuhn and White (1977) investigated the role of a nucleoside diphosphatase involved in the synthesis of lactose in rat mammary gland where a similar inhibitory effect of UDP at low concentrations was reported. In this system lactose is synthesised by the reaction:

and UDP was found to strongly inhibit the reaction. Kuhn and White (1977) reported that a nucleoside diphosphatase in the golgi apparatus could catalyse the reaction:

and thus maintain the UDP concentration in the golgi lumen at a sufficiently low level to prevent inhibition of galactosyltransferase. Furthermore a compartmentation of the enzyme and its products was proposed in that UDP-Galactose is provided to the enzyme on the cytosol side of the golgi membrane and lactose is synthesised by the membrane-bound enzyme of the golgi lumen. The UDP product from transferase activity is dephosphorylated by the nucleoside diphosphatase and released back to the cytosol as UMP. The UMP is then reconverted to UDP by a UMP kinase. Both membranes of the golgi apparatus are impermeable to UDP and therefore UDP in the cytosol is not capable of inhibiting transferase. In the plant

cell, UDP-Galactose available from the cytosolic side of the chloroplast envelope is utilised by a membrane-bound galactosyltransferase for the synthesis of MGDG. UDP or a corresponding uridine nucleotide may be released back to the cytosolic side of the envelope for reconversion to UDP-Galactose from ATP and glucose-1-phosphate (Konigs and Heinz, 1974). Consequently there is an interesting parallel between lactose synthesis in the rat mammary gland and MGDG synthesis in the plant cell. Both pathways involve membrane-bound enzymes utilizing UDP-Galactose as a substrate and catalysing a reaction giving UDP as a product, moreover both transferases are inhibited by low concentrations of UDP.

However, there are differences in the permeability of the chloroplast envelope and the golgi membrane. Heldt and Sauer (1971) reported that the inner of the two membranes of the chloroplast envelope was a permeability barrier and that the outer membrane was relatively permeable. Therefore unlike the golgi apparatus, UDP in the cytosol of the plant cell may be capable of interacting with galactosyltransferase unless this enzyme is situated on the inner side of the inner envelope membrane and consequently inside the permeability barrier for UDP.

Okamoto and Katoh (1977) reported that the addition of linoleic acid to a preparation of chloroplasts led to an inhibition of the electron transport system of the chloroplast and they postulated that this inhibition was due to penetration of the fatty acid into the hydrophobic region of the chloroplast lamellae membrane. The membrane-bound nature of galactosyltransferase of the chloroplast envelope prompted an investigation into the effect of an unsaturated fatty acid on its activity. Linoleic acid, at 0.7 mM, in the incubation mixture completely inhibited galactosyltransferase activity. Inhibition was partially removed by addition of 10 mM Ca^{2+} or Ba^{2+} but not 10 mM Mg^{2+}. The effect of the cations Ca^{2+} and Ba^{2+} on the inhibition by linoleic acid may be due to the ability of these cations to form insoluble salts of free fatty acids, e.g. in pancreatic lipase digestion Ca^{2+} is used to form insoluble salts of the released free fatty acids and so prevent inhibition of the enzyme by the fatty acid. This may account for the failure to

observe a reduction in inhibition when Mg^{2+} was used as the cation as salts of this cation are generally soluble. BSA was reported to be an effective scavenger of free fatty acids (Knudsen et al 1976 ; Okamoto and Katoh, 1977) but this protein failed to remove inhibition by linoleic acid. Variations in the effect of BSA on inhibition of the photochemical activities of chloroplasts have been reported. Friedlander and Neumann (1968) showed that BSA stimulated chloroplast electron transport in the presence of lipolytic enzymes, however, Wasserman and Fleischer (1968) suggested that BSA was not stimulating activity by binding free fatty acids. Okamoto and Katoh (1977) reported that at high free fatty concentrations BSA was not effective in reversing all of the inhibition by free fatty acids of the electron transport system.

From the results of the present study it is not clear whether inhibition by linoleic acid is a result of a general disruption of the integrity of the envelope or due to a direct effect of the fatty acid on galactosyltransferase.

The fatty acid composition of the endogenous diacylglycerol which is substrate for galactosyltransferase in the synthesis of MGDG is unknown. Joyard and Douce (1976c) reported that diacylglycerol isolated from envelopes of spinach chloroplasts contained high amounts of 18:3 and 16:3, however these workers gave no supporting data. Previous workers have reported differing effects of added DG on galactosyltransferase of an acetone powder of spinach chloroplasts. Although Mudd et al (1969) found that the addition of unsaturated DG to an acetone powder gave the highest stimulation of galactosyltransferase activity, Eccleshall and Hawke (1971) showed that diacylglycerols of differing degrees of unsaturation of the fatty acids gave a similar stimulation of galactosyltransferase activity.

The ratios of galactolipid and diacylglycerol in the chloroplasts and chloroplast envelopes are in close agreement with those obtained by Kuiper (1970) for chloroplasts and Douce et al (1973), Poincelot, (1973) and Joyard and Douce, (1976b) for chloroplast envelopes. MGDG, DGDG and DG are present in whole spinach leaves in the ratio of about 10:8:1 (MGDG:DGDG:DG). This ratio in chloroplasts is 60:20:1 and in the chloroplast envelope is 5:8:1. The envelope appears to be rich in DGDG (Mackender and Leech, 1974) and DG. The ratio of DG found

in the present study is considerably lower than the (MGDG:DGDG:DG) 1.5:2:1 figure reported by Joyard and Douce (1976b).

The fatty acid composition of MGDG and DGDG from whole tissue, chloroplasts and chloroplast envelopes reported by Kuiper (1970) and Douce et al (1973) and the results of the present study are in general agreement. MGDG contained characteristically high amounts of 18:3 and 16:3 and DGDG contained high amounts of 18:3 and smaller amounts of 16:0. DG from whole tissue and chloroplasts was rich in 18:1 and 16:0. Joyard and Douce (1976c) reported that DG from envelopes of spinach chloroplast envelopes contained high amounts of 18:3 and 16:3. In contrast, DG from envelopes of the present study was found to contain significant amounts of 18:1 and 16:0 in addition to 18:3 but very small amounts of 16:3. Therefore DG from chloroplast envelopes would appear to contain a mixture of saturated and unsaturated fatty acids. This may suggest that highly unsaturated diacylglycerols are not available as substrates for galactosyltransferase and may indicate that desaturation steps occur after galactosylation of the DG to give the characteristic highly unsaturated MGDG.

These results would be consistent with the report by Eccleshall and Hawke (1971) as to the non-specificity of stimulation of galactosyltransferase activity by DG of varying fatty acid composition. Furthermore these results are supported by the recent work of McKee and Hawke (pers.comm.) on the appearance of 18:1 and 16:0 in MGDG synthesised by spinach chloroplasts. However, these results are in direct contrast to the work of Mudd et al (1969) which suggested that transferase was specific towards the fatty acid composition of DG and to the work of Joyard and Douce (1976c) who reported that DG from chloroplast envelopes contained high amounts of 18:3 and 16:3.

Ongun and Mudd (1968) demonstrated that incorporation of radioactivity from UDP-^{14}C-Galactose into MGDG by an acetone powder of spinach chloroplasts was stimulated by addition of exogenous diacylglycerol (DG) and Mudd et al (1969) then reported that DG containing polyunsaturated fatty acids gave maximum stimulation of incorporation. However, they were unable to prepare a ^{14}C-DG of sufficiently high specific activity to measure incorporation of radioactivity from

^{14}C-DG into MGDG by the acetone powder. Ongun and Mudd (1968) and Eccleshall and Hawke (1971) adsorbed the exogenous DG directly to the acetone powder preparation. Veerkamp (1974) successfully used a similar procedure in experiments with an acetone powder of a bacterial transferase.

Following unsuccessful attempts at preparation of an acetone powder of envelopes of spinach chloroplasts, the effect of exogenous DG on galactosyltransferase of the chloroplast envelope was examined using a suspension of envelopes in buffer. Exogenous DG was not a requirement for activity by galactosyltransferase of the envelope and addition of 1-palmitoyl, 2-oleoyl glycerol or 1,2-di-linoleoyl glycerol to the envelope preparation gave no stimulation of incorporation of radioactivity from ^{14}C-DG into MGDG. However the low specific activity (0.005 μCi per mole) of the ^{14}C-DG may account for this lack of incorporation. Alternatively the diacylglycerols may have been unsuitable substrates for incorporation by transferase because the fatty acid composition of the lipid or the method of dispersion of the lipid in the incubation mixture was unsuitable.

Most of the endogenous DG is removed from the chloroplasts during preparation of an acetone powder and therefore transferase of an acetone powder is dependent on added DG for activity. In contrast Joyard and Douce (1976bc) reported that envelopes from spinach chloroplasts contained up to 0.4 mg DG per mg envelope protein (16 % of the total envelope lipid). In the present study a considerably lower figure, 0.035 mg DG per mg protein was obtained, i.e. 2 % of the total envelope lipid. Early reports by Douce (Douce, 1974 ; Douce et al 1973) on the lipid composition of chloroplast envelopes did not give values for the DG content of envelopes. Other workers (Poincelot, 1973 ; Mackender and Leech, 1974 ; Hashimoto and Murakami, 1975) also have not included values for DG in their detailed studies on the lipid composition of chloroplast envelopes. Therefore the high DG content found by Joyard and Douce (1976bc) is somewhat surprising. Furthermore these workers (Joyard and Douce, 1976c) have described a close relationship between changes in the levels of endogenous DG and MGDG after addition of UDP-Galactose. If such high concentrations of endogenous lipid are present in the envelope fraction it is perhaps not unexpected that added exogenous

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DG (addition of up to 11 mg DG per incubation) does not stimulate the incorporation of radioactivity from UDP-^{14}C-Galactose into MGDG. The endogenous DG rich in the fatty acids 16:0, 18:1 and 18:3 may have a more suitable fatty acid composition and be better dispersed than added DG and therefore the endogenous DG may be utilised in preference to exogenous DG.

The membrane-bound nature of the chloroplast envelope galactosyltransferase posed problems of heterogeneity of the micro-environment of the assay. Electronmicroscopy showed that a high proportion of the envelope membranes were present as vesicles which in many cases appeared to be resealed. Consequently added substrates and effectors may not have been freely accessible to galactosyltransferase. The failure to achieve stimulation of transferase by sonication before and after addition of DG prompted the use of the detergents Triton X-100 and sodium cholate. These were similarly unsuccessful in solubilizing an active transferase. The addition of DG along with the detergent did not stimulate incorporation and no radioactivity from ^{14}C-DG was incorporated into MGDG of the envelope. Furthermore no galactosyltransferase activity was detected in any soluble fraction derived from detergent action on the chloroplast or the chloroplast envelope. Preincubation of the envelope with phospholipase C or D prior to addition of UDP-^{14}C-Galactose showed that both enzymes inhibited transferase activity, perhaps indicating a phospholipid requirement of transferase. Berry _et al_ (1978) regarded the inhibitory effect of phospholipase C on the activity of guinea pig microsomal enzymes involved in glucuronidation of O-amino-phenol as indicating that these enzymes were phospholipid-dependent. The results of these workers are similar to the results of the present study and this may suggest that galactosyltransferase of the chloroplast envelope is dependent for activity on a phospholipid component of the membrane or a spatial relationship provided by a phospholipid component. Addition of exogenous phospholipid to the phospholipase digested membranes did not stimulate transferase activity. It is possible that phospholipase C or similar lipolytic enzymes may provide an endogenous source of DG and this may be the source of the high levels of DG reported by Joyard and Douce (1976bc) in their envelopes preparation. Although negative results were obtained when phospholipases were added to the incubation mixture, inhibition of transferase activity

may have not been entirely due to phospholipid depletion of the membrane but also to inhibition through an accumulation of the products of the reaction, i.e. phosphatidic acid, choline, etc.

The polypeptide composition of the chloroplast envelope has been reported (Pineau and Douce, 1974 ; Joy and Ellis, 1975 ; Mendiola-Morgenthaler and Morgenthaler, 1974) and about 20 major polypeptides have been identified. The polypeptide patterns of the envelope preparation before tryptic digestion were similar to that obtained by previous workers (Sprey and Laetsch, 1975 ; Joy and Ellis, 1975) and the pattern after tryptic digestion was similar to that reported by Joy and Ellis (1975) after pronase digestion of envelopes or pea chloroplasts.

Neither trypsin or protease (from B.subtilus) completely removed all of the detectable galactosyltransferase activity from the spinach envelope preparation which may indicate that the vesicular or lipid nature of the envelope preparation restricted the accessibility of the proteolytic enzymes to transferase. Polyacrylamide gel electrophoresis of the tryptic digest envelope preparation which contained about 50 % of the original transferase activity indicated that most of the polypeptides of the envelope had been digested and therefore it is concluded that galactosyltransferase is not a major polypeptide component of the chloroplast envelope.

From the present study it appears that treatments which contribute to disruption of the integrity of the envelope membrane (e.g. detergents, sonication, lipases) inhibit transferase activity. This suggests that galactosyltransferase is dependent on the membrane or some component of the membrane for activity as a multienzyme array or in order to satisfy a lipid cofactor or substrate requirement. The apparent membrane-dependence of the enzyme, the requirement for a lipid substrate and the failure to achieve stimulation or incorporation from added diacylglycerol, are the major difficulties in the study of solubilization of transferase. Furthermore the low yields of total envelope protein, only a portion of which would be galactosyltransferase and the rapid inactivation of the membrane-bound enzyme by detergent action complicates the study of this enzyme.

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