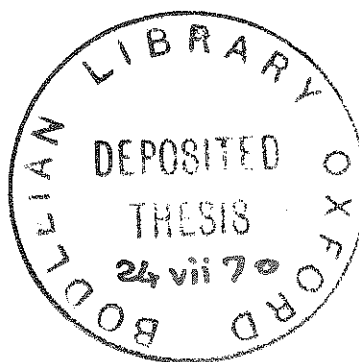


CONTROL OF CHLOROPHYLL SYNTHESIS

A thesis presented for the
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by

T.K. TEWIAN



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ABSTRACT

1. Peperomia magnoliaefolia, the main plant material used in the work reported in this thesis, possesses succulent leaves which show a periclinal chimera. The anatomy of the leaves, and the occurrence of 'bud-variations' which were found in the course of the work seem to indicate that their periclinal structure is a mesochimera.
2. Greening in the yellow leaves which takes place in winter has been shown to be due, not to the short-days, but to the reduced light intensities during the winter months.
3. Both green and yellow *Peperomia* leaves can synthesize chlorophylls and carotenoids. Their different appearance is due to the difference in the relative ratios of these pigments they contain.
4. A study of chloroplast differentiation in *Peperomia* leaf tissues has been reported. This showed a peculiar chloroplast structure in greened-yellow tissue - the chloroplasts here are incompletely differentiated.
5. The pigments formed in the yellow leaves at low light intensities, like those in the normal green leaves, are functional (i.e. photosynthetic).
6. Some evidence has been given to show that the occurrence of non-green (yellow) leaves is not the result of faster chlorophyll degradation of some leaf-tissues.

7. Studies on the incorporation of glycine-2- ^{14}C into chloroplast pigments of *Peperomia* leaf discs have been reported. These showed unexpectedly higher levels of labelling of a yellow terpenoid 'spot' (carotenes) than of chlorophylls. A tentative (but plausible) explanation has been given for this observation (i.e. the almost complete absence of incorporation of ^{14}C from the labelled glycine into chlorophylls).
8. In terms of the incorporation of ^{14}C from labelled glycine into 'chlorophyll precursors', chlorophyll synthesis is faster in green than in yellow leaves.
9. The rate of bleaching in the yellow leaves appears to be too high, compared with the rate at which chlorophyll is synthesized in them. It is therefore probable that these leaves appear yellow at high light intensities because the rate at which the pigment breaks down is too fast to make chlorophyll accumulation possible.
10. The control mechanism probably affects chloroplast differentiation too. Where (as in green tissue) complete chloroplast differentiation is possible, chlorophyll synthesis is high enough to permit chlorophyll accumulation. In yellow tissue, the control factor (or defect) inhibits complete chloroplast differentiation and/or reduces the rate of chlorophyll synthesis, thus making chlorophyll accumulation at high light intensities impossible.

C O N T E N T S

SECTION I	Page
GENERAL INTRODUCTION	1
ABBREVIATIONS AND SPECIAL TERMINOLOGY	13
MATERIALS	16
GENERAL METHODS	18
 SECTION II	
CONDITIONS FOR GREENING	27
 SECTION III	
CHANGES ACCOMPANYING GREENING	37
 SECTION IV	
CHLOROPHYLL DEGRADATION	59
 SECTION V	
CHLOROPHYLL BIOSYNTHESIS IN GREENING TISSUES	74
 SECTION VI	
FINAL DISCUSSION, ADDENDUM, BIBLIOGRAPHY	90

SECTION I

	Page
1. GENERAL INTRODUCTION	1
2. ABBREVIATIONS	13
SPECIAL TERMINOLOGY	15
3. MATERIALS	
(a) Plant materials	16
(b) Chemicals	16
4. GENERAL METHODS	
(a) Plant cultivation	18
(b) Selection of material	18
(c) Tissue-culture	19
(d) Sterilization procedure	20
(e) Pigment extraction and determination	22
(f) Chromatography	
(i) Column Chromatography	24
(ii) Paper Chromatography	24

1. GENERAL INTRODUCTION

The chloroplasts of higher plants contain the photosynthetic pigments chlorophylls and carotenoids. The carotenoids are yellow, orange or red pigments which are divided into carotenes, containing only carbon and hydrogen atoms, and xanthophylls, which possess hydroxyl or ketonic groups.

The chlorophylls of higher plants are chlorophylls a and b, chlorophyll a occurring, as far as is known, in all photosynthetic higher plants. It is usually associated with smaller quantities of chlorophyll b except in the saprophytic orchid, Neottia nidus-avis, which apparently has no chlorophyll b (see Kirk and Tilney-Bassett, 1967a). The distribution of chlorophylls amongst different groups of plants is shown below (from Kirk and Tilney-Bassett, 1967a).

Plant Group	Chlorophyll			
	a	b	c	d
Vascular plants, pteridophytes, bryophytes	+	+	-	-
Algae				
Chlorophyta	+	+	-	-
Euglenophyta	+	+	-	-
Chrysophyta				
Chrysophyceae	+	-	±	-
Xanthophyceae	+	-	-	-
Bacillariophyceae	+	-	+	-
Pyrrhophyta	+	-	+	-
Phaeophyta	+	-	+	-
Cryptophyta	+	-	+	-
Rhodophyta	+	-	-	±
Cyanophyta	+	-	-	-

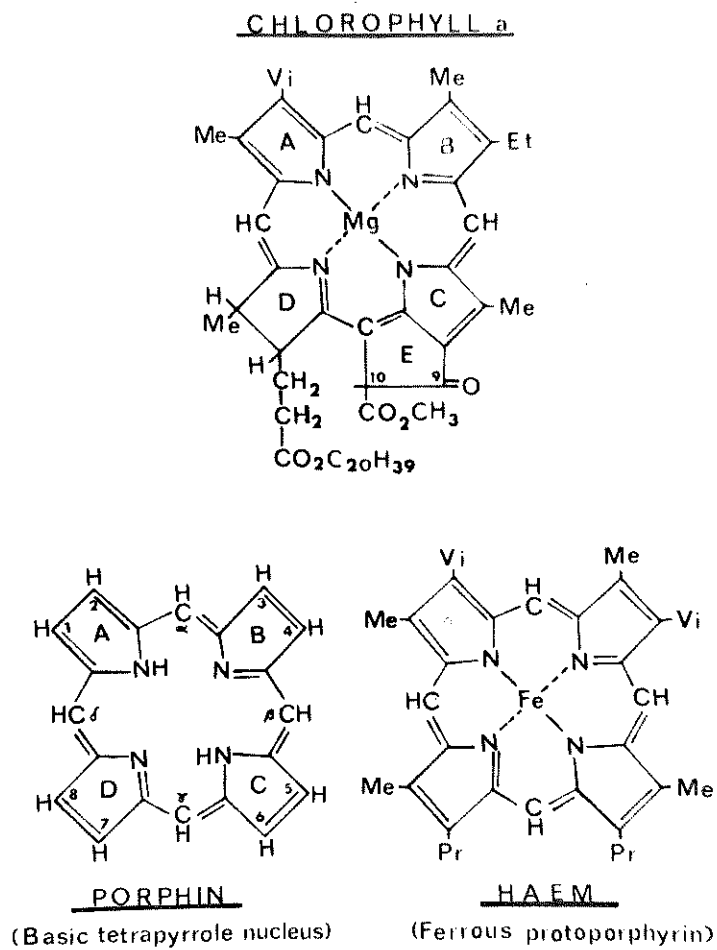


Fig. 1

Chemical structure of related tetrapyrroles.

(Me - CH_3 ; Vi - $\text{CH}=\text{CH}_2$; Et - CH_2CH_3
Pr - $\text{CH}_2\text{CH}_2\text{COOH}$)

The basic structure of the chlorophyll molecule is a tetrapyrrole nucleus (porphin), with substitutions in the β -carbon atoms 1 to 4. This is chelated with magnesium as opposed to iron in haem, the prosthetic group of haemoglobin and other haemoproteins like catalase, peroxidase and cytochromes. The chlorophyll molecule also differs from the iron porphyrins in having ring D (in chlorophyll a and b), and rings D and B (in bacteriochlorophyll), completely reduced. The propionic acid side-chain at C-7 is also esterified with a long chain alcohol, phytol (farnesol in the case of bacterioviridin), while the propionic acid-chain at C-6 forms an additional ring, E (cyclopentanone ring) with the γ -methane carbon. All chlorophylls in vivo are bound to lipoprotein complexes.

The early studies in tetrapyrrole biosynthesis were confined to the mechanism of protoporphyrin or haem synthesis in animal erythrocytes, mainly in man, ducks and rats. (Shemin and Rittenberg, 1945, 1946; Altman et al., 1948; Shemin et al., 1948 a and b; Radin et al., 1950; Shemin and Rittenberg, 1951; Shemin and Kumin, 1952).

The work of Granick and colleagues with mutant strains of the unicellular alga Chlorella vulgaris, was the first indication that the early steps of haem and chlorophyll synthesis share a common pathway that branches at protoporphyrin (Fig. 2). By X-ray irradiation, mutants were produced

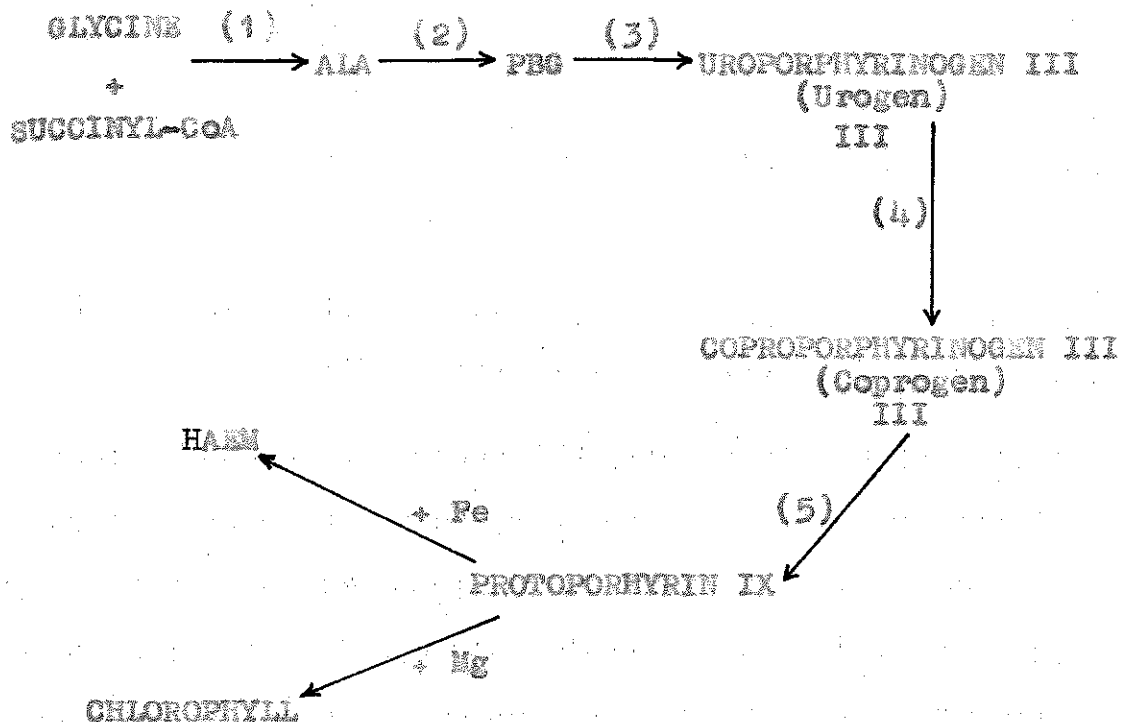


Fig. 2. PATHWAY FOR PROTOPORPHYRIN BIOSYNTHESIS

- (1) ALA Synthetase
- (2) ALA Dehydrase
- (3) Urogen I synthetase and Urogen III Cosynthetase
(PBG Deaminase) (Pyrroymethane isomerase)
- (4) Urogen Decarboxylase
- (5) Coprogen Oxidative Decarboxylase (Coprogen Oxidase)

in which chlorophyll synthesis was blocked at various stages in the biosynthetic pathway, but which were still able to grow heterotrophically in a medium of glucose and inorganic salts. The first of these mutants to be described, *Chlorella* #5-Brown, accumulated large amounts of protoporphyrin IX (Granick, 1948a). Re-irradiation of this strain produced another mutant, #5-B17 that accumulated a mixture of porphyrins with 2 to 8 carboxyl groups, dicarboxylic porphyrins being predominant (Bogorad and Granick, 1953a). Protoporphyrin, uro- and coproporphyrinogen III were also identified in these mixtures (Granick, Bogorad and Jaffe, 1953).

Accumulation of these porphyrins by the mutants provided evidence that chlorophyll was formed by a pathway involving these compounds. Later work showed that the tetrapyrrole nucleus of these porphyrins was formed by the same pathway already outlined in animals. (Della Rosa, Altman and Salomon, 1953; Bogorad and Granick, 1953b; Granick, 1950, 1954; Bogorad, 1958 a and b).

In animal tissues it has been found that the enzymes which catalyse the first and last reactions in the porphyrin biosynthetic chain up to protoporphyrin, ALA synthetase and coproporphyrinogen oxidase respectively (see Fig. 2), are mitochondrial enzymes. The enzymes which catalyse the 2nd, 3rd and 4th reactions occur in the soluble fraction of erythrocytes and liver. (Granick, 1963). ALA must therefore be released from mitochondria before becoming available for

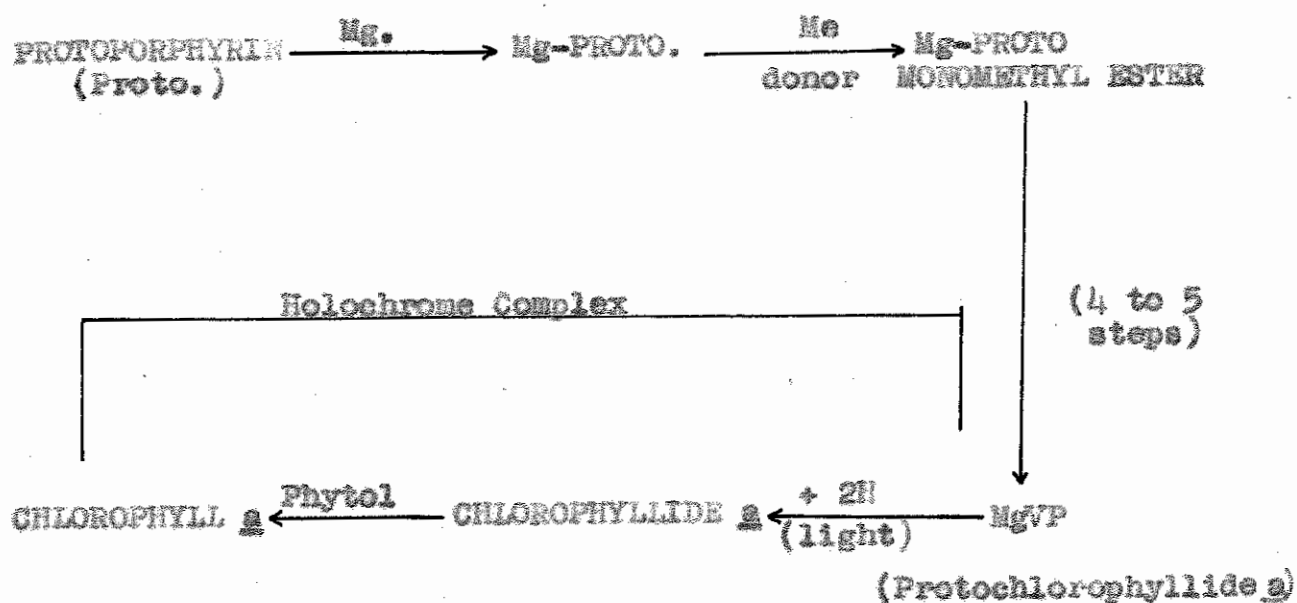


Fig. 3. POSSIBLE PATHWAY FOR CHLOROPHYLL a SYNTHESIS IN HIGHER PLANTS

(Modified from Lascelles, 1964a)

tetrapyrrole biosynthesis. In plants, protoporphyrin formation can be shown to occur in plastids as well as in mitochondria, but it is not known whether only some or all the enzymes of the biosynthetic chain to protoporphyrin are localized in both of these bodies (Granick, 1963).

The steps between protoporphyrin and protochlorophyllide are largely unknown (see Fig. 3). The available information about intermediates here has come mainly from the isolation of presumed chlorophyll precursors accumulated by mutant strains of algae and photosynthetic bacteria which are unable to form chlorophyll. (Granick, 1948b; Lascelles 1964a). There is however considerable information about the way in which magnesium protoporphyrin monomethylester is formed. The specific incorporation of ^{14}C -formate by *Chlorella* into the methyl ester group of chlorophyll suggested that formic acid was an indirect precursor of the methoxyl group. (Green, Altman, Richmond and Salomon, 1957). This step has also been studied at an enzymic level (Gibson, Neuberger and Tait, 1963).

Magnesium vinyl pheoporphyrin a_5 (MgVP), also known as protochlorophyllide a, is the next intermediate after the monomethylester; it has been found to occur in a protein-bound form in etiolated leaves, and this combination of pigment with protein has been termed "holochrome" by Smith (1963). This MgVP-holochrome is probably the true intermediate in chlorophyll synthesis rather than the free pigment.

By the use of etiolated seedlings the role of light in chlorophyll synthesis in higher plants has been found to be the reduction of protochlorophyllide (MgVP) to chlorophyllide (Smith, 1960, 1963; Granick, 1963). In most higher plants and in some algae the reduction takes place only in light (Lascelles, 1964a), but chlorophyll formation in the absence of light has been found to be possible in seedlings of the water lotus (*Nelumbo*), and the cotyledons of citrus embryos (Meyer et al., 1961a).

Other studies on etiolated plants (Wolff, and Price, 1956; Smith, 1961), suggest that the final step in chlorophyll a biosynthesis is the conversion of chlorophyllide a to chlorophyll a i.e. the esterification of the propionic acid side-chain on C-7 with phytol. More comprehensive reviews of tetrapyrrole biosynthesis have been provided by Rimington, 1957; Granick & Mauzerall, 1961; Smith & French, 1963; Lascelles, 1965; Bogorad, 1960, 1965a, 1966a; Aronoff, 1966; Marks, 1966.

Unlike etiolated plants in which chlorophyll is formed on exposure to light, in variegated leaves, under conditions which are favourable for chlorophyll synthesis in some plastids, other plastids on the same plant are unable to form the pigment. The work reported in this thesis was carried out in an attempt to elucidate the control mechanism preventing greening in the yellow parts of the variegated leaf of Peperomia magnoliaefolia. Abnormal plastids are usually invisible in homogeneous plants,

but they can be preserved in variegated plants and thus provide useful material for studies in plastid biology.

Variegation in plants is the result of a variety of causes, virus infection, defective tissue such as the occurrence of air blisters beneath the epidermis (false variegation) or mutation, which can be nuclear or cytoplasmic. True variegated plants show varying shades of colour in their leaves or other organs, owing to variations in the pigment content of their different plastid types (Tilney-Bassett, 1962).

Plastids can mutate and, like the nuclei, are self-propagating (Baur, 1909). From reciprocal crosses between *Pelargoniums* with white and green layers, Baur (1909) concluded that during successive cell divisions, mutant and normal plastids were able to 'sort-out' from a mixed zygote (derived from biparental plastid transmittance), into cells containing only normal or only mutant plastids. Sorting-out of defective and normal plastids into tissue layers, leads to the formation of Chimeras (tissues composed of genetically different cells).

In some plants e.g. *Humulus japonica*, (Winge, 1919), variegation (chimera formation) is caused by cytoplasmic difference from normal. But sometimes variation in plastid development (i.e. into defective plastids or plastids with chlorophyll), is gene induced as in *Lunaria annua* (Correns, 1909). Genes can also cause plastid mutation as in *Hordeum vulgare* (Imai, 1928) rice (Pal and Ramanujam, 1941),

and maize (Rhoades, 1943). Such mutant plastids are then transmitted, usually maternally, but sometimes biparentally (as in Nepeta cataria - Woods and DuBuy, 1951, and in Pelargonium - Tilney-Bassett, 1962), independently of the nucleus. Thus a gene which causes mutation of plastids is not necessary for the reproduction of the mutant plastid type. Occasionally as in Mirabilis jalapa (Correns, 1909) and Hordeum vulgare (Imai, 1928) mutation affects a whole growing point and hence the germ layers.

The first step in the origin of chlorophyll chimeras is mutation, and this must affect the plastid phenotype and at the same time occur in the germ layers. The growing point of the shoot has been shown to be built up of three germ layers (LI, LII and LIII) in most dicotyledonous plants and two layers in most monocotyledonous plants, and these are superimposed one above the other like a series of cones from which the body structure of the plant is derived (Tilney-Bassett, 1962). The pattern found in the leaf is a development of the pattern already present at the growing point.

Buds formed on true roots arise by growth from the central tissue and thus grow into plants exhibiting the characters of the core tissue. So that if plants from root-cuttings differ from those from stem-cuttings of the same plant, then the parent plant is a periclinal chimera, having

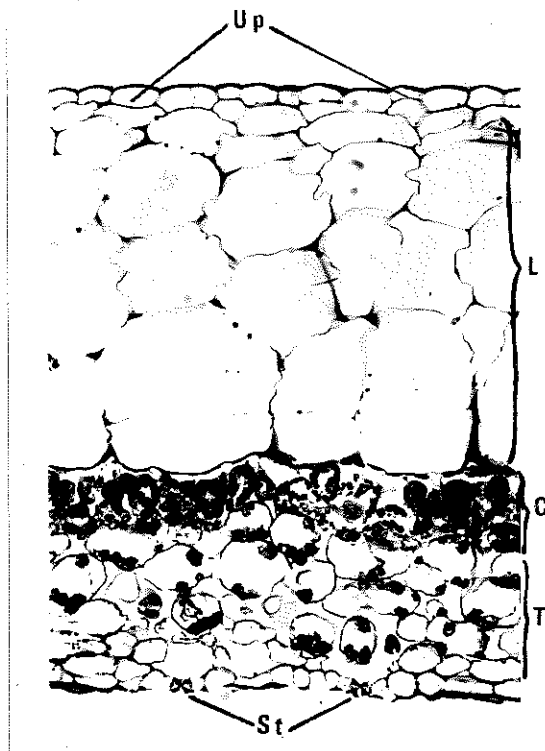


Fig. 4. T.S. of Green Peperomia leaf,
to show tissue and chloroplast
distribution.

- Up - Upper epidermis.
- L - Large water-storage cells.
(contain few scattered chloroplasts)
- C - Chlorenchyma tissue. (chloroplasts
concentrated here)
- T - Lower mesophyll tissue.
(some chloroplasts present here)
- St - Stomata.

core tissue genotypically different from the outer germ layer tissue (Tilney-Bassett, 1962). i.e. the different tissues are arranged in concentric layers. Chimeras where the distinct tissues are arranged in cross sections, as in the sections of a circle are known as sectorial chimeras i.e. they have sectorially divided growing points (Kirk and Tilney-Bassett, 1967b).

According to Darlington and Tilney-Bassett (1964), Peperomia magnoliaefolia is a White-over-green periclinal chimera; (this appears phenotypically as Yellow-over-green). This type of chimera structure is consistent with the following observations made while doing the work reported in this thesis.

Anatomical examination of Peperomia leaves shows no palisade cells, but the mesophyll tissue is differentiated into three distinct regions. There is an upper mesophyll tissue made up of large water-storage cells, and a lower mesophyll tissue of comparatively smaller cells. Separating these two tissues is a region of very small cells which is green in green leaves, but colourless in yellow leaves. The upper and lower mesophyll tissues contain a few scattered chloroplasts, many more occurring in the latter in green and greening-yellow leaves. Though stomata are only present on the lower surface, the chloroplasts are not only confined to guard cells here; all epidermal cells, even in yellow leaves, contain isolated chloroplasts. (Leaf structure is shown in Fig. 4).

During the period of this investigation, green leaves have occasionally been found to develop from variegated shoots while reversal from W/G to G/W has also been found (see Fig. 5a, b). From these observations and from the leaf structure described above, it appears that Peperomia magnoliaefolia might be a mesochimera (G - W - G), i.e. a periclinal chimera in which the middle layer is different (Winkler, 1935). That is, there is "a potentially green epidermis, a colourless sub-epidermis, and a green core" (Renner, 1936).

The above bud-variations (i.e. reversal and the formation of a green leaf from a variegated shoot), can be accounted for by displacement of LIII (GWG to GGW) and LII (GWG to GGG) by duplication of LI (Tilney-Bassett, 1962). Yellow leaves have also been found to develop occasionally from variegated shoots (Fig. 5c); it thus looks as if perforation of LI - which is very rare - but which has been reported in Pelargonium zonale "Flower of Spring" (Tilney-Bassett, 1962), can occur in Peperomia (i.e. GWG to WWG, which can then give rise to WWW by displacement of LIII).

The structure of plastid types in variegated leaves of Peperomia (Section III), shows that there is incomplete development and differentiation of plastids in the yellow tissue. Since a favourable interaction of all cell components is necessary for normal plastid development, the occurrence of

Fig. 5 Bud variations.

- (a) - Green shoot (G) from a variegated Peperomia shoot (V).
- (b) - Reversal (i.e. W/G to G/W) in Pelargonium. (This occurs in Peperomia too).
- (c) - Yellow leaf from a variegated Peperomia plant.



a



b



c

defective plastids in some cells is indicative of abnormal conditions; this could be due to a genetic or cytoplasmic lesion. To determine the site of the defect requires "biochemical studies to identify the lesion ...; and finally to determine by genetic experiments whether the inheritance of the defect is Mendelian or non-Mendelian, i.e. whether the gene is located in the nucleus or in the cytoplasm". (Kirk and Tilney-Bassett, 1967c).

Unfortunately *Peperomia magnoliaefolia* does not set seed, and is normally propagated by cuttings. Genetic studies can therefore not be made on this plant, and so some other method of approach is necessary. Localization of the factor(s) preventing normal plastid development in yellow tissue and/or how it operates, can provide useful information which will make it possible to speculate on the in vivo control mechanism involved.

Peperomia is a tropical plant which, in temperate countries, can be successfully cultivated by cuttings in warm greenhouses, and has been found to green in winter (i.e. in winter, variegation is less pronounced because the yellow tissue also greens). The first part of this investigation is concerned with the elucidation of this 'winter effect'.

The aim of the work reported in this thesis is to examine morphological, physiological and biochemical

differences and similarities of chloroplasts and other cell organelles from green and yellow leaves of *Peperomia*, which might help to speculate on the in vivo control of chlorophyll synthesis in these tissues. Chlorophyll synthesis (especially at high light intensities), is usually accompanied by chlorophyll degradation. It is the net result of these two processes (i.e. chlorophyll synthesis and chlorophyll degradation), which determines the rate of pigment accumulation.

Willstätter and Stoll (1910), showed that chlorophylls could be hydrolysed in aqueous acetone solution by the dried powder of leaves, free from pigments. This reaction was enzymic and the enzyme responsible for it was named chlorophyllase. It catalyses the hydrolysis of chlorophylls, pheophytins, purpurins, bacteriochlorophyll and chlorobium chlorophyll (bacterioviridin). Protochlorophyll and other porphyrins lacking the carbomethoxy group on C-10 and the hydrogens on C-7 and C-8 are not hydrolysed by the enzyme. (Smith and French, 1963). The enzyme has now been shown to be located in the chloroplast (Krossing, 1940; Ardao and Vennesland, 1960).

The first step in the enzymatic breakdown of chlorophyll in vivo is also thought to be the removal of the phytol side-chain to yield water-soluble chlorophyllide which can then undergo further degradation (Noack, 1943; Ardao and

Vennesland, 1960; Holden, 1961). Noack (1943), considered that chlorophyllase had an essential role in the 'photo-decomposition' of chlorophyll. Koeki and Smith (1951), working with a mutant white seedling of Zea mays also concluded that albinism arose from the rate of destruction of chlorophyll exceeding its rate of synthesis.

If under conditions where pigment accumulation is possible in some leaf tissues of *Peperomia* making them appear green, other tissues appear yellow, then from the above discussion, the latter tissues either do not form pigments at all, or the pigments they synthesize are breaking down faster than they are formed. The latter part of the thesis is concerned with determining the relative rates of chlorophyll degradation and chlorophyll biosynthesis in green and yellow *Peperomia* tissues, in an attempt to elucidate the mechanism controlling greening in *Peperomia* leaves.

2.

ABBREVIATIONS

ADP	Adenosine diphosphate
ALA	δ -aminolaevulinic acid
Asp.	Aspartic acid
ATP	Adenosine triphosphate
CoA	Coenzyme-A
2,4-D	2,4-Dichlorophenoxyacetic acid
DNA	Deoxyribonucleic acid
EMP Pathway	Embden-Meyerhof-Parnas pathway (Glycolysis)
Glu.	Glutamic acid
Gly.	Glycine
IAA	Indoleacetic acid
IPP	Isopentenyl pyrophosphate
HVA	Mevalonic acid
NAA	Naphthaleneacetic acid
NAD	Nicotinamide adenine dinucleotide (DPN)
NADP	Nicotinamide adenine dinucleotide phosphate (TPN)
OD	Optical density
OAA	Oxaloacetic acid
PBG	Porphobilinogen
PEP	Phosphoenolpyruvic acid
Pi	Orthophosphate
TCA cycle	Tricarboxylic acid cycle (Krebs or Citric acid cycle).

The following abbreviations have been
used in Tables and elsewhere

App. Photo.	Apparent Photosynthesis
Chl. cont.	Chlorophyll content
Exp.	Experiment
Fr. wt.	Fresh-weight
Gr.	Green
Lvs.	Leaves
L.P.	Low power
Pet. ether	Petroleum ether
Plts.	Plants
S.E.	Standard deviation
Sp. gr.	Specific gravity
T.S.	Transverse section
Yell.	Yellow

Apart from the above abbreviations and others which have been explained later (where they are used), chemical symbols and formulae, and other conventional abbreviations have been used.

SPECIAL TERMINOLOGY

C-1....C-10	β -carbon 1 to 10 of the chlorophyll molecule.
Day-neutral (regime)	12 hours light, 12 hours dark.
High light intensity	When used in connection with Peperomia experiments it means 200-800 ft-c. (All light intensity measurements were made with a Weston Master II light-meter).
Long-day (regime)	16 hours light, 8 hours dark.
Low light intensity	Light intensity from 20-50 ft-c.
Roof light cabinet	Light cabinet (on roof of Botany School), with intensity 200-800 ft-c, long-day regime of 16 hours light, and temperature of 25°C.
Short-day (regime)	8 hours light, 16 hours dark.
Terminal leaves	True terminal leaves, or where these are not fully developed, the second leaves on a plant.
Total radia- tion	Total light energy (light intensity $\times$ duration of exposure).

3. MATERIALS

(a) Plant Materials

Plant materials used in this work were all obtained from the Oxford Botanical Garden. Some of them, Rhynchosia phaseoloides and Buonymus japonicus, were used directly after collection, for the preparation of "standard chlorophyll solutions" for chlorophyllase activity determinations. Other plant materials were cultivated and maintained in the 'roof greenhouse' of the Botany School, or in the roof (long-day) light cabinet. This group was made up of the following variegated plants: Pedilanthus tithymaloides, Abutilon striatum, Buonymus japonicus, Veronica, (Hebe) elliptica, Hedera helix, Chlorophytum capensis and Pelargonium zonale.

The main plant material used for this work, is Peperomia magnoliaefolia. It is a succulent, perennial herb, a member of the Piperaceae. They are easy to cultivate (by cuttings), and their thick leaves make the cutting of leaf discs easy.

All plant materials have hereafter been referred to only by their generic names.

(b) Chemicals

Except those shown below, all reagents were obtained from B.D.H., and the Analar grade was used where available.

Glycine-2-¹⁴C

The Radiochemical Centre, Amersham,
Bucks, England.

δ -Aminolaevulinic	Sigma Chemical Company Ltd., London England.
Gibberellic acid	L. Light and Co. Ltd., Colnbrook, England.
2,4-D and IAA	Mann Research Laboratories, New York.
Kerosene (Alladin pink)	National Petrol Station, Oxford.
Kinetin (6- furfurylamino purine)	California Foundation for Biochemical Research, Los Angeles 63.
Yeast extract	Difco Laboratories, Detroit 1, Michigan, U. S. A.
Cellulose Powder (standard grade)	W & R Balston, Ltd., England.
Agar (Davis type "p")	Davis Gelatine Ltd., Warwick, England.
Coconut milk	Liquid endosperm from fresh coconuts bought locally in Oxford. The 'milk' was drained through muslin to remove debris, and stored in polythene containers at - 15° until required.

4. GENERAL METHODS

(a) Plant Cultivation

Plants used in these investigations were cultivated in the greenhouse on the roof of the Botany School. From this source, stem-cuttings were periodically made and kept in a glass-box in the greenhouse for 3 weeks to root. The cuttings were then transferred into, and maintained in a light cabinet which was lighted by standard 5 ft. fluorescent strips, fitted with day-light reflector tubes.

Conditions in this light cabinet which were found to favour rapid growth and maintain variegation in the cuttings were as follows:

- (i) a long-day regime of 16 hours light,
- (ii) a temperature of 25°C and
- (iii) light-intensity between 200 and 800 ft.c.

The plants used in all experiments were cultivated in the above conditions for at least 3 months in pots containing a 50-50 mixture of peat and John Innes potting compost No. 1. In *Peperomia*, by the end of this period, the cuttings had developed one or two suitable sized leaves, (i.e. leaves with large enough yellow portions from which leaf discs - diameter 7 mm. - could be obtained), above the original leaves of the stem-cuttings.

(b) Selection of material

For experiments involving whole plants, a suitable

number of potted plants was selected and divided into the required number of groups in such a way, that each group contained as far as possible, equal numbers and sizes of leaves. For experiments with whole-leaves, a suitable number of leaves were cut off at the bases of the petioles and sorted out into groups with equal numbers of leaves, with respect to size and position on the plant.

Leaf disc experiments were done with discs cut from green or yellow leaves, randomly sampled, and a known fresh-weight or number of discs, used.

(c) Tissue-culture

An attempt was made to get uniform leaf material from *Peperomia* by trying to induce callus formation in green and yellow leaves.

In these tissue-culture experiments, sterilized leaf-bits from very young green and yellow leaves were set up in semi-solid cultures in petri dishes and maintained in the growth cabinet described above. The cultures contained graded concentrations of kinetin and IAA, in White's medium (salt + vitamin - White's, 1943), with 0.6% agar, 10% or 15% coconut milk, and various concentrations of sucrose (1.0%, 1.5% and 2%). Combinations of various concentrations of Kinetin with, 2,4-D and NAA, were also tried, (see Fig. 6a, b). None of the trials produced any callus tissue, and so these tissue-culture experiments were abandoned.

Fig. 6a

Lay-out, showing concentrations of growth substances
in petri dishes (numbered 1 to 16)
used in tissue culture experiments.

		Kinetin 			
		0.0mM.	0.01mM.	0.1mM.	1.0mM.
IAA or NAA or 2,4-D 	0.0mM.	<u>1</u>	<u>2</u>	<u>3</u>	<u>4</u>
	0.01mM.	<u>5</u>	<u>6</u>	<u>7</u>	<u>8</u>
	0.1mM.	<u>9</u>	<u>10</u>	<u>11</u>	<u>12</u>
	1.0mM.	<u>13</u>	<u>14</u>	<u>15</u>	<u>16</u>

Fig. 6b

WHITE'S MEDIUM (Whites, 1943)

(Stock solutions for tissue-culture medium)

I. Mineral Solution
(Stored in the dark)

II. Vitamin Solution
(Stored in the cold)

	gm./l		mg./100 ml.
$\text{Ca}(\text{NO}_3)_2$	2	Glycine	300
Na_2SO_4	2	Nicotinic acid	50
KCl	0.8	Thiamine	10
$\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$	0.215	Pyridoxine	10
$\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$	0.066		
$\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$	0.027		
H_3BO_3	0.015		
KI	0.0075		
$\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$	36		

For Culture Medium

Add 100 ml. of I, + 10 ml. of II, + 2.5 ml. 1% $\text{Fe}_2(\text{SO}_4)_3$,
and make up to a litre.

An attempt was also made to establish leaf discs in sterile cultures but this too had to be abandoned. This was because it was not possible to get enough discs remaining sterile over the long periods of at least 4 weeks, required for greening in *Peperomia*. A 1:10 solution of sodium hypochlorite in water was found to be better than absolute alcohol in sterilizing the leaf bits. But in spite of all the precautions taken to ensure that the cultures remained sterile, the longest period all the discs in a petri dish remained sterile was about 2 weeks. The experiments in these studies therefore had to be done using whole leaves. It was only in short-period experiments (not more than 24 hours) that leaf discs were used.

(d) Sterilization procedure

This was the procedure used for preparing cultures of leaf materials for tissue culture experiments, and for experiments involving the maintenance of leaf discs and whole leaves under sterile conditions.

The culture media, distilled ^{water} and all necessary glassware were autoclaved at 20 lbs/sq.in. for 15 minutes. All these materials were then transferred into a small room which had been sterilized by spraying with absolute alcohol and heated by two bunsen burners for at least one hour. The washed leaf materials contained in a covered 'crystallization dish' were also taken to the sterile room. Enough 10% sodium

hypochlorite solution was poured into the dish to cover up all the leaves.

After 5 - 10 minutes, the hypochlorite solution was poured out from the dish and replaced by cooled, sterilized distilled water. The leaves were washed in the distilled water by gently shaking the covered dish. They were then transferred into, and shaken in another dish of sterilized distilled water. The leaves were finally transferred into a third covered dish. The large forceps used for these transfers, as well as the knives, cork borers, glass rods, needles and small forceps used for cutting out leaf-bits and leaf discs and for transferring these leaf materials into the culture media were all sterilized. This was done by dipping them into 70% alcohol, flaming them and allowing them to cool. (Before commencing the sterilization procedure, I washed my hands with 70% alcohol).

The cutting of leaf material was done on a glass slab between two bunsen burners. This slab had previously been rubbed with alcohol and flamed, and at short intervals during the cutting-out process, it was rubbed with cotton wool soaked in alcohol. All leaf-bits or leaf discs were transferred into dry, sterile petri dishes as soon as they were cut. After cutting out all the required leaf materials, they were transferred from the petri dishes into the culture media. These were either liquid media contained in small

glass tubes or semi-solid media contained in covered petri dishes. Liquid media containing whole leaves were put into covered, sterilized glass jars, while the petri dishes containing leaf-discs cultured in semi-solid media were taped (with 'scotch tape') to exclude outside air.

Modifications of the above procedure were tried, but these did not produce better results than could be obtained previously. For instance, instead of sterilizing the room by spraying and heating, ultra-violet radiation was tried (separately and in addition to the spraying and heating). Leaf materials were sterilized in hypochlorite after, instead of before cutting (leaf-bits for 1 minute, and leaf discs for 2 minutes). Even doing all the cutting-out and culturing behind an 'inoculation hood' did not make the cultures stay sterile for longer periods than before.

(e) Pigment extraction and determination

A known fresh-weight of leaf material or number of leaf discs with diameter 7 mm. was finely ground in a mortar with washed sand and about 0.5 g. calcium carbonate in 85% acetone. The brei was filtered in a Buchner funnel and washed with more acetone to extract as much of the pigments as possible. (A total of 30 ml. of acetone was used for the grinding and extraction of each sample). The filtrate was concentrated by transferring into 4, 5 or 10 ml.

petroleum ether (60° to 80°C), the volume of petroleum ether used depending on the leaf-material and on the nature of the experiment.

The transfer of the pigments from acetone into petroleum ether was carried out as follows: (Zscheile and Comar, 1940 - 41; McKinney, 1940). The acetone extract was carefully added to the petroleum ether contained in a 100 ml. glass separating funnel, and distilled water was slowly added down the inside of the separating funnel until 2 distinct layers were formed. The lower aqueous acetone layer was drained off and discarded. Traces of acetone in the petroleum ether solution were then removed by allowing the solution to flow by gravity through the long narrow stem of the separating funnel, so that it emerged below the surface of the water and rose to the top. The water was drained off and the 'scrubbing' process repeated three times. (A small amount of calcium carbonate was added to all distilled water used for washing purposes).

The petroleum ether solution was dried overnight over anhydrous sodium sulphate before chromatographic separation or spectrophotometric measurements were made. (The Spectrophotometer used was the Beckman Photoelectric Spectrophotometer Model DB).

For chromatographic analysis, the pigments from 40 green, or 80 yellow leaf discs (or the equivalent of 1 or 2 g. fresh-weight respectively), were transferred into 4 ml.

petroleum ether. For the determination of total pigment content, the volume of petroleum ether used was 5 ml/500 mg. fresh-weight leaf material for yellow leaves, and 10 ml/500 mg. fresh-weight for green leaves.

(f) Chromatography

(i) Column chromatography

1 ml. of pigment extract (prepared as described above) was adsorbed on a cellulose powder column and developed with a solvent mixture made up of petroleum ether (100° - 120°C), petroleum ether (40° - 60°C) and acetone (20 : 5 : 4). The pigments were eluted from the column in the following order, carotenes, xanthophylls, chlorophyll a, chlorophyll b, and were collected as separate fractions. The various pigment fractions were made up to known volumes and their optical densities determined on the Beckman Spectrophotometer. The absorption spectrum of each fraction was recorded, and pigment concentration was measured at an appropriate wave-length.

(ii) Paper chromatography

Various solvent systems for separating photosynthetic pigments by paper chromatography were tried (Bauer, 1952; Lind et al., 1953; Aronoff, 1953), and the following method was found to be best.

Whatman No. 1 chromatography paper, cut into sheets

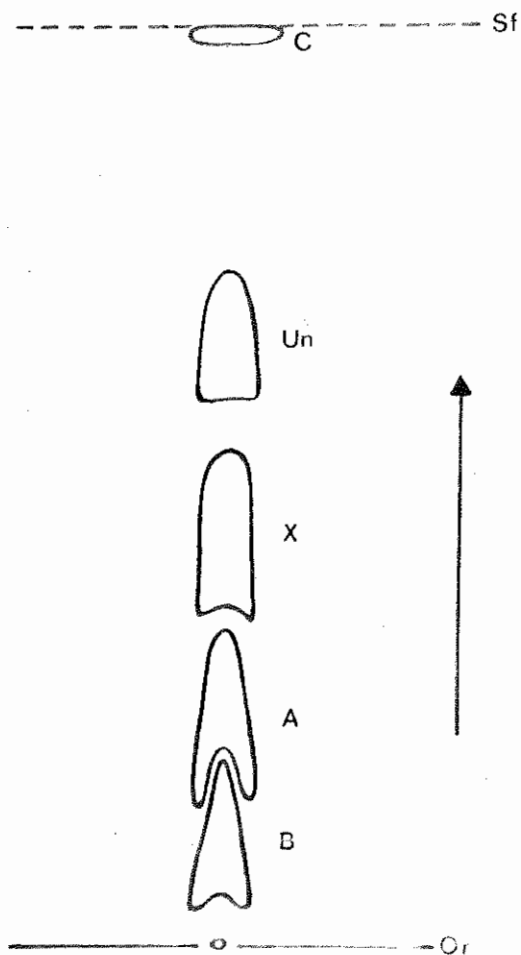


Fig. 7 Chromatography of chloroplast pigments
from *Peperomia* leaves.

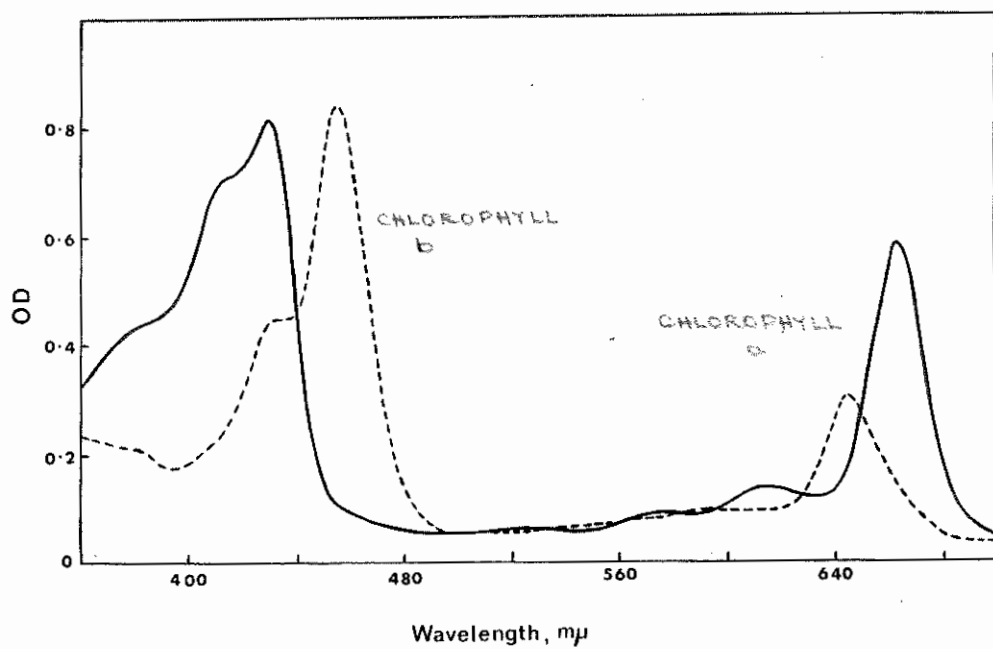
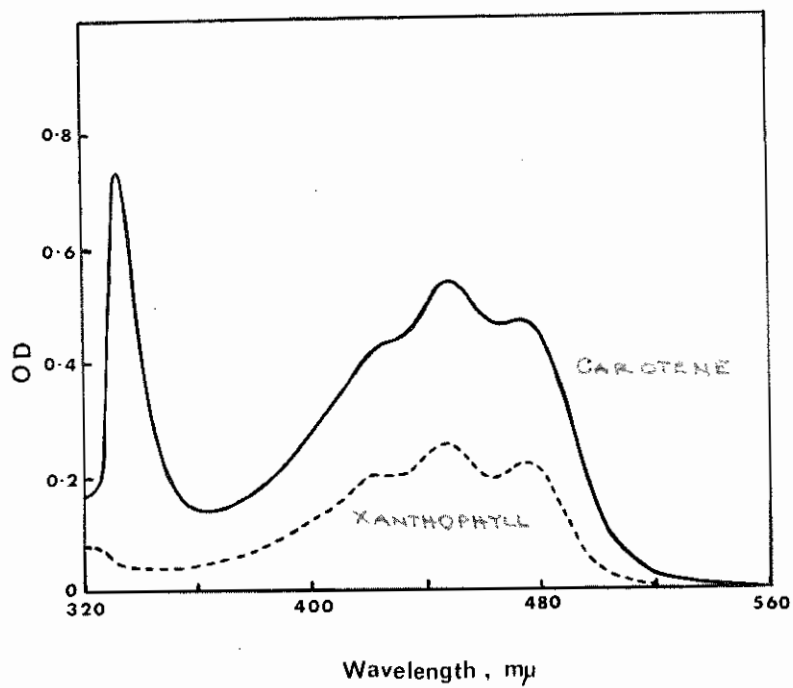
Or - Origin; B - Chlorophyll b;
A - Chlorophyll a; X - Xanthophylls;
C - Carotenes; Sf - Solvent front;
Un - See page 49.

17 x 29 cm. was used. The pigment extract in petroleum ether was spotted along a basal line drawn 3 cm. from one end of the paper. The paper was formed into a cylinder and stitched along the overlap. It was then put into the developing tank previously saturated with petroleum ether ($60^{\circ} - 80^{\circ}\text{C}$), and equilibrated for about twenty minutes before being developed (ascending, one-directional), for about 2 hours. The developing medium (a modification of the one used for column chromatography), was made up of petroleum ether ($100^{\circ} - 120^{\circ}\text{C}$), petroleum ether ($40^{\circ} - 60^{\circ}\text{C}$) and acetone (20 : 5 : 3). The transference of pigments to the paper, as well as the development of the chromatograms, was done in a 'dimly lighted room' at a temperature of about 20°C .

The distribution of pigments, starting from the solvent-front was as follows: a bright yellow spot of carotenes, a greyish spot designated Un, a yellow xanthophyll spot, a bluish-green spot of chlorophyll a, and a dark-green spot of chlorophyll b (Fig. 7). Each of these spots was eluted in 80% acetone and its optical density measured in a final volume of 5 ml. acetone, at a wave-length previously determined from its absorption spectrum.

When the pigment content of *Peperomia*, and of two other variegated plants, *Chlorophytum* and *Pelargonium* was determined by column chromatography (method described on page 24),

Fig. 8 Absorption spectra of Peperomia leaf
chloroplast pigments in acetone.



the concentration of chlorophylls in the yellow leaves was so small, that it was not possible to elute them from the column quantitatively. Absorption spectra of the four fractions obtained from the column also showed them to be contaminated by other pigments, e.g. there were traces of chlorophyll a in the carotenoids and vice versa. Paper chromatography was found to be quicker and more accurate, and was therefore used in studies of changes in pigment concentration during greening in *Peperomia* leaf tissues. The absorption spectra of the pigment fractions obtained by this method are shown in Fig. 8. These show no contamination by other pigments.

SECTION II
CONDITIONS FOR GREENING

	Page
1. INTRODUCTION	27
2. METHODS	
(a) Day-length effects	27
(b) Day-length/light intensity effects	28
(c) Fresh and dry-weight determinations	28
(d) Chemical treatment	29
3. RESULTS	30
4. DISCUSSION	34

1. INTRODUCTION

As mentioned in the General Introduction, the leaves of *Peperomia* have been found to remain variegated during the summer and autumn months, but in winter and early spring, the yellow parts show marked greening. The major changes in environmental conditions in the greenhouse during this greening period are:

- (i) decreased day-length
- (ii) decreased light intensity and
- (iii) decreased total radiation.

Experiments were therefore carried out in an attempt to find out if any of these environmental conditions could be correlated with the observed greening in winter.

2. METHODS

(a) Day-length Effects.

Three light cabinets, maintained at 25°C, were set up, one on a short-day (8 hours light), a second on a long-day (16 hours light), and the third on a day-neutral regime (12 hours light).

Six pots of 3-year old *Peperomia* plants from the greenhouse, eight pots of rooting stem-cuttings (without new shoots), and six pots of newly-shooting cuttings were put into each of these three light cabinets. The plants in all the cabinets were watered regularly and closely observed for a period of about six months.

(b) Day-length/light intensity effects.

Several pots of variegated Peperomia plants were put into two light cabinets, one with a photo-period of eight hours, and the other with a photo-period of sixteen hours a day. The plants in each cabinet were at two different light intensities - 200 ft-c. and 25 ft-c. Chlorophyll content was determined at 10 day intervals for a period of 40 days, using samples of 1 g. yellow leaf-material extracted in 30 ml. 85% acetone and transferred into 10 ml. petroleum ether. Two determinations of optical density at 663 mμ, were made from each extract.

(c) Fresh and dry-weight determinations.

Green and variegated plants were maintained at 25 and 300 ft-c. in the roof light cabinet. At 20 day intervals, 80 leaf discs were cut from the yellow parts of the variegated leaves, and 80 from the green leaves, at each of the two light intensities (the leaves were washed and blotted dry before the discs were cut). Each group of 80 discs was thoroughly mixed and divided into two replicate samples of 40 discs each. Each sample was weighed in a weighing bottle (of known weight), and was then oven-dried to constant weight at about 100°C. The dried discs were also weighed. From the above results, the fresh and dry-weights of 20 green, and of 20 yellow

leaf discs, at 25 and 300 ft-c. were calculated.

(d) Chemical treatment.

The effects of chemical factors in inducing greening was studied in entire *Peperomia* leaves growing in sterilised solutions. These solutions were changed (under sterile conditions), as soon as signs of infection were detected in them - the solutions, as well as the small glass-tube containers were replaced with sterile ones. Similar studies were made on leaf discs growing in semi-solid cultures. The cultures were made up of White's mineral and vitamin medium (White, 1943), 0.6% agar and different concentrations of sucrose. Sucrose concentrations from 1 to 8% were used in experiments to find out the effect of sucrose on greening. In experiments to study the effects of other compounds the semi-solid culture contained 2% sucrose.

The following amino acids and growth substance were used: Leucine and aspartic acid (in concentrations from 5 to 100 ppm.), gibberellie acid (from 5 to 50 ppm.) kinetin (from 2 to 10 ppm.), NAA and 2,4-D (from 0.2 to 2 ppm.). Solutions of yeast extract and casein hydrolysate (in concentrations, varying from 100 to 2,000 ppm.), as well as urea (from 1 to 20 ppm.), and ammonium nitrate (0.1 M and 0.2 M) were also tried.

3. RESULTS

During the 6-month period during which the *Peperomia* plants were kept under three different day-length regimes, the following general observations were made: Greening occurred in all three light cabinets in about five weeks, and usually started in the terminal leaves; (no sign of greening was found in the lower leaves until after about four weeks, in the short, and day-neutral cabinets). Terminal leaves formed after greening, started off variegated and greened later. Thus greened variegated plants always had one or two 'non-greened' terminal leaves (see Fig. 9a, b, c). Excising the terminal leaves did not alter the above pattern of greening - greening still started in the youngest leaves left on the plant.

Greening in the long-day cabinets was only found in the leaves which were shaded by other plants. Here, the observed regular pattern of greening, starting from the terminal leaves and later spreading to the lower ones, was not evident. Measurement of light intensity showed that greening only occurred at intensities lower than 200 ft-c. Thus while all the plants in the short, and day-neutral cabinets greened (light intensity here was between 100 and 150 ft-c.) the plants in the long-day cabinets where light intensity was above 200 ft-c., remained variegated, except (as mentioned above), those shaded by other plants.

Fig. 9

(a) - Variegated Peperomia plant.

(b) - Normal Green Peperomia plant.

(note 'yellow' terminal leaf -
see text).

(c) - Greening variegated Peperomia
plant (R).

Greened variegated Peperomia
plant (D). (New terminal leaf
here starts off variegated and
greens later - see text).



a



b

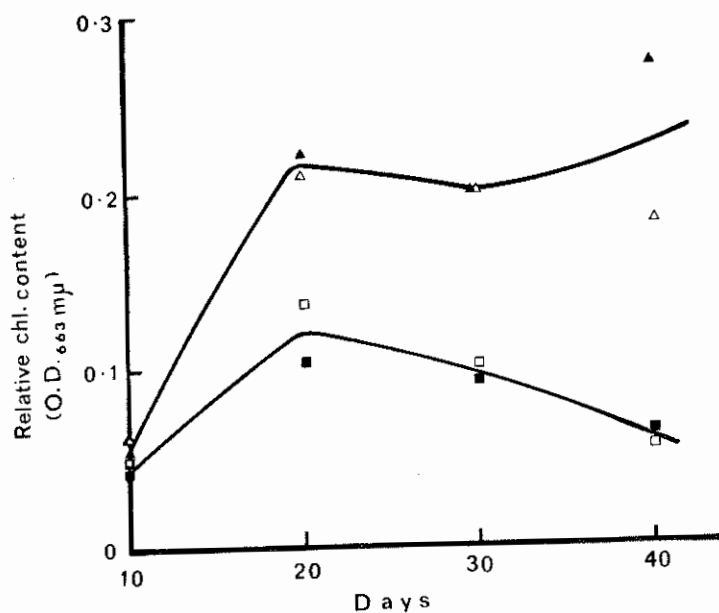


c

Other variegated plants were tried for the above light intensity effects found in *Peperomia*, but none of them responded in the same way. *Abutilon* and *Pelargonium* appeared to require high light intensity for good growth, and they died at the low light intensity level which induced greening in *Peperomia*. The other plants (*Chlorophytum*, *Pedilanthus*, *Euonymus*, and *Hedera*), also appeared to do better at high light intensity, and though they did not die at the low intensities, very little greening was found in *Chlorophytum* and *Hedera*, and no evidence of greening at all, in *Pedilanthus* and *Euonymus* at 25 ft-c.

Another experiment in which some *Peperomia* plants were kept in polythene bags and maintained at high humidity showed that high humidity had no observable effect in inducing greening. Vegetative growth was found to be slower under this condition of high humidity; i.e. though the plants maintained at high humidity produced a lot of adventitious roots and were more succulent, 'control plants' in drier conditions (not in bags) grew faster, and became larger.

Changes in chlorophyll content of yellow *Peperomia* leaves under long, and short-day conditions are shown in Fig. 10. The plants in each light-regime were at 25 and 200 ft-c. These results suggest that it is low light intensity rather than short day-length, which is responsible



**Fig. 10: Effect of day-length and light intensity
on chlorophyll content
in YELLOW Peperomia leaves.**

- Δ 25 ft-c. } — Short Days (8 hrs. light)
 □ 200 ft-c. }
 ▲ 25 ft-c. } — Long Days (16 hrs. light)
 ■ 200 ft-c }

for greening in yellow *Peperomia* leaves. Thus at 200 ft-c., under both long, and short-day conditions, though there was an initial increase in chlorophyll content up to about 20 days, there was a subsequent decrease in chlorophyll content (on fresh weight basis). At 25 ft-c. on the other hand, the increase appeared to continue beyond twenty days.

To check up on the apparent discrepancy in changes in chlorophyll content at 25 ft-c. (Fig. 10), the experiment was repeated under short-day conditions with smaller fresh-weight leaf samples, but more determinations. Fresh and dry-weight determinations under short and long-day conditions were also made. The pigments in 0.5 g. leaf-samples were extracted in 30 ml. 85% acetone and transferred into 5 ml. petroleum ether by the method already described on page 22 and each determination was done in triplicate. This and all subsequent determinations of chlorophyll content were confined to the 'terminal leaves'.

The results (Fig. 11), showed that chlorophyll content in yellow *Peperomia* leaf tissue continued to rise beyond 20 days in short-day conditions, as it did in the long-day regime. But at 200 ft-c., as in Fig. 10, there was a fall in chlorophyll content (per unit leaf area or unit fresh-weight), after a short initial period of about a fortnight, when there was roughly the same rate of increase as at 25 ft-c. In green leaves chlorophyll accumulation occurred both at high and low light intensities (Fig. 12).

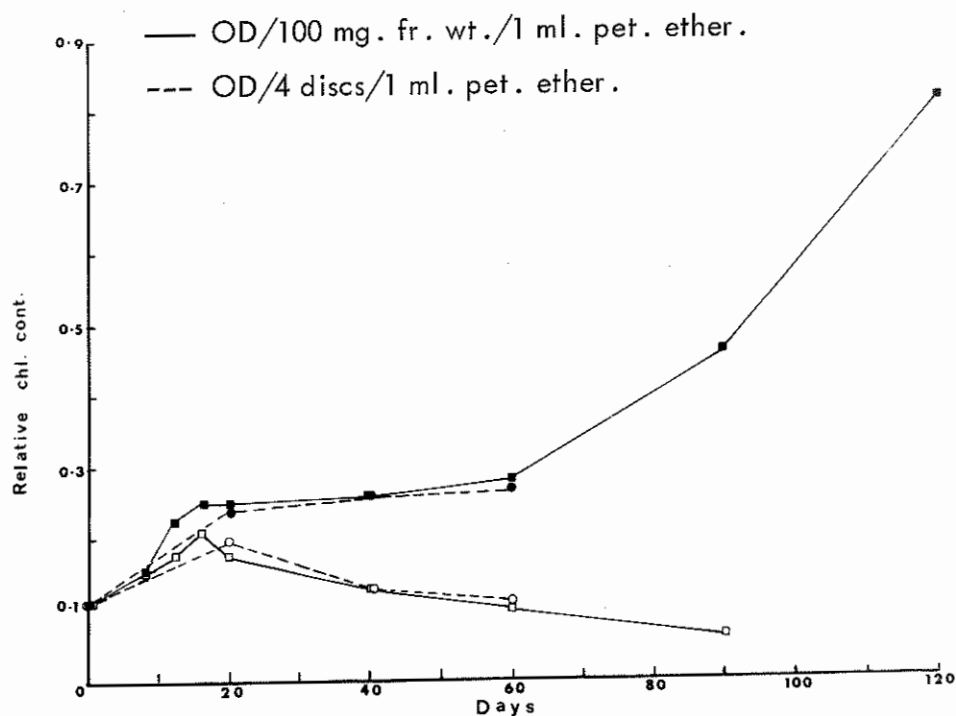


Fig. 11 Effect of light intensity on chlorophyll content in YELLOW *Peperomia* leaves in short-days.

Dark points ■●- 25 ft-c.

Light points □○- 200 ft-c.

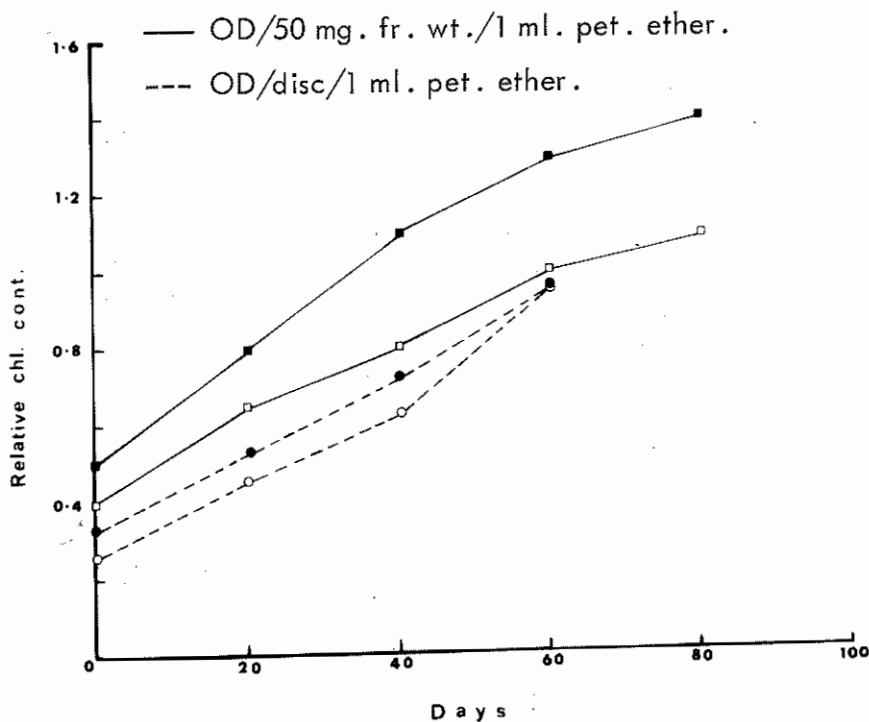


Fig. 12 Effect of light intensity on chlorophyll content in GREEN *Peperomia* leaves in short-days.

Dark points ■● - 25 ft-c.

Light points □○ - 200 ft-c.

Experiments using various growth-substances and compounds which have been reported to induce chlorophyll synthesis or chloroplast differentiation did not produce any greening in yellow *Peperomia* leaves. Leucine and aspartic acid, as well as gibberellic acid, which have been reported to induce normal chloroplast differentiation in some barley mutants, and regreening in *Valentia* orange respectively (von Wettstein, 1961; Coggins and Lewis, 1962), did not appear to induce any greening. Kinetin, NAA and 2,4-D were also tried but no evidence of greening was found.

To find out if variegation was the result of nutritional deficiency, whole-leaf cultures of *Peperomia* were set up in solutions of yeast extract, casein hydrolysate, urea ammonium nitrate. Here too, no greening occurred.

Though fungal and bacterial infection made the results of the above experiments rather difficult to interpret, no definite greening induced by chemical treatment was found after maintaining the cultures for periods of from 8 to 14 weeks. Profuse rooting occurred in the whole-leaf cultures, while adventitious roots were produced by the leaf discs, the latter only occurring in discs which were placed upside-down in the semi-solid culture medium.

4. DISCUSSION

The above results show that while greening in yellow *Peperomia* leaves occurs at 25 ft-c. in both long-and short-day conditions, no greening occurs at 200 ft-c., whether the plants are kept in long or short days. It is thus evident, that it is light intensity, rather than day-length which controls greening in yellow *Peperomia* leaves.

The results of fresh and dry-weight determinations (Fig. 13) show that the above conclusion is still valid. There is very little change in fresh and dry-weight of yellow *Peperomia* leaves at high and low light intensities over the period of the experiment. So that as can be seen from Fig. 11 (where chlorophyll content has been expressed per unit surface area as well as on fresh-weight basis), the fall in chlorophyll content at high light intensity cannot be attributed to 'dilution' caused by more rapid growth. Even in green leaves where there is a significant increase in fresh-weight at high light intensity, (Fig. 13), chlorophyll content per 'unit' fresh-weight continues to rise.

Though the determination of chlorophyll content was not repeated under long day conditions, the initial increase and subsequent fall in chlorophyll content in yellow *Peperomia* leaf tissue reported above, had been found to occur in all new stem-cuttings in the 'controlled conditions' in the roof light cabinet where they were cultivated (i.e. in long-days and at high light intensity). It might be a

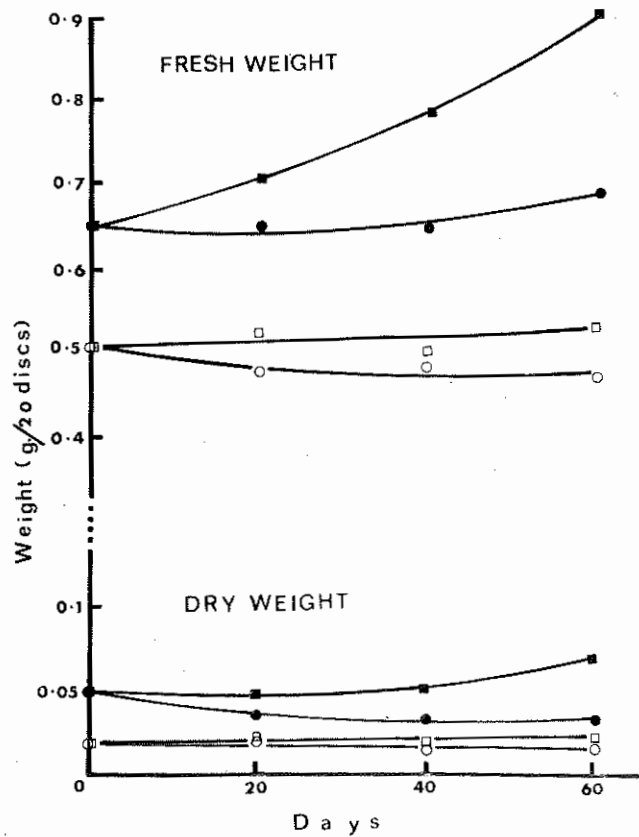


Fig. 13 Growth rates in Peperomia leaves

- High light intensity
 - Low light intensity
 - High light intensity
 - Low light intensity
- } — Green leaf discs
- } — Yellow leaf discs

'developmental phase' in the growth of yellow *Peperomia* leaves. The leaves of green *Peperomia* plants also show a peculiar developmental pattern. They start off yellow, but soon green, whether they are kept at high or low light intensity; i.e. unlike yellow *Peperomia* leaves, chlorophyll accumulation occurs in green leaves, not only at low, but also at high light intensities.

The pattern of greening found in *Peperomia* leaves, (i.e. starting from the terminal leaves) appeared to suggest that greening here was under hormonal control. But since greening still occurred even when the terminal leaves were cut off, it can be concluded that greening is not the result of the synthesis in the young leaves, of a diffusible compound required for chlorophyll synthesis. (It has also not yet been possible to induce greening by chemical treatment). That greening in *Peperomia* is not controlled by a hormone formed in the young leaves is again supported by the observation that in the long-day cabinet, all shaded leaves on any shoot greened, whether or not the terminal leaves of the shoot had already greened.

Because it was not possible to induce greening by chemical treatment, it was not easy to offer any explanation for the low light intensity effect in inducing greening in *Peperomia* leaves. If such induction of greening had become possible (or if greening by some chemical

treatment can later be achieved), then by studying the effect of light intensity on the relevant chemical factor, an attempt at explaining this light effect might have been possible (or might become possible). Studies of temperature effect, or of the effect of total radiation (both of which are yet to be done), might also help to elucidate the mechanism of greening in *Peperomia* leaves. Without successful results from the above studies, a speculative explanation is all that can be provided at this stage.

Since greening is the result of a higher rate of chlorophyll synthesis than the rate at which the pigment breaks down, low light intensity can cause greening by slowing down chlorophyll degradation, or by initiating chlorophyll synthesis or increasing the synthetic rate. But as has already been reported, some initial greening occurs in yellow *Peperomia* leaves, even at high light intensities. Thus greening at low light intensity might be the result of decreased chlorophyll degradation and/or increased formation of the pigment. Studies of these two effects have been reported in later Sections.

SECTION III
CHANGES ACCOMPANYING GREENING

	Page
1. INTRODUCTION	37
2. METHODS	
(a) Changes in pigment content	38
(b) Photosynthesis	39
(c) Respiration	40
(d) Anatomical studies	40
3. RESULTS	43
4. DISCUSSION	52

1. INTRODUCTION

Though greening is the result of increase in chlorophyll content, the process is also characterized by an increase in other photosynthetic pigments. The types of pigments formed, and the changes in total pigment content during greening, were determined for *Peperomia* leaves.

Photosynthetic rates in greening leaves were also measured. These involved the determination of oxygen uptake in the dark and gas exchange in light. Since dark oxygen uptake was unexpectedly found to be higher in yellow than in greened-yellow *Peperomia* leaves, it was decided to follow changes in respiration rates in the greening leaves as well as the photosynthetic rates.

As has been mentioned in the General Introduction (page 9), green *Peperomia* leaves are made up of a potentially green epidermis, a colourless sub-epidermis and a green core or chlorenchyma tissue. In yellow leaves the core tissue is colourless instead of being green. Anatomical investigations were therefore undertaken to study the changes which take place during greening in these yellow tissues. The main features studied were chloroplast structure and chloroplast morphology (the latter covering changes in chloroplast size, number and distribution). In addition, general leaf anatomy (i.e. tissue-types and distribution, and changes in cell size) was also studied.

2. METHODS

(a) Changes in pigment content

The total content of chloroplast pigments in *Peperomia* leaves under greening conditions was followed by determining the amount of each of the four pigment-types formed (chlorophyll a chlorophyll b, carotenes and xanthophylls). Green and variegated *Peperomia* plants were kept at 25 ft-c, and at 20-day intervals, their leaf pigments were separated by paper chromatography (page 24) and the concentrations of the pigments determined.

1 ml. samples of the petroleum ether extracts of leaf pigments (prepared as described on page 22 and used in the above determinations), were also measured out, evaporated under vacuum, dissolved in 5 ml. 80% acetone, and their optical densities at 663 mμ and 645 mμ measured. From these determinations, the concentrations of chlorophyll a and b in mg/g. fresh weight leaf material were calculated, using the following equations derived by using the specific absorption coefficients of McKinney (1940) for chlorophyll a and b. (MacLachlan and Zalik, 1963):

$$C_a = \frac{(12.3 D_{663} - 0.86 D_{645}) V}{d \times 1000 \times W}$$

$$C_b = \frac{(19.3 D_{645} - 3.6 D_{663}) V}{d \times 1000 \times W}$$

where C_a is conc. of chl. a in mg/g.fr.wt. leaf material,
 C_b is conc. of chl. b in mg/g.fr.wt. leaf material,
 D is optical density at wave-length indicated,
 V is volume of extract in ml., d . length of light
path in cm, and W the fr.wt. of leaf material in grams.

(b) Photosynthesis.

A Warburg apparatus adjusted to 30°C and 100 oscillations per minute, kept in an air-conditioned room was used for all photosynthetic determinations. The apparatus was illuminated by two 1000 watt bulbs supported in long retort stands at such height that the light intensity on the flasks containing the photosynthesizing material was about 800 ft-c. To overcome the heating effect of the strong light from the bulbs (i.e. to maintain the bath temperature at 30°C), water from a large plastic tank cooled in the air-conditioned room, was circulated inside the bath and the excess water drained off.

Each flask contained 20 leaf discs in 2 ml. Warburg No. 9 buffer solution (carbonate - bicarbonate solution with CO_2 concentration of 9.1×10^{-5} g/l.). The flasks were fitted to their corresponding manometers, covered with black bags and tested to ensure there was no leakage. They were then mounted in the bath and equilibrated for about 20 minutes. Oxygen absorption in the dark was measured at

10-minute intervals for one hour. At the end of this period, the black bags were removed from the flasks and gas exchange was again measured at 10-minute intervals for another hour.

Each determination was done in triplicate and the total chlorophyll content (in mg/g.fr.wt.) for each sample was also determined from optical density measurements of acetone extracts at 663 m μ and 645 m μ as described on page 38.

(c) Respiration.

20 leaf discs (diameter 7 mm.) were cut from washed leaves, blotted dry and put into the outer well of a Warburg flask. 1.8 ml. phosphate buffer (pH.6.4) were added to the leaf discs in each flask and 0.2 ml. 10% NaOH put into the centre well (into which had been inserted a rolled-up 2 cm. sq. No. 54 filter paper). The total volume of leaf discs and solutions in each flask was 3 ml. and the temperature of the bath, 30°C.

The flasks were tied up in black bags and after equilibrating for about 20 minutes, oxygen uptake was measured at 10-minute intervals for one hour. Total chlorophyll content of each sample was determined as above.

(d) Anatomical Studies

Potted variegated Peperomia leaves were kept in a light cabinet with a light intensity of 25 ft-c., a

temperature of 25°C and a daily photoperiod of 16 hours light. At intervals over a period of 10 weeks, samples of leaf material from green and yellow tissues were taken from the potted plants for anatomical investigations. Each sample was made up of leaf-bits from the terminal leaves of 5 plants so selected that their terminal leaves were about the same age, (the initial cell-sizes of these 5 selected terminal leaves were very similar).

Unstained transverse sections were prepared from the leaf-bits and 5 good sections (one from each leaf) constituted a sample for the above anatomical studies. The sections were mounted in water and were studied under the high power of a Zeiss Winkel microscope. For each sample, the distribution of chloroplasts in the different cell-types was studied and counts were made of chloroplast numbers. Measurements of chloroplast-size and cell-size were also made.

Electron microscopic investigations were also carried out. Electron micrographs (to show chloroplast structure) were prepared from normal green, yellow, and greened-yellow *Peperomia* leaf tissues. Some more electron micrographs were later prepared to complete the studies on chloroplast differentiation. The material used for the latter studies was obtained from a yellow leaf and a yellowish leaf-bud produced from a normal green plant at low light intensity (25 ft-c).

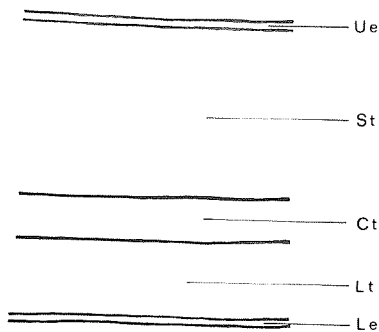
After electron micrographs had been prepared to show their initial chloroplast structure, the yellow leaf and the yellowish leaf-bud were kept at low light intensity for 4 weeks, and further electron microscope preparations were made from them. Electron micrographs were again prepared from the above tissues after they had remained in the low light intensity cabinet for a total of 10 weeks,

3. RESULTS

The structure of Peperomia leaf studied during this work, has been fully described on page 6. As can be seen from the low-power distribution diagram below, it shows two mesophyll regions, a lower mesophyll tissue of smaller cells separated from an upper mesophyll tissue by a chlorenchyma tissue. The upper mesophyll tissue is made up of large, water-storage cells (hereafter referred to as storage tissue). Both the lower mesophyll tissue and the storage cells are about 5 cell-layers thick in both yellow and green leaves. The chlorenchyma tissue consists of about 3 layers of very small cells and it is here that most of the chloroplasts are concentrated. (i.e. in green and greened-yellow leaves).

Fig. 14

L.P. distribution diagram of the T.S. of Peperomia leaf.



Ue - Upper epidermis.

St - Storage tissue.
(Upper mesophyll tissue).

Ct - Chlorenchyma tissue.

Lt - Lower mesophyll tissue.

Le - Lower epidermis.

Microscopical examination of transverse leaf-sections showed that both the storage tissue and the lower mesophyll tissue contained chloroplasts, many more occurring in the lower mesophyll than in the storage tissue. The chloroplasts in the chlorenchyma tissue of green leaves (even in very young green leaves which look yellow) were found to be so closely packed that it was not possible to make reliable chloroplast counts, or follow changes in cell size in this tissue. In the lower mesophyll and storage tissue, chloroplast numbers and sizes were also so variable that it was evident that quantitative analysis of chloroplast numbers and sizes was not going to provide any useful information concerning changes in chloroplast morphology under greening conditions. Only qualitative results of chloroplast distribution and morphology have therefore been reported in this thesis. It was however possible to make quantitative studies of changes in cell-size in the lower mesophyll and storage tissues, and statistical analysis of these results have been reported (page 47).

As has already been mentioned above, some chloroplasts could be found in all the 3 'tissue-types' of *Peperomia* leaves. In both green and yellow leaves, very few chloroplasts were present in the storage tissue, and these were usually small chloroplasts, under 3 μ in diameter. These occurred in singles or in groups of up to about

8 or more in isolated cells, and they were paler green in colour.

In yellow leaves, the lower mesophyll tissue and chlorenchyma region contained very few small chloroplasts. Here too chloroplast numbers varied from about 2 to 8, and were present in isolated cells. Some leucoplasts (colourless plastids) were also found in these tissues.

The lower mesophyll tissue of green leaves showed numerous chloroplasts (about 4 to 12 per cell). These occurred mostly in the 3 to 4 layers of lower mesophyll tissue nearest the chlorenchyma region, very few occurring in the 2 remaining lower mesophyll cell-layers. Some of these chloroplasts were the small-sized types which were found in the storage tissue and in the lower mesophyll and chlorenchyma region of yellow leaves, but most of them were large ones, with diameters of about 6 to 8 μ . All the chloroplasts in this tissue and in the chlorenchyma region, were of a distinctly dark-green colour. The chlorenchyma tissue of green leaves showed the highest concentration of chloroplasts. Here, in cells of about 24 x 32 μ were found, up to 10 or more closely packed large chloroplasts (6 to 8 μ diameter).

During the ten-week experimental period, no definite pattern of changes in chloroplast morphology or distribution was evident in green leaves kept under greening conditions. (i.e. light intensity of 25 ft-c). In the chlorenchyma

and the 4 layers of lower mesophyll tissue (immediately below the chlorenchyma tissue) where large numbers of chloroplasts occurred, no definite pattern of change in chloroplast number or size was found. The chloroplasts in these tissues were mostly the large-sized type, and some of them were found to be dividing by about the third week. The only regular, visible change was in the colour of the chloroplasts - they were progressively becoming darker-green.

During the same period as above, and under the same conditions, no quantitative change was found in the numbers and sizes of the chloroplasts in the storage tissue of the yellow leaves too. A distinct change in chloroplast colour was however found to be occurring in these yellow tissues. The chloroplasts, and some of the leucoplasts in the chlorenchyma region and in the lower mesophyll tissue were becoming greener and greener, though here too, no significant change in chloroplast size or number was found. The change in colour was particularly striking in the chlorenchyma tissue, which by the tenth week, was almost indistinguishable from that of normal green leaves. The only difference in chloroplast morphology between a section of normal green and greened-yellow leaf was that the chloroplasts in the former were larger and darker-green in colour.

Table 1 shows the changes in cell size in green and

Time (Weeks)	Storage Tissue		Lower Mesophyll	
	Green Lvs.	Yellow Lvs.	Green Lvs.	Yellow Lvs.
0	104 x 124 (9) (11)	60 x 72 (10) (12)	44 x 60 (12) (12)	36 x 40 (8) (6)
1	100 x 116 (7) (10)	60 x 72 (7) (9)	44 x 56 (7) (10)	36 x 44 (7) (8)
5	96 x 116 (14) (16)	64 x 88 (14) (13)	44 x 56 (7) (10)	44 x 56 (7) (13)
10	108 x 136 (19) (15)	88 x 108 (15) (21)	52 x 64 (7) (12)	56 x 76 (10) (17)

Table 1

Changes in cell-size in Green &
Yellow Peperomia leaf-tissues
at low light intensity
(25 ft-c).

Results are in μ . Each figure is
the average from about 40 counts.
(Figures in brackets are S.E.).

yellow leaf-tissues under greening conditions during the ten-week experimental period. From these results, green leaves showed no apparent increase in cell size, either in the storage tissue or in the lower mesophyll tissue. In yellow leaves on the other hand, there was clear evidence of cell enlargement in both the lower mesophyll and storage tissues, the increase in cell size being slightly more marked in the former tissue.

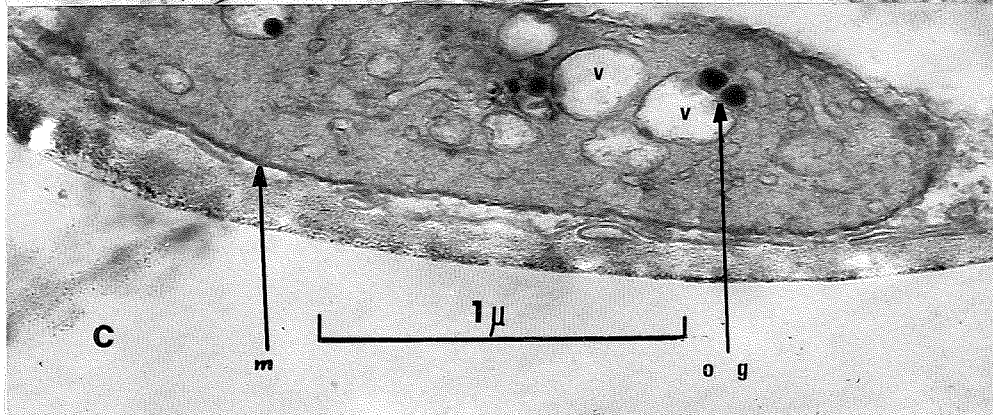
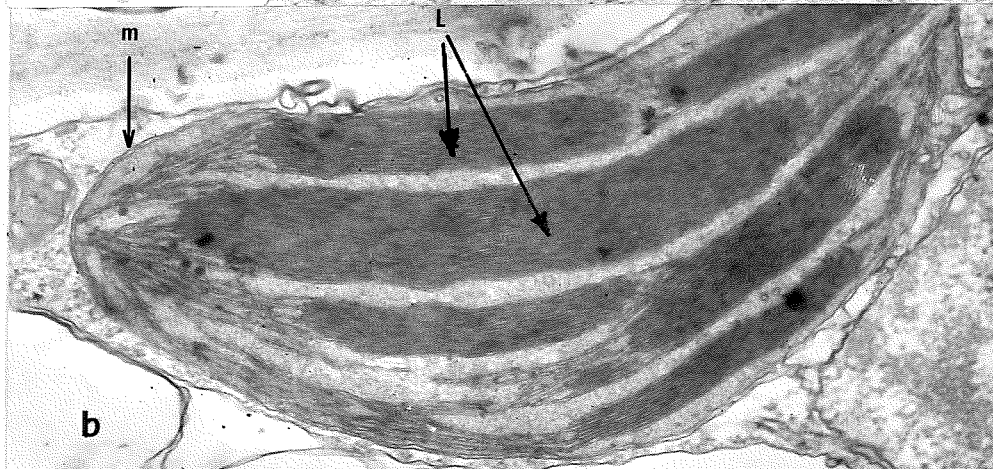
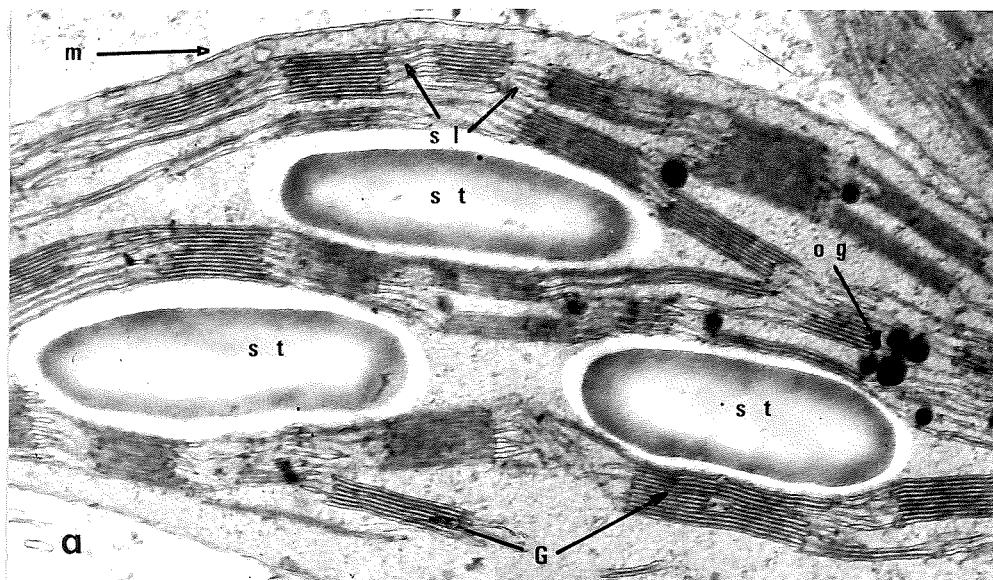
Electron microscope examination of mature green *Peperomia* leaf-tissue showed normal chloroplasts with well differentiated grana and stroma (intergrana) lamellae, and large starch grains (Fig. 15a). Chloroplasts from yellow leaves were completely devoid of lamellae and had no starch grains (Fig. 15c). They were also smaller than those found in the green cells. During greening these chloroplasts (from originally yellow tissue) formed lamellae extending from one end of a chloroplast to the other, but which showed no differentiation into grana and stroma lamellae (Fig. 15b). They also showed a complete absence of starch grains.

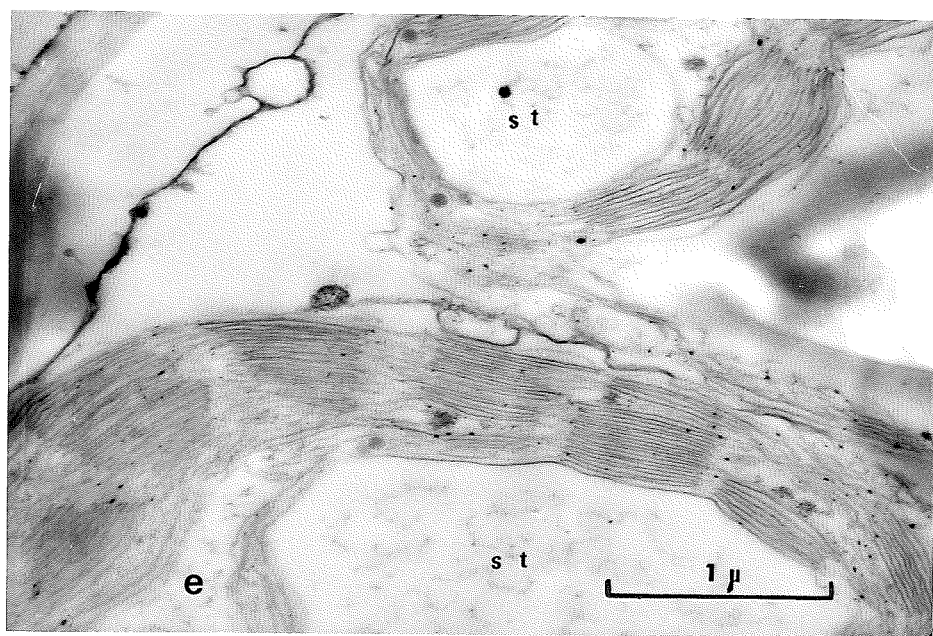
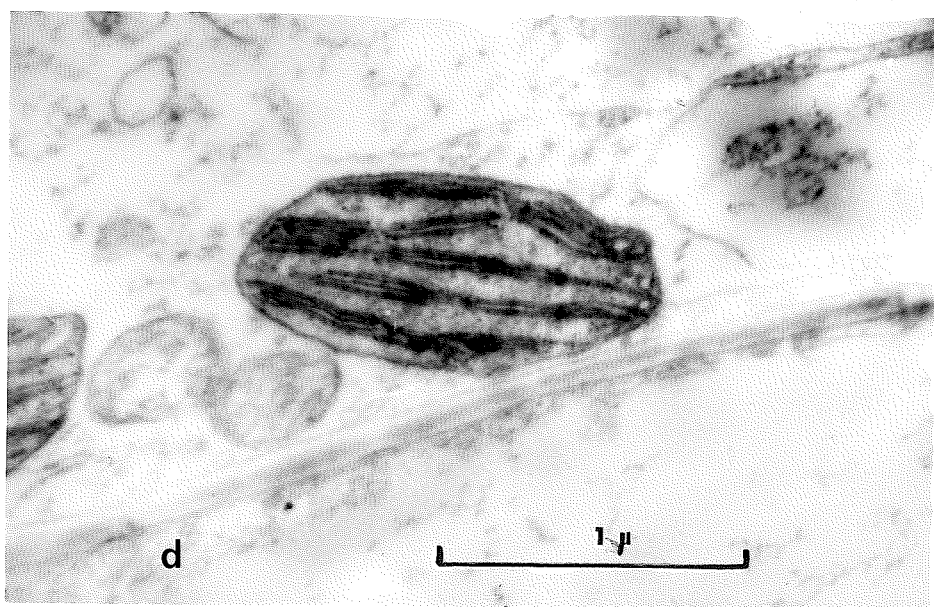
Chloroplast structure from a yellowish, fully developed leaf-bud produced from a normal green plant at low intensity is shown in Fig. 15d. Even though the tissue from which this type of chloroplast was prepared was yellowish in colour, and the chloroplasts were smaller than chloroplasts from normal yellow tissue, differentiation into stroma and grana

Fig. 15 Chloroplast differentiation in Peperomia leaves.

L - lamellae. G - grana. m - outer membrane.
sl - stroma lamellae. st - starch grains.
og - osmiophilic granules. v - vacuoles.

- a - Normal Green Chloroplast.
- b - Chloroplast from Greened-Yellow tissue.
- c - Chloroplast from Yellow tissue.
- d - Chloroplast from yellow leaf-bud produced from Green shoot in low light intensity cabinet.
- e - Chloroplast from above leaf-bud (now young, greenish leaf) 4 weeks later.





was already becoming evident, and they also contained starch grains. After about 4 weeks at low light intensity (by which time the yellowish leaf-bud had developed into a small, pale-green leaf), chloroplast differentiation was almost complete (Fig. 15c). By the tenth week, chloroplast structure in the now dark-green leaf (from the above yellowish leaf-bud at low light intensity) was as shown in Fig. 15a. In the same period (10 weeks), and under the same conditions, a chloroplast from a greening-yellow tissue was incompletely differentiated and contained no starch grains (its structure is as shown in Fig. 15c).

The changes in pigment content during greening in yellow *Peperomia* leaves are shown in Table 2a & b, which also show the results of pigment changes in green *Peperomia* leaves under the same conditions. From these results, the ratio of chlorophyll a to b is about 3 for both yellow and green leaves. (Table 2a).

The ratio of chlorophyll to carotenoids is higher in green, than in yellow leaves. (Table 2b). This most probably explains the difference in absorption spectra of chloroplast pigments from green and yellow *Peperomia* leaf tissues shown in Fig. 16a, b. From these spectra, the ratio $O.D._{430}/O.D._{663}$ is 3.1 for yellow leaf pigments and 2.3 for pigments from green leaves. According to Anderson and Calvin (1962), the presence of carotenoids

Days	Leaf Tissues	Chlorophyll <u>a</u>	Chlorophyll <u>b</u>	Chlorophyll <u>a</u> Chlorophyll <u>b</u>
0	Green	0.11	0.04	2.8
	Yellow	0.009	0.003	3.0
20	Green	0.144	0.045	3.2
	Yellow	0.019	0.006	3.2
40	Green	0.18	0.062	2.9
	Yellow	0.024	0.009	2.7
60	Green	0.208	0.069	3.0
	Yellow	0.034	0.011	3.1

Table 2a

Changes in chlorophyll a & b content
in Peperomia leaves at 25 ft-c.

(Values for chl.cont. are in mg.chl./g./fr.wt.)

Days	Leaf Tissue	Total Chlorophylls	Total Carotenoids	<u>Chlorophylls</u> Carotenoids
0	Green	0.325	0.13	2.5
	Yellow	0.055	0.05	1.1
20	Green	0.44	0.19	2.5
	Yellow	0.13	0.115	1.2
40	Green	0.51	0.22	2.3
	Yellow	0.185	0.115	1.6
60	Green	0.625	0.235	2.7
	Yellow	0.255	0.145	1.8

Table 2b

Changes in Pigment content in
Peperomia leaves at 25 ft-c.

(Values for pigment cont. are O.D. of
pigments from 50 mg.fr.wt. Green leaves/ml.
acetone, 100 mg.fr.wt. Yellow leaves/ml.
acetone).

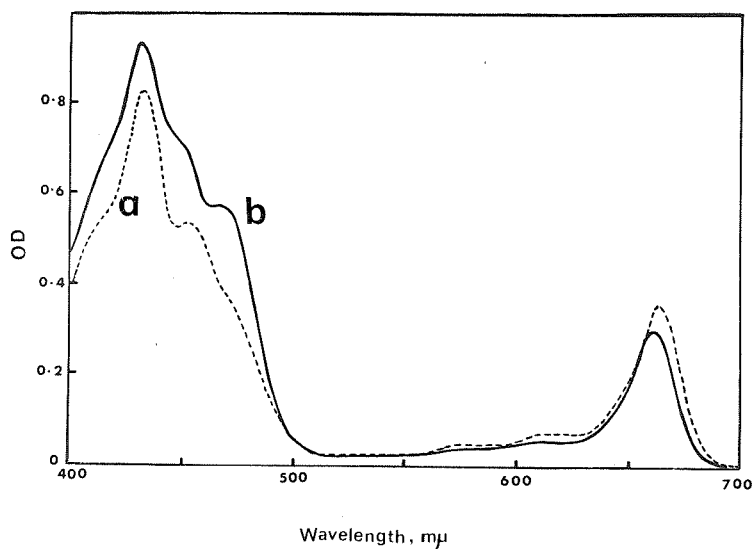


Fig. 16 Absorption spectra of Green and Yellow
Peperomia leaf chloroplast pigments in
acetone.

$$a - \text{Green leaf: } \frac{OD_{430}}{OD_{663}} = 2.3$$

$$b - \text{Yellow leaf: } \frac{OD_{430}}{OD_{663}} = 3.1$$

can produce an enhanced apparent intensity value in the blue band of the chlorophyll a spectrum. Thus a higher carotenoid to chlorophyll content (as is found in the yellow leaves), can result in the above spectral difference.

Chromatography of *Peperomia* leaf pigments (Fig. 7) showed in addition to the four components, chlorophyll a chlorophyll b carotenes and xanthophylls, another spot designated Un. When this pigment was eluted and its absorption spectrum determined, (Fig. 17), it was found to be almost identical to that of ethyl pheophorbide a (Holt and Jacobs, 1954). The close similarity between Holt and Jacobs spectrum and that of Un seemed to indicate that this pigment was pheophorbide a. But from the position of the pigment on the chromatogram shown in Fig. 7, chlorophyll a and b are 'more hydrophilic' than Un. This suggests that Un must still contain the lipophilic phytol unit of the chlorophyll molecule. In other words, while the absorption spectrum seems to suggest that Un is pheophorbide a, the position of the pigment after chromatographic separation of chloroplast pigments indicates that Un is more likely to be a pheophytin.

When pheophytin a and b, (prepared by addition of HCl to chlorophyll a and b) were chromatographed with the same solvent system used above, the position and colour of one of the 2 spots formed corresponded to that of Un. The

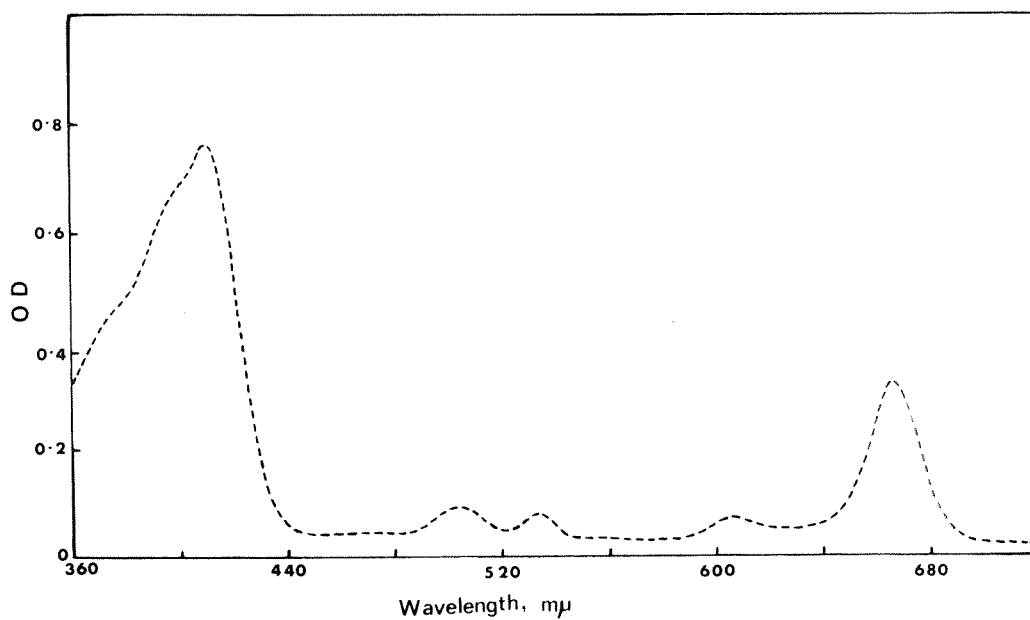


Fig. 17 Absorption spectrum of *Peperomia* leaf pigment designated Un (in acetone).

- See text.

absorption spectrum of this spot was also identical with that of Un, while the absorption spectrum of the other spot corresponded to that of ethyl pheophorbide b (Holt and Jacobs, 1954). The above results (though they could not help to determine the chemical structure of Un), suggest that this pigment is most probably a breakdown product of chlorophyll a. The sum of the optical densities of chlorophyll a and Un was therefore taken to represent total chlorophyll a content.

As has been reported above, the formation of chlorophyll in yellow *Peperomia* plastids is not accompanied by complete chloroplast differentiation. No starch grains were also found in chloroplasts from greened-yellow tissues. Hence though absence of starch is not necessarily indicative of lack of photosynthesis, photosynthetic determinations were made in green and yellow *Peperomia* leaves to find out if the unusual chloroplast structure of greened-yellow tissue is due to the formation in these plastids, of the non-photosynthetic type of chlorophyll reported in *Oenothera* leaves by Schoetz (1956).

Oxygen uptake in the dark (respiration) and oxygen evolution in light (apparent photosynthesis) for green and greened-yellow leaves are shown in Fig. 18 a and b. From these graphs, and from determinations of leaf-area, leaf-weight, and chlorophyll content per sample, the rates of

Fig. 18 Photosynthesis in Peperomia leaves.

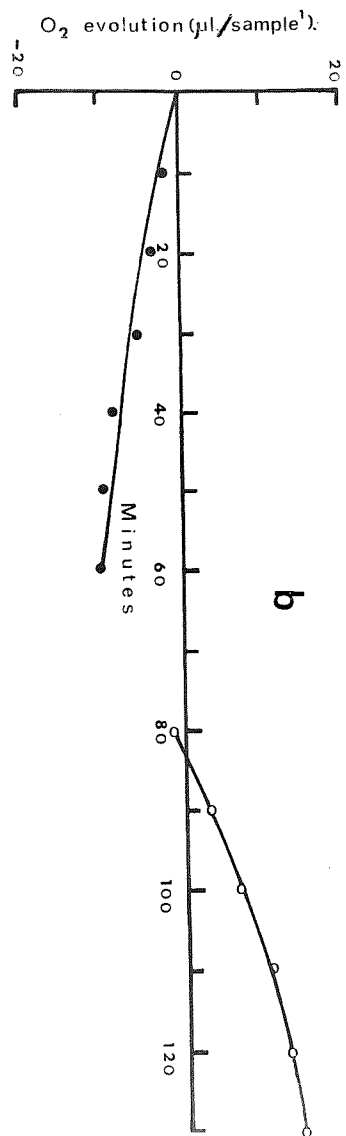
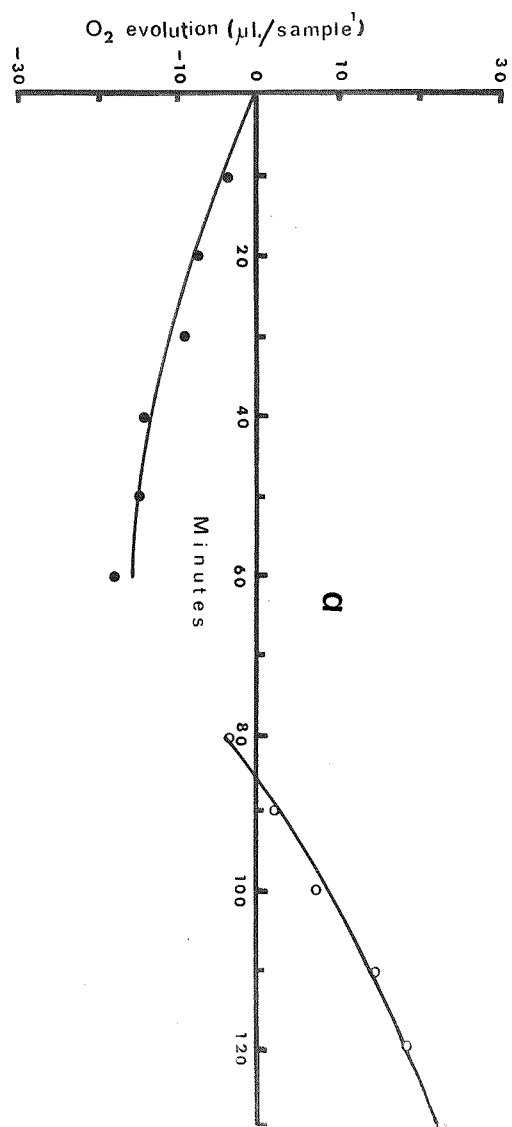
(a) - Normal Green leaves.

(b) - Greened-Yellow leaves.

● - Oxygen uptake in dark.
 (Respiration)

○ - Oxygen evolution in light.
 (App. photosynthesis)

¹ A sample is 20 leaf-discs
 (diameter 7 mm).



Leaf Discs	Photosynthetic Rates	
	$\mu\text{l. O}_2/\text{cm. /hr.}$	$\mu\text{l. O}_2/\text{mg. chl./hr.}$
Green	9	300
Greened-Yellow	6	1,700

Table 3

Relative Photosynthetic Rates
in Peperomia leaf discs.

Leaf Discs	(Photosynthetic Rates ($\mu\text{l. O}_2/20$ leaf discs/hr.)	
	0 Weeks	10 Weeks
Green	56	42
Yellow	-3	45

Table 4

Relative Photosynthetic Rates
in Peperomia leaves at low
light intensity.

photosynthesis in green and greened-yellow leaves shown in Table 3 were calculated.

Instead of evolving oxygen, yellow *Peperomia* leaves continue to take up oxygen in light. According to Curtis and Clark, (1950a) absorption of oxygen in light could be due to photo-oxidation. If it is due to photo-oxidation (bleaching has been found to occur in *Peperomia* leaves at about 800 ft-c.), then the rates of photosynthesis shown in Table 3, should be corrected for oxygen uptake in light due to photo-oxidation. But even without making this correction, it is evident that the chlorophyll formed in yellow *Peperomia* leaves during greening is functional (i.e. photosynthetic).

Table 4 shows the rates of photosynthesis in green and yellow leaves at low light intensity. In green leaves photosynthesis fell by about a quarter of the initial rate in about 10 weeks. On the other hand, photosynthetic rate in yellow leaf-tissue rose so markedly that by the end of 10 weeks the rate was about the same as in green leaves kept under the same conditions. From Fig. 19 the rise is most rapid from about the second to the fourth week; thereafter, though photosynthetic rate continues to increase, it does so even more slowly than within the first fortnight. In terms of photosynthesis rate per mg. chlorophyll, there is a fall in photosynthesis in yellow

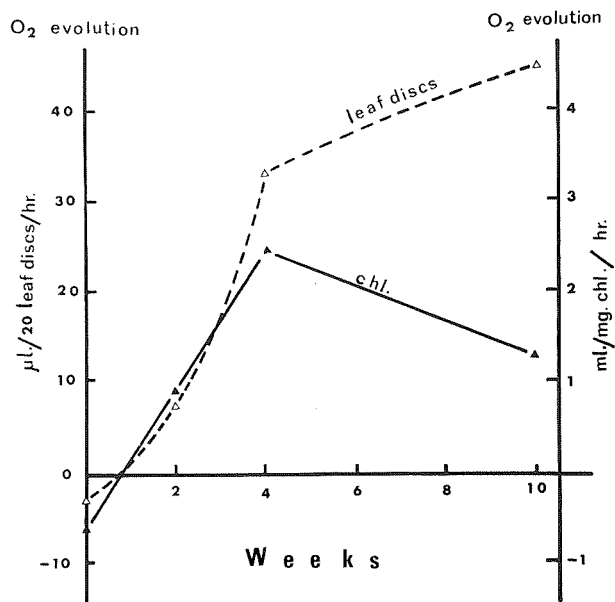


Fig. 19 Photosynthetic rates in Yellow Peperomia leaves
at low light intensity.

Days	Leaf Tissue	Rate of O ₂ uptake (μl./g./hr.)	Chl. cont. (mg/g./fr. wt.)	Chl. cont. (mg/g. fr. wt) / O ₂ uptake (ml. O ₂ /g/hr)
0	Green	175	0.146	0.83
	Yellow	92	0.013	0.14
20	Green	118	0.189	1.60
	Yellow	88	0.025	0.28
40	Green	112	0.242	2.16
	Yellow	58	0.033	0.57
60	Green	97	0.277	2.86
	Yellow	58	0.045	0.78

Table 5

Changes in rate of O₂ uptake (Respiration), and Chlorophyll content in Peperomia leaves at 25 ft-c.

leaves after about a month of greening.

Changes in respiration rates (rates of oxygen uptake) and chlorophyll content in green and yellow *Peperomia* leaves (Table 5) show that the ratio of chlorophyll content to oxygen uptake increases at 25 ft-c. in both green and yellow leaves. The increase is however faster in yellow leaves - the increase here is by a factor of over 5 in 60 days while in the same period, the ratio increases just over 3 times in green leaves.

DISCUSSION

The results from the determination of chloroplast pigments in *Peperomia* leaves show that both green and yellow leaves can synthesize chlorophyll a, chlorophyll b, carotenes and xanthophylls (the composition of the carotenoids formed in the green and yellow tissues has not been investigated). It is evident that it is the high proportion of carotenoids to chlorophylls in the latter tissue (i.e. compared with the ratio of these two pigments-types in green leaves) which gives them their yellow appearance.

As greening proceeds in these yellow leaves, the ratio of chlorophylls to carotenoids increases and after about 2 months of greening (when the leaves are distinctly green)

the total chlorophyll content is nearly twice as much as the carotenoids. These greened-yellow leaves are still very much paler green in colour than the normal green leaves which, in the same period, and under the same conditions, have about $2\frac{1}{2}$ times as much chlorophylls as carotenoids. (Table 2b).

The incompletely identified pigment Un, occurred both in yellow as well as in green leaves. By analogy to an observation reported by Shimizu and Tamaki (1963), it appears that the breakdown of chlorophyll a to produce Un could have occurred during the chromatographic separation of chloroplast pigments by removal of magnesium. It is however not easy to explain why during the chromatographic procedure, removal of magnesium was not found to occur in chlorophyll b as well.

Franz Schoetz (1956), found that young green leaves of Oenothera hookerilevata with mutated Lamarekiana plastids, could not photosynthesize, while green leaves from the same plant with normal biennis plastids, photosynthesized normally. The formation of non-functional chlorophyll was also found to occur in a species of Oenothera biennis with mutated plastids, as well as in certain chlorophyll-rich mutants of Chlorella pyrenoidosa (Davis, 1948). But as reported in the results, the chlorophyll found in the plastids of yellow Peperomia leaf-tissue during greening

is functional; i.e. it is not the non-photosynthetic type of chlorophyll reported in *Oenothera* leaves.

In terms of $\mu\text{l. O}_2/\text{mg. chlorophyll}$, the rate of photosynthesis is even higher in the greened-yellow *Peperomia* leaves than in the normal green ones, though the rate per unit leaf surface-area is higher in the green leaves (Table 3). Results from other plant species show that the rate of photosynthesis (carbon dioxide assimilation) per unit surface-area is less in yellow-green varieties than in dark-green ones, but the rate per unit chlorophyll (assimilation number) is higher in the yellow-green leaves - commonly by as much as ten times that in the normal green leaves (Curtis and Clark 1950b).

Determination of respiratory rates and chlorophyll content shows an interesting correlation between oxidative metabolism and chlorophyll accumulation. Porphyrin synthesis is known to be initiated by condensation between glycine and succinyl-CoA. The intracellular concentration of succinyl-CoA or succinate may be important in regulating tetrapyrrole biosynthesis, and the rate at which the tricarboxylic acid cycle operates could influence chlorophyll synthesis. Succinate/fumarate oxidation has been shown to be the limiting step in the TCA cycle under semi-anaerobic conditions (Krebs and Lowenstein, 1960).

Thus while highly aerobic conditions would favour metabolism

of succinate by the TCA cycle, with low oxygen concentration, more succinyl-CoA may become available for diversion towards tetrapyrrole formation.

Table 5 shows that oxygen uptake in *Peperomia* leaves decreases at low light intensity which has been shown to increase chlorophyll accumulation. It is thus probable that an oxidative mechanism such as succinate oxidation by the TCA cycle, may play an important role in controlling chlorophyll synthesis in *Peperomia*. Whatever the mechanism, it produces a more profound effect in yellow, than in green leaves, since the increase in the ratio of chlorophyll content to oxygen uptake is faster in the former. (The effect of oxygen uptake in *Peperomia* leaves is further discussed on page 88).

The principal changes that take place in a chloroplast during greening are increasing differentiation and chlorophyll accumulation. Anatomical studies have shown that in *Peperomia* leaves the increase in chlorophyll accumulation occurs mainly in the chlorenchyma tissue. No evident pattern of change in chloroplast number or increase in chloroplast size was found. The only definite anatomical change that was found to take place was increase in cell size in yellow leaves. Increase in cell size could lower the chlorophyll content of the yellow leaves, but from the determination of changes in chlorophyll

content at low light intensity (Figs. 11 & 12), chlorophyll content/unit leaf area increased both in green as well as in yellow leaves. Thus the 'dilution effect' due to increase in cell size in yellow leaves cannot be the explanation for the formation of yellow tissue in *Peperomia* leaves.

According to von Wettstein (1958, 1959), and Klein and Poljakoff-Mayber (1961), organization of lamellae can take place to a limited extent even without chlorophyll, though grana formation cannot. Yet even though the yellow leaves of *Peperomia* being studied in this work can accumulate fairly large quantities of chlorophyll at low light intensities, their chloroplasts never become as fully differentiated as those in the normal green leaves. (After 60 days at 25 ft-c., yellow leaves contain about 20% of the chlorophyll content of green leaves kept at the same intensity).

Studies of chloroplast structure in green and yellow *Peperomia* leaves have shown, that as is to be expected, chloroplasts from green tissue are completely differentiated into grana and stroma lamellae and they also contain some starch grains. Chloroplasts from yellow tissue on the other hand, show no differentiation at all.

When yellow tissues are induced to green at low light intensity, some differentiation is found to take place in

their chloroplasts. Lamellae are formed, but these mostly extend across the full length of the chloroplasts, showing no clear differentiation into grana and stroma regions. So though these chloroplasts contain large quantities of chlorophyll, they do not become as fully differentiated as chloroplasts from green tissue. This incomplete differentiation is either the result of a peculiar developmental pattern found in yellow *Peperomia* tissue chloroplasts, or else it could be due to the low light intensity requirement for chlorophyll formation and/or accumulation in these tissues.

Examination of chloroplasts from green and yellow leaves formed and developed at low light intensity showed that the peculiar structure of chloroplasts from greened-yellow tissue was not the result of low light intensity. Hence while yellow tissue chloroplasts could not show complete differentiation after 10 weeks at low light intensity, chloroplast differentiation was complete in green tissue after 4 weeks at low light intensity, even though the latter tissue was paler-green in colour than the former. Chloroplast differentiation, even in very young 'green' leaves (with smaller chloroplasts and distinctly yellowish tissue) was more advanced than was found in the yellow leaves which had been greening for 10 weeks.

All this clearly shows that the incomplete chloroplast differentiation found in greened-yellow leaves is not a low light intensity effect, and is therefore due to the physiological abnormality of the yellow *Peperomia* leaf-tissue.

The results discussed so far (i.e. in Sections II and III), show that there is no 'irreversible block' in the chlorophyll biosynthetic pathway in yellow *Peperomia* leaves. If they do not green at high light intensities, this could be due to one or both of the following reasons: High light intensity might result in (a) a faster rate of chlorophyll degradation than chlorophyll synthesis, and (b) a reduced rate or cessation of chlorophyll synthesis. These possibilities are examined next.

SECTION IV
CHLOROPHYLL DEGRADATION

	Page
1. INTRODUCTION	59
2. METHODS	
(a) Chlorophyllase activity	60
(i) Preparation of enzyme	60
(ii) Preparation of substrate	61
(iii) Determination of chlorophyllase activity	61
(b) Bleaching	
(i) Bleaching in chloroplast suspensions	62
(ii) Bleaching in whole leaves	63
3. RESULTS	65
4. DISCUSSION	67

1. INTRODUCTION

When green and greened-yellow *Peperomia* plants are exposed to high light intensities (about 800 ft.c.), their leaves become bleached. Experiments were therefore carried out to examine chlorophyll degradative processes in these leaves. The two processes examined were chlorophyllase activity and bleaching. The experiments were to find out if there was any correlation between the rate of chlorophyll breakdown and variegation. i.e. if variegation is the result of a faster rate of chlorophyll degradation in some of the leaf-tissues.

As has already been shown, low light intensity causes greening in yellow *Peperomia* leaves, and it has been reported that chlorophyllase activity is lower in leaves grown in the dark than in those grown in light (Holden, 1961). Determinations of the activity of the enzyme at high and low light intensities were therefore also made to see if greening at low light intensity can be explained in terms of the effect of light intensity on chlorophyllase activity.

Three groups of methods for determining chlorophyllase activity have been used by various workers. In one group, the pigments were extracted into ether and then into alkali. This was used by Willstatter and Stoll, (1910) and was also used by Peterson and McKinney (1938). Another method (a chromatographic method), was described by Sironval (1954),

and used by Gage and Aronoff (1956). A third method was based on the insolubility of chlorophyllides in light petroleum ether, (Weast and McKinney, 1940; Ardao and Vennesland, 1960; Holden 1961).

Though the chromatographic method is supposed to be the most accurate, it is time consuming, especially where large numbers of determinations of enzyme activity are envisaged. The third method, based on the insolubility of chlorophyllides in light petroleum ether is accurate enough to be used in experiments to compare enzyme activity in different tissues under similar conditions, and because it is quicker too, it was used in all chlorophyllase activity determinations reported in this work.

2. METHODS

(a) Chlorophyllase Activity

(1) Preparation of enzyme

The enzyme was prepared by quickly grinding fresh leaves in cold 85% acetone in a mortar and filtering the suspension in a Buchner funnel. The residue was washed with more acetone to remove as much of the chlorophyll as possible. The acetone-insoluble leaf residue was reground and washed with more acetone. The fine residue left was dried in a desiccator at 0°C and used as the enzyme extract.

(11) Preparation of substrate

A 'standard chlorophyll solution' was prepared as follows: Fresh *Rhynchosia* or *Eucynmus* leaves were quickly ground in a mortar with 85% acetone. The brei was filtered and washed with enough acetone to give the filtrate an optical density of around 2 at 660 m μ . The filtrate was kept for at least three days in a deep-freeze and carotenes which precipitated out, were filtered off.

(111) Determination of chlorophyllase activity

0.2 gm. of the fine, dry, powdered enzyme extract was incubated at room-temperature in the dark, with 6 ml. of the standard chlorophyll solution and 4 ml. 0.01 M sodium citrate. Optimum conditions for this enzyme action which were maintained in this experiment were, pH - 8; temperature 20° to 30°C; acetone concentration - 40 to 50%.

At the end of the incubation period, the 'reacting medium' was filtered and the residue washed with 30 ml. 80% acetone. 10 ml. of 2% sodium chloride were added to the filtrate and shaken up. 20 ml. of this solution was shaken up with 2 lots of 10 ml. light petroleum ether in a separating funnel, and the rest kept for determination of total chlorophyll. The petroleum ether layer was discarded and the acetone layer, containing the chlorophyllides, made up to 20 ml. with 80% acetone.

The optical densities of total chlorophyll and chlorophyllides at 660 m μ were determined on a Beckman spectrophotometer. From these readings and those from a Control with no enzyme, chlorophyllase activity was calculated. (Each result is the average of two determinations). Chlorophyllase activity was expressed in units/200 mg. enzyme powder, 1 unit being the amount which catalyses the breakdown of 10% of substrate in six hours. Enzyme activity determinations were confined to terminal leaves. (Activity of the enzyme was found to be higher in terminal, than in older leaves - 2.2 & 1.3 units respectively).

(b) Bleaching

Because the chlorophyll content of yellow *Peperomia* leaf-tissues is very low, greened-yellow tissues (from variegated leaves kept for about three months at 25 ft.c) were used for the following experiments:

(1) Bleaching in chloroplast suspensions

Peperomia leaf-tissues were ground in a sucrose-phosphate buffer solution (0.5 M sucrose, pH. 8). The suspension was filtered through muslin to remove coarse cellular debris and the filtrate centrifuged at 200 x g. for 1 minute. The sediment made up mainly of small cellular fragments, intact cells and starch grains was discarded and the supernatant centrifuged at 800 x g. for 5 minutes. The

sediment (mainly whole chloroplasts and a few smaller starch grains) was resuspended in sucrose-phosphate buffer solution and used for the bleaching experiments.

Each sample was made up of 100 leaf discs of green or greened-yellow tissues (i.e. equal leaf-area of green and greened-yellow material), or of equal weight of the two types of leaf tissues - 3 g. each. The whole-chloroplast sediment from each sample was resuspended in 15 ml. sucrose-phosphate solution, and this was divided into two small test tubes, one of which was kept in a dark cupboard and the other exposed to light of about 800 ft-c. The initial optical density of the chloroplast suspension from each sample was measured in a Gallenkamp photoelectric colorimeter with 608 filter, and the measurements repeated every 2 hours for a total of 8 hours.

For each experiment, chloroplast suspensions from 3 samples of green, and 3 samples of greened-yellow tissues were used, (either using equal leaf-weight or equal leaf-area).

(ii) Bleaching in whole leaves

Potted plants with normal green and 'greened variegated leaves' were maintained at 800 ft.c. for two months in the 'roof light cabinet'. At fixed intervals, three 0.5 g. leaf-samples were taken from the terminal and second

leaves of the green plants, and three 0.5 g. samples taken from the greened-yellow parts of the terminal and second leaves of the variegated plants. The chlorophyll content of these two sets of samples was determined by the method on page 22.

3. RESULTS

The first part of this work on chlorophyll degradation was to find out if yellow *Peperomia* leaves had higher chlorophyllase activity than green leaves. It was actually to ascertain whether it was because of higher chlorophyllase activity resulting in a higher rate of chlorophyll breakdown, which was responsible for some *Peperomia* leaf-tissues appearing yellow. From Fig. 20 b & c, chlorophyllase activity in two other variegated plants which were studied (*Pelargonium* & *Hedera*) is about the same in the green and yellow leaves. Results for *Peperomia* (Fig. 20a) also show that the activity of the enzyme is not higher in the yellow leaves - here chlorophyllase activity is lower in yellow than in green leaves.

Results of chlorophyllase activity in *Peperomia* and other variegated plants at different light intensities are shown in Table 6. These show that chlorophyllase activity is completely lost in *Peperomia* leaves after about a month in the dark. The initial activity of the enzyme in *Peperomia* during this study was so low (1.1 'units'), that no definite light intensity effect was evident. Various attempts failed to reproduce the high activities obtained during the study of the relative rates of chlorophyllase activity in green and yellow leaf-tissues. (Fig. 20a). Several determinations were made using different batches of *Peperomia* plants growing

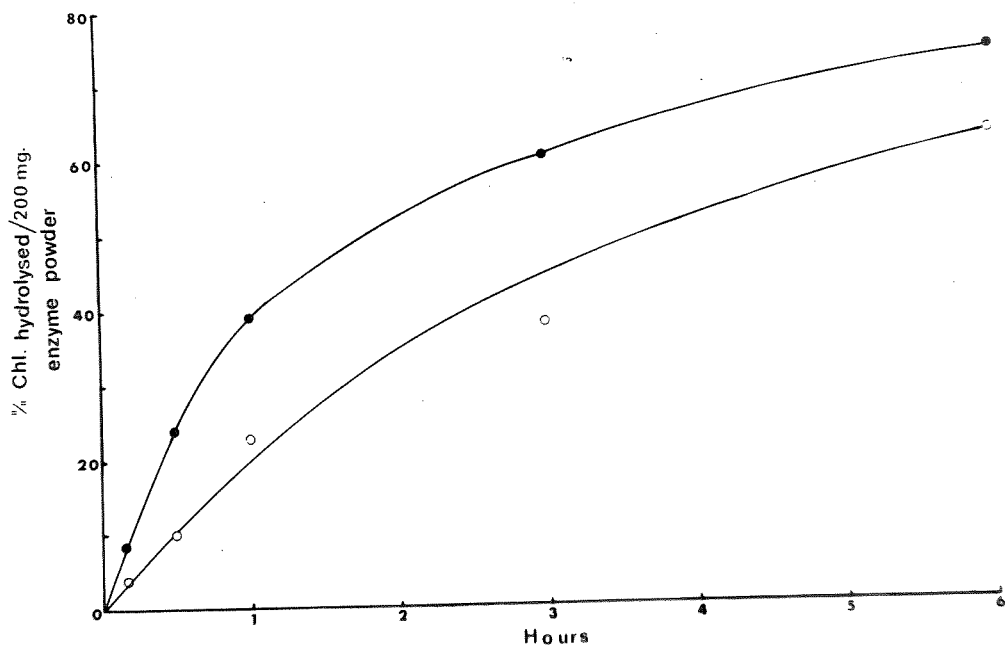


Fig. 20a Chlorophyllase activity in *Peperomia* leaves.

● - Green leaves; ○ - Yellow leaves.

(Each point is average from 2 determinations).

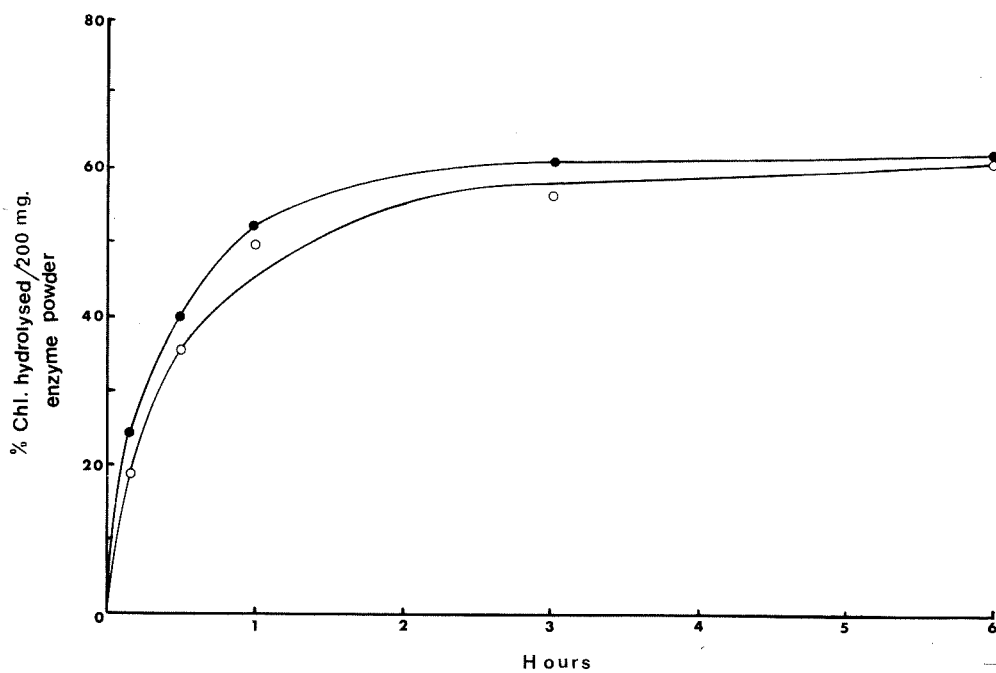


Fig. 20b Chlorophyllase activity in *Pelargonium* leaves.

● - Green leaves; ○ - Yellow leaves.
(Each point is average from 2 determinations).

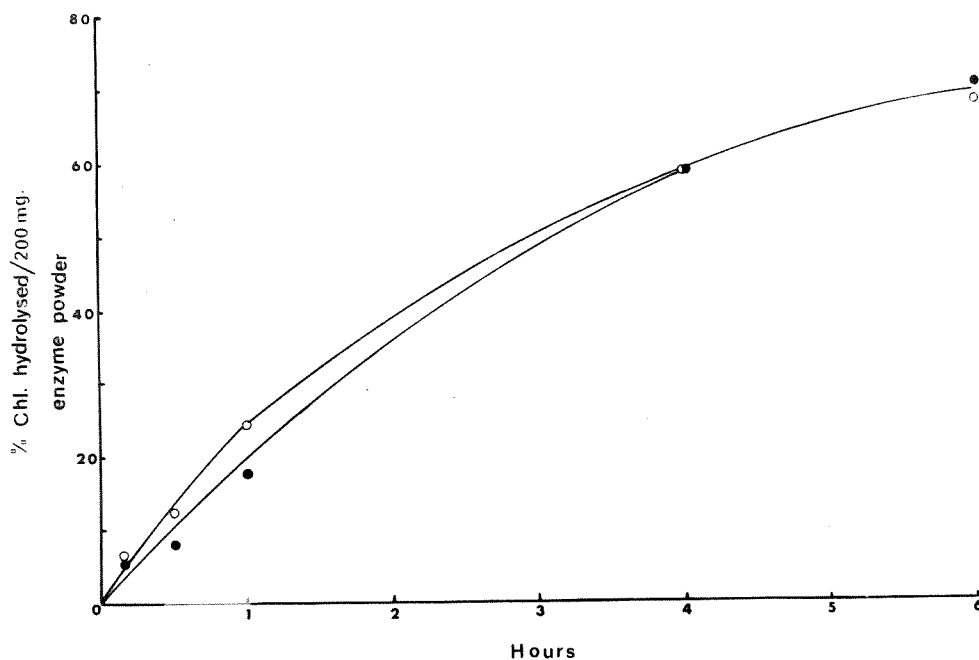


Fig. 20c Chlorophyllase activity in *Hedera* leaves.

● - Green leaves; ○ - Yellow leaves.
(Each point is average from 2 determinations).

PLANTS	HIGH LIGHT INTENSITY	LOW LIGHT INTENSITY	DARKNESS
PEPEROMIA	2 months at 200 ft-c	2 months at 25 ft-c	30 days (in the dark)
	1.2	1.1	0
CHLOROPHYTUM	2 months at 250 ft-c	6 months at 25 ft-c.	15 days (in the dark)
	6.5	6.5	6.1
PELARGONIUM	3 months at 250 ft-c	Greenhouse material in March	No results here (plants die in the dark)
	5.8	6.7	
HEDERA EUONYMUS VERONICA	Greenhouse material in August	-	28 days (in the dark)
	2.8		0.5
	4.5	-	2.4
	2.2		0.7

Table 6

CHLOROPHYLLASE ACTIVITY IN SOME
VARIEGATED PLANTS

(Activity expressed in units/200 mg. enzyme power, 1 unit being the amount which hydrolysis 10% of substrate in 6 hrs., under the conditions of the exp. - see text).

under controlled environmental conditions (in light cabinets). Determinations using different 'standard chlorophyll solutions' (from the Leguminous plants Rhynchosia phasecoloides and Lathyrus heterophyllus as well as from Euonymus japonicus) were also tried. All these determinations showed very low activity (below 1.5 'units').

Results in *Pelargonium* and *Chlorophytum* on the other hand (Table 6), clearly show very little light intensity effect on chlorophyllase activity. In *Hedera*, *Euonymus* and *Veronica*, there is a significant decrease in chlorophyllase activity in the dark, while in *Chlorophytum*, 15 days in darkness does not cause a significant change in activity.

Fig. 21 a & b and Fig. 22 show the results of experiments on bleaching in *Peperomia* leaves. As can be seen from Fig. 21a where chloroplast suspensions were prepared from equal fresh-weight of green and greened-yellow leaf-tissues, the chloroplast preparations which were kept in the dark show very little chlorophyll degradation. After 8 hours at room temperature, they still retain over 90% of their initial chlorophyll content. The suspensions exposed to light on the other hand, lose about 50% of their chlorophyll in 7 hours. Similar results were obtained when the chloroplast preparations were made from equal leaf-area of green and greened-yellow leaf-tissues (Fig. 21b).

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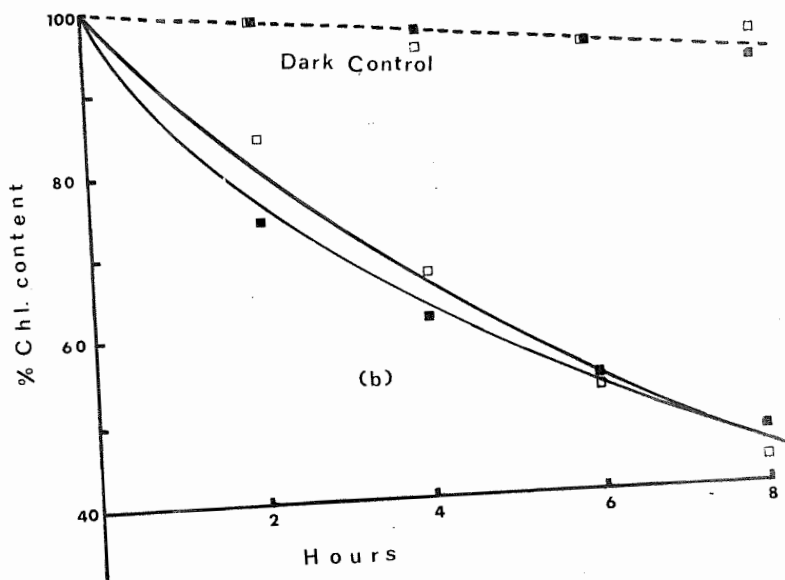
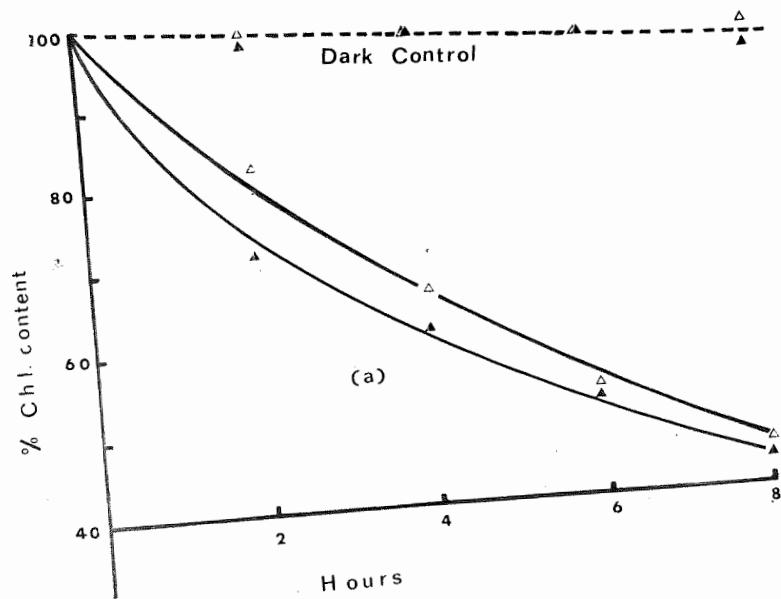
and the other two are the same as the first two.

**Fig. 21. Bleaching in chloroplast suspensions
from Peperomia leaves**

(a) - On leaf-weight basis }
(b) - On leaf-area basis } Explained in text.

▲ ■ - Green leaves

△ □ - Yellow leaves



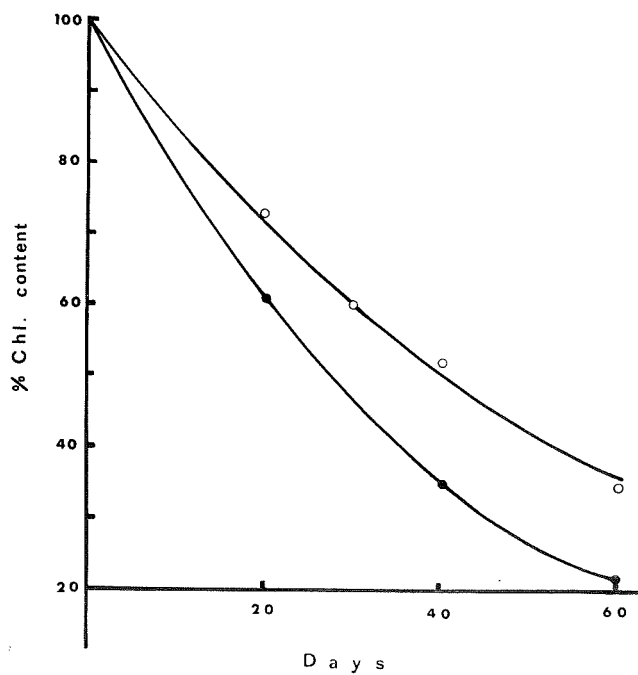


Fig. 22. Bleaching in Green & Yellow
Peperomia leaves (at 800 ft-c).

- - Green leaves
- - Yellow leaves,

It is quite clear from these results that chlorophyll degradation by light (bleaching), is not faster in yellow, than in green leaves. From experiments in which whole leaves, instead of chloroplast suspensions were used, the rate of bleaching was found to be higher in green than in greened-yellow leaves (Fig. 22). i.e. here too, bleaching is not faster in yellow, than in green leaves.

4. DISCUSSION

Results of chlorophyllase activity in *Peperomia* leaf-tissues have shown that the activity of the enzyme is not higher in yellow than in green leaves. Thus if the role of the enzyme in vivo is chlorophyll degradation, then it is not responsible for variegation in these plants.

However, though the rate of activity is less in yellow *Peperomia* leaves, it is high enough (compared with the rate in green leaves), for chlorophyllase activity to be an indirect factor preventing greening in yellow *Peperomia* leaves. As has been pointed out in the General Introduction, pigment accumulation is the net result of synthetic and degradation processes i.e. greening is only possible if chlorophyll is formed faster than it breaks down. Thus if the rate of chlorophyll synthesis is very much lower in

yellow than in green *Peperomia* leaves, then even the comparatively lower chlorophyllase activity in the former tissue can prevent greening in the yellow leaves, (i.e. if the role of the enzyme in plants is chlorophyll degradation).

Though the action of chlorophyllase has been demonstrated under various conditions in many plants, the role of the enzyme in chlorophyll synthesis in higher plants has been clearly demonstrated in only a few cases. Shimizu and Tamaki (1962) predicted increase in chlorophyllase activity to be the main cause of the yellowing of mature tobacco leaves, but the results of their study disproved that assumption. A similar result was reported by Gage and Aronoff (1956), who found that chlorophyllase activity was not related to chlorosis in a soybean mutant.

Sudyina, (1963) found that in various fruits (tomato, raspberry, garden strawberry, apricot, cherry) reduction in the amount of chlorophyll in the fruit was 'connected' with decreased chlorophyllase activity. This is supported by the report of Shimizu and Tamaki (1962), who also found that decrease in chlorophyll content of mature tomato leaves was caused, not by increasing enzyme activity in the degradative direction, but by decreased activity of the enzyme in the biosynthetic pathway.

In vitro, chlorophyllase has been shown to catalyse the removal of phytol from the chlorophylls and their pheophytins,

but Willstätter and Stoll (1910) were able in vitro, to carry out both the splitting of phytol from chlorophyll, and the back reaction of attaching phytol to chlorophyllide with the formation of chlorophyll. Phytylation of chlorophyllide and pheophorbide in vitro, has also been achieved by Shimizu and Tamaki (1963), using a purified preparation of tobacco leaf chlorophyllase and added phytol.

The above observations suggest that the normal function of chlorophyllase may be the attachment of the phytol side-chain to chlorophyllide (also see Bogorad, 1965b & c). As reported by Ardao and Vennesland (1960), it is only after chlorophyll has been removed from the lipoprotein (with which it is normally closely associated in the chloroplast), that it can be hydrolysed by chlorophyllase. So though it is possible to demonstrate the 'hydrolytic action' of chlorophyllase by separating the substrate from lipoprotein using organic solvents (as is usually done in in vitro experiments), the in vivo function of the enzyme might not necessarily be a degradative one.

It is nevertheless clear, that the enzyme can catalyse both hydrolysis and esterification, and by manipulating the reacting system, the net accumulation of either chlorophyll or chlorophyllide can be effected. (Bogorad, 1965c, 1966b). So that though indirect evidence favours assigning chlorophyllase a synthetic, and not a degradative role (Bogorad 1965c),

the normal role of the enzyme in plants has not been conclusively elucidated. It is not even certain that chlorophyllase is involved in the in vivo chlorophyll synthetic pathway at all, for according to Granick (1967), "the mechanism of phytylation is probably via phytol pyrophosphate rather than by the hydrophytic enzyme, chlorophyllase".

Thus the results of chlorophyllase activity in Peperomia and other plants reported in this study may not reflect chlorophyll breakdown rates in these plants. The results show no significant effect of light intensity on chlorophyllase activity in Peperomia and Chlorophytum (Table 6), nor do they show any definite correlation between changes in chlorophyllase activity in light, and greening. In Chlorophytum where light intensity does not seem to affect chlorophyllase activity, the outer portions of the yellow parts of the variegated leaves greened at low light intensity, while the yellow tissue along the midrib still remained yellow, (i.e. parts of the leaf greened at low light intensity).

In Peperomia, Hedera, Euonymus and Veronica, though light intensity affects chlorophyllase activity (as shown by the effect of darkness), no definite correlation between this effect of light on chlorophyllase activity and the ability (or lack of ability) of these plants to green at

low light intensity was found. Hedera and Veronica show slight greening, while no evidence of greening has yet been found in Euonymus, at low light intensities. On the other hand, Peperomia shows marked greening at low light intensities.

Since variegation in different plants might not be due to the same defect, even if chlorophyllase activity were responsible for producing yellow tissue in some of the above plants, it would not necessarily have been the cause in all of them. But from the above discussion, it can be seen that there is no definite correlation between the activity of the enzyme and variegation in any of the plants mentioned. The one positive conclusion which emerges is that chlorophyllase activity increases from dark to light conditions in most of the variegated leaves used here. Once the plants are in light, changes in the activity of the enzyme appear to be controlled by factors other than light intensity.

Microscopical examination of the chloroplast suspensions used for the bleaching experiments showed a few small starch grains in the preparations from green tissue. Apart from the starch grains, green tissue also contains more chloroplast material per unit leaf-area than greened-yellow leaves. Thus since fresh-weight/unit leaf-area is higher in green than in yellow leaves, the difference in solid material content in green leaf suspensions and suspensions from yellow leaves would be higher in preparations made on leaf-area basis than

in those prepared from equal leaf-weights. This means that light scattering effect due to suspended particles in the chloroplast preparations will be higher in the bleaching experiments carried out on leaf-area basis. Such an effect (by increasing optical density), will lower the rate of bleaching in green tissue, and should be more pronounced in the leaf-area experiments.

The results in Fig. 21 a and b however suggest that light scattering effect is not of sufficient magnitude to obscure the effect of bleaching. These results from the two types of chloroplast suspensions, as well as those from whole leaves (Fig. 22), all show that bleaching is not faster in yellow leaf-tissues. Bleaching is therefore not the factor directly responsible for preventing chlorophyll accumulation in the chloroplasts of yellow *Peperomia* leaves. But, as has been pointed out above for chlorophyllase activity, it can prevent greening in tissues with a lesion which markedly reduces the rate of chlorophyll formation.

It has been established (at least in terms of bleaching), that chlorophyll degradation cannot be the direct cause of variegation in *Peperomia* leaves. If some *Peperomia* leaf-tissues appear yellow above 200 ft-c, then unless no chlorophyll is formed in them at all at high light intensity (and this is not likely - see page 36), it must necessarily

mean that the rate of chlorophyll synthesis in these tissues is too low to cope with chlorophyll degradation at light intensities of over 200 ft-c. An attempt has been made to test the above hypothesis by trying to determine the rate of chlorophyll formation in green and yellow *Peperomia* leaf-tissues. This is reported in the next Section.

SECTION V

CHLOROPHYLL BIOSYNTHESIS IN GREENING TISSUES

	Page
1. INTRODUCTION	74
2. METHODS	
(a) Leaf disc incubation	76
(b) Extraction of labelled compounds from leaf discs	76
(c) Determination of ^{14}C in extracted leaf fractions	78
(d) Chromatography of petroleum ether fractions	79
(e) Chromatography of acetone fractions	79
(f) Chromatography of porphyrins	79
3. RESULTS	81
4. DISCUSSION	85

1. INTRODUCTION

It has been shown in Section II that if light intensity is not too high, yellow *Peperomia* leaves can accumulate chlorophylls. It has also been shown in Section IV that chlorophyll degradation is not faster in the yellow than in the green leaves. Thus, since greening can be induced in yellow *Peperomia* leaves at low light intensities, there must be a mechanism slowing down chlorophyll synthesis in yellow leaves at high light intensities. It is also likely however, that the rate of chlorophyll synthesis in yellow leaves is not markedly different at high and low light intensities, but that (as just pointed out on page 72), it is too low to make chlorophyll accumulation at high light intensities possible. It was in an attempt to elucidate this control mechanism that the work described in this Section, involving the use of radioactive glycine, was undertaken.

From known metabolic reactions, glycine metabolism relevant to the present studies can be summarized as shown in Fig. 23. It is clear that by using labelled glycine, it is not only possible to follow chlorophyll synthesis, but also to establish correlations between chlorophyll biosynthesis and other metabolic reactions, such as might help to speculate on the mechanism and localization of control of chlorophyll synthesis in *Peperomia* leaves. Thus glycine was used instead of substrates such as acetate, TCA cycle acids, and propionic acid which have been used by other workers in studies of porphyrin biosynthesis. Besides, several workers (see Bogorad, 1965d, and Lascelles, 1964b),

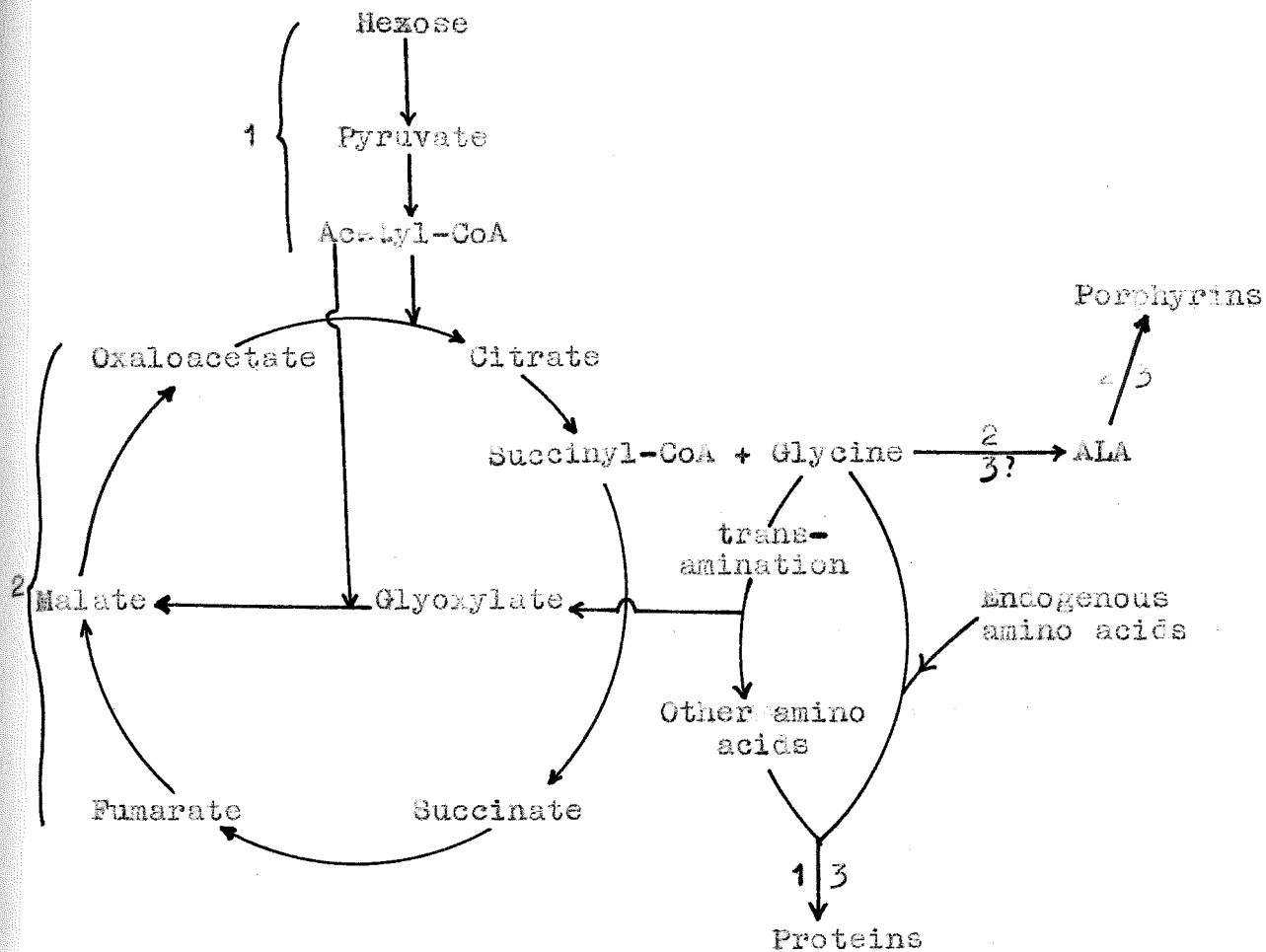


Fig. 23. Glycine Metabolism.

- 1 - Reactions in cytoplasm
- 2 - Reactions in mitochondria
- 3 - Reactions in chloroplasts

have shown that a very important step at which chlorophyll synthesis can be controlled is the succinyl-CoA $\rightarrow$ ALA reaction, catalysed by the enzyme ALA synthetase. This means that results obtained by using labelled ALA or some other later chlorophyll intermediates (and thereby eliminating the above control mechanism, if it operates in *Peperomia* leaves), will not give a true measure of the normal rate of chlorophyll synthesis in *Peperomia* leaves. Hence the choice of glycine as the labelled substrate for the radioactive work.

It has been established that while the carboxyl-carbon of glycine can be utilized in protoporphyrin synthesis in *Chlorella vulgaris* (Della Rosa, Altman & Salomon, 1953), it does not participate in the biosynthesis of protoporphyrin in mammalian tissues and avian red blood-cells. (Grinstein, Kamen & Moore, 1949). The α -carbon of glycine, on the other hand, has been shown to be utilized in both plant and animal tissues in the formation of protoporphyrin, the tetrapyrrole nucleus of chlorophyll and haem. Glycine-2-¹⁴C was therefore used in all the radioactive work reported in this thesis.

From previous experiments (page 33), cultured *Peperomia* leaf discs were found to root only if the discs were put in the semi-solid agar culture with the abaxial side up (i.e. lower side up). Preliminary experiments also showed that the incorporation of labelled glycine into these discs

(as well as radioactivity in the petroleum ether and acetone fractions), was higher if the discs were incubated with the lower side up. Because of these observations the leaf discs were incubated upside-down.

2. METHODS

(a) Leaf disc incubation

30 leaf discs (7 mm. diameter) were incubated on filter paper in a petri dish containing White's (1943) solution, 1% sucrose and glycine-2- ^{14}C in a total volume of 3 ml. The discs were cut from terminal leaves and were sorted out such that each of the petri dishes used in an experiment contained about equal numbers of discs from each of the leaves used, thus eliminating differences introduced by using different leaf-samples. Incubation was done for 24 hours at 25°C with 5 μc , or 2.5 μc . glycine-2- ^{14}C , and all light incubation was at 20 ft-c and in light regimes mentioned in the results.

(b) Extraction of labelled compounds from leaf discs

After the incubation period, the 30 discs from each petri dish were transferred into a small beaker and quickly washed about six times with water. They were then thoroughly ground in cold 85% acetone plus 0.2 ml. ammonium hydroxide (sp. gr. 0.880), and filtered in a Buchner funnel, a total

of 30 ml. of acetone being used for each extraction. The filtrate was transferred into a separating funnel containing 10 ml petroleum ether (60° - 80° C) and gently washed with water. The acetone layer was emptied out into a 50 ml. measuring cylinder and the petroleum ether fraction washed twice more, the water layer from each washing being added to the acetone layer.

The petroleum ether layer was then transferred from the separating funnel into a small specimen tube and evaporated to dryness under vacuum. It was then dissolved in 1 ml. petroleum ether (60° - 80° C) in the covered specimen tube and four 0.2 ml-samples measured out into separate specimen tubes.

The acetone fraction was made up to 50 ml. with acetone and four 10 ml-samples were measured out into separate specimen tubes and evaporated to about 2 ml. over a water bath. The four samples were then evaporated to dryness under vacuum. (This procedure was necessary because of spilling of the acetone fraction if this water-acetone solution was directly evaporated under vacuum.)

The acetone-insoluble leaf residue left after filtration was transferred into a 50 ml. quickfit round-bottomed flask and hydrolysed under reflux, in 10 ml 6 N hydrochloric acid over a sand-bath for about 24 hours. The resulting protein hydrolysates were filtered (using a Whatman No. 50 filter

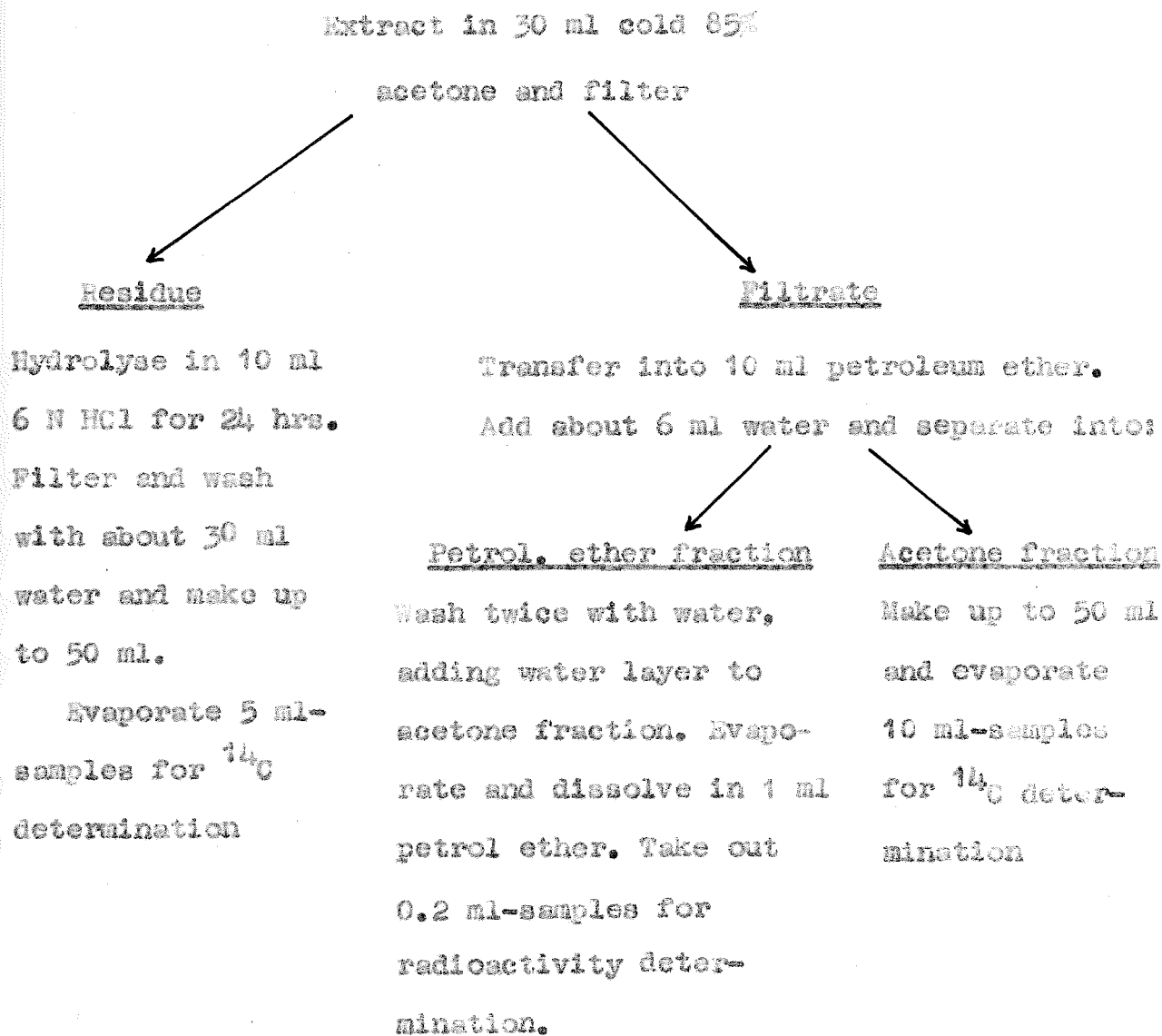


Fig. 24

Scheme for the extraction of leaf fractions.

paper), washed with about 30 ml. water and made up to a final volume of 50 ml. Three 5 ml-samples were measured out into specimen tubes and evaporated to dryness under vacuum. Each sample was dissolved in 1 ml. 90% alcohol and 0.5 ml. of it measured out into another specimen tube.

A summary of the above extraction procedure is shown in Fig. 24.

(c) Determination of ^{14}C in extracted leaf-fractions

Two protein hydrolysate samples, and two samples each from the acetone and petroleum ether fractions were spotted on 4 cm. strips of Whatman No. 1 filter paper and the total radioactivity in each spot determined as counts/100 secs. using an I.D.L. Chromatogram Scanner in conjunction with an I.D.L. Programme and Read-out unit, and I.D.L. Scaler 1700 and an Addo-X print-out unit, so that the counts were recorded directly. The efficiency of the scanner was about 5%. From the average counts/100 secs. of the 2 determinations, obtained by adding up the counts from each spot (less the background counts), the total activity/30 leaf-discs, in each of the 3 fractions was calculated.

The activity in the various compounds in the petroleum ether and acetone fractions was similarly determined after paper chromatographic separation of the compounds in each of the two remaining samples from each fraction. The chromatograms

were cut into strips 4 cm. wide, and the counts/100 secs for every $\frac{1}{4}$ inch of each strip determined as already described. A plot of counts against distance revealed peaks of radioactivity along the length of the chromatogram.

(d) Chromatography of petroleum ether fractions was done as already described for chloroplast pigments in General Methods (page 25).

(e) Chromatography of acetone fractions

This was carried out according to the method described by Gordon and Eastoe (1964). Water, n-butanol and glacial acetic acid (1:1:0.11) were shaken together and allowed to separate into two layers. The lower layer was used for saturating the chromatography chamber and the upper layer for development. The samples were spotted on Whatman No. 1 paper, 23 x 28 cm; the paper was stapled together at the overlap, and after saturation in the chamber, it was developed (ascending) at $22^{\circ}\text{C} \pm 2^{\circ}\text{C}$ for about 7 hours.

(f) Chromatography of porphyrins

Using their red fluorescence in ultra-violet light, the porphyrins from the 'acetone-fraction chromatograms' were cut off and eluted with 80% acetone. The method used here was that of Chu et al. (1951). The acetone solution

was concentrated by evaporation and spotted on Whatman No. 1 paper 12.5 x 14.5 cm, the paper stapled into a cylinder and equilibrated in a chromatography chamber saturated with chloroform. Development at $24^{\circ}\text{C} \pm 2^{\circ}\text{C}$, was with 4 ml. kerosene and 2.6 ml. chloroform, and it continued until the solvent-front was about 12 cm. from the basal line (about 30 mins.). The paper was taken out, and after allowing to drain for a few seconds and marking the solvent-front by pencil, it was dried between 105° - 110°C for about 5 mins. and then cooled to room temperature. It was then equilibrated in a second chamber saturated with kerosene, and developed (in the same direction as before), with a mixture of 5 ml. kerosene and 1 ml. iso-propyl alcohol. The paper was withdrawn from this chamber when the developing medium ascended to the same front as in the first chamber, and was again dried.

The chloroform used here was washed 3 times over water and dried over calcium chloride, and the kerosene redistilled from commercial paraffin between 190° and 250°C .

3. RESULTS

The results of incubating *Peperomia* leaf discs in 3 ml. media containing 2.5 or 5 μ c glycine-2- ^{14}C at 20 ft-c are shown in Table 7 and Fig. 25. Each medium in a petri dish contained 30 leaf discs. The aim of this 'trial experiment' and some preliminary experiments done earlier, was to find out what compounds would become labelled. It was also to determine what activity of glycine-2- ^{14}C , incubated for what period, would provide high enough labelling in the various leaf fractions, to make a comparison of the rate of chlorophyll synthesis in green and yellow leaf discs feasible.

Though the incorporation of glycine-2- ^{14}C into chloroplast pigments (petroleum ether fraction) reached a maximum in about 6 hours, an incubation period of 24 hours was used in the radioactive experiments. This was because while activity in these compounds was about the same after 6 hours as after 24 hours incubation, the latter period was found to be more convenient - since changes in the activity of other labelled metabolites like amino acids and proteins were not being investigated, results from 24 hours incubation should provide as reliable data for comparing the rate of labelling of chloroplast pigments in green and yellow leaves, as results from 6 hours incubation.

An incubation medium containing 2.5 μ c labelled glycine in

	GREEN		YELLOW
Total activity in inoculating medium	5 μ c. (1)	2.5 μ c. (2)	2.5 μ c. (2)
Activity in Pet. ether fraction	30 $\pm$ 5	11 $\pm$ 1	18 $\pm$ 1
Activity in Protein fraction	5,059 $\pm$ 180	1,073 $\pm$ 74	1,293 $\pm$ 55

Table 7

Total radioactivity (counts/sec./30 discs) after 24 hrs. incubation of Green & Yellow leaf discs in glycine-2-¹⁴C. Incubation in long-day cabinet (16 hrs. light).

Each result is the average from one exp.(1), or from two exps.(2).

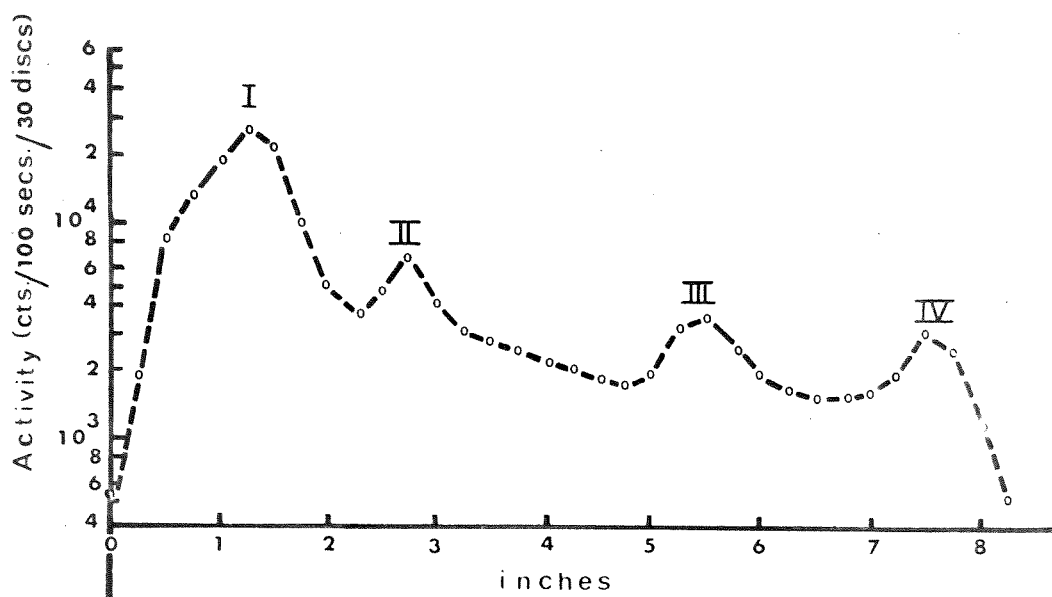


Fig. 25 Scan of radioactivity in chromatogram of acetone fraction from Green Peperomia leaf discs incubated in 5 μ c glycine-2- 14 C in roof light cabinet.

Distance (inches) shows position of peaks on chromatogram.

I - Amino acids; II - ALA; III - 'PBG';
IV - Porphyrins (see Figs 26 & 27).

3 ml. of solution was found to provide high enough activity in the various leaf fractions. Incubation in media with twice the above specific activity ($5 \mu\text{c}/3 \text{ ml}$) increased the activity in the petroleum ether fractions, but did not produce a significant increase in labelled chlorophylls. The incubation medium was prepared from $100 \mu\text{c}$ glycine-2- ^{14}C with specific activity of $287 \mu\text{c}/\text{mg}$.

Table 7 shows very high labelling of the protein fractions. The petroleum ether fractions (which contain the chloroplast pigments) show comparatively little activity, and the greater part of this (over 80%), was in a yellow spot. From its position at the solvent-front on a chromatogram of the petroleum ether fraction (c in Fig. 7) and from its absorption spectrum (Fig. 8), the yellow spot must be carotene. The labelling of the chlorophylls present in this fraction was barely above background level. Chromatography of the acetone fractions from the leaf-disc extracts in the trial experiment (Fig. 25) shows peaks of activity which have been identified as follows.

The acetone fraction should contain amino acids, and the early precursors in chlorophyll biosynthesis. From Fig. 29 (discussion on page 87) aspartate and glutamate are two amino acids which will be readily labelled in the discs incubated in glycine-2- ^{14}C . ALA, the first identifiable porphyrin precursor might also be labelled; and there should be some

Fig. 26.

Chromatography of amino acid and ALA markers.

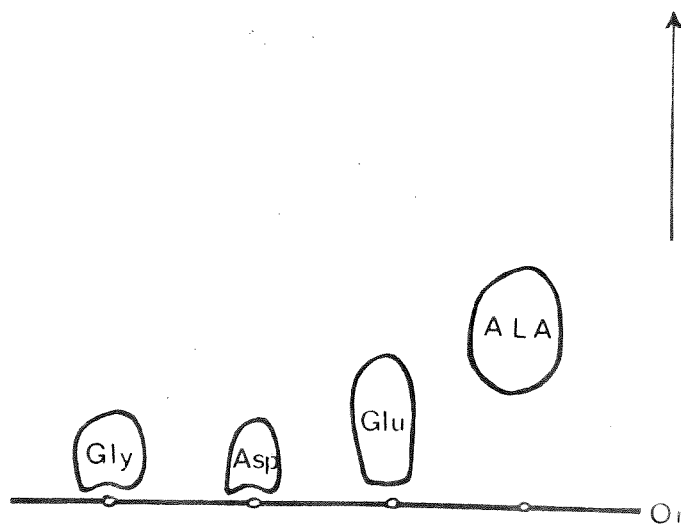
(or - origin; Sf - Solvent front; Gly - L-glycine
Asp - L-aspartic acid; Glu - L-glutamic acid;
ALA - ALA-hydrochloride).

Berlin et al (1956), using butanol-water-acetic acid as developing medium, had the following Rf values: Glycine - 0.13, ALA - 0.23, PBG - 0.48.

From their Rf values (shown below), relative to those of the markers (Fig. 26), the peaks in the chromatograms of acetone fractions from incubated *Peperomia* leaf discs (see Fig. 25), were tentatively identified as follows:

I -	Amino Acids	{	Gly - 0.06 - 0.08
			Asp - 0.04 - 0.05
			Glu - 0.08 - 0.1
II	_____		ALA - 0.2 - 0.3
III	_____		PBG - 0.5 - 0.6

----- Sf



free (not metabolized) labelled glycine too. A chromatogram of the above amino acids and ALA, using the same solvent system and development conditions as were used above (in the chromatography of the acetone leaf-extracts), is shown in Fig. 26. From the Rf values of these markers, the first peak (I) in Fig. 25 most probably represents the activity in the amino acids (mainly glycine, aspartate and glutamate), the second peak (II) corresponding to the activity in ALA.

From their positions of the chromatogram (i.e. from their Rf values) and from their red fluorescence in ultra-violet light, the other peaks were strongly suspected to be PBG and porphyrins. Chromatography of the eluate from the spots designated porphyrins (IV in Fig. 25, cut out in ultra-violet light), show peaks of activity with Rf values very close to those of uroporphyrin, coproporphyrin and protoporphyrin IX. (see Fig. 27). Thus the peak labelled IV in Fig. 25, is most probably made up of porphyrins involved in chlorophyll formation (uroporphyrinogen and coproporphyrinogen, the reduced products of uroporphyrin and coproporphyrin respectively, as well as protoporphyrin IX, are all involved in chlorophyll synthesis).

To sum up the above identification of labelled compounds, chromatography of the acetone leaf-extracts (Fig. 25), cross-checked by the use of markers showed that glycine, aspartate,

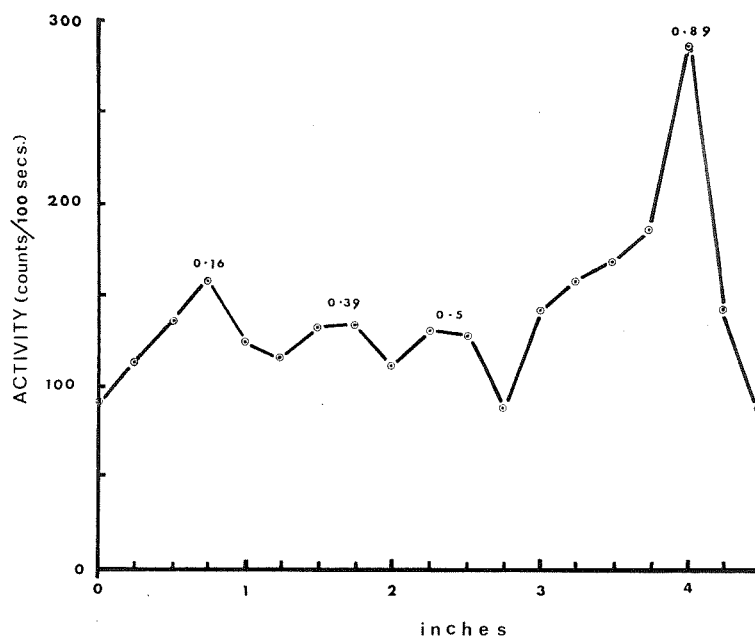


Fig. 27 Scan of radioactivity in chromatogram of porphyrins eluted from acetone fraction chromatograms (see page 83).

Values shown on peaks are Rf values.

Chromatography was done by the method of Chu et al. (1951), who gave the following values:

Uroporphyrin I - 0.17

Coproporphyrin I - 0.47

Coproporphyrin III - 0.67

Protoporphyrin IX - 0.84

Mesoporphyrin IX - 0.89

glutamate and ALA were all labelled. The identity of other labelled amino acids was not investigated, nor was the identity of the spot in the above chromatogram designated PBG confirmed by a marker (no PBG was easily available). Chromatography of the eluate of the porphyrin spot showed that glycine-2-¹⁴C was probably incorporated into some porphyrins in the chlorophyll synthesis pathway (Fig. 27). Thus, though the identity of the labelled compounds mentioned above (especially PBG) is not beyond question, there is some evidence to suggest the incorporation of labelled glycine into some chlorophyll precursors.

Activity in the acetone fraction after 24 hours light incubation of green and yellow leaf discs in labelled glycine is shown in Table 8. This shows that the total incorporation of glycine-2-¹⁴C into ALA, 'PBG' and porphyrins (identified as above) is higher in green than in yellow leaves (by about 60%). Incubation in 24 hours continuous darkness shows an even higher incorporation of labelled glycine into the above compounds in green than in yellow leaves (by over 100%).

LEAF DISCS	L i g h t		D a r k	
	GREEN	YELLOW	GREEN	YELLOW
Activity in AIA, 'PBG' & Porphyrins ('Chl. precursors')	354 $\pm$ 32	217 $\pm$ 15	300 $\pm$ 17	137 $\pm$ 10
Activity in acetone fraction (mainly amino acids)	584 $\pm$ 42	278 $\pm$ 11	417 $\pm$ 29	238 $\pm$ 18

Table 8

Total radioactivity (counts/sec./30 discs)
after 24 hrs. light & 24 hrs. dark incubation
of Green & Yellow Peperomia leaf discs in
2.5 μ c. glycine-2-¹⁴C.

Each result is the average from two
experiments.

4. DISCUSSION

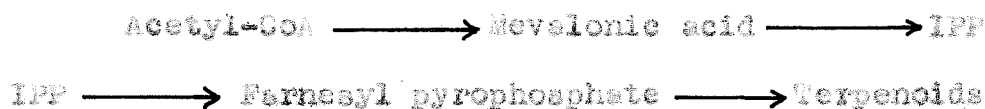
The radioactive experiments show that while there is very high incorporation of glycine-2-¹⁴C into proteins, there is comparatively little labelling of chloroplast pigments formed in *Peperomia* leaves. The greater porportion of the labelling in the petroleum ether leaf extracts which contain these pigments, is confined to a yellow terpenoid spot (carotene). Because hardly any labelling was found in the chlorophylls, the total activity in the 'chlorophyll intermediates' (ALA, 'PBG' and porphyrins, identified by methods already described) has been used as a probable measure of the amount of chlorophyll synthesized in *Peperomia* leaves. In terms of these 'chlorophyll precursors' chlorophyll synthesis is faster in green than in yellow leaves.

The reasons for using labelled glycine instead of other substrates used by other workers in studies of tetraphyrrole biosynthesis have been given on page 74. But since the labelling of the chlorophyll molecule could not be achieved by using labelled glycine as the source of labelled carbon, it might be worth while trying other labelled substrates (such as those mentioned on page 74). If such attempts are able to produce high labelling of chlorophylls in *Peperomia* leaf tissues, then the rate of chlorophyll synthesis can be more directly measured.

The incubation of *Peperomia* leaf discs in glycine-2-¹⁴C

has been shown to result in the labelling of a yellow terpenoid compound(s). Elucidation of the pathway by which the incorporation of glycine into terpenoids proceeds, seems to suggest that *Peperomia* has certain enzymes systems which will enable it to carry out dark CO_2 fixation. This indication, discussed with respect to the leaf material used for the radioactive work, appears to provide a plausible explanation for the almost complete lack of labelling of chlorophylls reported above.

The "biological isoprene unit" (from which terpenoids are formed) is isopentenyl pyrophosphate (IPP), and the main building unit from which IPP is synthesized is acetyl-CoA (Goodwin, 1965). The first step which is specific for terpenoid synthesis is the formation of mevalonic acid (MVA) which represents the "point of no return" because it has no metabolic future other than being converted into terpenoids (Popjak and Cornforth, 1960). i.e.



It is clear from the above pathway that the incorporation of ^{14}C from glycine-2- ^{14}C into terpenoids can be explained by a mechanism by which the carbon isotope can enter the acetyl-CoA molecule. A possible pathway is shown in Fig. 26. According to this scheme, labelled glycine can change into labelled glyoxylic acid by transamination or oxidative

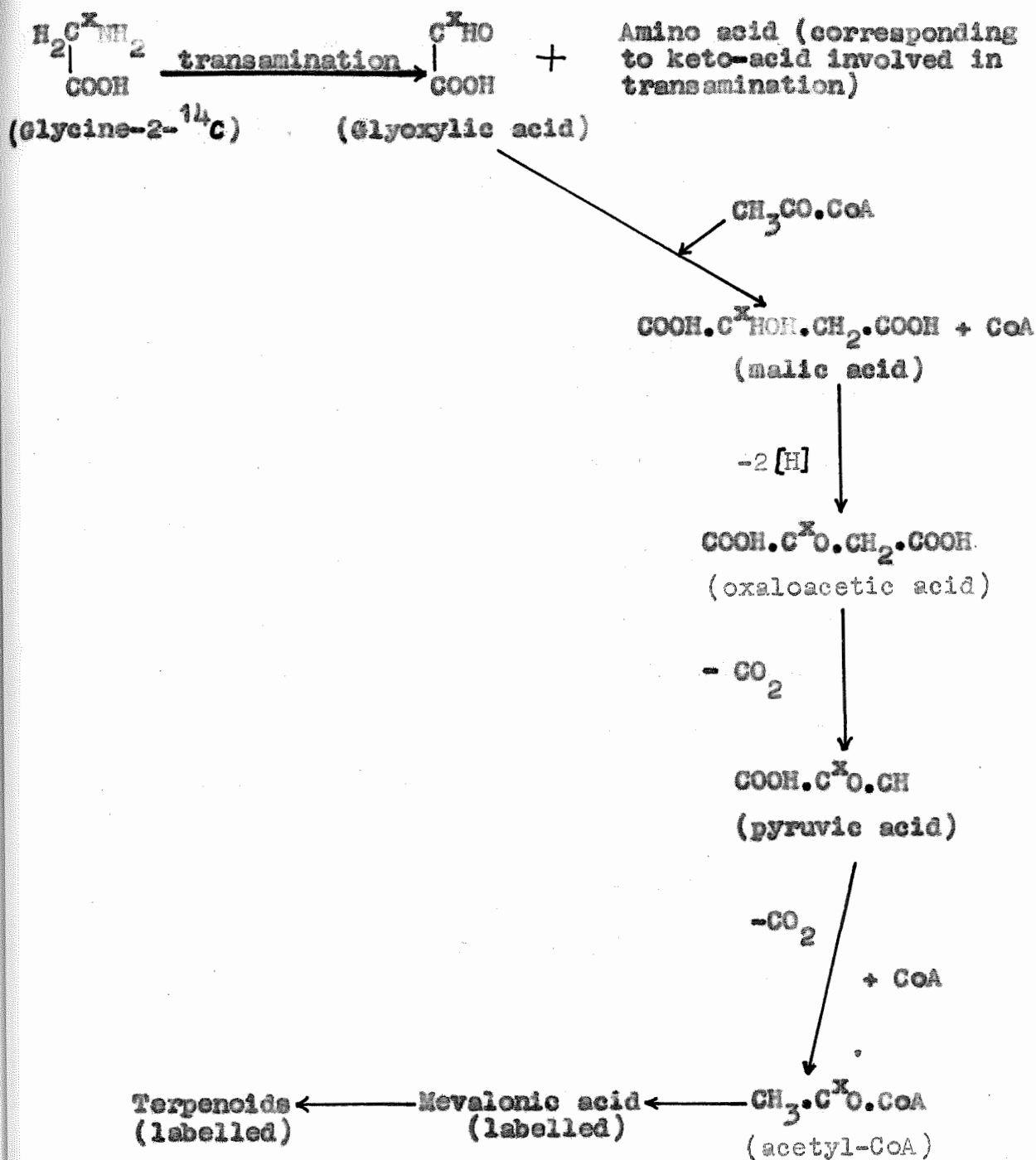


Fig. 28. Incorporation of ^{14}C from glycine-2- ^{14}C into terpenoids ($\text{C}^{\text{X}} = ^{14}\text{C}$)

deamination (Karlson, 1963). The glyoxylic acid can react with acetyl-CoA to give rise to labelled malic acid (Krebs and Kornberg, 1957), which can be oxidized into oxaloacetic acid (OAA). Some of this labelled OAA can be decarboxylated in the presence of ATP to give PEP (Whittingham, 1964), while part of it continues the TCA cycle. The PEP can then be converted by the EMP pathway, through pyruvate into acetyl-CoA. From this labelled acetyl-CoA, labelled terpenoids can be formed.

If the labelling of terpenoids proceeds by the pathway outlined above, then it means that *Peperomia* leaves show malate synthetase activity, in addition to possessing an enzyme system for converting malate or OAA into pyruvate. It also means that pyruvic acid, as well as all the acids in the TCA become labelled when *Peperomia* leaf discs are incubated in glycine-2-¹⁴C -see Fig. 29. The possession of the above enzyme system may enable *Peperomia* leaves to carry out the dark fixation of CO₂ found in succulent plants ("crassulacean or succulent metabolism" - Bennet-Clark, 1933).

Peperomia shows features of succulent plants (page 96); thus under normal growth conditions, there must be rigid control of gaseous exchange. In the radioactive investigations reported in this thesis, discs cut from *Peperomia* leaves were used. The cutting of leaf discs, by removing the 'impermeable cover' from part of the leaf can increase the oxygen content in the discs. Such increase in oxygen tension probably explains why very little labelling was found in the chlorophylls.

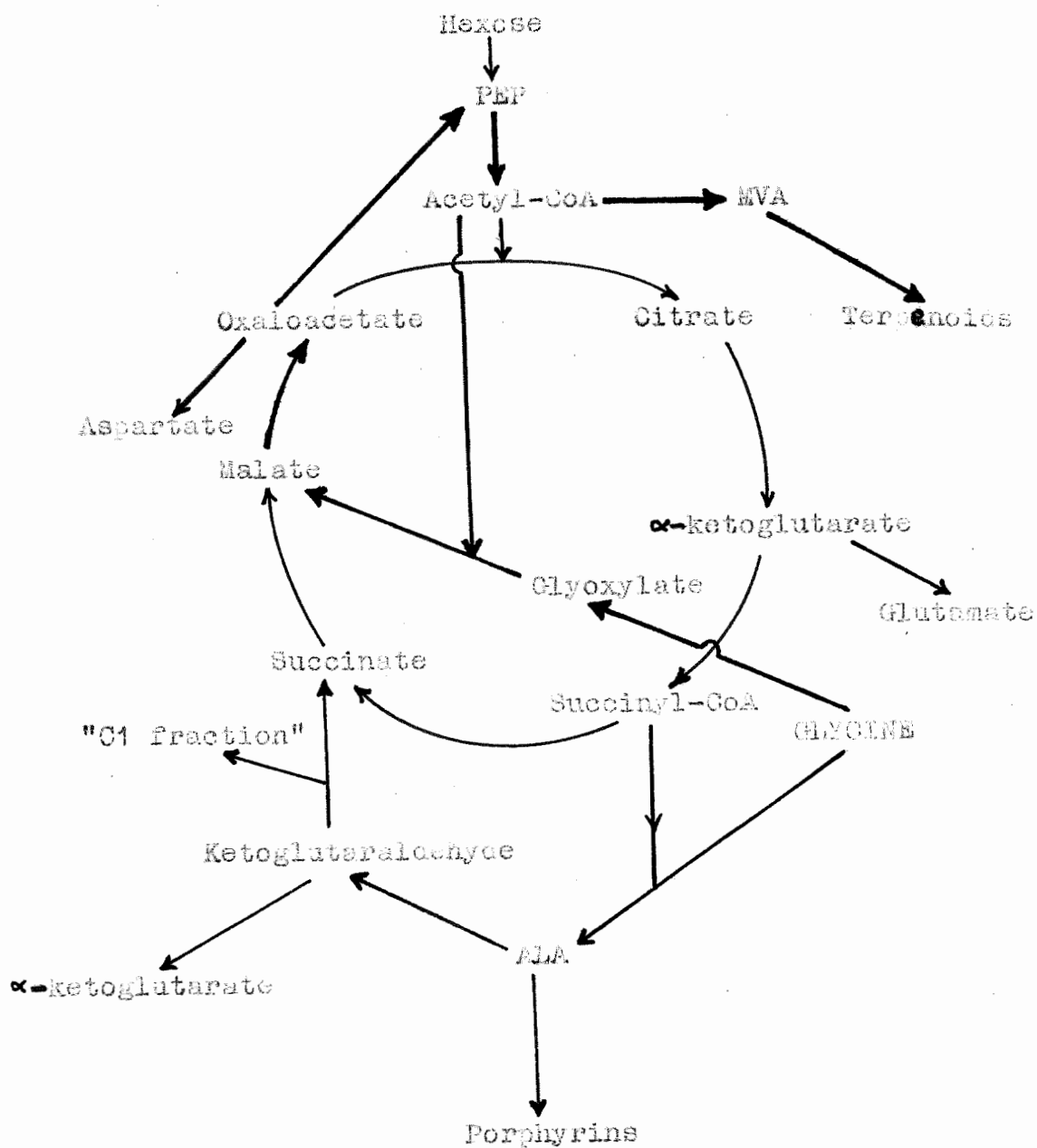


Fig. 29. Spread of ^{14}C from Glycine-2- ^{14}C .
shown
(all compounds here are labelled)

The operation of such a control mechanism in *Peperomia* leaves is highly plausible. Cohen-Bazire et al. (1957) have reported that in the non-sulphur purple bacteria (*Athiorhodaceae*), syntheses of chlorophyll and carotenoids appear to be linked and are affected by both light and oxygen tension. Under anaerobic conditions, increase of light intensity causes an immediate suppression of pigment synthesis while decrease of light intensity immediately accelerates pigment synthesis. Under aerobic conditions, on the other hand, pigment synthesis is permanently and almost completely suppressed at atmospheric oxygen tension, only carotenoid synthesis continuing, at an extremely low rate.

It has been reported in Section II that greening in intact yellow *Peperomia* leaves takes place at low light intensities. (Under such conditions, though not completely anaerobic, if *Peperomia* in fact carries out succulent metabolism, oxygen tension will be very low in the leaves). Decrease in chlorophyll content with increase in oxygen uptake in *Peperomia* leaves has also been reported in Section III (see Table 5). In the radioactive experiments too, where cut-discs of *Peperomia* leaves were incubated in glycine-2-¹⁴C, higher levels of activity were found in terpenoids relative to chlorophylls. Since all the above results from the present investigations are in very close agreement with the foregoing report of Cohen-Bazire et al., it appears reasonable to

suggest that it could be the increase in oxygen tension, the result of cutting out leaf discs, which suppressed the incorporation of labelled glycine into chlorophylls.

It is clear from the above discussion that the radioactive work reported in this Section could not provide a direct measure of the rate of chlorophyll synthesis in green and yellow *Peperomia* leaves. One molecule of chlorophyll contains 8 α -carbon atoms from glycine. It was therefore expected that incubation of *Peperomia* leaf discs in glycine-2-¹⁴C would result in high labelling of chlorophylls. But from the result of the experiments, very little labelling of chlorophylls occurred.

The above discussion of the work of Cohen-Bazire et al in relation to some of the results from the work reported in this thesis suggests (as already mentioned), that a possible explanation for this unexpected result could be high oxygen tension in the leaf discs. Further work is necessary to assess the validity of this hypothesis. A possible approach will be to repeat the radioactive experiments using intact leaves instead of leaf discs, or to incubate leaf discs in varying concentrations of oxygen. If high labelling could be achieved then rates of the synthesis and degradation in *Peperomia* leaves might be directly measurable by short term experiments.

SECTION VI

	Page
FINAL DISCUSSION	90
ADDENDUM	95
BIBLIOGRAPHY	98

FINAL DISCUSSION

It has been shown in this thesis that both green and yellow *Peperomia* leaf tissues can synthesize photosynthetic pigments and that their different appearance is the result of different relative ratios of the pigments they form. Both yellow and normal green leaves start off yellow, and they both show some greening in two to three weeks, whether they are kept at high or low light intensities. But while chlorophyll accumulation continues in normal green leaves at high or low light intensities, chlorophyll accumulation continues in yellow tissues only at low light intensities.

It thus appears that yellow tissue is capable of accumulating chlorophyll, but that after about three weeks, this capacity is affected by light intensity (i.e., high light intensity decreases the rate, and vice versa). Accumulation of chlorophyll in any tissue is a balance between synthesis and degradation, and it has been shown in both green and yellow tissue at high light intensity, that degradation proceeds at about the same high rate. In terms of total 'chlorophyll precursors' formed (at low light intensity where yellow tissue can accumulate chlorophyll), the rate of chlorophyll synthesis is higher in green than in yellow tissue. Thus if the above provides a reliable measure of chlorophyll formation in *Peperomia* leaves, then at high light intensity, while the rate of chlorophyll synthesis is high enough to make pigment accumulation

possible in green tissue, the rate of chlorophyll synthesis is too low in the yellow tissue compared with the high breakdown rate, to permit chlorophyll accumulation.

An attempt to find out if chlorophyllase activity was responsible for variegation in *Peperomia* leaves showed that the activity of the enzyme in vivo was probably concerned, not with chlorophyll degradation, but with chlorophyll synthesis. Young *Peperomia* leaves which have been found to be the first to green on a shoot were found to show higher chlorophyllase activity than older leaves. Thus since the activity of the enzyme was found to be higher in green than in yellow leaves, it is possible that chlorophyllase activity might be a factor controlling chlorophyll content in *Peperomia* leaves, increase in activity resulting in high rate of chlorophyll formation, rather than an increase in the rate of chlorophyll degradation.

An attempt to determine the relative rates of chlorophyll synthesis in green and yellow *Peperomia* leaves was not successful i.e. it was not possible to get a direct measure of the synthetic rate in terms of the incorporation of ^{14}C into chlorophylls. But using total activity in compounds separated by one-directional chromatography, chlorophyll formation is at least $1\frac{1}{2}$ times faster in green leaves.

But in view of the very low chlorophyll content of yellow leaves and the rather high rate of bleaching (compared with bleaching in green leaves), the normal rate of

chlorophyll formation might be lower in the yellow, or higher in the green leaves, than was suggested by the indirect method used to determine it. But whether it is lower in yellow or higher in green leaves, it appears that the chlorophylls formed in the yellow leaves break down too fast at high light intensities to make their accumulation possible. Greening of yellow *Peperomia* tissue at low light intensity might therefore be the result of decreased bleaching.

The chloroplasts of greened-yellow *Peperomia* leaves have been found not to become as fully differentiated as those of normal green tissue even though the chlorophyll they form has been shown to be photosynthetic. It is thus possible that the lesion in the yellow tissue also affects chloroplast differentiation. This means that the defect either directly affects both chloroplast differentiation and rate of chlorophyll synthesis, or indirectly affects one of these two processes through its effect on the other.

"Restriction of porphyrin metabolism in higher plants through plastid development is illustrated by studies on barley mutants albina 7 and xantha 23 which have only partially developed plastids. Seedlings of these varieties produce normal amounts of pigment when supplied aspartate or leucine, respectively (Eriksson et al., 1961). These observations also illustrate the interaction between cellular activity controlled by nuclear genes, e.g. leucine metabolism, and

plastid development with attendant chlorophyll synthesis" (Bogorad 1965c). In other words chloroplasts are sensitive indicators of the state of health of the plant cell, and some mutations which act primarily on cellular processes other than those belonging specifically to the chloroplast, affect chloroplast formation (Kirk & Tilney-Bassett, 1967d).

Another example of nuclear control of plastid nutrition is a recessive character found in maize (Bell et al., 1962). In such mineral deficiency chlorosis, it is generally not clear whether the primary effect is directly on the production of an enzyme of porphyrin synthesis, or upon plastid development in general.

Studies on the induction of complete chloroplast differentiation in yellow *Peperomia* leaf-tissues and/or identification or characterization of their leaf proteins might help to resolve the nature of the defect occurring in these leaves. For such and other studies, pure or uniform tissue would be invaluable. An ideal material will be callus tissue, but since this has proved very difficult to induce in *Peperomia* leaves, the establishment of leaf discs in sterile culture appears to be the only resort. Studies concerned with changes which take place over short periods can be done using leaf discs because they can be easily kept sterile for a week, and some, even for periods of two to three weeks.

Another useful and more direct method of approach can be suggested for future work. If chemical, nutritional or other environmental conditions which can induce the development of normal plastids in yellow tissue can be found, this might also throw some light on the problem of control of chloroplast differentiation. Since greened-yellow *Peperomia* leaves show incomplete plastid development, such studies might also help to explain the mechanism controlling chlorophyll synthesis in *Peperomia* leaves.

ADDENDUM

A 'Peperomia plant' which might be suitable for future study of chloroplast differentiation is described here. An interesting problem which came out during the course of the work has also been outlined.

In the tissue-culture experiments, some yellow *Peperomia* leaf discs were maintained in sterile culture long enough for them to green. These discs greened whether they were placed on the semi-solid media with their adaxial or their abaxial surface upwards. But adventitious roots which were found to be produced by these greening discs only occurred if the leaf discs were placed up-side down (i.e., abaxial surface up). When some of these rooted, greened, yellow discs were transferred into soil in pots maintained in the roof light cabinet at about 300 ft-c, they produced new shoots.

The leaves of these 'new plants' started off yellow and behaved like the leaves of normal green plants in greening at the above light intensity. But unlike normal green plants, their green leaves later became more severely bleached. (Fig. 30). Chloroplast development can be studied in these 'new plants' and if normal plastid formation is found to be possible here, then these 'new plants' will be ideal for studies on the control of chloroplast differentiation and possibly also on the control of chlorophyll synthesis in *Peperomia* leaves.

A feature which needs further investigation in *Peperomia* is the dark green coloration, found in succulent plants. *Peperomia* has thick fleshy stems, leaves and spires, and shows a number of features characteristic of succulent plants. Like most plants, whose leaves possess only a very small number of stomata or have very limited surface area (Doby, 1965), *Peperomia* has a very small upper surface, and a very small lower surface, which they account for mainly in the thick cuticle (Doby, 1965). Instead of the thick cuticle, the leaves have a thick cuticle. They can withstand long periods of desiccation, and the *Piperaceae* (to which *Peperomia* belongs), are entirely tropical plants (Kendall, 1959).



Fig. 30 *Peperomia* plant developed from succulent plants (due to small yellow leaf disc, and the presence of a thick cuticle on the epidermis). "Both photosynthesis and respiration occur to a limited extent" (Doby 1965). Photosynthetic and respiration rates in *Peperomia* leaves (measured in leaf discs), are very low (Table 9).

The families in which succulent *Peperomia* has been studied, do not seem to include the *Piperaceae*. They include the *Crossulaceae*, the *Cactaceae*, the *Euphorbiaceae*, the *Milliaceae*, and *Alzateaceae*, the *Amariyllidaceae*, the *Asclepiadaceae*.

A feature which needs further investigation in *Peperomia* tissue is the dark CO_2 fixation, found in succulent plants. *Peperomia* has thick fleshy stems, leaves and spikes, and shows a number of features characteristic of succulent plants. Like such plants, whose leaves possess only a very small number of stomata or have very limited surface area (Doby, 1965a), *Peperomia* leaves have no stomata at all on the upper surface.

Succulent plants are able to survive dry periods because of the relatively large quantities of water which they accumulate in their fleshy stems or leaves. They grow mainly in arid soils and in a tropical or subtropical climate (Doby, 1965a). The leaves of *Peperomia* have no palisade tissue; instead they show large parenchymatous water-storing cells. They can withstand long periods of dessication, and the Piperaceae (to which *Peperomia* belongs), are mainly tropical plants (Kendle, 1959).

Because of the reduced gaseous exchange in succulent plants (due to small numbers of stomata and the presence of a thick cuticle on the epidermis), "both photosynthesis and respiration occur to a limited extent" (Doby 1965a). Photosynthetic and respiration rates in *Peperomia* leaves (measured in leaf discs), are very low (Table 9).

The families in which crassulacean metabolism has been studied, do not seem to include the Piperaceae. They include the Crassulaceae, the Cactaceae, the Euphorbiaceae, the Liliaceae, and Aizaceae, the Amaryllidaceae, the Asclepiadaceae,

Material	Rate of App. Photo ($\mu\text{l.O}_2/\text{dm.}^2/\text{hr.}$)	Rate of Respiration ($\mu\text{l.O}_2/100 \text{ mg.fr.wt./hr.}$)
1 Green Peperomia leaf discs	0.6 at 800 ft-c	19 at 30°C
2 Lemon (Citrus limonia)	3-5 at 1,300 ft-c	-
2 Orange (Citrus sinensis)	4-6 at 1,300 ft-c	-
Alfafa (a) Resting (seeds) (Medicago (b) Germinating (seeds) sativa		38 at 18°C 106 at 18°C
Intact Kidney Bean (Phaseolus vulgaris)		26-57 at 26°C
Corn seedlings (Zea mays)		15 at 18°C

Table 9. Photosynthetic and Respiration rates
in *Peperomia magnoliifolia*
and other Angiosperms.

1. Determined in Warburg No. 9
buffer solution

2. In 1.5% CO_2 in air.

(Except for *Peperomia*, all values above
are from Altman & Dittmer. 1964).

the Begoniaceae and the Compositae. (Meyer et al., 1961b; Bonner, 1950). But from the close similarity in structure and habit between Peperomia and the succulent members of the above families, it is very likely that Peperomia also shows crassulacean metabolism. (Succulent plants are a morphological, rather than a taxonomic group - Bonner, 1950).

According to Ranson (1965), there is strong evidence that acid synthesis by succulent plants (which accumulate malate) is through OAA produced, when PEP is carboxylated under the agency of PEP carboxylase. Activity of such an enzyme system has been demonstrated in plants - in parsley root, in spinach leaves, in barley root and in higher plant leaves (Gollub and Vennesland, 1947; Bandurski and Greiner, 1953; Doby, 1965b). As has been postulated in Section V, the incorporation of ^{14}C from glycine-2- ^{14}C into terpenoids could be explained if decarboxylation of OAA or malate takes place in the incubated leaf discs. Thus Peperomia should possess an enzyme system for the decarboxylation of OAA or malate and the reversal reaction necessary for acid synthesis. Studies to find out if acid accumulation is possible in Peperomia leaves in the dark, and what these acids are, will help to establish whether crassulacean acid metabolism occurs in the Piperaceae too, and in Peperomia magnoliifolia in particular.

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