Phytochemistry, 1967, Vol. 6, pp. 187 to 192. Pergamon Press Ltd. Printed in England

SOME FACTORS WHICH INFLUENCE FATTY ACID ACCUMULATION IN LEAVES*

D. W. NEWMAN

Botany Department, Miami University, Oxford, Ohio

(*Received* 20 *April* 1966)

Abstract—The effect of age, flowering and nitrogen deficiency on the fatty acid composition of lettuce leaves has been investigated. Nitrogen deficiency increases the ratio of saturated to unsaturated acids as well as decreasing the total amount present. Aging shows a more marked effect in the flowering shoots where the older leaves contain a higher proportion of saturated acids but lower overall totals. Squash leaf discs showed a higher incorporation of acetate-2-^{14}C into linolenate in the presence of UDPG than in its absence, in both normal and nitrogen-deficient plants. UDPG-^{14}C was incorporated into two unidentified lipid components in lettuce leaves. 8-Azaguanine and actinomycin-D, as expected, both inhibited acetate-^{14}C incorporation into lipids in lettuce leaf.

CHLOROPLASTS isolated from nitrogen-deficient or senescent leaf tissue may contain a reduced amount of unsaturated fatty acids,[1] especially linolenic acid, presumably because of a concomitant reduction in the amount of monogalactosyl diglycerides.[2] This is often true for plastids isolated from squash leaves[3] but not for plastids isolated from lettuce leaves of apparently non-flowering plants. The following studies were an attempt to determine some of the factors which affect the accumulation of saturated and unsaturated fatty acids in intact leaf tissue.

RESULTS AND DISCUSSION

Relationship between Tissue Age and Fatty Acid Accumulation

The fatty acids of lettuce leaves from plants grown on a complete mineral nutrient and from plants grown on a nitrogen-deficient, but otherwise complete, nutrient were determined. Leaves from both vegetative and flowering shoots were used for the analysis (Table 1). In general, there seemed to be little relationship between the ratio of saturated to unsaturated fatty acids and the age of the tissue from which the lipids were extracted, if the leaves were harvested from shoots which had not given rise to a definite flowering stalk. The lower ratio of saturates to unsaturates in the youngest nitrogen-deficient leaf tissue is an exception to this. It is interesting that the youngest leaf tissue seems to have the highest percentage of linoleic acid (C18:2)

Younger leaves of nitrogen-deficient flowering shoots and of the flowering shoots of plants grown on a complete nutrient had a lower ratio of saturated to unsaturated fatty acids than did older leaves. This is primarily due to the decreased percentage of palmitate (C16) and/or increased percentage of linolenate. The percentage of linoleate did not vary markedly with the age of the tissue–relative position of the leaf on the flowering shoot (Table 1).

* Supported by Grant GB-2717 from The National Science Foundation, U.S.

[1] J. W. WALLACE and D. W. NEWMAN, *Phytochem.* **4**, 43 (1965).
[2] P. S. SASTRY and M. KATES, *Biochim. Biophys. Acta* **70**, 214 (1963).
[3] D. W. NEWMAN, *Plant Physiol.* **41**, 328 (1966).

TABLE 1. FATTY ACID COMPOSITION OF LEAVES FROM APPARENT VEGETATIVE AND FLOWERING SHOOTS OF LETTUCE

Sample	Position on stalk	Acid, mole % C16	C18	C16:1	C18:1	C18:2	C18:3	μmoles × 10 / 40 leaf discs	Saturated/ unsaturated material
Vegetative stalk									
Complete	Mid	15·2	2·2	1·4	4·9	15·1	61·1	25·7	0·21
Complete	Top	18·2	1·9	1·2	3·4	29·2	46·1	37·5	0·25
−N	Bottom	29·3	3·4	4·2	5·9	19·6	37·7	21·6	0·48
−N	Mid	28·4	4·1	3·6	7·8	15·8	40·3	17·8	0·48
−N	Top	21·7	2·4	1·8	4·7	27·4	42·0	9·9	0·32
Flowering stalk									
Complete	Bottom	20·1	3·9	2·1	5·5	11·7	56·6	25·1	0·32
Complete	Mid	13·6	1·5	0·9	2·2	9·7	72·0	48·3	0·18
Complete	Top	15·0	2·0	1·2	2·7	16·3	62·8	60·7	0·20
−N	Bottom	39·2	3·4	—	3·7	14·2	39·5	24·9	0·74
−N	Lower-mid	27·1	2·0	—	3·2	11·6	56·1	36·5	0·41
−N	Upper-mid	25·0	3·3	1·5	3·4	11·3	55·5	48·0	0·39
−N	Top	18·2	4·1	3·1	2·3	12·1	60·3	56·4	0·29

Some Factors which Influence the Accumulation of Fatty Acids

A bewildering array of factors may influence or control the rate of fatty acid accumulation, especially in the plastid. Two of the most obvious would be: (1) the capacity of the tissue to synthesize the substrate(s) and (2) the capacity to synthesize the enzymatic systems. Any change in one or both of these would most probably result in a change in the total accumulation of each fatty acid observed at any time. In an attempt to partially regulate the above-mentioned factors (1) UDP-glucose was provided to leaf tissue, since it has been observed previously that UDP-glucose may serve as a precursor for the synthesis of the galactolipids[4] and since previous work with isolated chloroplasts has demonstrated that UDP-glucose will enhance acetate incorporation into linolenate as compared to that into palmitate and (2) greened leaf discs were incubated with 8-azaguanine and actinomycin D, in addition to acetate-2-^{14}C, in an attempt to alter nucleic acid and protein synthesis. It has been demonstrated previously that actinomycin D inhibits greening of etiolated leaves.[5]

(1) Squash leaf discs which were treated with UDP-glucose and allowed to react with acetate-2-^{14}C in a sodium bicarbonate–phosphate buffer medium appeared to exhibit a higher incorporation into linolenate, as compared to that into palmitate, than did the non-treated leaf discs (Table 2). The specific activity of palmitate with respect to that of linolenate decreased with increased time of incubation with acetate-2-^{14}C. This was expected since, even though the most abundant fatty acid in the leaf is linolenate, linolenate is less actively labeled during a short period of incubation.[6] The results confirm those obtained using isolated chloroplasts from lettuce leaves.[3] The added UDP-glucose enhanced incorporation of acetate into linolenate, as compared to that into palmitate, in the nitrogen-deficient leaf discs as well as in the leaf discs from plants grown on a complete nutrient. The ratio of specific activity of palmitate to that of linolenate was higher in the nitrogen-deficient tissue

[4] E. F. NEUFELD and C. W. HALL, *Biochem. Biophys. Res. Commun.* **14**, 503 (1964).
[5] L. BOGORAD and A. B. JACOBSON, *Biochem. Biophys. Res. Commun.* **14**, 113 (1964).
[6] A. T. JAMES, *Biochim. Biophys. Acta* **70**, 9 (1963).

than in the tissue grown on complete nutrient, when incubated for a comparable period of time.

Since the accumulation of the "galactolipids" in the chloroplast is dependent upon the immediate products of photosynthesis, then the amount of linolenate found is also dependent upon the capacity for photosynthesis. This is probably true since linolenic acid is the predominant fatty acid of the plastid galactolipids and since little free linolenic acid seems to exist in the mature chloroplast. Consequently, the rate of linolenate synthesis appears to be influenced by the factors which affect photosynthesis or the accumulation of photosynthate.

(2) Leaf discs from plants grown on a complete nutrient and from plants grown on a nitrogen-deficient nutrient were incubated for varying periods of time with UDP-glucose-^{14}C. The total lipid extracts from these leaf discs were separated on thin layers of silica gel. Autoradiograms were made in order to identify the regions of the layers containing radioactive materials. The two-dimensional chromatograms contained only two radioactive spots, neither of which was mono- or digalactosyl diglyceride (Fig. 1). Neufeld and Hall[4] found eight

TABLE 2. INFLUENCE OF UDP-GLUCOSE ON THE INCORPORATION OF ACETATE-2-^{14}C INTO C18:1, C18:2, AND C18:3 FATTY ACIDS AND INTO LINOLENATE, AS COMPARED TO THAT INTO PALMITATE, IN SQUASH LEAF DISCS. THE INCORPORATION INTO Δ^3-*trans* C16:1 IS ALSO GIVEN

Sample	Time treated	UDP-glucose	Specific activity*				
			C18:1	C18:2	C18:3	C16/C18:3	Δ^3 *trans* C16:1
Complete	30 min	+	100	20	0·4	14·5	2·6
	5 hr	+	105	79	5·5	7·3	16·0
	30 hr	+	26	169	29·0	1·2	159·0
	30 min	−	71	50	0·3	47·9	5·6
	5 hr	−	107	48	2·6	14·1	18·0
−N	30 min	+	26	10	0·1	63·5	2·2
	5 hr	+	93	4	0·3	17·0	2·0
	30 min	−	17	4	0·02	219·0	1·4
	5 hr	−	68	51	0·5	30·4	7·5

* CPM/mμmole.

radioactive components after thin-layer chromatography. Samples of mono- and digalactosyl diglyceride, kindly supplied by Dr. M. Kates, were chromatographed in the same solvent systems. The radioactive materials were counted with a liquid-scintillation counter and the results are given in Table 3. Discs cut from leaves grown on a complete nutrient had a greater capacity, per unit leaf surface area, to incorporate the ^{14}C of UDP-glucose-^{14}C into these two lipid fractions. In each case the activity of the lipid fraction increased with increasing time of incubation. Mono- and digalactosyl diglycerides contained a negligible amount of radioactivity. The nature of these two labeled substances and the significance of these in galactolipid synthesis are now being investigated.

(3) Previously greened lettuce leaf discs were incubated for varying periods of time with solutions containing 8-azaguanine or actinomycin D, in addition to acetate-2-^{14}C, in order to alter the rate of aging. Increased concentrations of 8-azaguanine caused a reduction in the incorporation of acetate-2-^{14}C into lipid components of the plastid (Table 4). High concentrations of actinomycin D (30 and 90 μg/ml) also caused a reduction of acetate incorporation into the lipid-soluble fraction of green leaf discs (Table 5).

Perhaps one of the most obvious indications of senescence in Angiosperms is yellowing of the leaves followed by separation of the leaf from the plant. The yellow condition reflects an alteration in the plastid membrane system. In turn, the condition of the plastid membrane

TABLE 3. INCORPORATION OF RADIOACTIVITY OF UDP-GLUCOSE-^{14}C INTO LIPID-SOLUBLE FRACTION OF LETTUCE LEAF DISCS

Treatment	Relative age of tissue	Reaction time	CPM		
			Prior to chromatography	50% of sample-substance 1*	50% of sample-substance 2†
Complete	Young	30 min	37	—	—
		3 hr	156	1115	1142
		8 hr	356	2545	3149
	Older	30 min	19	—	—
		3 hr	190	2022	1751
		8 hr	394	3113	2920
−N	Young	30 min	—	—	—
		3 hr	58	438	708
		8 hr	109	796	1143
	Older	30 min	13	—	—
		3 hr	65	418	592
		8 hr	161	829	1138

The reaction mixture was: sixty leaf discs (1 cm in dia.), 10 ml of pH 7·4, 0·2 M phosphate buffer containing 30 μmoles/ml of sodium bicarbonate, 0·25 μc/ml of UDP-glucose-^{14}C, and 30 μmoles/ml of sodium acetate. The leaf discs were incubated for 30 min, 3 hr, or 8 hr prior to extracting the lipids.

* Has R_f value near but does not correspond to monogalactosyl diglyceride.

† Has higher R_f value in both directions than substance 1.

TABLE 4. INCORPORATION OF ACETATE-2-^{14}C INTO THE LIPIDS OF LETTUCE LEAF DISCS TREATED WITH VARIOUS CONCENTRATIONS OF 8-AZAGUANINE

8-Azaguanine (mM)	Total time of treatment	Time with acetate	CPM/total extract
0	30 hr	10 hr	7565
0·05	30 hr	10 hr	6030
0·5	30 hr	10 hr	5942
5·0	30 hr	10 hr	1965
0	58 hr	10 hr	2163
0·05	58 hr	10 hr	1606
0·5	58 hr	10 hr	1270
5·0	58 hr	10 hr	1165
0	74 hr	2 hr	467
0·05	74 hr	2 hr	293
0·5	74 hr	2 hr	147
5·0	74 hr	2 hr	25

The leaf discs, 0·6 cm in diameter, were treated for 48 and 72 hr with 8-azaguanine prior to the addition of acetate-2-^{14}C (2 μc; specific activity, 20 mc/mmole). The total volume of the fluid in the incubation dish was 5 ml.

system is related to the lipid composition and physical state. It follows, therefore, that inhibitors of nucleic acid and protein synthesis would also alter the lipid composition of the plastid, since the time of appearance of the senescent state is dependent upon the rate of change of the RNA and protein fractions of the leaf.

TABLE 5. INCORPORATION OF ACETATE-2-^{14}C INTO THE LIPIDS OF LETTUCE LEAF DISCS TREATED WITH VARIOUS CONCENTRATIONS OF ACTINOMYCIN D

Actinomycin D μg/ml	CPM/total extract
0	222
10	355
30	164
90	85

The leaf discs, 0·6 cm in diameter, were treated for 72 hr with actinomycin D prior to the addition of acetate-2-^{14}C (2 μc; specific activity, 20 mc/mmole) in a total volume of 5 ml. The discs were allowed to remain with the acetate for 2 hr prior to extracting the lipids.

EXPERIMENTAL

Plant Material and Reaction Mixtures

Leaves from two species (*Lactuca sativa* L., var. Burpee's Ruby Red and *Cucurbita maxima* Duchesne, var. Burpee's Blue Hubbard) were used for this study. (1) A survey was made of the fatty acids in lettuce leaves, of varying age, from vegetative and flowering stalks. For each sample, forty leaf discs (1 cm in dia.) were selected from either side of the midrib, placed immediately in 200 ml of isopropanol and boiled for 4 min, and allowed to stand under nitrogen in the cold until extracted.[7] (2) Squash leaf discs, from relatively immature leaves, were incubated with acetate-2-^{14}C in the presence or absence of added UDP-glucose for varying periods of time (30 min to 30 hr). The reaction mixture included sixty leaf discs (1 cm in dia.) and 10 ml of pH 7·4, 0·2 M phosphate buffer containing 30 μmoles/ml of sodium bicarbonate and 10 μc of acetate-2-^{14}C (specific activity, 20 mc/mmole). Some of the flasks contained, in addition, 6·6 μmoles of UDP-glucose per flask. The contents of the flask were placed under a slight vacuum in order to cause the infiltration of the reaction mixture. The flasks were illuminated with about 13,000 lx incandescent light in a water bath at 20·3°. The leaf discs were boiled with 150 ml of chloroform–methanol (2:1, v/v) for 4 min. (3) Lettuce leaf discs, from plants grown on a complete nutrient and from plants grown on a nitrogen-deficient nutrient, were incubated in flasks containing sodium bicarbonate (30 μmoles/ml), phosphate buffer (pH 7·4, 0·2 M), UDP-glucose-^{14}C (0·25 μc/ml), and acetate (30 μmoles/ml). The total volume was 10 ml. The flasks were illuminated at 13,000 lx intensity and were contained in a water bath maintained at 20·3°.

Leaf discs were removed at varying times and extracted in chloroform–methanol (2:1, v/v). The lipid-soluble components were washed and chromatographed in thin layers of silica gel G (Merck). The UDP-glucose-^{14}C was obtained from New England Nuclear (specific activity—137 mc/mmole; radiochemical purity—98·4%; enzymatic purity—100% as determined by UDP-glucose dehydrogenase assay). The amount of UDP-galactose-^{14}C was less than 1·2 per cent. (4) Small discs (0·6 cm in dia.), from comparatively young lettuce leaves, were incubated with varying concentrations of 8-azaguanine or actinomycin D. Each petri dish contained sixty-five leaf discs, 5 ml of liquid, and one circle of Whatman No. 1 filter paper. The discs were illuminated at 13,000 lx.

[7] M. KATES, *Can. J. Biochem. Physiol.* 35, 127 (1957).

Extraction of the Lipids

The leaf discs were homogenized; the homogenate was filtered through fat-extracted, glass-fiber filter paper, and the glass-fiber paper was washed with solvent. The extract was reduced to dryness (60°, N_2), taken up in chloroform, and the chloroform extract passed through a slightly acidified layer of water. This procedure was repeated twice and the extract was taken to dryness after the addition of anhydrous sodium sulfate. The fatty acids were methylated by refluxing with dry methanolic HCl.[8]

Gas Chromatography and Counting

The fatty acid methyl esters were chromatographed isothermally (196°) using a 5 ft column of 15% Reoplex 400 on HMDS-treated Chromosorb W.[9] In most cases, the sample was passed through the column and the entire sample, except for the hydrocarbons with low retention volume, was collected in a cooled U-tube. The sample was again chromatographed and individual components collected. The sample was transferred to 20 ml liquid-scintillation vials. A dimethyl-POPOP and PPO or a BBOT scintillator in toluene was used; the counting efficiency was about 55 per cent. The lipid extracts from 8-azaguanine- or actinomycin D-treated tissue were counted directly with a thin-window G-M tube (counting efficiency, *c.* 2·1 per cent).

Thin-layer Chromatography

The lipid extracts obtained from leaf discs incubated with UDP-glucose-^{14}C were chromatographed in thin layers of silica gel G (Merck). The solvent for the first dimension was chloroform–methanol–water (65:25:4, v/v) and that for the second dimension was diisobutyl ketone–acetic acid–water (80:50:10, v/v).[9]

Acknowledgements—The author wishes to thank W. Loescher and R. Donaldson for their expert assistance. Sincere thanks are extended to Dr. M. Kates for samples of mono- and digalactosyl diglyceride and to Merck Sharp and Dohme for samples of actinomycin D.

[8] M. KATES, *J. Lipid Res.* **4**, 132 (1964).
[9] C. F. ALLEN, P. GOOD, H. F. DAVIS, P. CHISUM and S. D. FOWLER, *J. Am. Oil Chemists' Soc.* **43**, 223 (1966).