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LIPID CHANGES IN SENESCING CUCUMBER COTYLEDONS

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Abstract—Analyses of the fatty acids of yellowing cucumber cotyledons show that changes occur in the composition of both the free and esterified fatty acid fractions. There is an increase in the percentage of linolenic acid in the free acid fraction and this may be accounted for by the 60 per cent loss of galactolipid that occurs during the same period. The final stages of senescence are marked by a loss of all classes of lipid.

INTRODUCTION*

THE COTYLEDONS of cucumber (*Cucumis sativus* L.) become fully green about 2 weeks after germination. The subsequent yellowing process is convenient for study as it occurs within a short period. The changes in protein and nucleic acid metabolism which accompany the loss of chlorophyll show that specific biochemical changes occur during senescence.[1] The experiments described here are concerned with the changes in the lipid composition of this tissue as ageing occurs.

The fatty acid composition of green leaves is characterized by a high level of linolenic acid; this is due to the galactolipids of the chloroplast.[2] In leaf tissue lacking chlorophyll, the content of linolenic acid is much lower than in green tissue of the same plant.[3] As the chloroplast has a relatively high lipid content[4] and as the structure of the chloroplast changes considerably during senescence[5] it is to be expected that large changes in the lipid composition of whole tissue will occur during the period of yellowing.

A lipid fraction was obtained by extraction of cucumber cotyledons with chloroform–methanol. The composition of the free and esterified fatty acid fractions and of the isolated galactolipids was determined by GLC. A quantitative estimation of the galactolipids was obtained by assay of the galactose moiety while a comparative study of the levels of each lipid class was carried out by using a densitometric method for analysis of thin-layer plates.

RESULTS

The age of the cotyledon was taken as the time from sowing; germination occurred within several days. The highest content of chlorophyll was observed at 13 days (Fig. 1). During

* *Abbreviations used:* FFA = free fatty acid; EFA = esterified fatty acid; PC, PE, PI and PG = phosphatidyl choline, -ethanolamine, -inositol and -glycerol respectively; SG = sterol glycoside; SL = sulpholipid; DGDG = digalactosyl diglyceride; MGDG = monogalactosyl diglyceride.

[1] R. J. LEWINGTON, M. TALBOT and E. W. SIMON, *J. Exp. Botany* **18**, 526 (1967).

[2] F. T. WOLF, J. G. CONIGLIO and R. B. BRIDGES, in *Biochemistry of Chloroplasts* (edited by T. W. GOODWIN), Vol. I, p. 187, Academic Press, London and New York (1966).

[3] W. M. CROMBIE, *J. Exp. Botany* **9**, 254 (1958).

[4] W. MENKE and E. JACOB, *Z. Phys. Chem.* **272**, 227 (1942).

[5] R. D. BUTLER, *J. Exp. Botany* **18**, 535 (1967).

the period 20–30 days there is an almost complete loss of chlorophyll. After 30 days there is a rapid loss of fresh weight as the cotyledon becomes less turgid. Subsequent experiments are particularly concerned with changes in the lipid composition which occur during the period of yellowing.

The fatty acid composition of the EFA and FFA fractions was determined (Table 1*a* and *b*). The percentages given are of the major fatty acids which were all identified by comparison with standards; other minor components were observed but these are not recorded. At 13 days the cotyledon was fully green and the predominant esterified fatty acid was linolenic, present as 69·5 per cent of the total. Palmitic and linoleic acids were the next main components. In contrast, when the FFA fraction from cotyledons at this age was examined it was found that the level of palmitic exceeded the level of linolenic acid. Again the next largest component was linoleic acid. After the rapid decline in chlorophyll content commences

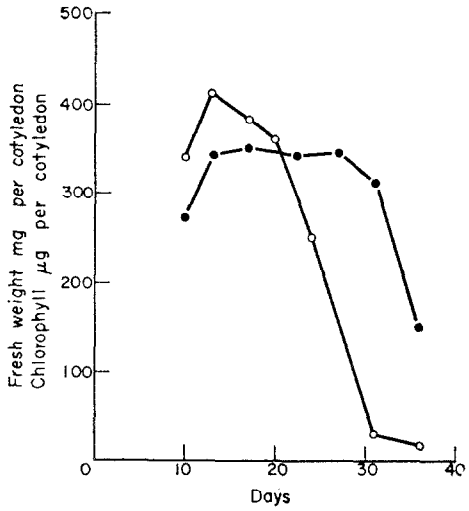

FIG. 1. FRESH WEIGHT AND CHLOROPHYLL CONTENT OF CUCUMBER COTYLEDONS.
The age is given as the time in days from sowing. Closed circles = fresh weight; open circles = chlorophyll.

at 20 days there is a progressive rise in the percentage of free linolenic acid and throughout the period of senescence it is the main free acid. By 36 days the percentage of esterified linolenic acid has dropped to 43·8 per cent.

The next series of experiments was carried out during the summer months and the yellowing process occurred more rapidly, the main loss of chlorophyll occurring between 14 and 21 days (Table 2). Separations of the lipid classes on thin-layer plates showed eight major constituents. These were identified by chromatographic mobility and by the use of various spray reagents.[6] In particular, after spraying with 25% H_2SO_4 and partially charring the plates at 220°, the galactolipids appeared as purple-brown spots and sterol glycoside gave a positive Lieberman–Burchard colour. The other lipids gave pale-brown spots. Standards of PC, PE, PI and neutral lipid were used to locate these classes. Ninhydrin spray was used to confirm the position of PE. The solvent system used did not resolve DGDG and PG and

[6] B. W. NICHOLS, *Thin Layer Chromatography*, p. 55, United Trade Press.

the existence of two components in peak 5 (Fig. 2) was shown by the separation of acidic and non-acidic lipids prior to TLC.[7]

TABLE 1. THE PERCENTAGE COMPOSITION OF THE FFA AND EFA FRACTIONS FROM CUCUMBER COTYLEDONS

(*a*) *Esterified fatty acids*

	Days from sowing				
	10	13	22	27	36
Palmitic	22·9	18·1	23·9	22·9	42·7
Palmitoleic	1·7	1·1	0·8	0·6	0·9
Stearic	3·2	2·7	2·8	3·7	4·3
Oleic	3·1	2·1	1·5	0·9	2·8
Linoleic	8·4	7·7	8·1	5·9	5·4
Linolenic	60·9	69·5	62·9	66·0	43·8

(*b*) *Free fatty acids*

	Days from sowing				
	13	21	27	31	36
Palmitic	35·2	30·3	29·3	27·9	33·1
Palmitoleic	9·0	9·4	1·4	1·7	6·6
Stearic	7·4	7·2	5·7	3·5	5·4
Oleic	7·8	11·3	6·8	3·2	8·3
Linoleic	10·2	10·4	4·5	5·6	6·5
Linolenic	30·6	31·7	52·8	58·4	40·2

The percentages are of the major fatty acids; other minor components were observed but these are not recorded.

TABLE 2. ASSAY OF THE GALACTOLIPIDS OF CUCUMBER COTYLEDONS BY ESTIMATION OF THE GALACTOSE MOIETY

Age (days)	Fresh weight (mg)	Per cotyledon		
		Chlorophyll (μg)	MGDG (μmoles)	DGDG (μmoles)
8	303	434	1·71	0·47
14	472	459	1·35	0·63
21	464	59	0·36	0·28
28	140	7	0·20	0·05

After spraying with 25% H_2SO_4 and completely charring the plates the spots were scanned with a densitometer. Such analyses gave a comparative estimation of the lipids of cotyledons at various ages. At 15 days, ignoring the neutral lipids and pigments, the largest peaks were

[7] P. G. ROUGHAN and R. D. BATT, *Analyt. Biochem.* **22**, 74 (1968).

MGDG and DGDG+PG (Fig. 2*a*). During the period of yellowing, considerable changes occur. There is a large decrease in the amount of MGDG which at 20 days appears only as a shoulder on the peak containing pigments and neutral lipids (Fig. 2*b*). The DGDG+PG peak decreases in height by about 40 per cent and the amount of sulpholipid decreases. The levels

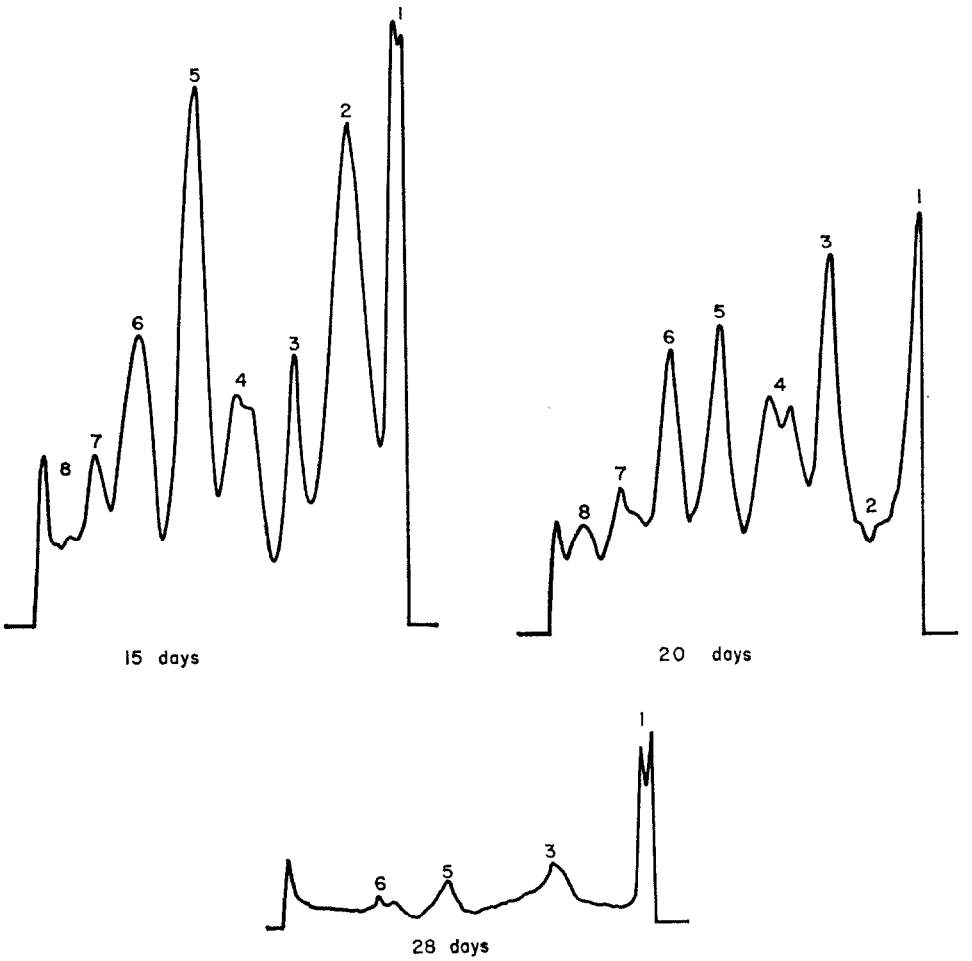

FIG. 2. DENSITOMETRIC ANALYSIS OF THIN-LAYER SEPARATIONS OF THE LIPIDS OF CUCUMBER COTYLEDONS.

The thin layers of silica gel G were developed from left to right; the peaks correspond to charred spots which appear after spraying the plate with 25% H_2SO_4 and heating at 220°. In each separation the plate was loaded with 10 μl of a chloroform solution of lipids containing the equivalent of five cotyledons per ml. The positions of the various lipids are as follows: (1) pigments+neutral lipid, (2) MGDG, (3) SG, (4) PE, (5) DGDG+PG, (6) PC, (7) SL, (8) PI.

of PC and PE remain constant and there is a relative increase in SG. During the final stages of senescence the levels of all classes of lipid decrease and by 28 days the largest peaks are due to SG and the pigments (Fig. 2*c*).

Estimations of MGDG and DGDG by isolation on thin-layer plates and subsequent estimation of galactose[7] confirm that during the period of yellowing there is a considerable

loss of these lipids (Table 2). The proportion of the galactolipids lost, as indicated by this assay, compares very closely with the results from the comparative densitometric analyses.

The fatty acid composition of the galactolipids was determined by GLC. Both lipids contain a large amount of linolenic acid (Table 3).

TABLE 3. THE PERCENTAGE FATTY ACID COMPOSITION OF THE GALACTOLIPIDS OF GREEN CUCUMBER COTYLEDONS

	Palmitic	Palmitoleic	Stearic	Oleic	Linoleic	Linolenic
MGDG	4·7	2·4	1·9	2·4	2·8	85·5
DGDG	19·1	2·5	4·0	3·0	9·1	62·9

DISCUSSION

The composition of the EFA fraction prepared from fully green cotyledons follows a pattern which is to be expected from the known fatty acid composition of mature green leaves and of chloroplasts. They contain high levels of linolenic acid and linoleic and palmitic acid are important components.[3,8] In comparison, the FFA fraction is found to contain a smaller percentage of linolenic acid; this has also been reported for plastids isolated from green leaves of deciduous treees.[9] The changes in the composition of the FFA fraction which occur during yellowing may be interpreted in terms of the observed changes in the levels of the different lipid classes.

All photosynthetic organisms with Hill Reaction capacity have in common the four lipids, MGDG, DGDG, SL and PG. Although PC and PE occur in leaf extracts they are absent from washed isolated chloroplasts.[10] In contrast these two lipids are found as major components in non-green tissue.[11,12]

The galactolipids are present in mature cucumber cotyledons in amounts intermediate between those found in barley leaves[13] and in leaves of some other species.[7] During the phase of rapid yellowing the main change is that the amount of galactolipid is considerably reduced. The quantity of sulpholipid also falls. The levels of PC and PE remain constant while the level of SG rises. In fact accompanying the loss of chlorophyll there is a specific breakdown of the lipids which are known to be constituents of the photosynthetic apparatus. In later stages of senescence, occurring between 20 and 28 days, there is a widespread loss of all lipids. These changes may be compared with the breakdown of lipids which accompanies storage of alfalfa leaves at room temperature,[14] autumnal yellowing of leaves of *Acer*,[15] and cold storage of frozen peas.[16] Lipid hydrolysis is also observed during ageing of isolated chloroplasts.[17]

[8] F. T. WOLF, J. G. CONIGLIO and J. T. DAVIS, *Plant Physiol.* **37**, 83 (1962).
[9] D. W. NEWMAN, *Plant Physiol.* **41**, 328 (1966).
[10] A. T. JAMES and B. W. NICHOLS, *Nature, Lond.* **210**, 372 (1966).
[11] T. GALLIARD, *Phytochem.* **7**, 1907 (1968).
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[13] H. W. GARDNER, *J. Lipid Res.* **9**, 139 (1968).
[14] J. VAN DER VEEN and H. S. OLCOTT, *J. Agr. Food Chem.* **15**, 682 (1967).
[15] E. C. GROB and L. CSUPOR, *Experientia* **23**, 1004 (1967).
[16] B. BENGTSSON and I. BOSUND, *J. Food Sci.* **31**, 474 (1966).
[17] G. CONSTANTOPOULOS and C. N. KENYON, *Plant Physiol.* **43**, 531 (1968).

Galactolipids of leaves usually contain large amounts of linolenic acid[18] and the galactolipids of cucumber are no exception (Table 3). The increase in the percentage of free linolenic acid may be accounted for by the breakdown of chloroplast lipids and in particular the galactolipids. These biochemical events may be related to the fact that ultrastructural changes in the chloroplast are recognisable at an early stage in senescence.[19] Among the proposed causes of sequential senescence is a translocation of substances from the leaf or cotyledon to newly formed leaves nearer the apex.[20] If sugars were to be lost in this way the rate of biosynthesis of the galactolipids might be reduced as a loss of hexose containing material would be equivalent to a direct loss of one of the substances required for galactolipid formation. Such an event would be likely to lead to an overall loss of galactolipid which would disrupt the structure of the chloroplast.

EXPERIMENTAL

Plant Material

Plants of cucumber (*Cucumis sativus* L.) variety Long Green Trailing were grown in John Innes potting compost under greenhouse conditions. Although artificial lighting was provided more rapid growth rates were observed during the summer than during the winter.

Chlorophyll and Fresh Weight

Fresh weight measurements were made on samples of 30–40 cotyledons. Chlorophyll was estimated after extraction in 80% acetone.[21]

Lipid Extraction

Tissue (10 g) was disrupted in 150 ml of a 2:1 $CHCl_3$-MeOH mixture using an overhead homogenizer. The homogenate was warmed to 50° and insoluble material was removed on a sintered-glass filter. The residue on the filter was washed with $CHCl_3$-MeOH until it was colourless and the pooled washings added to the original filtrate. The solution of lipids was then freed of water-soluble impurities.[22]

Fatty Acids

The $CHCl_3$ solution of lipids was reduced to dryness. The lipids were redissolved in 25 ml of 1:1 Et_2O–light petroleum (b.p. 40–60°) and extracted with four separate volumes of 1% Na_2CO_3. The organic phase was then taken as the esterified fatty acid fraction. The pooled aqueous extracts were acidified with conc. H_3PO_4 and the free fatty acids recovered in 4 × Et_2O–petroleum mixture. Samples of either free or esterified fatty acids were evaporated to dryness under N_2 and converted to their Me esters using BF_3–MeOH reagent.[23] The esters were estimated by GLC using an instrument fitted with a flame ionization detector (series 104, W. G. Pye & Co. Ltd., Cambridge). Glass columns were packed with 10% polyethylene glycol adipate on 100/120 mesh celite. The operating temperature was 184·5°.

Thin-Layer Chromatography

TLC was carried out on 250 μ silica gel G with $CHCl_3$/MeOH/acetic acid/H_2O (85/15/10/3·5). For densitometric analyses 10 μl of 5 cotyledons/ml $CHCl_3$ were used and the dried plates were sprayed with 25% H_2SO_4 and charred for 10 min at 220°. The plates were scanned with a Vitatron densitometer (Fisons Scientific Apparatus Ltd., Loughborough). The intensity of charring varies between lipid classes and even between lipids of the same class with different fatty acid composition and so only comparative results were obtained. To quantify the galactolipid changes, the galactose moiety of these lipids were assayed as described by Roughan and Batt.[7] To determine the fatty acid composition of the galactolipids, samples were isolated by a combination of column chromatography on DEAE cellulose[7] and TLC. The preliminary column separation was

[18] C. F. ALLEN, D. HIRAYAMA and P. GOOD, in *Biochemistry of Chloroplasts* (edited by T. W. GOODWIN), Vol. I, p. 195, Academic Press, London and New York (1966).
[19] R. D. BUTLER and E. W. SIMON, in press.
[20] E. W. SIMON, *Types of Leaf Senescence*, S.E.B. Symposium XXI. 215 (1967).
[21] G. MACKINNEY, *J. Biol. Chem.* **140**, 315 (1941).
[22] J. FOLCH, M. LEES and G. H. SLOANE STANLEY, *J. Biol. Chem.* **226**, 497 (1957).
[23] V. A. KNIVETT and J. CULLEN, *Biochem. J.* **96**, 771 (1965).

necessary as PG cochromatographs with DGDG on thin-layer plates. After the thin-layer separation the appropriate area of silica gel was scraped from the plate and the lipid was eluted with 100 ml of 2:1 $CHCl_3$–MeOH. The constituent fatty acids were estimated by GLC.

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