CHROMATOGRAPHIC CHARACTERIZATION OF POLAR-LIPIDS OF *PISUM SATIVUM L.* VAR. BONNEVILLE

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PREFACE

The research work embodied in this dissertation has been carried out in the School of Life Sciences, Jawaharlal Nehru University, New Delhi. The work is original and has not been submitted so far, in part or full for any other degree or diploma of any university.

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ABBREVIATIONS

DGDG	:	Digalactosyl diglyceride
DPG	:	Diphosphatidylglycerol
LPC	:	Lysophosphatidylcholine
LPE	:	Lysophosphatidylethanolamine
MGDG	:	Monogalactosyldiglyceride
PA	:	Phosphatidic acid
PC	:	Phosphatidylcholine
PE	:	Phosphatidylethanolamine
PG	:	Phosphatidylglycerol
PI	:	Phosphatidylinosital
PS	:	Phosphatidylserine
SL	:	Sulpholipids
TLC	:	Thin layer chromatography

INTRODUCTION

In recent years, the functional role of membrane lipids have received considerable attention. Plant lipids consist of both polar and neutral lipids (Hitchcock and Nichols, 1971). Membrane lipids of plants are predominantly polar lipids such as phospholipids, galactolipids and sulpholipids (Benson, 1965). Sterols occur as a minor constituent and they represent the neutral lipids of the membrane. In addition to the above membrane lipids chlorophylls and carotenoids are also present in chloroplast membrane. Extramembranous lipids in plants mostly constitute neutral lipids and they occur in the form of oil droplets in fat-storing seeds or in the form of wax in the cuticle (Hitchcock and Nichols, 1971). Since polar lipids constitute the majority of membrane lipids, Benson (1965) has introduced the usage of the term 'membrane lipids' as a synonym for polar lipids. In the present study also the term membrane lipids has been used to represent polar lipids.

Membrane lipids have been observed to undergo changes during different stages of growth (Quinn and Williams, 1978) which suggests involvement of lipids in membrane functions. Membrane lipids have also been shown to undergo changes during seed germination (Wilson and Rinne, 1974; Macher et al., 1975), greening (Kates, 1970), senescence of flower buds (Bentelmann

and Kende, 1977) and due to injury caused by freezing and thawing (Yamaki and Uritani, 1973; Yoshida, 1979).

Recently work has been started in various laboratories on the changes in lipid composition in plant tissue cultures (Radwan and Mangold, 1976; Moore, 1977; Yamada, 1979; Phillips and Butcher, 1979) or in isolated protoplasts (Fischer, 1979).

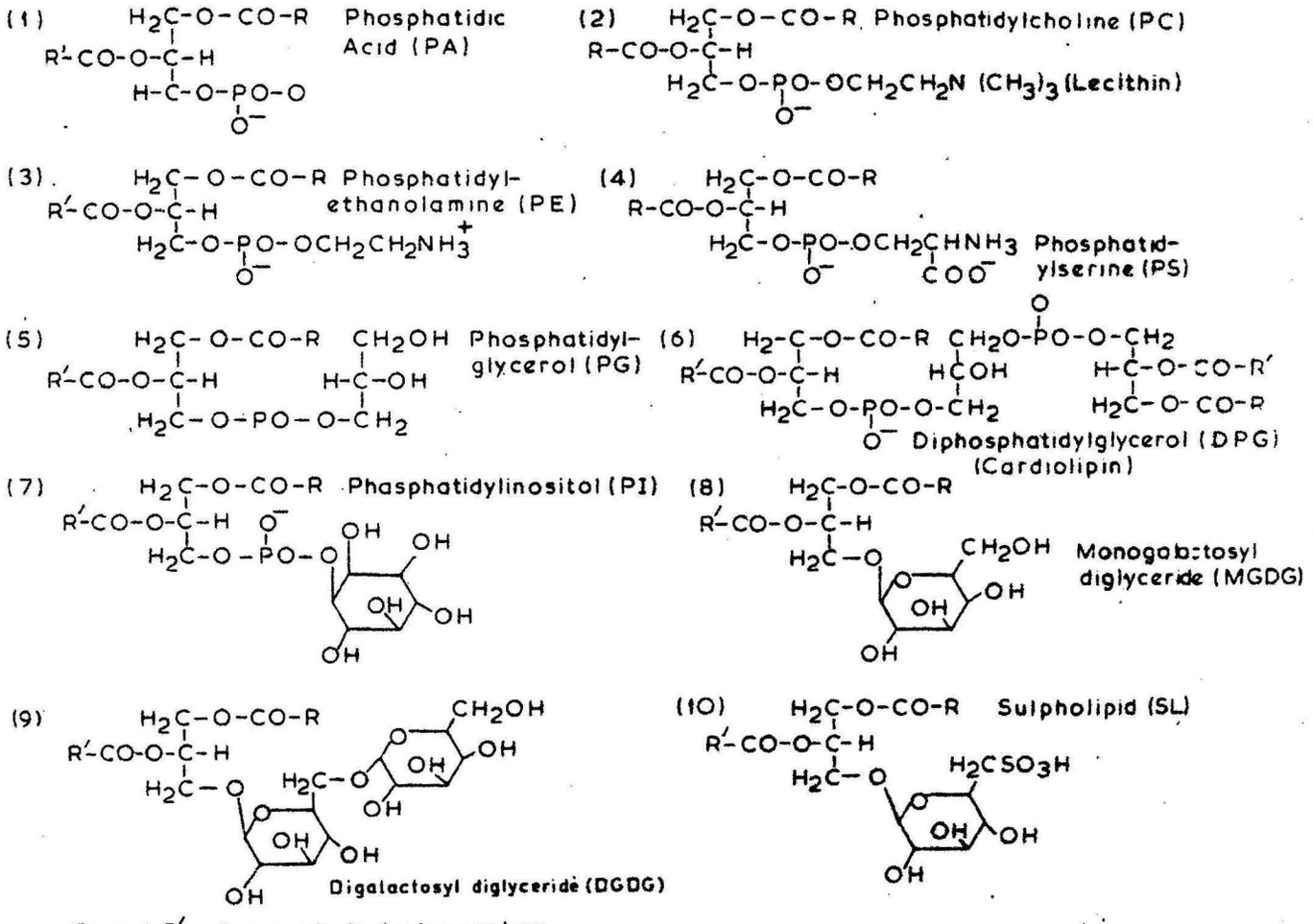
The present work was undertaken to characterize chromatographically the polar lipids of pea, with a view to apply this knowledge in understanding their involvement in morphogenesis. As a system, isolated protoplasts have been employed because of the simplicity and non-interference of the cell wall.

REVIEW OF LITERATURE

Lipid Involvement in Membrane Functions

Polar lipids which are highly concentrated in membranes provide structural matrix to them (Bangham, 1972). Phospholipids of plant-membranes include phosphatidic acid (PA), phosphatidylcholine (PC), phosphatidylethanolamine (PE), diphosphatidylglycerol (DPG) and phosphatidylinositol (PI), Monogalactosyl-diglyceride (MGDG) and digalactosyldiglyceride (DGDG) constitute the galactolipids (Kates, 1970). Sulpholipid (SL) is represented by a single member of the group (Benson, 1965). Chemical structure of different polar lipid molecules found in the plant membrane is given in Fig. 1.

It is known that anyone kind of polar-lipid molecule could serve the purpose of providing a structural matrix for membranes (Lucy, 1974; Kruijff, 1979). Therefore, the presence of various kinds of polar lipids in biomembrane has been considered necessary for providing different forms and functions (Ansell, 1972; Klausner et al., 1980). Fourcans and Jain (1974) observed changes in membrane-lipid composition during different stages of growth which lent support to the view that the lipids have functional significance in the biomembrane. Studies with microorganisms lacking specific step in the biogenesis of membrane lipids provided first positive



R and R′ = Long chain hydrocarbon

1-7: Phospholipids

8&9: Galactolipids

10 · Sulpholipid

–from Kates (1970)

FIG. 1 MEMBRANE LIPIDS OF PLANTS

evidence for the functional role of lipids in biomembrane (Fox, 1969). Later, studies with the reconstituted systems demonstrated the specificity of lipid involvement in various membrane functions (Fourcans and Jain, 1974; Walker and Wheeler, 1975). Based on the observations reported in the above studies, lipid involvement in various membrane functions has been shown to have different levels of specificity: (1) at the fatty acid level (2) at the molecular level irrespective of fatty acid composition and (3) at the head group as well as fatty acid level.

Effect of lipids on membrane bound enzymes - The galactoside-transport system of E. coli is a good example of the specificity at the fatty acid level (Fox, 1969). Galactoside transport has been found to be the function of the y-gene of the inducible lactose operon. This gene for the membrane protein (M-protein or permease) maps between the structural genes for β-galactosidase (Z) and galactoside acetylase (a). In the case of increasing gene dosage, as in the construction of lac/Flac homogenotes, increased activity of only β-galactosidase and β-galactoside acetylase were observed. Though the synthesis of M-protein was comparable to the products of other two structural genes, increase in uptake was not observed. Fox (1969) by employing fatty acid auxotrophs of E. coli

showed that the failure in M-protein function was due to the fatty acid requirement. For the short-term induction of galactoside-uptake, coordinate synthesis of M-protein and fatty acid component of membrane lipids was found to be necessary.

In the reconstituted system of rat-liver ATPase an overall specificity at the molecular level irrespective of fatty acid composition has been reported (Pittotti et al., 1972). Delipidation by nonionic detergent like deoxycholate (DOC) rendered the ATPase inactive. The activity was shown to be restored by providing the crude lipid extract to the delipidated ATPase preparation. In this system no absolute specificity of lipid-protein interaction was observed as the reactivation was more or less equally achieved by the different classes of phospholipids. In the case of cytochrome oxidase, a membrane bound enzyme, diphosphatidylglycerol (DPG) requirement was shown to be specific (Fry et al., 1980).

In another membrane bound enzyme system viz. D-β-butyric dehydrogenase, specificity operating both at the level of head group and fatty acid level has been reported (Nielsen and Fleisher, 1973). The delipidated enzyme showed significant loss of activity and the activity was restored only by

phosphatidylcholine (PC). Among the two different molecular species of PC tested for the reactivation of the enzyme, the one which had unsaturated fatty acid alone was found to be effective. Lipid requirement was thus found to be both specific and absolute for various systems (Gazzotti and Peterson, 1977).

Role of Lipids in Plants

In higher plants, the evidences for the functional role of membrane lipids are mostly indirect. Changes in membrane lipids, during different stages of growth, in different physiological conditions or under nutritional stress have been considered to reflect changes in membrane functions (Kates, 1970; Yamaki and Uritani, 1973; Wilson and Rinne, 1974; Bentelmann and Kende, 1977). Some evidences from reconstitution systems of mitochondria and chloroplasts are available for establishing the functional role of membrane lipids (Livine and Racker, 1969; Yamaki and Uritani, 1973).

Lipid changes during development and germination of seeds - To gain an insight into the role of membrane lipids, several studies have been made to find out the changes in membrane lipids during different stages of plant development. Mazliak (1973) found that changes in lipid composition during various stages of plant development to be only of a quantitative

nature. Wilson and Rinne (1974) analysed lipid composition in developing seeds during the period of 30-60 days after fertilization. They observed a significant decrease in the concentration of PA and increase in PI, PC, PE, PG and DPG. Decrease in the amount of total PA - a precursor for the synthesis of other phospholipids - has been suggested to represent the utilization of this molecule for the synthesis of other phospholipids. Macher et al. (1975) reported an increasing level of PC during the early stage of seed germination in cotyledons of cucumber. They made a comparative study of four enzymes involved in phospholipid synthesis with that of soluble enzymes of glyoxysomes, such as catalase and malate synthetase. Increase in the activity of choline phosphotransferase-the enzyme catalysing the final step in the synthesis of PC-was shown to be parallel with that of the glyoxysomal enzymes. The similar behaviour of one of the membrane- lipid synthesizing enzymes and glyoxysomal enzymes was shown to reflect the synthesis of new glyoxysomes (Wilson and Rinne, 1976). The conversion of fat reserve of the seeds by the gluconeogenic enzymes of the newly synthesized glyoxysomes was shown to mark the onset of seed germination. Thus, this report suggests the possibility of lipid synthesis triggering the events of seed germination.

Lipid changes during greening - Light induced changes in lipid composition was shown to occur during the process of "greening" (Appelqvist, 1968; Kates, 1970; Poincelot, 1973; Hawke et al., 1974; Hendriques and Park, 1974; Kasemir, 1979). These changes were mainly in the levels of galactolipids and sulpholipids. These two lipids have been shown to increase mainly in the chloroplast membrane (Kannangara et al., 1971; Tremolieres, 1971; Roughan and Boardman, 1972; Leech et al., 1973). Such specificity in the distribution of galactolipids and sulpholipids was considered to represent their functional role in the chloroplast membrane. Roughan and Boardman (1972) observed in pea and bean that an increase in the level of chloroplast lipids was associated with the expansion of the grana membrane of the chloroplast. Specially, a steady increase in the proportional distribution of monogalactosyl diglyceride (MGDG) to digalactosyl diglyceride (DGDG) was observed during the process of greening. This was shown to be due to the more specific distribution of MGDG in the grana membrane (Lichtenthaler and Park, 1963; Nielsen et al., 1979). The chloroplast envelope membrane was shown to have higher concentration of DGDG (Mackender and Leech, 1974; Bahl et al., 1976).

Lipid changes during senescence - Senescence induced changes in lipids have been reported in flower buds of Ipomoea tricolor (Bentelmann and Kende, 1977) where a

drop in the level of phospholipids preceeded the appearance of visible symptoms of senescence. Decrease in the level of PC, PE, PI, PA and PG was also noted during senescence. Changes in lipid composition has been suggested to cause changes in membrane integrity and intracellular compartmentation leading to senescence. Wade and Bishop (1978) reported change in lipid composition during the ripening of fruits . In banana overall drop in the level of phospholipids and increase in the level of PA were shown to occur during the ripening process. They related the lipid composition to the increased activity of ATPase, probably due to a change in the cell membrane permeability. Castelfranco et al. (1972) reported an increase in the level of PC during the process of 'aging' in potato tuber slices which was correlated with the increasedrespiratory rate due to a change in the mitochondrial membrane.

Lipid changes under stress - In Robinia sps. the injury due to freezing and thawing has been shown to result in an increased activity of phospholipase D, a membrane bound enzyme catalyzing the hydrolysis of phospholipids (Yoshida and Sakai, 1974; Yoshida, 1979). The activity of phospholipase-D has been found to be induced by suboptimal temperatures which leads to an increased degradation of various phospholipids. In frost-resistant varieties of

wheat, it has been shown that membrane-lipids play a role in frost-hardening process (Willemot, 1975). Increase in the overall level of phospholipids was implicated to reflect an adaptation at the membrane level towards the acquisition of frost hardening. Yamaki and Uritani (1973) reported that ~~due to~~ the injury due to chilling was the cause of disorganization of mitochondrial membrane in sweet potato. The activities of mitochondrial membrane bound enzymes, NADH cytochrome C oxidoreductase, succinate-cytochrome C oxidoreductase and succino-oxidase were found to be reduced due to chilling.

Nutritional stress has also been reported to induce changes in membrane lipids (Muller and Santarius, 1978; Kuiper and Kuiper, 1979). Muller and Santarius (1978) reported that the adaptation of barley to saline-stress alters various chloroplast membrane lipids, specially galactolipids. The activity of UDP galactose-diglyceride transferase-enzyme converting diglycerides to galactolipids-has been shown to be affected by high concentration of intracellular sodium chloride. Reversal of the changes in the enzyme activity and lipid contents has also been shown to occur when the plants were relieved off saline stress.

Lipid changes in isolated tissue and protoplasts - Besides the changes in lipid composition in intact plant tissues recently some studies with plant tissue cultures have also been reported. Age of culture, composition of nutrient medium, aeration, illumination (photoperiod) and various temperatures were shown to induce changes in lipid composition of various plant tissues in culture (Redwan and Mangold, 1976; Yamada et al., 1979; Phillips and Butcher, 1979). Transformation induced changes in membrane lipids have also been reported in cultures of Vinca rosea, transformed by Agrobacterium tumefaciens (Cockerham and Lundeen, 1979). Such transformed cultures were shown to have reduced level of phospholipids. Recently, protoplasts have been employed to understand involvement of lipids in the function of plasma membrane (Fischer, 1979).

Methodology of Characterizing Polar-lipids

Eventhough the lipids have acquired prominence among plant physiologists, the following section would reveal that the separation and identification of plant lipids is very difficult. Chromatographic fractionation of the complex mixtures of lipid-extract are widely in use (Spanner, 1973; Rouser et al., 1976). These procedures are based on either adsorption-partition or on ion exchange - partition. No single step chromatographic fractionation has been found to be ideal for the separation of various plant lipids. The difficulty

has been mainly attributed to the overlapping pairs of components and trailing peaks. To avoid such shortcomings combination of different column chromatographic procedures alongwith thin layer chromatography has been found to be satisfactory. By having thin layer chromatographic step in combination with column chromatography, rapidity in separations were also achieved (Rouser et al., 1976).

In thin layer chromatographic procedures, multi-component solvent systems, consisting of chloroform, methanol and water are used as mobile phase (Renkonen and Luukkonen, 1976). Modification to this commonly used solvent system have been made by the addition of either an acid or alkali. There are several different combinations of multicomponent solvent systems used by various group of workers (Table 1). Different solvent systems vary in the proportion of the individual components of the solvent system. The choice for a particular solvent system has been observed to be due to the complement of polar-lipid components present in a particular biological system (Hitchcock and Nichols, 1971). Besides this, manupulations in an established solvent system was found to be essential due to certain undefined variables (Snyder, 1966). The use ofdifferent solvent systems for the separation of similar lipids may provide evidence for such a situation. Reports are available where ideal conditions

TABLE 1

COMMONLY USED TLC SYSTEMS FOR THE SEPARATION OF MEMBRANE LIPIDS OF PLANTS

Plant material	Mobile phase Composition	Proportion (v/v)	Stationary phase	Separated components of phospholipids
A. Separation of Phospholipids:				
Castor bean seeds (Sparce and Moore, 1979)	Chloroform Methanol Water	65 25 4	Silica gel H*	PC, DPG.
	&			
	Chloroform Methanol 7M ammonium hydroxide	65 30 4	Silica gel H	PA
Soybean tissue culture (Moore, 1977)	Chloroform Methanol 7M ammonium hydroxide	65 25 4	Silica gel G**	PC, PE, PG, PS & PA.
Soybean seeds (Wilson and Rinne, 1974)	Chloroform Methanol 7M ammonium hydroxide	70 20 1.5	AdsorbsoSil-5 (silicic acid adsorbant)	PC, LPC, PE, LPE & PI.
Castor bean endosperm (Mackender, 1979)	Chloroform Methanol Acetic acid Water	65 50 5 3	Silica gel G	PC, PE, PS & PI.
Pea leaves (Tremolieres, 1970)	Chloroform Acetone Methanol Acetic acid Water	5 2 1 1 1	Silica gel G.	PC, PE, DPG, PG, PI & PA.

Contd...../-

TABLE 1(contd.)

Plant material	Mobile phase Composition	Mobile phase Proportion (v/v)	Stationary phase	Separated components of galactolipids
(B) Separation of Galactolipids:				
Chlamydomonas (Nichols, 1964)	Chloroform Methanol Acetic acid Water	170 30 20 7	Silica gel H	SL, DGDG & MGDG.
Vicia faba leaves (Williams et al. 1975)	Acetone Benzene Water	91 30 8	Silica gel G containing $(NH_4)_2SO_4$	SL, DGDG
Maize leaves (Mackender, 1979)	Dimethyl-ketone Benzene Water	91 30 8	Silica gel G.	SL, DGDG.

* - Commercial preparation of adsorbant, a polymer of silicic acid.

** - Commercial preparation of silicic acid adsorbant containing about 15% calcium sulphate (designation G refers to gypsum content).

for separation in a particular biological system have been worked out by trying several solvent systems in succession (Wilson and Rinne, 1974; Sparce and Moore, 1979). None of the reports have considered the acid-base nature of the adsorbant and the acid-base nature of the solvent systems.

MATERIALS AND METHODS

Storage of Seeds

Seeds of Pisum sativum L. var. Bonneville were obtained from National Seeds Corporation, Indian Agricultural Research Institute, New Delhi and stored in desiccator.

Growth and Maintenance of Plants

Seeds of apparently uniform size were selected for germination. Seeds were soaked in running tap-water for 24 hrs. After thorough washing with distilled water, seeds were planted on a layer of wet absorbant cotton in petri dishes and grown in dark. Petri dishes were screened regularly and ungerminated seeds and infected plants, if any, were removed. Watering was done under dim green safe light during the dark period of growth. Growth temperature was maintained at 26 $\pm$ 1^{o}C. Age of plant was counted from the time of soaking.

Light Treatment

12-day-old dark grown pea plants were transferred to white light for studying the light induced changes. Light treatment was given to plants for a period of 4 days. Controls for the above experiments were maintained in dark. Only the leaf laminae were employed for the analysis of lipids and preparation of protoplasts.

Light Sources

Green safe light was obtained through eight layers of green cellophane papers wrapped on a 40 watt cool white fluorescent tube light. The intensity of light at the plant level was never more than 1 $\mu W\ cm^{-2}$. White light with an intensity of 1200 $\mu W\ cm^{-2}$ was obtained from white fluorescent lamps.

Isolation of Protoplasts from Leaves

Protoplasts were isolated according to the procedures developed by Bhalla-Sarin (1977). Sterile conditions were ensured during the protoplast isolation to control the possible microbial contamination. Leaves from healthy plants (approx. 2 gms) were collected and they were surface-sterilised in 0.1% (aq) $HgCl_2$ for about 5 min. After surface sterilization, they were thoroughly rinsed with sterile water to ensure complete removal of $HgCl_2$. Later, leaves were chopped into smaller pieces and suspended in a mannitol solution (0.6 M, pH 5.6). Chopped-fragments of leaf tissue were then washed with an excessive amount of mannitol and transferred to the enzyme solution. Macerozyme 1,5% and micellase 3% were prepared in 0.6M mannitol solution (pH 5.6). For approx. 2 gms of leaves 50 ml of enzyme solution were used. Incubation in the enzyme mixture was carried out for 4 hrs in a shaking water bath at 26°C. After the incubation period, the incubation mixture was

filtered through a sieve (pore size: 100 μm) to remove the cell debris and undigested leaf-tissues. The protoplasts in the enzyme mixture was pelleted down by centrifuging at 500 x g for 5 min. The supernatant was removed and the protoplasts were resuspended in the mannitol solution (pH 5.6). Counting of protoplasts was done in a haemocytometer.

Extraction of Lipids

Leaves or protoplasts from leaves were fixed by boiling in 5 volumes of isopropanol (w/v) for 5 minutes to inhibit the activity of various lipases and phospholipases (Yang et al., 1967; Roche, 1973; Roughan and Slack, 1976). The boiled tissue was transferred to a mortar and initial grinding was done in iso-propanol with a pestle using inert washed sand to aid maceration. The leaf lipids from the ground extract were extracted in 20 volumes (w/v) of chloroform:methanol (2:1 v/v) according to the procedure of Bligh and Dyer (1959). The lipid extract was filtered through Whatman No.1 filter paper to remove the residual matter. The extract was washed with 0.2 volume of 0.9% (aq) NaCl, to wash away the nonlipid-contaminants (Folch, 1957). The extract was transferred to a separating funnel, to separate the two phases of solvent and water. The lower solvent phase containing the total lipid was withdrawn and the upper water phase (containing methanol)

was discarded. The lipid extract was concentrated in a vaccum flash evaporator under reduced pressure and temperature. The traces of water was removed by repeated addition of benzene during the concentration. Lipid was resuspended in chloroform and the concentrated lipid-extract was transferred quantitatively from the flask of the evaporator to a pre-weighed tube and stored at -20°C till further use.

Column Chromatographic Fractionation

Silica gel column chromatography was performed according to the procedure of Lis et al. (1968). (61 of 68) Bibliography check.

(a) Washing of the gel - Silica gel (BDH-coarse) was washed successively in 1N HCl, 1N KOH and methanol. The gel was rinsed thoroughly in distilled water and dried in an oven at 120°C for 4 hrs. It was then stored in a desiccator.

(b) Activation of gel - Just before use, the gel was activated for one hr at 120°C.

(c) Packing of the column - Glass column tubes of 0.6 cm internal diameter and 22 cm height were used. The base of the column tube was plugged with glasswool. All tubings were of silicon material as the PVC-tubes were found to be corroded by the organic solvents. Activated silica gel was taken in chloroform and the slurry was poured

into the column tube. When the gel got settled, the column was washed with excessive volume of chloroform.

Amount of gel packed to the column	= 1 gm
Height of the column bed	= 12 cms
Bed volume	= 3 ml
Void volume	= 2.4 ml

(d) Sample loading - The chloroform above the column bed was brought down just above the bed surface. The lipid extract was gently loaded over the column bed with a microsyringe. The injection-volume was always 200 µl (about 6 mg total lipid).

(e) Elution scheme - The elution scheme adopted in the present study was as that of Lis et al. (1968). Successive elution with chloroform, acetone and methanol was carried out as given below :

	Flow rate/ minute	Elution volumes
I Elution : Chloroform	1.3 ml	35 ml
II Elution : Acetone	1.1 ml	80 ml
III Elution : Methanol	0.8 ml	35 ml

Individual fractions were concentrated in the flash-evaporator and the contents were dissolved in chloroform for further analysis.

Thin Layer Chromatography

(a) Preparation of thin layer plates - 6 gm of silica gel H were used for each of the 20 x 20 cm plates. The gel was made into a slurry by the addition of 18 ml of distilled water or the indicated solution with the impregnation agent. The plates were kept on a horizontal surface and allowed to dry at room temperature. The dried plates were stored in a chamber.

(b) Activation of plates - Just before use, the plates were activated for one hr at 120°C.

(c) Sample application - Activated plates were brought to room temperature. Plant-lipid extract or reference compounds of phospholipids in chloroform was loaded onto the plate with a microsyringe. Sample was applied either as a spot or a band. To avoid the exposure of the TLC plates to the atmospheric humidity, a glass plate closely covering the surface of the TLC-plate was placed. A hair drier was used in the viscinity of the exposed area.

(d) Development of plates - Solvent tank of 25 x 11 x 25 cm was used. The TLC plates were gently placed in the solvent mixture (composition as indicated in the results) and allowed to develop. When the solvent front reached 2-3 cm below the top edge of the plate the chromatogram was

taken out and dried. The total running time of TLC plates in different solvent systems was 80 to 120 min.

(e) Visualization of spots on the chromatogram - The chromatogram was placed in a chamber saturated with iodine vapours. The margin of the visualised spots was outlined with a needle.

(f) Detection of separated components - Specific detection tests for phospholipids (molybdenum - spray), α-aminogroup containing phospholipids (ninhydrin - spray) and galactolipids (orcinol spray) were carried out to ascertain the identity of phospholipids and galactolipids.

Composition of Various Spray Reagents

(a) Molybdenum blue reagent for the detection of phospholipids - This reagent was prepared according to the procedure of Dittmer and Lester (1964), as follows:
Solution I - To 125 ml of 25N H_2SO_4, 5 gm of molybdenum trioxide (MoO_3) was added and the mixture was boiled gently until the MoO_3 was dissolved.
Solution II - To 62 ml of solution I, 0.22 gm of powdered molybdenum was added and the mixture was boiled for 15 minutes. The solution was cooled and decanted from the residue.

Equal volumes of solution I and II were mixed and the combined solution was mixed with two volumes of water.

The final solution was greenish yellow in colour. The reagent was kept in a dark container and stored in a refrigerator.

(b) Orcinol-reagent for detection of galactolipids and sulpholipids - This reagent was prepared according to the procedure of Buchanan et al. (1950). 0.2% oricinol was dissolved in 75% H_2SO_4 (v/v). The spray reagent was sprayed to make the surface of the chromatogram moist. The plate was heated at 100°C for 15 minutes.

(c) Ninhydrin spray for the detection of α-aminogroup containing phospholipids - This reagent was prepared according to the procedure of Nichols (1964). 0.2% solution of ninhydrin in acetone was diluted with an equal volume of water immediately before use. After spraying, the chromatogram was allowed to stand at room temperature for 2 hours or it was heated for 5 min at 100°C.

Another criterion used for phospholipid identification was based on their mobility. The Rf values for each spot was calculated and compared with simultaneously running standards.

$$Rf = \frac{\text{distance of spot-centre from origin}}{\text{distance of solvent front from origin}}$$

Quantitative Determinations

(a) Estimation of total chlorophyll - Total chlorophyll was estimated according to the procedure of Arnon (1949). 80% acetone extract of leaves was centrifuged at 500 x g for 5 min to settle the cell debris. The clear supernatant was monitored at 663 and 645 nm in a Carl Zeiss PMQII spectrophotometer. Total chlorophyll was calculated as follows :

$$\text{mg. total chlorophyll (gm. fresh wt.)}^{-1} = 20.2(D_{645}) + 8.02(D_{663}) \times \frac{V}{1000 \times w}$$

where D_{645} and 663 were absorbance at respective wave lengths; V was the volume of extract and w was the fresh weight of leaves taken.

(b) Estimation of phospholipids - Lipid-phosphorous was estimated according to the prodedure of Wagner et al. (1962).

The total lipid extract or the scraped phospholipid-spots from thin layer chromatogram were collected in assay tubes. 1 ml of 60% perchloric acid was added to each tube. The tubes were transferred to a digestion rack and the contents digested at 180°C for 2.5 hrs. The tubes were brought to room temperature. Reagent mixture containing 10% ascorbic acid, 2.5% ammonium molybdate and 4% perchloric acid in the proportion of 1:1:8 (v/v) was prepared fresh

before use. 8 ml of the above reagent were added to each tube and the contents were thoroughly mixed in a vortex mixture. Incubation was carried out in a water bath at 30°C for 2 hrs. The tubes were centrifuged at 500 x g for 5 min to settle the gel. The contents were monitered at 660 nm.

Standard curve was prepared by using potassium dihydrogen orthophosphate (KH_2PO_4). There was no detectable interference due to the presence of TLC adsorbant during phospholipid estimation (Fig. 2).

(c) Estimation of galacto- and sulpholipids - Lipid-galactose was estimated according to the procedure of Roughan and Batt (1968).

The total lipid extract or acetone fraction or the spot area scrapped from thin layer chromatogram was estimated for galactose contents with the TLC adsorbant. To the sample with the adsorbant 2 ml of 2N H_2SO_4 was added. The tubes were covered with the glass marbles. The tubes were incubated in a boiling water bath for 60 min with occasional shaking. After cooling, the contents were centrifuged and 1 ml of aliquot was withdrawn from each tube. 50 µl of 80% (aq) phenol and

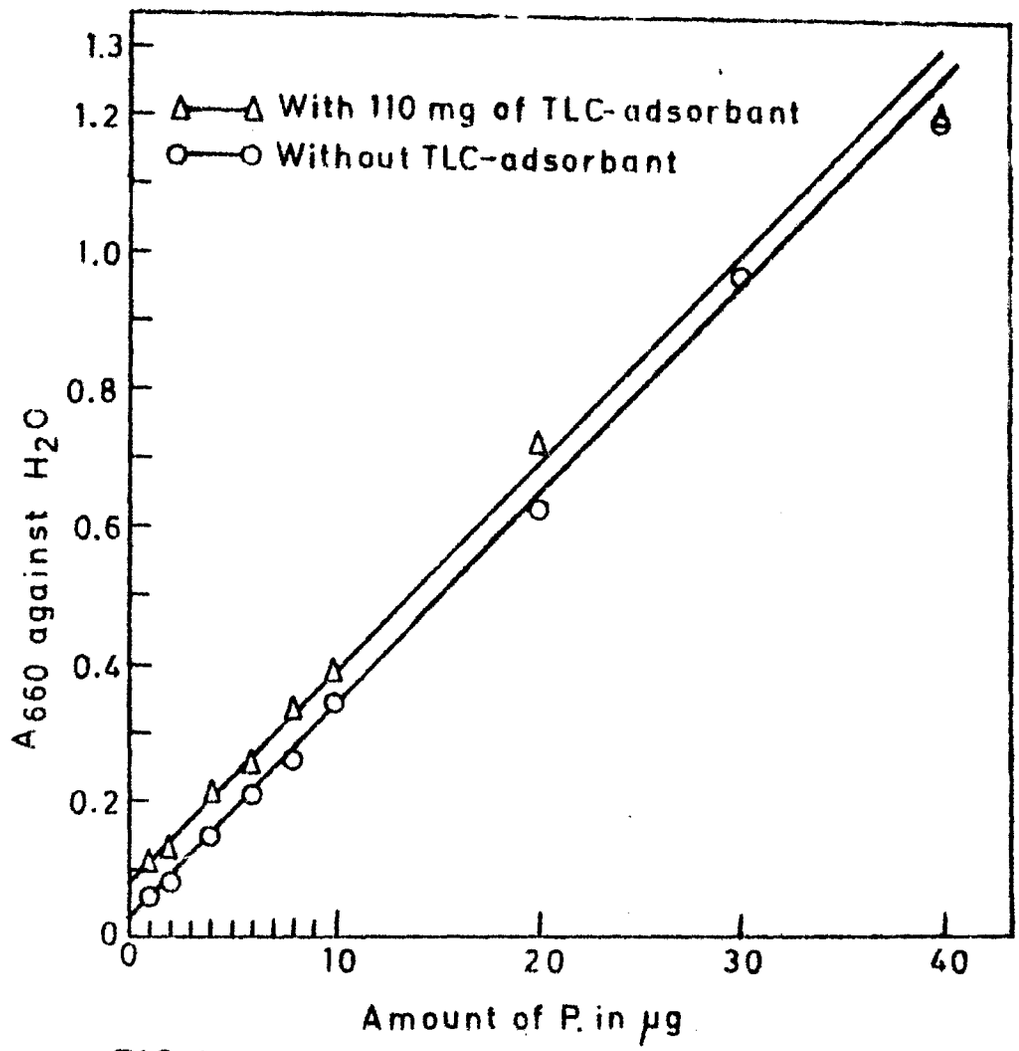


FIG. 2

Fig. 2: Standard curve for the estimation of lipid phosphorous.

	A_{660} against water
Assay blank	= 0.03
Assay blank + 110 mg of TLC-adsorbant	= 0.08

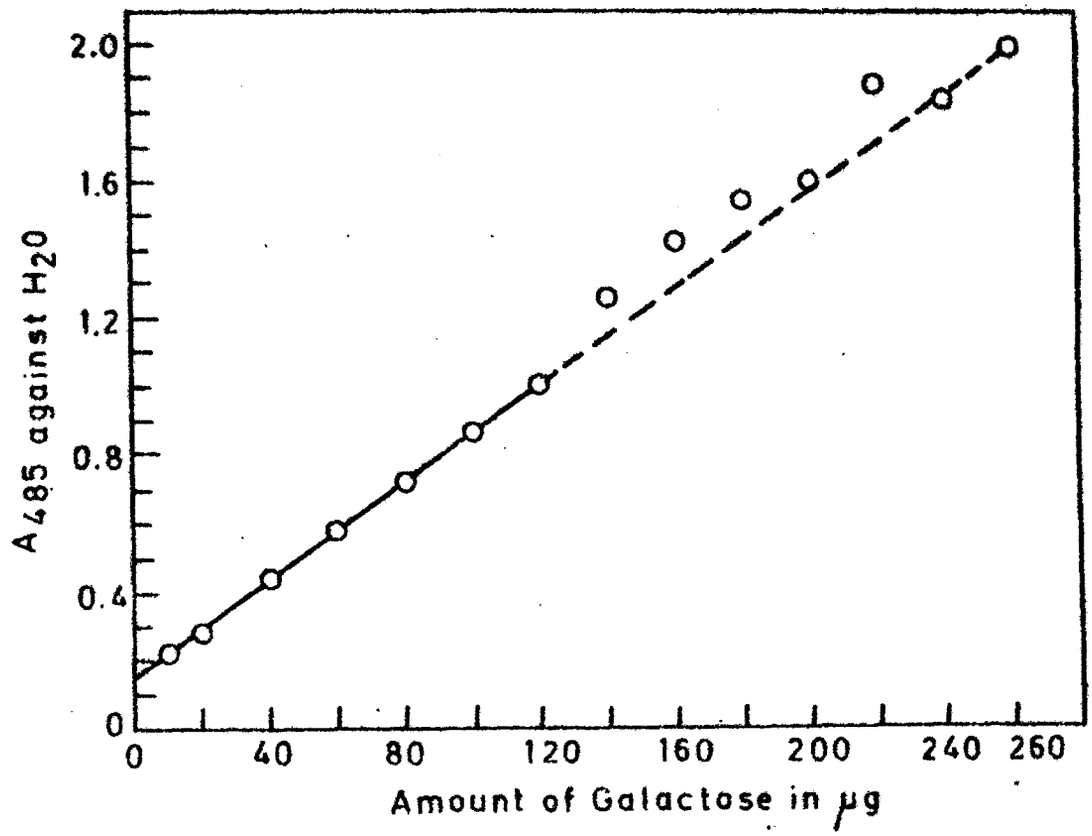


Fig. 3: Standard curve for the estimation of lipid galactose.

		A_{485} against water:
Assay blank	=	0.05
Assay blank + 110 mg TLC adsorbant	=	0.15

4 ml of conc. H_2SO_4 were then added. The assay mixture was allowed to stand for 15 min at room temperature before measuring absorbance at 485 nm. Standard graph was prepared with α-D-galactose (Fig. 3). In this case there was no significant interference due to the presence of TLC-adsorbant.

Chemicals

Silica gel H used in TLC and silica gel (coarse) used in column chromatography were from BDH, Bombay, India. Mannitol, reference compounds of phospholipids, α-D-galactose, orcinol and ninhydrin were from Sigma Chemical Company, St. Louis, Mo., USA. Macerozyme and micellase were from Welding & Co., Hamburg. Molybdenum (metal powder) and molybdenum trioxide were from BDH, Poole, England. All the organic solvents and inorganic chemicals were of the locally available grade of highest purity.

RESULTS

Levels of Different Classes of Membrane-lipids in the Leaf Tissues of P. sativum

Total lipids from leaf-tissue of 16 day old seedlings P. sativum (12 days dark + 4 days light and 12+4 days dark) were extracted. The analysis of total lipid extract to quantitate various classes of lipids was done as described in Materials and Methods. Table 2 describes the level of various classes of lipids which is in general agreement with that of leaves from other plants (Wintermans, 1960; Allen et al., 1966; Roughan and Batt, 1969).

The leaf tissue of both dark and light grown seedlings had higher content of neutral lipids (82% and 65% of total lipids in the case of 16 days dark grown and 12 days dark + 4 days light grown plants, respectively). The level of total phospholipids and galactolipids + sulpholipids were in the range of 12-23%. The amount of galactolipids and sulpholipids in 16 days dark grown seedlings was very low (4%) as compared to the seedlings grown in light for 4 days (23%).

Fractionation of Total Lipid Extract

Since the total lipid extract of leaves from light grown seedlings contained a significant amount of chlorophyll

TABLE 2

ANALYSIS OF TOTAL LIPID EXTRACT OF PISUM SATIVUM

Total lipid was extracted from leaf tissue and quantitated gravimetrically as described in Materials and Methods. Different group of membrane lipids viz. phospholipids and galactolipids + sulpholipids were quantitated colorimetrically. 16 day old pea seedlings (16 days dark grown and 12 days dark + 4 days light grown) were used.

Lipid class	Dark (16 days)		Dark + Light (12 days dark + 4 days light)	
	Amount+	Percent	Amount+	Percent
Total lipids	23.0	100	30.4	100
Neutral lipids	18.5	82	19.8	65
Phospholipids*	3.6	14	3.6	12
Galactolipids** + Sulpholipids**	0.9	4	7.0	23

\+ - mg/gm fresh weight.

* - Total phospholipid content was calculated on the basis of molecular weight (MW) of dipalmitoylphosphatidylcholine where lipid-phosphorous amounts to 1/25th of its MW.

** - Total content of galactolipids + sulpholipids was calculated on the basis of average molecular weight of plant-galactolipids viz. monogalactosyl diglyceride (MGDG), digalactosyl diglyceride (DGDG) and sulpholipid(SL) where lipid-galactose amounts to 1/4.7th of the MW of the above lipids.

(6% of the neutral-lipids) it was essential to remove it from the total lipid extract. Chlorophylls and other pigments have been shown to interfere in the quantitative determination of galactolipids (Roughan and Batt, 1968). The removal of chlorophyll was achieved by passing the total lipid-extract through a silica gel column using chloroform as the eluent. By using the same silica gel column, the left over lipid can be quantitatively separated into galactolipids + sulpholipids and phospholipids fraction.(Lis et al., 1961; Vorbeck and Marinetti, 1965). The above groupwise fractionation was accomplished by using different eluents viz. acetone and methanol. First, the lipids were eluted with acetone (85 ml) which contained 80% of the mixture of sulpholipids and galactolipids. The phospholipids were eluted completely with methanol (35 ml). The elution profile is shown in the histogram (Fig. 4). Thus silica gel column chromatographic fractionation was employed to accomplish a sequential separation of individual classes of lipids in a single column. Both the methanol and acetone fraction ware analysed for phospholipids and galacto- and sulpholipids respectively as follows.

Analysis of Methanol Fraction

Light induced change in the level of total phospholipids - There was no significant change in the level of

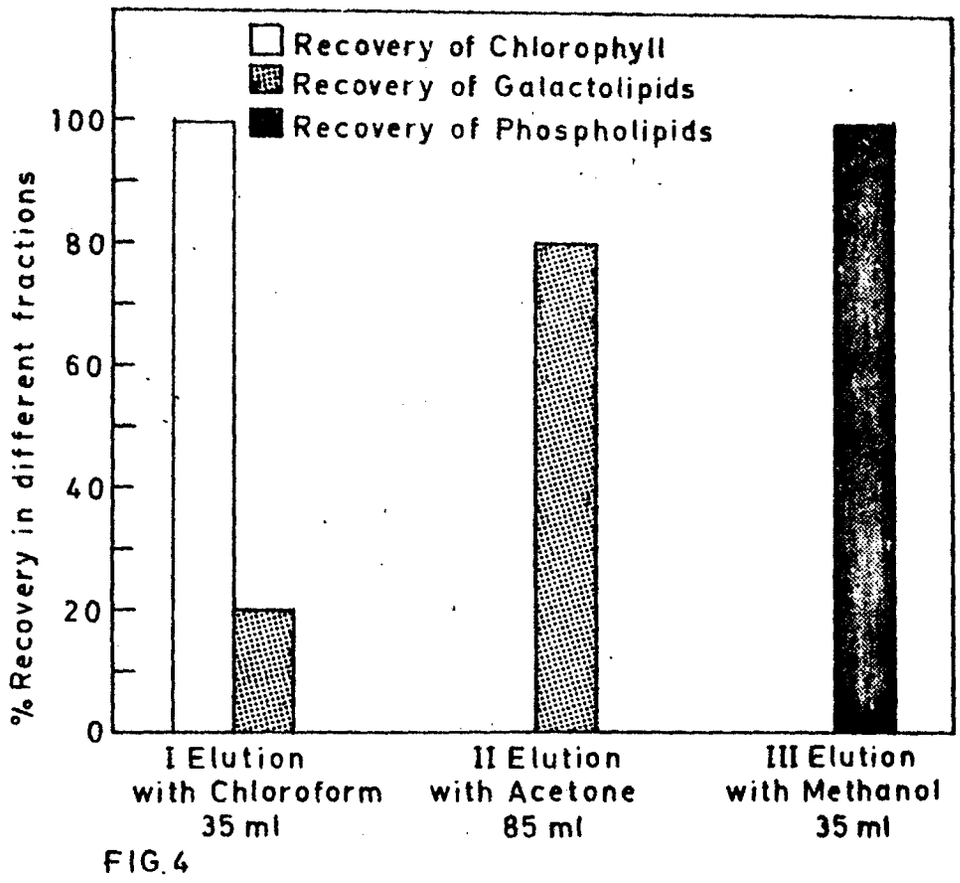


FIG. 4

Fig. 4: Elution profile of total lipid extract of pea in silica gel column.

total phospholipids of leaf-tissue of light treated plants (12 days dark + 4 days light grown). Similarly in the case of protoplasts prepared from the leaf tissue of these seedlings, there was no significant change in the level of total phospholipids (Table 3).

Fractionation and characterization of phospholipids - Since there was no available single solvent system for TLC or no elution scheme for column chromatography to fractionate various plant phospholipids, a set of solvent systems using TLC with silica gel H was attempted in the present study. The acidity and alkalinity of the mobile and stationary phases has been shown to affect the resolution of different phospholipids (Dallas, 1965; Snyder, 1966). Keeping this in view, a series of five different combinations of solvent and adsorbant systems with varying acidity and alkalinity were used to separate various phospholipids (Table 4). In all the different solvent systems a 10% aqueous slurry of silica gel H having an acidic pH (pH 6.1) was used in combination with different solvent systems.

I. With neutral mobile phase - In this TLC system, a mixture of chloroform:methanol: water (65:25:4 v/v) was used as the mobile phase which was neutral while the

TABLE 3

LIGHT INDUCED CHANGES IN THE LEVEL OF TOTAL PHOSPHOLIPID IN LEAF-TISSUE AND PROTOPLASTS FROM LEAVES

Methanol fraction phospholipids from silica gel column were analysed for the level of total phospholipids.

Treatments	Amount of total phospholipid	
	Leaf tissue*	Protoplasts**
12 + 4 days Dark	4.9	1.3
12 days Dark + 4 days Light	5.1	1.1

* - µmole (gm fresh wt.)$^{-1}$.

** - µmole (10 x 10^6 protoplasts)$^{-1}$.

TABLE 4

DEVELOPMENT OF A SINGLE DIMENSIONAL TLC-SYSTEM FOR THE SEPARATION OF PHOSPHOLIPIDS

Adsorbant	Solvent system		Designation of mobile phase
	Composition	Proportion*	
Silica gel H	Chloroform Methanol Water	65 25 4	Neutral
Silica gel H	Chloroform Methanol 30% (aq.) ammonia	65 25 4	Basic
Acidified silica gel H (with oxalic acid):			
0.05N oxalic acid	Chloroform Methanol Water	65 25 4	Neutral
0.07N oxalic acid	Chloroform Methanol Water	65 25 4	Neutral
0.1N oxalic acid	Chloroform Methanol Water	65 25 4	Neutral

* - Proportion of the solvent was on v/v basis.

adsorbant (stationary phase) was silica gel H (pH 6.1). As can be seen from Fig. 5A, the methanol fraction could not be resolved into any phospholipid components. The total extract was developed as a streak. Similar pattern was observed in the case of the mixture of reference compounds (Fig. 5A).

II. With alkaline mobile phase - In this case the mobile phase was changed from neutral to alkaline by adding 30% aqueous ammonia to the solvent system of the earlier mobile phase (chloroform:methanol:water). The adsorbant was, however, same as in the earlier case. Here also none of the phospholipids were resolved from both methanol fraction as well as from the mixture of reference compounds (Fig. 5B).

III. With acidic stationary phase - In this set of conditions, only the acidity of the stationary phase was changed by using different concentrations of oxalic acid. The stationary phase of the earlier attempts i.e. silica gel H was impregnated with 0.05, 0.07 and 0.1N oxalic acid. Impregnation was done by adding the required concentration of oxalic acid to the silica gel H slurry before making the TLC-plates. The mobile phase was kept the same (chloroform:methanol: water) at all the concentrations of impregnation-agent (oxalic acid).

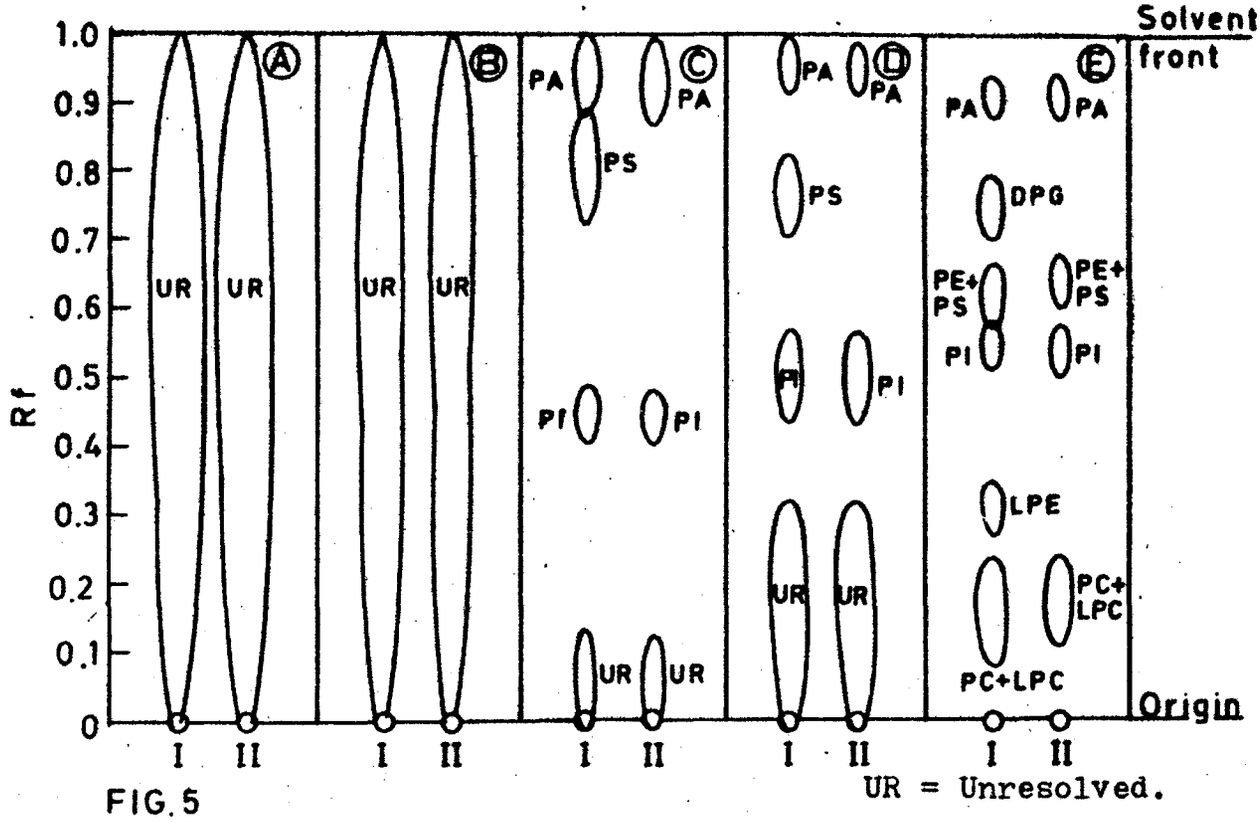


FIG. 5

Fig. 5: Diagramatic representation of separated phospholipids in different TLC-systems.

The stationary phase in A and B was silica gel H; in C, D and E acidified silica gel H with 0.05N, 0.07N and 0.1N oxalic acid respectively. Mobile phase in all cases was chloroform:methanol:water (65:25:4 v/v) exeept in B where water was replaced by 30% (aq) ammonia. I and II of each lane refers to mixture of reference compounds (PC, LPC, PE, LPE, DPG, PS, PI and PA) and methanol fraction of silica gel column respectively.

In both the lower concentrations of impregnation agent i.e. 0.05 and 0.07, only PA, PS and PI of the methanol fraction phospholipids could be resolved (Fig. 5 C & D). But the picture was different when slightly higher concentration of oxalic acid (01N) was used under similar set of conditions. Most of the phospholipids present in the methanol fraction were optimally resolved (Fig. 5E). Since this was found to be the ideal set of conditions, the further characterization of phospholipids was done using 0.1N oxalic acid impregnated silica gel H in combination with the neutral mobile phase (chloroform:methanol:water).

Identification of various phospholipids - The separated components of phospholipids of the methanol fraction were identified by (a) comparing their mobility (Rf values) with reference compounds; (b) by their response to various group specific spray reagents such as Dittmer and Lester's reagent for general phospholipids (Dittmer and Lester, 1964) and ninhydrin spray for α-aminogroup containing phospholipids (Nichols, 1964) (Table 5).

A total of five resolved spots were observed on the chromatogram. Four of the spots showed positive response to phosphate-specific spray. They had Rf values comparable

TABLE 5

CHARACTERIZATION OF PHOSPHOLIPIDS OF PEA LEAF-TISSUE

Phospholipids from the methanol fraction of silica gel column was developed in chloroform:methanol:water (65:25:4 v/v) and acidified silica gel H TLC system. Standard phospholipids were also run parallely to compare the mobility of reference compounds with that of separated components. Separated components were further tested with different group-specific reagents to establish their identity.

Rf value of separated components	Reference component(s) of comparable Rf values	Response to spray reagents		Identified component
		Phosphate specific	α-amino group specific	
0.20	PC+LPC	+ve	-ve	PC+LPC
0.55	PI	+ve	-ve	PI
0.63	PE+PS	+ve	+ve	PE+PS
0.74	Not comparable	-ve	-ve	UI*
0.94	PA	+ve	-ve	PA

*unidentified Component.

to reference compounds of PC+LPC, PI, PE+PS and PA. Among the above four spots the one having Rf value of 0.65 gave positive response to ninhydrin test and so that was characterised as PE or PS. One of the spots with Rf value of 0.71 showed no response to either phosphate-test or α-amino group test and so it could not be identified under the present set of conditions.

The other spots which had comparable Rf values of PC+LPC and PE+PS were further resolved in an another solvent system (chloroform:methanol:acetic acid:water 170:30:20:7 v/v) and it was found to be homogeneous of PC and PE only. There was no detectable level of LPC or PS in those comigrating pairs.

Light induced quantitative changes in phospholipid composition - Since there was no detectable change in the total phospholipid contents of light versus dark grown leaf-tissue and their protoplasts an attempt was further made to evaluate if any individual phospholipid component may have changed with the altered growth conditions.

In the leaf-tissue of light treated seedlings (12 days dark + 4 days light grown) a drastic fall in the level of PA was observed. The PA content was 35% of the total phospholipids which was reduced to 12% in

TABLE 6

LIGHT INDUCED CHANGES IN THE DISTRIBUTION OF DIFFERENT PHOSPHOLIPIDS FROM LEAF-TISSUE AND PROTOPLAST FROM LEAVES

Relative distribution of different phospholipids were analysed. Methanol fraction phospholipids were fractionated in acidified silica gel H - chloroform:methanol:water (65:25:4 v/v) TLC system. Separated components of phospholipids were estimated and the level of the components are expressed as percent of total phospholipids.

(A) Leaf-tissue:

Treatments	Percent of total phospholipids			
	PC	PI	PE	PA
12+4 days Dark	15	35	16	35
12 days Dark + 4 days Light	20	49	20	12

(B) Leaf-protoplasts:

Treatments	Percent of total phospholipids			
	PC	PI	PE	PA
12+4 days Dark	17	33	33	17
12 days Dark + 4 days Light	15	40	20	25

the light treated leaves. There were some minor changes in the other phospholipid levels (Table 6A).

In the case of protoplasts, almost similar pattern of changes were observed in ~~case of~~ PC and PI+PG levels. However, the situation was different in the case of PE and PA contents of the protoplast -phospholipids (Table 6B). Whether the changes in protoplasts reflect the changes in the plasmamembrane of P. sativum leaves is not clear from these results since the protoplasts used for phospholipid analysis were not fractionated into subcellular fractions. The isolation of plasmamembrane rich fraction from protoplasts from subsequent phospholipid analysis remains to be done.

Analysis of Acetone Fraction

Light induced changes in the total level of galactolipids + sulpholipids - The acetone fraction from silica gel column was analysed for total galactolipids + sulpholipids under various treatments. The estimation of these two classes of lipids was done as described in Materials and Methods. There was about 6-fold light induced increase in the level of total galactolipids + sulpholipids in leaf-tissue (Table 7). It is pertinent to mention that these lipids could be estimated by colorimetric procedure which is based on the sugar content of the lipids.

TABLE 7

LIGHT INDUCED CHANGES IN THE LEVEL GALACTOLIPIDS AND SULPHOLIPIDS OF LEAF TISSUE/PROTOPLASTS

Total galactolipids + sulpholipids from the acetone fraction of silica gel column was quantitated colorimetrically. Lipid extracts from leaves and protoplasts of 16 days old pea seedlings (12+4 days dark grown and 12 days dark + 4 days light) was used.

Treatments	Amount of Galactolipids + sulpholipids (μ mole)	
	Leaf tissue*	Protoplasts**
12 + 4 days Dark	1.2	0.53
12 days Dark + 4 days light	8.2	2.1

* - per gm fresh weight.

** - per 10×10^6 protoplasts.

When the protoplast-lipids were analysed for total galactolipids + sulpholipids, the pattern of change was same. However, the extent of change was less (0.53 µmole/ 10×10^6 protoplasts in dark grown seedlings and 2.1 µmole/ 10×10^6 protoplasts in the light grown seedlings) (Table 7).

Fractionation and characterization of galactolipids and sulpholipids - The acetone fraction containing galactolipids and sulpholipids was fractionated on TLC using a mixture of chloroform:methanol: acetic acid:water (170:30: 20:7 v/v) as the mobile phase. A reproducible resolution was possible in this set of solvent system. Various galactolipids and sulpholipids were identified by comparing their Rf values with known standard Rf values and by using specific spray reagent for galactose (Table 8). Only three spots were identified as sulpholipid (SL), digalactosyl diglyceride (DGDG) and monogalactosyl diglyceride (MGDG) and the other spots could not be identified under the present set of conditions and so designated as UI_1 and UI_2 (unidentified components).

Light induced quantitative changes in galactolipids and sulpholipids composition - Light induced changes in the content of different galactolipids and sulpholipids were very significant in the leaf tissues as well as in the protoplasts isolated from them (Table 9). There was an increase in the level of SL and a decrease in DGDG. MGDG level was not signifi-

TABLE 8

IDENTIFICATION OF GALACTOLIPIDS AND SULPHOLIPIDS

Galactolipids and sulpholipids from the acetone fraction of silica gel column was developed in chloroform: methanol:acetic acid:water (170:30:20:7 v/v) and silica gel H TLC system. Separated components were identified by comparing their Rf value with known standard components.

Rf values of separated components	Rf values from Roughan and Batt (1968)	Response to galactose specific spray	Idenfified component
0.17	0.21 (SL)	+ve	SL
0.25	Not comparable	-ve	UI_1 ★
0.37	0.35(DGDG)	+ve	DGDG
0.59	Not comparable	-ve	UI_2
0.90	0.94 (MGDG)	+ve	MGDG

★ UI_1 and UI_2 = Unidentified Components.

TABLE 9

LIGHT INDUCED CHANGESIN THE DISTRIBUTION OF SULPHOLIPIDS AND GALACTOLIPIDS OF LEAF TISSUE AND PROTOPLASTS

Relative distribution of different galactolipids and sulpholipids isolated from leaves and protoplasts were analysed. Galactolipids from the acetone fraction of silica gel column was developed in chloroform:methanol: acetic acid:water (170:30:20:7 v/v) using TLC. Separated components were quantitated by estimating lipid-galactose.

(A) Leaf-tissue:

Treatments	Percent distribution SL	DGDG	MGDG
12+4 days Dark	4	44	52
12 days Dark + 4 days Light	29	17	55

(B) Protoplasts:

Treatments	Percent distribution SL	DGDG	MGDG
12 + 4 days Dark	1	40	60
12 days Dark + 4 days Light	15	22	63

TABLE 10

LIGHT INDUCED CHANGES IN THE RATIO OF MGDG AND DGDG IN THE LEAF-TISSUE AND PROTOPLASTS

The ratio of MGDG/DGDG was calculated from the data given in Table 9A and B.

Condition	Ratio of MGDG/DGDG	
	Leaf tissue	Protoplasts
4 days Dark	1.2	1.5
4 days Light	3.2	2.9

cantly changed in the case of both leaves and leaf protoplasts. Increase in SL was 7-fold and 15-fold in the case of leaves and protoplasts respectively. The observed pattern of changes for SL, DGDG and MGDG was almost similar in leaf-tissue and their protoplast lipids (Table 9).

The ratio of MGDG/DGDG level has been shown to change in response to growth conditions (specially illumination) (Roughan and Boardman, 1972). To ascertain this in our set of conditions if such changes can be demonstrated, this ratio was determined. The ratio of MGDG/DGDG was significantly increased due to light-treatment indicating a significant reduction in the level of DGDG. This was reflected in both leaf-tissue and the protoplasts isolated from them (Table 10).

DISCUSSION

Lipids are one of the prominent integral components of biological membranes. The currently accepted model of biological membrane involves a lipid bilayer as the basic structural unit, with proteins embedded partly or fully into the interior of the lipid bilayer (Singer, 1974). It has been shown that changes in lipid content and composition in membrane could affect not only the properties of lipid bilayer, as such, but also have considerable effect on the bound enzyme activities which are found to decline or to be lost completely when membrane lipids are extracted or modified (Coleman, 1973). Some of these enzymes have been fully or partly restored by the addition of lipids.

In bacterial membrane, lipids have been demonstrated to be required for the functions of membrane-located enzymes, for example, those related to oxidative phosphorylation (Cunningham and Hager, 1971) and active transport (Fox, 1969; Milner and Kaback, 1970; Prasad *et al.*, 1975; Cox *et al.*, 1975; Ohta *et al.*, 1977). Several workers have investigated the relationship between unsaturated fatty acids and the activities of sugar and amino acid transport system of *E. coli* (Linden *et al.*, 1973). Fox (1969) reported that an abortive induction of lipid transport system was due to a nonfluid and immobile state of membrane lipids. Pittoti *et al.*

(1972) showed in rat liver ATPase preparation that membrane phospholipids form an essential constituent of this membrane bound enzyme.

Most of the earlier work related to membrane lipids and their functional role came from bacterial and mammalian systems. The work on plant lipids and their relation to various membrane functions has only started recently (Yamaki and Uritani, 1973; Wilson and Rinne, 1974; Wade and Bishop, 1978; Cockerham and Lundeen, 1979). Before assessing any role of plant lipids in membrane function, their characterization and localization is absolutely essential. In spite of several attempts (Wilson and Rinne, 1974; Sparce and Mode, 1979) to isolate and characterize plant lipids, a rapid, reproducible separation method for plant lipids has not yet been developed (Hitchcock and Nichols, 1971; Roughan et al., 1978).

Therefore, in the present investigation an attempt was made to develop a rapid quantitative method for the characterization of various polar lipids of Pisum sativum. As can be seen from results that the total lipid extract could be fractionated into various polar lipids e.g. galactolipids, sulpholipids and phospholipids. The main interfering pigment of the total lipid extract (chlorophyll) could also be removed by silica gel column chromatography (Fig. 4). The methanol fraction could be resolved into PC, PI, PE and PA

components by using TLC-system with acidified silica gel H and chloroform, methanol, water (65:25:4 v/v) (Fig. 5 and Table 5). Similarly galactolipid and sulpholipid could be separated on TLC using (silica gel H and chloroform, methanol, acetic acid and water system (Table 6). The identification and characterization of various polar lipids were done by using various specific spray reagents and by comparing the Rf values of known standards ran simultaneously under similar set of conditions (Table 5 and 8). Thus by using a combination of silica gel column and thin layer chromatography, it was possible to resolve and identify most of the polar lipids extracted from P. sativum.

Lipids have earlier been shown to be altered during various physiological conditions e.g., germination, differentiation and senescence. In most of the cases the evidences for the functional role of lipids have been indirect (Bentelmann and Kende, 1977; Wade and Bishop, 1978; Cockerham and Lundeen, 1979). An attempt was made in the present study to characterize and quantitate lipid changes under one set of conditions in pea (dark grown versus light grown seedlings). There was no noticable change in the total phospholipid and the content of individual kind of phospholipid molecules (Table 3 and 6). The content of sulpholipid and galactolipids demonstrated a significant increase when the dark grown seedlings were exposed to

4 day-light period (Table 7 and 9). The ratio of MGDG/ DGDG which has earlier been shown to respond to growth conditions (Tremoliefs, 1970; Roughan and Batt, 1972) was found to increase during light exposure of the seedlings (Table 10). The interesting part of the observation was that when the protoplasts were isolated from the leaf tissues of seedlings grown in different conditions, a similar pattern of changes was observed (Table 3,6,7,9 and 10). This suggests that these changes may reflect true changes of membrane lipids. However, since the protoplasts were still full of cellular milieu, the observed changes may not be entirely due to the plasma membrane. The isolation of pure plasma membrane for the analysis of various lipids remains to be done.

Recently, an indirect correlation between lipid composition and cellular permeability of plant cells have been pointed out (Bentelmann and Kende, 1977; Cockerham and Lundeen, 1979). It is known that the plasma membrane of plants house many of the carrier-mediated transport systems (Higginbotham, 1974). However, studies related to functional role of lipids of plasma membrane in relation to uptake have not taken any serious turn. In plant cells, the presence of cell wall has been observed to prevent direct access to the plasma membrane (Guy, 1978). For example, uptake studies in intact plants has been observed to cause

nonspecific adsorption of metabolities to the cell wall (Mettler and Leonard, 1979). This has been observed to be a serious obstacle in interpretting the characteristics of different transport systems involved in various uptake. However, with the employment of plant protoplasts, these obstacles appear to have been overcome (Guy, 1978). The effect of lipid changes observed in the protoplasts of P. sativum on the transport of various neutrients/metabolites is being initiated in our laboratory. This would give us an opportunity to assess the role of lipids in cellular transport in this membrane system. These studies may be related to the changes in growth pattern in plants under different physiological conditions.

SUMMARY

With a view of understanding the role of membrane lipids in morphogenesis the present study was initiated. As a primary task, characterization of membrane lipids was undertaken. Lipid analysis was carried out in leaf tissue and protoplasts isolated from them. A two step fractionation procedure involving silica gel column chromatography and silica gel thin layer-chromatography was employed for the purpose. By the silica gel column chromatography, the interfering pigments in the crude lipid extract was separated from the membrane lipids. The membrane-lipids were fractionated into galactolipids + sulpholipids and phospholipids containing fractions. The above mentioned fractionation was achieved by employing a sequential elution with chloroform, acetone and methanol.

Individual fractions of the membrane lipids were further fractionated in thin layer chromatography. A schematic approach involving different TLC-systems were attempted to resolve individual components of phospholipids. The acidity and alkalinity of the mobile phase and stationary phase were considered in developing a one dimensional TLC-system for the separation of phospholipids. Yet another one dimensional TLC-system was standardised for the separation of galactolipids and sulpholipids. Separated components were characterized by their mobility

(Rf values) in comparison with that of known reference compounds and by using several group specific spray reagents. Four components of phospholipids viz. phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylinositol (PI) and phosphatidic acid (PA) were identified from the methanol fraction of silica gel column. Three components were identified from the acetone fraction of the silica gel column viz. sulpholipid (SL), digalactosyl diglyceride (DGDG) and monogalactosyl diglyceride (MGDG).

Study was made to find out the light induced changes in the membrane lipids. Total content and level of individual components of phospholipids and galactolipids + sulpholipids were analysed to observe the light induced changes. There was no significant change in either the total content of phospholipids or level of PC, PE, PI and PA in the leaf tissue. However, protoplasts showed a significant decrease in the level of PA.

Changes observed in the galactolipids and sulpholipids were very significant. Total content as well as level of individual components of galactolipids and sulpholipids showed marked change due to light treatment. There was an increase in the level of sulpholipid and a decrease in digalactosyl diglyceride. The ratio of monogalactosyl diglyceride to digalactosyl diglyceride (MGDG/DGDG) showed a significant increase.

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