THE LIPID METABOLISM OF PLANTS

A thesis presented in partial fulfilment of the requirements for the degree of Doctor of Philosophy in Biochemistry at Massey University.

by

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ABSTRACT

A method, based on the isolation of pure compounds by a combination of DEAE-cellulose and thin-layer chromatography, has been developed for the rapid and quantitative estimation of the major glycerolipids of plant tissues. The method has been used 1) for the analyses of the major glycerolipids of a wide variety of plant species and 2) as part of a detailed chromatographic analysis of the glycerolipid constituents of the green alga Mesotaenium caldariorum during which two unknown lipids, probably glycerolipids, were isolated.

On the basis of the incorporation of radiocarbon from ^{14}C-labelled precursors into the glycerolipids of both Mesotaenium cells and pumpkin leaves, the likelyhood of relatively low turnover rates for the various glycerolipids, with the possible exceptions of phosphatidyl glycerol in Mesotaenium and phosphatidyl choline in pumpkin leaf, is discussed.

The unusual growth requirements of Mesotaenium caldariorum in liquid culture is discussed briefly.

PREFACE

In this study, a considerable amount of time has been spent on the development of techniques for the routine separation and analysis of all of the major glycerolipids of plant tissues. These techniques were considered an essential prerequisite for obtaining the type of results envisaged in the planning of the topic. Time limitations have subsequently dictated that a lesser period than would have been desired was available for metabolic studies, so that the isotope incorporation experiments reported here should be regarded as preliminary in nature. Nonetheless, these experiments do point the way for further investigations which could provide reliable measurements of the turn-over of the individual glycerolipids of algae and leaves.

I wish to express my appreciation to Professor R.D. Batt for his advice and guidance during the course of this work and to Dr K.J. Mitchell for his constant encouragements. To Dr A.O. Taylor go my thanks for his patience and attentiveness during our discussions of some aspects of this work.

I am indebted to the Department of Scientific and Industrial Research for ensuring me employment at the Plant Physiology Division of the Department while this investigation was carried out.

P.G. Roughan.

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   P.G. Roughan and C.G. Tunnicliffe (1967).

   J. Lipid Res. 8; 511.

2. Quantitative analysis of sulfolipid (sulfoquinovosyl diglyceride) and galactolipids (monogalactosyl and digalactosyl diglycerides) in plant tissues.

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3. The glycerolipid composition of leaves.

   P.G. Roughan and R.D. Batt (1969).

   Phytochemistry, 8; In Press.

A man would do nothing if he waited until he could do it so well that no one could find fault with what he had done.

Cardinal Newman.

PART 1

SEPARATION, IDENTIFICATION AND ANALYSIS OF PLANT GLYCEROLIPIDS

INTRODUCTION

1. Glycerolipids of Photosynthetic Tissue.

Studies on the nature of the lipid constituents of photosynthetic tissue have been undertaken only recently. Benson and co-workers have been the major contributors in this field since 1958 and the group has been responsible for the discovery of plant sulpholipid (Benson, Daniel and Wiser, 1959), phosphatidyl glycerol (Benson and Maruo, 1958) and first reported the presence of diphosphatidyl glycerol in plant tissue (Benson and Strickland, 1960). Phosphatidyl choline, phosphatidyl glycerol, phosphatidyl ethanolamine and phosphatidyl inositol were found in all leaves examined but phosphatidyl serine was encountered only rarely (Benson, Wintermans and Wiser, 1959). The two galactolipids, which had been discovered originally in wheat flour (Carter, McCluer and Slifer, 1956), were shown to be the major constituents of leaf, chloroplast and algal lipids (Benson, Wintermans and Wiser, 1959; Ferrari and Benson, 1961) and sulpholipid has also been found in all photosynthetic tissues so far examined (Benson, 1963; James and Nichols, 1966).

2. Extraction of Lipids.

a) Using Ethanol.

A wide variety of methods have been used for the extraction of lipids from fresh plant tissues. Benson and co-workers (Benson et al., 1959) normally extracted with hot ethanol while Wintermans (1960) has followed boiling ethanol with repetitive ethanol/toluene extractions until the residue was colourless. Zill and Harmon (1962) extracted spinach leaves with boiling ethanol and followed this with an acetone extraction at room temperature. In this instance the residue was finally extracted with chloroform/methanol (2:1). Bailey (1962) and later Russell (1966) used boiling 80% ethanol for extracting grasses and clovers.

b) Using Isopropanol.

Kates and Eberhardt (1957) extracted runner bean leaves with boiling isopropanol followed by isopropanol/chloroform after first powdering the leaves under liquid nitrogen. This procedure is said to eliminate artifacts such as the formation of phosphatidic acid from phospholipase activity known to occur during other extraction procedures (Jordan and Chibnall, 1933; Hanahan and Chaikoff, 1948; Smith and Chibnall, 1932). Extraction with hot isopropanol was later used by Nichols (1963) with leaves and by Nichols and James (1964) with

narcissus bulb but in each of these cases the freezing step was omitted.

c) Using Chloroform/Methanol.

In subsequent reports (Wood, Nichols and James, 1965; Nichols, 1965) algal cells were extracted with chloroform/methanol (2:1) and this solvent mixture has also been used by O'Brien and Benson (1964) for the extraction of alfalfa and Chlorella lipids. Allen et al. (1964) extracted spinach leaves with mixtures of methanol in chloroform (1:2, 1:4) and finally with chloroform alone. More recently Haverkate and Van Deenen (1965a, 1965b) and Allen et al. (1966) extracted algal and leaf lipids into chloroform/methanol (1:1) by the method devised by Bligh and Dyer (1959) for fish muscle.

3. Removal of non-lipid material.

a) Aqueous washing of organic phases.

A considerable amount of non-lipid material is extracted with the lipids by all of the methods described above. Earlier procedures for the removal of these non-lipid compounds involved concentration of the extract to an aqueous emulsion, partitioning with chloroform (Benson et al., 1959; Wintermans, 1960; Eberhardt and Kates, 1957) or diethyl ether (Weenink, 1964), and repeated

water washing of the organic phase. Both of these methods however, tend to produce difficult and persistant emulsions and subsequent repetitive washing may lead to lipid loss. The chloroform/methanol extraction and purification procedures developed for brain (Folch et al., 1957) and fish muscle (Bligh and Dyer, 1959) have the advantages that no concentration of the extract is required and separation of aqueous and organic phases is sharp and rapid. These methods have been used with plant tissues (Zill and Harmon, 1962; Nichols and James, 1964; Nichols, 1965; Haverkate and Van Deenen, 1965b).

In 1962 a radically different procedure was reported by Bailey and later used by Russell (1966). An 80% ethanol extract of pasture plants was evaporated to a thick syrup which was subsequently diluted with water and the lipids recovered by centrifugation.

b) Column partition methods.

Separation of lipid and non-lipid material by column partitioning on cellulose (Lea and Rhodes, 1953) or Sephadex (Wells and Dittmer, 1963; Siakatos and Rouser, 1965; Wuthier, 1966) have been used infrequently for the removal of non-lipid components from leaf extracts. Rouser et al. (1967) used a Sephadex column to purify a spinach leaf extract.

c) Column adsorption method.

Rouser et al. (1963) thoroughly dried an unwashed chloroform/methanol extract of brain tissue and extracted the residue with chloroform. The chloroform solution was placed on a column of DEAE-cellulose and neutral and zwitterionic lipids were eluted in mixtures of methanol in chloroform. Non-lipid material was then eluted from the column in methanol and the acidic lipid subsequently eluted in chloroform/methanol/ammonia mixtures. Although not specifically stated this technique was probably used by Allen et al. (1964) with spinach leaf lipid extracts.

4. Qualitative analyses.

a) The deacylation technique.

In this technique, the washed lipid is subjected to a controlled, mild-alkaline hydrolysis which splits fatty acids from glycerol leaving the rest of the molecule intact. The resultant water-soluble glycerol esters or glycosides can then be separated from the fatty acids by partitioning between aqueous and chloroform phases and from each other by two-dimensional paper chromatography (Benson and Maruo, 1958). It was with this technique and the use of isotopically labelled lipids that Benson and co-workers identified the new

glycerolipids of photosynthetic tissue referred to previously.

b) Paper adsorption chromatography.

Kates (1960) adapted the silicic acid impregnated paper chromatographic technique of Marinetti and Stotz (1956) for the separation of runner bean leaf lipids into eleven components and similar papers were used by Ferrari and Benson (1961) in a study on the composition and turnover of Chlorella glycerolipids.

c) Column adsorption chromatography.

i) Silicic acid.

The earliest attempts to separate intact leaf lipids were made on silicic acid columns. Kates and Eberhardt (1957) separated runner bean leaf lipids into four fractions on columns of silicic acid/celite. Zill and Harmon (1962) applied the Hirsch and Ahrens (1958) silicic acid column technique to the separation of spinach leaf lipids but could achieve only a limited resolution of the more polar glycerolipids. Better separation of the acetone insoluble lipids (a mixture of galacto-, sulpho- and phospholipids) from cabbage leaves was obtained by Wheeldon (1960) using silicic acid columns and chloroform/methanol mixtures for elution.

Vorbeck and Marinetti (1965) have separated glyco-

lipids from the phospholipids of Gram positive bacteria by eluting silicic acid columns with acetone/chloroform and acetone. Phosphatides were retained on the column and could be eluted with chloroform/methanol mixtures. This technique was adopted by Rosenberg et al. (1966) for the separation of the galactolipids of Euglena and by Rouser et al. (1967) for the separation of spinach glyco- and phospho- lipids.

ii) Florisil (Magnesium silicate)

Florisil column chromatography was employed by O'Brien and Benson (1964) for the separation of glycolipids of Chlorella and alfalfa from phosphatides. By using a combination of Florisil, DEAE-cellulose and silicic acid column chromatography these authors were able to obtain the galactolipids and sulpholipid in pure form. A batchwise Florisil procedure was later used by Russell (1966) for the separation of glycolipids and phospholipids of pasture species.

d) Ion-exchange chromatography.

i) On paper.

The major phosphatides of Chlorella have been separated by Mumma and Benson (1961) using aminoethyl cellulose paper chromatography and the technique was also used by Zimmerer and Hamilton (1965) for the

separation of the phosphatides from Avena coleoptile.

ii) On columns.

DEAE-cellulose column chromatography, modified for the separation of brain glycerolipids (Rouser et al. 1961), was applied by a) Nichols and James (1964) to the separation of Narcissus bulb lipids b) Allen et al. (1964) to separate spinach leaf lipids and c) Weenink (1964) for the fractionation of the acetone insoluble lipids of red clover leaves. The main advantage of this technique is the clear separation of neutral and dipolar lipids from acidic lipids, a separation which cannot be achieved with silicic acid columns.

e) Thin-layer chromatography.

Thin-layer chromatography of plant glycerides was first described by Nichols (1963) who separated 19 components from cabbage and lettuce leaf lipid extracts. As mentioned previously, Kates (1960) resolved 11 components from runner bean leaf lipids by paper adsorption chromatography. Subsequent reports by Nichols (1964) describe extensions of the thin layer technique to two dimensions giving a clear separation of the 19 components of lettuce leaf. La Page (1964) has also described a two-dimensional thin-layer method which separated 17 lipid components from potato tuber.

5. Quantitative Analyses.

In a recent review of plant lipid metabolism, Mudd (1967) has pointed out that there are few complete quantitative analyses of glycerolipids in either plant tissues or subcellular fractions. In 1966, James and Nichols had collected information on the occurrence of particular glycerolipids in a variety of photosynthetic organisms but could not list relative concentrations. In fact, only four reports have attempted to give figures for the glycerolipid concentrations in leaves, algae or subcellular fractions.

a) Leaves and chloroplasts.

Total lipids, isolated from leaves of spinach, beet, elder and bean seedlings and from chloroplasts of spinach and beet, were subjected to mild alkaline hydrolysis and the water soluble, deacylation products separated by two-dimensional paper chromatography (Wintermans, 1960). The separated glyceryl compounds were eluted from the paper and estimated by phosphorus or sugar content. Recoveries of phosphoryl compounds were variable (60 to 90%) and it is possible that the sugar estimations were complicated by contamination of eluted spots with cellulose fibres (Wintermans, 1962).

b) Algae.

Chlorella pyrenoidosa was grown in the presence of $^{14}CO_2$ to give uniformly labelled lipids which were then deacylated and separated by paper chromatography (Ferrari and Benson, 1961). Phosphoryl compounds were estimated by neutron activation (Benson et al., 1958) and the concentration of the glycosyl compounds were calculated from ^{14}C contents compared with the values in known phosphoryl compounds. While considerable accuracy might be expected with this procedure, it is unnecessarily complicated for routine use and probably could not be used with higher plants.

c) Bean chloroplast and pepper fruit plastids.

Bean chloroplast and pepper fruit plastid lipids were fractionated by silicic acid column and silicic acid impregnated paper chromatography and estimated by sugar or phosphorus content (McArthur et al., 1964). This chromatographic procedure cannot adequately separate all the glycerolipids of a photosynthetic tissue however (Kates, 1960), and results were given in percentage composition rather than in relation to fresh or dry weight of tissue or chlorophyll content.

d) Spinach chloroplast lamellae.

The glycerolipids of spinach chloroplast lamellae

were separated on a column of DEAE-cellulose and molar concentrations derived from the weights of column fractions (Allen et al., 1966). Reliable results might be expected with those components present in high concentrations (monogalactosyl and digalactosyl diglycerides and phosphatidyl glycerol) but other fractions (weighing 3 to 4 mg) would be extremely difficult to determine accurately in view of the hygroscopic nature of polar glycerolipids. It is not clear from this report whether ammonium acetate, eluted along with the acidic lipids, was removed before weighing the individual fractions.

e) Partial analyses.

In 1959, Benson et al. measured the relative concentrations of phosphatides in a variety of leaf and algal species by neutron activation of the chromatographically separated, deacylated lipid. O'Brien and Benson (1964) gave values for the concentrations of the two galactolipids and sulpholipid which had been isolated by a combination of Florisil, DEAE-cellulose and silicic acid column chromatography. Semi-quantitative measurements of galactolipids from runner bean leaves were reported by Sastry and Kates (1964). Bailey (1962) described a method for the measurement of lipid bound galactose and Russell (1966) later reported a

method for the estimation of both lipid-bound galactose and sulphoquinovose.

6. Summary.

The results reported by Wintermans represent the only complete comprehensive analysis of the major glycerolipids of whole leaves and chloroplasts. The need for a rapid and reproducible method for the quantitative analyses of the major glycerolipids from plant tissues is apparent from the literature reports. The information available suggested that leaf lipids might be separated effectively by two-dimensional thin-layer chromatography (Nichols, 1964) or by unidimensional thin-layer chromatography following preliminary fractionation into a) glyco- and phospholipids by Florisil column chromatography (O'Brien and Benson, 1964) or b) acidic and non-acidic lipids using DEAE-cellulose (Nichols and James, 1964). Although the silicic acid/acetone procedure described by Vorbeck and Marinetti (1965), Rosenberg et al. (1966) and Rouser et al. (1967) might be used, the large volumes of acetone required and relatively slow flow rates (Rouser et al., 1967) would be disadvantages in a technique specifically designed for routine analyses.

7. Aim of the present investigations.

The aim of the present investigation was firstly, to evaluate existing methods for the extraction and separation of the glycerolipids of photosynthetic tissues and then, with methods judged satisfactory on the basis of recoveries and convenience, develop a technique specifically for rapid, routine separations. In addition, simple and rapid methods for quantitative chemical analyses of the separated lipids would also be sought.

The following section describes the development of methods for the rapid and accurate estimation of the major glyco- and phospho- lipids from photosynthetic tissues. The results of the application of the new technique to a wide variety of species are also presented.

METHODS

1. Selection of plant material.

Wherever possible only young, fully expanded leaves were sampled and extraction was begun within 2 to 5 min after harvesting. No attempt was made to dissect out the vascular tissue but as little petiole as possible was included in the sample. For the comparison of glycerolipid concentrations in a variety of species the youngest fully expanded leaves were taken from a number of plants in the spring and early summer of 1967-1968. In the case of ryegrass and cocksfoot, flag leaves were taken from plants in which the inflorescence was just emerging from its sheath. With compound leaves, only leaflets were taken. Pine needles, 5 to 6 cm in length, were gathered from the bases of new shoots of about 18 cm in length. The liverwort, *Marchantia*, was grown in a mist house under natural daylength. As much of the rhyzoid material as possible was removed from thalli before weighing and extracting. The green alga, *Mesotaenium caldariorum*, was grown in liquid culture at 18°C under continuous illumination from fluorescent tubes. This organism was harvested in the middle of the log phase of growth and the results given in terms of fresh weight are based on the assumption that 1 ml of packed cells weighed 1 g. The moss used, was a mixture

of equal amounts of Furoria and Leptobyrum pyriforme. Parsnip root was washed free of soil before samples were cut from the cortex for weighing and extracting.

2. Extraction and purification of lipid.

a) Technique adopted in this study.

Fresh leaves were weighed and homogenized with 10 volumes of methanol/chloroform (7:3) in a 'Waring blendor' (10 to 50 g leaves) or in a high-speed, overhead blender (0.5 to 5 g). Smaller amounts (0.25 to 0.5 g) were normally homogenized manually in a glass tissue grinder. The homogenate was filtered under reduced pressure and 10 volumes of chloroform were used to rinse out the homogenizer and to wash the residue on the filter. After this treatment the residue was colourless.

The combined filtrate was shaken with 0.2 volumes of 0.73% NaCl and stored in the freezer while the phases separated. If the extract was required immediately the phases were separated by centrifugation. The lower, chloroform phase was recovered, evaporated under reduced pressure and traces of water were removed from the lipid residue by adding absolute ethanol/benzene (90:10) and re-evaporating. This dry lipid was then redissolved in chloroform so that 1 ml of solution was equivalent to 1 g fresh weight of original leaf tissue.

This procedure is a slight modification of that described by Folch et al. (1957) for brain tissue.

b) Other methods tested.

For purposes of comparison, lipid extracted (but not washed) by the method outlined above was freed of non-lipid material by the Sephadex method of Wuthier (1966) and was also subjected to DEAE-cellulose column chromatography without any washing (Rouser et al., 1963).

The extraction and purification methods described by Bligh and Dyer (1959) for fish muscle and by Bailey (1962) for pasture plants were also tested for their recovery and convenience.

3. Preliminary separation of glycerolipids.

a) DEAE-cellulose semi-micro column.

Diethylaminoethyl cellulose (DEAE-cellulose, Whatman DE-11) was washed with HCl, NaOH, methanol and chloroform as recommended by Rouser et al. (1963), and converted to the acetate form by standing overnight in glacial acetic acid. The acetic acid was replaced by methanol and the adsorbent stored as a dilute slurry in methanol. Aliquots of this slurry were pipetted into a 1 cm (i.d.) glass chromatographic tube fitted with a glass wool plug and a teflon stopcock to make a column

3 cm in height. The column was washed with methanol (5 ml) and the methanol carefully replaced by chlcroform (10 to 15 ml) before lipid, (equivalent to 2 g fresh weight of leaves) in chloroform was applied.

Neutral and zwitterionic lipids were eluted in 25 ml of chloroform/methanol (6:4) at a flow rate of 2 to 3 ml/min, and acidic lipids were eluted in 20 ml of chloroform/methanol (6:4) containing 1.5 ml of concentrated ammonia solution (Fig. 1.1). Both fractions were evaporated to dryness under reduced pressure, using ethanol/benzene to remove water from the second fraction, and finally made up to 2 ml (non-acidics) and 1.0 ml (acidics) in chloroform.

When it was considered desirable to remove the ammonium acetate, generated in the ion-exchange process, from the second fraction the following method was used. The lipid and ammonium acetate were quantitative transferred to a centrifuge tube in 2 ml of methanol followed by 2 ml of chloroform, and thoroughly shaken with 1.8 ml of water (Bligh and Dyer, 1959). The phases were separated by centrifugation and the lower, chloroform layer recovered by aspiration. A further 2 ml of chloroform was used to rinse out the evaporating flask and to re-extract the aqueous methanol phase. The combined chloroform solutions were evaporated to dryness under nitrogen

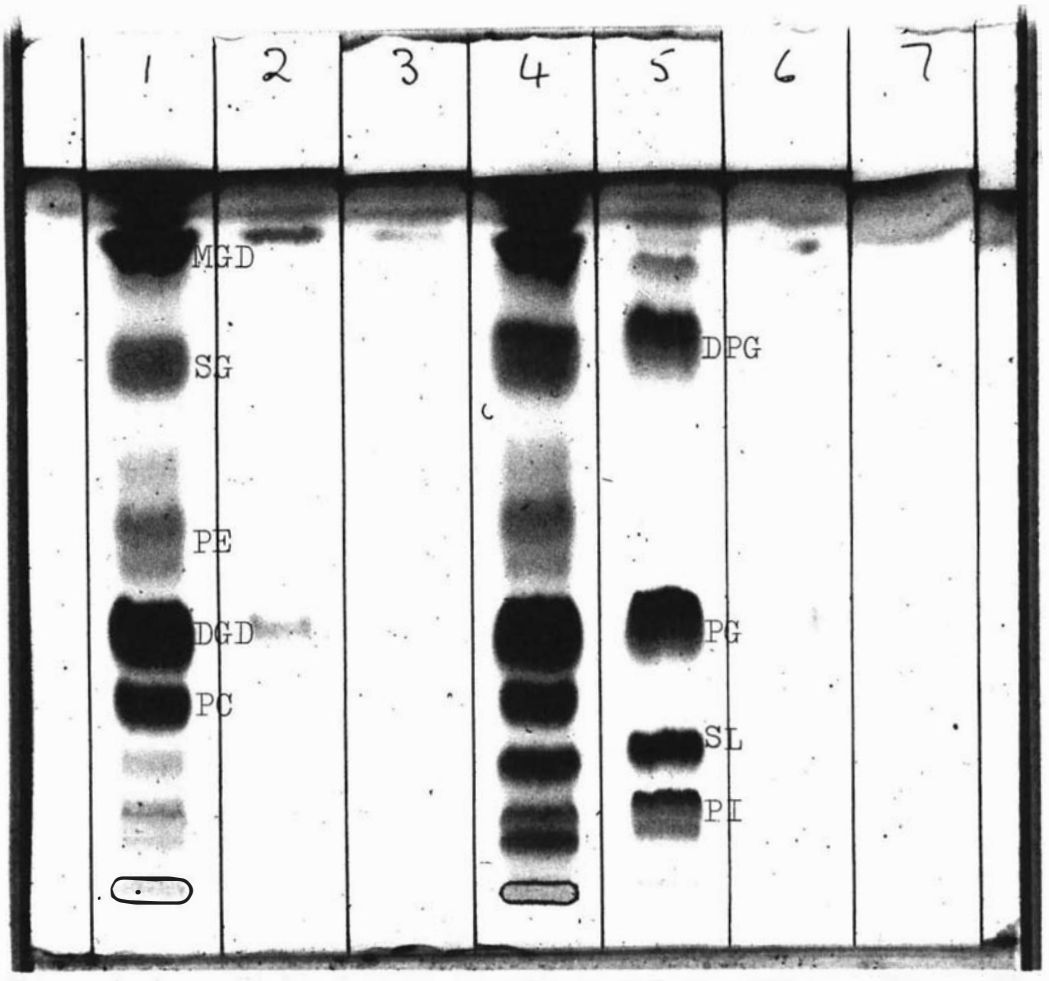

Fig. 1.1. Thin-layer monitor of fractions from the DEAE-cellulose column. Two ml (≡2 g fresh weight of leaf) of a chloroform solution of white clover lipid were placed on the column and 8 ml fractions of each eluting solvent collected. Fractions were concentrated and made up to volume in the same way as bulk eluates (see text); 25 µl were streaked across 1 cm on Silica Gel G. Lanes 1 to 3 are sequential fractions of the chloroform/methanol (6:4) eluting solvent while lanes 5 to 7 are sequential fractions of the chloroform/methanol/conc. ammonia (6:4:0.75) solvent. Lane 4 is the lipid before column chromatography. Development, chloroform/methanol/acetic acid/water (85:15:10:3); detection and identification of separated components as for Fig. 1.2. This chromatogram demonstrates phasic separations at the solvent front.

and the residue made up to 1.0 ml in chloroform.

b) Florisil column chromatography.

Florisil (a highly activated magnesium silicate, Floridin Co., West Virginia) was washed with water, activated and stored under methanol as described by O'Brien and Benson (1964). When this washing procedure was found to have little, if any, effect on the separations of glyco- and phospholipids the adsorbent was also used unwashed. The adsorbent was originally tested in column procedures which were essentially one-fifth to one-tenth of the scale reported by O'Brien and Benson (1964) but in later work this was scaled down even further in an attempt to decide whether a Florisil column could be used in a routine procedure. Pertinent details on the preparation and elution of semi-micro column of Florisil have appeared in a publication (Roughan and Batt, 1968), a reprint of which is bound in the back of this thesis.

4. Thin-layer chromatography.

a) Activation and development.

Thin-layer chromatography (TLC) was performed on 250 μ layers of Silica Gel G, H and HR (Merck, Darmstat, Germany) and one or two dimensional separations achieved

using the solvent systems recommended by Nichols (1963, 1964). Layers were activated at 120°C for 30 min immediately before use and development was normally carried out in tanks lined with filter paper.

Consistent two dimensional thin-layer separations were obtained (Fig. 1.2) when suitable essential precautions were taken. After development in the first direction (chloroform/methanol/conc. ammonia; 65:25:2) it was necessary to reactivate the layers under high vacuum for 30 min to prevent serious phase separation of the solvent (chloroform/methanol/acetic acid/water; 170:25:25:4) during development in the second direction. Phase separation was sometimes encountered during development with the solvent (chloroform/methanol/acetic acid/water; 85:15:10:3) recommended for unidirectional chromatography also and this was thought to be caused by deactivation of the adsorbent during cooling of the activated layer and application of samples. It could be prevented or controlled by ensuring that activated layers were not exposed to the laboratory atmosphere for more than 20 min prior to beginning development.

b) Application of samples.

For maximum resolution of components in one-dimensional TLC it was better to apply samples as narrow streaks rather than as spots and to obtain sufficient of

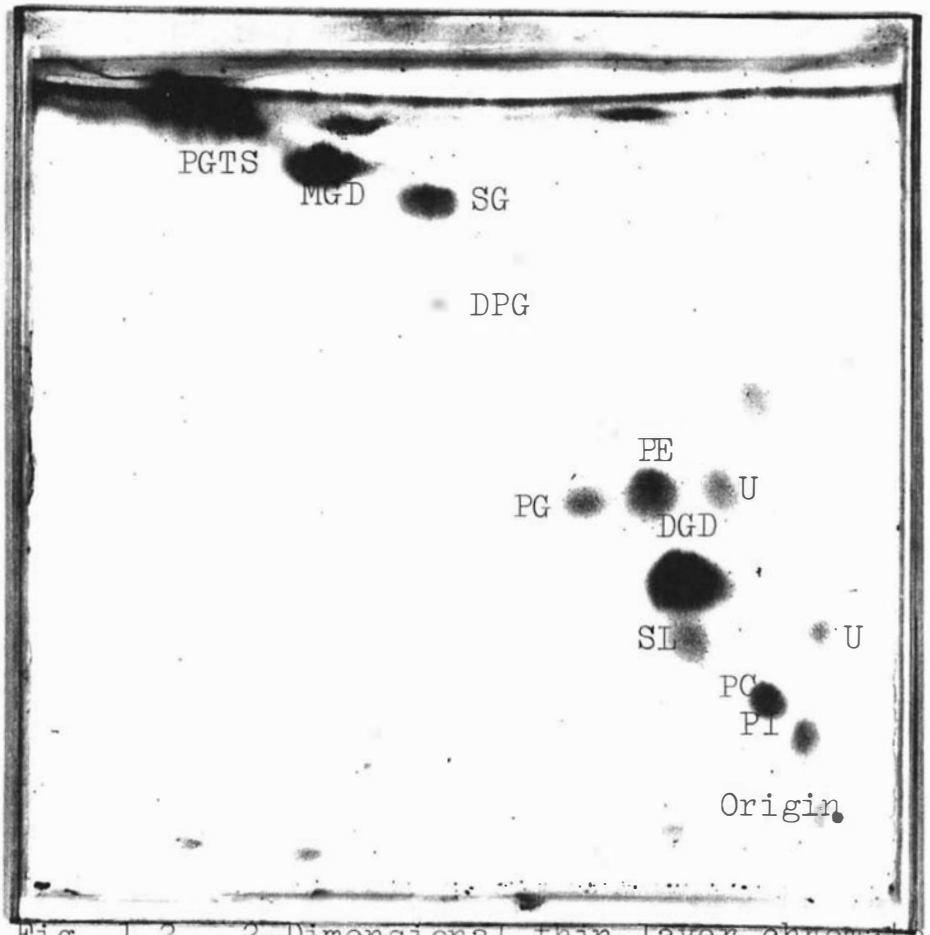

Fig. 1.2. 2-Dimensional thin-layer chromatogram of red clover leaf lipids after Nichols (1964). Lipid equivalent to 20 mg of leaf was applied and the separated compounds were detected with the acid dichromate spray of Rouser et al. 1964 and charring at 180 for 30 min.

PGTS, Pigments; MGD, Monogalactosyl diglyceride; SG, Sterol glycoside; DPG, Diphosphatidyl glycerol; PG, Phosphatidyl glycerol; PE, Phosphatidyl ethanolamine; U, Unknown; SL, Sulpholipid (sulphoquinovosyl diglyceride); PC, Phosphatidyl choline; PI, Phosphatidyl inositol; DGD, Digalactosyl diglyceride.

each glycerolipid for chemical analyses it was necessary to apply 25 to 200 µl of solution across a 2 to 4 cm streak. The search for a suitable method for making such applications culminated with the construction of trough-type streakers (Fig. 1.3) which were very much faster than existing designs. An account of the construction and testing of these devices has been published as a short communication (Roughan and Tunnicliffe, 1967), a reprint of which is included at the end of the thesis.

Samples (25 to 50 µl, equivalent to 25 to 50 mg of leaf) were applied as spots for separation by two-dimensional TLC.

c) Detection of separated components.

Lipids separated by TLC were detected and identified by the use of a variety of spray reagents. Phospholipids were identified with the molybdendum blue reagent of Dittmer and Lester (1965) and glycolipids with the α-naphthol/sulphuric acid reagent of Saikatos and Rouser (1965). The most useful reagent for the localization and identification of separated lipids however, was a 50% sulphuric acid spray followed by heating in an oven at 120°C. Sterols produced a characteristic pink colouration in the first few minutes of heating and subsequently (10 to 20 min later) the glycolipids showed as red-purple zones while phospholipids turned a

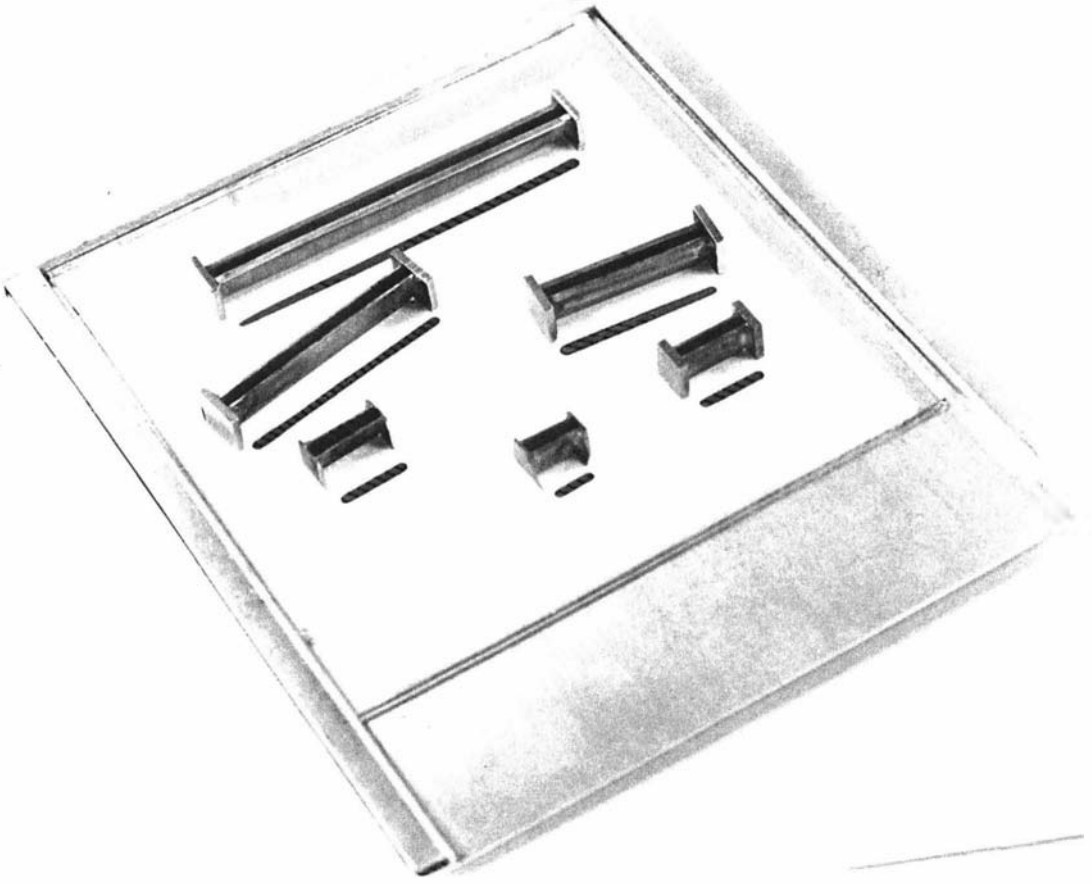

Fig. 1.3. Some of the sample streakers in routine use in this laboratory arranged on a $(20\ cm)^2$ layer (250 μ) of Silica Gel H. The thin streaks produced by the devices are displayed in front of each one.

pale brown (Nichols, 1963, 1964). With prolonged heating all spots turned black or shades of grey depending on initial concentration. The sulphuric acid preferentially reacted with unsaturated lipids; methyl stearate on Silica Gel G did not react while methyl palmitoleate, linoleate and linolenate gave quite strong reactions. To obtain an indication of relative amounts of separated lipids therefore, the acid dichromate charring procedure of Rouser et al. (1964) was used.

For routine use and where quantitative analyses were involved, iodine staining was used and identifications were based on chromatographic mobilities in acid and alkaline solvents. Developed chromatograms were placed in a tank containing iodine vapour until the separated lipids had reacted; the stained zones were outlined with a needle before the iodine evaporated.

d) Separation for glycerolipid analyses.

For the routine analyses of all the major glycerolipids in an extract, a 20 cm^2 layer of Silica Gel G or HR was divided into five equal lanes. Duplicate aliquots of non-acidic (50 µl ≡ 50 mg of leaf) and acidic (100 µl ≡ 200 mg of leaf) lipid fractions from DEAE cellulose columns were streaked across 2 cm in the four outer lanes while the middle lane was left blank.

Chromatograms were developed with chloroform/methanol/acetic acid/water (85:15:10:3) and separated lipids were detected with iodine vapour. Appropriate zones were quantitatively transferred to digestion tubes (phospholipids) or centrifuge tubes (glycolipids) for analyses as described below.

5. Quantitative estimation of phospholipids.

a) Method currently in use.

Phospholipids separated by a combination of DEAE-cellulose column chromatography and TLC (Fig. 1.4) were analysed without elution from the adsorbent by a slight modification of the method of Rouser et al. (1966). The lipid-loaded adsorbent was digested in the presence of 60% perchloric acid (0.6 ml) at 180°C to 200°C in an electrically heated aluminium block until the acid became colourless (15 to 30 minutes). Ammonium molybdate (3.5 ml of 0.31%) was added to the cool digests followed by ascorbic acid (0.5 ml of 10%) and the contents of the digestion tubes were transferred to centrifuge tubes which were placed in a boiling bath for 5 min to complete colour development. After cooling and centrifuging the absorbance of the blue colour was measured at 660 mμ. Adsorbent blanks and standards were included with every analysis.

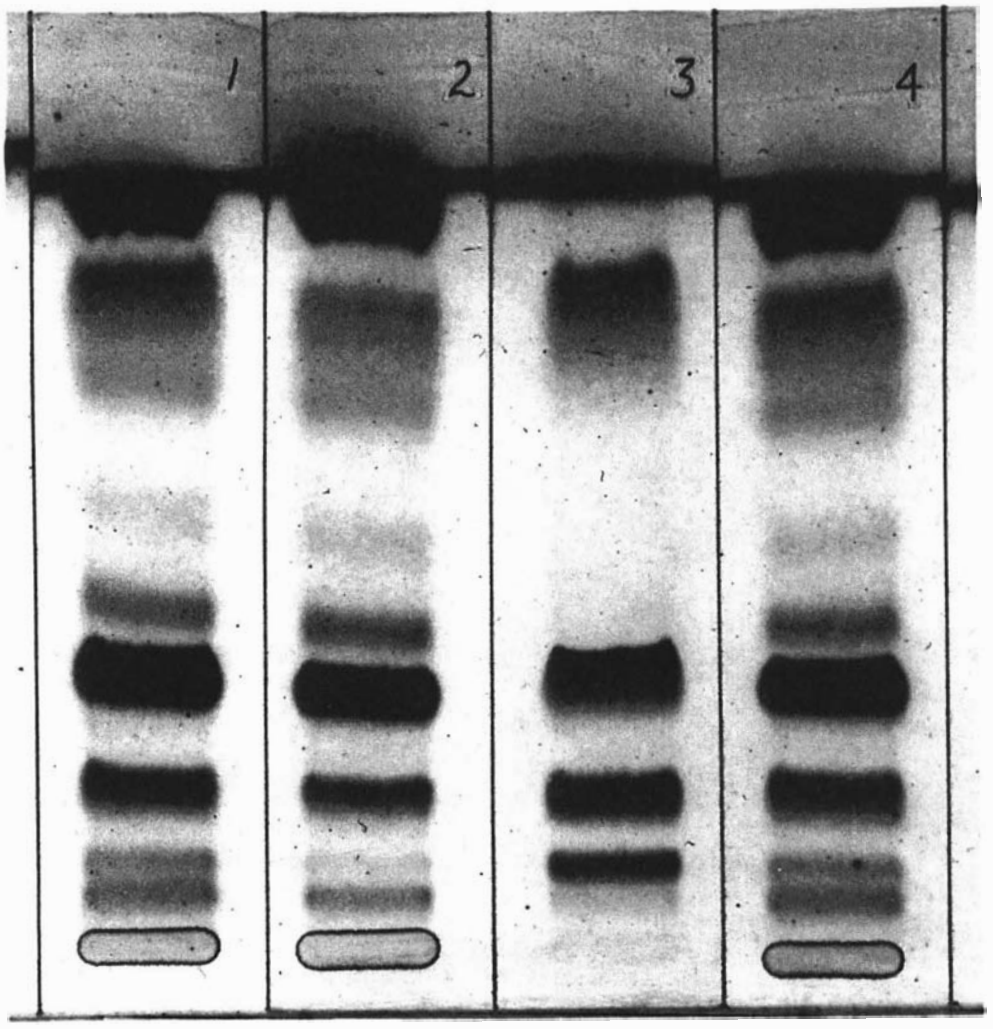

Fig. 1.4. Typical thin-layer separation prior to quantitative glycerolipid analysis. In lanes 1 and 4, 50 µl (≡ 50 mg fresh weight of leaf) of white clover leaf total lipid was streaked across 2 cm while in lanes 2 and 3, 50 µl non-acidic and acidic lipid fractions (≡ 50 mg and 200 mg fresh weight of leaf respectively) from the DEAE-cellulose column, were also streaked across 2 cm. Development, chloroform/methanol/acetic acid/water (85:15:10:3); layer, Silica Gel G; detection, acid dichromate spray.

b) Method previously used.

Since quantitative elution of polar lipids from thin-layer chromatograms can be very time consuming (Privette et al., 1965; Abramson and Blecher, 1964) it was decided early in this study to adopt a method which would permit chemical estimation to be made in the presence of adsorbent. In the first instance the method of Marinetti (1962) was used for the digestion of phospholipids in the presence of adsorbent and for the assay of liberated phosphorus. Although this method was quite satisfactory in the absence of adsorbent very high blanks were always obtained when silica gel was present during digestion. These high blanks were attributed to an interaction between perchloric acid and silica gel and accordingly the digestion was modified as follows.

A mixture of sulphuric and 60% perchloric acids (3:2 v/v; 0.5 ml) was added to the adsorbent in 1.5 x 15 cm digestion tubes and moderate heat was applied (electric micro-Kjeldahl rack) until the liquid turned a pale yellow. High temperatures were then used until the digest became colourless by which time most of the perchloric acid had been removed. To the cooled digests were added 8 ml of water, 1.5 ml of 2.5% ammonium molybdate and 0.2 ml of an aminonaphthol sulphuric acid

reducing reagent (Bartlett, 1959). The tubes were placed in a boiling bath for 7 min and the adsorbent removed by centrifugation before the absorbance of the resulting blue colour was measured at 660 mμ. Silica gel blanks and standards were included with every analysis. This method gave low blanks and a linear response to added phosphate but suffered from the disadvantage that bumping frequently occurred during distillation of the perchloric acid. Such bumping could not be prevented by adding alundum chips to the digestion mixture and was presumably due to a combination of the high temperature and the high ratio of solids to liquid.

This procedure was discontinued when the method of Rouser et al. (1966) became available.

6. Analysis of glycolipids.

Glycolipids, separated by a combination of DEAE-cellulose chromatography and TLC were also estimated in the presence of adsorbent.

a) Phenol/sulphuric acid reaction.

i) Direct method.

Galactolipids, from the equivalent of 25 to 50 mg of leaf (Fig. 1.2 and 1.4) were transferred on the thin-layer adsorbent into straight-sided centrifuge tubes and 1 ml of 2% phenol added (Fig. 1.5). This

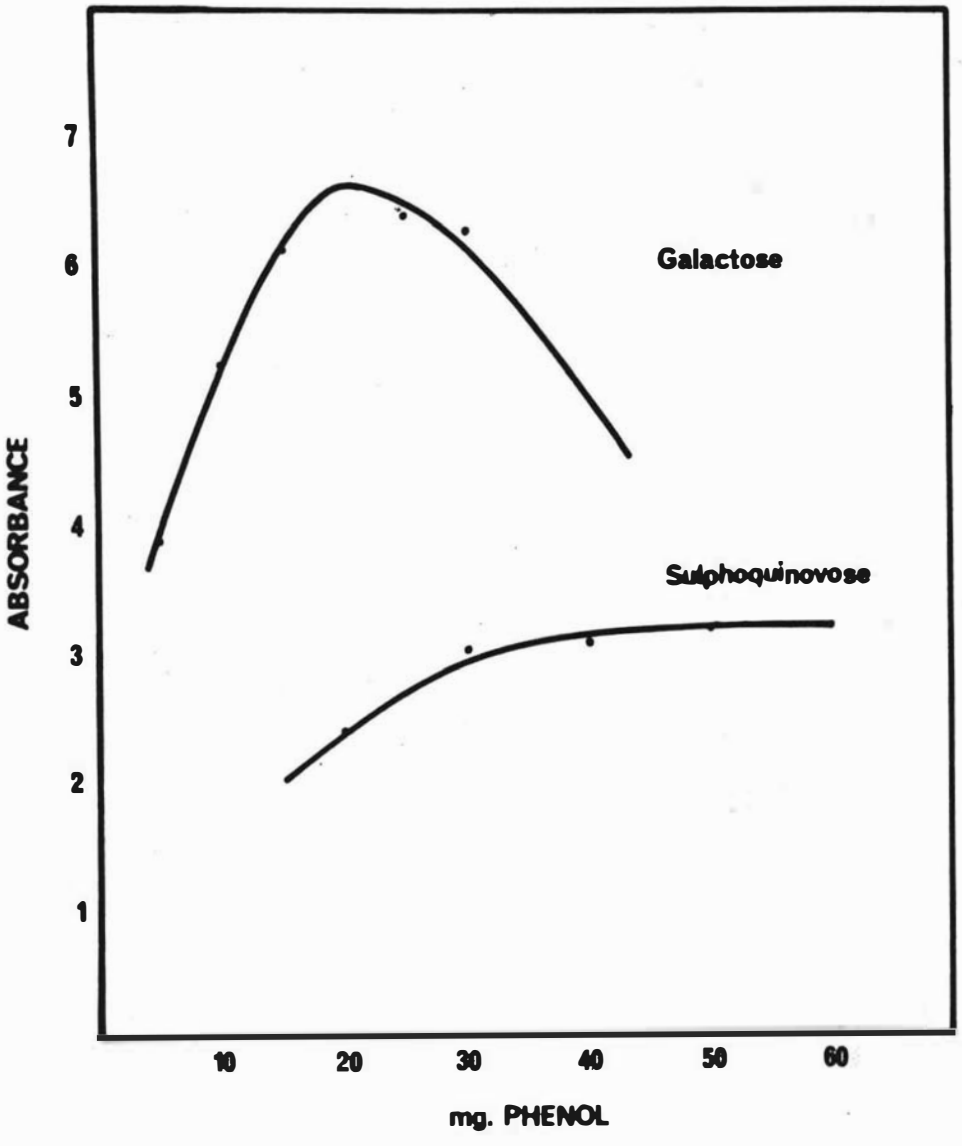

Fig. 1.5. The effect of phenol concentration on the colour produced by 50 µg of galactose (480 mµ) and 30 µg of sulphoquinovose (485 mµ) in the phenol/sulphuric acid estimation.

was followed by 4 ml of concentrated sulphuric acid, which was added rapidly and directed onto the surface of the aqueous phenol solution to ensure maximum mixing and heating. The reactants were thoroughly mixed (vortex mixer) and the tubes were allowed to stand for 15 min before centrifugation. The absorbance of the clear yellow-brown solution was measured at 485 mμ against water, corrected for blanks and compared with standards containing silica gel from a blank lane of the chromatogram (Roughan and Batt, 1968).

Sulpholipid, isolated from the equivalent of 200 mg of leaf (Fig. 1.4) was analysed in the same way except that 1 ml of 5%, instead of 2%, phenol (Fig. 1.5) was added to the adsorbent and absorbances were measured at 490 mμ.

Calibration curves for both galactose and sulphoquinovose in these assay systems were linear up to at least, 100 μg (Fig. 1.6) and the presence of silica gel in the reaction mixture had no effect on the colour produced.

ii) Indirect method.

When the amount of galactolipid separated, exceeded the equivalent of 0.6 μmole of galactose (see results), it was necessary either to scale up the reagent volumes or use a less direct method of analysis. In the indirect

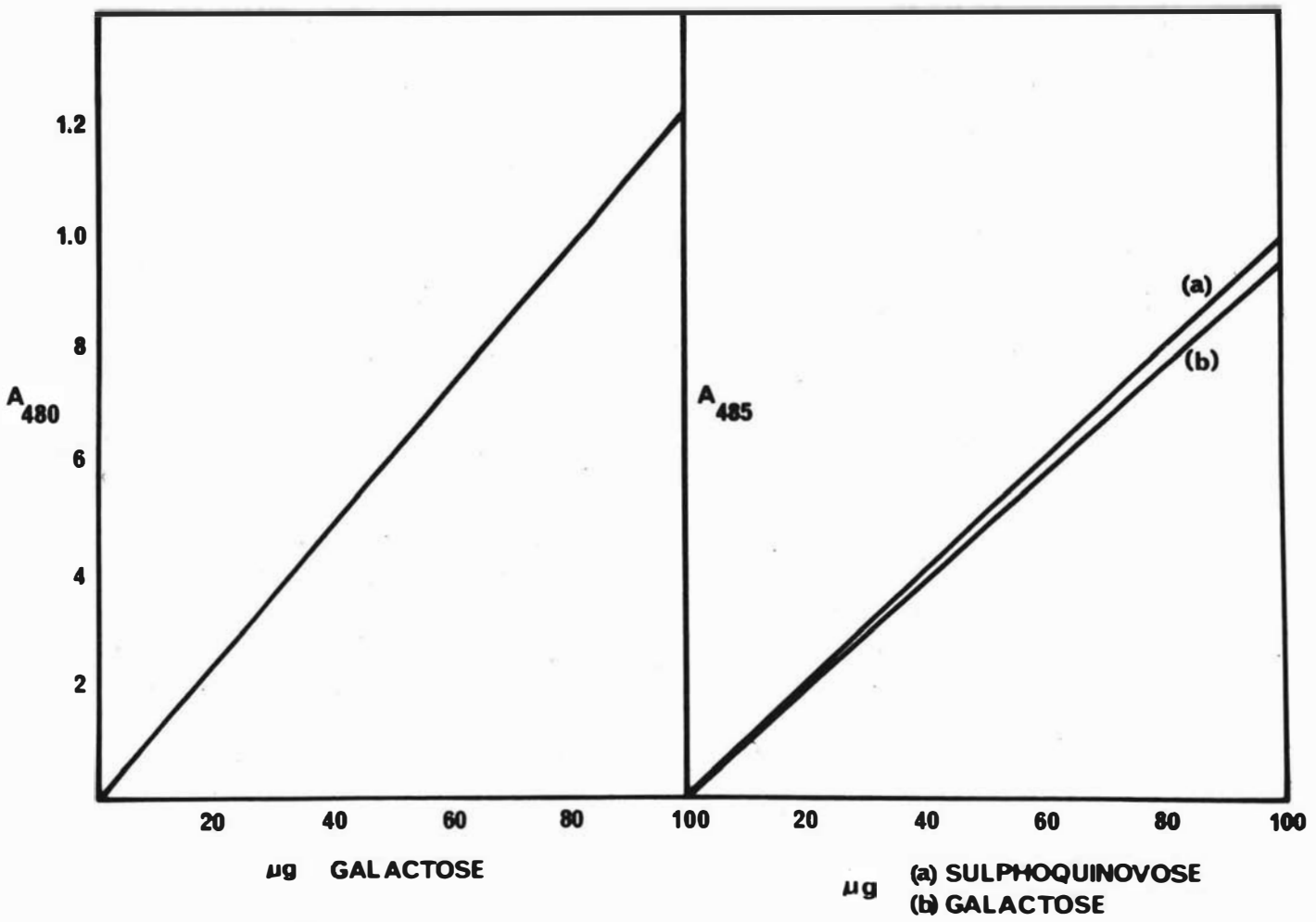

Fig. 1.6. Standard curves for galactose and sulphoquinovose and their respective assay systems. The curve for galactose in the sulphoquinovose system is also shown.

method the galactolipid zones were scraped into centrifuge tubes, 2 ml of 2N sulphuric acid was added and the lightly stoppered (glass marbles) tubes were placed in a boiling bath with occasional shaking for 60 min. After cooling and centrifuging, aliquots of the hydrolysate (0.6 ml) were added to 5% phenol (0.4 ml). Concentrated sulphuric acid (4 ml) was then added, the reactants thoroughly mixed, and after 15 min absorbances were measured at 485 mu. Blanks and standards were always included.

Sulpholipid zones were also scraped up into 2N sulphuric acid and the lipid hydrolysed as for galactolipids. In this case 1 ml aliquots of hydrolysates were taken, 50 µl of 80% phenol was added followed by 5 ml of sulphuric acid and absorbances were measured at 490 mµ.

Both the direct and indirect methods produced identical results.

b) Alternative methods.

Acid hydrolysates of glycerolipids on the adsorbent were initially analysed for reducing sugar by the method of Nelson (1944) and later by the method of Dygert et al. (1965).

An anthrone method (Russell, 1966) was also used for the analysis of glycolipids by direct and indirect methods similar to those used for the phenol/sulphuric acid procedure.

7. Application of the new method to a variety of species.

Common pasture species (clovers, ryegrasses) and squash plants were the main source of leaves used in the development of the methods described here. The amounts of extract taken for column chromatography and the amounts of column fractions taken for TLC apply to this group of plants. However large variations occur in the lipid content of different leaves relative to fresh weight, and this is reflected in the chlorophyll content of the leaf. In the study of the glycerolipid composition of different species, the amount of an extract required for column and thin-layer chromatography was judged from chlorophyll content rather than fresh weight. For example, an amount of extract containing 5 mg of chlorophyll (equivalent to 2.5 g of white clover leaflets, 5 g of squash leaves or 20 g of lettuce leaves) was within the capacity of the 3 x 1 cm (i.d.) DEAE-cellulose column and an amount of the non-acidic lipid fraction containing 100 to 150 µg of chlorophyll was as much as could be applied as a 2 cm streak to a 250 µ layer of Silica Gel G or HR without overloading the chromatogram.

Total chlorophyll was measured in methanol solutions of total lipids using the spectrophotometric factors reported by Comar and Zscheile (1942).

8. Chemicals.

Chloroform and methanol were either "Analar" or redistilled commercial grade solvents. Commercial grade 95% ethanol was refluxed over $KMnO_4$/KOH and redistilled. All other solvents were of "Analar" quality as were reagents unless another grade was recommended in the appropriate references. Solvents other than those listed above were of "Analar" grade.

Sulphoquinovose was prepared by the method of Miyano and Benson, 1962.

RESULTS

1. Lipid extraction and purification.

a) Recoveries in the modified Folch et al. (1957) procedure.

The modified Folch et al. (1957) procedure for the extraction and purification of leaf lipids as outlined under METHODS was the simplest and most convenient of the methods tested. Re-extraction of the residue from this treatment with boiling 95% ethanol and of the aqueous methanol washings with chloroform showed that the recovery of the major glycerolipids in the washed chloroform phase was essentially quantitative (Table 1.1). In this procedure the phases separated more rapidly and more sharply and there was less interfacial material compared with the similar technique of Bligh and Dyer (1959).

b) Effect of different washing procedures.

Since aqueous washing of chloroform/methanol extracts has been criticised (Nazir and Rouser, 1967) because of possible losses of polar lipids, the recoveries of DGD and SL were measured (a) after two different washing techniques and (b) when washing was omitted. Analyses of DGD and SL were identical in the Folch-washed and Sephadex-washed lipids but the recovery of DGD was comparatively high (x 2) and SL comparatively low (x 0.5) in the unwashed lipids taken

TABLE 1.1

Recovery of DGD, PG and SL from red clover leaves by the modified Folch extraction and purification procedure

	DGD*	PG*	SL*
Chloroform phase	98.5+	99	99
Aqueous Methanol phase	0.5	1	1
Residue	1	Trace	Trace

* Analysed after DEAE cellulose and thin-layer chromatography (methods).

\+ Percent of total lipid recovered from the three phases.

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through the same procedure (Table 1.2). The low recovery of SL was probably due to overloading of the DEAE-cellulose column accentuated by the presence of non-lipid material in the chloroform solution. When only half the quantity of unwashed lipid (i.e. 1 ml) was chromatographed, SL recovery became identical to that obtained with the other two procedures (Table 1.2). The recovery of DGD however, remained comparatively high. This could have been due to the presence of water soluble, sugar-like compounds that were eluted from the DEAE-cellulose column with non-acidic lipids and co-chromatographed subsequently with DGD. Supporting this supposition is the finding that DGD isolated by

TABLE 1.2

Effect of different lipid-washing procedures on the recovery of DGD and SL from the DEAE-cellulose column

Treatment	µmole lipid/g fresh weight of leaves	
	DGD	SL
Folch wash	3.44	0.60
Sephadex column	3.40	0.58
Unwashed (2 ml on column)	7.50*	0.34
(1 ml on column)	7.50*	0.58
(2-D TLC)	4.75*	

A chloroform/methanol extract (300 ml) of white clover leaves (15 g) was divided into three equal portions. One was washed with 0.2 vol of 0.73% NaCl (Folch wash), one was concentrated and passed through a Sephadex column (Wuthier, 1966) and one was evaporated to dryness and the residue extracted with chloroform (Rouser et al., 1963). The material from each treatment was finally made up to 5 ml in chloroform and 1 or 2 ml of this was placed on the DEAE-cellulose column. DGD and SL were analysed after TLC.

* DGD spot contaminated with sugar-like substances?

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two-dimensional thin-layer chromatography of the unwashed extract gave a value for DGD that was less than 50% higher than that obtained with the Folch and Sephadex washed lipids. For its speed and convenience the Folch wash was preferred over the Sephadex method.

Large losses of glycerolipids were encountered

during water washings in the method of Bailey (1962) which is discussed in detail in Appendix 2.

2. Preliminary separations.

a) DEAE-cellulose column.

Sharp separations of non-acidic and acidic lipids were achieved with comparatively small volumes of eluting solvents on DEAE-cellulose, semi-micro columns. (Figs. 1.1 and 1.4).

Removal of ammonium acetate (generated in the ion-exchange process) from the acidic lipid fraction was not essential but the insolubility of the salt in chloroform made pipetting of the final acidic-lipid solution difficult on occasions. The washing procedure described here for the removal of ammonium acetate was rapid and the recovery of acidic lipids quantitative.

b) Florisil column.

Attempts to obtain a clear separation of plant glycolipids from phospholipids by the Florisil column chromatographic technique of O'Brien and Benson (1964) were unsuccessful. The more polar glycolipids, DGD and SL, tailed through a large volume of eluting solvent (Fig. 1.7) such that in a large number of trials, involving i) variations of lipid loading, ii) different solvent volumes, ratios and types and, iii) different degrees of activation of the adsorbent, it was never possible to

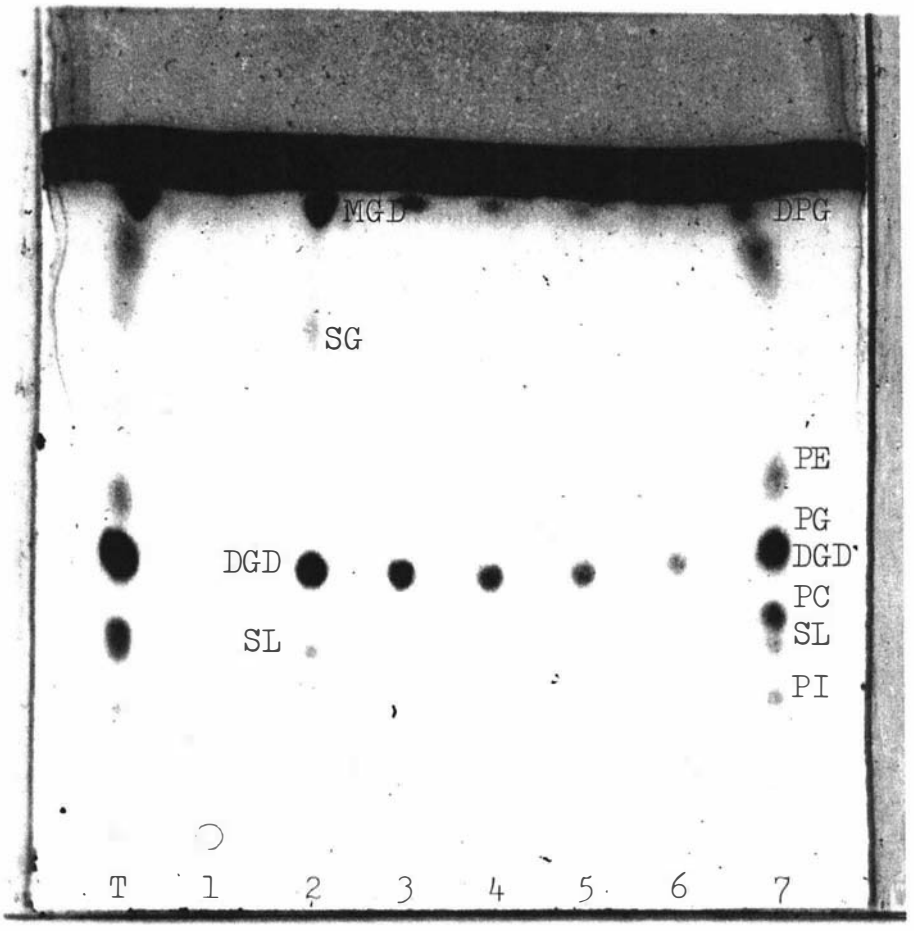

Fig. 1.7. Thin-layer monitor of fractions from a 20 g Florisil column. White clover leaf lipid. T, total lipid; 1, 100 ml chloroform (5% DMP); 2-6, 80 ml fractions of chloroform/methanol (2:1 5% DMP); 7, 200 ml chloroform/methanol/water (1:1:0.2). Silica Gel H with chloroform/methanol/acetic acid/water (85:15:10:3) as developing solvent. Detection, 50% sulphuric acid.

quantitatively recover the glycolipids free of phospholipid.

c) Florisil "batchwise" technique.

A thorough examination (Appendix 1) of the Florisil batchwise technique of Russell (1966) for the quantitative estimation of leaf galactolipids and sulpholipid showed that large losses of glycolipids were to be expected when only the "glycolipid" fraction was retained for analysis. Up to 60% of the sulpholipid and more than 50% of the two galactolipids were lost even when great care was taken with the technique.

3. Thin-layer chromatography.

a) Two-dimensional TLC.

Total lipid equivalent to 50 mg of clover leaf was the maximum that could be applied to Silica Gel G for two dimensional TLC. This amount did not give sufficient SL, DPG, PE or PI for accurate analyses and was only marginal for PC and PG. However, analyses of MGD and DGD separated by two-dimensional TLC could be performed by the phenol/sulphuric acid technique.

b) One-dimensional TLC.

With the solvent used for unidirectional TLC, MGD was sometimes localised with the pigments near the solvent front (Fig. 1.4) and could not be estimated by the direct phenol/sulphuric acid technique. If demixing

of the solvents was controlled so that the solvent fronts were 1 to 1.5 cm apart at the end of development, the pigments appeared between the two fronts and MGD immediately behind the second solvent boundary (Fig. 1.1). Demixing occurred more readily on Silica Gel G than on HR. When MGD was localised with the pigments, estimations were performed by the indirect phenol/sulphuric acid method.

4. Quantitative analyses of phospholipids.

The amount of a non-acidic lipid fraction from a DEAE-cellulose column required for accurate analyses of PC and PE was equivalent to 100 to 200 mg of leaf depending on the species being examined. For this reason it was better to judge the amount of this fraction required for TLC by chlorophyll content rather than by fresh weight of leaf (cf Tables 1.3 and 1.4). In most cases an aliquot containing the equivalent of 150 to 200 µg chlorophyll contained sufficient PC and PE for reproducible phospholipid assays but some exceptions were found. Xanthium, paspalum and lucerne contained low levels of PC and PE relative to chlorophyll (Table 1.4) and an amount of column fraction equivalent to 300 to 400 µg of chlorophyll was required for these analyses.

In general, amounts of the acidic lipid fraction,

equivalent to four times that of the non-acidic fraction (in terms of fresh weight of leaf) were required for the accurate analyses of glycerolipid components. The minor component in this fraction was usually PI.

5. Quantitative analyses of glycolipids.

a) Phenol/sulphuric acid method.

The phenol/sulphuric acid estimation (Dubois et al., 1956) is one of the most sensitive methods for sugar analysis. When tested with standard sulphoquinovose and galactose the sensitivity of this method was equivalent to that of the reducing sugar procedure of Dygert et al. (1965) and more than twice as sensitive as the anthrone estimation (Russell, 1966). Silica gel markedly depressed colour development in the reducing sugar assay but had no effect on the phenol/sulphuric acid reaction.

Sulphoquinovose in phenol/sulphuric acid had a single absorption peak (A_{max} = 490 mμ) while sulpholipid showed two peaks, 490 and 425 mμ (Fig. 1.8) of which the latter, smaller peak was found to be due to the fatty acid components of the sulpholipid (Fig. 1.8).

Galactose produced a major absorption peak at 485 mμ and a smaller peak at about 420 mμ (Fig. 1.9) in this assay system. Absorption due to a fatty acid reaction was superimposed on this minor peak when intact galactolipids were reacted with phenol/sulphuric acid (Fig. 1.9).

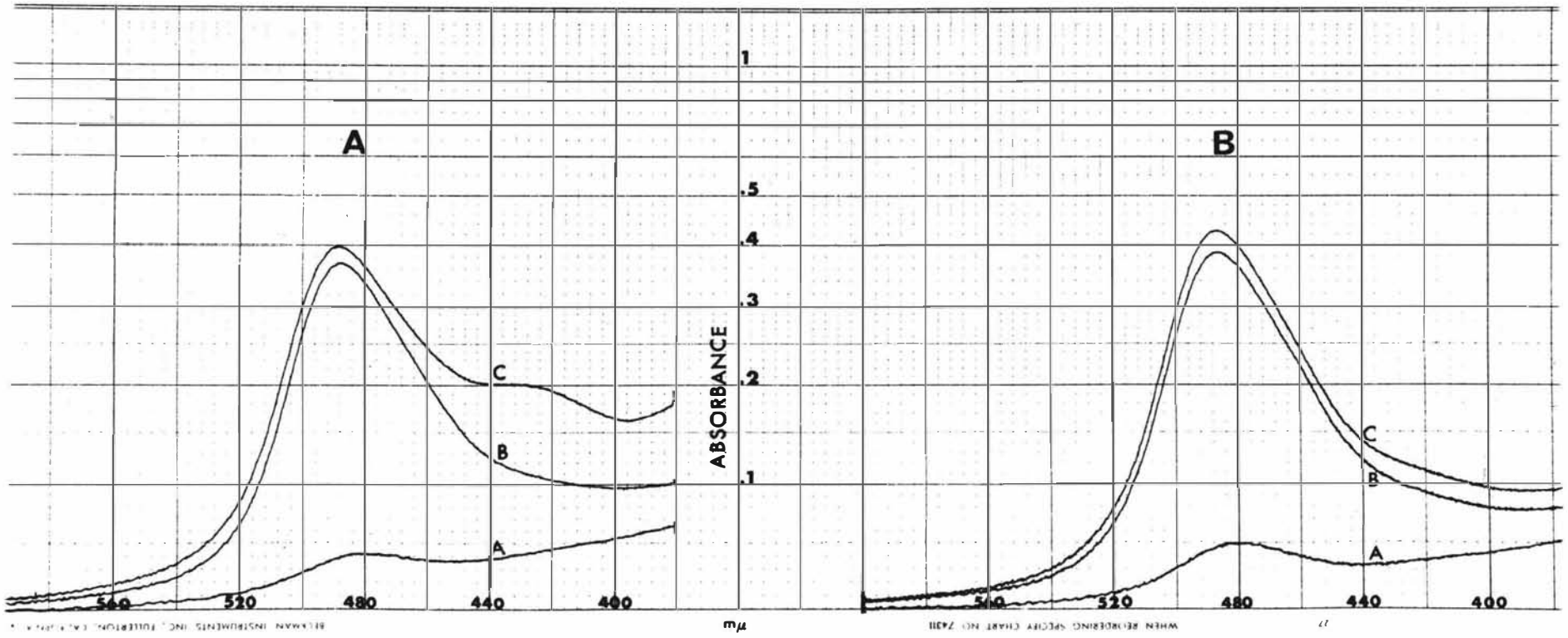

Fig. 1.8A. Absorption spectra of sulphoquinovose and sulpholipid in phenol/sulphuric acid in the presence of Silica Gel HR: (A) silica gel blank; (B) 30 μg sulphoquinovose; (C) sulpholipid isolated from the equivalent of 200 mg of red clover leaves.

Fig. 1.8B. Absorption spectra of deacylated sulpholipid in phenol/sulphuric acid: (A) acid hydrolysis blank; (B) 30 μg sulphoquinovose; (C) sulphoquinovosyl glycerol from the equivalent of 200 mg of the same red clover leaf extract as in Fig. 1.8A.

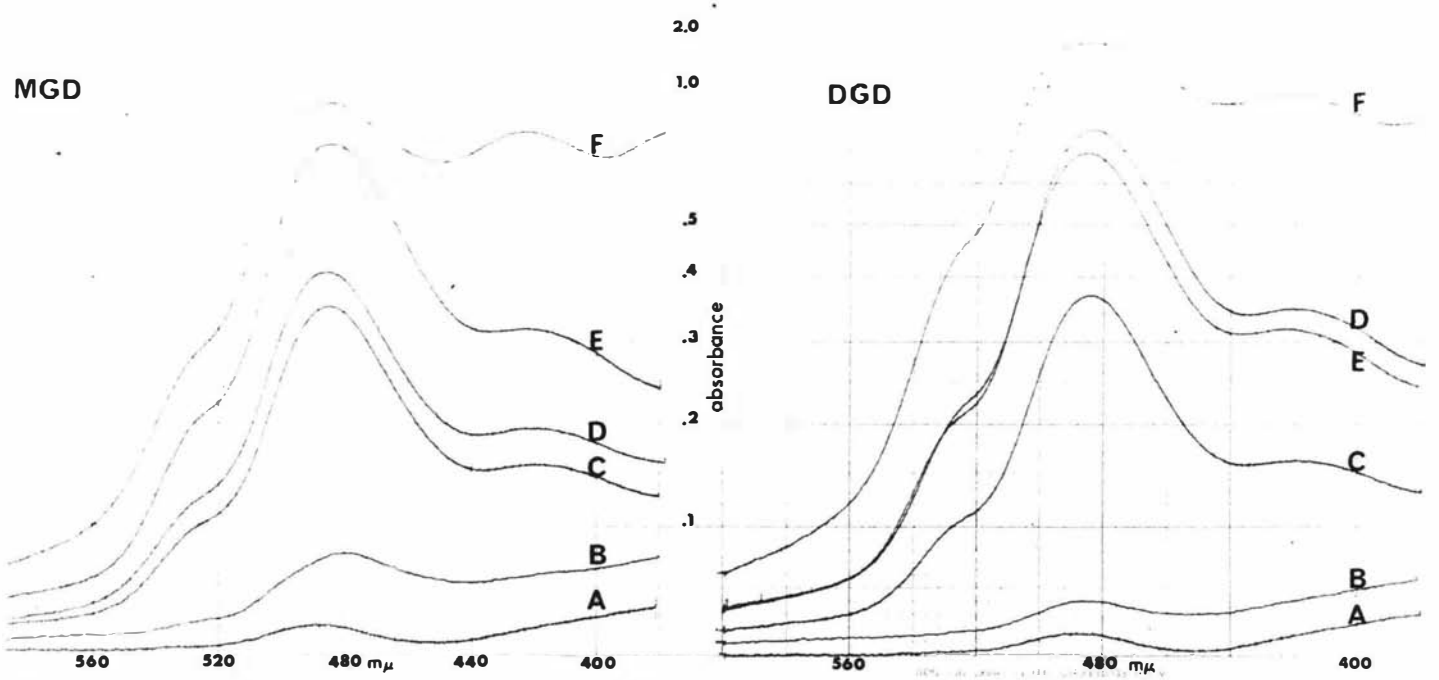

Fig. 1.9. Absorption spectra from the direct and indirect methods for galactolipid analysis in phenol/sulphuric acid: (A) hydrolysis blank; (B) silica gel blank; (C) hydrolysis standard (25 μg galactose); (D) galactolipid, equivalent to 50 mg of squash leaf, by indirect method; (E) 50 μg galactose plus Silica Gel HR; (F) galactolipid, isolated from 100 mg of squash leaf, by the direct method. MGD, monogalactosyl diglyceride. DGD, digalactosyl diglyceride.

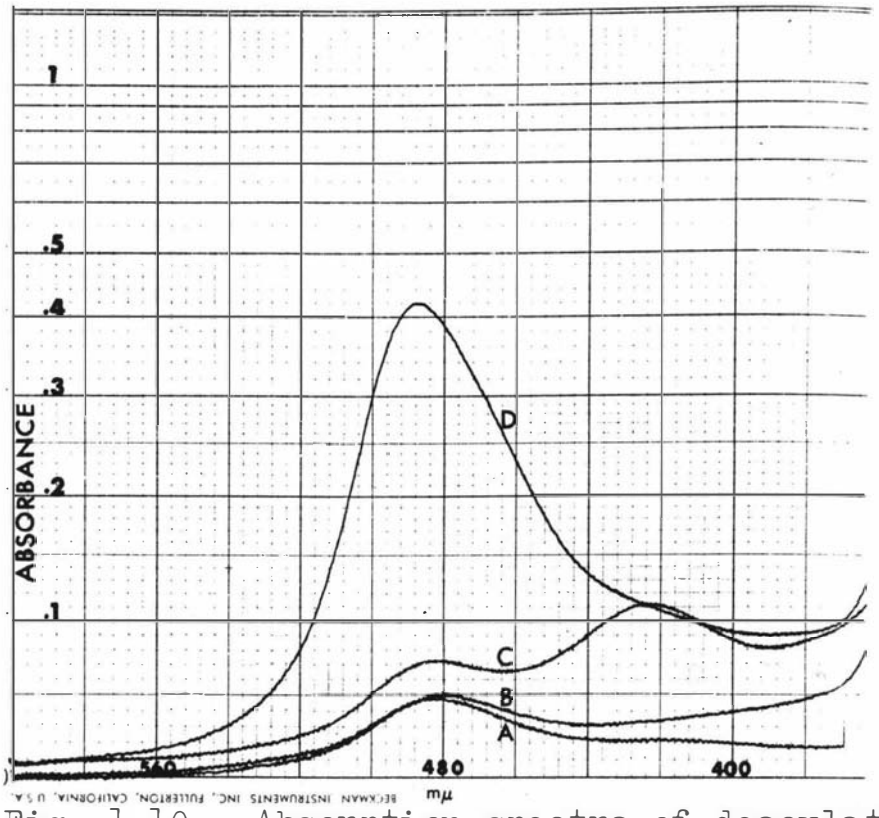

Fig. 1.10. Absorption spectra of deacylation products of sulpholipid in phenol/sulphuric acid: (A) silica gel blank; (B) hydrolysis blank; (C) fatty acids from the total isolated sulpholipid on Silica Gel HR; (D) sulphoquinovosyl glycerol from one-half of the isolated sulpholipid (see text).

Glycerol did not interfere with the analyses and fatty acids of intact lipids contributed to the absorption at 485 or 490 mμ to an extent of less than 2% for SL and DGD and less than 3% for MGD (Fig. 1.10 and Roughan and Batt, 1968).

Deacylation of MGD (for analysis by the indirect method) was necessary when this component was mixed with the pigments after TLC (Fig. 1.4). Sixty minutes at 100°C in 2N sulphuric acid was sufficient for the complete deacylation of MGD; fatty acids and pigments were retained on the adsorbent while galactose or galactosyl glycerol was liberated into aqueous solution.

b) Anthrone method.

The anthrone method (Russell, 1966) could not be used for the estimation of glycolipids in the presence of adsorbent. During colour development (7 min at 100 °C), tubes containing lipid turned a muddy brown colour which was possibly caused by charring of the fatty acids. The insensitivity of the method was a disadvantage in the analysis of acid hydrolysates.

c) Reducing sugar method.

Comparative analyses of acid hydrolysates of galactolipids by the reducing sugar method (Dygert et al., 1965) and by the phenol/sulphuric acid method showed that, while deacylation was complete in 60 min at 100°C,

quantitative release of reducing sugar required at least 90 min. For reducing sugar assays, acid hydrolysates were neutralized with 2N sodium hydroxide. The sodium sulphate formed, further depressed colour formation so that the sensitivity of the method was only 63% that of the phenol/sulphuric acid method.

Quantitative release of reducing sugar (sulphoquinovose) from the sulpholipid hydrolysed on the adsorbent was not obtained even after 3 hr at 100°C in 2N sulphuric acid although it could be shown (Fig. 1.8) that deacylation was complete within 60 min.

6. Simultaneous analyses of glyco- and phospholipids.

For simultaneous analyses of the glyco- and phospholipids in the DEAE-cellulose column fractions, an equivalent of 200 to 400 mg of leaf (0.2 to 0.6 mg of chlorophyll) of the acidic lipid fraction and 50 to 100 mg (about 100 µg of chlorophyll) of the non-acidic lipid fraction were required for TLC. Where the concentrations of PC and PE were low relative to the galactolipids (Xanthium, paspalum, lucerne) it was necessary to apply the equivalent of 200 to 300 µg of chlorophyll (200 mg of leaf) as a 4 cm streak to a thin-layer to obtain sufficient of these glycerolipids for accurate analysis. Galactolipids, in these instances, were determined by the indirect phenol/sulphuric acid procedure.

7. Glycerolipid composition of a variety of species.

The method of analysis adopted in this study ((a) lipid extraction and purification after Folch et al. (1957), (b) DEAE-cellulose/TLC separation of individual glycerolipids and (c) chemical analyses of the glycerolipids in the presence of adsorbent) was used to examine the glycerolipid composition of a range of species. Results are presented in terms of both fresh weight (Table 1.3) and chlorophyll content (Table 1.4) of tissue; the plants are listed in phylogenetic sequence according to Hutchinson (1964).

Although there are considerable species differences in the amounts of individual lipids present, the average values (in μmole/g) agree reasonably well with values calculated from the results of Wintermans (1960) (Table 1.5). Variability between species was less pronounced when the amounts were calculated relative to chlorophyll (Table 1.4) instead of fresh weight of tissue (Table 1.3). The excessively high values for camellia were due to the very low chlorophyll content of young leaves.

Variations between species were considerable even when chloroplast lipids were considered separately. The molar ratios of these lipids (MGD, DGD, PG and SL) were recalculated based on phosphatidyl glycerol equivalent to 1.0 (Table 1.6) and while these lipids may be the

only glycerolipids of chloroplasts (James and Nichols, 1966; but see Allen et al., 1966), these organelles show quite different molar ratios of the four compounds. The large standard deviations are due mainly to the results for Xanthium which had a very low level of PG but even when Xanthium was omitted from the analyses the standard deviations were still relatively high. The ratio $^{MGD}/_{DGD}$ varied from 2.4 in the fern Blechnum to 1.0 in camellia and lettuce but DGD did not exceed MGD in molar concentration in any leaf sample.

No relationship between evolutionary status and glycerolipid composition was observed.

TABLE 1.3

GLYCEROLIPID COMPOSITION OF PLANT TISSUES I

μ Moles of lipid per gram fresh weight of leaf

PLANT	MGD	DGD	SL	PC	PG	PE	PI	DPG
Mesotaenium caldariorum	10.00	5.50	1.30	1.10	1.67	0.80	0.80	Tr
Marchantia berteroana	1.07	0.66	0.19	0.18	0.16	0.09	0.07	0.16
Moss (see text)	2.68	1.50	0.48	1.26	0.40	0.40	0.15	0.05
Blechnum fluviatile	5.60	2.30	0.38	0.80	0.60	0.40	0.13	0.35
Ginkgo biloba	4.70	2.80	0.30	1.80	0.85	0.55	0.25	0.27
Pinus radiata	2.80	1.95	0.52	0.81	0.55	0.28	0.37	0.29
Rose Rosa	5.60	4.60	0.54	1.50	0.60	0.55	0.27	0.20
Rowan Sorbus aucuparia	10.20	7.16	0.46	2.20	0.97	1.70	0.37	0.30

Table 1.3 (cont.)

White clover Trifolium repens	8.60	5.20	0.76	1.41	1.13	0.87	0.25	0.28
Lucerne Medicago sativa	8.60	5.20	1.72	0.75	0.67	0.50	0.28	0.58
Poplar Populus itilica	4.95	3.80	0.62	1.80	1.10	0.80	0.50	0.35
Camellia japonica	3.10	3.10	0.23	2.00	0.65	0.90	0.20	0.90
Squash Cucurbita pepo	4.10	2.70	0.30	1.60	0.91	1.00	0.14	0.09
Tomato Solanum lycopersicum	5.08	2.46	0.31	1.10	0.43	0.45	0.10	0.10
Lettuce Lactuca sativa	0.68	0.68	0.03	0.31	0.10	0.21	0.06	0.13
Xanthium orientale	6.10	5.90	0.62	0.50	0.25	0.15	0.05	0.04
Cocksfoot Dactylis glomerata	8.00	5.10	0.62	1.10	1.10	0.80	0.10	0.28
Perennial ryegrass Lolium perenne	5.10	3.95	0.95	1.35	0.75	0.55	0.20	0.20
Paspalum dilatatum	6.00	3.60	0.62	0.38	0.48	0.18	0.10	0.10

Table 1.3 (cont.)

Maize Zea mays	3.10	2.30	0.35	0.45	0.48	0.24	0.12	0.24
RANGE	0.68 -10.20	0.68 -7.16	0.03 -1.72	0.18 -1.00	0.10 -1.67	0.09 -1.70	0.05 -0.80	0.01 -0.90
Parsnip Root	0.17	0.34	Tr	0.33	0.07	0.18	0.11	0.19

These results are the means of duplicate and in some cases triplicate and quadruplicate analyses of a single extract. For PE and PI duplicates that agreed to within 10%, were considered acceptable but for the other components only those duplicates that agreed to within 5% were taken.

TABLE 1.4

GLYCEROLIPID COMPOSITION OF PLANT TISSUES II

μ Moles of lipid per mg of total chlorophyll

PLANT	MGD	DGD	SL	PC	PG	PE	PI	DPG
Mesotaenium	6.25	3.42	0.81	0.69	1.04	0.50	0.50	Tr
Marchantia	3.25	1.90	0.53	0.50	0.45	0.25	0.19	0.45
Moss	2.60	1.46	0.47	1.22	0.39	0.39	0.14	0.05
Blechnum	3.08	1.26	0.21	0.44	0.33	0.22	0.07	0.19
Ginkgo	2.90	1.70	0.19	1.12	0.53	0.34	0.16	0.17
Pinus	5.10	3.50	0.95	1.47	1.00	0.51	0.67	0.53
Rose	3.97	3.26	0.38	1.06	0.43	0.39	0.19	0.14
Rowan	3.95	2.82	0.18	0.86	0.38	0.67	0.15	0.11
White clover	3.98	2.40	0.35	0.66	0.52	0.40	0.12	0.13
Lucerne	4.30	2.60	0.86	0.38	0.33	0.25	0.14	0.28
Poplar	4.20	3.30	0.53	1.55	0.94	0.68	0.43	0.30

Table 1.4 (cont.)

Camellia	6.74	6.74	0.50	4.44	1.95	1.41	0.44	1.95
Squash	4.10	2.70	0.30	1.60	0.90	1.00	0.14	0.05
Tomato	5.08	2.46	0.31	1.10	0.43	0.45	0.10	0.10
Lettuce	2.76	2.76	0.12	1.48	0.40	0.96	0.24	0.52
Xanthium	3.80	3.70	0.39	0.31	0.16	0.09	0.03	0.03
Cocksfoot	3.30	2.10	0.24	0.46	0.46	0.33	0.02	0.11
Perennial ryegrass	3.08	2.35	0.58	0.81	0.44	0.32	0.12	0.10
Paspalum	4.46	2.70	0.46	0.28	0.36	0.13	0.08	0.13
Maize	2.70	2.05	0.31	0.40	0.43	0.22	0.11	0.22
RANGE	2.60 -6.74	1.26 -6.74	0.12 -0.95	0.28 -4.44	0.16 -1.95	0.09 -1.41	0.02 -0.44	0.01 -1.95

TABLE 1.5

COMPARISON OF RESULTS WITH THOSE OF WINTERMANS[3]

μ Mole lipid per g fresh weight of leaf

	MGD	DGD	SL	PC	PG	PE	PI	DPG
Present study*	5.30	3.57	0.57	1.12	0.69	0.57	0.22	0.25
Wintermans+	3.97	1.79	0.53	1.80	1.18	0.73	0.38	-

* Averages of 20 species

+ Averages of three species
Elder, beet and bean (8 hr seedlings)

TABLE 1.6

MOLAR RATIOS OF "CHLOROPLAST" LIPIDS

(PG = 1.0)

	MGD	DGD	SL	PG
Mesotaenium	6.00	3.30	0.78	1.0
Marchantia	6.70	4.12	1.19	1.0
Moss	6.70	3.75	1.20	1.0
Blechnum	9.25	3.83	0.63	1.0
Ginkgo	5.54	3.29	0.35	1.0
Pinus	5.10	3.54	0.94	1.0
Rose	9.35	7.65	0.90	1.0
Rowan	9.50	7.00	0.45	1.0
White clover	7.60	4.60	0.67	1.0
Lucerne	12.90	7.80	2.58	1.0
Poplar	4.50	3.16	0.56	1.0
Camellia	4.77	4.77	0.35	1.0
Squash	4.50	2.96	0.33	1.0
Tomato	11.80	5.72	0.72	1.0
Lettuce	6.80	6.80	0.30	1.0
Xanthium	24.10	23.60	3.28	1.0
Cocksfoot	7.28	4.64	0.56	1.0
Perennial ryegrass	6.80	5.28	1.28	1.0
Paspalum	12.50	7.50	1.29	1.0
Maize	6.35	4.80	0.73	1.0
Means	8.40	5.90	0.95	
± Standard Deviation	±4.23	±4.67	±0.80	
Xanthium omitted from analysis				
Means	7.05	4.97	0.83	
± Standard Deviation	±4.14	±2.85	±0.57	

DISCUSSION

1. The New Technique.

The aim of this study has been to evaluate methods for the separation and analysis of leaf lipids. The method finally adopted is considered to be the most convenient, the most rapid and provides the most accurate analyses of the major glycerolipids of leaves, of the methods so far available. The lipid washing and extraction techniques are rapid and give excellent results. The chloroform/methanol extraction is preferred over sequential extraction schemes involving ethanol or acetone which do not break lipid-protein associations as effectively as methanol. The DEAE-cellulose, semi-micro column separation of neutral and dipolar lipids from acidic lipids can be accomplished quite rapidly and uses a minimum of solvents. With the thin-layer step taking approximately 60 min, an experienced laboratory worker can expect to isolate the eight major glycerolipids of leaves within 2 to 3 hr and to complete the glycolipid analyses within a further 0.5 to 1.0 hr. The complete analysis of glyco- and phospho- lipids may require up to 4.5 hr if it is necessary to use the indirect method for galactolipid analysis.

The speed and convenience of the separations can be judged from the studies on the incorporation of labelled

precursors into the glycerolipids of leaves and algae reported in Parts 2 and 3. For example, from 6 algal samples taken on one day, 60 glycerolipids were isolated for counting by the end of the following day; an estimated 8 hr of laboratory working time for one person.

The methods developed here for the separation of plant lipids are based on techniques which have been used for the qualitative, but not quantitative study of these lipids. In one instance (O'Brien and Benson, 1964) analytical figures were given for glycolipids in alfalfa and Chlorella following a combination of Florisil and DEAE-cellulose column chromatography but the values for Chlorella were in marked contrast to those reported earlier from the same laboratory (Ferrari and Benson, 1961). Classic silicic acid column chromatography (Zill and Harmon, 1962) proved unsatisfactory for the separation of plant lipids as did the silicic acid-impregnatednated paper technique of Marinetti and Stotz (1956) (Kates, 1960; Ferrari and Benson, 1961). Even careful thin-layer chromatography on silica gel does not adequately resolve some major constituents in a single development (Nichols, 1963). In this procedure for instance, PG co-chromatographs with DGD, SG with DPG and PC often with SL in the thin-layer system used. Florisil column chromatography has been reported to separate plant glyco-

lipids from phospholipids (O'Brien and Benson, 1964; Russell, 1966) but despite repeated tests here Florisil columns have not been found to be satisfactory. It is considered that the technique described by O'Brien and Benson should give low recoveries of MGD, which would account for the discrepancies reported by Ferrari and Benson (1961) and O'Brien and Benson (1964); in the earlier report Chlorella was claimed to show a MGD/DGD ratio of 2:1 while the later paper gave the ratio as 1:2. Modifications of the procedure gave quantitative recoveries of MGD but no method was found to correct the tailing of DGD and SL that was encountered. Similar tailing with Florisil columns has been reported in the separation of polar lipids (Rouser, O'Brien and Heller, 1961) and even neutral lipids (Carroll, 1961) of mammalian origin.

A recent method for the analysis of sulpholipid and galactolipids (Russell, 1966) used a batchwise Florisil technique to concentrate the glycolipid but when this was tested large losses of glycolipid was observed. It was found that even when great care was taken with this "batchwise" chromatography procedure, losses of sulpholipid of up to 60% and galactolipids of up to 40% for MGD and 50% for DGD were to be expected.

The technique of eluting silicic acid columns with

acetone (Vorbeck and Marinetti, 1965) has not been used in this study in the separation of leaf glycolipid and phospholipid (Rouser et al., 1967) but has given excellent results with Mesotaenium lipids (Part 2). However, even with very small columns, the slow flow rates and relatively large volumes of eluting solvents required are disadvantages for routine analyses.

DEAE-cellulose column chromatography adapted for the separation of polar lipids (Rouser et al., 1961) proved of great value in the separation of leaf lipids. The components which co-chromatograph on silica gel are quickly and easily separated on the column. In earlier applications of the DEAE-cellulose column to the separation of lipids from plant tissues (Allen et al., 1964; Nichols and James, 1964; Allen et al., 1966), attempts were made to fractionate the individual non-acidic lipids by using increasing concentrations of methanol in chloroform. This procedure has not been followed in this study since the fractionation was more simply and effectively accomplished by TLC.

The main analytical advantage in using DEAE-cellulose is the sharp and unequivocal separation of acidic from non-acidic lipids; the method is both rapid and simple. An additional advantage is that several of the more minor components appear in the acidic lipid fraction and

this results in a useful concentration of these substances. It was possible to apply an amount of the acidic lipid fraction from 200 to 400 mg fresh weight of leaf (depending on the source) across a 2 cm streak and this improved the convenience and economy of the thin-layer step. As a disadvantage, low levels of PE sometimes complicated the simultaneous analysis of phospho- and galacto- lipids from the non-acidic lipid fraction (see Results) but once the relative concentrations were known approximately, then the operation was simplified by resorting to the indirect method of galactolipid analysis.

Galactolipids of photosynthetic tissue have attracted a good deal of interest for a variety of reasons and a number of methods have been used for their analyses (Bailey, 1962; O'Brien and Benson, 1964; Rosenberg and Pecker, 1964; Rosenberg et al., 1966; Russell, 1966; Gray et al., 1967). Some of these methods have been examined in this study (Bailey, 1962; O'Brien and Benson, 1964; Russell, 1966) and they are referred to in more detail in Appendices 1 and 2. Some methods have used hydrolytic cleavage of the whole lipid (Rosenberg and Pecker, 1964), but in these cases there can be no indication of the relative amounts of MGD and DGD in the samples. Alternatively, MGD and DGD have

been separated by column chromatography (Rosenberg et al., 1966) or eluted from thin-layer chromatograms (Gray et al., 1967) prior to hydrolysis. When galactolipid analyses only were required it was found most convenient to use the DEAE-cellulose/TLC method or even 2-dimensional TLC although the latter took a little longer and was less convenient when dealing with a number of samples. For example, up to nine lanes could be drawn on a single 20 cm^2 layer of silica gel and 25 µl of D1 streaked across 1 cm in each of eight lanes. Up to four samples could be analysed in duplicate on a single chromatogram.

In earlier attempts to measure galactolipids by the reducing sugar method of Nelson (1944) it was noted that the presence of silica gel during hydrolysis markedly depressed the colour produced by galactose standards. It was also very difficult to get a measurable response for the sulpholipid. When the more reliable method of Dygert et al., (1965) became available this was also tried but with essentially the same results. Because of the acid stability of the sulphoquinovose-glycerol linkage (Benson, Daniel and Wiser, 1959) it seemed unlikely that reliable results would be obtained by using the reducing sugar method in combination with acid hydrolysis of the sulpholipid. Both the phenol/H_2SO_4 and the anthrone/H_2SO_4 reagents effectively split this bond however, and provided the basis for the first

reliable estimations of sulpholipid concentrations in leaves.

Of some significance and interest was the finding that adsorption to silica gel enhanced the rate of deacylation of the galactolipids in 2N H_2SO_4. Earlier work (Appendix 2) had shown that deacylation of leaf lipid samples in methanolic alkali prior to acid hydrolysis gave higher and more reproducible recoveries of galactose than when the deacylation step was omitted (Bailey, 1962). On the basis of reducing sugar and phenol/H_2SO_4-reacting galactose differences in the acid hydrolysates these low recoveries were considered to be caused by the slow deacylation of the glycolipid. This in turn was attributed to the tendency of the lipid to form globules into which the acid could not readily penetrate. Higher andreproducible recoveries of galactose could also be obtained if the lipid was first adsorbed onto silica gel and then subjected to acid hydrolysis. The pigments and other fatty materials remained on the adsorbent while the glyceryl compounds were released into solution.

By using the phenol/H_2SO_4 assay it has been possible to show that the galactolipids of a leaf lipid sample adsorbed on silica gel are completely deacylated in 2N H_2SO_4 at 100° in less than 45 min. This does not mean

however, that galactose would be released at this rate. The hydrolysate at this time could contain a mixture of digalactosyl glycerol, galactosyl glycerol and galactose, and measurement of the reducing sugar in such a mixture would lead to low estimations of galactolipid concentration. It would seem that if acid hydrolysis of a lipid sample is to be followed by estimation of the reducing sugar as a measure of the original glycolipid then at least 90 min should be allowed for the quantitative release of reducing sugar in 2N H_2SO_4 at 100°.

2. The glycerolipids of different species.

Glycerolipids, other than those listed in Tables 1.3 and 1.4 may have been present in some of the extracts and were either not detected by the light iodine-staining technique or appeared in only trace amounts. Typical of such constituents would be phosphatidyl serine which has been found in sweet clover leaves (Benson et al., 1959). Lysophosphatidyl choline was found in significant amounts only in Paspalum and maize (0.14 and 0.17 μmole/g respectively) and two unknown lipids together with triglyceride were found in high concentrations in Mesotaenium. From their charring reactions (Marsh and Weinstein, 1966) compared with known glycolipids and phospholipids, the unknown lipids were estimated at 2.4 and 0.9 μmole/g and, from its glycerol content (Renkonen,

1962) the triglyceride was estimated at 2.3 μmole/g.

The results of this study extend the range of species for which quantitative and detailed glycerolipid analyses are available from four (Chlorella, beet, bean and elder leaves) to twenty four and in addition provides the first analysis of root tissue.

The large variations between species in the concentrations of glycerolipids relative to chlorophyll was unexpected, especially with respect to the lipids considered to be confined to chloroplasts. Even when camellia is omitted from consideration (because of the relatively low chlorophyll content of the sample taken) only the galactolipids show a reasonably constant distribution varying by approximately 50% from the average over the range of species examined. Both of the remaining chloroplast glycerolipids, SL and PG, showed variations around the mean value of more than 100%. Extrachloroplastic lipids also showed high variations but this might be expected with chlorophyll content as the basis for comparison since variable amounts of non-photosynthetic tissue (i.e. vascular tissue) would be encountered in different leaves. The most variable of the glycerolipids was DPG, which was undetectable in Mesotaenium (cultured under continuous light) even by radioisotope techniques and yet was a major phospholipid

(by weight) of camellia leaves. It is not impossible that these variations portent seasonal or diurnal variations in the concentrations of these components in leaves especially since analyses of red clover leaves have suggested sulpholipid levels in the early morning may be reduced three fold compared with later in the day (Roughan, unpublished).

"Chloroplast" lipids have been detected in potato tuber (La Page, 1964) and it is possible that they are present in roots (e.g. carrot) which have the ability to become green in response to light. However, parsnip root does not develop chloroplasts and yet has relatively high levels of these lipids. It seems likely that these lipids are localized in the proplastids of root tissue although these organelles do not contain the prolamella bodies typical of chloroplast precursors in etiolated leaves. Instead, root proplastids are apparently involved with the synthesis of storage starch and, in mature carrot root tissue, can be recognized only as a thin membrane surrounding large starch grains (Toyama, 1967). A similar situation is likely to exist in mature parsnip root cells.

It has been suggested that the galactolipids of chloroplasts may be involved in sugar transport across the chloroplast membrane (Benson, 1963) and these

lipids could play a similar role in the starch storing plastids of root tissue.

PART 2

THE GLYCEROLIPIDS OF MESOTAENIUM CALDARIORUM AND THEIR TURNOVER

INTRODUCTION

Few kinetic studies of the uptake of ^{14}C-labelled precursors into the glycerolipids of leaves and algae have been carried out, primarily through a lack of suitable methods for the routine separation of the glycerolipids of photosynthetic tissue. In 1957, Eberhardt and Kates demonstrated the incorporation of label from $^{14}CO_2$, 2-^{14}C-pyruvate, 1-^{14}C-acetate and ^{32}P-orthophosphate into the four "phosphatide" fractions of runner bean leaves. Subsequently, Kates (1960) detected incorporation of radioactivity from a similar range of precursors into more specific glycerolipid fractions of runner bean leaf, using a more effective separation procedure. All of the labelled substrates except $^{14}CO_2$ were taken up into whole leaves through the cut petiole. When radioactivity levels in the separated lipids were determined after 1.5 to 2 hr, most of the activity was found in the pigment plus neutral (i.e. non-saponifiable) lipid fraction and, of the glycerolipids, MGD, DGD and PA showed the highest level of labelling. From incorporation studies

with ^{32}P-orthophosphate, PI was found after 5 hours to have the highest specific activity with decreasing activities in PA, PE, PG and PC. In 1965, Sastry and Kates followed the incorporation of ^{32}P-orthophosphate into the phosphatides of Chlorella vulgaris for 42 hrs. After 3 hr PG and DPG showed the highest specific activities with lower incorporations into PI and little activity in PC and PE. The general pattern was unchanged at 6 hr but by 18 hr the specific activity of PG began to level off and the order of decreasing specific activities, which remained unchanged up to 42 hrs, was DPG > PG = PE > PI = PC. The 6 hr results suggested relative turnover rates in the order DPG and PG > PE > PI > PC. The uptake of ^{32}P-orthophosphate into phosphatides of elongating and non-growing Avena coleoptile has been reported by Zimmerer and Hamilton (1965). They suggested that PI and PG had the highest turnover rates since these lipids contained relatively more label (compared with PE and PC) at 8 hr than at 24 hr. The only other phospholipids detected in this study were PE and PC which contained relatively more label at 24 hr than at 8 hr su gesting that they took a longer time to equilibrate with the orthophosphate in the medium.

Working with the green alga Chlorella pyrenoidosa, Ferrari and Benson (1961) reported the most detailed

study, to that date, on the kinetics of incorporation of a labelled precursor into the glycerolipids of a photosynthetic tissue. Algal suspensions were treated with $^{14}CO_2$ (as bicarbonate) and lipid extracts from samples which had been removed at different time intervals were deacylated for the separation of the individual glyceryl compounds by paper chromatography. After 15 sec, radioactivity was detected only in galactosyl and digalactosyl glycerols but within 30 seconds phosphoryl glycerol and sulphoquinovosyl glycerol also contained appreciable amounts of radioactivity. Of the remaining deacylated compounds, glycerophosphoryl inositiol showed the highest turnover of ^{14}C and the glycerol moiety of the galactosyl glycerols was shown to become labelled more slowly than the galactose moiety. For longer labelling periods (up to 6 hr) cultures were connected with a closed system and 500 uc of $^{14}CO_2$ in 5% CO_2 in air was circulated for the duration of the experiment. Intact lipids were separated by silicic acid paper chromatography and, although complete separations of all components were not achieved, the results were in general agreement with those for the deacylated compounds. Isotope incorporation into the fractions was linear for 6 hr; radioactivity levels in the individual fractions were in the order MGD > TG > DGD > PG + PC > SL + PI > PE. The fatty acids of all fractions were found to

become labelled at a much slower rate than the hydrophilic moieties.

More recently, Nichols et al. (1967), using techniques similar to those described in Part 1, followed the incorporation of 2-^{14}C-acetate into the glycerolipids of dark-grown Chlorella vulgaris both in the light and in the dark. Their results showed that the lipids could be grouped in the following way, on the basis of turnover rates:- (a) those which showed a high turnover rate of certain fatty acids (including MGD, PG, PC and TG) and (b) those which had a low turnover of constituent fatty acids (including DGD, SL, PI and PE). These findings seemed to be characteristic of both steady state conditions (i.e. dark-grown cells incubated in the dark) and the developing chloroplast system (dark grown cells incubated in the light).

With the exception of this recent study of Nichols et al. (1967), where 2-^{14}C-acetate incorporation was followed into the separate glycerolipids and into the various fatty acids of each glycerolipid, fatty acid biosynthesis in leaf tissue and green algae (James, 1963; Hawke and Stumpf, 1965; Harris and James, 1965; Harris et al. 1965) has been studied independently of glycerolipid metabolism. For the examination of fatty acid biosynthetic mechanisms, labelled acetate has usually

been found to be the most useful precursor. The kinetics of incorporation of ^{14}C-acetate into the fatty acids of leaves and green algae have been reported by James and his co-workers (James, 1963; Harris et al., 1965; Nichols et al., 1967). In castor leaf (James, 1963), oleic acid was found to have the highest specific activity within 60 min of supplying labelled acetate to a detached leaf, while palmitic, linoleic and stearic acids had relatively low specific activities. Linolenic acid, which represents 60% of the total fatty acids of castor leaf, was labelled very slowly; it took at least 24 hrs for appreciable radioactivity to be detected in the acid. In some respects, this low rate of labelling is surprising. The galactolipids of higher plants are largely dilinolenyl esters (O'Brien and Benson, 1964; Sastry and Kates, 1964; Weenink, 1964) and are the predominant glycerolipids of photosynthetic tissue (see Part 1). Taken in conjunction with the findings of Ferrari and Benson (1961) and James (1963), the possibility is suggested that a rapid turnover of the galactose and glycerol moieties occurs with an associated slow turnover of the fatty acids in the galactolipids of higher plants.

Initially, it was planned that leaves would be used here in the study of the incorporation of labelled

precursors into the glycerolipids of a photosynthetic tissue, partly because no kinetic experiments using leaf tissue had been reported previously. However, technical difficulties arose in both the handling and reproducible labelling of leaves which directed attention towards a photosynthetic micro-organisms, as the test biological system. The green alga, Mesotaenium caldariorum, was already being cultured in the laboratory for other purposes and it presented several clear advantages for study, each related to the possibilities of growing it in a liquid medium. Growth conditions could be well defined, uniform labelling and sampling of a culture could be effected readily and the calculation of relative turnover rates from isotope incorporation appeared to be more straightforward with a unicellular system.

Previous incorporation studies with algae had shown that $^{14}CO_2$ was a good precu sor for both glycerolipids (Ferrari and Benson, 1961) and fatty acids (Harris et al., 1965) while labelled acetate was incorporated into Chlorella fatty acids to a lesser extent that $^{14}CO_2$ (Harris et al., 1965).

It was considered that some quantitative data for turnover of glycerolipids might be obtained from experiments similar to those described by Ferrari and Benson (1961). In such cases, a linear incorporation

was demonstrated of ^{14}C from $^{14}CO_2$ into the glycerolipids of Chlorella between 30 min and 6 hrs after supplying $^{14}CO_2$ at a constant specific activity. The ratio of labelling of galactose to fatty acids in MGD did not change after 30 min which suggested carbon was being incorporated into the galactose and fatty acid components of the glycerolipids at the same rates. Knowing that the incorporation of ^{14}C from $^{14}CO_2$ is linear for the individual glycerolipids and given the specific activity of $^{14}CO_2$ in the medium and the number of carbon atoms in each of the glycerolipids, it should be possible to calculate rates of replacement of carbon atoms in the lipids, assuming that the specific activity of $^{14}CO_2$ in the medium was the same as the activity in the cells.

METHODS

1. Growth Conditions.

Mesotaenium caldariorum cultures were originally obtained from the culture collection of algae at Indiana University, where the organism had been propagated on unenriched agar slopes. It had been demonstrated by Dr Taylor (personal communication) that, although the organism is an obligate phototroph, maximum growth in liquid culture is obtained only when the media is enriched with glucose and yeast extract. Accordingly cultures have been routinely subcultured under sterile conditions into 30 ml of enriched media in 100 ml conical flasks fitted with cotton wool plugs. Continuous illumination (11 watt.meter^{-2} of photosynthetic irradiance) was provided by a bank of ("daylight") fluorescent tubes in mirror-lined growth cabinets maintained at 18^{o}C. Flasks were not agitated during growth except for an occasional inspection of some cultures and the non-motile cells normally proliferated on the bottom of the flask. The growth medium was the same as that described by Sager and Granick (1953) for *Chlamydomonas reihhardii* except for the addition of glucose (1%) and yeast extract (Difco., 0.2%). In the present work, stock cultures were routinely maintained as described while larger cultures (400 ml) were grown in 1000 ml

culture vessels (Fig. 2.1) for experimental purposes. These flasks were placed on a rocking stage which was designed to swirl the contents gently and prevent the cells from settling. Two procedures were used for collecting aliquots of the cultures (Fig. 2.1); an earlier device consisted of a glass syphon which was fixed into the flask through the cotton wool plug but later it was found to be more satisfactory to use an outlet fitted into the side of the flask near the base so that larger aliquots (40 ml) could be drawn off quickly. Cells were harvested by centrifugation at 2500 rpm (1600 x g) for 10 min and washed once with tap water. The packed cell volume was measured by using graduated centrifuge tubes and it has been assumed that 1 ml of packed cells weighed 1 g.

2. Analysis of Growth.

For growth analysis, 10 ml of culture was normally sampled and centrifuged in a graduated, 10 ml Kolmer tube (Kontes, Vineland, New Jersey). The packed cells were extracted with 200-300 vol of methanol, centrifuged after a few minutes and the chlorophyll content of the supernatant measured by applying the spectrophotometric factors of Comar and Zschiele (1944). Glucose in the media was normally measured in suitable

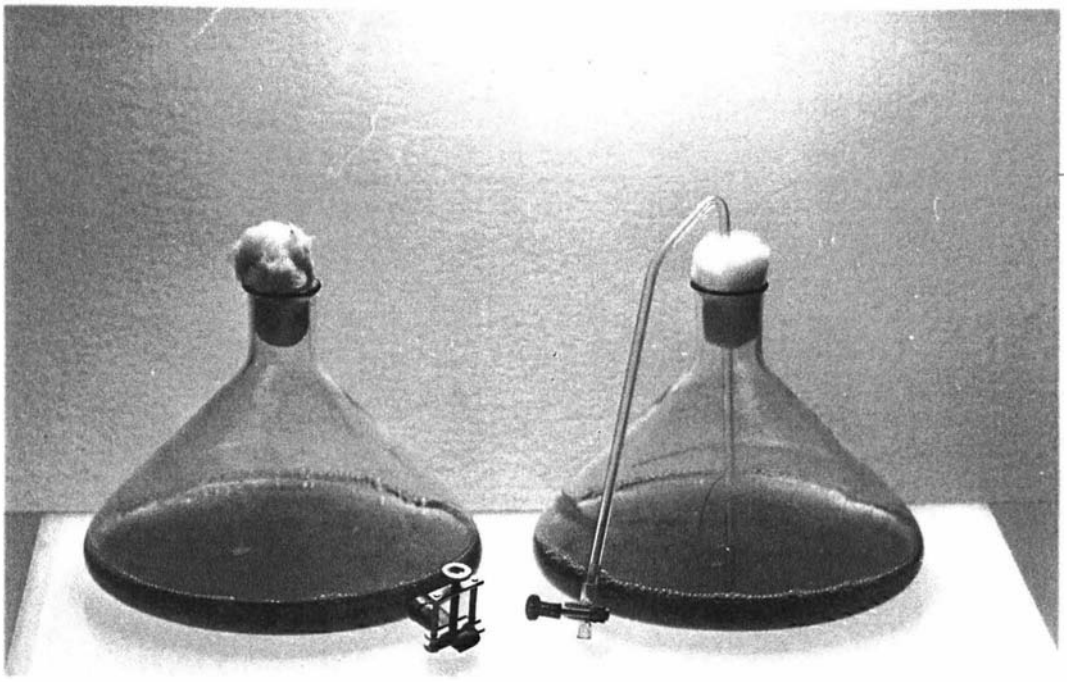

Fig. 2.1. Flasks (1000 ml) in which Mesotaenium cultures were grown, showing the two types of sampling outlets employed in these studies. Both flasks contained 400 ml of media.

dilutions by the reducing sugar method of Dygert et al. (1965). In some cases the glucose was separated from other constituents in the media by thin-layer chromatography on Silica Gel G using the solvent system n-butanol/acetic acid/water (5:4:1; Jacin and Mishkin, 1965) and estimated in the presence of absorbent by the phenol/H_2SO_4 method as outlined in Part 1. Both methods gave essentially similar results.

3. Measurement of Photosynthetic Potential.

Photosynthetic oxygen evolution was measured manometrically by incubating 1% (1 g wet weight of cells/100 ml buffer) suspensions of cells in 0.1 M $KHCO_3/NaHCO_3$ buffer, pH 8.3 (Pratt, 1943) at 25°C. Carbon dioxide fixation was measured at the same time by adding known amounts of $NaH^{14}CO_3$ to the incubations and measuring the $^{14}CO_2$ fixed in a given time. Cells were thoroughly dark adapted (12 to 18 hrs) before the addition of the $NaH^{14}CO_3$ and were incubated in the dark for 30 mins, to establish dark CO_2-fixation rates to correct for photosynthetic CO_2 fixation. Aliquots of the labelled cultures were quickly withdrawn under dim light and the cells collected under reduced pressure on a paper in a Millipore filter holder. The cells and filter were washed repeatedly with water, freeze dried overnight

and finally subjected to combustion in an O_2 atmosphere to give $^{14}CO_2$ which was collected in methanolic ethanolamine for scintillation counting (Kalberer and Rutschmann, 1961). Total CO_2 uptake was calculated from the proportion of added ^{14}C recovered in the cells.

4. Glycerolipid Analyses.

For lipid analyses, cultures were harvested when the cells reached a level of 1 to 1.5 g (wet weight)/100 ml medium, which approximated to the midpoint on the growth curve. The packed cells were extracted with methanol and chloroform as described for leaves in Part 1.

Total glycerolipids were initially examined by 2-dimensional TLC using the procedure of Nichols (1964) and by 1- and 2-dimensional TLC of the non-acidic and acidic fractions from DEAE-cellulose columns (Part 1). Separated components were located and identified by the procedures described in Part 1. As an aid in the detection and identification of components present in small amounts, cells were grown in the presence of ^{32}P-orthophosphate and ^{35}S-sulphate, and the extracted lipids were then subjected to 2-dimensional TLC for the preparation of radioautographs.

Qualitative column chromatography was carried out on a much smaller scale than that normally used. In

the present work, column fractions were dried, made up in chloroform to the volume of the lipid solution applied to the column, and aliquots were then taken for TLC. The most useful method for detecting separated components was to spray developed chromatograms with 50% sulphuric acid followed by charring of the lipids in an oven at 120°C. This provided a means of distinguishing glycolipids from phospholipids as well as localizing all lipids on the layers and allowed for the analysis of very small amounts of material.

Silicic acid (Mallinckrodt, A.R., 100 mesh) was washed to remove fines by the method of Galanos and Kapoulas (1965), air dried and then activated at 120° for 16 hr. Florisil (Floridin Co., Talahassee) was washed by the method of O'Brien and Benson (1964) and also air dried and activated at 120° for 16 hr immediately before use.

Quantitative analyses of the glycerolipids were performed after TLC of DEAE-cellulose and silicic acid column fractions by the methods described in Part 1. Triglyceride, separated by TLC from the non-acidic fraction of the DEAE-cellulose column or from the neutral lipid fraction of the silicic acid/acetone column, was estimated by a colorimetric determination of glycerol (Renkonen, 1962) released from acid hydrolysis of the material in the presence of silica gel.

5. Fatty Acid Analyses.

For analysis of the total fatty acids of Mesotaenium by gas/liquid chromatography, washed lipid extracts were saponified in isopropanol/KOH (Noll and Bloch, 1955) and the fatty acids released were methylated by refluxing in methanol/benzene/conc. sulphuric acid (150:75:10) (Nichols, 1965 a) for 60 min. Individual glycerolipids, isolated by TLC, were transesterified by refluxing in the methanol/benzene/sulphuric acid mixture. The fatty acid methyl esters formed in these treatments were separated on an $\frac{1}{8}$ inch x 5 ft column of ethylene glycol succinate (17%) on Chromabsorb W at a temperature of 155°C and using nitrogen (30 ml/min) as the carrier gas. The response of the flame ionization detector was determined using a standard mixture of methyl linolenate, linoleate, oleate, stearate and palmitate (Applied Science Laboratories). Relative masses of individual fractions were estimated from the product of peak height and peak width (at half peak height).

As an additional aid to identification, the methyl esters were separated on silver nitrate impregnated Silica Gel G (Morris, 1964) into saturated, monoenoic, dienoic and trienoic compounds prior to gas chromatography.

6. ^{14}C-Incorporation Studies.

U-^{14}C-glucose (2.9 mc/mmole), sodium 2-^{14}C-acetate (27.4 mc/mmole) and ^{14}C-sodium bicarbonate (26.7 mc/mmole) were obtained from The Radiochemical Centre, Amersham, England and added to enriched, 400 ml cultures which were nearing the midpoint of the growth phase. At intervals after these additions, aliquots (20-40 ml) of the cultures were drawn off and the cells, recovered by centrifugation, were washed once before extraction with chloroform/methanol. After filtration through a glass sinter, the chloroform/methanol extract was washed with dilute NaCl (Folch et al., 1957) yielding a total of three fractions, namely (1) a residue fraction - the cell debris which represented cell wall material and most of the macromolecular constituents; (2) a water soluble fraction - the aqueous methanolic phase of the Folch-washed, chloroform/methanol extract which represented the small molecular weight substances in the cells and; (3) lipids - the chloroform phase of the Folch-washed, chloroform/methanol extract. Radioactivity in aliquots of each of these fractions was determined as outlined below. Individual, ^{14}C-labelled glycerolipids were separated by a combination of DEAE-cellulose column chromatography and TLC.

7. Measurement of Radioactivity.

The residue fraction recovered from the sintered glass filter was burned in an atmosphere of O_2 yielding CO_2 which was trapped in methanolic ethanolamine for scintillation counting (Kalberer and Rutschmann, 1961). Total lipids were plated at infinite thinness on corrugated, copper planchets (Nuclear-Chicago Corp., Des Plaines, Ill., U.S.A.) and counted at 25% efficiency in a Nuclear-Chicago gas flow counter. Aqueous solutions were added to a dioxane/naphthalene based solution (Snyder, 1964) for counting. Glycerolipids, separated by TLC, were transferred on the adsorbent to vials containing 8 ml of a dioxane/naphthalene/water scintillation fluid (Snyder, 1964) and counted at 55 to 60% efficiency in a Packard liquid scintillation spectrometer. When using the scintillation method, counting efficiencies were continually checked by the channels ratio technique (Bush, 1963).

Radioautographs were obtained by laying developed thin-layer chromatograms on double layered X-ray film (10 x 8 in.) for periods of up to 48 hr for ^{32}P- and 7 days for ^{35}S- labelled lipids.

RESULTS

1. Growth of Mesotaenium caldariorum in liquid culture.

a) Light requirement.

There was no discernible growth in glucose-yeast extract media which were placed in complete darkness immediately after inoculation while control cultures in the light grew vigorously. Growth phase cultures ceased growth upon transfer to total darkness. However, inocula survived at least two weeks of total darkness and established vigorous cultures within 4-5 days of being transferred into light.

b) Glucose requirement.

Satisfactory growth rates were obtained only when glucose was added to the basal salts medium (Table 2.1). Sucrose supported a limited amount of growth while galactose appeared to be slightly inhibitory. The sodium and potassium salts of acetic, succinic and citric acids completely inhibited growth. Experiments to determine the optimum levels of yeast extract for growth stimulation were not reproducible (cf experiments 1 and 4 in Table 2.1). In general however, a level of 0.2% yeast extract in the glucose-enriched medium was found to produce a greater yield of cells than when the yeast extract was omitted (e.g. Fig. 2.3). The growth promoting effect of glucose is illustrated graphically in Fig. 2.2

TABLE 2.1

Growth of *Mesotaenium caldariorum* in enriched and non-enriched media

Additions to Basal Salts medium	µg Chlorophyll per culture 16 days aft inoculation
Experiment 1* (400 ml cultures)	
None	2.3
Yeast extract (0.2%)	3.2
Glucose (1%)	43.2
Glucose (1%) + Yeast extract	60.6
Experiment 2+ (30 ml cultures)	
None	0.8
Glucose (1%)	11.1
Sucrose "	4.9
Galactose "	0.8
Glycine "	1.9
K Acetate "	0
K Succinate "	0
K Citrate "	0
Experiment 3* (400 ml cultures)	
Yeast extract (0.2%)	2.7
" " + Galactose (1%)	0.4
" " + Sucrose (1%)	4.7
" " + Glucose (1%)	58.5
" " (0.1%) + Glucose	55.0
Experiment 4* (400 ml cultures)	
Glucose (1%)	40
" + Glycine (0.01%)	34.4
" + Yeast extract (0.01%)	46.0
" + " " (0.05%)	42.0
" + " " (0.1%)	47.6

* Cell inoculum containing an equivalent of 35-55 mg chlorophyll (from enriched cultures).

\+ Cell inoculum containing an equivalent of 45 mg chlorophyll (from basal salts cultures).

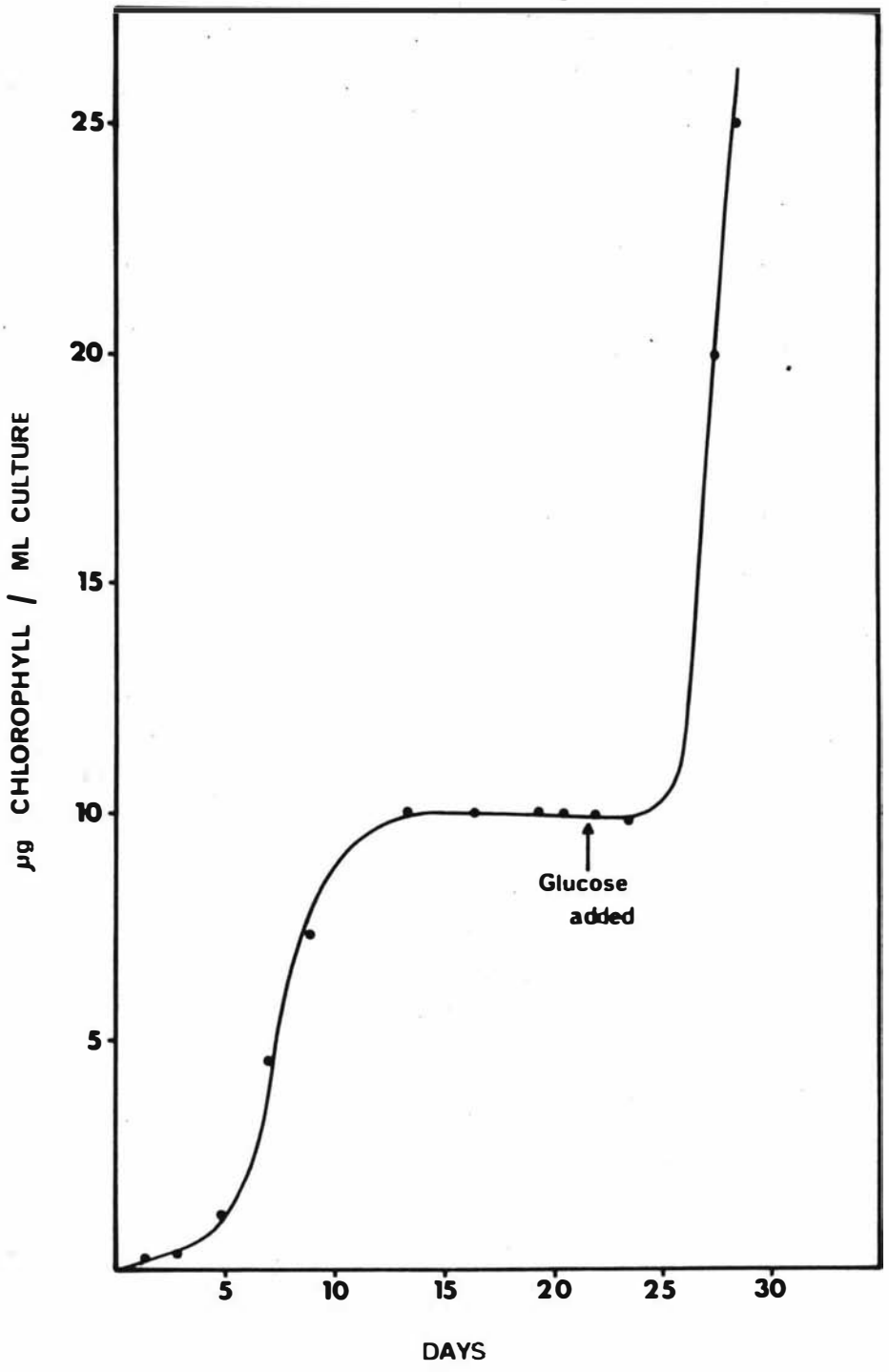

Fig. 2.2. Glucose-dependent growth of Mesotaenium. The medium initially contained 0.1% glucose and, after growth had stopped, glucose was added to give a concentration of 1% in the medium.

The medium in this experiment contained yeast extract (0.2%) and a relatively low level of glucose (0.1%) so that the culture ceased net growth when the cells reached 16% to 20% of the concentration normally obtained in 1% glucose media. Six days after growth had stopped, glucose was added to the culture to bring the level up to 1% and after a lag of 3 days the culture resumed exponential growth.

c) Glucose utilization.

Growth in media containing 1% glucose and 0.2% yeast extract ceased when the cell concentration of a culture reached approximately 2 g wet weight/100 ml media. (8 ml of packed cells in a 400 ml culture) and before all of the glucose of the medium had been consumed (Fig. 2.3A).

It was noted that glucose utilization lagged behind growth so that, up to about half way along the growth curve, less than 1 g of glucose had disappeared from the medium. In a subsequent experiment it was found that the disappearance of 1.04 g of glucose from the medium was accompanied by the production of 0.83 g dry weight of cells. Since it could be shown that CO_2-fixation did not contribute significantly to the carbon of the new growth, at least in the upper half of the growth curve (see below), this represents an assimilation of 80% of

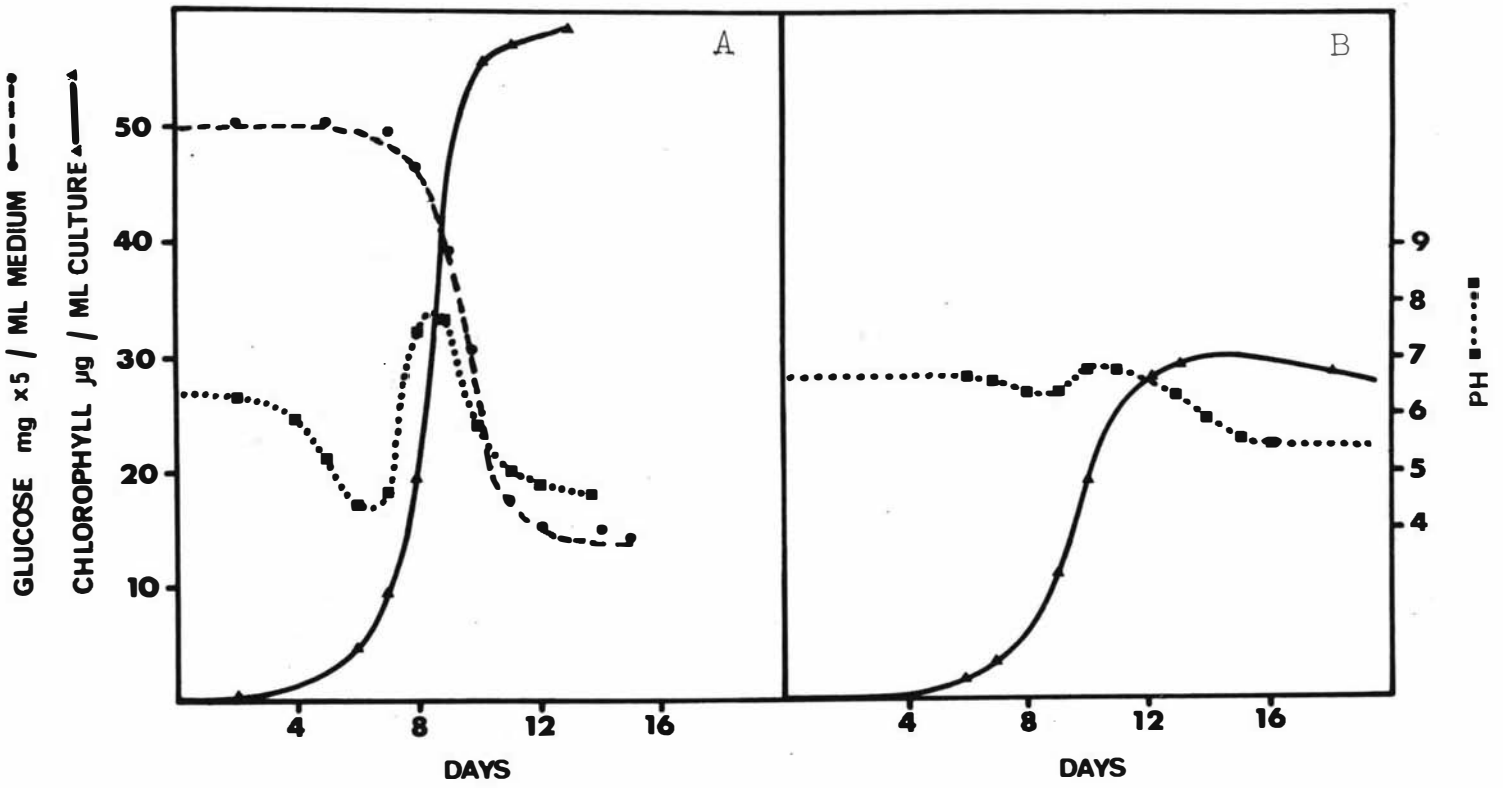

Fig. 2.3. Growth characteristics of Mesotaenium in 400 ml liquid cultures. Left (A); growth in medium containing glucose (1%), yeast extract (0.2%) and salts. Right (B); growth in medium containing glucose (1%), sodium phosphate buffer (0.01M, pH 6.6) and salts.

exogenous glucose into cellular material[+]. This is a much higher value than would be expected from simple oxidative assimilation which would provide for the conversion of 20-50% of the glucose to cell material (see review by Gunsalus and Shuster, 1961).

d) Medium pH variations.

During isotope incorporation studies it was noted that the pH of the medium varied with cell concentrations. A comparison of glucose utilization with pH changes in the medium was therefore made (Fig. 2.3A). As the culture entered the log phase of growth the pH dropped from 6.5 to 4.3 but rose again to about 8.0 with continuing growth. The cessation of growth was also accompanied by a subsequent sharp drop in the pH of the medium from 8.0 to 4.7. These pH variations could be reduced by including phosphate buffer in the medium, at a final concentration of 0.01 M (Fig. 2.3B). Higher concentrations of phosphate (0.05 M), completely inhibited growth.

[+] A conversion factor for weight of glucose into weight of cellular material of 1.09 was calculated using the generalized composition of green plants given by Buckman and Brady (1960).

e) Oxygen evolution.

In the small culture systems (30 ml) cells proliferated on the bottom of the flasks and could reach a thickness of several millimeters. There was rarely any sign of effervescence. A sample of mixed algae (obtained from an open drain), which had been cultured in the basic salts medium under the same conditions as those employed for Mesotaenium effervesced vigorously at cell concentrations much lower than those of the Mesotaenium cultures.

However, Mesotaenium cells did evolve oxygen, when they were incubated in the presence of high concentrations of bicarbonate (Table 2.2) even at light intensities (15W lamps, expt 3) close to those used in the growth cabinets, and with glucose concentrations the same as those in normal culture media.

f) CO_2-fixation and growth rate.

The CO_2-fixation rate measured in cell suspensions incubated in the presence of high concentrations of CO_2 (Table 2.2) was sufficient to account for the observed growth rate of cultures in the glucose-yeast extract, enriched media. However, isotope incorporation studies during growth in enriched, 400 ml cultures did not show a comparable utilization of exogenous CO_2 (see below). Under normal growth conditions, exogenous CO_2 would be

TABLE 2.2

Oxygen evolution and Carbon Dioxide Fixation in Mesotaenium

	μ mole/mg chlorophyll/hr	
	O_2 evolved	CO_2 fixed
Experiment 1*		
-Glucose	120	160
Experiment 2+		
-Glucose	200	
+Glucose	220	250
Experiment 3* (15W Lamps)		
-Glucose	54	
+Glucose	59	
Experiment 3* (40W Lamps)		
-Glucose	140	
+Glucose	126	

The reaction mixture (final volume, 2 ml) consisted of 1.6 ml of 0.1 M $NaHCO_3/KHCO_3$ buffer, pH 8.3, containing approximately 1% (wet weight) of cells together with (a) 0.2 ml of 10% glucose or 0.2 ml H_2O and (b) 0.2 ml $NaH^{14}CO_2$ (1 μc) or 0.2 ml H_2O. The temperature was maintained at 18°C and flasks were illuminated from below by 40W lamps unless otherwise stated (Braun Photosynthetic Warburg apparatus).

* Cells from the post-growth phase.

+ Cells in the growth phase.

available almost exclusively from the atmosphere and it can be calculated that the amount of CO_2 dissolved in the medium and being replenished from the atmosphere under these conditions would be insufficient to provide a significant source of carbon for the observed growth.

2. The lipids of Mesotaenium caldariorum.

a) Lipid content of the cells.

The average yield of washed lipid from log-phase Mesotaenium cells was 26 mg/g fresh weight and the level of chlorophylls a + b was 2.0 to 2.2 mg/g (2.75 µmole/g). Slight variations in these values were encountered possibly as a result of the packing characteristics of cells of different physiological status. For instance, cells from cultures, which had ceased growth could not be packed into a compact pellet, even at 5000 x g for 20 min, but formed a watery, jelly-like precipitate. The most reliable and consistant results were obtained with cultures that contained 1 g wet weight of cells/100 ml medium.

b) The glycerolipids of Mesotaenium.

i) 2-Dimensional Thin-Layer Chromatography.

A typical 2-D thin-layer chromatogram of the total lipids of Mesotaenium is shown in Fig. 2.4. For convenience, the two unknowns have been labelled U1 and U2. The mobilities of U1 suggested that the compound

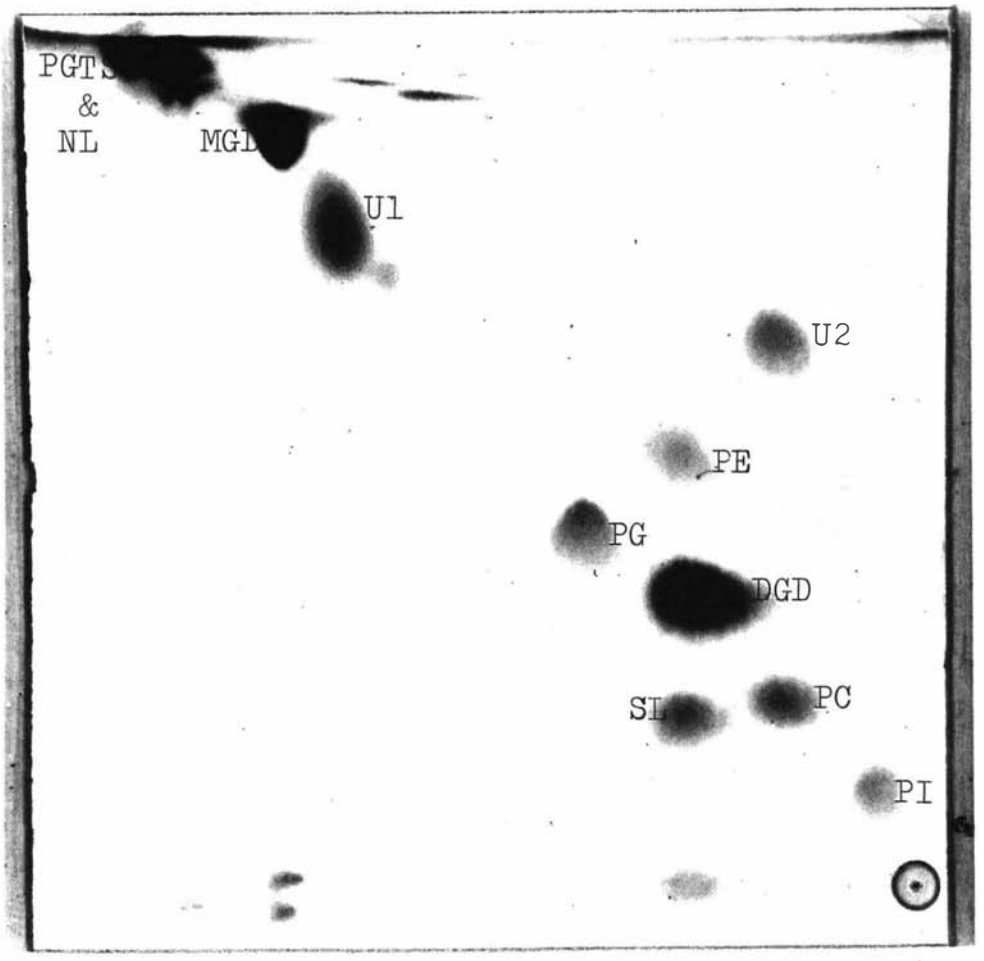

Fig. 2.4. 2-Dimensional thin-layer chromatogram of the glycerolipids of Mesotaenium caldariorum after Nichols (1964). An amount of lipid in $CHCl_3$ equivalent to 50 mg of packed cells was applied at the origin. Spots were located by spraying the chromatogram with the dichromate/sulphuric acid reagent of Rouser et al. (1964) and heating in an oven at 180°C for 30 min. Layer, Silica Gel G.

was only slightly more polar than MGD. By contrast, U2 had a much greater mobility in acidic as distinct from an alkaline developing solvent and presumably it was an acidic lipid. Neither of the unknowns contained phosphorus or sulphur. Figure 2.5 shows both a chromatogram and radioautograph of the lipids from cells which had been grown in the presence of ^{32}P-orthophosphate. Apart from the four major phosphatides only traces of PA, DPG and an unknown material, with high mobility in both solvents, were observed. Similar findings had been obtained using TLC with a phospholipid spray. A radioautograph (Fig. 2.6) prepared from a 2-dimensional chromatogram of lipids from cells grown in the presence of ^{35}S-sulphate showed only one spot, which corresponded to sulpholipid.

ii) DEAE-Cellulose Column Chromatography.

A thin-layer chromatogram of the non-acidic and acidic lipid fractions from a DEAE-cellulose column is shown in Fig. 2.7. This is typical of the chromatograms on which quantitative analyses were carried out (see Part 1) and although the unknowns did not react significantly with either the phosphorus or sugar assays both gave good responses with the charring assay of Marsh and Weinstein (1966). U2 reacted as a glycolipid both to the H_2SO_4/charring method of detection and to the

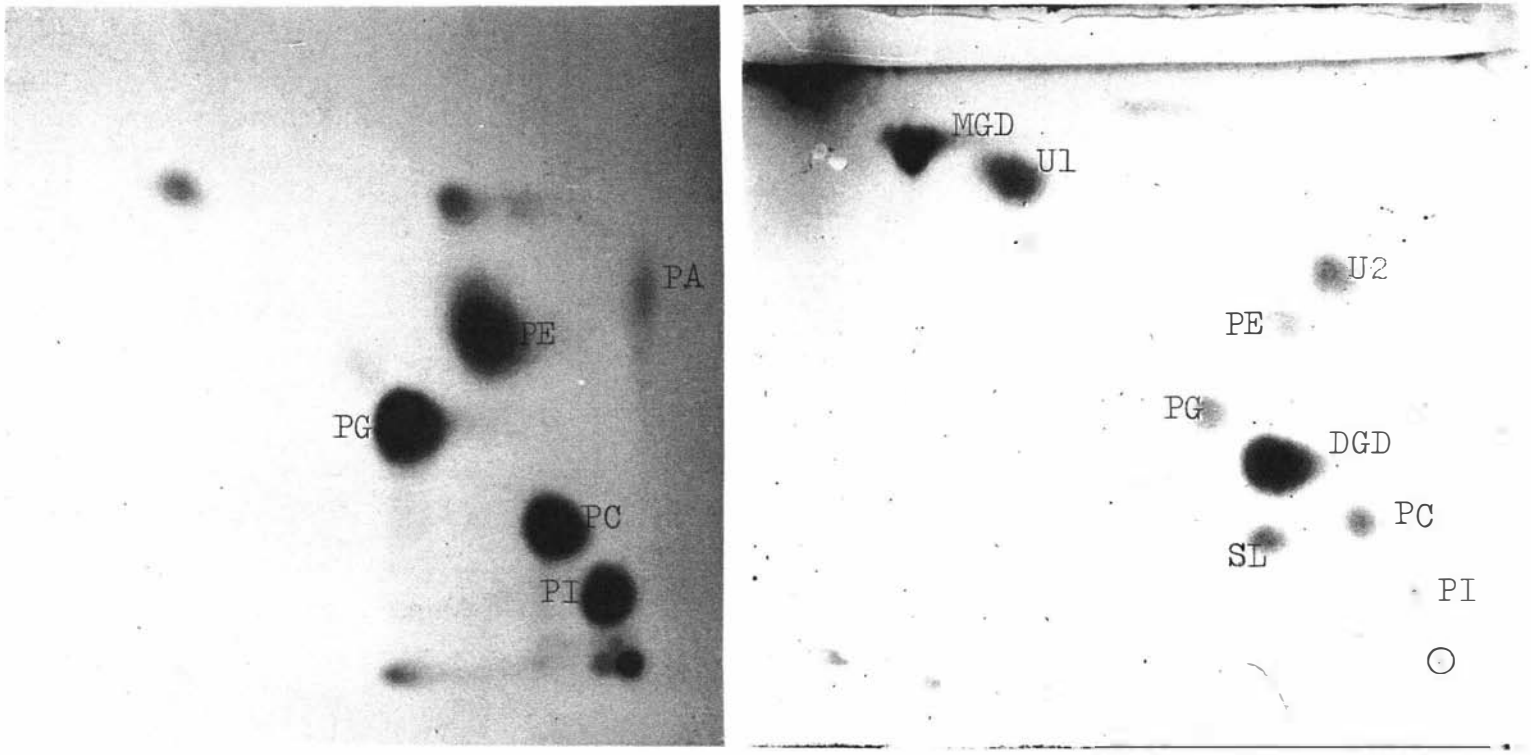

Fig. 2.5. 2-Dimensional thin-layer chromatogram of ^{32}P-orthophosphate labelled lipids of Mesotaenium (right) and the radioautograph of the same chromatogram (left). Lipid equivalent to 25 mg of packed cells was applied at the origin. The X-ray film was exposed to the chromatogram for 12 hr. Separated lipids were visualized by spraying the chromatogram with 50% H_2SO_4 and charring at 120^{o}C. Layer, Silica Gel G.

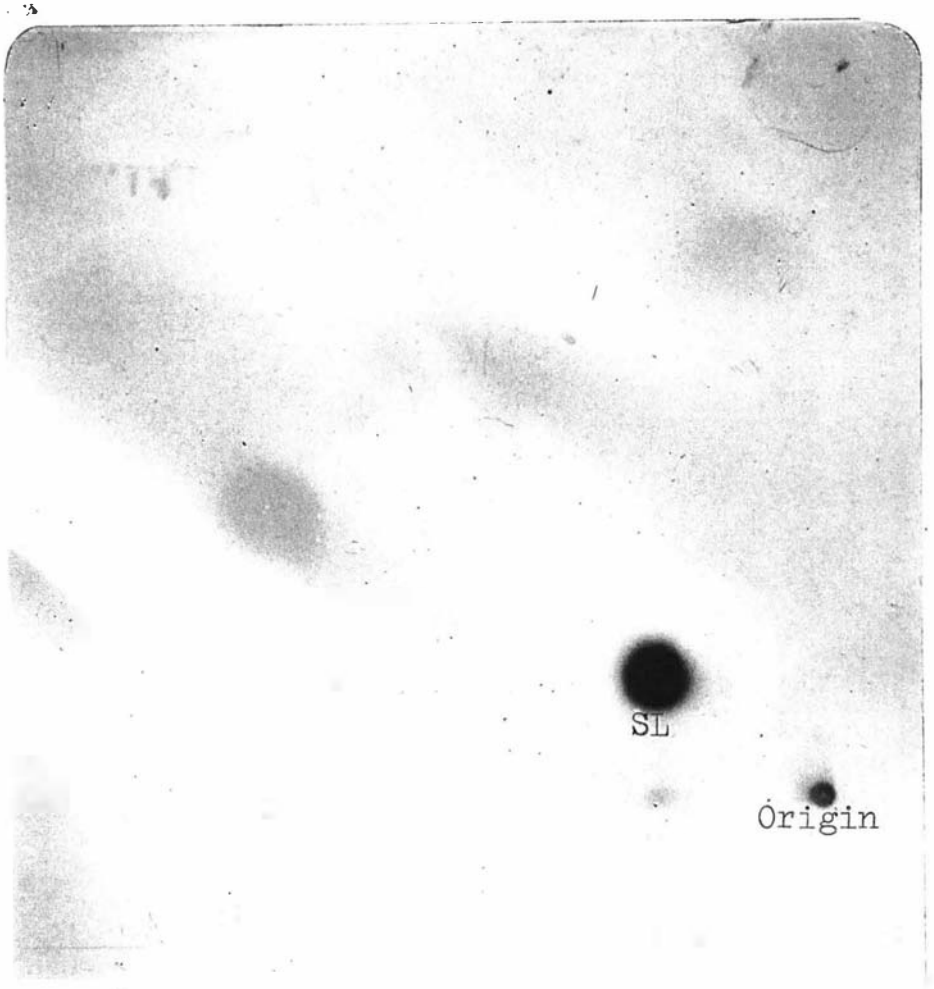

Fig. 2.6. Radioautograph of 2-dimensional, thin-layer separation of ^{35}S-sulphate-labelled, Mesotaenium lipids. Lipid equivalent to 50 mg of packed cells was applied at the origin. The X-ray film was exposed to the chromatogram for 48 hrs and the single spot corresponded to SL on the stained (50% H_2SO_4 and charred) chromatogram.

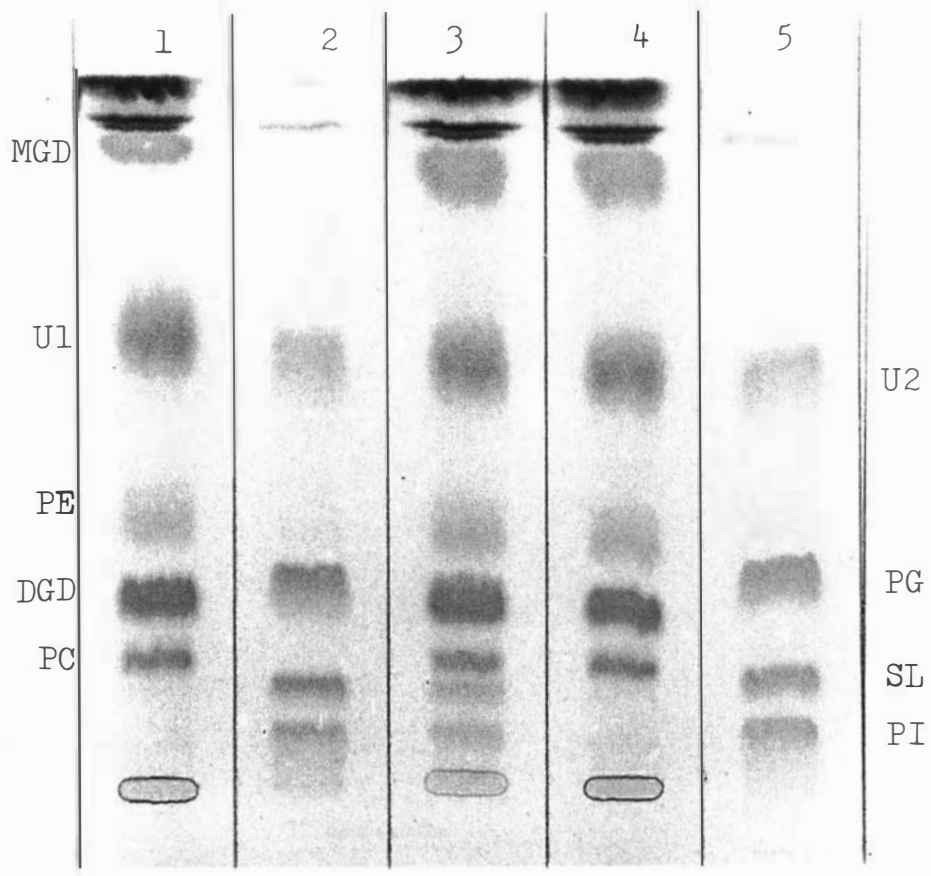

Fig. 2.7. Separation of Mesotaenium total lipids (lane 3) and of the non-acidic (lanes 1 and 4) and acidic (lanes 2 and 5) lipid fractions from a DEAE-cellulose column by a single development with chloroform/methanol/acetic acid/water (85:15:10:3). Lipid equivalent to 25 mg of packed cells was streaked across 2 cm in lanes 1, 3 and 5 and equivalent to 100 mg in lanes 2 and 5. Visualization: Iodine vapour. Layer: Silica Gel G.

α-napthol/H_2SO_4 spray of Saikatos and Rouser (1965) although it did not respond significantly in the phenol/H_2SO_4 assay.

When a DEAE-cellulose column was eluted with $CHCl_3$ and increasing concentrations of CH_3OH in $CHCl_3$ the results (Fig. 2.8) were as expected from the results obtained by TLC (see Nichols and James, 1964) except that U1 appeared to elute from the column before MGD. This elution property was later used in an isolation procedure for the compound.

iii) Silicic acid column Chromatography.

"Neutral" and "polar" lipids were separated on a column of silicic acid using stepwise elution with solvents of increasing polarity and partial resolution within the classes was obtained (Fig. 2.9). The "neutral" lipid" fraction contained a comparatively large amount of triglyceride which was characterised by its glycerol and fatty acid content and by mass spectrometry. In these separations, U1 was retained on the column more strongly than would have been expected from its mobility with 2-dimensional TLC (Fig. 2.4) and on DEAE-cellulose columns (Fig. 2.7). The effect was shown even more markedly with the silicic acid/acetone column procedure of Rouser et al. (1967) (Fig. 2.10A). Using this technique, the acidic lipid U2 was eluted with MGD in

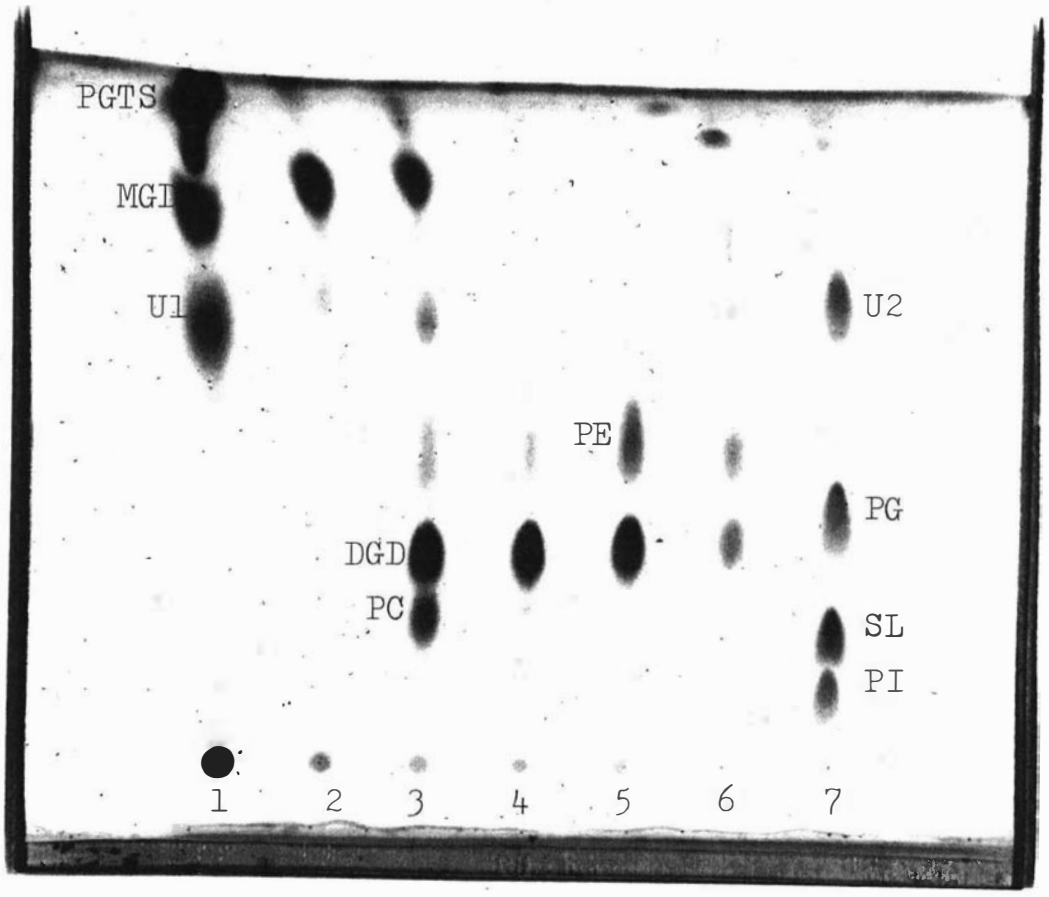

Fig. 2.8. Thin-layer chromatographic analysis of fractions from an 8 x 1 cm (i.d.) column of DEAE-cellulose. Mesotaenium total lipid (85 mg) in chloroform was placed on the column and fractions collected corresponding to elution with 1. 25 ml chloroform; 2. 25 ml chloroform; 3. 25 ml 5% methanol in chloroform; 4. 25 ml 5% methanol in chloroform; 5. 25 ml 10% methanol in chloroform; 6. 25 ml 40% methanol in chloroform; 7. 50 ml chloroform/methanol/conc. ammonia (6:4:0.75). Each fraction was evaporated to dryness, the residue redissolved in 2 ml chloroform and 20 µl of this spotted for TLC. Development: chloroform/methanol/acetic acid/water (85:15:10:3). Visualization: 50% H_2SO_4. Layer: Silica Gel G.

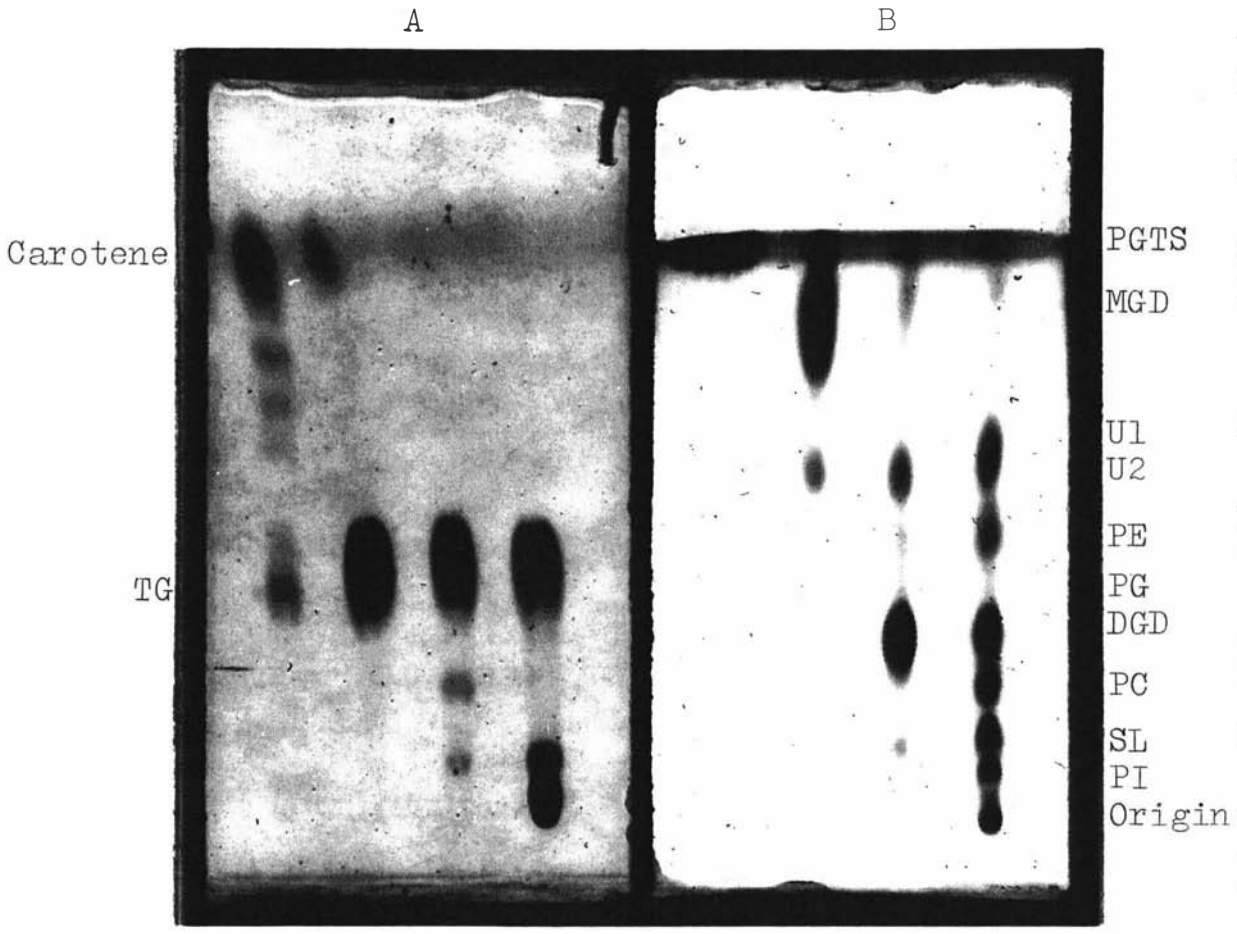

Fig. 2.9. Thin-layer chromatographic analysis of the "neutral" (A) and "polar" (B) lipid fractions from a silicic acid column. Mesotaenium total lipid (46 mg) in benzene was placed on a 12 x 1 cm (i.d.) column and fractions collected corresponding to elution with 1. 30 ml benzene; 2. 40 ml benzene/chloroform (1:1); 3. 40 ml chloroform; 4. 40 ml chloroform; 5. 40 ml 5% methanol in chloroform; 6. 40 ml 10% methanol in chloroform; 7. 50 ml methanol. Each fraction was evaporated to dryness and the residue redissolved in 2 ml chloroform. 20 µl of this solution was then spotted. Development: A, Benzene and B, chloroform/methanol/acetic acid/water (85:15:10:3). Visualization: 50% H_2SO_4. Layer: Silica Gel G.

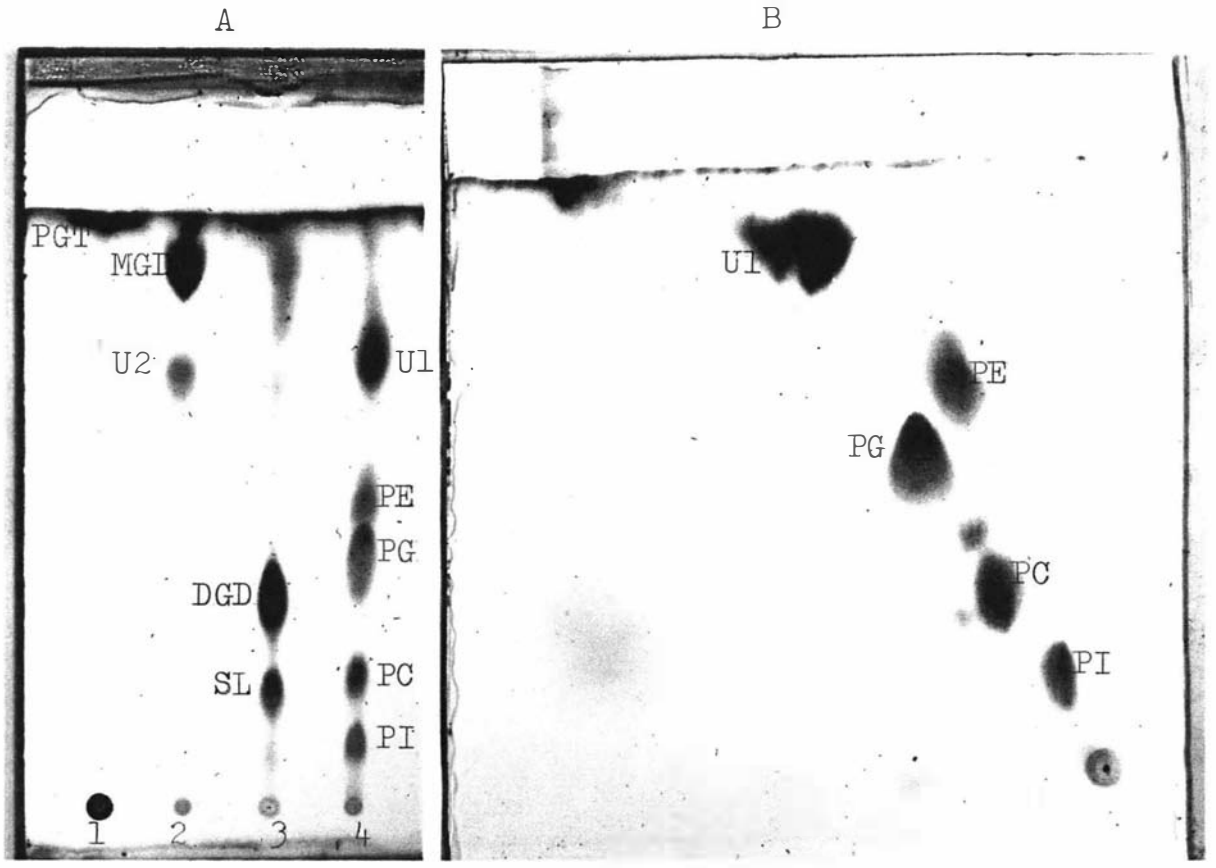

Fig. 2.10A. Thin-layer analysis of column fractions obtained by the silicic acid/acetone technique. Mesotaenium lipid (25 mg) in chloroform was placed on a 3 x 1.5 cm (i.d.) column and fractions collected corresponding to elution with 1. 42 ml chloroform; 2. 32 ml chloroform/acetone; 3. 180 ml acetone; 4. 55 ml methanol. All fractions were dried and finally made up to 1 ml in chloroform. 20 µl of each fraction was spotted. Development: chloroform/methanol/acetic acid/water (85:15:10:3). Fig. 10B. 2-Dimensional thin-layer separation of the phospholipids (fraction 4). Visualization: 50% H_2SO_4.

chloroform/acetone (1:1) while U1 was strongly retained during prolonged development with acetone and was finally removed, along with the phospholipids, by using methanol. The phospholipid fraction obtained from this procedure was subjected to 2-D TLC (Fig. 2.10B) to give better separation of the components for identification. Unknown 1 behaved as a less polar compound than MGD with DEAE-cellulose column chromatography but like a phospholipid using the silicic acid/acetone column procedure.

iv) Florisil Column Chromatography.

In spite of its limitations for quantitatively separating phospholipids and glycolipids (Part 1), the property of Florisil to adsorb phospholipids strongly made it useful as a column packing for checking the results obtained from the silicic acid/acetone technique. Both U1 and U2 were strongly adsorbed on the column and were eluted with the phospholipids (Fig. 2.11A). The U1-U2-phospholipid fraction was subsequently separated on a DEAE-cellulose column (Fig. 2.11B) as a further step towards compound identification.

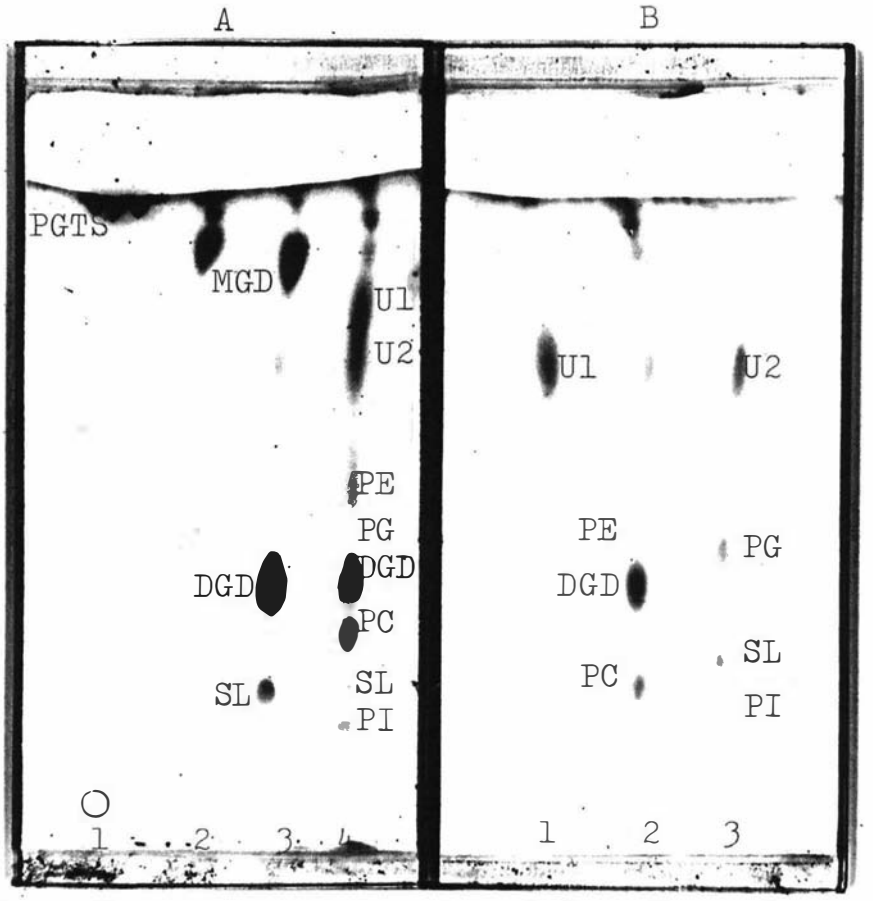

Fig. 2.11A. Thin-layer analysis of fractions from Florisil column chromatography of Mesotaenium lipids. Total lipid (25 mg) in chloroform was placed on an 8 x 1 cm (i.d.) column and fractions collected corresponding to elution with 1. 20 ml chloroform; 2. 25 ml chloroform/methanol (85:5); 3. 60 ml chloroform/methanol (6:4); 4. 25 ml chloroform/methanol/water (1:1:0.2). All fractions were finally made up to 1 ml in chloroform with 20 µl taken for TLC. Development: chloroform/methanol/acetic acid/water. Visualization: 50% H_2SO_4.

Fig. 2.11B. Fraction 4 from the Florisil column subjected to DEAE-cellulose column chromatography. All of this fraction was placed on a 6 x 1 cm (i.d.) column and fractions collected corresponding to elution with 1. 20 ml chloroform; 2. 25 ml chloroform/methanol (6:4); 3. 20 ml chloroform/methanol/conc. ammonia (6:4:0.75). All fractions were made to 1 ml in chloroform and 20 µl taken for TLC.

c) Quantitative analysis of the glycerolipids of Mesotaenium.

The molar concentrations of the various glycerolipids of Mesotaenium are shown in Table 2.3. The results are the means of five determinations made on separate cultures harvested near the middle of the growth phase. Two of these were analysed by both the DEAE-cellulose/TLC and the silicic acid/acetone/TLC techniques which gave almost identical results. The most accurate measurements of phospholipids were obtained by TLC (Silica Gel G; chloroform/methanol/7M ammonia, 65:25:4) of the phospholipid fraction from the silicic acid-acetone column; SL was best measured after TLC of the acidic lipid fraction from the DEAE-cellulose column. On the assumption that both of the unknown lipids were of the α,β-diglyceride type (an assumption based on fatty acid and glycerol content) approximate molar concentrations of U1 and U2 were obtained by comparing their response in the charring assay of Marsh and Weinstein (1966) with those of known amounts of phospho- and glycolipids.

d) Fatty Acids of Mesotaenium.

Growth phase cells contained more polyunsaturated fatty acids (39%) than cells from non-growing cultures

TABLE 2.3

Concentrations and fatty acid compositions of the glycerolipids of growth-phase Mesotaenium

Glycerolipid	μ mole/g wet weight cells	% of total fatty acid										
		12:0	14:0	16:0	16:1	16:2	16:3	18:0	18:1	18:2	18:3	(17:3)
MGD	9.6	-	-	3	1	9	25	-	5	18	40	
DGD	5.9	-	-	21	tr	3		-	17	24	35	
U1	2.4	-	-	50	-	-		tr	9	17	6	18
TG	2.3	-	-	16	tr	-		tr	66	17	-	
PG	1.2	-	-	49	5	-		tr	2	26	17	
SL	1.2	10	1	43	-	-		1	6	19	18	
U2	1.2			61	-	-		tr	3	26	10	
PC	0.9	4	tr	47	-	-		tr	15	30	2	1
PE	0.8	tr	-	48	-	-		tr	23	29	tr	
PI	0.7	-	-	46	-	-		tr	15	37	2	

* 18:1 = 18:1 + 16:3 for all lipids except MGD.

(8%) (Table 2.4; cf also Figs. 2.12 and 2.13). The decrease in the proportion of polyunsaturated acids was accompanied by an increase in the proportion of oleic acid (18:1). The triunsaturated, 16-carbon fatty acid ester was not separated from oleic acid on the EGS column used in these studies but its contribution to the oleic acid peak could be calculated by a comparison of the 16:3 with the 18:3 peak after separation of the methyl esters by $AgNO_3$-impregnated silica gel TLC. A minor component of the trienoic fraction had a retention time consistant with a 17 carbon fatty acid (17:3) (Korn, 1964).

Fatty acid components of the various glycerolipids are shown in Table 2.3. The minor component listed as 17:3 was found predominantly in fraction U1.

e) Attempted characterization of unknown lipids.

Milligram amounts of both unknowns were isolated by a combination of preparative column (DEAE-cellulose, silicic acid/acetone) and thin-layer chromatography (Silica Gel G). Infra-red spectra were obtained from (a) solid samples on NaCl discs, (b) carbon tetrachloride solutions of the lipids (Fig. 2.14) and (c) from KBr pellets containing the substances. The pellets, however, tended to be waxy in appearance and scattered or absorbed a good deal of the shorter wavelength energy. The spectra

TABLE 2.4

Fatty acids of growing and non-growing Mesotaenium cultures

		% of total fatty acids									
		14:0	16:0	16:1	16:2	16:3	18:0	18:1	18:2	18:3	(17:3)
Growing		tr	22	tr	3	13	tr	12	24	26	
Non-Growing		tr	28	tr	tr	3	tr	39	25	4	
Growing	Saturated	1	98				1				
	Monoenoic			12				88			
	Di				12				88		
	Tri					31				62	(7)
Non-Growing	Saturated		99				1				
	Mono			2				98			
	Di				15				85		
	Tri					30				51	(19)

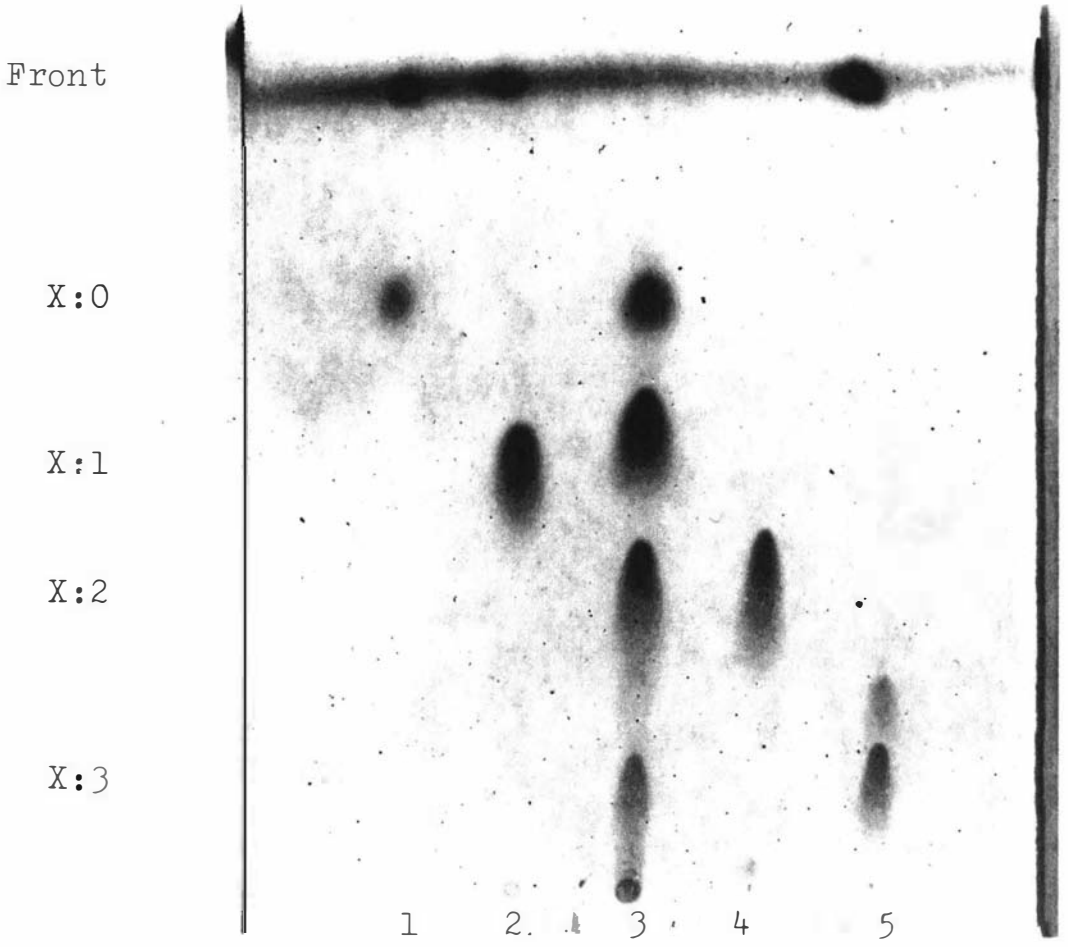

Fig. 2.12. Thin-layer chromatograph of Mesotaenium (post growth phase cells) fatty acid methyl esters on $AgNO_3$-impregnated Silica Gel, showing the relatively low proportion of polyunsaturated fatty acids. 1. Methyl stearate; 2. Methyl palmitoleate; 3. Mesotaenium fatty acid esters; 4. Methyl linoleate; 5. Methyl γ-linolenate. Development: Benzene. Visualization: 50% H_2SO_4. Layer: 2.5% $AgNO_3$ in Silica Gel H.

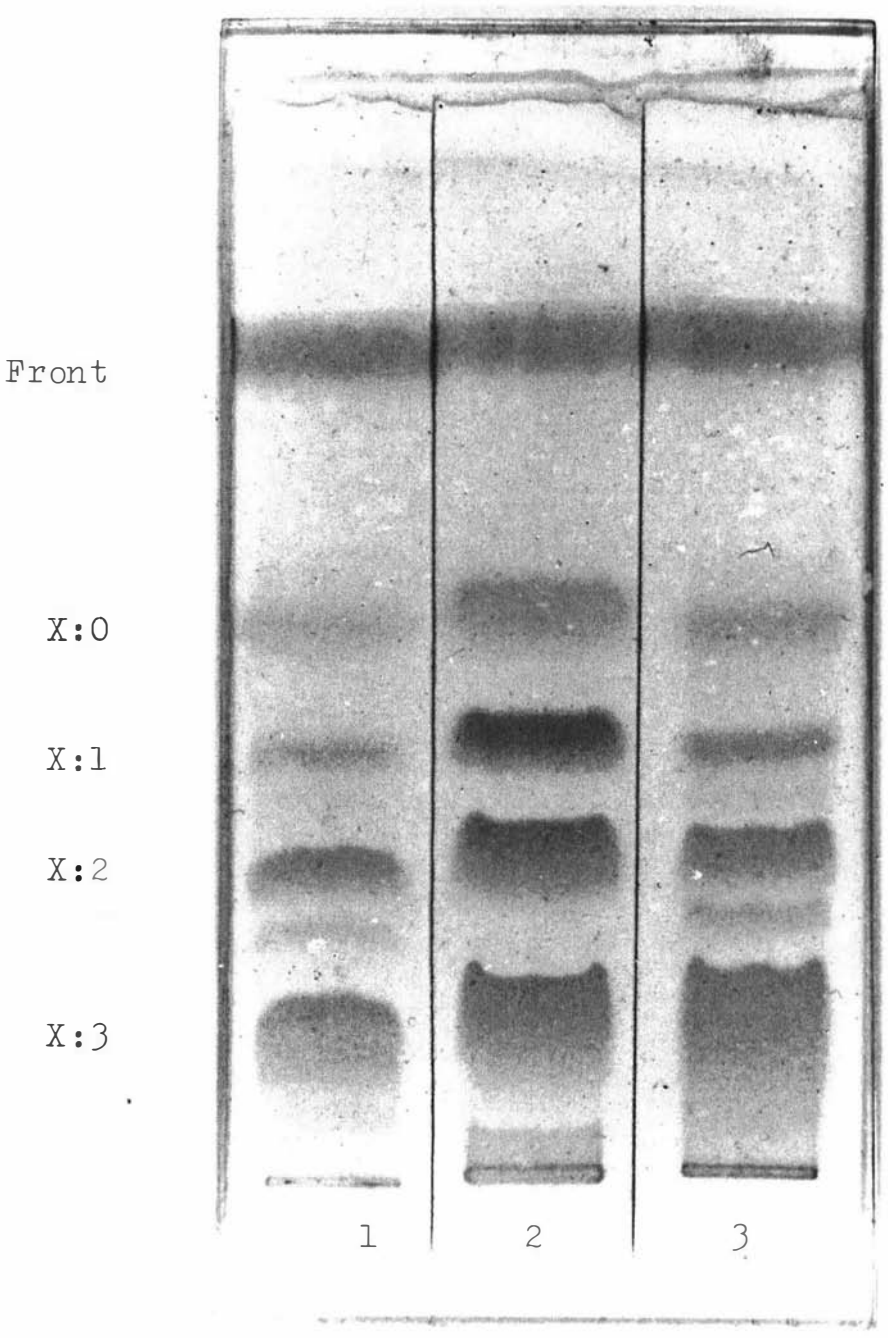

Fig. 2.13. Separation of fatty acid methyl esters from growth-phase cells of Mesotaenium on silver nitrate impregnated Silica Gel H (as in Fig. 2.12). In lanes 1 and 3 are the esters prepared from Mesotaenium lipids and in lane 2 is a standard mixture of equal weights of palmitic acid (16:0), palmitoleic acid (16:1), linoleic acid (18:2) and α-linolenic acid (18:3). (Mann Laboratories). This chromatogram shows the relatively high proportion of polyunsaturated fatty acids in growing cells.

of the two unknowns were similar and unlike those published by Rouser et al. (1963) or Allen et al. (1966) for a variety of glycerolipids. Both possessed bands characteristic of the ester carbonyl group (5.70 - 5.75 μ), methylene group (3.45 μ) and hydroxyl group (3.00 μ) and the relative heights of the ester carbonyl band and the methylene band were comparable with those obtained for glycerolipids. Unusual features of the spectra were the two complex groups of bands with peaks at 6.2 - 6.4 μ and 7.1 - 7.2 μ; the former could indicate an amide group (Rouser et al., 1963) while the latter appears to be characteristic of ammonium salts of acidic lipids (Rouser et al., 1963; Allen et al., 1966). The assymetry of the hydroxyl band indicates the possibility of additional absorption at 3.15 μ which would be consistent with ammonium salts (Rouser et al., 1963) even though U1 behaved as a non-acidic lipid on DEAE-cellulose columns.

Attempts to detect nitrogen in perchloric acid digests of the unknown lipids by Nesslerization or by the recent method of Sloane-Stanley (1968) were unsuccessful.

Of the water soluble, acid hydrolysis (4-48 hr at 100°C) products, only glycerol has been positively identified as a constituent of both unknown lipids. Glycerol was identified both by the method of Renkonen (1962) and by paper and cellulose thin-layer chromatography in combination with a periodate/permanganate spray reagent (Sastry and Kates, 1964).

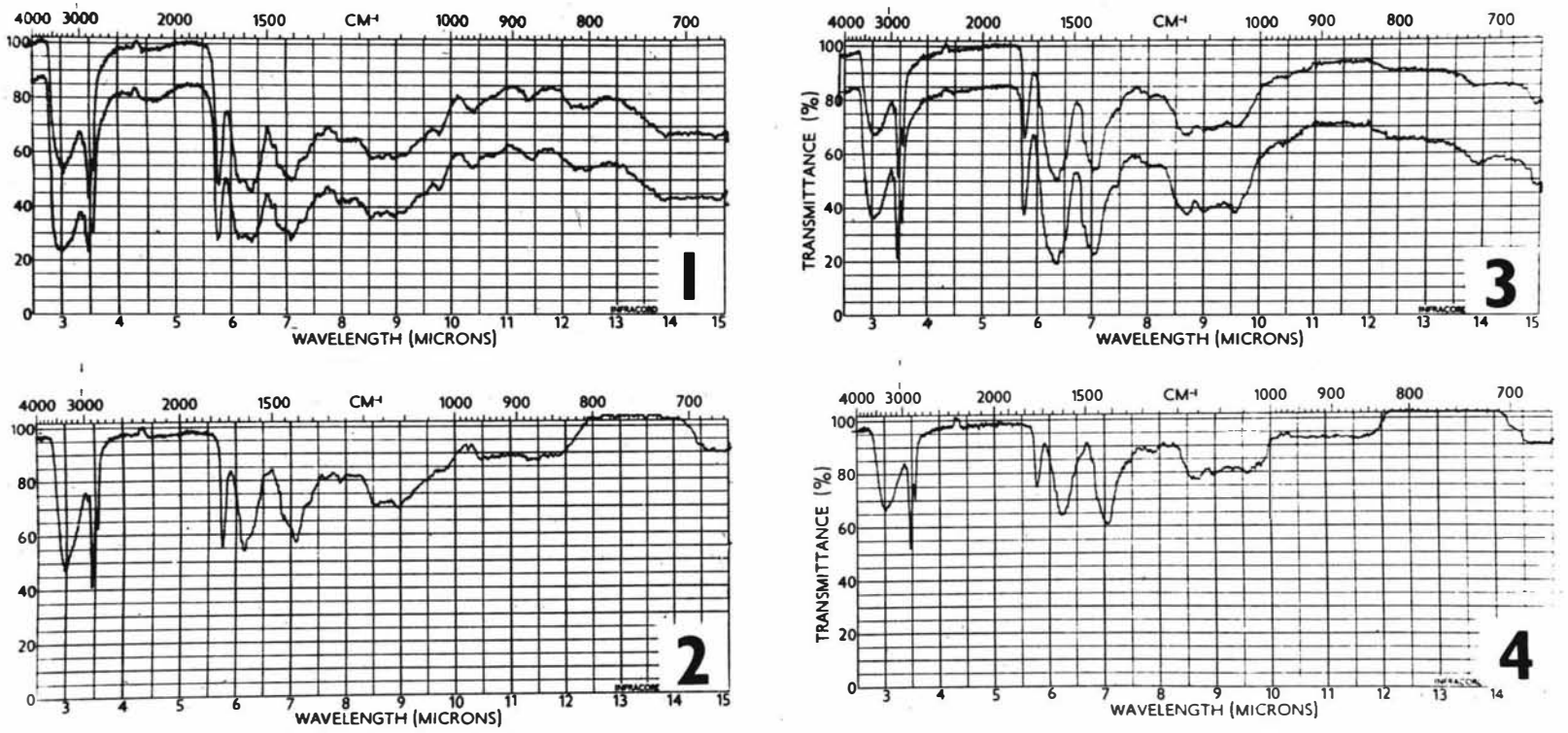

Fig. 2.14. Infra-red spectra of the unknown glycerolipids of Mesotaenium. 1. U1 as a film (2 different thicknesses) on a NaCl disc; 2. U1 in carbon tetrachloride solution; 3. U2 as a film on NaCl disc, and 4. U2 in carbon tetrachloride solution.

3. Isotope incorporation studies.

a) Incorporation into whole cells.

i) Using $NaH^{14}CO_3$.

Rates of incorporation of ^{14}C from $NaH^{14}CO_3$ into the three cell fractions obtained after chloroform/methanol extraction of washed cells recovered from an enriched culture are shown in Fig. 2.15. Ninety minutes after adding the labelled substrate, 75% of the ^{14}C recovered in the cells was in the residue fraction, 16% was in the aqueous phase of the chloroform methanol extract and 9% was in the lipids. The sum of the activities in the fractions corresponded to an uptake of 14% of the added radioactivity in 30 min, 46% in 90 min and 89% in 6 hrs.

A CO_2-fixation rate for the culture was calculated from the measured uptake of $^{14}CO_2$ in the first 30 min. The maximum amount of CO_2 which could be retained by the medium was calculated to be 230 µg and the culture at that point contained 3g fresh wt (0.6 g dry wt) of cells. Fourteen percent incorporation of the added $^{14}CO_2$ in 30 min corresponds to a fixation rate of 107 µg CO_2/g dry wt cells/hr so that, using the empirical formula,

$$CO_2 + H_2O \longrightarrow CH_2O + O_2$$

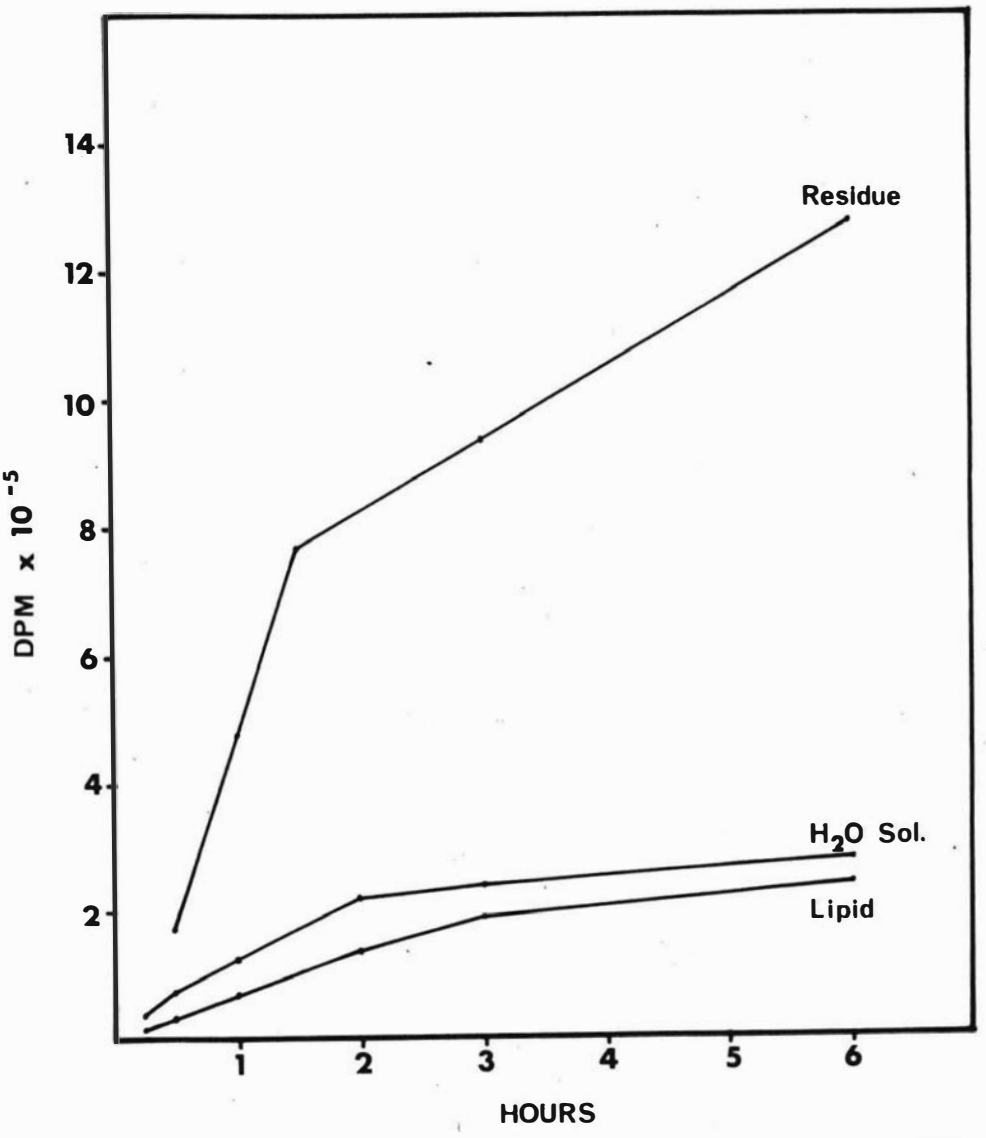

Fig. 2.15. Incorporation of ^{14}C from $NaH^{14}CO_3$ into the three fractions recovered after chloroform/methanol extraction of Mesotaenium cells. 9 μc (0.33 μ mole) of $NaH^{14}CO_3$ was added at zero time when the glucose/yeast extract-enriched culture (400 ml) was nearing the mid-point of its growth phase. Samples (40 ml) were taken at the times indicated and cells recovered by centrifugation. Ordinate; total radio-activity recovered in each fraction of each sample.

as a close approximation, CO_2 contributed 73 µg/g dry wt cells/hr. However, the growth rate of the culture was 4%/hr or 40 mg/g dry wt cells/hr. Accordingly, CO_2 contributed a maximum of only 0.18% to the dry wt increase of the culture.

ii) Using U-^{14}C-glucose.

Incorporation of ^{14}C into the three cell fractions after the addition of 20 µc (7 µmole) of U-^{14}C-glucose to an enriched culture at mid-growth phase is shown in Fig. 2.16. Twenty-four hours after addition of the label, when the culture had reached the end of the growth phase, 84% of the ^{14}C recovered in the cells was in the residue, 10% in the aqueous phase of the chloroform/methanol extract and 6% was in the lipids.

The rate of assimilation of glucose was calculated from the ^{14}C recovered in the 24 hr sample, which was equivalent to 2.2 µc ^{14}C for the cells of the whole culture. The specific activity of the glucose in the medium at zero time was estimated to be 6.0 µc/g (Fig. 2.3) so that the ^{14}C recovered in the cells after 24 hrs represents an assimilation of 0.36 g of glucose.

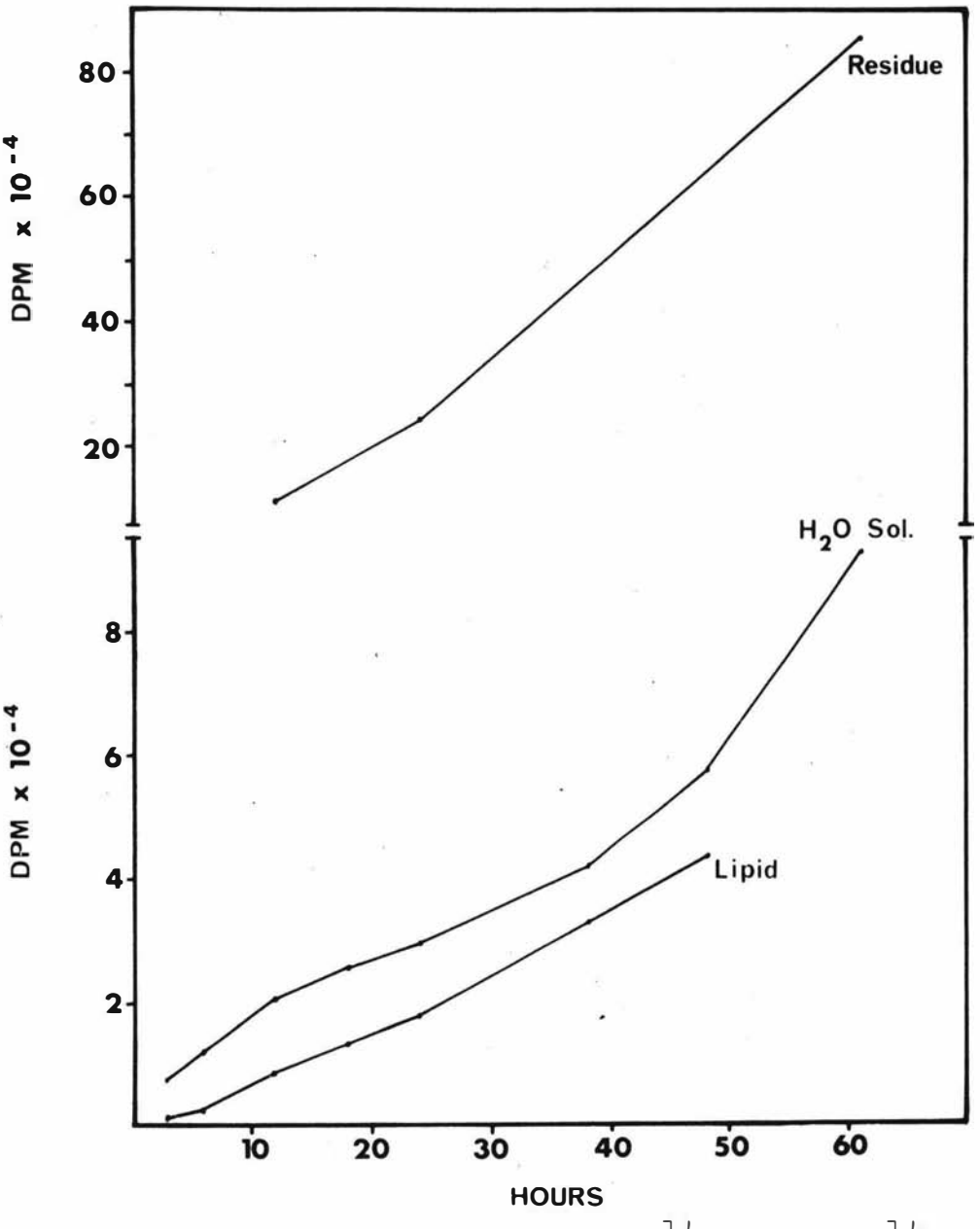

Fig. 2.16. Incorporation of ^{14}C from U-^{14}C-glucose into the three fractions recovered from chloroform/methanol-extracted Mesotaenium cells. At zero time 20 μc (7 μ mole) of radioglucose was added to a culture in which the cell concentration had reached 1% (wet wt/volume) and aliquots of the culture sufficient to give 0.5 - 0.6 ml of packed cells were drawn off at the times indicated. Ordinate; total radioactivity recovered in each fraction of each sample.

During this period however, the cell mass increased by 0.7 - 0.8 g dry weight and, since only half of this is new growth, glucose contributed 90-100% of the weight for this increase. This figure provided additional evidence that growth of the alga was by the assimilation of exogenous glucose rather than by CO_2 fixation.

b) Incorporation into glycerolipids.

i) Using $NaH^{14}CO_3$.

The various glycerolipids incorporated ^{14}C at a linear rate between 30 min and 2 hr (Fig. 2.17) after adding $NaH^{14}CO_3$ to an enriched culture (same experiment as for Fig. 2.15). A feature of these results is the relatively high incorporation into PG and TG and the low incorporation into the galactolipids. During the 2 hr in which the specific activities of these lipids increased from zero to the values shown in Fig. 2.17, the total amount of each lipid in the culture would be expected to have increased by a constant amount in parallel with the growth the culture i.e. by approximately 8%. The different rates of ^{14}C incorporation may therefore indicate the relative rates of synthesis of the various glycerolipids from the supplied, labelled precursor.

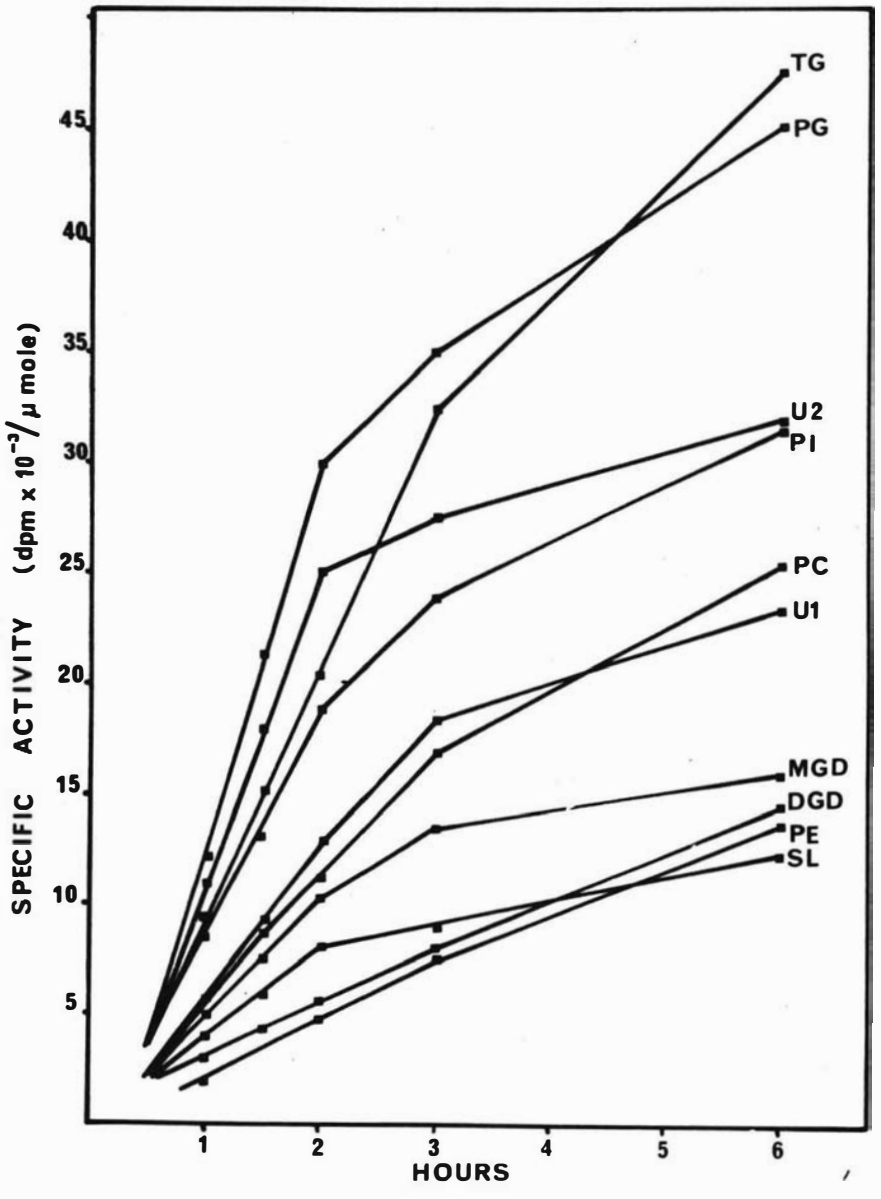

Fig. 2.17. Rate of incorporation of ^{14}C from $NaH^{14}CO_3$ into the glycerolipids of Mesotaenium.

It was calculated that lipid contributed 12.5% of the dry weight of Mesotaenium cells so that the specific activities of the "residue" plus "H_2O soluble" fractions and of the lipid fraction were the same at 30 and 60 min after adding the $^{14}CO_2$. It seemed likely therefore, that the cellular constituents were uniformly labelled within 30 min and that if turnover of the constituents was taking place, the turnover rates would be of the same order as incorporation rates.

ii) Using U-^{14}C-glucose.

When 20 µc of U-^{14}C-glucose was added to a mid-growth phase culture, ^{14}C was incorporated slowly into the lipid fraction. Only after 24 hr had sufficient label been taken up into the glycerolipids to give reliable specific activities for many of the compounds (Table 2.5). However, it was possible to demonstrate a linear incorporation of ^{14}C into MGD between 6 and 24 hrs.

Although there was little growth between 24 and 48 hrs it was notable that radioactivity continued to be incorporated into all of the lipids and into some at a much higher rate than into others. This continued incorporation of ^{14}C is probably associated with changes in the intracellular concentrations of

TABLE 2.5

Incorporation of ^{14}C from U-^{14}C-glucose into the glycerolipids of Mesotaenium

	Specific activity (dpm x 10^{-3}/μmole)	
	24 hr	48 hr+
PG	7.2	8.8
U2	4.9	8.6
MGD	3.4	4.0
PC	2.8	7.9
PI	2.8	7.4
SL	2.5	3.6
U1	2.3	6.5
DGD	2.1	6.2
TG	1.1	12.2
PE	1.1	1.9

+ Post-growth conditions. Apparent specific activities calculated using glycerolipid concentrations measured in growth phase cells.

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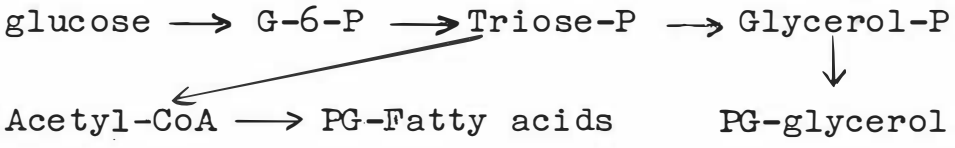
these components as the cells adapt to post-growth conditions. It was obvious from the relative intensity of staining on chromatograms, for instance, that the concentration of TG was higher in the post-growth than in the growth phase.

As in the $^{14}CO_2$ incorporation experiment PG and U2 had the highest specific activities in the shorter time intervals and PE and DGD were among those components with the lowest specific activity.

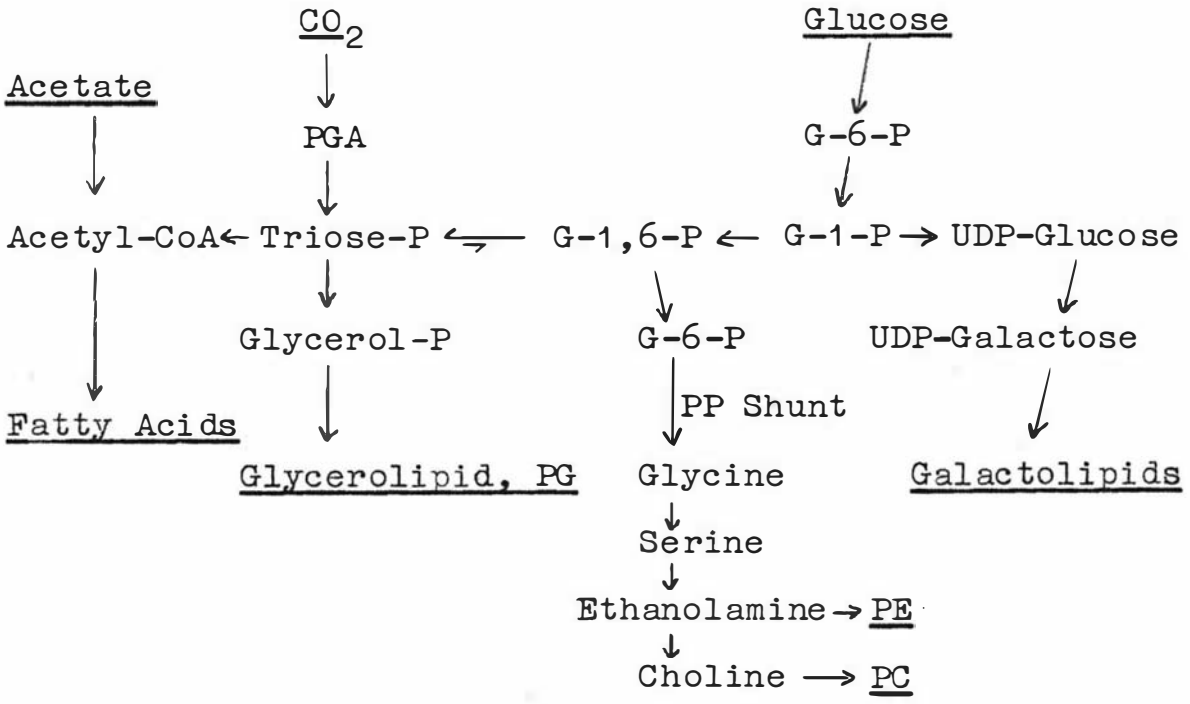
The high relative labelling of MGD from U-^{14}C-glucose compared with that from $^{14}CO_2$ was consistent with the incorporation sequence proposed in Fig. 2.18, but the high incorporation into PG and very low incorporation into TG are more difficult to understand. The incorporation into PG is consistent with the sequence,



and suggests that glycolysis may be a major pathway for glucose assimilation in the cells but this scheme should also lead to high labelling of TG.

Fig. 2.18

Probable routes of carbon from labelled precursors into glycerolipids



iii) Using 2-^{14}C-acetate.

Five hrs after adding 40 µc (1.6 µmole) of 2-^{14}C-acetate to a growth phase culture (0.75% with respect to fresh weight of cells), 20% of the added ^{14}C was recovered in the individual glycerolipids separated by TLC. Specific activities of these glycerolipids were determined at 5, 17 and 24 hr after addition of the labelled precursor. The results (Table 2.6) indicated that all of the added acetate had been taken up by the cells within 5 hr since, at 5 hr, all of the glycerolipids had their highest specific

activities which were subsequently diluted by the growth of the culture. The ^{14}C-acetate was incorporated predominantly (>90%) into the fatty acids of the glycerolipids so the rate of dilution of the specific activities gave an indication of the turnover of the fatty acid moieties of the individual glycerolipids. In this respect the fatty acids of PG and TG had the highest turnover rate; their specific activities were diluted 2.5 fold while the growth of the culture increased about 2 fold. In both U2 and PI too, the specific activities were diluted at a slightly greater rate than the growth of the culture. The constituent fatty acids of the two galactolipids had lowest turnover rates probably as a result of the relatively high concentration in these comounds of α-linolenic acid which has been shown to have a low rate of synthesis (James, 1963; Harris and James, 1965).

TABLE 2.6

Specific activities of glycerolipids after adding 2-^{14}C-acetate to a growing culture.

	dpm x 10^{-5}/μmole		
	5 hr	17 hr	24 hr
PG	13	6.4	4.9
TG	12.5	6.7	5.2
U2	5.4	2.9	2.0
PI	5.0	2.5	2.1
U1	4.7	2.8	2.6
PE	4.6	3.1	2.7
PC	4.0	2.6	2.3
SL	3.3	2.2	1.9
MGD	3.1	1.9	1.8
DGD	1.3	1.0	1.8
Cell concentration of culture (% wet wt, w/v)	0.75	1.2	1.5

DISCUSSION

1. Growth of Mesotaenium caldariorum.

The glucose and light requirements for rapid growth of Mesotaenium caldariorum cultured under conditions of relatively low CO_2 concentrations have been confirmed in this study. Although cell suspensions were capable of high rates of CO_2 fixation and O_2 evolution when incubated in the presence of 0.1 M bicarbonate buffer, it appeared that CO_2 fixation was not a significant source of carbon during growth in the normal, glucose enriched media. In fact, under the growth conditions used here (i.e. low CO_2) the cells were incapable of fixing sufficient CO_2 to support any growth (Fig. 2.2). This lack of utilization of CO_2 was probably due to the low levels of CO_2 available in the medium, although some algae (e.g. Chlorella vulgaris; Nichols, 1965; Sastry and Kates, 1966) can apparently be grown successfully in unenriched media with atmospheric CO_2 as the sole carbon source. Attempts to grow cells in 0.1 M bicarbonate buffer plus basal salts resulted in the rapid death of the inocula. Aerating cultures with mixtures of 5% CO_2 in nitrogen or in air may provide a means for determining whether rapid rates of autotrophic growth of Mesotaenium are possible.

The absolute requirement for light for the assimilation of glucose in supporting growth is noteworthy (see review by Danforth, 1962). Several diatoms have been found to grow in the light, in CO_2-free environments and in the presence of substrates which would not support dark growth (Lewin, 1953) and it has been suggested that the obligate phototroph, Chlamydomonas dysosmos, is incapable of coupling the oxidative release of energy with assimilation (Lewin, 1954). These findings suggest that an adequate supply of ATP can be produced in these cases only by photophosphorylation.

2. Glycerolipids of Mestoaenium.

All of the glycerolipids normally found in photosynthetic tissues (Ferrari and Benson, 1961; Allen and Good, 1965; James and Nichols, 1966) were detected in Mesotaenium caldariorum. Triglyceride, which is not normally found in significant amounts in leaves, was present in Mesotaenium in concentrations in excess of any of the phospholipids and there was some evidence to suggest that its concentration increased in post-growth cultures. In addition to the usual glycerolipids, two unknown compounds appeared

during chromatographic analysis. The first of these (U1) was the most abundant lipid, apart from galactolipids, in growing cells and appeared to be only slightly more polar than MGD in the 2-dimensional TLC system; it was less polar on DEAE-cellulose and yet behaved like a phospholipid on silicic acid and Florisil columns. The second unknown (U2) was present in concentrations comparable with the major phospholipids and behaved as an acidic lipid in 2-dimensional TLC and on DEAE-cellulose columns. Since this compound could be eluted from DEAE-cellulose columns in chloroform/acetic acid (4:1) it seemed likely that the functional acidic group in ion-exchange chromatography was a carboxylic acid. In the silicic acid/acetone column technique this compound chromatographed as a glycolipid but on Florisil it behaved as a phospholipid. Although U2 stained as a glycolipid with the α-napthol/H_2SO_4 reagent, neither unknown gave a significant response in the phenol/H_2SO_4 glycolipid assay and neither contained phosphorus or sulphur. The presence of glycerol in acid hydrolysates and of fatty acid esters in transesterification mixtures indicated that both unknowns were glycerolipids.

Apart from the triglyceride, the concentrations of the various glycerolipids of Mesotaenium were similar to those measured in some leaves (Table 2.7), although "chloroplast" lipids were relatively more concentrated in Mesotaenium. Diphosphatidyl glycerol was not detected in Mesotaenium, even by radiochemical methods which did however, reveal traces of phosphatidic acid (PA) and two other phospholipids demonstrating high mobilities during TLC in acid developing solvents (Fig. 2.5). The only other green alga for which comparable glycerolipid figures are available is Chlorella pyrenoidosa (Ferrari and Benson, 1961). In this organism, the values for the lipids MGD, DGD, SL, PE and PI were about twice as high as the values for Mesotaenium on a cell fresh weight basis, while the value for PG was five times as high (Table 2.7). The percentage lipid yield from Chlorella was three times that from Mesotaenium.

TABLE 2.7

Comparison of Glycerolipid Concentrations
(μmole/g fresh weight)

Glycerolipid	White Clover	Mesotaenium	Chlorella
MGD	8.6	10.0	25
DGD	5.2	5.51	10
TG	tr	2.30	25
PC	1.41	1.10	3.9
PG	1.13	1.60	8.6
PE	0.87	0.80	1.7
SL	0.76	1.31	2.5
PI	0.25	0.8	2.0
DPG	0.28	0	-
Lipid content mg/g fresh wt	1.5	2.6	7.7

3. Fatty Acids.

Growth phase Mesotaenium cells have relatively more linolenic and oleic acids and less linoleic acid compared with Chlorella vulgaris (Nichols, 1965), but the proportion of trienoic fatty acids to total fatty acids is lower in Mesotaenium than in leaves of higher plants (see Part 3). The relatively high level of polyunsaturated fatty acids, compared with autotrphically grown Chlorella (Nichols, 1965), is in agreement with the theory linking functions of polyunsaturated fatty acids with photosynthetic

oxygen evolution (Erwin and Bloch, 1963) but the very low level of trans-$\Delta 3$ - hexadecenoic acid in phosphatidyl glycerol is in contrast to the amounts normally found in algae (Nichols, 1965; Haverkate and van Deenen, 1965) and higher plants (Allen et al., 1964; Weenink, 1964; Haverkate and van Deenen, 1965 b; Nichols, 1965). This acid which has also been implicated in the photosynthetic process, is found only in the photosynthetic tissue of higher plants and algae but is absent from some blue green algae (James and Nichols, 1966). The unknown polyunsaturated fatty acid has been tentatively identified as 17:3 (Korn, 1964), and could presumably arise from α-oxidation (Hitchcock and James, 1966) of α-linolenic acid.

4. Glycerolipid, ^{14}C-incorporation studies.

The incorporation figures for all three ^{14}C-labelled precursors into the glycerolipids of Mesotaenium suggested a relatively high turnover rate for PG and U2, while, with $^{14}CO_2$ and ^{14}C-acetate, relatively high rates also were obtained for TG. The incorporation patterns obtained with $^{14}CO_2$ and ^{14}C-acetate were remarkably similar but differed from that obtained using ^{14}C-glucose (Table 2.8). It is possible that $^{14}CO_2$ assimilation had been biased towards the formation of acetyl-CoA

TABLE 2.8

Specific activities of glycerolipids after addition of labelled precursors

Radioactive Precursor	Order of decreasing specific activity →									
CO_2	PG	U2	TG	PI	U1	MGD	PC	SL	DGD	PE
Acetate	PG	TG	U2	PI	U1	PE	PC	SL	MGD	DGD
Glucose	PG	U2	MGD	U1	SL	PC	DGD	PI	TG	PE
CO_2(Chlorella)+	PE	SL+PI		DGD	PG+PC		MGD			

+ Calculated from data of Ferrari and Benson (1961).

rather than hexose phosphate (Fig. 2.18). The relatively low incorporation of ^{14}C from $^{14}CO_2$ into the galactolipids does not correlate with the proposal of Benson (1963) the galactolipids are involved in the transport out of the chloroplast of galactose produced in photosynthesis. Had the $^{14}CO_2$ fixed been passing through the galactose moieties of the galactolipids it seems certain that the galactolipids would have had specific activities at least higher than those of PC and PI and probably higher than those of any other glycerolipid, particularly at shorter time intervals. In their study of the photosynthesis of Chlorella pyrenoidosa glycerolipids from $^{14}CO_2$, Ferrari and Benson (1961) stressed the high turnover of the galactolipids and phosphatidyl glycerol. However these interpretations were based on gross ^{14}C incorporations into the lipids and when relative specific activities were calculated from their published data (Table 2.8) it was found that MGD had less than half the specific activity of that for the most highly labelled lipid, namely, PE.

A convincing demonstration of the turnover of the fatty acid moieties of the individual glycerolipids of Mesotaenium was obtained in the ^{14}C-acetate experiment where the initial, high specific activities, 5 hrs after the addition of the labelled precursor, subse-

quently decreased, partially through the growth of the culture and partially by turnover. In the cases of PG, TG and , to a lesser extent, U2 and PI a net turnover of fatty acid carbon must have occurred since the specific activities of these lipids decreased at a greater rate than could be accounted for by the growth of the culture. At the other end of the scale, MGD and DGD fatty acids must have had a very slow turnover since the decrease in specific activities proceeded more slowly than the growth of the culture. In these cases there would have been a continual slow incorporation of ^{14}C into the fatty acids had the culture been a stady state, non-growing system such as a leaf.

In a recent study on the incorporation of 2-^{14}C-acetate into the individual glycerolipids of Chlorella vulgaris, Nichols et al., (1967) found that the turnover rate of each lipid varied with the fatty acid content of the lipid. The decreasing order of turnover rates of the fatty acids was stearic, myristic, oleic, palmitic, palmitoleic, linoleic, linolenic and the 16 carbon, dienoic acids. From a consideration of the relative proportions of palmitic, oleic and stearic acids in the glycerolipids of Mesotaenium (Table 2.3) it might therefore be expected that the order of

decreasing specific activities of these glycerolipids 5 hrs after adding the ^{14}C-acetate would be TG, PE, U2, PC, PI, U1, PG, SL, DGD and MGD. That this sequence does not agree with the results obtained with Mesotaenium could be due to Mesotaenium cultures being fully light-adapted while the Chlorella cells were still developing functional chloroplasts. Alternatively, an individual fatty acid of a glycerolipid may not exchange readily with the metabolic pool of that fatty acid so that a particular fatty acid may have different turnover rates depending on the glycerolipid to which it is attached.

Although the results of this study have shown up large differences in the relative turnover of the different glycerolipids, there is a general impression that the glycerolipids as a class show low turnover rates. To enlarge on this briefly, consider the incorporation of U-^{14}C-glucose into PG. At the time of the addition of the ^{14}C-glucose to the culture the specific activity of the glucose in the medium was calculated to be 1.1 μc/m mole. After 24 hrs the specific activity of PG was found to be 3.3×10^{-3} μc/umole which is equivalent to the incorporation of 3 μmole glucose/μmole PG. On a carbon for carbon basis this incorporation accounts for most

of the carbon required for the synthesis of new PG in 24 hrs. Had there been a significant turnover of the carbon of PG as suggested by Benson (1963) a much higher level of radioactivity would be expected in the lipid. However, the results obtained with ^{14}C-acetate indicated that the fatty acids of PG did undergo some turnover.

PART 3

TURNOVER OF THE GLYCEROLIPIDS OF CUCURBITER LEAVES

INTRODUCTION

In the early stages of this work, before analytical techniques had been refined to the degree described in Parts 1 and 2 of this thesis, attempts were made to introduce radiocarbon from ^{14}C-labelled precursors into the glycerolipids of leaves. In the first instance these experiments were confined to the uptake of the labelled precursors via cut petioles (Kates, 1960; James, 1963) but it was later found simpler, with squash leaves in particular, to introduce the precursors by applying their aqueous solutions to the upper surfaces of intact leaves. From experience gained during studies on the incorporation of labelled precursors into the glycerolipids of *Mesotaenium caldariorum* (Part 2) it was realized that it would be possible to isolate sufficient of each of the glycerolipids of a leaf, sufficient for counting and analysis, from as little as 0.5 g (wet wt) of leaf tissue. It should be possible, therefore, to perform incorporation studies using single, large leaves by applying the ^{14}C-precursors to the leaf surface and, at suitable intervals thereafter, repeatedly and representatively sampling

(8-12 discs of about 1 cm diameter) the leaf for extraction and separation of the labelled glycerolipids. Providing the ^{14}C-precursor could be applied uniformly over the surface of the leaf, such an experimental system should give reliable measurements of the physiological turnover of the glycerolipids of leaves.

This section describes the experiments which were performed in an endeavour to obtain results which could be interpreted in terms of glycerolipid turnover in intact leaves.

METHODS

1. Squash leaves.

Squash plants (Cucurbiter pepo; variety butternut) were grown in glasshouses under natural lighting.

Labelled precursors (1- and 2- C^{14}-acetate, 2-^{14}C-malonate and U-^{14}C-palmitate, all as the sodium salts) were initially taken up into detached leaves via the cut petiole (Eberhardt and Kates, 1957; James, 1963). The detached leaves were then placed in growth cabinets at 20°C under a bank of fluorescent and tungsten lights, delivering approximately 80 watt.metre^2 of photosynthetic irradiance at the leaf surface (Dr D. Scott, personal communication. This figure is to be compared with the 550 watt.meter^2 produced by full sunlight in the New Zealand summer), for the duration of the incorporation period. Whole leaves were extracted and individual glycerolipids separated by 2-dimensional TLC (Nichols, 1964) or by DEAE-cellulose/TLC (Part 1).

Difficulty was experienced in preventing cut leaves from wilting, even when the petioles were cut under water. Some cut leaves recovered after an initial wilt but others remained flaccid and could not be used.

When the fate of the added radioactivity was to be followed over several days, labelled precursors in

aqueous solution were spread over the surface of the leaves. Two techniques were used for this: a) The solution was applied as evenly as possible to the upper surface of a leaf in a large number of minute drops and, b) the solution was streaked along the lines of the major veins on the upper surface of the leaf.

Fifty to one hundred µl of solution was used to treat a leaf of about 80 cm^2, this volume being considered the minimal amount for even application. Solutions spread best when traced along the lines of the veins; when 100 µl was applied in this way and the plant returned to the growth cabinet no visible evidence of the application was apparent after 15 min.

2. Pumpkin leaves.

Pumpkin plants (Cucurbiter pepo, Whangaparaoa strain) were grown in the open in the season of 1967-68. The leaves used were at least 33 cm in width and had surface areas of approximately 880 cm^2.

a) $^{14}CO_2$ labelling experiment.

For the $^{14}CO_2$-labelling experiment an air circulating system, consisting of a reservoir (a 250 ml flask) in which the $^{14}CO_2$ was generated, two rubber

tubes and a rubber bulb ("Pro-pipette"), was set up. The leaf was enclosed in a large plastic bag (35 x 45 cm) which was attached around the petiole and the two rubber connecting tubes. One of the tubes ran through onto the upper surface of the leaf ("input tube") and the other terminated under the lower surface ("outlet tube"). The tubes were connected to the reservoir through a rubber bung. The "Propipette" was attached in the "outlet tube" and provided the means for circulating air through the system. At zero time 2N sulphuric acid (2 ml) was tipped from a vial in the reservoir onto 50 µl (200 µc) of $NaH^{14}CO_3$ and for 10 min the air was circulated around the system by means of the hand operated pump.

This experiment was conducted in the early morning (8.30 a.m.) to avoid the excessive heating of the enclosed leaf which could occur in full sunlight. The air temperature at this time was 18°C and rose to 25°C by midday. From 8.50 a.m. the leaf was exposed to full sun for the remainder of the day.

b) ^{14}C-acetate experiments.

The leaf was sprayed with a fine mist of 0.001% Tween 80 in water until the upper surface was uniformly wet (2 to 3 ml). When the leaf had dried, 0.5 ml (100 µc) of 1-^{14}C-acetate was evenly and carefully spotted

about the leaf surface giving very even spreading. The tracer was applied at 1.00 p.m. in full sun with the air temperature at 26°C.

c) Sampling.

At intervals, three discs (1.4 cm dia.) were taken from each quadrant of the leaf, the selection of sites being random except that care was taken to avoid cutting a main vein. The 12 discs, weighing approximately 0.5 g were ground with methanol (3 ml) in a glass tissue grinder, chloroform (3 ml) was added and the slurry filtered under reduced pressure. Chloroform (3 ml) was used to rinse out the grinder and to wash the residue on the filter and the filtrate was washed with 0.2 vol of 0.73% NaCl.

All other preparations and manipulations were as described for Mesotaenium lipids (Part 2).

RESULTS

The lipid composition of squash leaves was determined in young, fully expanded leaves which had been exposed to continuous light in a growth cabinet (20°C) for 24 hr. The results are shown in Table 3.1 which also shows the fatty acid composition of the individual glycerolipids. The molar ratios of these lipids were similar to those found in squash leaves grown in the open (Table 1.3).

TABLE 3.1

The glycerolipids of squash leaf and their fatty acid composition

Glycero-lipid	µMoles/g fresh Wt.	% of total fatty acid							
		12:0	14:0	16:0	16:1	18:0	18:1	18:2	18:3
MGD	4.0	1	tr	5	tr	1	tr	tr	93
DGD	2.5	3	tr	14	tr	3	tr	tr	79
SL	0.3	tr	tr	40	tr	11	7	tr	42
PC	1.5	1	1	27	tr	9	tr	8	54
PG	0.9	2	tr	33	28	8	11	5	13
PE	0.9	1	1	28	tr	5	2	8	55
PI	0.14	7	4	47	tr	17	8	tr	17
DPG	0.08	1	tr	32	tr	9	tr	8	49
Whole lipid+		1	1	11	3	2	2	5	75

+ means of six determinations on separate leaf extracts.

1. Incorporation studies using squash leaves.

a) Detached leaves.

i) U-^{14}C-palmitate.

Attempts to induce U-^{14}C-palmitate incorporation into squash leaf glycerolipids were unsuccessful. When the compound was fed to detached leaves via the cut petiole no activity was found in the leaf lipids even after 24 hr. Slices of the petiole, however, were found to be radioactive, with the activity concentrated around the cut region. Lipid extracted from this portion of the petiole was chromatographed on Silica Gel G using the solvent petroleum ether/diethyl ether/ glacial acetic acid (90:10:1). Radioautographs of these chromatograms showed that most of the radio-activity was in the free fatty acid spot but that some of the activity remained at the origin and possibly represented incorporation of palmitic acid into polar lipids.

When the incorporation period was increased to 3 days there was still no activity in the leaf but considerably more than half of the ^{14}C in the lipid fraction from the lower region of the petiole was present as polar glycerolipid. The remainder was as fatty acid and in none of these experiments was any activity detected in the region of chromatograms where triglyceride would be. Labelled, polar lipids from petioles were separated by 2-dimensional TLC

and radioautographs showed that most of the activity was in MGD; label could also be detected in PC and PG.

ii) ^{14}C-Acetate and ^{14}C-malonate.

1- and 2- ^{14}C-acetate and 2-^{14}C-malonate were incorporated into leaf glycerolipids when supplied via cut petioles. Within 2 to 3 hours the major phospholipids (PC and PG) had incorporated the bulk radioactivity in the glycerolipids but of the remaining glycerolipids only MGD possessed detectable (by radioautography) label. A greater proportion of ^{14}C from the supplied acetate than from malonate was incorporated into the lipid fraction of leaves.

b) Intact leaves.

i) ^{14}C-Palmitate.

U-^{14}C-Palmitate penetrated slowly into squash leaves when applied as an aqueous solution to the upper or lower surface. Eight hours after application 25% of the original activity could be recovered from the leaf surface in a petroleum ether rinse (10 ml) followed by 50% aqueous methanol (10 ml). Lipids extracted from treated leaves were subjected to 2-dimensional TLC but only one radioactive spot was ever observed and that corresponded to free fatty acid.

ii) ^{14}C-Acetate.

1-^{14}C-Acetate was rapidly incorporated into the glycerolipids when applied to the upper surface of the leaf in aqueous solution. In periods of up to 4 hours most of the activity was again concentrated in PC and PG with MGD the only other component with a significant contribution. It was noted that 6 to 8 hrs after application of the labelled acetate to the leaf surface 13% of the original activity could be recovered in the leaf lipid fraction when plants were subsequently placed in growth cabinets.

Two to three hours after application of 1-^{14}C-acetate the monoenoic fraction of leaf fatty acids had the highest activity.

2. Turnover studies using pumpkin leaves.

a) Labelling with $^{14}CO_2$.

When a large pumpkin leaf was treated with 200 µc of $^{14}CO_2$ approximately 1% of the recovered activity was located in the lipids after 60 min (Fig. 3.1). It was calculated that the 12 discs taken at each sampling represented approximately 2% of the total area of the leaf and, with an original level of 200 µc of $^{14}CO_2$, slightly more than 4 µc should be recovered in the 15 min sample. In fact, 4.8 µc was detected in this

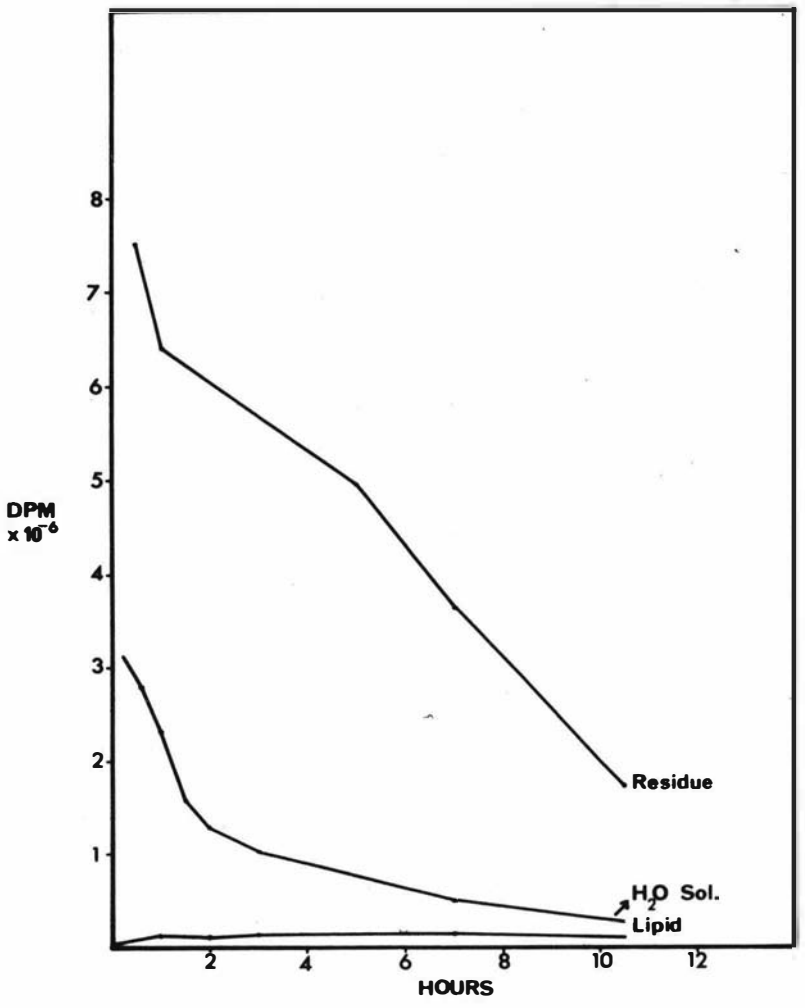


Fig. 3.1. Radiocarbon recovered in the three fractions of pumpkin leaf at intervals after labelling the leaf with $^{14}CO_2$.

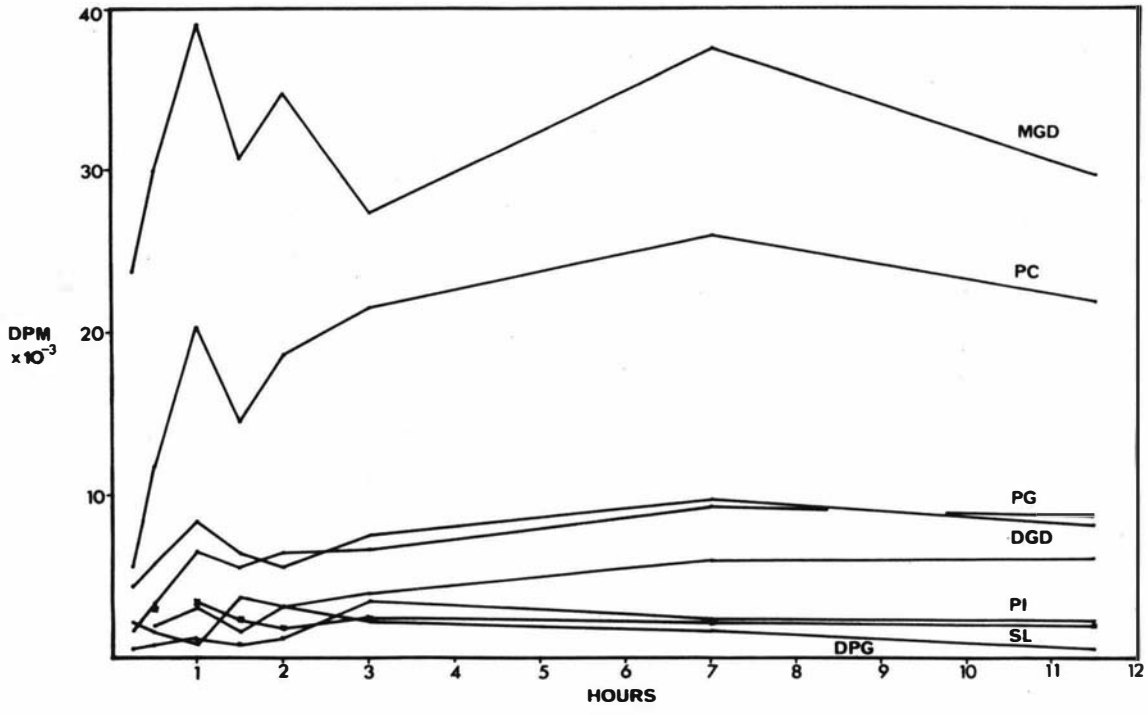


Fig. 3.2. Labelling of the glycerolipids of pumpkin leaf after treating the leaf with $^{14}CO_2$. These curves show the gross ^{14}C content of the lipids.

sample (Fig. 3.1) and the bulk of this activity was found in the residue from the chloroform/methanol extraction. The ^{14}C level in both the residue and the water-soluble fractions declined steadily over the 11.5 hr of the experiment but the labelling of the lipids did not change significantly after 1 hr.

Of the individual glycerolipids, PC and MGD were distinctive in incorporating a high proportion of the total activity in the fraction (Fig. 3.2). In contrast to the findings of Ferrari and Benson (1961), DGD and PG respectively incorporated less than half of the amount of ^{14}C which appeared in PC.

The pattern of uptake was the same for all glycerolipids except DPG. There was an almost linear incorporation of ^{14}C over the first 60 min followed by a slight drop and then further incorporation with maximum labelling being reached in about 7 hr. DPG was the only component to show a decline in activity between 15 and 60 min; from chromatograms this seemed to be due to a decrease in the concentration of this lipid over that period.

Analyses were performed on the acidic lipids separated from the equivalent of 100 mg of leaf for each sample. The results (Table 3.2) showed that there was a considerable variation in the concentrations

TABLE 3.2

Variations in the concentrations of acidic lipids during the day ($^{14}CO_2$ experiment)

Time	μmole/sample		
	PG	DPG	SL
8.45 a.m.	.94	.75	.36
9.00	.93	.16	.66
9.30	.97	.07	.55
10.00	.90	.21	.19
10.30	.97	.16	.41
11.30	.90	.11	.33
3.30 p.m.	.91	.05	.19
7.00	.98	.01	.27

of DPG and to a lesser extent SL; PG was maintained at a fairly constant level over the same period. Concentrations of MGD and DGD varied by 10% and 15% around their respective mean values during the course of the day. In addition, it seemed likely that the drop in labelling of MGD between 2 and 3 hrs was not due to a decrease in the total amount of the lipid during this period.

Deacylation of MGD, DGD and PG by acid hydrolysis in the presence of adsorbent (Part 1) was used to show that 95% of the ^{14}C of MGD in the 15 min sample was in the water soluble portion of the molecule. By 60 min this percentage had dropped slightly to 90%. The

figures for DGD were 92% and 90% respectively while at 60 min 85% of the radioactivity of PG was recovered in the aqueous hydrolysate.

b) Labelling with 1-^{14}C-acetate.

Incorporation of 1-^{14}C-acetate (100 µc; 2.25 µ mole) into the three fractions of pumpkin leaf is shown in Fig. 3.3 where it can be seen that the lipids took up the bulk of the ^{14}C. This was in contrast to earlier experience with squash leaves where only 13% of added 1-^{14}C-acetate was found in the lipid fraction after 4 hrs. A likely explanation for this difference was differing light intensities (e.g. see Eberhardt and Kates, 1957; Mudd and McManus, 1965). The squash experiments had been carried out in growth cabinets where the light intensity was about one-seventh of that of full sunlight.

From Fig. 3.3 it can be calculated that almost 35% of the ^{14}C which should appear in the sample (assuming no translocation of isotope) was recovered in the lipid fraction after 5 hr. However, if it is assumed that there has been no translocation of ^{14}C away from the leaf within 60 min of application and that there had been a uniform spread of the labelled material across the leaf surface then approximately 2 µc of ^{14}C

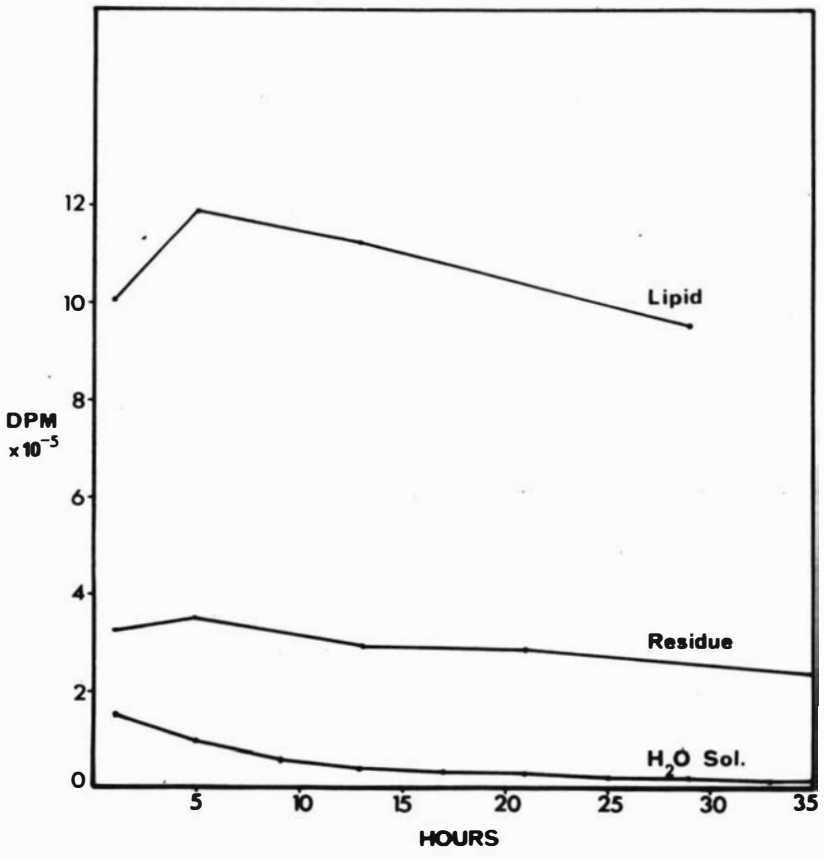


Fig. 3.3. Radiocarbon recovered in the three fractions of pumpkin leaf at intervals after labelling the leaf with 1-^{14}C-acetate.

should have been recovered in the 60 min sample. In fact it can be seen from Fig. 3.3 that after 60 min 0.7 and after 5 hr 0.8 μc were recovered.

These results suggested both an uneven application of the labelled material since more ^{14}C was recovered at 5 hr than at 1 Hr and also some significant translocation (or some other form of loss such as transpiration or evaporation of ^{14}C-acetic acid) of ^{14}C away from the leaf since less than half of the theoretical activity was recovered at either time.

Incorporation of 1-^{14}C-acetate into the glycerolipids of pumpkin leaf was followed over 45 hr and the results are shown in Figs. 3.4 and 3.7. As in the experiments of Nichols et al. (1967) with Chlorella two distinct groups of lipids could be distinguished. The first group, consisting of PC, MGD, PG and PE, had a high uptake of ^{14}C while the second group (DGD, PI and SL) had a relatively low incorporation rate. An anomalous curve was again produced by DPG (Fig. 3.4). Sometime between 17 and 21 hr after application of the tracer (i.e. between 6 a.m. and 10 a.m. on the following morning) there was a sharp increase in the labelling of DPG and this was accompanied by a sharp decrease in the labelling of PC. Staining of chromatograms and the experience of the previous experiment indicated that the increase in

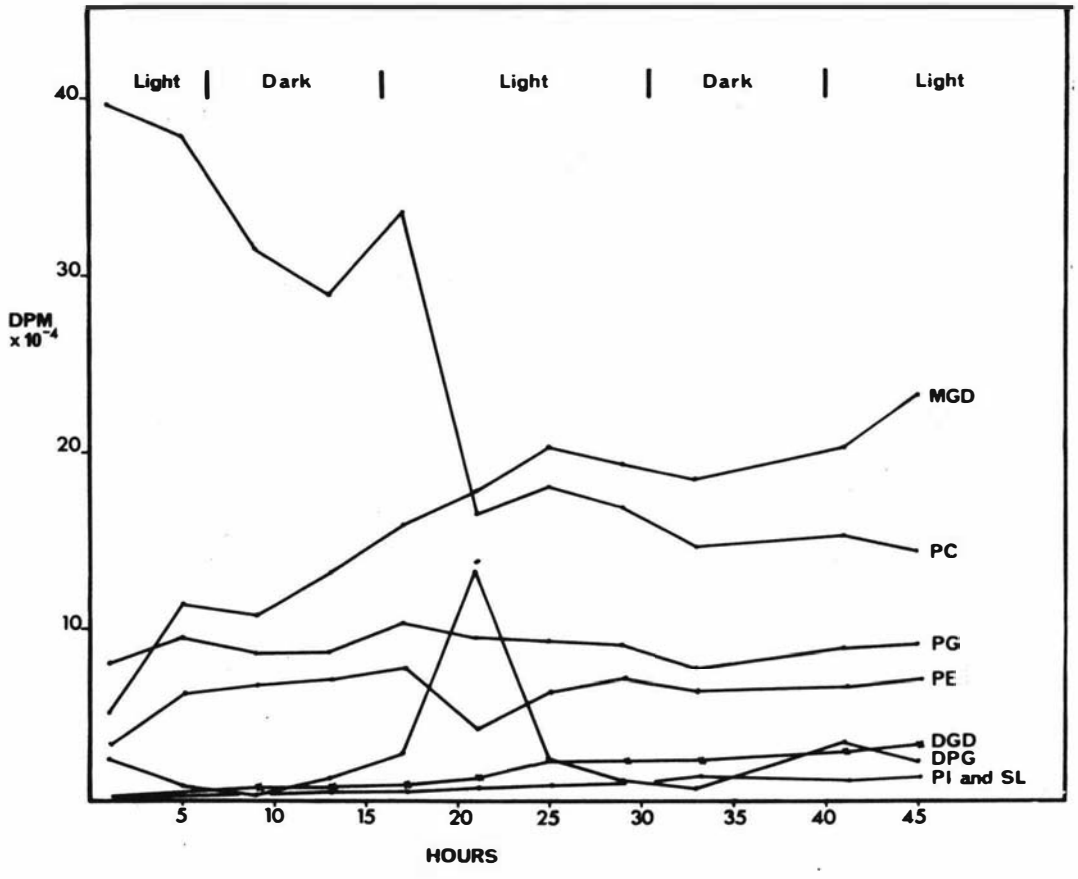


Fig. 3.4. Incorporation of 1-^{14}C-acetate into the glycerolipids of pumpkin leaf and the fate of the incorporated label as a function of time.

labelling of DPG was probably a result of an increase in the concentration of the lipid so analyses of the individual glycerolipids in each fraction were performed.

Diurnal variations in the concentrations of the various glycerolipids are shown in Fig. 3.5. The most significant variation was that of DPG which was scarcely detectable at 10 p.m. but which was a major phospholipid constituent at 10 a.m. the following morning. Accompanying the increase in concentration of DPG were significant decreases in the levels of PC and PE and when the DPG concentration returned to its normal low level the concentrations of PC and PE increased.

Changes in the specific activities of the glycerolipids (Fig. 3.6) were calculated from Figs. 3.4 and 3.5.. Several important features of these results are as follows.

1. Of all the glycerolipids only PC showed a significant decline in specific activity during the experiment.

2. The major glycerolipids of leaves MGD and DGD had relatively low specific activities which increased very slowly during the experiment.

3. After reaching maximum specific activity PG (in 1 hr) and PE (in 10 to 15 hr) did not alter

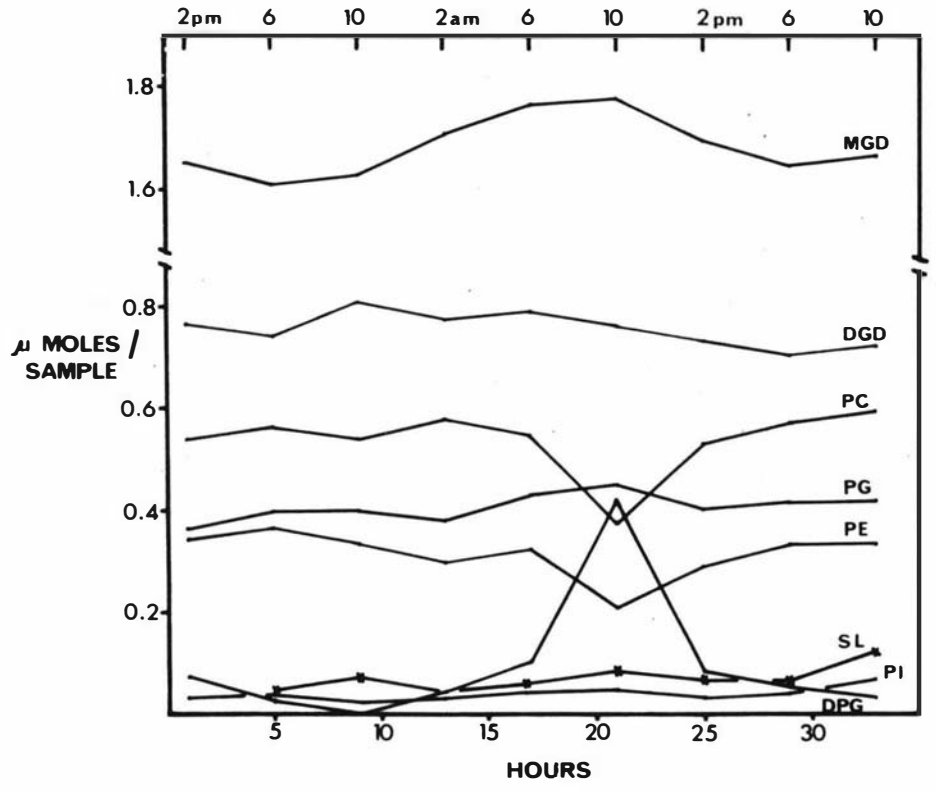


Fig. 3.5. Diurnal variations in the concentrations of the glycerolipids of pumpkin leaf (^{14}C-acetate experiment). The values of DPG are given in μ mole x 2 since this lipid is almost twice the size of the other glycerolipids. Each sample contained 0.76 (± 2%) μ mole of chlorophylls a + b.

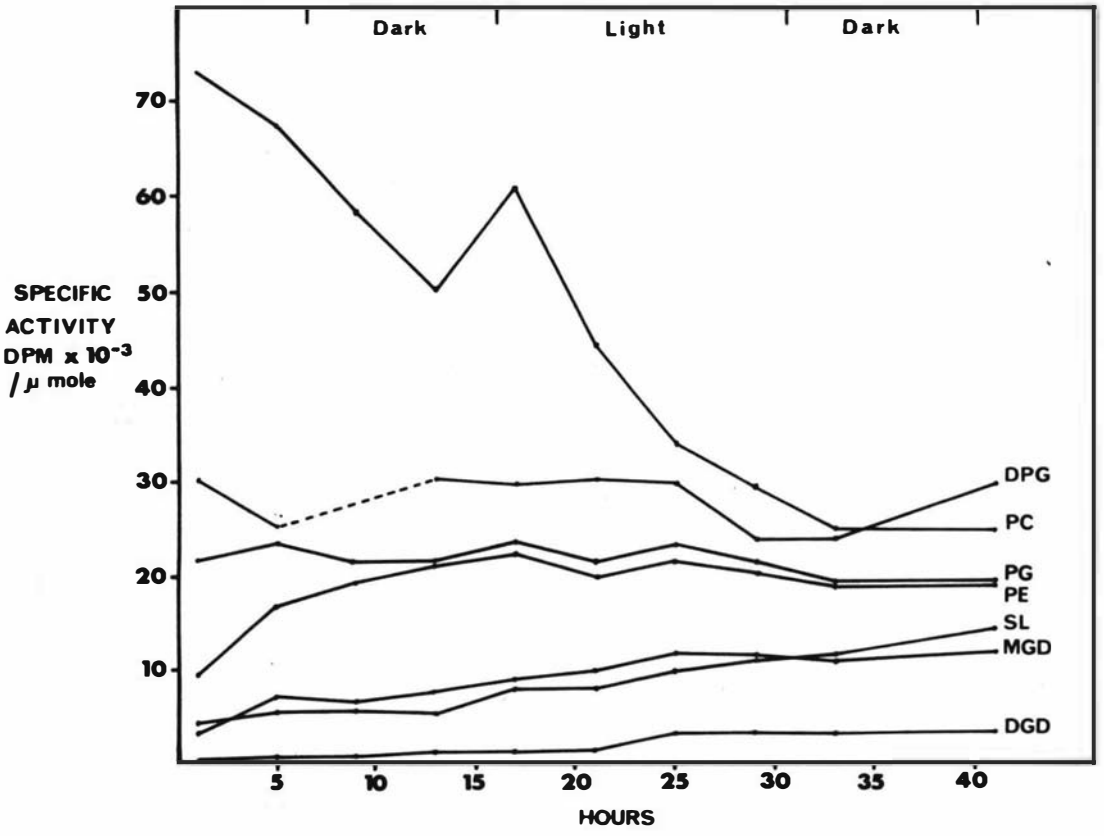


Fig. 3.6. Changes in the specific activities with time of the ^{14}C-acetate-labelled glycerolipids of pumpkin leaf. The ^{14}C in PC at 1 hr is equivalent to the incorporation of 7.3×10^{-3} μ mole of ^{14}C-acetate/μ mole PC.

significantly for the remainder of the experiment.

4. The specific activity of PC increased immediately prior to the decrease in the concentration of this glycerolipid but other than this the concentration and radioactivity changes observed in DPG, PC and PE (Figs. 3.4 and 3.5) combined to produce relatively smooth curves in terms of specific activity.

3. Labelling of fatty acids in PC and MGD.

In view of the slow incorporation of ^{14}C-acetate into the linolenic acid fraction of leaves (James, 1963) it was of some interest to determine the distribution of radioactivity among the fatty acids of PC and MGD. The bulk of the radioactivity in these lipids was in the fatty acids (Table 3.3) but a relatively high proportion of ^{14}C in the polar region of MGD at 5 hr compared with 17 hr may be an indication of a high turnover of the galactose moiety of this molecule (Ferrari and Benson, 1961).

Methyl esters of the constituent fatty acids were prepared from PC and MGD isolated by TLC from the 5, 13, 17, 21, 33 and 41 hr samples. These esters were separated according to their number of double bonds on silver nitrate impregnated Silica Gel G and the ^{14}C content of each fraction determined by scintillation counting.

TABLE 3.3

Proportion of label from 1-^{14}C-acetate in polar and fatty acid regions of PC and MGD

		polar dpm	fatty acids+	% in polar
PC	5 hr	302	57000	.53
	17 hr	495	47600	1.04
	21 hr	105	31500	.30
	41 hr	324	20000	1.62
MGD	5 hr	2360	25800	9.2
	17 hr	176	33500	.19
	21 hr	176	36400	.21
	41 hr	310	41500	.75

+sum of the counts in the various fractions separated by silica gel/$AgNO_3$ TLC.

Of the fractions the trienoic (X:3) and dienoic (X:2) were the most homogeneous consisting almost exclusively of linolenate (18:3) and linoleate (18:2) respectively. The monoenoic (X:1) fractions consisted of almost equal mounts of palmitoleate (16:1) and oleate (18:1) and the saturated (X:0) fractions consisted of palmitate (16:0) and stearate (18:0); 75 and 25% respectively in PC and 83 and 17% in MGD (Table 3.1).

The results of these analyses are shown in Fig. 3.7. Five hours after application of the tracer the linolenic acid fraction of MGD contained 66% of the ^{14}C of the fatty acids. By 41 hr this had increased to 91% which

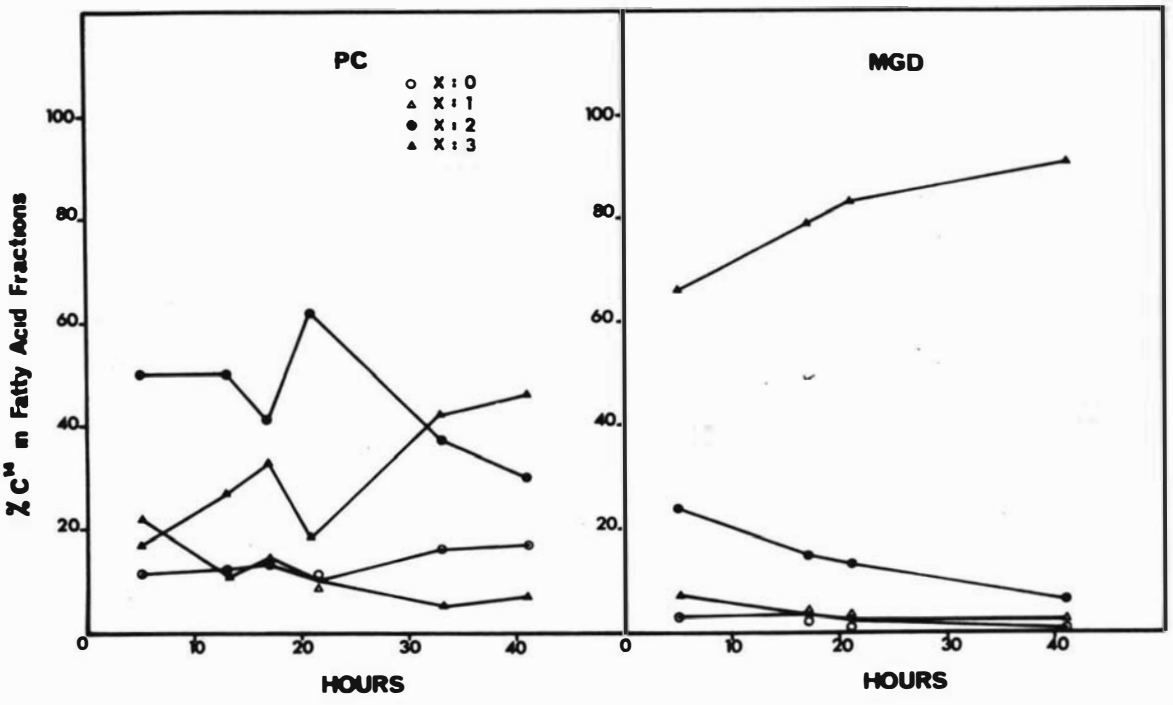


Fig. 3.7. Distribution of radioactivity among the fatty acid fractions of PC and MGD isolated from pumpkin leaf at intervals after labelling the leaf with 1-^{14}C-acetate.

was very close to contribution by weight of this acid to the total. At all times linoleic acid had a higher specific activity than linolenic acid and from the shapes of their respective curves the former appeared to be the precursor of the latter. The saturated fatty acids of MGD (16:0 and 18:0) had much lower specific activities at all times than the monoenoic acids and linoleic acid.

The contribution of ^{14}C by the saturated fatty acid fraction of PC changed very little from 5 to 41 hrs while that of the monoenoic acid fraction declined steadily. As had been the case in MGD the tendency was for the proportion of ^{14}C in the linoleic acid of PC to decrease while that of linolenic acid increased. However, coincident with the drop in concentration of PC between 17 and 21 hr (Fig. 3.5) there was a sharp reversal of this tendency, i.e. the proportion of ^{14}C in linoleic acid increased while that of linolenic acid decreased.

From a consideration of Table 3.1 and Fig. 3.7 it was calculated that the monoenoic fatty acid fraction of PC had the highest specific activity. The ratios of specific activities at 5 hrs were monoenoic, 78; dienoic, 21; trienoic, 1.2 and saturated, 1.0. These

results suggested that ^{14}C-acetate incorporation into the fatty acids was a result of the chain elongation mechanism rather than de novo synthesis and are consistent with the sequence

^{14}C-acetate
+
16 carbon fatty acid → oleic acid → linoleic acid → linolenic acid

Even if all of the ^{14}C in the saturated fatty acid fraction of PC at 5 hr was in stearic acid, the specific activity of that acid would be only 25% of that of linoleic acid which suggested that stearic acid was not the direct precursor of oleic and linoleic acids in PC. However, Nichols et al. (1967) produced evidence for a very fast turnover of stearic acid in PC of Chlorella and it is possible that, at shorter periods, the stearic acid in PC of pumpkin leaves may have had a higher specific activity than oleic or linoleic acids.

DISCUSSION

The results of this study, while preliminary in nature, indicate that the glycerolipids of leaf tissue undergo a relatively slow turnover.

a) $^{14}CO_2$-labelled leaves.

The $^{14}CO_2$ labelling experiment was expected to provide information on the turnover of the hydrophilic portions of the glycerolipids (Ferrari and Benson, 1961).

i) Distribution of ^{14}C in leaf fractions.

Fifteen minutes after beginning the administration of $^{14}CO_2$ to a large pumpkin leaf the radioactivity was recovered almost quantitatively in the residue and H_2O soluble fractions while the lipid fraction contained a small part of 1% of the recovered ^{14}C (Fig. 3.1). The ^{14}C in the residue would be mainly in macromolecules (protein, starch, nucleic acids) and the rapid dilution of the label in this fraction is probably due to the turnover of these constituents. Translocation would be responsible both for the loss of radioactivity from the water soluble fraction (i.e. the small molecular weight substances) and for the overall loss of label from the leaf. The lipid fraction, on the other hand showed no significant change in ^{14}C content between 1 and 10 hrs following treatment with $^{14}CO_2$ in spite of the fact that photosynthesis would be proceeding at maximum

physiological rates for most of this period.

ii) Distribution of ^{14}C in glycerolipids.

This apparent low turnover of the total lipid was also evident in the labelling of the individual glycerolipids (Fig. 3.2). Apart from some small variations, which may have been a result of experimental error, the labelling of the glycerolipids did not alter significantly between 1 and 10 hrs. This suggests that the polar regions of these lipids do not participate directly in the CO_2 assimilation processes of the leaf.

In terms of gross ^{14}C incorporation the results with pumpkin leaves show some significant differences compared with those for Chlorella (Ferrari and Benson, 1961). On the basis of this gross ^{14}C incorporation from $^{14}CO_2$ into Chlorella lipids, Ferrari and Benson (1961) emphasised the metabolic importance of the galactolipids, phosphatidyl glycerol and sulpholipid whereas for leaf, sulpholipid becomes of minor importance and phosphatidyl choline assumes a major role. More significantly, the specific activities of all of the phospholipids were higher than those of the glycolipids; PC had the highest specific activity and DGD the lowest.

b) ^{14}C-acetate labelled leaves.

Using 1-^{14}C-acetate, the fatty acid moieties of the

glycerolipids became preferentially labelled leaving the hydrophilic regions of relatively, very low activity.

i) Distribution of ^{14}C in leaf fractions.

Rapid assimilation of the labelled acetate was indicated by the proportions of the recovered ^{14}C in the leaf fractions at 60 min (Fig. 3.3). A puzzling feature however, was the retention of label in the residue fraction considering the rapid loss of $^{14}CO_2$ labelled materials from this fraction in the previous experiment. Slow loss of ^{14}C from the lipids between 5 and 30 hrs indicated low turnover of this fraction.

ii) Distribution of ^{14}C among glycerolipids.

In the labelling of the glycerolipids some similarities to the $^{14}CO_2$ results were evident.

1) Low turnover rates for all glycerolipids, except PC, were indicated by the retention or by the continuous slow incorporation of label over prolonged periods.

2) Phospholipids had higher specific activities than the glycolipids; PC and DGD distinguished themselves by their very high and very low specific activities respectively. The slow incorporation of ^{14}C into the glycolipids was not simply a function of their 18:3 content (i.e. James (1963) had demonstrated the very slow incorporation of ^{14}C-acetate into linolenic acid of castor leaves) since SL had a lower proportion of 18:3 than any glycerolipid except PI and PG.

REFERENCES

Abramson, D. and M. Blecher (1964). J. Lipid Res. 5; 628.

Allen, C.F., P. Good, H.F. Davis and S.D. Fowler (1964). Biochem. Biophys. Res. Comm. 15; 1964.

Allen, C.F., P. Good, H.F. Davis, P. Chisum and S.D. Fowler (1966). J. Amer. Oil Chems' Soc. 43; 1.

Allen, C.F. and P. Good (1965). J. Amer Oil Chems' Soc. 42; 610.

Bailey, R.W. (1962). Anal. Biochem. 3; 178.

Bartlett, G.R. (1959). J. Biol. Chem. 234; 466.

Benson, A.A. (1963). Proc. 5th Intern. Congress in Biochem. Vol.6, Pergamon Press, London. p.340.

Benson, A.A., H. Daniel and R. Wiser (1959). Proc. Nat. Acad. Sci. 45; 1582.

Benson, A.A. and B. Maruo (1958). Biochim. Biophys. Acta. 27; 189.

Benson, A.A., B. Maruo, R.J. Flipse, H.W. Yurow and W.W. Miller (1958). Proc. 2nd Intern. Conf. on Peaceful uses of Atomic Energy, Geneva. Vol. 24, p.289.

Benson, A.A. and E.H. Strickland (1960). Biochim. Biophys. Acta. 41; 328.

Benson, A.A., J.F.G.M. Wintermans and R. Wiser (1959). Plant Physiol. 34; 315.

Bligh, E.G. and W.J. Dyer (1959). Can. J. Biochem. 37; 911.

Buckman, H.O. and N.C. Brady (1960). The Nature and Properties of Soils, The Macmillan Co., New York. p.130.

Bush, E.T. (1963). Anal. Chem. 35; 1024.

Carroll, K.K. (1961). J. Lipid Res. 2; 135.

Carter, H.E., R.H. McCluer and E.D. Slifer (1956). J. Amer. Chem. Soc. 78; 3735.

Comar, C.L. and P.F. Zscheile (1942). Plant Physiol. 17; 198.

Danforth, W.F. (1962). in Physiology and Biochemistry of Algae. Ed. R.A. Lewin, Academic Press, New York, 1962. p.99.

Dittmer, J.C. and R.L. Lester (1965). J. Lipid Res. 5; 126.

Dubois, M., K.A. Gillies, J.K. Hamilton, P.A. Rebers and F. Smith (1956). Anal. Chem. 28; 250.

Dygert, S., L. Li, D. Florida and J.A. Thoma (1965). Anal. Biochem. 13; 367.

Dawson, R.M.C. (1958). Biochem. J. 68; 357.

Erwin, J. and K. Bloch (1963). Biochem. Zeitschrift 338; 496.

Eberhardt, F.M. and M. Kates (1957). Can. J. Biochem. 35; 907.

Ferrari, R.A. and A.A. Benson (1961). Arch. Biochem. 93; 185.

Folch, J., M. Lees and G.M. Sloane-Stanley (1957).

J. Biol. Chem. 226; 497.

Galanos, D.S. and V.M. Kapoulas (1965). Biochim. Biophys. Acta. 98; 278.

Gray, I.K., M.G. Rumsby and J.L. Hawke (1967). Phytochem. 6; 107.

Gunsalus, I.C. and C. W. Shuster (1961). in "The Bacteria" Vol.2, (Eds. I.C. Gunsalus and R.Y. Stanier), Academic Press, New York, pp.30-51.

Hanahan, D.J. and I.L. Chaikoff (1948). J. Biol. Chem. 172; 191.

Harris, R.V., P. Harris and A.T. James (1965). Biochim. Biophys. Acta. 106; 465.

Harris, R.V. and A.T. James (1965). Biochim. Biophys. Acta. 106; 456.

Haverkate, F. and L.L.M. Van Deenen (1965a). Koninkl. Nederl. Akademie van Wetenschappen, Amsterdam. 68; 141.

Haverkate, F. and L.L.M. Van Deenen (1965b). Biochim. Biophys. Acta. 106; 78.

Hawke, J.C. and P.K. Stumpf (1965). J. Biol. Chem. 240; 4746.

Hawke, J.C. and P.K. Stumpf (1965). Plant Physiol. 40; 1023.

Hirsch, J. and E.H. Ahrens (1958). J. Biol. Chem. 233; 311.

Hitchcock, C. and A.T. James (1966). Biochim. Biophys. Acta 116: 413.

Hutchinson, J. (1964). The Genera of Flowering Plants, Vol.1, Oxford University Press, London, p.9.

Jacin, H. and A.R. Mishkin (1965). J. Chromatog. 18; 170.

James, A.T. (1963). Biochim. Biophys. Acta 70; 9.

James, A.T. and B.W. Nichols (1966). Nature 210; 372.

Jordon, R.C. and A.C. Chibnall (1933). Ann. Botany 47; 163.

Kalberer, F. and J. Rutschmann (1961). Helv. Chimica Acta 44; 1956.

Kates, M. (1960). Biochim. Biophys. Acta 41; 315.

Kates, M. and F. Eberhardt (1957). Can. J. Botany 35; 895.

Korn, E.D. (1964). J. Lipid Res. 5; 352.

LaPage, M. (1964). J. Chromatog. 13; 99.

Lea, C.H. and D.N. Rhodes (1953). Biochem. J. 54; 467.

Lewin, J.C. (1953). J. Gen. Microbiol. 9; 305.

Lewin, R.A. (1954). J. Gen. Microbiol. 11; 459.

Lichtenthaler, H.K. and R.B. Park (1963). Nature 198; 1070.

McArthur, J.A., T.V. Marsho and D.W. Newmann (1964). Plant Physiol. 18; 229.

Marinetti, G.V. (1962). J. Lipid Res. 3; 1.

Marinetti, G.V. and E. Stotz (1956). Biochim. Biophys.

Acta 21; 168.

Marsh, J.B. and D.B. Weinstein (1966). J. Lipid Res. 7; 574.

Miyano, M. and A.A. Benson (1962). J. Amer. Chem. Soc. 84; 59.

Morris, L.J. (1964). in "New Biochemical Separations" (Eds. A.T. James and L.J. Morris), Van Nostrand, London, p.295.

Mudd, J.B. (1967). Ann. Rev. Plant Physiol. 18; 229.

Mudd, J.B. and T.T. McManus (1965). Plant Physiol. 40; 340.

Nazir, D. and G. Rouser (1966). Lipids 1; 159.

Nelson, N. (1944). J. Biol. Chem. 153; 375.

Nichols, B.W. (1963). Biochim. Biophys. Acta 70; 417.

Nichols, B.W. (1965). Biochim. Biophys. Acta 106; 274.

Nichols, B.W. (1965a). Phytochem. 4; 769.

Nichols, B.W. (1964). in "New Biochemical Separations" (Eds. A.T. James and L.J. Morris) Van Nostrand, London. p.321.

Nichols, B.W. and A.T. James (1964). Fette Seifen Anstrichmittel 66; 1003.

Nichols, B.W., A.T. James and J. Breuer (1967). Biochem. J. 104; 486.

Noll, H. and K. Bloch (1955). J. Biol. Chem. 214; 251.

O'Brien, J. and A.A. Benson (1964). J. Lipid Res. 5; 432.

Pratt, R. (1943). Amer. J. Botany 30; 626.

Privett, O.S., M.L. Blank, D.W. Codding and E.C. Nickell (1965). J. Amer. Oil Chems' Soc. 42; 1.

Renkonen, O. (1962). Biochim. Biophys. Acta 50; 367.

Rosenberg, A. and M. Pecker (1964). Biochemistry 3; 254.

Rosenberg, A., J. Gouaux and P. Milch (1966). J. Lipid Res. 7; 733.

Roughan, P.G. and C.G. Tunnicliffe (1967). J. Lipid Res. 8; 581.

Roughan, P.G. and R.D. Batt (1968). Anal. Biochem. 22; 74.

Rouser, G., A.J. Bauman, G. Kritchevsky, D. Heller and J.S. O'Brien (1961). J. Amer. Oil Chems' Soc. 38; 544.

Rouser, G., G. Kritchevsky, D. Heller and E. Leiver (1963). J. Amer. Oil Chems' Soc. 40; 425.

Rouser, G., C. Galli, E. Leiver, M.L. Blank and O.S. Privett (1964). J. Amer. Oil Chems' Soc. 41; 836.

Rouser, G., G. Kritchevsky, G. Simon and G. Nelson (1967). Lipids 2; 37.

Russell, G.B. (1966). Anal. Biochem. 14; 205.

Sager, R. and S. Granick (1953). Ann. N.Y. Acad. Sci. 56; 831.

Saikatos, A.N. and G. Rouser (1965). J. Amer. Oil Chems' Soc. 42; 913.

Sastry, P.S. and M. Kates (1964). Biochemistry 3; 1271.

Sloane-Stanley, G.H. (1968). Biochem. J. 104; 293.

Smith, J.A.B. and A.C. Chibnall (1932). Biochem. J. 26; 1345.

Snyder, F. (1964). Anal. Biochem. 9; 183.

Toyama, S. (1967). Sci. Rep. Tokyo Kyoiku daigaku 12; 245.

Vorbeck, M.L. and G.V. Marinetti (1965). J. Lipid Res. 6; 3.

Weenink, R.O. (1964). Biochem. J. 93; 606.

Wells, M.A. and J.C. Dittmer (1963). Biochemistry 2; 1259.

Wheeldon, L.W. (1960). J. Lipid Res. 1; 439.

Wintermans, J.F.G.M. (1960). Biochim. Biophys. Acta 44; 49.

Wintermans, J.F.G.M. (1962). Colloq. intern. cent. Nat. Recherche Sci. (Paris) 119; 381.

Wood, B.J.B., B.W. Nichols and A.T. James (1965). Biochim. Biophys. Acta 106; 261.

Wuthier, R.E. (1966). J. Lipid Res. 7; 558.

Zill, L.P. and E.A. Harmon (1962). Biochim. Biophys. Acta 57; 573.

Zimmerer, R.P. and R.H. Hamilton (1965). Plant and Cell Physiol. 6; 681.

APPENDICES

1. AN EXAMINATION OF A RECENTLY PUBLISHED METHOD FOR THE QUANTITATIVE ESTIMATION OF PLANT SULPHO- AND GALACTO- LIPIDS

2. SOME NOTES ON THE LIPID EXTRACTION AND LIPID HYDROLYSIS METHODS USED IN A PUBLISHED METHOD FOR THE ANALYSIS OF LIPID-BOUND GALACTOSE

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AN EXAMINATION OF A RECENTLY PUBLISHED METHOD FOR THE QUANTITATIVE ESTIMATION OF PLANT SULPHO- AND GALACTO- LIPIDS

INTRODUCTION

The major plant glycolipids, monogalactosyl diglyceride (MGD), digalactosyl diglyceride (DGD) and the sulpholipid (sulphoquinovosyl diglyceride, SL) along with phosphatidyl glycerol (PG) are restricted to and are probably the only glycerides occurring in chloroplasts (Nichols, 1963; James and Nichols, 1966). These lipids and chlorophyll constitute the bulk of the lipid phase of chloroplast membranes (Benson, 1964; Benson, 1966). Until recently analyses of these compounds in various leaves and chloroplast preparations reported in the literature always show the sulpholipid to be the minor component of the group (Table I).

The ratio MGD to DGD is of the order of 2 to 1 (see also Weenink, 1961 for red clover) and the ratio galactolipids to sulpholipid is almost 17 to 1 for elder leaves, beet chloroplasts and chlorella and 27 to 1 in bean leaves.

TABLE I

MOLAR RATIOS OF POLAR LIPIDS IN LEAVES, CHLOROPLASTS AND CHLOROPLAST LAMELLA

Starting Material	MGD	DGD	PG	SL
Beet Leaves (1)	12	7	5	5
Beet Chloroplasts (1)	12	7	2.3	1.5
Elder Leaves (1)	11	5	3	1
Bean Leaves (1)	20	7	5	1
Alfalfa Leaves (2)	4.7*	9.3	-	2.3
Chlorella (2)	2.3*	4	-	1
Chlorella (3)	12.5	5	4.3	1.3
Spinach Lamellae (4)	7	2	2.7	2
Spinach Lamellae (5)	17.3	7.2	2.6	2.4

*These results almost certainly are too low (see below).
(1) Wintermans, 1960; (2) O'Brien and Benson, 1964;
(3) Ferrari and Benson, 1961; (4) Allen et al., 1966;
(5) Lichtenthaler and Park, 1963.

In a recently published method for the quantitative analyses of plant sulpho- and galacto- lipids (Russell, 1966) a much higher ratio of sulpho- to galacto-lipid than might be expected from the foregoing evidence is reported. Data in this paper have been

recalculated in molar concentrations (Table II) by assuming a MGD to DGD ratio of 2 to 1 in the species analysed so that both compounds contributed equally to the lipid bound galactose.

TABLE II

RECALCULATION OF RUSSELL'S RESULTS TO GIVE MOLAR CONCENTRATIONS OF GALACTOLIPIDS AND SULPHOLIPIDS

		µM gal-lipid/10g dry wgt	µM SL/10g dry wgt	gal-lipid / SL
Lolium perenne	2.10.64	33	25	1.32
	10.12.64	60	16.4	3.9
L.perenne x multiflorum	2.10.64	94.5	53	1.77
	10.12.64	61.0	18.8	3.25
Trifolium repens	2.10.64	186	24	7.77
Trifolium pratense	30.9.64	125	33	3.8
	14.10.64	212	36.5	5.8
	24.10.64	158	34	4.64
	19.11.64	230	28	8.2
	2.12.64	128	15.5	8.25
	14.1.65	155	37	4.2
	4.2.65	142	29	4.9

This low ratio of galactolipid to sulpholipid (or high ratio of SL to gal-lipid) reported (Russell, 1966) is surprising, and the variation of concentration encountered is also unexpected. Separations performed in this laboratory using the same species and established techniques (Nichols, 1964; Nichols and James, 1964) gave results in accordance with the general body of evidence in the literature, indicating that SL concentration was but a small proportion of the combined galactolipid concentrations (e.g. Fig. 1.2). It has been possible to perform quantitative analysis on galactolipids separated by 2-dimensional thin-layer chromatography but we have not been able to apply sufficient lipid at the origin to get reliable analyses of sulpholipid without overloading the chromatogram. This also points to a high galactolipid to sulpholipid ratio. Since large variations (2- or 3- fold) within a species have not been observed in this laboratory, a close evaluation of the experimental technique was made.

The method involved extracting the leaf lipid by the procedure of Bailey (1962), dissolving the water-washed lipid in chloroform and fractionating the glyco-lipids with Florisil using the batchwise technique of stirring the adsorbent into the $CHCl_3$ solution. In the published flow diagram, part of which is reproduced

below, it is assumed that the separation of glycolipids is the same as that achieved by O'Brien and Benson (1964) using Florisil column chromatography. In achieving this separation, however, O'Brien and Benson used

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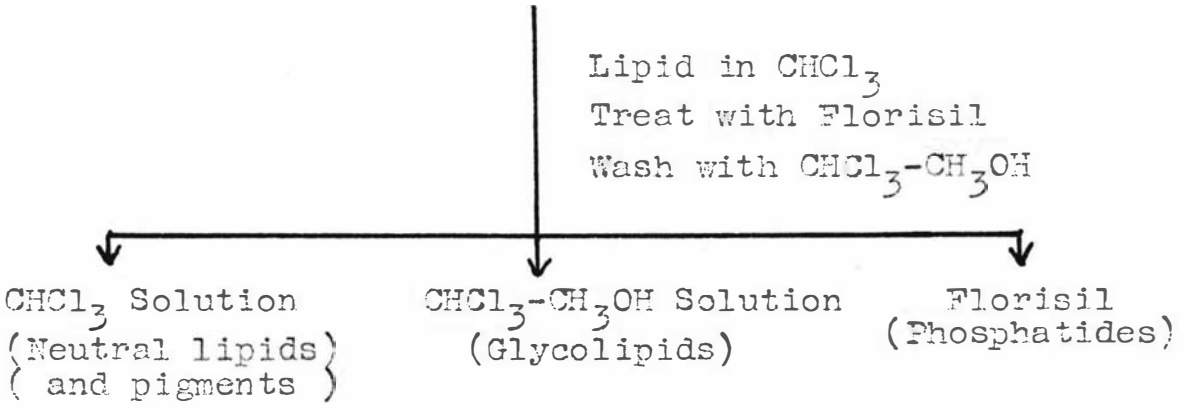
Reproduction of part of Russell's flow diagram



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highly activated Florisil and added dimethoxypropane (DMP) to their solvents as a water scavenger, since Rouser et al. (1961) had reported optimum separation of polar lipids on Florisil only when this adsorbent was highly activated and water excluded from the solvents. In this laboratory it has been found that quantitative recovery of the glycolipids in the $CHCl_3$-CH_3OH (2:1 + 5% DMP) eluate from a Florisil column prepared according to O'Brien and Benson is unsatisfactory even when these precautions are taken as some MGD is lost in the $CHCl_3$-CH_3OH (9:1 + 5% DMP) fraction and the DGD tails so badly

that significant quantities remain on the adsorbent. This loss of MGD in the first fraction probably explains the conflicting results from Benson's laboratory concerning the ratio of MGD to DGD in chlorella. Ferrari and Benson (1961) found a ratio of 2 to 1 using silicic acid impregnated paper chromatography, but in 1964 O'Brien and Benson using Florisil column chromatography reported the ratio to be 1 to 2.

Since no information was provided by Russell (1966) as to the activity of the Florisil nor of the quality of the $CHCl_3$ used in the batchwise scheme it seemed likely that MGD could have been lost in the $CHCl_3$ solution. Also, DGD could have been lost by retention on the adsorbent. Assuming quantitative recovery of the sulpholipid in the $CHCl_3$-CH_3OH eluate, losses of this nature could account for the low galactolipid to sulpholipid ratios. The batchwise and column Florisil methods have been repeated in this laboratory and the results evaluated by thin-layer chromatography.

MATERIALS AND METHODS

Florisil (Floridin Co., Tallahassee, U.S.A.) was washed with water according to O'Brien and Benson (1964), dried at 120°C for 16 hours and had been stored in a glass-stoppered bottle for 5 months. Chloroform was

'ANALAR' reagent grade containing 2% ethanol or this reagent washed with water, dried over anhydrous Na_2SO_4 and redistilled. Methanol was 'ANALAR' reagent grade. Dimethoxypropane (DMP, added to some solvents as a water scavenger) was supplied by Distillation Products. All solvent mixtures given are volume/volume.

Lipids were extracted from 10 g (fresh weight) of leaves and washed free of non-lipid material by the method of Bailey (1962) or that of Folch et al. (1957). The latter method was slightly modified as follows: Fresh leaves were sliced into hot (50°C) methanol (70 ml) and after 2-3 minutes, homogenized in a Waring blender. This material was filtered on a Buchner funnel. The blender was washed out with 70 ml of chloroform which was also passed through the residue on the filter. Finally a further 70 ml of $CHCl_3$ was used to wash the residue giving a $CHCl_3$-CH_3OH solution of lipids according to the recommendations of Folch et al. This extract was shaken against 0.2 vol. of 0.73% NaCl and either allowed to separate overnight in the refrigerator, or, if the extract was required immediately, centrifuged. The lower $CHCl_3$ layer was collected and dried on a rotary evaporator using benzene-absolute ethanol (9:1) to remove traces of water. Lipids prepared by the method of Bailey (1962) were dried in the same way. The dry lipid residues were

taken up in 25 ml $CHCl_3$, 5 g Florisil added and the separation of the glycolipids performed according to Russell (1966). Filtration was carried out over a solvent-washed, glass wool plug in a glass filter-funnel. Since we have found $CHCl_3$-CH_3OH-H_2O (1:1:0.2) to strip the phospholipids from a Florisil column, 75 ml of this mixture was used to wash the adsorbent following the $CHCl_3$-CH_3OH (2:1) treatment. Three fractions, 1, 2 and 3 were thus obtained and these were equivalent to 1. $CHCl_3$ solution (neutral lipids and pigments), 2. $CHCl_3$-CH_3OH solution (glycolipids) and 3. Florisil (phosphatides) in Russell's (1966) flow diagram. Each of the fractions was dried in a rotary evaporator using benzene-ethanol to remove the water from fraction 3. Residues 1 and 2 were redissolved in 5 ml $CHCl_3$ and residue 3 redissolved in 5 ml of $CHCl_3$-CH_3OH (2:1). These solutions were stored at -15°C.

Separation of the components of each fraction was achieved on thin layers (250 μ) of silica gel G or H (Merck) in tanks lined with filter paper using the solvent $CHCl_3$-CH_3OH-CH_3COOH-H_2O (85:15:10:2) for development. Quantitative analysis of the MGD and DGD separated in this manner from 50 μl of each fraction (applied as a thin streak along 5 cm) was performed after detecting the lipids with iodine vapour.

Appropriate zones were scraped into centrifuge tubes, then 1 ml water, 0.2 ml of 30% phenol in ethanol and 3 ml conc H_2SO_4 added. The tubes were mixed after each addition, given a thorough mix on a Vortex mixer, then allowed to stand at room temperature for 15 mins before centrifugation. The absorbence of the resulting solution was measured at 490 mμ. Blanks and galactose standards were carried through the same modified Dubois et al. (1956) procedure (see also Galanos and Kapoulas, 1965), which was chosen for its simplicity and speed. Silica gel H gave somewhat higher blanks than did silica gel G, but the separation achieved on the former was more satisfactory. Both silica gel G and H gave somewhat variable blanks so it was found necessary to take at least three blank zones and average the resulting absorbences. However, in the present work the absorbences produced by the galactolipids were sufficiently high that the blank variation could be ignored.

Phosphorus was determined on 50 μl aliquots of each fraction by the method of Marinetti (1962).

Florisil column chromatography was performed as described by O'Brien and Benson (1964), the absorbent being stored dry after washing and reactivated at 120°C for 18 hours immediately before use.

RESULTS AND DISCUSSION

In one-tenth scale repetitions of the column procedure (O'Brien, 1964) it has been found; 1) the presence of even a small proportion of CH_3OH in the first eluting solvent (e.g. $CHCl_3$-CH_3OH 19:1 v/v + 5% DMP) will cause some MGD to be eluted with the pigments. 2) DGD tails to such an extent that passing a four-fold excess of $CHCl_3$-CH_3OH (2:1 + 5% DMP) through the column will not elute the compound quantitatively, and 3) phosphatides can be stripped from the column using a mixture of $CHCl_3$-CH_3OH-H_2O (1:1:0.2).

In Fig. 1.7 the tailing of DGD and SL through a three-fold excess (compared with O'Brien and Benson 1964) of the $CHCl_3$-CH_3OH (2:1 + 5% DMP) eluate is demonstrated. The bulk of the pigments had first been eluted in $CHCl_3$ (+ 5% DMP).

The distribution of glycolipids among the three fractions recovered from the "Batchwise" Florisil treatment (Russell, 1966) of eight separate leaf lipid preparations is shown in Fig. I.

Details of extractions and fractionations are as follows:-

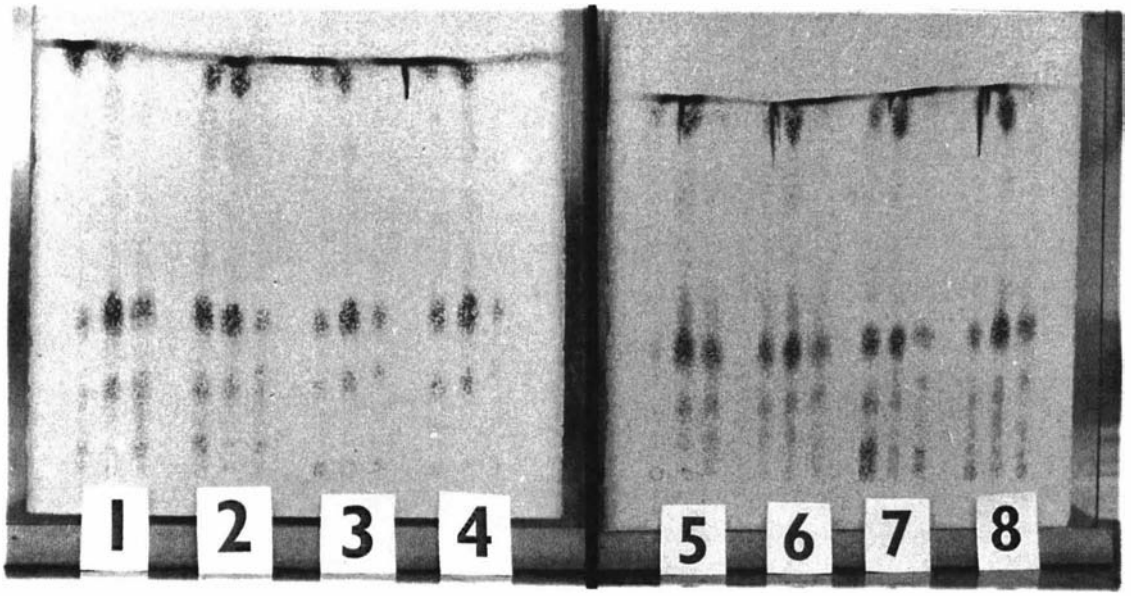

Fig. I. Thin layer chromatographic monitoring of the three fractions from the Florisil treatment of eight separate lipid extracts. 10 µl of fractions, 1, 2, and 3 of each of 8 treatments were spotted in groups of 3, numbering from left to right. Glycolipids were detected with the α-naphthol-H_2SO_4 reagent of Saikatos and Rouser (1965).

Experiment 1.

Tissue: white clover leaflets.

Extraction: Folch et al.

Florisil: no further activation.

Fractions: 1. 25 ml $CHCl_3$ stirred with Florisil for 5 min prior to filtration. One hundred mls $CHCl_3$ collected; washings still slightly green. Chloroform was analar reagent (see Materials and Methods).
2. 75 ml $CHCl_3$-CH_3OH (2:1).
3. 75 ml $CHCl_3$-CH_3OH-H_2O (1:1:0.2).

Experiment 2.

Tissue: white clover leaflets.

Extraction: Folch et al.

Florisil: activated 16 hrs at 130°C before use.

Fractions: 1. as for expt. 1.
2. 150 ml $CHCl_3$-CH_3OH.
3. 75 ml $CHCl_3$-CH_3OH-H_2O.

Experiment 3.

Tissue: perennial ryegrass.

Extraction: Bailey.

Florisil: activated 4 hrs at 170°C.

Fractions: 1. as for expt. 1. except that $CHCl_3$ was redistilled and contained 5% (v/v) DMP.
2. 75 ml $CHCl_3$-CH_3OH.
3. 75 ml $CHCl_3$-CH_3OH-H_2O.

Experiment 4.

Tissue: perennial ryegrass.

Extraction: Folch et al.

Florisil: activated 14 hrs at 130°C.

Fractions: 1. as for expt. 3, except that A) no DMP in $CHCl_3$ and B) absorbent stirred into $CHCl_3$ solution for 30 min prior to filtering.
2. 75 ml $CHCl_3$-CH_3OH.
3. 75 ml $CHCl_3$-CH_3OH-H_2O.

Experiment 5.

Tissue: red clover leaflets.

Extraction: Folch et al.

Florisil: no further activation.

Fractions: 1. Florisil stirred into $CHCl_3$ solution (as in expt. 4) for 15 min. 75 ml collected.
2. 75 ml $CHCl_3$-CH_3OH.
3. 75 ml $CHCl_3$-CH_3OH-H_2O.

Experiment 6.

As for expt. 5 except that Florisil activation 24 hrs at 120°C.

Experiment 7.

Tissue: white clover leaflets.

Extraction: Bailey.

Florisil: 16 hrs at 120°C.

Fractions: 1. as for expt. 5 except that 40 ml only collected.
2. 75 ml $CHCl_3$-CH_3OH.
3. 75 ml $CHCl_3$-CH_3OH-H_2O.

Experiment 8.

As for expt. 7 except that red clover leaflets were used.

Significant amounts of galactolipids were revealed in all three fractions in most cases. In every case had the "glycolipid" fraction alone been retained for analysis serious error would clearly have resulted. The sulpholipid spot also shows a variable distribution although this is more difficult to assess as it is present in relatively low concentrations and co-chromatographs with phosphatidyl choline (PC).

Analysis of each fraction for lipid phosphorus (Table III) confirmed the inefficiency of the batchwise adsorption process. The phosphatides were more or less randomly distributed among the three fractions, the highest recovery in fraction 3 ("phosphatides") being 43% (expt. 8). Mono- and di- galactosyl diglyceride were analysed in the 3 fractions in every case (Fig. II), and the results shown in Table III compare favourably with a visual assessment of Fig. I. The sulphclipid co-chromato-

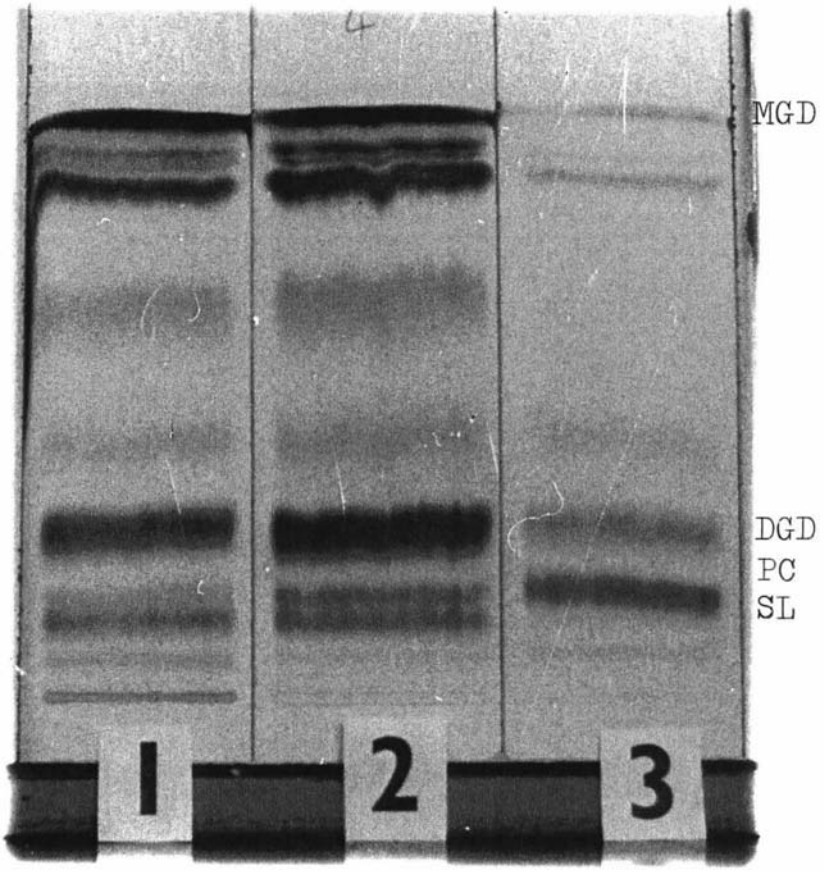

Fig. II. Thin-layer separation of the type from which analyses of MGD and DGD performed. Zones detected with iodine vapour and then photographed through a blue filter. Expt. 4.

TABLE III

DISTRIBUTION OF LIPID COMPONENTS BETWEEN THE THREE FRACTIONS FROM THE FLORISIL TREATMENT

	Lipid Phosphorus			MGD			DGD		
Fraction	1	2	3	1	2	3	1	2	3
Expt. 1.	9.4	28.7	22.6	35.2	29.2	4.6	4.8	38.8	15.4
2.	32.6	18.0	17.1	28.5	29.3	4.9	20.3	23.4	6.7
3.	6.8	10.0	13.5	9.3	32.9	2.8	4.3	15.0	3.2
4.	14.8	22.6	13.5	8.8	49.6	3.9	11.0	27.8	3.5
5.	4.5	33.9	27.1	7.4	48.7	6.7	4.8	37.8	21.5
6.	19.0	19.7	17.1	12.3	41.7	1.9	19.8	32.4	14.4
7.	21.9	16.8	14.2	6.7	69.0	0	19.0	29.7	8.0
8.	14.8	16.8	23.9	11.6	73.0	5.3	11.2	35.2	20.2

All results in μMoles/fraction

graphed with phosphatidyl choline in most cases (but see Fig. II) and analytical results using the glycolipid assay cannot be given with any confidence.

The presence of sulpholipid in all three Florisil fractions of red and white clover lipids was demonstrated as follows. Lipids (= 10 g fresh weight leaves) were fractionated by the Florisil method (Russell, 1966) using activated Florisil and adding DMP to the appropriate solvents. A total of 100 ml of fraction 1, 75 ml of 2, and 75 ml of 3 were collected, evaporated to dryness and finally made up to 5 ml with $CHCl_3$. A 2 ml aliquot of each fraction was then separated into neutral plus zwitterionic and acidic lipids on a column of DEAE cellulose (see O'Brien and Benson, 1964; Nichols and James, 1964). Thus each Florisil fraction was separated into two further fractions, one of which contained MGD, DGD, PC and phosphatidyl ethanolamine as major components while the other contained predominantly PG, SL, phosphatidyl inositol and an unidentified lipid. These two groups of compounds were easily resolved by a single thin-layer run (Fig. III). The sulpholipid was thus found in significant amounts in all three Florisil fractions. Analyses of the major components were performed after TLC and the results are shown in Table IV.

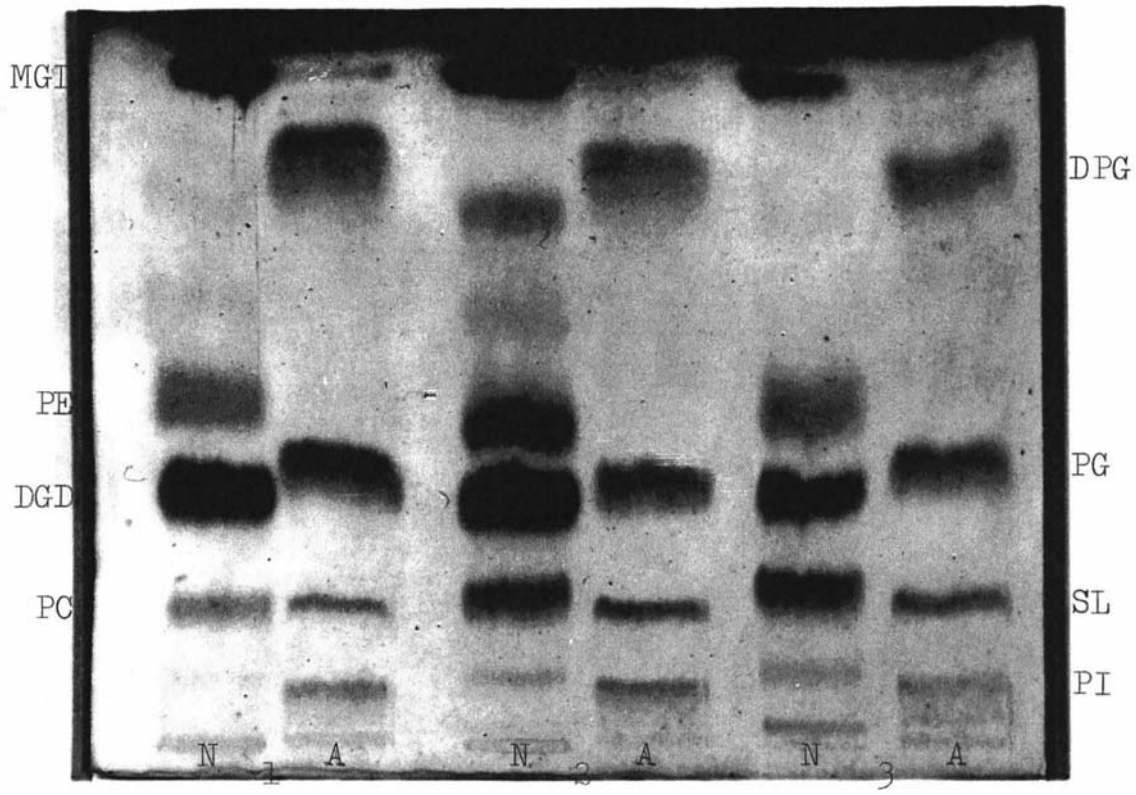

Fig. III. Thin-layer chromatography of Florisil fractions 1, 2 and 3 separated by DEAE cellulose column chromatography into N, neutral (plus zwitterionic) and A, acidic lipids showing the presence of sulpholipid in all three Florisil fractions. Spots in the neutral lipid fraction are identified on the left hand side of the photograph and those in the acidic lipid fraction on the right hand side. Red clover lipid. Detection of bands by acid dichromate spray and charring at 180^{o}C for 30 min.

TABLE IV

PERCENTAGES OF THE MAJOR COMPONENTS IN THE THREE FLORISIL FRACTIONS OF RED AND WHITE CLOVER LEAF LIPIDS

	Component	Florisil fraction 1	2	3
White Clover	MGD	8	61	31
	DGD	14	54	33
	SL	44	40	16
	PC	48	30	22
	PG	50	44	6
Red Clover	MGD	22	73	5
	DGD	27	59	14
	SL	25	42	33
	PC	9	38	53
	PG	39	35	26

CONCLUSIONS

On the basis of this evidence it is concluded that serious error must arise from the use of the batchwise Florisil technique. Even when the adsorbent is highly activated and good quality solvents, containing dimethoxypropane to ensure anhydrous conditions (Rouser et al., 1961), are used the adsorption of polar lipids from chloroform is extremely inefficient. This conclusion is supported by a comparison (Table V) of lipid-bound galactose levels reported by Bailey in 1962 and by Russell in 1966. In every case the values reported by Russell are considerably lower than those

of Bailey whose method did not include any preliminary purification of the washed lipid extract.

TABLE V

COMPARISON OF LIPID-BOUND GALACTOSE IN RYEGRASS AND CLOVER AS MEASURED BY BAILEY AND RUSSELL

	Lipid-Galactose (mg/g dry wt.)	
	Bailey	Russell
Lolium perenne	25 - 103 (66*)	12.0, 21.6
L. perenne x multiflorum		21.9, 34.0
Trifolium repens	95	67
T. pratense	91	45 - 83 (56+)

* Mean of 6 determinations

+ Mean of 7 determinations.

SOME NOTES ON THE LIPID EXTRACTION AND LIPID HYDROLYSIS METHODS USED IN A PUBLISHED METHOD FOR THE ANALYSIS OF LIPID-BOUND GALACTOSE.

In Appendix 1 experiments are described which show that the Florisil chromatographic procedure, used in a recently published method for the quantitative analyses of plant sulpho- and galacto- lipids, is unsatisfactory. It was found that up to 60% of the sulpholipid and 25-50% of the galactolipid were lost in this step (cf. Tables III and IV of Appendix 1). When examining this chromatographic step, lipid extraction and purification was based on the procedure of Folch et al. However, when it was found that the Florisil separation was giving unsatisfactory results, it became necessary to use the extraction and purification method used by Russell and his analytical procedure to determine whether this influenced the result. White clover, red clover and perennial ryegrass were extracted by this method which proved to be less convenient than older, more sophisticated techniques.

Among the properties that characterize the group of biological molecules commonly known as lipids is that of a specific gravity of less than one. It is surprising therefore to find a method for the preparation and purification of lipids from aqueous suspension by high speed centrifugation. Since aqueous solutions have a specific gravity of one or more (except in the case of

aqueous solution of some organic solvents) it would be expected that lipid material separated by centrifugation would float on the surface of the aqueous phase. That the "lipid" extracted from leaves by the method of Bailey does in fact sediment from aqueous suspension at 25,000 x g has been confirmed in this laboratory. It is concluded therefore, that the material separated by this procedure is not purified lipid but is an association of lipid with some other material, probably protein, with a specific gravity greater than one. It was noted that in some cases, the supernatant solution remaining after the sedimentation of the "lipid" was clear but deep green in colour. It was also found that significant quantities of lipid could be extracted from this solution.

The following describes a typical extraction in which this occurred. Five gm of white clover leaves were homogenized in 100 ml of 80% ethanol, the slurry boiled for 5 min and then filtered on a Buchner funnel. The leaf residue was re-extracted into 60 ml of boiling 80% ethanol for 5 min and the slurry was again filtered. Finally 40 ml of boiling 80% ethanol was poured over the residue on the filter. The combined ethanol extracts were concentrated on a rotary evaporator until only H_2O was condensing on the cooling coil. Lipids were suspended in the water with the aid of glass beads and the mixture poured into a centrifuge tube. Ten ml of H_2O

were used to rinse out the flask and this was also transferred to the centrifuge tube. This was then centrifuged at 20°C for 30 min at 25,000 x g. The clear, deep green supernatant was carefully aspirated, the "lipid" resuspended in 10 ml H_2O with the aid of glass beads and the suspension re-centrifuged.

To the supernatant from the first centrifugation (25 ml) was added 28 ml CH_3OH and then 28 ml $CHCl_3$. The mixture was vigorously shaken in a separatory funnel and the lower $CHCl_3$ phase collected after separation of the layers. The $CHCl_3$ was removed by rotary evaporation and the lipid residue made up to 1 ml in $CHCl_3$. This solution is referred to as "washings" in Table VI of this addendum. The sedimented "lipid" was transferred to an evaporating flask in diethyl ether-methanol, the solvent removed under reduced pressure and the dry residue extracted with $CHCl_3$ and finally made up to 5 ml in $CHCl_3$.

A $CHCl_3$-insoluble residue in the flask which was also largely insoluble in water was heated at 100°C for 60 min in 5 ml of N HCl, diluted with 5 ml H_2O and the soluble fraction tested for carbohydrate by a reducing sugar method and by the method of Bath. The insoluble residue was digested in 6N HCl for 6 hours and the soluble portion of this treatment gave a strong positive reaction for amino acids with ninhydrin.

Aliquots (50 ul) of the $CHCl_3$ solutions were streaked across 2 cm in 4 cm lanes on layers of silica

gel G (Fig. IV) and the lipid components resolved with the solvent system $CHCl_3$-CH_3OH-CH_3COOH-H_2O (85:15:10:3). One plate was sprayed with an acid dichromate solution and charred at 180°C for 30 min to show the presence of galactolipids (Fig. IV). Another plate was stained with iodine vapour, the galactolipid zones outlined with a needle and, after evaporation of the iodine, galactolipids were estimated by a direct reaction with phenol and sulphuric acid.

TABLE VI

RECOVERY OF GALACTOLIPIDS FROM THE WASHINGS OF THE SEDIMENTED "LIPID"

	uM Galactolipid found		
	"Lipid"	Washings	% Recovery in Washings
MGD	38.0*	5.3	12.2
DGD	31.0	3.3	9.6

Results are means of analyses performed on separate white clover extracts prepared on successive days. The results agreed to within 10% in the case of "lipid" MGD and to within 5% in the remaining measurements.

The losses reported here (11%) are five times more than those claimed for the method. Figure IV shows a thin-layer chromatogram similar to the type from which the results shown in Table VI were obtained. It can be seen from this figure that the extraction procedure was

essentially quantitative in that insignificant lipid was recovered from the 80% ethanol-extracted leaf residue by a re-extraction into 100 ml of $CHCl_3$-CH_3OH (2:1). The chromatogram also demonstrates the reproducibility of the loss of lipid in the aqueous washings.

After hydrolysis in N HCl for 30 min at 100°C the $CHCl_3$-insoluble residue of the sedimented "lipid" gave high sugar values by both of the analytical methods used. With galactose as a standard in both cases, the Bath procedure gave a value of 966 ug galactose in this fraction and the reducing sugar method gave a value of 1420 ug. From Table VI it can be calculated that complete acid hydrolysis of the galactolipids present in 5 g fresh weight of white clover leaves would yield 20.0 mg of galactose. Hence the sugar liberated by the hydrolysis of the $CHCl_3$-insoluble residue represents a 5 to 8% contribution to the "lipid"-bound galactose value.

Acid hydrolysis of the lipid gave low and variable results for lipid-bound galactose. Chloroform aliquots of leaf lipids were placed in test tubes and the solvent removed under nitrogen. Three to four ml of N H_2SO_4 were added and the tubes heated for 60 min with occasional shaking in a boiling water bath. Lipids have a lower specific gravity than, and hence float on the surface of, water. It was not surprising therefore, that the lipid tended to rise to the surface of the acid and to accumulate in a ring adhering to the wall of the test tube

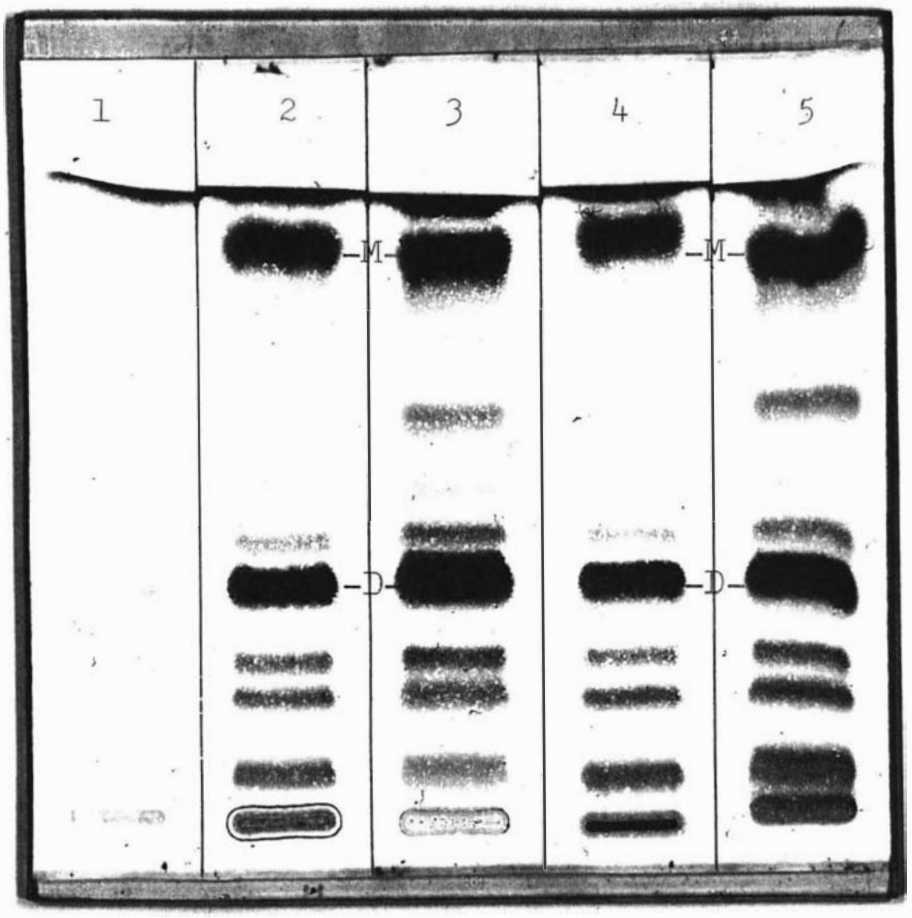

Fig. IV. Thin-layer chromatogram showing the recovery of galactolipids from the water washings of the sedimented "lipid". Lane 1 is the lipid recovered from the 80% ethanol-extracted-leaf residue by re-extraction into $CHCl_3$-CH_3OH (2:1). Lanes 2 and 3 and lanes 4 and 5 are the lipids from the water washings and sedimented lipids respectively of separate white clover leaf extracts. Silica gel G was the adsorbent and $CHCl_3$-CH_3OH-CH_3COOH-H_2O (85:15:10:3) the developing solvent. 50 µl of each fraction was streaked across 2 cm.

M = monogalactosyl diglyceride;

D = digalactosyl diglyceride.

just above the surface of the acid, which was thus in contact with this lipid only when the tubes were shaken. In a few cases a globule of lipid would adhere to the wall of the test tube below the surface of the acid and would resist attempts to dislodge it. It is difficult to see how the acid could penetrate this droplet and hydrolyze the sugar residues contained in it. It is worth mentioning at this point that Russell found that a sulpholipid preparation was undergoing only 70% hydrolysis in N HCl when heated at 100°C (for 60 min). It is our experience that the sulpholipid is readily deacylated in N H_2SO_4 when it is adsorbed to silica gel but that the glycerol-sulphoquinovose bond is extremely acid stable. Since the anthrone procedure used by Russell would not distinguish between sulphoquinovose and sulphoquinovosyl glycerol we interpret his results as implying an incomplete deacylation of the sulpholipid rather than the incomplete degradation to fatty acids, glycerol and sulphoquinovose as was suggested. The variable results we had obtained were most likely caused by incomplete liberation of the water soluble moieties of the lipids into the aqueous phase. This was tested as follows.

Chloroform solutions of leaf lipids (0.2 ml) were dried in test tubes, taken up in 1 ml of methanolic KOH

(0.2N KOH in methanol) and incubated at 37°C for 15 min. This procedure deacylated the lipids and at the end of the incubation the methanol was removed under reduced pressure. Normal H_2SO_4 (4 ml) was added to the tubes and hydrolyses performed as usual. A control set of tubes in which the lipid had not been subjected to this initial mild-alkaline hydrolysis were run concurrently. After the acid hydrolysis 3 ml of petroleum ether was added to each tube and the fatty acids, non-saponifiable lipid and unhydrolysed lipid extracted into the organic phase. Analyses of the reducing sugar present in the aqueous phase showed that prior deacylation of the lipid resulted in a 25-50% increase in the recovery of reducing sugar and that only in the cases where deacylation was performed was satisfactory duplication achieved.

In a more specific experiment, 0.2 ml of a lipid solution was deacylated in 0.8 ml of $CHCl_3$ plus 0.2 ml of 0.5N $NaOCH_3$ in CH_3OH for 20 min at room temperature. The solvent was removed on a rotary evaporator, 4 ml of N H_2SO_4 added and hydrolysis performed at 100°C for 60 min. A control group was set up in which no sodium methoxide hydrolysis was performed initially. Tubes were shaken at 10 min intervals during the hydrolysis. Unhydrolysed lipid and fatty acids were extracted into petroleum ether as before and 1 ml aliquots of the aqueous phase were analysed for reducing sugar. Blanks and standards were

taken through the same procedure. In these cases where acid hydrolysis was not preceded by the sodium methoxide treatment the values obtained were 103, 165, 162, 170 and 157 µg of galactose in 4 ml of acid while the values obtained when deacylation was performed were 278, 281, 286, 278 and 284 µg of galactose in 4 ml of acid. The same type of experiment was performed by an independent operator using the phenol/H_2SO_4 sugar estimation as described in the main report. The values obtained in this case were 226, 192, 214, 94 and 233 µg galactose in 4 ml of acid solution when deacylation was not first performed and 290, 295, 309, 280 and 310 when deacylation was performed. These results suggest that the lipid is only slowly deacylated under the conditions described by Bailey and hence the water soluble galactosyl glycerol is slowly liberated into aqueous solution where the acid can split the glycosidic linkage. The relatively low values obtained with the reducing sugar method compared with those obtained with the phenol/H_2SO_4 method would tend to confirm this belief.

In his paper Bailey claimed that there was a marked seasonal effect on the concentration of lipid bound galactose (galactolipids) in ryegrass, and that the concentration of the sugar could vary between quite wide limits (0.25 - 1.03%). However a glance at the published table reveals that in one month a fluctuation of from

0.39 - 1.03% was encountered. The present evidence indicates that differences shown up by this method are more likely to be a result of analytical error than of real differences in the plant.

ABBREVIATIONS

A_{max}	Absorbance maximum
DEAE-	Diethylaminoethyl-
DGD	Digalactosyl diglyceride
DI	DEAE-cellulose fraction I (non-acidic lipids)
DMP	Dimethoxy propane
DPG	Diphosphatidyl glycerol
EGS	Ethylene glycol succinate
i.d.	Internal diameter
MGD	Monogalactosyl diglyceride
NL	Neutral lipid
PA	Phosphatidic acid
PC	Phosphatidyl choline
PE	Phosphatidyl ethanolamine
PG	Phosphatidyl glycerol
PI	Phosphatidyl inositol
PGTS	Pigments
SG	Steryl glycoside
SL	Sulpholipid
TG	Triglyceride
TLC	Thin-layer chromatography
U1	Unknown neutral lipid from Mesotaenium
U2	Unknown acidic lipid from Mesotaenium
18:3 etc	Shorthand notation for fatty acids. The first figure denotes the number of carbon atoms and the second the number of double bonds in the molecule. In this case the notation stands for linolenic acid.