THE BIOSYNTHESIS OF GALACTOLIPIDS IN PHOTOSYNTHETIC TISSUE

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ABBREVIATIONS

ACP	-	acyl carrier protein
AES	-	automatic external standard
ATP	-	adenosine 5' - triphosphate
BSA	-	bovine serum albumin
C	-	Curie
C/M	-	chloroform - methanol solution
CMU	-	3-(p-chlorophenyl) - 1:1-dimethyl urea
CoA	-	coenzyme A
cpm	-	counts per minute
DCMU	-	3-(3:4-dichlorophenyl) - 1:1-dimethyl urea
DCPIP	-	2,6-dichlorophenolindophenol
DEGS	-	diethylene glycol succinate
DGDG	-	digalactosyl diglyceride
dpm	-	disintegrations per minute
FRS	-	ferredoxin reducing substance
g.	-	gram
GLC	-	gas liquid chromatography
GSH	-	L-glutathione
IUPAC-IUB	-	International Union of Pure and Applied Chemistry - the Internationsl Union of Biochemistry
l.	-	litre
M	-	molarity
MGDG	-	monogalactosyl diglyceride
NADP	-	nicotinamide adenine dinucleotide phosphate
p.	-	page
PC	-	phosphatidyl choline
PCMB	-	p-chloromercuribenzoic acid
PE	-	phosphatidyl ethanolamine

PG - phosphatidyl glycerol

PGA - 3-phosphoglyceric acid

PI - phosphatidyl inositol

POPOP - 1,4 bis /~2- (5-phenyloxazolyl) 7- benzene

PPO - 2,5 - diphenyloxazole

PS I, II - photosystem I, II

rac - racemo

sn - stereospecific numbering

SQDG - sulphoquinovosyl diglyceride

TCPIP - 2,3', 6 - trichlorophenolindophenol

TGDG - trigalactosyl diglyceride

TLC - thin layer chromatography

Tris - Tri - (hydroxymethyl) methylamine

UDP-D-galactose - uridine 5' - diphosphate D-galactose

UDPG - uridine 5' - diphosphate α-D-glucose

v. - volume

w., wt. - weight.

NOMENCLATURE OF LIPIDS

For the specific structural designation of complex lipids containing a glyceryl moiety, the nomenclature suggested by the IUPAC-IUB Commission on Biochemical Nomenclature (European J. Biochem (1967) 2, 127) has been followed. However, although a diglyceride prepared by chemical synthesis is correctly named as, e.g., rac-1-O-oleoyl, 2-O-linoleoyl glycerol, it is referred to as, in this case, 1-oleoyl, 2-linoleoyl glycerol, for sake of brevity. As a group the synthetic diglycerides are designated 1,2 (2,3)-diglycerides. The trivial names of complex lipids are used when it is more appropriate to do so e.g. phosphatidyl choline.

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

Chapter 1.

REVIEW OF LITERATURE

Section 1.I : Occurrence.

The galactolipids under study are the galactosyl diglycerides found extensively throughout the plant kingdom. Monogalactosyl diglyceride (MGDG) and digalactosyl diglyceride (DGDG) occur in all cells capable of the photosynthetic evolution of oxygen, as well as in the non-chlorophyllous tissue of higher plants and some Gram-positive bacteria. Monogalactosyl diglyceride is also present in vertebrate spinal tissue.

Hexosyl diglycerides occur quite generally in Gram-positive bacteria (Shaw and Baddiley, 1968). As a part of the total lipid, they vary from a few per cent in lactobacilli and staphylococci to more than 40% in Microbacterium lacticum and Mycoplasma laidlawii B. The mono- and dihexosyl diglycerides, present singly or together, contain the hexose moieties, glucose, galactose, monnose or rhamnose, each of which can occur independently or in combination with another. MGDG and DGDG have been identified in Arthrobacter globiformis (Walker and Bastl, 1967, and in Bifidobacterium bifidium (Exterkate and Veerkamp, 1969), the latter also containing the tri-homologue (TGDG). Although the glycosidic linkages of these latter DGDG and TGDG were not determined, in general, bacterial diglycosyl diglycerides have glycosidic linkages that differ from those in DGDG of photosynthetic organisms both in the carbon atoms involved and/or the configuration of the bond.

The green photosynthetic bacterium Chloropseudomones ethylicum contains glycolipids which include a monogalactolipid which had an identical configuration and structure with the monogalactolipid from plant tissue (Constantopoulos and Bloch, 1967a). Nichols and James

(1965) found both galactosyl diglycerides in the green photosynthetic bacterium Chlorobium limicola but the photosynthetic non-sulphur purple bacteria they examined lacked any galactolipids. The galactolipids of Chlorobium limicola were not chemically identified.

As suggested above, all non-bacterial photosynthetic organisms have galactose exclusively as the hexose moiety of their glycosyl diglycerides; the glycosidic linkage involving the glycerol is always in the β anomeric configuration and the inter-galactosyl linkage is $\alpha(1 \rightarrow 6)$. (See Section I.II).

Blue-green algae, which are prokaryotic like bacteria, have photosynthesizing lamellae as their only subcellular particles and these contain both MGDG and DGDG (Nichols, Harris and James, 1965). Blue-green algae, unlike photosynthetic bacteria, are capable of the Hill reaction as are all photosynthetic eukaryotic organisms. Tables 1 and 3 refer to species in which galactolipids have been identified.

The trigalactosyl diglyceride (TGDG), previously tentatively identified in spinach lamellae (Allen, Hirayama and Good, 1966) and in radiochemical experiments using Chlorella (Benson et al, 1958) and spinach chloroplasts (Neufeld and Hall, 1964; Ongun and Mudd, 1968), was recently characterized in potato tubers by Galliard (1969).

Galactolipids, first isolated from wheat flour (Carter, McCluer and Slifer, 1956), were recognized by Benson, Wintermans and Wiser (1959) and Weenink (1959) as major glycerolipid components of leaves. They contributed 23% of the total (ether-soluble) lipids of red clover (Trifolium pratense) leaves (Weenink, 1961) and purified MGDG and DGDG accounted for 16.6% and 4%, respectively, of the total lipids of scarlet runner bean (Phaseolus multiflorus) leaves (Sastry and Kates, 1964a).

The glycerolipid content of a range of unfractionated plant tissues

Table 1.

Galactolipid content of plant tissues and other sources.

Plant	μMoles of lipid per g. fresh weight of leaf		μMoles of lipid per mg. of total chlorophyll		Percentage contribution to complex glycerolipids‡		Reference
	MGDG	DGDG	MGDG	DGDG	MGDG	DGDG	
Non-angiosperms:							
Mesotaenium caldariorum	10.00	5.50	6.25	3.42	47.2	26.0	Roughan and Batt (1969)
Marchantia berteroana	1.07	0.66	3.25	1.90	41.5	25.6	
Moss*	2.68	1.50	2.60	1.46	38.7	21.7	
Blechnum fluviatile	5.60	2.30	3.08	1.26	53.0	21.8	
Ginkgo biloba	4.70	2.80	2.90	1.70	40.8	24.3	
Pinus radiata	2.80	1.95	5.10	3.50	37.0	25.8	
Mean ± standard deviation	-	-	3.5 ± 1.3	2.0 ± 0.9	43.0 ± 6.0	24.2 ± 2.8	
Angiosperms:							
Rose (Rosa cv)	5.60	4.60	3.97	3.26	40.4	33.2	
Rowan (Sorbus aucuparia)	10.20	7.16	3.95	2.82	43.7	30.7	
White clover (Trifolium repens)	8.60	5.20	3.98	2.40	46.5	28.1	
Lucerne (Medicago sativa)	8.60	5.20	4.30	2.60	47.0	28.4	
Poplar (Populus italica)	4.95	3.80	4.20	3.30	35.6	27.3	
Camellia japonica	3.10	3.10	6.74	6.74	28.0	28.0	
Squash (Cucurbita pepo)	4.10	2.70	4.10	2.70	37.8	24.9	
Tomato (Solanum lycopersicum)	5.08	2.46	5.08	2.46	50.7	24.5	
Lettuce (Lactuca sativa)	0.68	0.68	2.76	2.76	30.9	30.9	

continued

Table 1 continued

Plant	μMoles of lipid per g. fresh weight of leaf		μMoles of lipid per mg. of total chlorophyll		Percentage contribution to complex glycerolipids		Reference
	MGDG	DGDG	MGDG	DGDG	MGDG	DGDG	
Xanthium orientale	6.10	5.90	3.80	3.70	44.8	43.4	
Cocksfoot (Dactylis glomerata)	8.00	5.10	3.30	2.10	46.8	29.8	
Perennial ryegrass (Lolium perenne)	5.10	3.95	3.08	2.35	39.1	30.3	
Paspalum dilatatum	6.00	3.60	4.46	2.70	52.4	31.4	
Maize (Zea mays)	3.10	2.30	2.70	2.05	42.6	31.6	
Mean ± standard deviation	-	-	3.4 ± 0.6 Δ	2.4 ± 0.4 Δ	41.8 ± 7.1	30.2 ± 4.5	
Range	0.68 - 10.20	0.68 - 7.16	2.6 - 6.74	1.26 - 6.74	28.0 - 53.0	21.7 - 43.4	
Previous values:							
Red clover (Trifolium pratense)	8.76	5.84	-	-	-	-	Roughan and Batt (1968)
Soybean (Glycine soja)	7.12	4.84	-	-	-	-	
Xanthium orientale	3.64	2.74	-	-	-	-	
Perennial ryegrass (Lolium perenne)	6.76	4.12	-	-	-	-	
Perennial ryegrass (Lolium perenne)	3.61	2.03	-	-	-	-	calculated from Gray, Rumsby and Hawke (1967)
Barley seedlings (Hordeum vulgare)							
1) light-grown	2.73	0.96	-	-	-	-	
2) dark-grown	1.03	0.32	-	-	-	-	
Sugar beet (Beta vulgaris)							calculated from Wintermans (1960)
1) leaf	2.4	1.3	1.92	1.04	25.8	14.0	
2) chloroplasts	12.1	6.8	1.88	1.06	44.2	24.9	

continued

Table 1 continued

Plant	Moles of lipid per g. fresh weight of leaf		Moles of lipid per mg. of total chlorophyll		Percentage contribution to complex glycerolipids†		Reference
	MGDG	DGDG	MGDG	DGDG	MGDG	DGDG	
Elder (Sambacus nigra)							
1) green leaves	5.6	2.6	2.24	1.04	41.8	19.9	
2) yellow leaves	1.7	1.1	0.76	0.49	26.2	16.9	
Bean (Phaseolus vulgaris)	3.9	1.4	1.62	0.58	43.3	15.6	
Non-chlorophyllous tissue:							
Potato tuber (Solanum tuberosum)	0.092	0.185			8.8	17.7	Galliard (1968a)
Apple pulp							Galliard (1968b)
1) pre-climacteric	0.054	0.114			7.9	16.6	
2) post-climacteric	0.015	0.052			2.6	9.1	
Parsnip root (Pastinaca sativa)	0.17	0.34			12.2	24.5	Roughan and Batt (1969)

† calculated values

* Moss - equal amounts of Funaria and Leptobryum pyriforme

△ neglecting value for Camellia

were quantitatively analysed by Roughan and Batt (1969). The values they obtained for the galactolipid content of various species are shown in Table 1. Their technique, which required a minimum of manipulation, gave values that were consistently higher than the small number of values already in the literature.

For the species studied by Roughan and Batt (1969), the concentrations of MGDG and DGDG range from 0.68 to 10.20 and 0.68 to 7.16 μmoles/g. fresh weight, respectively. Expressing these values as μmoles of lipid/mg. of total chlorophyll reduced the range of values. The value obtained for Camellia was higher than the others because the sample analysed was young and contained little chlorophyll.

If the glycerolipid concentrations, given as μmoles/g. fresh weight, are analysed and the contribution of MGDG and DGDG to the total glycerolipid content derived, MGDG is found to contribute 28.0 to 53.0% and DGDG 21.7 to 43.4%. When the tissues are divided into angiosperms and non-angiosperms, the mean DGDG contribution is higher for angiosperms than for non-angiosperms while the MGDG contribution is about equal in both groups.

	MGDG	DGDG	
non-angiosperms	43.0 ± 6.0	24.2 ± 2.8	(mean ± standard deviation)
angiosperms	41.3 ± 7.1	30.2 ± 4.5	

This finding is consistent with the higher α-linolenic acid content of the chloroplasts of angiosperms compared to the level in mosses, ferns and gymnosperms (Wolf, Coniglio and Bridges, 1966). A slight difference between the two groups exists, with respect to DGDG content, if the ratio of the number of molecules of galactosyl diglyceride per molecule of chlorophyll (assuming total chlorophyll consists of only chlorophyll a) is calculated.

	MGDG	DGDG	
non-angiosperms	3.5 ± 1.3	2.0 ± 0.9	(mean ± standard deviation)
angiosperms ǂ	3.4 ± 0.6	2.4 ± 0.4	

Since MGDG and DGDG have been shown to occur in the mitochondria of avocado mesocarp and sweet potato root (Biale, Yang and Benson, 1960), and chlorophyll is known to be localised in chloroplasts, any difference in galactolipid : chlorophyll ratios, determined from analysis of unfractionated tissues, may not accurately reflect the true relationship between chlorophyll and the galactosyl diglycerides. That green leaves of elder (Sambacus nigra) and bean (Phaseolus vulgaris) contained more galactolipids than their respective yellow leaves (aurea form for elder) was shown by Wintermans (1960). Moreover, the dark green outer leaves of cabbage had a higher galactolipid content than the paler inner leaves (Nichols, 1963). The relationship of chlorophyll and galactosyl diglycerides is discussed further in Section 1.VI.

The molar ratio of MGDG to DGDG for the green unfractionated tissues examined by Roughan and Batt (1969) varied from 1.0 for lettuce, to 2.4 for blechnum. In the green alga, Euglena gracilis, this ratio was between 2 and 3, depending on the culture conditions (Rosenberg, Gouaux and Milch, 1966).

Galactolipids are especially concentrated in chloroplasts (Wintermans, 1960). Ongun, Thomson and Mudd (1968) found that the aqueous-extracted chloroplasts of tobacco (Nicotiana tobacum) leaves contained

83% of total cellular MGDG
88% of total cellular DGDG
76% of total cellular SQDG
74% of total cellular PG.

These four glycerolipids contribute 30% of the total dry weight of chloroplasts (quoted by Nichols, 1965a).

ǂ neglecting the Camellia value because of the low chlorophyll content of the young leaf.

Quantitative data for the glycerolipid content of fractionated photosynthetic cells is limited. Table 2 presents the data compiled by Hirayama (1967) who analysed the lamellae of Anacystis nidulans and spinach.

Table 2.

Glycerolipid content of fractionated tissues (Hirayama, 1967)

Glycerolipid	Anacystis nidulans		Spinach		Chlorella
	whole cells	lamellae	lamellae	chloroplast	
MGDG	32*	30	37	80	92
DGDG	12	9	19	33	88
TGDG	-	-	2	-	-
PG	9	8	14	9	33
SQDG	10	10	10	10	10
PC	-	-	4	8	15
PI	-	-	3	3	8
PE	-	-	-	0.3	6

* all values relative to SQDG.

Allen, Hirayama and Good (1966) obtained a similar composition for spinach lamellae. The data of Table 2 shows that the galactosyl diglycerides are the main glycerolipid components of chloroplasts and lamellae. It is also illustrative of the greater simplicity of the glycerolipid composition of blue-green algae which generally lack PC, PE and PI (Nichols, Harris and James, 1965).

The occurence of galactosyl diglycerides in non-chlorophyllous tissue has been widely documented, for example

dark-grown Euglena cells - Rosenberg and Pecker (1964)

etiolated castor leaves - Nichols, Stubbs and James (1967)

wheat flour - Carter, Hendry and Stanacev (1961b)

avocado mesocarp - Ongun and Mudd (1968)

potato tubers - Lepage (1968)

starch grains - Duncan and Rees (1965)

also see Tables 1 and 3.

Etiolated tissue has a relatively lower content of galactolipids than green photosynthetic tissue (Gray, Rumsby and Hawke, 1967). Galliard (1968a,b) found the galactolipid content of potato tubers of the same order as that in etiolated tissue but higher than in apple pulp.

The DGDG content of parsnip root, potato tubers and apple pulp is greater than the MGDG content of the same tissue; the reverse situation was seen in green leaves, above.

MGDG, identical in structure with that of plant origin, was isolated by Steim (1967) from mammalian nervous tissue. It was present in bovine spinal cord and in the brains (white matter) of cow, cat, pig, rat and man, but absent in most of the other organs. Rumsby (1967) showed its occurrence in sheep brain.

Table 3.

Fatty acid composition of galactolipids from various sources.

Source		Fatty acid																					Reference
		14:0	14:2	14:3	15:0	16:0	16:1 Δ^7 or Δ^9	16:2	16:3	16:4	17:0	18:0	18:1	18:2	18:3 ω3	18:3 ω6	18:4	19:0	20:0	20:1	20:4	20:5	22:0	Others	
Bacteria:																								
Arthrobacter globiformis	MGDG	1.4				66.8	14.2				17.6													1
Chloroseudomonas ethylicum	PGDG						46.0	49.0					3.7										2.1	2
Blue-green Algae:																								
Anabaena variabilis	MGDG						27.1	27.5	1.0				12.0	18.7	14.7									3
	DGDG						26.4	26.0	2.3			2.1	9.0	20.4	17.0									
Anacystis nidulans	MGDG	1.2					42.6	33.6				4.0	20.4											3
	DGDG	1.7					51.8	28.3				3.6	16.2											
Spirulina platensis	MGDG						45.0	1.9				3.1	1.3	5.6	4.9	42.9								4
	DGDG						42.8	9.4				2.0	7.9	4.8		33.7								
Marine Algae:																								
Batrachaspermum moniliforme	MGDG	1.2					18.0	5.0	0.3	2.7		0.7	6.7	2.0	1.9		2.8				4.4	51.4	2.9		5
	DGDG	0.8					38.4						9.3	2.0	1.1						4.3	35.2	8.9		
Fucus vesiculosus	MGDG	2.7					4.9	0.7			6.2		8.2	5.3	13.4		30.2				4.4	24.7			5
	DGDG	4.0					7.8	0.8			0.8		13.1	6.6	15.7		23.0				4.7	25.3			

continued

Table 3 continued

Source		Fatty acid																					Reference
		14:0	14:2	14:3	15:0	16:0	16:1 Δ^7 or Δ^9	16:2	16:3	16:4	17:0	18:0	18:1	18:2	18:3 $\omega 3$	18:3 $\omega 6$	18:4	19:0	20:0	20:1	20:4	20:5	22:0	Others	
Freshwater Algae:																								
Chlorella pyrenoidosa	MGDG					2.7	9.7				1.5	0.3	40.5	4.5	26.8		2.6	12.0						6
	DGDG					11.6	9.5				1.2	0.4	36.8	5.8	27.0		3.3	3.3						
Chlorella vulgaris																								7
1) photoautotrophic	MGDG					4.5	1.5	18.9	10.0			1.9	2.8	16.8	44.6									
	DGDG	2.1	3.5			7.6	2.7	5.6	1.4			3.4	2.6	34.9	36.6									
2) photoheterotrophic	MGDG	1.4		3.2		3.7	14.3	21.4	1.8			0.7	18.4	29.9	8.0									
	DGDG	3.5				15.0	4.3	5.9	1.9			3.6	14.3	39.7	7.1									
3) dark-grown heterotrophic	MGDG					3.3	10.6	28.4					17.5	33.7	5.0									
	DGDG	1.6	0.5			9.6	6.8	3.9				1.7	16.5	44.9	3.5									
Euglena gracilis																								8
1) photoautotrophic	MGDG	1.7				3.2	3.3	6.6		32.3		2.4	6.1	5.1	39.4									
	DGDG	7.4				15.8	13.9	8.3		0.8		3.3	16.5	11.6	18.0									
2) photoheterotrophic	MGDG					5.0	6.6	14.5	15.4	8.2			8.8	12.2	26.7									
	DGDG	7.6				10.2	21.3	20.0				0.6	26.0	4.9	9.3									
Chlamydomonas mundana	MGDG			3.3		8.1	8.0	9.1	27.1				9.5	7.6	26.1									9
	DGDG			1.1		11.5	8.5	14.0	17.8				16.4	10.0	20.0									
Moss:																								
Hypnum cupressiforme	MGDG					2.3	0.8	2.0	10.7				1.2	4.1	48.3						28.5	11.2	3.1	10
	DGDG					6.2	1.8	1.2	1.7			1.6	2.5	4.6	62.2						13.8	4.4	2.6	

continued

Table 3 continued

Source		Fatty acid																				Reference
		14:0	14:2	14:3	15:0	16:0	16:1 Δ^{7} or Δ^{9}	16:2	16:3	16:4	17:0	18:0	18:1	18:2	18:3 ω3	18:3 ω6	18:4	19:0	20:0	20:1	20:4	20:5	22:0	Others	
Higher plant leaves:																								
Alfalfa (Medicago sativa)	MGDG					2.7						0.2	0.3	1.7	95.0									6
	DGDG					14.0					0.7	0.3	0.4	0.8	82.0									
Castor (Ricinus gibsoni)	MGDG			2.3		6.0									91.0									9
	DGDG					11.1								3.7	85.3									
Runner bean (Phaseolus multiflorus)	MGDG					2.3								2.2	95.5									11
	DGDG					4.5						1.0		1.3	93.2									
Spinach																								
1) leaf	MGDG								30.0				1.0	1.0	67.0									12
	DGDG					6.0			3.0			1.0	4.0	3.0	84.0									
2) chloroplast lamellae	MGDG								25					2	72									13
	DGDG					3			5				2	2	87									
	TGDG[3]					9		1	15				1	1	70									
Nymsotis scorpioides	MGDG	0.2				3.0	0.2					0.4	0.7	3.4	41.5	6.1	44.0					0.3		14
	DGDG	0.3				18.6	1.7				0.1	2.5	1.8	10.9	43.4	7.9	11.9		0.2			0.2		
Artemisia princeps	MGDG					1.5	0.4						0.3	3.7	94.1									15
	DGDG					16.3	5.0					2.5	5.7	14.1	56.4									
Holly (Ilex aquifolium)	MGDG					1.0								2.3	96.7									10
	DGDG					13.4								6.9	79.7									

continued

Table 3 continued

Source		Fatty acid																				Reference
		14:0	14:2	14:3	15:0	16:0	16:1 Δ^7 or Δ^9	16:2	16:3	16:4	17:0	18:0	18:1	18:2	18:3 ω3	18:3 ω6	18:4	19:0	20:0	20:1	20:4	20:5	22:0	Others	
Non-chlorophyllous tissue:																								
Apple pulp																								16
1) pre-climacteric apples	MGDG					2.0						0.6	0.6	5.4	91.4									
	DGDG					16.9						4.2	2.2	16.9	59.8									
2) post-climacteric apples	MGDG					6.1						0.8	7.1	12.7	73.0									
	DGDG					19.5						6.7	7.0	23.8	43.0									
Narcissus bulbs	MGDG					4.4	2.2	0.8	0.8			2.2	5.3	73.8	10.3									17
	DGDG					12.7	2.1	0.7	0.7			2.0	8.1	68.6	4.6									
Potato tuber	MGDG					0.8							0.9	58.0	40.3									18
	DGDG					14.1						6.3	7.7	46.5	25.4									
	TGDG					24.9						10.5	10.3	42.5	11.8									
Wheat flour																								
1)	MGDG	0.5				13.9	3.4					1.3	17.2	57.0	2.0							5.1		19
	DGDG					41.6						4.4	12.1	29.3								12.6		
2)	MGDG					8.2	0.3				0.1	0.5	8.3	79.0	3.6									20
	DGDG					15.9	0.1				0.1	1.1	8.0	71.6	3.2									
Animal tissue:																								
Bovine spinal cord	MGDG	2.6			0.4	53.2	2.0				2.1	7.9	22.3	1.0	5.4			0.9				1.9*		21
Sheep brain	MGDG	10.3			2.6	56.4	2.6					18.0	5.1					5.1						22

* tentative.

continued

Table 3 continued

1 Walker and Bastl (1967)

2 Constantopoulos and Bloch (1967a)

3 Nichols, Harris and James (1965)

4 Nichols and Wood (1968)

5 Radunz (1968)

6 O'Brien and Benson (1964)

7 Nichols (1965a)

8 Constantopoulos and Bloch (1967b)

9 Nichols, Stubbs and James (1967)

10 Nichols (1965b)

11 Sastry and Kates (1964)

12 Allen et al (1964)

13 Allen, Hirayama and Good (1966)

14 Jamieson and Reid (1969)

15 Hoda and Fujiwara (1967)

16 Galliard (1968b)

17 Nichols and James (1964)

18 Galliard (1968a)

19 Carter et al (1961a)

20 De Stefanis and Ponte (1969)

21 Steim (1967)

22 Rumsby (1967)

Section 1.II : Structure

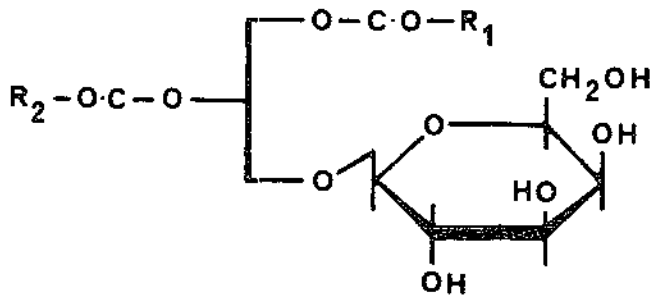
The structures of the galactosyl diglycerides of wheat flour were elucidated by Carter and co-workers (1956, 1961a,b) and Sastry and Kates (1964a) demonstrated that the galactosyl diglycerides from runner-bean (Phaseolus multiflorus) leaves had identical structures, viz. 1,2 di-O-acyl, 3-O- (β-D-galactopyranosyl) -sn-glycerol and 1,2 di-O-acyl, 3-O- [β-D-galactopyranosyl - (1→6)-O-α-D -galactopyranosyl]- sn - glycerol. (Figure 1).

1.II.1 Characterization of the carbohydrate moiety:

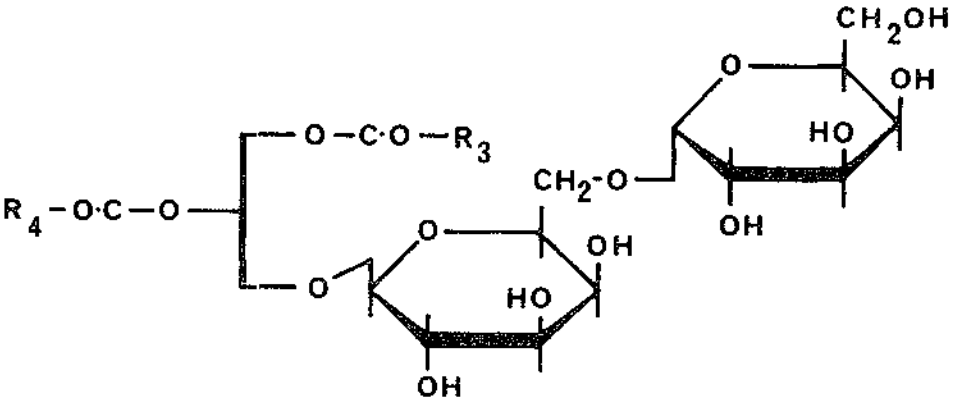
Carter, McCluer and Slifer (1956) fractionated a benzene extract of wheat flour by counter-current distribution. The lipocarbohydrate fraction was further subdivided into acetone-soluble and acetone-insoluble fractions, each of which was subjected to alkaline hydrolysis. The two carbohydrate fractions, obtained from each of the acetone fractions by carbon-Celite column chromatography, contained only galactose and glycerol on acid hydrolysis. Chemical analysis of the compounds that crystallized from each carbohydrate fraction showed that one was monogalactosyl glycerol and the other digalactosyl glycerol.

The monogalactosyl glycerol consumed 3.0 moles of periodate and produced 1.0 mole of formic acid and 1.2 moles of formaldehyde per mole, whereas the digalactosyl glycerol consumed 5.0 moles of periodate and yielded 1.9 moles of formic acid and 1.3 moles of formaldehyde per mole. These data are consistent with the structures galactopyranosyl-1-glycerol and galactopyranosyl (1→6) galactopyranosyl-1-glycerol for the mono- and digalactosyl glycerols, respectively.

Enzymatic hydrolysis with α- and β -galactosidases established that the glycosidic linkage between the galactose and glycerol moieties had a β-configuration in both of the galactosyl glycerols while the second



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



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

Figure 1

The structures of MGDG and DGDG (Carter et al, 1956, 1961a, b)

(R_1, R_2, R_3, R_4 are long chain fatty acyl residues)

galactose moiety was linked by an α-glycosidic linkage in the digalactosyl glycerol. Supplementary evidence for these assignments was obtained by comparing the specific rotations of the galactosyl glycerols with other α- and β-galactosides.

1.II.2 Analysis of the intact lipid:

The structure of the intact wheat flour galactolipids was further examined by Carter and co-workers (1961a,b) in two later papers.

The MGDG and DGDG, which were purified by silicic acid column chromatography, had saporification equivalents that indicated the occurrence, in each, of two fatty acyl residues per molecule.

Acid hydrolysis of the galactolipids, subsequent to complete methylation and followed by deacylation, yielded products which were identifiable by paper chromatography. Glycerol and 2,3,4,6-tetra-methyl-galactose were the derivatives obtained from MGDG while DGDG gave the products glycerol, 2,3,4-tri-O-methyl- and 2,3,4,6-tetra-O-methyl-D-galactose. These results are consistent with the structures assigned previously.

When digalactosyl glycerol, obtained by alkaline hydrolysis of DGDG, was subjected to a Kuhn methylation, a nona-O-Me-digalactosyl glycerol resulted. Acid hydrolysis of this product yielded two compounds that were identified, by paper chromatography using an ammoniacal silver nitrate spray, as 2,3,4,6-tetra-O-methyl- and 2,3,4-tri-O-methyl-D-galactose. This indicated that the glycerol moiety had been methylated and liberated on hydrolysis as a 1,2-di-O-methyl glycerol.

The methylated then deacylated MGDG and DGDG consumed 1.03 moles and 1.34 moles of periodate per mole respectively, and each released formaldehyde (Figure 2).

This evidence is consistent only with a 1,2 diacyl glyceryl glycoside structure for the intact lipid.

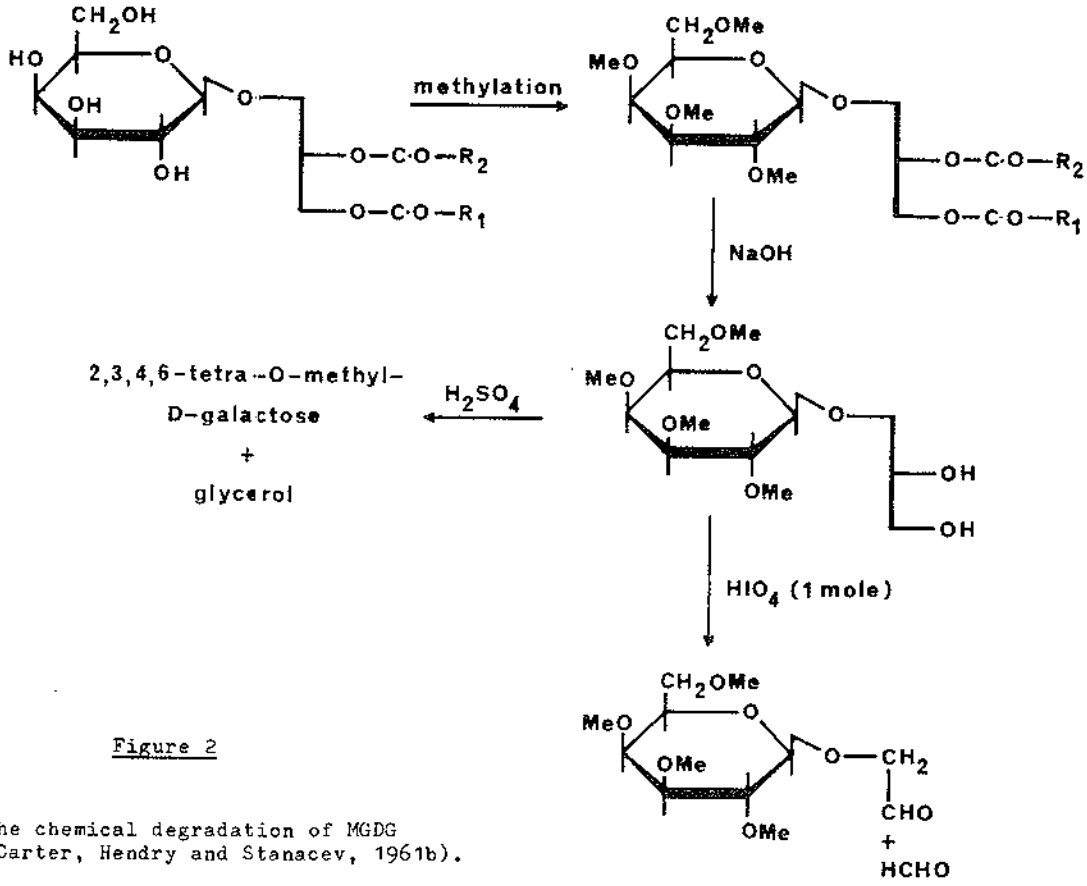


Figure 2

The chemical degradation of MGDG (Carter, Hendry and Stanacev, 1961b).

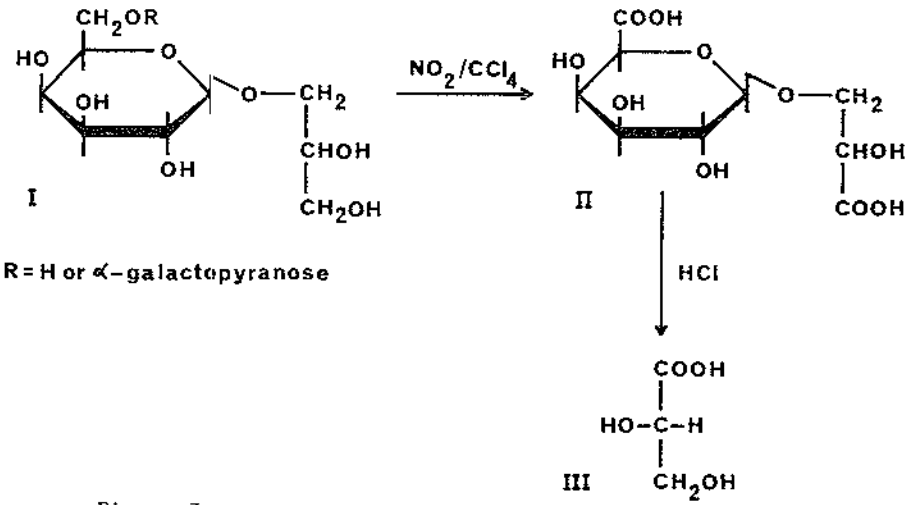


Figure 3

The conversion of deacylated galactolipid to L(+)-glyceric acid (Miyano and Benson, 1962)

1.II.3 Configuration of the glycerol moiety:

For the characterization of the mono- and digalactosyl glycerols from red algae, Wickberg (1958a,b) synthesized a series of α- and β-galactosides of 1- and 3-O-sn-glycerol. These were shown to have different and characteristic infra-red absorptions. Using infra-red spectra, and other physical properties, the digalactosyl glycerol isolated from red algae was deduced to be a 3-O-sn-glycerol derivative. Wickberg demonstrated that the mono- and digalactosyl glycerols of Carter, McCluer and Slifer (1956) also had this configuration. However, the monogalactosyl glycerol isolated from the red algae was found to be an isomorphous mixture of 1- and 3-O-sn-glycerol galactosides.

Carter, Hendry and Stanacev (1961b), on the basis of its IR spectrum, confirmed Wickberg's assignment for the wheat flour monogalactosyl glycerol as a 3-O-sn-glycerol derivative.

Miyano and Benson (1962) also reaffirmed this configuration using a radiochemical technique. The ^{14}C-labelled galactosyl glycerols, formed on the deacylation of the photosynthetic lipid products of Chlorella exposed to $^{14}CO_2$ (Ferrari and Benson, 1961), were oxidized with nitrogen dioxide in carbon tetrachloride to a mixture of oxidation products e.g. II (Figure 3). Acid hydrolysis of these, followed by chromatographic separation, gave ^{14}C - glyceric acid in 36% yield. Repeated crystallizations of the authentic asymmetric glyceric acid in the presence of the ^{14}C - glyceric acid gave quantitative retention of the radioactivity in calcium DL-glycerate and in calcium L(+)-glycerate but not in the quinine salt of D(-)-glyceric acid. Thus the conclusion that the galactosyl glycerols were 3-O-sn-glycerol derivatives was correct.

1.II.4 The identity of galactolipids from green leaves:

The MGDG and DGDG isolated from scarlet runner-bean (Phaseolus

multiflorus) leaves (Sastry and Kates, 1964a), contained galactose, glycerol and fatty esters in the ratio 1:1:2 and 2:1:2, respectively. Alkaline hydrolysis of MGDG and DGDG (unsaturated or hydrogenated) yielded two moles of fatty acids and, 1 mole of monogalactosyl glycerol or 1 mole of digalactosyl glycerol, respectively. Acid hydrolysis of these latter products gave glycerol and galactose, only, on the paper chromatograms (except for a trace of glucose in the digalactosyl glycerol because of cerebroside contamination).

The attachment of the galactose through C-1 of the glycerol was proved for both deacylation products by the molar ratio of the periodate consumed to formaldehyde liberated which were near the theoretical values of 3:1 for monogalactosyl-1-glycerol and 5:1 for digalactosyl-1-glycerol.

The crystalline specimens of mono- and digalactosyl glycerols had melting points and infra-red spectra that were consistent with the structures:

3-O-[β-D-galactopyranosyl]-sn-glycerol and

3-O-[β-D-galactopyranosyl-(1→6)-O-α-D-galactopyranosyl]-sn-glycerol

Hence Sastry and Kates (1964a) concluded that the MGDG and DGDG of runner-bean leaves had identical structures to the corresponding compounds from wheat flour.

1.II.5 The trigalactosyl diglyceride:

The TGDG, isolated from potato tubers (Galliard, 1969), had molar ratios of sugars to fatty acids of 1.53 ± 0.06, and of D-galactose to glycerol of 2.9, which are close to theoretical for a trigalactosyl diglyceride. The infra-red spectra of MGDG, DGDG and TGDG in carbon disulphide were basically similar; the former two having absorptions in agreement with those of MGDG and DGDG isolated from leaves by Sastry and Kates (1964a). The straight line obtained by plotting a function of R_F,

from each of two solvent systems, of the deacylation products of MGDG, DGDG and TGDG against the number of galactose units/molecule in each lipid, suggested that the TGDG was a higher homologue of MGDG and DGDG, containing three galactose units per molecule.

The molar ratio of periodate consumed to formaldehyde liberated for the deacylated TGDG was near the theoretical of 7:1 expected for a compound in which three galactopyranose units were linked together to C-1 of glycerol.

No reducing sugar was released when the deacylated TGDG (or DGDG) was incubated with β-D-galactoside galactohydrolase, although the deacylated MGDG from potato tuber was hydrolysed to galactose and glycerol.

These results are consistent with TGDG having the following structure:

1,2-di-O-acyl, 3-O-$\{\beta$-D-galactopyranosyl-(1$\rightarrow$6)-O-α-D-galactopyranosyl-(1$\rightarrow$6)-O-α-D-galactopyranosyl$\}$-<u>sn</u>-glycerol.

Section 1.III Fatty acid composition

1.III.1 General considerations:

A survey of the fatty acid compositions of galactolipids from various sources is contained in Table 3. Considerable data are available concerning the fatty acid composition of the galactosyl diglycerides from unfractionated tissues but there is scant information for the fatty acid composition of galactolipids isolated from chloroplast preparations, except that for spinach chloroplasts and lamellae (Allen et al, 1964; Allen, Hirayama and Good, 1966). However, since galactolipids are primarily concentrated in chloroplasts, the fatty acid compositions given in Table 3 are likely to have a good correspondence with those of chloroplast galactolipids. The similarity of composition of spinach leaf and spinach lamellae supports this view. The data in Table 3 also reflects the finding that the major fatty acids of the chloroplasts of higher plants are palmitic, oleic, linoleic and linolenic acids (Wolf, Coniglio and Bridges, 1966).

The galactolipids of photosynthetic tissue are usually the most unsaturated class of glycerolipids. In spinach lamellae, they contain over 70% of the trienoic acids bound to complex lipids (calculated from the data of Allen et al, 1966). The fatty acid, α-linolenic acid (cis 9,12,15-octadecatrienoic acid) is the most abundant fatty acid in both the MGDG and DGDG of green plants. The MGDG and DGDG of green leaves may possess up to 97% and 93%, respectively, of their fatty acids as α-linolenic acid. In general, the MGDG fraction contains a higher percentage of polyunsaturated fatty acids than the DGDG fraction. This is evident in Table 4.

Whereas α-linolenic acid seems to be the preferred polyunsaturated fatty acid in higher plants for both galactolipids it is not the exclusive choice. Spinach leaves contain 7,10,13-hexadecatrienoic acid which

Table 4.

The degree of unsaturation of the fatty acids of galactolipids from various sources*

Source	percentage of fatty acids with two or more double bonds			percentage of fatty acids with three or more double bonds		
	MGDG	DGDG	TGDG	MGDG	DGDG	TGDG
Bacteria:						
Arthrobacter globiformis	0	-	-	0	-	-
Chloropseudomonas ethylicum	0	-	-	0	-	-
Blue-green algae:						
Anabaena variabilis	34.4	39.7	-	14.7	17.0	-
Anacystis nidulans	0	0	-	0	0	-
Spirulina platensis	53.4	38.5	-	47.8	33.7	-
Marine algae:						
Batrachaspermum gonliforme	65.5	42.6	-	63.2	40.6	-
Fucus vesiculosis	84.2	76.1	-	79.1	69.5	-
Freshwater algae:						
Chlorella pyrenoidosa	33.9	36.1	-	29.4	30.3	-
Chlorella vulgaris						
1) photoautotrophic	90.3	82.0	-	54.6	38.0	-
2) photoheterotrophic	64.3	54.6	-	13.0	9.0	-
3) dark-grown heterotrophic	67.1	52.8	-	5.0	3.5	-
Euglena gracilis						
1) photoautotrophic	83.4	38.7	-	71.7	18.8	-
2) photoheterotrophic	77.0	34.3	-	50.3	9.3	-
Chlamydomonas mundana	73.2	62.9	-	56.5	38.9	-
Moss:						
Hypnum cupressiforme	93.4	85.8	-	88.0	80.0	-
Higher plant leaves:						
Alfalfa	96.7	82.8	-	95.0	82.0	-
Castor	93.3	89.0	-	93.3	85.3	-
Runner bean	97.7	94.5	-	95.5	94.0	-

continued

Table 4 continued

Source	percentage of fatty acids with two or more double bonds			percentage of fatty acids with three or more double bonds		
	MGDG	DGDG	TGDG	MGDG	DGDG	TGDG
Spinach						
1) leaf	98.0	90.0	-	97.0	87.0	-
2) chloroplast lamellae	99.0	94.0	87.0	97.0	92.0	85.0
Myosotis scorpioides	95.0	74.1	-	91.6	63.2	-
Artemisia princeps	97.8	70.5	-	94.1	56.4	-
Holly	99.0	86.3	-	96.7	79.7	-
Non-chlorophyllous tissue:						
Apple pulp						
1) pre-climacteric apples	96.8	76.7	-	91.4	59.8	-
2) post-climacteric apples	85.7	66.8	-	73.0	43.0	-
Narcissus bulbs	85.7	74.6	-	11.1	5.3	-
Potato tuber	98.3	81.9	54.3	40.3	25.4	11.8
Wheat flour						
1	59.0	29.3	-	2.0	0	-
2	82.6	3.6	-	74.8	3.2	-
Animal tissue:						
Bovine spinal cord	6.4	-	-	5.4	-	-
Sheep brain	0	-	-	0	-	-

* calculated from Table 3

contributes 30% of the fatty acids in the MGDG fraction, apparently being equivalent to α-linolenic acid (Allen et al, 1964). The DGDG fraction contains little of this acid. This specific enrichment of hexadecatrienoic acid in MGDG from spinach leaves was confirmed by Constantopoulos and Bloch (1976b) and it was also found in the green algae Chlorella vulgaris Chlamydomonas reinhardi, and Scenedesmus.

γ-Linolenic acid (cis 6,9,12-octadecatrienoic acid), which is usually associated with animal-type fatty acid metabolism, is abundant in the Boraginaceae family of herbaceous plants (Jamieson and Reid, 1969). The MGDG and DGDG of one member, Myosotis scorpioides, contains 6.1 and 7.9%, respectively, of their fatty acids as γ-linolenic acid, in addition to a high proportion of α-linolenic acid. However, the blue-green alga, Spirulina platensis, is almost devoid of α-linolenic acid and its galactolipids have a considerable content of γ-linolenic acid (Nichols and Wood, 1968). If the preference for α-linolenic acid by higher plants is because of its molecular configuration, then it is surprising that its substitution by γ-linolenic acid occurs, since the latter has a different molecular shape.

The unicellular photosynthetic marine dinoflagellate, Gonyaulax polyedra, also has galactolipids which have very little α-linolenic acid content (Patton et al, 1966). Although the organism contains less than 1% of its total fatty acids as α-linolenic acid, octadecatetraenoic, eicosapentaenoic and docosahexaenoic acids contribute 14, 14 and 23%, respectively, of the total fatty acids of the organism. Octadecatetraenoic acid is also a major fatty acid component of the galactolipids of the brown alga, Fucus vesiculosus, (Radunz, 1968) and of Myosotis scorpioides. Both the red and brown algae, given in Table 3, contain large concentrations of eicosapentaenoic acid as well as some eicosatetraenoic acid. The moss, Hypnum cupressiforme, also contains these two fatty acids (Nichols, 1965b),

although in lesser amounts, and with more tetraenoic than pentaenoic acid.

In those tissues which contain galactolipids with tri-, tetra-, penta- or hexaenoic acids in addition to α-linolenic acid, the fatty acids seem generally to belong to the $\omega 3$ fatty acid series rather than the $\omega 6$ series. However, this aspect has not been investigated.

The data for the fresh-water algae show that unsaturated hexadecanoic acids make significant contributions to the galactolipid fatty acids. The hexadecatrienoic and hexadecatetraenoic acids tend to be concentrated in the MGDG fraction, as also occurs for Hypnum cupressiforme and spinach. An effect of the environment on fatty acid composition is evident for green algae. Under heterotrophic conditions, whether in light or in dark, the content of tri- and tetraenoic acids is lower than when the algae are dependent on light as the exogenous source of metabolic energy, i.e. under photoautotrophic conditions. The influence of light on fatty acid composition is discussed in paragraph 1.III.3.

The galactosyl diglycerides from non-chlorophyllous tissues of higher plants contain mainly linoleic and α-linolenic acids. Their α-linolenic acid levels are lower than for the galactolipids of green leaves but the levels of palmitic, stearic, oleic and especially linoleic acids are higher.

Polyunsaturated fatty acids are entirely absent from the galactolipids of the blue-green alga, Anacystis nidulans, as they are lacking from the whole organism (Holton, Blecker and Onore, 1964). Three other blue-green algae have also been found to lack polyenoic acids and two to lack α-linolenic acid (Parker, van Baalen and Maurer, 1967; Holton, Blecker and Stevens, 1968). Linoleic and α-linolenic acids were the usual polyunsaturated acids of the blue-green algae. The photosynthetic bacterium Chloropseudomonas ethylicum lacked polyenoic acids (Constantopoulos and Bloch, 1967a), as is the general rule for bacteria,

and its MGDG contained almost 50% of palmitic and hexadecaenoic acids. The MGDG from nervous tissue contained a large percentage of palmitic acid, too (Steim, 1967).

The occurrence of a seasonal variation in the fatty acid composition of galactolipids of plants has not been explored. Jamieson and Reid (1969) observed a variation in the contribution of the octadecapolyenoic acids to the total fatty acid composition of Myosotis scorpioides, with the time of year. Indeed, the level of galactolipids may also vary, as a seasonal change in the level of lipid-bound sugar in red-clover (Trifolium pratense) leaves was noted to be independent of plant maturity (Bailey, 1964).

1.III.2 Specific composition of MGDG and DGDG:

The MGDG from photoheterotrophically-cultured Chlorella vulgaris cells was fractionated into five different molecular species by argentation TLC (Nichols and Moorhouse, 1969). The separation depended on the total number of double bonds in the molecule, thus the five species isolated contained one, two, three, four and five double bonds per molecule. A molecule species with six double bonds would no doubt be isolated from photoautotrophic cells in which the concentration of trienoic acids is greater. The significant feature of the fatty composition of each of the molecular species was that, in every case, the major component acids did not differ by more than one double bond in their degree of unsaturation. Hence the fractions containing two or four double bonds per molecule consisted almost exclusively of monoenoic or dienoic acids, respectively, and the fractions containing one or three double bonds per molecule were composed of saturated and monoenoic or monoenoic and dienoic acids, respectively. The only fraction containing α-linolenic acid was the one with five double bonds per molecule. The

small quantities of saturated and monoenoic acids in the four and five double bond molecular species could not be explained. The principal fatty acids in all fractions were of the 16- and 18-carbon series, the 14-carbon fatty acids occurred at greater than 1% only in the one double bond molecule species.

The positional specificity of the fatty acids for the one- or two-position in the glycerol moiety was not determined. However, it was evident that the fatty acid distribution differed from that of the phospholipids in which the saturated fatty acid predominate in the one-position and the more highly unsaturated fatty acid, especially linoleic acid in the two-position (Sastry and Kates, 1964a). This conclusion was drawn despite the results of Noda and Fujiwara (1967) suggesting a similarity of the galactolipid fatty acid distribution with that of phospholipids. These workers subjected the galactolipids isolated from Artemisia princeps leaves to incubation with pancreatic lipase which removed the fatty acid from the one-position. Most of the saturated and monoenoic acids appeared to be liberated by the enzyme in contrast to the higher concentration of α-linolenic acid in the mono- and digalactosyl monoglycerides. Thus it was concluded that, when the MGDG and DGDG molecules contained different fatty acids, the saturated and monoenoic fatty acids were esterified to the one-position and α-linolenic acid in the two-position. The MGDG and DGDG of Artemisia princeps leaves contain 94% and 56% of their total fatty acids as α-linolenic acid, respectively.

1.III.3 The influence of light:

The stimulation, by illumination, of the unsaturated fatty acid synthesis in developing chloroplasts and greening leaves has been well noted (Wolf, Coniglio and Bridges, 1966; Wallace and Newman, 1965; Appelqvist et al, 1968).

The etiolated leaves of castor (Ricinus gibsoni) contain galactolipids that contain considerable amounts of α-linolenic acid (Nichols, Stubbs and James, 1967). Illumination of these leaves for 20 hours increased the concentration of α-linolenic acid in both galactosyl diglycerides, primarily at the expense of linolenic acid. Other than the appearance of trans, 3-hexadecenoic acid in the phosphatidyl glycerol, the changes in the other glycerolipids were small.

Table 5.

The change in fatty acid composition of etiolated castor leaf galactolipids upon illumination (Nichols, Stubbs and James, 1967).

condition of the leaf		fatty acid				
		16:0	Δ^9-16:1	18:1	18:2	18:3
etiolated	MGDG	7	3	7	14	67
	DGDG	14	3	8	21	51
after 20 hr. illumination	MGDG	4	-	3	4	88
	DGDG	15	2	7	7	65

The MGDG fraction of barley (Hordeum vulgare) seedlings grown in the dark contained a considerable percentage of α-linolenic acid (Gray, Rumsby and Hawke, 1967). The growth of the seedlings in low light and in natural light proportionately increased the level of α-linolenic acid in the MGDG fraction. The fatty acid composition of the DGDG fraction was not followed. The concentration of both galactolipids in the seedlings was also increased by the light intensity. The level of galactolipids in the plastids of light-grown bean (Phaseolus vulgaris) leaves was observed to be higher than that in the plastids of dark-grown leaves (Wallace and Newman, 1965). In greening barley leaves, an

enrichment in ∝-linolenic acid was observed in MGDG (and in SQDG) but not in DGDG. There was no apparent reason for this (Appelqvist et al, 1968).

The fatty acid compositions of Chlorella galactolipids show that, for this alga, light induces a change in fatty acid composition only if the alga is made dependent on light as a source of energy (Table 3). Transferring Chlorella from dark heterotrophic to photoautotrophic conditions induces the synthesis of polyunsaturated galactolipids (Nichols, 1965a). The effect of the culture medium, as well as of light, on the galactolipid fatty acid content of Euglena gracilis was noted by Rosenberg, Gouaux and Milch (1966). When heterotrophic cells were grown under illumination, the relative amounts, compared with dark-grown heterotrophic cells, of hexadecatrienoic acid, which doubled, and ∝-linolenic acid increased in both MGDG and DGDG while hexadecatetraenoic acid appeared in MGDG. Photoautotrophic cells had twice the percentage of hexadecadienoic and hexadecatetraenoic acids in the MGDG fraction and three times as much hexadecadienoic and linoleic acids in the DGDG fraction as did photoheterotrophic cells. The levels of ∝-linolenic acid and hexadecatrienoic acid in each galactolipid were similar in these latter two cultures, as was its level in the MGDG fraction from dark-grown heterotrophic cells. Dark-grown heterotrophic cells contained high percentages of monoenoic acids in both MGDG and DGDG.

Rosenberg and Gouaux (1967) exposed cells of Euglena gracilis, which had been grown in the dark, to illumination in a mineral medium and followed the changes in fatty acid composition as a function of total time of illumination. On greening, the percentages of hexadecatetraenoic, oleic and hexadecadienoic acids increased, the former two from zero to 15 and 20%, respectively, of the fatty acids of MGDG. The saturated and monoenoic acids decreased in concentration. For the DGDG fraction the

major increases occurred in the concentration of hexadecadienoic and oleic acids. The galactolipid content of the cells increased ten-fold for both MGDG and DGDG under photosutotrophic conditions. In absolute values, the major increases in the fatty acids of MGDG occurred in the hexadecadi-, -tri- and -tetraenoic acids and in linoleic and linolenic acids while for DGDG, the increases occurred in hexadecadienoic, hexadecatrienoic, oleic and linolenic acids. In both the Rosenberg papers cited, the illumination of the cells was at an intensity of about 30 foot-candles.

When photoautotrophic cultures of Euglena gracilis were grown under light intensities varying from 120 to 610 foot-candles, the concentrations of hexadecatetraenoic and linolenic acids in the MGDG fraction increased by about 50% whereas the actual content of the galactolipids decreased (Constantopoulos and Bloch, 1967b).

The above observations indicate that light is not obligatory for polyenoic acid or galactolipid synthesis but that the degree of unsaturation of the galactolipid fatty acids depends especially on the light intensity. The structural changes that occur in, or result in, the lamellar chloroplast on illumination, may facilitate, or be dependent on, the synthesis of highly unsaturated fatty acids. The more dramatic changes appear to occur in the MGDG fraction.

Section 1.IV : Biosynthesis.

Studies designed to elucidate the biosynthetic pathway of galactolipids have, to date, invariably involved the use of radioactive substrates. The enzymatic synthesis of lipids in aqueous environments *in vitro*, is complicated by dispersion and substrate accessibility problems so that the ability to detect small net synthesis is advantageous.

1.IV.1 Use of $^{14}CO_2$

When Ferrari and Benson (1961) analysed the water-soluble deacylation products of the lipids isolated from *Chlorella pyrenoidosa* after steady-state photosynthesis in $^{14}CO_2$, radioactivity appeared in both the mono- and digalactosyl glycerols. The considerable amount of label occurring in the galactose moieties suggested that these could be readily exchanged with the intermediates of hexose photosynthesis. The glycerol and fatty acids of the galactolipids contained relatively less radioactivity than the galactose. The fatty acids of MGDG (as with those of phosphatidyl glycerol and the triglycerides) were more rapidly labelled than other esterified fatty acids.

The galactolipids together incorporated more than half the radioactivity that appeared in the glycerolipids, and this proportion appeared to be independent of the time of exposure to $^{14}CO_2$. For exposure times shorter than 30 minutes the decrease in the percentage of radioactivity appearing in the monogalactosyl glycerol was matched by a concomitant increase in the digalactosyl glycerol. This observation suggested that the MGDG could be converted to DGDG by galactosylation, and the following sequence was suggested:

$CO_2 \longrightarrow$ PGA $\longrightarrow$ $\longrightarrow$ $\longrightarrow$ UDPG $\rightleftharpoons$ UDP-D-galactose $\xrightarrow{\text{1,2-diacyl-sn-glycerol}}$ MGDG $\xrightarrow{\text{UDP-D-galactose}}$ DGDG.

Photosynthesis by runner bean (Phascolus multiflorus) leaves in $^{14}CO_2$ for two hours resulted in galactose as the only radioactive lipid-bound sugar (Kates, 1959). When glucose - ^{14}C was administered to the leaves a similar pattern of incorporation to that obtained with $^{14}CO_2$ occurred (Kates, 1960). This evidence supported the scheme shown above.

1.IV.2 Experiments using chloroplast suspensions:

Isolated spinach chloroplasts, suspended in 0.1M Tris buffer, pH 7.4, transferred galactose to an endogenous acceptor when incubated with UDP-D-galactose - ^{14}C, at 37°C (Neufeld and Hall, 1964). The alkali-labile products were similar, though not identical to the galactolipids isolated from plant material. The deacylated lipids yielded four bands on a radioautogram, consistent with members of a homologous series. Two bands were identified as β-D-galactosyl glycerol and digalactosyl glycerol, and the other two suggested as trigalactosyl- and tetragalactosyl glycerols. While the digalactosyl glycerol had the chromatographic mobility of 3-O-{β-D-galactosyl-(1⟶6)-O-α-D-galactosyl}-sn-glycerol, only 17% of the terminal galactose could be hydrolysed with α-galactosidase, the remainder being susceptible to β-galactosidase attack. The inner linkage was entirely β. Water-soluble galactosides were also isolated from the chloroplasts and identified as hexose, β-galactosyl glycerol, digalactosyl glycerol (of which approximately half had a terminal β-linkage) and a trace of trigalactosyl glycerol. Thus some galactolipase activity occurred in the chloroplasts.

The optimum pH for galactolipid synthesis was about pH 7 in either 0.1M Tris or phosphate buffers. Whether the reaction was performed in light or in darkness had no effect on the amount of incorporation. Added cations (3.8mM) were either partially or completely inhibitory except Mg^{++} which, like EDTA or cysteine (6mM), had no effect on the reaction.

Nucleoside triphosphates (5mM) had little effect on the reaction, except UTP which was almost completely inhibitory.

Incubation of the chloroplasts with UDP-D-galactose-^{14}C and UDP-D-glucose-^{14}C gave 30% and 15% incorporation, respectively, into chloroform-soluble products, while the other sugar nucleotides tested were ineffective glycosyl donors. The effectiveness of UDP-D-glucose as a donor was attributed to the UDP-D-galactose epimerase activity in the cloroplasts.

Ongun and Mudd (1968) achieved more than 95% incorporation of radioactivity into galactolipids when they incubated spinach (Spinacia oleracea) leaf chloroplasts, suspended in 0.1M Tris pH 7.4, with UDP-D-galactose - ^{14}C for 2 hours. A labelled compound obtained thus, tentatively identified as trigalactosyl diglyceride, was not detectable in lipid extracts of whole leaves. The rate of incorporation was shown to be proportional to the concentration of UDP-D-galactose - ^{14}C. The MGDG to DGDG ratio declined from 7.5 in the early stages of the incubation to a constant value of 1.9 after about one hour.

Young leaf chloroplasts were twice as active as mature leaf chloroplasts on a chlorophyll basis in incorporating label into galactolipids. However, the difference was smaller on a protein basis, mature leaf chloroplasts having 66% of activity of young leaf chloroplasts. In each chloroplast preparation, MGDG was synthesized without a time lag, whereas DGDG and TGDG were synthesized after a lag which was most pronounced for TGDG. The authors suggested that the occurrence of more galactolipid in the mature portions of perennial ryegrass (Lolium perenne) leaves than in the younger portions of the same leaves (Gray, Rumsby and Hawke, 1967) was due to a limiting UDP-D-galactose concentration rather than a low enzyme activity. However, the level of galactosyl diglycerides may be dependent on the structural presence of chlorophyll rather than the effect of chlorophyll to enhance the photosynthetic efficiency of the leaf to provide UDP-D-galactose

When the incorporations of radioactivity from UDP-D-galactose-^{14}C into galactolipids by chloroplasts, chloroplast fragments (the 20,000 x g particle from a diluted chloroplast suspension) and the 20,000 x g supernatant were compared, the chloroplast fragments contained a much higher proportion of the total radioactivity in MGDG than in DGDG than was observed in the galactolipids of unfractionated chloroplasts. The reverse was true for the soluble enzyme system which synthesized a greater proportion of TGDG as well. The combined 20,000 x g pellet and supernatant gave a distribution that was similar to that for chloroplasts. Thus it was suggested that galactolipid synthesis involved two enzymes, the one synthesizing the MGDG, i.e. forming a β-glycosidic linkage, being more tightly bound to the chloroplast membrane than the enzyme forming DGDG (and TGDG) and α-glycosidic linkages. While this deduction seems inherently logical, the configuration of the inter-galactosidic linkages of the DGDG and TGDG were not checked. One must assume that they were α-linkages despite the results of Neufeld and Hall (1964).

The galactose incorporation from UDP-D-galactose - ^{14}C into chloroplast lipids increased with temperature to about 45°C (Mudd, van Vliet and van Deenen, 1969) and the proportion of MGDG also increased as the temperature was raised. The optimum pH for galactolipid synthesis was re-determined as pH 7.2, at 37°C, in either 0.1M Tris or phosphate buffers. The higher pHs tended to favour the incorporation into MGDG than into DGDG or TGDG.

1.IV.3 Non-chloroplastic synthesis:

When the galactolipid-synthesizing activities of the chloroplasts, 15,000 x g particles and 15,000 x g supernatant of spinach leaves were compared, not all the incorporation by the latter fractions could be explained by chloroplast contamination (Ongun and Mudd, 1968). The

contamination of the 15,000 x g supernatant with soluble chloroplast enzymes could not be estimated. This fraction had the lowest MGDG-^{14}C to DGDG-^{14}C ratio.

Mudd, van Vliet and van Deenen (1969) were unable to localize the galactolipid-synthesizing activity to any particular subcellular fraction of spinach leaves; thus supporting the notion of extra-chloroplastic synthesis although the enzymes concerned may be easily removed from the chloroplast. It may be significant that the 100,000 x g supernatant, in which 30-40% of the galactosyl diglyceride-synthesizing activity was recovered, had a greater ability for DGDG synthesis than other fractions.

The 20,000 x g particles from the inner mesocarp of avocado (Persea americana), pea (Pisum sativum)root and cauliflower (Brassica cauliflora) florets, all had the capacity for galactolipid synthesis (Ongun and Mudd, 1968). Most of the radioactivity incorporated into lipid from UDP-D-galactose - ^{14}C by avocado particles appeared in the DGDG fraction whereas the distribution of label for cauliflower particles was similar to that of spinach with most of the radioactivity in the MGDG fraction.

The 20,000 x g partciles of completely etiolated pea leaves also incorporated radioactivity from UDP-D-galactose - ^{14}C into MGDG and DGDG. Illumination of the leaves gave a two-fold increase in enzyme activity even though the protein increase was very small during the same period.

1.IV.4 The nature of the diglyceride acceptor:

The dissociation of the endogenous acceptor from the enzyme was achieved by preparing an acetone powder of chloroplasts (Neufeld and Hall, 1964). The acetone powder and acetone-soluble material were active in transferring galactose - ^{14}C from UDP-D-galactose - ^{14}C to lipid acceptors when incubated in combination but not separately. Magnesium ions, but not sn-glycero-3-phosphate (10mM), gave a slight stimulation. Acetone

powder preparations have thus been used to explore the nature of the lipid substrate of the galactosylating enzyme.

When an acetone powder of spinach chloroplasts was incubated with UDP-D-galactose - ^{14}C and diolein, of which only 1% was 1,2-isomer, nearly 20% incorporation of the label into MGDG occurred while no DGDG or TGDG was synthesized (Ongun and Mudd, 1968). Dipalmitin, which was at least 90% 1,2-isomer, did not act as an acceptor. The effect of adding diglycerides to acetone powder preparations was further investigated by Mudd, van Vliet and van Deenen (1969) who also demonstrated that diglycerides gave a stimulation only when incubated with the acetone powder but not when they were added to chloroplast suspensions. These workers prepared a range of diglycerides from spinach leaf phospholipids and the molecular species of diglycerides were separated according to the number of double bonds per molecule. The diglycerides possessed the fatty acid compositions shown in Table 6.

Table 6.

The fatty acid composition of the diglycerides prepared from spinach leaf phospholipids (Mudd, van Vliet and van Deenen, 1969).

sample	fatty acid				
	16:0	16:1	18:1	18:2	18:3
total	23	7	8.5	25	34
1	46.5	7.5	45	-	-
2	46	-	3	51	-
3	40	9	2	-	49
4	7.5	-	9	83.5	-
5	6	-	-	46	48
6	4	-	-	5.5	90.5

When each of these diglycerides was incubated with an acetone powder preparation and UDP-D-galactose - ^{14}C, the more highly unsaturated diglyceride species gave a greater stimulation than the unfractionated diglycerides, on a weight basis. A similar result occurred when the diglycerides were derived from egg phosphatidyl choline.

The technique of Mudd, van Vliet and van Deenen (1969) of adding lipid back to the acetone powder was to suspend the diglyceride in 0.1M Tris buffer, pH 7.2, which was 0.03% with respect to Tween 20, and to add an appropriate aliquot to the acetone powder preparation homogenized in the same medium. On the other hand, Ongun and Mudd (1968) homogenized the diglyceride and acetone powder in a small volume of acetone, evaporated the acetone and homogenized the residue in 0.1M Tris buffer, pH 7.4.

The preference of the MGDG-synthesizing enzyme for the more highly unsaturated diglycerides correlates well with the highly unsaturated MGDGs extant in spinach leaves. However, the authors (Mudd, van Vliet and van Deenen, 1969) acknowledged that the method of adding the diglyceride, either in aqueous solution with detergent or in organic solvent, might have an influence on the apparent enzyme specificity.

In all incubations involving diglyceride and acetone powder to date, little or no DGDG has been synthesized. That the added diglyceride acts as a substrate rather than by some physical effect was shown by the lack of stimulation given by the diglyceride which had had its free hydroxyl group methylated. (Mudd, van Vliet and van Deenen, 1969).

1.IV.5 Galactosylation of MGDG:

When spinach leaf chloroplasts were incubated with UDP-D-galactose - ^{14}C for one hour, in which time 96% of the added radioactivity was incorporated into the galactolipids, and then incubated with 100 times

the amount of unlabelled UDP-D-galactose for two hours, the MGDG - ^{14}C to DGDG - ^{14}C ratio decreased from 2.22, at the end of the first incubation, to 1.27, at the end of the second incubation (Ongun and Mudd, 1968). This decrement was greater than that which occurred without the addition of unlabelled UDP-D-galactose. The MGDG - ^{14}C to TGTG - ^{14}C ratio similarly decreased.

The incubation of radioactive galactosyl diglycerides with acetone powder preparation of spinach leaf chloroplasts and unlabelled UDP-D-galactose yielded radioactive galactosyl homologues. Use of MGDG as the acceptor gave DGDG and TGDG which contained 9% and 3.5%, respectively, of the added radioactivity while incubating DGDG - ^{14}C gave TGDG containing 9% of the added radioactivity. (In each case, 30% of the added radioactivity was extracted in the aqueous phase.) These experiments seem to confirm the sequential galactosylation of MGDG to yield DGDG, and TGDG, despite the doubt cast because of the different fatty acid profiles of MGDG and DGDG (e.g. Bloch *et al*, 1967).

1.IV.6 Incorporation of fatty acyl thioesters:

In cell-free extracts of photoautotrophic *Euglena gracilis* the fatty acyl moiety of 9.10-^{3}H-oleoyl-ACP was preferentially transferred to glycolipids to the extent of 4-10% incorporation (Renkonen and Bloch, 1969). The incorporation was stimulated by UDP-D-galactose and up to a 9-fold stimulation was obtained with *rac*-glycero-1-phosphate. Hence this suggested *de novo* synthesis of the glycolipids. The stearoyl moiety from 1-^{14}C-stearoyl-ACP or from acetylated 1-^{14}C-stearoyl-ACP was also incorporated into glycolipids but not significantly into phospholipids. However, 9,10-^{3}H-palmitoyl-ACP, at the same concentration, was not utilized for glycolipid or phospholipid synthesis.

The glycolipids formed using ACP derivatives were 61-84% MGDG and

only traces of DGDG were synthesized. The MGDG fraction of Euglena had been shown to have a β-galactoside structure (C. Kenyon, 1967).

The acyl groups of 1-^{14}C-oleoyl-, 1-^{14}C-stearoyl- and 1-^{14}C-palmitoyl-CoA were incorporated into phospholipids as well as into glycolipids though they appeared to be more effective donors for the former lipid class. The transfer of the oleoyl- group from oleoyl-CoA to MGDG was also stimulated by UDP-D-galactose. The glycolipids derived from oleoyl-CoA were 66% MGDG and again only a trace of DGDG was present.

Analysis of the fatty acids of the glycolipids formed from acetylated 1-^{14}C-stearoyl-ACP, 9,10-^{3}H-oleoyl-ACP, 1-^{14}C-stearoyl-CoA and 1-^{14}C-oleoyl-CoA showed that these contained considerable amounts of oleic acid. Each of the stearoyl-thioesters were equally effective as precursors of glycolipid-bound monoene. The transofrmations of the acyl groups into dienes and polyenes was marginal in all cases.

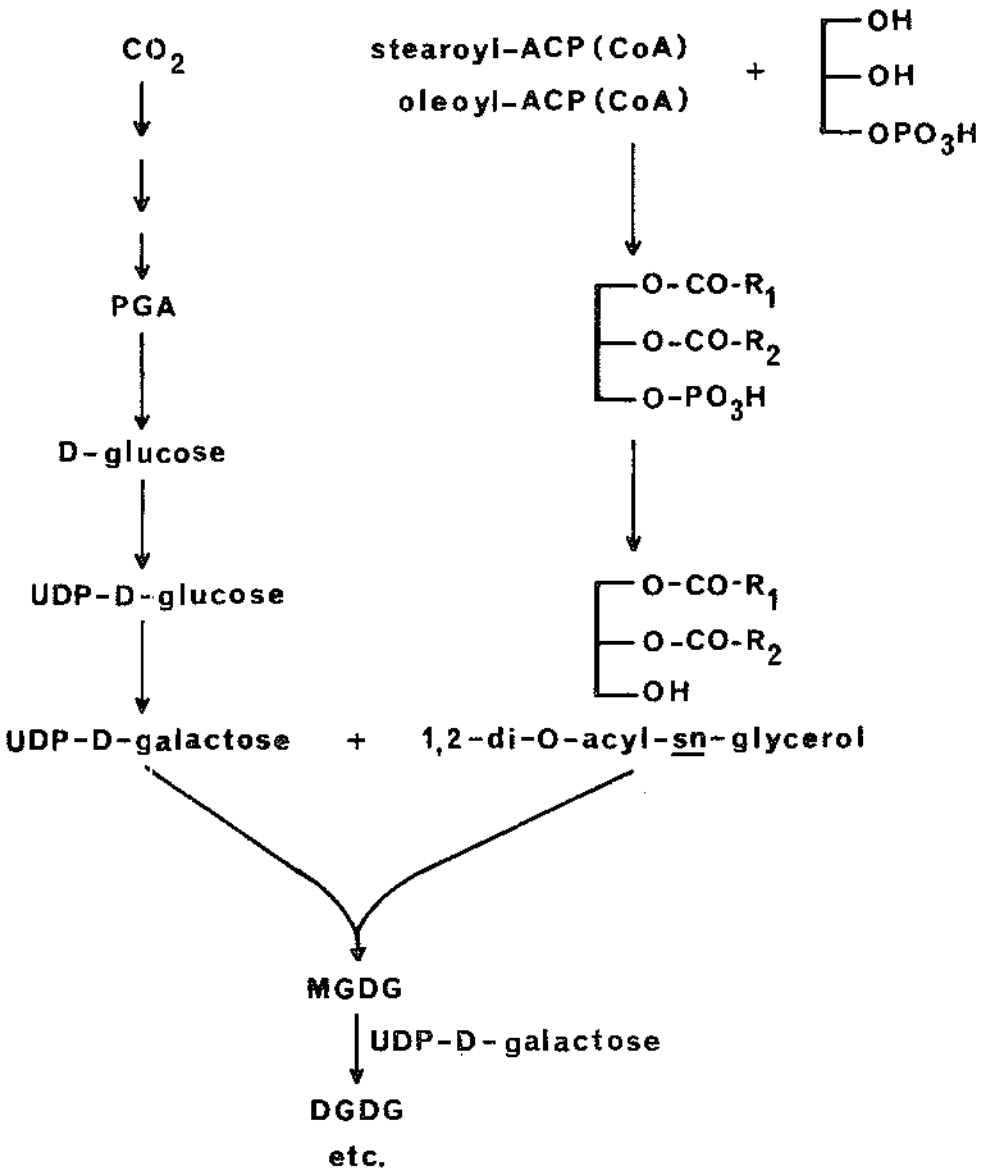
These experiments indicate that the fatty acyl-ACP thioesters are more specifically incorporated into MGDG than the corresponding CoA thioesters. This correlates with the chloroplast localization, in Euglena, of the fatty acid synthetase that yields stearoyl-ACP, but not palmitoyl-ACP (J. Delo), and the stearoyl-ACP desturase which does not act on palmitoyl-ACP (Nagai and Bloch 1965, 1967). The less specific utilization of fatty acyl-CoA thioesters may be related to the occurrence in Euglena of a second fatty acid synthetase, which yields palmitoyl-CoA (J. Delo). It was speculated that ACP thioesters are used for de novo galactolipid synthesis and the CoA thioesters used for deacylation-reacylation reactions. However, the experiments did not indicate whether one or two fatty acid chains were transferred from the ACP, or CoA, thioesters to the glycerophosphate.

1.IV.7 The possible biosynthetic pathway:

These data discussed above are consistent with the pathway for galactolipid biosynthesis shown in Figure 4. It is assumed that the reactions shown for plant leaves and green algae are identical in all photosynthetic eukaryotic organisms.

Figure 4

The possible biosynthetic pathway of galactolipids in plants.



Section 1.V : Degradation.

1.V.1 Characterization of a galactolipase:

Although galactolipids could be extracted with isopropanol from runner-bean (Phaseolus multiflorus) leaves in high concentrations, they were completely absent in water homogenates and chloroplast preparations of the leaves (Sastry and Kates, 1964b). The incubation of MGDG and DGDG, isolated from runner-bean leaves, with the total water homogenate of runner-bean leaves gave marked decreases in the acyl ester content of the homogenate. Most of the activity was associated with the broken-chloroplast fraction and to a lesser extent the cell-sap cytoplasm, little activity being recovered in the "microsome" pellet.

The cell-sap cytoplasm fraction was used for further work because it contained soluble enzymes. The microsome particles were precipitated from the cell-sap cytoplasm and the fraction that precipitated between 0 and 70% saturation of ammonium sulphate taken as the crude enzyme preparation. The preparation exhibited optimal galactolipid hydrolytic activity at pH 7.0 when the substrate was MGDG and at pH 5.6 for DGDG. The activity, at 30°C, toward DGDG at pH 5.6 was greater than that t ward MGDG at pH 7.0. The enzyme was susceptible to substrate inhibition by both galactolipids. A greater affinity by the enzyme for DGDG than for MGDG was indicated by the apparent Michaelis-Menten constants of 1.5mM and 7.8mM for the respective substrates.

Although the enzyme preparation did not hydrolyse the fully hydrogenated galactosyl diglycerides, this was attributed to the inaccessibility of the substrate for the enzyme. No significant phospholipids or lipase activity was evident in the enzyme preparation.

Using a fraction that precipitated from the cell-sap cytoplasm on dialysis against distilled water, as well as the above preparation, the

galactolipids were shown to be hydrolysed to the corresponding galactosyl glycerol without any detectable accumulation of lyso- intermediates. Thus the reaction rates for the lyso- compounds must be much faster than for the parent galactolipid. Sastry and Kates (1964b) also argued that a separate enzyme acted on each galactosyl diglyceride because

(a) storage at 4°C of the ammonium sulphate-precipitated enzyme fraction decreased its activity towards DGDG but not toward MGDG,

(b) the supernatant from the enzyme fraction prepared by dialysis alone was only active towards DGDG, and

(c) the apparent K_m's and pH optima were different for each substrate.

The appearance of free galactose, when employing the enzyme preparation precipitated by ammonium sulphate, suggested that the cell sap cytoplasm also contained enzymes that hydrolysed the mono- and digalactosyl glycerols to galactose and glycerol.

The leaf supernatant fractions of three species of Phaseolus readily manifested lipolytic activity towards the galactolipids whereas the other plant sources tested showed no activity. The exception was spinach leaves which gave a supernatant active on MGDG and DGDG to the extent of 1% and 3%, respectively, in reduction of acyl ester content as compared to over 20% reductions obtained using bean leaf supernatants. The galactolipase activity of spinach leaves was further investigated by Helmsing (1967). A crude enzyme fraction was prepared from young spinach (Spinacia oleracea) leaves by ammonium sulphate precipitation (as per Sastry and Kates, 1964b). The activity of the preparation toward MGDG was shown by the reduction in acyl ester content (about 1%) of the reaction mixture while the activity towards DGDG was much higher than noted by Sastry and Kates (1964b). The enzyme activity towards MGDG rose to a maximum after 10-11 days of storage at 4°C whereas the activity towards DGDG decreased in this period. (The preparation was assayed at

pH 7.0 and 5.6, at 30°C, for MGDG and DGDG as respective substrates). This seemed to argue against the two-enzyme hypothesis of Sastry and Kates (1964b) for spinach, at least. The spinach enzyme also appeared different from the runner-bean enzyme in that the pH optima were 7.5 and 5.9 for MGDG and DGDG, respectively, and the activity towards MGDG was greater than towards DGDG.

That both galactolipase activities could reside in one protein molecule emerged with the isolation and purification of the galactolipase from the cell-sap cytoplasm of runner-bean (Phaseolus multiflorus) leaves (Helmsing, 1969). With a molecular weight of 110,000, as determined using a Sephadex column, the enzyme appeared pure by gel electrophoresis under various conditions. Also throughout the purification, the ratio of monogalactolipase to digalactolipase activity was 2:1, except after the last purification procedure when it increased to 3:1. These activities were measured at 30°C and at the appropriate pH optima i.e. either pH 7.0 or pH 5.6. The enzyme appeared to be reasonably stable to storage and temperature, though the digalactolipase activity was more thermolabile. The greater affinity for DGDG was confirmed by the Michaelis-Menten constants for the pure galactolipase; they were 0.65mM and 0.31mM for MGDG and DGDG, respectively. These values are a factor of 10 lower than those obtained for the crude enzyme preparation (Sastry and Kates, 1964b), thus indicating a stronger affinity of the pure enzyme for the galactolipids.

The inhibitors of the enzyme were GSH, PCMB and 2-mercaptoethanol which never gave more than 50% inhibition and cysteine which was completely inhibitory for both enzyme activities at 1mM. Sodium dithionate and sodium metabisulphite activated the galactolipase, possibly by reducing quinones that normally inhibit the enzyme.

Since the isoelectric point of the protein is pH 7.0, the enzyme is

neutral when it acts optimally on MGDG and negatively charged for optimal digalactolipase activity. Thus a pH-dependent allosteric transformation was suggested to account for the double action of the enzyme.

Even though no lyso- compounds have been isolated they are assumed to be intermediates in the reaction pathway to the galactosyl glycerols which are the result of galactolipase activity:

MGDG ⟶ (galactosyl monoglyceride) + fatty acid
↓
galactosyl glycerol + fatty acid
↓ β galactosidase
galactose + glycerol

DGDG ⟶ (digalactosyl monoglyceride) + fatty acid
↓
digalactosyl glycerol + fatty acid
↓ α-galactosidase
galactosyl glcyerol
↓ β-galactosidase
galactose + glycerol

Evidence for an extracellular galactolipase of Chlorella ellipsoidea was obtained by adding ^{14}C-galactolipids to an illuminated culture, incubating for a period of time, then observing that ^{14}C-galactosyl glycerol and ^{14}C-digalactosyl glycerol were always detected in the culture medium (Miyachi, Miyachi and Benson, 1965).

1.V.2 Identification of acylated galactolipids:

A glycolipid which was isolated from leaf homogenates of higher plants was absent if the leaves were first placed in boiling water before homogenization (Heinz, 1967a). The enzymatically-formed glycolipid from spinach leaves was composed of glycerol, galactose and fatty acids in a molar ratio of 1:1:3, was more hydrophobic than MGDG and yielded crystalline

3-O-{β-D-galactopyranosyl}-sn-glycerol after saponification. This acylated MGDG appeared to be a mixture of isomers but reinvestigation of its structure, using NMR and synthetic compounds for comparison, showed that the compound was wholly an 6-O-acyl isomer (Heinz and Tulloch, 1969) with the structure 1,2,-di-O-acyl-3-O {6-O-acyl-β-D-galactopyranosyl} - sn-glycerol. Thus the enzyme involved esterified the primary hydroxyl group of the galactose moiety. The optimal pH for the enzymatic formation of the acylated MGDG in spinach leaf homogenates was about pH 4.6. The acyl group was apparently transacylated from either MGDG or DGDG.

Two acyl derivatives of MGDG and one of DGDG were identified in isolated spinach chloroplasts (Wintermans et al, 1969). One of the acylated MGDGs was assumed to be identical with that characterized by Heinz although the ester to galactose ratio was variable and usually greater than three. Although these acylated galactolipids are probably isolation artifacts of leaf homogenates, the 6-O-acyl isomer of MGDG was isolated from wheat flour (Myhre, 1968).

1.V.3 Factors influencing the expression of galactolipid degradation:

The effect of isolation media on the manifestation of galactolipase activity was examined by Wintermand et al (1969). The monogalactolipase activity was observed when spinach leaf chloroplasts were isolated in media containing high concentrations of NaCl. In media containing sucrose or mannitol, the DGDG content of the chloroplasts also decreased and acylated galactolipids identified. Isolation of chloroplasts in NaCl solutions results in the loss of stromal protein and it is supposed that the transacylating enzyme, localized in the plastid stroma, is easily lost. On lowering the pH from 7.5 to 6.0 no significant differences in the pattern of galactolipid breakdown were observed even though such a change influenced the activity of the partially purified galactolipase (Helmsing,

1967). Hence the pattern of lipid transformation depended on the major osmotic component of the suspension medium rather than on the pH.

1.V.4 Consequences of galactolipase activity in isolated chloroplasts:

The chloroplasts of bean (Phaseolus vulgaris) leaves isolated in 0.35 M NaCl at pH 6 were strongly inhibited in their ability to photoreduce either DCPIP or ferricyanide or to catalyse ATP formation (McCarty and Jagendorf, 1965). When bean chloroplasts were isolated at pH 8, as for spinach chloroplasts isolated at either pH, these activities were unimpaired. The bean chloroplasts isolated at pH 6 had lost 40-50% of their non-chlorophyll lipid and had the appearance of salt-burst chloroplasts. Their DCPIP reaction exhibited a pH dependence which was optimal at pH 4.5.

Spinach chloroplasts isolated at pH 6 in 0.35 M NaCl and aged for 24 hours displayed the same behaviour and appearance as bean chloroplasts isolated at pH 6. Aging spinach chloroplasts at pH 6 for 21 hours caused the loss of twice as much lipid as did aging at pH 8. Dialysed bean homogenates accelerated the changes in spinach chloroplasts.

The adding of linolenic acid to freshly isolated spinach chloroplasts caused them to have the characteristic behaviour of bean chloroplasts isolated at pH 6, for example, 5×10^{-5} M linolenic acid induced a pH-dependent optimum in DCPIP reduction at pH 4.5. Increases in linolenic acid content of bean chloroplast homogenates ground at pH 6 and in spinach chloroplasts aged for 21 hours at pH 6 were shown. Thus, the changed biochemical characteristics of bean chloroplasts isolated at pH 6 or of spinach chloroplasts aged at pH 6 could be accounted by linolenic acid released by the galactolipase.

A crude galactolipase preparation, isolated from bean leaves using method of Sastry and Kates (1964b), caused a linear increase in the

intensity dependence of the quantum requirement of the DCPIP Hill reaction, when incubated with spinach chloroplasts in low concentrations (Bamberger and Park, 1966). Since the order of magnitude of the qunatum requirement at very low light intensities remained at between 4 and 5 for all three concentrations of enzyme used, it was concluded that one of the dark reactions in the electron transport chain became rate limiting after a short treatment of the chloroplasts with the enzyme preparation. The authors could not be sure that galactolipid hydrolysis, per se, was responsible for the loss of acitivity. Indeed, as pointed out by Constantopoulos and Kenyon (1968), the changes in Hill activity in isolated chloroplasts may be the product of two processes, the structural changes in the chloroplasts brought about by lipases, and the effect of free fatty acids. Prolonged incubation of chloroplasts with the runner bean enzyme preparation resulted in morphological changes within the chloroplast membranes (Bamberger and Park, 1966).

Incubating spinach chloroplasts at room temperature for 2 hours increased the free fatty acid content of the chloroplasts by 42% and decreased the Hill activity (measured spectrophotometrically as the reduction of TCPIP) by 70% (Constantopoulos and Kenyon, 1968). Incubation at 37°C caused three-fold increase in free fatty acids and completely inhibited the Hill activity. Both the free fatty acids released and the Hill reaction were pH-dependent, with the greatest losses at pH 8 and least at pH 5.8. The variance of these results with those of McCarty and Jagendorf (1965) was attributed to the different suspending medium and the higher temperature used here. More saturated fatty acids were released at pH 5.8 than at pH 7.0 or 8.0 when mainly trienoic acids were released. The galactolipase of spinach leaves had pH optima at pH 5.9 and 7.5 for DGDG and MGDG as respective substrates (Helmsing, 1967). This enzyme probably accounts for the release of the unsaturated fatty

acids but another is implicated to explain the quantity and type of fatty acid released at pH 5.8.

The relationship of lipid transformation and photochemical behaviour of fresh and aged spinach chloroplasts were re-examined by Wintermans et al (1969). They found that the Hill reaction of photosystem II and the $NADP^+$ reduction of photosystem I were not directly affected by lipid transformations. However, lipid transformations could contribute to the lability of photophosphorylation and the Hill reaction at limiting light intensities. The greater resistance of photoreductions in photosystem II than obtained by Constantopoulos and Kenyon (1968), was attributed to the differences in the osmotic values of the suspending mediums. The decay in the DCPIP reaction was more rapid as the chlorophyll content of the suspension was decreased and, in this case, the activity of galactolipase did not appear relevant. The authors were unable to decide whether or not the free fatty acid concentration in aging chloroplasts was sufficient to block the electron flow through photosystem I and II at saturating intensities. Nevertheless, very low free fatty acid concentrations could contribute to the uncoupling of photophosphorylation and be responsible for the low quantum yield of the Hill reaction.

Section 1.VI : Raison d'être.

1.VI.1 Structural features:

Any account of the function of galactosyl diglycerides must consider their unique structural features. They are amphipathic asymmetric molecules possessing a neutral hydrophilic region and a highly unsaturated hydrophobic region. The hydrophilic region consists of one or two galactopyranosyl moieties which bear four or seven hydroxyl groups, of which in each case, only one is a primary hydroxyl group. The hydrophobic region consists of two fatty acyl residues which typically have 16 to 18 carbon atoms each, though they may have as many as 22 carbon atoms. The fatty acyl moieties, linked through ester bonds to vicinal hydroxyl groups, have typically six double bonds divided equally between them in such a manner that the fatty acids belong to the ω3 series. The double bonds have a cis configuration and are separated by methylene groups, that is, they are non-conjugated. Thus, the π electrons of the double bonds are localised in the region of the bond in a higher density than if the double bonds were conjugated.

The backbone of the molecule is a glycerol moiety. In common with the phospholipids and sulpholipid of the cell, the hydrophilic group is attached specifically through the hydroxyl of C-3 of sn-glycerol. Whether this configuration has more than biosynthetic importance is unclear.

1.VI.2 Structural components of membranes:

Nichols and James (1968) enunciated two possible functions of lipids as components of the protein-enzyme-pigment-lipid complex of chloroplast.

1. Lipid could act as "specific structural components which maintained lipid-soluble pigments in the correct spatial alignment with one another and their associated enzymes".
2. They might be "relatively non-specific micellar elements which exclude

water and in which the pigment-protein-enzyme complexes could be partially or completely embedded. Such water-free areas could allow the operation of electron-transport chains that would be inhibited by free water and the lipid would thus act as an organized medium of low dielectric constant".

Because the same basic acyl lipid composition occurs in all photosynthetic apparatus which perform the Hill reaction, even though the stoichiometry and fatty acid composition of the lipids may vary, one would conclude that they have rather specific functions.

Chloroplast membranes are conceived to consist of asymmetric lipoprotein subunits stabilized by the hydrophobic association of branched and unsaturated hydrocarbon chains with the hydrophobic internal structure of the lamellar protein (Weier and Benson, 1966; Benson, 1966). In this model, the chlorophyll, carotenoids and quinones would be bound in the highly hydrophobic region between two rows of subunits in a partition. It was proposed that the highly surfactant galactolipids predominated at the membrane interfaces with the stroma, loculi and fret channels. The linolenyl residues might strengthen the hydrophobic association with the membrane protein by associating with the π orbitals of the aromatic amino acids. The three olefinic groups can easily assume a helical orientation which could be conducive to a close association with a single protein chain. In this way the galactolipid molecule would be stabilized at the interface.

The unsaturated fatty acids of galactosyl diglycerides are thought to be important in electron transport in the chloroplast (discussed further in paragraph 1.VI.4). It is therefore relevant, since galactolipids contribute a significant proportion of chloroplast lipids, that lamellar lipid and protein was separated and then sufficiently reaggregated to reconstitute the molecular structure essential for electron transport,

as assayed by the Hill reaction (Shibuya and Maruo). Hence it was possible to restore some of the natural conformation of the membrane.

An alternative explanation for the widespread distribution of polyunsaturated fatty acids might be that, since leaves and to a lesser degree algae, sometimes need to function at low ambient temperatures, the high proportion of unsaturated fatty acids may ensure fully mobile lipoprotein structures over a wide temperature range (Nichols and James, 1968).

Despite the apparent specificity of lipid and protein association reasoned above, in the reassociation of lamellar protein and chloroplast lipids observed experimentally, the combining ratio of lipid with lamellar protein was 29-36 hydrocarbon chains per molecule of lamellar protein, regardless of significant differences in ionic or hydrophobic character of the lipids (Ji and Benson, 1968). Saturation of lipid association occurred when the following amounts of lipid were bound per mole of lamellar protein:

36 moles of palmitic acid
30 moles of chlorophyll
16 moles of MGDG
15 moles of DGDG
15 moles of PG
1 mole of β-carotene.

The reassociation of MGDG or chlorophyll with the protein in the presence of competitors was dependent on the nature of the hydrophobic moiety of the competing amphipathic lipid and independent of their hydrophilic groups. The most effective competitor of MGDG was phytyl sulphate, and palmitic acid competed with chlorophyll. These data were presented as evidence for the hydrophobic association between lipid and protein in the chloroplast.

Morphological changes within the thylakoid membrane were apparent

after prolonged treatment of thylakoids with a crude runner-bean galactolipase preparation (Bamberger and Park, 1966). The changes suggested that thylakoids contain a smooth inner galactolipid layer with which chlorophyll was not associated. The chlorophyll was thought to be associated with the large 175 Å lipoprotein particles and embedding matrix which were lying on the galactolipid surface. The galactolipid layer, which was removed by galactolipase action, was conceived as being completely surrounded by the thylakoid lipoprotein membrane. A stack of such thylakoids make up a granum.

The occurrence of galactolipids in non-chlorophyllous tissues and in mitochondria may emphasize their structural role in plant cells. Galliard (1968a) suggested that the galactolipids of potato tubers may be components of membranes of plastid nature because an electron microscope examination of potato tubers showed the presence of structures resembling the prolamellar bodies of etiolated tissues and membranes of amyloplasts are presumably of plastid origin.

1.VI.3 Relationship to chlorophyll:

(a) In vivo evidence:

Illumination of dark-grown Euglena gracilis cells, transferred to a mineral medium, with a light intensity of 30 foot-candles, induced, after a lag, an almost linear accumulation of chlorophyll and galactolipids (Rosenberg and Gouaux, 1967). At first, the DGDG content increased more rapidly than the MGDG content but when the rate of accumulation of DGDG decreased, before the completion of greening, the increase of the rate of MGDG accumulation was compensatory. The ratio of MGDG to DGDG remained between 2 and 3 throughout greening.

Both the chlorophyll and the total galactolipid fraction started

to increase simultaneously. The rate of accumulation of the galactolipids was proportional to the rate of chlorophyll increase but the galactolipid level was not a function of the chlorophyll level.

It was previously shown that the accumulation of galactolipid was linear and independent of the linear rise of the chlorophyll level by exposing Euglena cells to illumination at 90 foot-candles (Rosenberg and Pecker, 1964). Increased proportions of MGDG and DGDG with photosynthetic growth were also noted in Chlorella vulgaris (Nichols, 1965a). Prolonged cultivation of Chlorella in the dark did not produce etiolated cells devoid of galactolipids, hence light is not a prerequisite for their formation.

Bloch et al (1967) also observed the synchronous increase in chlorophyll and galactolipids in Euglena, so that the ratio of chlorophyll to glycolipid was fairly constant. Even though the formation of chloroplasts in Chlorella vulgaris is not light induced, as they are in Euglena, the same relationship between chlorophyll and galactolipids occurred, with both increasing on illumination. However, in Chorella only the MGDG showed a sharp rise, suggesting that the DGDG levels are less critical than the levels of MGDG for photosynthetic activity. During the period when the chlorophyll and galactolipid content of illumination etiolated cells of Euglena showed the greatest increase, on a dry weight basis, the content of α-linolenate and hexadecatetraenoic acid in the galactolipids rose most steeply. Illumination of the dark-grown cells of Scenedesmus D_3, a green alga which produces chloroplasts in the dark, did not change in chlorophyll or galactolipid content significantly.

Incubation of whole leaves of barley seedlings with [1 - ^{14}C]-acetate gave a greater proportion of incorporation into MGDG than

when leaf slices were used (Appelqvist et al, 1968). In the whole leaves, but not in the leaf slices, chlorophyll was synthesized during the incubation. It was also found that the increase in α-linolenic acid content of the total lipid, and the enrichment of MGDG with linolenic acid, in barley seedlings, occurred in the first 15 hours of greening, with little change after that. This coincided with the early portion of the exponential phase of chlorophyll synthesis and the formation of lamellae.

Hence, available evidence indicates that an inter-relationship exists between the increase and polyunsaturation of galactolipids chlorophyll synthesis and the transition from the crystalline proplastid to the lamellar configuration of chloroplasts. This transition is induced by exposure to light (Nichols, Stubbs and James, 1967). The relatively constant ratio of galactolipids to chlorophyll for a wide range of photosynthetic species (Roughan and Batt, 1969) also indicates an interaction between these molecules.

(b) Lock-and-key hypothesis:

A possible function for the galactosyl diglycerides of Euglena was proposed by Rosenberg (1967). He suggested that the unsaturated fatty acyl chains of the galactolipids could provide a stable lock-and-key fit with the phytol chains of the chlorophyll in such a way that the porphyrin structures of chlorophyll would be spaced for efficient photoreception. Chlorophyll molecules, which consist of a hydrophilic (relatively) chlorophyllide and a hydrophobic phytol tail. must be spread in a thin film to give an efficient photoreceptive surface. Galactolipids were considered the lipid class with the most suitable fatty acids for forming a stable interaction with the phytol tail. The 16-carbon phytol bears four methyl groups at

positions 3, 7, 10 and 15 while the methylene-interrupted cis-double bonds of α-linolenic acid cause pockets of twists in the conformation of the fatty acid. Thus phase-mixing between the branched-chain phytols and the twisted fatty acid chains should be possible. Additional stability would be gained by the induced polar interactions, which are of the order of a hydrogen bond, between the methyl groups and the double bonds. The close association of the two chains would also enable the backbones of these moieties to be further stabilized by London - van der Waals dispersion-attraction forces. Models of linolenic acid and phytol can be arranged to give such a space-saving arrangement. A film of chlorophyll can be stabilized by superposition on a film of oleoyl alcohol but not on a film of stearoyl alcohol. Relative to the pure chlorophyll film, the stabilized film had enhanced ability to react with light.

A ratio of one phytol chain for 4 cis-double bond fatty acyl chains from galactosyl diglycerides would space the chlorophyll sufficiently for good photoreception. Illuminated Euglena cells maintain a ratio of 2 to 3 molecules of galactosyl diglycerides/ molecule of chlorophyll.

Rosenberg also suggested that other methyl-bearing groups, e.g. plastoquinones, might be similarly stabilized by galactolipids.

(c) Molecular studies.

With increasing chlorophyll concentration in mixed monolayers with purified chloroplast glycolipids, and other colourless lipids e.g. oleoyl alcohol, the chlorophyll a fluorescence exhibited a decrease in quantum efficiency (Trosper, Park and Sauer, 1968). The fluorescence which was strongly polarized in dilute films, became progressively depolarized as the area fraction of chlorophyll

increased, and in the pure chlorophyll a monolayer it was completely depolarized. This behaviour was consistent with an inductive resonance mechanism of energy transfer among the chlorophyll molecules with a critical transfer distance of 20-90 Å. The fluorescence polarization of high chlorophyll concentrations in mixed monolayers indicated that several of the lipid diluents facilitated local ordering of the pigment molecules.

Purified MGDG, DGDG, and probably SQDG, formed relatively strong complexes with chlorophyll a, as measured by their ability to dissociate chlorophyll dimers in carbon tetrachloride solution (Trosper and Sauer, 1968). The extent of the dimerization of chlorophyll in carbon tetrachloride was dependent on the pigment concentration and the presence of polar solvents or Lewis bases. The chloroplast lipids interacted with chlorophyll a to form one-to-one complexes. It was concluded that galactolipids would compete effectively with water for the pigment, on considering the relative free energies of interaction, and that in the presence of excess lipid, chlorophyll a complexes would form at the expense of chlorophyll a aggregation, even in an environment containing water molecules. The authors suggested that the bulk fraction of the randomly-oriented pigment in the chloroplast lamellae may be associated with MGDG because chlorophyll was randomly dispersed by MGDG in monolayers and MGDG randomly broke up pigment dimers in solution; and also that aggregated, oriented forms of chlorophyll a, *in vivo*, were probably not in such an environment. They realized that extrapolation of their results to chloroplast lamellar surfaces were somewhat tenuous.

The electronic absorption spectra of chlorophyll molecules in aqueous DGDG and phospholipid dispersions showed a shift for the red peak of chlorophyll of some 10 millimicrons to a longer wave-

length to 671 mμ and 673 mμ, respectively, compared with the spectrum of chlorophyll in ether (Chapman and Fast, 1968). Chlorophyll a in ether shows a strong absorption band at 661 mμ but it is observed at 675 mμ in Chlorella suspensions. The significance of these shifts could not be assessed.

1.VI.4 Implication in electron transport:

For the chemical involvement of acyl lipids in the electron-transport chain, the unsaturated fatty acids would be the most likely candidates as readily oxidizable components. Over 80% of MGDG molecules and over 60% of DGDG molecules (calculated from Table 4) of higher plants usually have at least six double bonds (two α-linolenic molecules) per molecule, thus they are the most likely acyl lipid to be involved.

The essentiality for α-linolenic acid, either chemically or by virtue of some physico-chemical properties, in the operation of oxygen-evolving systems in green plants and photosynthetic protists was argued by Erwin and Bloch (1964). They observed:

1. Scenedesmus mutant that was defective in the Hill reaction had morphologically normal chloroplasts but its α-linolenic content was very much less than in the parent wild-type but otherwise it had a normal fatty acid spectrum.
2. In short term experiments, light specifically stimulated incorporation of ^{14}C-acetate into α-linolenic acid by green Euglena. This stimulation, but not the synthesis of other fatty acids, was inhibited by Hill reaction inhibitors, e.g. CMU.
3. The α-linolenic acid content of green Euglena was shown to depend on other environmental variables that affect photosynthetic evolution of oxygen e.g. atmospheric CO_2.

However, when polyunsaturated fatty acids were shown to be absent

from the blue-green alga Anacystis nidulans (Holton, Blecker and Onore, 1964), which evolves oxygen in photosynthesis, there could be no absolute correlation between the distribution of unsaturated fatty acids and the occurrence of the Hill reaction. Nevertheless, evidence exists that suggests that the efficiency of the Hill reaction correlates with the chloroplast polyunsaturated fatty acid content i.e. the degree of unsaturation of the galactolipids.

The periodic changes in photosynthetic competence of whole cells of Chlorella vulgaris monitored as photosynthetic oxygen evolution per mg. of chlorophyll, were paralleled by changeds in α-linolenic acid per mg of chlorophyll (Appleman, Fulco and Shugarman, 1966). In general, the magnitude of the photosynthetic competence of chlorophyll was directly proportional to the magnitude of the ratio of α-linolenic acid to chlorophyll.

Illumination of photoautotrophic Euglena gracilis cells, at light intensities varying between 120 and 610 foot-candles, caused the chlorophyll and galactosyl diglyceride content to decrease with increasing light intensity (Constantopoulos and Bloch, 1967b). In contrast, the content of highly unsaturated fatty acids increased with the light intensity, especially in MGDG. The increase of Hill reaction activity of isolated Euglena chloroplasts per mg of chlorophyll, or mg dry weight of chloroplast, was in direct proportion to the increase in light intensity at which the cells were grown. This response demonstrated a high degree of correlation with the level of polyunsaturated fatty acids in the chloroplast MGDG isolated from the same cells. The $\omega 3$ fatty acids, α-linolenic acid and hexadecatetraenoic acid, contributed 31% and 15%, respectively, of the total fatty acids of the MGDG of cells grown at the highest light intensity.

Constantopoulos and Bloch (1967a) suggested that "plant fatty acids linked to the galactosyl diglycerides may have evolved to provide an especially favourable environment for the electron transport processes in higher plant photosynthesis".

The occurrence of polyunsaturated fatty acids, other than α-linolenic acid, in galactolipids, suggested to Patton et al (1966) that the function of α-linolenic acid could be equally well effected by other members of the linolenic acid family i.e. fatty acid belonging to the ω3 series. Another suggestion was that the overall degree of unsaturation of the galactolipid molecules was more important than the actual fatty acyl residues (Kates and Volcani, 1966).

Galactosyl diglycerides had a stimulatory effect on the rate of cytochrome c photoreduction by intact spinach chloroplasts (Chang and Lundin, 1964). DCMU inhibited this stimulation, suggesting that the galactolipids might participate in photosystem II. Both MGDG and DGDG stimulated equally well on a molar basis and the phosphorylating condition had no effect on the activity of the galactolipids in cytochrome c reduction.

The participation of galactolipids in the overall organization of electron transport in the photochemical system of plants was revealed by galactolipase treatment of chloroplasts as interpreted from fluorescence spectra (Ostrovskaya, Kochubei and Manuil'skaya, 1969). However, as mentioned in paragraph 1.V.4, the effect of galactolipase on photochemical activities may be due to both the loss of molecular integrity of the galactolipids and the resultant free fatty acid concentration.

If galactolipids are somehow involved in electron transport chain the available evidence would indicate photosystem II as the most likely site of their involvement (Figure 5).

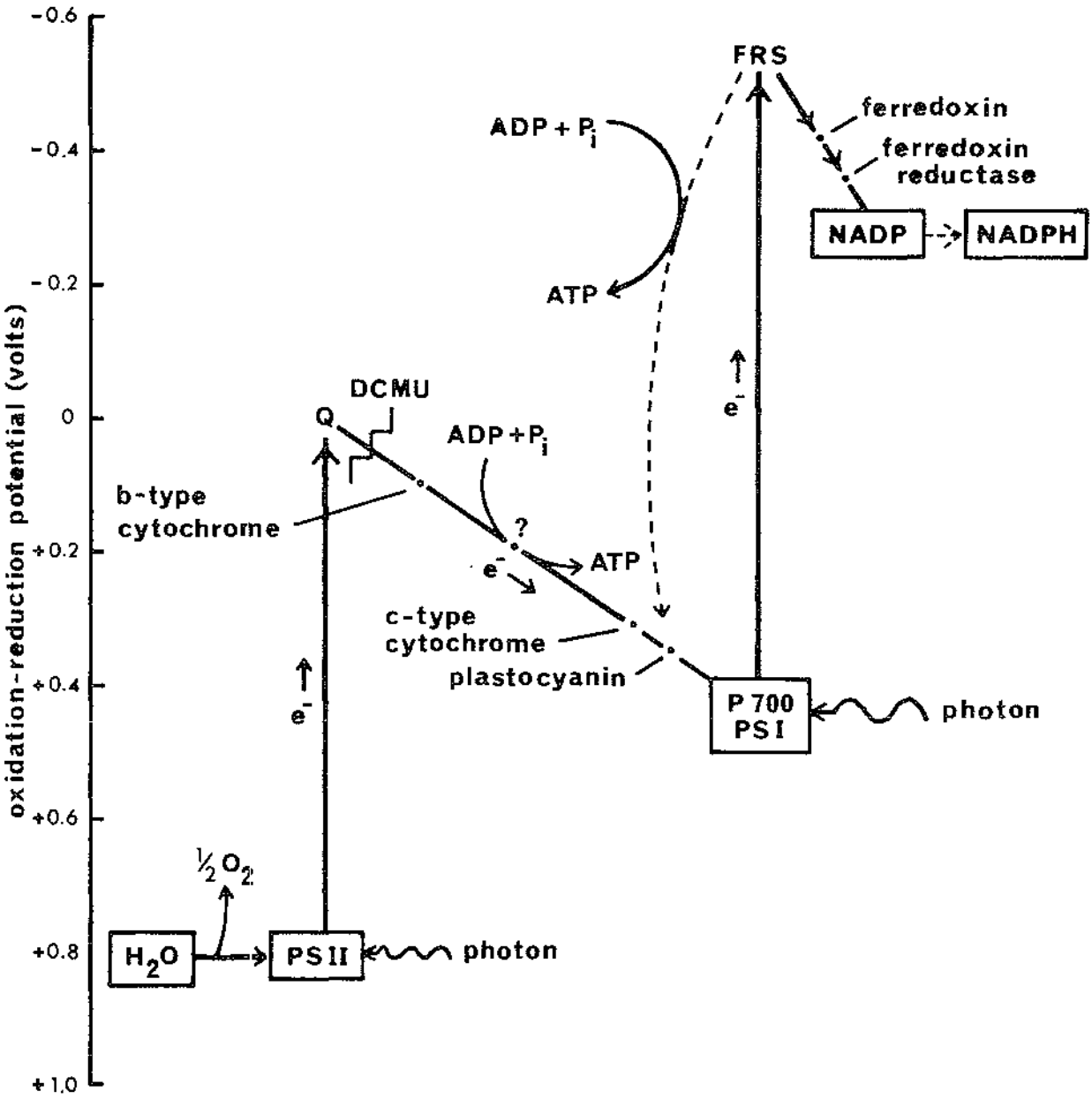


Figure 5

The electron-transport chain in photosynthesis
(Levine, 1969)

1.VI.5 Participation in carbohydrate metabolism:

High amounts of radioactivity were incorporated into the galactolipids of Chlorella during photosynthesis in $^{14}CO_2$, with the deacylated MGDG and DGDG containing about 34% and 17% of the total amount of label of the deacylated lipid products (Ferrari and Benson, 1961). The authors proposed that the galactolipids were metabolically active, the galactosyl moieties being exchangeable with the intermediate of hexose photosynthesis. An association with the enzyme of saccharide synthesis was suggested. The high incorporation of $^{14}CO_2$ in galactolipids was confirmed using runner-bean leaves for which ^{14}C-glucose gave a similar labelling pattern (Kates, 1960). Radioactivity was also significantly incorporated from $^{14}CO_2$ into the galactosyl diglycerides of the fresh-water diatom, Navicula pelliculosa (Kates and Volcani, 1966). The proportions of labelling of the deacylated lipids after 4 days in the light were glycerol > monogalactosyl glycerol > digalactosyl glycerol > 6-sulfoquinovosyl glycerol. These four deravitives contained 80% of the incorporated radioactivity, which was almost equally shared.

No more substantial evidence for an involvement of galactolipids in carbohydrate metabolism is available. It would be interesting to know if the α-glycosidic linkage of DGDG has any significance in this regard.

The occurrence of the TGDG, which has been detected in radiochemical experiments (Ongun and Mudd, 1968), identified in spinach lamellae (Allen, Hirayama and Good, 1966) and characterized for potato tubers (Galliard, 1968a), can not be evaluated as more than a curiosity at this stage. Galactolipids could play a role in the starch-storing plastids of root tissue (Roughan and Batt, 1969) similar to the involvement in sugar transport across the chloroplast membrane as suggested by Benson (1963).

The participation of bacterial glycolipids in polysaccharide synthesis was speculated by Distler and Roseman (1964); however, this is less likely since, in the biosynthesis of lipopolysaccharides (Wright et al, 1967) and peptidoglycans (Hiyashi, Stromin.r and Sweeley, 1967) in bacteria, the sugar residues are attached to an intermediate lipid pyrophosphate in which the lipid was a polyisoprenoid alcohol.

1.VI.6 Participation in fatty acid metabolism:

A study of the uptake of $[2\text{-}^{14}C]$ - acetate into the acyl lipids of Chorella vulgaris showed two distinguishable groups of lipids in both the dark- and light- incubated cells (Nichols, James and Breuer, 1967). Group one, consisting of PG, MGDG, PC and neutral glyceride, had a very turnover rate of certain fatty acids while group two, consisting of DGDG, SQDG, PE and PI had a slow turnover of fatty acids. The maximal labelling of Group one was usually evident in first four hours of the incubation in either dark or light; this peak was followed by a slight decline and recovery with time. The major changes in fatty acid composition were completed within 24 hours (about one cell generation). At the end of 48 hours the proportion of label in DGDG matched that in MGDG. The high specific activities of the stearic acid and palmitic acid of MGDG in the first four hours showed that MGDG had a very rapid flux in these acids. Quantitatively, the most important fatty acid in MGDG was oleic acid which contained 45% of incorporated label after one hour, but this had declined to about 10% after 24 hours with a compensatory rise in linoleic and linolenic acids. The major fatty acid compositional changes occurred in MGDG, DGDG and PG, in which trans-3-hexadecanoic acid appeared. When the total fatty acids of MGDG were analysed, only linolenic acid made a dramatic rise.

MGDG had a high turnover of tetradecanoic, palmitic, hexadecenoic,

stearic, oleic and linoleic acids while DGDG had either a low turnover rate or a steady increase in specific activities of fatty acids which suggested a slow synthesis. The fatty acids with a very fast turnover are intermediates in the sequence

$$14{:}0 \longrightarrow 16{:}0 \longrightarrow 18{:}0 \longrightarrow \Delta^{9}18{:}1 \longrightarrow \Delta^{9,12}18{:}2$$

$$\downarrow \qquad\qquad\qquad\qquad\qquad\qquad \downarrow$$

$$\Delta^{9}16{:}1 \qquad\qquad\qquad\qquad \Delta^{9,12,15}18{:}3$$

The maximum turnover rates were so high as to suggest that the acyl lipid was acting in a manner similar to that expected for a true intermediate in a fatty acid synthetic sequence. Thus, MGDG could act as a transporting agent, moving the fatty acid between enzyme locations, or be the true substrate of the desaturase. The labelling between groups one and two was so different as to make acyl-migration between the groups unlikely although migration was possible within each group.

The results were consistent with galactosylation of MGDG and DGDG since the specific activities of all the major fatty acids in the two lipids were the same at the end of the incubation. The observed difference in fatty acid profiles of MGDG and DGDG may be due to deacylation - reacylation or specific conversion of distinct MGDG species.

The incubation of heterotrophic Chlorella vulgaris with [2-^{14}C]-acetate and analysis of the individual MGDG species, separated according to number of double bonds per molecule, showed that at any one time the specific activity of a single fatty acid could vary considerably among the various species in which it occurred (Nichols and Moorhouse, 1969). This was consistent with the hypothesis that modification of the fatty acid composition of MGDG occurred after its de novo synthesis. The results are not conclusive as certain species of MGDG may be more metabolically active than others.

Thus for Chlorella, both MGDG and DGDG may have structural functions

with MGDG having an additional metabolic role. Whether this is also true for higher plants awaits verification. However, the differential light-induced synthesis of MGDG and DGDG in Chlorella, unlike Euglena, may be relevant (Bloch et al, 1967).

The pattern of uptake of radioactivity into the blue-green algae Anabaena cylindrica and Anacystis nidulans, when incubated with [2 - ^{14}C] - acetate differed from that observed for Chlorella vulgaris under similar conditions (Nichols, 1968). DGDG (and SQDG) were labelled more quickly in the blue-green algae than in Chlorella. In all three algae, only slight variations in the proportion of activity in each lipid class throughout the course of the reaction. Hence, transfer of fatty acids between the lipid classes did not occur to any great extent because the specific activities of different lipid classes, at any moment, were sufficiently different that significant acyl transfer would have resulted in marked differences in total activity in the lipids. The changes in distribution of activity among each fatty acid of MGDG of Anacystis nidulans showed a consistent desaturation of tetradecanoic and palmitic acids to the corresponding monoenes. The increase in total activity in hexadecanoic acids was balanced by the loss from tetradecanoic acids. Both sets of results for Anabaena cylindrica and Chlorella vulgaris for the MGDG (and SQDG) analysed were compatible with progressive desaturation of hexadecanoic and octadecanoic acids coupled with some conversion to the long-chain acids. Thus the results were consistent with an intermediary role of lipids in fatty acid synthesis, though the metabolic behaviour of individual lipids may vary according to the class of alga.

When barley leaf slices were incubated with [1 - ^{14}C]- acetate, the amount of labelling of α-linolenic acid was highest in the MGDG fraction and very low in DGDG fraction (Appelqvist et al, 1968). This could indicate the more active role for MGDG. On the addition of UDPG

to a reaction mixture containing lettuce chloroplasts, [2 - ^{14}C]- acetate and non-cyclic phosphorylation cofactors, an increased proportion of the incorporation appeared in the total α-linolenic acid fraction relative to the palmitic acid fraction (Newman, 1966). This may be further evidence for the participation of galactolipids in fatty acid metabolism.

1.VI.7 Miscellaneous:

A novel proposal was made for the function of bacterial glycolipids (Brundish, Shaw and Baddiley, 1967). The glycolipids would form clusters in which the hydrophilic regions of the molecules would come together to form a "pore" extending through the membrane and allowing the passage of small ions and charges water-soluble metabolites into the cell.

The MGDG of the nervous tissue is likely to have primarily a structural role. Its concentration increased with the onset of myelination in developing rat brain (Wells and Ditmer, 1967).

Chapter 2

THE AIM OF THE PRESENT WORK

This study was undertaken to further elucidate the biosynthetic pathway of galactolipids in plant tissue - especially in Gramineae species. Previous workers (Neufeld and Hall, 1964; Ongun and Mudd, 1968) had shown that spinach leaf chloroplasts were active in galactolipid synthesis. Thus, the object was to show similar activity in the chloroplasts isolated from the leaf tissue of Gramineae species and to find the specificity of the enzyme(s) concerned for the diglyceride substrates. Synthetic 1,2 (2,3) - diglycerides were prepared for this purpose.

Since this work was commenced, Mudd and his coworkers have explained much of the biosynthetic pathway of galactolipids in spinach leaves. Consequently, the present study parallels and supplements their work.

Chapter 3

MATERIALS AND METHODS

Section 3.I : Materials and analytical techniques.

3.I.1 Reagents and solvents:

All the chemicals used were either Analytical, Laboratory or Technical Reagents from the British Drug Houses Ltd. (Poole, England) or from May and Baker Ltd. (Dagenham, England), except for the following:

Dextran 40T, from Pharmacis, Uppsala, Sweden.

Dioxane, from Nuclear Enterprises (G.B.) Ltd., Edinburgh, Scotland.

Ficoll, from Pharmacia.

Linoleic acid (Puriss), from Koch-Light Laboratories Ltd., Colnbrook, England.

γ -Linolenic acid (Puriss), substrate for lipoxidase, from Koch-Light Lab. Ltd.

POPOP (1,4 bis [2 - (5-phenyloxazolyl)]-benzene) and

PPO (2,5 diphenyloxazole), from either Nuclear Enterprises (G.B.) Ltd., or Sigma Chemical Co., St. Louis, U.S.A.

Tris [tri-(hydroxymethyl) methylamine], Puriss, from Koch-Light Lab.Ltd.

All solvents, except dioxane, were distilled before use.

3.I.2 Radioactive materials:

D - Galactose -1- ^{14}C, specific activity of 35.4 or 55.7 mC/mM

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

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

These radioactive materials were obtained from the Radiochemical Centre (Amersham, England).

3.I.3 Chromatography:

(a) Thin-layer chromatography (TLC)

Glass plates, either 5cm. x 20cm. or 10cm. x 20cm., were spread with a slurry of Silica Gel G (according to Stahl), from E. Merck A.G. (Darmstadt, Germany), at a suitable thickness (0.25mm to 0.50mm) with a spreader made by Desaga (Heidelberg, Germany). The thin layers were activated by heating at 110°C for one hour.

(b) Solvent mixtures employed in TLC and paper chromatography.

Compounds separated	solvent mixture
i. by TLC	
diglycerides	hexane-diethyl ether - glacial acetic acid, 70:30:1 v/v
	† hexane-diethyl ether, 70:30 or 50:50 v/v
galactolipids	toluene - ethyl acetate - 95% ethanol 2:1:1 v/v
phospholipids	chloroform - methanol - glacial acetic acid-water, 85:15:10:3 v/v
ii. paper chromatography	
polyhydric compounds	pyridine - ethyl acetate - water 2:5:5 v/v, upper phase

† used for boric acid impregnated plates.

(c) Detection of compounds on chromatograms.

Acyl lipids were usually detected on thin-layer chromatograms by spraying with 2,7 - dichloro(R) fluorescein, 0.2% (w/v) in ethanol, and viewing under ultraviolet light, or alternatively, exposing the chromatogram to iodine vapour.

The water-soluble products of galactolipids were detected on the paper chromatogram by spraying with a freshly prepared solution of 2% sodium metaperiodate and 1% potassium permanganate, 2:1 (v/v). As the spots developed, the excess reagent was washed from the chromatogram with running water. The spots appeared as brown areas on a white background.

(d) Gas Liquid Chromatography (GLC)

The fatty acid methyl esters obtained from the synthetic diglycerides were analysed in a Packard Gas Chromatograph on a 2mm x 6 ft. column of 15% DEGS (diethylene glycol succinate), supported on 60 - 70 mesh Anakrome G (Analabs, Hamden, Connecticut, U.S.A.), at an oven temperature of 160°C.

The analyses of samples obtained in the fractionation of methyl oleate were performed using a Varian Aerograph (model 1520) Chromatograph on a $\frac{1}{4}$-inch x 5 ft. column of 15% DEGS, at 165°C, with a flow rate of the carrier gas, nitrogen, of about 45 ml/min.

For all other fatty acid analyses, the Varian Aerograph was used with a $\frac{1}{8}$-inch x 6 ft. column of 12% DEGS, at a temperature of 150°C.

3.I.4 Quantitative and qualitative measurement of radioactivity:

(a) Radioisotope counting

An aliquot of radioactive extract in chloroform was evaporated to dryness in a counting vial with a current of air and 10 mls of toluene scintillation fluid (toluene containing PPO (0.6%) and POPOP (0.05%)) added. If the radioactive sample was in aqueous solution, 10mls. of Bray's scintillation solution [naphthalene (60g), PPO (4g), POPOP (200mg), methanol (100ml), ethylene

glycol (20ml) and p-dioxane to make 1 litre_/ were added to the aqueous aliquot.

All aliquots, of up to 50 µl., of radioactive solutions mentioned in this thesis, were measured with syringes (Hamilton Co. Ltd., Whittier, California, U.S.A., or Scientific Glass Engineering Pty. Ltd., Melbourne, Australia).

Duplicate sample vials were counted through the preset -C^{14} channel in a Packard Tri-carb Liquid Scintillation Spectrometer, model 3375. The counting rates had a percentage standard error which was invariably less than two and usually around one. The counting efficiency for each sample vial was read from curves of AES (automatic external standard) value against observed counting efficiency. These curves were constructed by finding the effect of increasing amounts of quenching agent (non-radioactive total lipid extract) with a measured amount of n - hexadecane -1- ^{14}C. Alternatively, for experiments 4.I.1, 2 and 3, the counting efficiency was obtained by counting a radioactive standard vial with the sample vials.

(b) Radioisotope scanning.

The developed chromatogram was scanned for radioactivity using a Packard Radiochromatogram Scanner, model 7200, with a mixture of 1.3% isobutane - 98.7% helium as carrier gas.

Optimum conditions were:

gas flow 110 c.c./min; high voltage 1.15 kv.; time constant 30 sec.; scale 0-300 cpm.; speed 6 or 12 cm/hr.; slit width 2.5mm.

For paper chromatography, the gas flow was 150 cc/min and sometimes a scale of 0-100 cpm (time constant 100 sec.) was preferred.

The contributions of different compounds to the total radioactivity of the sample were assessed from the radio-chromatogram using a planimeter (G. Coradi A.G., Zurich, Switzerland).

3.I.5 Sonication

A MSE (Measuring and Scientific Equipment Ltd., London, England) 100 watt ultrasonic disintegrator was utilized in the auto mode at a power output of about 8 microns peak-to-peak.

Section 3.II : Experimental procedures.

3.II.1 Plant tissues:

Barley (Hordeum vulgare) seedlings were grown under sterile conditions in Agee jars in dark at 23°C. The seedlings were greened for 24 hours, by illumination from a 250 watt IR reflector lamp held at 60 cm., immediately prior to harvesting.

Meadow fescue (Festuca elation) and phalaris (Phalaris arundinacea x tuberosa) leaf tissue was gathered from clones grown in the open. The samples of ryegrass (Lolium perenne) and Yorkshire fog (Holcus lanatus) were obtained from paddocks of each. Spinach (Spinacia oleracea) was bought at a local greengrocer's shop.

3.II.2 The incubation of leaf slices and extraction of lipids:

(a) Barley.

The whole leaves of barley seedlings were harvested, washed with water, blotted dry, and cut into 1mm slices with a dewaxed stainless steel razor blade. The incubation mixture was composed of:

1 gm sliced leaf tissue

0.2 ml M phosphate buffer, pH 7.4

1.4 ml water

0.1 ml $KHCO_3$ (50 μmoles/0.1 ml).

0.05 ml Na acetate (200 μmole/0.1 ml)

and galactose-1-^{14}C

The incubation was performed at 25°C with shaking.

The reaction was stopped by adding sufficient chloroform-methanol (C/M) 1:2 (v/v), to give one phase. The supernatant was decanted after refluxing the solution for one hour, and the residue re-extracted twice with C/M 2:1 (v/v). The combined

extracts were washed with 0.2 volumes of 0.73% aqueous NaCl made 1% with respect to D-galactose (after Folch, Lees and Sloane-Stanley, 1957). The aqueous salt-methanol layer was re-extracted with chloroform and the combined chloroform fractions dried with anhydrous Na_2SO_4. The clear lipid extracts were evaporated to dryness under reduced pressure and redissolved in 1ml of chloroform. Suitable aliquots were taken for radioisotope counting and scanning.

(b) Fescue.

The leaf tissue was washed and sliced as for barley. The incubation flasks contained,

0.5 g tissue slices

0.30 ml M phosphate buffer, pH 7.4

2.60 ml water

0.10 ml $KHCO_3$ (50 μmoles/0.1ml)

0.05 ml Na acetate (200 μmoles/0.1ml)

0.10 ml cysteine hydrochloride (1.2 μmoles/0.1ml)

0.10 ml D-galactose-1-^{14}C (containing 2.11 x 10^6 dpm)

After a 4-hour incubation at 25°C, with shaking and in room light, the incubation was stopped by adding sufficient C/M, 2:1, to give one phase and the solution refluxed for one hour. The supernatant was decanted, the residue re-extracted twice with C/M, 2:1 (v/v), and the combined chloroform extracts washed with 0.58% aqueous NaCl (0.2 vol.). The aqueous layer was extracted with chloroform and the combined chloroform fractions washed with 0.58% aqueous NaCl. The chloroform layer was taken to dryness, with, if necessary, the aid of the mixture absolute alcohol-benzene, 90:10 (v/v), and the lipid extract redissolved in 2 mls of chloroform. Aliquots were taken for radioisotope

counting and scanning.

3.II.3 Isolation and hydrolysis of radioactive galactolipids:

(a) Isolation.

Three of the total lipid extracts from experiment 4.I.2, involving barley leaf slices, were resolved by preparative TLC. The MGDG and DGDG bands, identified by standards run at the sides of the plate, were scraped into centrifuged tubes and extracted with C/M, 2:1 (v/v). (The MGDG and DGDG standards were isolated from red-clover lipid extracts using the standards of R.O. Weenink). The radioactive galactosyl diglycerides were re-isolated by TLC and to check purity, areas in front of and behind the sample bands were also extracted. The extracts were taken to dryness, redissolved in 1ml of chloroform and aliquots counted for radioactivity. Table 7 shows the results.

Table 7.

The distribution of radioactivity on the TLC plates after re-chromatography of the isolated radioactive galactolipids

area	total dpm	remarks
in front of the MGDG band	600	
MGDG band	68,400	
behind the MGDG band	200	included area of DGDG band
in front of the DGDG band	3,500	included area of MGDG band
DGDG band	97,200	
behind the DGDG band	700	included the origin area

The re-isolated galactolipid samples were concluded to be reasonably pure.

(b) Alkaline hydrolysis.

Each sample was refluxed with 5 mls of 0.3M NaOH in 90% methanol for one hour. The solution was shaken with DOWEX-50 (H^+ form), to remove sodium ions, and the resin filtered off using a filter paper pre-washed with petroleum ether. The fatty acids were extracted with petroleum ether and the neutral methanolic solution taken to dryness, below 30°C. The products were redissolved in a small amount of water.

(c) Acid hydrolysis.

The water-soluble products of alkaline hydrolysis were refluxed for 4 hours with 5 mls of 2M HCl. After 2 hours, 2 mls of 2M HCl were added to maintain acid strength. The solution was evaporated to dryness and the residue placed over KOH sticks in a vacuum dessicator for 4 hours. The acid hydrolysis products were dissolved in water, the solution adjusted to neutrality with dilute NaOH, evaporated to dryness, and the products redissolved in a small volume of water.

(d) Analysis of the hydrolysis products.

Aliquots of water-soluble and ether-soluble hydrolysis products were counted for radioactivity in the appropriate scintillation fluid. Samples of the water-soluble hydrolysis products were spotted onto Whatman 3MM chromatography paper with standards of galactose, glucose and glycerol. The chromatogram was developed by a descending technique for 5½ hours and the appropriate strips were scanned for radioactivity.

3.II.4 Isolation of chloroplasts:

Crude chloroplast preparations were prepared by two methods. All operations were carried out at 0 - 4°C. Chlorophyll content was

estimated, using 80% aqueous acetone solutions, according to Arnon (1949). The spinach leaves were deveined before use.

(a) Method I.

(after Spencer and Wildman, 1964).

The leaf tissue was chilled on ice for 1 - 2 hours, then 7 g, wet weight, of tissue were minced as finely as possible with dewaxed razor blades in a medium (about 20 mls.) consisting of

25 mM Tris - HCl buffer, pH 7.4

250 mM sucrose

1 mM magnesium acetate

4 mM mercaptoethanol

2.5% Ficoll

5% Dextran 40T

The resulting brei was strained through 2 layers of nylon stocking and centrifuged for 2 min. at 30 x g and 10 min. at 3020 x g (Sorval RC - 2B centrifuge). The crude chloroplast pellet was suspended in 1 ml of 0.1M Tris - HCl buffer, pH 7.4.

(b) Method II.

The leaf tissue, prechilled on ice for 1 - 2 hours, was ground with acid-washed sand in a medium (2 volumes, v/w) consisting of

0.01 M Na_2HPO_4 - KH_2PO_4 buffer, pH 7.4

0.5 M sucrose

The homogenate was strained through three layers of nylon stocking and centrifuged for 2 min. at 120 x g and 2 x 10 min. at 3020 x g. The crude chloroplast pellet was suspended in 0.1M Tris - HCl buffer, pH 7.4.

3.II.5 Preparation of crude enzyme preparations from chloroplasts:

(a) Acetone powder preparation

(after Ongun and Mudd, 1968).

Chloroplasts were isolated by method II from approximately 100 g., wet weight, of leaf tissue and the pellet suspended in 10 ml of 0.1M Tris - HCl buffer, pH 7.4. The suspension was pipetted quickly into 80 mls of acetone at -15 to -20°C and stirred vigorously. The mixture was filtered under suction at -13°C and the suction continued for one hour. The green precipitate was stored on filter paper in a vacuum dessicator for two days at -20°C, then ground to a fine powder in a glass mortar and stored at 4°C.

Table 8 summarizes the data concerning the acetone powder preparations.

Table 8.

Summary of the acetone powder preparations of chloroplasts

tissue	approx. wet weight of leaf tissue used (g.)	date	preparation number	weight (mg)	chlorophyll content of chloroplast suspension (mg)
fescue	70	9-9-69	1	52	not determined
fescue	100	15-9-69	2	461	16.1
fescue	100	25-9-69	3	409	16.3
spinach	100	9-9-69	1	321	19.1
spinach	100	15-9-69	2	380	19.7
spinach	100	25-9-69	3	312	18.8
spinach	100	10-10-69	4	365	26.6
spinach	100	10-10-69	5	389	24.7
spinach	100	24-10-69	6	388	25.5
spinach	100	24-10-69	7	259	18.4
spinach	100	7-11-69	8	245	16.9
spinach	100	7-11-69	9	287	18.2

(b) Freeze-dried chloroplast preparations.

The chloroplast fraction from about 100g., wet weight, of spinach leaves were isolated by method II, except that only one centrifugation at 3020 x g was performed. About 70g. of fescue leaf tissue was also treated as in method II to yield a crude chloroplast pellet. The chloroplast pellets were suspended in 0.1M Tris - HCl buffer, pH 7.4, freeze dried, and stored at $4^{\circ}C$.

3.II.6 Incubation procedures of chloroplast preparations:

(a) Chloroplast suspensions.

Aliquots of chloroplast suspensions were incubated with microlitre aliquots of UDP-D-galactose - ^{14}C solution (5 μC/ml) at $30^{\circ}C$, with shaking, in room light.

(b) Acetone powder preparations.

Acetone-soluble lipids were homogenized with 20.0mg. of acetone powder in about 0.2ml of acetone in a glass homogenizer with a teflon plunger. The acetone was quickly evaporated in a stream of nitrogen and the mixture homogenized in 1 ml. of 0.1M Tris - HCl buffer, pH 7.4. The suspension was slowly bubbled with nitrogen for 30-45 seconds and then sonicated for up to 5 minutes in an atmosphere of nitrogen. An aliquot (0.7ml.) was incubated with UDP-D-galactose - ^{14}C at $30^{\circ}C$, with shaking. Acetone-insoluble lipids were transferred to the homogenizer in diethyl ether and the ether evaporated with a current of nitrogen. The acetone powder was then added and rubbed with the lipid using the plunger. With the addition of buffer, the mixture was treated as above.

(c) Freeze-dried chloroplasts.

The freeze-dried chloroplast preparation (25mg) was homogenized in 1 ml. of 0.1M Tris - HCl buffer, pH 7.4, and an aliquot

(0.7ml.) was incubated with UDP-D-galactose - ^{14}C at 30°C, with shaking. Acetone-soluble lipids were homogenized with dry freeze-dried preparation in acetone, which was evaporated before homogenization in buffer.

3.II.7 Extraction of lipids from reaction mixtures:

The reaction was stopped by adding sufficient C/M, 1:2 (v/v), to give one phase and heating to boiling, or to near boiling for 5 minutes. After low speed centrifugation, the supernatant was decanted and the residue was agitated with C/M, 2:1 (v/v), on a vortex mixer and heated to boiling. The bulked extracts were washed three times with 0.58% aqueous NaCl. The chloroform layer was evaporated to dryness and the total lipid extract redissolved in 0.5ml. of chloroform. Suitable aliquots were taken for radioisotope counting and scanning.

Section 3.III : Preparation of lipid substrates

3.III.1 Chemical synthesis of diglycerides.

(adapted from Mattson and Volpenheim, 1962)

(a) Fatty acids.

Oleic acid was prepared from methyl oleate which had been purified by fractionation on a spinning band column (Nester-Faust Co., Delaware, U.S.A.). The average composition of the fractions used to yield oleic acid was 92.1% methyl oleate and 7.9% methyl linoleate, as analysed by GLC. The bulked fractions were refluxed for 2 hours with 5% methanolic KOH, and, after removal of the methanol, the residue dissolved in water. On acidification, the solution was extracted three times with hexane, and the combined extracts washed until neutral. Evaporation of the hexane, under reduced pressure, yielded the oleic acid.
The oleic acid employed in the preparation of 1-linoleoyl, 2-oleoyl glycerol was the gift of Mr. O. Faruque.
The linoleic and linolenic acids were used directly.

(b) Preparation of acyl chlorides.

One part of fatty acid and 1.2 parts of oxalyl chloride, by weight, were mixed and allowed to react for 3 days at room temperature, the mixture was heated on a steam bath for 30 minutes under the vacuum of a water aspirator and the fatty acid chloride taken up in 20 volumes of hexane. After washing three times with ice-cold water, the solution was dried with anhydrous Na_2SO_4, and the solvent evaporated under reduced pressure. The fatty acyl chloride was stored at $-20^{o}C$ under vacuum.

(c) Preparation of 1,3-benzylidene glycerol.

The glycerol was dehydrated by vacuum distillation, in which the

first and last 15% were rejected.

Equimolar amounts of glycerol and benzaldehyde, three volumes of toluene and a catalytic amount of p-toluene sulfonic acid (2% of glycerol, by weight) were stirred and then refluxed, with the condensed vapours passing through a water trap. When the water evolution stopped, the flask was immersed in an ice water bath, allowed to cool, and the contents seeded with 1,3 benzylidene glycerol which was crystallized overnight. The crystals were filtered off and, while still cold, stirred with eight volumes of toluene containing sufficient sodium methoxide to neutralize the catalyst. The toluene solution, obtained by warming, was washed with 1% aqueous Na_2HPO_4, dried with anhydrous Na_2SO_4, and the 1,3 benzylidene glycerol crystallized at $0^{\circ}C$. The isolated crystals were recrystallized from benzene - hexane, 1:1 (v/v).

(d) Preparation of 2-monoglyceride.

1,3 - Benzylidene glycerol, in 20% excess, was dissolved in a minimum amount of ethanol-free chloroform, and pyridine and then fatty acid chloride added in equimolar amounts. After 24 hours at room temperature, the mixture was dissolved in 2.5 volumes of diethyl ether and the solution washed first with an equal volume of ice-cold 0.4% HCl, then with ice-cold water until neutral. On drying with anhydrous Na_2SO_4, the solvent was evaporated under reduced pressure. The removal of the blocking group by borate replacement was effected by dissolving the product in 2-methoxy-ethanol and heating with an excess of finely powdered boric acid on a boiling water bath for 30-40 minutes. The solution was taken up in diethyl ether, washed three times with water, dried quickly with Na_2SO_4 and the ether evaporated under reduced pressure at less than $25^{\circ}C$. This residue, containing

the 2-monoglyceride, was used immediately for the diglyceride synthesis.

(e) Preparation of 1,2 (2,3)-diglyceride.

The 2-monoglyceride was dissolved in ethanol-free chloroform, then pyridine and fatty acid chloride were added in equimolar amounts. After the mixture had stood at room temperature for 4 hours, it was diluted with 20 volumes of petroleum ether - diethyl ether, 1:1 (v/v). This solution was washed twice with ice-cold water, three times with ice-cold 1% HCl, three times with water and dried with anhydrous Na_2SO_4. Upon evaporation of the solvent, the diglyceride was isolated by preparative TLC from the residue.

Figure 6 summarizes the reactions for the synthesis of the diglyceride.

The diglycerides were estimated to contain less than 5% impurity of 1,3-diglyceride, as judged by analytical TLC. They were stored under nitrogen at -20°C.

A summary of the diglyceride preparations is given in Table 9.

Table 9.

Summary of the 1,2 (2,3) - diglyceride preparations

no. of double bonds/molecule	diglyceride	wt. of fatty acid chloride* (g.)	wt. of diglyceride (mg.)
2	1,2 dioleoyl glycerol	1.8	351
3	1-oleoyl, 2-linoleoyl glycerol	2.2	375
3	1-linoleoyl, 2-oleoyl glycerol	2.0	289
4	1,2 dilinoleoyl glycerol	2.0	297
5	1-γ-linolenoyl, 2-linoleoyl glycerol	2.2	286

* the weight of fatty acid chloride taken in the preparation of 2-monoglyceride.

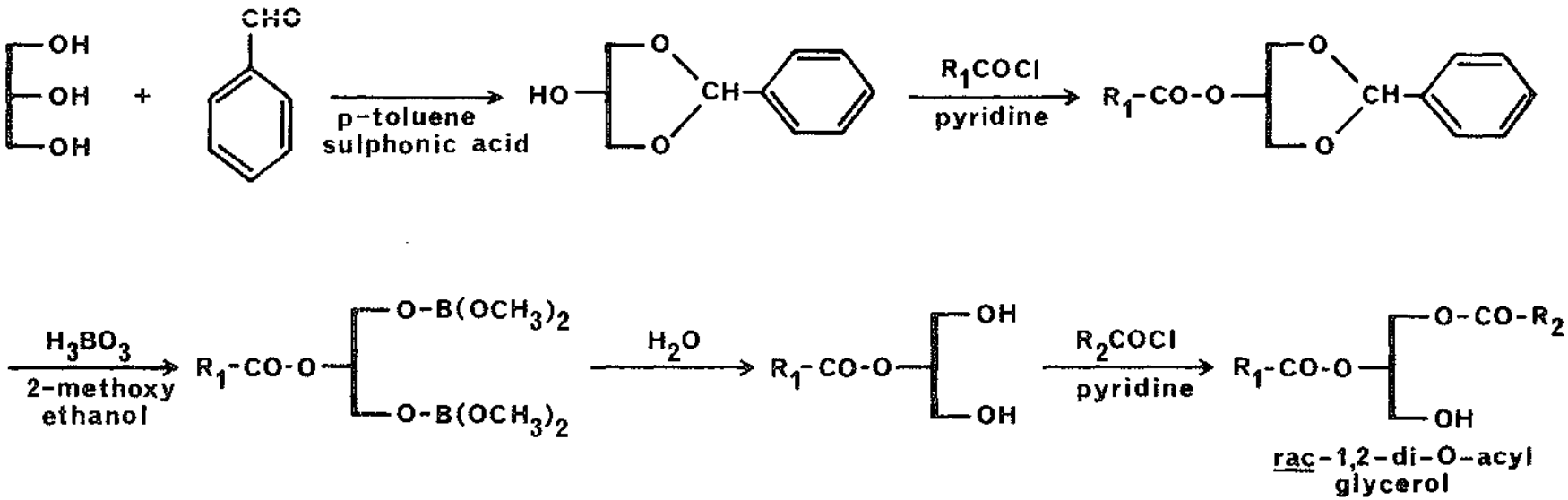


Figure 6

The chemical synthesis of rac - 1,2 -di-O-acyl glycerol
(Mattson and Volpenhein, 1962).

(f) Fatty acid analysis of the diglycerides.

The diglycerides containing two different fatty acyl residues were subjected to enzymic hydrolysis by pancreatic lipase to remove the acyl group from the α-position. An emulsion was prepared consisting of

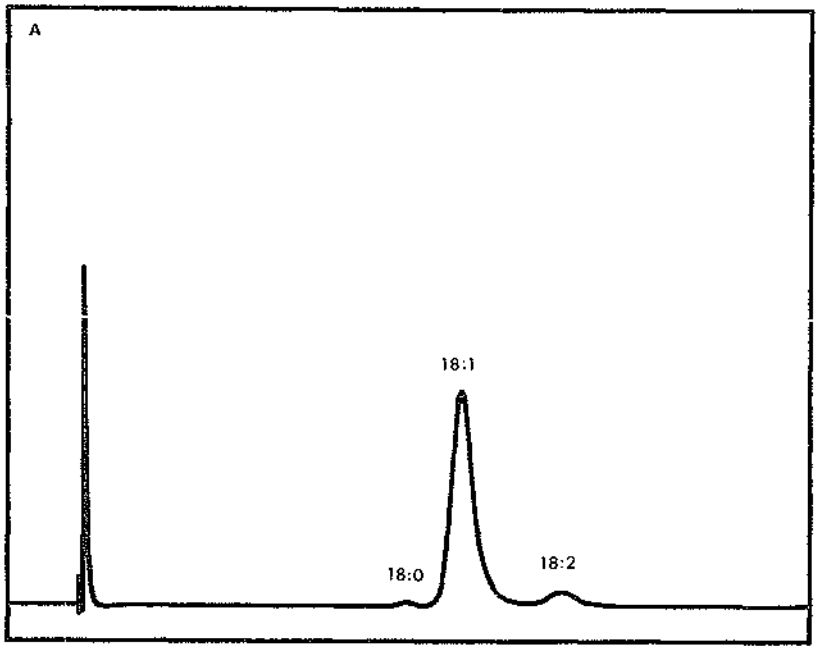
10mg. diglyceride;

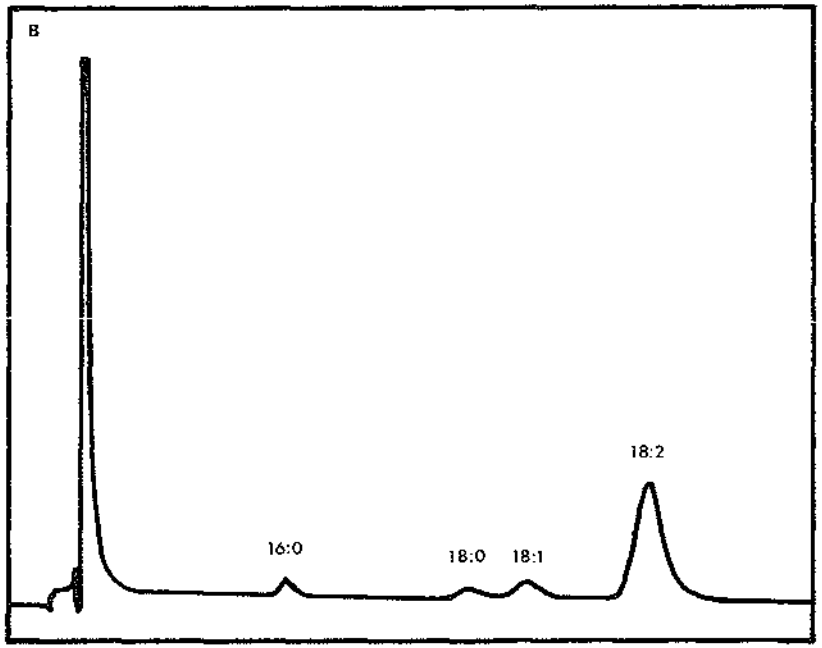
5.0 ml. 1M Tris - HCl buffer, pH 7.5;

0.5 ml. 22% $CaCl_2$

1.0 ml. 0.1% sodium cholate.

One ml. of this emulsion was incubated with 100mg. of lipase (from hog pancreas, type II, Sigma Chemical Co.) for $2\frac{1}{2}$ min., at $37^{o}C$ and the reaction stopped with ethanol. The liberated fatty acid and 2-monoglyceride were isolated by TLC. The 2-monoglyceride, and the 1,2-dioleoyl- and 1,2-dilinoleoyl glycerols, were deacylated by refluxing with methanolic KOH and the fatty acid was converted to the methyl ester by reaction with freshly-prepared diazomethane. The methyl esters were analysed by GLC and identified by comparison of their retention times with standard compounds. The fatty acid compositions were obtained from the GLC chromatographs (Figure 7) by measuring the peak areas by planimetry. The results are given in Table 10.





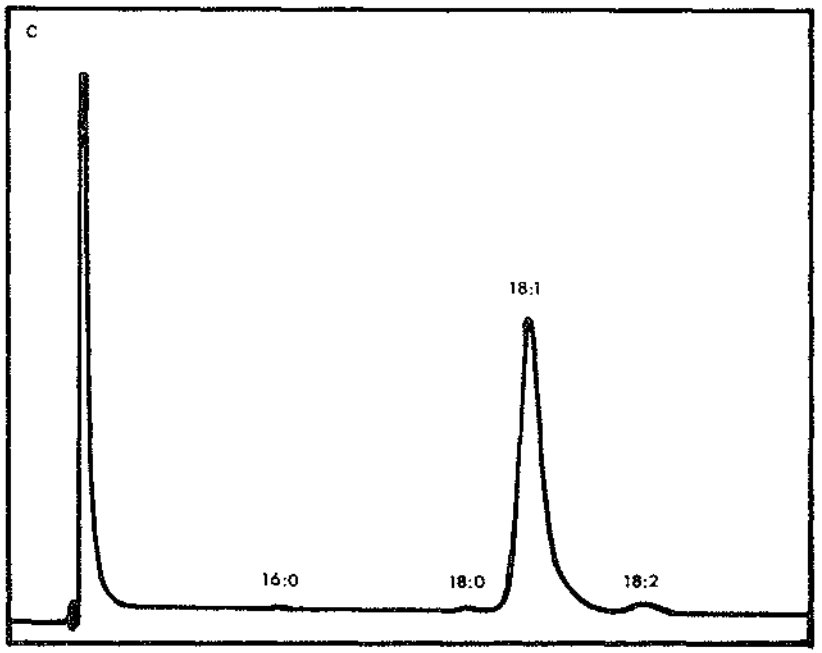


Figure 7

Examples of the GLC chromatographs obtained in the fatty acid analysis of the synthetic diglycerides:

A - total fatty acids of 1,2 -dioleoyl glycerol.
B - 1-position fatty acid of 1-linoleoyl, 2-oleoyl glycerol.
C - 2-position fatty acid of 1-linoleoyl, 2-oleoyl glycerol.

Table 10.

The fatty acid composition of the synthetic 1,2 (2,3)-diglycerides

diglyceride		fatty acid		
		18:1	18:2	18:3
1,2-dioleoyl glycerol		91.4	8.6	-
1-oleoyl, 2-linoleoyl glycerol				
	Position 1	86.1	13.9	-
	Position 2	2.2	97.8	-
1-linoleoyl, 2-oleoyl glycerol				
	Position 1	7.7	92.3	-
	Position 2	96.7	3.2	-
1,2 dilinoleoyl glycerol		1.0	99.0	-
1-linolenoyl, 2-linoleoyl glycerol				
	Position 1	-	11.2	88.8
	Position 2	-	100.0	-

The appearance of small amounts of palmitic and stearic acids on the chromatographs for those fatty acids liberated by enzymic hydrolysis was attributed to a contamination of the enzyme preparation and thus these peaks were not considered in calculating the fatty acid compositions.

3.III.2 Isolation of diglyceride and MGDG from the acetone extracts of chloroplasts.

(a) Isolation of diglyceride.

The acetone extracts from the spinach acetone powder preparations 1 through 4 were bulked and passed through a carbon-Celite, 1:1, column, having the dimensions of 11cm. x 1cm., to decolorize the extract (van der Veen, Hirota and Olcott, 1967). The lipids

were eluted with chloroform (about 40 mls.) and with C/M, 9:1 (v/v), until green pigment appeared. The fractions lacking green pigment were bulked and the diglyceride isolated by preparative TLC using silica gel G impregnated with 3% (w/v) boric acid. By analytical TLC, this diglyceride fraction contained only the 1,2-isomers and, since it had been enzymically synthesized, the diglycerides would be expected to be specifically 1,2-diacyl-sn-glycerols. The total fatty acid composition of the diglyceride fraction (Figure 8) was

16:0 16.8%; 16:1 15.3%; 18:0 1.6%; 18:1 and/or 16:3 8.4%; 18:2 14.0%; 18:3 43.9%.

(b) Isolation of MGDG.

The MGDG from the acetone extract of spinach acetone powder preparation no.9 was isolated by preparative TLC.

3.III.3 Isolation of phospholipids:

(a) From egg-yolk.

The yolks of two fresh eggs were extracted with C/M 2:1, the extract taken to dryness and redissolved in a minimum volume of diethyl ether. Ten volumes of acetone were added and the acetone-soluble fraction decanted. The crude phospholipid (acetone-insoluble fraction) was resolved by preparative TLC into phosphatidyl choline (PC) and phosphatidyl ethanolamine (PE) fractions, using known standards.

The fatty composition of the egg phosphatidyl choline (Figure 9), which was similar to that of the total phospholipid fraction, was

16:0 34.7%; 16:1 2.0%; 18:0 12.5%; 18:1 39.8%; 18:2 10.9%.

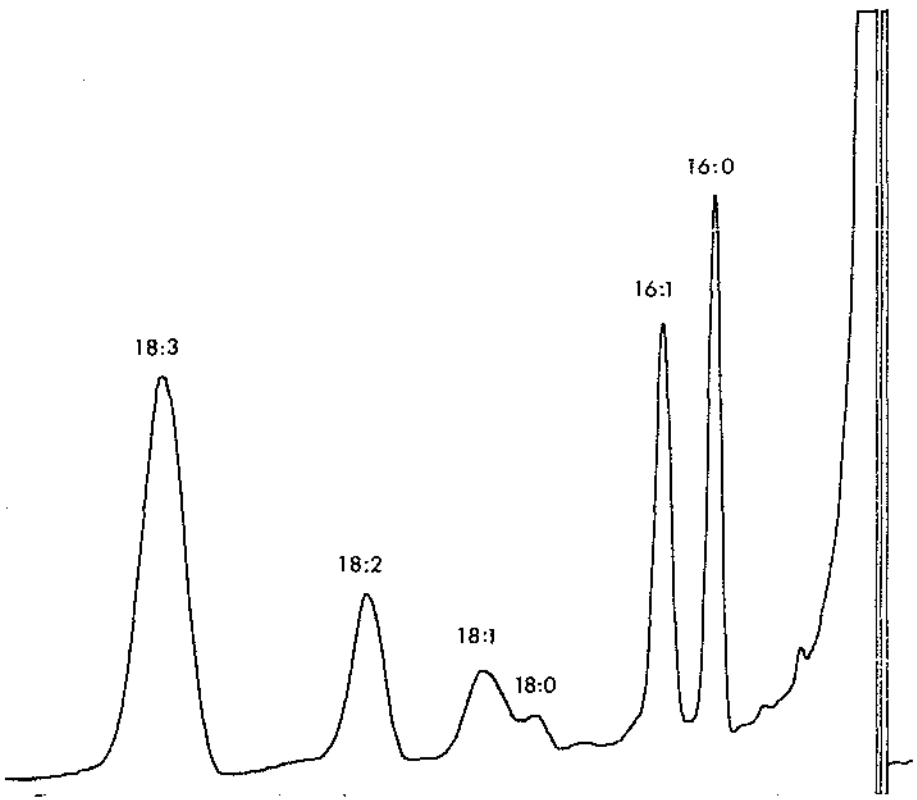


Figure 8

The GLC chromatograph obtained for the total fatty acids of the diglyceride isolated from the acetone extracts of chloroplasts.

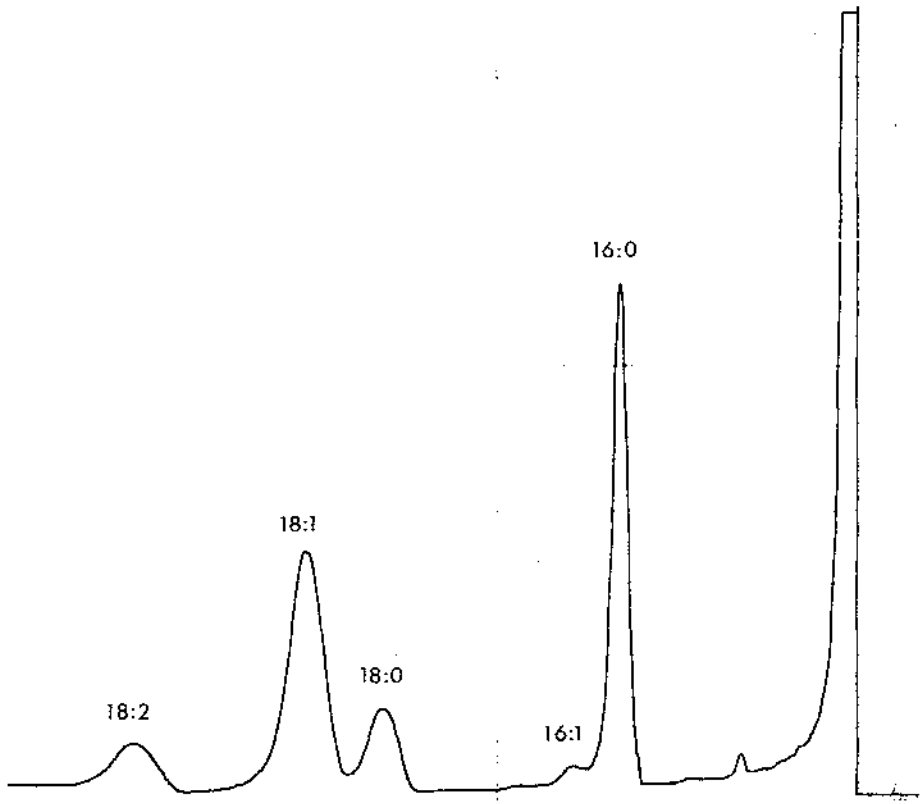


Figure 9

The GLC chromatograph obtained for the total fatty acids of the phosphatidyl choline isolated from egg yolk.

(b) From spinach.

Spinach leaf and stalk (approx. 200g.) were blended in alcohol and heated to boiling. The alcohol extract was decanted and the residue re-extracted with C/M, 2:1 (v/v). Both extracts were evaporated to dryness, dissolved in diethyl ether and then combined. Phosphatidyl choline and phosphatidyl ethanolamine were resolved by preparative TLC.

Chapter 4

RESULTS

Section 4.I : Experiments with leaf slices.

4.I.1 The incorporation of radioactivity, from D-galactose -1- ^{14}C into the galactolipids of barley leaf slices, with respect to time.

To find the dependence of the incorporation of D-galactose -1- ^{14}C into MGDG and DGDG of barley leaf slices on time, barley seedlings were harvested on the 5th day after planting and leaf slices incubated with 0.11ml. of D-galactose -1- ^{14}C, containing 2.3×10^6 dpm, for periods up to 7 hours, in room light. The results are given in Table 11 and shown graphically in Figures 10 and 11.

The percentage incorporation of radioactivity into the MGDG and DGDG fractions were determined by measuring the peak areas on the radiochromatogram scans (Figure 12) of each lipid extract and appropriately dividing the total incorporation value. Equal amounts of radioactivity appeared in both galactolipid fractions after 1 hour but then the rate of accumulation of label into the MGDG fraction slowed so that the percentage incorporation into DGDG was greater than that into MGDG at 4 hours. With further incubation the amount of radioactivity into the DGDG fraction decreased whereas the amount of label into the MGDG fraction continued to increase. The ratio of the percentage incorporation of radioactivity into MGDG to that into DGDG exhibited a dish-shaped curve with respect to time (Figure 11). The ratio had about the same value for the incubation times, $\frac{1}{2}$-hour and 7 hours and a minimum value at $3\frac{1}{2}$ hours.

The difference between the total incorporation for the lipid extract and the sum of the incorporations of label into the MGDG and DGDG fractions

Table 11.

The incorporation of radioactivity from D-galactose -1- ^{14}C into the lipids of barley leaf slices with respect to time.

Reaction mixture:

1g. sliced leaf tissue; 1.4ml. water; 0.2ml M phosphate buffer, pH 7.4; 0.1ml $KHCO_3$ (50 μmoles); 0.05ml Na acetate (100 μmoles); 0.11ml D-galactose -1- ^{14}C (containing 2.3×10^6 dpm); total volume 1.86ml. Incubation at 25°C in room light.

incubation time (hrs)	total incorporation (dpm)	% total incorporation	% incorporation into MGDG	% incorporation into DGDG	sum*	ratio‡
0	2,500	0.11	-	-	-	-
½	12,500	0.54	0.22	0.16	0.38	1.38
1	25,200	1.10	0.44	0.41	0.85	1.07
2	46,900	2.04	0.55	0.67	1.22	0.82
2½	45,000	1.96	0.76	1.09	1.85	0.70
3	50,000	2.18	0.83	1.05	1.88	0.79
3½	52,700	2.29	0.80	1.23	2.13	0.65
4	50,900	2.21	0.86	1.30	2.16	0.66
5	52,700	2.29	1.02	1.11	2.13	0.92
7	66,100	2.88	1.66	1.20	2.86	1.32

* The sum of the percentage incorporations into MGDG and DGDG.

‡ The ratio of the percentage incorporation of radioactivity into MGDG to that into DGDG.

Figure 10

The percentage incorporation of radioactivity from D-galactose -1- ^{14}C into MGDG and DGDG by barley leaf slices with respect to time. (see Table 11 for the reaction mixture.)

key: ▣ - MGDG; □ - DGDG; ⊛ - MGDG + DGDG (sum)

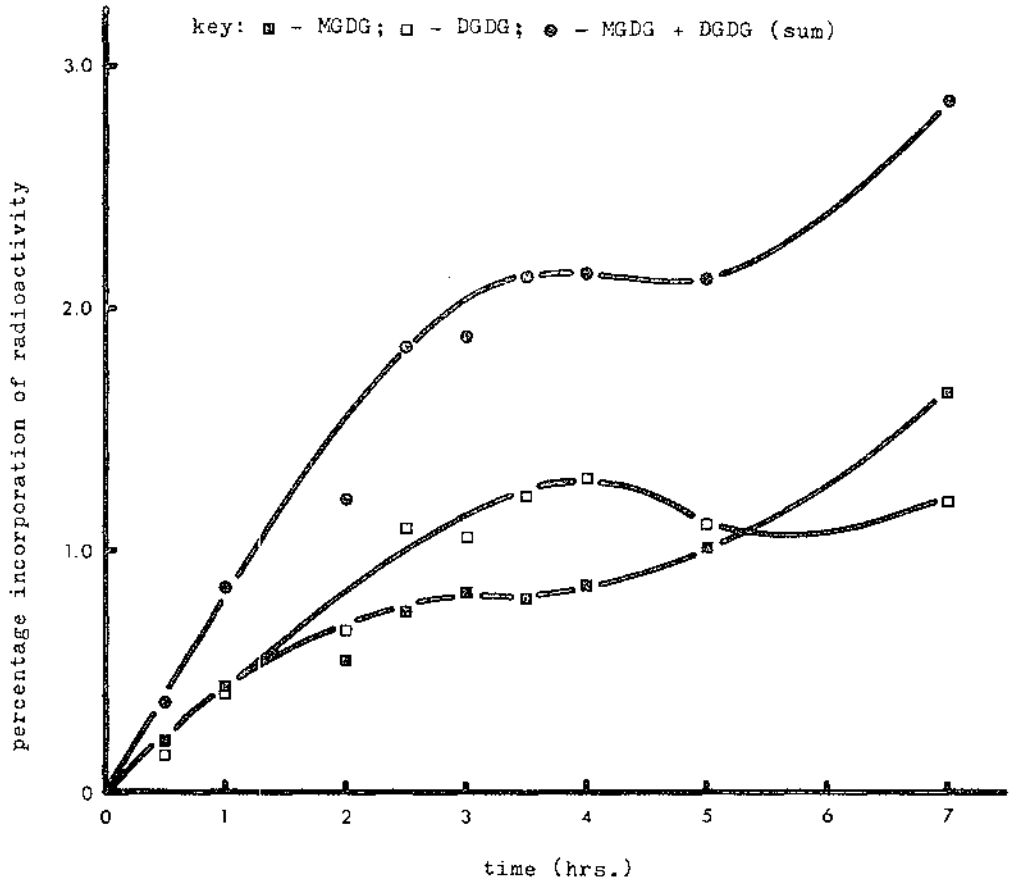
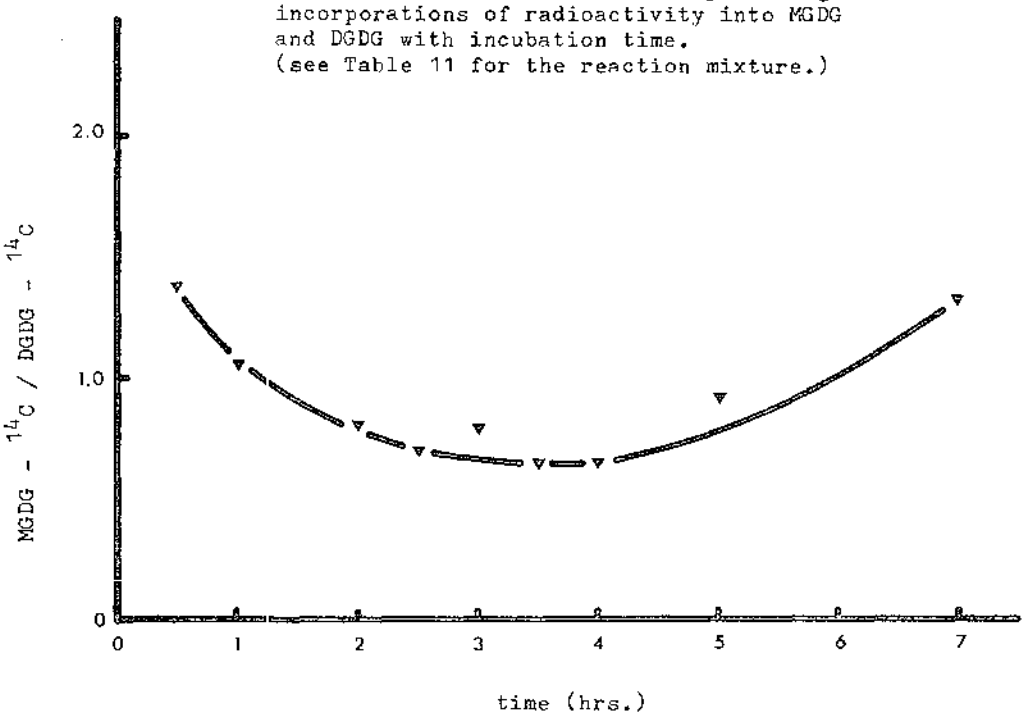


Figure 11

The change in the ratio of the percentage incorporations of radioactivity into MGDG and DGDG with incubation time.
(see Table 11 for the reaction mixture.)



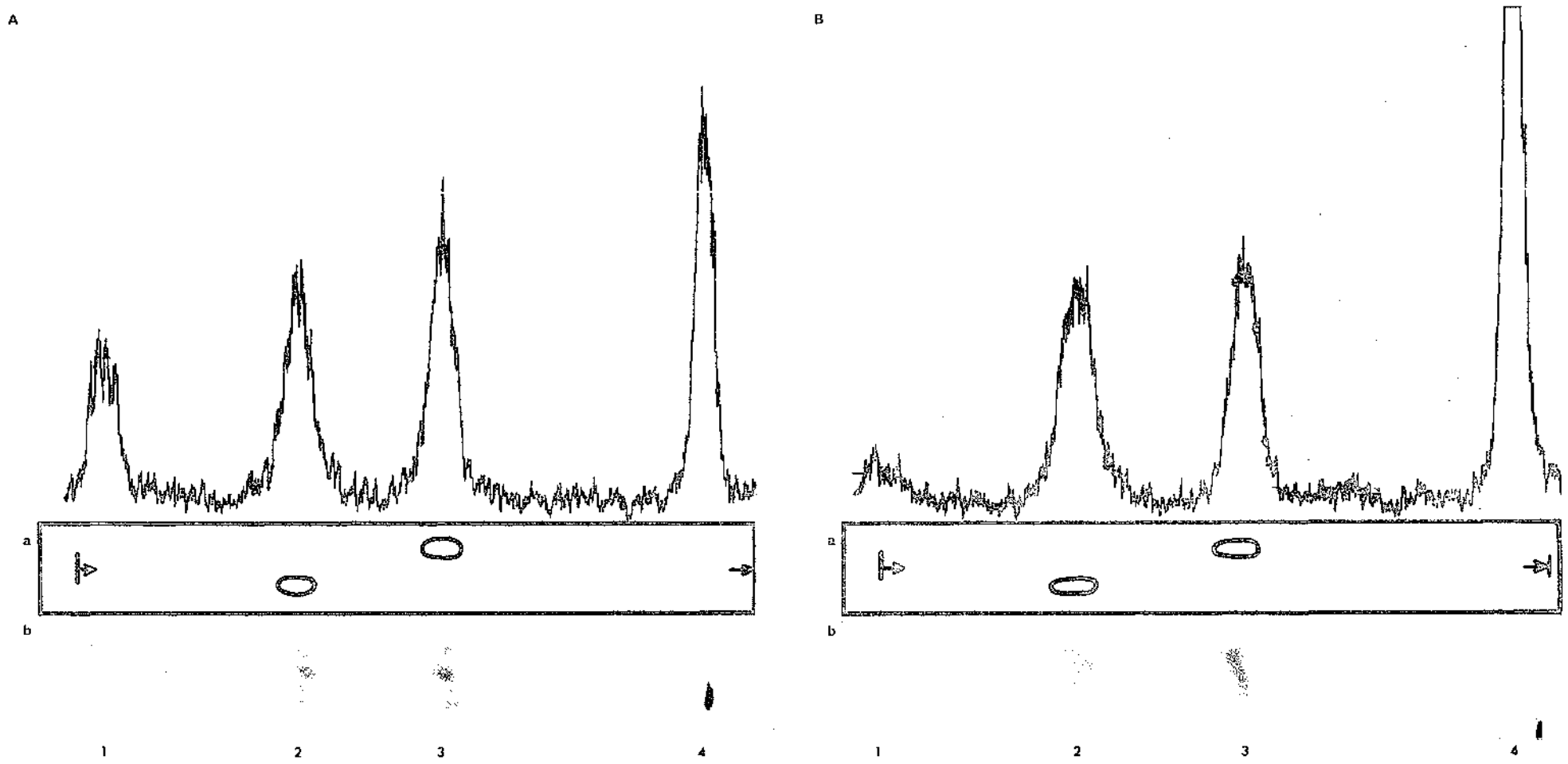


Figure 12

The radiochromatogram scans for the lipid extracts obtained after the incubation of barley leaf slices with D-galactose -1- ^{14}C for 1 hr., (A), and 5 hrs., (B). With each scan the positions of the standard MGDG and DGDG (a), and the radio-autograph, (b), are shown. (experiment 4.I.1)

key: 1, galactose (tentative); 2, DGDG;
3, MGDG; 4, marker.

tended to be variable. These differences were attributed to the D-galactose -1- ^{14}C which had not been removed from the total lipid extract in the washing step and this showed as a radioactive area at the origin on the radiochromatogram scans (Figure 12).

4.I.2 The variation in the incorporation of radioactivity from D-galactose -1- ^{14}C into MGDG and DGDG with the age of the barley seedlings:

To find the variation in the incorporation of D-galactose into MGDG and DGDG by barley leaf slices with the age of the barley seedlings, the dark-grown barley seedlings were harvested from different jars on the 4th, 5th, 6th and 7th days after planting and the leaf slices incubated with 0.135 ml. of D-galactose -1- ^{14}C (containing 2.3×10^6 dpm) for 4 hours under the illumination of a 250 watt IR reflector lamp held at 60 cms. Duplicates were incubated except when there was insufficient tissue in the jar. By the 7th day, the height of the seedlings equalled the height of the Agee jar and the experiment was discontinued. Prior to harvesting, each jar was illuminated for 24 hours. The results are given in Table 12 and shown graphically in Figure 13.

The greater incorporation of label into the DGDG fraction than into the MGDG fraction in the 4 hour incubation of barley leaf slices seen in experiment 4.I.1 was confirmed in this experiment. The increasing percentage incorporation of radioactivity into the DGDG fraction showed a linear relationship for the 4-, 5- and 6-day old seedlings. The 6-day old seedlings had the greatest incorporation of label for both the MGDG and DGDG fractions and the amount of incorporation for the 5-day old leaf slices was greater than in experiment 4.I.1.

The duplicate lipid extracts exhibited good agreement for the percentage incorporation of radioactivity into the galactolipids, although the total lipid incorporation varied as in experiment 4.I.1.

Table 12.

The variation in the incorporation of radioactivity from D-galactose -1- ^{14}C into MGDG and DGDG by barley leaf slices with the age of the barley seedlings.

Reaction mixture:

1g. sliced leaf tissue; 1.4ml. water; 0.2ml M phosphate buffer, pH 7.4; 0.1ml $KHCO_3$ (50 μmoles); 0.05ml Na acetate (100μmoles); 0.135ml. D-galactose -1- ^{14}C (containing 2.3×10^6 dpm); total volume 1.89mls. Incubation at 25°C, under illumination, for 4 hours.

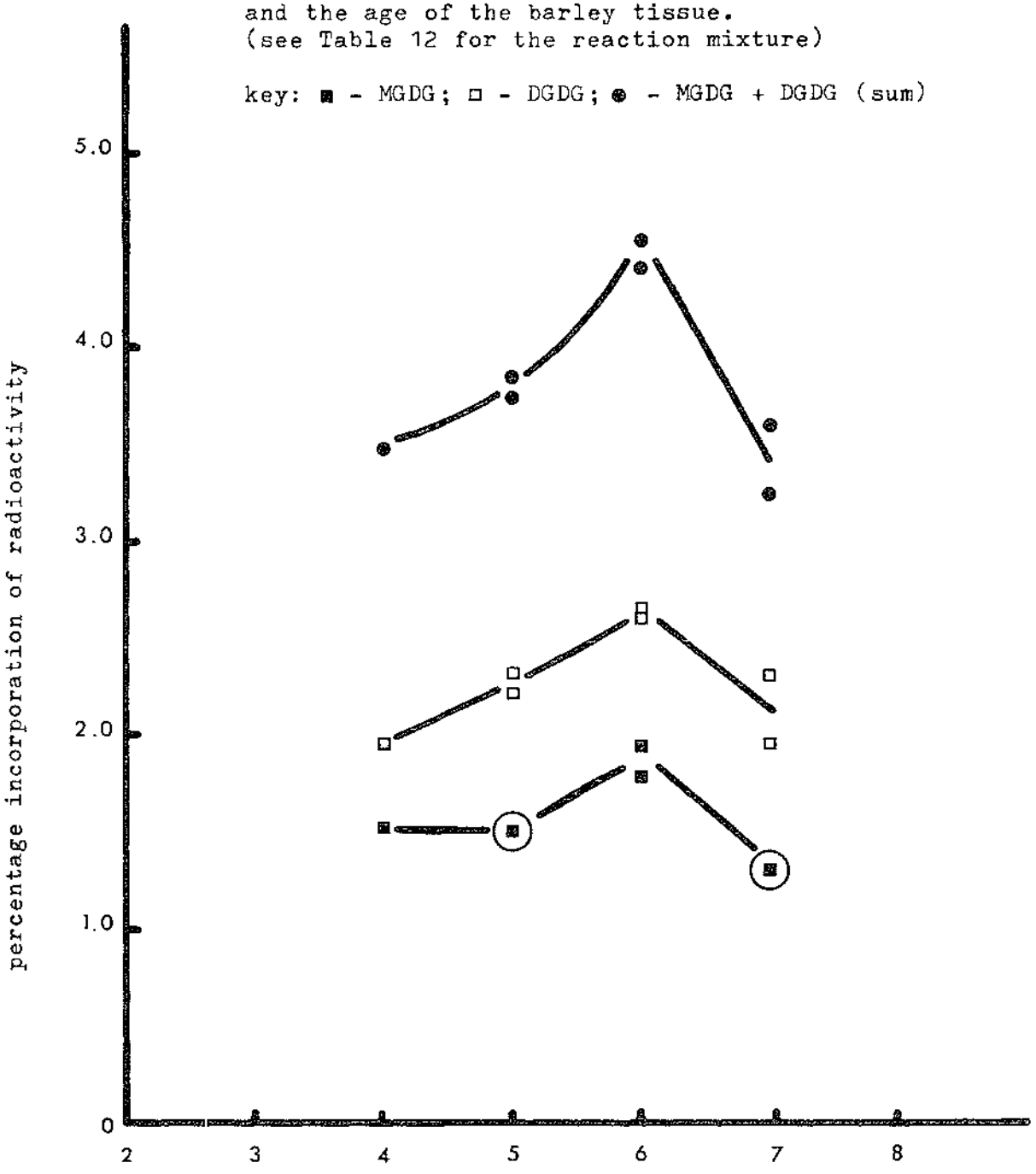
age of tissue (days after planting)		total incorporation (dpm)	% incorporation into MGDG	% incorporation into DGDG	sum †
4		93,000	1.52	1.95	3.47
5	a	90,900	1.51	2.32	3.83
	b	81,100	1.51	2.22	3.73
6	a	118,400	1.94	2.65	4.59
	b	104,900	1.79	2.61	4.40
7	a	80,000	1.29	1.94	3.23
	b	89,200	1.28	2.34	3.59

† The sum of the percentage incorporations of radioactivity into MGDG and DGDG.

Figure 13

The relationship between the percentage incorporation of radioactivity from D-galactose -1- ^{14}C into MGDG and DGDG by barley leaf slices and the age of the barley tissue.
(see Table 12 for the reaction mixture)

key: ■ - MGDG; □ - DGDG; ● - MGDG + DGDG (sum)



4.I.3 The incorporation of radioactivity from D-galactose -1- ^{14}C into the galactosyl moieties of MGDG and DGDG:

The distribution of radioactivity between the fractions obtained by deacylation and acid hydrolysis of the galactolipids was determined to demonstrate that the radioactivity incorporated from D-galactose -1- ^{14}C into the galactolipids, by barley leaf slices, appeared in the galactosyl moieties of the galactolipids. The MGDG and DGDG were isolated from three of the total lipid extracts of experiment 4.I.2. The distribution of radioactivity within the galactolipid molecules is indicated in Table 13 and Figure 14.

Table 13.

The distribution of radioactivity between the fractions obtained by the hydrolysis of the radioactive galactolipids formed by incubating barley leaf slices with D-galactose -1- ^{14}C.

fraction		radio-activity (dpm)	remarks
starting materials	MGDG	68,400	radioactivity present after isolation by TLC
	DGDG	97,200	
water-soluble, alkaline hydrolysis products	MGDG	65,100	
	DGDG	87,000	
ether-soluble, alkaline hydrolysis products	MGDG	0	
	DGDG	0	
acid hydrolysis products	MGDG	20,100	0.6 vol. of the water-soluble, alkaline hydrolysis products taken for acid hydrolysis
	DGDG	39,800	

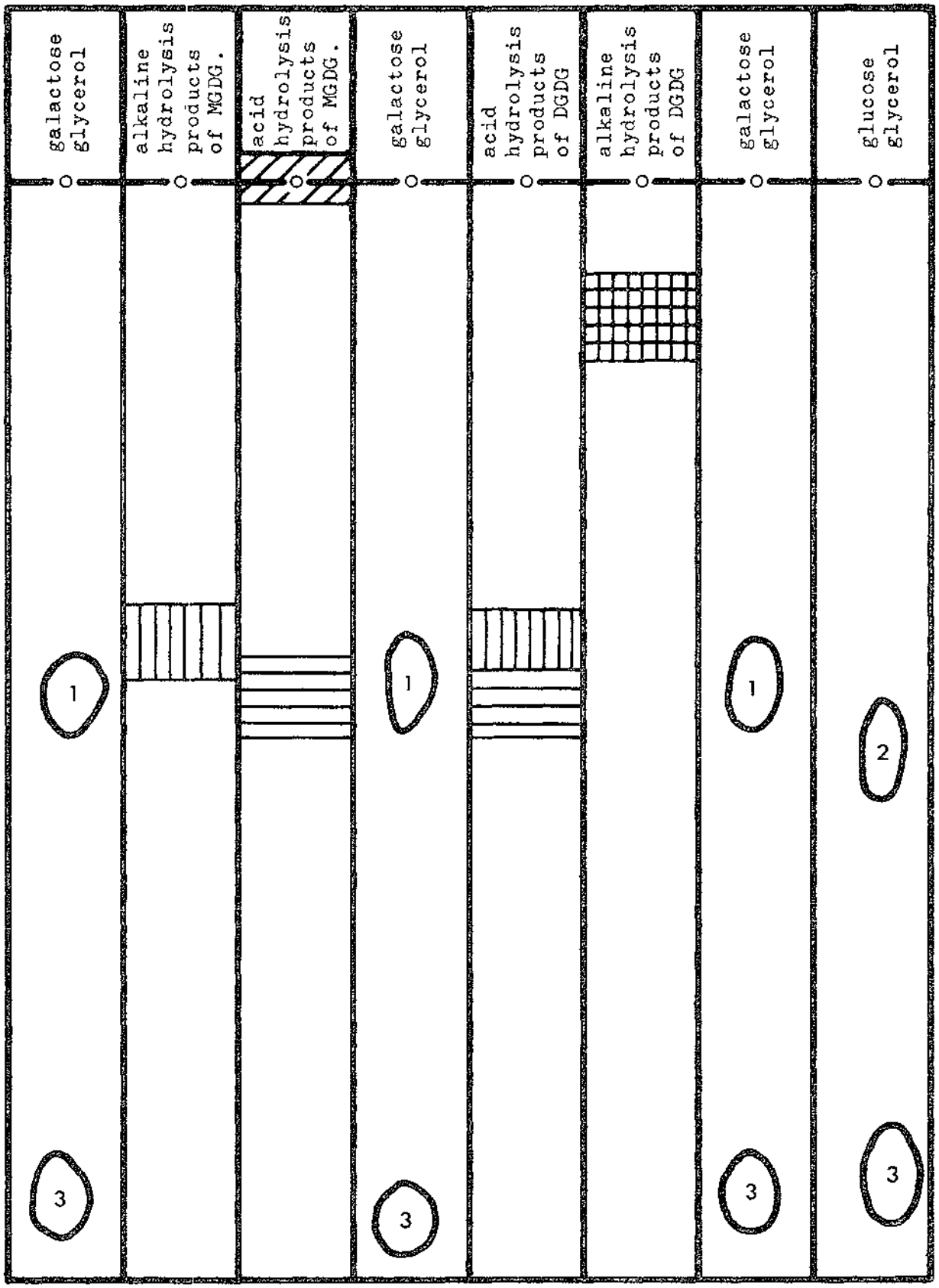


Figure 14

The chromatogram of the water-soluble hydrolysis products from radioactive MGDG and DGDG (experiment 4.I.3). The barred areas indicate the radioactive peaks and the standard compounds were galactose, (1), glucose, (2), and glycerol, (3).

The data in Table 13 shows that after alkaline hydrolysis of the galactolipids the radioactivity was associated with the water-soluble products and thus indicates that the fatty acids of the galactosyl diglycerides were not labelled during the incubation. When the appropriate strips of the paper chromatograph of the water-soluble hydrolysis products were scanned for radioactivity, they showed that no radioactivity was associated with the glyceryl moiety of the galactolipids (Figure 14). A comparison with the chromatogram of the galactolipid hydrolysis products given by Sastry and Kates (1964a) shows that the positions of the radioactive peaks, relative to the standards on the chromatogram shown in Figure 14, are consistent with the following radioactive hydrolysis products:

MGDG: alkaline hydrolysis - monogalactosyl glycerol

acid hydrolysis - galactose

DGDG: alkaline hydrolysis - digalactosyl glycerol

acid hydrolysis - galactose and monogalactosyl glycerol.

The radioactive area at the origin for the acid hydrolysis products of monogalactosyl glycerol was not identified.

The above results prove that the radioactivity incorporated from D-galactose -1- ^{14}C into the galactolipids of barley leaf slices was contained wholly within the galactosyl moieties of the galactolipids.

4.I.4 The incubation of fescue leaf slices with D-galactose -1- ^{14}C:

The incubation of fescue leaf slices with D-galactose -1- ^{14}C resulted in incorporation of radioactivity into the lipid fraction. The results for the duplicate flasks are shown in Table 14.

Table 14.

The incorporation of radioactivity from D-galactose -1- ^{14}C into the lipids of fescue leaf slices.

Reaction mixture:

0.5g. fescue leaf slices; 0.30ml M phosphate buffer, pH 7.4; 2.60ml water; 0.10ml $KHCO_3$ (50 μmoles); 0.05ml Na acetate (100 μmoles); 0.10ml cysteine hydrochloride (1.2 μmoles); 0.10ml D-galactose-1-^{14}C (containing 2.11 x 10^6 dpm); total volume 3.25ml. Incubation at 25°C for 4 hours in room light.

flask	total incorporation (dpm)	% incorporation
1	33,000	1.6
2	19,500	0.9

A radiochromatogram scan of the lipid extract obtained from flask (1) showed that the distribution of radioactivity was: pigment peak 9%; MGDG 65%; DGDG 20%; others 6%.

In previous incubations of fescue leaf slices with D-galactose-1-^{14}C, when the reaction mixture lacked cysteine hydrochloride, no incorporation of radioactivity into the lipid fraction was obtained.

Section 4.II : Experiments with chloroplast suspensions.

4.II.1 The incorporation of radioactivity from UDP-D-galactose - ^{14}C into the lipids of chloroplasts isolated from different plant species.

(a) The incorporation of radioactivity from UDP-D-galactose - ^{14}C into the lipids of chloroplasts, which were isolated from various sources, was shown by incubating an aliquot of 0.2ml of a chloroplast suspension with 2 μl. of UDP-D-galactose - ^{14}C solution, containing 22,200 dpm, for 2 hours at 30°C in room light. The chloroplasts from 7g., wet weight, of leaf tissue from spinach fescue, Yorkshire fog, ryegrass, phalaris and barley seedlings were isolated using method I and resuspended in 1ml of 0.1M Tris - HCl buffer, pH 7.4. (The dark-grown 8-day old barley seedlings had had a light treatment of 24 hours illumination followed by 12 hours of darkness prior to harvesting.) The amount of radioactivity that was incorporated into the total lipid extracts of the chloroplasts is given in Table 15.

The incorporation of label by the plastids isolated from barley seedlings was very small and variation of the age of the tissue used, or different light treatments (e.g. returning seedlings to darkness after illumination to reduce the starch content of the chloroplasts), did not lead to the incorporation of radioactivity into the lipid fraction. The chloroplast preparations from the leaf tissue of fescue, ryegrass and spinach gave the best incorporations of radioactivity from UDP-D-galactose - ^{14}C into the lipid fraction.

Table 15.

The incorporation of radioactivity from UDP-D-galactose - ^{14}C into the lipids of chloroplasts isolated from different plant species.

Reaction mixture:

0.2ml chloroplast suspension (0.1M Tris - HCl buffer, pH 7.4)† ;

2μl UDP-D-galactose - ^{14}C solution, containing 22,200 dpm.

Incubation at 30°C for 2 hours in room light.

leaf source	total incorporation (dpm)	% incorporation
barley	300	1.3
fescue	5,700	25.7
fog	1,900	8.7
phalaris	800	3.6
ryegrass	3,500	15.8
spinach	11,800	53.2

† equivalent to the chloroplasts of 1.4g. leaf tissue.

(b) To confirm the level of incorporation of ^{14}C into the chloroplast lipids of fescue and ryegrass, a further 14g., wet weight, of leaf tissue from each was treated as for method I and the chloroplast pellet suspended in 1ml. of 0.1M Tris - HCl buffer, pH 7.4. Duplicate aliquots (0.2ml) of this suspension were incubated with 4μl of UDP-D-galactose - ^{14}C solution (containing 44,400 dpm) at 30°C for 2 hours. The results are given in Table 16.

Table 16.

The incorporation of radioactivity from UDP-D-galactose - ^{14}C into the lipids of fescue and ryegrass leaf chloroplasts.

Reaction mixture:

0.2 ml chloroplast suspension (0.1M Tris - HCl buffer, pH 7.4) ⁺; 4μl UDP-D-galactose - ^{14}C solution, containing 44,400 dpm.

Incubation at 30°C for 2 hours.

leaf source	flask	total incorporation (dpm)	% incorporation
fescue	1	4,700	10.6
	2	5,600	12.6
ryegrass	1	5,000	11.3
	2	4,900	11.0

⁺ equivalent to the chloroplasts of 2.8g. leaf tissue.

Despite the addition of twice the amount of radioactive substrate, the more concentrated chloroplast suspensions gave smaller percentage incorporations of radioactivity than found in (a) above. This was particularly evident for fescue chloroplasts. A radiochromatogram scan of lipid extract (2) of fescue chloroplasts showed that the major portion of the incorporated radioactivity was in the MGDG

fraction. Although no radioactive DGDG was apparent, two additional regions of radioactivity occurred, one corresponding to the pigment region of the chromatograph and the other slightly ahead but unresolved from the MGDG peak. Unlike the scans obtained in experiments 4.I.1 and 4.I.2, only a trace of radioactivity appeared at the origin.

4.II.2 The relationship between the chloroplast concentration and the percentage incorporation of radioactivity from UDP-D-galactose - ^{14}C into chloroplast lipids.

The chloroplasts of fescue leaf tissue were isolated to find the relationship between the chloroplast concentration and the percentage incorporation of radioactivity from UDP-D-galactose - ^{14}C into chloroplast lipids. Using buffer in which cysteine hydrochloride had been substituted for mercaptoethanol, the chloroplasts from 15g., wet weight, fescue leaf were isolated by method I and the chloroplast pellet suspended in 1.0ml. 0.1M Tris - HCl buffer, pH 7.4. Aliquots of this suspension were incubated, in a reaction mixture of 0.2ml., with 8 μl of UDP-D-galactose - ^{14}C solution (containing 88,800 dpm) for 40 minutes at 30°C. Chlorophyll content (as determined by Arnon, 1949) was used as a measure of chloroplast concentration. The results are given in Table 17 and shown graphically in Figure 15.

Table 17.

The effect of chloroplast concentration on the incorporation of radioactivity from UDP-D-galactose - ^{14}C into chloroplast lipids.

Reaction mixture:

x ml. chloroplast suspension; (0.2 - x) ml. 0.1M Tris - HCl buffer, pH 7.4; 8 µl UDP-D-galactose - ^{14}C solution, containing 88,800 dpm. Incubation at 30°C for 40 min.

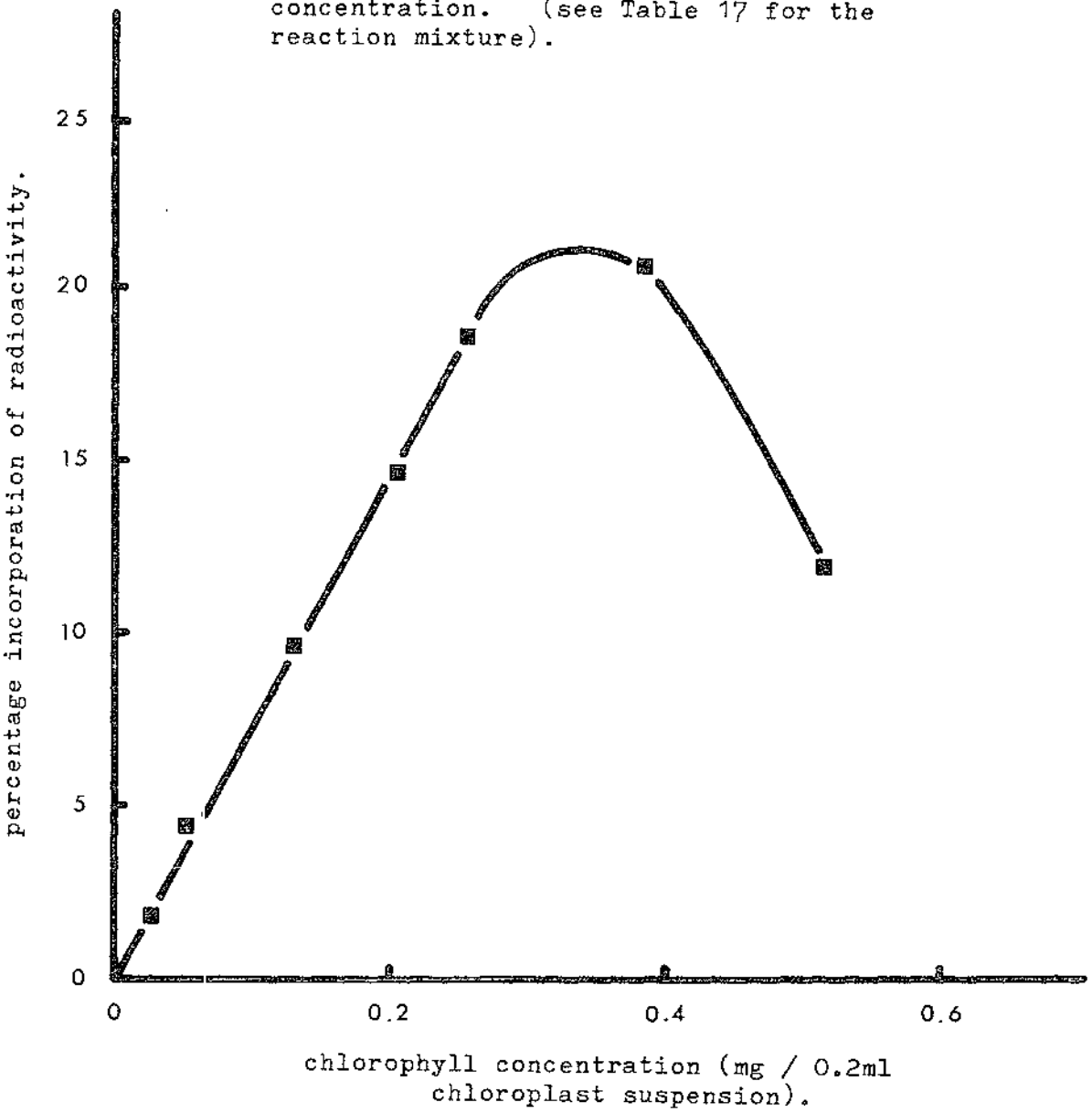
tube	chlorophyll content of the reaction mixture (mg.)	total incorporation (dpm)	% incorporation
1	0.026	1,600	1.8
2	0.051	3,900	4.4
3	0.128	8,500	9.6
4	0.204	13,000	14.6
5	0.255	16,500	18.6
6	0.383	18,300	20.6
7 ‡	0.511	10,600	11.9

‡ contained chloroplasts equivalent to 3g. leaf tissue.

From Figure 15, it is seen that the percentage incorporation of radioactivity is linearly dependent on the chloroplast concentration up to a chlorophyll concentration of 1.25mg/ml (0.25mg/0.2ml). At higher chloroplast concentrations, the percentage incorporation decreased. The percentage incorporation obtained in tube 7 was approximately that shown in Table 16 for fescue chloroplasts; the reaction mixtures contained about the same amount of chloroplast material although the incubation times and amount of radioactive substrate used for each were different.

Figure 15

The relationship between the percentage incorporation of radioactivity from UDP-D-galactose - ^{14}C into lipids and chloroplast concentration. (see Table 17 for the reaction mixture).



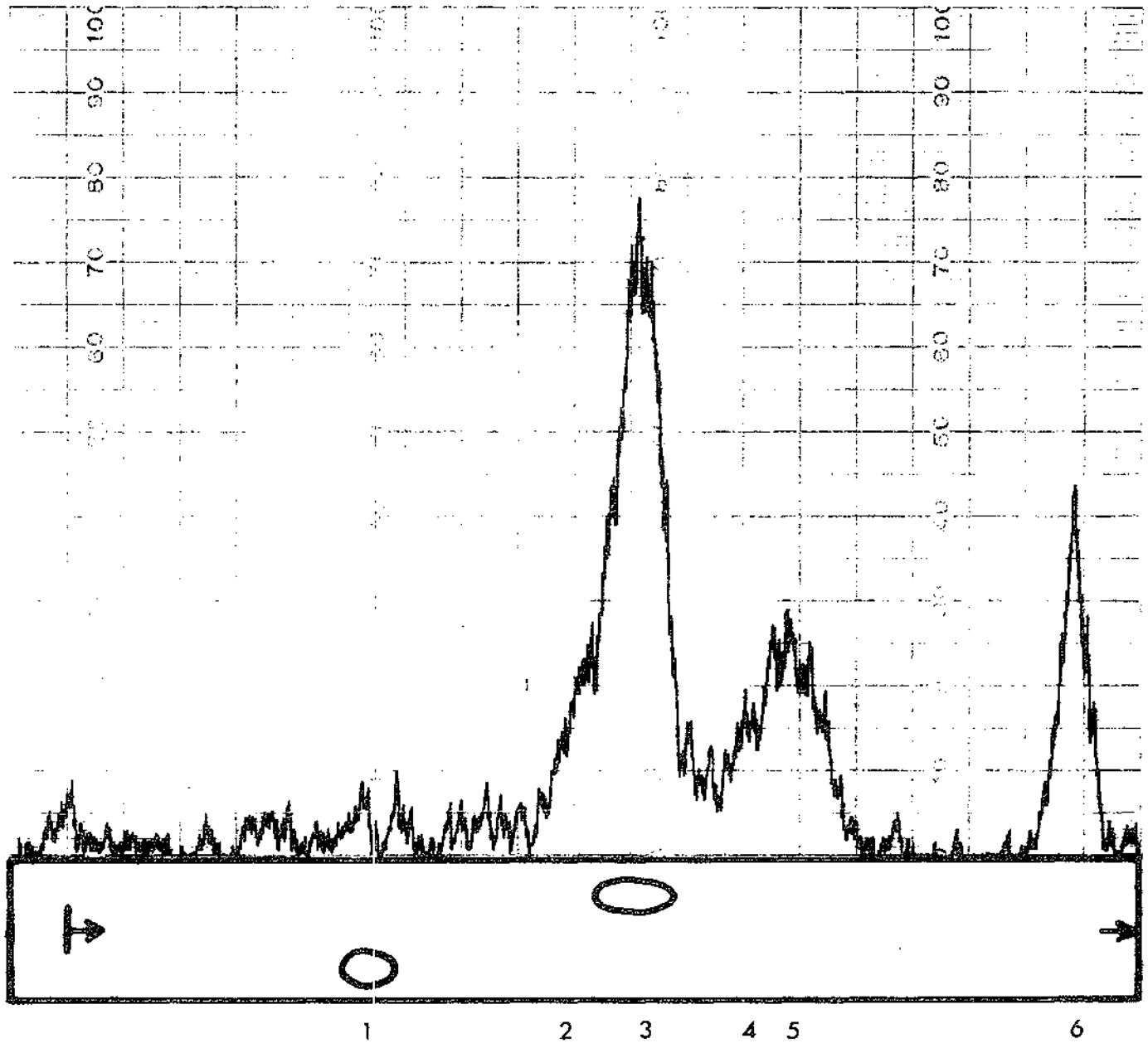


Figure 16

The radiochromatogram scan of the lipid extract (no.5) obtained after incubating fescue chloroplasts with UDP-D-galactose - ^{14}C. (see Table 17 for the reaction mixture).

key: 1, DGDG; 2, sterol glycoside (tentative); 3, MGDG; 4, unidentified; 5, acyl MGDG (tentative); 6, marker.

A radiochromatogram scan of lipid extract 5 (Figure 16) showed that the radioactivity appeared to be distributed among two and possibly four compounds. The major component was MGDG and the larger peak of greater mobility than MGDG was tentatively designated as an acyl MGDG. The trailing edge of the MGDG peak was tentatively identified as a sterol glycoside, but no designation of the shoulder on the acyl MGDG peak was possible. No radioactive DGDG was detected. Analysis of the DGDG, MGDG and acyl MGDG regions of the chromatogram for sugar (determined as galactose), using the method of Roughan and Batt (1968), indicated that the acyl MGDG region had the higher sugar content:

acyl MGDG region - 48 μg galactose

MGDG region - 37 μg galactose

DGDG region - 33 μg galactose.

4.II.3 The effect of chloroplast isolation procedure on the incorporation of radioactivity from UDP-D-galactose - ^{14}C into lipids by chloroplasts.

Two different techniques of isolating chloroplasts were used to find whether the chloroplast isolation procedure had an effect on the ability of chloroplasts to incorporate radioactivity from UDP-D-galactose - ^{14}C into lipids. The chloroplasts from 7 g., wet weight, of both spinach and fescue leaf tissue were prepared by method I (using cysteine hydrochloride as the thiol reagent) and method II. Each chloroplast pellet was suspended in 2 mls. of 0.1M Tris - HCl buffer, pH 7.4, and 0.1ml aliquots were incubated, in duplicate, with 4 μl of UDP-D-galactose - ^{14}C solution (containing 44,400 dpm) at 30°C for 2 hours. The results are given in Table 18.

Table 18.

The incorporation of radioactivity from UDP-D-galactose - ^{14}C into lipids by chloroplasts isolated by methods I and II.

Reaction mixture:

0.2 ml chloroplast suspension (0.1M Tris - HCl buffer, pH 7.4); 4 μl. UDP-D-galactose - ^{14}C solution, containing 44,400 dpm. Incubation at 30°C for 2 hours.

extract	leaf source	isolation method	incorporation (dpm)	% incorporation	chlorophyll content of the reaction mixture (mg)
1	fescue	I	8,700	19.6	0.25
2	fescue	I	8,200	18.5	0.25
3	fescue	II	15,950	35.9	0.15
4	fescue	II	15,900	35.8	0.15
5	spinach	I	5,300	11.9	0.19
6	spinach	I	5,400	12.2	0.19
7	spinach	II	13,700	30.8	0.20
8	spinach	II	13,500	30.4	0.20

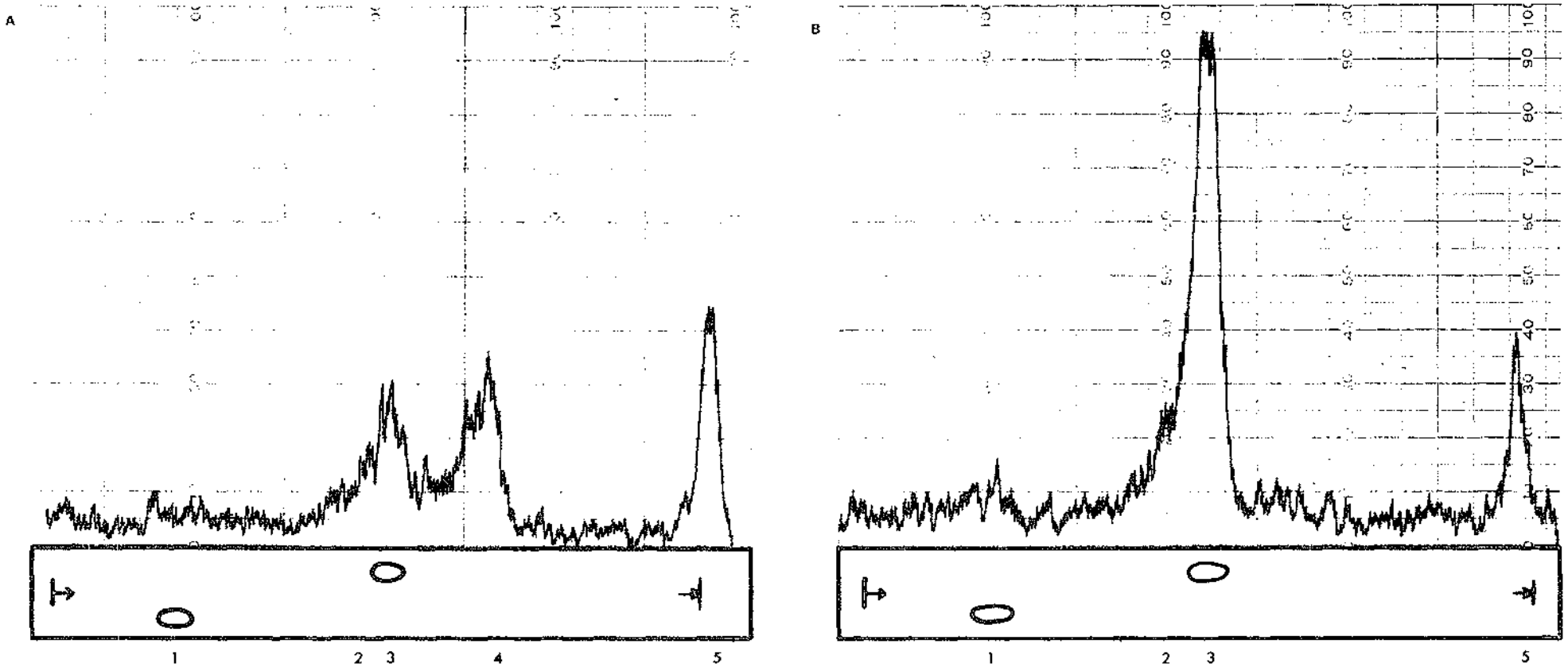


Figure 17

The radiochromatogram scans of the lipid extracts obtained after the incubation of fescue chloroplasts, isolated by method I, A, and method II, B, with UDP-D-galactose - ^{14}C. (see Table 18 for the reaction mixture).

key: 1, DGDG; 2, sterol glycoside (tentative); 3, MGDG; 4, acyl MGDG (tentative); 5, marker.

The more vigorous isolation procedure (method II) yielded the more active chloroplasts which would be expected to have less stromal protein, i.e. less integrity, than chloroplasts isolated by method I. The supernatants in the isolation of chloroplasts by method II contained chlorophyll whereas chlorophyll was absent in the supernatants obtained in method I. This indicated that very few chlorophyll-containing membranes were broken during method I.

The lipid extracts 1, 3, 6 and 7 were resolved by TLC and scanned for radioactivity (Figure 17). The major peaks were assigned to MGDG and the "acyl MGDG" which was essentially absent in the extract from fescue chloroplasts isolated by method II. Extracts from method II chloroplasts had a higher proportion of radioactivity as MGDG than did the lipid extracts from chloroplasts isolated by method I. Thus chloroplasts isolated by method II incorporated more radioactivity from UDP-D-galactose - ^{14}C into MGDG than the chloroplasts isolated by method I. No significant label was found in the DGDG fraction in any extract. The chloroplasts from fescue leaf tissue gave better incorporations of radioactivity than the chloroplasts from spinach leaves in both isolation methods.

4.II.4 The effect of treatment of the chloroplast suspension on the incorporation of radioactivity from UDP-D-galactose - ^{14}C into chloroplast lipids:

In an attempt to increase the amount of incorporation of radioactivity from UDP-D-galactose - ^{14}C into lipids by chloroplasts, and to stimulate incorporation of label into DGDG, the effect of sonication and the addition of deoxycholate to chloroplast suspensions was examined.

(a) Sonication:

The chloroplasts from fescue and spinach leaves (7g., wet weight) were isolated by method I (buffer medium containing cysteine

hydrochloride) and the chloroplast pellets suspended in 2 mls. of 0.1M Tris - HCl buffer, pH 7.4. Two 0.2 ml aliquots were removed from the suspension and the remainder sonicated for 5 minutes. Duplicate 0.2 ml aliquots were withdrawn and all the aliquots incubated with 4 µl of UDP-D-galactose - ^{14}C solution (containing 44,400 dpm) at 30°C for 2 hours. The results are given in Table 19.

The chloroplast suspension which had been sonicated gave a 20-30% increase in the incorporation of radioactivity from UDP-D-galactose - ^{14}C into chloroplast lipids compared to unsonicated chloroplast suspensions. This increase occurred for the chloroplasts derived from both fescue and spinach leaf tissue. The radiochromatogram scans of extracts 2, 3 and 7 showed that most of the radioactivity was divided between MGDG and the 'acyl MGDG' for extracts 2 and 7, and extract 3 contained radioactive MGDG but not a significant amount of radioactive "acyl MGDG". Thus sonication appeared to dissociate or destroy the ability of isolated fescue chloroplasts to form this latter compound. No extract contained any radioactive DGDG.

Table 19.

The effect of sonication of the chloroplast suspension on the incorporation of radioactivity from UDP-D-galactose - ^{14}C into chloroplast lipids.

Reaction mixture:

0.2 ml chloroplast suspension (0.1M Tris - HCl buffer, pH 7.4); 4 μl UDP-D-galactose - ^{14}C solution, containing 44,400 dpm. Incubation at 30°C for 2 hours.

extract	leaf source	treatment	incorporation (dpm)	% incorporation	chlorophyll content of reaction mixture (mg)
1	fescue	none	7,100	16.0	0.31
2	fescue	none	6,700	15.1	0.31
3	fescue	sonication	9,300	20.9	0.31
4	fescue	sonication	8,700	19.6	0.31
5	spinach	none	4,600	10.4	0.18
6	spinach	none	4,200	9.5	0.18
7	spinach	sonication	5,400	12.2	0.18
8	spinach	sonication	5,300	11.9	0.18

(b) Addition of deoxycholate.

The addition of deoxycholate (potassium salt) to 0.2 ml aliquots of chloroplast suspensions, to give a final concentration of 2% (w/v), effectively inhibited any incorporation of radioactivity from UDP-D-galactose - ^{14}C into lipids. (Chloroplasts were isolated by both methods I and II from 7g., wet weight, of fescue and spinach leaf tissue and suspended in 2mls. of 0.1M Tris - HCl buffer, pH 7.4). The idea of adding deoxycholate was to facilitate the entry of radioactive substrate, which appeared to be rate limiting in experiment 4.II.3, to the site of galactolipid synthesis, by the action of deoxycholate of weakening membranes. However, at the concentration of deoxycholate used, no galactolipid synthesis occurred either because of the binding of all the diglyceride acceptors or the denaturation of the enzyme(s).

Section 4.III : Experiments with chloroplast enzyme preparations.

4.III.1 The incubation of acetone powder preparations and freeze-dried chloroplasts with UDP-D-galactose - ^{14}C.

The first acetone powder and freeze-dried preparations of fescue and spinach chloroplasts were incubated, with and without added lipid, with 3μl of UDP-D-galactose - ^{14}C solution (containing 33,300 dpm) at 30°C for 90 minutes. In this experiment, the sonication step, mentioned in paragraph 3.II.6(b), was omitted in the preparation of the reaction mixtures. The results are given in Table 20.

The enzyme preparations from fescue chloroplasts showed no activity (Table 20) whereas the spinach preparations were able to incorporate label into lipids. The spinach freeze-dried preparation showed considerable activity when incubated without added lipid and the addition of diolein had no effect on the amount of label incorporated. The addition of lipid to the spinach acetone powder preparations increased the incorporation by up to 80%.

A radiochromatogram scan of the lipid extract from the spinach freeze-dried preparation which was incubated without diolein, showed four peaks (Figure 18). The major one corresponded to MGDG, and the other three were apparently those observed in experiment 4.II.1(b). The scan of the bulked lipid extract from the spinach acetone powder preparations which were incubated with diolein indicated that MGDG was the only component that was significantly labelled.

Table 20.

The incorporation of radioactivity from UDP-D-galactose - ^{14}C into lipids by acetone powder and freeze-dried chloroplast preparations.

Reaction mixture:

14mg acetone powder preparation no.1 (or 15.5mg freeze-dried chloroplast preparation); 0.7ml. 0.1M Tris - HCl buffer, pH 7.4; 3 μl UDP-D-galactose - ^{14}C solution (containing 33,300 dpm); and the lipid indicated below. Incubated at 30°C for 90 minutes.

leaf source	type of chloroplast preparation	mg. of lipid added ‡ acetone extract	mg. of lipid added ‡ diolein*	incorporation (dpm)	% incorporation
spinach	acetone powder no.1	-	-	1,600	4.8
spinach	acetone powder no.1	4.4	-	1,900	5.7
spinach	acetone powder no.1	-	1.08	2,900	8.7
spinach	acetone powder no.1	-	2.16	2,300	6.9
spinach	freeze-dried preparation	-	-	11,500	34.5
spinach	freeze-dried preparation	-	1.08	10,800	32.4
fescue	acetone powder no.1	-	-	0	-
fescue	acetone powder no.1	4.5	-	100	0.3
fescue	freeze-dried preparation	-	1.08	0	-
fescue	freeze-dried preparation	-	-	100	0.3
fescue	freeze-dried preparation	-	1.08	100	0.3

‡ mg. of lipid added per 20mg. acetone powder (or per 25mg. of freeze-dried preparation)

* 1,2-dioleoyl glycerol.

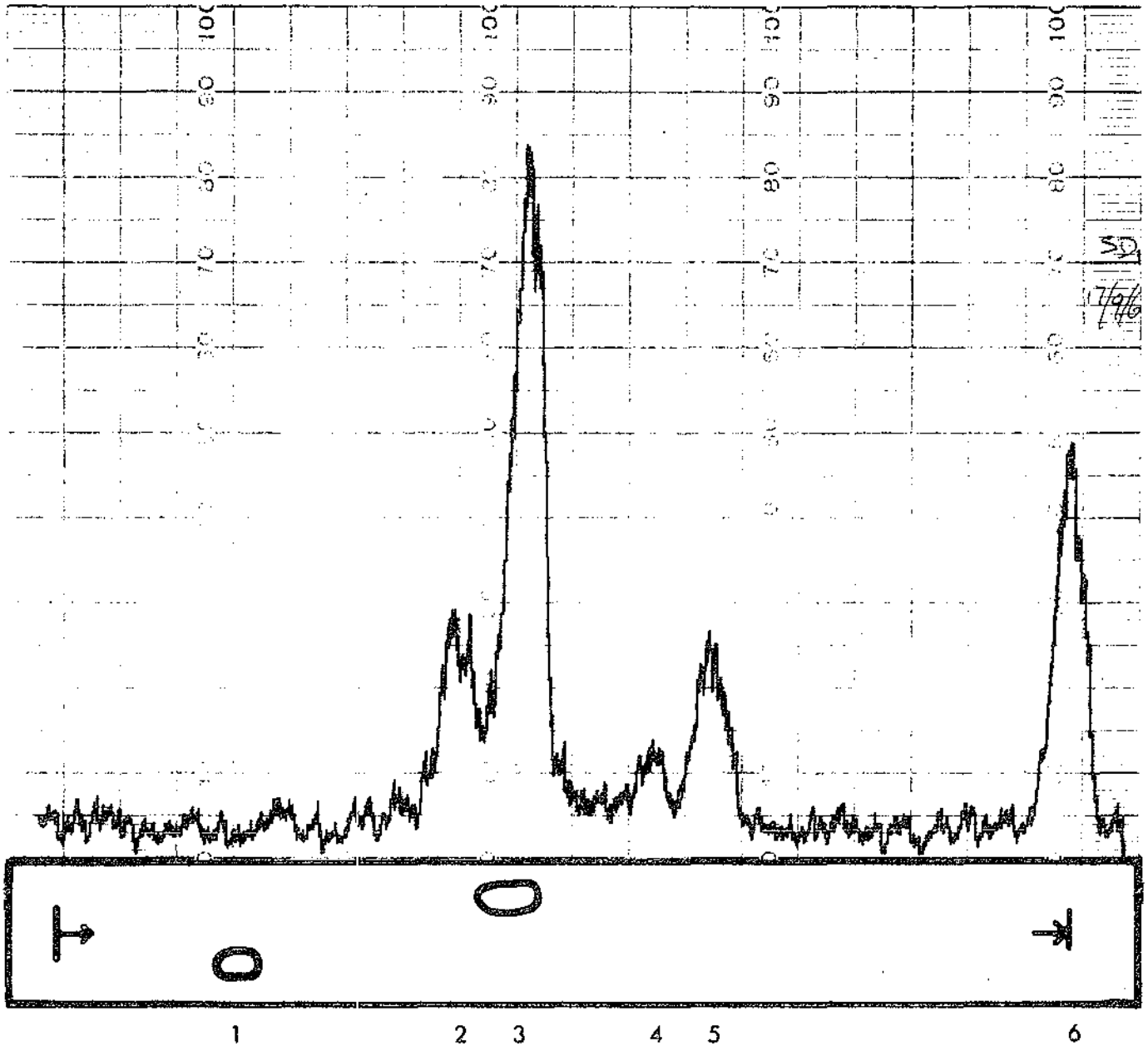


Figure 18

The radiochromatogram scan of the lipid extract obtained after incubating a freeze-dried spinach chloroplasts with UDP-D-galactose - ^{14}C. (see Table 20 for the reaction mixture).

key: 1, DGDG; 2, sterol glycoside (tentative); 3, MGDG; 4, unidentified; 5, acyl MGDG (tentative); 6, marker.

4.III.2 The effect of adding increasing amounts of 1,2-dioleoyl glycerol on the incorporation of radioactivity from UDP-D-galactose - ^{14}C into lipids by a spinach acetone powder preparation:

If added diolein acted as an acceptor of the galactose moiety from UDP-D-galactose - ^{14}C in MGDG synthesis, then it was expected that increasing amounts of diolein would give increasing incorporation of radioactivity from UDP-D-galactose - ^{14}C into lipids by spinach acetone powder preparations. The second spinach acetone powder preparation (20.0mg) was homogenized with from 0.125 - 2.5 mg. of diolein, the suspension sonicated for 5 minutes and 0.7 ml aliquot incubated with 4 μl. of UDP-D-galactose - ^{14}C solution (containing 44,400 dpm) at 30°C for 90 minutes. Since all tubes were incubated simultaneously, some tubes stood up to 4 hours in ice before the UDP-D-galactose - ^{14}C solution was added.

The percentage incorporation of radioactivity into lipids increased as the amount of diolein was increased (Table 21, Figure 19). A radiochromatogram scan of extract 5 indicated that MGDG was the only radioactive component of significance in the reaction products (Figure 20).

A similar experiment with fescue acetone powder preparation no.2, confirmed the inactivity of the enzyme responsible for the synthesis of MGDG in acetone powder preparations, as observed in experiment 4.III.1. While the small amount of incorporation increased slightly as the amount of diolein increased, the radiochromatogram scan of the bulked extracts demonstrated that the incorporation was into the sterol glycoside.

Table 21.

The effect of adding increasing amounts of 1,2-dioleoyl glycerol on the incorporation of radioactivity from UDP-D-galactose - ^{14}C into lipids by a spinach acetone powder preparation.

Reaction mixture:

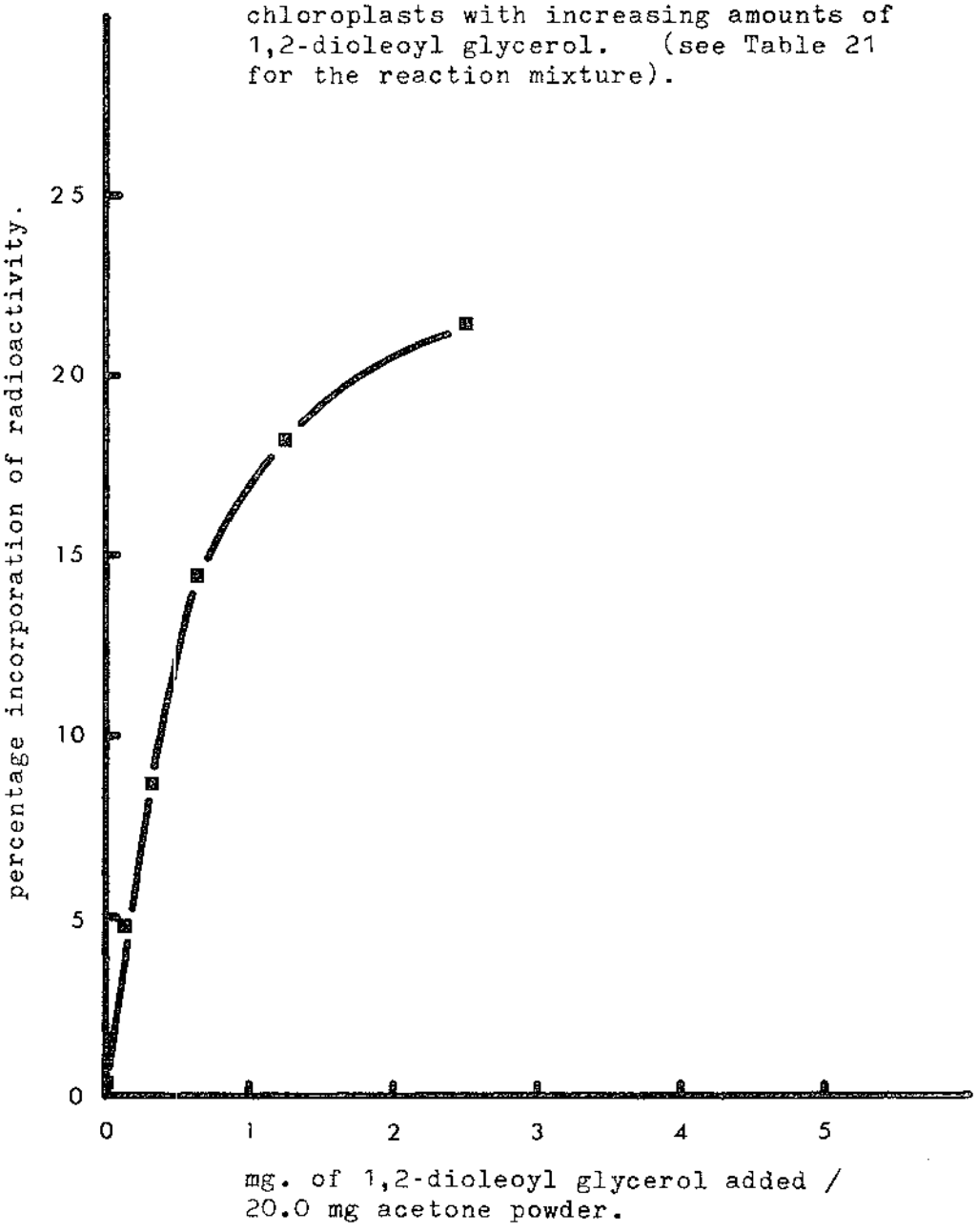
0.7 ml aliquot of a suspension made of 20.0mg spinach acetone powder preparation no.2, 0.125 - 2.5 mg diolein and 1ml 0.1M Tris - HCl buffer, pH 7.4; 4 μl UDP-D-galactose - ^{14}C solution, containing 44,400 dpm. Incubation at 30°C for 90 minutes.

extract	mg of diolein ‡	incorporation (dpm)	% incorporation
1	0	150	0.3
2	0.125	2,100	4.7
3	0.31	3,800	8.6
4	0.63	6,400	14.4
5	1.25	8,100	18.2
6	2.50	9,500	21.4

‡ mg of 1,2-dioleoyl glycerol added to 20.0mg acetone powder preparation.

Figure 19

The incorporation of radioactivity from UDP-D-galactose - ^{14}C into lipids on incubating the acetone powder of spinach chloroplasts with increasing amounts of 1,2-dioleoyl glycerol. (see Table 21 for the reaction mixture).



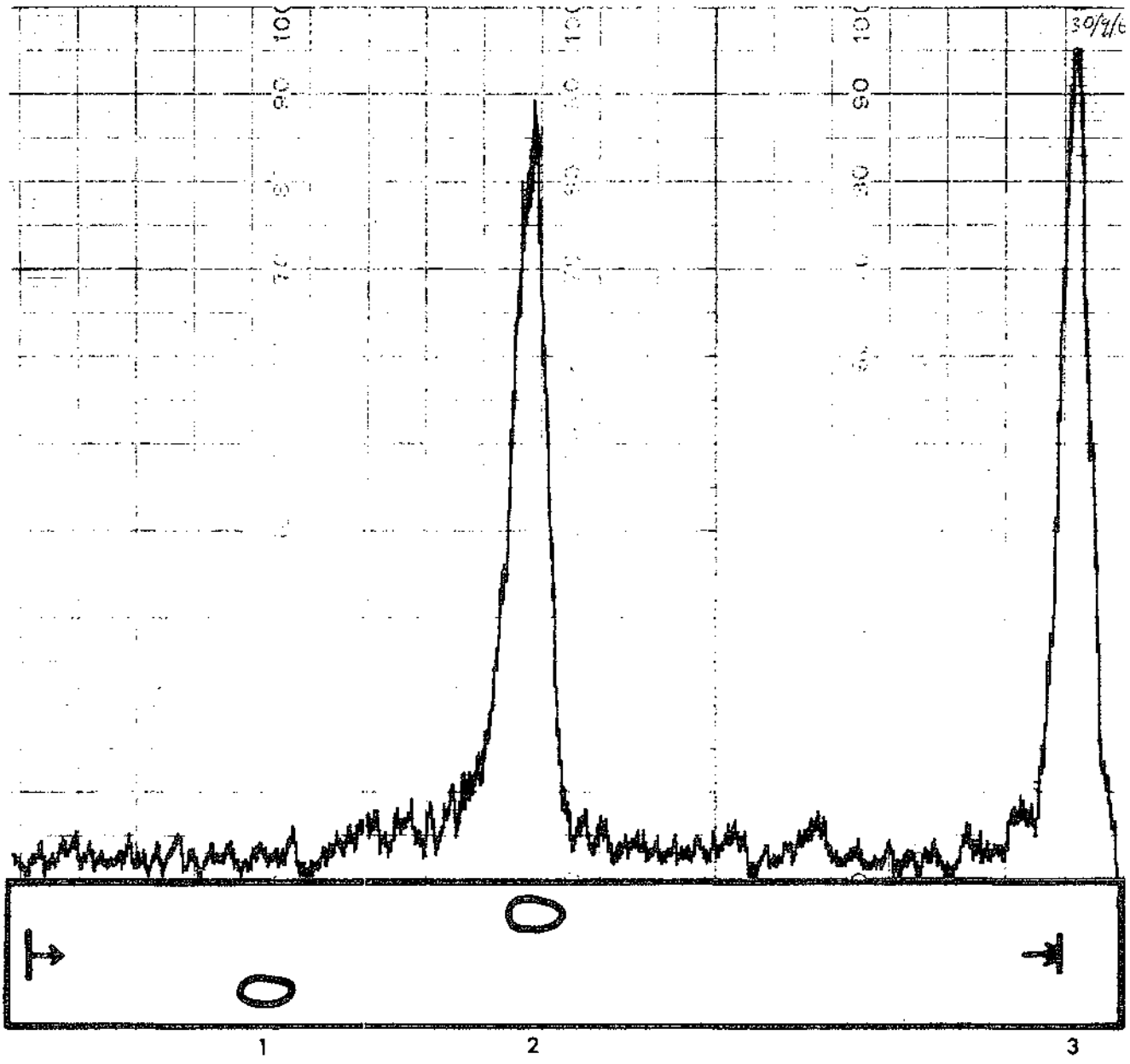


Figure 20

The radiochromatogram scan of the lipid extract (no.5) obtained after incubating the acetone powder of spinach chloroplasts with 1,2 - dioleoyl glycerol and UDP-D-galactose - ^{14}C. (see Table 21 for the reaction mixture).

key: 1, DGDG; 2, MGDG; 3, marker.

4.III.3 The effect of holding the reaction mixtures at 0°C before incubation with UDP-D-galactose - ^{14}C.

It was suspected that holding the suspensions of the acetone powder preparation and diolein in Tris buffer at 0°C before incubation with UDP-D-galactose - ^{14}C might affect the incorporation of radioactivity into lipids. Hence, spinach acetone powder preparation no.2, (80.4mg) was homogenized with 5.0mg of diolein in acetone and in 4 mls of 0.1M Tris - HCl buffer, pH 7.4, and the suspension sonicated for 5 minutes. After sonication, five 0.7ml aliquots were withdrawn and one was immediately incubated with 4μl of UDP-D-galactose - ^{14}C solution (containing 44,400 dpm) at 30°C for 90 minutes. A further three aliquots were incubated at 1, 2 and 4 hour intervals after having stood in ice, and the fifth aliquot was held at room temperature for 4 hours before incubation. The reaction mixture lacking diolein was incubated immediately after its preparation. The results are given in Table 22 and Figure 21.

A lower incorporation of radioactivity into lipids was obtained for those reaction mixtures held at 0°C before incubation with radioactive substrate. Thus, this experiment suggested that the reaction mixtures should be incubated immediately after preparation.

Table 22.

The effect of holding the reaction mixtures at 0°C before incubation with UDP-D-galactose - ^{14}C

Reaction mixture:

0.7ml aliquot of a suspension consisting of 80.4mg spinach acetone powder preparation no.2, 5.0mg diolein and 4 mls 0.1M Tris - HCl buffer, pH 7.4; 4 µl UDP-D-galactose - ^{14}C solution containing 44,400 dpm. Incubation at 30°C for 90 minutes. (The reaction mixture lacking diolein consisted of, 14.0mg spinach acetone powder preparation no.2; 0.7ml. 0.1M Tris - HCl buffer, pH 7.4 and 4 µl. UDP-D-galactose - ^{14}C solution, containing 44,400 dpm.)

period of delay before incubation (hrs.)	ambient temperature during delay (°C)	incorporation (dpm)	% incorporation
0	-	1,600 ‡	3.6
0	-	9,000	20.3
1	0	6,600	14.9
2	0	6,200	14.0
4	0	6,000	13.5
4	20	7,500	16.9

‡ no diolein added.

Figure 21

The effect of standing the acetone powder - diglyceride suspensions before incubating with UDP-D-galactose - ^{14}C. (see Table 22 for the reaction mixture).

key: ■ - suspension held at 0°C;
□ - suspension held at 20°C.

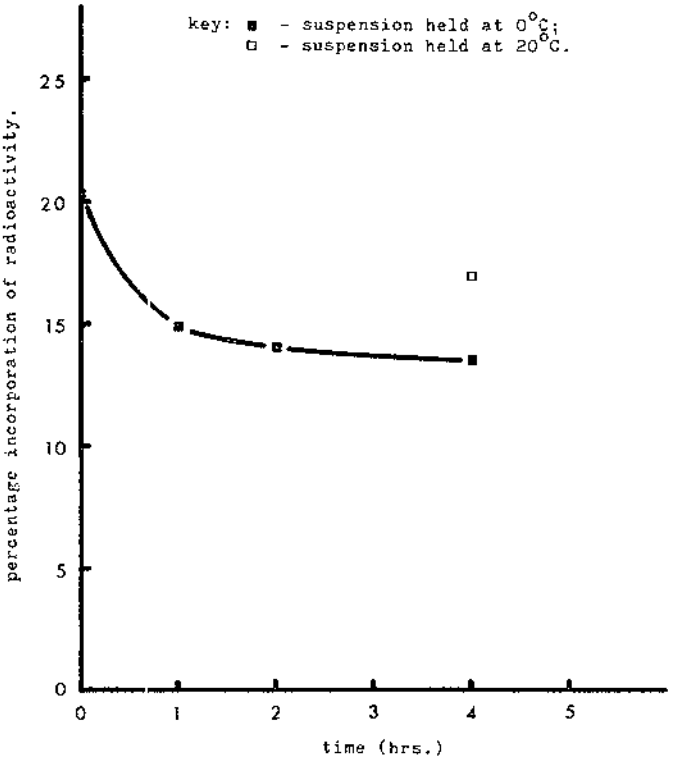
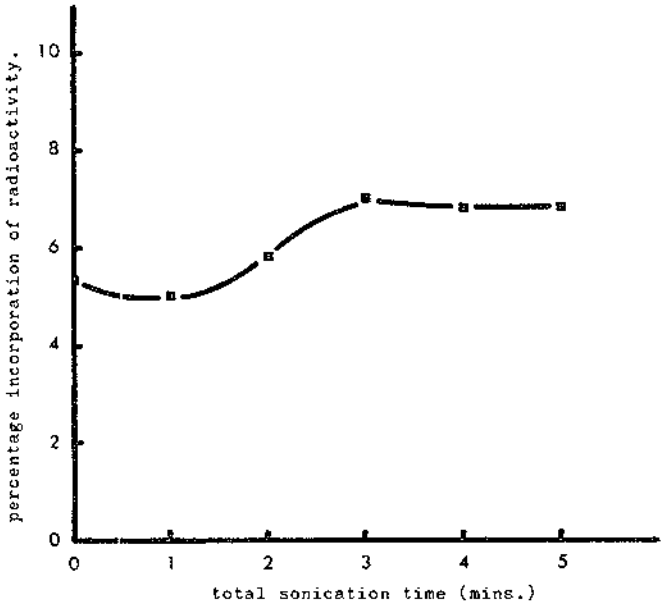


Figure 23

The effect of sonication time on the incorporation of radioactivity from UDP-D-galactose - ^{14}C into lipids by an acetone powder preparation of spinach chloroplasts. (see Table 25 for the reaction mixture).



4.III.4 The effect of different diglycerides on the incorporation of radioactivity from UDP-D-galactose - ^{14}C into lipids.

(a) To find whether the nature of the fatty acid constituents of the diglyceride acceptors in MGDG synthesis affected the incorporation of radioactivity from UDP-D-galactose - ^{14}C into lipids, different 1,2-diacyl glycerols were incubated with a spinach acetone powder preparation and UDP-D-galactose - ^{14}C. The reaction mixture contained spinach acetone powder preparation no.3 (20.0mg), synthetic 1,2 (2,3) - diglycerides (1.64mg) or 4.8mg of acetone extract total lipid, and 4μl of UDP-D-galactose - ^{14}C solution (containing 44,400 dpm) were incubated in duplicate at 30°C for 90 minutes. Each sample was incubated immediately after preparation. The results are given in Table 23.
It is apparent that, for the particular weight ratio of diglyceride to acetone powder preparation chosen (cf Figure 19), the 1,2 (2,3) - diglycerides with an oleoyl residue in the 1-position give the best incorporations. Despite the large variation for the duplicates containing diolein, the mean value is in reasonable agreement with the percentage incorporation obtained in experiment 4.III.2. Diolein was as good as the total lipid of the acetone extract in stimulating incorporation of radioactivity into lipids from UDP-D-galactose - ^{14}C.
One of each of the duplicate extracts was resolved by TLC and scanned for radioactivity. All the scans showed MGDG to be the major radioactive lipid and, except when diolein and 1-oleoyl, 2-linoleoyl glycerol were the added diglycerides, a partially resolved sterol glycoside peak was present containing about 20% of the radioactivity (Figure 22). No significant radioactivity was found in the DGDG fraction.

Table 23.

The incorporation of radioactivity, from UDP-D-galactose - ^{14}C into lipids, stimulated by different 1,2(2,3)-diglycerides when incubated with a spinach acetone powder preparation.

Reaction mixture:

0.7ml aliquot of a suspension consisting of 20.0mg spinach acetone powder preparation no.3, lipid (either 1.64mg 1,2(2,3)-diglyceride or 4.8mg total lipid of the acetone extract), and 1ml 0.1M Tris - HCl buffer, pH 7.4; 4 µl UDP-D-galactose - ^{14}C solution, containing 44,400 dpm. Incubation at 30°C for 90 minutes.

lipid added	number of double bonds /molecule	incorporation (dpm)	% incorporation	% incorporation mean value ± deviation
none	-	1,500	3.4	3.4
		1,500	3.4	
1,2-dioleoyl glycerol	2	10,000	22.5	19.9 ± 2.6
		7,700	17.3	
1-oleoyl,2-linoleoyl glycerol	3	7,900	17.8	18.0 ± 0.2
		8,100	18.2	
1-linoleoyl,2-oleoyl glycerol	3	5,100	11.5	12.5 ± 0.8
		5,800	13.1	
1,2-dilinoleoyl glycerol	4	4,400	9.9	10.7 ± 0.8
		5,100	11.5	
1-γ-linoleoyl 2-linoleoyl glycerol	5	5,300	11.9	11.5 ± 0.5
		4,900	11.0	
acetone extract	-	9,200	20.7	19.7 ± 1.0
		8,300	18.7	

(b) To confirm the different stimulations given by 1-oleoyl, 2-linoleoyl glycerol and 1-linoleoyl, 2-oleoyl glycerol, amounts of spinach acetone powder preparation no.4 (20.0mg) were incubated with each substrate and 5 .1 of UDP-D-galactose - ^{14}C solution, containing 55,500 dpm, at 30°C for 90 minutes.

Table 24.

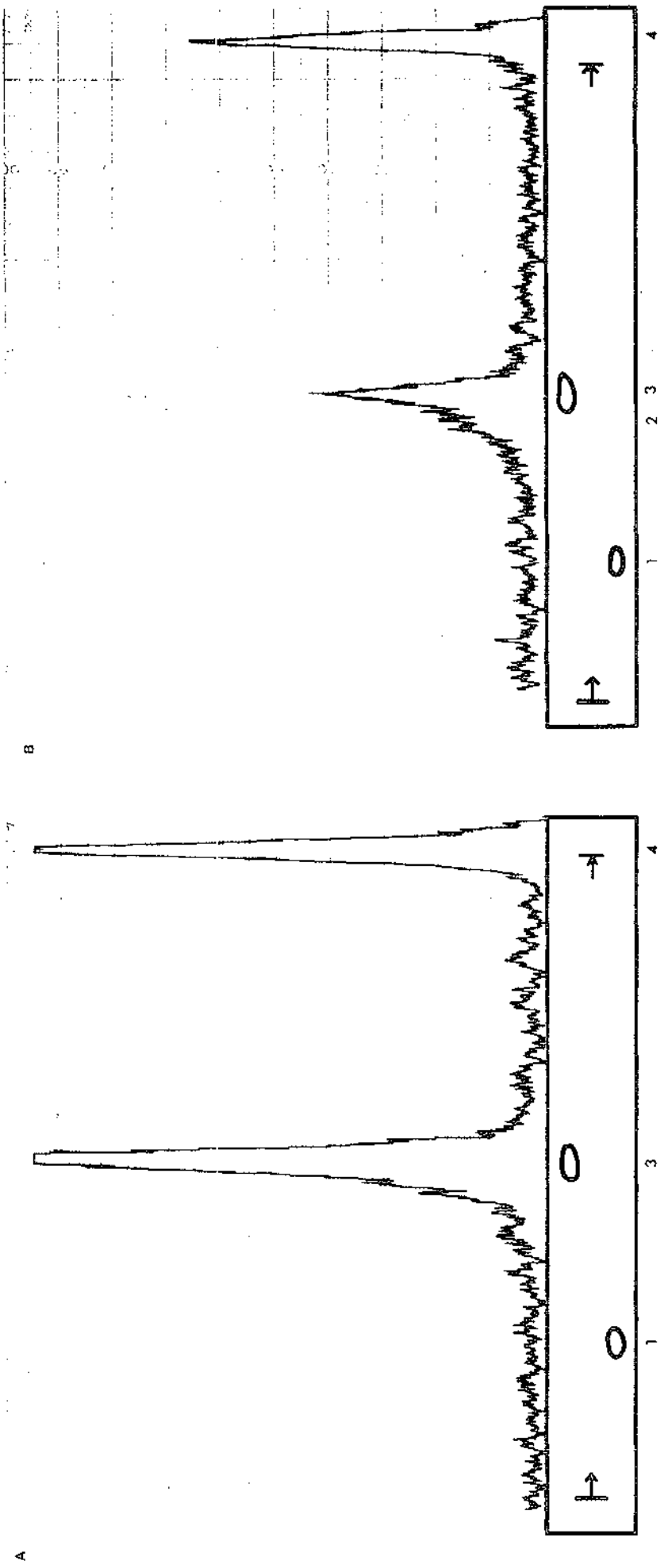
The stimulation of incorporation of radioactivity into lipids from UDP-D-galactose - ^{14}C given by 1-oleoyl, 2-linoleoyl glycerol and 1-linoleoyl, 2-oleoyl glycerol incubated with a spinach acetone powder preparation.

Reaction mixture:

0.7ml aliquot of a suspension consisting of 20.0mg spinach acetone powder preparation no.4, lipid (either 1.58mg 1-oleoyl, 2-linoleoyl glycerol or 1.60mg 1-linoleoyl, 2-oleoyl glycerol) and 1ml 0.1M Tris - HCl buffer, pH 7.4; 5 l UDP-D-galactose - ^{14}C solution, containing 55,500 dpm. Incubation at 30°C for 90 minutes.

diglyceride	incorporation (dpm)	% incorporation	mean value ± deviation
none	800	1.4	
1-oleoyl,2-linoleoyl	5,900	10.6	11.7 ± 1.1
glycerol	7,100	12.8	
1-linoleoyl, 2-oleoyl	4,900	8.8	8.1 ± 0.7
glycerol	4,100	7.4	

Thus the greater stimulation of 1-oleoyl,2-linoleoyl glycerol was confirmed (Table 24), even though the spinach acetone powder preparation no.4 was less active than the no.3 preparation.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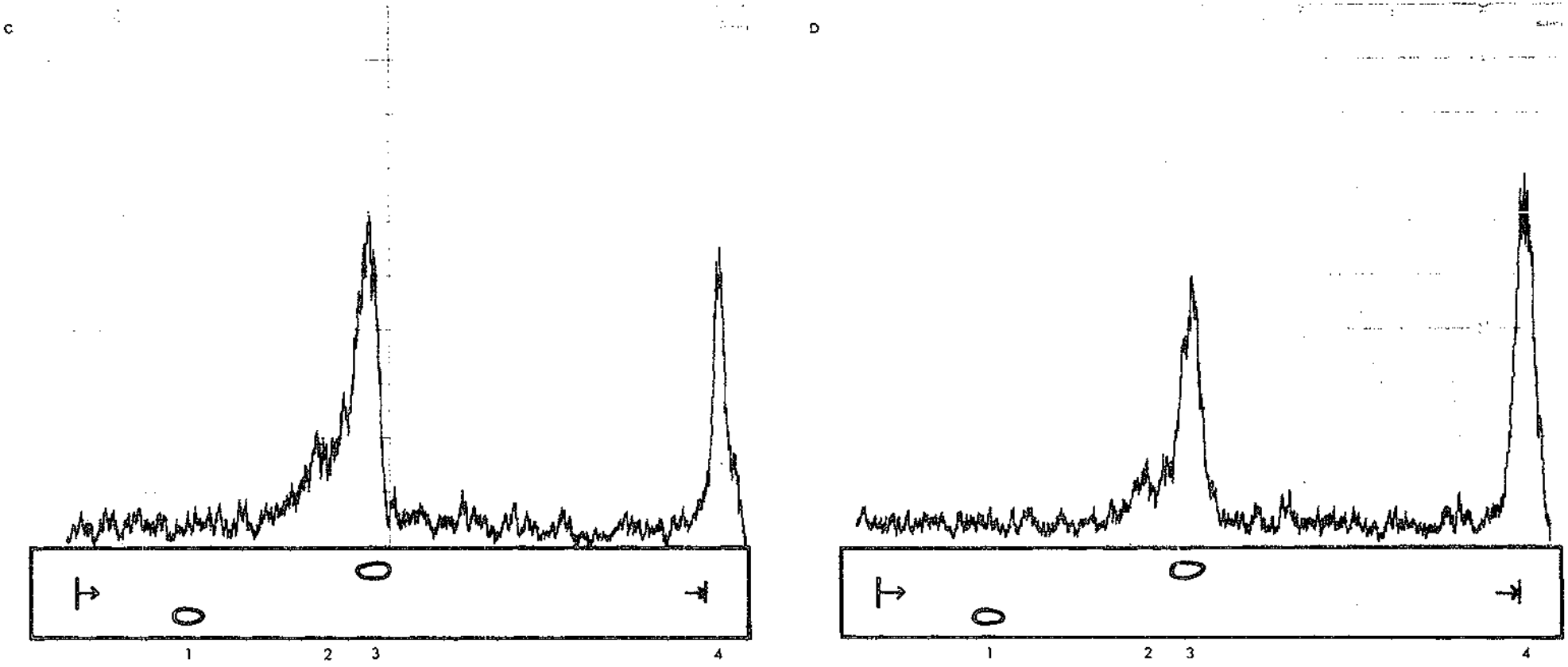


Figure 22

Some of the radiochromatogram scans of the lipid extracts obtained after incubating the acetone powder of spinach chloroplasts with UDP-D-galactose - ^{14}C and different diglycerides. (see Table 23 for the reaction mixture).
The lipid substrates were: A, 1-oleoyl,2-linoleoyl glycerol; B, 1-linoleoyl,2,oleoyl glycerol; C, 1-γ-linolenoyl,2-linoleoyl glycerol; D, acetone extract from chloroplasts.

key: 1, DGDG; 2,sterol glycoside (tentative); 3, MGDG; 4, marker.

4.III.5 The effect of sonication time on the incorporation of radioactivity from UDP-D-galactose - ^{14}C into lipids by spinach acetone powder preparations:

The effect of increasing sonication times on the incorporation of radioactivity from UDP-D-galactose - ^{14}C into lipid by a spinach acetone powder preparation was examined. The spinach acetone powder preparation no.4 (99.6mg) was homogenized with 1-γ-linolenoyl, 2-linoleoyl glycerol (75.5mg) in acetone and in 5mls of 0.1M Tris - HCl buffer, pH 7.4. Aliquots (0.7ml) of the suspension were removed after sonication for 0, 1, 2, 3, 4 and 5 minutes. These samples, held for a short time at room temperature, were incubated simultaneously with 4μl of UDP-D-galactose - ^{14}C solution, containing 44,400 dpm, at 30°C for 90 minutes. The results are given in Table 25 and Figure 23.

Sonication was thus shown to aid incorporation and it was noted that the acetone powder preparation used exhibited low activity in incorporating radioactivity into lipids compared to the previous preparations. From the increased incorporation with time of sonication it would appear that prolonged sonication had no harmful effects on the components of the reaction mixture.

When 1.6mg of 1-linoleoyl,2-oleoyl glycerol was sonicated for 4 minutes in 0.1M Tris - HCl buffer, pH 7.4, under nitrogen, the re-extracted lipid contained a considerable amount of 1,3-diglyceride and very little 1,2-diglyceride. However, repetition of this procedure in the presence of 20,g of acetone powder gave only a negligible amount of 1,3-diglyceride as judged by analytical TLC.

Table 25.

The effect of increasing sonication time on the incorporation of radioactivity from UDP-D-galactose - ^{14}C into lipids by a spinach acetone powder preparation.

Reaction mixture:

0.7ml aliquots of a suspension consisting of 99.6mg spinach acetone powder preparation no.4, 75.5mg 1-α-linolenoyl,2-linoleoyl glycerol and 5mls 0.1M Tris - HCl buffer, pH 7.4; 4 μl UDP-D-galactose - ^{14}C solution, containing 44,400 dpm. Incubation at 30°C for 90 minutes.

total time of sonication (minutes)	incorporation (dpm)	% incorporation
0	2,400	5.4
1	2,200	5.0
2	2,600	5.8
3	3,100	7.0
4	3,000	6.8
5	3,000	6.8

4.III.6 The effect of added phospholipid on the incorporation of radioactivity from UDP-D-galactose - ^{14}C into lipids by a spinach acetone powder preparation.

To find the effect of added phospholipid on the incorporation of radioactivity from UDP-D-galactose - ^{14}C into lipids, egg and spinach phospholipids were incubated, with and without diolein, with spinach acetone powder preparation no. 5, (20.0mg) and 4 l of UDP-D-galactose-^{14}C solution (containing 44,400 dpm) at 30°C for 90 minutes under nitrogen in stoppered test tubes. The reaction mixture was sonicated for 4 minutes before the addition of the radioactive substrate. The results are given in Table 26.

Both the recently isolated phospholipids from egg yolk, phosphatidyl choline (PC) and phosphatidyl ethanolamine (PE), stimulated incorporation of label into lipids when incubated alone or with diolein. The net incorporation obtained when egg phosphatidyl choline and diolein were incubated together was more than the sum of the net incorporation given by these lipids incubated separately. The total phospholipid fraction from egg yolk gave a slight increase in incorporation when incubated alone but when incubated with diolein the incorporation was less than that given by diolein alone, for the amounts used. The small amounts of spinach phospholipids available did not stimulate incorporation when incubated alone with the acetone powder preparation and UDP-D-galactose - ^{14}C.

The radiochromatogram scans of each extract showed the same pattern of radioactivity observed previously, i.e. most of the radioactivity was present in the MGDG fraction and a small amount in sterol glycoside (Figure 24).

Table 26.

The effect of added phospholipid on the incorporation of radioactivity from UDP-D-galactose - ^{14}C into lipids by a spinach acetone powder preparation.

Reaction mixture:

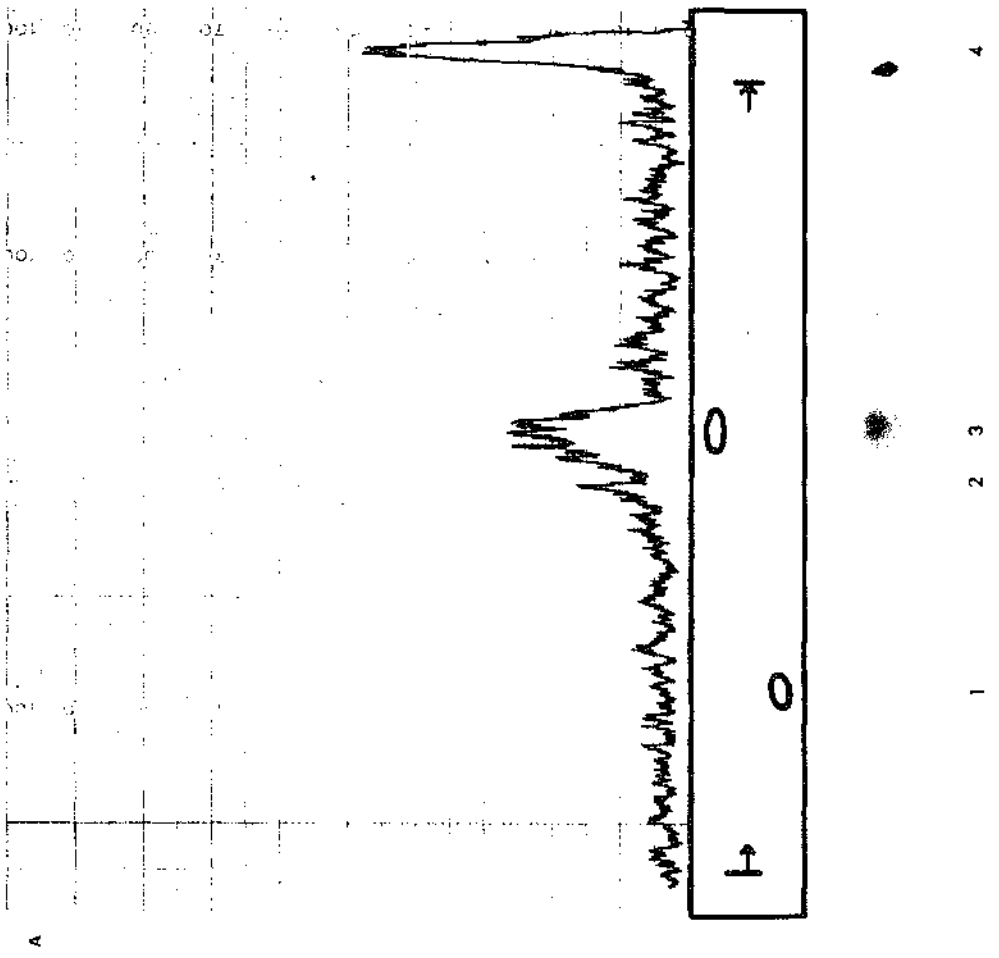
0.7ml aliquot of a suspension consisting of 20.0mg spinach acetone powder preparation no.5, lipid, and 1ml 0.1M Tris - HCl buffer, pH 7.4; 4 μl UDP-D-galactose - ^{14}C solution, containing 44,400 dpm. Incubation at 30°C for 90 minutes.

source and type of phospholipid	amount of lipid (mg)*		incorporation (dpm)	% incorporation
	phospholipid	diolein		
-	-	-	1,800	4.0
PC from egg yolk	1.60	-	2,700	6.1
-		1.61	9,400	21.1
PC from egg yolk	1.60	1.61	12,400	27.9
PE from egg yolk	2.02	-	3,400	13.3‡
PE from egg yolk	1.73	1.61	10,700	24.1
total phospholipid ▽	5.14	-	2,300	5.2
total phospholipid	5.14	1.61	7,000	15.8
PC from spinach	1.7	-	1,400	3.2
PE from spinach	0.7	-	1,700	3.8

* mg of lipid added to 20.0mg spinach acetone powder preparation.

‡ 0.4ml aliquot incubated with 2.3 μl UDP-D-galactose - ^{14}C solution, containing 25,500 dpm.

▽ total phospholipid fraction from egg yolk.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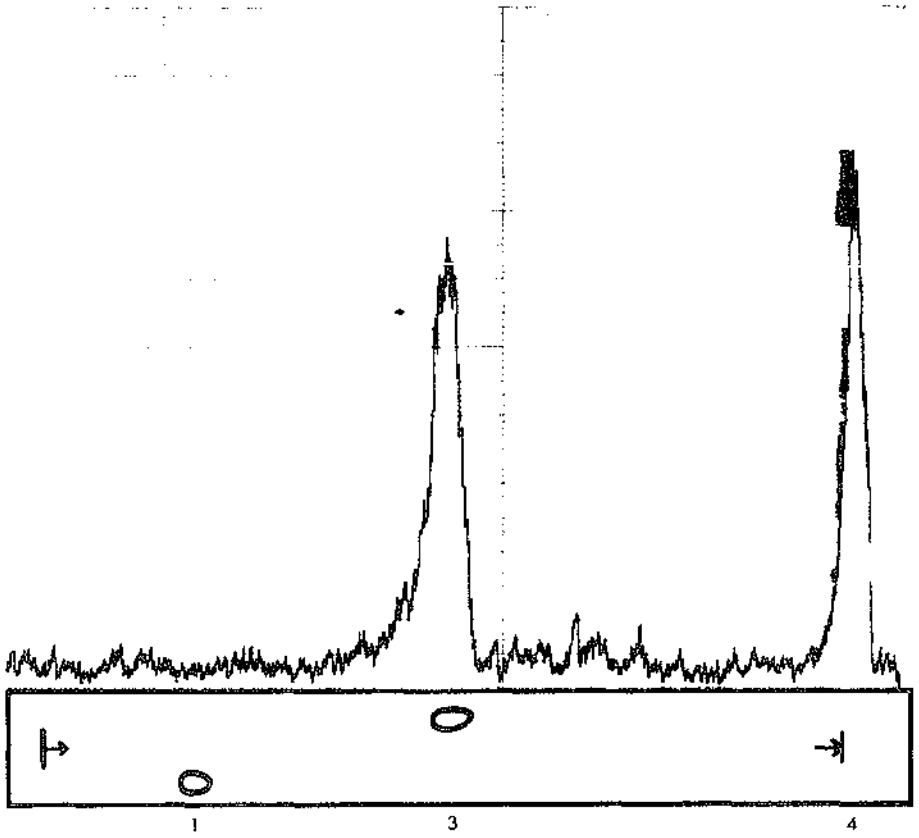

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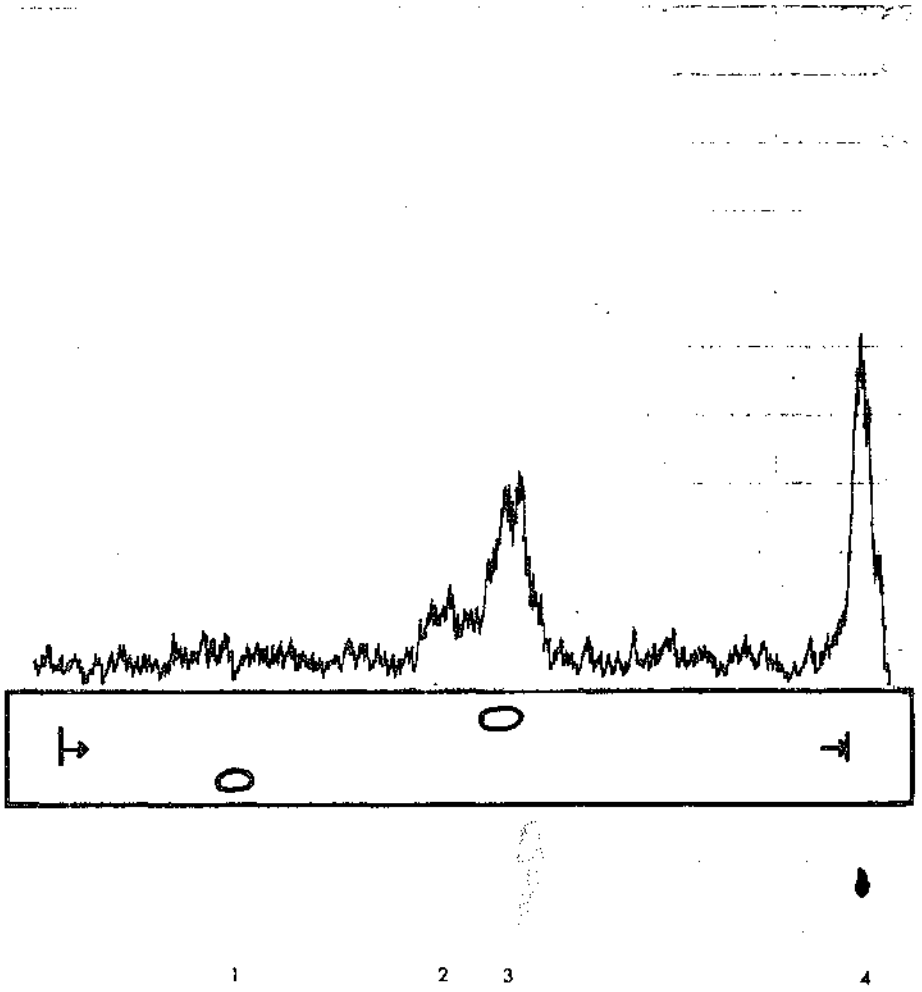


Figure 24

Examples of the radiochromatogram scans of the lipid extracts obtained after incubating phospholipids with the acetone powder and UDP-D-galactose - ^{14}C (see Table 26 for the reaction mixture).
The lipid substrates were: A, phosphatidyl ethanolamine; B, phosphatidyl ethanolamine and 1,2 dioleoyl glycerol; C, the total phospholipid fraction from egg yolk.

key: 1, DGDG; 2, sterol glycoside (tentative); 3, MGDG; 4, marker.

4.III.7 The effect of time of incubation on the incorporation of radioactivity from UDP-D-galactose - ^{14}C into lipid by a spinach acetone powder preparation.

The dependence on incubation time of incorporation of radioactivity from UDP-D-galactose - ^{14}C into lipids by a spinach acetone powder preparation was studied using 1-oleoyl, 2-linoleoyl glycerol and 1-linoleoyl, 2-oleoyl glycerol as substrates.

(a) Using 1-oleoyl,2-linoleoyl glycerol as the lipid substrate.

Spinach acetone powder preparation no.5 (118.7mg) was homogenized with 1-oleoyl,2-linoleoyl glycerol (10.03mg) in acetone and in 6mls of 0.1M Tris - HCl buffer, pH 7.4. The suspension was sonicated for 5 minutes and 0.7ml aliquots were incubated with 4 μl of UDP-D-galactose - ^{14}C solution, containing 44,400 dpm, at 30°C under nitrogen in stoppered test tubes.

From Table 27 and Figure 25 it is seen that the incorporation of radioactivity into lipids was still increasing after 2 hours. Two near-linear regions are discernible in Figure 25, with a break at about 45 mins. The percentage incorporation at 90 mins. was lower than that expected from previous incubations of 1-oleoyl, 2-linoleoyl glycerol with acetone powder preparations.

Table 27.

The effect of time of incubation on the incorporation of radioactivity from UDP-D-galactose - ^{14}C into lipids by a spinach acetone powder preparation using 1-oleoyl,2-linoleoyl glycerol as the lipid substrate.

Reaction mixture:

0.7ml aliquots of a suspension consisting of 118.7mg spinach acetone powder preparation no.5, 10.03mg 1-oleoyl,2-linoleoyl glycerol and 6mls 0.1M Tris - HCl buffer, pH 7.4;
4 µl UDP-D-galactose solution, containing 44,400 dpm. Incubation at 30°C under nitrogen.

incubation time (minutes)	incorporation (dpm)	% incorporation
5	500	1.1
10	900	2.0
20	1,800	4.0
30	2,400	5.4
45	3,400	7.7
60	4,000	9.0
90	5,000	11.3
120	6,200	14.0

Figure 25

The relationship between the percentage incorporation of radioactivity, from UDP-D-galactose - ^{14}C into lipids by the acetone powder of spinach chloroplasts, and incubation time, using 1-oleoyl, 2-linoleoyl glycerol as the lipid substrate. (see Table 27 for the reaction mixture).

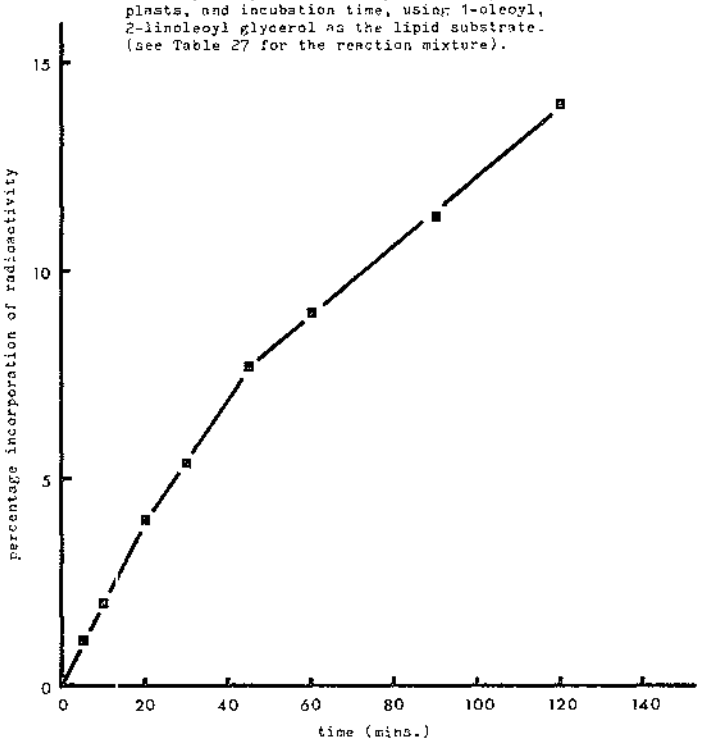
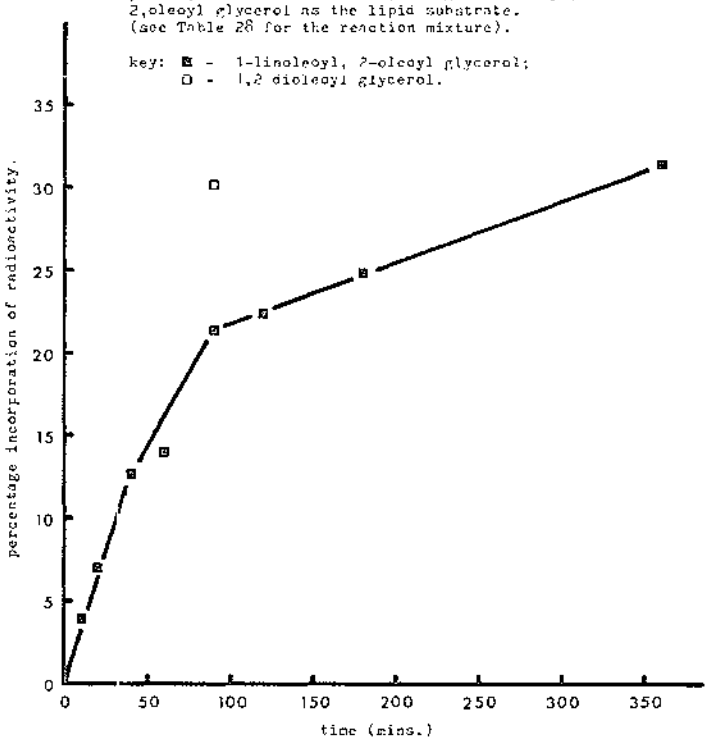


Figure 26

The relationship between the percentage incorporation of radioactivity, from UDP-D-galactose - ^{14}C into lipids by the acetone powder of spinach chloroplasts, and incubation time, using 1-linoleoyl, 2,oleoyl glycerol as the lipid substrate. (see Table 28 for the reaction mixture).

key: ■ - 1-linoleoyl, 2-oleoyl glycerol;
□ - 1,2 dioleoyl glycerol.



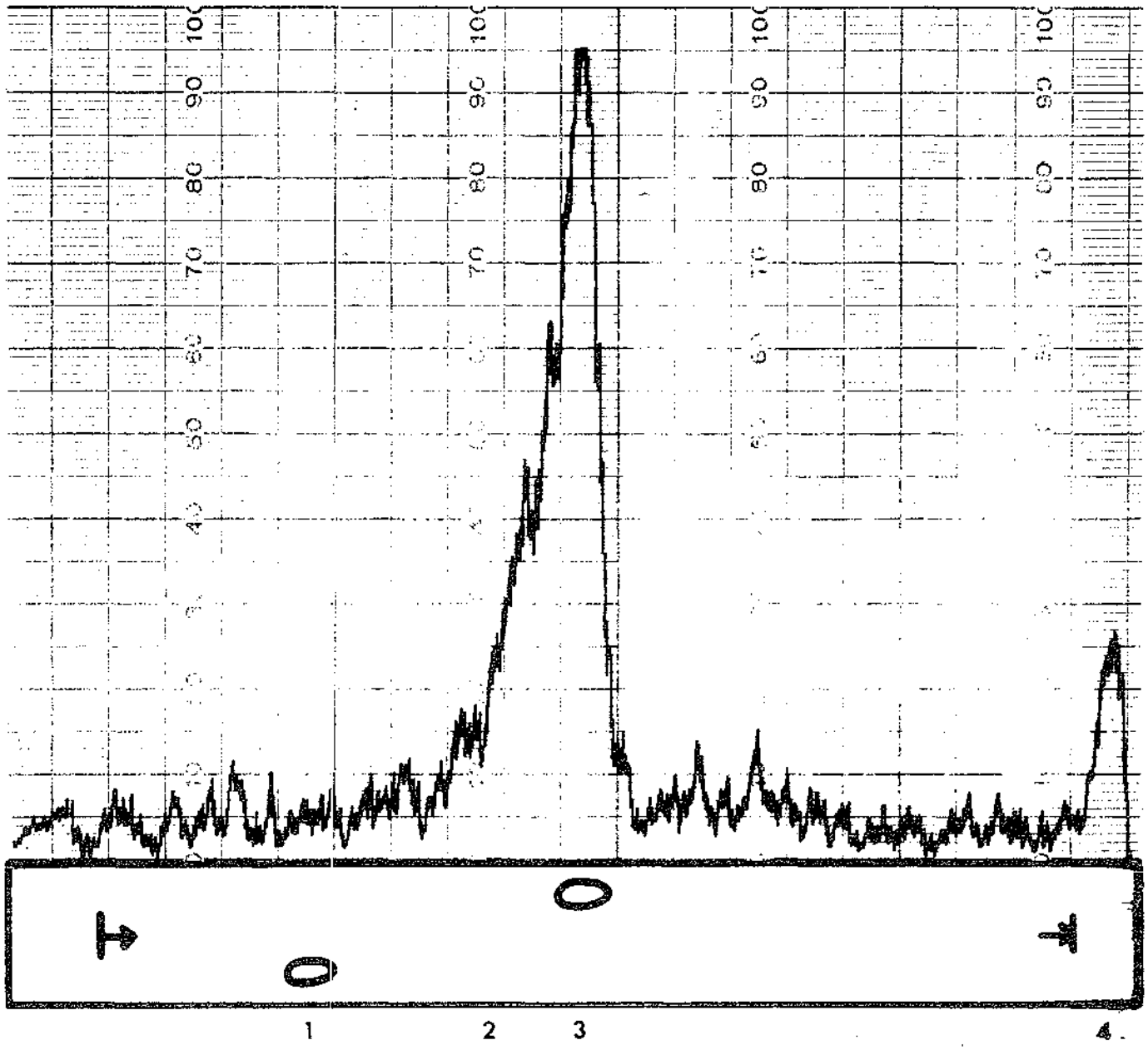


Figure 27

The radiochromatogram scan of the lipid extract obtained after incubating the acetone powder of spinach chloroplasts with UDP-D-galactose - ^{14}C and 1-linoleoyl, 2-oleoyl glycerol for 6 hrs. (see Table 28 for the reaction mixture).

key: 1, DGDG; 2, sterol glycoside (tentative); 3, MGDG; 4, marker.

(b) Using 1-linolecyl, 2-oleoyl glycerol as the lipid substrate.

Part (a) was repeated using spinach acetone powder preparation no.7 (120.0mg), and 1-linoleoyl,2-oleoyl glycerol (10.03mg). The range of the incubation times was extended to 6 hours. A control, containing spinach acetone powder preparation no.7, and diolein was also incubated.

Again, incorporation of radioactivity into lipids was still occurring when the experiment was terminated at 360 minutes (Table 28, Figure 26). Two near linear regions were again evident but on this occasion the break occurred around 90-100 mins. The high incorporation of radioactivity stimulated by diolein showed that this acetone powder preparation was particularly active in galactolipid biosynthesis. A radiochromatogram scan of the lipid extract from the reaction mixture incubated for 360 minutes (Figure 27) demonstrated the MGDG component contained most of the radioactivity with perhaps 20% in the sterol glycoside, which appeared as an unresolved peak behind the MGDG peak.

Table 28.

The effect of time of incubation on the incorporation of radioactivity from UDP-D-galactose - ^{14}C into lipids by a spinach acetone powder preparation, using 1-linoleoyl,2-oleoyl glycerol as the lipid substrate.

Reaction mixture:

0.7ml aliquot of a suspension consisting of 120.0mg spinach acetone powder preparation no.7, 10.03mg 1-linoleoyl,2-oleoyl glycerol and 6mls 0.1M Tris - HCl buffer, pH 7.4; 4 μl UDP-D-galactose - ^{14}C solution, containing 44,400 dpm. Incubation at 30°C under nitrogen. (The reaction mixture for the diolein control was a 0.7ml aliquot of a suspension of 20.0mg spinach acetone powder preparation no.7, 1.67mg diolein and 1ml 0.1M Tris - HCl buffer, pH 7.4. Incubation with 4 μl UDP-D-galactose - ^{14}C solution, containing 44,400 dpm, at 30°C for 90 minutes).

incubation time (minutes)	incorporation (dpm)	% incorporation
10	1,700	3.8
20	3,100	7.0
40	5,600	12.6
60	6,200	14.0
90	9,500	21.4
120	9,900	22.3
180	11,000	24.8
360	13,900	31.3
90†	13,400	30.2

† the diolein control

4.III.8 The effect of increasing amount of 1,2 (2,3)-diglycerides on the incorporation of radioactivity from UDP-D-galactose - ^{14}C into lipids by spinach acetone powder preparations.

To find the dependence of the incorporation of radioactivity from UDP-D-galactose - ^{14}C into lipids on various amounts of different diglycerides, increasing amounts of 1,2 (2,3)-diglycerides were incubated with spinach acetone powder preparations and UDP-D-galactose - ^{14}C. Spinach acetone powder preparation (20.0mg) was homogenized with each diglyceride substrate in acetone and 1ml of 0.1M Tris - HCl buffer, pH 7.4, and the suspension sonicated for 4 minutes. Aliquots of 0.7ml were incubated with 4μl of UDP-D-galactose - ^{14}C solution, containing 44,400 dpm, at 30°C for 90 minutes, under nitrogen. As in experiment 4.III.7(b), a control containing diolein as the lipid substrate, was also incubated. The results are given in Tables 29, 30 and 31, and Figure 28.

Table 29.

The effect of increasing amounts of 1-oleoyl,2-linoleoyl glycerol on the incorporation of radioactivity from UDP-D-galactose - ^{14}C into lipids by a spinach acetone powder preparation.

Reaction mixture:

0.7ml aliquot of a suspension consisting of 20.0mg spinach acetone powder preparation no.6, 0 - 5.37mg 1-oleoyl,2-linoleoyl glycerol (or 1.61mg diolein), and 1ml 0.1M Tris - HCl buffer, pH 7.4; 4 μl UDP-D-galactose - ^{14}C solution, containing 44,400 dpm. Incubation at 30°C for 90 minutes, under nitrogen.

amount of lipid † (mg.)	incorporation (dpm)	% incorporation	net % incorporation ▽
0	2,900	6.5	0
0.13	5,000	11.3	4.8
0.34	6,100	13.7	7.2
0.67	9,900	22.3	15.8
1.34	11,000	24.8	18.3
2.69	11,300	25.5	19.0
5.37	9,900	22.3	15.8
1.61*	11,000	24.8	18.3

† mg. of 1-oleoyl,2-linoleoyl glycerol added to 20.0mg spinach acetone powder preparation.

▽ net % incorporation due to added diglyceride.

* diolein.

Table 30.

The effect of increasing amounts of 1-linoleoyl,2-oleoyl glycerol on the incorporation of radioactivity from UDP-D-galactose - ^{14}C into lipids by a spinach acetone powder preparation.

Reaction mixture:

0.7ml aliquot of a suspension consisting of 20.0mg spinach acetone powder preparation no.6, 0 - 5.73mg 1-linoleoyl,2-oleoyl glycerol (or 1.61mg diolein), and 1ml 0.1M Tris - HCl buffer, pH 7.4;
4 μl UDP-D-galactose - ^{14}C solution, containing 44,400 dpm.
Incubation at 30°C for 90 minutes, under nitrogen.

amount of lipid† (mg.)	incorporation (dpm)	% incorporation	net % incorporation
0	2,900	6.5	0
0.14	4,700	10.6	4.1
0.36	6,600	14.9	8.4
0.72	9,600	21.6	15.1
1.43	7,800	17.6	11.1
2.87	6,600	14.9	8.4
5.73	5,400	12.2	5.7
1.61*	11,200	25.2	18.7

† amount of 1-linoleoyl,2-oleoyl glycerol added to 20.0mg spinach acetone powder preparation.

* diolein.

Table 31.

The effect of increasing amounts of 1-γ-linolenoyl,2-linoleoyl glycerol on the incorporation of radioactivity from UDP-D-galactose - ^{14}C into lipids by a spinach acetone powder preparation.

Reaction mixture:

0.7ml aliquot of a suspension consisting of 20.0mg spinach acetone powder preparation no.8, 0 - 5.44mg 1-γ-linolenoyl,2-linoleoyl glycerol (or 1.66mg diolein), and 1ml 0.1M Tris-HCl buffer, pH 7.4; 4 μl UDP-D-galactose - ^{14}C solution, containing 44,400 dpm.
Incubation at 30°C for 90 minutes, under nitrogen.

amount of lipid ‡ (mg.)	incorporation (dpm)	% incorporation	net % incorporation	adjusted ▽ % incorporation
0	1,800	4.1	0	0
0.16	3,800	8.6	4.5	5.2
0.39	5,400	12.2	8.1	9.4
0.78	6,000	13.5	9.4	10.9
1.32	4,700	10.6	6.5	7.6
2.72	4,500	10.1	6.0	7.0
5.44	4,700	10.6	6.5	7.6
1.66 *	8,900	20.0	15.9	18.5

‡ mg of 1-γ-linolenoyl,2-linoleoyl glycerol added to 20.0mg spinach acetone powder preparation.

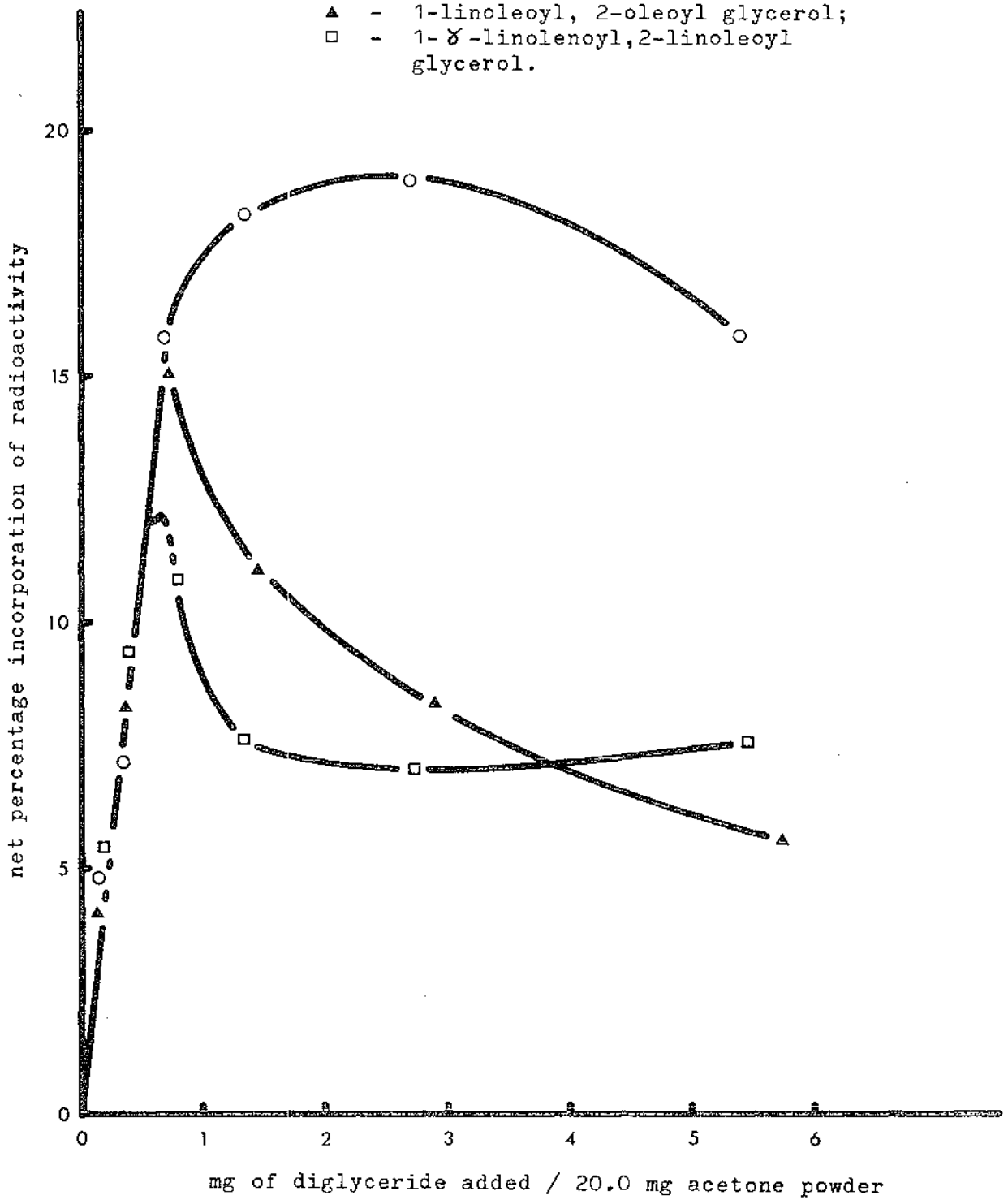
* diolein.

▽ net % incorporation adjusted to compensate for the lower activity of spinach acetone powder no.8 than the no.6 preparation, i.e. the values in the previous column were multiplied by 18.5/15.9 after comparison of the net % incorporations obtained for the diolein controls in Tables 29, 30 and 31. (It was assumed that 1.61 and 1.66mg diolein give equivalent incorporation: see Figure 19).

Figure 28

The net percentage incorporation of radioactivity from UDP-D-galactose - ^{14}C into lipids by the acetone powder of spinach chloroplasts incubated with UDP-D-galactose - ^{14}C and increasing amounts of diglyceride. (see Tables 29, 30 and 31 for the reaction mixtures).

key: ○ - 1-oleoyl, 2-linoleoyl glycerol;
▲ - 1-linoleoyl, 2-oleoyl glycerol;
□ - 1-γ-linolenoyl, 2-linoleoyl glycerol.



These data show that up to about 0.6mg of diglyceride added per 20.0 mg of acetone powder preparation, each diglyceride gave approximately equivalent stimulation of net incorporation of radioactivity from UDP-D-galactose - ^{14}C into lipids (The net percentage incorporations stimulated by 1-δ-linolenoyl,2-linoleoyl glycerol as the lipid substrate were adjusted because of the lower activity of spinach acetone powder preparation no.8; Table 31). The incorporation of radioactivity decreased sharply as the amount of diglyceride increased beyond about 0,8 and 0,6mg per 20,0mg of spinach acetone powder preparation when 1-linoleoyl,2-oleoyl glycerol and 1-δ-linolenoyl, 2-linoleoyl glycerol were used as the respective lipid substrates. Only very high amounts of 1-oleoyl,2-linoleoyl glycerol were inhibitory. Despite the fact that slightly different experimental conditions were used when diolein was incubated with spinach acetone powder preparation no.2 (experiment 4,III.2) the values obtained (Table 21) are consistent with the above conclusions.

The shape of the curve given with diolein as substrate (Figure 19) more closely follows that for 1-oleoyl,2-linoleoyl glycerol than the curves obtained with the other two diglycerides as substrates (Figure 28). The results in this experiment "explain" the different stimulations of incorporation by diglycerides seen in experiment 4.III.4, when an arbitrary amount of diglyceride was used.

A radiochromatogram scan of the lipid extract of the reaction mixture containing 5.44mg of 1-δ-linolenoyl,2-linoleoyl glycerol per 20.0mg of spinach acetone powder preparation (Table 31) confirmed the pattern of radioactivity observed previously i.e. mainly MGDG with some sterol glycoside.

4.III.9 The incubation of other lipid substrates with a spinach acetone powder preparation and UDP-D-galactose - ^{14}C.

The diglyceride and MGDG isolated from the acetone extracts of chloroplasts, as well as the phosphatidyl choline from egg yolk and diolein, were each homogenized with spinach acetone powder preparation no.9 (20.0mg), and incubated with 4 μl of UDP-D-galactose - ^{14}C solution, containing 44,400 dpm, at 30°C for 90 minutes, under nitrogen. The suspensions were sonicated for 4 minutes before the addition of the radioactive substrate. The results are given in Table 32.

Both the isolated diglyceride and MGDG obtained from the acetone extracts of chloroplasts stimulated significant incorporations of label. However, in the radiochromatogram scans of the lipid extracts (Figure 29), each extract exhibited a considerable amount of sterol glycoside, i.e. more than 50%, as well as the MGDG component. No radioactive DGDG was formed in either case.

At the level used, the egg yolk phosphatidyl choline inhibited incorporation when incubated alone with the acetone powder preparation or with diolein. One of the values for the incorporation of radioactivity obtained using diolein as the lipid substrate is regarded as anomalous. While the diglyceride from the acetone extract gave equivalent or better stimulation of incorporation as diolein, it gave more radioactive sterol glycoside and thus was not as good an acceptor for MGDG biosynthesis as diolein.

Table 32.

The incubation of other lipid substrates with a spinach acetone powder preparation and UDP-D-galactose - ^{14}C.

Reaction mixture:

0.7ml aliquot of a suspension consisting of 20.0mg spinach acetone powder preparation no.9, lipid, and 1ml 0.1M Tris - HCl buffer, pH 7.4; 4 μl UDP-D-galactose - ^{14}C solution, containing 44,400 dpm. Incubation at 30°C for 90 minutes, under nitrogen.

lipid	amount of lipid ⁺ (mg)		incorporation (dpm)	% incorporation
	diolein	other lipid		
none	-	-	1,700	3.8
MGDG	-	1.36	5,100	11.5
			5,700	12.8
diglyceride *	-	1.61	8,300	18.7
			10,500	23.7
PC from egg yolk	-	0.79 ▽	400	1.0
PC from egg yolk and diolein	0.81	0.79 ▽	1,600	3.6
diolein	0.81		8,500	19.1
diolein	1.61		7,700	17.3

⁺ mg of lipid added to 20.0mg spinach acetone powder preparation.

* the diglyceride isolated from the acetone extracts of chloroplasts.

▽ amount expressed as the weight of diglyceride expected on hydrolysis.

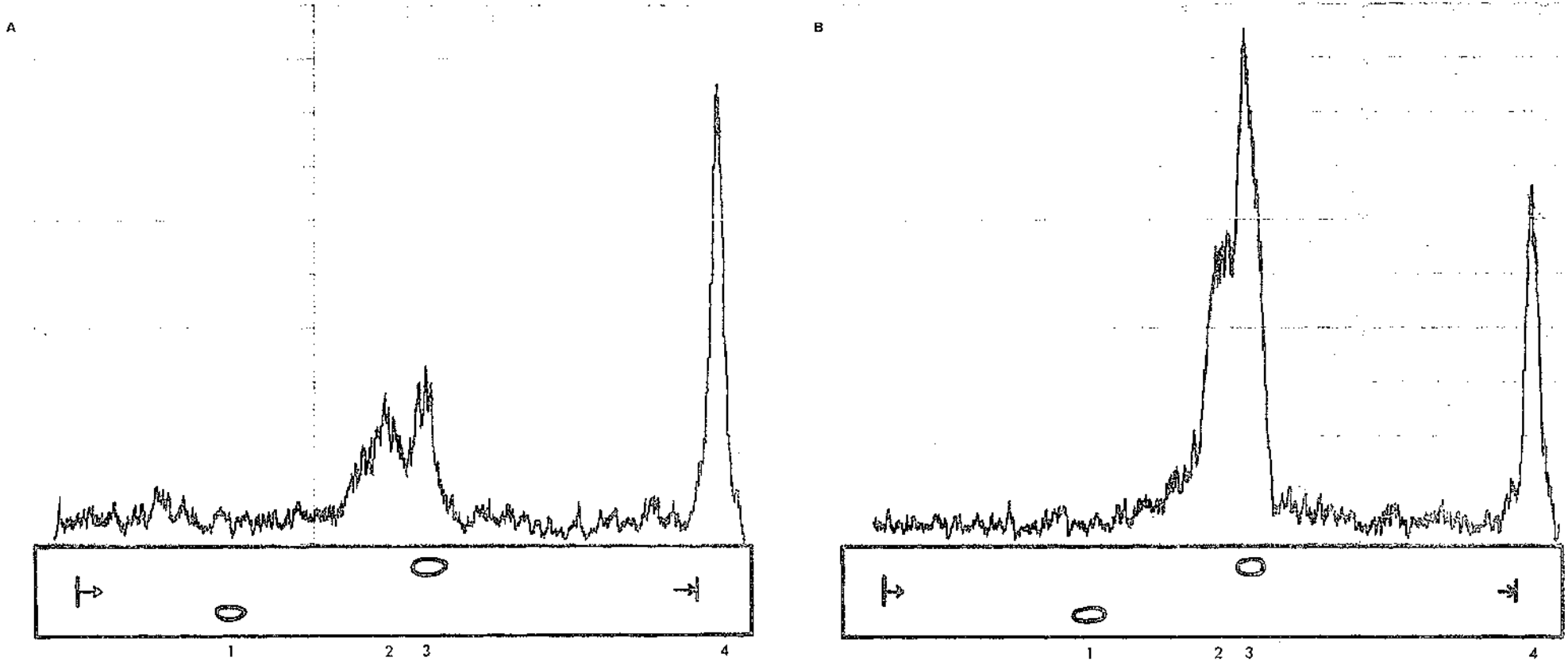


Figure 29

The radiochromatogram scans of the lipid extracts obtained after the incubation of the acetone powder of spinach chloroplasts with UDP-D-galactose - ^{14}C and the MGDG, A, or the diglyceride, B, which were isolated from the acetone extract of chloroplasts. (see Table 32 for the reaction mixture).

key: 1, DGDG; 2, sterol glycoside (tentative); 3, MGDG; 4, marker.

Chapter 5

DISCUSSION

Section 5.I : The incorporation of D-galactose -1- ^{14}C into the lipids of leaf slices.

The incorporation of radioactive galactose into the galactolipids of barley leaf slices was assumed to involve UDP-D-galactose. The incorporation noted may be interpreted to represent de novo synthesis of galactolipids and the higher amounts of radioactivity in the DGDG fraction were probably due to a proportion of the DGDG molecules possessing two radioactive galactosyl moieties. However, for de novo synthesis, the amount of radioactivity in the DGDG fraction might be expected to lag behind the amount in the MGDG fraction as shown when chloroplasts were incubated with UDP-D-galactose - ^{14}C (Ongun and Mudd, 1968). Thus, the amount of incorporation of radioactivity in the DGDG fraction, rather than showing de novo synthesis, may indicate a rapid equilibration of the α-linked galactosyl moiety of the pre-existing DGDG with the hexose pool. The maximum incorporation of label into DGDG occurred after 4 hours and the subsequent decrease with time (Figure 10) could indicate the transfer of the terminal galactose residue to a non-lipid acceptor or specific galactolipase activity for the DGDG. The role of DGDG as a galactose transport molecule may be correct whether or not the label in DGDG was due to exchange reactions or net de novo synthesis because, relative to the amount of labelled MGDG, the amount of labelled DGDG increased to a maximum at $3\frac{1}{2}$ hours then declined to the previous level at 7 hours (Figure 11). This might be evidence for the involvement of galactolipids in hexose metabolism as suggested by Ferrari and Benson (1961). The incorporation of radioactive glucose into the galactolipids of whole

runner bean leaves was shown by Kates (1960).

The ability of leaf slices to incorporate galactose into galactolipids increased with the age of the tissue up to the sixth day after planting. Decreased incorporation after this could be attributable to the condition of the seedlings since at this stage they had outgrown their container. Whether the higher incorporation obtained in experiment 4.I.2 than in experiment 4.I.1 was due to incubation in light was not investigated further. However, galactolipid synthesis in leaf slices is unlikely to be sensitive to light because they are incapable of net chlorophyll formation (Appelqvist et al, 1968). Thus the possibility of a cooperative template relationship suggested by Patton (1968) can not occur. Still, de novo synthesis of galactosyl diglycerides is conceivable in leaf slices because their synthesis is not dependent on chlorophyll formation as evidenced by their concentration in etiolated tissue (Gray, Rumsby and Hawke, 1967).

Fescue leaf slices also had the ability to incorporate radioactive galactose into MGDG and DGDG and the necessity for cysteine in the reaction mixture indicated galactolipase activity in the tissue (Helmsing, 1969). The latter activity is probably associated with the extra-chloroplast fraction since fescue leaf chloroplasts could incorporate galactose for UDP-D-galactose into MGDG, though not into DGDG. Incorporation of galactose into galactolipids by barley tissue could be obtained without the addition of cysteine but the effect of cysteine on this incorporation was not investigated.

Section 5.II : The incubation of chloroplasts with UDP-D-galactose - ^{14}C.

The crude chloroplast pellets of different plant species exhibited different activities in incorporating label from UDP-D-galactose - ^{14}C into lipid compounds. Spinach, fescue and ryegrass leaves yielded the most active preparations. The lower activity of the other preparations, viz. barley, Yorkshire fog and phalaris, may have been due to a lower number of isolatable intact chloroplasts, lower intrinsic enzyme activity, less accessibility of the radioactive substrate to the enzyme, diversion of UDP-D-galactose - ^{14}C to the other reactions, or a combination of these.

The inability to prepare active plastid preparations from barley seedlings could have resulted from galactolipase activity in the leaf homogenate. Appelqvist, Stumpf and von Wettstein (1968) found that barley leaf homogenates at pH 6 contained galactolipase activity and gave a low yield of intact chloroplasts. The use of 0.5% sucrose buffer, pH 8, gave the highest number of intact chloroplasts. In the present work, plastid preparations were not active in galactolipid synthesis when isolated from barley seedlings between 5 and 10 days after planting or from seedlings subjected to different light treatments. The fragility of the plastid membranes, galactolipase activity and the starch content of the plastids probably all weighed against the isolation of intact plastids.

The incorporated label in the chloroplast lipids of fescue and spinach was mainly confined to MGDG and a compound tentatively identified as acyl MGDG. This compound contained galactose and had a greater chromatographic mobility than MGDG in toluene - ethyl acetate - 95% ethanol, 2:1:1 (v/v). Acyl derivatives of galactolipids were also found in spinach chloroplasts isolated in sucrose media by Wintermans et al (1969). At no time did more than a trace of label appear in DGDG. This could also be attributed to the transacylase activity because acyl MGDG is formed primarily at the expense of DGDG (Heinz, 1967b).

Using similar methods, previous workers (Neufeld and Hall, 1964; Ongun and Mudd, 1968) obtained incorporation of radioactivity from UDP-D-galactose - ^{14}C into DGDG and TGDG as well as into MGDG in chloroplasts. They did not note any formation of acyl MGDG although the radioautograms of Ongun and Mudd (1968) showed traces of a compound with greater chromatographic mobility than MGDG. The synthesis of DGDG and TGDG, using spinach chloroplasts, could explain why the incorporations of radioacttivity from UDP-D-galactose - ^{14}C were much higher (Ongun and Mudd, 1968) than in the present experiments.

Different treatments, i.e. alternative isolation procedure, sonication, deoxycholate addition, of the chloroplasts failed to stimulate any incorporation into DGDG even though the percentage incorporation into lipids increased after the former two treatments. The chloroplast preparations isolated by the more vigorous isolation procedure of grinding the leaf tissue in a sucrose buffer with sand showed increased incorporation and thus indicated that the accessibility of the enzyme for the UDP-D-galactose - ^{14}C might be limiting for chloroplasts prepared using the Spencer-Wildman technique. The sonication of chloroplast suspensions of fescue, prepared by this latter technique, produced a decreased synthesis of acyl MGDG but again no label appeared in the DGDG fraction even though the percentage incorporation was increased. The transacylase activity was not labile to sonication in spinach chloroplasts. Acylated galactolipids are artifacts of the chloroplast isolation technique rather than normal chloroplast constituents and the enzyme(s) responsible for the transacylase activity have not been identified (Heinz, 1967b; Wintermans et al, 1969).

Fescue chloroplasts, isolated using the Spencer-Wildman technique, exhibited a linear increase in incorporation of radioactivity, when incubated with UDP-D-galactose - ^{14}C, with increasing chloroplast

concentration up to a concentration of 1.3mg chlorophyll per ml. but higher concentrations of chloroplasts gave lower incorporations. Ongun and Mudd (1968) obtained a linear relationship between incorporation of label and chloroplast concentration for spinach chloroplasts, isolated in sucrose buffer, to a chloroplast concentration of 0.65mg chlorophyll per ml. A non-linearity was also observed by Neufeld and Hall (1964) at the higher chloroplast concentrations but no explanation has been offered.

Section 5.III : The incubation of chloroplast enzyme preparations.

Active acetone powder and freeze-dried preparations could be derived from spinach, but not from fescue, chloroplasts. The lability of the fescue chloroplast enzymes was not studied further and the large quantity of tissue required for these enzyme preparations precluded the use of the Spencer-Wildman technique for the isolation of chloroplasts for such a study.

The addition of lipid in organic solvent was chosen as the best method of achieving intimate association between the substrate and the acetone powder containing the enzyme system. Since 88mg of acetone-soluble lipid was extracted in the preparation of 321mg of acetone powder (i.e. 5.5mg lipid per 20mg acetone powder), some hydrophobic interaction between the added lipid and the acetone powder would be expected (Ji and Benson, 1968). The protection of the 1,2-diglycerides from isomerization to 1,3-diglycerides by acetone powder during sonication is evidence of this. Sonication of the acetone powder - lipid suspensions gave consistently good incorporation of radioactivity from UDP-D-galactose - ^{14}C into lipids. No harmful effects of sonication were noticed provided appropriate precautions were taken, i.e. the suspension sonicated under nitrogen at $0^{o}C$.

The conclusion drawn from the incubations of acetone powder preparations with 1,2-diglycerides and UDP-D-galactose - ^{14}C was that, up to a level of approximately 0.6mg diglyceride per 20mg acetone powder preparation, the enzyme responsible for MGDG synthesis exhibited no specificity for the diglyceride substrate. The sharp decrease in incorporation given by some 1,2-diglycerides at levels above 0.6mg diglyceride per 20mg acetone powder might be interpreted as substrate inhibition in which case this would indicate a greater affinity of the

enzymes for the more unsaturated diglycerides. However, this conclusion can not be drawn with certainty because at these diglyceride levels the incorporation of radioactivity into the sterol glycoside is greater for the more unsaturated diglycerides than for the 1-oleoyl diglycerides. The increased incorporation may be indicative of the role of polyunsaturated fatty acids in the conformation of chloroplast enzymes. The change in stimulation by the more unsaturated diglycerides was not because of the sonication step in the reaction procedure since 1-oleoyl,2-linoleoyl glycerol and 1-linoleoyl, 2-oleoyl glycerol have the same fatty acid composition but gave different stimulations.

The unfractionated acetone extract and the diglyceride isolated from the acetone extract gave equivalent or slightly greater stimulations than diolein. Whether the isolated 1,2-diglyceride, which contained over 40% linolenic acid, reflected the composition of the diglyceride pool in the chloroplasts, if this pool exists, or whether the diglyceride was primarily formed by degradation of some chloroplast lipids upon isolation is uncertain. A comparison of the Michaelis constants of the enzyme for different diglyceride substrates is not possible because the concentration of the diglyceride in aqueous solution cannot be determined.

It should be noted that the linolenoyl residue in the synthetic 1,2(2,3)-diglyceride containing five double bonds was the 6,9,12-isomer of linolenic acid whereas the 9,12,15-isomer is the one commonly found in plant tissues. Since these isomers possess different molecular conformations, the utilization of 1-δ-linolenoyl, 2-linoleoyl glycerol to the same extent as other diglycerides tested would emphasize the non-specificity of the UDP-D-galactose : 1,2-di-O-acyl-sn -glycerol galactosyltransferase for acyl groups in the 1,2-diglycerides.

These conclusions appear to contradict those of Mudd, van Vliet and van Deenen (1969) who found that the greatest stimulation of monogalacto-

lipid synthesis occurred with the more highly unsaturated diglyceride species. Since their diglycerides were derived from spinach and egg phospholipids, the diglycerides were wholly 1,2-isomers i.e. the 2,3-isomers were not present. These workers added the diglycerides to the acet ne powder using a different method, i.e. as an emulsion made by shaking the lipid in Tris buffer which was 0.03% with respect to Tween 20, to that employed in the present study or by Ongun and Mudd (1968). Aliquots of the stock lipid-water suspension were incubated with the acetone powder preparation, at a level of 0 to 0.6mg diglyceride/10mg acetone powder preparation, for 20 minutes at 37°C. Thus these authors chose a narrower range of diglyceride:acetone powder ratios, a shorter incubation time and a higher incubation temperature than in the present study. Mudd, van Vliet and van Deenen (1969) compared their results for monogalactolipid synthesis with those for phosphatidyl choline synthesis by rat liver particles using the same method of lipid addition (Mudd, van Golde and van Deenen, 1969). Whereas the diglycerides, derived from egg phosphatidyl choline, of increasing unsaturation produced, in general, increasing stimulations of monogalactolipid synthesis, they gave equivalent stimulation of phosphatidyl choline synthesis. However, the diglycerides in the two series of experiments were not shown to be of indentical composition. From this it might be argued that the solubility of the diglyceride is not a limiting factor and that the technique of adding the diglyceride in aqueous solution allows determination of the enzyme specificity.

Both the above techniques of adding lipid substrates have been employed by previous authors in studying the biosynthesis of lipids in bacteria and animal tissue. For monomannosyl dilgyceride synthesis in Micrococcus lysodeikticus, Lennarz and Talamo (1966) prepared a suspension of 1,2-diglyceride in water, at a concentration of 10 μmoles/ml, by sonic dispersion (performed under nitrogen in the case of diglycerides containing

polyunsaturated fatty acids). In this case, the best substrates were the 1,2-diglycerides, containing branched chain fatty acids, isolated from the bacterium or from Bacillus megaterium. Pieringer (1968) preferred to saturate the dry enzyme preparation with an aliquot of benzene solution containing the diglyceride at room temperature, evaporate the benzene and incubate the enzyme in buffer, when he examined the diglyceride requirements for the synthesis of glucolipids of Streptococcus faecalis. Here distearin and diolein were the best precursors of the monoglucosyl diglyceride while dilinolein was the best for the diglucosyl dilgyceride and a third compound which was apparently derived from the other glucolipids. The shorter chain length and the greater unsaturation of the fatty acids of the diglycerides appeared to favour the conversion of monoglucosyl diglyceride to the other two. This change in activity seemed to be directly related to the relative solubility of the diglyceride substrates and thus the greater activity of dicaprin and dilinolein was probably attributable to this physicochemical property. In contrast, the synthesis of monoglucosyl diglyceride from diglyceride appeared to increase with chain length and with degree of saturation of the diglyceride, indicating that the initial conversion of diglyceride to monoglucolipid was not nearly as sensitive to solubility of the lipid as subsequent steps. Pieringer (1968) concluded that the greater amounts of measurable monoglucolipid synthesized from the more saturated diglycerides with the longer chain lengths may have been caused by an inability of the relatively more insoluble monogluco-diglyceride product to be converted to the higher glycolipid, or possibly by true enzyme specificity, or both. (It was also shown that 1,2 dipalmitoyl-sn-glycerol but not 2,3 dipalmitoyl-sn-glycerol stimulated the synthesis of monoglucolipid).

Microsomal enzyme preparations of brain tissue from 13- to 20-day old rats catalysed the synthesis of MGDG from UDP-D-galactose and

1,2-diglyceride (Wenger, Petitpas and Pieringer, 1968). Using the same technique for adding the diglyceride as described by Pieringer (1968), they found that the diglycerides having long chain saturated fatty acid constituents were preferred. It was noted also that the incorporation of galactose from UDP-D-galactose was linear up to at least two hours of incubation time. For the spinach acetone powder system in the present work the incorporation was almost linear up to about 40 or 90 minutes incubation, depending on the substrate used, and then a new linear rate was established. The same phenomenon was observed by McCaman and Cook (1966) who studied the synthesis of phosphatidyl choline in brain tissue. The synthesis of phosphatidyl choline occurred at a linear rate with time during the first 10 minutes but between 15 to 60 minutes a new rate (70% of that found during the initial 10 minutes) appeared. The results of Mudd, van Golde and van Deenen (1968) also showed a change in the incorporation rate after 20 minutes. These rate changes serve to emphasize the importance of the stability of lipid emulsions and, hence, of the relative lipid solubility and accessibility to the enzyme, to the observed synthesis of lipids.

The techniques of McCaman and Cook (1966) of adding the dilgyceride to the enzyme was as an aqueous suspension in the presence of Tween 20 (0.03%) and BSA (0.05%). The most active substrates were those diglycerides with a high proportion of unsaturated fatty acids, though the results are difficult to interpret because inhibition was observed at higher concentrations of diglycerides. The addition of detergents to the reaction mixture to increase the miscibility of diglycerides was only of limited success (McCaman and Cook, 1966). Tween 20, Cutscum and Triton X-100 were more than 90% inhibitory at concentrations in excess of 0.1% (w/v). An increase in the concentration of Tween 20 from 0.02% to 0.03% (w/v) reduced the apparent activity of the phosphocholine-glyceride transferase

by 28%. In the study of the biosynthesis of glucolipids, none of the 18 adjuvants (including neutral, anionic and cationic detergents, and organic solvents) tried, aided in the stimulation of incorporation of radioactivity from UDP-glucose - ^{14}C into glycolipid (Pieringer, 1968). On the other hand, Lennarz and Talamo (1966) found that the specificity for an adjuvant of a particular type was rather high and that a fatty acid salt was required.

In this regard, adding phospholipids with the diglyceride seemed relevant to the present studies. The incubation of phospholipid alone with the acetone powder preparation and UDP-D-galactose - ^{14}C did not yield significant MGDG synthesis although with egg phosphatidyl ethanolamine the result was suggestive that phosphatidyl ethanolamine acted as a diglyceride source. When phosphatidyl choline (1.60mg) was incubated with diolein (1.61mg) acetone powder (20.0mg) and UDP-D-galactose - ^{14}C, the net incorporation of radioactivity into lipid was more than occurred in the reaction mixture having diolein as the only added lipid substrate. However, when a lower level of each lipid was used no incorporation whatsoever was stimulated. Phosphatidyl ethanolamine also caused more net incorporation when incubated with diolein than when diolein was the only added lipid in the reaction mixture but the incorporation of label decreased when diolein was incubated with a large amount of crude egg phospholipid. Thus in some cases the added phospholipid may have had a beneficial effect on the incorporation of radioactivity stimulated by diolein because of its detergent action. This effect was not consistent but might still indicate that the accessibility of the diglyceride to the enzyme is a limiting factor in MGDG synthesis.

The results of P.G. Roughan (personal communication) suggested that phospholipids were intimately associated with the unsaturation of fatty acids and thus be a source of diglyceride moieties in the biosynthesis

of other complex lipids. This possibility could not be positively confirmed or denied with the present experiments.

The effect of adjuvants indicate that the interaction between lipid and the enzyme in aqueous systems can be influenced by factors other than substrate - enzyme affinity. Thus the results of experiments in which the lipid was added in aqueous suspension must be interpreted with caution. The addition of the lipid to the enzyme preparation in organic solvent, provided the solvent did not in some way harmfully alter the enzyme system, would be expected to allow maximum interaction between the two and the resultant enzyme activity be affected less by, for example, the relative solubilities of the lipid substrates. Hence the results obtained using the latter procedure are probably more meaningful in determining enzyme specificity. However, the physicochemical properties of diglycerides in aqueous systems need quantitative analysis.

The characteristic fatty acid composition of the MGDG fraction in plants could be obtained by

1. a specific selection of the appropriate unsaturated diglycerides from the diglyceride pool by the UDP-D-galactose : 1,2 di-O-acyl-sn-glycerol galactosyltransferase and some adjustments to the fatty acid composition of the MGDG effected by deacylation-reacylation reactions, or
2. a nonspecific selection of diglycerides by the galactosyltransferase and extensive desaturation of the partially unsaturated MGDG molecules, possibly involving deacylation-reacylation reactions.

The conclusions drawn from the present work are more consistent with the second possibility. Furthermore, Nichols, James and Breuer (1967) showed that in Chlorella vulgaris, MGDG had a high turnover of the fatty acids intermediate in the sequence leading to linolenic acid and they argued that MGDG, and some other lipids, had an active metabolic role in fatty

acid metabolism. The MGDG fractions of photoheterotrophic Chlorella vulgaris, separated according to the total number of double bonds per molecule, had constituent fatty acids which differed by a maximum of one double bond (Nichols and Moorhouse, 1969). Cell-free extracts of Euglena gracilis preferentially transfer stearoyl- and oleoyl- groups from ACP-thioesters into MGDG whereas the fatty acyl groups of CoA-thioesters were incorporated into MGDG and into phospholipids (Renkonen and Bloch, 1969). Partial desaturation of the acyl moieties to more unsaturated residues was concomitant with the transfer. The chloroplasts of spinach and lettuce contain an ACP-dependent fatty acid synthetase which produces stearic acid while isolated intact chloroplasts yield mainly oleic and palmitic acids from acetate (Stumpf, Brooks, Galliard, Hawke and Simoni, 1967). Although oleic acid was shown to be a precursor of linolenic acid in leaf tissue (Harris and James, 1965), no in vitro system has been shown to effect this desaturation. In the apparent desaturation of galactosyl diglycerides, in algae and in higher plants, on exposure to light (Nichols, Stubbs and James, 1967) it is not known whether the change in fatty acid composition was due to the desaturation of pre-existing galactolipids, the de novo synthesis of polyunsaturated galactolipids, or both. The effect of increasing light intensities on the lipids, specifically MGDG, of Euglena gracilis. (Constantopoulos and Bloch, 1967b), would support the alternative that pre-existing galactolipids were desaturated. These considerations would be consistent with the formation of MGDG from diglycerides of low unsaturation and the subsequent desaturation of the fatty acyl residues to trienoic acids, assuming the metabolic pathways of green algae and higher plants are similar. Moreover, a tight coupling of oleate desaturation with phosphatidyl choline synthesis was shown in Chlorella vulgaris (Gurr, Robinson and James, 1969). The higher degree of unsaturation of MGDG than DGDG

and the characteristic hexadecatrienoic acid content of spinach MGDG would also be more easily explained by fatty acid changes after the formation of MGDG and DGDG. It is pertinent that dipalmitin did not stimulate MGDG synthesis by a spinach acetone powder preparation (Ongun and Mudd, 1968), but that diglycerides containing 40-50% palmitic acid were able to be utilized (Mudd, van Vliet and van Deenen, 1969). The MGDG from spinach chloroplasts contains 30% of its total fatty acids as hexadecatrienoic acid (Allen et al, 1964). This might also indicate that fatty acid chain length or some degree of desaturation of the diglyceride is required by the galactosyltransferase.

As in the present experiments, Mudd, van Vliet and van Deenen (1969) were unable to obtain DGDG synthesis by incubating diglycerides with acetone powder preparations of chloroplasts. They did not note any significant incorporation of radioactivity into sterol glycosides. In this connection, Renkonen and Bloch (1969) were also unable to stimulate DGDG synthesis using cell-free extracts from Euglena gracilis. The inability to obtain DGDG synthesis with these enzyme preparations may be caused by a translocation barrier since MGDG and DGDG are apparently synthesized by separate enzymes, the one synthesizing MGDG being more tightly membrane-bound (Ongun and Mudd, 1968). All the chloroplast proteins are likely to be present in the acetone powder preparation, whether denatured or not, because Bottrill and Possingham (1969) found that 80% aqueous acetone solutions precipitate all the nitrogen of concentrated chloroplast suspensions. Two enzymes were dissociable and shown to be active for monomannosyl diglyceride and dimannosyl diglyceride synthesis in Micrococcus lysodeikticus (Lennarz and Talamo, 1966). The enzyme responsible for monomannosyl diglyceride synthesis was associated with a particulate fraction whereas the one forming dimannosyl diglyceride was present in the soluble cell fraction. The results of Pieringer (1968)

for the synthesis of the glucolipids of Streptococcus faecalis are also consistent with this two-enzyme scheme for glycolipid synthesis.

When Ongun and Mudd (1968) fractionated the acetone extract of chloroplasts and incubated a fraction containing all the MGDG, 90% of the DGDG and a small amount of the phosphatidyl glycerol of the total extract, with the spinach chloroplast acetone powder preparation and UDP-D-galactose - ^{14}C, a considerable amount of radioactivity appeared in MGDG and DGDG as well as some into TGDG. The incubation of MGDG with acetone powder preparation and UDP-D-galactose - ^{14}C (experiment 4.III.9) yielded radioactive MGDG and sterol glycoside. The incorporation of label into the galactolipids was probably occurring by exchange reactions and would imply that the enzyme responsible for DGDG synthesis was not necessarily inactivated during the preparation of the acetone powder in the case of Ongun and Mudd (1968). It is unlikely that the MGDG was degalactosylated and the diglyceride released since the normal degradative pathway involves the galactolipase. The high incorporation of radioactivity into the sterol glycoside may be indicative of the structural importance of MGDG for optimal activity of the enzyme responsible for sterol glycoside synthesis.

SUMMARY

1. The incubation of barley leaf slices with D-galactose-1-^{14}C gave up to 4.6% incorporation of radioactivity into the galactosyl residue(s) of MGDG and DGDG. The DGDG fraction generally contained more of the radioactivity than the MGDG fraction and the ratio of MGDG - ^{14}C to DGDG - ^{14}C decreased to a minimum and then increased to its former value with incubation time. The percentage incorporation was maximum in the leaf slices of barley tissue harvested 6 days after planting.

2. The incorporation of radioactivity from UDP-D-galactose - ^{14}C into the lipids of crude chloroplast fractions, isolated using the Spencer-Wildman technique from fescue (Festuca elation), ryegrass (Lolium perenne) and spinach (Spinacia oleracea) leaves, was shown to occur mainly into MGDG and a compound tentatively identified as an acylated MGDG. The plastid preparations from barley seedlings did not incorporate label into the lipid fraction. The adoption of different experimental techniques e.g. alternative chloroplast isolation procedure, sonication or addition of deoxycholate, failed to stimulate the incorporation of radioactivity into DGDG when fescue and spinach chloroplasts were incubated with UDP-D-galactose - ^{14}C even though the percentage incorporation increased when the former two treatments were used. The fescue chloroplasts, isolated by the Spencer-Wildman technique, showed a linear increase in incorporation with increasing chloroplast concentration except at chloroplast concentrations greater than 1.3mg chlorophyll/ml.

3. The synthesis of MGDG was shown to be stimulated by synthetic 1,2(2,3)-diglycerides, and the diglyceride isolated from the acetone extract of chloroplasts, when they were incubated with an acetone powder preparation of spinach chloroplasts and UDP-D-galactose - ^{14}C.

Depending on the activity of the acetone powder preparation, and incubation time, percentage incorporations of up to 31% were achieved. Increasing the weight ratio of 1,2(2,3)-diglyceride to acetone powder preparation gave an increasing incorporation of radioactivity with the diglycerides giving equivalent stimulation up to about 0.6mg diglyceride/20.0mg acetone powder preparation. Beyond this level of addition, 1-oleoyl diglycerides stimulated further incorporation whereas other diglycerides showed decreased stimulation. The incorporation into a compound, tentatively identified as sterol glycoside increased using these latter diglycerides.

4. These results are consistent with the enzyme, UDP-D-galactose : 1,2 - di -O- acyl-sn-glycerol galactosyltransferase, having no specificity for diglycerides of a particular fatty acid composition. Thus the role of MGDG as an active metabolite in the desaturation of fatty acids, suggested by Nichols, James and Breuer (1967), is supported. The results are, however, at variance with those of Mudd, van Vliet and van Deenen (1969) who found that diglycerides of increasing unsaturation gave, in general, increasing incorporation of galactose from UDP-D-galactose into MGDG. The possible reasons for this difference are discussed.

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