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#2

Ultrastructural and Lipid Changes Associated With the Aging of Citrus Leaves[1]

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With 6 Figures

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Summary

During maturation and senescence of leaves of navel orange (*Citrus sinensis* L.), total lipids per gram of leaf steadily decline. The decline is attributable to the galactolipid and phospholipid partners, while chlorophylls, carotonoids and tocopherols increase during maturation. The phospholipid/galactolipid ratio declines steadily during maturation and senescence but the monogalactosyldiglyceride/digalactosyldiglyceride ratio remains relatively constant. The phospholipid composition remains relatively constant even though the total phospolipid declines markedly.

Ultrastructural changes concomittant with changes in lipid composition include the development of an extensive internal membrane system (grana-fretwork system), and several large-plastoglobuli in the chloroplasts of mature green leaves. With the conversions of chloroplasts to chromoplasts in the senescing leave the internal membrane system is reduced and numerous, large plastoglobuli appear.

1. Introduction

It is well recognized that senescence in plants follows different patterns in the whole plant, and in particular organs, tissues and cells (Leopold 1964, Woolhouse 1967, Butler and Simon 1971, Sacher 1973). Leaf senescence occurs naturally or can be induced when leaves are detached and supplied with water. In many ultrastructural and physiological studies of leaf senescence,

[1] Abreviations used in the text: carot, carotenoid; chl, chlorophyll; DGB, digalactosyldiglyceride; GL, glycolipid; MGDG, monogalactosyl diglyceride; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PI, phosphatidylinositol; PL, phospholipid, PS, phosphatidylserine; quin, quinones.
Abbreviations on plates: B = microbody, C = chloroplasts, G = granum, M = mitochondria, N = nucleus, O = plastoglobuli, S = starch.

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the detached system has been preferred since it offers means to control, experimentally, conditions which affect senescent processes, and can be used to examine the sequential changes with reasonable accuracy. However, it is not well understood whether the senescent changes in attached leaves correspond with those of detached leaves, and valid extrapolations between the changes in intact leaves and excised leaves are difficult to evaluate until precise comparisons are made. Only a few studies of ultrastructural changes associated with leaf senescence have been reported with attached leaves (Barton 1965, Toyama and Ueda 1965, Dodge 1970, Harris and Arnott 1973, Wuttke 1976). In this paper we are reporting our observations on the ultrastructural and lipid changes associated with aging and senescence of attached citrus leaves. The significance of these changes relative to other studies on plant senescence is developed in the discussion.

Table 1. *Composition of Lipid Extracts From Leaves of Different Ages From Naval Orange Trees*

Leaf age	mg Lipid per g leaf	mg Lipid per g leaf						
		Galacto-lipid	Phospho-lipid	Toco-pherols	Caro-tenoids	Chloro-phyll	Cuticle	% Recovery
A	16	6.1	8.15	0.019	0.128	0.57	0.16	94
B	13	5.3	7.05	0.029	0.127	0.59	0.17	102
C	11	3.8	4.20	0.031	0.167	0.84	0.44	86
D	10	4.6	3.70	0.048	0.122	0.16	0.50	91
E	3	0.75	0.24	0.023	0.026	0.04	0.51	53

Tocopherols, carotenoids and chlorophyll were determined on the lipid extracts as described in Materials and Methods. Galactolipid and phospholipid were determined by assay of galactose or phosphate in the individual lipids after thin-layer chromatography. Weight of galactolipid was calculated from galactose using molecular weights of 846 and 1006 for MGDG and DGDG respectively. Calculations of phospholipid weight from phosphate analysis used an average molecular weight of 771. Values were first obtained in relation to weight of lipid analyzed and were computed on a per g leaf basis by use of the figures in the first column.

2. Materials and Methods

2.1. Materials

Leaves were taken from mature, orchard grown Navel orange trees (*Citrus sinensis* L.) at Riverside, California. Leaves at five different stages of development and age were selected on the following basis:

A. Small developing leaves near the shoot apex. These leaves were approximately 24 mm in length, pale green in color, and had a soft texture.

B. Young expanding leaves approximately 60 mm in length, light green in color, and with a soft texture.

C. Mature leaves, deep green in color, but with a smooth texture.
D. Mature, deep green leaves, with a hard, rougher texture.
E. Mature leaves which had turned yellow but were still attached to the branch.
Several samplings were made and the observations described represent the characteristic features for each group. Figure 1 is a photograph of representative leaves.

2.2. Fractionation of Lipids

Leaves of ages A through E were picked in 40 gram lots, and washed in distilled water. After the midribs had been removed, half of each lot of the weighed leaves was cut into 1 cm^2 sections and the lipids were extracted in boiling methanol for 10 minutes, three extractions were made, and the lipids were isolated according to BLIGH and DYER (1959).

Table 2. *Phospholipid Distribution in Lipids From Naval Orange Leaves*

	% Distribution				
Leaf age	PS	PI	PC	PG	PE
A	0.5	19	30	14	34
B	2	14	39	13	31
C	9	3	45	17	23
D	12	10	23	23	28
E	8	13	21	29	20

Percentage distribution was determined by phosphate assay of areas scraped from silia gel thin layer plates. Small amounts of phosphatidic acid which were found are not included in the table. PS = phosphatidylserine, PI = phosphatidylinositol, PC = phosphatidylcholine, PG = phosphatidylglycerol, PE = phosphatidylethanolamine.

This method depends on the formation of a monophasic system by obtaining a ratio of methanol : chloroform : water of 2 : 1 : 0.8. A biphasic system is then obtained by addition of 1 part chloroform and 1 part water. The lower, chloroform phase contains the lipids. The chloroform extract was used for phospholipid, galactolipid and chlorophyll determinations.
The other half of each lot of leaves was frozen in liquid nitrogen and ground with a mortar and pestle. The ground material was weighed, placed into an extraction thimble, and the lipids extracted with methanol in a Soxhlet extraction apparatus (Kimax brand, from Scientific Products). The lipids were extracted according to BLIGH and DYER (1959), dried under reduced pressure, weighed, and redissolved in absolute ethanol. Sufficient 60% (w/v) KOH was added to bring the KOH concentration to 6—10% overall. The mixture was allowed to saponify under nitrogen at 5 °C for 12—15 hours in the dark. An equal volume of anhydrous diethyl ether was added and water was added until two layers formed. The ether phase was saved and the aqueous phase was reextracted with ether. The combined ethereal extract were washed with destilled water until pH 6 or pH 7 was achieved. The extract then was dried over 2 grams anhydrous Na_2SO_4 and all traces of ether were then redissolved in a known volume of absolute ethanol and stored under nitrogen at 5 °C in the dark (BRITTON and GOODWIN 1971). This extract was used for total carotenoid

and tocopherol determinations. On some occasions the lipid extract used for phospholipid determination was also used as the starting material for tocopherol and carotenoid determination. The results were identical using either method.

Phospholipids and galactolipids were separated by two-dimensional thin-layer chromatography on 0.25 mm Silica Gel G (Brinkmann Instruments Inc., Westbury, N.Y.) on 20 × 20 cm Pyrex plates, the gel being activated at 100 °C for 30 minutes before chromatography. The plates were developed in chloroform-methanol-7N NH_4OH (65 : 30 : 4) in the first direction and in chloroform-methanol-acetic acid-H_2O (170 : 25 : 25 : 6) in the second direction (NICHOLS and JAMES 1964). The developed plates were dried in air for 30 minutes and sprayed with 0.01% (w/v) 8-anilo-1-napthalene sulfonic acid in H_2O. Lipid spots were viewed under UV light and marked. Weight of the cuticle was estimated by washing 10 g of leaves in hexane at 60 °C for 2 minutes. The hexane layer was evaporated to dryness and the residue was weighed.

Table 3. *Ratios of Lipid Components From Naval Orange Leaves of Different Ages*

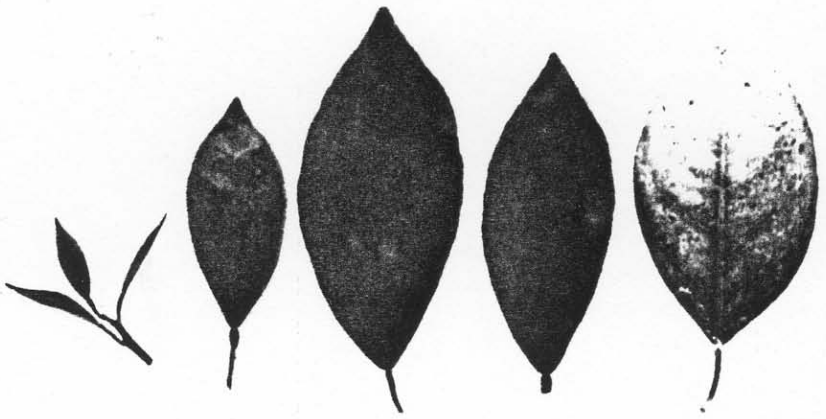
Leaf age	PL/GL	MGDG/ DGDG	chla/chlb	GL/chl	PL/chl	tocopherols/ chl	carotenoids/ chl
A	1.34	0.73	2.31	10.7	14.4	0.034	0.23
B	1.32	1.27	2.45	9.0	11.9	0.048	0.22
C	1.22	1.00	2.24	4.4	4.95	0.037	0.20
D	0.81	1.09	2.60	29.4	23.7	0.310	0.78
E	0.32	1.07	1.92	18.6	5.95	0.580	0.63

The MGDG/DGDG ratios were determined after galactose analysis of areas scraped from silica gel thin layer plates. Chlorophyll ratios were calculated from chlorophyll absorbance spectra. All other ratios are calculated from data presented in Table 1. PL = phospholipid, GL = glycolipid, MGDG = monogalactosyl diglyceride, DGDG = digalatosyl diglyceride, chl = chlorophyll.

2.3. Lipid Identification

For quantitative analysis of phospholipids and galactolipids, the spots were scraped from 7 plates and the lipids were extracted with 4 ml chloroform-methanol 1 : 1, 4 ml chloroform-methanol 1 : 2, and twice with 4 ml 100% methanol. This gave sufficient lipid for duplicate measurements. Galactolipid extracts were evaporated to dryness under reduced pressure and the residue was dissolved in 3 ml methanol and 3 ml 2 N HCl. An inert carbon boiling chip was added to each sample and the lipids were hydrolyzed at 100 °C for 1 hour in open tubes. Fatty acids and 8-anilo-1-napthalene sulfonic acid were removed with ether and the aqueous phase was evaporated to dryness under reduced pressure. The residue was dissolved in 2 ml distilled water and the amount of sugar was determined according to the method of DUBOIS *et al.* (1956) using galactose as the standard.

Phospholipid extracts were evaporated to dryness under reduced pressure in 15 × 150 mm Pyrex test tubes. The sides of the tubes were washed with 2 ml chloroform and reevaporated under vacuum. Phosphate was then determined by slight modifications of the procedures of CHEN *et al.* (1956) and AMES und DUBIN (1960). Two drops of 10% Mg $(NO_3)_2 \cdot 6\,H_2O$ in absolute ethanol was added to the residue in the tubes, along with tubes containing standard phosphate solutions. The samples were then dried in an oven at 150 °C for 20 minutes, and ashed over a flame until the brown fumes disappeared and all organic material turned white. After cooling, 0.9 ml 0.5 HCl was added and the samples were heated



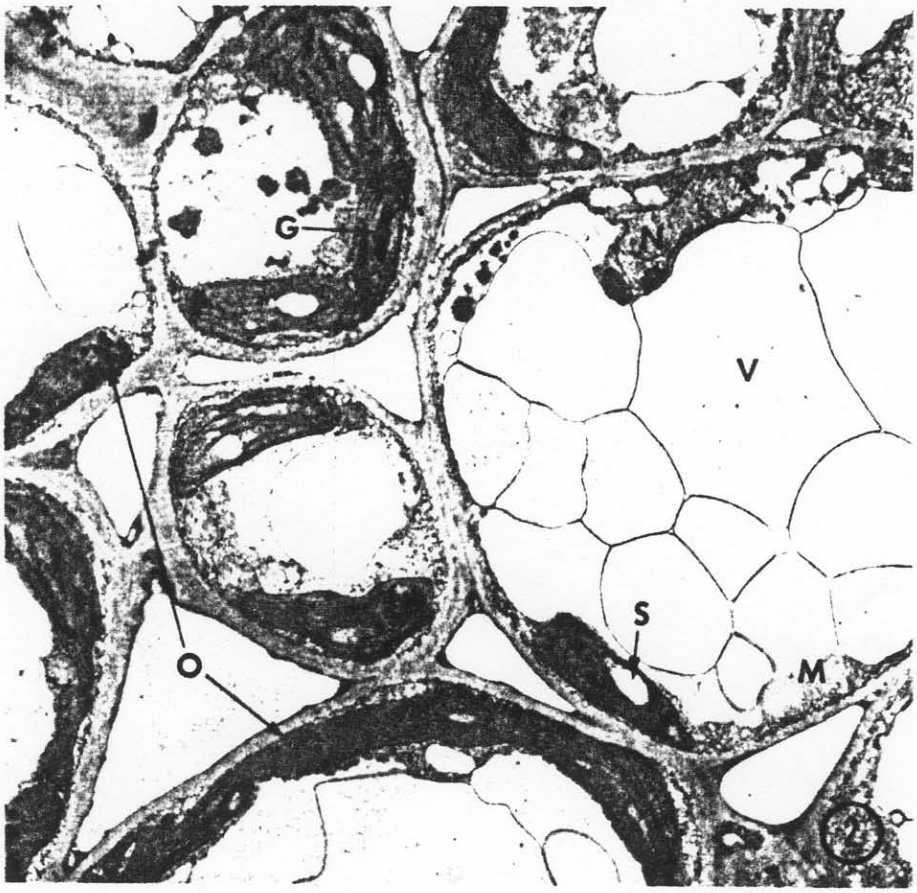


Fig. 1. A photograph illustrating the representative leaves of different age and development from A to E

Fig. 2. Mesophyll cells of a young leaf of sample *A* of illustrating the grana *G*, starch *S*, the osmiophilic globules *O*, in the chloroplasts and other cellular constituents: mitochondrion *M*, vacuole *V*, nucleus *N*. ×7,000

for 15 minutes in a boiling water bath to hydrolyze any pyrophosphate formed during the ashing procedure. Inorganic phosphate was then determined by adding 2.1 ml ascorbate-molybdate solution, incubating at 45 °C for 20 minutes and reading the absorbance at 820 nm against a blank containing no phosphate, which also had been taken through ashing and hydrolysis procedures. The ascorbate-molybdate solution, made up daily, contained 1 part 10% (w/v) ascorbic acid to 6 parts 0.42% ammonium molybdate · 4 H_2O (w/v) in 1 N H_2SO_4.

Chlorophylls a and b were determined according to ARNON (1949).

Carotenoids were determined using the following equation:

CAROTENOIDS = 40 × E_{max} (µg/10 ml) LICHTENTHALER, 1965.

The maximum for carotenoid absorbance was 448 nm.

Tocopherols were determined using a modification of the method of EMMERIE and ENGEL proposed by TSEN (1961). Standard curves were prepared using α tocopherol. The maximum for the bathophenanthroline-tocopherol complex was found to be 534 nm.

2.3. Electron Microscopy

For electron microscopy small sections were out from leaves of each group and fixed in 0.1 M phosphate buffered (pH 7.0) glutaraldehyde. Fixation was carried out for 1 hour at room temperature followed by a short rinse with 0.1 M phosphate buffer and post-fixation in 1% phosphate-buffered (pH 7.0) osmium tetroxide for 1 hour. The material was dehydrated with a acetone series and embedded in epoxy resin (SPURR 1969). Thin sections were cut with a Porter-Blum MT-2 ultramicrotome and mounted on uncoated copper grids. The thin sections were stained with aqueous uranyl acetate followed by 1–2 minute exposure to lead citrate (REYNOLDS 1963). The sections were examined using a Philips 300 electron microscope.

3. Results

3.1. Lipids

The lipid analyses of the navel orange leaves of different ages are shown in Tab. 1. The data are expressed on the basis of one gram of leaf tissue, but the amounts per unit weight of lipid may be calculated from the figures of mg lipid per g leaf. It should be emphasized that chlorophyll is at a maximum at stage C and is apparently degraded thereafter. Carotenoids are degraded less rapidly and tocopherols even continue to be synthesized to stage D. Galactolipids and phospholipids are not degraded precipitously until the period between stages D and E. At stages D and E, phospholipid has been degraded more rapidly than galactolipid. Cuticular lipids increase up to stage C but there is little change after that. By stage E, the cuticular lipid forms a large proportion of the total lipid. Low recovery of lipids at stage E may indicate that other components are present besides those analyzed.

The distribution of phospholipid is shown in Tab. 2 The major phospholipid is phosphatidylcholine at most ages, but phosphatidylethanolamine also contributes a large proportion of the lipid. The results indicate increasing proportions of PS and PG as the age increases. Considering that the amount of phospholipid per g leaf at stage E is less than 5% of earlier stages, however, the percentage composition is remarkably well retained.

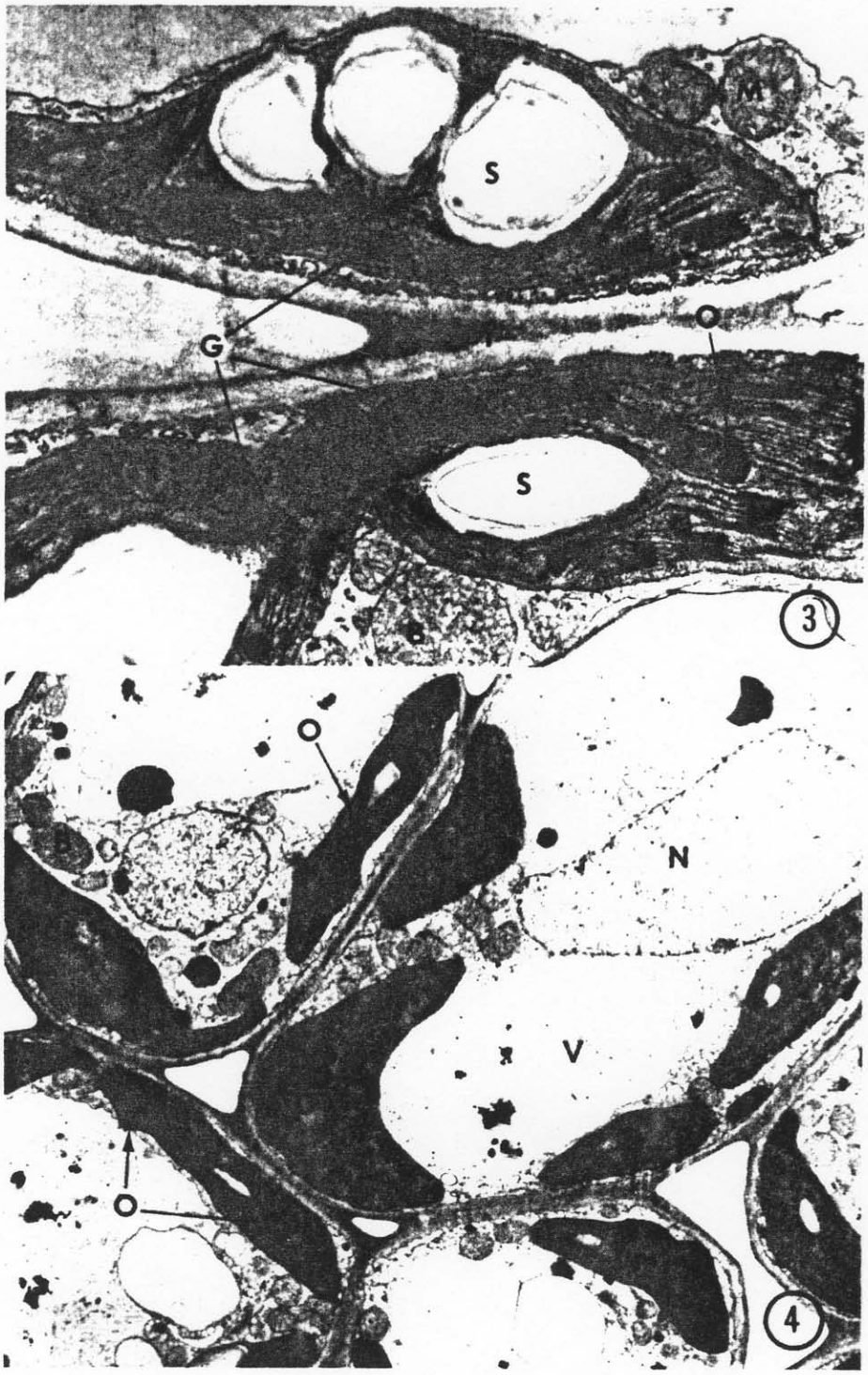


Fig. 3. Chloroplasts with grana *G*, osmiophilic globules *O*, and starch *S*, mitochondria *M*, and microbodies *B*, of mesophyll cells of a young, expanding leaf of samples B. ×20,000

Fig. 4. Mesophyll cells of a mature leaf of sample C illustrating the chloroplasts *C*, and osmiophilic globules *O*, and other cellular organelles: microbody *B*, vacuole *V*, and nucleus *N*. ×7,500

We have computed various ratios of lipid components for presentation in Tab. 3. Neither the MGDG/DGDG ratio nor the chl a/chl b ratio changes notably during maturation and senescence even though chlorophyll is considerably degraded in stages D and E, and galactolipid is degraded by stage E. The PL/GL ratio declined steadily throughout the observation period, this reflects the more rapid decrease in phospholipid. Both GL/chl and PL/chl decrease to a minimum at stage C since chlorophyll is accumulating up until this point. The tocopherol/chl and carotenoid/chl ratios increase throughout the observation period. In the early stages this reflects the greater accumulation of tocopherols and carotenoids, but in the later stages reflects their slower degradation as compared to chlorophyll.

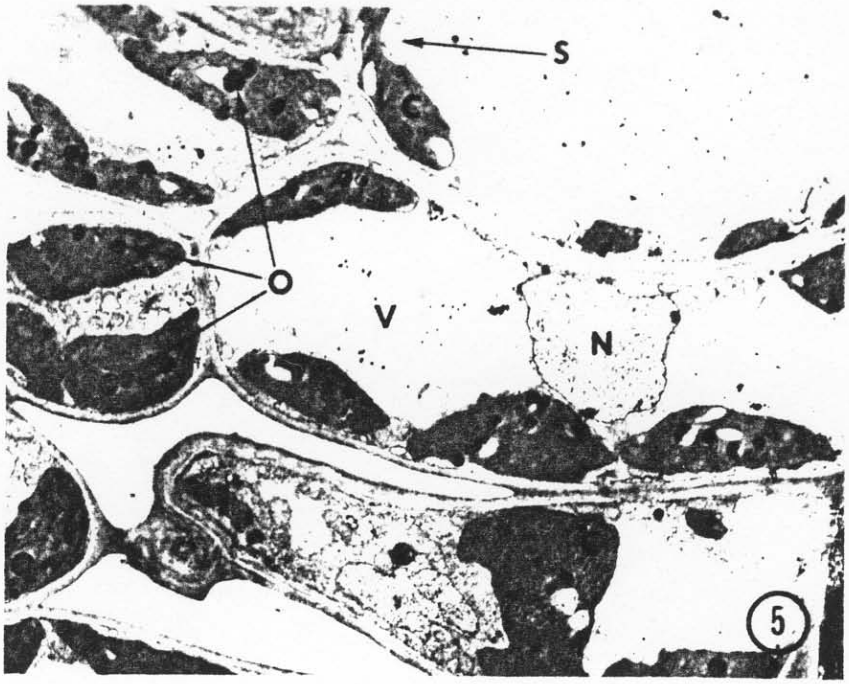
3.2. Ultrastructure

The chloroplasts in young leaves (sample A) were often exceedingly elongate, contained small starch grains but the internal membrane system was not fully developed (Fig. 2). A common characteristic was the presence of 1–3 very large grana per section of a chloroplast (Fig. 2). Plastoglobuli were present in all the chloroplasts. They were relatively small and the numbers varied from one to as many as 20 per section of a chloroplast. Frequently the plastoglobuli were localized in one region, often near the ends of the chloroplasts (Fig. 2). The cytoplasm contained the normal complement of organelles and the vacuoles contained membranous vesicles of various sizes.

In the young, expanding leaves (sample B) the chloroplasts were relatively well developed and contained many grana (Fig. 3). The presence of relatively large starch grains was a common feature as were plastoglobuli (Fig. 3). The plastoglobuli were more uniform in size than in the younger leaves and the number of plastoglobuli varied from 2–12 in any one section of a chloroplast. Small vesicles, dictyosomes with associated vesicles, mitochondria, microbodies and elements of endoplasmic reticulum were distributed throughout the cytoplasm (Fig. 3). The vacuoles tended to be generally devoid of material and although not shown, membrane vesicles within the vacuoles and the apparent protuberances of membrane vesicles from the cytoplasm through the tonoplast of the vacuole were frequently observed.

The ultrastructure of the mesophyll cells of leaves from sample C differed significantly from A and B. The chloroplasts in these cells contained an extensively developed internal membrane system; however, few starch grains were observed in these chloroplasts (Fig. 4). Several plastoglobuli were observed per section of a chloroplast and they were larger than in the younger leaves (Fig. 4). As with the younger leaves, the cytoplasm contained the usual complement of organelles.

In the older, deep-green leaves (sample D) the chloroplasts were often variable in contour and contained an extensively developed internal membrane system (Fig. 5). Small starch grains were frequently present in the plastids and the



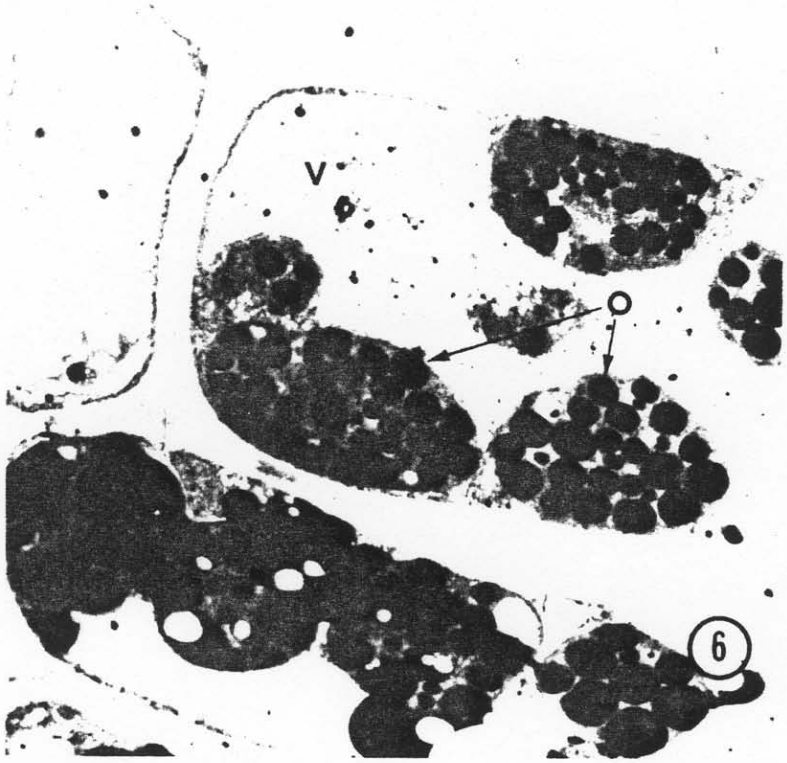


Fig. 5. Mesophyll cells from an older, mature leaf representative of sample D illustrating the chloroplasts *C*, with osmiophilic globules *O*, starch *S*, and other cellular constituents: vacuole *V*, nucleus *N*. ×4,400

Fig. 6. Mesophyll cells of a leaf from sample E illustrating the numerous plastoglobuli *O*, in the plastids of these yellow leaves: vacuole *V*. ×13,000

plastoglobuli ranged from small to quite large (Fig. 5). The number of plastoglobuli also varied widely with counts up to as many as 20 or more per section of a chloroplast. The other cytoplasmic organelles were generally similar to those in the younger leaves and the vacuoles contained vesicles of various sizes.

Table 4. *Lipid Composition of Senescing Leaves:* PL = phospholipid, GL = glycolipid, MGDG = monogalactosyl diglyeride, DGDG = digalatosyl diglyeride, Chl = chlorophyll, Quin = quinones, Carot = carotenoid

System	Age	PL/GL	MGDG/ DGDG	Chl a/ Chl b	GL/Chl	PL/Chl	Quin/Chl	Carot/Chl	Reference
Fig leaves	2 days			2.94			0.106	0.230	Lichtenthaler and Weinert (1970)
	30 days			2.86			0.191	0.200	
	1 year			3.03			0.452	0.192	
	old			2.94			0.785	0.200	
Cucumber	11 days	0.40	1.49		7.17	2.88			Ferguson and Simon (1973)
	14 days	0.25	1.38		3.63	0.91			
	18 days	0.29	1.56		2.80	0.78			
	22 days	0.44	1.47		5.67	2.49			
	25 days	0.47	1.58		6.85	3.30			
Impatiens balsamina cotyledons		0.27	1.81	2.4	5.7	1.51			Tevini (1972)
Hordeum leaves		0.44	1.76	2.62	8.4	3.67			Tevini (1971)

The ultrastructure of the mesophyll cells in the yellow leaves (sample E), showed many of the characteristics of senescent cells (Fig. 6). The plastids or chromoplasts had a reduced internal membrane system, and the dominant feature of these plastids was the presence of numerous large plastoglobuli. These plastoglobuli were often irregular in shape and frequently images were observed of plastoglobuli apparently protruding into the cytoplasm as well as the vacuole. Similar observations have been reported by Simpson and Lee (1976). The cytoplasm was much reduced although mitochondria, microbodies and nuclei were still observed.

4. Discussion

The analytical data we have collected for citrus leaves of different ages cannot be compared in their entirety with any single other study. Comparisons can

be made with data from other laboratories using quite different materials and a selection has been recalculated for presentation in Table 4. In comparison to LICHTENTHALER's (1970) results from senescing fig leaves, the citrus leaves exhibit the same constancy of the chl a/chl b ratio and steady increases in the quinone/chl ratios. The results of FERGUSON and SIMON (1973) from cucumber cotyledons show little change in the MGDG/DGDG ratio and the minima in GL/chl and PL/chl ratios that we have observed in citrus leaves. Therefore, the three rather disparate senescing systems show similar features in lipid synthesis and degradation. The citrus leaf lipid data is most noticeably different in the relatively high amount of phospholipid. Data from two analyses of TEVINI are included in Tab. 4 which further indicate that the amount of phospholipid in citrus leaves is unusually high (1971, 1972). An analysis of lipids in senescing fig leaves (TEVINS 1976) has shown PL/GL ratios of 0.04—0.120, so this ratio may vary with species.

The accumulation of osmiophilic globules in the plastids is a common feature of senescing leaves (BUTLER and SIMON 1971) and citrus is not unusual in this respect. LICHTENTHALER's studies have shown that these osmiophilic globules contain carotenoids and various quinones (1970). Studies by GREENWOOD *et al.* (1963) and BAILEY and WHYBORN (1963) have described the isolation and analysis of osmiophilic globules. Thin-layer chromatography of the lipids of the globules and the chloroplast lamellae showed that in addition to neutral lipids, the globules were also rich in polar lipids characteristic of the granal membranes: the galactosyl diglycerides and sulfoquinovosyl diglycerides. Our results show prominent globules at stage D while galactolipid and phospholipid are major lipid components of the leaf. It appears likely that the globules incorporate all types of lipids and not only the neutral lipids. However the large decrease in all the measured lipids does not agree well with the accumulation of osmiophilic globules. One aspect that was somewhat unexpected is that although chlorophyll decreased in the mature leaves of sample D there did not appear to be a diminution in the internal membrane system of the chloroplasts. The disappearance of the internal membrane system occurred during the stage from D to E when the major lipid, galactolipid, of this membrane system also decreased. A detailed examination of the development of the magnograna in the young chloroplasts and the subsequent extensively developed internal membrane system in the older mature leaves is now in process.

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