LIGHT AND PROCESSING EFFECTS ON PROTEINS, LIPIDS, CARBOHYDRATES, CAROTENOIDS, VITAMIN C, AND ON PLASTID ULTRASTRUCTURE OF Phaseolus aureus Roxb. (MUNG BEAN) SEEDLINGS

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ABSTRACT

1. EM and Nutrient Studies of Light-Treated Etiolated Mung Bean Seedlings

The prolamellar bodies of leaf plastids of 4-day-old dark-grown mung bean seedlings were gradually being converted to grana membranes, and after 8 h of a 24 h illumination period this conversion was complete. In parallel studies, an increase in nutrients was observed mostly after 6 h illumination. By the end of 24 h, total lipids, total available carbohydrates and total proteins showed a marked increase compared to those of dark-grown seedlings. Carotenoids, on the other hand, started to increase after 1 h illumination, and chlorophylls, which were absent from etiolated seedlings, showed an immediate increase on exposure to light. The vitamin C content of seedlings did not change significantly with light. Twenty simple and complex lipids, three free sugars, seventeen amino acids and seven carotenoids were identified in etiolated and greened seedlings.

2. Nutrient Studies of Processed Mung Bean Seedlings Stored at Three Temperatures

Blanching of dark-grown mung bean seedlings prior to packing them into jars and cans had a marked effect on vitamin C, on total available carbohydrates, on free sugars and on starches and dextrins, whereas total proteins and amino acids were not severely affected by the blanching process; lipids and carotenoids were only slightly affected. The blanched seedlings were packed in jars and in cans. Bottled (jars) seedlings were stored for six months under three temperatures: 10°C, room temperature (RT) and 35°C, while canned seedlings were stored for the same period at RT only. Generally speaking, the seedlings packed in jars and kept at 35°C sustained the most losses in nutrients, while the seedlings kept at 10°C retained the most. There were no marked differences in nutrients of bottled and canned seedlings stored at RT.

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Introduction

Mung beans, 'Golden grana' or 'Green gram' or Phaseolus aureus Roxb., are grown on a small scale in many parts of tropical and subtropical Asia, Africa and America, but are widely cultivated in India and are probably native to this part of the world where they are chiefly used as pulse (Schery, 1972; Masefield et al., 1973). In Iran they have been used in bread-making for a long time and as a supplementary food ingredient in various stews on a small scale.

The Chinese developed the sprouting of mung beans centuries ago and sprouting mung beans are now one of the principal ingredients in chop suey, chow mein and other Chinese dishes. With the growing popularity of Chinese food, many western countries e.g. America, are becoming familiar with mung beans (Hsuen et al., 1972; Fordham et al., 1975; Kylen and McCready, 1975). Dried mung bean seeds can be stored conveniently for an extended length of time and the sprouts are obtained by germinating the seeds in the dark until the sprouts reach the desired length (usually 3-6 cm). The sprouting of seeds does not require sunshine or soil and is not limited to seasonal growth. Furthermore, the sprouting time is short (3 - 4 days) and large amounts of sprouts can be obtained in a short time. They can be produced easily at home and then can be used as a fresh salad vegetable or cooked with certain foods. Germination seems to increase the nutritive value of seeds (Ahlberg, 1935, 1945; Fordham et al., 1975; Kylen and McCready, 1975) and modify the flavour and digestibility of seeds (Patwardhan, 1962; Ajei-Twum et al., 1976). Young (1782) first observed that germinating seeds acquired antiscorbutic properties (Chayen, 1953). Several studies have shown that dry seeds have measurable amounts of ascorbic acid (Ahlberg, 1935; Bhagvat and Rao, 1942; Cravioto et al., 1945; Fordham et al., 1975), but the amount is increased by germination. Germinated seeds may therefore be a cheap source of vitamin C in the diet. Certain nutrients of mung bean seeds and seedlings have been noted by Fordham et al. (1975), and Kylen and McCready (1975), but chemical analysis of the nutrients was not done in a systematic manner and a further study was therefore necessary.

The present study will be divided into two sections: Section I comprises the effect of light on various constituents of mung bean seeds germinated for 4 days in the dark and then given various light treatments up to 24 h. In Chapter II the effect of continuous light on the changes in the ultrastructure of plastids (prolamellar body into grana) will be investigated, and in the following five Chapters the relationship of these changes to the following nutrients will be discussed: Chapter III (Lipids), Chapter IV (Carbohydrates), Chapter V (Proteins), Chapter VI (Vitamin C) and Chapter VII (Chlorophylls and Carotenoids and provitamin A activity) over the same period of illumination. It is well known that plastids contain most of these constituents (Robard, 1970) and although a substantial amount of work relating to plastid interchange has been carried out, comparative studies with all the above nutrients have not been reported so far. Section II. In this section the effect of blanching and processing on the nutrient composition of 4-day-old dark-grown mung bean seedlings will be examined as well as the effect of different containers (glass jars and cans) and storage temperature on these nutrients over a six-month period:

(1) Bottled seedlings were kept under three different temperatures: 10°C, RT (room temperature) and 35°C
(2) Canned ones were kept only at RT over the same six-month storage time.

The advantages and benefits of food processing are numerous (Hardenburg, 1975) and can be summarised as follows:

(1) they serve as an efficient handling unit
(2) they serve as a convenient warehouse or home storage unit
(3) they protect quality and reduce waste:
    (a) they protect from mechanical damage
    (b) they protect against moisture loss
    (c) they provide clean and sanitary products
    (d) they may provide a beneficial modified atmosphere
(4) they facilitate service and sales marketing
(5) they reduce cost of transport and marketing
(6) they facilitate use of new modes of transportation

As mung beans contain trypsin inhibitors, the heating inactivates these compounds and so protein utilisation is improved (Wolf and Cowan, 1975). For these reasons and since mung bean shoots are perishable, most of those produced in the United States are canned and only seldom are fresh shoots found on the retail market (Hsuen et al. 1972). No systematic study of the nutrients present in processed and stored mung bean shoots has yet been undertaken and the object of the present work was to remedy this. The following chapters will deal with the effect of processing and storage of processed materials at different temperatures in the following nutrients: Chapter VIII (Lipids), Chapter IX (Carbohydrates), Chapter X (Proteins), Chapter XI (Vitamin C) and Chapter XII (Carotenoids and Provitamin A activity). Finally, Chapter XIII will cover conclusions on this study.

SECTION I

EFFECT OF LIGHT ON VARIOUS PARAMETERS IN 4-DAY-OLD DARK-GROWN MUNG BEAN SEEDLINGS

CHAPTER I

GERMINATION

Germination of mung bean seeds

Unbroken seeds were washed and soaked in five times their volume of water for 5 h at room temperature in a dark cupboard. By the end of the soaking time seeds were soft, or broken testa were observed. Cellulose sponge sheets, 3 cm thick (Fordham *et al.*, 1975), boiled in water for 10 min were placed in sterilised plastic trays (100 cm x 90 cm) and allowed to cool to room temperature. The soaked seeds were washed thoroughly with water, placed in a layer over the sponge, and water was poured to half the height of the sponge (1.5 cm) to provide moisture during sprouting. The trays were then covered with aluminium foil and held at 25°C in a dark cupboard. Sprouts 3 - 6 cm long were produced during 4 days before the development of rootlets. During the various transfers light was excluded as far as possible, but some light did fall on the seeds. Since the seeds at this stage are not under photocontrol, the small amount of light they received did not have any significant effect on them.

The seeds showed about 85% yield by this procedure and no washing was needed. The advantage of using this method was that the moisture content could be regulated, whereas with other growing media, e.g. cheesecloth (Chen, 1970) or paper towels (Courter, 1972), the seeds needed to be washed every 4 h to prevent the growth of moulds.

CHAPTER II

THE EFFECT OF CONTINUOUS LIGHT ON THE CHANGES IN THE ULTRASTRUCTURE OF PLASTIDS OF 4-DAY-OLD DARK-GROWN MUNG BEAN SEEDLINGS (GRANA FORMATION)

Introduction

It is well known that different parts of plants react differently to the same stimulus, for example, when certain etiolated seedlings are illuminated the hypocotyls stop growth almost immediately whereas the leaves start to green and expand. It would have been ideal then to study the different parts of mung bean seedlings to observe what happens in each part as a response to light.

However, the etiolated mung bean seedlings used in this study were made up of large hypocotyls, of small cotyledons, and of very small first leaves and radicles. For most biochemical studies it was not convenient to separate each seedling into its different parts and so the whole seedling was extracted except where otherwise stated.

In the case of electron microscopy, it was more convenient to use the leaves since they contain very large numbers of etioplasts compared to the hypocotyls. In preliminary studies, hypocotyls were used but although the etioplasts were similar to those of leaves they were not numerous and very often the sections contained no such organelles. It was therefore decided to use leaves for e.m. studies.

Most plastids investigated have been found to store starch but they may also store oil or protein. It is well known that dark-grown plastids of higher plants do not contain any chlorophyll but under adequate illumination etioplasts are converted to chloroplasts. Etioplasts contain a structure known as the prolamellar body. This body is made up of a symmetrical association of tubules which in section frequently appear as vesicles.

Etioplast development to mature chloroplasts cannot take place in the absence of sufficient light. However, if adequate illumination is available the internal membranes elaborate to form a stack of discs (grana), which normally become more-or-less completely separated from the plastid envelope. The grana are joined to each other by membranes. The synthesis of chlorophylls and the structural elaboration of the chloroplasts are both depdendent upon light. This is not surprising in view of the fact that chlorophyll is an integral part of the chloroplast membranes (Robards, 1970).

The onset of photosynthetic activity and the formation of photosynthetic enzymes in parallel with prolamellar body transformation to grana structure has been studied extensively by many workers, namely Gyldenholm (1968); Bradbeer (1969, 1973); Bradbeer et al. (1974); Whatley (1977).

In the present study 4-day-old dark-grown mung bean seedlings were subjected to 1000 lux continuous illumination over a 24 h period. The changes in the fine structure of plastids from prolamellar body to grana formation were completed in 8 h illumination. At the same time proteins, lipids, carbohydrates, pigments and vitamin C of dark-grown seedlings, and changes caused by illumination over a 24 h light period, have been estimated and will be discussed in the appropriate sections.

Materials and Methods

Leaves of mung bean seedlings were prepared for electron microscopy with slight modifications of the methods of Nunn (1970). Leaves were chopped into small pieces and kept in 2.5% glutaraldehyde in 0.1M phosphate buffer (pH 7.4) overnight at 4°C. They were washed thoroughly in buffer and post-fixed in 2% osmium tetroxide in 0.15M phosphate buffer (pH 7.3) for 2 h. Dehydration was carried out in a series of ethanol-propylene oxide mixtures (30%, 50%, 70% and 100% v/v). The material was next embedded in Spurr's resin for 6 h at 45°C under 15 lb sq in^{-1} in a vacuum oven and the temperature increased to 60°C until the blocks had cured to hardness (up to 36 h). Samples

were sectioned in an LKB ultratome and after staining in 5% uranyl acetate and lead citrate the sections were examined in an AEI EM6B, a Zeiss EM9A or a JEM100 CX electron microscope.

Results and Discussion

Plastids of 4-day-old dark-grown mung bean seedlings contain few crystalline prolamellar bodies, with less contracted membranes that resemble a typical prolamellar body, described by Weier and Brown (1970). This prolamellar body appeared crystalline with a half-open structure (Fig. 1). When seedlings were subjected to 1000 lux continuous light, rearrangement of internal membranes of the etioplast took place by the conversion of the prolamellar body into thylakoids (grana), a process which took almost 8 hours for completion under experimental conditions. During the first 5 h under light no obvious changes were observed except that crystalline structure of prolamellar bodies was being converted into the open form (Figs. 2, 3 & 4). This decrease in size implied that prolamellar bodies were gradually being converted to thylakoids which then made up most of the plastids, especially after 6 h (Figs. 5, 6 & 7), presumably indicative of the beginning of grana formation which was completed by 8 h light (Figs. 8, 9 & 10). At this stage no prolamellar bodies were observed, indicating in fact the presence of grana. More grana were formed over the next 16 h illumination. They had obvious membranes which were separated from each other (Fig. 11). The plastids of dark-grown leaves contain a few thylakoids but newly-synthesised ones presumably do not appear until 6 to 8 h after illumination. This is shown in Fig. 5 when large numbers of thylakoids were then visible.

It has been suggested by Bradbeer *et al.* (1974) working on *Phaseolus vulgaris*, that under continuous light the pre-existing thylakoids may be rearranged to give grana. It seems unlikely that the onset of photosynthesis is dependent on the commencement of *de novo* thylakoid formation. It would seem more likely that the attainment of a substantial rate of membrane assembly is dependent on the occurrence of photosynthesis. However,

Bradbeer (1969) and Bradbeer *et al.* (1973) have suggested that light treatment would appear to have induced *de novo* protein synthesis and/or inhibition of protein degradation rather than enzyme activation. This has also been observed in the present study with mung bean seedlings (p. 109). However, light is known to convert protochlorophyllide to chlorophyllide by a photochemical reaction (Bradbeer, 1969) and, from the magnitude of chlorophyll synthesis in comparison with the low protochlorophyllide content of etiolated leaves, there may also be an increase in the activity of the enzyme responsible for chlorophyll synthesis. This substantiates the findings with mung bean seedlings that chlorophyll synthesis (as measured by total chlorophyll) was greatly increased on illumination.

Abbreviations

PB	prolamellar body
G	grana
PLS	porous lamellar sheet (thylakoid)
Cr	crystalline
O	open
OG	osmiophilic globules
S	starch

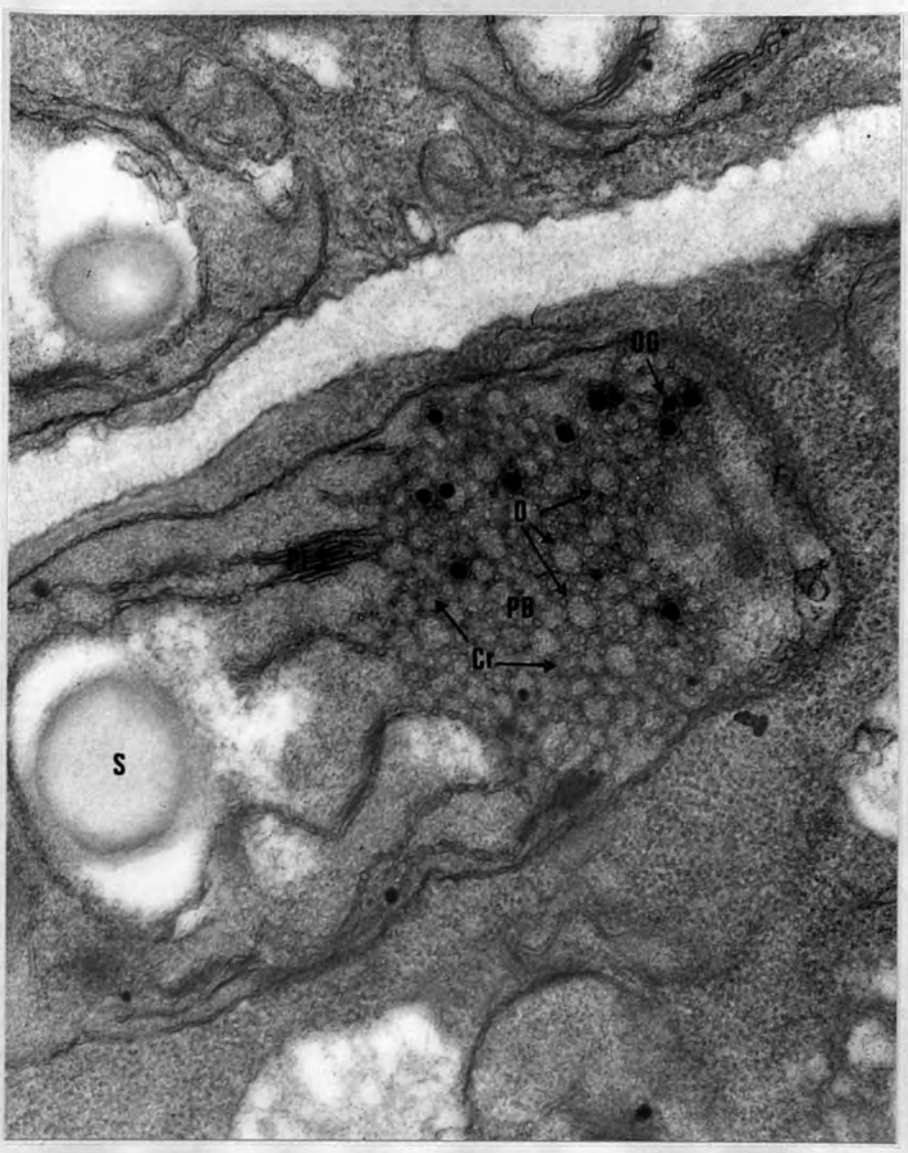

Fig. 1. Plastids of 4-day-old dark-grown mung bean leaf (x 40,000). Some prolamellar bodies (PB) show crystalline (Cr), while others show open (O) forms. There are large starch granules (S) present.

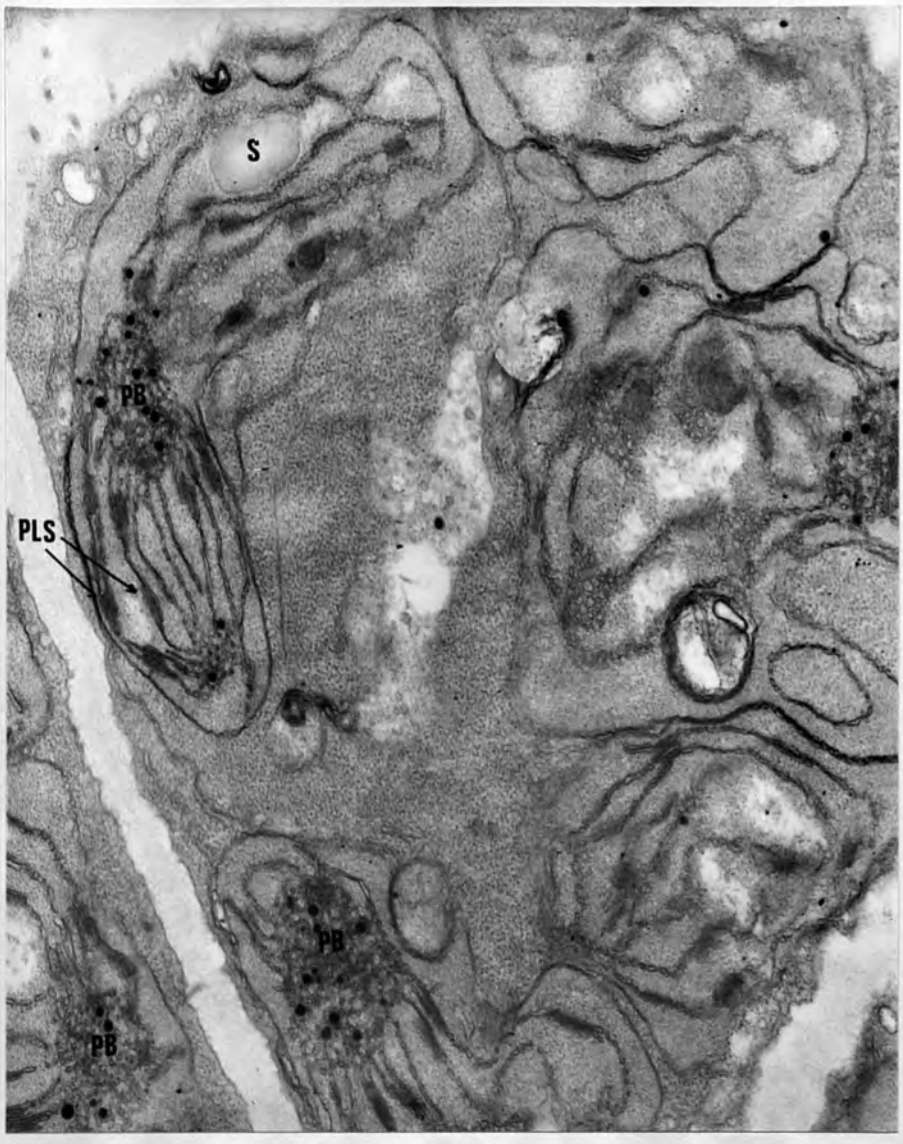

Fig. 2 , Plastids of 4-day-old dark-grown mung bean leaf after exposure to 4 h light (x 24,000). Some plastids still contain at least two prolamellar bodies (PB).

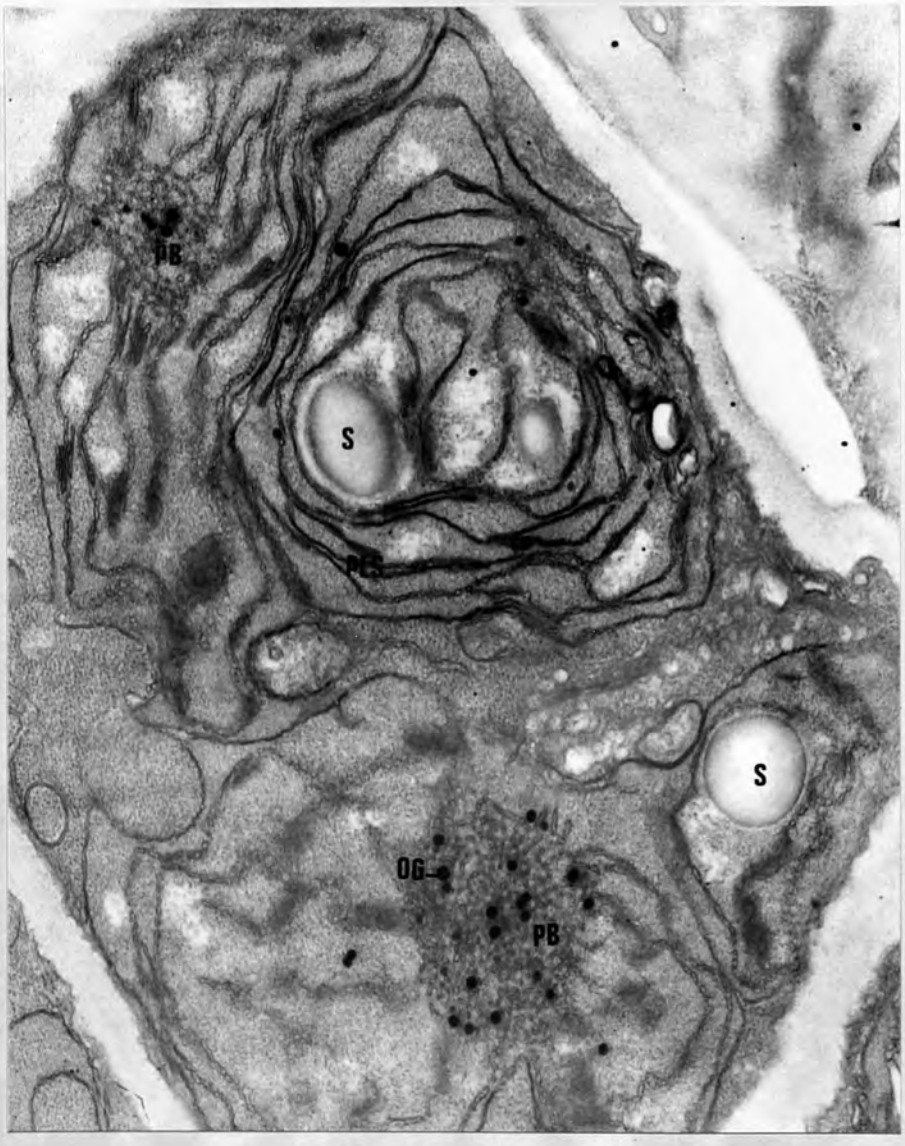

Fig. 3 . Plastids of 4-day-old dark-grown mung bean leaf after exposure to 5 h light (x 24,000). Some of the plastids are condensed with porous lamellar sheet (PLS), and prolamellar bodies (PB) have started to open.

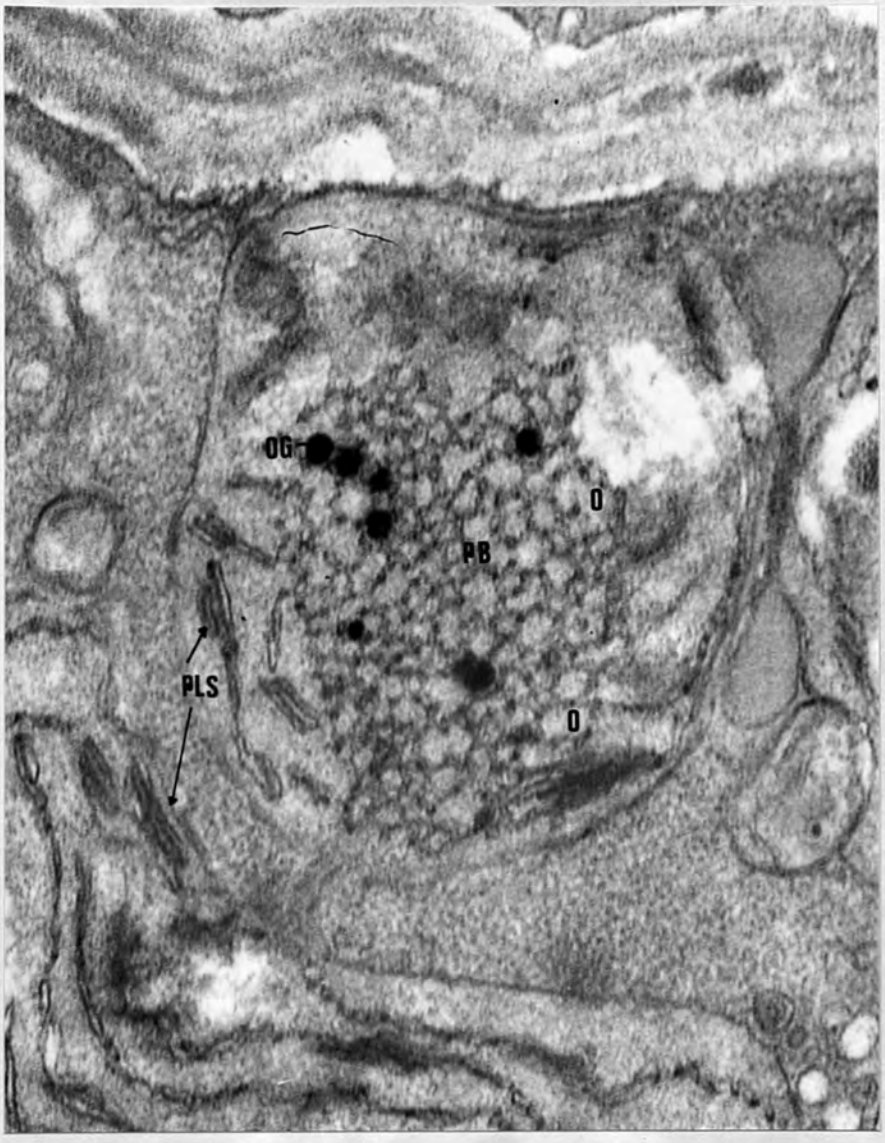

Fig. 4 . A plastid of 4-day-old dark-grown mung bean leaf after exposure to 5 h light (x 63,000). One of the plastids of fig. 3 . greatly enlarged. Most of the prolamellar bodies have swollen and some have already opened (O).

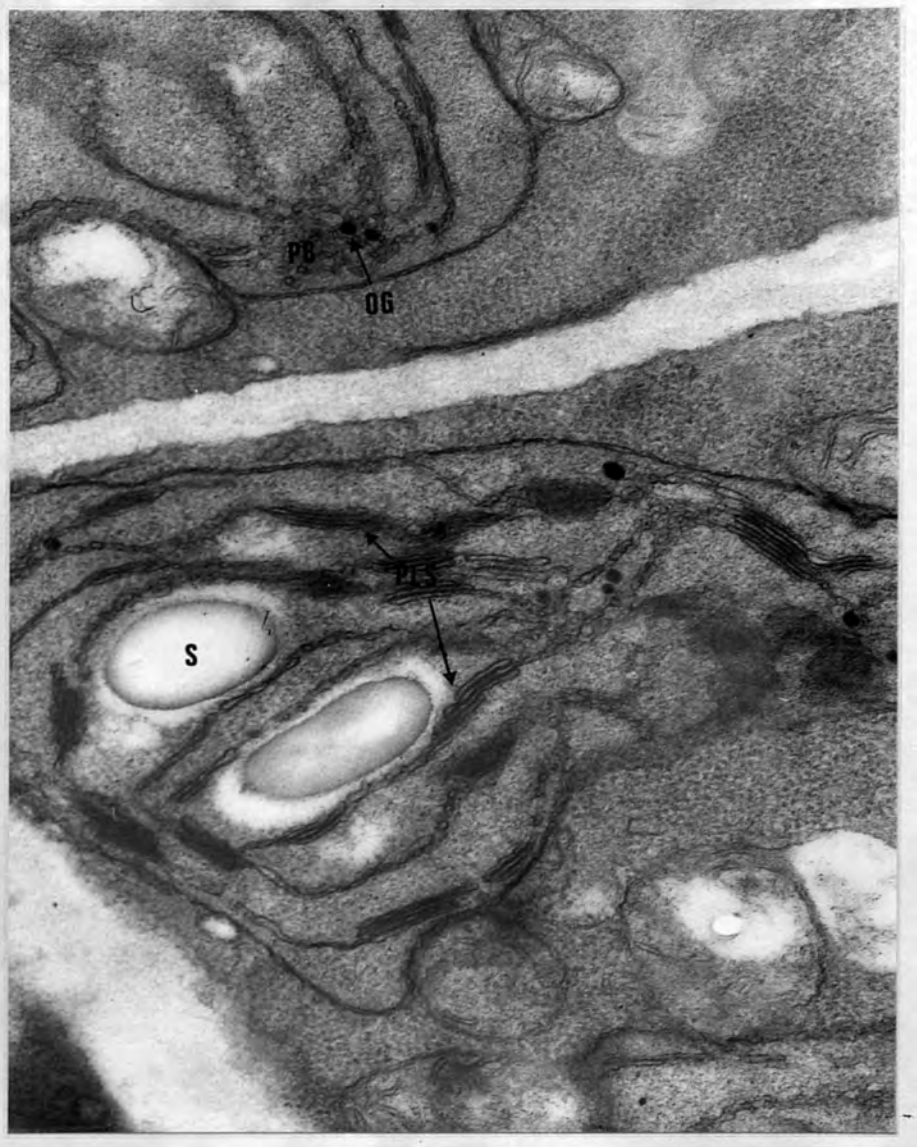

Fig. 5 . Plastids of 4-day-old dark-grown mung bean leaf after exposure to 6 h light (x 40,000). Prolamellar bodies (PB) are beginning to open to form grana.

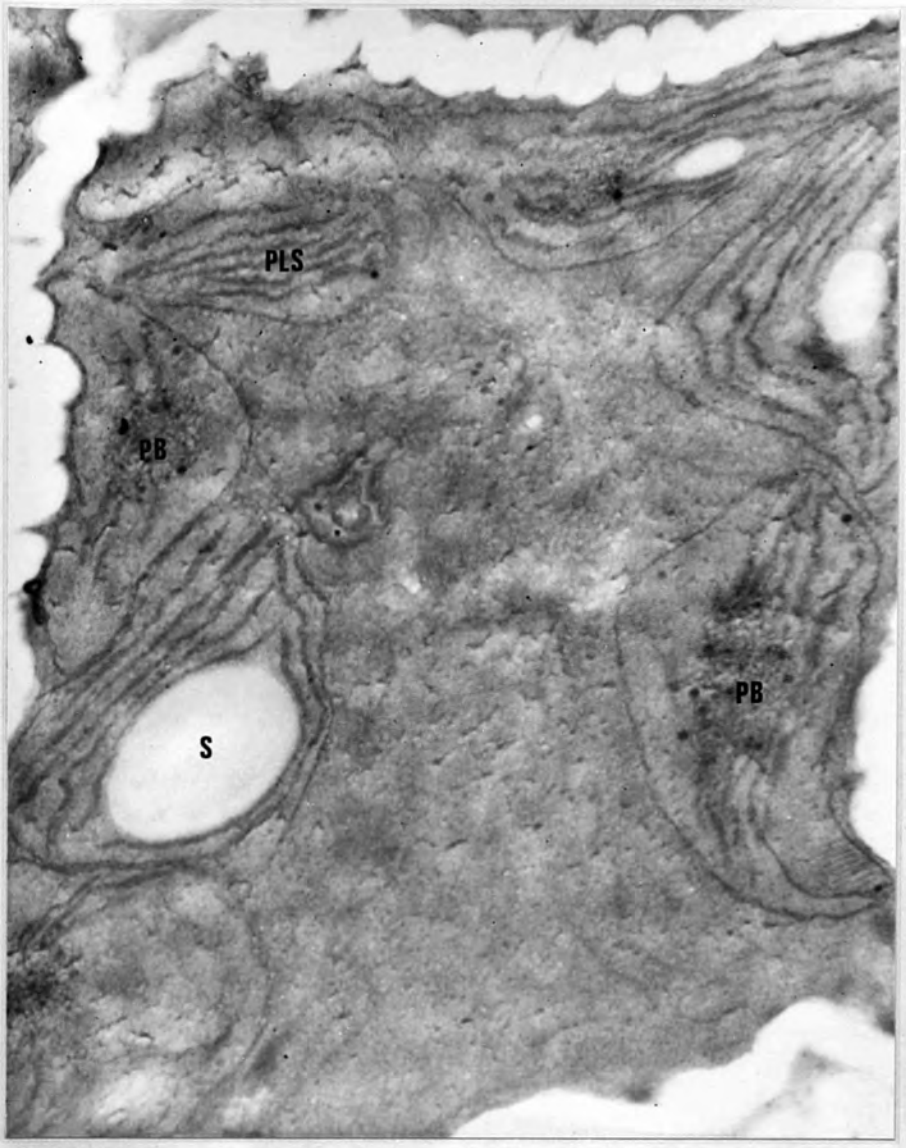

Fig. 6 . Plastids of 4-day-old dark-grown mung bean leaf after exposure to 7 h light (x 24,000). Some prolamellar bodies (PB) are reduced in size and others show grana formation.

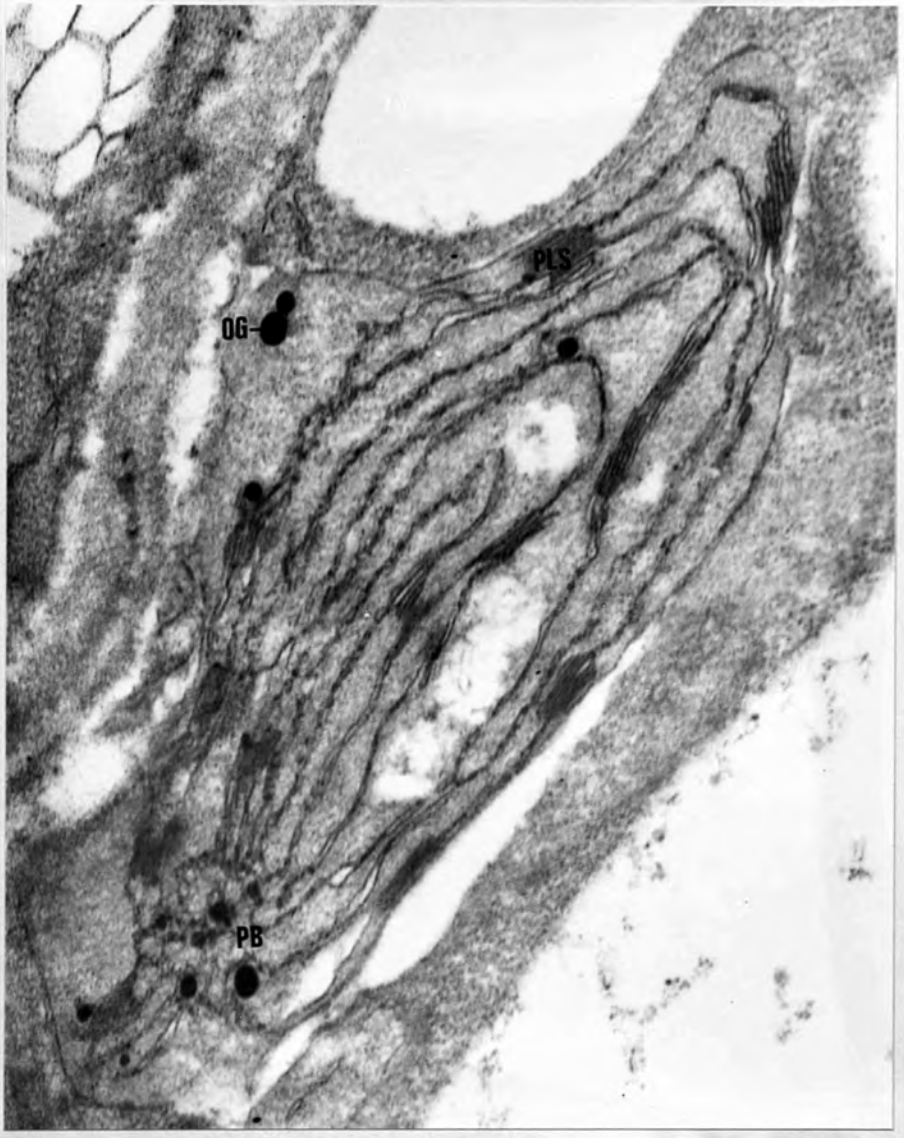

Fig. 7 . Plastid of 4-day-old dark-grown mung bean leaf after exposure to 7 h light (x 31,500). The prolamellar bodies (PB) are almost open and porous lamellar sheet (PLS) is seen on the increase.

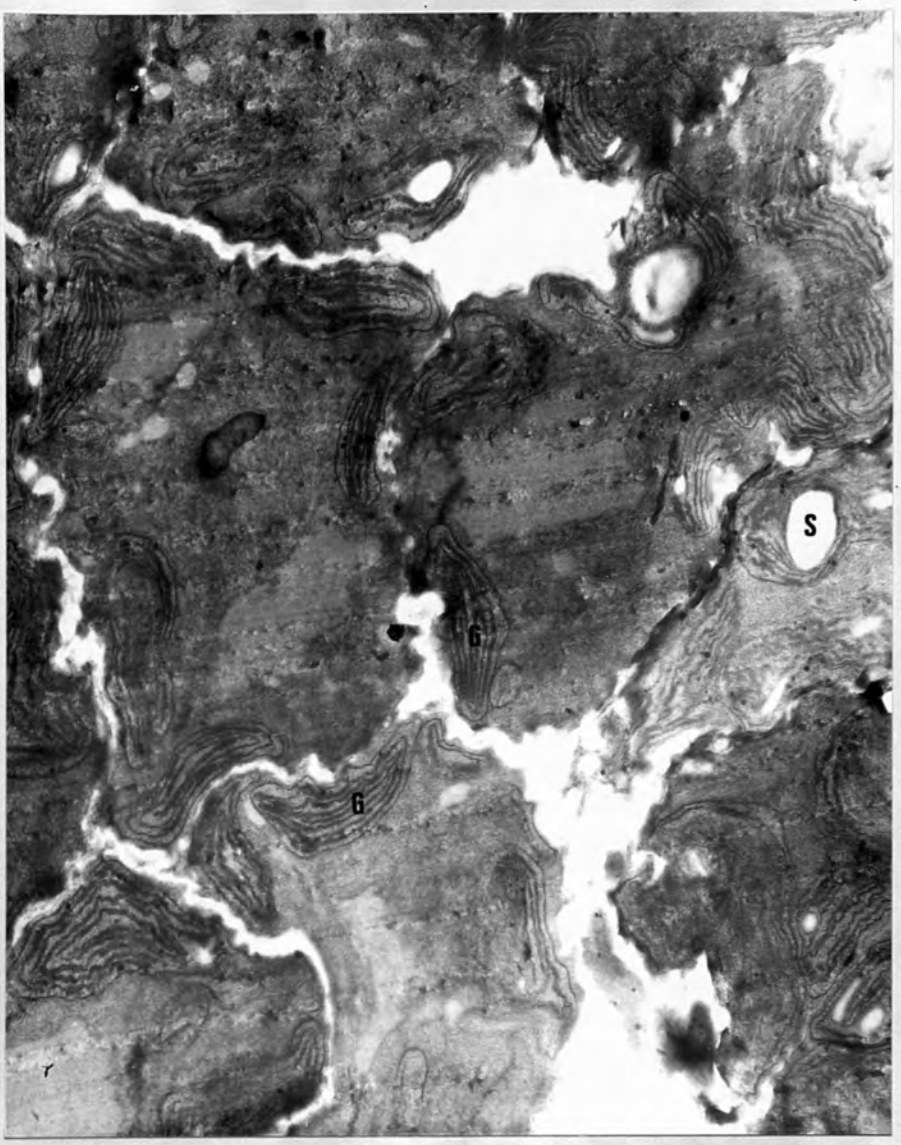

Fig. 8 . Plastids of 4-day-old dark-grown mung bean leaf after exposure to 8 h light (x 12,800). The grana membranes are almost complete.

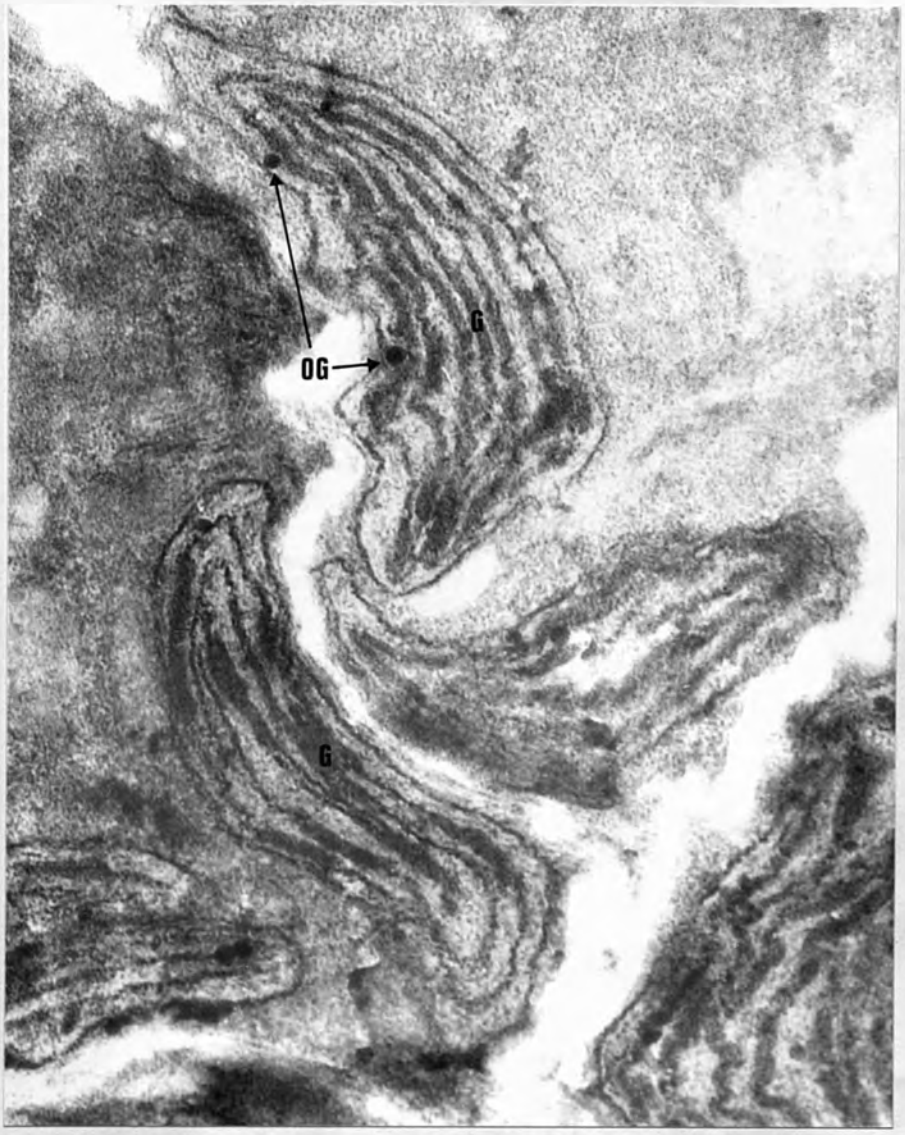

Fig. 9 . Plastids of 4-day-old dark-grown mung bean leaf after exposure to 8 h light (x 51,200). Grana (G) are being formed and fill almost the whole of the plastids.

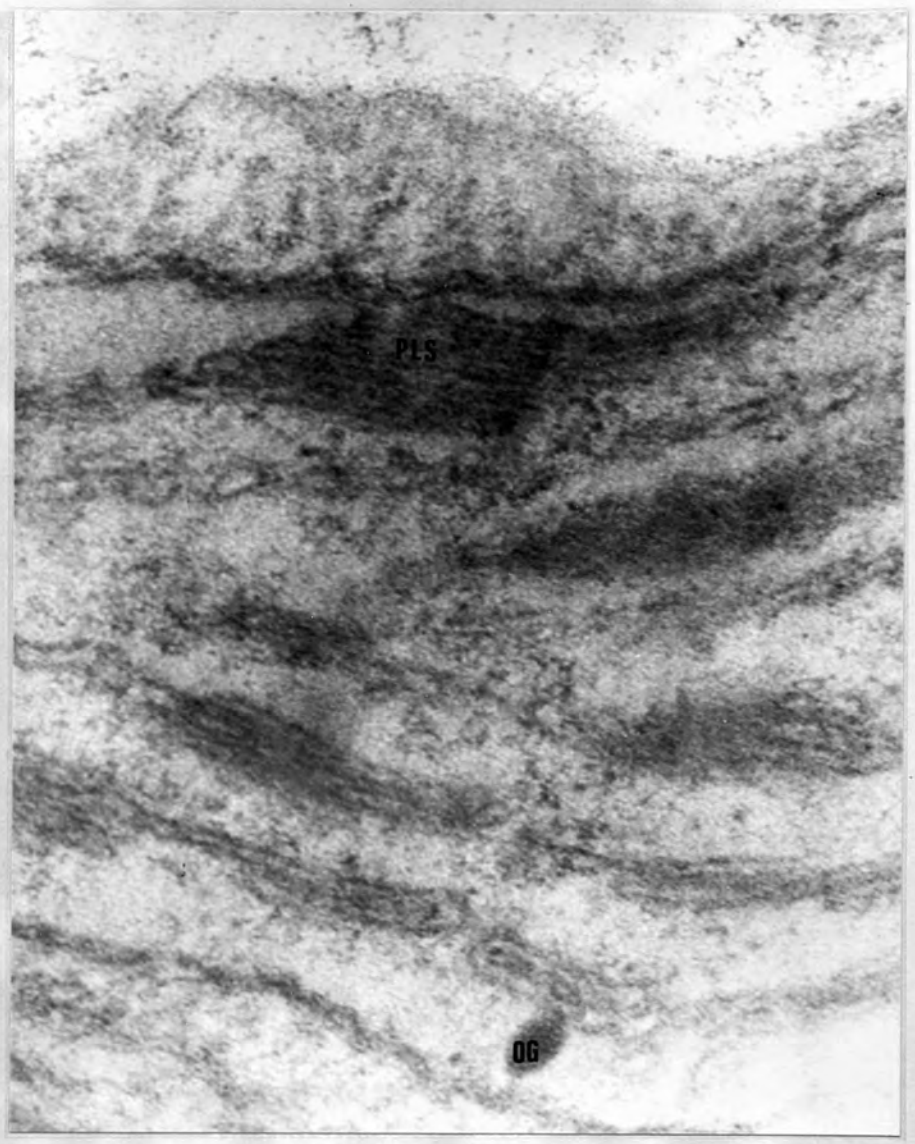

Fig. 10 . High magnification of grana of 4-day-old dark-grown mung bean leaf which has been illuminated for 8 h (x 172,800). The membranes are formed but are still attached to each other.

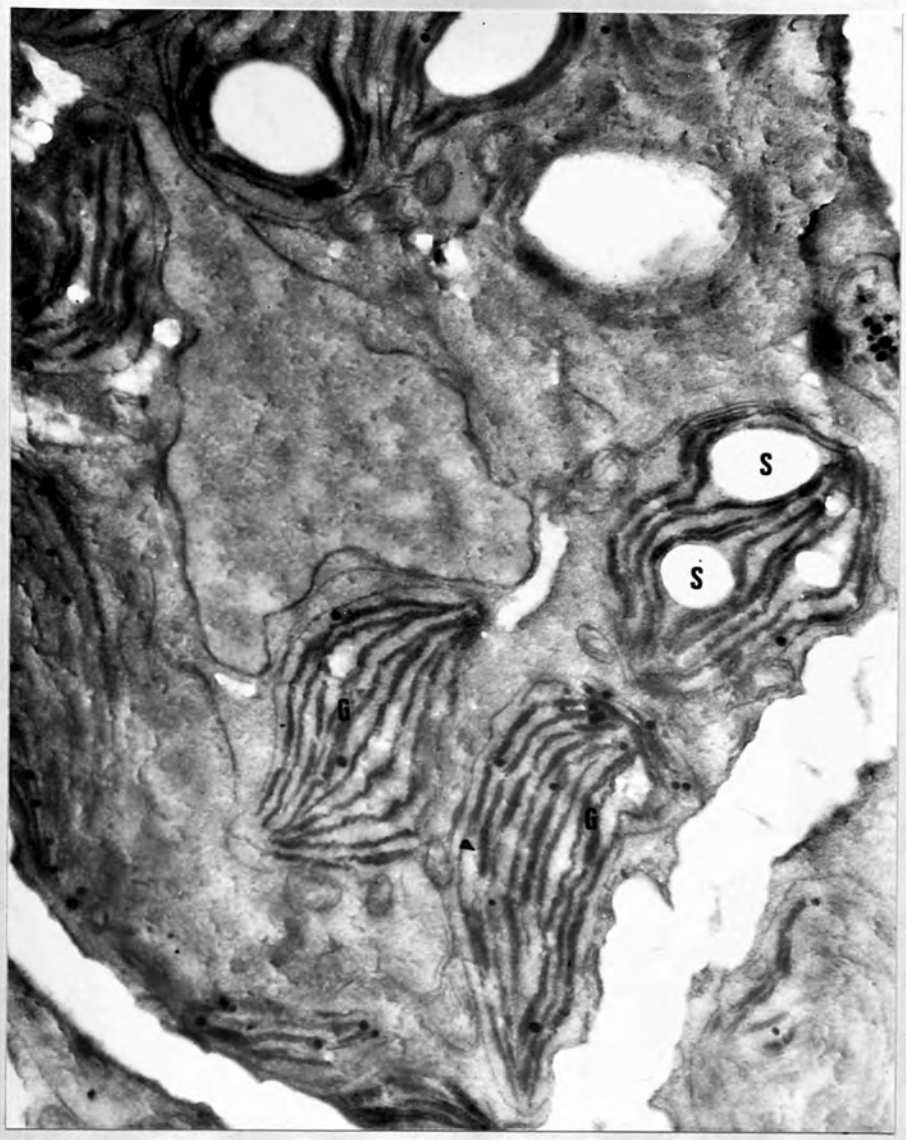

Fig. 11. Plastids of 4-day-old dark-grown mung bean leaf after exposure to 24 h light (x 24,000). Grana membranes (G) are well formed and there are no signs of prolamellar bodies.

CHAPTER III

EFFECT OF LIGHT ON LIPIDS

Introduction

Fatty acids, as is well known, are the backbone of all lipids (p. 34). A variety of fatty acid structures are found in nature and each must be due to a number of enzyme reactions resulting in the accumulation of a particular molecular species. In general terms however a natural fatty acid has a straight chain usually composed of an even number of carbon atoms with a few chemical features at specific positions. C_{16} and C_{18} acids predominate, with unsaturated acids characterised by monoene or methylene-interrupted polyene structures near the centre of the chain (Hitchcock and Nichols, 1971). Nearly all the fatty acyl groups produced by fatty acid synthesising systems are eventually incorporated into acyl lipids via fatty acid transferases, which transport the acids from their site of synthesis to the site of acyl lipid synthesis. Most of the available evidence (Barron and Stumpf, 1962; Sastry and Kates, 1966; Hitchcock and Nichols, 1971; Privett et al., 1973) indicates that L-α-glycerophosphate is the initial acceptor of acyl groups at the primary site of glyceride synthesis, and that all classes of lipids (neutral, phospholipids and glycolipids) are derived from the diacyl-L-(α)-glycerophosphoric acid (PA) thus formed (Fig. 12).

The synthesis of lipid classes through phosphatidic acid (PA) and their interconversions is illustrated in Fig. 13 . In the present study the lipid classes of 4-day-old dark-grown mung bean seedlings, and their changes over a 24 h light period, have been investigated and any correlation with changes in the ultrastructure of plastids attempted.

It has been suggested by Roughan and Boardman (1972) that grana formation is not dependent on a substantial amount of lipid synthesis as the prolamellar bodies of etiolated plants seem to

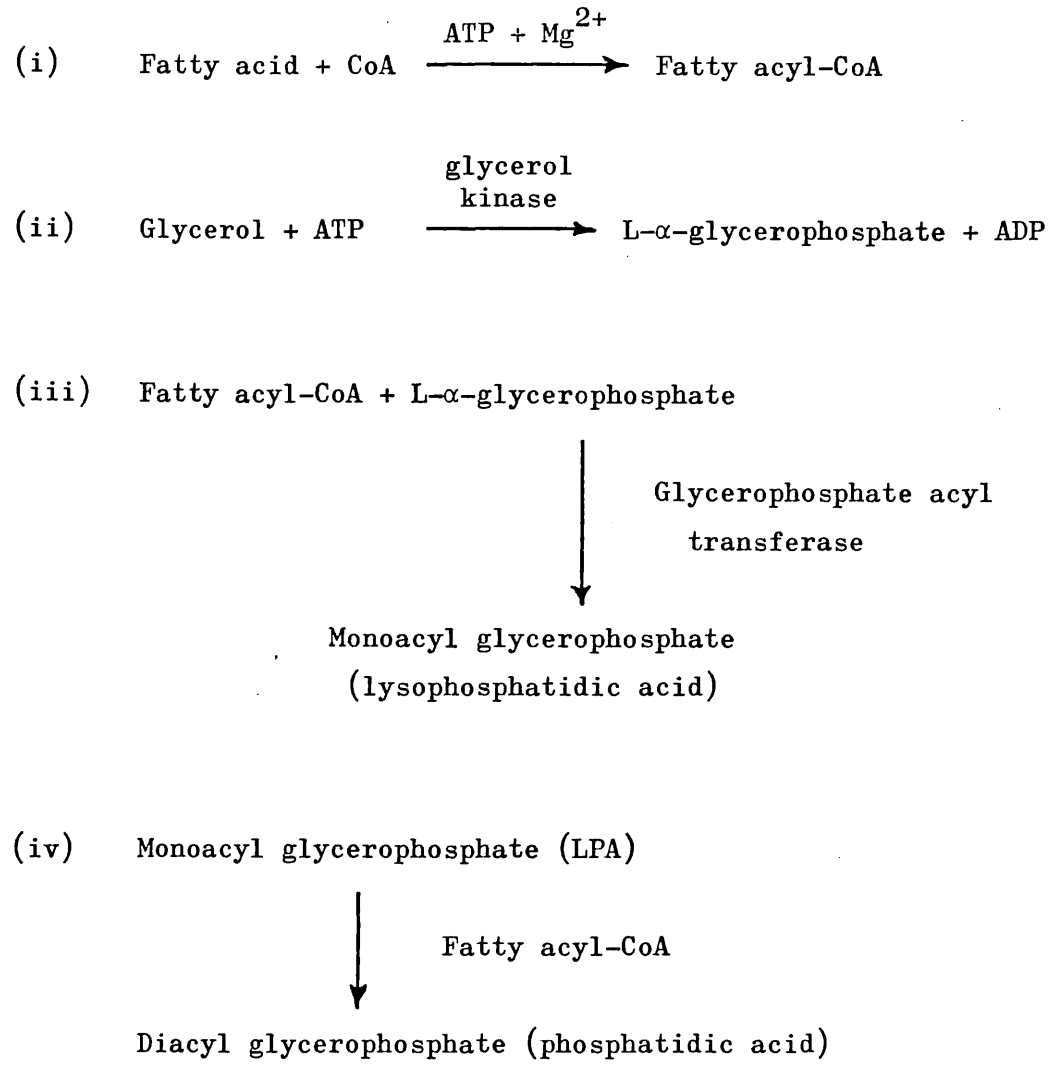

Fig. 12. Biosynthesis of phosphatidic acid (PA)

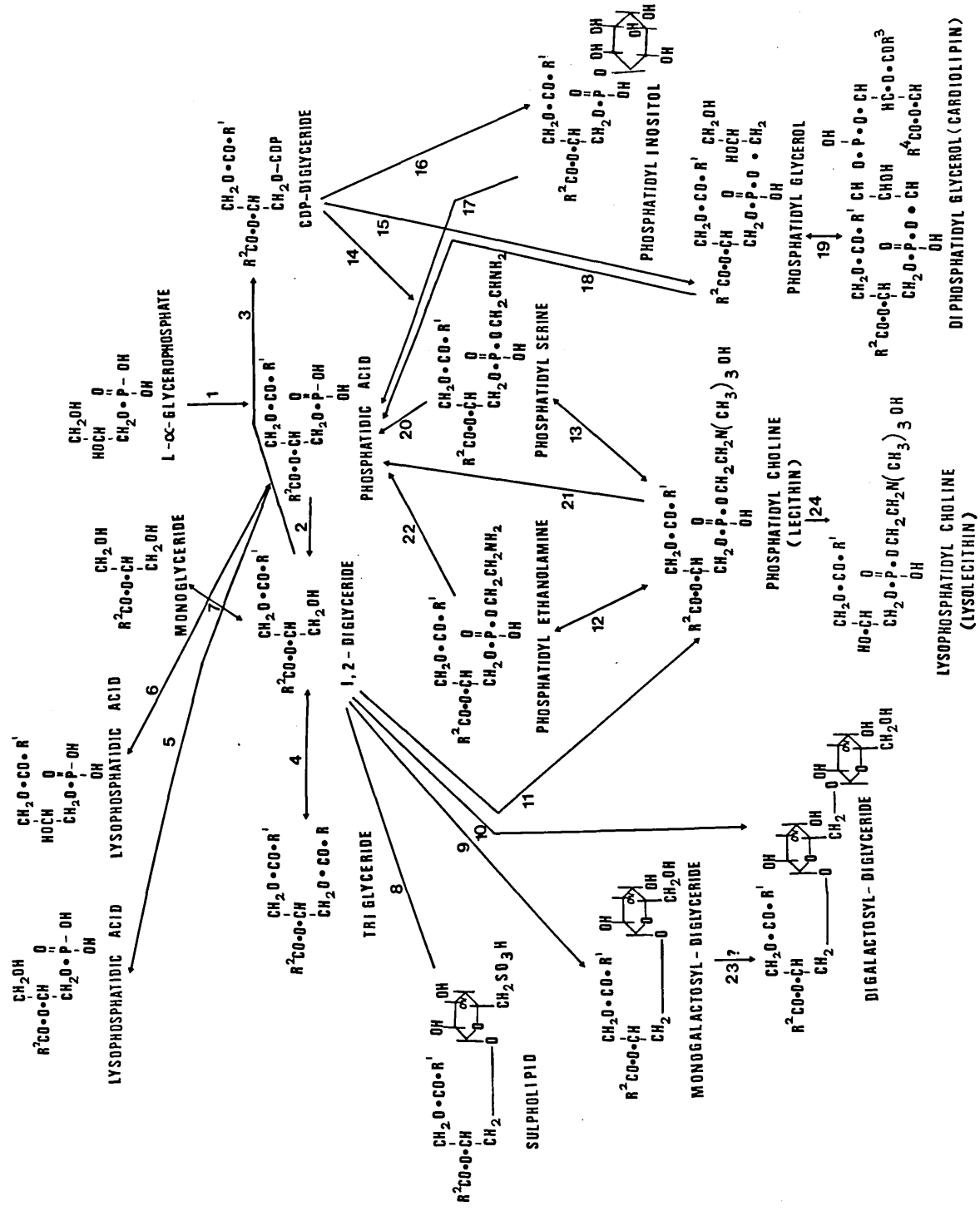

Fig.13. Established and postulated interconversions between classes of plant glycerides. Numbers indicate how the appropriate reactions are taking place. (After Hitchcock and Nichols, 1971).

contain all the essential lipid components. There is presumably therefore an interconversion of these components rather than synthesis of new lipid components. Support for this viewpoint comes from work on etiolated pea seedlings by Trémoliéres and Lepage (1971) who have shown that there are only small changes in lipid components on illumination.

Lipid Extraction

Reagents: Chloroform:methanol, analytical grades mixture (2:1) (Bligh and Dyer, 1959)

Materials: 40 g shoots or seed
400 ml chloroform
200 ml methanol

Procedure:

The lipid extraction of seedlings due to enzyme activity in the leaves and in the hypocotyls is more difficult than from seeds. The most common active enzymes during extraction are phospholipase-D and those which catalyse the hydrolysis of the galactosyl diglycerides. Phospholipase-D activity is activated by many organic solvents including chloroform so in order to minimise lipase activity during the operation, cold isopropanol or hot methanol was used.

The seedlings were macerated in 100 ml of hot methanol for 2 - 3 min and transferred to a Waring blender. 200 ml Chloroform was added and homogenised for 2 min. The homogenate was filtered through a defatted Whatman No. 1 filter paper on a Buchner funnel No. 3 with suction. The residue and filter paper were blended with 150 ml chloroform and 100 ml methanol for 30 seconds and filtered. Then blender jar and residue were rinsed with 50 ml chloroform which was added to the filtrate.

The above procedure was applied directly to sample with 80% water content (i.e. shoots). In cases where the moisture content was much less than 80% (i.e. seeds ≡ about 10%) more water had to be used and it was necessary to add 30 ml distilled water to 40 g seeds.

Washing of crude extract

The extract was transferred into a separating funnel and mixed thoroughly with 80 ml (i.e. 20% of 400 ml chloroform) of 0.9%

NaCl solution. The volume of this solution is very critical as excessive amounts would form emulsion and insufficient volume would leave non-lipid material behind in the final extract.

The above mixture was left for 3 - 4 h to allow two layers to form. The lower phase (chloroform layer) contained the lipids, and the upper phase (methanol-water) contained non-lipid material. The lower phase was collected in a measuring cylinder and the last few millilitres were left behind to ensure complete separation. One-third of the total chloroform layer was taken for determination of total lipids and reduced on a rotary evaporator at about 40°C to 2 - 3 ml and then reduced to dryness under nitrogen (Folch et al., 1956). After weighing, a small volume of chloroform was added to the flask to detect the presence of (insoluble) non-lipid material. If non-lipids were present the chloroform was carefully decanted and the flask rinsed three times with chloroform. The dry weight of the residue was determined and subtracted from the initial weight. The lipid content of the sample was calculated as follows:

$$\text{Total lipid} = \frac{\text{wt. of lipid in aliquot x vol. of chloroform layer}}{\text{vol. of aliquot}}$$

Preparation of sample for thin-layer chromatography (TLC) and gas-liquid chromatography (GLC)

The remaining two-thirds of total chloroform layer was reduced to dryness as above and dissolved in 2 ml of benzene : amyl alcohol : chloroform (1:1:1 v/v) with 0.01% butylated hydroxy-toluene (BHT) to prevent oxidation, and stored at -20°C.

Separation, identification and quantitative estimation of lipid classes by thin-layer chromatography (TLC)

Lipid classes both simple and complex were separated by TLC. Simple lipids such as monoglycerides, diglycerides, triglycerides, sterols, free fatty acids, fatty acid esters and sterol esters

were separated by single-dimensional TLC from complex ones such as phospholipids and glycolipids. Complex lipids were further separated either by single-dimensional TLC using one or two solvent systems or by two-dimensional TLC. Individual lipid classes were identified using different reagents. Charring procedure, followed by scanning densitometry, were used to estimate lipids separated by TLC.

Chromatographic procedure

Thin-layer plates (20 cm x 20 cm) plastic-backed, coated with a layer of 0.25 mm thick silica gel H with fluorescent indicator type Polygram Sil G PG 22/2020 supplied by Macherey-Nagel & Co. Ltd. were used.

The plate margins were scraped free of silica gel at the sides (0.5 cm to 1.0 cm) and the top (0.5 cm) to prevent contamination of plates during development and handling. The samples were applied by graduated microsyringes as bands 1.5 cm long and 0.5 cm apart along a line of origin, 1 cm above the lower edge of each plate. Application of between 10 - 15 µl were found to be suitable. When applying samples the application 'band' was kept narrow (less than 2 mm wide) in order to obtain the best resolution. The drying of samples at the application zone was facilitated by a cold-air blower. Heating was avoided as oxidation would undoubtedly take place.

Separation of simple lipids into different fractions

For this purpose two different solvent systems were used:

A - Hexane : diethyl ether : formic acid (80:20:2 v/v)
B - Benzene : diethyl ether : acetic acid (80:10:0.2 v/v)

Better separation was achieved by solvent system A and different fractions from origin line to the solvent front were as indicated (Fig. 14).

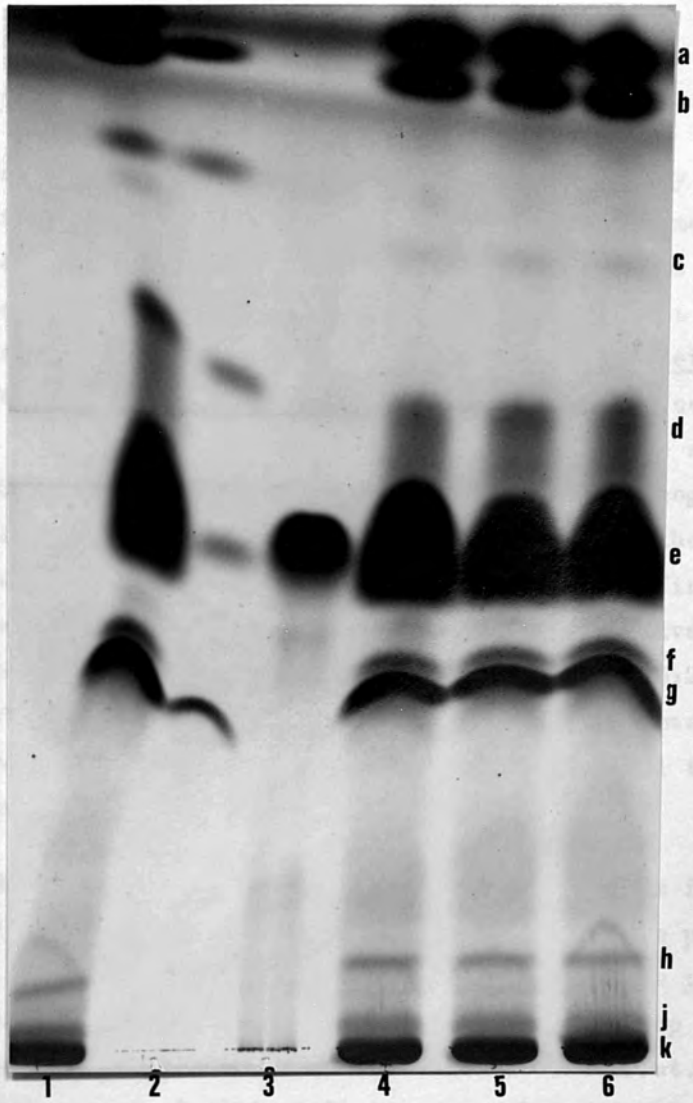

Fig. 14. TLC separation of simple lipids of mung bean seedlings on silica gel H layers. a, hydrocarbon; b, sterol esters; c, fatty acid esters; d, triglycerides; e, free fatty acids; f, stigmasterol; g, β-sitosterol; h, diglycerides; j, monoglycerides; k, complex lipids.

1, sample + b, c, d and g; 2, b, c, d and g; 3, e; 4, sample + e; 5, sample + d; 6, sample.

Developing solvent: hexane-diethyl ether-formic acid (80:20:2 v/v).

One-dimensional TLC of complex lipids

One-dimensional TLC of complex lipids was necessary for the quantitative estimation of lipid classes. Therefore more work had to be carried out and a variety of solvent systems with slight modificationshave been used. The first solvent system was chloroform : methanol : water (65:25:4) (Biserte et al., 1964). With this solvent quite a number of problems occurred. Firstly, the lipid classes tended to move near to the solvent front. Secondly, cardiolipin, phosphatidic acid, monogalactosyl-diglyceride and cerebrosides were overlapping each other, and worse, were not separated from neutral lipids. The first problem was overcome by reducing the water in the solvent, so a new mixture of chloroform : methanol : water (65:25:2) was tried and the lipids were better separated, but the overlapping problem was still there. Therefore a one-dimensional two-step system was found to be necessary.

In this procedure the plates were developed in the first solvent (pre-washing mixture) right to the top of the plate and dried in a vacuum oven for 30 min at room temperature then placed into the second solvent system and developed up to 3 cm below the plate top in the same direction as the first. A substantial amount of neutral lipid was washed off and a better separation was achieved by this system. Several different solvent systems were used as follows:

First solvent: (pre-washing mixture) of pyridine : petroleum ether (60° - 80°) (3:1 v/v)

Second solvent: chloroform : methanol : pyridine : 2M NH_4OH (35:12:65:1) (Skipski et al., 1966)

This solvent system did not give good separation so it was discontinued. The best separation was achieved by the following systems:

System I

First solvent: petroleum ether (60° - 80°) : acetone (3:1 v/v)

Second solvent: chloroform : methanol : water (65:25:3 v/v)

With this system the lipid classes above phosphatidylethanolamine such as cardiolipin, phosphatidic acid, monogalactosyl diglyceride, cerebrosides and digalactosyl diglyceride, tended to stay close to each other and the lipids below phosphatidyl ethanolamine such as phosphatidyl serine, phosphatidyl inositol, phosphatidyl glycerol, phosphatidyl choline and lysophosphatidyl choline, separated well from each other (Fig. 15).

System II

First solvent: (pre-washing mixture) petroleum ether (60° - 80°) : acetone (3:1 v/v)

Second solvent: chloroform : methanol : acetic acid : water (80:13:8:0.3) (Skipski and Barclay, 1969)

With this system the reverse of the previous solvents was observed, i.e. the lipids above phosphatidyl ethanolamine separated well, while those below did not separate so well (Fig. 16), so quantitative measurements were taken using a combination of these two solvents. Lipids above phosphatidyl ethanolamine were determined using solvent system II and those below, solvent system I.

Two-dimensional TLC for separation of complex lipids

It was shown in the previous section that none of the one-dimensional TLC systems separate all phospholipids and glycolipids. Therefore several two-dimensional systems were used for better separation, identification and confirmation that single

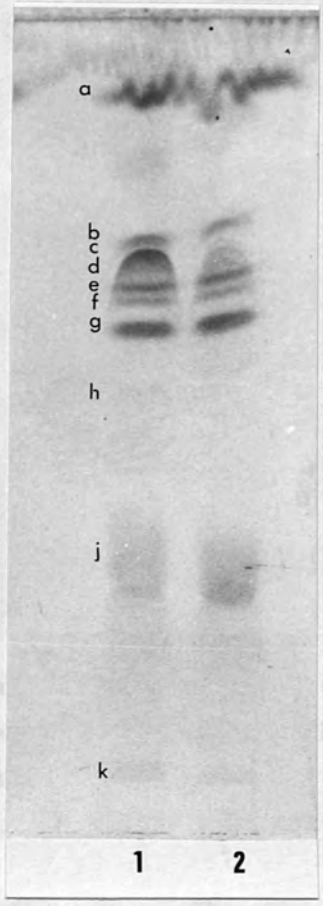

Fig. 15 . One-dimensional two-step TLC separation of complex lipids of mung bean seedlings on silica gel H layer. a, NL; b, CL; c, PA; d, MGDG; e, CMH; f, PG; g, PE; h, DGDG; j, PC; k, LPC. 1 and 2 are both samples. Prewashed solvent: petroleum ether (60°-80°)-acetone (3:1 v/v). Developing solvent: chloroform-methanol-water (65:25:3 v/v).

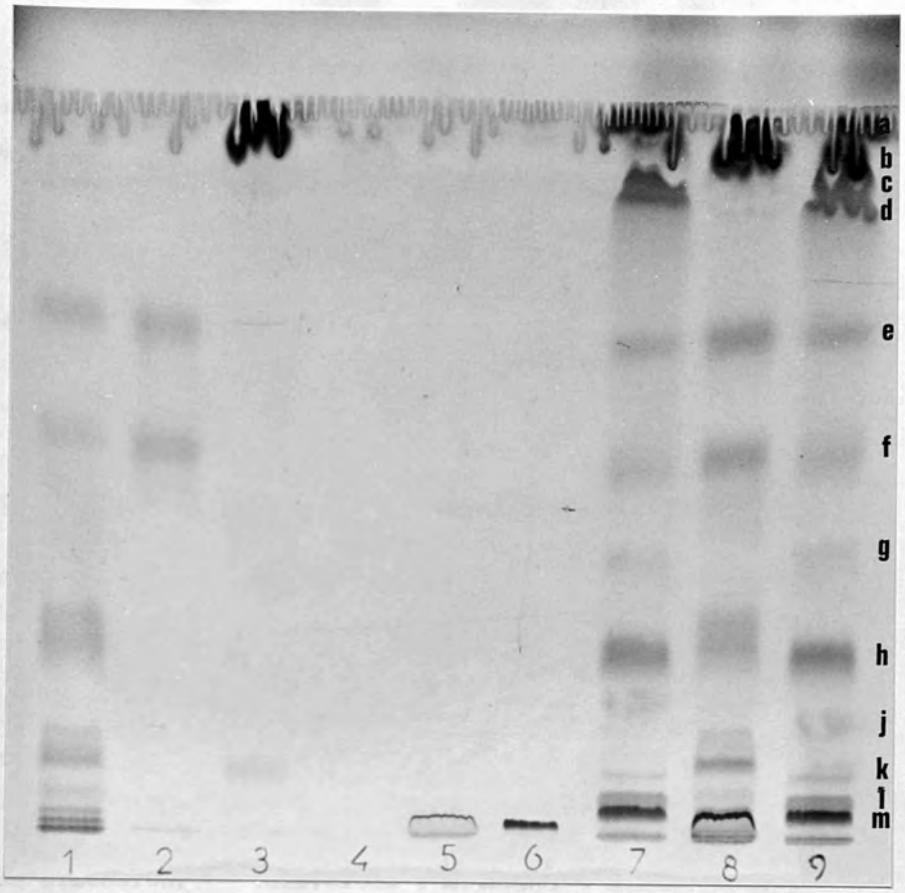

Fig.16. One-dimension two-step separation of complex lipids of mung bean seedlings on silica gel H layers. a, NL; b, SG; c, PA; d, MGDG; e, CMH; f, PG; g, DGDG; h, PE; j, SL; k, PI; l, PC; m, LPC.

1-6 are standards as follows: 1, PE + impurities; 2, CMH; 3, C; 4, CL; 5, LPC; 6, SP; 7, sample; 8, sample + all the standards 1-6; 9, a duplicate sample.

Prewash solvent: petroleum ether (60°-80°)-acetone (3:1 v/v).

Developing solvent: chloroform-methanol-acetic acid-water (80:13:8: 0.3 v/v).

spots did not conceal more than one compound. These different solvent systems are as follows:

First dimension:	chloroform : methanol : NH_4OH (65:35:5)
Second dimension:	chloroform : acetone : methanol : acetic acid : water (100:40:20:20:4) (Privett, et al., 1973)
First dimension:	chloroform : methanol : NH_4OH (75:25:3)
Second dimension:	chloroform : methanol : acetic acid : water (170:25:25:4) (Deven and Manocha, 1975)
First dimension:	chloroform : methanol : 7 N ammonium hydroxide (65:30:4)
Second dimension:	chloroform : methanol : acetic acid : water (170:25:25:6) (Nichols, 1964)

Best separation was achieved by the solvent system of Nichols (1964) as shown in Fig. 17. The plates were first developed in the solvent chloroform : methanol : 7 N ammonium hydroxide (65: 30:4) and the solvent frontwas allowed to run to 3 cm below the top edge of the plates. The plates were then removed from the chromatography tank and dried for 3 min in a vacuum oven at RT or under N_2. After that the plates were rotated anticlockwise 90° and placed in the second solvent, a mixture of chloroform : methanol : acetic acid : water (170:25:25:6).

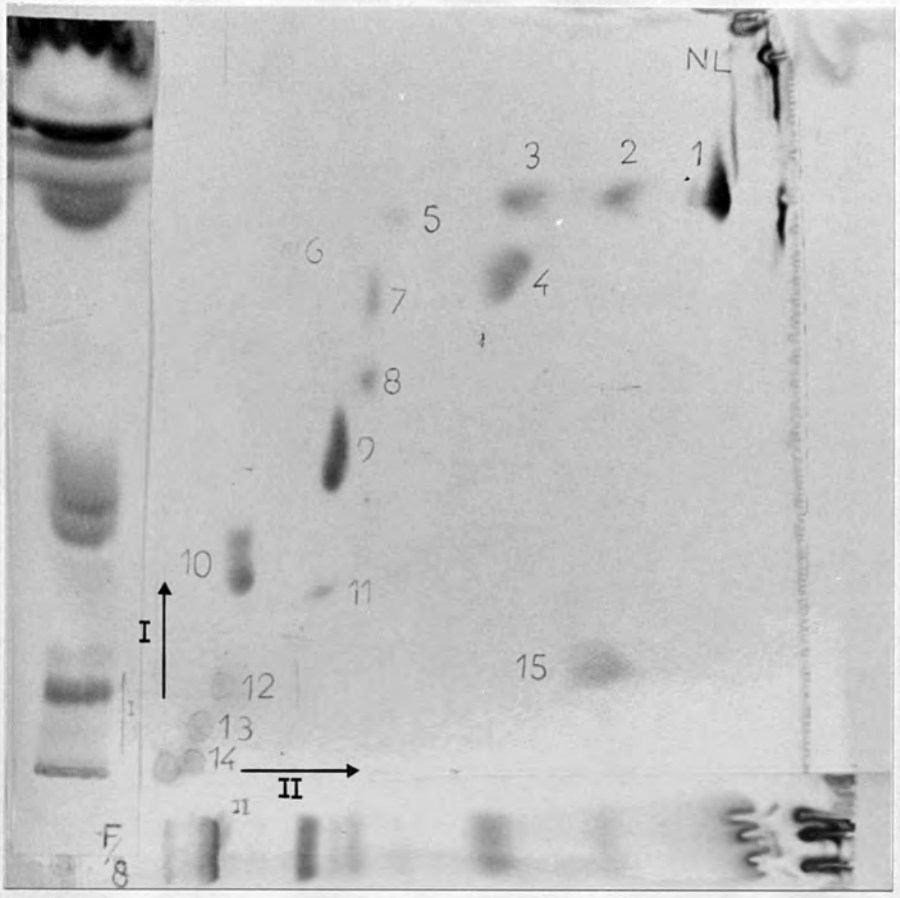

Fig.17. Two-dimensional TLC separation of complex lipids of mung bean seedlings on silica gel H layers.
Solvent I: chloroform-methanol-7N ammonium hydroxide (65:30:4)
Solvent II: chloroform-methanol-acetic acid-water (170:25:25:6)
NL, neutral lipids; 1, SG; 2, CL; 3, CMH; 4, PE; 5, SL; 6, unidentified; 7, MGDG; 8, DGDG; 9, PC; 10, PG; 11, PS; 12, PI; 13, LPC; 14, LPE + LPS; 15; PA. The side parts show the separation of the same samples which were developed in either solvent I or solvent II. The plate has been sprayed with cupric acetate reagent.

Detection and identification of lipids

Lipid classes were detected by destructive or non-destructive specific or non-specific reagents and identified by their migration characteristics relative to authentic standards that were chromatographed under the same conditions as the samples. Where a specific non-destructive reagent was used for identification, it was necessary to remove polar solvents such as water and acetic acid in a vacuum oven at RT or by N_2, otherwise no reaction would be visible.

A. General detection tests

1. Sulphuric acid

The chromatogram plate was sprayed lightly with 50% sulphuric acid (v/v) followed by heating in an oven at 180°C for 20 min. All lipids, as well as other organic non-volatile compounds, formed dark brown spots on the white background. This method is very sensitive for the detection of lipids (Privett and Blank, 1962).

2. 2',7'-Dichlorofluorescein

When a spray of 0.1% (w/v) solution of 2',7'-dichlorofluorescein in 95% methanol was used, most lipids showed up immediately as yellow spots under UV light (Christie, 1973).

3. Iodine vapour

The plates were hung in a development tank containing a trough filled with crystals of iodine. In a few minutes most lipids appeared as brown spots on a pale-yellow background. Iodine vapour was used to distinguish between glycolipids and phospholipids as only the latter were stained significantly under these conditions (Skipski and Barclay, 1969).

4. Rhodamine B

Most neutral lipids and phospholipids were detected by spraying the chromatogram with a 0.05% solution of rhodamine B in 95% ethanol. Red-violet spots with red fluorescence appeared in UV light on a pale-pink background (Biserte et al., 1964).

5. Ammonium molybdate-Perchloric acid

A spray of ammonium molybdate-perchloric acid coloured most lipid spots blue-grey or dark-blue on a white background (Skipski and Barclay, 1969). After spraying, the chromatogram was heated at 80°C for development of colour. This spray was prepared by dissolving 3 g of ammonium molybdate in a mixture of 25 ml of water, 30 ml of NHCl and 13 ml of 60% perchloric acid. The ammonium molybdate-perchloric acid spray was usually used after identifying free amino acid groups by the ninhydrin spray. In this case the background became grey while the spots remained dark-blue.

6. Cupric acetate

The chromatogram was sprayed with a solution of 3% cupric acetate in 8% phosphoric acid and the spots were charred by heating the plate in an oven for 25 - 30 minutes at 180°C. This procedure minimises the evaporation of the volatile material and therefore there is no difference in the intensity of spots between saturated and unsaturated compounds (Privett et al., 1973). This procedure was used for the quantification of lipids by the scanning densitometer (Fig. 16).

B. Specific detection tests

1. Spray for free sterol and sterol esters

Ferric chloride ($FeCl_3.6H_2O$,50 mg) was dissolved in water (90 ml) with acetic acid (5 ml) and H_2SO_4 (5 ml). Plates were sprayed with the ferric chloride reagents and heated at 100°C for 5 - 10 min. The red-violet colour for sterols appeared slightly before that of their esters (Christie, 1973). It was shown that mung beans contained β-sitosterol and stigmasterol.

2. Free fatty acids (FFA)

Free fatty acids were identified by means of a specific sequence of three sprays. Spray 1 was made up of 0.1 g of 2' 7'-dichloro-fluorescein in 95% methanol, spray 2 was a solution of 1% aluminium chloride in ethanol and spray 3 was a solution of 1% aqueous ferric chloride. The plates were warmed up after each spray. Free fatty acids gave a rose-violet colour (Christie, 1973).

3. Esterified fatty acids

Reagents

The hydroxylamine-ferric chloride reagent was used for the detection of esterified fatty acids.

Reagent I: 10 g of hydroxylamine hydrochloride ($HONH_2.HCl$) was dissolved in 25 ml water and diluted to 100 ml with ethanol. It was then mixed with 26 ml of a saturated aqueous solution of NaOH diluted to 200 ml with ethanol. The NaCl precipitates were removed by filtration.

Reagent II: 10 g of ferric chloride ($FeCl_3.6H_2O$) and 20 ml concentrated HCl were ground together in a mortar. This solution was shaken with 300 ml of diethylether.

The plates were sprayed with Reagent I, dried briefly and then sprayed with Reagent II. Purple spots appeared on a yellowish

background (Skipski and Barclay, 1969). Mung beans had very small amounts of esterified fatty acids.

4. Phospholipids

Reagents

The specific reagent for the detection of phospholipids is the molybdenum blue reagent.

Reagent I: 40.11 g of molybdic anhydride (MoO_3) was added to one litre of 25 N H_2SO_4 and boiled gently until the molybdic anhydride was dissolved.

Reagent II: 1.78 g of powdered molybdenum was added to 500 ml of Reagent I and the mixture was boiled gently for 15 min. The solution was cooled and decanted.

Reagent III: Equal volumes of Reagent I and Reagent II were mixed and the combined solution was mixed with 2 volumes of water. This final Reagent III (molybdenum blue reagent) had a greenish-yellow colour and was stable for months.

Procedure

The plates were moistened lightly with the molybdenum blue spray (Reagent III). Phospholipids showed up immediately as blue spots on a white or light blue-grey background (Dittmer and Lester, 1964). The intensity of the spots increased on standing but after several hours the background became dark and obscured the spots. However, the treated plates could be kept for months in the refrigerator after the spots had reached a certain colour intensity (Fig. 18).

5. Glycolipids (orcinol test)

The reagent was prepared by dissolving orcinol (200 mg) in 75% sulphuric acid (100 ml). The whole surface of the plate was wetted by a fine spray of the solution and the plate then heated in an oven at 100°C for 10 - 15 min. Glycolipids appeared

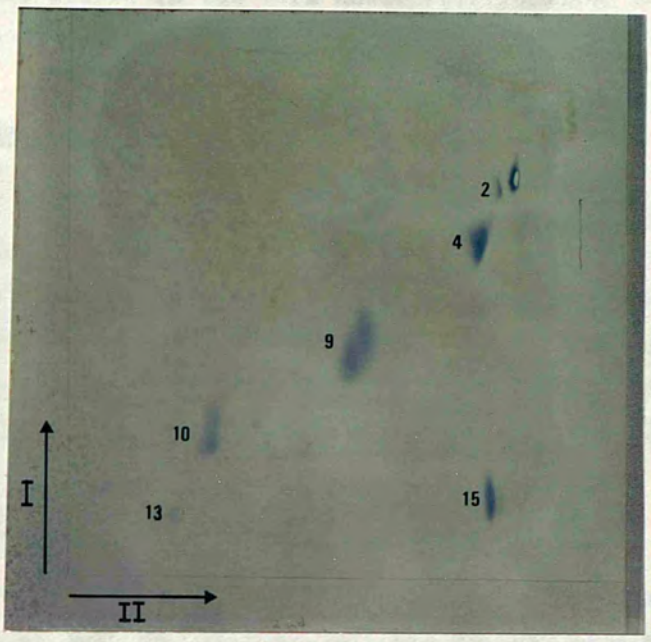

Fig.18 . Two-dimensional TLC separation of complex lipids of mung bean seedlings on silica gel H layer. The plate has been sprayed with molybdenum blue reagent, and the following phospholipids showed as blue spots: 2, CL; 4, PE; 9, PC; 10, PG; 13, LPC; 15, PA.

Solvents: see Fig.17.

as blue-violet spots on a white background (Svennerholm, 1956). The solution was stored in the dark under refrigeration and it was stable for about a week (Fig. 19).

6. Glycolipids (α-naphthol test)

Half a gram of α-naphthol or diphenylamine was dissolved in methanol : water (1:1 v/v) and sprayed on the plate until the surface became wet. After air drying the plate was sprayed lightly with 95% sulphuric acid then heated at 120°C until glycolipids appeared as purple-blue spots, and other complex lipids as yellow spots (Siakotos and Rouser, 1965) (Fig. 20).

7. Free amino groups of phospholipids

The ninhydrin spray was used to detect phospholipids containing free amino groups such as phosphatidyl ethanolamine, phosphatidyl serine and their lyso-derivatives.

The plate was sprayed with a solution of 0.2% ninhydrin in butanol saturated with water. Lipids with free amino groups appeared as red-violet spots when the plate was heated in an oven at 100°C for about 5 min (Skipski and Barclay, 1969).

The ninhydrin spray was also used as a non-lipid (impurities) detection test in the lipid sample extract. If any impurities were present in the sample it would have given a positive reaction with the ninhydrin on the TLC plates, but a negative one with the Cu acetate spray (Privett et al., 1973) (Fig. 21).

8. Sphingolipids

Ceramides and cerebrosides gave positive tests with the Clorox-benzidine spray.

Reagents

Reagent I: 50 ml benzene was mixed with 5 ml Clorox bleach (trade name of commercial bleach active reagent sodium hypochlorite) and 5 ml of glacial acetic acid.

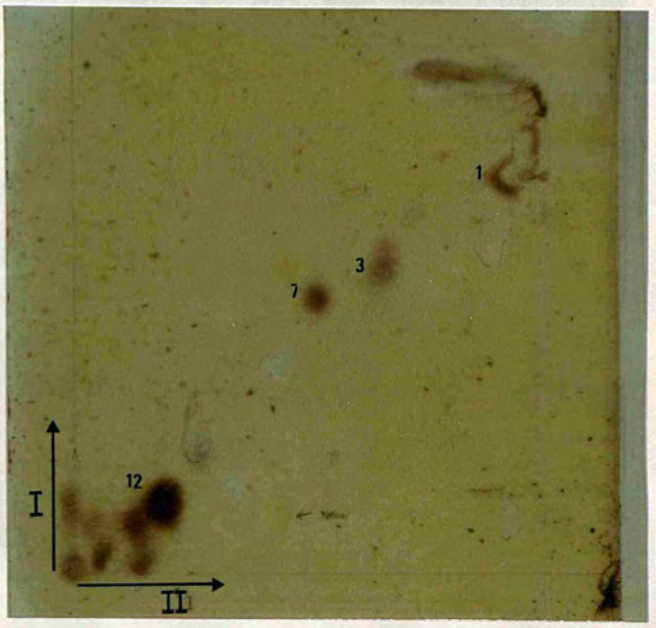

Fig.19. Two-dimensional TLC separation of complex lipids of mung bean seedlings on silica gel H layer. The plate has been sprayed with orcinol reagent and the following glycosides showed up as brown-violet spots: 1, SG; 3, CMH; 7, MGDG; 12, PI. The other spots are impurities.

Solvents: see Fig.17.

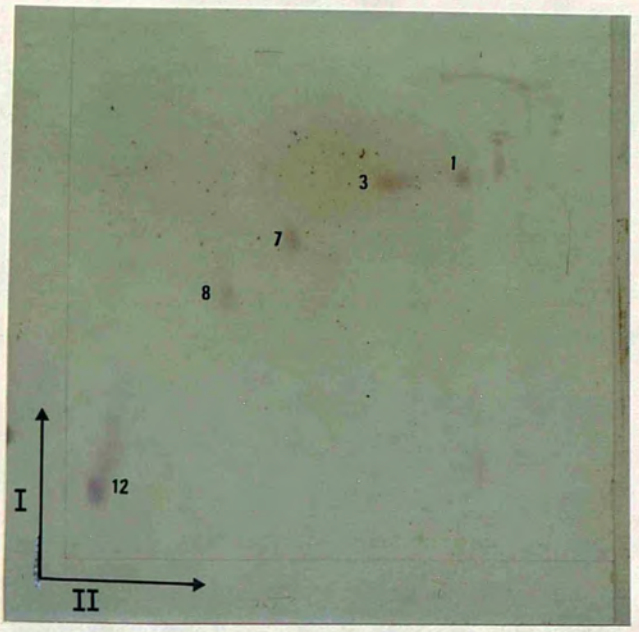

Fig. 20. Two-dimensional TLC separation of complex lipids of mung bean seedlings on silica gel H layer. The plate has been sprayed with α-naphthol reagent, and the following glycosides showed up as purple-blue spots: 1, SG; 3, CMH; 7, MGDG; 8, DGDG; 12, PI.

Solvents: see Fig. 17.

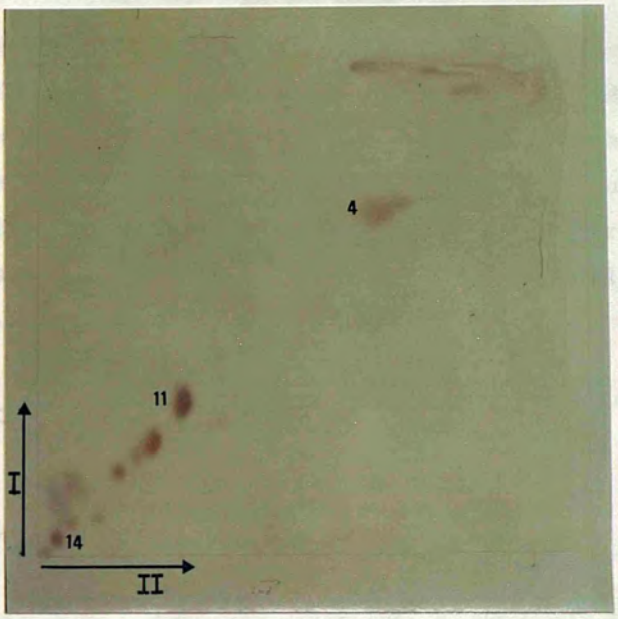

Fig. 21. Two-dimension TLC separation of complex lipids of mung bean seedlings on silica gel H layer. The plate has been sprayed with ninhydrin reagent, and the following phospholipids containing free amino groups showed up as red-violet spots: 4, PE; 11, PS; 14, LPE + LPS. The other spots are impurities.

Solvents: see Fig.17.

Reagent II: 200 ml of benzidine dihydrochloride (benzidine) and a small crystal of potassium iodide were dissolved in 50 ml of 50% ethanol, and filtered.

Procedure

The plate was sprayed immediately with freshly-prepared Reagent I, left at room temperature for 30 min, dried in hot air (this was done in the fume cupboard) for 10 min and then sprayed with Reagent II (this reagent was used within two hours of preparation and protected from direct light). Ceramides and cerebrosides appeared as blue spots almost immediately (Skipski and Barclay, 1969).

9. Choline-containing phospholipids

Phosphatidylcholine and lysophosphatidylcholine gave a positive reaction with the following reagents.

Reagents

Reagent I: 1.7 g basic bismuth nitrate was dissolved in 100 ml of 20% acetic acid.

Reagent II: 40 g of potassium iodide was dissolved in 100 ml water.

Reagent III: 20 ml Reagent I was mixed with 5 ml Reagent II and 70 ml water just before use.

Procedure

After the plate was sprayed with Reagent III the choline-containing phospholipids appeared as orange to orange-red spots immediately or after warming at 40°C for between 5 and 10 min. (Wagner et al., 1961).

10. Compounds with vicinal-diol groups (lipids containing glycol groups)

All lipids of this type which contain glycol groups (two -OH

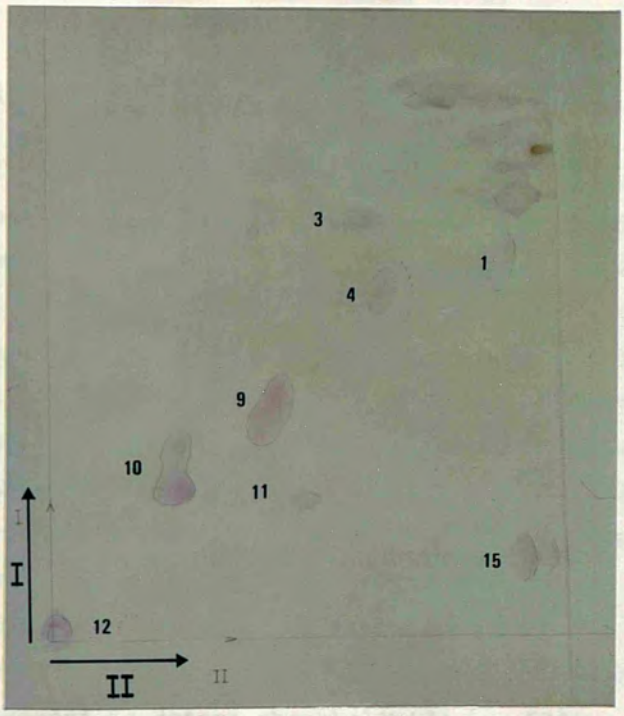

Fig.22. Two-dimensional TLC separation of complex lipids of mung bean seedlings on silica gel H layer. The plate has been sprayed with Schiff's reagent, and the following complex lipids with vicinal diol groups showed up as blue-purple spots: 1, SG; 3, CMH; 4 PE; 9, PC; 10, PG; 11, PS; 12, PI; 15, PA.

Solvents: see Fig.17.

groups on adjacent carbons, e.g. phosphatidylinositol, phosphatidylglycerol, 1-monoglyceride and glycolipids) were detected with the periodate-Schiff's reagent (Shaw, 1968).

Reagents

Reagent I: 0.2% aqueous sodium periodate.

Reagent II: (Schiff's reagent) 0.2 g of pararosaniline (Fuchsin) was dissolved in 85 ml water and 5 ml of 10% sodium bisulphite was added. The mixture was allowed to stand overnight and then decolourised with charcoal and filtered. The plate was sprayed with the periodate solution and left at RT for 15 min after which it was treated with sulphur dioxide to destroy excess reactant. The plate was then sprayed with the Schiff's reagent and treated once more with sulphur dioxide. After a short time glycol-containing lipids appeared as blue-purple spots while some other complex lipids appeared as yellow spots (Fig. 22). After the glycolipids had been made visible by the above technique, the same plates were sprayed with the molybdenum reagent to detect phospholipids (p. 50).

Quantification of lipids

Charring procedure

The plate was sprayed with 3% cupric acetate in 8% phosphoric acid and heated at 180°C for 20 min. All lipid classes were charred (Marsh and Weinstein, 1966) (p. 48).

Measurement of chromatographic zones

Each series of zones was cut out and scanned in a Chromoscan double-beam recording and integrating densitometer (Joyce-Loebl & Co. Ltd.) with quartz iodine light source, blue filter, and 20 cm x 1 cm light slit and sample-holder (Drive in gear ratio was 1:1). The areas of the peaks on the recorder trace are proportional to the amount of lipid originally present in the

sample (Christie, 1973) and authentic standards. Individual spots were estimated using the following formula:

$$\frac{\text{Peak area of standard}}{\text{Peak area of sample}} \equiv \frac{\text{Amount of lipid in standard}}{\text{Amount of lipid in sample}}$$

Charring procedure followed by scanning photodensitometry has a number of disadvantages - the samples are destroyed and lipid classes, which are not always available, are necessary for calibration. Further, there are considerable doubts that there is a linear relationship between the density of a spot and the amount of material present (Christie, 1973). The important feature of this technique is that it gives constant yields of carbon within each class of compounds (Privett et al., 1973), and in these studies where a large number of similar samples had to be analysed routinely this was probably the best method. In preliminary experiments weighings of the spots were carried out and the results obtained by this method were not very different from those obtained by the photodensitometric method.

Gas-liquid chromatography (GLC) of fatty acids

One of the most important applications of GLC are the determination and identification of the fatty acid composition of lipids.

Fatty acids were esterified by the boron trifluoride-methanol (BF_3-methanol) reagent to give their methylesters which were then injected into the GLC columns.

A Pye 'Series 104' with flame ionisation detector, nitrogen carrier gas and full-scale drift per hour of 10^{-12} was used.

Column:

Glass columns (1.5 m long and 2 mm i. diameter) were used. A number of columns with different mesh filters of two polyester stationary phases, polyethylene glygol adipate (PEG-A) and diethylene glycol succinate (DEG-S), were used.

The columns were packed and supplied by Pye Unicam Ltd. and Phase Separation Ltd. Pye Unicam Ltd. supplied three columns with the following descriptions:

1 PEG-A 10% diatomite M. AW (acid washed) 100-120 mesh

2 PEG-A 10% diatomite C. AW 100-120 mesh

3 DEG-S 10% diatomite C. AW 100-120 mesh

Phase Separation Ltd. supplied one column as follows:

1 PEG-A 10% Chromosorb W. AW.DMCS 60-80 mesh (DMCS = dimethyldichlorosilane)

These columns were operated under different conditions such as isothermal temperatures of 150°C, 160°C, 170°C or 180°C, and a temperature programme of 80°C - 180°C and 80°C - 175°C at 8°C min^{-1}. Carrier gas flow rates of 5.4, 12 and 13.3 cm min^{-1} and recorder chart speeds of 25.4 or 76.2 cm h^{-1} were tried. Some problems were encountered with the Pye Unicam Ltd. columns, e.g. the peaks were broad so the fractions did not completely separate from each other, and often gaps appeared in the column phase. The best separation was achieved by the Phase Separation Ltd. PEG-A column under the following conditions:

Temperature: injection 80°C, final 175°C at 8°C min^{-1}

Carrier gas flow rate: 13.3 cm min^{-1}

Chart speed: 76.2 cm h^{-1}

H_2 pressure: 15 p.s.i.

Air pressure: 12 p.s.i.

A typical chromatogram is shown in Fig. 23.

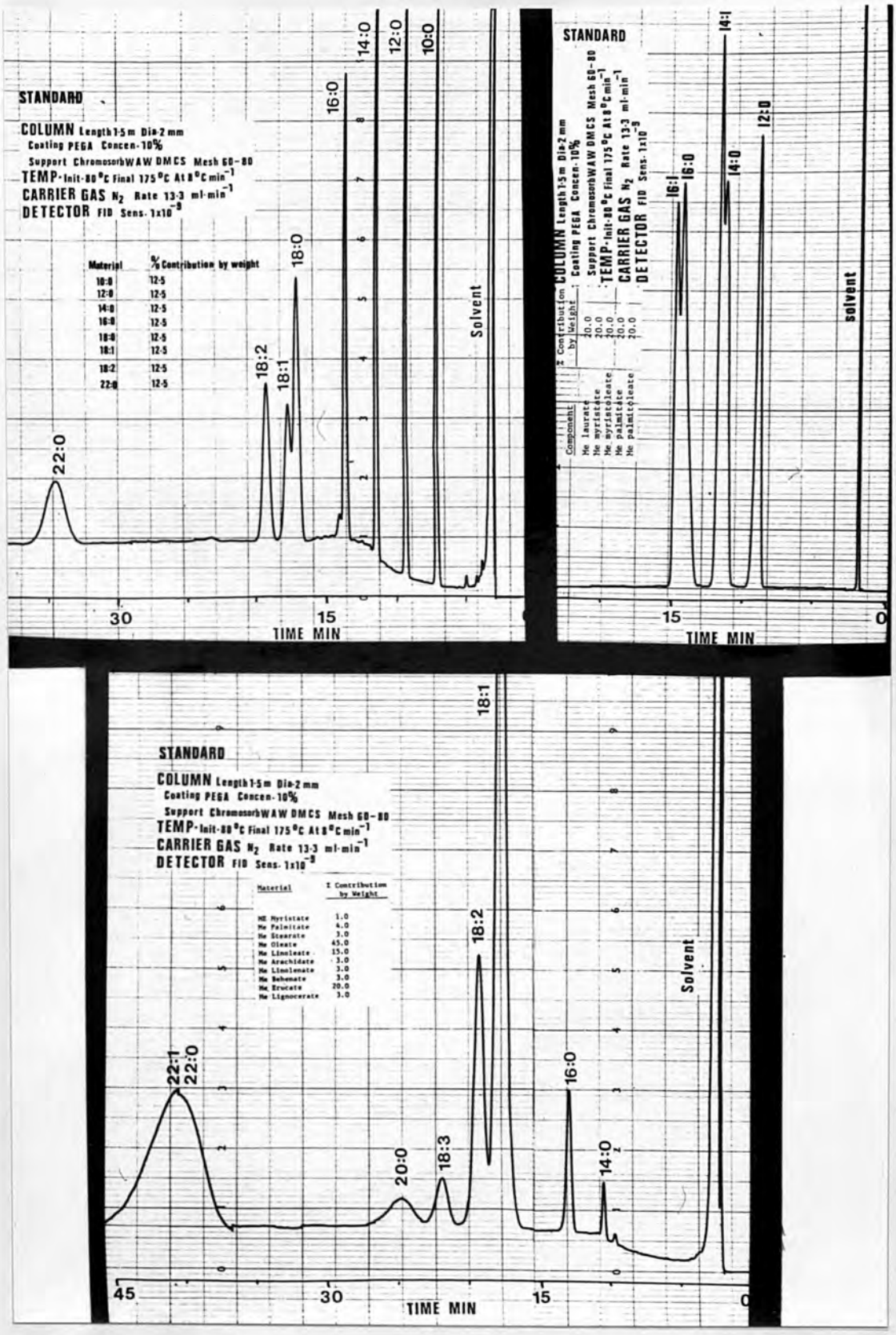

Fig. 23. Gas-liquid chromatogram of standard fatty acid methyl esters which were analysed under the same conditions as the samples.

Fatty acid esterification

One ml of prepared lipid sample (p. 38) was placed into a 5 ml screw-cap bottle and 3 ml of BF_3-methanol reagent, 14% BF_3 in methanol, was added. The mixture was boiled on a water bath for 2 min. The boiled mixture was washed into a 125 ml separating funnel with 30 ml of petroleum ether (BP 40°C - 60°C) reagent grade and 20 ml of distilled water was added. The funnel was shaken vigorously and the layers were allowed to separate. The aqueous-methanol layer was drained off and discarded while the petroleum ether layer was drained through filter paper into a 50 ml evaporating flask and was evaporated on a 60°C water bath. The last traces of solvent were removed under reduced pressure and the residue was taken up in 0.5 - 1 ml benzene and injected into the GLC column (Metcalfe and Schmitz, 1961).

Identification of fatty acids

Fatty acids were identified by their retention time relative to the standards which were analysed under the same conditions as the sample (Fig. 23), but at the low final operating temperature of 175°C limit imposed by the particular columns used, it was difficult to separate C20:0 from C20:1, and C22:0 from C22:1. Nevertheless the following fractions were identified:

C12:0, C14:0, C14:1, C14:2, C16:0, C16:1, C18:0, C18:1, C18:2, C18:3, C20:0, C20:2 and C22.0, although there was some doubt about 14:2 and 20:2 (Fig. 24).

The standards of fatty acid methylesters were supplied by Applied Science Europe B.V. Holland.

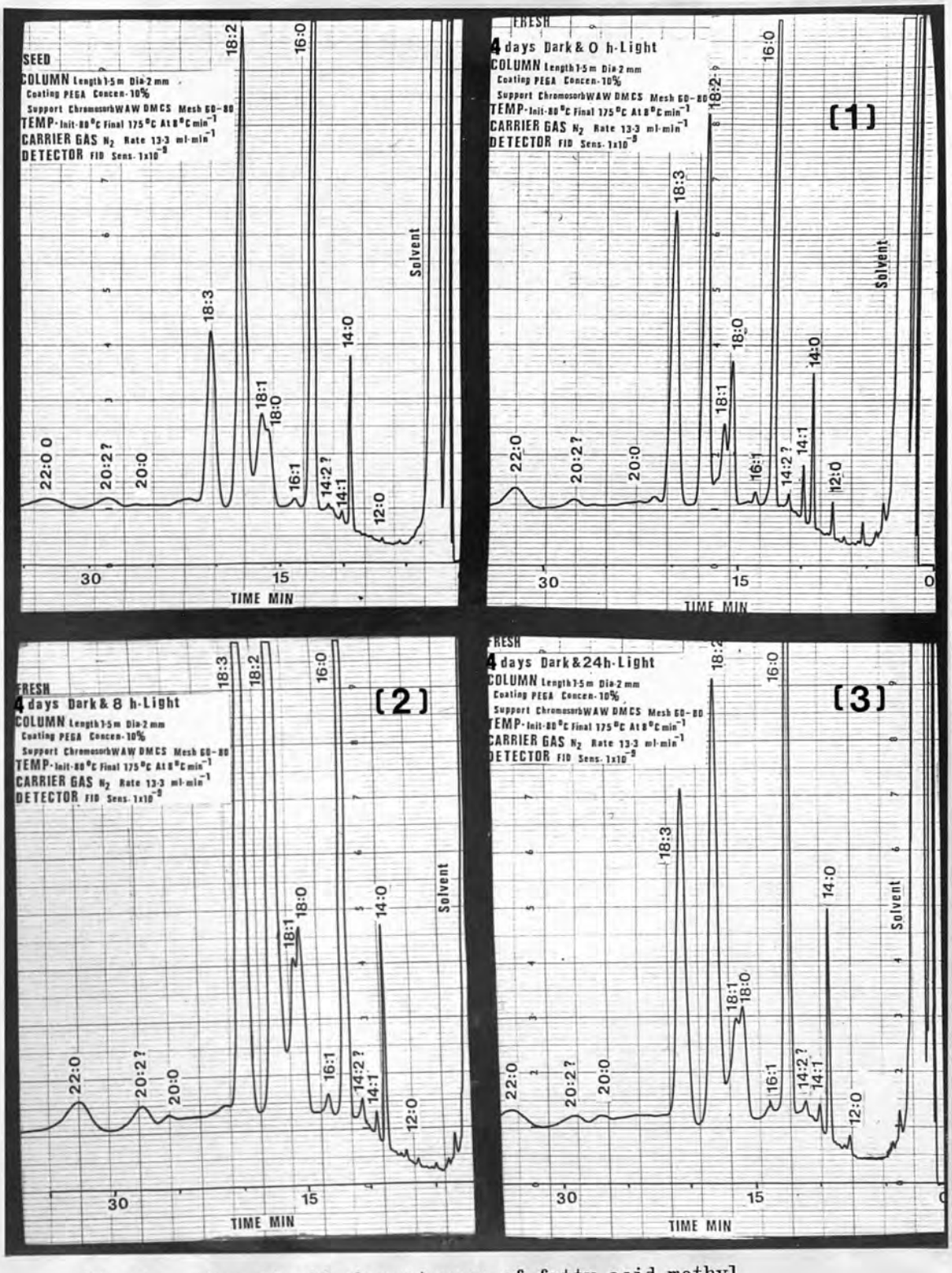

Fig. 24. Gas liquid chromatogram of fatty acid methyl esters of 4-day-old dark-grown mung bean seedlings (1), those subjected to 8 h (2) and to 24 h (3) illumination.

Quantitative estimation of fatty acids by GLC

The areas of the peaks on the recorder trace are proportional to the amount of fatty acid originally present in the lipid sample and standards. So amounts of fatty acid esters were calculated as follows:

$$\frac{\text{Peak area of standard}}{\text{Peak area of sample}} \equiv \frac{\text{Amount of fatty acid in standard}}{\text{Amount of fatty acid in sample}}$$

Peak area = peak height x width at half height

Results

Total lipids of 4-day-old dark-grown seedlings were 0.35 g 100 g^{-1} fresh weight. There was no significant change up to 6 h after illumination but after this total lipids increased and reached 0.64 g 100 g^{-1} fresh weight after 24 h (Fig. 25 and Table 1).

Total weight of simple and complex lipids of 4-day-old etiolated mung bean seedlings increased from 33 mg 100 g^{-1} to 53 mg 100 g^{-1}, and from 317 to 593 mg 100 g^{-1} respectively after 24 h illumination. On the other hand the percentage of each in relation to total lipids showed no significant increase over the same period of illumination, being approximately 10% for simple and 90% for complex lipids.

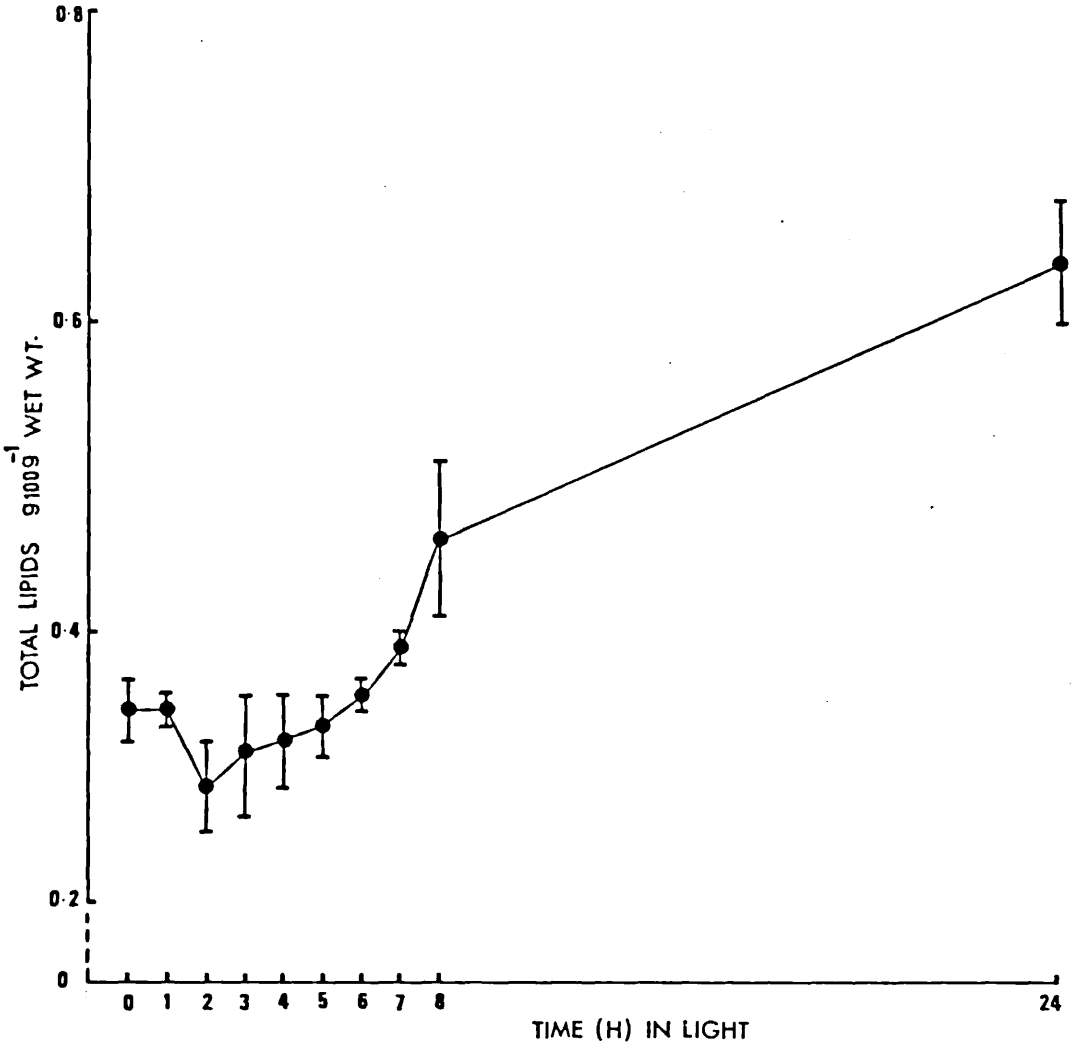

Fig. 25. Total lipids of 4-day-old dark-grown mung bean seedlings illuminated for various times within 24 h

Table 1. The simple and complex lipids of 4-day-old dark-grown mung bean seedlings illuminated for various times within 24 h. The results are expressed as percentage of total unless otherwise stated.

Hours in Light	0	1	2	3	4	5	6	7	8	24
SIMPLE LIPIDS										
Sterol esters	15 ± 1.5	15 ± 1.4	14 ± 1.4	13 ± 2.1	14 ± 1.5	14 ± 1.2	14 ± 1.1	14 ± 1.3	14 ± 2.0	17 ± 1.4
Fatty acid esters	1 ± 1.1	3 ± 1.2	3 ± 0.8	4 ± 0.9	5 ± 0.1	6 ± 0.4	6 ± 0.6	7 ± 0.3	7 ± 0.2	7 ± 0.1
Triglycerides	9 ± 0.4	8 ± 0.6	10 ± 0.4	11 ± 0.2	11 ± 0.5	11 ± 0.6	12 ± 0.4	12 ± 0.3	12 ± 0.2	14 ± 0.1
Free fatty acids	15 ± 0.7	15 ± 0.8	15 ± 0.6	14 ± 0.5	13 ± 0.4	13 ± 0.1	12 ± 0.5	12 ± 0.3	11 ± 0.9	7 ± 1.1
Stigmasterol	10 ± 0.5	10 ± 0.3	11 ± 0.4	12 ± 0.5	12 ± 0.3	12 ± 0.2	12 ± 0.1	12 ± 0.2	13 ± 1.1	15 ± 0.5
β-Sitosterol	36 ± 0.5	36 ± 0.4	35 ± 0.3	33 ± 0.6	33 ± 0.6	32 ± 1.1	32 ± 1.2	31 ± 1.0	31 ± 1.3	28 ± 1.8
Diglycerides	7 ± 0.5	6 ± 0.4	5 ± 0.3	5 ± 0.1	4 ± 0.5	3 ± 0.4	3 ± 0.2	3 ± 0.1	3 ± 0.3	3 ± 0.2
Monoglycerides	7 ± 0.1	7 ± 0.2	7 ± 0.5	8 ± 0.3	8 ± 0.6	9 ± 0.5	9 ± 0.1	9 ± 0.1	9 ± 0.2	9 ± 0.3
Total (mg 100 g^{-1})	33 ± 1.5	32 ± 1.2	33 ± 1.0	40 ± 0.9	42 ± 1.6	45 ± 1.0	46 ± 0.5	46 ± 0.9	46 ± 2.2	53 ± 2.8
COMPLEX LIPIDS										
Sterol glycosides	8 ± 0.5	8 ± 0.2	8 ± 0.1	8 ± 0.3	7 ± 0.2	6 ± 0.7	6 ± 0.5	6 ± 0.3	6 ± 0.2	5 ± 1.0
Cardiolipin	8 ± 0.6	8 ± 0.2	8 ± 0.4	7 ± 0.4	6 ± 0.1	6 ± 0.5	6 ± 0.2	6 ± 0.3	6 ± 0.1	6 ± 0.2
Phosphatidic acid	12 ± 0.6	12 ± 0.4	12 ± 0.8	10 ± 0.3	10 ± 0.6	10 ± 0.5	10 ± 0.4	10 ± 0.3	9 ± 1.3	9 ± 1.1
Monogalactosyl diglyceride	5 ± 0.4	5 ± 1.2	5 ± 1.8	9 ± 1.2	9 ± 1.8	9 ± 1.7	9 ± 0.5	9 ± 0.6	9 ± 1.7	9 ± 2.8
Ceramide monohexoside	6 ± 1.1	6 ± 1.2	6 ± 0.8	4 ± 0.2	3 ± 0.4	3 ± 0.6	3 ± 0.7	3 ± 0.5	3 ± 0.4	3 ± 0.6
Phosphatidyl glycerol	5 ± 0.2	5 ± 0.1	6 ± 0.7	6 ± 0.6	7 ± 0.5	7 ± 0.6	6 ± 0.5	6 ± 0.6	6 ± 0.8	9 ± 0.1
Phosphatidyl ethanolamine	12 ± 0.4	12 ± 0.3	10 ± 0.2	10 ± 0.1	10 ± 0.4	10 ± 0.5	10 ± 0.3	10 ± 0.2	10 ± 0.5	9 ± 0.4
Digalactosyl diglyceride	3 ± 0.2	3 ± 0.1	4 ± 0.5	5 ± 0.2	5 ± 0.3	6 ± 0.4	7 ± 0.5	7 ± 0.4	7 ± 0.3	10 ± 1.2
Sulpholipid	1 ± 0.5	1 ± 0.6	2 ± 0.2	2 ± 0.1	2 ± 0.5	2 ± 0.6	2 ± 0.7	2 ± 0.2	3 ± 0.1	4 ± 0.5
Phosphatidyl inositol	2 ± 0.1	2 ± 0.2	2 ± 0.4	2 ± 0.5	4 ± 0.4	4 ± 0.3	5 ± 0.2	5 ± 0.1	6 ± 0.9	5 ± 0.5
Phosphatidyl choline	32 ± 0.5	32 ± 0.6	31 ± 0.2	31 ± 0.1	31 ± 0.3	31 ± 0.2	30 ± 0.5	30 ± 0.1	29 ± 0.2	26 ± 1.2
Lysophosphatidyl choline	6 ± 0.1	6 ± 1.0	6 ± 1.1	6 ± 0.5	6 ± 0.6	6 ± 0.8	6 ± 0.8	6 ± 0.9	6 ± 0.5	5 ± 1.9
Total (mg 100 g^{-1})	317 ± 1.9	321 ± 1.8	272 ± 1.7	281 ± 2.1	292 ± 1.5	302 ± 2.5	317 ± 1.7	337 ± 2.4	411 ± 2.8	593 ± 2.6

Table 1. (contd.) Simple and complex lipids of 4-day-old dark-grown mung bean seedlings illuminated for various times within 24 h. Results are expressed as percentage of total unless otherwise stated.

Hours in Light	0	1	2	3	4	5	6	7	8	24
Stigmasterol/ Sitosterol ratio	0.28	0.28	0.31	0.31	0.36	0.36	0.36	0.39	0.42	0.54
Total sterols (SE + St + Si + SG)	69 ± 0.6	69 ± 0.7	68 ± 0.1	66 ± 0.2	66 ± 0.3	64 ± 0.4	64 ± 0.4	63 ± 0.1	64 ± 0.5	65 ± 0.6
Simple lipids as % of total lipids	10	10	11	13	13	13	13	12	11	9
Complex lipids as % of total lipids	90	90	89	87	87	87	87	88	89	91
Phospholipids as % of complex lipids	77	77	75	72	74	74	73	73	72	69
Glycolipids as % of complex lipids	23	23	25	28	26	26	27	27	28	31
Total lipids ($g\ 100\ g^{-1}$)	0.35 ± 0.02	0.35 ± 0.01	0.30 ± 0.04	0.32 ± 0.03	0.33 ± 0.03	0.34 ± 0.02	0.36 ± 0.01	0.39 ± 0.01	0.46 ± 0.05	0.64 ± 0.04

Simple lipids

The components of simple lipids of etiolated seedlings are β-sitosterol which makes up 36% total simple lipids, free fatty acids and sterol esters 15% each, stigmasterol 10%, triglycerides 9%, mono- and di-glycerides 7% each and fatty acid esters only 1%. After 24 h illumination however there is a significant increase in fatty acid esters, triglycerides and stigmasterol, a significant decrease in fatty acids, β-sitosterol and diglycerides, and in the case of monoglycerides and sterol esters the percentage stayed uniformly constant. Overall, the decrease in β-sitosterol could be accounted for by an increase in stigmasterol since sterol esters stayed constant. In terms of glycerides the increase in triglycerides of 5% is almost wholly accounted for by the decrease in diglycerides of 4%. This suggests that there is a straightforward relationship between the two glycerides since monoglycerides stay constant. Finally, the substantial increasesin fatty acid esters may be explained solely in terms of decreases in free fatty acids. This is in direct contrast to the state in sterols and their esters when the latter stays constant. This may be explained by the fact that β-sitosterol is the precursor of stigmasterol and that esterification proper occurs more than 24 h after light treatment.

Total sterols including sterol esters show a gradual decrease from 69% to 64% after 5 h and then they stay uniformly constant up to the 24 h of study. A closer look at the sterols in the present study (Table 1 and Fig. 26) shows that there is a gradual decrease in β-sitosterol from 36% in dark to 28% after 24 h illumination. On the other hand stigmasterol shows the reverse trend with a gradual increase from 10% in dark to 15% after 24 h light.

Free fatty acids

Free fatty acids as a percentage of lipids of mung bean seedlings decrease over the 24 h period (Table 1 and Fig. 27). During the

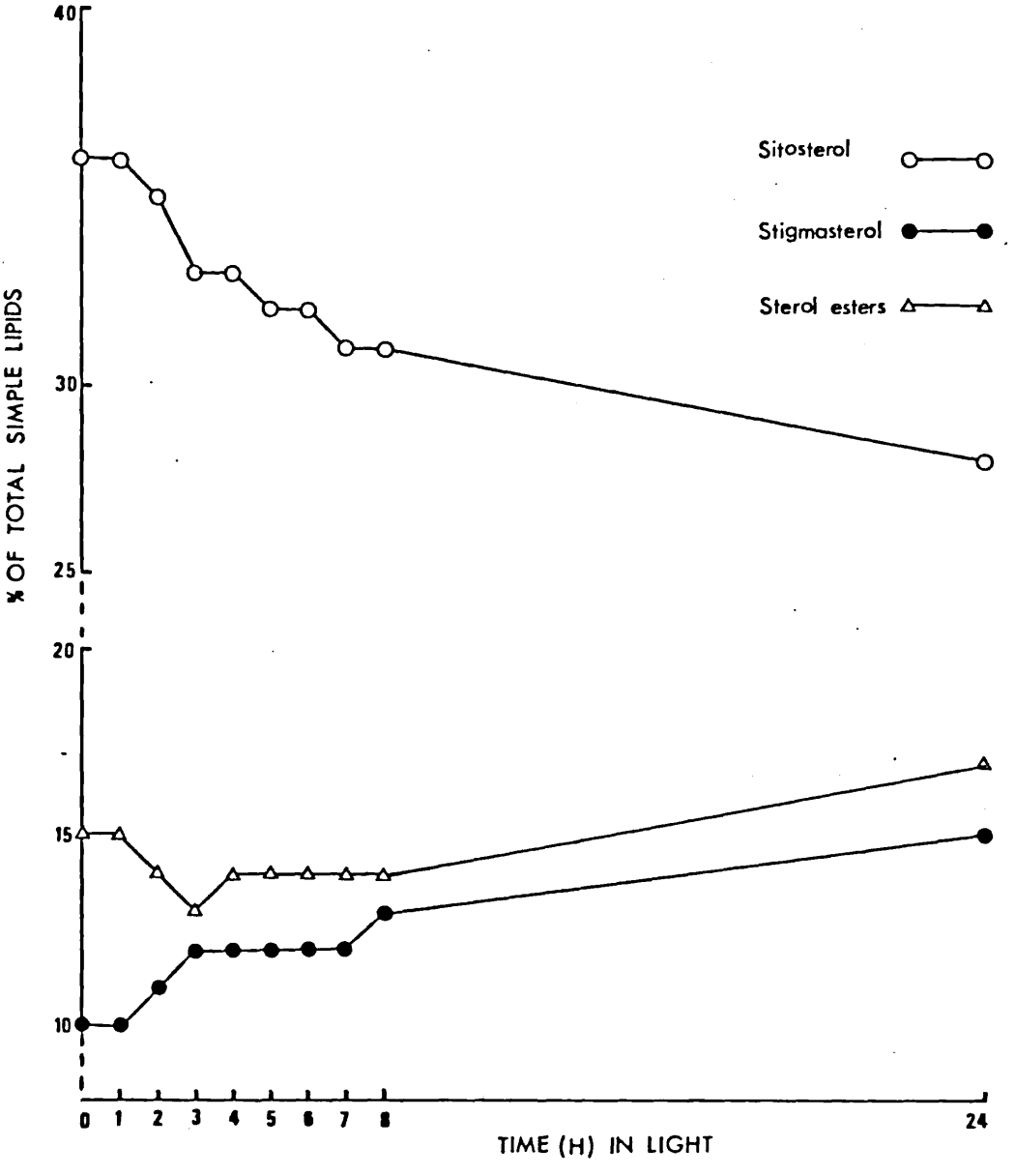

Fig. 26. Simple sterols of 4-day-old dark-grown mung bean seedlings illuminated for various times within 24 h

Fig. 27. Simple lipids of 4-day-old dark-grown mung bean seedlings illuminated for various times within 24 h

first 3 h of illumination the percentage stays the same as the dark controls, and thereafter it is decreasing slightly over the next 5 h, and even more so until 24 h. In terms of weight there is a marked increase over the first 2 h, a low at 3 and 4 h and a marked increase to 7 h with a very gradual increase then to 24 h (Table 2).

The ratio of unsaturated fatty acids to saturated, defined as desaturation ratio by Hitchcock and Nichols (1971), is not uniform over the 24 h period. From a ratio of 1.50 in the dark it goes to a low 1.22 after 3 h and then increases to a high 1.86 after 7 h and eventually to the same starting ratio of 1.50 after 24 h (Table 2).

Individual free fatty acids

The major free fatty acids of etiolated seedlings were 18:2 (26%), 18:3 and 16:0 each 20%, 18:0 (17%), 18:1 (14%), 14:0 (3%), but 12:0, 14:1, 14:2?, 16:1, 20:0, 20:2? and 22:0 had also been identified in fairly small amounts. After the 24 h illumination period the major free fatty acids were 18:2, 18:3, 16:0 each 24%, 18:0, 18:1 each 12%, and 14:0 (4%) (Table 2). The percentage of the 18:2 fatty acid was constant for the first 2 h after illumination then it decreased for the next 4 h followed by an increase to the original dark level (Fig. 28).

The percentage of the 18:3 fatty acid decreased on illumination and for the first 4 h followed by a significant increase to reach the maximum level after 7 h, then staying constant at this level (Fig. 28). The percentage of the unsaturated fatty acid 18:1 only increased between the second and third hour of illumination, reaching a maximum then, followed by decreases to dark level after 5 h, thereafter staying constant (Fig. 28). The percentage of the 14:0 fatty acid remained constant over the 24 h period of this experiment. The percentage of the 16:0 fatty acid stayed the same as the dark controls over the first 8 h, thereafter increasing slightly. The percentage of the 18:0 fatty acid increased on illumination, reaching a maximum after 3 h, thereafter decreasing.

Table 2. Major free fatty acids of 4-day-old dark-grown mung bean seedlings illuminated for various times within 24 h. Results are expressed as percentage total FFA ($\mu g\ g^{-1}$). Desaturated ratio is unsaturated divided by saturated fatty acids

Hours in light	0	1	2	3	4	5	6	7	8	24
Fatty acids										
14:0	3 ± 0.5	3 ± 1.5	3 ± 1.0	3 ± 1.8	3 ± 1.7	3 ± 1.2	3 ± 0.8	3 ± 0.9	3 ± 0.6	4 ± 1.8
16:0	20 ± 1.8	18 ± 1.9	18 ± 2.0	19 ± 1.1	19 ± 1.8	18 ± 1.5	18 ± 1.7	17 ± 2.1	17 ± 2.7	24 ± 1.1
18:0	17 ± 1.8	21 ± 0.6	21 ± 0.4	23 ± 0.4	23 ± 0.5	20 ± 1.1	21 ± 1.2	15 ± 1.1	15 ± 1.3	12 ± 1.7
18:1	14 ± 0.9	15 ± 1.9	15 ± 0.5	19 ± 1.1	18 ± 0.8	15 ± 2.1	14 ± 1.8	13 ± 2.1	13 ± 2.2	12 ± 2.3
18:2	26 ± 1.3	28 ± 1.8	28 ± 1.5	20 ± 0.8	20 ± 0.6	22 ± 0.9	22 ± 0.3	26 ± 1.7	26 ± 1.6	24 ± 1.0
18:3	20 ± 1.2	15 ± 1.1	15 ± 1.8	16 ± 1.0	17 ± 1.1	22 ± 1.3	22 ± 1.4	26 ± 0.8	26 ± 1.8	24 ± 0.4
Total FFA ($\mu g\ g^{-1}$)	43 ± 1.7	66 ± 1.1	64 ± 1.8	53 ± 1.2	54 ± 1.3	65 ± 1.1	64 ± 1.5	73 ± 1.2	73 ± 0.5	83 ± 2.6
Desaturation ratio	1.50	1.38	1.38	1.22	1.22	1.44	1.38	1.86	1.86	1.52

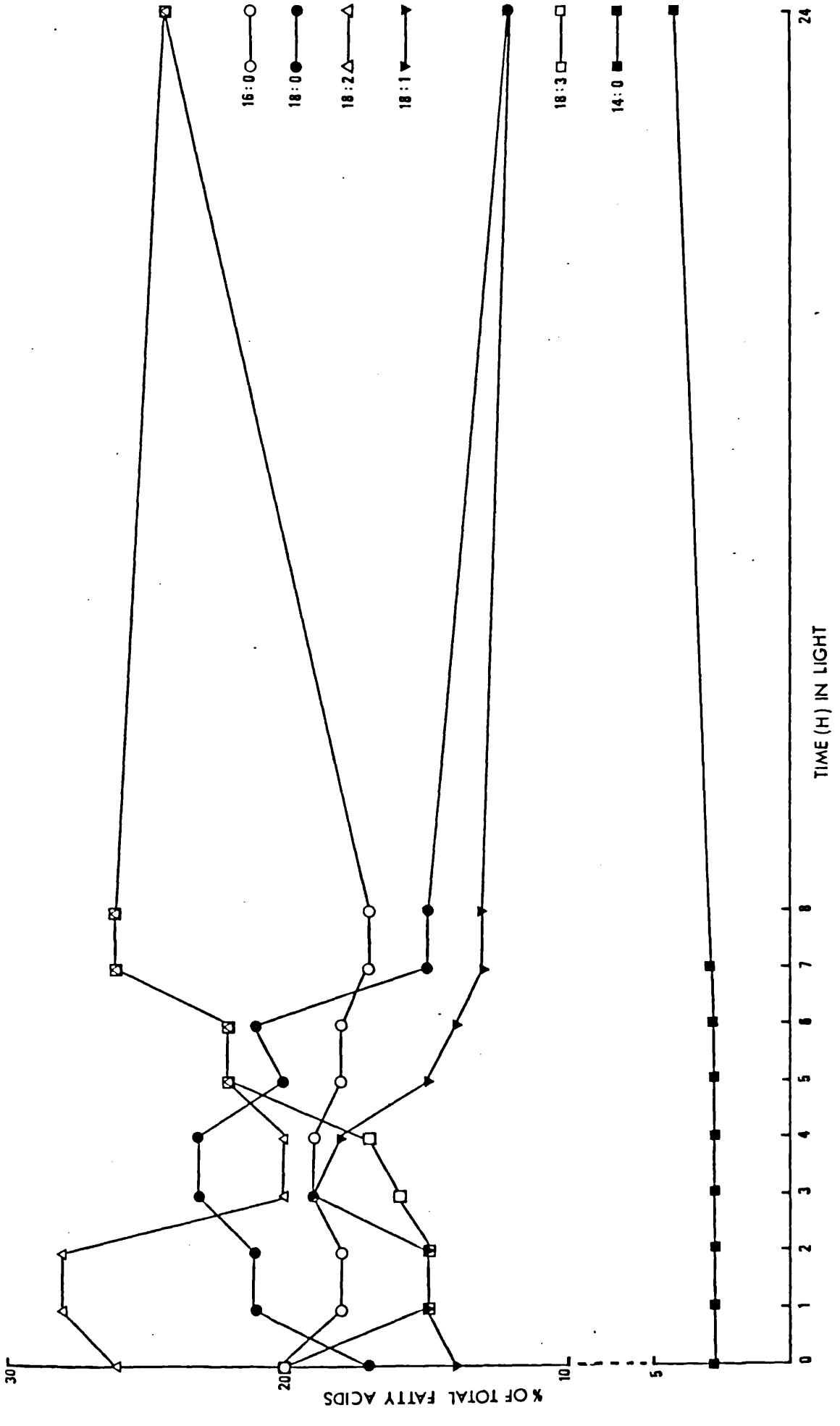

Fig. 28. Free fatty acids of 4-day-old dark-grown mung bean seedlings illuminated for various times within 24 h

Complex lipids

The major complex lipids of etiolated mung bean seedlings are phosphatidyl choline (PC) which makes up 32% of total complex lipids, then phosphatidyl ethanolamine (PE) and phosphatidic acid (PA) 12% each, closely followed by cardiolipin (CL) and by sterol glycosides 8% each. The other complex lipids identified were lysophosphatidyl choline (LPC) and ceramide monohexosides 6% each. Phosphatidyl glycerol (PG) and mono-galactosyl diglycerides (MGDG) 5% each, digalactosyl diglycerides (DGDG) 3%, phosphatidyl inositol (PI) 2% and finally sulpholipids (SL) 1% (Table 1).

Generally there is a significant gradual decrease in total phospholipids from 77% to 69%, with an increase in total glycolipids from 23% to 31% (Table 1 and Fig. 29).

Phospholipids

These will be discussed in order of decreasing relative amounts.

The major phospholipid in etiolated seedlings is phosphatidyl choline (32%) which decreases gradually over a 24 h period of illumination to 26%. It is still the major fraction of 24 h illuminated seedlings and is also the complex lipid which decreases the most (Table 1 and Fig. 30).

Phosphatidyl ethanolamine (PE) and Phosphatidic acid (PA)

These two complex lipids are found in the same concentration (12%) in etiolated seedlings and both decrease within the first 3 h illumination, after which they stay constant at 9% total complex lipids.

Phosphatidyl ethanolamine (PE) decreases 1 hour before PA but apart from this these two lipids follow the same trend. The decrease in PE earlier than in PA may suggest that PE is giving rise to PA (Table 1 and Fig. 30).

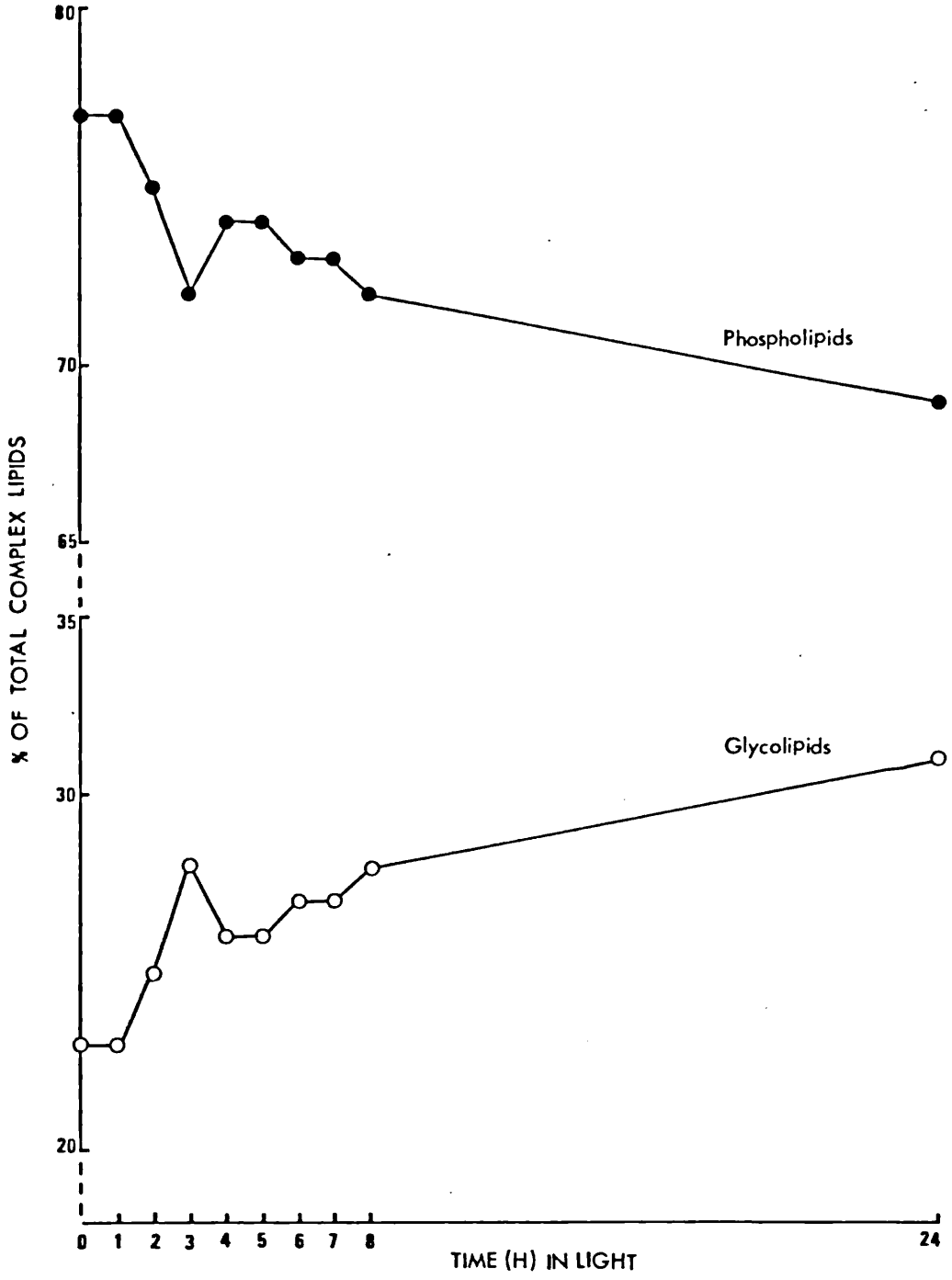

Fig. 29. Total phospholipids and glycolipids of 4-day-old dark-grown mung bean seedlings illuminated for various times within 24 h

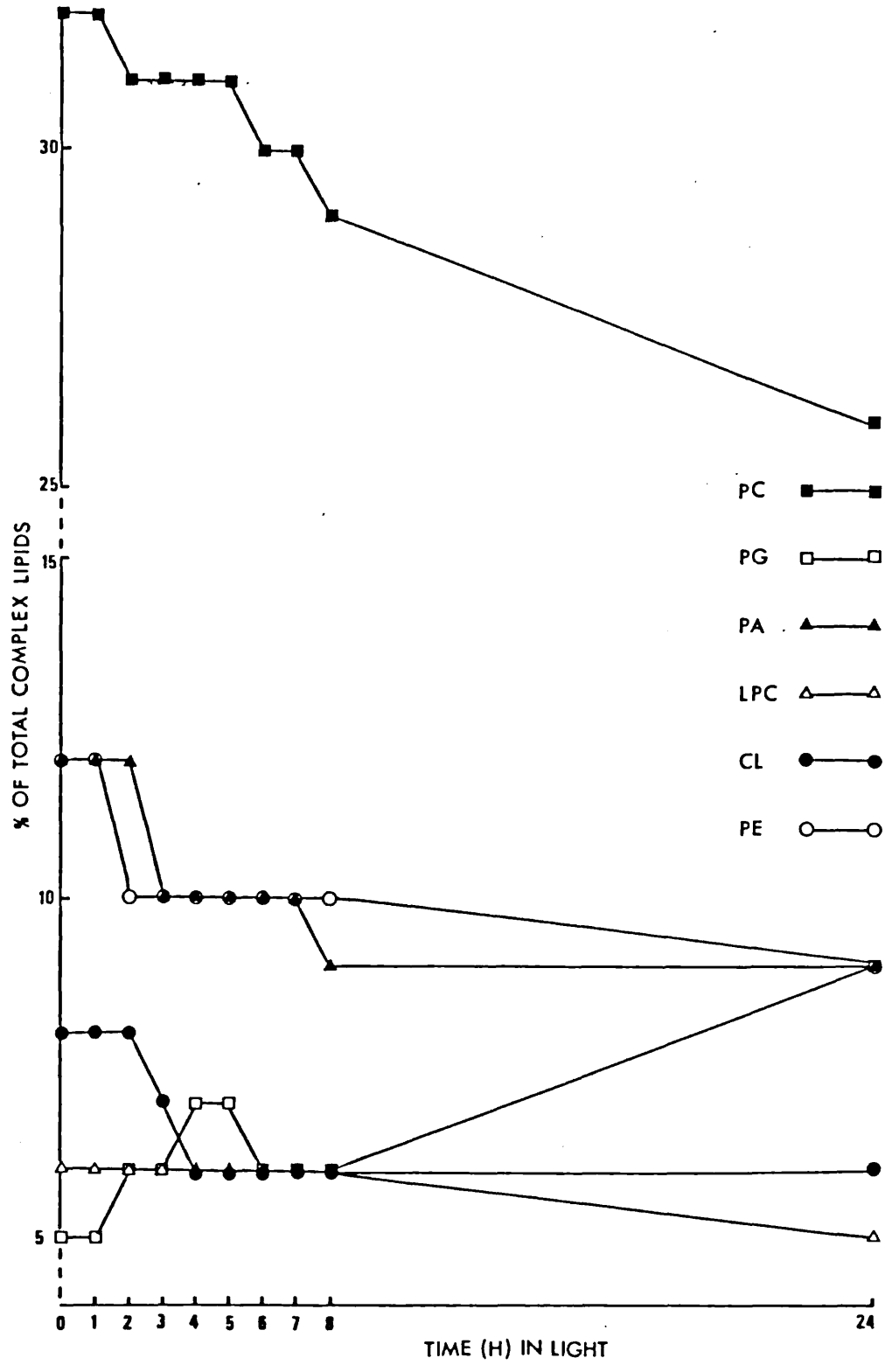

Fig. 30. Phospholipids of 4-day-old dark-grown mung bean seedlings illuminated for various times within 24 h

Cardiolipin (CL)

Cardiolipin (= diphosphatidyl glycerol) follows the same trend as PA, i.e. it decreases after 2 h illumination from 8% to 6% and then stays constant over the next 20 h (Table 1 and Fig. 30).

Lysophosphatidyl choline (LPC)

Lysophosphatidyl choline remains uniformly constant over the 24 h illumination period. It neither increases nor decreases from its initial 6% total complex lipids. It is likely therefore that LPC is an end-product (Table 1 and Fig. 30).

Phosphatidyl glycerol (PG)

This is the only phospholipid to show any increase over the 24 h illumination period. It remains constant at 5% up to 8 h light and then increases significantly to 9% at the end of 24 h. Since the increase is after 8 h, when most of the changes have taken place, and since diphosphatidyl glycerol (CL) is constant during that time, it cannot be produced here by hydrolysis of CL. It is most likely therefore that there is actual synthesis of PG from CDP-diglyceride (Table 1 and Fig. 30).

Glycolipids

These are found in small amounts compared to phospholipids, this being usual for plant tissues. These compounds are very varied in structure, e.g. ceramide monohexosides, sulpholipids and sterol glycosides, and only the presence of glycosides is common here. There is no simple relationship among this group as there is among phospholipids. They are very important as membrane components though.

Sterol glycoside (SG)

Sterol glycoside is the major glycolipid of etiolated mung bean seedlings but it decreases mainly between 3 and 5 h of illumination from 8% to 6%, then to a low 5% after 24 h (Table 1 and Fig. 31).

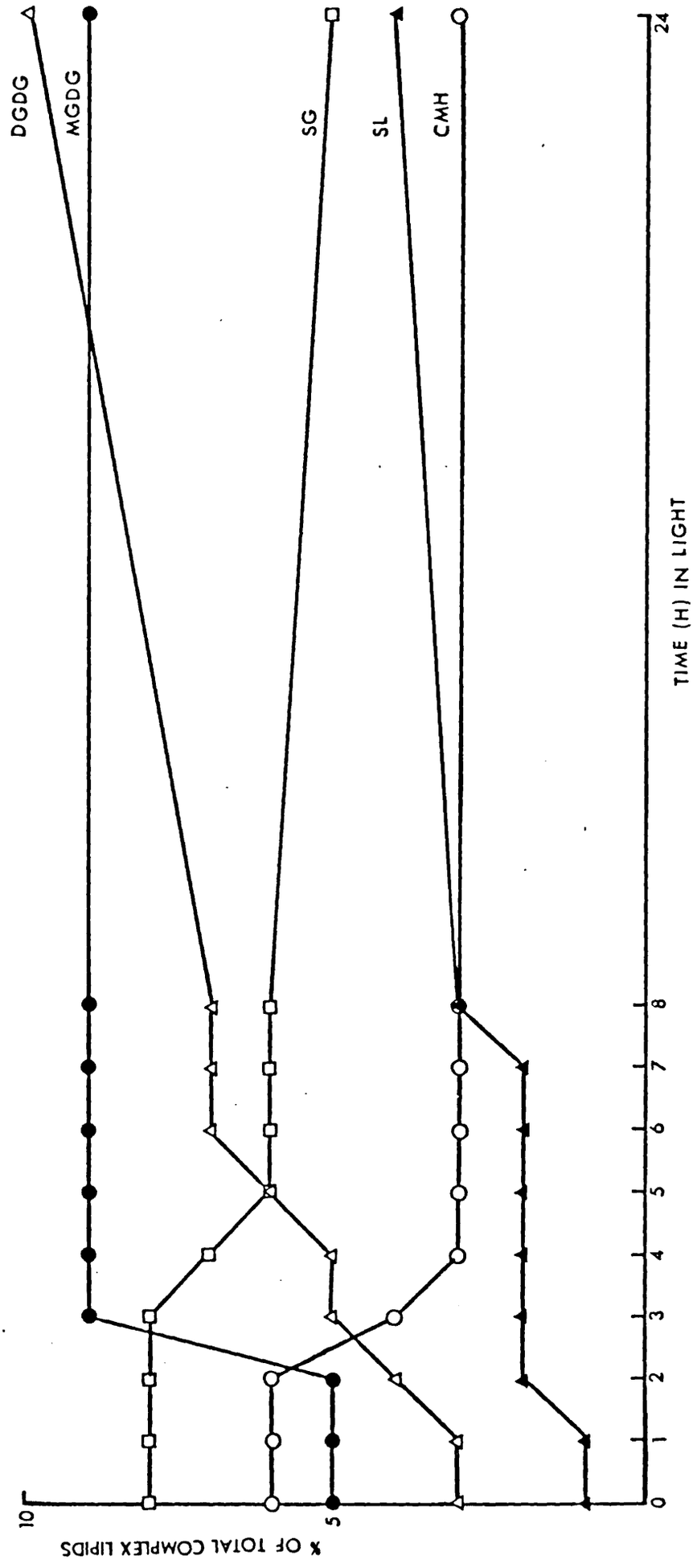

Fig. 31. Glycolipids of 4-day-old dark-grown mung bean seedlings illuminated for various times within 24 h

Ceramide monohexoside (CMH)

Ceramide monohexoside is the next major glycolipid which also decreases on illumination from 6% to 3% between 2 and 4 h, thereafter staying constant (Table 1 and Fig. 31).

Monogalactosyl diglyceride (MGDG)

This is one of the three remaining glycolipids which all increase on illumination. MGDG stays constant at 5% for the first 2 h, increases maximally to 9% in the next hour, thereafter remaining constant. This compound follows the reverse pattern for CMH and this inverse relationship may suggest that one is formed from the other (Table 1 and Fig. 31).

Digalactosyl diglyceride (DGDG)

DGDG and MGDG are chemically related compounds and one would expect MGDG to give rise to DGDG. However, although they both increase over 24 h illumination, the increase in DGDG is more gradual but more than trebles itself over that period. There is therefore no simple relationship between these two glycolipids as has been shown by other workers (Table 1 and Fig. 31).

Sulpholipid (SL) (= sulphoquinosyl diglycerides)

This glycolipid is found in most green tissues in relatively small amounts and mung bean seedlings are no exception. SL starts to increase slightly between the first and second hour from 1% to 2% when it stays constant up to 7 h illumination. Thereafter it increases gradually to 4% by 24 h of light treatment (Table 1 and Fig. 31).

Discussion

It is to be expected that under different conditions the same seeds would yield different nutrient values. Mung bean seedlings of different ages have been analysed for their lipid content by a number of investigators, and the values obtained are not always the same. Watt and Merrill (1963) obtained 0.2 g 100 g^{-1} fresh weight total lipids for 4-day-old etiolated seedlings, while Kylen and McCready (1975) obtained the same amount for 3-day-old sprouts. On the other hand, Fordham, Wells and Chen (1975), using 3 to 4-day-old sprouts (before the development of rootlets) obtained a lower value of 0.1 g 100 g^{-1} and in the present study 4-day-old seedlings contained 0.35 g 100 g^{-1}, the highest value recorded to date.

Varietal differences may account for the varying amounts obtained. When etiolated mung bean seedlings were exposed to light there was no significant increase in total lipids up to 6 h but thereafter there was a very significant increase. It may be suggested here that during the 6 h period the rate of anabolic and catabolic processes was the same, although in other systems it is known that lipid synthesis takes place in growing tissues such as hypocotyls and radicles, and lipid is being hydrolysed in reserve organs such as cotyledons (Hitchcock and Nichols, 1971). Between 6 and 8 h the prolamellar bodies are actively changing into the chloroplast grana (p. 19) and a significant increase in total lipid to 0.46 g 100 g^{-1} is then apparent. Although anabolic processes dominate at this stage, namely prior to grana formation, initial formation of thylakoids does not seem to require the synthesis of substantial amounts of new lipid material, as was shown previously by Roughan and Boardman (1972) in both pea and bean seedlings. Furthermore, Roughan and Boardman (1972) suggested that all the essential lipid components (with the exception of chlorophyll) were already present in the etioplast, possibly in the prolamellar bodies, the grana being formed initially by a light-induced rearrangement of constituent lipid and protein. In other words the tubular membranes of

prolamellar bodies appear to be building-blocks for the formation of photosynthetically-active thylakoids. The substantial lipid increase between 8 and 24 h is due to the high photosynthetic activity of the now fully functional chloroplasts.

Trémoliéres and Lepage (1971) working with etiolated pea seedlings noted a slight increase in the percentages of simple lipids and a decrease in complex lipids under a 96 h light period. With mung bean seedlings, no such increases or decreases were observed as simple lipids made up about 10% and complex lipids 90% total lipids over the 24 h period of study.

A. Simple Lipids

Sterols

The decrease in total sterols which include stigmasterol, β-sitosterol, sterol esters and sterol glycosides shown in the present study during illumination has already been observed by Bush, Grunwald and Davis (1970) in etiolated barley shoots, and Geuns (1973) in etiolated mung bean seedlings; also that younger tissues contain more sterols than older ones. However there is no clear explanation for sterol decrease during seed germination and the role of sterol in this process is very complex (Mayer and Shain, 1974).

It seems that the initial sterols which accumulated in the seeds are metabolised in the early stages of growth. There is a gradual decrease in total sterols for the first 8 h illumination. Membrane transformation is taking place at the time that prolamellar bodies are converting to grana, allowing for changes in permeability to water and gases (Grunwald, 1975). Between 8 and 24 h illumination there is a steady level of total sterol content probably due to the fact that the rate of metabolism and synthesis is the same during that period.

Table 1 shows that there is a gradual decrease in the percentage of β-sitosterol and an increase in the percentage of stigmasterol over the first 24 h of illumination, and the ratio of stigmasterol to β-sitosterol 0.28 after the first hour increases to 0.54 after 24 h, an increase favouring stigmasterol synthesis. It should be pointed out that only free sterols are effective plant membrane stabilisers (Grunwald, 1975).

Stigmasterol and β-sitosterol are C29 sterols, the former differing from the latter in having one double bond at C_{22} - C_{23} (*trans*). It is known that a C_{22}, C_{23} dehydrogenase transforms β-sitosterol to stigmasterol (Fig. 32).

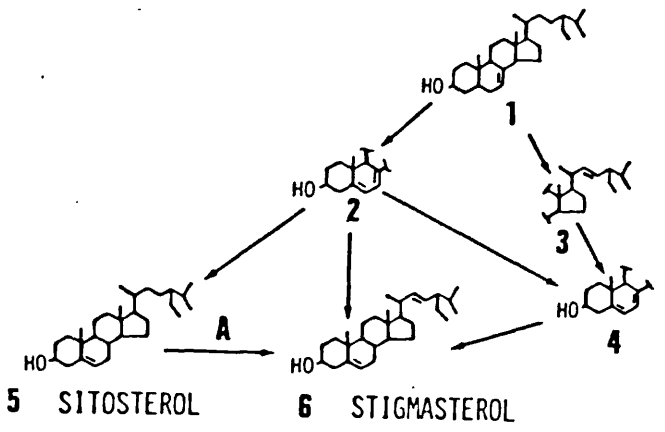

Fig. 32. Biosynthetic pathways leading to β-sitosterol and stigmasterol formation

It has been suggested that β-sitosterol is the precursor of stigmasterol (Bush, Grunwald and Davis, 1971; Bush and Grunwald, 1973; Grunwald, 1975) (Part A Fig. 32). Direct conversion of labelled β-sitosterol to stigmasterol has generally been very low and this has resulted in the suggestion that there is another pathway for stigmasterol and β-sitosterol biosynthesis (Goad and Goodwin, 1972). It has also been suggested that stigmast-7-en-3β -ol (1) may be the starting point for these two 4-dimethyl sterols. A $\Delta^{7(8)} \longrightarrow \Delta^{5,7} \longrightarrow \Delta^{5,6}$ migration occurs to give β-sitosterol (5), and to form stigmasterol (6) a double bond is first introduced at C_{22-23} followed by the same above Δ migrations (Fig. 32). Recent work with ^{14}C-mevalonic acid, however, supports the precursor-product relationship (Grunwald, 1975).

Although in the present study no labelling was used, there is overwhelming evidence for a direct relationship between β-sitosterol and stigmasterol under the influence of light. However evidence to date does not always substantiate the finding that light only is responsible for the interconversion of these two

sterols. Whereas Davis (1972) with tobacco plants, and Kemp, Goad and Mercer (1967) with maize seedlings, are in favour of the above suggestion, the results of Geuns (1973) favour an ageing effect. He observed that the younger tissues (roots, leaves and cotyledons) contained more β-sitosterol than stigmasterol but that as the plant ages there is a gradual decrease in the percentage of β-sitosterol and an increase in that of stigmasterol compared to younger tissues (first hypocotyl fraction, the plumular hook and stem tips). The results of the present study support the findings of Davis (1972) and Kemp, Goad and Mercer (1967), though those of Geuns (1973) cannot be ruled out since ageing etiolated tissues were not investigated in the present study.

Sterol esters of mung bean seedlings remain uniformly constant as has been observed previously by Bush and Grunwald (1974), Dupérons (1965) and Shewry et al., (1974) with different tissues. These results do not therefore support the suggestion that sterol esters are involved in the transport of sterols (Kemp, Goad and Mercer, 1967).

Glycerides

Several groups of workers (Barron and Stumpf, 1962; Cheniae, 1965; Sastry and Kates, 1966; Mazliak, 1967) have shown that phosphatidic acid is the precursor of plant di- and tri-glycerides, and that the metabolic sequence involved is probably through reactions 2 and 4 (Fig. 13).

In this study, taking the above into consideration over the 24 hour illumination period, the formation of triglycerides may be at the expense of PA. However, the simple lipids mono-, di- and triglycerides by themselves can account for its metabolism and synthesis.

Monoglycerides stay constant over the period under discussion while triglycerides increase at the expense of diglycerides. The major decrease in the latter is after 5 h which does correspond to the major increase in triglycerides. This is evidence that diglycerides are the immediate precursors of triglycerides

(Hitchcock and Nichols, 1971). On the evidence presented, the fact that monoglycerides remain constant over the 24 h period does not necessarily mean that they are not involved in the synthesis of the other two glycerides, but simply that their anabolism and catabolism are in equilibrium.

Free Fatty Acids

The weight of free fatty acids expressed as $\mu g\ g^{-1}$ fresh weight increased on illumination suggesting that cellular interaction then caused some release of free fatty acids which were not used for the synthesis of other lipids. The following decrease after 2 h illumination may be explained on the grounds that fatty acids are being used to build up other lipids. The final increase from 4 to 24 h suggests that the fatty acids pool is being built up during that time. Although on a weight basis there is a doubling increase over the 24 h, based on percentages there is a corresponding decrease to half what it was in the dark. This suggests that the increase in free fatty acids over the 24 h period is not keeping pace with other lipids. Decrease in the percentage free fatty acids is accompanied by some synthesis of lipids during that time, especially triglycerides; glycolipids (MGDG, DGDG, and SL). In fact the presence of diglycerides and PA would seem to indicate that the major route of lipid synthesis is through phosphatidic acid (Privett *et al.*, 1973)(Fig. 13).

Phosphatidic acid is the precursor of many lipids including monogalactosyl diglyceride, digalactosyl diglyceride and sulpholipid, and triglyceride, discussed in their appropriate sections. The distribution of the various molecular species of fatty acids among some neutral, phospholipids and glycolipids of green and etiolated cells have been investigated by many workers including Nichols (1965) on *Chlorella vulgaris*, Rosenberg and Gouaux (1967) on *Euglena gracilis*, Trémoliéres and Lepage (1970) on pea seedlings, and Roughan and Boardman (1972) on pea and bean leaves. In most cases they found that the same major fatty acids 14:0, 16:0, 18:0, 18:1, 18:2 and 18:3 were incorporated into the

neutral lipids, phospholipids and glycolipids. In the present study the 16:0 and 18:3 fatty acids showed a significant increase, while 14:0, 18:1 and 18:2 stayed constant and 18:0 decreased slightly over the 24 h illumination period. Similar results have been obtained by Trémolières and Lepage (1971) for the fatty acids of pea seedlings during the same period. The decrease in the 18:2 fatty acid during the present study could be correlated with increases of 18:0 and 18:1 especially between 2 and 3 h after illumination. These results may be interpreted in that hydrogenation of the 18:2 fatty acids could give rise to 18:1 and finally to 18:0 fatty acids. Nevertheless the results of individual fatty acids which have been described above indicate that no definite fatty acid pattern can be considered characteristic of mung bean seedlings. Various hydrogenation and dehydrogenation reactions of plant fatty acids may take place depending on the experimental conditions as has been suggested by Hitchcock and Nichols (1971) and Radwan and Mangold (1976).

The desaturation ratio fluctuated all through the experiments and these results are not in agreement with those of Newman (1962) who showed that there was an increase in saturated fatty acid in bush bean leaves over the maturity period.

B. Complex Lipids

It has already been observed that there was an increase in the glycolipids and a decrease in phospholipids in mung bean seedlings over the 24 h illumination. Similar results have been obtained by many workers on other tissues, e.g. Roughan and Boardman (1972) on peas and beans; Trémoliéres and Lepage (1971) on peas. No significant changes in complex lipids were observed during the first 8 h. Subsequent changes in relative concentration of the complex lipids, notably an increase in glycolipids and a decrease in phospholipids, probably reflect a combination of chloroplast growth and cell expansion. In the cell of an etiolated plant the plastids are relatively small and make a very small contribution to the effective (nonvacuolar) volume of the cell. The dominant complex lipid in such a cell is phosphatidyl choline. However, as cell expansion and vacuolation proceeds with an accompanying increase in the size of the chloroplasts, the contribution of the latter to the cytoplasmic volume increases to the extent that the glycolipids become the predominant complex lipids.

The results of the present study are very much in keeping with those of Roughan and Boardman (1972) in that PG and SL increased between 1 and 24 h illumination but whereas they showed that MGDG was increasing at a greater rate than DGMG, the reverse was observed with mung bean seedlings.

Phospholipids

On the basis of biochemical evidence obtained from all classes of living tissues, it is clear that there are alternative biosynthetic pathways for most classes of lipids and it would be misleading to suggest that there exists a single 'Plant Pathway' for each class (Fig. 13). Even within a single type of cell or subcellular particle alternative pathways to a single phosphoglyceride are possible and evidence for the activity of one biosynthetic mechanism does not necessarily exclude the concomitant (or alternative) operation of another (Hitchcock and Nichols,

1971). However, the PC decrease may be correlated with increases in MGDG and DGDG following pathways 2, 8 and 23 of Figure 13.

Glycolipids

Sterol glycosides (SG)

Sterol glycosides are a storage form of sterols and very little is known about their changes during germination (Grunwald, 1975). In the present study there was an apparent decrease of 2% over the 24 h illumination period although sterol glycosides were actually increasing over this period. Acylated sterol glycosides were not present in this study and therefore there is no evidence for the suggestion that acylated sterol glycosides are synthesised from sterol glycosides as was reported by Ongun and Mudd (1970) for peas, spinach, avocado fruits and cauliflower, and by Hou *et al.* (1968) for immature soya beans.

The sugar moiety of sterol glycosides has been identified as glucose and mannose (Swift, 1952; Wright *et al.*, 1962). Glucose is the major reducing sugar found in mung bean seedlings (p.99) and is therefore very likely to be the sugar moiety of the sterol glycosides here, especially as no mannose has been identified in the present study.

Ceramide monohexoside (CMH)

Decreases in CMH over the 24 h illumination period could be explained on the basis of its breakdown into its fatty acids and sugar components, the fatty acids being used up during phosphatidic acid synthesis. PA has been shown to undergo further changes at the same period of illumination.

Monogalactosyl diglyceride (MGDG) and Digalactosyl diglyceride (DGDG)

MGDG and DGDG increase over the 24 h illumination period but the increase in amounts of DGDG (x 3) is found to be much greater than that of MGDG (x 2), PG (x 2) and SL (x 2). These results are in sharp contrast with those of Roughan and Boardman (1972)

who reported a disporportionate increase in MGDG compared with DGDG at the later stages of greening. Costes et al. (1971) had earlier shown that the agranal bundle sheath chloroplasts of maize had a lower MGDG/DGDG ratio compared to the grana containing mesophyll chloroplasts. This had led Roughan and Boardman (1972) to suggest that MGDG may play a part in cementing together of thylakoids in grana stack. Results obtained here suggest that this may not be so in mung bean seedlings.

Sulpholipid (SL) ≡ Sulphoquinovosyl diglyceride

This glycolipid is found in most green tissues and it increases over the 24 h illumination period nearly twofold at the time when total chlorophyll is increasing (p. 124). Sulpholipid synthesis appears then to bear a temporal relationship to chlorophyll synthesis.

Sulpholipid molecules may be necessary for the orientation and possibly the functions of chlorophyll. Nevertheless its ability to undergo oxidation and photoreduction (Ibanez-Martini and Lindstrom, 1959) should not be overlooked as possible clues to the mechanisms involved in the photoinduction of chlorophyll synthesis in etiolated cells (Rosenberg and Pecker, 1964).

CHAPTER IV

EFFECT OF LIGHT ON CARBOHYDRATES

Introduction

In this section 4-day-old dark-grown mung bean seedlings were subjected to a 24 h illumination period. The total dietary carbohydrates available to man is usually referred to as total available carbohydrates. These have been determined by the Clegg Anthrone method. Total available carbohydrates include starch and dextrins as well as the free soluble sugars which are well known plant constituents. The free sugars of mung bean seedlings were identified as fructose, glucose and sucrose, and were estimated quantitatively by TLC as was an unidentified monosaccharide. The changes in these carbohydrates were determined in the hope of finding a correlation between these compounds and grana formation which is actively taking place between 6 and 8 h after illumination (p. 19).

Estimation of total available carbohydrates (Clegg Anthrone Method)

The tissues were digested with perchloric acid, and the hydrolysed starches, together with soluble sugars, were determined colorimetrically by the anthrone method. The anthrone-sugar complex forms a green colour which has maximum absorption at 630 nm.

Reagents:

Perchloric acid 52%. 270 ml of perchloric acid (sp. gr. 1.70) was added to 100 ml water and was kept cold before use.

Sulphuric acid. 760 ml of sulphuric acid (sp. gr. 1.84) was added to 330 ml of water and was kept cold before use.

Anthrone reagent. Sufficient 0.1% anthrone was made up in the above sulphuric acid for each day's requirements. This solution had to be made daily.

Glucose standard solution. 100 mg glucose was dissolved in 1 litre distilled water (1 ml $\equiv$ 0.1 mg glucose).

Procedure: Extraction

Ten grams of fresh shoots or seeds were macerated thoroughly in a mortar with 10 ml water and then transferred into a 100 ml

measuring cylinder. Thirteen millilitres of 52% perchloric acid were added, and the mixture was stirred frequently with a glass rod for 20 min. After mixing, the glass rod was washed with water and the cylinder contents diluted to 100 ml. The mixture was filtered through Whatman No. 542 into a 250 ml graduated flask. The measuring cylinder was washed with water which was then added to the filtrate and made up to 250 ml.

Sample preparation:

10 ml of the above filtrate was diluted to 100 ml with water and 1 ml of this solution was placed in a test tube.

Standard preparation:

One millilitre of diluted glucose standard solution (1 ml ≡ 0.1 ml glucose) and 1 ml of each sample were placed in a separate test tube. Five millilitres of freshly-prepared anthrone 0.1% reagent were added rapidly to all tubes, the tubes stoppered and the contents mixed thoroughly. The tubes were then placed in a boiling water bath for exactly 12 mins and cooled quickly to room temperature. A green colour was formed with the anthrone reagent which was stable for at least 2 h. The coloured solutions were transferred to glass cuvettes and the absorbance of the samples and standard were read against a blank solution containing 1 ml distilled water plus 5 ml anthrone reagent using a Unicam SP-800 spectrophotometer.

Calculations:

W ≡ weight (g) of sample

a ≡ absorbance of diluted glucose standard (1 ml ≡ 0.1 mg glucose)

b ≡ absorbance of diluted sample

According to Beer and Lambert's Law, the total available carbohydrate was calculated as a percentage using the following formula:

$$\text{Total available carbohydrate as } \% \equiv \frac{25 \times b}{a \times W}$$

Quantitative estimation of neutral sugars by thin-layer chromatography (TLC)

Extraction of free sugars

Procedure:

Twenty grams of shoots or seeds were placed in a Waring blender, covered with 100 ml ethanol and homogenised for 2 min. The homogenate was filtered through a Buchner funnel (No. 3) with suction.

The residue was further blended with 50 ml ethanol for 1 min and filtered. The volume of the pooled filtrate, approximately 150 - 170 ml, was reduced to 20 ml on a rotary evaporator at 40°C, and centrifuged at 4000 rpm at -3°C for 10 minutes. The clear supernatant was collected and transferred to a 2 ml screw-cap bottle, and a few drops of the preservative merthiolate (Thiomersol) was added and kept in the refrigerator at 4°C.

Standard solution for sugars

The sugar standards of sucrose, glucose, fructose and xylose were each made up to contain 400 mg per 100 ml of water. The latter was used as an internal marker.

Sample preservation

Ersser and Andrews (1971) found that a few drops of merthiolate (Thiomersol) adequately preserved the sugar standards and samples against bacterial growth. However the presence of protein in the samples was shown by Menzies (1973) to interfere with the antibacterial activity of merthiolate, but not if samples were stored at -20°C. Therefore all samples under study were always kept at -20°C.

Sample preparation for TLC

One millilitre of the sample was placed in a 2 ml plastic centrifuge tube with stopper, and Zerolit DM-F (Biodeminrolit) BDH Limited was used as a desalting resin.

Zerolit DM-F was added to the tube until the volume occupied approximately 1.5 ml and it was shaken for about 3 min. The tube was then centrifuged at 4000 rpm for 5 min and the clear supernatant was ready for application to TLC plates.

TLC procedure

Thin-layer plates (20 cm x 20 cm) were plastic-backed and coated with F.1500 silica gel, 0.25 mm thick. The absorbant was acid resistant and was without fluorescent indicator (Art. No. 355100 Dassel, West Germany). The margins of the plates were scraped and sample applied as a streak 1 cm long (p. 39). The application of 5 - 10 µl was found suitable for sugars.

Development of TLC plates

The solvent system of Menzies and Mount (1975) was modified as follows:

ethyl acetate	60 ml
pyridine	30 ml
acetic acid (glacial)	15 ml
water	5 ml

The original system contained 5 ml of acetic acid and 15 ml of water. Each plate was rolled into a 250 ml beaker and the beaker was inverted within the chromatography tank (Fig. 33). The 'inverted beaker' technique maintained a constant concentration of solvent vapour across the surface of the plate and reduced the bowing of the solvent front (Menzies, Mount and Wheeler, 1978). The plate was developed twice with the same solvent system right to the top of the plate. Between the runs the plate was air dried in the fume cupboard overnight to remove the pyridine completely as it inhibits many colour reactions for sugar (Menzies and Mount, 1975).

Colour reaction

4-aminobenzoic acid locating reagent

Seven grams of 4-aminobenzoic acid were dissolved in 400 ml methanol and 17.5 ml orthophosphoric acid (90% w/v) was added

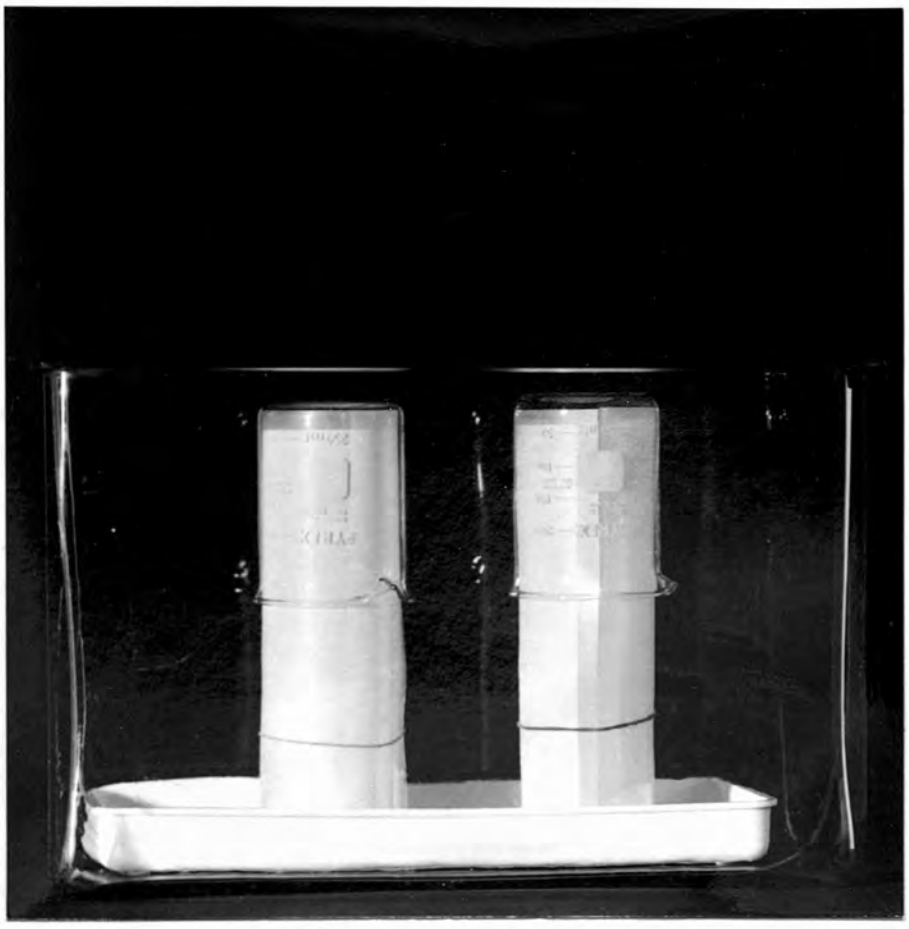

Fig.33. The 'inverted beaker' technique for developing TLC plates. The plates were rolled into a 250 ml beaker and the beaker was inverted within the chromatography tank.

and the solution was made up to 500 ml with methanol. This locating reagent was stable for a long time when stored in a refrigerator.

Dipping procedure

A specially constructed dipping chamber was used to overcome irregular 'hesitation lines' of non-uniform reagent distribution resulting from a traditional 'dipping tray' technique. This consists of two rectangular glass plates 24 cm x 22 cm mounted in a frame and held apart by a U-shaped spacer made of thick-walled 'tygon' tubing reinforced with a wire core. Adjustable wing-nuts on the frame enabled the plates to be compressed against the spacer thus sealing the chamber (Menzies, Mount and Wheeler, 1978). The plate dipping was as brief as possible to prevent zone trailing (Fig. 34).

Colour Reaction and identification of spots

The excess reagent was then wiped out from the back of the plate, and colour was developed by heating the plate in the oven at 100 - 110°C for 5 - 10 min. Sucrose, glucose, fructose and an unknown fraction near the top of the plate showed up as bright yellow spots and xylose, the internal marker, appeared pink (Fig. 35). The unknown fraction had different chromatographic properties to erythrose and D(-) 3-phosphoglyceric acid, with the erythrose spot showing up below and D(-) 3-phosphoglyceric above the unknown fraction. However it seems that it could be a 3-carbon compound. The colours were relatively stable. To prevent variable background discolouration due to extended exposure to the laboratory atmosphere the plates were placed in polyethylene bags and kept in the refrigerator if densitometry readings could not be performed immediately.

Quantitative determination

Each series of chromatographic zone was cut out and scanned in a Chromoscan (Joyce-Loebl Limited) as described (p. 58). The

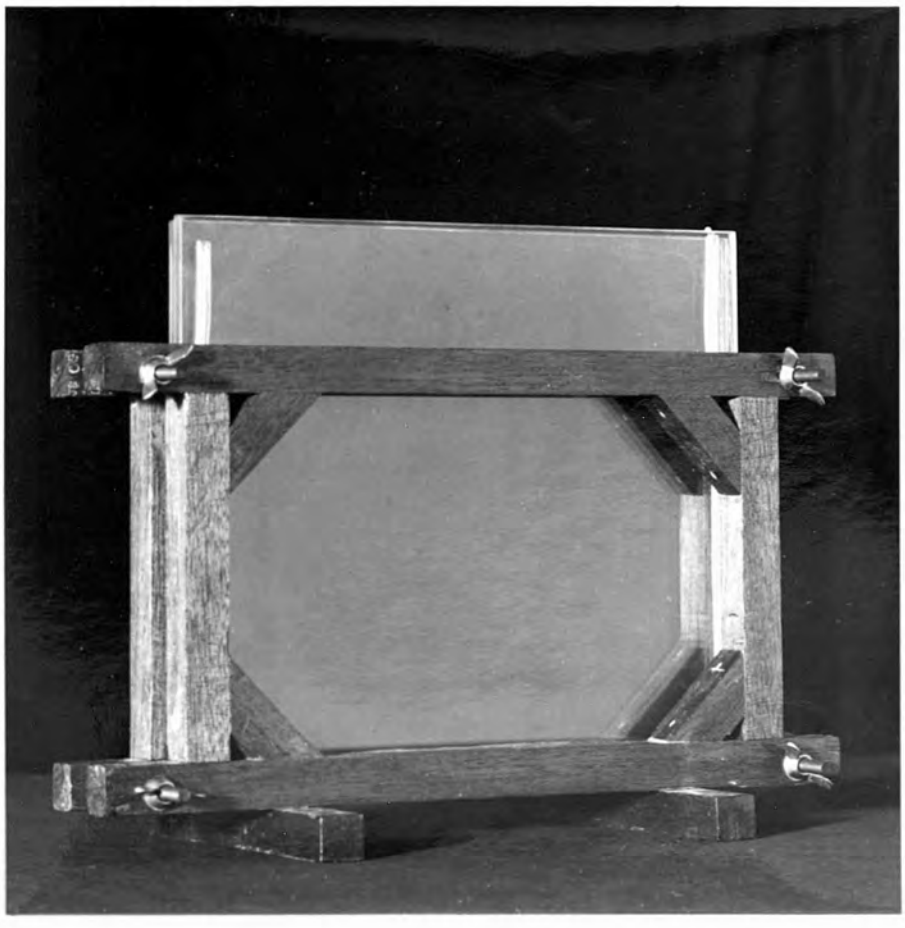

Fig. 34. Dipping chamber. This consists of two rectangular glass plates (24 cm x 22 cm) mounted in a frame and held apart by a U-shaped spacer.

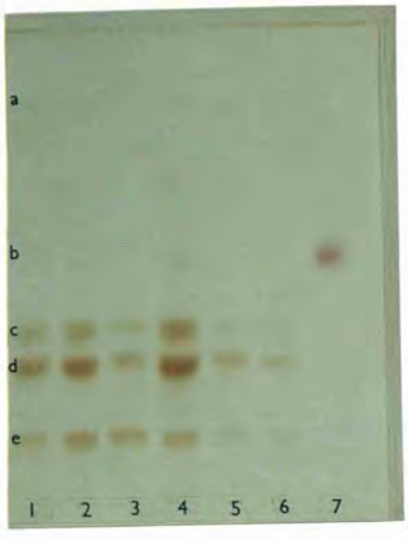

Fig. 35. TLC separation of free sugars of mung bean seedlings on F1500 silica gel layer. a, unidentified; b, xylose (as internal marker); c, fructose; d, glucose; e, sucrose.

1, four-day dark-grown (4 DD); 2, 4 DD + 8 h light; 4, 4 DD + 24 h light; 3, 5, 6, standards; 7, xylose.

Developing solvent: ethyl acetate-pyridine-acetic acid-water (60:30:15:5 v/v).

amount of sugar was calculated as follows:

$$\frac{\text{Corrected peak height of standard}}{\text{Corrected peak height of sample}} \equiv \frac{\text{Amount of sugar in standard}}{\text{Amount of sugar in sample}}$$

Corrected peak height ≡ peak height x marker factor (X/M)

X/M is a constant figure for each plate

X is the average peak height of xylose which was found to be 90

M is the actual peak height for xylose for each plate (Menzies, Mount and Wheeler, 1978).

Results and Discussion

The total free sugars and total available carbohydrates of 4-day-old etiolated mung bean seedlings were 1.9 and 5.1 g 100 g^{-1} of fresh weight respectively. These values did not increase significantly over the first 5 h of light, but they increased significantly over the next 19 h in a 24 h light period (Table 3 and Fig. 36). The increase in free sugars, which almost trebled by 24 h, was much greater than the increase in total available carbohydrate (only approximately 50%) over the same period of time.

The increase in carbohydrates as a result of illumination is not altogether unexpected, since during photosynthesis carbohydrates are manufactured; hence an increase of these compounds would be expected in light. Similar results have already been obtained by Ozburn (1974) who observed increases in carbohydrates of cowpeas with light. On a percentage basis, dark-grown mung bean seedlings have 37% total free sugars while total starches and dextrins make up 63% of the total available carbohydrates. So, as seen previously, up to 5 h illumination did not alter this relationship but by the end of 24 h total free sugars had increased to 76% at the expense of total starches and dextrins, which were then only 24% (Table 4). The increase in free sugars and the decrease in starches and dextrins at the same period of time may be explained on the basis of enzymic breakdown of the complex carbohydrates releasing the free sugars.

Four free sugars were obtained in fairly large amounts during the course of this study, namely fructose, glucose, sucrose and an unknown. This latter sugar stayed uniformly constant at 0.2% fresh weight over 24 h (Table 5) while the other three sugars did not increase over the first 5 h of light. Thereafter, fructose and sucrose increased maximally up to 8 h and then stayed constant. On the other hand, glucose continued to increase over the 24 h period of this experiment. However, the increase in carbohydrates after 5 h of light suggests that they were contributing to the structure of grana membranes which were actively being formed between 6 and 8 h after the seedlings had been subjected to light.

Table 3. Total free sugars and total available carbohydrates (starches, dextrins and free sugars) of 4-day-old dark-grown mung bean seedlings subjected to illumination for a period of 24 h. Results are given as percentage fresh weight (g 100 g^{-1} fresh weight)

Hours in light	0	1	2	3	4	5	6	7	8	24
Total free sugars	1.9 ± 0.1	1.9 ± 0.2	1.9 ± 0.1	1.9 ± 0.1	2.0 ± 0.1	2.1 ± 0.1	2.8 ± 0.2	3.2 ± 0.1	4.9 ± 0.3	5.3 ± 0.1
Total available carbohydrates	5.1 ± 0.2	5.3 ± 0.1	5.2 ± 0.2	5.4 ± 0.1	5.5 ± 0.1	5.6 ± 0.1	5.9 ± 0.1	6.0 ± 0.2	6.4 ± 0.1	7.0 ± 0.2

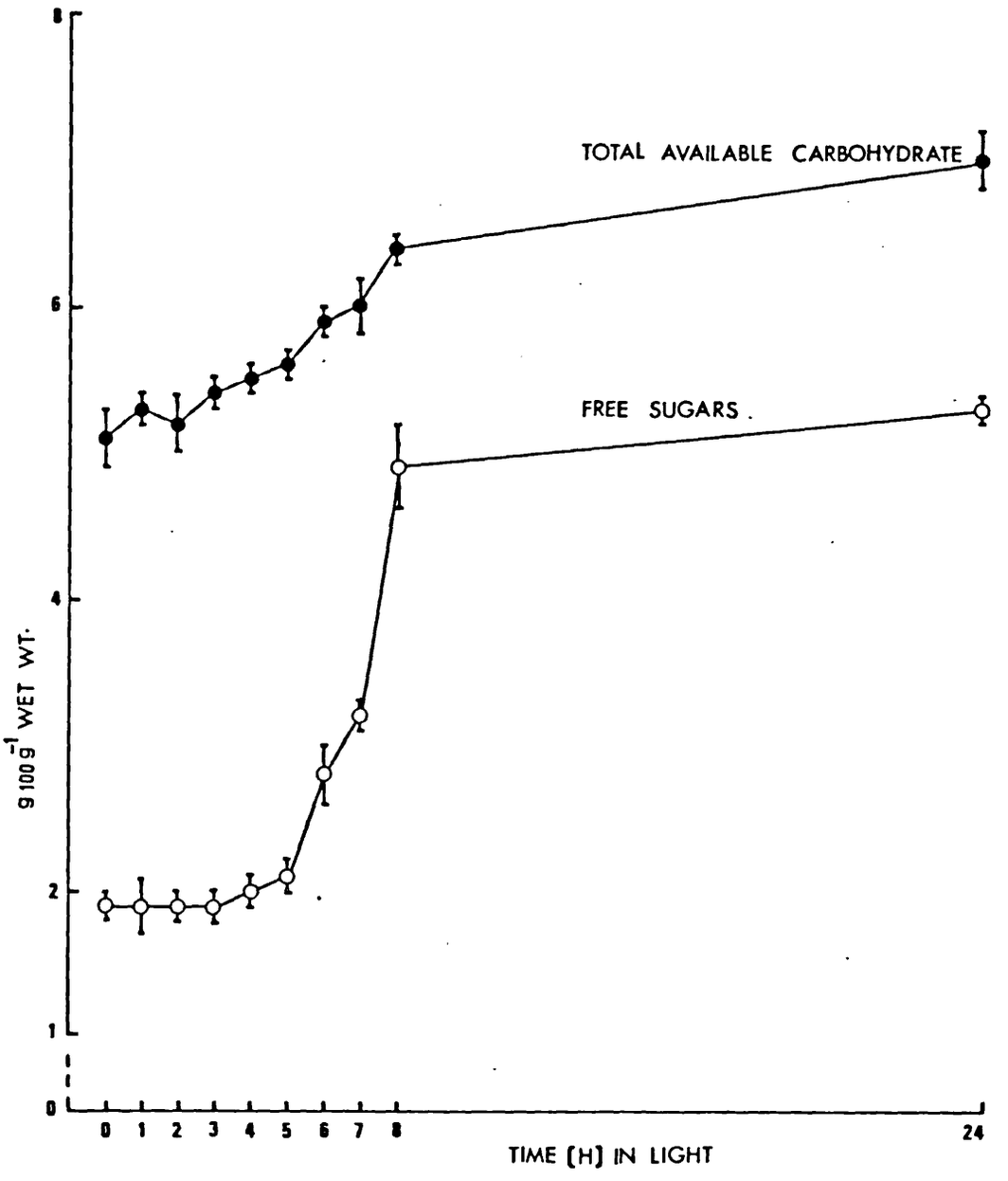

Fig. 36. Total available carbohydrate and free sugars of 4-day-old dark-grown mung bean seedlings illuminated for various times within 24 h

Table 4. Percentage contribution of total free sugars and total starches and dextrins to the total available carbohydrates of 4-day-old etiolated mung bean seedlings subjected to illumination over a period of 24 h.

Hours in light	0	1	2	3	4	5	6	7	8	24
Total free sugars	37	36	37	35	37	37	47	53	77	76
Total starches + dextrins	63	64	63	65	63	63	53	47	23	24

Table 5. Individual free sugars of 4-day-old dark-grown mung bean seedlings subjected to illumination over a 24 h period. Results are given as percentage fresh weight of seedlings (g 100 g^{-1})

Hours in light	0	1	2	3	4	5	6	7	8	24
Unidentified	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2
Fructose	0.5	0.5	0.5	0.5	0.6	0.6	0.9	1.1	1.6	1.7
Glucose	0.7	0.7	0.7	0.7	0.7	0.7	1.0	1.2	1.7	2.0
Sucrose	0.5	0.5	0.5	0.5	0.5	0.6	0.7	0.7	1.4	1.4
Total	1.9 ± 0.1	1.9 ± 0.2	1.9 ± 0.1	1.9 ± 0.1	2.0 ± 0.1	2.1 ± 0.1	2.8 ± 0.2	3.2 ± 0.1	4.9 ± 0.3	5.3 ± 0.1

CHAPTER V

EFFECT OF LIGHT ON PROTEINS

Introduction

No thorough systematic investigation has yet been undertaken on total protein and protein synthesis of germinating seeds subjected to different light periods. Therefore in a series of studies total protein, ^{14}C-leucine incorporation (protein synthesis) and amino acids in both the free and total forms have been determined over a 24 h light period. The objective of this exericse was to observe any correlations between these data and changes in the ultrastructure of plastids from prolamellar bodies into grana membranes over the same period of time.

Protein determination

The Folin-phenol reagent of Lowry, Rosebrough and Farr (1957), with slight modification, was used. The protein solution gave maximum colour absorbance at 750 nm with the Folin-phenol reagent, and a Unicam SP500 spectrophotometer was used to monitor absorbance at this wavelength. This technique actually measures the phenol-containing amino acid tyrosine present in most proteins and an assumption is then made of its proportion in relation to other amino acids present in proteins. From this total protein is estimated. However, since individual amino acids varied during the experiments, total protein obtained by this method must be viewed with caution. In most cases, by adding the various individual amino acids obtained by the amino acid analyser, it was possible to double-check the total protein content obtained by this the Folin-phenol reaction.

Reagents:

A 2% Na_2CO_3 in 0.1N NaOH

B 0.5% $CuSO_4.5H_2O$ in 1% solution tartrate

C mixture of 50 ml of reagent A and 1 ml of reagent B

D Folin-Ciocalteu's phenol reagent, diluted 1:1 v/v in water

Reagents C and D had to be made up fresh for each test.

Procedure:

Half a gram of seedlings were incubated with 10 ml N NaOH for 12 h at 20°C (Fraser and Loening, 1974). The solution was centrifuged at 20,000 rpm for 20 min at -5°C, 2 ml of the supernatant was collected and 1 ml of 3M trichloroacetic acid (TCA) was added to precipitate the proteins and to prevent the interference of any phenolic compounds in the test. The precipitate was isolated by centrifugation (20,000 rpm for 20 min at -5°C), and dissolved in 2 ml of distilled water, and 5 ml of reagent C was added. After the solution had stood for exactly 10 min, 0.5 ml of reagent D (Folin-Ciocalteu's phenol) was added and the colour absorbance after exactly 30 min was read at 750 nm against a blank containing 2 ml of water instead of protein solution. The amount of protein in $\mu g\ ml^{-1}$ was read from a standard curve prepared by using bovine serum albumin as standard.

Calculation:

The amount of protein in $\mu g\ ml^{-1}$ was read from the standard curve, giving the amount of protein in 2 ml of sample solution. From this the concentration of protein in $mg\ g^{-1}$ fresh weight was calculated.

^{14}C amino acid incorporation studies (protein synthesis)

Amino acid incorporation was studied with modifications of methods used by Key (1964), and Valadon, Travis and Key (1975). Seedlings (1.0 g) were incubated at 30°C for 2 h in 15 ml of medium containing sucrose, 30 mM; K-phosphate buffer adjusted with alkali to pH 6.0, 1 mM; streptomycin, 20 $\mu g\ ml^{-1}$; asparagine, 0.2 $mg\ ml^{-1}$; thiamine, 2 $\mu g\ ml^{-1}$; ^{14}C-leucine, 1 ml

containing 0.5 μC. Tissues were homogenised in a Virtis 45 homogeniser at 2-4°C for 2 min in 10 ml Tris buffer (10 mM, pH 7.5), and filtered through glass wool. Protein was precipitated from small aliquots of the filtrate with an equal volume of 10% TCA. After centrifugation at 4000g for 10 min the pellet was suspended in 10 ml of 5% TCA and recentrifuged. The pellet was dissolved in 5 ml of 1 mM NaOH solution containing 200 mg ^{12}C-leucine, reprecipitated with TCA, heated to 90°C for 30 min, filtered on a Whatman GFA glass-fibre disc, washed with 5% TCA, dried and counted in a liquid scintillation counter, Beckman LS-230. Samples with ^{14}C-leucine added at homogenisation were free of radioactivity.

Determination of amino acids

Free amino acids and total amino acid composition were extracted with modification of methods used by Russell (1944) and Naguib (1964). Fresh or processed mung bean seedlings were dried in an oven at 70-80°C overnight, and ground to a fine powder. To 50 mg of this powder was added 5 ml of 2% phenol and 10 ml of 30% TCA (trichloroacetic acid); the mixture was left overnight and then filtered through filter paper. The pH of the solution which contained free amino acids was adjusted to 2 $\pm$ 0.1 with NaOH.

Protein amino acids

The filter paper containing the precipitate was kept in an oven at 50°C overnight. Five mg of the dried residue was collected to which 5 ml of 10N NaOH was added. This was hydrolysed in a boiling water bath for 5 h, filtered and the pH of the solution adjusted to 2 $\pm$ 0.1 with 5N H_2SO_4. This solution contained amino acids after protein hydrolysis.

Estimation of amino acids

The separation and identification of the amino acids were carried out on a Jeol Model JLC 6AH fully automatic amino acid analyser. The amount of amino acid in each sample was

Table 6. Total proteins (g 100 g^{-1}) of 4-day-old dark-grown mung bean seedlings which have been subjected to illumination over a period of 24 h, and protein synthesis as x 10^{-3} CPM g^{-1} protein. Results are based on fresh weight.

Hours in light	0	1	2	3	4	5	6	7	8	24
Protein	5.3 ± 0.1	5.3 ± 0.2	5.3 ± 0.1	5.4 ± 0.1	5.5 ± 0.1	5.6 ± 0.1	6.0 ± 0.1	5.9 ± 0.1	6.1 ± 0.1	6.7 ± 0.2
x 10^{-3} CPM g^{-1} protein	2.7 ± 0.2	3.6 ± 0.1	3.4 ± 0.1	2.8 ± 0.1	2.6 ± 0.1	2.5 ± 0.1	2.5 ± 0.2	2.3 ± 0.2	2.2 ± 0.2	2.2 ± 0.2

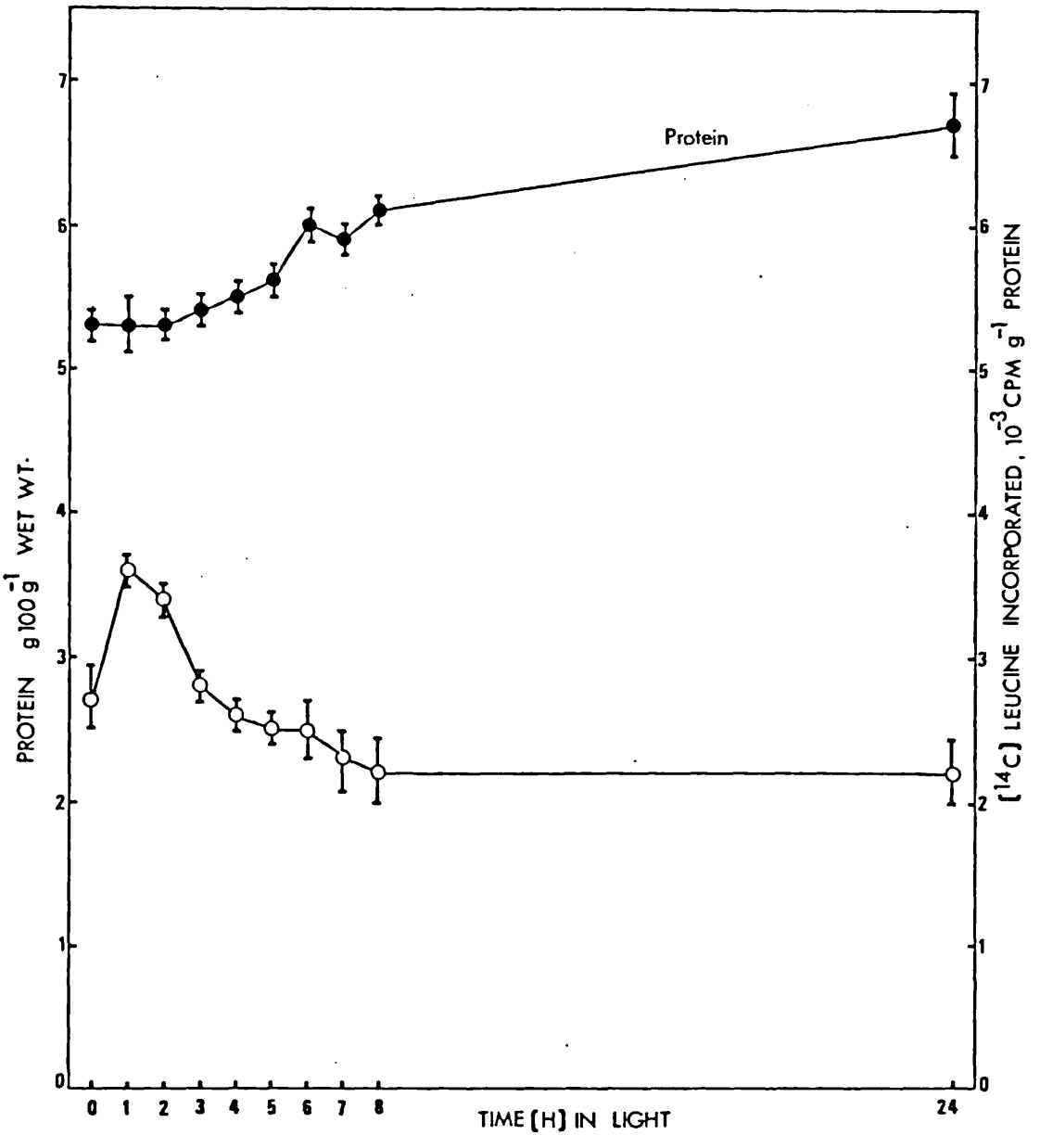

Fig. 37. Total protein (•——•) and (^{14}C) leucine incorporation (o——o) of 4-day-old mung bean seedlings illuminated for various times within 24 h

calculated by comparison of peak areas with those obtained using a calibration mixture as described by Eveleigh and Winter (1970).

Results and Discussion

In the present study the total protein of 4-day-old dark-grown mung bean seedlings estimated by the Folin-phenol method was 5.3 g 100 g^{-1} fresh weight (Table 6), compared to 5.2 g 100 g^{-1} using total amino acid content. These results are not unlike those of Kylen et al. (1975), who reported 4.3% protein for 3-day-old etiolated mung bean seedlings, and Fordham et al. (1975) who obtained 2.4% for 3 - 4-day-old dark-grown mung bean seedlings. The differences obtained in these three studies may be due to age differences, seed varieties or indeed the methods used for determining total protein. However, the percentage of protein in etiolated seedlings subjected to light did not alter significantly up to 5 h in light (= approximately 5.5%) compared to the etiolated controls (Table 6 and Fig. 37). Between 5 and 6 h of light a significant increase to 6% was observed, this value increasing gradually to 6.7% at the end of the 24 h illumination period. Once more, total protein estimated by either of the two methods used, namely Folin-phenol or by adding individual amino acids, gave very similar results (Fig. 39). ^{14}C-Leucine incorporation on the other hand gave very different results. The maximum activity was shown in the first hour of illumination and it remained the same for another hour, thereafter decreasing gradually to the same as the dark value after 3 h (Fig. 37 and Table 6). These results show that even though total protein was increasing, protein synthesis as measured by ^{14}C-leucine incorporation gave a very different picture, the largest increase in protein synthesis occurring up to 2 h after illumination.

Effect of light on amino acids

The free amino acids (i.e. prior to protein hydrolysis) present in mung bean seedlings over a 24 h period of illumination are

Table 7. Free amino acids of etiolated mung bean seedlings subjected to illumination over a period of 24 h. Results are given as mg 100 g^{-1} protein.

Amino acid	Time (h) in light									
	0	1	2	3	4	5	6	7	8	24
Lysine	5.7	3.8	5.7	5.6	5.4	5.4	8.3	8.5	8.2	9.0
Histidine	1.9	1.9	3.8	3.7	3.6	1.8	5.0	5.1	4.9	3.0
Ammonia	17.0	17.0	17.0	18.5	20.0	17.9	18.3	17.0	16.4	19.4
Arginine	9.4	7.5	7.5	7.4	9.1	8.9	13.3	13.6	11.5	11.9
Aspartic acid	13.2	13.2	13.2	13.0	12.7	12.5	16.7	22.0	24.6	9.0
Threonine	9.4	9.4	9.4	9.3	7.3	8.9	6.7	8.5	8.2	6.0
Serine	11.3	11.3	11.3	9.3	10.1	10.7	10.0	10.2	8.2	7.5
Glutamic acid	13.2	17.0	22.6	20.4	23.6	26.8	31.7	30.5	31.1	31.3
Proline	5.7	5.7	3.8	5.6	3.6	5.4	10.0	10.2	9.8	9.0
Glycine	1.9	1.9	3.8	5.6	3.6	5.4	5.0	5.1	3.3	4.5
Alanine	5.7	5.7	5.7	5.6	7.3	5.4	6.7	6.8	6.6	6.0
Valine	13.2	13.2	13.2	13.0	12.7	12.5	13.3	13.6	11.5	11.9
Methionine	1.3	1.3	1.4	1.5	1.5	1.8	1.7	1.7	1.6	3.0
Isoleucine	9.4	9.4	11.3	9.3	9.1	8.9	10.0	10.2	9.8	9.0
Leucine	7.5	7.5	7.5	7.4	7.3	8.9	6.7	8.5	6.6	7.5
Tyrosine	3.8	3.8	3.8	3.7	3.6	3.6	5.0	5.1	3.3	4.5
Phenylalanine	5.7	5.7	5.7	5.6	5.4	5.4	6.7	6.8	6.6	6.0
Total	135.3 ± 4.1	135.3 ± 6.2	146.7 ± 12.0	144.5 ± 4.5	145.9 ± 2.3	150.2 ± 5.5	175.1 ± 2.4	183.4 ± 4.6	172.2 ± 3.9	158.5 ± 2.5

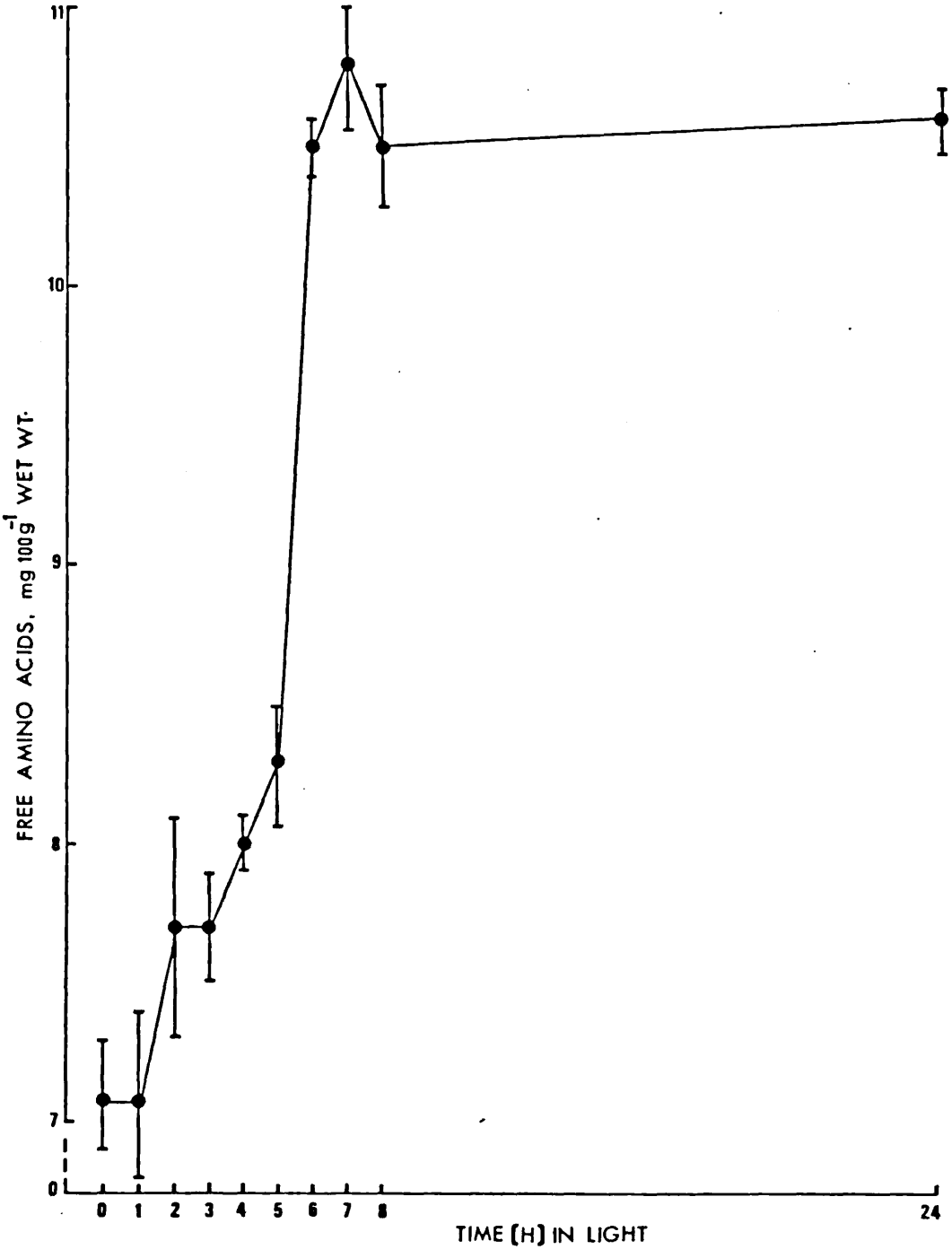

Fig. 38. Total free amino acids of 4-day-old dark-grown mung bean seedlings illuminated for various times within 24 h

shown in Table 7. Of the twenty-one amino acids usually present in plant proteins, seventeen were identified as free amino acids, the missing four being cysteine, cystine, tryptophan and hydroxyproline. On the other hand, amino acids produced after hydrolysing proteins also gave seventeen amino acids but with methionine (free amino acid) replaced by cystine (Tables 7 & 8). Ammonia was detected in fairly large amounts under the conditions used. Although it is not strictly an amino acid, for the purpose of this study it was considered as one.

Total free amino acids based on mg 100 g^{-1} fresh weight did not increase significantly for up to 5 h of illumination but then in the next hour they increased significantly from approximately 7 mg to 10.5 mg 100 g^{-1} fresh weight. Then they stayed constant over the next 18 h (Fig. 38). However, if the results are placed on a mg 100 g^{-1} protein basis (a better parameter) the same general trend is observed in that there is a highly significant increase between 5 and 8 h after illumination, followed by a gradual decrease at the end of the 24 h period.

Individual free amino acids

Light appears to have some effect on the seventeen individual free amino acids (based on mg amino acid 100 g^{-1} protein). The following essential amino acids present in mung bean seedlings; histidine for infants and isoleucine, leucine, lysine, methionine, phenylalanine, threonine and valine for adults, are of special interest. At the highest increase of total amino acids i.e. after 7 h light, there are significant increases in only lysine and histidine and a decrease in threonine (Table 7). The increase in histidine is particularly important, from 1.9 mg in the dark-grown seedlings to 5.1 mg 100 g^{-1} protein.

Amino acids from hydrolysed protein

There were marked differences between the amino acids of proteins and free amino acids, not only in composition (as already noted) but also in quantity (Tables 7 & 8). Whereas there were only 135 mg 100 g^{-1} protein in the latter, amino acids of proteins

Table 8. Amino acid composition of protein of etiolated mung bean seedlings subjected to illumination over a period of 24 h. Results are given as g 100 g^{-1} protein.

Amino acid	Time (h) in light									
	0	1	2	3	4	5	6	7	8	24
Lysine	4.8	4.8	4.7	4.8	4.7	4.6	4.8	4.9	4.7	4.7
Histidine	1.9	1.9	1.8	1.9	1.8	1.8	1.9	2.0	1.9	1.9
Ammonia	2.9	2.8	2.9	2.9	2.9	2.9	2.9	2.9	2.8	2.8
Arginine	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1
Aspartic acid	23.1	22.9	23.2	22.7	22.3	21.9	23.2	23.4	22.9	22.6
Threonine	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1
Serine	8.7	8.7	8.9	8.8	8.7	8.6	8.7	8.9	8.6	8.5
Glutamic acid	15.4	15.4	15.5	15.3	15.1	15.0	15.4	15.6	15.2	15.0
Proline	3.8	4.0	4.0	3.9	3.8	3.7	3.9	4.0	3.8	3.8
Glycine	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1
Alanine	14.4	14.6	14.7	14.6	14.4	13.9	14.6	14.8	14.3	14.1
Cystine	6.7	6.6	6.7	6.7	6.4	6.4	6.5	6.8	6.7	6.6
Valine	1.9	1.9	1.9	1.9	1.8	1.8	1.9	2.0	1.9	1.9
Leucine + Isoleucine	6.7	6.9	6.8	6.7	6.7	6.8	6.7	6.9	6.7	6.6
Tyrosine	2.9	2.8	2.9	2.9	2.7	2.7	2.8	2.9	2.8	2.7
Phenylalanine	2.9	3.0	3.0	3.0	2.9	2.9	2.9	3.0	2.8	2.8
Total	96.4 ± 1.0	96.6 ± 0.9	97.3 ± 3.5	96.4 ± 4.2	94.5 ± 3.3	93.3 ± 1.9	96.5 ± 4.4	98.4 ± 2.9	95.4 ± 3.6	94.3 ± 2.7

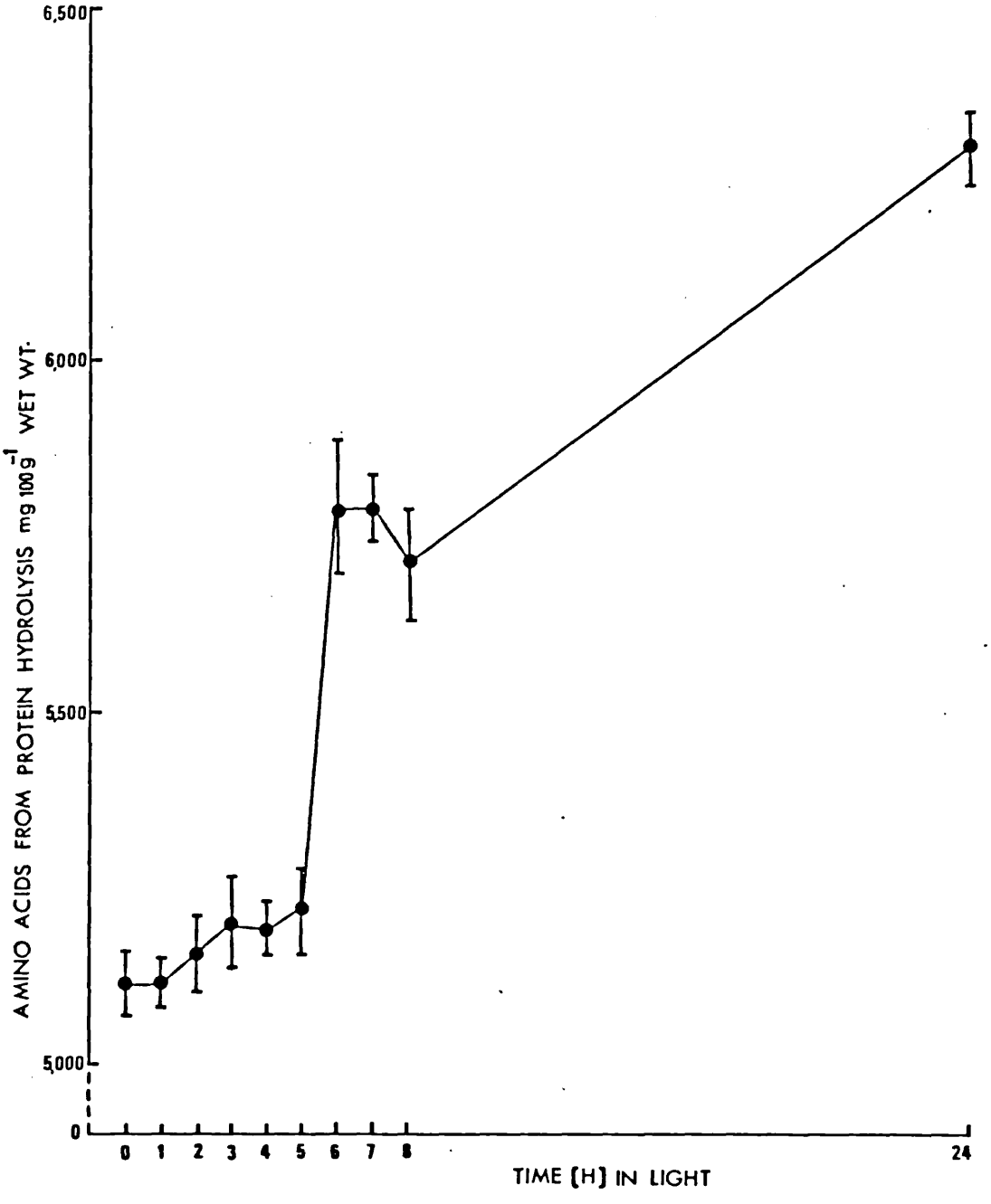

Fig. 39. Total amino acids from hydrolysed protein of 4-day-old dark-grown mung bean seedlings illuminated for various times within 24 h

made up 96.4 g 100 g^{-1} protein (x 715) of dark-grown seedlings. Light however did not affect total amino acids of hydrolysed proteins, nor did it affect individual amino acids making up these proteins.

Although no work has yet been reported on the effect of light on free amino acids and total amino acid composition, Tkachuk (1979), working with wheat, showed that free amino acids increased when wheat seed germinated in the dark. In the present studies the increase in free amino acids of mung bean seedlings may be due to an increase in proteolytic enzyme activity between 6 and 8 h after illumination. This is the time when grana membrane is actively being formed (p. 19), as are large increases in the following essential amino acids: lysine and histidine, and the non-essential ones aspartic acid, glutamic acid and proline. These must be contributing to small increases in total protein and consequently become incorporated into grana membranes.

CHAPTER VI

EFFECT OF LIGHT ON VITAMIN C

Introduction

Germination of mung bean seedlings leads to a marked increase in vitamin C (Fordham et al. 1975; Bender, 1978). Therefore in this section the amount of vitamin C of mung bean seeds has been determined, and it showed a sixfold increase four days after the seeds had germinated in the dark. The etiolated seedlings were then subjected to 1 000 lux continuous light over a 24 h period. There was not a significant change in the vitamin C content of seedlings during this time, which suggests the synthesis of this vitamin is not dependent on light.

Materials and Methods

Determination of 'total' vitamin C

Vitamin C was determined by the 2,4-dinitrophenylhydrazine procedure of Freed (1966) which is based upon the oxidation of ascorbic acid to dehydroascorbic acid. Subsequent transformation of dehydroascorbic acid to diketogulonic acid, followed by coupling with 2,4-dinitrophenylhydrazine under carefully controlled conditions gives red-coloured osazones. A comparison of colour produced in samples and ascorbic acid of standard solutions was used as a means of determining ascorbic acid content.

Reagents:

1. 9N Sulphuric acid.
2. 2% 2,4-Dinitrophenylhydrazine in 9N sulphuric acid, stored at 3°C when not in use.
3. 10% Metaphosphoric acid. This solution was kept under refrigeration when not in use.

4. 5% Metaphosphoric acid.

5. 2% Thiourea in 5% metaphosphoric acid.

6. 85% Sulphuric acid.

7. Bromine. This reagent was used in the fume cupboard.

8. Nitrogen. A cylinder of N_2 with facilities for saturating the gas with moisture.

9. Ascorbic acid standard: 100 mg ascorbic acid and 100 ml of 5% HPO_3. This solution therefore contains 1 mg ascorbic acid per ml.

Procedure:

This procedure describes the determination of total vitamin C-like compounds (referred to as 'total' vitamin C) represented by vitamin C, dehydroascorbic acid and diketogulonic acid, if present.

1. Extraction

(a) 100 g of seedlings (fresh or processed) were homogenised with 100 g of 10% HPO_3 for 2 - 5 min in a Waring blender.

(b) 40 g of the above homogenised slurry were weighed into a 100 ml flask and diluted to 100 ml with 5% HPO_3 solution.

(c) The suspended solids were removed by centrifugation and the supernatant liquid was decanted.

2. Oxidation to dehydroascorbic acid

(a) To the solution from 1 (c), 1 - 2 ml bromine was added and gently shaken until the solution appeared slightly yellow, decanted from excess bromine, and nitrogen gas saturated with water was passed through the solution until all the dissolved bromine was expelled, i.e. when the solution became colourless.

(b) To a 10 ml aliquot of oxidised extract 2 (a), 10 ml of 2% thiourea in 5% HPO_3 (Reagent 6) was added and mixed thoroughly.

3. Formation of osazone

Aliquots (4 ml) of the sample dilution 2 (b) were pipetted into three test tubes: one of the tubes was set aside to serve as a blank, and to each of the remaining tubes 1.0 ml of 2% 2,4-dinitrophenylhydrazine reagent was added. All the tubes were placed in a water bath at 37 $\pm$ 0.5°C for exactly 3 h. The tubes were then removed from the water bath and placed in an ice bath.

4. Treatment with 85% H_2SO_4 (Formation of soluble pigment)

While the tubes were in the ice bath, 5.0 ml of 85% H_2SO_4 was added to each, followed by 1.0 ml of 2% 2,4-dinitrophenyl-hydrazine. The tubes were removed from the ice bath and allowed to stand for exactly 30 min at room temperature. The 30 min waiting period is critical because many interfering osazones will slowly decompose in H_2SO_4, while shorter waiting time may result in serious over-estimation of the samples.

5. Measurement of colour

A Unicam SP500 Spectrophotometer was used to determine the percentage transmittance in the region of 490 - 550 nm.

6. Calibration of spectrophotometer and calculation

The vitamin C standard solutions used were in the range 1 - 12 $\mu g\ ml^{-1}$. A calibration chart was obtained by plotting percentage transmittance against concentration of vitamin C as abscissa on semilogarithmic paper. The total vitamin C content of each aliquot was obtained from this chart according to the formula:

$$\frac{R}{W} \times \frac{100}{1000} = \text{mg total vitamin C per 100 g of fresh weight}$$

where R = mg of total vitamin C per ml of solution obtained by reading from the calibration chart

and W = g of sample per ml of the diluted solution.

Results and Discussion

It is common practice in many Eastern countries to sprout mung bean seeds before consumption. This leads to a marked increase in the vitamin C content (Fordham et al. 1975). The vitamin C of mung bean seeds was 2.5 mg per 100 g and after 4 days etiolated seedlings contained 15 mg per 100 g vitamin C. Kylen et al. (1975) reported 20 mg per 100 g for 3-day-old etiolated mung bean seedlings and Fordham et al. (1975) found 35.3 mg per 100 g for total vitamin C for 3 - 4-day-old etiolated seedlings; both using the same determination procedure of Freed (1966) as was the case in the present study. However total vitamin C of etiolated mung bean seedlings in this study showed no significant change over the 24 h light regime (Table 9). The result is in agreement with those of Eggleton and Harris (1925) and of Matsuoka (1931) who suggest that the maximal yield was in the dark and that light had no great effect on vitamin C biosynthesis. It is also known that low light intensity and low nutrient level yield low vitamin C content in plants (Carroll, 1943). From these results it is obvious that there is no improvement in amounts of vitamin C after mung bean seeds have germinated. Three to 4-day-old dark-grown seedlings are usually used in such places where they are consumed in large quantities and these do provide a good source of vitamin C.

Table 9. Vitamin C content of seeds and seedlings in darkness and in light over a period of 24 h.

Sample	mg 100 g^{-1}
Seed	2.5 ± 1.3
Dark	15.0 ± 1.1
Light (h)	
1	15.8 ± 1.2
2	15.7 ± 1.3
3	16.1 ± 1.7
4	16.3 ± 0.9
5	16.0 ± 0.8
6	15.8 ± 1.8
7	16.7 ± 2.1
8	16.2 ± 1.1
24	16.6 ± 1.3

CHAPTER VIII

EFFECT OF LIGHT ON CHLOROPHYLLS AND CAROTENOIDS

Introduction

The biogenesis of photosynthetically-active membranes in the plastids of many higher plants is controlled by light. The plastids of the dark-grown plant (etioplasts) are devoid of chlorophyll but they contain protochlorophyllide. On illumination of the plants protochlorophyllide is rapidly converted to chlorophyll which is then esterified with phytyl alcohol. After a very short lag phase there is continued synthesis of chlorophyll to be found (Boardman et al. 1970). On the other hand etiolated seedlings are known to contain small amounts of carotenoids which increase rapidly when exposed to light (Goodwin and Phagpolngarm, 1960; Wolf 1963; Kay and Phinney, 1956; Valadon and Mummery, 1969). There is unrefuted evidence that carotenoids are derived from mevalonic acid (MVA):

$$\text{MVA} \longrightarrow \text{40C components} \longrightarrow \text{carotenoids}$$

These reactions are enzymatic and are stimulated by light. Different light intensity causes different rates of stimulation of the production of carotenoids (Britton, 1976). α- and β-carotene syntheses are on two different pathways (Fig. 40), as α-carotene cannot be derived from β-carotene (Goodwin and Williams, 1965), and vice versa (Williams, Britton and Goodwin, 1967). Carotenoids are located in the inner membranes of cells in the form of chromoproteins or probably in oil droplets (Goodwin, 1976). Plastids of dark-grown mung bean seedlings contained a substantial amount of prolamellar body and a small amount of porous lamellar sheet (thylakoids), but when they were subjected to light, thylakoid membranes increased substantially in number and finally formed grana by the end of an 8 h illumination period (p.19). This increase seems to be

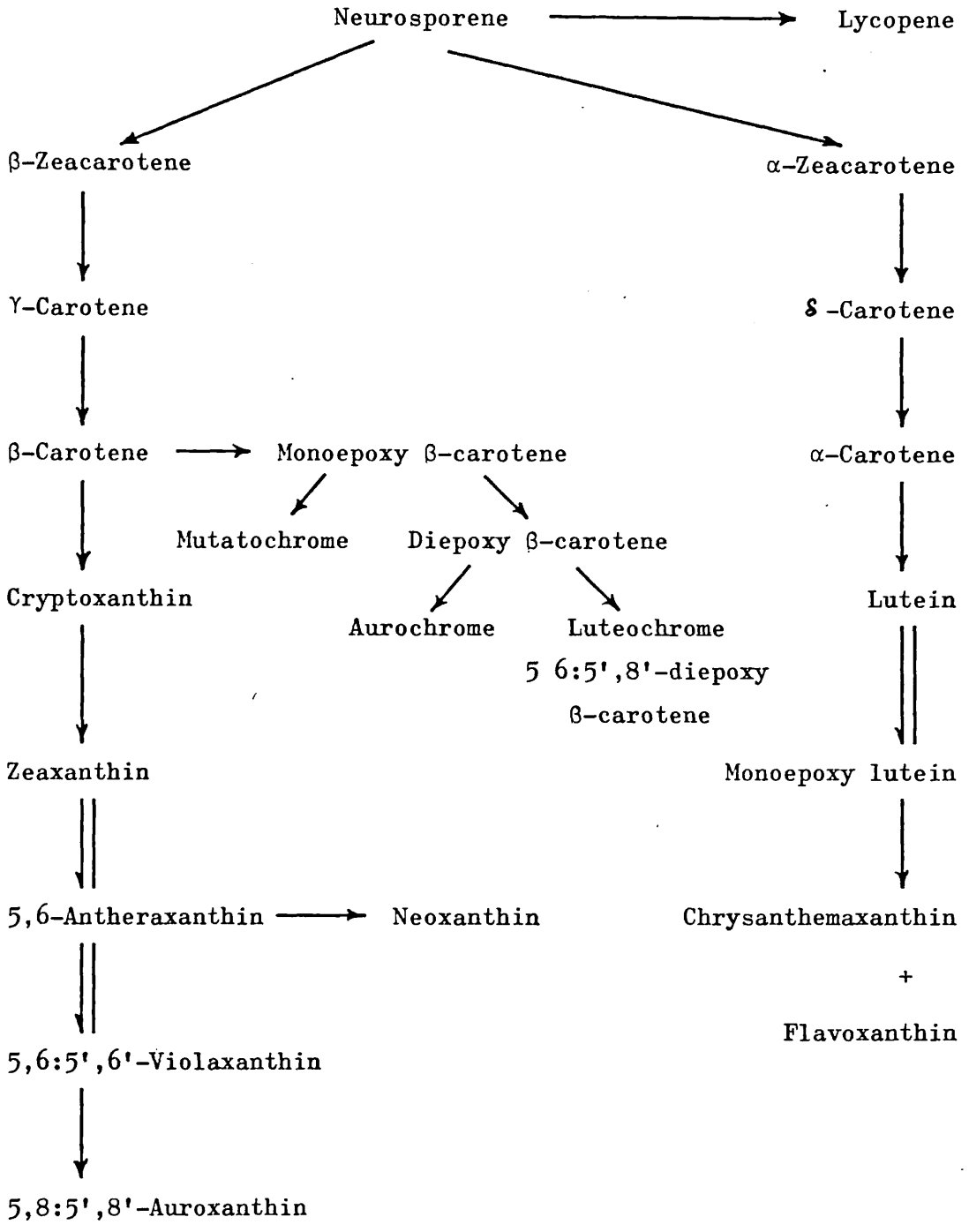

Fig. 40. Suggested pathways of the synthesis of α- and β-carotenes and of their derivatives

correlated with an increase in pigments which again suggests that carotenoids are probably located in this membrane.

Extraction and quantification of chlorophylls and carotenoids

Eighty grams of mung bean seedlings were extracted several times with methanol in a Waring blender until no more colour emerged. The colour absorbance at 650 nm and 665 nm was measured by a Unicam spectrophotometer Model SP800, using Mackinney's (1941) equations: total chlorophylls (mg l^{-1})$\equiv$25.5 D_{650} + 4.0 D_{665} where D is the absorbance. Partition with diethylether was performed when all colour was transferred to the epiphasic layer, which was concentrated under reduced pressure at about 35°C. Saponification of extracts and removal of sterols were performed by 10% KOH in methanol (v/v) (Valadon and Mummery, 1968, 1975). Unsaponifiable material was extracted with diethylether, washed free of alkali while chlorophylls present in these extracts were removed by this treatment. The extracts were taken up in 10% (v/v) ether in n-hexane and then chromographed on a magnesium oxide-celite (1:1 v/v) column (Jungalwala and Cama, 1962; Valadon and Mummery, 1968, 1975). Individual carotenoids were identified by comparing their chromatographic properties and UV and visible spectra with those of authentic carotenoids (Goodwin, 1954).

Carotenoids having epoxy groups were characterised by a modified 10% (v/v) HCl-methanol test (Jungalwala and Cama, 1962). On addition of a few drops of HCl-methanol (10% v/v) to compounds containing a 5,6-epoxide group, it was converted to a 5,8-furanoid epoxide which showed a lower wavelength shift of about 15 - 20 per epoxide group.

The concentrations of individual carotenoids were determined by measuring E_{max} and comparing it with known $E^{1\%}_{1\ cm}$ values at λ_{max} for pure pigments (Goodwin, 1955). For those pigments whose $E^{1\%}_{1\ cm}$ values were not known, λ_{max} was assumed to be 2500 (Goodwin, 1954). All results were calculated on a µg per 100 g wet weight basis.

Results and Discussion

As expected, mung bean seedlings which have been grown for four days in the dark did not have any chlorophylls but on transfer to light, greening was observed. After 1 h illumination the seedlings developed 11 mg 100 g^{-1} chlorophyll and after 8 h light, chlorophyll increased to 46 mg 100 g^{-1} and at 24 h it reached the highest amount of 160 mg 100 g^{-1} wet weight (Table 10). Similar observation of a dramatic increase in chlorophylls of Phaseolus vulgaris from early illumination up to 35 h have been noted by Boardman et al. (1970). On the other hand, dark-grown seedlings contained 284 µg 100 g^{-1} total carotenoids which increased more than five times to 1502 µg 100 g^{-1} after 8 h light and a further increase to 1676 µg 100 g^{-1} wet weight by the end of the 24 h illumination period (Table 11). The rate of increase, and total chlorophyll and carotenoids, were greater after 5 h than the preceding period and after that they continued to increase evenly over the next 19 h (Tables 10 and 11).

Flavoxanthin, which was present in etiolated seedlings, decreased fivefold from 15 to 3 µg 100 g^{-1} after only 1 h light, and disappeared completely after 3 h light, whereas other carotenoids increased at different rates over the 24 h illumination period. Lutein showed the greatest increase from 109 µg 100 g^{-1} in the dark to 761 µg 100 g^{-1} after 24 h; β-carotene from 18 to 321 µg 100 g^{-1}; violaxanthin from 101 to 267 µg 100 g^{-1}, and zeaxanthin from 31 to 134 µg 100 g^{-1} wet weight over the 24 h illumination period (Table 11).

It is well known that not all carotenoids are active as vitamin A precursors: the prerequisite for activity seems to be at least one β-ionone ring which must not be hydroxylated (Goodwin, 1962). β-Carotene has the highest biological potency of all carotenoids and cryptoxanthin is roughly one half as active as β-carotene. On this basis and using the conversion 1 mg β-carotene = 1667 IU vitamin A, 4-day-old dark-grown seedlings contained 32 IU per 100 g wet weight provitamin A,

Table 10. Total chlorophyll of mung bean seedlings grown in darkness for 4 days then transferred to light for a 24 h period.
Results are given as mg 100 g^{-1} wet weight.

Hours in light	0	1	2	3	4	5	6	7	8	24
Chlorophyll	0	11	11	17	19	22	35	40	46	160

Table 11. Carotenoids of mung bean seedlings grown in darkness for 4 days then transferred to light for a 24 h period.
Results are given as μg 100 g^{-1} wet weight and provitamin A content of seedlings as I.U. 100 g^{-1} wet weight.

Hours in light	0	1	2	3	4	5	6	7	8	24
β-Carotene	18	19	50	73	71	75	188	244	246	321
Cryptoxanthin	2	3	5	12	14	15	23	22	23	26
Violaxanthin	101	112	163	182	211	225	282	351	360	367
Lutein	109	118	193	231	268	285	501	630	670	761
Neoxanthin	8	9	10	12	28	38	59	57	63	67
Zeaxanthin	31	47	81	97	113	113	122	130	131	134
Flavoxanthin	15	3	2	1	-	-	-	-	-	-
Total	284	311	504	607	705	751	1175	1434	1502	1676
Provitamin A	32	34	87	132	130	137	333	425	429	557

which increased dramatically to 429 IU after 8 h light and a further increase to 557 IU per 100 g wet weight by the end of 24 h illumination period. The rate of increase of provitamin A showed the same trend as chlorophylls and total carotenoids over the same period of time (Table 11). Seedlings germinated in the dark do not produce chloroplasts and thus, as would be expected, contain no chlorophyll but do contain small amounts of carotenoids, mainly xanthophylls (Goodwin and Phagpolngarm, 1960; Valadon and Mummery, 1969). Carotenoids were found either in the etioplasts, which contained small yellowish granules, or outside the plastids (Granick, 1961). Illumination of etiolated seedlings rapidly results in the development of chloroplasts and the simultaneous synthesis of the characteristic green leaf pigments (Goodwin, 1958; Wieckowski, 1961; Wolf, 1963; Valadon and Mummery, 1969).

The results in the present study show that there was a substantial increase of chlorophylls and carotenoids after 5 h light which corresponds with the formation of grana between 6 h and 8 h after illumination. This increase in both chlorophyll and carotenoid syntheses could be due to the increase in the activity of plastids RNA polymerase (Bogorad, 1967). Increase in the activity of some enzymes of the photosynthetic carbon reduction cycle notably ribulose 1,5-diphosphate carboxylase (Chen et al. 1967; Keller and Huffaker, 1967), may build up grana membranes by formation and/or incorporation of some lipids and previously lacking polypeptides (Bogorad, 1976). The disappearance of flavoxanthin in light which has also been reported by Valadon and Mummery (1969) suggests that the steps from lutein to flavoxanthin may be dark-dependent ones, since it is known that the α-carotene pathway is independent of the β-carotene pathway (Fig. 40).

General Discussion on Section I

Compounds of nutritive value studied in mung bean seedlings were lipids, carbohydrates, proteins and the vitamins A and C. Changes have been observed in these nutrients as dark-grown seedlings were illuminated and these will be discussed more fully. However, one must bear in mind that although photosynthesis takes place in the presence of light, respiration too is going on at the same time. During growth some compounds are being synthesised and at the same time they are being metabolised, either being converted to other essential compounds or being degraded. It must be borne in mind therefore that any compound observed at any particular time is the result of these two processes occurring simultaneously.

Fats and oils are sources of energy in the diet. They are the most concentrated form of energy in foods. No recommended dietary allowance (RDA) has been established for fat, but it has been suggested that fat should not comprise more than 35% of a person's daily total kilocalories of which less than 10% should come from saturated and up to 10% from polyunsaturated fatty acids (Lapedes, 1977). Total lipids of mung bean seedlings increased from 350 mg in the dark to 646 mg 100 g^{-1} fresh weight after 24 h light.

The essential fatty acid, linoleic acid (18:2), found in mung bean seedlings is very important nutritionally as it cannot be synthesised by humans from either saturated fatty acids or their precursors. So, linoleic acid and other highly unsaturated fatty acids must be present in the diet. These compounds may be interconvertible and in 4-day-old dark-grown mung bean seedlings the three unsaturated fatty acids are oleic (18:1), linoleic (18:2) and linolenic (18:3), making up 2.5 mg 100 g^{-1} fresh weight, while the saturated ones are 1.7. Both the saturated and unsaturated fatty acids only double after 24 h in light and these increases to 4.8 mg 100 g^{-1} of unsaturated fatty acids are not very important nutritionally, although some linoleic acid is present here.

Carbohydrates are required by man to provide energy but at present there is no recommended dietary allowance (RDA) for these compounds because they can be produced in the body. It is desirable to include 50 to 100 g of carbohydrate in the diet daily to avoid ketosis, excessive breakdown of body protein and involuntary dehydration (Lapedes, 1977). Although 24 h illumination increases total available carbohydrate from 5.1 to 7 g 100 g^{-1} in mung bean seedlings, it is not a large enough increase to be important nutritionally.

All animal proteins, except gelatin, are complete as is the protein of the soybean in that they contain all the essential amino acids. Vegetable proteins (except for soybeans) are incomplete and mung beans are no exception, although tryptophan is the only essential amino acid not present here. Total protein increases in the light from 5.3 to 6.7 g 100 g^{-1} and again light-treated seedlings are not much better nutritionally than etiolated seedlings.

Light does not seem to have any major effect on the essential amino acids present in mung bean seedlings. Isoleucine + leucine, methionine + cystine, phenylalanine + tyrosine, and threonine and valine of both free amino acids and amino acids derived from proteins are not altered by light. It is worth mentioning that histidine, an essential amino acid for infants, increases in the free form from 1.9 mg to 5.1 mg 100 g^{-1} protein after 7 h illumination. When compared with the amount of histidine present in protein (1.9 g 100 g^{-1} protein) however, this increase in negligible.

In many parts of the world there is widespread vitamin A deficiency, resulting in thousands of cases of blindness. The daily requirement varies from 1500 international units (IU) for infants to 8000 IU for lactating women. In the present study it has been shown without any doubt that light is very important in increasing vitamin A from 32 IU in 4-day-old dark-grown mung bean seedlings to 557 IU 100 g^{-1} after 24 h light. This increase in vitamin A after 24 h will go a long way towards

providing this essential vitamin in a diet where it is in short supply.

Vitamin C has been known for a long time to be essential to humans. Infantile scurvy usually occurs between the sixth and twelfth months of life and is due to a lack of dietary vitamin, especially in artificially fed infants. In adults, the disease is often related to chronic or severe gastrointestinal disease or to food idiosyncrasies. The RDA are 30 mg per day for infants, 80 mg for growing boys and girls and 100 mg for lactating women. Four-day-old dark-grown mung bean seedlings contain 15 mg vitamin C 100 g^{-1} fresh weight which is not affected by light. In this case, dark-grown seedlings can provide a substantial amount of this vitamin (about one-fifth) to growing boys and girls.

In conclusion then, the only compound to benefit, by increasing, when mung bean seedlings are exposed to light is vitamin A. The other compounds with nutritive values are not affected by light and therefore the custom of eating etiolated mung bean seedlings provides some very useful nutritional compounds in a diet sadly lacking in some of them. However a diet lacking in vitamin A will benefit much more if the seedlings are exposed to light, even for 24 h. There are problems in this case. The etiolated seedlings are tender and are of an acceptable appearance but light-treated ones are tough, not so easily digestible and do not look appetising. Such are food preferences of human beings that they will refuse to eat what would otherwise be a good source of vitamin A.

For these reasons, only etiolated mung bean seedlings were used in blanching and processing, to be discussed in the following Section.

SECTION II

EFFECT OF PROCESSING ON VARIOUS CONSTITUENTS OF FOUR-DAY-OLD DARK-GROWN MUNG BEAN SEEDLINGS

CHAPTER VIII

METHODS OF PROCESSING AND EFFECT OF PROCESSING ON LIPIDS

Processing of mung bean seedlings (sprouts)

The processing method for 4-day-old dark-grown mung bean seedlings was kindly provided by Mr. K. W. Evans of the Campden Food Preservation Research Association (Chipping Campden, Glos.).

The fresh seedlings (500 g seedlings to 5 l water) were blanched for 1 minute in boiling water, the temperature of which never fell below 95°C, then water-cooled. Before packing, the seedlings were well drained. One-pound (454 g) jam jars with twist-off caps were used with a filled weight of 170 g bean-sprouts. The fresh seedlings had a pH of ~6.2.

In order to acidify the seedlings and to bring down the pH of the final product to ~3.25, the sprouts in the jars were covered with distilled vinegar (diluted by 4 parts to 1 part water) to give an overall acetic acid content in the final pack of 1.5%. The vinegar was added at 70°C and the capped jar had an equilibrium temperature of ~54°C.

The jars were pasteurised in water at 85°C for 13 min until the temperature of the jar centre reached 74°C. The jars were then air-cooled. Some of the seedlings would obviously have reached 85°C and all would have reached 74°C.

The dated, commercially sterilised, canned seedlings were

prepared by Tung Chun Soy and Canning Company, Hong Kong, and citric acid was the additive used to bring down the pH of the final product to $\sim$ 4.5.

Sterilisation of the seedlings was carried out at 120°C for 20 min for a container of similar dimensions to the jars, and the final temperature of the seedlings reached 115 - 120°C.

Blanching prior to packing was essential in order to destroy micro-organisms, to inactivate enzyme systems, which would otherwise cause deterioration during storage, to wilt bulky seedlings and reduce their volume prior to packing, to expel gases that might create excessive pressure in the can, and to maintain colour of product. Carotenoids become dissolved in small oil droplets during blanching, and in this way are protected from oxidative breakdown during processing (Duckworth, 1966).

The bottled seedlings (in jars) were stored under three different temperature conditions for six months, i.e. in the cold room at 10°C, at room temperature (about 25°C) and in an incubator at 35°C. Canned seedlings were only stored at room temperature (RT) over the same period of time.

For convenience, from now on the two methods of processing will be referred to as bottled (processed and stored in glass jars), and canned (processed and stored in tin containers).

Effect of processing on lipids

Introduction

Heat treatment (blanching) and the storage conditions of processed vegetables influence the nutritive components present e.g. proteins, carbohydrates, vitamins and mineral salts. Very little is known concerning the fate of small amounts of lipids (0.35%) in a vegetable such as the beansprout and it would be most informative to find out exactly what would become of such small amounts during processing and storage.

Ghanem and Hassan (1970) reported no significant changes of total lipids for a variety of pickled vegetables and Fricker et al. (1975) showed an increase of monogalactosyl diglyceride (MGDG) compared to digalactosyl diglyceride (DGDG) in processed spinach.

In the present study no significant changes in lipids during blanching of mung bean seedlings were observed and this will not be discussed further. However, the following experiments were carried out to determine the effect of six-months storage on lipids of processed mung bean seedlings either in (1) jars at three temperatures: 10°C, room temperature (RT) and 35°C, or in (2) cans at RT only.

It is likely that any degradation of lipids during this period would be related to oxidation and autoxidation, which would eventually produce hydroperoxide.

The study of the oxidation of lipids in foods is very complicated because the rate of reaction is influenced by the different components of foods present - carbohydrates, proteins, natural antioxidants, free amino acids and metals - as well as by the physical conditions under which the food has been prepared

and/or stored. Water activity, pH, temperature, availability of oxygen and light are among some of the physical factors which are important here (Scarborough and Watts, 1949; Olcott and Kuta, 1959; Mabrouk and Dugan, 1961; Bank et al. 1961; Sherwin, 1972).

Results and Discussion

The total lipids of fresh mung bean seedlings were 0.35 g per 100 g fresh weight and did not change after blanching of seedlings before packing. This percentage of total lipids in jars packed and stored for 6 months at 10°C and at RT, and cans at RT, did not vary significantly from fresh seedlings. However the total lipids of seedlings in jars at 35°C decreased significantly to 0.24 g per 100 g fresh weight (Table 12). Simple lipids as a percentage of total lipids in jars at 10°C and cans at RT was approximately 10%, very similar to that of fresh seedlings; that for jars at RT increased significantly to 14%, while the greatest increase was observed in jars at 35°C to 26% (Table 12), correspondingly the percentage of complex lipids of total lipids decreased in the latter two cases. Phospholipids as a percentage of complex lipids of processed seedlings decreased in all cases under the storage conditions of the experiment and it had fallen from 77% in fresh seedlings to 67% in seedlings stored at 10°C and those in cans at RT. In jars at RT and at 35°C there was the same significant decrease in percentage of phospholipids to about 60% (Table 12).

On the other hand glycolipids as a percentage of complex lipids had the opposite effect, namely, there were continued significant increases under all conditions of the experiment - jars at 10°C and cans at RT to 33%, and jars at RT and at 35°C to approximately 40% - compared to controls of 23% (Table 12).

Free Fatty Acids (FFA)

The percentage of free fatty acids of total simple lipids was 15% in fresh seedlings while it increased significantly to 28%

Table 12. Simple and complex lipids of fresh mung bean seedlings, and of those packed and stored at three temperatures over six months. Results are expressed as % total unless otherwise stated.

	Fresh	Bottled 10°C	Bottled RT	Canned RT	Bottled 35°C
SIMPLE LIPIDS					
Sterol esters	15 ± 1.5	9 ± 1.0	9 ± 1.8	9 ± 1.3	10 ± 1.1
Fatty acid esters	1 ± 0.4	1 ± 0.2	1 ± 0.2	1 ± 0.1	1 ± 0.3
Triglycerides	9 ± 0.4	9 ± 1.0	9 ± 1.1	9 ± 0.8	5 ± 1.1
Free fatty acids	15 ± 0.7	28 ± 1.1	33 ± 1.1	29 ± 0.8	51 ± 2.8
Stigmasterol	10 ± 0.5	6 ± 1.0	7 ± 1.1	6 ± 1.2	3 ± 0.9
β-Sitosterol	36 ± 0.5	14 ± 1.1	13 ± 1.2	14 ± 1.8	12 ± 1.2
Diglycerides	7 ± 0.5	12 ± 1.1	11 ± 1.0	9 ± 1.2	5 ± 1.0
Monoglycerides	7 ± 0.1	21 ± 1.0	15 ± 1.2	23 ± 1.2	13 ± 1.8
Total (mg 100 g^{-1}	33 ± 0.8	33 ± 1.1	43 ± 1.2	34 ± 0.8	62 ± 1.7
COMPLEX LIPIDS					
Sterol glycoside	8 ± 0.5	8 ± 1.5	8 ± 1.2	8 ± 1.1	5 ± 0.9
Cardiolipin	8 ± 0.6	-	-	-	-
Phosphatidic acid	12 ± 0.6	6 ± 0.8	-	-	-
Monogalactosyl diglyceride	5 ± 0.4	16 ± 1.1	22 ± 1.3	17 ± 1.2	29 ± 1.7
Ceramide monohexoside	6 ± 1.1	4 ± 0.8	3 ± 1.0	3 ± 0.6	2 ± 1.2
Phosphatidyl glycerol	5 ± 0.2	12 ± 1.9	15 ± 1.8	15 ± 1.9	14 ± 1.8
Phosphatidyl ethanolamine	12 ± 0.8	12 ± 0.6	9 ± 1.0	10 ± 0.6	8 ± 0.6
Digalactosyl diglyceride	3 ± 0.2	2 ± 0.4	2 ± 0.5	1 ± 0.2	1 ± 0.3
Sulpholipid	1 ± 0.5	1 ± 1.6	2 ± 1.1	2 ± 1.1	1 ± 1.2
Phosphatidyl inositol	2 ± 1.0	1 ± 1.8	1 ± 1.1	2 ± 1.2	1 ± 1.8
Phosphatidyl choline	32 ± 0.5	20 ± 1.2	12 ± 1.1	13 ± 1.4	10 ± 1.7
Lysophosphatidyl ethanolamine + Lysophosphatidyl inositol	-	1 ± 1.1	3 ± 1.8	3 ± 1.7	2 ± 1.8
Ceramide	-	2 ± 1.2	3 ± 1.1	4 ± 0.9	4 ± 1.2
Lysophosphatidyl choline	6 ± 0.1	15 ± 1.1	20 ± 1.2	22 ± 1.2	23 ± 1.1
Total (mg 100 g^{-1})	317 ± 4.1	302 ± 2.1	264 ± 3.4	286 ± 4.1	182 ± 2.9
Simple % of total lipids	10 ± 1.1	10 ± 0.8	14 ± 1.1	11 ± 1.2	26 ± 2.1
Complex % of total lipids	90 ± 1.1	90 ± 0.8	86 ± 1.1	89 ± 1.2	74 ± 2.1
Phospholipids % of complex	77 ± 1.2	67 ± 1.1	60 ± 0.5	66 ± 1.2	58 ± 1.0
Glycolipids % of complex	23 ± 1.2	33 ± 1.1	40 ± 0.5	34 ± 1.2	42 ± 1.0
Total lipids (g 100 g-1)	0.35 ± 0.02	0.33 ± 0.01	0.30 ± 0.01	0.32 ± 0.01	0.24 ± 0.02

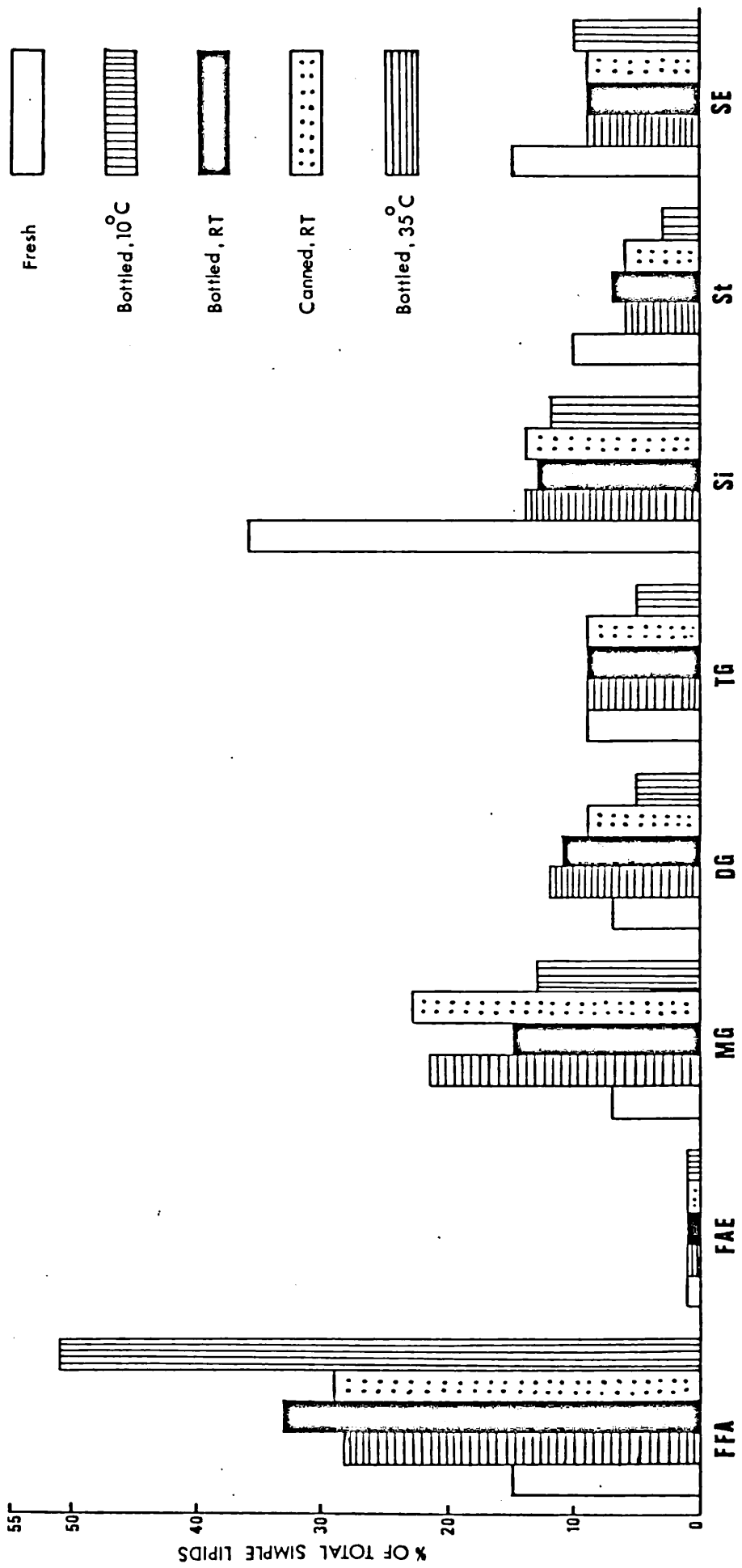

Fig. 41. Simple lipids of fresh 4-day-old dark-grown mung bean seedlings, and of those canned, bottled and stored for six months at different temperatures

in both jars at 10°C and cans at RT, and to 33% in jars at RT, with the greatest increase to 51% in jars at 35°C (Fig. 41 and Table 12). On the other hand fatty acid esters were found in small amounts, about 1% under all conditions tested and there were no significant differences under these conditions, as the percentage of those esters of total simple lipids did not vary significantly from that of control (1%) (Table 12 and Fig. 41).

Individual fatty acid components of FFA

The major free fatty acid components of processed mung bean seedlings were 14:0, 16:0, 18:0, 18:1, 18:2 and 18:3 (Fig. 42). In terms of $\mu g\ g^{-1}$ fresh weight, there was an increase in all fatty acid components at all conditions of storage compared to controls, the greatest increases being in 18:2 and 18:3 (Table 13 and Fig. 43). For these reasons the desaturation ratio, i.e. unsaturated/saturated, increased from 1.50 in fresh seedlings to 1.77 in canned seedlings stored at RT, the latter being very similar to that of seedlings in jars stored at 35°C (Table 13). Total free fatty acids in jars (132 $\mu g\ g^{-1}$) was higher than that in cans (121 $\mu g\ g^{-1}$) when both were stored at RT (Table 13). The fatty acid components that varied a great deal were the saturated 16:0 and the unsaturated 18:2 and 18:3 under all conditions of these experiments, but all these values were significantly higher compared to those of controls. On the other hand 14:0, 18:0 and 18:1 did not vary significantly under the four conditions of storage although once again these values were significantly higher than those of controls. The temperature of these experiments therefore did not seem to have any effect on the 14:0, 18:0 and 18:1 fatty acid components of seedlings in cans or jars under storage for six months. Whereas there were always increases in fatty acid components in terms of $\mu g\ g^{-1}$ fresh weight under the conditions of the experiments, this was not always the case if the percentage of the individual components of total FFA was taken into account (Table 13 and Fig. 44). There were similar increases in 14:0 under the four conditions of storage to approximately 8% compared to 3% in controls, in 18:3 to 26% compared to 20% in controls. The percentages of

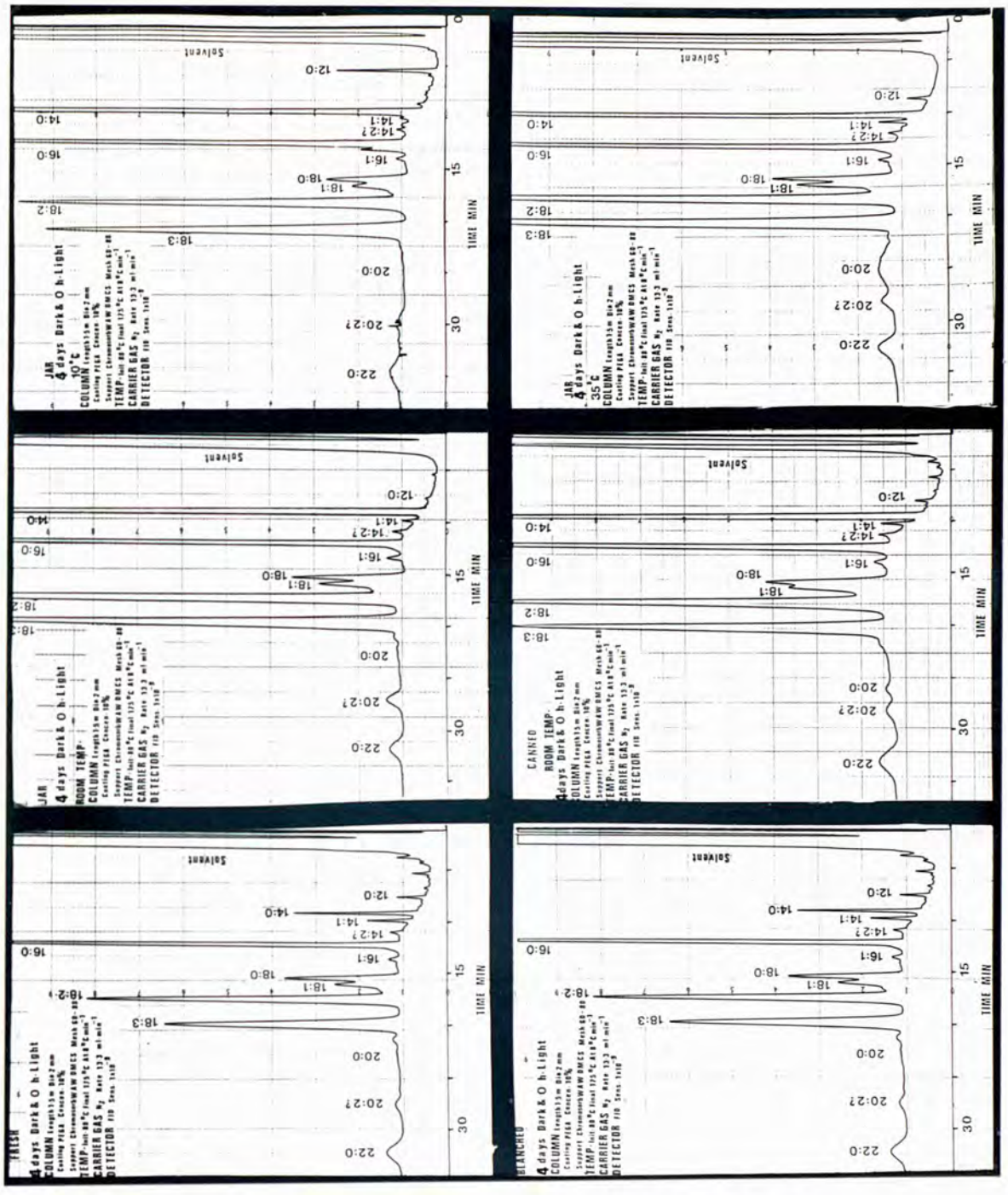

Fig. 42 . Gas-liquid chromatogram of fatty acid methyl esters of fresh mung bean seedlings, also bottled seedlings stored at 10°C, RT and 35°C, and canned seedlings stored at RT for a six-month period.

Table 13. Major free fatty acids of fresh 4-day-old dark-grown mung bean seedlings, and of those bottled and canned which were kept at various temperatures over six months. Results are expressed as $\mu g\ g^{-1}$ wet weight and as % of total fatty acids

	Fresh		Bottled 10°C		Bottled RT		Canned RT		Bottled 35°C	
Fatty acids	$\mu g\ g^{-1}$	%	$\mu g\ g^{-1}$	%	$\mu g\ g^{-1}$	%	$\mu g\ g^{-1}$	%	$\mu g\ g^{-1}$	%
14:0	1.3 ± 0.1	3 ± 1.2	8 ± 0.9	7 ± 0.8	10 ± 1.3	8 ± 0.9	10 ± 1.5	8 ± 0.4	10 ± 1.2	7 ± 0.7
16:0	8.6 ± 0.6	20 ± 1.6	20 ± 1.1	18 ± 1.7	26 ± 1.2	19 ± 1.5	20 ± 0.6	16 ± 1.6	30 ± 1.2	20 ± 1.8
18:0	7.3 ± 0.5	17 ± 1.1	13 ± 1.2	12 ± 0.6	15 ± 1.9	11 ± 0.7	14 ± 1.5	12 ± 0.9	16 ± 1.8	10 ± 1.1
18:1	6.0 ± 0.6	14 ± 1.1	10 ± 1.1	9 ± 0.7	10 ± 1.4	8 ± 1.2	10 ± 0.4	8 ± 0.4	10 ± 0.8	6 ± 0.6
18:2	11.2 ± 0.7	26 ± 1.3	30 ± 0.9	28 ± 1.8	37 ± 1.8	28 ± 1.4	37 ± 0.4	31 ± 2.1	46 ± 1.9	31 ± 2.6
18:3	8.6 ± 1.1	20 ± 0.8	32 ± 0.4	26 ± 1.0	34 ± 0.9	26 ± 0.4	31 ± 1.1	25 ± 1.1	40 ± 1.3	26 ± 0.8
Total FFA ($\mu g\ g^{-1}$)	43.0 ± 1.8		113.0 ± 2.5		132.0 ± 1.9		121.0 ± 2.1		152.0 ± 1.7	
Desaturation ratio	1.50		1.60		1.64		1.77		1.72	

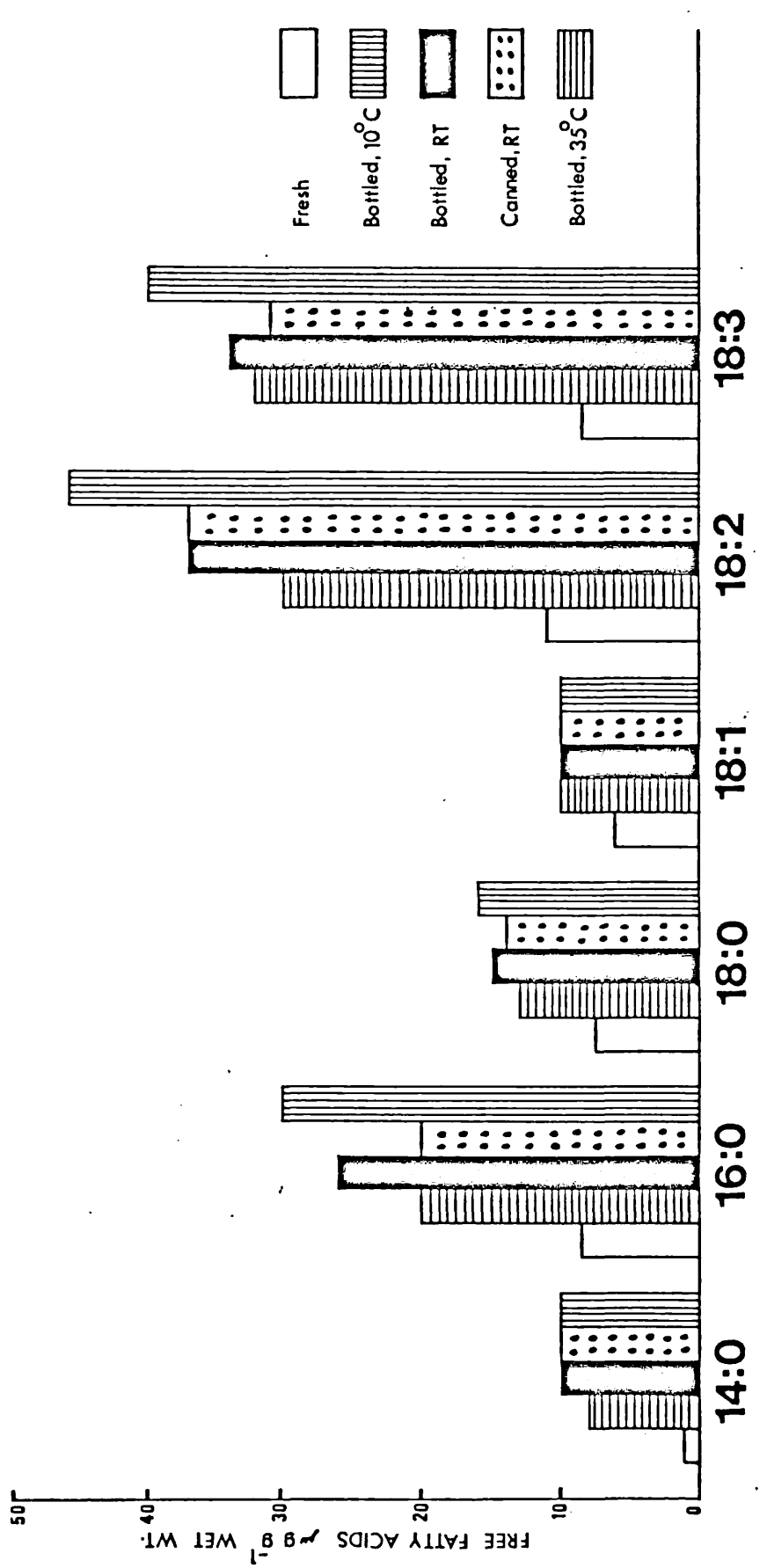

Fig. 43. Free fatty acids of fresh 4-day-old mung bean seedlings and of those bottled, canned and stored for six months at different temperatures

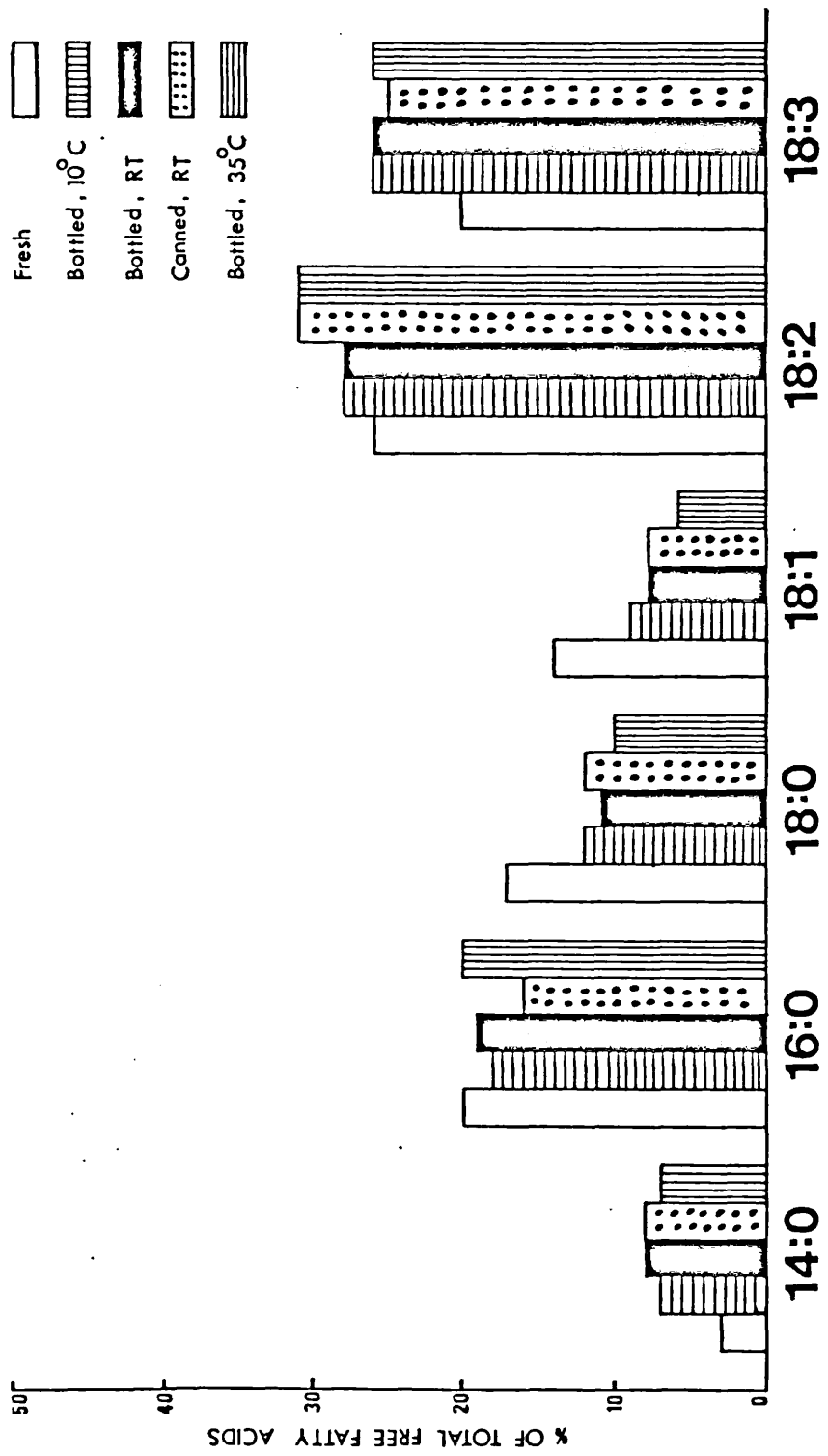

Fig. 44. Free fatty acids of fresh 4-day-old mung bean seedlings and of those bottled, canned and stored for six months at different temperatures

16:0 and 18:2 did not vary significantly from controls, approximately 20% in the former and 28% in the latter, while there were obvious decreases in 18:0 and in 18:1.

Acyl glycerides

Monoglycerides

The percentage of monoglycerides with respect to total neutral lipids in fresh seedlings was 7% and it increased under all four conditions tested. Seedlings in jars at 10°C and in cans at RT showed a 200% increase in both cases to approximately 21%, while those in jars at RT and at 35°C showed only a 100% increase (15%) over controls (Table 12 and Fig. 41).

Diglycerides

In the case of this diacyl glyceride its percentage of total neutral lipids in fresh seedlings was 7% and there were increases under three conditions of this experiment (never as high as in monoglycerides), and only a decrease in that of seedlings in jars at 35°C. The percentage of this neutral lipid increased to very similar values (approximately 11%) in all three conditions under discussion, namely jars stored at 10°C and at RT and cans at RT (Table 12 and Fig. 41).

Triglycerides

Fresh mung bean seedlings had a percentage of triglycerides of total neutral lipids of 9% which did not vary at all under three of the conditions used but in the fourth, i.e. in jars at 35°C, there was a definite decrease to 5% (Table 12 and Fig. 41).

Sterols

Sterols identified in fresh seedlings were β-sitosterol, stigmasterol and also sterol esters (SE), a neutral lipid fraction and sterol glycerides (SG) a complex lipid fraction. All these were also identified in processed seedlings. The results to be

discussed are presented in terms of β-sitosterol, stigmasterol and sterol esters as a percentage of total neutral lipids and the complex lipid fraction (SG) as a percentage of total complex lipids, unless otherwise stated (Fig. 41).

β-Sitosterol

There was 36% β-sitosterol in fresh seedlings which decreased to approximately 13% in all the other four conditions tested (Table 12 and Fig. 41).

Stigmasterol

This free sterol was in small amounts compared to β-sitosterol and only made up 10% total neutral lipids in fresh seedlings. This percentage decreased to a similar value (approximately 6%) under three of the conditions used, namely jars stored at 10°C and at RT, and cans at RT. The greatest decrease (3%) was observed in jars stored at 35°C (Table 12 and Fig. 41).

Sterol esters (SE)

Once again there were similar decreases of this neutral fraction under the four conditions tested (approximately 9%) compared to fresh seedlings (15%). This trend is slightly different to that observed for stigmasterol since even the jars at 35°C had similar values to those under the other three conditions (Table 12 and Fig. 41).

Sterol glycosides (SG)

The picture for this sterol fraction found as complex lipids is different from that of the other sterols discussed. It made up 8% complex lipids in fresh seedlings and this stayed the same under three of the conditions used but decreased to 5% in jars at 35°C (Table 12). The overall decrease in total sterols of processed compared to fresh seedlings seems to be the same at 10°C and at RT regardless of whether they were in cans or in jars. However at 35°C there is an even greater decrease due mostly to decreases in stigmasterol and in sterol glycosides.

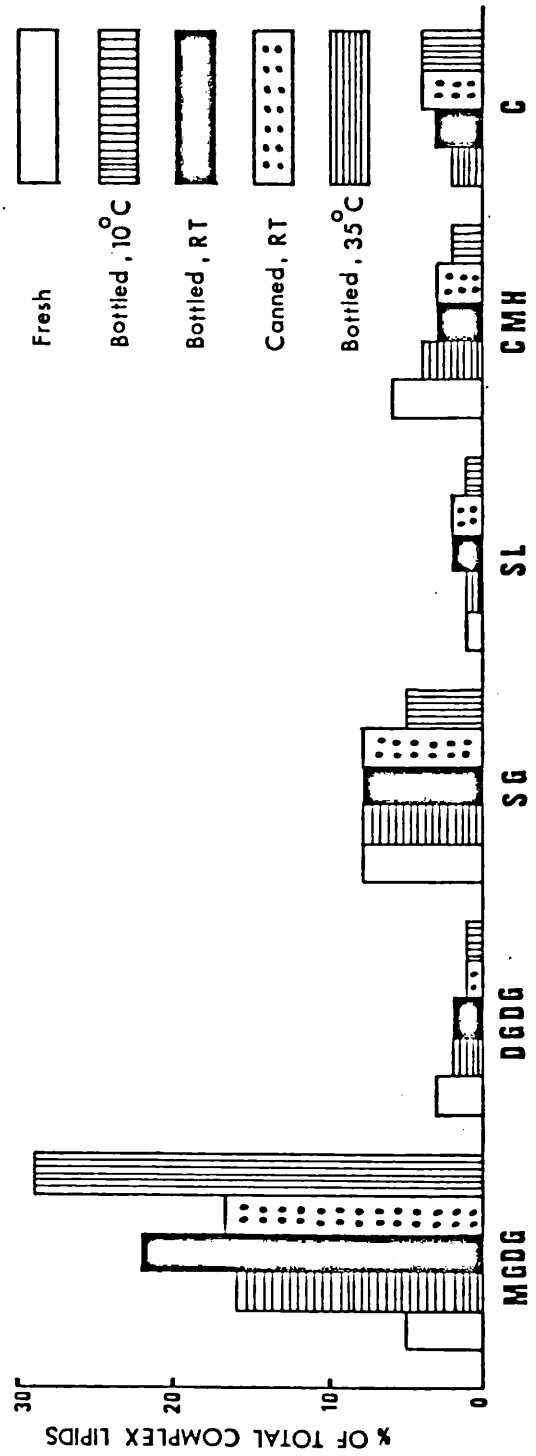

Fig. 45. Glycolipids of fresh 4-day-old dark-grown mung bean seedlings, and of those canned, bottled and stored for six months at different temperatures

So the higher temperature used (35°C) has a greater effect on sterol breakdown than the other lower temperatures (Fig. 45).

Other complex lipids

The results will be interpreted as a percentage of each fraction with respect to total complex lipids. In the first instance the complex lipids will be divided into two main fractions: glycolipids and phospholipids.

Overall, glycolipids increase while phospholipids decrease under the four conditions tested. The fraction which contributes most to the increase in glycolipids is monogalactosyl diglyceride (MGDG). This compound increases from a low 5% in fresh seedlings to a similar 16% in jars at 10°C and in cans at RT, to 22% in jars at RT and to a high 29% in jars at 35°C (Table 12). Digalactosyl diglycerides (DGDG) are also very low in fresh seedlings (3%) decreasing further under all four conditions used to a very low 1% in both cans at RT and jars at 35°C (Table 12). Sulpholipids make up a very small percentage of the complex lipids of fresh seedlings (1%) and this value does not change under the other four conditions of the experiment (Table 12 and Fig. 45).

Ceramide

Ceramide was not identified in fresh seedlings but is found in processed seedlings. This compound is derived undoubtedly from the glycoside complex ceramide monohexoside (CMH) since the increase in the former may be accounted for by a decrease in the latter under all the four conditions under study (Table 12 and Fig. 45).

Phospholipids

The major phospholipid in fresh seedlings is phosphatidyl choline (PC) which makes up about a third of the complex lipids.

Phosphatidyl choline (PC)

The phosphatidyl choline content of processed seedlings decreases

significantly under the four conditions of storage from 32% in fresh seedlings to 20% in jars at 10°C, while under the other three conditions it was at a similarly low 10% (Table 12 and Fig. 46).

Lysophosphatidyl choline (LPC)

It is not surprising to find that LPC increases during processing in proportion to PC decrease. Fresh seedlings had a percentage of 6% LPC which increased to 15% in jars at 10°C and to a high 22% in the other three conditions of storage. These results are in complete agreement with the pathway suggesting that LPC is formed from PC by the loss of one fatty acid residue from the β-carbon atom (Table 12 and Fig. 46).

Phosphatidyl ethanolamine (PE)

There were very small decreases in this phospholipid which made up 12% complex lipids in fresh seedlings, the greatest decrease being in jars at 35°C to 8%. It is to be noted that there was an insignificant difference in jars at 10°C compared to controls (Table 12 and Fig. 46).

Monophosphatidyl glycerol (PG) and Diphosphatidyl glycerol ≡ Cardiolipin (CL)

The phospholipid PG increased from 5% in fresh seedlings to 15% in three of the conditions of storage but only to 12% in jars at RT. Cardiolipin on the other hand was only observed in fresh seedlings as 8% of complex lipids. The trebling effect of processing on phosphatidyl glycerol can be accounted for simply by the decrease in cardiolipin. This suggests that processing, regardless of temperature and container, hydrolysed cardiolipin (diphosphatidyl glycerol) to the monophosphatidyl glycerol (PG) with the loss of one phosphatidyl glycerol molecule attached to the α-carbon atom of one of the glycerol residues (Table 12 and Fig. 46).

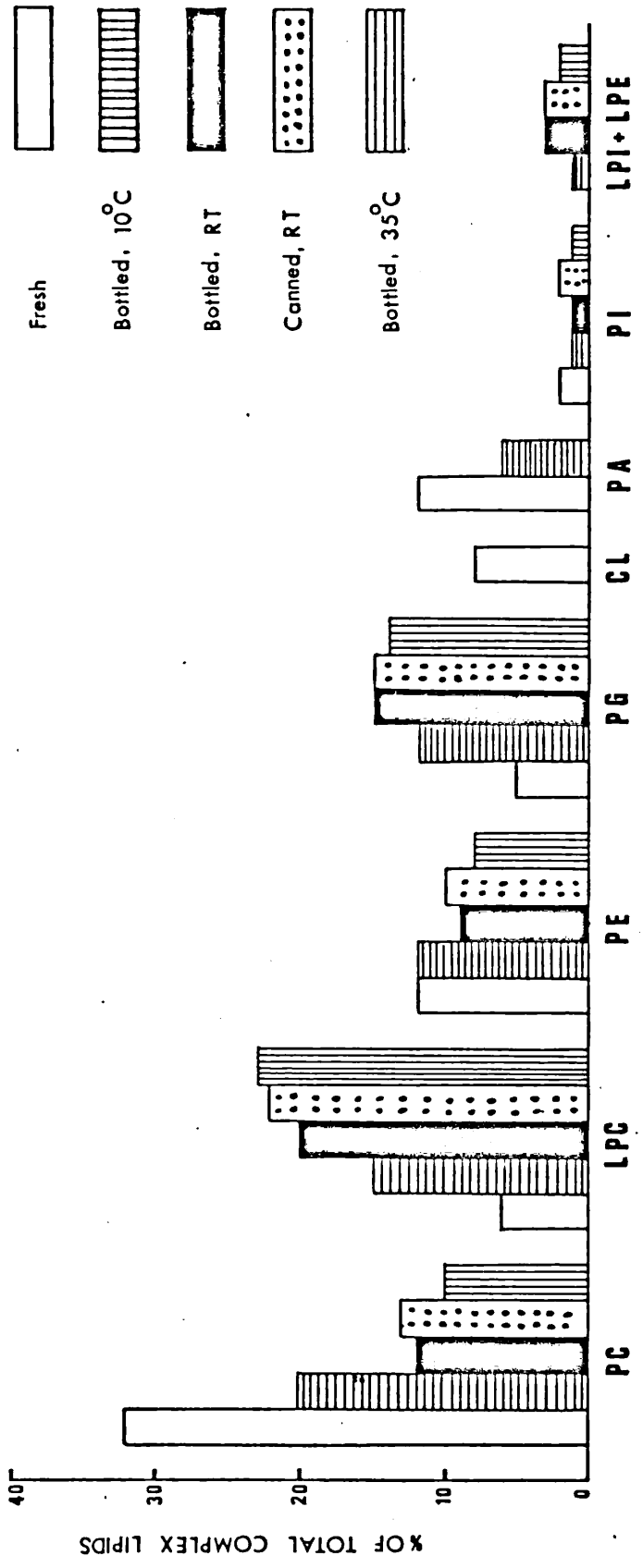

Fig. 46. Phospholipids of fresh 4-day-old dark-grown mung bean seedlings, and of those canned, bottled and stored for six months at different temperatures

Phosphatidic acid (PA)

This phospholipid made up 12% complex lipids of fresh seedlings. Although it was found halved (6%) in jars at 10°C it disappeared completely under the other conditions of storage. Phosphatidic acid can be easily hydrolysed to diglycerides but is nevertheless a very important compound in the building of the more complex phospholipids (Table 12 and Fig. 46).

Phosphatidyl inositol (PI)

There were very small amounts of this compound in fresh seedlings (2%) and their value varied only slightly under the conditions of storage (Table 12 and Fig. 46).

Lysophosphatidyl inositol (LPI) and Lysophosphatidyl ethanolamine (LPE)

These two were not separated further but were grouped together. They were found in processed seedlings in small amounts and in unmeasurable amounts in fresh seedlings, and would have arisen from the hydrolysis of the corresponding phosphatidyl compound, i.e. PI and PE respectively (Table 12 and Fig. 46).

Discussion

Total lipids of fresh seedlings was 0.35 g per 100 g wet weight and this value was fairly similar to that in jars at 35°C (0.24 g 100 g^{-1}). The pH of the liquid in jars was 3.25 and in cans 4.15, therefore a pH within this range does not seem to have much effect on total lipids. Similar results were obtained by Ghanem and Hassan (1970) studying the effect of pickling on a number of vegetables including peppers, onions and cauliflowers. Total lipids by themselves are not meaningful in this context since changes have been observed in a number of different components making up total lipids. Under the range of acidic pH in these experiments some of the complex lipid components were hydrolysed to smaller molecules and so were

some of the neutral lipids. In general, therefore, chemical degradation increased in response to storage at higher temperatures. In other words there were increases among simple lipids and a decrease in the more complex ones, especially in phospholipids as the storage temperature increased from 10°C to 35°C (Table 12). The increase in simple lipids of processed seedlings was due to a very substantial increase in free fatty acids. Since all complex lipids contain fatty acid, it is not surprising therefore that this component will vastly increase when the other complex lipids become hydrolysed. One other effect of this fatty acid increase is probably at the expense of sterols. Sterols are well known membrane components (Grunwald, 1975) and during processing there is no doubt that cell permeability is increased and this as a result of sterol degradation. The mono- and di-glycerides increase may be related to the decrease in phosphatidic acid, this latter compound disappearing completely at storage temperatures of 20°C and above. Also, as has already been discussed, phosphatidic acid is mainly responsible for the decrease in phospholipids under the storage conditions in these experiments.

Phosphatidylcholine (PC) seems to be converted to its lysoform (LPC) as expected and the amount of conversion increased as the temperature of storage increased from 10°C to 35°C.

Cardiolipin (= diphosphatidylglycerol) disappears in processed seedlings due to its hydrolysis to the corresponding monophosphatidylglycerol, which therefore increases as the storage temperature increases from 10°C to 35°C.

Glycolipids of processed seedlings increased mostly due to increases in MGDG; the higher the storage temperature (35°C) the greater was the increase in MGDG. Digalactosyldiglyceride (DGDG) on the other hand, although found in smaller amounts, decreased slightly under the conditions of storage. Fricker *et al.* (1975), working on spinach at temperatures of up to 100°C, suggested that lipid fractions were affected by heat treatment;

with increasing heat MGDG increased and DGDG decreased. This relationship however was not stoichiometric and it was possible that further reactions were leading to other unidentified products. The same results were obtained in the present study and there was no doubt whatsoever that the small decrease in DGDG could not by itself give rise to the large increases in MGDG. It has been suggested recently that MGDG and DGDG may be on separate pathways but may be formed from the same precursor 1,2-diglyceride. It may be possible that under the conditions of the present experiment, 1,2-diglyceride could combine with free galactose (p. 35) to give rise to MGDG. Fricker et al. (1975) also suggested that the influence of heat may change the plant cells and their membranes in such a way that lipids not accessible to the solvent in the fresh product become more readily extractable. This would account for the large increases in MGDG in processed mung bean seedlings without the corresponding large decreases in DGDG. This is the most likely explanation and is not unlike the suggestion put forward by Grunwald (1975) for sterols. Ceramide was not identified in fresh seedlings but only in processed ones. This molecule could very well be produced by a simple loss of the sugar moiety mostly from CMH because there was an inverse relationship between CMH and ceramide, the total of these two being very similar under all conditions of the present study.

CHAPTER IX

EFFECT OF PROCESSING ON CARBOHYDRATES

Introduction

As free sugars are soluble components, they would be affected by the blanching process and some may leach out into the blanching water (Adam, 1941). Other available carbohydrates on the other hand seem to be unaffected at this stage. It would be of interest to discover what happens when bottled and canned seedlings are stored at different temperatures. Processed mung bean seedlings were therefore stored at different temperatures over a six-month period and it was observed that the more complex carbohydrates were hydrolysed to the simpler forms e.g. reducing sugars. These components seem to be more under Maillard reaction control (p. 161) which makes some of them unavailable to the food system.

The order of reactivity of these sugars seems to be glucose then fructose, the latter being one-tenth as reactive as glucose (Lewis and Lea, 1959). Sucrose can undergo Maillard reactions presumably as a result of the splitting of its glycosidic bonds to yield glucose and fructose.

Certain carbohydrates e.g. cellulose, cannot be used as nutrients by man and the total dietary carbohydrate available to man will be referred to as total available carbohydrate.

Results and Discussion

A. Blanching

The percentage of free sugars of blanched seedlings had decreased slightly while that of starches and dextrins had decreased by half compared to fresh mung bean seedlings (Table 14).

The percentage contribution of simple sugars to total available carbohydrates of blanched seedlings increased, whereas that of starches and dextrins decreased compared to fresh seedlings (Table 14). Twenty-one percent of free sugars was lost during

Table 14. Total sugars, starches and dextrins, and available carbohydrates expressed as g 100 g^{-1} wet weight of fresh and blanched mung bean seedlings, the percentage contribution and percentage retention of each of the carbohydrates of blanched compared with fresh seedlings.

	g per 100 g		% Contribution		% Retention
	Fresh	Blanched	Fresh	Blanched	Blanched
Simple sugars	1.9	1.5	37	53	79
Starches and dextrins	3.2	1.7	63	47	53
Total available carbohydrates	5.1	3.2	100	100	63

Table 15. Individual simple sugars of fresh and blanched seedlings as g per 100 g wet weight, and percentage retention of sugars of blanched seedlings compared with fresh seedlings

	Fresh	Blanched	% Retention
Unidentified	0.2	0.2	100
Fructose	0.5	0.4	80
Glucose	0.7	0.6	85
Sucrose	0.5	0.3	60
Total	1.9	1.5	70

blanching, 47% starches and dextrins and 37% total available carbohydrates, so the results show that the blanching process facilitated the breakdown of complex carbohydrates (starches and dextrins) into free sugars. The percentage of individual free sugars retained is shown in Table 15 as well as the amounts of each sugar expressed as g 100 g^{-1} fresh weight. The largest decrease in free sugars is in sucrose, suggesting that it would probably be hydrolysed into its constituent monosaccharides, glucose and fructose. However the loss in free sugars may have been due to their solubility in the blanching water which was discarded here.

Adam et al. (1942) had reported an average of 81% of sugars retained after blanching from a wide variety of vegetables including fresh peas, stringless beans, runner beans, carrots and swedes. Thus the results with mung bean seedlings are comparable with those given by Adam et al. (1942). The effect of blanching on carbohydrates has been widely reviewed by many authors, namely Meyer (1969), Bender (1978) and Priestley (1979) who have all found similar results to those described here.

B. Storage

The total free sugars of bottled seedlings kept at 10°C and at RT for a period of six months had decreased from 15 mg g^{-1} in blanched seedlings to 6 mg g^{-1} in both cases while canned seedlings stored at RT or bottled and stored at 35°C showed a greater loss over the same period of storage time to about 5 mg g^{-1} (Table 16). Starches and dextrins of bottled seedlings stored at 10°C decreased to 7 mg g^{-1} over the six months storage period compared with 17 mg g^{-1} of blanched ones. Bottled and canned seedlings stored at RT and bottled seedlings at 35°C all showed a decrease in starch and dextrins to approx. 5 mg g^{-1} over the same period of storage. The percentage retention of free sugars, starches and dextrins, and total available carbohydrates for the bottled seedlings stored at 10°C were approximately 40% of each component. Forty percent of free sugars of bottled seedlings stored at RT were retained

Table 16 Total free sugars, total starches and dextrins, and total available carbohydrates (sugars, starches and dextrins) as mg g^{-1} wet weight of blanched, bottled and canned seedlings stored at different temperatures over a period of six months

		10°C	R.T.	R.T.	35°C
	Blanched	Bottled	Bottled	Canned	Bottled
Free sugars	15 ± 0.1	6 ± 0.1	6 ± 0.2	5 ± 0.3	5.4 ± 0.1
Starches and dextrins	17 ± 1.2	7 ± 0.6	5 ± 1.3	4.5 ± 1.0	4.6 ± 0.8
Total available carbohydrates	32 ± 1.1	13 ± 0.8	11 ± 0.1	9.5 ± 1.1	10 ± 1.1

Table 17 Percentage retention of carbohydrates of processed (bottled and canned) seedlings stored under different temperatures over a six-month period compared with blanched seedlings.

	% Retention			
	10°C	R.T.	R.T.	35°C
	Bottled	Bottled	Canned	Bottled
Free sugars	40 ± 1.2	40 ± 0.9	34 ± 1.8	36 ± 1.6
Starches and dextrins	41 ± 0.8	29 ± 1.8	26 ± 1.3	27 ± 1.2
Total available carbohydrates	41 ± 1.1	34 ± 1.5	30 ± 1.8	31 ± 1.1

while there were further decreases of starch and dextrins and of total available carbohydrates (Table 17). The results obtained for canned seedlings at RT and bottled seedlings at 35°C were very similar for all three parameters, approximately 35% free sugars were retained, 26% of starches and dextrins, and 30% total available carbohydrates (Table 17), all these values being lower than the corresponding ones for bottled seedlings stored at 10°C.

The losses of individual free sugars of bottled seedlings stored at 10°C and at RT over a six-month period were as follows: a loss of about 70% of the unknown sugar, 55% of fructose, 60% each of glucose and sucrose compared with values for blanched seedlings. The trend of individual free sugars lost when stored at 10°C or at RT were the same in both cases (Table 18); on the other hand canned seedlings stored at RT and bottled seedlings stored at 35°C showed very similar losses to one another but increased losses compared to bottled seedlings stored at 10°C and at RT respectively (Table 19). Total carbohydrates too follow these trends closely. In other words it has been observed that canned (RT) and bottled(35°C) seedlings lost more carbohydrates than bottled (10°C) and bottled (RT) seedlings over the same period of storage (Tables 18 and 19).

Differences between canned and bottled seedlings may be explained to a certain extent by the fact that canned seedlings received a higher initial heat treatment of 115 - 120°C for 20 min whereas bottled seedlings received a milder heat treatment of 85°C for the shorter time of 13 mins. Later however bottled seedlings had been stored at the higher temperature of 35°C compared to the canned seedlings (RT). Higher temperatures, as expected, hydrolysed the complex carbohydrates to give rise to simple sugars, and finally, further loss of reducing sugars can be accounted for in part by the browning reaction. So what has been observed can be summarised thus: under the storage temperatures used, regardless of the packing material (be it bottles at pH 3.25 or cans at pH 4.5), there is

Table 18. Individual free sugars of blanched and processed (bottled and canned) seedlings as mg g^{-1} fresh wet weight stored at different temperatures over a six-month period.

		10°C	R.T.	R.T.	35°C
	Blanched	Bottled	Bottled	Canned	Bottled
Unidentified	2 ± 0.1	1.2 ± 0.1	1.2 ± 0.1	0.9 ± 0.3	1.0 ± 0.1
Fructose	4 ± 0.2	2.4 ± 0.3	2.3 ± 0.4	1.8 ± 0.3	2.0 ± 0.3
Glucose	6 ± 0.8	1.8 ± 0.2	1.9 ± 0.3	1.8 ± 0.4	1.9 ± 0.5
Sucrose	3 ± .1	0.6 ± 0.1	0.6 ± 0.1	0.5 ± 0.1	0.5 ± 0.1
Total	15 ± 0.1	6.0 ± 0.1	6.0 ± 0.2	5.0 ± 0.5	5.4 ± 0.1

Table 19. Percentage retention of individual free sugars of processed (bottled and canned) seedlings over a six-month storage period at different temperatures compared with that of blanched seedlings

	% Retention			
	10°C	R.T.	R.T.	35°C
	Bottled	Bottled	Canned	Bottled
Unidentified	30 ± 1.0	30 ± 1.2	25 ± 1.1	25 ± 1.3
Fructose	45 ± 1.2	47 ± 1.8	45 ± 1.7	47 ± 1.9
Glucose	40 ± 1.1	39 ± 1.2	30 ± 1.2	33 ± 1.8
Sucrose	40 ± 1.1	40 ± 1.2	30 ± 1.5	33 ± 1.6

a series of hydrolyses of complex carbohydrates: starches are first converted to dextrins, then to reducing sugars. These reactions could be slowed down by storing at the low temperature of 10°C when there was less breakdown of starches into reducing sugars. The free sugars or reducing sugars which have been released by the hydrolysis process of starches may well be involved further as reducing agents. These sugars may be then decomposed to furfuraldehydes or related compounds which then polymerise or react with nitrogen compounds (amino acids) to form brown pigments (Meyer, 1969) through the Maillard reaction. General browning effects obtained during processing have been reviewed extensively by a number of authors, including Meyer (1969), Bender (1978) and Priestley (1979). Salem (1975), working specifically with broad beans, came to the conclusion that the browning effect was due mainly to the Maillard reaction.

CHAPTER X

EFFECT OF PROCESSING ON PROTEIN

Introduction

As degradation of proteins and carbohydrates (p. 160) during processing and storing are somewhat related through the Maillard reaction, this will be dealt with in this section.

Maillard reactions (non-enzymic browning) occur between proteins, amino acids and amines on the one hand, and sugars, aldehydes and ketones on the other. These reactions appear to be the major cause of browning during the heating or prolonged storage of foods containing proteins and carbohydrates. Although this browning is necessary in certain foods for the development of flavours and odours, it can cause a severe reduction in nutritive values of the protein components (Hurrell and Carpenter, 1977). Maillard reactions are complex and as yet not fully understood, although they do appear to follow certain common pathways.

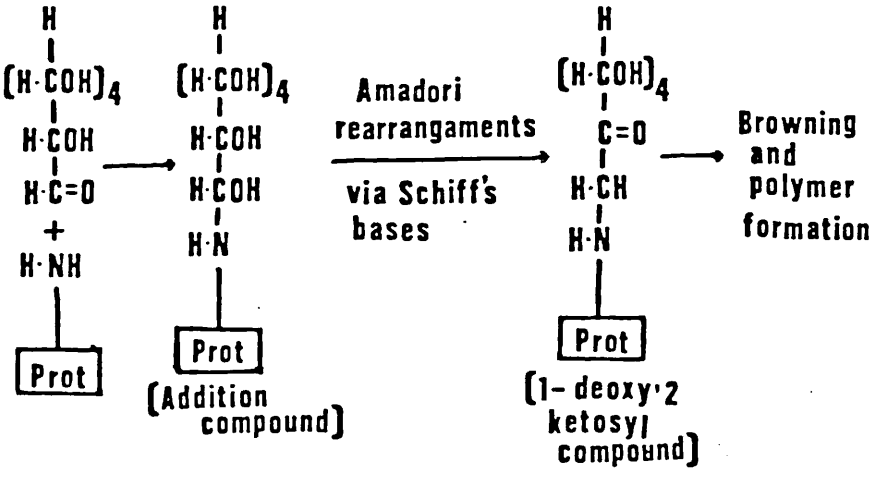

Fig. 47. Simplified scheme of Maillard reaction

The preliminary steps are shown in a simplified form in Fig. 47.

The first step involves a condensation reaction between the carbonyl group of a reducing sugar with the free amino group from an amino acid or protein. The condensation product formed immediately loses a molecule of water and is converted into a Schiff's base. This is converted into the N-substituted aldosyl-amine which is immediately converted to the 1-amino-1-deoxy-2-ketose by Amadori rearrangement.

Following the formation of this deoxy-ketosyl derivative, the reactions leading to the formation of brown pigments are not too well defined. In these later stages however amino acids can be degraded directly by reactions with other carbonyl compounds which are formed during these reactions.

It is now well known that Maillard reactions are more active under high pH, high amounts of free sugars and low moisture content.

This sections deals with a study of total protein as well as the seventeen amino acids obtained from mung bean seedlings when these were (1) bottled and stored under three different temperatures (10°C, RT and 35°C), and (2) canned and stored at room temperature for a period of six months.

Results and Discussion

A. Blanching

Blanched seedlings of mung beans had lost about 6% protein compared with fresh ones (Table 20). Adam *et al.* (1942) have also reported a slight loss of protein (average 4%) after water-blanching in a wide range of vegetables including stringless beans, broad beans, brussels sprouts, peas and carrots. The slight loss of protein could be due to hydrolysis of protein into free amino acids that would couple with carbohydrates, especially reducing sugars, to form brown pigments. However, heating of seedlings during the blanching process tends to make the protein more susceptible to hydrolysis during further processing.

Table 20. Total protein (g 100 g^{-1}) of fresh and blanched mung bean seedlings, and protein percentage retention during blanching

	g 100 g^{-1}	% Retention	% Loss
Fresh	5.3 ± 0.1	100	-
Blanched	5.0 ± 0.1	94	6

B. Storage

The loss in protein of bottled and canned seedlings ranged from 52% to 70% under the different temperature conditions over the six-month period (Table 21). The pH of canned (pH 4.5) and of bottled (pH 3.25) seedlings was low and under these conditions some of the protein would be hydrolysed to release amino acids which would react with reducing sugars to form a brown colour, the so-called Maillard reaction. Moreover the Maillard reaction involves condensation not only between the amino groups of amino acids in protein but also of the groups in peptide linkage or even in free amino acids with glycosidic sugars. The first stage of this reaction leads to the formation of colourless compounds which later complex to form brown pigments, hence the Maillard reaction is also termed non-enzymic browning (Bender, 1978). The percentage retention of protein in bottled and canned mung bean seedlings is highly dependent on the storage temperature regardless of the container. Compared to blanched seedlings, there is a loss of 52% in bottled seedlings stored at 10°C, increasing to approximately 60% at RT (both for bottled and canned seedlings), and to a very high loss of 70% at 35°C (Table 21). These results suggest once again that the Maillard reaction is positively dependent on high temperatures (Meyer, 1969; Bender, 1978; Priestley, 1979). It was obvious that under all the temperatures used that browning had taken place but the largest effect was observed in the bottled seedlings stored at 35°C for six months.

Effect of processing on amino acids

A. Blanching

The total free amino acids of blanched mung bean seedlings were 90.7 mg 100 g^{-1} protein compared with 135.3 mg 100 g^{-1} fresh ones, and therefore only 67% was retained during the blanching process (Table 22). All 17 amino acids decreased after blanching, with glycine disappearing totally (Table 22). So

Table 21. Total protein (g 100 g^{-1}) of blanched, canned and bottled mung bean seedlings, and protein percentage retention over a six-month storage period under various temperature conditions.

	g 100 g^{-1}	% Retention	% Loss
Blanched	5.0 ± 0.2	100	-
Bottled 10°C	2.4 ± 0.1	48	52
Bottled RT	2.0 ± 0.1	40	60
Canned RT	1.9 ± 0.1	38	62
Bottled 35°C	1.5 ± 0.2	30	70

Table 22. Free amino acids contained in fresh and blanched mung bean seedlings as mg 100 g^{-1} protein, and percentage retention of blanched compared to fresh seedlings

Amino acid	Fresh	Blanched	% Retention
	mg 100 g^{-1} protein		
Lysine	5.7	4.0	70
Histidine	1.9	1.8	95
Ammonia	17.0	10.0	59
Arginine	9.4	6.0	64
Aspartic acid	13.2	10.0	76
Threonine	9.4	8.0	85
Serine	11.3	8.0	71
Glutamic acid	13.2	12.0	91
Proline	5.7	4.0	70
Glycine	1.9	T	0
Alanine	5.7	2.0	35
Methionine	1.3	0.9	69
Valine	13.2	8.0	61
Isoleucine	9.4	6.0	64
Leucine	7.5	4.0	53
Tyrosine	3.8	2.0	53
Phenylalanine	5.7	4.0	70
Total	135.3	90.9	67

T = trace

Table 23. Amino acid composition of protein of fresh and blanched seedlings as mg 100 g^{-1} protein, and percentage retention of blanched compared with fresh mung bean seedlings

Amino acid	Fresh	Blanched	% Retention
	mg 100 g^{-1} protein		
Lysine	4.8	4.7	98
Histidine	1.9	1.9	100
Ammonia	2.9	2.8	96
Arginine	0.1	0.1	100
Aspartic acid	23.1	23.1	100
Threonine	0.1	0.1	100
Serine	8.7	8.7	100
Glutamic acid	15.4	15.4	100
Proline	3.8	3.8	100
Glycine	0.1	0.1	100
Alanine	14.4	14.4	100
Cystine	6.7	6.6	98
Valine	1.9	1.9	100
Leucine + Isoleucine	6.7	6.7	100
Tyrosine	2.9	2.9	100
Phenylalanine	2.9	2.9	100
Total	96.4	96.1	99

blanching has a marked effect on individual free amino acids from the extreme, labile glycine to histidine which was retained the most (95%).

The total amino acids from hydrolysed protein of blanched seedlings were 96.1 g 100 g^{-1} protein compared to 96.4 g of fresh seedlings. As can be seen (Table 23) very little total amino acid (= 1%) was lost, if any, and this is reflected in individual amino acids where no significant loss was observed except for possibly cystine. These results are rather different to those of Kulesza and Gertig (1976) who showed that 92% total amino acids were retained after blanching, with glycine the most susceptible amino acid.

These results showed that the loss of free amino acids was greater than that from hydrolysed protein. In other words, there may be more leaching of free amino acids into the blanching water than of proteins. The blanching water was not investigated here and therefore the possibility of the first stage in non-enzymic browning cannot be ruled out. During the first stage of the Maillard reaction (non-enzymic browning), a variety of groups such as aldehydes, ketones and reducing sugars combine with amino groups in aldol condensation to form first a Schiff's base and then an N-substituted glycosylamine. These undergo Amadori rearrangement at which stage the compounds formed are colourless (Bender, 1978). So the loss of free amino acids could also be accounted for by these means.

B. Storage

The total free amino acids of bottled seedlings stored for six months at 10°C were 41.1 mg 100 g^{-1} protein, for bottled and canned at RT approximately 30 mg 100 g^{-1} each, and for bottled at 35°C 18.6 mg 100 g^{-1} (Table 24), retaining 45%, 34% and 20% respectively compared with blanched seedlings (Table 24). The absence of free isoleucine suggests that it may have been converted into leucine during storage in all the conditions under study, while the least retained free amino acid of processed

Table 24. Free amino acids of blanched, bottled and canned mung bean seedlings stored under different temperature conditions over a six-month period. Results are expressed as mg 100 g^{-1} protein, and as percentage retention compared to blanched seedlings.

Amino acid	Blanched	Bottled 10°C		Bottled RT		Canned RT		Bottled 35°C	
	mg per 100 g	mg per 100 g	% Retention	mg per 100 g	% Retention	mg per 100 g	% Retention	mg per 100 g	% Retention
Lysine	4.0	1.0	25	0.8	20	0.8	20	0.6	15
Histidine	1.8	0.7	40	0.4	20	0.4	20	0.2	10
Ammonia	10.0	8.2	82	8.0	80	8.0	80	4.0	40
Arginine	6.0	3.0	50	1.4	24	1.4	23	1.2	20
Aspartic acid	10.0	8.2	82	8.0	80	8.0	80	4.0	40
Threonine	8.0	0.8	10	0.2	2.5	0.2	2.5	0.2	2.5
Serine	8.0	2.4	30	2.0	25	2.0	25	1.6	20
Glutamic acid	12.0	7.0	58	4.0	33	4.1	34	2.0	17
Proline	4.0	0.8	20	0.4	10	0.2	5	0.2	5
Alanine	2.0	1.4	70	1.4	70	1.4	70	1.0	50
Methionine	0.9	1.4	70	0.4	50	0.4	50	0.4	40
Valine	8.0	2.2	28	1.4	18	1.2	15	1.2	15
Isoleucine	6.0	-	-	-	-	-	-	-	-
Leucine	4.0	2.2	55	1.6	40	1.6	40	1.2	30
Tyrosine	2.0	1.2	60	0.4	20	0.4	20	0.2	10
Phenylalanine	4.0	1.4	35	0.6	15	0.6	15	0.6	15
Total	90.7 ± 1.8	41.4 ± 0.7	45	31.0 ± 1.6	34	30.7 ± 1.1	34	18.6 ± 0.8	20

seedlings was threonine with 10% for bottles at 10°C and 2.5% under the other three conditions. There was a considerable loss in proline and lysine as well but not as much as for threonine (Table 24). The free amino acids most retained in bottles stored at 10°C and at RT, and in cans at RT were ammonia and aspartic acid (approx. 80% in all three cases), while for bottles stored at 35°C, alanine was the most retained (50%) with methionine next best at 44% (Table 24).

The total amino acids of hydrolysed protein for bottles stored at 10°C, RT and at 35°C and cans at RT were as follows: 47.4, 38.9, 29.8 and 37.6 g 100 g^{-1} fresh weight (Table 25), retaining respectively 49%, 40%, 31% and 39% of blanched seedlings.

Serine was the least retained amino acid from the hydrolysed proteins under all four conditions of storage followed very closely by aspartic acid, cystine, leucine and alanine (Table 25). The most retained amino acid on the other hand was ammonia followed closely by proline (Table 25). It is to be noted that as the temperature of storage increased from 10°C, through RT to 35°C, the destruction rate of free amino acids and proteins increased regardless of the container in which the seedlings were stored. On the whole, there is very little difference in amino acids composition between seedlings stored in cans or jars at RT. It has been shown earlier (p. 157) that under similar conditions free sugars decrease. One would expect that a decrease in total proteins (as the storage temperature increases) would cause an increase in free amino acids. Free amino acids have been found in very small amounts compared to the amino acids obtained on hydrolysis of protein. If, as expected, increases in storage temperatures cause increases in free amino acids, then total free amino acids would increase enormously after canning or bottling. However, the reverse was observed; free amino acids decreased. This may be explained once more by the reaction of amino acids with reducing sugars through the Maillard reaction or by other reactions including hydroperoxides formed with unsaturated fat (Carpenter and Booth, 1973) (p.133). The reaction between

Table 25. Amino acid composition of proteins of blanched mung bean seedlings, and of those packed and stored at three temperatures over a six-month period. Results are expressed as g 100 g^{-1} protein, and as percentage retention compared to blanched seedlings.

Amino acid	Blanched	Bottled 10°C		Bottled RT		Canned RT		Bottled 35°C	
	g per 100 g	g per 100 g	% Retention	g per 100 g	% Retention	g per 100 g	% Retention	g per 100 g	% Retention
Lysine	4.8	3.4	70	2.8	58	2.6	55	2.0	43
Histidine	1.9	0.8	49	0.8	41	0.7	39	0.5	27
Ammonia	2.9	2.6	89	2.2	75	2.0	68	1.6	57
Arginine	0.1	-	-	-	-	-	-	-	-
Aspartic acid	23.1	8.1	35	6.0	26	6.5	28	5.0	22
Threonine	0.1	-	-	-	-	-	-	-	-
Serine	8.7	2.5	29	2.3	26	2.3	26	1.9	22
Glutamic acid	15.4	10.8	70	9.4	61	8.9	58	7.2	47
Proline	3.8	3.3	86	2.3	64	2.2	58	1.8	47
Glycine	0.1	-	-	-	-	-	-	-	-
Alanine	14.4	5.6	39	4.9	34	4.5	31	3.3	23
Cystine	6.7	2.3	35	2.0	30	1.9	28	1.5	23
Valine	1.9	1.4	74	1.2	64	1.1	58	1.0	51
Leucine	6.7	2.3	35	1.9	28	1.9	28	1.6	24
Tyrosine	2.9	1.8	63	1.5	53	1.5	52	1.1	39
Phenylalanine	2.9	2.4	82	1.6	54	1.5	53	1.3	45
Total	96.4 ± 1.9	47.4 ± 1.0	49	38.9 ± 1.3	40	37.6 ± 1.7	39	29.8 ± 0.8	31

oxidised fat and protein depends on temperature and may explain, to a certain extent, why processed seedlings stored at 35°C lost most protein. Furthermore, protein may well act as an antioxidant (Angelo and Ory, 1975) and therefore may no longer be available to the system. This can further explain protein losses.

CHAPTER XI

EFFECT OF PROCESSING ON VITAMIN C

Introduction

It is well known that blanching of vegetables prior to packing is essential. Among many beneficial features it inactivates enzymes which may cause deterioration during storage. On the other hand the cells may be ruptured and allow the water-soluble vitamin C to leach out from the system (Adam, 1941; Guerrant and O'Hara 1953; Meyer, 1969; Woollen, 1969; Lapedes, 1977; Bender, 1978). In this study about half the vitamin C of mung bean seedlings was lost during the blanching process.

Temperature, light, oxygen availability, high pH and the presence of reducing sugars are the other factors which may bring about the vitamin C destruction (Guerrant and O'Hara, 1953; Meyer, 1969; Bender, 1978). In the present study the effectsof two factors, pH and storage temperature on the vitamin C content of processed mung bean seedlings, were investigated. It will be shown that processed seedlings at the lower storage temperature and lower pH retained more vitamin C than when stored at higher temperatures with higher pH.

Results and Discussion

A. Blanching

Half the total vitamin C of fresh mung bean seedlings was lost during blanching (Table 26). The results were not entirely unexpected since it is well known that there are other activities apart from enzyme deactivation during blanching. Vitamin C oxidase will have been completely deactivated since the blanching temperature used was well above 65°C, the temperature required for complete inactivation of this enzyme (Bender, 1978). At the same time the tissues rupture due to blanching and the soluble

Table 26. The amounts of vitamin C (mg 100 g^{-1} fresh weight) and percentage retention during blanching of mung bean seedlings compared with fresh

Sample	mg 100 g^{-1}	% Retention
Fresh	15 ± 1.1	100
Blanched	7 ± 1.3	47

vitamin C is leached out. Birch and Parker (1974) have pointed out that two reactions appear to be occurring simultaneously during heating, namely breakdown of the cell structure allowing contact between enzyme and substrate, and destruction of the enzyme. As a result, leaching losses of vitamin C into the cooking water become greater as the cells become more and more disrupted. The results of the vitamin C content of seedlings after blanching when 47% was retained (Table 26) are in close agreement with those of Guerrant and O'Hara (1953) working with peas and lima beans.

The amount of vitamin C lost may depend upon another factor, namely on the volume of water present rather than the heat treatment. However this subject has been reviewed extensively by many authors (Meyer, 1969; Woollen, 1969: Lapedes, 1977; Bender, 1978) and will not be discussed further. Suffice it to say that the possibility exists and that the volume of water present may greatly increase vitamin C destruction.

B. Storage

The vitamin C of processed seedlings in jars and cans during a six-month storage period was investigated. The results (Table 27) show that there was an approximate loss of 70% in jars stored at 10°C, RT and 35°C for six months compared to controls. There was no significant difference between the jars at those three temperatures. Cans kept for the same period at RT showed a 90% loss which was significantly higher than for jars kept at the same temperature (Table 27).

Three hypotheses have been put forward to explain vitamin C destruction during processing:

1. Destruction of ascorbic acid by an oxidation process

This could occur immediately after bottling and canning due to dissolved headspace air (Bender, 1978). The stability of vitamin C in the presence of oxygen decreases with increases in temperature and pH (Lapedes, 1977).

Table 27. The amounts of vitamin C (mg 100 g^{-1} fresh weight) and percentage retention of bottled and canned mung bean seedlings which had been stored under different temperatures over six months, compared with blanched seedlings.

Sample	mg 100 g^{-1}	% Retention
Blanched	7.0 ± 1.3	100
10°C Bottled	2.5 ± 0.4	35
RT Bottled	1.8 ± 0.7	26
RT Canned	0.8 ± 0.5	12
35°C Bottled	1.7 ± 0.6	24

In the present study, temperatures of up to 35°C do not have any marked effect on the vitamin C content of bottled seedlings, whereas canned seedlings at room temperature showed a decrease. The only differences between these were (1) that the latter had a pH of 4.5 compared to 3.3 for seedlings in jars, and (2) the cans were made of metal and the jars of glass. Both of these differences could account for the greater loss of vitamin C in canned compared to bottled seedlings. However, residual oxygen is normally used up in the electrochemical process of corrosion and so disappears rapidly in cans and much more slowly in bottles (Adam, 1941). Therefore one would have expected more breakdown due to continued oxidation in bottles, the converse of what has been observed in the present study. It is possible therefore that the metal itself can become reactive and thus destroy a certain amount of vitamin C.

2. Anaerobic destruction of vitamin C phosphates

This leads to the formation of furfurals (Kefford et al., 1959). The reaction is largely independent of pH but is slightly increased in the range pH 3 - 4, which is the range of pH used in the present experiment.

3. Non-enzymic browning

This is known as the Maillard reaction and occurs between carbohydrates or carbohydrate acid (vitamin C) and amino acids, resulting in the formation of brown pigments (Meyer, 1969). As the colour of processed seedlings had darkened over the storage time this reaction could also have taken place here.

CHAPTER XII

EFFECT OF PROCESSING ON CAROTENOIDS

Introduction

Carotenoids form one of the most important groups of natural pigments which are to be found in small amounts in etiolated mung bean seedlings (p.124). The important role of carotenoids in foodstuffs is their potency as vitamin A, which is a vital dietary substance. It is well known that not all carotenoids are active as vitamin A precursors (Goodwin, 1962). β-Carotene has the highest biological potency (1 mg β-carotene = 1667 IU) of all carotenoids, and cryptoxanthin is one half as active as β-carotene.

Carotenoids are insoluble in water and they are relatively stable to heat (Hollingsworth, 1970), which makes them resistant to the blanching process. In fact they have been shown to increase after this treatment (Guerrant et al., 1947; Feaster et al., 1949; Heberlein et al., 1950; Geurrant and O'Hara, 1953). Carotenoids are readily oxidised in the presence of oxygen which is accelerated by high temperature and light availabilities, and acidic pH assists the conversion of 5,6-monoepoxy- and 5,6:5',6'-diepoxy-carotenoids e.g. violaxanthin, to the 5,8- and 5,8:5',8'-furanoid epoxide forms (Panalaks and Murray, 1970; Moss and Weedon, 1976; Woolfe, 1979) (Fig. 48).

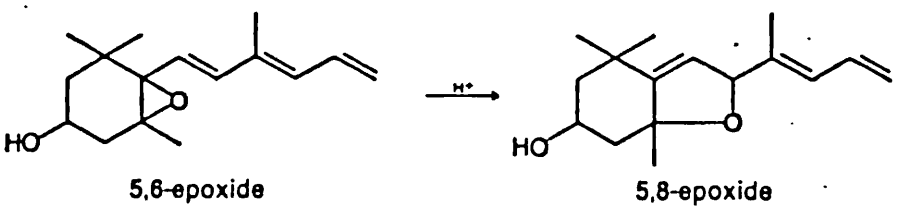

Fig. 48. Isomerisation of a 5,6-monoepoxide carotenoid into 5,8-epoxide form

In the present study processed seedlings which had been packed in cans and jars were subjected to different storage temperatures ranging from 10°C to 35°C over a six-month period. It seems that the temperature of storage has a greater significant effect on the extent of carotenoid destruction than the initial heat processing (blanching) (Lund, 1975).

A. Blanching

The total carotenoids of blanched mung bean seedlings was 295 μg 100 g^{-1} compared to 284 μg 100 g^{-1} of fresh ones; i.e. a 4% increase which may not be significant. However there was a rearrangement of certain individual carotenoids. The amount of lutein and zeaxanthin was 172 and 50 μg 100 g^{-1} respectively, compared to 109 and 31 μg 100 g^{-1} for fresh seedlings with a corresponding increase of 58% and 61% respectively. On the other hand cryptoxanthin did not change at all and β-carotene not very much, while the greatest decreases were in neoxanthin (48%), violaxanthin (53%) and flavoxanthin (87%). Therefore the latter three compounds were most susceptible to the blanching process. Provitamin A content of fresh seedlings was 32 IU which decreased to 28 IU per 100 g wet weight (Table 28). Similar observations related to increase in carotenoids content of vegetables after blanching have been noted previously by Guerrant et al. (1947), on various vegetables, Feaster et al. (1949) and Heberlein et al. (1950) on peas, Guerrant and O'Hara (1953) on peas and lima beans.

The differences may be attributed to a number of factors, among which may be mentioned the fact that certain carotenoids are generally stable under the conditions for blanching and to a certain extent blanching may increase the extractability of the carotenoids present (Guerrant and O'Hara, 1953). In the present study the increase in lutein and zeaxanthin is difficult to explain but has also been observed by Mummery and Valadon (personal communication) in orange juices. It may be possible that under the conditions used the epoxy compounds, violaxanthin

Table 28. Carotenoids of fresh and blanched mung bean seedlings (μg 100 g^{-1} wet weight), and percentage retention of individual carotenoids of blanched compared to fresh seedlings. Provitamin A content of seedlings is expressed as IU 100 g^{-1} wet weight.

Carotenoids	Fresh	Blanched	% Retention
	μg 100 g^{-1}		
β-Carotene	18	16	89
Cryptoxanthin	2	2	100
Violaxanthin	101	48	47
Lutein	109	172	158
Neoxanthin	8	5	62
Zeaxanthin	31	50	161
Flavoxanthin	15	2	13
Total	284	295	104
Provitamin A	32	28	87

and neoxanthin on the one hand, and flavoxanthin on the other may yield their parent compounds zeaxanthin and lutein respectively. It is known that under certain conditions of treatment with acid chloroform, although most of the epoxy compounds yield other furanoid derivatives, some are converted to their original parent molecules (Liaaen-Jensen, 1971). In the present study the whole of the loss in neoxanthin and violaxanthin will have to account for the increase in zeaxanthin which is just possible, but loss in flavoxanthin of 13 µg cannot possibly account for the increase in lutein of 63 µg 100 g^{-1} wet weight. It is now well established that α- and β-carotene syntheses are on two separate pathways (p. 121) and that β-carotene and its derivatives zeaxanthin, neoxanthin and violaxanthin cannot give rise to α-carotene and its derivatives lutein and flavoxanthin, and vice versa (Fig. 40). So the high increase in lutein and zeaxanthin may be due to increases in the extractability of carotenoids when seedlings are blanched. (Guerrant and O'Hara, 1953).

B. Storage

The total carotenoid content of canned mung bean seedlings stored at RT for six months was 162 µg 100 g^{-1}, whereas bottled ones at the same storage temperature (RT) contained 18 µg 100 g^{-1} and at 10°C 42 µg 100 g^{-1} (Table 29). Bottled seedlings stored at 35°C for six months no longer contained carotenoids. Canned seedlings stored at RT retained the most carotenoids (55%) followed by bottled seedlings stored at 10°C (14%) and at RT (6%). Although blanched seedlings contained seven individual carotenoids, namely β-carotene, cryptoxanthin, violaxanthin, lutein, neoxanthin, zeaxanthin and flavoxanthin, a number of these disappeared during storage. Bottled seedlings stored at 10°C for six months contained 5 IU, and bottled and canned at RT 2 IU and 19 IU per 100 g wet weight respectively (Table 29). These results show that canned seedlings retained more carotenoids than did bottled seedlings.

Similar results have been obtained by Guerrant and O'Hara

Table 29. Carotenoid content of blanched, bottled and canned mung bean seedlings as μg 100 g^{-1} wet weight, provitamin A as IU 100 g^{-1} wet weight, and percentage retention of canned and bottled seedlings stored at different temperatures over six months compared to blanched seedlings

Carotenoids	Blanched	Storage Temperature					
		Bottled 10°C		Bottled RT		Canned RT	
	wt.	wt.	% Retention	wt.	% Retention	wt.	% Retention
β-Carotene	16	3	19	1	6	11	69
Cryptoxanthin	2	-	-	-	-	1	50
Violaxanthin	48	-	-	-	-	-	-
Lutein	172	28	16	12	7	104	60
Neoxanthin	5	-	-	-	-	2	40
Zeaxanthin	50	11	22	5	10	44	88
Flavoxanthin	2	-	-	-	-	-	-
Total	295	42	14	18	6	162	55
Provitamin A	28	5	18	2	7	19	68

(1953) on peas and lima beans. They suggested that in cans there is the complete elimination of light and less oxygen available, resulting in less destruction of carotenoids compared to bottled seedlings. Storing bottled seedlings at 10°C is better than at RT since more of the carotenoids are retained at the lower temperature. The fact that β-carotene, lutein and zeaxanthin are the only compounds retained is most interesting. This may be explained on the grounds that they are not as labile as their other derivatives.

Bottled seedlings at the two temperatures (10°C and RT) had lost cryptoxanthin, violaxanthin, neoxanthin and flavoxanthin, whereas the canned seedlings stored at RT lost the least, only violaxanthin and flavoxanthin disappearing (Table 29). In canned seedlings stored at RT 50% cryptoxanthin and 40% neoxanthin had been retained as had 69% β-carotene, 60% lutein and 88% zeaxanthin (Table 29) compared to carotenoids of blanched seedlings. In the case of bottled seedlings stored at 10°C and at RT, only the following three carotenoids remained: β-carotene, lutein and zeaxanthin and these were retained at 19%, 16% and 22% respectively at 10°C, and 6%, 7% and 10% respectively at RT (Table 29).

The compound that disappeared the most, apart from cryptoxanthin, flavoxanthin and neoxanthin (all found in very small amounts in blanched seedlings), was none other than violaxanthin which is the 5,6:5',6'-diepoxy derivative of zeaxanthin. Under acid conditions this gives rise to the 5,8:5',8'-difuranoid compound auroxanthin which is very labile and is easily degraded under the conditions used. Bottled (pH 3.25) and canned (pH 4.5) seedlings are in an acidic medium and the conversion takes place readily, especially when the storage temperature is increased (RT). Carotenoids in processed seedlings seem first to undergo an all-*trans* to *cis*- or 5,6-epoxy to 5,8-furanoid isomerisations which are facilitated by low pH, raising temperatures and light accessibility, all available in glass jars (Panalaks and Murray, 1970; Sweeney and March, 1971; Moss and Weedon, 1976). All these

play a positive role in the final destruction of these compounds. In the present study the change from all-*trans*- to *cis*- compounds was not observed, presumably because the amount of carotenoid in fresh seedlings was not very high, even before bottling. So had there been any *trans*- *cis*- isomerisation it would not have been detected on this scale. In addition to the above conditions favouring the breakdown of carotenoids, the role of oxidation of lipids or proteins may have a marked effect on the losses in carotenoids because the latter are dependent on their association with lipids or proteins to protect them from oxidation (Woolfe, 1979). Consequently under the conditions used, complexes between lipids and carotenoids are broken down. Firstly lipids become oxidised, followed by the carotenoids which are therefore degraded as a result of these oxidation reactions.

General Discussion on Section II

The same compounds, namely lipids, carbohydrates, proteins and the vitamins A and C were studied in relation to their behaviour after blanching and after the storage of processed mung bean seedlings.

Total lipids of fresh mung bean seedlings were 350 mg 100 g^{-1} fresh weight and this value did not change very much after blanching, nor when the processed seedlings were stored for 6 months at 10°C, nor at RT. However, there was a 66% decrease at the highest storage temperature of 35°C. The essential fatty acid, linoleic acid and the other C18 unsaturated compounds C18:1 and C18:3 increase after processing and under the three temperatures of storage, reaching a maximum of 9.6 mg 100 g^{-1} at 35°C compared to 2.5 mg in fresh seedlings. So, on processing and storage there is an increase in essential fatty acids, due to the hydrolysis of other lipids which then release some fatty acids present in their molecules. It may be advantageous then to use stored processed seedlings since some of the essential fatty acids are now present in the free form and therefore more readily available to humans.

Total available carbohydrates are also affected after blanching and after the storage of processed seedlings at the three temperatures used in the present study. Total available carbohydrates of fresh seedlings of 5.1 g 100 g^{-1} fresh weight decreased to 3.2 g after blanching and to approximately 1.0 g under all four conditions of storage. So, the temperatures of storage had the same effect on total available carbohydrates which are therefore temperature insensitive.

It has been shown earlier that the proteins of fresh mung bean seedlings, although incomplete, contained all the essential amino acids except tryptophan. After blanching there was a slight decrease in total proteins of fresh seedlings (5.3 g 100 g^{-1}), but greater decreases were observed when the processed seedlings were stored over a period of six months, to

approximately 2 g 100 g^{-1} at all three temperatures used in this study. Since there is a decrease in total protein, decreases in some or all individual amino acids must follow. All essential amino acids from hydrolysed protein were affected during storage, some more than others. The most labile essential amino acids were found to be isoleucine + leucine, cystine, closely followed by histidine (essential for infants), valine, and phenylalanine + tyrosine. There is very little threonine, which disappears under all four storage conditions. During storage at RT the containers had very little effect on the essential amino acids present. Further, apart from phenylalanine + tyrosine, which had decreased the most (x $\frac{1}{2}$) compared to processed seedlings stored at 10°C, the other essential amino acids did not decrease so much. So storing processed seedlings for six months at RT is only slightly detrimental to the essential amino acids compared to those stored at 10°C. Since free amino acids only represent approximately a thousandth of amino acids obtained from hydrolysed protein, they are very insignificant in terms of nutritive value and will not be discussed further.

Vitamin A increases were shown to be strongly light-dependent and dark-grown mung bean seedlings when exposed to light for 24 h produced over a third of an infant's daily requirement for this essential vitamin. On blanching the etiolated seedlings though, there was very little decrease in vitamin A. On storage in glass jars for six months however large amounts disappeared and at the higher storage temperature of 35°C it disappeared completely. Canned seedlings stored at RT retained the most vitamin A (55%) and this result can be explained on the grounds that in cans there is the complete elimination of light and less oxygen available, and therefore less carotenoids are destroyed.

Blanching destroys about half the vitamin C content of fresh-grown mung bean seedlings. This vitamin is reduced further when processed seedlings are stored at the three different temperatures used for six months, reaching the lowest level in canned seedlings at RT. In this case, the metal itself may have become reactive and thus may have caused the destruction of a certain amount of vitamin C.

So although the advantages and benefits of food processing are numerous (p. 14), it is important that certain precautions are taken during the storage of processed mung bean seedlings. On the whole, tin cans are no better than glass containers for the six-month storage of mung bean seedlings since at the same RT proteins, carbohydrates, fatty acids and total available carbohydrates are equally affected by the two containers, the exception being vitamin A which is less affected in tin cans. On the other hand glass containers are better than tin cans in that less vitamin C is destroyed during the same storage period at RT.

Storage temperature for a six-month period as expected has an effect on the nutritional value of bottled mung bean seedlings. It would be advantageous from a nutritional viewpoint then to store them at 10°C. On the other hand, 10°C is only fractionally better than storing at RT (approximately 25°C) and the cost of storing at the lower temperature will make it more attractive to store the glass jars at RT. It must be borne in mind also that at the highest temperature used (35°C) there is a much greater loss of almost all the nutrients under study. It is strongly recommended therefore not to store bottled mung bean seedlings at 35°C if at all possible.

CHAPTER XIII

Conclusions

The first part of this study dealt with investigating the effect of continuous illumination (1000 lux) on 4-day-old mung bean seedlings over a 24 h period.

1. Under electron microscopy it was observed that after 6 h light the prolamellar bodies of plastids of etiolated leaves were gradually converted to grana membranes, and this was completed by the end of 8 h. More intact grana were formed over the next 16 h illumination period.

2. The increase in nutrients was mostly observed after 6 h light, and by the end of 24 h the following increases compared to dark-grown seedlings were observed: total lipids (from 0.35 to 0.64 g 100 g^{-1}), carbohydrates (from 5.1 to 7 g 100 g^{-1}) and proteins (from 5.3 to 6.7 g 100 g^{-1}) (wet weight).

3. Carotenoids, on the other hand, started to increase after one hour illumination (from 32 to 557 µg 100 g^{-1} wet weight). Chlorophylls, which were absent from etiolated seedlings, showed an immediate increase on exposure to light and by the first hour the seedlings contained 11 mg 100 g^{-1} which then increased to 160 mg 100 g^{-1} by the end of the 24 h illumination period.

4. The vitamin C content of mung bean seedlings did not change significantly with light, being approximately 16 mg 100 g^{-1}.

5. Total lipids were unchanged over the 24 h illumination period. Twenty simple and complex lipids were identified. Simple lipids made up 10% and complex lipids 90% of total lipids: an increase in glycolipids (23% - 31%) was observed at the expense of phospholipids (77% - 69%). Only two free simple sterols were identified, namely stigmasterol and β-sitosterol. The decrease in β-sitosterol from 36% to 28% was balanced by an increase in stigmasterol from 10% to 15%.

6. Total free sugars (fructose, glucose and sucrose) increased from 37% to 76% at the expense of starches and dextrins (63% to 24%) which were being hydrolysed at the same time.

7. Seventeen amino acids were identified and all of them had increased over the 24 h illumination period. The following amino acids found in fairly large amounts are shown in decreasing order of magnitude: aspartic acid, glutamic acid, alanine, serine and cystine; whereas methionine, histidine, arginine and glycine were found in much smaller amounts.

8. Seven carotenoids; β-carotene, cryptoxanthin, violaxanthin, lutein, neoxanthin, zeaxanthin and flavoxanthin were identified in dark-grown seedlings. With the exception of flavoxanthin, which had disappeared after 3 h illumination, all the others were observed in greening seedlings and they had all increased during the 24 h light period.

The second part of this study dealt with the effect of storage of processed dark-grown mung bean seedlings. These were blanched prior to packing them into jars and cans. Blanching had a marked effect on vitamin C, total available carbohydrates, simple sugars and on starches and dextrins as only 50%, 63%, 79% and 53% respectively were retained in each case.

9. Total proteins and amino acids from total hydrolysed proteins were not severely affected as they were mostly retained in each case, whereas only 67% was retained after blanching of total free amino acids.

10. Lipids and carotenoids were affected only slightly. In fact total carotenoids showed 104% retention, with flavoxanthin, violaxanthin and neoxanthin the most labile, and zeaxanthin, lutein, cryptoxanthin and β-carotene the most resistant carotenoids under these conditions.

11. The blanched seedlings were packed in jars (pH 3.25) and cans (pH 4.5). Whereas the jars were pasteurised (85°C for 13 minutes), the cans were sterilised (120°C for 20 min). Differences obtained between the seedlings in the two containers are not only due to their make up (i.e. glass or tin) but also to the fact that the temperatures used during processing were much higher in the canned seedlings. Bottled (jars) seedlings were stored for six months under three temperatures: 10°C, RT and 35°C, and canned seedlings were stored for the same period at RT only. Generally speaking the seedlings packed in glass containers and kept at 35°C suffered the most losses of all, while the seedlings kept at 10°C retained the most nutrients. Bottled and canned seedlings which were at RT showed more or less similar results.

12. Carotenoids in bottled seedlings stored at 35°C disappeared completely but 24% vitamin C was retained, whereas canned seedlings at RT contained the highest carotenoids of all the processed seedlings with 55% retained though only 12% vitamin C was retained. Bottled seedlings stored at 10°C and RT retained 14% and 6% of their carotenoids respectively while 35% and 26% respectively of their vitamin C were retained. Flavoxanthin and violaxanthin were completely lost in all cases, and neoxanthin and cryptoxanthin only appeared in canned seedlings, while zeaxanthin, lutein and β-carotene were the most resistant carotenoids.

13. Both canned seedlings stored at RT and bottled seedlings at 35°C retained about 27% and 35% respectively of starches and dextrins, and free sugars, whereas seedlings at 10°C and RT respectively showed 41% and 29% retention of starches and dextrins while 40% of free sugars were retained at both temperatures. Sucrose was the most labile sugar under the storage conditions used followed by glucose then by fructose.

14. Forty-eight percent of total proteins was retained in bottled mung bean seedlings stored at 10°C, 40% for both bottled and canned at RT and 30% for bottled at 35°C. The least retained free amino acid of processed seedlings under the storage conditions used was threonine with 10% retention for bottled seedlings

at 10°C and 2.5% retention under the other three conditions (bottled and canned at RT, and bottled at, 35°C). The most retained free amino acids were ammonia and aspartic acid in bottles stored at 10°C, and bottles and cans at RT (with approximately 80% in all three cases). The most retained free amino acid of bottled seedlings stored at 35°C was alanine (50%). The least retained amino acid of hydrolysed protein was serine and the most retained was ammonia with proline a close second in all four cases studied.

15. Total lipids of processed (bottled and canned) seedlings over the six-month storage period were unaffected compared to blanched seedlings. There was an increase in simple lipids and a decrease in the more complex ones, especially phospholipid, as phosphatidic acid disappeared completely at all storage temperatures above 10°C. However, as the storage temperature increased from 10°C to 35°C, simple lipids increased at the expense of complex lipids which decreased under the same conditions. The simple lipid increases were due to a very substantial increase in free fatty acids of processed seedlings. Since all complex lipids contain fatty acids, what was observed was that during storage some complex lipids e.g. phosphatidyl choline (PC) was converted to its lyso- form (LPC), and cardiolipin (= diphosphatidyl glycerol) disappeared completely due to its being hydrolysed to the corresponding mono- form with the loss of one molecule of fatty acid in each case. Finally, glycolipid increases were due mainly to increases in monogalactosyl diglyceride (MGDG) and in digalactosyl diglyceride (DGDG) during the six-month storage period.

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