VARIATIONS IN LIPID COMPOSITION OF PERENNIAL RYEGRASS (*LOLIUM PERENNE*) AND BARLEY (*HORDEUM VULGARE*) WITH SPECIAL REFERENCE TO GALACTOLIPIDS

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

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A C K N O W L E D G E M E N T S

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I N T R O D U C T I O N

In New Zealand, dairy cows are fed mainly on pasture *in situ*. This is in contrast to feeding practices in other countries where lactating cows are nor ally fed diets containing appreciable proportions of food products, other than fresh pasture. Although lipids form only 4 - 6% of the dry weight of leaf tissue (Hilditch and Williams, 1965) it has been estimated that a cow by eating 9 Kg. of dry matter of grass per day may consume up to 700 g. of lipid (Hawke, 1963). The nature of the dietary lipid and its modification in the rumen (Reiser and Reddy, 1956) is of particular interest as it may influence the composition of the depot and milk fats of ruminants. Only a few investigators have studied possible relationships between the composition of the milk fat and dietary lipids when ruminants are fed entirely on pasture species. (McDowall and McGillivray, 1963; Hawke, 1963). The latter worker demonstrated that young succulent short rotation ryegrass (*Lolium multiflorum* x *Lolium perenne*) contained a higher content of lipid (% of dry wt.) than mature grass which contained appreciable stalk material. Furthermore the lipid from the new growth contained higher proportions of linolenic acid and lower proportions of linoleic and palmitic acids. When pairs of monozygous twin cows were grazed on these two types of pasture, the cows feed on the young grass had higher levels of unsaturated fatty acids in the milk fat. This was thought to be related to higher levels of unsaturated fatty acids in the young grass and to the degree of hydrogenation of the unsaturated fatty acid components of the dietary lipid in the rumen.

A large proportion of the dietary lipid of ruminants fed on pasture would consist of galactosyl glycerides since these components are the major lipids of photosynthetic tissue (Weenink, 1961; Sastry and Kates, 1964). Consequently the comparative levels of galactosyl glycerides and changes in their fatty acid composition in L. perenne leaf tissue of varying age has been investigated. The present investigation also includes a study of the effect of the light environment on the levels and composition of lipid, especially the galactolipids, and on the biosynthesis of fatty acids.

REVIEW OF LITERATURE

1. Lipids of Photosynthetic Tissue

Lipids are a group of chemical compounds which are insoluble in water but soluble in fat solvents such as chloroform, diethyl ether and benzene.

They can be conveniently subdivided into three classes depending on their chemical and physical properties. These classes consist of:-

(1) Simple or Neutral Lipids

(2) Compound Lipids

(1) Simple or Neutral Lipids

These lipids contain carbon, hydrogen and oxygen, and in photosynthetic tissues consist of hydrocarbons, waxes, alcohols, quinones, sterols, sterol esters, and glycerides.

Hydrocarbons have been isolated from ryegrass by Pollard et al., (1931), and from cocksfoot (Dactylis glomerata) by Waldron et al., (1961) who has demonstrated the presence of normal hydrocarbons ranging from C_{27} to C_{33} in chain length. Weenink (1962) has also demonstrated that 0.38% of the acetone soluble lipids of red clover (Trifolium pratense) consisted of hydrocarbons from C_{15} to C_{25} in chain length with the latter compound predominating.

Waxes are esters of long chain fatty acids combined with high molecular weight alcohols and have been identified in cocksfoot (Dactylis glomerata) and perennial rye (Lolium perenne) by Pollard et al., (1931), and in spinach (Spinacia oleracea) by Zill and Harmon (1962).

Free alcohols such as ceryl alcohol (n-hexacosanol) have been identified in spinach by Zill and Harmon (1962) who consider it may possibly be a breakdown product from waxes.

The same workers have also identified a quinone from spinach (Spinacia oleracea) chloroplast lipids and they consider it may be associated with an electron transport system in the chloroplast.

Glycerides are esters of long chain fatty acids (palmitic, linoleic, linolenic acid, etc.) with glycerol. Mono, di and triglyceride have one two or three fatty acids esterified to the hydroxyl group of glycerol respectively. Until recently triglycerides were thought to constitute the major proportion of the acetone soluble fraction of plant lipids but Weenink (1962) demonstrated that the acetone soluble lipids of red clover contained about 60% galactosyl glycerides. He has since demonstrated that triglycerides constitute only 0.53% of the total acetone soluble lipids (Weenink, 1962).

Diglycerides were also identified in red clover (Trifolium pratense) by Weenink (1962) but their existence has been questioned as they could be formed by the enzymic breakdown of phospholipids (Kates, 1953) or be present as intermediates in phospolipid synthesis (Kennedy, 1956).

Sterols which have the fundamental carbon structure of cyclopentanophenanthrene are present only in trace amounts in plants. Stigmasterol and β sitosterol have been identified in pasture grasses by Pollard et al., (1936) and Zill and Harmon (1962) have identified β sitosterol, stigmasterol and spinasterol in spinach chloroplast lipids.

Sterol esters have a fatty acid esterified to the hydroxyl group on carbon three of the sterol ring structure and have been identified in red clover (Trifolium pratense) by Weenink (1962). An ester of β sitosterol appeared to be the main component constituting 0.57% of the total acetone soluble lipid.

(2) Complex Lipids

Complex lipids may in addition to carbon, hydrogen and oxygen, contain other elements such as nitrogen, phosphorus, sulphur or a carbohydrate moiety.

The complex lipid group may be subdivided into:-

(a) Phospholipids - Lipids which contain phosphorus

(b) Glycolipids - Lipids which contain a sugar residue but have no phosphorus.

(c) Sulpholipids - Lipids which contain a sulphuric acid residue.

(a) Phospholipids

Phosphatidic acid is the simplest phosphatide and its presence in photosynthetic tissue was first demonstrated by Chibnall and Channon (1927). These workers found high concentrations of this lipid in cabbage leaves but subsequent investigations of Kates (1956, 1957) demonstrated the presence of this lipid was probably due to the action of phospholipase D on other glycerol phosphatides. Traces of phosphatidic acid have been found in cabbage (Brassica oleracea) leaves (Benson and Maruo, 1958) and in runner bean (Phaseolus multiflorus) leaves (Kates, 1960) but its presence in Scendesmus, sweet clover (Meliotus alba) and barley (Hordeum vulgare) was not demonstrated (Benson and Maruo, 1958).

Diphosphatidyl glycerol was first observed by Benson and Strickland (1961) in Chlorella, Scendesmus and Rhodospirillum rubrum and higher plant tissue. The work suggests that this lipid is present only in low concentrations.

Reports of phosphatidyl serine in plant tissue are not numerous but it has been identified by Benson and Maruo (1958), Wheldon (1960) and Lepage (1964) in photosynthetic tissue.

The major phosphatides of photosynthetic tissue; phosphatidyl choline, phosphatidyl glycerol, phosphatidyl ethanolamine and phosphatidyl inositol have been identified by various workers: Benson and Maruo (1958) in tobacco (Nicotiana

tabacum), sweet clover (Meliotus alba), and barley (Hordeum vulgare); Kates (1960) in runner bean (Phaseolus multiflorus); Wintermans (1960) in elder (Sambucus nigra), bean (Phaseoulus vulgaris) and beet (Beta vulgaris); Ferrari and Benson (1961) in Chlorella pyrenoidosa; Lepage (1964) in alfalfa (Medicago sativa), potatoe (Solanum tuberosum) and chlorella; and by Weenink (1964) in red clover (Trifolium pratense).

TABLE 1

Weenink (1964)

The concentration of phospholipid components in the acetone insoluble fraction of red clover (Trifolium pratense) lipids

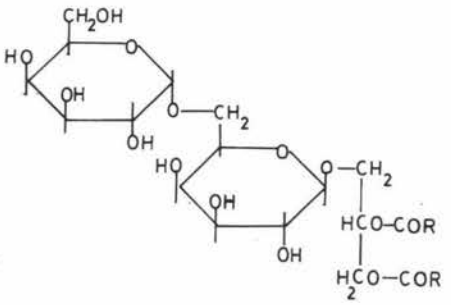
Component	% composition of acetone insoluble lipid fraction
Phosphatidyl choline	37
Phosphatidyl glycerol	23
Phosphatidyl ethanolamine	15
Phosphatidyl inositol	2
Unidentified acidic phospholipids	13
Unknown	10

(b) Glycolipids

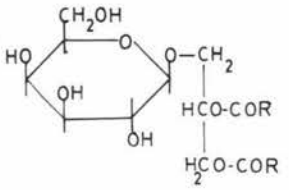
The glycolipids in photosynthetic tissue consist of the galactosyl glycerides and the minor glucocerebrosides recently isolated by Kates and Sastry (1964).

(i) Galactosyl glycerides

Monogalactosyl glyceride and digalactosyl glyceride were first isolated by Carter et al., (1956) from wheat flour and appear to be the major complex lipid of photosynthetic tissue.



α - D - Galactosyl - (1→6) - β - D - Galactosyl - 2', 3' - Diglyceride



β - D - Galactosyl - (1→1) - 2', 3' - Diglyceride

Figure 1

(ii) Gluco-cerebrosides

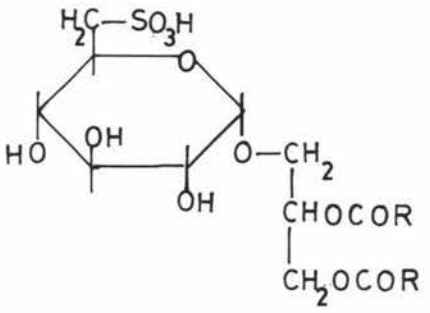
Gluco-cerebrosides containing a long chain fatty acid and a glucose moiety linked to a long chain alcohol (dehydrophytosphingosine, phytosphingosine, dihydrophytosphingosine or an isomer of sphingosine) have been isolated by Sastry and Kates (1964) from runner bean (Phaseolus multiflorus) leaves.

The gluco-cerebrosides have been shown to account for 1% of the total leaf lipids.

Similar components have been demonstrated by Carter et al., (1961a) from wheat flour. The gluco-cerebrosides contained similar long chain alcohols as observed in runner bean lipids but the concentration of dehydro-phytosphingosine was much reduced. Carter and Hendrickson (1963) have characterised the sphingosine isomer found in gluco-cerebrosides of wheat flour as a Δ 8, 9 phytosphingosine.

(c) Sulpholipids

The plant sulpholipid was first identified by Benson et al., (1959) who observed that $S^{35}O_4^=$ was rapidly incorporated into the lipids of the green algae (Rhodospirillum rubrum) and higher plants. It has been found in all photo-synthetic tissues so far investigated and its concentration appears similar to that of the major phosphatides (Hales, 1960, Wintermans, 1960).



6 sulpho α D quinovopyranosyl - (1 → 1')

2'3' diglyceride

Figure 2

The plant sulpholipid contains a sulphonic acid group having a direct carbon to sulphur bond which appears very stable to acid hydrolysis. This lipid differs from the animal tissue sulphatide which contains a sulphate ester group which is easily hydrolysed under acid conditions.

Lepage et al., (1961) finally established the structure of the deacylated sulpholipid isolated from plant tissue. The cyclohexylamine salt exhibited molecular rotation and dispersion curves characteristic of α glucosides. Elemental analysis and spectrophotometric data were consistent with the structure shown above.

2. Fatty acids of photosynthetic tissue

2.1. Fatty acid composition of total lipids

The levels of free fatty acids in photosynthetic tissue are low, as fatty acids are mainly esterified to neutral and complex lipid moieties.

Speer et al., (1929) in examining lipids of spinach (Spinacea oleracea) found 53% of the fatty acids in the free form and Shorland (1941) found 24.6% free fatty acids (expressed as a percentage of the total lipid) in mixed pasture grasses, but this worker attributes this high value to the action of enzymes on lipids during the cold storage of the grass prior to lipid extraction. More recent work has indicated that free fatty acids constitute only a small percentage of the total photosynthetic tissue lipids. Shorland (1953) has shown that a free fatty acid fraction did not exceed 10% in perennial rye (Lolium perenne) grass. The same worker (1961) has reported values of 14% and 9% for free fatty acids expressed as oleic acid for rape (Brassica napus) and cocksfoot (Dactylis glomerata) respectively. Free fatty acids constituting 7% of the total lipid has been reported by Van der Veen et al., (1964) in dehydrated alfalfa (Medicago sativa).

Fatty acids have been shown to constitute approximately one half of the total lipid (Garton, 1960; Hawke, 1963) when those fatty acids esterified to lipid moieties are considered.

Early studies on the fatty acids of leaf tissues were concerned with the glyceride fraction which has subsequently been shown to consist mainly of galactosyl glycerides (Weenink, 1961; Shorland, 1961). Smith and Chiball (1932), during the examination of the glycerides of cocksfoot (Dactylis glomerata) and perennial rye (Lolium perenne) grasses found a higher proportion of unsaturated (80 - 90%) than saturated fatty acids (10 - 12%). The saturated fatty acids consisted mainly of palmitate and stearate while unsaturated fatty acids consisted mainly of linolenate and linoleate. Similar results were obtained by Shorland (1941, 1944), in studies on the glycerides of mixed pasture and cocksfoot (Dactylis glomerata) lipids.

Fatty acids from the Cruciferae such as cabbage (Brassica oleracea) (Chiball and Channon, 1927) and rape (Brassica napus), Reyes and Shorland, 1951) showed similar trends to the Gramineae except Brassica napus was shown to contain a high proportion of a hexa-deca, 7, 10, 13 trienoic acid. In studies on the glycerides of Cruciferae and Gramineae the proportion of linolenic acid was observed to be lower than levels reported more recently and this may be thought due to the use of older methods for the analysis of fatty acids (Shorland, 1962).

Saponifiable fats of leaf meal fat from buckwheat (Polygonum fagopyrum) (Krewson, 1952) and the triglycerides of leaf fat from alfalfa (Medicago sativa) (Jackson and Kummerow, 1949) showed linolenic acid to be the major fatty acid although oleic acid was present in higher levels than in the grasses.

More recent work has confirmed earlier observations on the fatty acid composition of leaf tissue. Crombie (1958) using reverse phase chromatography and ultra violet spectrophotometric techniques has examined the fatty acids of

TABLE 2

The fatty acid composition (moles %), determined by gas-liquid chromatography, of the total lipids from leaf tissue of higher plants

Species and Reference	Fatty acid														
	12:0	13:0	14:0	15:0	16:0	16:1	16:1 trans	16:3	18:0	18:1	18:2	18:3	18:4	20:0	22:0
Short rotation ryegrass (*L.perenne* x *L.multiflorum*) (Hawke, 1963)					immature grass										
	0.24		0.48		9.95	1.22			0.73	1.46	6.80	79.12			
					mature grass										
	0.47		0.94		13.61	1.17			0.94	3.29	11.74	67.84			
Perennial ryegrass (*L.perenne*) Shorland (1961)[1]	0.4	0.2	0.9	tr	8.6	0.2			1.2	1.2	6.7	79.5		0.9	200 and above
Mixed pasture Garton (1960)			1.1		15.9	2.5			2.0	3.4	13.2	61.3		0.2	0.3
Alfalfa (*Medicago sativa*) Van der Veen *et al.*, (1964)[2]	1.0		2.0		19.0	3.0			1.0	2.0	14.0	58.0			
Spinach (*Spinacea oleracea*) Debuch (1962)			0.2	tr	12.9		2.6	4.6	tr	6.6	16.3	56.2	tr	0.6	
Spinach (*Spinacea oleracea*) Allen *et al.*, (1964)			tr		12.0		3.0	10.0	tr	4.0	13.0	57.6			
Snapdragon (*Antirrhinum majus*) Debuch (1961)			0.1		13.4		1.3	tr	2.4	1.8	17.7	57.9	3.6	1.8	

[1] traces of branched n-saturated acids reported

[2] traces of short chain 6:0, 8:0, 10:0 and 11:0 n-saturated acids reported

maple (Acer negundo), maize (Zea mais) holly (Ilex aquifolium) and privet (Ligustrum ovatifolium) tissue in different physiological states. Total lipid extracts of normal green leaves contained linolenic acid as the major component. Linoleic and oleic acid were the other major unsaturated fatty acids and palmitic acid was found to be the major saturated fatty acid although smaller proportions of stearic and myristic acids were identified. The white leaves of maize (Zea mais japonica variegata), maple (Acer negundo variegatum) and holly (Ilex aquifolium) were also examined for fatty acids and the linolenic acid level was found to be substantially reduced while the concentration of linoleic acid showed an increase.

In other recent investigations total lipid from leaves of several species of plants have been examined for fatty acids using gas-liquid chromatographic techniques. Linolenic acid has been shown to be the major fatty acid of the total lipid fractions, although smaller proportions of other unsaturated fatty acids such as linoleic, oleic and palmitoleic acid have been found to be present. Palmitic acid has been shown to be the major saturated fatty acid, although smaller proportions of stearic, myristic, lauric, arachidic and behenic acids have been recorded.

Garton (1960) examined the fatty acids of total lipid extracts from mixed pasture consisting of 50% perennial rye (Lolium perenne), 25% cocksfoot (Dactylis glomerata) 15% timothy (Phleum pratense) and 10% Italian rye (Lolium multiflorum) and demonstrated similar distributions of fatty acids.

Hawke (1963) examined the fatty acids from total lipid extracts of short rotation ryegrass at two stages of growth. The lipid from new growth was shown to constitute a higher percentage of the dry weight of the grass and the fatty acids contained higher proportions of linolenic acid but lower levels of linoleic and palmitic acids compared with lipid from mature growth which constituted a

lower percentage of the total dry weight of the grass.

Debuch (1961, 1962) examined the fatty acids from total lipid extracts of spinach (Spinacia oleracea) and snapdragon (Antirrhinum majus) and obtained similar results to earlier workers except that the presence of a trans hexadecenoic acid was demonstrated. The presence of this acid has since been confirmed by Allen et al., (1964) in spinach (Spinacia oleracea) and Weenink and Shorland (1964) in red clover (Trifolium pratense) perennial ryegrass (Lolium perenne) and pea (Pisa sativum). Weenink (1964) has stated that this fatty acid may have been previously identified as palmitoleic acid because of their similar retention times on adipate columns.

i.e. palmitoleic acid - retention time relative to stearate 0.64:

trans-hexadecenoic acid - retention time relative to stearate 0.67.

The fatty acids of lipid from dehydrated alfalfa (Medicago sativa) have been examined by Van der Veen et al., (1964) who have reported the presence of shorter chain saturated fatty acids containing 6, 8, 10 and 11 carbon atoms in addition to the longer chain length fatty acids reported by other workers. They consider that these fatty acids may have arisen by oxidative degradation of higher molecular weight fatty acids during the dehydrating process of the alfalfa prior to extraction of the lipid material.

2.2. Fatty acid composition of chloroplasts and related structures

Menke (1938) has reported that the chloroplasts of spinach (Spinacia oleracea) contained the highest proportion of the total leaf lipids by demonstrating that the chloroplasts had an average lipid content of 30.9% of the dry weight, while the cytoplasm contained 0.5% lipid. Other workers have confirmed the high lipid content of chloroplasts in different plant species and values between 18 - 40% of the chloroplast dry weight are recorded (Bot, 1942; Conar, 1942; Weber, 1962).

Several workers have examined the fatty acids of the chloroplasts and compared them with the fatty acids of the total leaf lipid Crombie (1958) found that fatty acids of the chloroplast lipids contained higher proportions of linolenic acid but lower proportions of linoleic acid compared with fatty acids of the total leaf lipids in maple (Acer negundo) and broad bean (Vicia faber).

Similar trends have been demonstrated by Debuch, (1961) in snapdragon (Antirrhinum majus) and spinach (Spinacia oleracea) although the same worker (1962) showed in spinach that chloroplasts contained less linolenic and linoleic acid compared with the whole leaf. This worker has also demonstrated that a 16 carbon trienoic acid is more concentrated in the chloroplasts.

Newman (1962) demonstrated that mature tissue of bush bean (Phaseoulus vulgaris) leaves contained plastids with lower chlorophyll levels and a higher ratio of saturated to unsaturated fatty acid compared with young tissue. Wallace and Newman (1964) isolated plastids from bush bean (Phaseoulus vulgaris) plants grown for 14 days under three different environmental conditions (in total darkness, in total darkness except for a final 36 hour light period and in a 20 hour photoperiod of 1,500 foot candles). The amounts of palmitic, stearic, oleic and linoleic acids appeared to decline on an increased exposure

to light whereas the relative amount of linolenic acid appeared to increase. Similarly the ratio of saturated to unsaturated fatty acids decreased with an increased exposure to light.

In the blue-green algae Anabaena variabilis a particulate fraction containing the photosynthetic apparatus contained the highest proportion of linolenic acid compared with the remainder of the cell (Levin et al., (1964) while Hulanicka et al., (1964) have also demonstrated in the photo-auxotrophic Euglena gracilis that 70% of all linolenate is localised in the galactolipid "pigment" fraction.

2.3. Fatty acid composition of component lipids

Wheldon (1960) separated cabbage (Brassica oleracea) phospholipids by silicic acid chromatography and although several phospholipid fractions contained small traces of contaminating lipids the fatty acid composition of the phospholipids were fairly uniform containing predominantly palmitic, linoleic and linolenic acids.

Allen et al., (1964) obtained pure lipid components from spinach leaves by utilizing countercurrent distribution to remove a considerable proportion of the pigments and give a preliminary separation of lipids. The lipid fractions were then fully separated on DEAE cellulose. Weenink (1964) separated the acetone insoluble lipids of red clover (Trifolium pratense) into pure pigment free fractions by utilizing a preliminary separation on DEAE cellulose followed by silicic acid chromatography. The fatty acids were isolated and examined by gas-liquid chromatography and both authors have demonstrated that the phospho-lipids contain more saturated fatty acids than the galactosyl glycerides which have higher proportions of linolenic acid. A hexadecatrienoic acid concentrated in monogalactolipid and a trans-3-hexadecenoic acid concentrated in phosphatidyl

TABLE 3

1 Allen et al., (1964)

2 Weenink (1964)

The fatty acid composition of spinach[1] (Spinacia oleracea) and red clover[2] (Trifolium pratense) lipid components

Fatty Acid X : Y	Monogalactolipid		Digalactolipid		Sulpholipid		Phosphatidyl glycerol		Phosphatidyl choline		Phosphatidyl ethanolamine		Phosphatidyl inositol	
	1	2	1	2	1	2	1	2	1	2	1	2	1	2
14 : 0								tr		tr	tr	tr	tr	
15 : 0								tr		0.9		tr		
16 : 0	tr	8.9	6.0	8.9	27.0		22.0	44.3	20.0	38.4	46.0	41.2	41.0	
16 : 1 (trans)	tr	0.7		0.7			35.0	31.0	tr	1.4		1.8		
16 : 3	30.0		3.0						tr		2.0			
18 : 0		1.9	1.0	1.9			tr	2.6		5.2	1.0	3.5	1.0	
18 : 1	1.0	2.2	4.0	2.2	6.0		2.0	5.2	11.0	5.8	2.0	2.6	6.0	
18 : 2	1.0	3.3	3.0	3.3	39.0		5.0	9.2	30.0	36.4	7.0	37.1	25.0	
18 : 3	67.0	83.0	84.0	83.0	28.0		36.0	7.7	40.0	11.9	43.0	13.8	27.0	

glycerol were reported by Allen et al., (1964) and in work on red clover (Trifolium pratense) Weenink, (1964) reported the presence of a unidentified sixteen carbon chain fatty acid concentrated in phosphatidyl glycerol. This acid has since been identified in more recent work (Weenink and Shorland, 1964) to be trans-3-hexadecenoic acid as found by Allen et al., (1964).

The fatty acids of the gluco-cerebroside fraction isolated by Sastry and Kates (1964) have been shown to consist entirely of α hydroxy fatty acids (predominantly C22 and C24 in chain length). The fatty acids were assumed to be α hydroxy fatty acids as they had identical relative retention times with pure markers of α hydroxy acids. Wheat flour cerebrosides have also been shown to contain α hydroxy acids with α hydroxy stearic acid as the major fatty acid component (Carter et al., 1961a).

3. Occurrence and structure of galactolipids

3.1. Discovery and occurrence of galactolipids

The initial report of the presence of a galactosyl glycerol component in plant extracts was presented by Collin et al., (1933) who isolated a compound from several species of marine red algae that could be hydrolysed by α galactosidase to yield equimolar proportions of glycerol and D galactose. From this data they considered the linkage between galactose and glycerol to be α in configuration. Collin et al., (1937) presented some evidence to suggest that the linkage in the compound occurred through the secondary alcohol group of the glycerol.

Puttman and Hassid (1954) confirmed the above work by an investigation using methylation and periodate oxidation procedures. Sun dried Irideae laminaroides (marine red algae) were extracted with 80% ethanol on a steam bath and after elimination of salts and inorganic material, an immobile syrup was obtained which on hydrolysis by α galactosidase yielded D galactose and glycerol. Methylation of the extract produced a hexamethyl galactoside which on hydrolysis yielded 2, 3, 4, 6 tetra-O-methyl D galactopyranose and 1, 3 di-O-methyl glycerol which were identified by melting points and co-chromatography with pure standards. Periodate oxidation of the galactoside gave the consumption of 2 moles of periodate with the production of 1 mole of formic acid. This data was consistent with a compound having the structure shown below:-

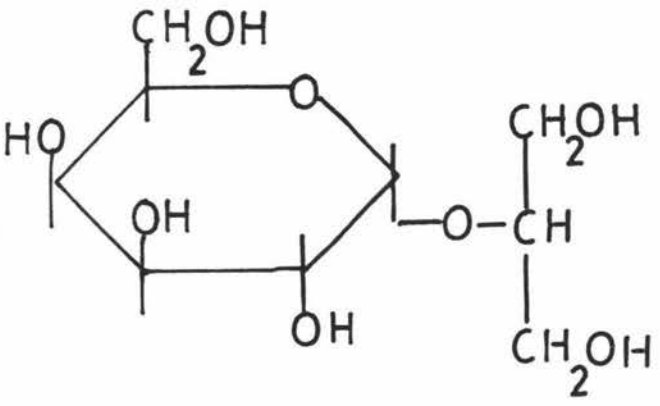
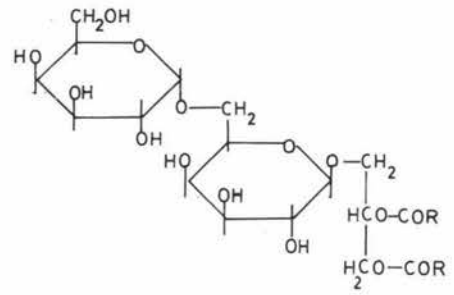
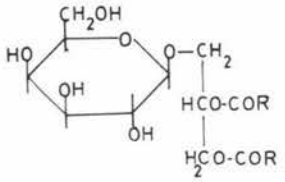


Figure 3

Carter et al., (1956) demonstrated that a benzene extract of wheat flour subjected to Craig Distribution between n-heptane and methanol fractionated into triglyceride, steroid and lipocarbohydrate fractions. The lipocarbohydrate fraction was found to be soluble in warm acetone but on cooling could be separated into acetone insoluble (fraction I) and acetone soluble (fraction II) material. These fractions were subject to alkaline hydrolysis and the fatty acids were extracted with petroleum ether. Following acid hydrolysis fractions I and II gave only galactose and glycerol identified by paper chromatography. Quantitative galactose and glycerol determinations indicated that fraction I was a monogalactose glycerol and fraction II was a digalactose glycerol. Periodate oxidation confirmed these results and enzymatic hydrolysis with α and β galactosides established the linkage of these compounds. All this data indicated compounds having the structure shown below:-



∝-D-Galactosyl-(1→6)-β-D-Galactosyl
-2', 3'-Diglyceride



β-D-Galactosyl-(1→1)-2',3'-
Diglyceride

Figure 4

Since 1956 galactosyl glycerides have been demonstrated in wheat flour by Zetner (1958), Daniels (1958) and Carter et al., (1961b) who also demonstrated their presence in corn gluten, wheat germ oil and green groat oil. The presence of galactolipids in potatoe starch grains has been recently demonstrated by Duncan and Rees (1965).

Benson et al., (1958) discovered galactosyl glycerides in Chlorella pyrenoidosa and since this date these components have been discovered in a wide variety of photosynthetic micro-organisms and higher plants. Anabaena variabilis (a primitive blue-green algae) has been shown to contain galactolipid compounds by Levin et al., (1964). The phytoflagellates have been studied extensively and galactosyl glycerides have been demonstrated in Euglena gracilis by Carter et al., (1961b); Rosenberg, (1963); Rosenberg and Pecker (1964); and Hulanicka et al., (1964). Chlorella pyrenoidosa has been shown by Benson et al., (1958);

TABLE IV

Occurrence of galactosyl glycerides in leaf lipids of higher plants

Species	Reference
Trifolium pratense (red clover)	Weenink (1961), (1964)
Medigaco sativa (alfalfa)	Lepage (1964); Obrien and Benson (1964); Van der Veen *et al.*, (1964)
Phaseoulus vulgaris (bush bean)	Wintermans (1960); McArthur *et al.*, (1964); Wallace and Newman (1964).
Phaseoulus multiflorus (runner bean)	Sastry and Kates (1964)
Solanum tuberosum (potatoe)	Lepage (1964)
(*Spinacia oleracea* (*Tetragonia expansa* (Spinach)	Wintermans (1960); Benson *et al.*, (1959); Zill and Harmon (1962); Allen *et al.*, (1964)
Brassica oleracea (cabbage)	Nichols (1964)
Capsicum annum (pepper fruits)	McArthur *et al.*, (1964)
Curcurbita maxima (squash)	Wallace and Newman (1964)
Sambucus nigra (elder)	Wintermans (1960)
Pisum sativum (pea)	Adhikari *et al.*, (1961)

Ferrari and Benson (1961), Lepage (1964) and O'Brien and Benson (1964) to contain galactosyl glycerides.

Reports of galactosyl glycerides in a wide variety of species of higher plants is recorded. (see Table 4).

Galactosyl glycerides have not been demonstrated in the photosynthetic bacteria which do not evolve oxygen during photosynthesis (Erwin and Bloch, 1964).

Galactosyl glycerides have not been demonstrated in bacteria although several other types of glycolipids have been reported. McFarlane (1961) found a manosyl diglyceride in Micrococcus lysodeiktieus and Bennarz (1964) found a dimanosyl diglyceride in another strain of the same organism. McFarlane (1962) demonstrated the presence of a glucosyl diglyceride in Staphylococcus aureus. This work was also confirmed by Polonovski et al., (1962) who found the diglucosyl diglyceride as well in the same organism. Vorbeck and Marinetti (1965) have isolated a glucosyl diglyceride and a glycosyl diglyceride containing both galactose and glucose from Streptococcus faecalis. Gram positive bacteria have been shown to contain glycolipids constituting 1 - 2% of the total lipids (Brundish et al., 1965). However, Pneumococcus was an exception in that glycolipids represented 30% of the total lipids. A galactosyl glucosyl diglyceride was demonstrated in Pneumococcus and Lactobacillus casei whereas 6 other species of Gram positive bacteria were shown to contain a diglucosyl diglyceride lipid. Reeves et al., (1965) have reported the isolation of galactofuranosyl glycerol from the lipids of Bacteroides symbiosus (a Gram negative organism).

3.2. Location of galactolipids in photosynthetic tissue

Wintermans (1960) determined the galactolipid level in the whole leaf and the chloroplast of several species of plants, and data suggested that these lipids were contained exclusively in the chloroplast. It was also demonstrated by the same worker that there was a possible relation between the level of chlorophyll and galactolipids as green leaves of elder (Sambucus nigra) contained higher levels of galactosyl glycerides than yellow leaves. This observation has been supported by Nichols (1963) who by utilizing thin layer chromatography found higher levels of galactosyl glycerides in the dark outer leaves of cabbage (Brassica oleracea) than the pale inner leaves and the stalk. McArthur et al., (1964) has also demonstrated that pepper fruit plastids (Capsicum annum) ripening from the green to the red state showed a general decrease in content of galactosyl glycerides.

The pigment fraction of photoauxotrophic Euglena gracilis cells have been demonstrated by Hulanicka et al., (1964) to contain six lipids yielding galactose on acid hydrolysis while Levin et al., (1964) demonstrated that α linolenic containing galactolipids were localised in the photosynthetic apparatus of Anabaena variabilis (a blue green algae).

Sastry and Kates (1964) have suggested that the lipid analysis of chloroplasts by Wintermans (1960), Benson et al., (1959) and other workers may have to be reviewed on their finding of specific enzymes in Phaseoulus species that catalyse the hydrolysis of galactosyl diglyceride, to galactosyl glycerols and 2 moles of linolenic acid. The presence of α and β galactosidase in the cell sap cytoplasm hydrolysing galactosyl glycerols to galactose and glycerol was also demonstrated.

3.3. Structural identification of galactolipids

Carter et al., (1956) prepared a crude mixture of galactolipids which on alkaline hydrolysis yielded substances other than just mono- and digalactosyl glycerol. A partial separation of the two galactolipids was achieved due to the greater solubility of the monogalactosyl glyceride in acetone.

Wickberg (1957) in an investigation on the red algae Polysiphonia fastigiata isolated a glycoside, which after acid hydrolysis yielded D galactose glyceritol identified by paper chromatography.

A similar glycoside was obtained from another red algae Corallina officinalis by Wickberg (1958a). The algae were extracted for three days with diethyl ether followed by fourteen days extraction with methanol and an aqueous solution of the concentrated methanolic extract was filtered through IR- 120 and IR- 4B resins, concentrated, and stored for two months. The material was then dissolved in 50% aqueous ethanol and crystalline material was filtered off. The filtrate was passed through a carbon column, concentrated, and the resulting syrup was fully separated on a carbon-celite column with water/ethanol mixtures which gave eluates that were identified by paper chromatography.

The eluate fraction containing the glycoside was concentrated, dissolved in ethanol and seeded with a trace of glycoside A. The crystals had a melting point of 194 - 196°C and this melting point was not depressed by a sample of digalactosyl glycerol isolated by Carter et al., (1956). From a quantitative determination of the hydrolysis products, analysis for carbon and hydrogen, results of periodate oxidation and enzymic studies with α and β galactosidases glycoside A was assigned the following structure:-

0 - α - D galactopyranose (1 → 6)

0 - β - D galactopyranose (1 → 1) - glyceritol

A second glycoside, O - β - D galactopyranosyl (1 → 1) glyceritol, having a melting point 139 - 140°C was also identified.

The configuration of the glyceritol residue was solved by synthesis of the D and L glyceritol forms of monogalactopyranose. The D glyceritol galactosyl was found identical with a sample of monogalactosyl glycerol (Carter, et al., 1956) so that glyceritol in glycoside A was assigned to be of D configuration.

A subsequent investigation of Wickberg (1958b) demonstrated that the monogalactosyl glycerol from Porphyra umbilicalis was an isomorphous mixture of D and L glyceritol residues.

Carter et al., (1961b), (1961c) conclusively established the structure of the galactosyl glycerides from wheat flour. Unbleached wheat flour was extracted with benzene at room temperature and concentrated in vacuo to a heavy syrup. The benzene extract was treated with acetone and left overnight at 4°C to precipitate the insoluble lipo-protein fractions. The precipitated material was redissolved in benzene and reprecipitated with acetone. The combined benzene acetone soluble fractions were pooled and the solvent was removed in vacuo yielding a yellow oil which was dissolved in n-heptane (pre-equilibrated with 95% methanol) and extracted twice with an equal volume of methanol. Distribution between n-heptane and 95% methanol gave a clear separation of all glycolipids into the methanol phase with triglycerides, sterols and sterol esters passing into the heptane phase.

The methanol fraction was further purified by silicic acid column chromatography using chloroform with gradually increasing concentrations of methanol as the elating solvents. The monogalactolipid was eluted with 98 : 2 C/M (V/V) followed by the digalactolipid and cerebroside with increasing methanol concentrations. Column products were identified by paper chromatography of

deacylation products. Some overlapping of the fractions was observed so further purification appeared necessary.

The monogalactolipid fraction was purified by rechromatography on silicic acid columns eluting with chloroform and chloroform/methanol mixtures. The purified monogalactolipid was obtained as a white amorphous powder after solvent evaporation.

The fraction containing the digalactolipid was chromatographed on a column of Amberlite R-MB-3 with methanol but a complete separation from cerebroside was not obtained. Separation of digalactolipid and cerebroside was only obtained by passing partially methylated material in ether through a silicic acid column three times.

Structural identification of the galactolipids was established by the following methods:- The saponification equivalents of the purified fractions were determined and results suggested the presence of two long chain fatty acid groups, per mole of lipid.

A lipid fraction was methylated and then saponified to yield partially methylated derivatives. Periodate oxidation of these substances demonstrated an uptake of one mole of periodate and the formation of formaldehyde. This evidence suggested the presence of 2 free hydroxyl groups on the glycerol moiety and also indicated that in the original lipid molecule two fatty acids were esterified to the hydroxyl groups of the glycerol. Acidic hydrolysis of the methylated deacylated lipids yielded 2, 3, 4, 6 tetra-O-methyl D galactose and unmethylated glycerol from the monogalactosyl derivative (see Figure 5); and 2, 3, 4, 6 tetra-O-methyl D galactose, 2, 3, 4 tri-O-methyl galactose (in equimolar proportions) and unmethylated glycerol from the digalactosyl derivative. These methylated derivatives and glycerol co-chromatographed with pure standards. Infared data indicated that the glycerol moiety had a D configuration.

GALACTOSYLGLYCEROL LIPIDS FROM WHEAT FLOUR

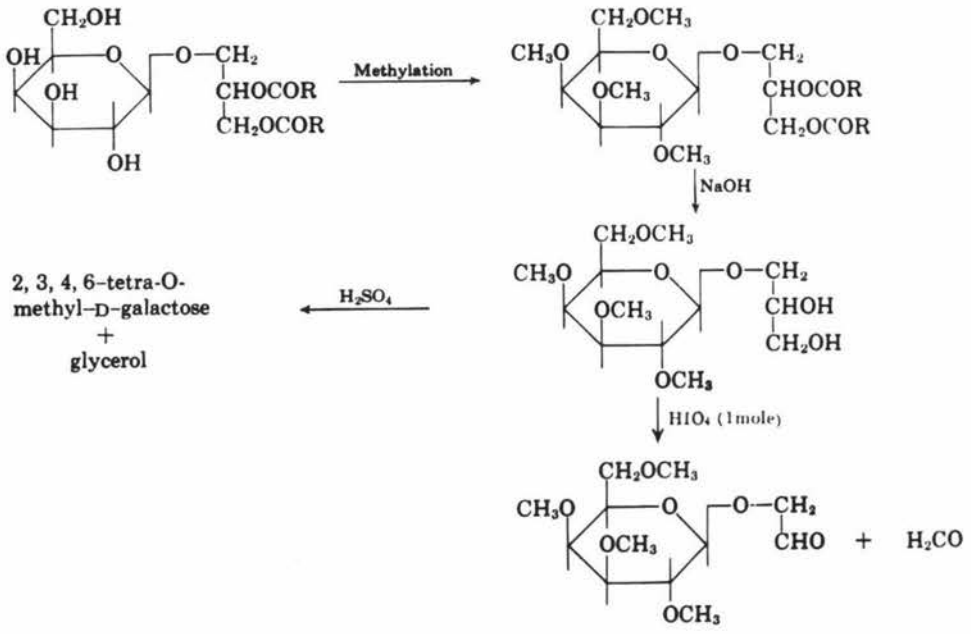


Figure 5

On the basis of this data Carter et al., (1961c) assigned the following structures to the galactosyl glycerides:-

2, 3 diacyl - 1 β - D galactopyranosyl - D - glycerol from the mono-galactosyl glycerol

and 2, 3 diacyl - 1 - (α - D galactopyranosyl - 1, 6 - β - D galactopyranosyl) - D - glycerol for the digalactosyl glycerol.

Sastry and Kates (1964) have since confirmed that the galactosyl glycerides from runner bean (Phaseoulus multiflorus) leaves were of similar structure to those isolated from wheat flour (Carter et al., 1961b, 1961c).

TABLE 2

The fatty acid composition (moles%) of the galactosyl glycerides from photosynthetic tissue of higher plants

Fatty Acid	Chorella pyrenoidosa 1		Trifolium pratense (Red clover) 2		Medicago sativa (alfalfa) 3		Spinacea oleracea (spinach) 4		Phaseoulus multiflorus (bean) 5	
	GDG	GGDG	GDG	GGDG	GDG	GGDG	GDG	GGDG	GDG	GGDG
14:0	tr	tr			tr	tr				
15:0	tr	tr			tr	tr				
16:0	2.7	11.6	17.0	8.9	2.7	14.0	tr	6	2.3	4.5
16:1	9.7	9.5			tr	tr				
16:1 (trans)			0.2	0.7			tr			
16:3							30	3		
17:0	1.5	1.2			tr	0.7				
18:0	0.3	0.4	1.4	1.9	0.2	3.3		1	tr	1.0
18:1	40.5	36.8	1.4	2.2	0.3	0.4	1	4	tr	tr
18:2	4.5	5.8	5.5	3.3	1.7	0.8	1	3	2.2	1.3
18:3	26.8	27.0	74.5	83.0	95.0	82.2	67.0	84.0	95.5	95.5
19:0	12.0	3.3								
18:4	2.6	3.3								

1 O'Brien and Benson (1964)
2 Weenink (1964)
3 O'Brien and Benson (1964)
4 Allen et al., (1964)
5 Sastry and Kates (1964)

GDG = monogalactolipid
GGDG = digalactolipid

3.4. Fatty acid composition of galactolipids

The fatty acids of the galactosyl glycerides have been examined by several workers. In photosynthetic tissue the predominate fatty acid appears to be α linolenic acid (9, 12, 15 octadecatrienoic acid) as shown in Table 5 below. Carter et al., (1961b) in examining the galactolipids from wheat flour showed that a higher percentage of saturated fatty acids was present.

4. Metabolism of galactolipids with special reference to photosynthetic tissue

4.1. Biosynthesis of galactolipids

Ferrari and Benson (1961) in studying the incorporation of $C^{14}O_2$ into the lipids of Chlorella pyrenoidosa during steady state photosynthesis demonstrated that the most rapidly labelled of the soluble deacylation products were in order: galactosyl glycerol, digalactosyl glycerol, diglycerol phosphate, sulphoglycosyl glycerol and glycerol phosphoryl inositol. Acid hydrolysis of the labelled galactosyl diglyceride indicated the fatty acids contained only 25% of the radioactivity of the galactose moiety. This suggested that the galactose moiety was metabolised far more rapidly than the diglyceride. These workers suggested that the relatively slow labelling of the fatty acids of the glycosides and diglycerol phosphate could indicate an active transfer of the galactose and glycerol phosphoryl groups between absorbed pools of intermediates in carbohydrate metabolism.

These workers suggest that the only precursor for the synthesis for galactolipids, present in sufficient quantities, would be uridine diphosphate galactose (U.D.P. galactose). This as well as U.D.P. glucose have been identified by Buchannan et al., (1953) who demonstrated that these compounds become rapidly labelled during photosynthesis, constituting a large proportion of the total labelled non-polysaccharide hexose. These workers state that U.D.P. galactose would probably arise via the photosynthetic cycle and a reverse of the glycolytic pathway.

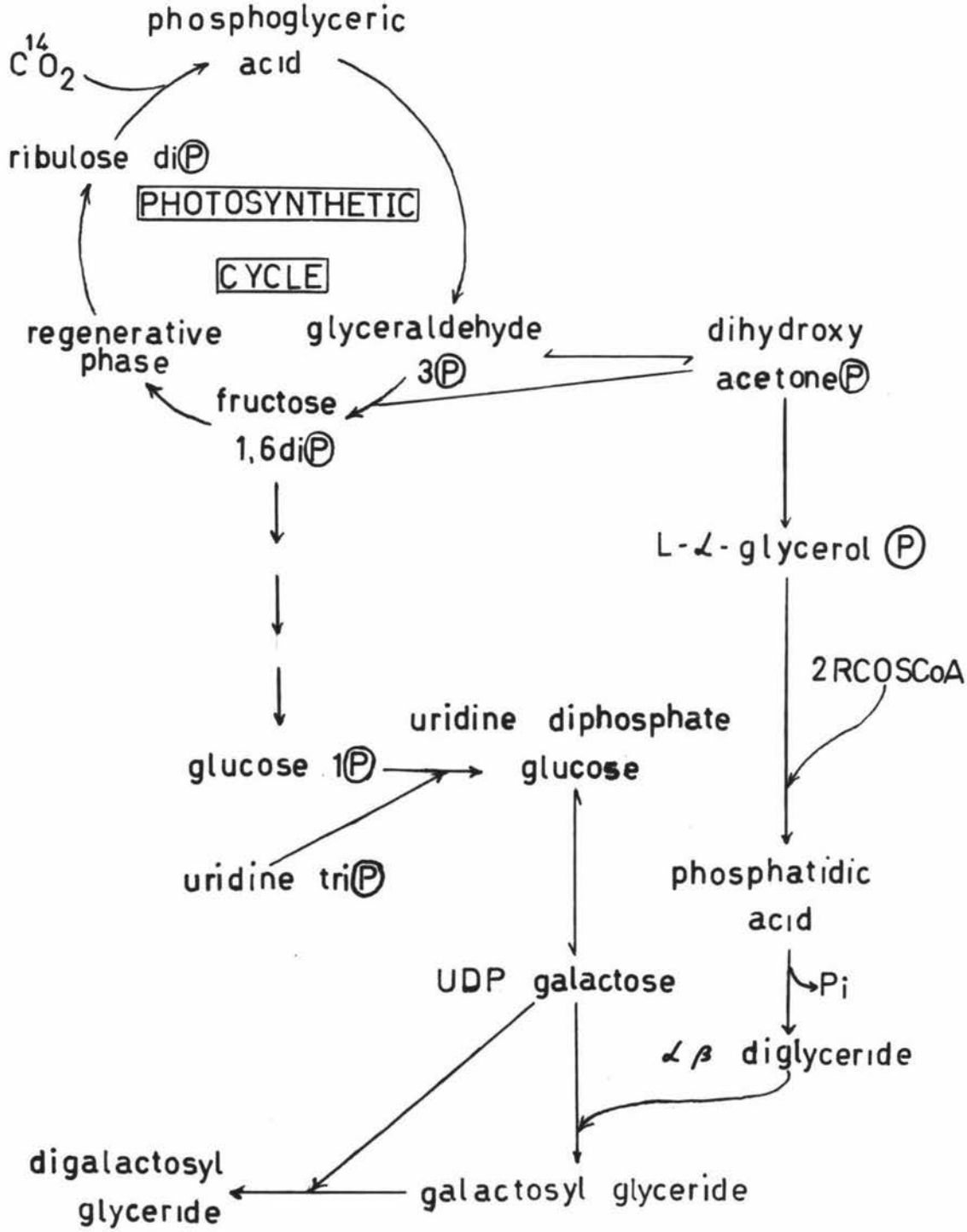
Neufeld (1963), Neufeld and Hall (1964) have also proposed that U.D.P. galactose is a precursor of the galactolipids. They demonstrated that by incubating C^{14} labelled U.D.P. galactose with spinach chloroplasts, a high percentage of the radioactivity appeared in the chloroplast lipids. This reaction proceeded equally well in either dark or light phases, and appeared

to be a function of chloroplast concentration and time. The sugar nucleotides (U.D.P. galactose and U.D.P. glucose) appeared specific for the reaction. The conversion of U.D.P. glucose to U.D.P. galactose could be attributed to U.D.P. galactose epimerase activity.

Radioactive products were subjected to paper electrophoresis and the neutral area (accounting for 7% of total radioactivity) contained several radioactive compounds identified as hexose (24%), β galactose glycerol (16%), digalactose glycerol (57%), and trigalactose glycerol (4%). Further radioactive material (68% of the total) was eluted with methanol, saponified, and the deacylation products were subjected to paper chromatography. Four compounds were identified, the fastest moving component being D galactosyl glycerol, followed by 0 - α - D galactose (1 → 6), 0 - β - D galactose (1 → 1) D glycerol. The nature of the third and fourth compounds presumed to be tri- and tetragalactosyl glycerol were not determined. In this connection, Lepage et al., (1961) have reported the presence of small amounts of polygalactosyl glycerols (in addition to the major lipid derivatives) in the deacylated products of alfalfa leaves.

Ferrari and Benson (1961) have proposed that the galactose from the sugar nucleotide would be transferred to a diglyceride (which would have arisen by established pathways) to form the monogalactolipid. These workers, by assuming the biosynthesis of the phosphatides to be independent of galactosyl glyceride synthesis, demonstrated that a decrease in the C^{14} content of monogalactolipid was matched by a concomitant rise in radioactive label appearing in digalactolipid. Hence they proposed a galactosylation step in which monogalactolipid is a precursor of the digalactolipid. O'Brien and Benson (1963) supported this proposal by showing that the fatty acids of the two galactolipids are closely similar.

GALACTOLIPID SYNTHESIS



4.2. The function of the galactolipids

4.2.1. The function of galactolipids in chloroplasts

The major lipid fractions within the chloroplasts of higher plants are the neutral (galactolipids) and anionic (sulpholipid and phosphatidyl glycerol) surfactants (Benson, 1964). It has been proposed, that these neutral and anionic lipids stabilize the large interfacial areas between pigment and protein phases within the chloroplast. (Benson, 1961). Also chloroplast laminae comprised of lipids with cationic or anionic functional groups would allow a possible function in a photosynthetic electron transfer system. (Benson, 1961).

4.2.2. The function of the galactose component

Benson et al., (1959) have suggested that the galactose moieties of the galactolipids form a carbohydrate reserve varying with the metabolic status of the plant. Evidence is presented on the basis of the high concentration of galactolipids in plant tissue, and the rapid labelling of the galactose moiety which occurs during photosynthesis. Ferrari and Benson (1961).

Greenwood et al., (1963) have demonstrated the presence of spheroidal osmiophilic globules in the stroma of chloroplasts from higher plants. Similar globules have been reported by Park and Pon, 1961; Murakami and Takamiya, 1962; and Bailey and Whyborn, 1963. Silica gel chromatography of lipid extracts from the globules of Vicia faba has demonstrated the presence of two galactolipids, plastoquinone, additional less polar lipids and β sitosterol. (Greenwood et al., 1963). β carotone and chlorophyll were not demonstrated in the globules. Greenwood et al., (1963) have suggested that the globules are a general deposit of insoluble lipid material just as starch is thought to be a deposit of insoluble carbohydrate. Considered as lipid depots, the globules may possibly represent a reserve source of energy to the plant (Bailey and Whyborn, 1963).

4.2.3. The function of linolenic acid

The synthesis of galactosyl glycerides containing high percentages of α linolenic acid appears to be dependent on the photosynthetic process in photosynthetic micro-organisms and higher plants.

Anabaena variabilis (a primitive blue green algae) synthesises α linolenate (Levin et al., 1964) whereas Beggiatoa a related colourless Cyanophyte synthesises only mono-unsaturated acids (Erwin and Bloch, 1964). However, Holton et al., (1964) demonstrated no linolenic acid in the blue-green algae Anacystis nidulans.

Lipid metabolism in the phytoflagellate Euglena gracilis has been studied extensively by the following workers: Rosenberg, (1963), Rosenberg and Pecker, (1964), Rosenberg et al., (1965), Erwin and Bloch (1963) and Hulanicka et al, (1964).

Different lipid patterns are found to be present in Euglena gracilis when grown as a photoauxotroph in the light or as a heterotroph in the dark. In the heterotrophic cell galactolipids were not found to be present and C_{20}, C_{22} and C_{24} chain length polyenoic acids typical of animal cells were identified in high percentages with α linolenate virtually absent. A similar fatty acid composition is also found in the colourless Euglenid Astasia longa. In the heterotrophic cell and the colourless Euglenid the galactolipids appear to be replaced by phospholipids (mainly phosphatidyl choline and phosphatidyl ethanolamine) characteristic of animal mitochondria and typical of heterotrophic metabolism (Erwin and Bloch, 1964) and Hulanicka et al., 1964). Morphologically the change of light to dark is accompanied by a disappearance of chloroplasts and an increase in mitochondrial type organelles (Wolken, 1961).

Rosenberg and Pecker, (1964) have demonstrated that illumination of etiolated Euglena cells resulted in the onset of galactolipid synthesis which proceeded at a linear rate and which did not appear to be dependent on chlorophyll level but on the products of photosynthesis.

In higher plants conditions that reduced the photosynthetic rate such as reduced light intensity and nitrogen deficiency (Newman and Wallace, 1965) appeared to cause a decrease in the galactosyl glycerides and α linolenic acid content.

The only photosynthetic organisms so far examined that do not contain α linolenic acid are the photosynthetic bacteria (Erwin and Bloch, 1964a) Nichols and James, 1965). These organisms are falcultative anaerobes and do not envolve oxygen during photosynthesis. Nichols and James (1965) examining the lipids of six species of photosynthetic bacteria showed that Chlorobium limicola contained both galactosyl glycerides and sulpholipid but Rhodopseudomonas spheroides contained only sulpholipid and no galactosyl glycerides. Other Rhodopseudomonas species and Rhodospirillum rubrum contained no galactosyl glycerides or sulpholipid. All these photosynthetic bacteria contained only mono-unsaturated and long chain saturated fatty acids, poly-unsaturated fatty acids being completely absent (Harris, et al., 1965). The major fatty acid isolated was cis-11-octadecenoic (vaccenic) acid.

Erwin and Bloch (1963, 1964) considering α linolenic acid to be a universal component of chloroplasts or related photosynthetic units extending from blue-green algae to higher plants have suggested that this fatty acid is required for one of the steps leading to oxygen production ducing the photosynthetic cycle, rather than for the formation of chloroplast structure which varies throughout the plant kingdom. Several lines of evidence have been proposed to support this hypothesis:-

(a) A Scendesmus mutant containing normal chloroplasts could carry out most of the photosynthetic processes but the cycle was blocked at the oxygen envolving stage. This mutant has been shown to contain 50% less α linolenic acid than normal photosynthetic algae.

(b) Light has been shown to stimulate the incorporation of acetate-I-C^{14} into α linolenic acid of photoauxotrophic Euglena gracilis cells. The results of adding 3 - (p - chlorophenyl) - 1, 1 - dimethyl urea (C.M.U.) and related compounds that inhibit the Hill reaction abolished the incorporation of acetate-I-C^{14} into the unsaturated fatty acids but the synthesis of animal type polyenoic acids was observed. These workers have proposed that C.M.U. or dark treatment cause a decrease in the synthesis of α linolenic acid which appears associated with the Hill reaction.

(c) Euglena cells grown in an atmosphere containing 0.5% CO_2 instead of 5% CO_2 yielded chloroplasts with essentially the same amount of chlorophyll but with a greatly diminished capacity for catalysing the Hill reaction. Simultaneously the α linolenic acid content of cells grown in 0.5% CO_2 was considerably reduced.

(d) The absence of α linolenate in photosynthetic bacteria which fail to envolve oxygen is considered to support the suggestion that α linolenate is concerned in the oxygen evolving stage of photosynthesis.

Bloch and Chang (1964) have shown that abolition of the Hill reaction in the algae Ankistrodesmus braunii brought about by growing the organism on manganese deficient media leads to the cessation of α linolenate synthesis. These workers suggest that since two algae mutants (mutant 11 of Scendesmus obliguus and mutant Ac 141 of Chlamydomonas reinhardi) lack the ability to evolve oxygen but contain some α linolenic acid but no dectable amounts of galactosyl glycerides, both galactose and α linolenate may be essential components associated with the photosynthetic evolution of oxygen.

Nichols and James (1965) have suggested that lipids are not concerned in the primary reactions of photosynthesis. The major lipids of the chloroplasts of higher plants (mono and digalactosyl glycerides, sulpholipid and phosphatidyl glycerol) were also found in significant proportions in roots, stems bulbs and etiolated tissues.

In these tissues the proportions of polyenoic acids were found to be lower than in normal photosynthetic tissues and it has been suggested that the polyenoic acids may still be required for photosynthesis. The observation that photosynthetic bacteria which do not evolve oxygen contain no glycolipids and polyenoic acids support this view. Phosphatidyl glycerol was found to be the only lipid common to both chloroplasts and photosynthetic bacteria.

An alternative explanation is that oxygen may be required for the synthesis of linolenic acid in yeast rather than linolenic acid being required for oxygen synthesis. (O'Brien and Benson, 1964) Yuan and Bloch (1961) found that in yeast (Torulopsis utilis) the transformation of oleic acid to linoleic acid and possibly to linolenic acid requires oxygen and involved a direct desaturation process.

Vandenheuvel (1963) who examined the shape of the linolenic acid chain suggested that the curved structure of this cis trienoic acid could contribute to hydrophobic binding of non-polar molecules (quinones, pigments) in the unit membrane of the chloroplast.

EXPERIMENTAL METHODS

1. Growth and harvesting of plant material

Ramets from a Lolium perenne clone (A83/147) were obtained from Grasslands Division, D.S.I.R., New Zealand; and planted in plastic pots in a 3 : 1 mixture of pumice and peat. The plants, retained near field capacity and irrigated with approximately 50 ml. of Modified Hoaglands solution (see Appendix I) three times weekly, were grown in a eight hour photoperiod in glasshouses (Plant Physiology Division, D.S.I.R., New Zealand) prior to experimental use.

The Lolium perenne plants were grown in controlled environment Growth Cabinets (Plant Physiology Division, D.S.I.R., New Zealand) for experimental purpose. The environmental conditions were as follows: a 12 hour photoperiod with a light intensity of 2,000 foot candles (obtained from a mixture of ten 125 watt reflector backed fluorescent tubes (TL/33) and ten 150 watt incadescent flood lamps) and light and dark temperatures of 21 $\pm$ 1 ^{o}C and 18.5 $\pm$ 1 ^{o}C respectively. Plants were sub-irrigated with 50% Modified Hoaglands solution daily. Average leaf length measured every 48 hours was used as a criterium of growth rate.

In an initial experiment L. perenne plants were harvested, after 40 days growth, when they were 28 cm. high. The leaf tissue was cut at 3 regions (see figure 7) at levels 1, 10 and 19 cm. above the pot. The 1 - 10 cm. region contained young leaves, leaf sheaths and basal sections of more mature tissue; while the 10 - 19 cm. and 19 - 28 cm. regions contained leaf tissue that was increasing in maturity. In a subsequent experiment leaves (10 cm. high) were harvested from the plants after 8 days growth.

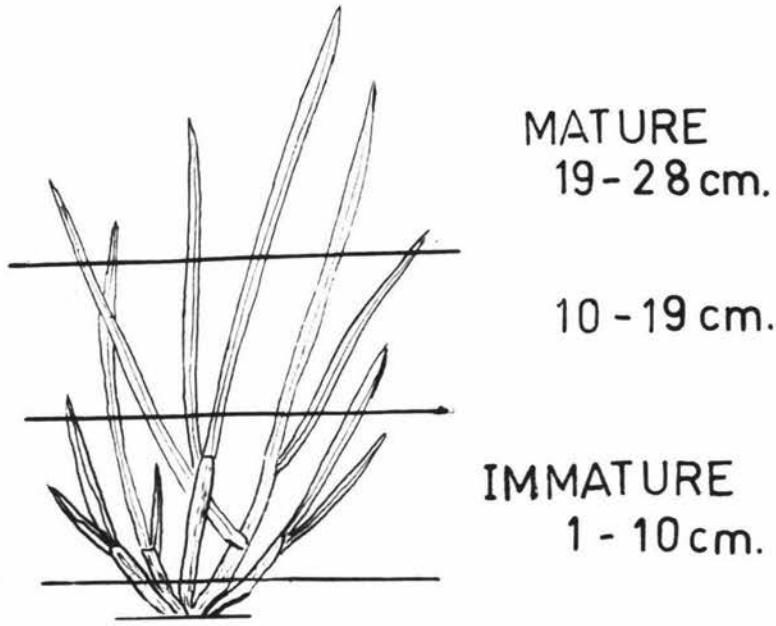


Figure 7

Seedlings of L. perenne (A2304 N.Z. Govt. Stock) and Hordeum vulgare var. Kenia (Hodder and Tolley Ltd.) were grown in seed boxes in a 3 : 1 : 1 pumice, peat, sand mixture in a glasshouse in darkness and high and low levels of natural light in August, 1965. Natural daylength was supplemented by incandescent lamps to bring the photoperiod to 14 hours. Low light was approximately 2 - 3% of full natural light, and was obtained by shading the seedling by several layers of scrim. The intensity relative to full natural light was checked by measurement with a photometer. The glasshouse was maintained at day and night temperatures of 21 - 25°C and 15 ± 2°C respectively. Seedlings were grown for seven days after germination, after which the primary leaf was cut from the seedling from a point just above the coleoptile in each treatment.

2. Extraction of lipids from plant tissue

Leaf tissue was cut from the plants or seedlings and the extraction process commenced as rapidly as possible to minimise the breakdown of lipid by enzyme action.

A weighed sample of tissue was taken for dry weight analysis. The grass was dehydrated and then dried to a constant weight in an oven at 110°C for 24 hours.

In preliminary experiments plant tissue was extracted with ether and ethanol mixtures but in subsequent work chloroform : methanol (C/M) (2 : 1, v/v) extraction was used as it was demonstrated that the latter solvent mixture extracted more lipid material. The extraction method involving alcohol and ether is reported in appendix II.

The grass sample was weighed, cut into short lengths and macerated in a Waring blendor with C/M (2 : 1, v/v) in a ratio of 100 ml. of solvent per 100 g. wet weight of grass. The resultant slurry was filtered through a sintered funnel into a Buchner flask and the extraction process was repeated three times with fresh aliquots of C/M (2 : 1, v/v). At the end of this process the residue contained no appreciable pigment colouration, but to ensure that all lipid material had been extracted, samples of residual material were extracted with hot C/M in a Soxhlet apparatus for 4 hours. Neglible quantities of lipid appeared to have been extracted as determined by thin layer chromatography.

The combined C/M extracts were then heated to 55°C to ensure the deactivation of lipolytic enzymes, (Sastry and Kates, 1964) prior to removal of the solvent *in vacuo* between 40 - 50°C. The resulting dark green residue was taken up in C/M (2 : 1, v/v) and non-lipid contaminants were removed according to the method of Folch *et al.*, (1957), in which the extract was mixed thoroughly with 20% of its volume of 0.1 M NaCl solution and left to equilibrate at 4°C

for 24 hours. The brown aqueous upper phase was removed and the interface washed with aliquots of upper phase from a mixture of 2 volumes of 0.1 M NaCl and 10 volumes of C/M (2 : 1, v/v). Finally the upper phase was removed and a single phase system formed by the addition of a minimum quantity of methanol. The extract was evaporated *in vacuo*, taken up in C/M (2 : 1, v/v) and the washing process repeated twice more.

After the third washing the lower solvent phase containing the majority of the lipid was separated from the light green interfacial fluff. Interfacial material was dried down by evaporation at 40 - 50°C *in vacuo* and extracted with C/M (2 : 1, v/v) to ensure removal of all lipid material. The extract was filtered through Whatman No. 1 paper (previously ether washed) and added to the main solvent extract.

The total pooled extracts were evaporated down *in vacuo* below 50°C and the dark green residue taken up in chloroform into a weighed flask. The chloroform was removed and the lipid extract weighed after drying for 18 hours *in vacuo*.

3. Studies on total lipid extracts

3.1. Determination of chlorophyll

In initial experiments levels of chlorophyll *a* and *b* in the total lipid extract were determined by measuring absorption of light in the 600 - 700 mμ region. The optical density of a known weight of lipid dissolved in diethyl ether (100 ml. was compared spectrophotometrically at the wavelengths of 662 mμ (maxima for chlorophyll *a*) and 644 mμ (maxima for chlorophyll *b*) as reported by Smith and Benitez (1955).

In later work chlorophyll levels were determined quantitatively by the method of Koski *et al.*, (1950) (see Appendix III).

3.2. Separation of lipid components

3.2.1. Thin layer chromatography (T.L.C.)

Thin layer chromatography was utilized in the work to give a rapid separation and identification of galactolipids. Other lipids present in the extracts were identified in many cases.

Thin-layer chromatoplates were prepared according to the method of Stahl (1958) using equipment manufactured by Desaga Co., Heidelburg, Germany. The glass plates (20 x 20 cm.) were coated with a slurry of silica gel G (E. Merck, Darmstadt, Germany.) in water to give a layer thickness of 250 μ. The plates were allowed to dry in air for 10 - 15 minutes prior to activation at 110°C for 1 hour.

Chromatographic tanks were lined with filter paper to ensure vapour saturation of the atmosphere within the tank. Solvent was added to a depth of approximately 0.5 cm.

The lipid mixture dissolved in chloroform was spotted onto the chromatoplates in bands 1 cm. wide using a capillary pipette. Preliminary work indicated that bands gave superior resolution of the lipid mixture compared with single spots which tended to produce streaking of the separated lipid components. On a single chromatoplate (20 x 20 cm.) up to 700 μg. of lipid could be successfully resolved for the isolation of a particular component.

For the measurement of R_f values 50 μg. of lipid was applied to each band on the plates which were subsequently chromatographed over a distance of 15 cm.

Several solvent systems were used to resolve the components of the lipid extract. Initially a solvent system of toluence - ethyl acetate - 95% ethanol (10 : 5 : 5, v/v) (Hawke, J.C., private communication) was utilized to separate pigments, neutral lipids and glycolipids from the phospholipids. Monogalactosyl glyceride and digalactosyl glyceride were identified by reference to pure marker

compounds of red clover galactolipids (supplied by Weenink, R.O., Fats Research Division, D.S.I.R., Wellington) and R_f values were recorded. The monogalactolipid component was observed to chromatograph close to minor lipid and pigment compounds and separation was not considered satisfactory for later work involving elution of the component from the chromatoplates.

The solvent system of Wagner et al., (1961) of chloroform : methanol : water (65 : 25 : 4, v/v) was also examined. This solvent resolved phospholipids as well as the neutral lipids and glycolipids and R_f values were recorded and components identified by the use of specific sprays and marker compounds. Again it was observed that monogalactolipid had not clearly separated from pigment or from an unidentified minor lipid component. In this solvent digalactolipid and phosphatidyl ethanolamine had similar R_f values.

The solvent system of Marinetti et al., (1957) of diisobutyl ketone : acetic acid : water (40 : 25 : 4, v/v) gave fair separations of lipids but diisobutyl ketone proved difficult to evaporate from the chromatoplates.

Accordingly a solvent system was developed to separate the monogalactolipid from pigment and accompanying minor lipids. A mixture of chloroform-methanol (185 : 15 v/v) proved satisfactory and although the solvent mixture was fairly volatile, renewal of the solvent daily was found satisfactory. This system was used for work involving the elution and examination of the monogalactosyl glyceride from leaf tissue.

Lipid components were identified by using the following spray reagents.

(a) General lipid sprays

Iodine (1% in chloroform)
(Sims and Larose, 1962)

Unsaturated lipid gave yellow brown spots but similar colours were given by saturated lipids containing nitrogen. An advantage of this reagent was that iodine would slowly evaporate from the plate used.

2, 7, dichlorofluoroscein (0.2% in 95% ethanol)
(Mangold and Mallins, 1960)

Saturated and unsaturated lipids were detected under ultra-violet (U.V.) light as yellow fluorescent spots on a yellow background. Glycolipids due to their higher concentration in plant lipids were easier to detect than the phospholipids. Following spraying it was often found necessary to warm the chromatoplates slightly for fluorescence to appear.

Rhodamine 6G (0.003% in N NaOH)
(Allen et al., 1964)

This proved to be more senitive than 2, 7, dichlorofluoroscein. Plates were sprayed to near saturation and were then viewed under U.V. light in which lipids fluoresced yellow on a purple-grey background although several lipids appeared as distinct purple bands.

Sulphuric acid (50%)

Plates were sprayed until the layer was saturated with acid and were then heated at 110°C for 30 minutes. Some initial colour development prior to charring was achieved by this method. For example, sterols, pigments and glycolipids initially appeared as coloured bands prior to charring.

(b) Specific spray reagents

Ninhydrin (0.2% in 95 ml. butanol and 5 ml. 10% acetic acid)
(Wagner et al., 1961)

This spray was used to detect lipids containing a free amino group. Plates were lightly sprayed with the reagent and left in the atmosphere for 30 minutes. A purple-blue colouration signified the presence of phosphatidyl ethanolamine or phosphatidyl serine.

Modified Dragendorf Reagent
(Wagner et al., 1961)

Solution A: 40% potassium iodide in water. Solution B: 1.7 g. bismuth subnitrate in 100 ml. of 20% glacial acetic acid. Prior to spraying solutions A and B were mixed as follows:- 20 ml. of solution A plus 5 ml. solution B and 70 ml. of water. Choline-containing phospholipids gave orange spots immediately while galactolipids gave similar coloured spots more slowly.

Phosphate spray
(Dittmer and Lester, 1964)

Solution I: To 1 litre of 25 N H_2SO_4 40.11 g. of MoO_3 was added and the mixture boiled gently until the MoO_3 was dissolved. Solution II: To 500 ml. of Solution I 1.78 g. of powdered molybdenum was added and the mixture boiled for 15 minutes. The solution was cooled and decanted from any residue that may be present. Equal volumes of solutions I and II were mixed with 2 volumes of water. Plates were sprayed lightly until absorbent was damp. Compounds with a phosphate ester group immediately appeared as blue spots on a white-blue grey background.

Aniline hydrogen phthalate (2.5% in water saturated butanol)
(Hough, 1954)

The reagent was sprayed onto the chromatoplates until the absorbent was damp. The plates were then heated approximately 1 hour at 110°C after which galactolipids appeared as brown spots on a white background. Other sugar-containing lipids were not detected.

3.2.2. Column chromatography

Partial separation of the galactolipids was achieved by using columns of diethylaminoethyl (DEAE) cellulose according to the procedure of Allen et al., (1964).

MN cellulose powder 300 DEAE (Macherey, Nagel Co., Germany) was prepared according to the technique of Rouser (1963) in which 20 g. of DEAE cellulose powder was washed on a buchner funnel with 200 ml. of 1N-HCl followed by 500 ml. distilled water, 200 ml. 1N-NaOH and 500 ml. of distilled water. This procedure was repeated three times and the DEAE cellulose was then washed with 200 ml. each of acetone, chloroform, methanol and acetic acid respectively. The DEAE resin was poured as a slurry in acetic acid into a column (50 x 2.5 cm.) and packed under a N_2 pressure of approximately 2 lb./sq. in. After equilibrating overnight the acetic acid was flushed from the column with 200 ml. methanol followed by chloroform to remove the methanol.

A lipid sample (0.34 g. in 5 ml. of chloroform) was carefully applied to the column and the elution sequence described by Allen et al., (1964) was followed (Table 6). Column fractions were examined by thin-layer chromatography.

TABLE 6

Separation sequence of a lipid extract of L. perenne on DEAE cellulose

Eluting solvent	Volume of eluant	Main components eluted
Chloroform	300 ml.	Majority of pigments
C/M (19 : 1, v/v)	500 ml.	Monogalactolipid
C/M (9 : 1, v/v)	400 ml.	Lecithin and digalactolipid
C/M (2 : 1, v/v)	400 ml.	Phosphatidyl ethanolamine
Linear gradient of C/M (2 : 1, v/v) against C/M (2 : 1 v/v) containing 1.5 g. ammonium acetate	600 ml.	Phosphatidyl glycerol Sulpholipid Phosphatidyl inositol

The monogalactolipid fraction was further purified according to the method of O'Brien and Benson (1964) on a silicic acid column using C/M (9 : 1, v/v) as the eluting solvent. Pigment was successfully separated from the monogalactolipid but traces of unidentified minor lipids were found to be present.

3.3. Identification of galactosyl glycerides

The galactosyl glycerides were identified on thin-layer chromatoplates by reference to marker compounds of red clover galactolipids (Weenink, Fats Research Division, D.S.I.R., Wellington) and by comparison with R_f values reported by Lepage (1964) from thin-layer chromatographic studies.

Further structural data on monogalactosyl glyceride was obtained by the chromagraphic examination on the water-soluble hydrolysis products obtained after alkaline and acid hydrolysis.

A lipid sample (approximately 500 µg.) prepared by thin layer chromatography was saponified according to the method of Sastry and Kates (1964). The sample was refluxed at 80 - 90°C for 1 hour in 5 ml. of 0.3N-NaOH in 90% aqueous methanol. The mixture was then shaken with sufficient Dowex 50 ion exchange resin (H^+ form) to remove the sodium ions and was filtered through Whatman No. 1 paper (previously ether washed) to remove the resin. The mixture was then extracted three times with 10 ml. aliquots of petroleum ether to remove liberated fatty acids, and the neutral methanolic water phase was evaporated to dryness at 30 - 35°C *in vacuo*. The residue was dissolved in 0.5 ml. of distilled water.

An aliquot of this solution was evaporated to dryness and hydrolysed at 80 - 90°C for one hour in 3 ml. of 1N-H_2SO_4. The hydrolysate was neutralised by the addition of 5 ml. of N-Ba CO_3 and the insoluble $BaSO_4$ filtered off. The excess barium ions were removed with Dowex 50 (H^+ form) and after filtration to remove the resin the neutral solution was evaporated to dryness *in vacuo* and the residue was dissolved in 0.5 ml. of distilled water.

The hydrolysis products were examined by paper chromatography and by cellulose thin-layer chromatography.

Cellulose thin-layer chromatoplates were prepared by homogenising 15 g. of cellulose powder 300 G (Macherey, Nagel and Co., Germany) with 120 ml. of distilled water in a Waring blendor and spreading the suspension on 20 x 20 cm. chromatoplates to give a layer thickness of 300 μ. Plates were dried for 1 hour at 110°C. Whatman No. 1 chromatography paper cut into strips (15 x 25 cm.) was used for paper chromatographic examination of hydrolysis products. Aqueous hydrolysis products and standards of glucose, galactose and glycerol were spotted onto either paper, or cellulose plates which were developed in the solvent system of pyridine-ethyl acetate - water (1 : 2.5 : 2.5, v/v), (upper phase) (Sastry and Kates, 1964). After drying, the chromatograms were sprayed with a freshly prepared mixture of 2% sodium metaperiodate and 1% potassium permanganate (2 : 1, v/v) (Sastry and Kates, 1964). Compounds gradually appeared as brown spots on a purple background. With this spray glycerol was identified immediately but sugars and other hydrolysis products took up to 20 minutes to develop. The excess spray was washed from the paper chromatograms and compounds remained as brown spots on a white background. Alkaline hydrolysis products were identified by reference to R_f values reported by Sastry and Kates (1964) from work undertaken under similar experimental conditions. Acid hydrolysis products were identified by reference to glucose, galactose and glycerol standards developed simultaneously on the chromatograms.

3.4. Determination of galactolipids in lipid extracts

Galactolipid level, following acid hydrolysis, was estimated as galactose by the orcinol method of Svennerholm (1956).

T.L.C. of 400 μg. of lipid in the solvent systems of chloroform : methanol (185 : 15, v/v) and chloroform : methanol : water (65 : 25 : 4, v/v) were used to separate mono- and digalactosyl glyceride respectively. The components were localised by spraying with 1% iodine in chloroform, marked and after

evaporation of the iodine, the silica gel was carefully scraped off the chromatoplate and transferred to micro-columns.

Preliminary work indicated that the sintered glass disc at the base of the columns did not retain all the silica gel G and subsequently a disc of Whatman No. 1 paper (previously ether washed), 2 - 3 cm. of cotton wool and a further disc of filter paper were placed above the sintered glass disc. Columns were washed with C/M (2 : 1, v/v) prior to addition of the silica gel G. Lipid was eluted with the following solvent sequence: 25 ml. chloroform, 50 ml. of C/M (2 : 1, v/v) and 25 ml. of methanol. The eluting solvents were then evaporated <u>in vacuo</u> at 40 - 50°C.

The lipid sample was dissolved in 0.5 ml. of alcohol and 3 ml. of 1N-H_2SO_4 was then refluxed for one hour. An acid strength of 1N was used as Bailey (1962) had demonstrated that hydrolysis for 60 minutes with 1N acid was sufficient for liberation of plant lipid-bound sugars. Pirt and Whelan (1951) have also demonstrated that losses of liberated sugars in H_2SO_4 become significant if the heating time was extended for more than two hours with acid stronger than 2 N. The hydrolysate was cooled and filtered through acid-washed filter paper into a 10 ml. volumetric flask and the digestion flask rinsed three times with 1 ml. aliquots of distilled water which was added to the total. The contents of the volumetric flask were finally made up to 10 ml. with distilled water.

Galactose was estimated by the orcinol method of Svennerholm (1956). Triplicate 2 ml. aliquots were transferred to stoppered test tubes and placed in an ice bath for 15 minutes. 4 ml. of orcinol (0.2 g. in 200 ml. conc. H_2SO_4) was added to the tubes which were mixed and left in the ice bath for a further 15 minutes. Colour was developed by heating for 20 minutes at 80°C and after chilling the tubes for a further 5 minutes, the absorbance was read at 505 mμ. In each run a blank and standards containing 20 μg, 40 μg, 60, 80 and 100 μg of galactose were processed through all steps.

4. Fatty acid analysis

4.1. Preparation of methyl esters

A sample 0.1 g. of the total lipid was refluxed in 25 ml. of 5% alcoholic KOH for 4 hours at 80 - 90°C. The saponification mixture was reduced to approximately half its volume and transferred to a separating funnel in which non-saponifiable material was extracted three times with 25 ml. aliquots of petroleum ether. The alcoholic phase containing the fatty acid salts was adjusted to pH 1 - 2 by the addition of 6N-HCl and the fatty acids were extracted three times with 25 ml. aliquotsof petroleum ether which after washing with distilled water until neutral was removed *in vacuo*. The fatty acids were weighed after drying *in vacuo* overnight.

The fatty acids were then dissolved in 25 ml. of 1% methanolic H_2SO_4 Hilditch (1956) and refluxed for 3 hours at 80 - 90°C. The mixture was reduced to half-volume and transferred to a separating funnel to which 5 ml. of 10% K_2CO_3 was added to neutralise the H_2SO_4. Methyl esters were extracted with three 25 ml. aliquots of petroleum ether and after washing with water the solvent was removed *in vacuo*.

Methyl esters of the galactolipid components were prepared directly by interesterification of the component (prepared by thin-layer chromatography) in anhydrous 5% methanolic HCl at 80 - 90°C for 1 hour (O'Brien and Benson, 1964). Methyl esters were extracted into petroleum ether and the solvent was removed *in vacuo*.

4.2. Gas-liquid chromatography (G.L.C.)

Methyl esters of the fatty acids were separated by G.L.C. in a PyeArgon chromatograph fitted with an ionisation detector with a 20 millicurrie strontium 90 radiation source (Lovelock, 1958).

Glass columns (6 ft. long and 4 mm. internal diameter) packed with either 20% polyethylene glycol adipate (P.E.G.A.) on 60-80 mesh/in acid-washed Celite or 12% diethylene glycol succinate (D.E.G.S.) on Chromosorb were used.

The chromatograph was operated at a detector voltage of 1250 volts, a column temperature of 160 - 165°C and an argon flow rate of 40 - 50 ml./minute.

A linear detector response was checked by chromatography of National Heart Institute Standards C and D.

Samples of methyl esters (dissolved in petroleum ether) were added to the column in 0.5 cm. sections of capillary tubing.

The proportion of each ester in a sample was obtained by the planimetric method of James (1960). Measurements were repeated ten times for small peaks and three times for larger peaks and the means calculated. The relative proportion of ester was expressed as a percentage of the total peak area on the chromatogram. The averages of three graphs a particular sample was used as the final figures.

4.3 Identification of fatty acids

Fatty acid esters were identified by the following methods.

(a) Co-chromatography with known standards of myristate, palmitate stearate and linolenate.

(b) Relative retention volumes (measured from the negative air peak) were compared with values recorded in the literature and values obtained after chromatography of National Heart Institute Standards C and D.

(c) The graphical relationship between the $\log_{10}$ relative retention volumes and the carbon numbers of the fatty acids of a homologous series (Hawke *et al.*, 1959) was used as a further means of identification.

(d) G.L.C. of the methyl esters of the saturated mono-, di and triunsaturated esters following separation by thin-layer chromatography on silica gel G impregnated with silver nitrate.

Silver nitrate (1.32 g.) in 16 ml. of distilled H_2O was mixed with 6.6 g. silica gel G (E. Merck, Darmstadt, Germany.) according to Mulanicka et al., (1964) and the resultant slurry was spread on chromatoplates by the method of Lees and De Muria (1962). Plates were activated for 1 hour at 110°C prior to use. Methyl esters and standards of palmitate, linoleate and linolenate esters were applied to the chromatoplates prior to development in hexane : diethyl ether (60 : 40, v/v) (Morris, 1962). Components were detected under U.V. light after spraying with 2', 7' dichlorofluorescein (0.2% in 95% ethanol) Mangold, and Mallins, 1960). Components were scraped off the plates into centrifuge tubes and the esters were extracted three times with 5 ml. aliquots of petroleum ether : diethyl ether (1 : 1, v/v). The ester fractions, after removal of solvent in vacuo, were then subjected to G.L.C.

5. The incorporation of acetate-1-C^{14} into the fatty acids of Lolium perenne

5.1. Incubation of leaf tissue

Cross sectional slices of perennial ryegrass (approximately 1 mm. thick) were incubated in a total volume of 4 ml. containing sodium phosphate buffer (200/u moles, pH 7.4), 50/u moles sodium bicarbonate, 50/u moles of potassium chloride and 13.76/u moles (4/u c) of sodium 1-C^{14} acetate (The Radiochemical Centre, Amersham, England) at 30°C for 4 hours according to the procedure of Hawke and Stumpf (1965).

5.2. Separation and analysis of fatty acids

The incubation mixture was saponified with 5 ml. of 15% KOH and fatty acids extracted three times with chloroform after adjustment to pH 1 - 2 with 6N HCl. The combined extracts were washed with 1% acetic acid, dried over anhydrous Na_2SO_4, and suitable aliquot was taken for radioactive measurement.

The extracted fatty acids were methylated as previously described and the methyl esters were subject to T.L.C. on silica gel G in a solvent system of hexane : diethyl ether (60 : 40, v/v) to remove pigments and hydroxy fatty acids. The extracted methyl esters were then separated by silica gel G $AgNO_3$ T.L.C. as previously described into mono-, di- and tri-unsaturated fractions.

These fractions were analysed on a gas-liquid chromatograph (G.L.C. (Aerograph, Model A-90-P2) fitted with a thermal conductivity detector. Columns of diethylene glycol succinate (D.E.G.S.) 12% on chromosorb held at 165°C with a flow rate of 50 - 60 ml. H_2/minute were used. Collection of individual fatty acids was carried out manually with tubes packed with siliconised glass. The methyl esters were recovered by washing the collection tubes with diethyl ether into counting vials.

5.3. Radiochemical analysis of methyl esters

Radioactivity was measured in a liquid scintillation counter (Assembly NE8301; Nuclear Enterprises Ltd.) in 5 ml. of dioxan solution (containing 0.66% para-diphenyl benzene). Individual esters were counted for sufficient time to give a 1,000 counts. Efficiency of counting was 50% with a background of 23 counts/minute.

RESULTS

1. The total lipid content of Lolium perenne and Hordeum vulgare leaf tissue

Table 7 lists the dry weight and lipid content of leaf regions (from 40 days growth) and whole leaves (from 8 days growth) of Lolium perenne plants grown in a 12 hour photoperiod of 2,000 F.C. and day and night temperatures of 21°C and 18.5°C respectively. The feature of the results of this Table is that the dry weight and lipid content increases from the 1 - 10 cm. to the 19 - 28 cm. leaf region of the 40 day old plants.

TABLE 7

The total lipid and dry weight content of Lolium perenne leaf tissue

Days of growth	Leaf region* (length in cm.)	Total lipid (% of wet wt.)	% dry wt.	Total lipid (% of dry wt.)
40	1 - 10	1.34	14.35	9.35
	10 - 19	1.52	15.00	10.12
	19 - 28	1.63	16.75	9.85
8	Whole leaf (length in cm.) 9	1.36	12.95	10.5

* measured from base of plant

Table 8 listing the lipid content of Hordeum vulgare seedlings grown in different light environments (dark, low and full light) for seven days demonstrates that an increase in light appears to stimulate lipid synthesis.

TABLE 8

The total lipid and dry weight of seven day old Hordeum vulgare seedlings grown in different light environments

Light treatment	Length of primary leaf (cm.)	Total lipid (% of wet wt.)	% dry wt.	Total lipid (% of dry wt.)
Dark[1]	11.55	0.60	7.3	8.2
Low light[1]	10.20	0.69	7.2	9.6
Full light[1]	8.38	1.04	9.15	10.4
Dark[2]	14.00	0.35	6.35	5.5
Low light[2]	12.50	0.65	6.24	10.4
Full light[2]	6.85	1.04	9.9	10.5

[1] light germinated

[2] dark germinated

2. Chlorophyll levels of Gramineae leaf tissue

2.1. Chlorophyll levels of Lolium perenne leaf tissue

The levels of chlorophyll a and b were compared by measurement of optical densities at 663 mμ and 644 mμ of a standard lipid solution (0.01 g. in 100 ml. diethyl ether) for each extract (Table 9).

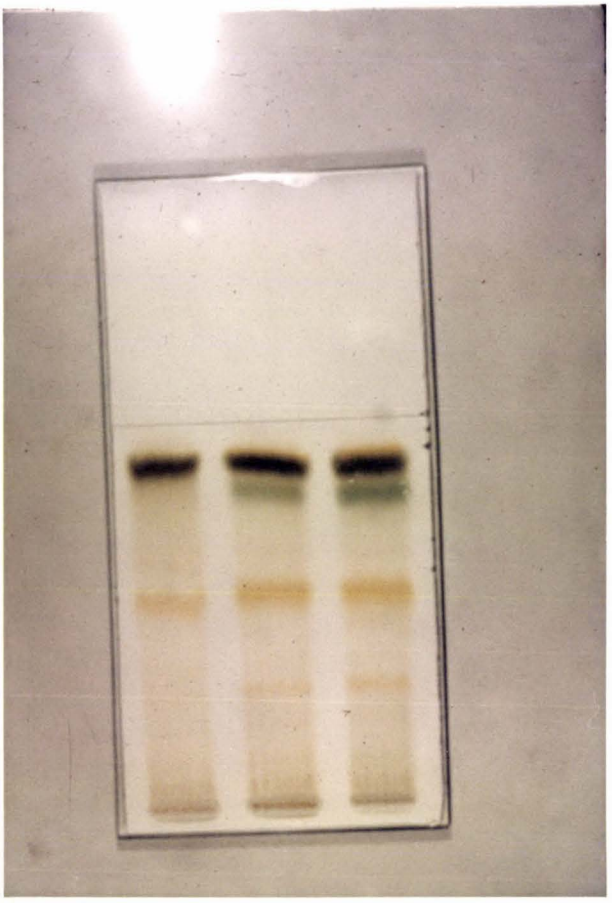
TABLE 2

The comparative levels of chlorophyll a and b in Lolium perenne tissue

Leaf region (length in cm.)	Optical density/g. wet wt. tissue	
	Chlorophyll a (663 mμ)	Chlorophyll b (644 mμ)
1 - 10	0.28	0.08
10 - 19	0.43	0.23
19 - 28	0.51	0.29
Whole leaf (length in cm.)		
9	0.46	0.09

There appears to be an increase in chlorophyll concentration from the 1 -10 to the 19 - 28 cm. leaf region. This relationship between leaf region and pigmentation was supported by the thin-layer chromatography of a standard amount of lipid from each extract. Comparison of the chlorophyll concentration of the 3 leaf regions demonstrates that chlorophyll a and b and other pigments increased from the basal leaf region (Figure 8). The concentration of chlorophyll a in the leaves from 8 day old plants appears to be similar to the 10 - 19 and 19 - 28 cm. leaf region of the older plants (Table 9).

Figure 8



Leaf region	1-10 cm.	10-19 cm.	19-28 cm.

The separation of pigments, from the lipid extracts obtained from leaf regions of L. perenne, by T.L.C. in the solvent system of chloroform-methanol (185 : 15 v/v). The chromatoplate was photographed on High speed Ektachrome Type B film (1/50 sec., $F/_4$).

2.2. The chlorophyll content of Hordeum vulgare leaf tissue

The chlorophyll content of barley seedlings grown in different light environments is shown in Table 10.

TABLE 10

The chlorophyll content of H. vulgare seedlings

Germination conditions	Light treatment	mg./100g. wet wt. tissue	
		Chlorophyll a	Chlorophyll b
Light Germinated	Dark	0.28	0.28
	Low light	3.60	1.40
	Full light	14.00	4.44
Dark Germinated	Dark	0.01	0.02
	Low light	5.75	1.33
	Full light	16.70	3.84

3. The identification of the methyl esters of the fatty acids of L. perenne and H. vulgare by G.L.C.

The retention times of the component fatty acids from two Gramineae species relative to methyl palmitate on P.E.G.A. are presented in Table 11. The relative retention times of National Heart Institute Standards and retention values as reported by Burchfield and Storrs (1962) are recorded in Table 11.

TABLE 11

Relative retention volumes on polyethylene glycol adipate at 163°C of the methyl esters of L. perenne and H. vulgare

Chemical name of fatty acid	Shorthand notation	Relative retention volumes			
		L.perenne	H.vulgare	N.H.I. Stds.	Burchfield & Storrs (1962)*
n - decanoic	10 : 0			0.12	0.134
n - dodecanoic	12 : 0	0.27	0.26	0.25	0.245
n - tetradecanoic	14 : 0	0.50	0.50	0.50	0.50
n - hexadecanoic	16 : 0	1.00	1.00	1.00	1.00
n - hexadecenoic	16 : 1	1.14	-	1.14	1.15
n - hexadecenoic	16 : 1	1.21	1.21	-	-
n - octadecanoic	18 : 0	2.02	1.99	2.01	1.99
n - octadecenoic	18 : 1	2.26	2.22	2.22	2.23
n - octadecadienoic	18 : 2	2.72	2.69		2.70
n - octadecatrienoic	18 : 3	3.54	3.49		3.51
n - eicosanoic	20 : 0			4.14	
unidentified	20. X ?	4.80			

* Measured at 173°C.

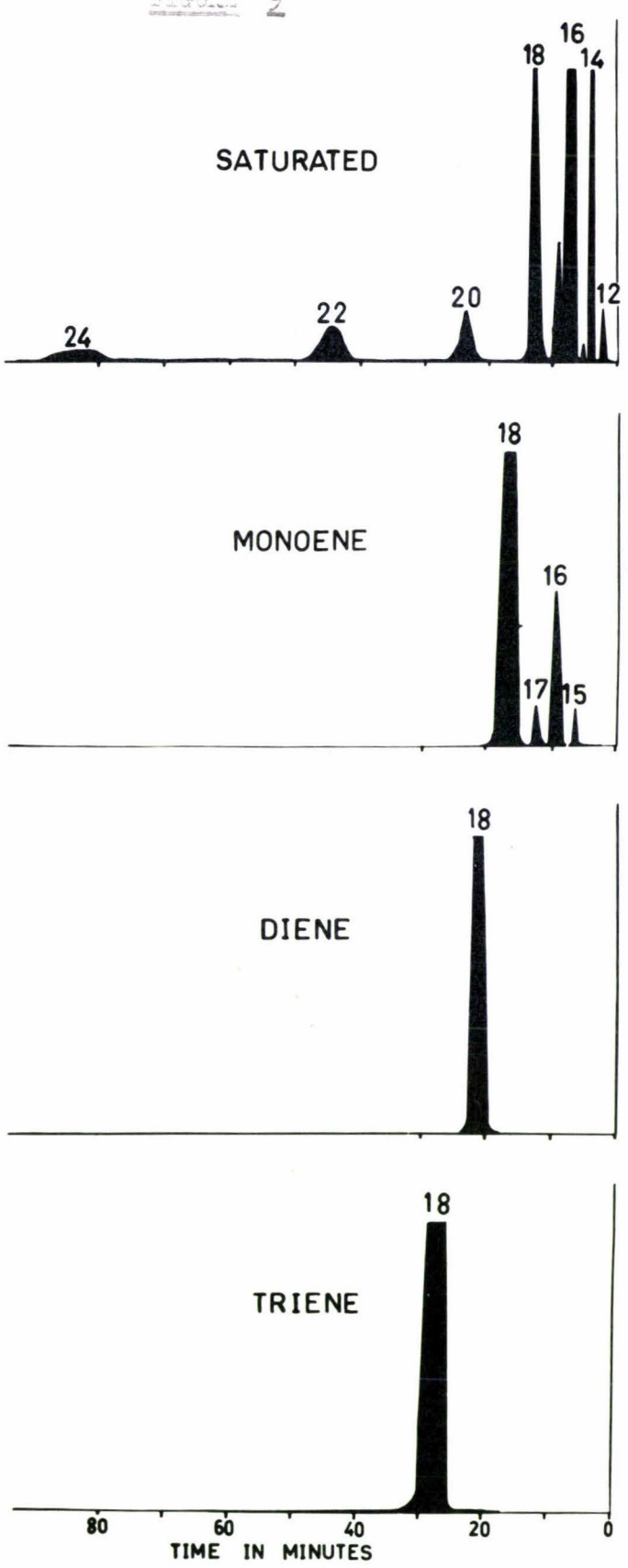
The relative retention volumes of a homologous series of the methyl esters exhibited the expected logarithmic relationship with the number of carbon atoms in the fatty acid (Hawke et al., 1959). As found by these workers a plot of the $\log_{10}$ of the relative retention volumes against carbon number for each homologous series fell along parallel straight lines.

Further confirmation on the identity of the fatty acids of L. perenne was obtained by G.L.C. of the esters after their separation into saturated, monoene, diene and triene fractions by silver nitrate/silica gel G thin-layer chromatography. The chromatographic traces are shown in Figure 9 while the relationship between the $\log_{10}$ relative retention volume versus carbon number is presented in Figure 10.

Saturated acids containing 12, 14, 15, 16, 18, 20, 22 and 24 carbon atoms were identified following preparative T.L.C. on $AgNO_3$ silica gel G. A further small peak was present in the gas-liquid chromatogram of the saturated fraction which corresponds to a 16 carbon compound in the monoene fraction. This could have been due to a incomplete separation between the monoene and saturated bands on the thin-layer chromatoplate. The fact that there was no contamination of oleate in the saturated fraction supports a proposal that this 16 carbon monoene fatty acid could be a trans-isomer, since Morris (1962) has reported that trans isomers of monoene fatty acids have higher R_f values than cis isomers on $AgNO_3$/ thin-layer chromatoplates. Further confirmation that this 16 carbon monoene acid was a trans-isomer was achieved by elution of the methyl esters corresponding to a standard of methyl elaidate from a $AgNO_3$ thin-layer chromatoplate. G.L.C. showed considerable concentration of this 16 : 1 fatty acid in this fraction. It was also demonstrated that traces of the 16 : 1 cis isomer were present as confirmed by G.L.C. following preparative $AgNO_3$/T.L.C. This fatty acid had a relative retention time that was similar to palmitoleic acid on P.E.G.A. and D.E.G.S. columns, compared to the higher relative retention time of the trans-isomer.

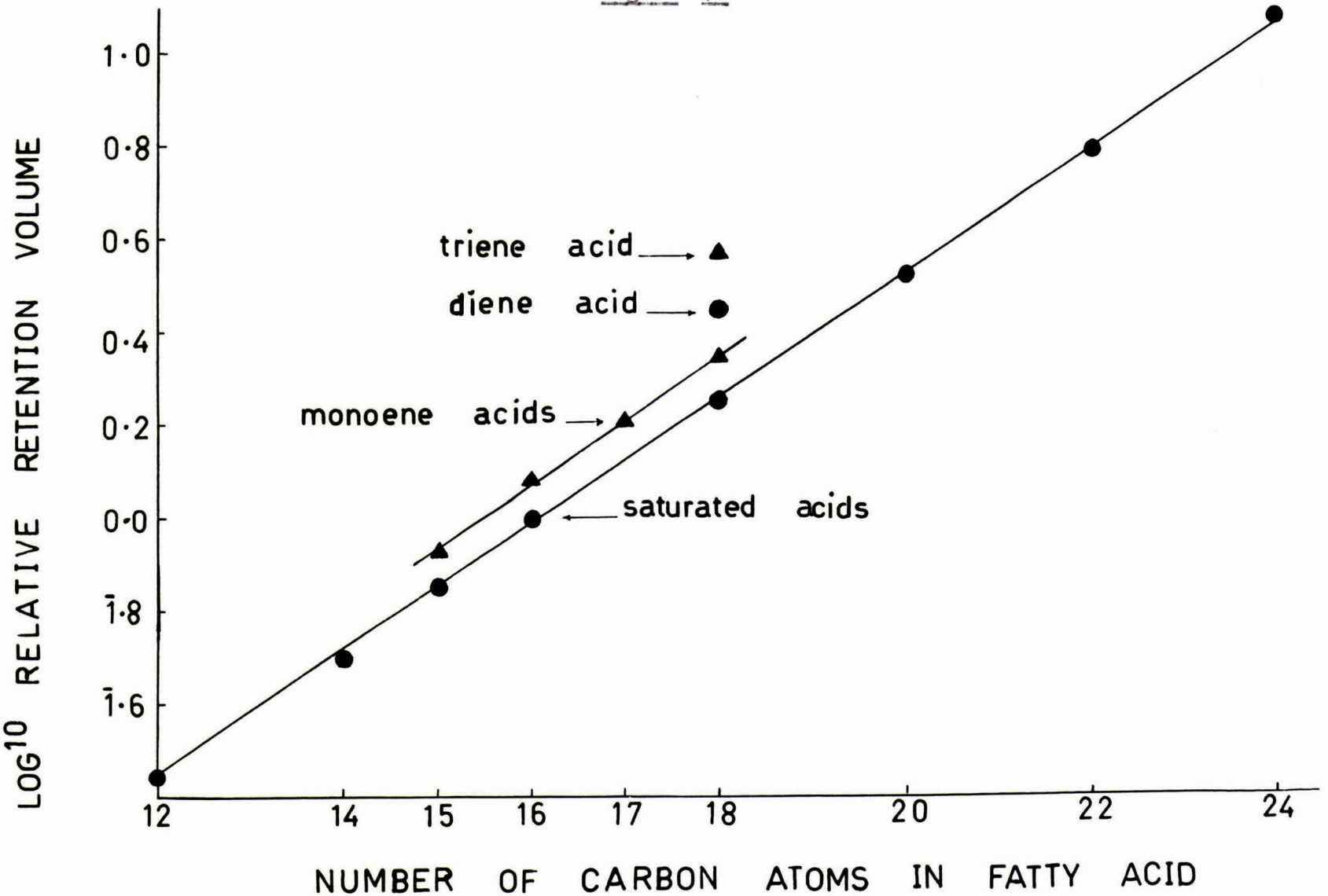
Other mono-unsaturated fatty acids with 15, 17, 18 carbon atoms were identified, as also were the 18 diene and triene acids in other fractions.

FIGURE 2



The gas liquid chromatographic traces of the methyl esters of L. perenne (on D.E.G.S. liquid phase at 165°) after separation into saturated mono-, di- and tri-unsaturated fractions by $AgNO_3$/silica gel G T.L.C.

Figure 10



The relationship between the carbon number of the fatty acid and the $\log_{10}$ relative retention volume, for the fatty acid fractions of L. peruana separated by $AgNO_3$/silica gel G T.L.C. prior to G.L.C. on D.E.G.S. liquid phase at 165°.

4. The yields of fatty acids and their composition from the total lipid of Gramineae

The yields of fatty acids from the lipid extracts, obtained from L. perenne leaf tissue, is shown in Table 12 while the fatty acid composition of the lipid is presented in Tables 13 and 14 and Figure 11. The gas-liquid chromatographic traces of the leaf regions of mature plants are shown in Figure 12.

TABLE 12

The yield of fatty acids from the total lipid extracts of L. perenne tissue

Days of growth	Leaf region (length in cm.)	Total lipid (% of wet wt.)	Fatty acids (% of total lipid)
	1 - 10	1.34	51.2
40	10 - 19	1.52	51.6
	19 - 28	1.63	42.3
	Whole leaf (length in cm.)		
8	9	1.36	51.2

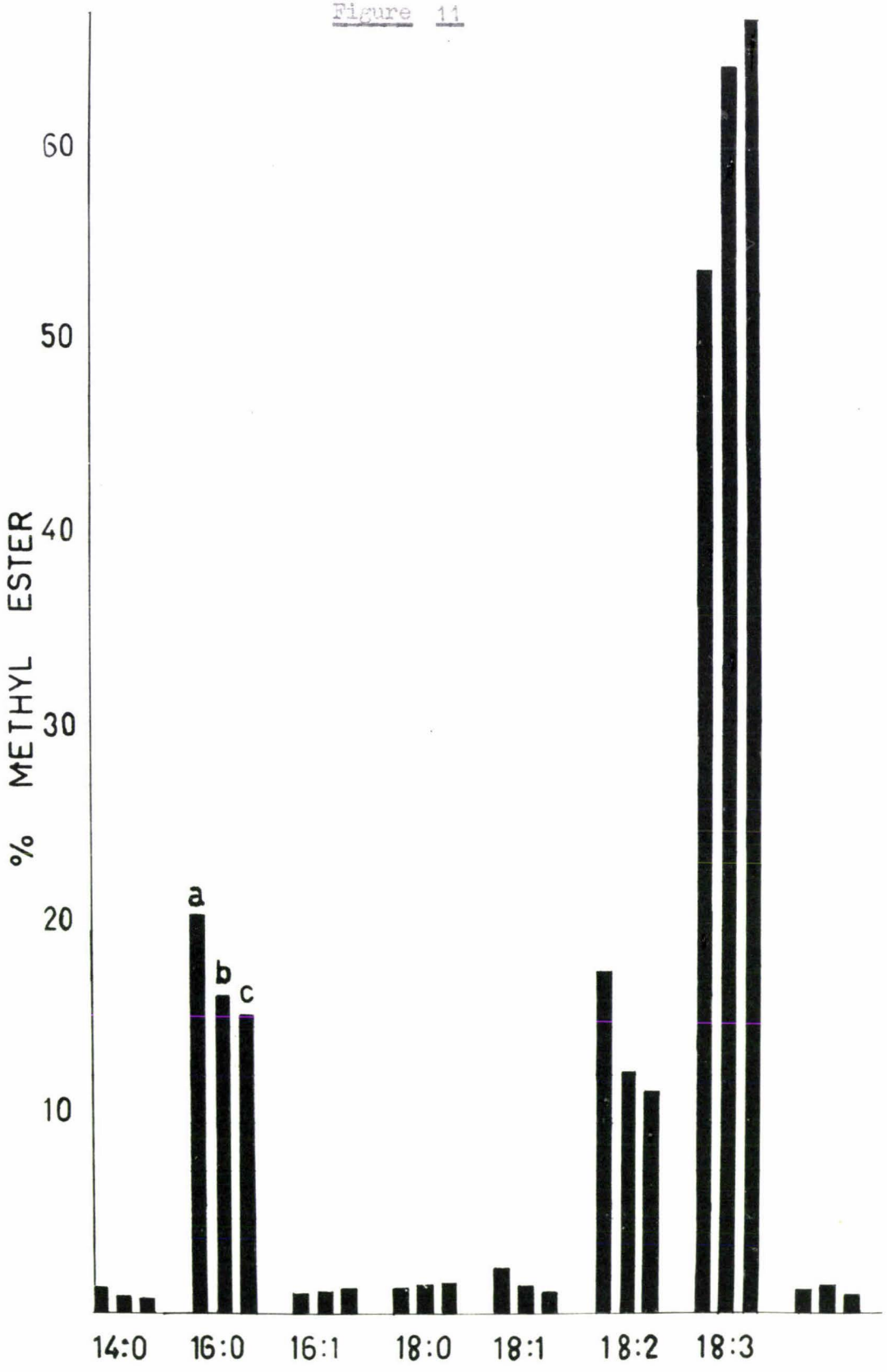
TABLE 13

The fatty acid composition (moles %) of the total lipids of *L. perenne* leaf regions

Chemical name of fatty acid	Shorthand notation	Leaf region of plant		
		0-9 cm.	9-18 cm.	18-27 cm.
n - dodecanoic	12 : 0	trace	-	-
n - tetradecanoic	14 : 0	1.30	0.90	0.70
n - hexadecanoic	16 : 0	20.90	16.60	15.50
n - hexadecenoic	16 : 1	1.00	1.10	1.40
n - octadecanoic	18 : 0	1.40	1.50	1.60
n - octadecenoic	18 : 1	2.40	1.30	1.20
n - octadecadienoic	18 : 2	17.90	12.60	11.70
n - octadecatrienoic	18 : 3	53.90	64.60	67.00
Unidentified		1.20	1.40	0.90

It will be seen from Table 13 that the main differences in the fatty acid composition of the leaf regions (from 40 days growth) are the increased proportions of linolenic acid and decreased proportions of palmitic and linoleic acid from the basal to the distal leaf region of the plant. Minor differences in the proportions of a hexadecenoic acid of uncertain structure and of oleic acid was also observed from different leaf regions. The central leaf region was found to give a value which was intermediate for each fatty acid.

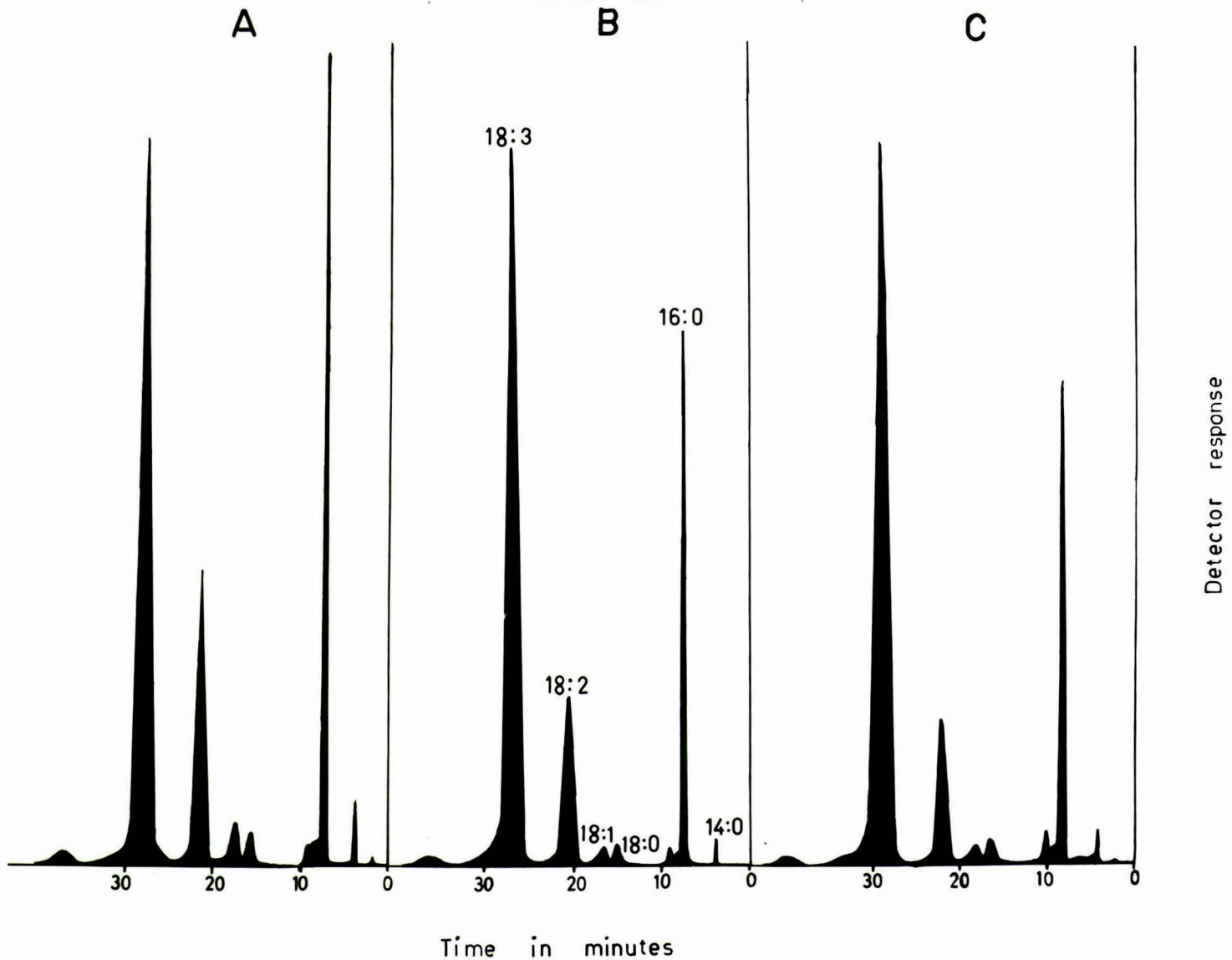
Figure 11



The fatty acid composition of leaf regions of L. perenne plants grown for 40 days. The unlabelled fatty acid was not identified.

a = 1 - 10 cm. leaf region; b = 10 - 19 cm. leaf region;
c = 19 - 28 cm. leaf region.

Figure 12



The gas-liquid chromatographic traces of the methyl esters of the fatty acids of L. perenne leaf regions on P.E.G.A. at 165°C.

A = 1 - 10 cm. leaf region
B = 10 - 19 cm. leaf region
C = 19 - 28 cm. leaf region

TABLE 14

The fatty acid composition (moles %) of the total lipid of eight day old L. perenne leaves

Chemical name of fatty acid	Shorthand notation	Moles %
n - tetradecanoic	14 : 0	0.20
n - hexadecanoic	16 : 0	11.90
n - hexadecanoic	16 : 1	1.70
n - octadecanoic	18 : 0	1.00
n - octadecenoic	18 : 1	2.20
n - octadecadienoic	18 : 2	14.60
n - octadecatrienoic	18 : 3	68.20
Unidentified		0.20

The fatty acid composition of 8 day old leaves from L. perenne plants (see Table 14) appears to resemble most closely the composition of the 18 - 27 cm. leaf region of L. perenne plants grown for 40 days.

TABLE 15

The yield of fatty acids from the total lipid of *H. vulgare* seedlings

Light treatment	Total lipid (% of wet wt.)	Fatty acids (% of total lipid)
dark	0.60	60.0
low light	0.69	54.5
full light	1.04	51.5

TABLE 16

The fatty acid composition (moles %) of total lipids of *H. vulgare* seedlings grown in different light environments

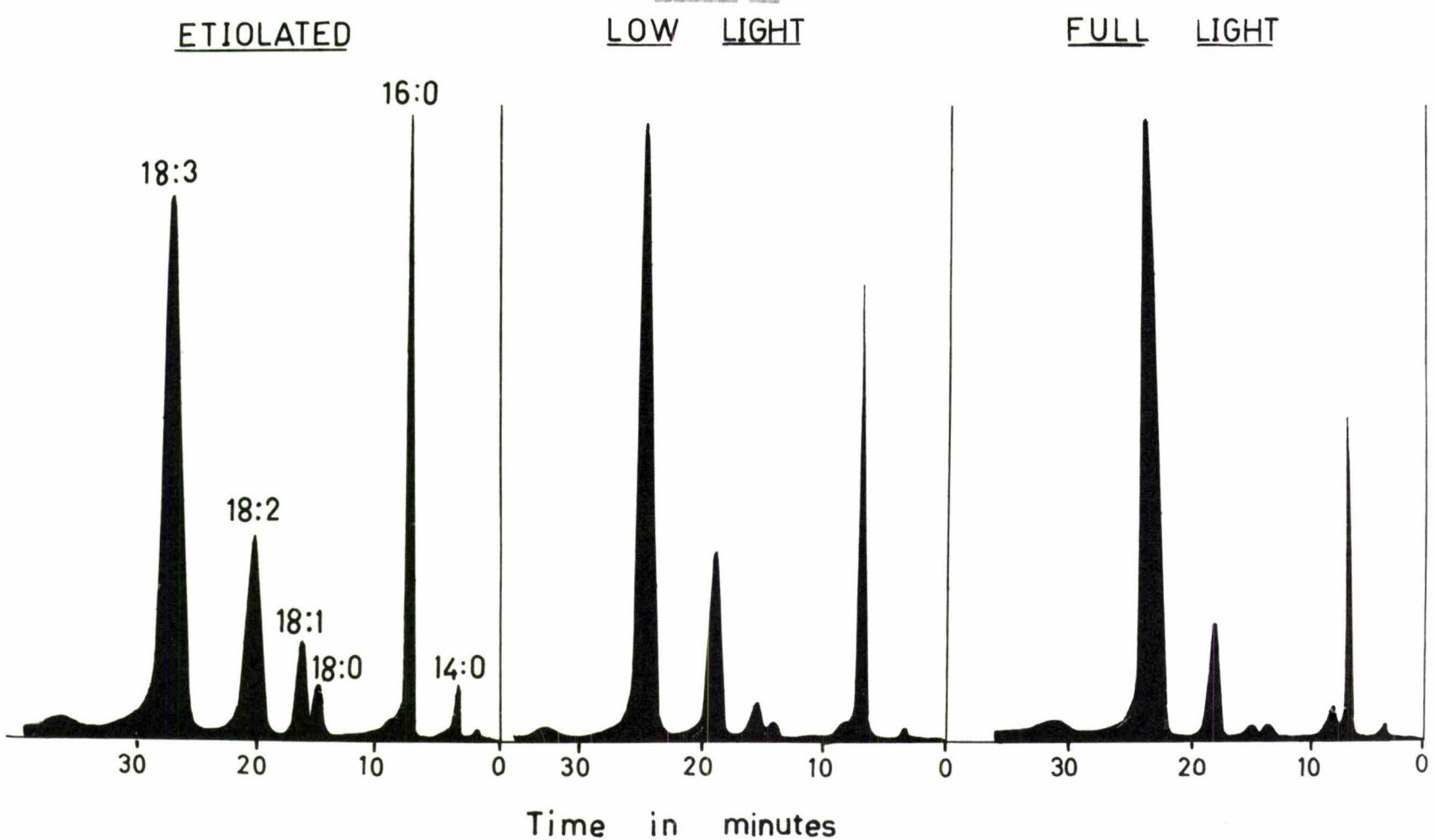
Chemical name of fatty acid	Shorthand notation	Light treatment		
		Dark	Low light	Full light
n - dodecanoic	12 : 0	0.30	trace	trace
n - tetradecanoic	14 : 0	1.40	0.30	1.30
n - hexadecanoic	16 : 0	19.50	17.50	12.40
n hexadecenoic	16 : 1	1.00	0.90	1.60
n - octadecanoic	18 : 0	3.00	1.00	1.00
n - octadecenoic	18 : 1	6.80	2.60	1.10
n - octadecadienoic	18 : 2	15.10	13.70	9.40
n - octadecatrienoic	18 : 3	51.80	61.60	71.10
Unidentified		1.10	2.40	3.10

The yield of fatty acids and composition from *H. vulgare* seedlings grown in different light environments isshown in Tables 15 and 16 respectively, while Figure 13 shows the gas-liquid chromatographic traces.

Increased exposure to light increased the relative proportions of linolenic acid while there were corresponding decreases in the relative percentages of stearic, palmitic, oleic and linoleic acids.

Trace amounts of 15 : 0, 17 : 0, 17 : 1, 22 : 0 and 24 : 0 were identified when comparatively large sample sizes of Gramineae esters were separated by G.L.C. Arachidic acid was not identified in the total lipid methyl esters since this fatty acid has a similar retention time to linolenic acid on adipate columns. However, G.L.C. following T.L.C. on $AgNO_3$/silica gel G showed that levels of the 20 : 0 fatty acid was similar to the 22 : 0 fatty acid (see Figure 9).

Figure 13



The gas-liquid chromatographic traces of the methyl esters of the fatty acids of Hordeum vulgare (grown in different light environments) on P.E.G.A. at 165°C.

5. The identification of the Glycolipids and phospholipids of L. perenne by thin-layer chromatography

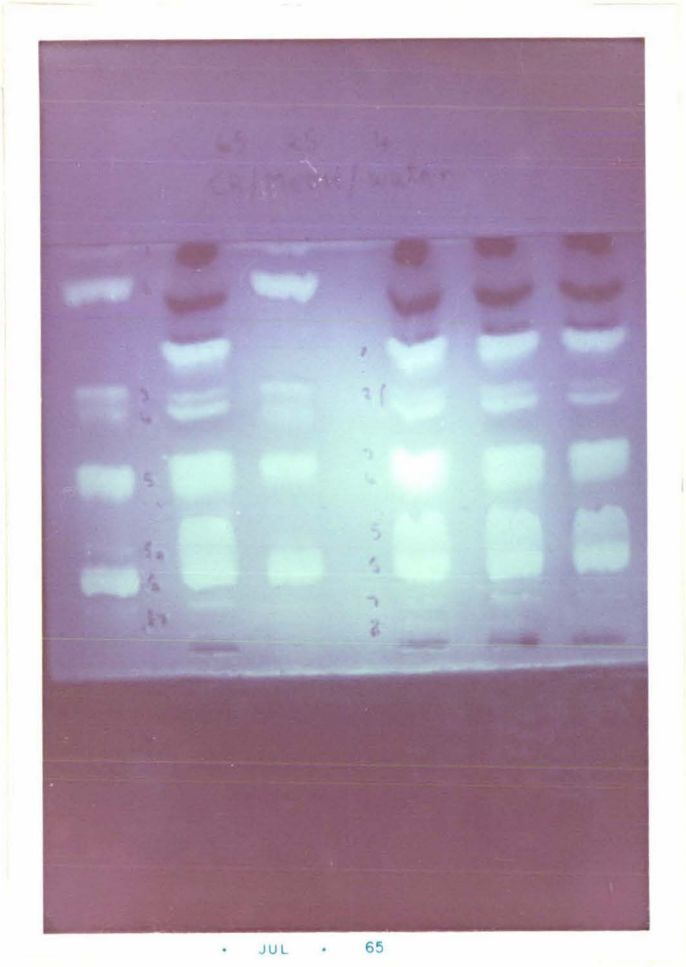
Thin-layer chromatography on silica Gel G using a solvent system of chloroform - methanol - water (65 : 25 : 4, v/v) was used to identify the lipids of L. perenne. Compounds were identified by reference to authentic samples of galactolipids, phosphatidyl choline and phosphatidyl ethanolamine, and by the use of specific sprays (Table 17). The relative retention values reported by Lepage (1964) were used as a further aid in identification.

Visual examination of the T.L. chromatoplates after charring with 50% H_2SO_4 showed that the galactosyl glycerides and phosphatidyl choline appeared to be the lipid components present in highest concentrations.

The positive identification of the components chromatographing at R_f 0.72 and R_f 0.69 was not made but it was observed that these two components chromatographed similarly to the two components in ovine brain cerebroside. (Rumbsby, M.G., personal communication). In this connection Sastry and Kates (1964) have reported the isolation of glucocerebrosides compounds from Phaseoulus bean species.

Typical separations of L. perenne lipids and ovine brain lipids are shown in Figure 14.

Figure 14



Band number 1 2 3 4 5 6

The separation of the lipids of L. perenne and ovine brain by T.L.C. on silica gel G in the solvent system of chloroform - methanol - water (65 : 25 : 4, v/v). The chromatoplate was sprayed with 0.003% Rhodamine 6G in N NaOH and photographed while still damp under U.V. light using High speed Ektachrome film (1 sec. at F/4).

The components of L. perenne lipids are as numbered on band 4, e.g.

1 monogalactosyl glyceride; 2 cerebroside; 3 digalactosyl glyceride; 4 phosphatidyl ethanolamine; 5 phosphatidyl glycerol and sulpholipid; 6 phosphatidyl choline; 7 phosphatidyl inositol; 8 minor component possibly phosphatidyl serine.

The black components above monogalactosyl glyceride are pigments that do not fluoresce. Ovine brain cerebroside is demonstrated as components 3 and 4 in band 1.

TABLE 17

The spray reactions and R_f values (of L. perenne) glycolipids and phospholipids resolved in chloroform : methanol : water (65 : 25 : 4, v/v) by thin-layer chromatography

Component	Reaction of spray reagents				R_f values	
	Dragendorf	Ninhydrin	Phosphate	Aniline phthalate	Observed	Lepage (1964)
monogalactosyl glyceride	+	-	-	+	0.77	0.77
unidentified	-	-	-	-	0.72	0.73
unidentified	-	-	-	-	0.69	0.70
digalactosyl glyceride	+	-	-	+	0.60	0.62
phosphatidyl ethanolamine	-	+	+	-	0.58	0.62
phosphatidyl glycerol	-	-	+	-	0.43	0.48
sulpholipid	-	-	-	-	0.40	0.42
phosphatidyl choline	+	-	+	-	0.37	0.33
phosphatidyl inositol	-	-	+	-	0.24	0.23
unidentified	-	-	-	-	0.17	
minor component (possibly phosphatidyl serine)	-	+	+	-	0.06	0.14

+ = positive reaction

- = negative reaction

6. The identification of galactosyl glycerides in plant lipid extracts

Confirmation that the galactolipids had been correctly identified by the previous techniques was carried out as follows:-

(a) Thin-layer chromatography with authentic samples in additional solvent systems. (See Table 18). The separation of the galactolipids by T.L.C. is demonstrated in Figures 15 and 16.

TABLE 18

The R_f values of the galactosyl glycerides of L. perenne determined by thin-layer chromatography in different solvent systems

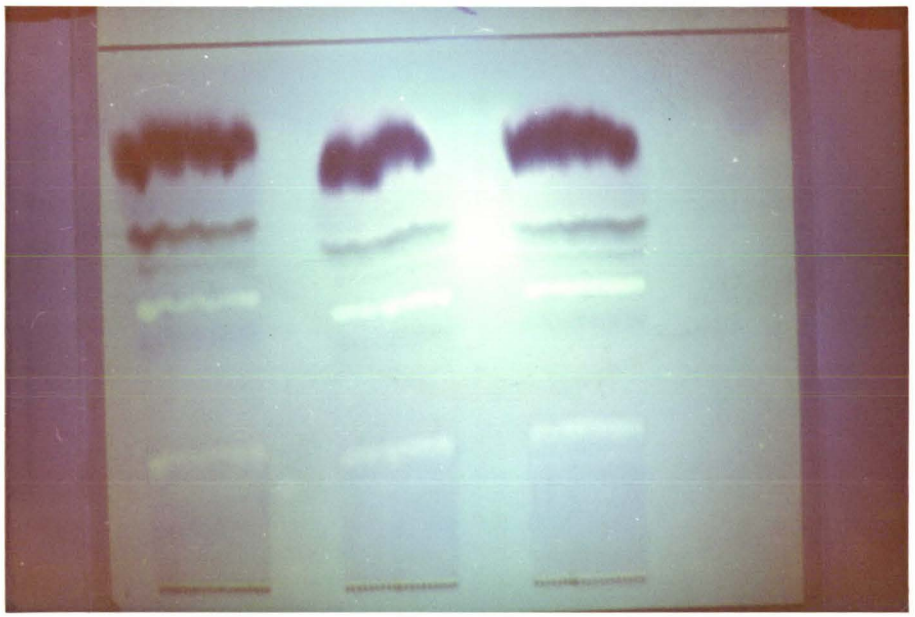
Component	Solvent A		Solvent B		Solvent C	
	Observed	Lepage (1964)	Observed	Lepage (1964)	Observed	Havre & Stumpf (1965)
monogalactosyl glyceride	0.78	0.77	0.50	0.51	0.17	0.20
digalactosyl glyceride	0.60	0.62	0.30	0.25	0.56	0.60

Solvent A Chloroform : methanol : water (65 : 25 : 4, v/v)

Solvent B Diisobutyl ketone : acetic acid : water (40 : 25 : 4, v/v)

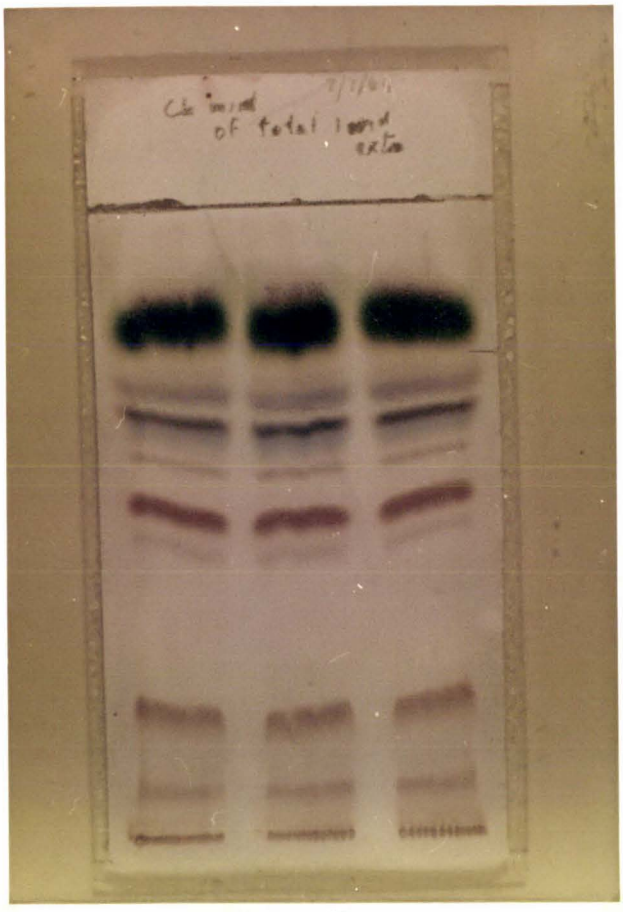
Solvent C Toluene : ethyl acetate : 95% ethanol (10 : 5 : 5, v/v)

Figure 15



The separation of L. perenne galactolipids by T.L.C. in the solvent system of toluene : ethyl acetate : 95% ethanol (10 : 5 : 5). The chromatoplate was sprayed with 2, 7, dichlorcfluoroscein (0.2% in 95% ethanol) and photographed under U.V. light on Kodacolor-X film (1 sec at $^{F}/_{1.8}$).

Figure 16



The separation of L. perenne galactolipids by T.L.C. in the solvent system of toluence : ethyl acetate : 95% ethanol (10 : 5 : 5). The chromatoplate was sprayed with 50% sulphuric acid, partly charred, and photographed under ordinary light on Kodacolor-X film at ($\frac{1}{2}$ sec. at $^{F}/_{1.8}$).

(b) Paper chromatography and cellulose thin layer chromatography of the alkaline and acidic hydrolysis products of the monogalactosyl glyceride in the solvent system of pyridine : ethyl acetate : water (1 : 2.5 : 2 : 5, v/v upper phase) (Figure 17).

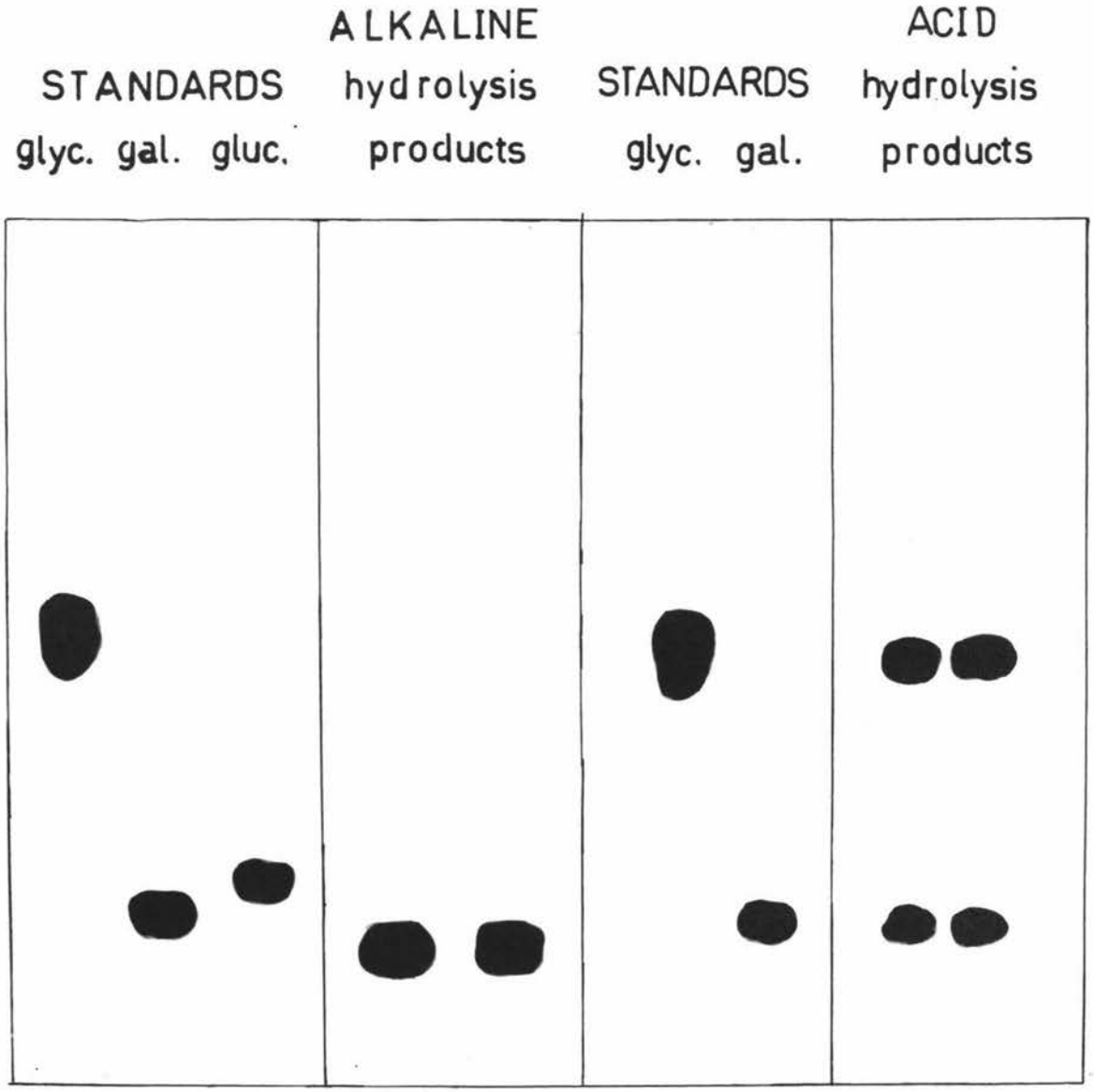
The alkali hydrolysis product was identified by the reference to the R_{gal} reported by Sastry and Kates (1964) while the acid hydrolysis products were identified by authentic standards chromatographed under similar conditions. The R_f values on cellulose thin-layers are reported in Table 19.

TABLE 19

The R_f and R_{gal} values of the hydrolysis products of L. perenne monogalactosyl glyceride separated on thin-layer cellulose chromatograms in the solvent system of pyridine : ethyl acetate : water (1 : 2.5 : 2.5, v/v, upper phase)

Component	R_f values		R_{gal} values	
	Observed	Standard	Observed	Sastry & Kates (1964)
monogalactosyl glycerol	0.15		0.79	0.80
galactose	0.19	0.19		
glucose		0.26		
glycerol	0.50	0.53		

FIGURE 17



The separation of the alkaline and acid hydrolysis products of L. perenne monogalactosyl glyceride, on a cellulose thin-layer chromatoplate in the solvent system of pyridine : ethyl acetate : water (1 : 2.5 : 2.5, v/v, upper phase).

glyc. = glycerol
gal. = galactose
gluc. = glucose

7. The galactolipid content of Gramineae leaf tissue

The galactolipid content of L. perenne expressed as a percentage of the wet weight of tissue and a percentage of the total lipid is reported in Table 20. This data is calculated from data shown in Appendix IV. Table 20 shows that the galactolipid content expressed as a percentage of the wet weight increases from the 1 - 10 cm. to the 19 - 28 cm. leaf region. These differences are not as evident when the galactolipid content is expressed as a percentage of the total lipid.

As with the chlorophyll a content the galactolipid content of the 8 day old leaves shows a similar relation to the 10 - 19 cm. leaf region from leaves grown for 40 days.

TABLE 20

The galactolipid content of L. perenne leaf tissue

Leaf region (cm.)	Total lipid (% of wet wt.)	Galactolipid content					
		% of wet wt.			% of total lipid		
		GDG	GGDG	Total	GDG	GGDG	Total
1 - 10	1.34	0.25	0.15	0.40	19.00	11.20	30.2
10 - 19	1.52	0.31	0.19	0.50	20.30	12.50	32.8
19 - 28	1.63	0.35	0.21	0.56	21.20	12.60	33.6
Leaf region (cm.)							
9	1.36	0.28	0.19	0.47	20.9	12.8	33.7

GDG = monogalactolipid GGDG = digalactolipid

The galactolipid content of barley seedlings (light germinated group) is reported in Table 21. The content of galactolipid when expressed as a percentage of the wet weight increases as the plant receives more light.

TABLE 21

The galactolipid content of H. vulgare seedlings

Light treatment	Total lipid (% of wet wt.)	Galactolipid content % of wet wt.			% of total lipid		
		GDG	GGDG	Total	GDG	GGDG	Total
dark	0.60	0.08	0.03	0.11	13.1	5.5	18.6
low light	0.69	0.12	0.04	0.16	20.4	7.1	27.5
full light	1.04	0.21	0.10	0.31	20.6	8.8	29.4

This relationship is also evident when the galactolipid content was expressed as a percentage of the total lipid. This data was supported by applying equal volumes of standard lipid solutions from each treatment to a chromatoplate, spraying with 50% H_2SO_4 and charring. The galactolipid content (especially the monogalactosyl glyceride) showed an increase in content with increased exposure to light. It was also observed that the phospholipids, especially phosphatidyl choline, decreased in concentration in the lipid extracts from the seedlings grown in the dark to the seedlings grown in full light.

8. The fatty acid composition of monogalactosyl glyceride isolated from Gramineae leaf tissue lipids

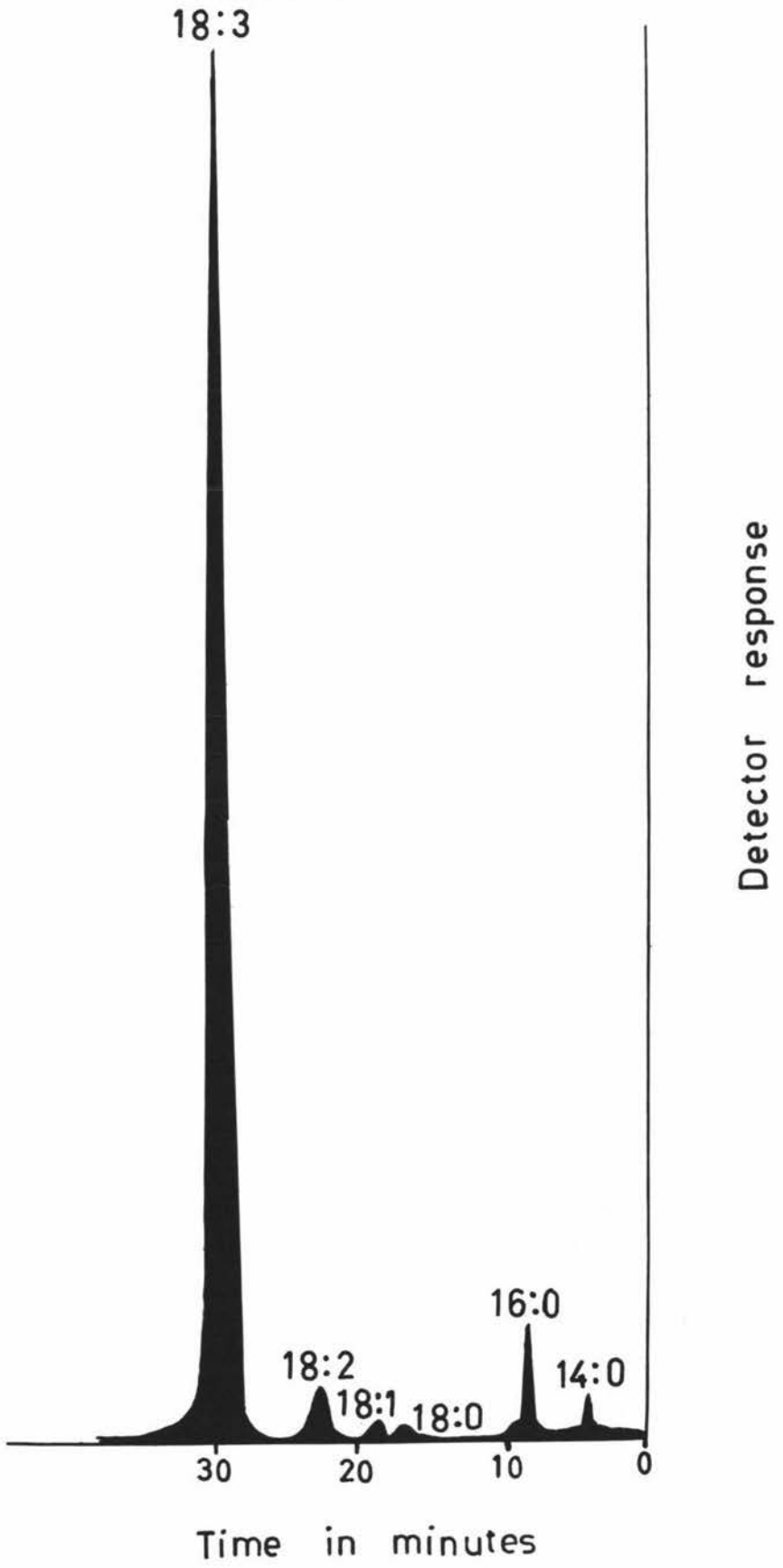
The fatty acid composition of the monogalactosyl glyceride component isolated from L. perenne lipids is reported in Tables 22 and 23. The feature of these tables is that there is little difference in the fatty acid composition in tissue of varying maturity. Compared with the fatty acid composition of the total lipids, higher percentages of linolenate, and lower percentages of palmitate and linoleate are reported. A gas-liquid chromatographic trace of the methyl esters of the fatty acids of L. perenne monogalactolipid is shown in Figure 18.

TABLE 22

The fatty acid composition (moles %) of monogalactosyl glyceride from L. perenne leaf regions

Chemical name of fatty acid	Shorthand notation	Leaf Region of Plant		
		1-10 cm.	10-19 cm.	19-29cm.
n - tetradecanoic	14 : 0	0.60	0.30	0.70
n - hexadecanoic	16 : 0	3.10	2.80	5.30
n - octadecanoic	18 : 0	1.30	1.00	1.50
n - octadecenoic	18 : 1	1.50	1.20	1.40
n - octadecadienoic	18 : 2	5.50	3.90	2.50
n - octadecatrienoic	18 : 3	88.00	90.80	88.60

FIGURE 19



A gas-liquid chromatographic trace of the methyl esters of the fatty acids of L. perenne monogalactolipid on P.E.G.A. liquid phase at 165°C.

TABLE 23

The fatty acid composition of monogalactosyl glyceride from eight day old leaves of L. perenne

Chemical name of fatty acid	Shorthand notation	Moles %
n - tetradecanoic	14 : 0	0.20
n - hexadecanoic	16 : 0	3.40
n - octadecanoic	18 : 0	0.90
n - octadecenoic	18 : 1	1.90
n - octadecadienoic	18 : 2	3.20
n - octadecatrienoic	18 : 3	90.4

The fatty acid composition of the monogalactolipid component isolated from H. vulgare seedlings, grown in the dark and full light is reported in Table 24. Only minor differences in fatty acid composition between treatments are demonstrated. This is in contrast to the major differences in fatty acid composition of the total lipids as reported in Table 16.

TABLE 24

The fatty acid composition of the monogalactolipid component from *H. vulgare* seedlings grow in dark and full light

Chemical name of fatty acid	Shorthand notation	Light treatment	
		dark	full light
n - tetradecanoic	14 : 0	0.5	trace
n - hexadecanoic	16 : 0	7.5	4.80
n - octadecanoic	18 : 0	3.8	1.30
n - octadecenoic	18 : 1	5.3	1.60
n - octadecadienoic	18 : 2	1.90	1.00
n - octadecatrienoic	18 : 3	78.0	85.60
Unidentified		3.0	5.70

9. The incorporation of acetate 1-C^{14} into the fatty acids of L. perenne

Table 25 demonstrates the influence of light on the incorporation and distribution of radioactivity in the fatty acids of L. perenne, after incubation of leaf tissue with acetate 1-C^{14}. The feature of the Table is that L. perenne is able to synthesise long chain saturated fatty acids with 20, 22, 24 and 26 carbon atoms. An appreciable proportion of acetate is incorporated into these acids, by either seedling tissue or tissue from mature plants, although these acids appear to constitute a minor percentage of the endogenous lipids. Increased light appears to stimulate the incorporation of acetate into unsaturated acids.

TABLE 25

The influence of light on the incorporation and distribution % of radioactivity in the fatty acids of L. perenne tissue

Light treatment	Seedling tissue						Tissue from mature plants	
	Dark		low light		full light		full light	
Experiment	A	B	A	B	A	B	A	B
Chlorophyll a (mg./100g. wet wt. tissue)	0.43	0.15	8.3	6.6		17.5	17.5	21.7
Chlorophyll b (mg./100g. wet wt. tissue)	-	-	1.82	1.65		3.2	5.32	5.85
% incorporation of radioactivity into long chain fatty acids	2.42	1.95	3.13	3.45		1.8	5.5	1.1
Fatty acid	Distribution of radioactivity(%)							
14 : 0	2.0	2.4	3.8	2.8		7.3	6.1	4.3
16 : 0	12.2	32.4	17.5	31.0		22.4	24.5	22.7
16 : 1			1.6			2.8	7.0	
18 : 0	8.8	1.9	16.0	16.4		10.8	12.2	25.7
18 : 1	8.8	13.0	6.7	15.7		14.4	27.4	5.8
18 : 2	18.4	tr.	5.1	5.5		tr.	3.6	tr.
18 : 3	0.8	tr.	3.9	4.8		tr.	1.6	tr.
20 : 0	6.0	7.2	6.4	8.8		5.7	5.6	6.1
22 : 0	10.7	11.8	14.6	4.6		8.6	2.6	6.5
24 : 0	7.0	11.6	6.8	3.2		3.7	5.6	5.8
26 : 0	25.3	19.7	17.6	7.2		24.3	1.8	23.1

D I S C U S S I O N

1. The lipid content of Gramineae tissue

In the present work values ranging from 9.35 to 10.5% lipid (of the dry wt.) were reported for L. perenne leaf tissue. This appears to be higher than previously reported for Gramineae leaf tissue.

Hilditch and Williams (1965) have summarised recent work on leaf lipids and have stated that values between 4 and 6% appear to be the average lipid content of leaves. In studies in Gramineae tissue Smith and Chiball (1932) and Pollard (1936) have reported values ranging from 3.8 to 6.5% lipid (of the dry wt.) for cocksfoot (Dactylis glomerata) while Shorland (1944), (1961) found values of 5.67 and 6.12% lipid in the same species. Shorland (1941) has stated that a mixed pasture consisting of mainly ryegrass, cocksfoot, Yorkshire fog, sweet vernal and white clover contained 5.95% lipid (of the dry wt.) while the same worker (1961) found 7.68% lipid in 6 - 7 in. luscious growth of L. perenne pasture.

The fact that earlier workers utilized extraction methods involving ethanol and ether for Gramineae leaf tissue while in the present work a chloroform-methanol extraction procedure was utilized may account for the higher lipid content extracted from the species studied.

The age and type of tissue extracted may affect lipid composition. In this connection, Fagan (1928) demonstrated a decrease in the concentration of the ether extractable material from Italian ryegrass (L. multiflorum) as the grass matured. Also Waite and Sastry (1949) have demonstrated that as timothy (Phleum pratense) grass matures the content of crude protein, ether extractable material and ash content decreased while the proportion of crude fibre and nitrogen free extractable material increased. These trends were probably due to a decrease in the leaf to stem ratio. The stem was shown

to contain lower proportions of protein, ether extractable material and ash while increased proportions of crude fibre and nitrogen free extract were observed.

More recently Hawke (1963) has demonstrated that young succulent short rotation ryegrass (L. multiflorum x L. perenne) containing entirely leaf tissue contained higher percentages of lipid (mean 8.1% of dry wt.) than mature grass which contained appreciable stalk (mean 5.1% of dry wt.) These results were reported in conjunction with data on the fatty acid composition of the milk fat from monozygotic cows grazed on these two types of pasture. Cows grazed on the younger grass had higher levels of unsaturated fatty acids in the milk fat which may have been related to the higher degree of unsaturated fatty acids in the young grass or to the degree of hydrogenation of fatty acids in the rumen.

In the present work the lipid content of mature L. perenne leaf tissue was found to be lower than 8 day old leaves when results were expressed on a % dry weight basis. However, the lipid content of mature tissue has been found to be greater than that of young tissue when results are expressed as % wet weight of tissue. The high dry weight content of mature leaf tissue could account for the lower lipid values obtained for mature tissue when results are expressed as % dry weight.

When considering the possible environmental factors that could affect lipid content of photosynthetic tissue the light environment may be important. Spoehr and Millner (1949) have shown that light stimulated the synthesis of lipid material in Chlorella. The effect of light levels on lipid content was confirmed in the present work in which photosynthesising barley seedlings contained an appreciably higher concentration of lipid compared with seedlings grown in the dark or exposed tolow levels of light. Furthermore, the observation that the basal tissue of L. perenne plants (which contained lower chlorophyll levels compared with the rest of the plant) had a lower content lipid content supports the proposal that light levels received by leaf tissue affects lipid content. Also since leaf sheaths

and stems of L. perenne contain fewer chloroplasts (Soper and Mitchell, 1956) which are reported to be rich in lipid material (Wolf et al, 1962) the data of lower lipid levels in the basal leaf region of mature plants is confirmed.

2. The constituent fatty acids of Gramineae leaf lipids

The constituent fatty acids found in L. perenne and H. vulgare leaf lipid are similar to those reported for other Gramineae species by Shorland (1944, 1961); Hawke (1963 and Carton (1960). In each case linolenic, linoleic, oleic, stearic and palmitic acids are the major fatty acids.

The nature of the hexadecenoic acid ($R_{palmitate}$ 1.20 on P.E.G.A.) is of particular interest as this study has shown that increased light appears to promote synthesis of this fatty acid in Gramineae leaf tissue. It is possible that this fatty acid is trans-3-hexadecenoic which has been identified in spinach (Spinacea oleracea) lipids by Debuch (1961) and Allen et al., (1964); and by Weenink and Shorland (1964) in L. perenne and pea (Pisum sativum). Nichols (1965) has reported the presence of this fatty acid in Chlorella vulgaris, and it was found that this acid was almost entirely absent when cells were grown on an organic medium, in light or darkness. Light was shown to stimulate synthesis of α linolenic acid when cells were grown on an inorganic media but light had little effect on the fatty acid composition of cells grown on an organic media. The trans-3-hexadecenoic acid appears to be concentrated in the phosphatidyl glycerol component (Weenink, 1964; Allen, et al., 1964; Nichols, 1965; Haverake and Van Deenen, 1965) and it is possible that this fatty acid may be important in the photosynthetic process since phosphatidyl glycerol appears to be the only lipid present in all photosynthetic micro-organisms (including photosynthetic bacteria which lack the usual poly-unsaturated acids) and higher plants. Nichols and James (1965). Confirmation of this acid as a trans isomer was achieved by elution of the fraction of methyl esters corresponding to methyl elaidate after $AgNO_3$/silica gel G thin-layer chromatography. Gas-liquid chromatography on P.E.G.A. showed considerable concentration of the slower moving 16 : 1 component in this fraction.

Of particular interest in light of recent work on the biosynthesis of fatty acids in plant tissue, was the finding of trace amounts of 20 : 0, 22 : 0 and 24 : 0 fatty acids. Hawke and Stumpf (1965) have demonstrated in Gramineae seedling tissue that these components comprise a large percentage of the total fatty acids synthesised from acetate.

The small amounts of fatty acids with odd numbers of carbon atoms in leaf tissue is confirmation of earlier work in which these fatty acids were components of photosynthetic tissue in short rotation ryegrass (Hawke, 1963), Chorella vulgaris, Schlenk et al., (1960), the phytoflagellate Euglena gracilis, Korn (1964) and in the blue-green algae Anacystis nidulans Holton, et al., (1964).

The nature of the fatty acid chromatographing at a $R_{palmitate}$ value of 4.8 on P.E.G.A. was not determined, but it, was observed that this relative retention volume would be similar to unsaturated fatty acids 20 carbons in chain length. In this connection, Shorland (1953) has reported minor percentages of 20 carbon unsaturated fatty acids in L. perenne lipids.

Although Keys and Shorland (1951) found a 16 carbon trienoic acid in rape (Brassica napus), also confirmed by Allen et al., (1964) and Debuch (1962) in spinach (Spinacea oleracea) and Debuch (1962) in Antirrhinum majus no evidence of this fatty acid in Gramineae leaf tissue was obtained in the present study. This discrepancy may represent a species difference.

3. The variation in the fatty acid composition of Gramineae leaf lipids

Variations in the fatty acid composition of leaf regions of L. perenne plants grown for 40 days in a controlled environment was demonstrated. The mature leaf regions (10 - 19, 19 - 28 cm.) were found to contain higher levels of chlorophyll compared with the immature leaf region which contained a high proportion of leaf sheaths and stem tissue. The mature leaf regions was shown to contain higher levels of linolenate with corresponding decreases in the proportions of palmitate, oleate, and linoleate compared with immature leaf tissue. Soper and Mitchell (1956) have reported that leaf sheaths and stems of L. perenne contained less photosynthetic tissue and since linolenate appears to be concentrated in chloroplasts (Debuch, 1962; Crombie, 1958) the above observation would be expected. Further, Newman (1962) has reported that the fatty acids of plastids containing reduced levels of chlorophyll contained a higher ratio of saturated to unsaturated fatty acids. Crombie (1958) has also demonstrated that variegated leaves of maple (Acer negundo variegatum) and maize (Zea mais japonica variegata) contained less linolenic acid.

If age of tissue was a criteria that determined fatty acid composition, it would have been expected to obtain similar results from the 8 day old leaves of L. perenne and the leaf region of corresponding age obtained from 40 days growth (i.e. the 1 - 10 cm. leaf region). However, it was demonstrated that the chlorophyll level and fatty acid composition of eight day old leaves was similar to the mature leaf regions of the older plants. Hence it is proposed that chlorophyll content is of greater importance than the age of tissue in determining fatty acid composition.

In this connection, Wallace and Newman (1964) have shown that illumination of dark grown bush bean (Phaseoulus vulgaris) increased the proportion of linolenic

acid with relative decreases in the proportion of palmitic, stearic, oleic and linoleic acids in the plastid lipids. Similarly Erwin and Bloch (1964) and Rosenberg and Pecker (1964) have shown that dark treatment of Euglena gracilis cells decreased the content of linolenic acid. The finding that H. vulgare seedlings grown in a normal light environment had increased relative proportions of linolenic acid and decreased relative proportions of palmitate, stearate, oleate, and linoleate compared with dark grown seedlings supports the above work.

Although light appears to be the major environmental factor affecting fatty acid composition in photosynthetic tissue, other environmental factors such as nutrient level, CO_2 concentration in the atmosphere and temperature levels, that may affect the photosynthetic rate, appear to affect fatty acid composition. Wallace and Newman (1964) have demonstrated that a nitrogen deficient media decreased the relative proportions of linolenic acid in the plastids of squash (Curcurbita maxima) while Erwin and Bloch (1964) have demonstrated changes in fatty acid composition by growing Euglena gracilis on different media. The same workers have demonstrated that Euglena gracilis grown in an atmosphere of 0.5% CO_2 compared with 5% CO_2 had an appreciably reduced content of linolenic acid. An increase of temperature from 26°C to 44.2°C was shown to bring about an elevation in the proportion of saturated acids in the blue-green algae, Anacystis nidulans Holton et al., (1964). Their work has confirmed observations on the effects of temperature on the fatty acid composition of non-photosynthetic micro-organisms such as Escherichia coli (Marr and Ingraham (1962) and Serratia marsecens (Bishop and Still, 1963).

4. Fatty acid biosynthesis

The biosynthesis of fatty acids from acetate 1-C^{14} in plant tissue confirms the work of Hawke and Stumpf (1965).

It was observed that a large proportion of the fatty acids synthesised de novo from acetate by L. perenne tissue were the very long chain saturated fatty acids containing 20, 22, 24 and 26 carbon atoms. These fatty acids appear to constitute only a minor percentage of the endogenous lipid. Seedling tissue as well as tissue of the same age from established plants appeared to be able to synthesise these fatty acids. Etiolated or green tissue, synthesised similar fatty acids although the latter appeared to contain a high percentage of unsaturated fatty acids. This is consistent with previous findings that light stimulated synthesis of unsaturated fatty acids (Rosenberg and Pecker, 1964; Wallace and Newman, 1965). Hawke and Stumpf (1965) have demonstrated that the biosynthesis of these long chain fatty acids involves the synthesis of palmitate as the primary fatty acid followed by chain lengthening by addition of C_2 units. The metabolic significance of these very long chain saturated fatty acids is not yet understood.

5. The galactolipid content of plant tissue

A high galactolipid content in the leaf tissues of red clover (Trifolium pratense), mixed pasture, runner bean (phaseoulus multiflorus) and alfalfa (Medicago sativa) has been reported by Weenink (1961); Garton (1960); Sastry and Kates (1964); and O'Brien and Benson (1964) respectively. The values reported for galactolipid content vary from 50% (expressed as per cent of total extractable lipid) for mixed pasture (Garton, 1960) to 13% for alfalfa (O'Brien and Benson, 1964). In the present work the galactolipids of photosynthetic Gramineae tissue were found to constitute approximately 30% of the total leaf lipids. The monogalactolipid component was present in higher amounts than the digalactolipid component as reported by Weenink (1961) in red clover (Trifolium pratense) and Sastry and Kates, 1964 in runner bean (Phaseoulus multiflorus). However, O'Brien and Benson, (1964) have reported a higher concentration of the digalactolipid in alfalfa (Medicago sativa) lipids.

According to the method of presenting results different trends can be shown in the galactolipid content of leaf tissue.

The galactolipid content of leaf regions obtained from L. perenne plants grown for forty days, is very similar when results of the galactolipid concentration are expressed as a percentage of the total lipid. However, when results are expressed on a wet weight basis, there is a noticeable increase in galactolipid content from the basal to the distal leaf region. This increase is matched by a similar rise in chlorophyll content, indicating a possible relationship between these two components. Similar relationships between galactolipid content and chlorophyll level have been demonstrated by Wintermans (1960); Nichols, 1963 and McArthur et al., 1964, in other plant species.

The above results were further confirmed by growing barley (H. vulgare) seedlings in different light environments. An increase in illumination has been

found to lead to an increase in the galactolipid content of the seedlings, when results are expressed on either a percent total lipid or a percent wet weight basis. Similarly, as shown for L. perenne, a possible relationship between galactolipid content and chlorophyll level has been demonstrated, and this would suggest that these lipid components are located in the chloroplast as found by Wintermans (1960).

The finding of galactosyl glycerides in etiolated and non-photosynthetic barley seedlings would suggest that these components are not entirely found in photosynthetic tissue as thought previously. Similarly Wallace and Newman (1964) have found galactosyl glycerides in etiolated bush bean plastids while Nichols and James (1964) confirmed the presence of these compounds in non-photosynthetic tissue such as Narcissus bulbs, stems, roots and etiolated plants.

The presence of galactolipids in etiolated barley seedlings would suggest that their synthesis does not depend entirely on products derived from the photosynthetic pathway as proposed in the scheme of Ferrari and Benson (1961). It is possible that these lipids are synthesised from the storage fat in the seed. Crombie and Comber, (1956) and Hardman and Crombie, (1958) have demonstrated that the storage fat of Citrullus vulgaris (containing predominantly linoleic, stearic and palmitic acids) was utilised in the same fashion whether seedlings were grown in dark or light conditions. The formation of linolenic acid in the protoplasmic fat in the leaf tissue, following the complete utilization of storage fat was demonstrated in both the dark and light grown seedlings. However, a lower proportion of linolenic acid was observed in the dark grown seedlings. Similarly, it was demonstrated that barley seedlings could synthesise linolenic acid in the dark from seed reserves. The galactose moiety required for galactolipid synthesis could arise from conversion of fat to carbohydrate via the glyoxylate cycle. Zill and Cheniae (1962). This pathway is well established in light grown seedlings but Bradbeer (1958) has demonstrated fat to carbohydrate conversion in etiolated sunflower seedlings. Since galactolipids may be important structural constituents of chloroplasts (Benson, 1964) these components

may have a similar function in the yellow protoplastids of etiolated plants.

Linolenic acid has been shown to be the major fatty acid of the monogalactolipid component isolated from Gramineae leaf lipids. The fatty acid composition of monogalactosyl glyceride, in contrast to the fatty acid composition of the total lipid did not appear to be greatly affected by the light environment. Hence, the low linolenic acid content of the total lipid extracts of Gramineae tissue containing low chlorophyll levels, may be a reflection of low galactolipid concentrations.

SUMMARY

1. Total lipid content, fatty acid composition and galactolipid level have been compared in leaf tissue of varying age obtained from Lolium perenne plants grown in a controlled environment. A similar study has been undertaken with Hordeum vulgare seedlings grown in different light intensities.

2. The total lipid content and the fatty acid composition of L. perenne photosynthetic tissue appeared to be dependent on chlorophyll levels, rather than on age of tissue. High levels of chlorophyll in plant tissue was paralleled by high lipid concentrations (expressed as % wet wt.). It was also demonstrated that a high chlorophyll level appeared to be related to high proportions of linolenic acid and corresponding low proportions of linoleic, oleic and palmitic acids.

3. Light intensity affected both the lipid level and fatty acid composition in H. vulgare seedlings. Increased exposure to light stimulated the synthesis of lipid material which contained relatively high proportions of linolenic acid.

4. The relative concentrations of the galactosyl glyceride lipid components in Gramineae were closely related to chlorophyll levels. The monogalactosyl glyceride component contained high concentrations of linolenic acid. However, in contrast to the fatty acid composition of the total lipid that of the monogalactosyl glyceride component did not appear to be greatly affected by the light level

5. Glycolipids including mono- and digalactolipid, cerebroside, sulpholipid and phospholipids including phosphatidyl glycerol, phosphatidyl ethanolamine, phosphatidyl choline and phosphatidyl inositol were identified in L. perenne lipids.

6. This biosynthesis of fatty acids from acetate-1-C^{14} in L. perenne seedling tissue was demonstrated. The biosynthesis of unsaturated fatty acids from acetate-1-C^{14} was greatest in tissues that contained the highest chlorophyll content.

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APPENDIX I

Modified Hoaglands Solution
(after Hoagland and Arnon, 1938)

Each micro or macro element was dissolved in 2 l. of water (see Table). The specified volume of each solution (see Table) was then taken, mixed together, and the resultant solution was then made up to 10 l. by addition of water to form the nutrient solution.

TABLE

Compound	g. in 2 l. water	Volume in ml. in 10 l.
	Macro - elements	
Ammonium dihydrogen phosphate	230	10
Potassium nitrate	202	60
Calcium nitrate	472	40
Iron chelate	20	100
	Micro - elements	
Boric acid	5.72	10
Manganese chloride	3.62	10
Zinc sulphate	0.44	10
Calcium sulphate	0.16	10
Sodium molybdate	0.24	10

A P P E N D I X I I

Extraction of lipid from leaf tissue
Hawke (1963)

The tissue was chopped into short lengths, mixed and then 100 g. was immediately macerated in ethanol with a Waring blender. The resultant slurry was warmed to about 55°C to deactivate lipolytic enzymes and then filtered through a sintered funnel. The fibrous residue was washed on the funnel several times with small volumes of ethanol and ether and then transferred to Soxhlet thimbles. Soxhlet extraction was carried out for 8 hours with diethyl ether.

The ethanolic and ether extracts were then evaporated to dryness separately, and each residue extracted with ether. These extracts were combined and shaken with water to remove water soluble impurities. To avoid the formation of emulsions shaking was not vigorous until the third and final extraction. The washed ether solution was then evaporated to dryness and the residue extracted with anhydrous ether. The solvent was then removed *in vacuo* and the lipid material obtained was weighed after drying for 18 hours *in vacuo*.

APPENDIX III

The measurement of chlorophyll content (Moshi et al., 1950)

Seedlings were cut into segments 1 cm. long and ground in 20 ml. of acetone with the addition of a little sand. After 5 minutes of grinding the acetone was decanted and the tissue was re-extracted with 20 ml. of ether. This extraction sequence with 10 ml. of acetone and 10 ml. of ether alternately was repeated four times. The combined extracts were filtered through a sintered glass funnel and the acetone was removed by washing with water. Ether was added to make a total extract of 25 ml. and after standing in a refrigerator for 1 hour to allow settling out of residual water, an aliquot of the ether extract was examined in a Unicam spectrophotometer at the wavelengths of 663 mμ ; 644 mμ and 624 mμ; the respective maxima for chlorophyll a, chlorophyll b and protochlorophyll respectively.

Pigment content in mg./g. leaf tissue was calculated as follows using the equations:-

$$\text{Chlorophyll } a = \frac{V\,(1.0151\,D_{663} - 0.0896\,D_{644} - 0.0037\,D_{624})}{0.095 \times 1000 \times W \times 1}$$

$$\text{Chlorophyll } b = \frac{V\,(-0.1610\,D_{663} + 1.0195\,D_{644} - 0.0301\,D_{624})}{0.0575 \times 1000 \times W \times 1}$$

where V = volume of solution in ml.

D = optical density of solution at wavelength designated

W = wet weight of leaves in grams

l = internal length of spectrophotometer cell in cm.

APPENDIX IV

Galactolipid values

By assuming the mole % of galactose in each galactolipid, the galactose values obtained from the acid hydrolysis products of the lipid components (obtained from a known weight of total lipid) were used to calculate values reported below. Each value reported is the result of separate experiment on the particular lipid sample. The mean value was used to calculate data reported in Tables 20 and 21.

TABLE

Lipid component	% galactolipid of the total lipids			
	L. perenne			
	Leaf Region			Whole leaf
	1-10 cm.	10-19 cm.	19-28 cm.	9 cm.
Monogalactolipid	18.25	19.60	23.0	23.80
	19.60	20.30	16.60	23.40
	17.40	21.50	21.80	18.70
	19.80	20.00	23.40	18.70
Digalactolipid	8.6	12.50	10.7	11.20
	9.35		11.20	11.90
	12.30		14.50	12.70
	14.70		14.20	11.70
	H. vulgare			
	Light treatment			
	dark	low light		full light
Monogalactolipid	10.40	21.40		20.80
	15.80	20.0		20.80
	12.80	17.40		20.00
	17.10	20.0		
	12.50	23.0		
Digalactolipid	3.58	5.95		9.35
	4.50	5.95		8.20
	6.10	8.70		
	4.50	7.60		
	7.55	7.60		
	6.45	6.60		