SYNTHESIS OF EPICUTICULAR PRIMARY ALCOHOLS AND INTRACELLULAR FATTY ACIDS BY TISSUE SLICES FROM *cer-j⁵⁹* BARLEY LEAVES

by

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The relative amounts of [$1-^{14}\text{C}$]-acetate incorporated by tissue slices from primary leaves of Bonus barley into intracellular and epicuticular lipids were determined. Dark grown tissue incorporates about 2.5 times more label into intracellular fatty acids than into epicuticular lipids. Light grown tissue synthesizes the same amount of epicuticular lipids but ten times more intracellular fatty acids. The same total and relative amounts of incorporation were found when tissue slices from *cer-j⁵⁹* were studied. Furthermore, no difference in the distributions of label among saturated, monoenoic, dienoic and trienoic fatty acids from the two genotypes were detected. This supports the contention that the block in *cer-j⁵⁹* is independent of intracellular fatty acid synthesis.

The locus *cer-j⁵⁹* blocks the production and use of C_{26} fatty acyl chains in the reductive pathway of epicuticular wax synthesis while increasing the relative importance of C_{22} chains. Wax on leaves of dark grown Bonus seedlings is a reasonable phenocopy of that present on *cer-j⁵⁹* light grown ones. The sites of control of wax synthesis by the locus *cer-j* and light as deduced from compositional analysis are confirmed by the present tissue slice incorporation experiments with [$1-^{14}\text{C}$]- C_{22} , C_{16} and C_{18} precursors. Although the distribution of label among the epicuticular lipid classes was not always a facsimile of the appropriate mass distribution, the distribution of label among the primary alcohols mimicked that of the corresponding mass distribution with respect to the relative importance of the C_{22} and C_{26} chain lengths. In wax synthesized by dark grown tissue the C_{22} alcohol was as well or better labelled than that with 24 carbons, whereas the latter was always more heavily labelled than the former in the wax from light grown tissue.

Abbreviations: C:M = chloroform : methanol; cpm = counts per minute; C_2 = acetate; C_{16} = palmitate; $\text{C}_{16:1}$ = palmitoleate (monoenoic); C_{18} = stearate; $\text{C}_{18:1}$ = oleate (monoenoic); $\text{C}_{18:2}$ = linoleate (dienoic); $\text{C}_{18:3}$ = linolenate (trienoic); radio-GLC = radio gas liquid chromatography; TLC = thin layer chromatography.

1. INTRODUCTION

The synthesis of plant epicuticular waxes involves fatty acid synthetase and elongases yielding the very long carbon chains as well as the enzymes of the reductive or decarboxylative pathways which give rise to the different lipid classes. Many if not all of these enzymes are possibly located in the plasmalemma and/or wall of the epidermal cells (24). Few attempts have been made to isolate them or the similar enzymes synthesizing internal waxes (18, 28, 36 and references in 16, 34). On barley leaves free primary alcohols synthesized via the reductive pathway are the major lipid class (9, 20, 33). Such alcohols are also present as moieties of the esters. Compositional studies have revealed that modifying the photoperiod under which the seedlings grow and/or the genotype leads to characteristic marked changes in the total amount as well as chain length distribution of these alcohols (9, 10, 33). As a first step toward preparing cell free systems containing the reductive or esterifying enzymes involved in alcohol and ester synthesis, we have studied the fate of labelled fatty acids fed to tissue slices from seedling leaves of the wild type Bonus and the *eceriferum* mutant *cer-j⁵⁹* grown under various photoperiods. The results presented substantiate our hope that manipulation of these two variables will prove useful tools in isolating the aforementioned enzymes. In addition to analyzing the label incorporated into the epicuticular lipids, we have simultaneously examined the amount and distribution of label present in the intracellular lipids. The latter, which include most but not all of the other lipids present, are those which can be recovered by extraction of the tissue slices subsequent to removal of the epicuticular waxes.

2. MATERIALS AND METHODS

2.1. Plant material and growth conditions

Seeds of barley (*Hordeum vulgare* L.) cv. Bonus and its mutant *cer-j⁵⁹* (19) were planted in vermiculite and germinated either in the light (14) or dark at 23 °C. Six days later the seedlings germinated in the dark were divided into two groups. At this time the primary leaves extended 7-10 cm above the coleoptile. One group of seedlings was transferred into the light, while the other group remained in the dark. Twelve hours

later the latter was again subdivided in an analogous manner. Samples were taken from the appropriate dark to light transferred seedlings at 3, 6 and/or 12 hour intervals, while those from the seedlings grown continuously in either the light or dark were taken at 6 and 7 days after planting.

2.2. Incorporation of labelled precursors

To prepare tissue slices for the incorporation experiments, the primary leaves from the various light, dark and dark to light transferred seedlings were excised just above the coleoptile and cut transversely into approximately 0.5 × 2 mm slices with a razor blade. Sufficient tissue slices were prepared so that 1 g aliquots could be weighed out for each individual experiment starting at the same time. Dark grown leaves were cut in the dark and weighed out either in the dark or in the light according to which light conditions the incorporations were to be carried out in. For whole leaf experiments the excised seedling leaves were trimmed at the base to a length of 3 cm, that is slightly less than the diameter of the erlenmeyer flask used for the incorporation. 0.5 g leaves were used per experiment, however, the results are expressed on a 1 g basis.

The tissue slices or whole leaves were added to 50 ml erlenmeyer flasks containing 5 ml water plus either 10 µCi of sodium-[1-¹⁴C]-acetate (0.177 µmoles, 56.5 mCi·mmol⁻¹), [1-¹⁴C]-palmitic acid (0.175 µmoles, 59.0 mCi·mmol⁻¹), [1-¹⁴C]-stearic acid (0.178 µmoles, 55.7 mCi·mmol⁻¹) or 3 µCi of [1-¹⁴C]-palmitoyl-CoA (0.05 µmoles, 60.0 mCi·mmol⁻¹) or [1-¹⁴C]-stearoyl-CoA (0.06 µmoles, 50.4 mCi·mmol⁻¹). All isotopes were purchased from the Radiochemical Centre, Amersham, England. The long chain fatty acids were dispersed in water (5 ml containing an equivalent amount of NaOH) by sonicating at full power 3 × 30 sec with a PG 100 MSE Scientific Instruments (Sussex, England) Model 150 W ultrasonic disintegrator. The flasks were shaken continuously for 2 hours. During this time the temperature of the water bath was held at 25 or 20 °C as specified. When the incubation was carried out in the light 24,000 lumen were supplied by an Osram HQI-E, 400 W/D lamp. For dark incubations the flasks were completely covered with a layer of black paper plus a second layer of aluminium foil. Three combinations of

photoperiods were used: light/light, dark/light and dark/dark where the first word refers to the light condition during growth of the seedlings and the second to that during incubation of the tissue slices.

2.3. Isolation of lipids

The epicuticular waxes were recovered by terminating the reaction with the addition of 100 μ l 10 N H_2SO_4 and shaking for 30 sec with 15 ml of chloroform. The chloroform extracts were concentrated under N_2 . In those experiments where the long chain substrates were used, the dried chloroform extracts were taken up in 2 ml of benzene and applied to Sep-pak Silica Cartridges (Water Associates Inc., Mildford, Massachusetts). Four fractions were eluted using: 20 ml benzene, 10 ml benzene, 20 ml chloroform:methanol (1:1, v/v) and 10 ml of the same chloroform:methanol solution, hereafter referred to as C:M. Thin layer chromatographic (TLC) analyses showed that the first fraction contained all the wax classes except the free acids which were present in the first C:M fraction (Figure 1). To ensure that no lipids were lost, however, the first two fractions were combined as were the last two. Passing 5 x 3 ml of C:M plus 5 x 3 ml of benzene through the Sep-pak regenerated it for the next sample.

After extraction of the epicuticular waxes, the tissue slices were transferred to a mortar and the internal lipids were recovered as follows. Excess water was removed from the mortar with a Pasteur pipette and the tissue slices homogenized with a pestle. Thereupon 20 ml of C:M were added and the extracted internal lipids were recovered at the end of an hour by the Bligh and Dyer method (5). The fatty acids of the complex lipids were converted to their methyl esters by transesterification using 10% BF_3 -methanol at 90 °C for 2 hours. The resulting total internal fatty acid methyl esters in benzene were purified with the aid of a Silica Sep-pak (Mikkelsen and Kanangara, unpublished). That is, the methyl esters were recovered from the Sep-pak cartridge by passing through it several aliquots of benzene totalling approximately 10 ml. All pigments as well as several minor, labelled, unknown compounds retained by the Sep-pak could then be eluted with

Figure 1. Radio-TLC traces of fractions eluted from a Sep-pak cartridge of ^{14}C -labelled epicuticular wax.

The wax was isolated from tissue slices prepared from six day old, light grown primary leaves after incubation with [$1-^{14}C$]-stearoyl-CoA for two hours at 25 °C. The TLC plate was first developed in hexane and thereafter in amylene stabilized chloroform. See Material and Methods (2.4) for additional information. Upper trace is first benzene fraction. Lower trace is first chloroform:methanol fraction. O = origin. FA = fatty acids. POH = primary alcohols. SH EST / ALD = short esters and possibly a trace of aldehydes (see 3.1). EST = Esters. 1, 2, and 3 = unknowns that are neither free acids, hydrocarbons nor primary alcohols, respectively.

two 10 ml volumes of C:M, and the Sep-pak regenerated as described above.

2.4. Analyses of the epicuticular waxes and internal lipids.

Subsequent to counting aliquots of the isolated epicuticular waxes (10 μ l/ml), the wax was applied to 5 x 20 cm preparative silica gel H type 60 (Merck) TLC plates which were developed first in hexane and then in amylene stabilized chloroform (23). The lipid classes were located on the

plates and the amount of label present quantitated as described previously (25) except that Insta-Fluor (Packard) was used as scintillant. The primary alcohols were recovered from replicated preparative TLC plates. They were then applied to new TLC plates and their acetate derivatives synthesized (32). The latter were purified by developing the plates with amylene stabilized chloroform. Radio-gas liquid chromatography (radio-GLC), separation and identification of the alcohol acetates with standards plus quantification were carried out as described (23) with the subsequent modifications. The flows of the radio-GLC were adjusted so that the helium carrier gas flow from the splitter to the flame ionization detector was $2.2 \text{ ml} \cdot \text{min}^{-1}$ and from the splitter to the proportional counter was $19.1 \text{ ml} \cdot \text{min}^{-1}$. Make up helium gas flow to the flame ionization detector was $21.7 \text{ ml} \cdot \text{min}^{-1}$. The flame ionization detector was operated at hydrogen and air gas flows of 29.1 and $250 \text{ ml} \cdot \text{min}^{-1}$, respectively, to give the optimal sensitivity. Quenching gas (propane) for the proportional counter was regulated by a Model 8286 pressure regulator (Porter Instrument Company, Halfield, Penn., USA) to $2.8 \text{ ml} \cdot \text{min}^{-1}$. The high voltage setting was 1650 volts. A stainless steel column, $162 \text{ cm} \times 2 \text{ mm}$, packed with 5% SE30 Anakrom ABS 110/120P (Analabs, Conn., USA) was used. The column temperature was programmed from 200°C at a rate of 1 or $2^\circ \text{C} \cdot \text{min}^{-1}$ to 280°C at which temperature it was held for 10 min. The injector port was maintained at 300°C and the flame ionization detector at 340°C .

The fatty acid methyl esters were separated into trienoic, dienoic, monoenoic and saturated constituents by preparative AgNO_3 -TLC (Figure 2). The plates were prepared by making a slurry of Silica gel GF₂₅₄ type 60 (Merck): $\text{AgNO}_3 \cdot \text{H}_2\text{O}$ (45:7.5:100, w/w/v). Mixtures of palmitate, stearate, oleate, linoleate and linolenate as methyl esters, purchased from Supelco (Bellefonte, Penn., USA), served as standards. The developing system was chloroform (Puranal, Riedel-de Hen, Hannover, W. Germany): 96% ethanol:acetic acid (150:1:0.1, v/v/v). Four techniques were used to conclusively identify the labelled fatty acids: (i) co-chromatography with standards on TLC plates coated with silica gel H type 60 and those containing AgNO_3 as de-

Figure 2. Separation of the ^{14}C -labelled fatty acid methyl esters derived from the complex lipids into tri-enoic (T), di-enoic (D), mono-enoic (M) and saturated (S) groups using radio- AgNO_3 -TLC as detailed in 2.4. O = origin.

tailed above; (ii) co-chromatography with standards on 5% SE30 as well as 10% Silar 10C on 100/120 mesh Gas Chrom Q and 15% HI EFF-2BP on 80/100 mesh Chromosorb W/AW (Applied Science, Penn., USA) 2 mm GLC columns which were 162, 152 and 162 cm long, respectively; (iii) hydrogenation (1) of the unsaturated fatty acids to yield the expected saturated homologues; and (iv) reductive ozonolysis (30) of the unsaturated fatty acids to yield the same derivatives as did standards when subjected to GLC using the 10% Silar-10C and 5% SE30 columns. The same radio-GLC and conditions were used as described above except that the temperature program was modified to best suit the fatty acid derivative or moiety thereof being studied. The distribution of label along the carbon chains of the purified fatty acids was determined with the aid of α -oxidation (23).

3. RESULTS

3.1. Effect of light on the incorporation of $[1-^{14}\text{C}]$ -acetate into the epicuticular wax by Bonus and *cer-j*⁵⁹ barley leaf tissue slices.

The data presented in Table I indicate that tissue slices prepared from dark and light grown primary seedling leaves of Bonus barley incorporate approximately the same amount of label from acetate into the wax lipids. When all similar

Table I

Effect of light on the ability of tissue slices prepared from Bonus primary leaves to incorporate $[1-^{14}\text{C}]$ -acetate into leaf wax lipids^a.

Illumination during			Total label in wax (cpm × 10 ⁻⁶)	Wax class (% total cpm)						
Growth		Incor- poration		Hydrocarbons	Esters	Short esters + aldehydes	Primary alcohols	Fatty acids	Un- knowns	
Days	Hours									
Dark	Light	Light								
6 ^b	+	0	Dark	1.4	—	13.1	—	60.3	26.6	—
6	+	0	Light	1.0 ^c	0.9	11.0	4.2	74.9	9.0	—
7	+	0	"	1.3	0.4	14.7	5.5	56.2	23.2	—
6	+	3	"	1.5	0.4	13.9	5.3	62.7	17.7	—
6	+	9	"	1.3	0.5	15.4	6.4	61.9	15.8	—
6	+	15	"	1.1	0.4	14.7	8.1	59.2	17.6	—
6	+	21	"	1.1	0.3	12.5	12.2	51.5	23.5	—
6	6	0	"	0.9	0.4	11.8	4.6	66.3	13.8	3.1
7	7	0	"	1.5	0.8	21.0	11.1	46.6	20.5	—

^a All incorporations were carried out as described in section 2.2.

^b The reported values for this treatment were carried out as part of another experiment.

^c Mean value of eight analogous experiments was 1.3; range 1.0 to 1.5.

— = not detected.

experiments are considered, however, tissue slices from dark grown leaves often incorporate considerably more label than do those from light grown ones, as illustrated in Table II. The opposite situation was never encountered. Exposing dark grown leaves to light for varying lengths of time before preparing the tissue slices had no dramatic effect on the level of acetate incorporation; neither did carrying out incorporations in the dark. The data in Table II demonstrate, however, that the amount of wax synthesized by tissue slices is dependent upon the temperature at which the incorporation is carried out. Analogous results were obtained with tissue slices from *cer-j⁵⁹* leaves (Tables II and III), except that the relative amount of label incorporated was significantly lower. This agrees with the earlier observations that light and dark grown leaves of *cer-j⁵⁹* have only 26 and 43%, respectively, as much wax $\cdot \text{cm}^{-2}$ as the corresponding wild type leaves (10).

On the basis of previous analyses of the ability of tissue slices from yellow and green portions from a developing maize, *Zea mays*, leaf to incorporate acetate into the wax (3), the present lack of a marked effect of the photoperiod during growth of the tissue was unexpected. Moreover why should tissue slices from dark grown barley

leaves incorporate acetate into wax as well as those prepared from the corresponding green leaves? From measurements of the amount of wax present on dark, light and dark to light transferred Bonus seedling leaves, the following rates of wax synthesis had been deduced: 6, 15 and 46 $\mu\text{g} \cdot \text{cm}^{-2} \cdot 24 \text{ h}^{-1}$, respectively. The higher rate for the transferred leaves was only maintained for 24 h, that is, until the amount wax $\cdot \text{cm}^{-2}$ had reached that characteristic for light grown leaves (9).

In seeking an explanation for these conflicting results, we compared the ability of tissue slices

Table II

Effect of temperature on the incorporation of $[1-^{14}\text{C}]$ -acetate in the light into waxes by tissue slices prepared from six day old Bonus and *cer-j⁵⁹* barley leaves grown either in the light or darkness (cpm $\times 10^{-6}$).

°C	Bonus		<i>cer-j⁵⁹</i>	
	Seedling growth light	dark	Seedling growth light	dark
25	0.8	1.5	0.5	0.7
20	0.4	0.5	0.1	0.2

Table III

Effect of light on the ability of tissue slices prepared from *cer-j⁵⁹* primary leaves to incorporate [1-¹⁴C]-acetate into leaf wax lipids^a.

Illumination during			Total label in wax (cpm × 10 ⁻⁶)	Wax class (% total cpm)				
Growth		Incor- poration		Hydrocarbons	Esters	Short esters + aldehydes	Primary alcohols	Fatty acids
Days	Hours							
Dark	Light							
6 ^b	+ 0	Dark	0.7	—	19.6	—	19.7	50.9
6	+ 0	Light	0.5	—	28.4	6.3	32.0	33.3
7	+ 0	"	1.2	—	14.1	3.3	52.2	30.4
6	+ 6	"	0.7	—	11.4	6.9	45.2	36.5
6	+ 12	"	0.7	—	33.1	11.0	20.0	35.9
6	+ 18	"	0.5	—	25.4	19.5	25.8	29.0
	6 + 0	"	0.6	—	35.9	9.6	21.9	32.6
	7 + 0	"	0.4	—	30.4	11.9	23.3	34.4

^a All incorporations were carried out as described in section 2.2.

^b The reported values for this treatment were carried out as part of another experiment.

— = not detected.

and excised whole leaves to incorporate acetate into the wax. Very interestingly whole leaves were able to incorporate just as much label into the wax as were tissue slices prepared from them: for example, dark grown Bonus whole leaves and tissue slices incorporated 44 and 42 × 10⁻⁴ cpm, respectively, while light grown *cer-j⁵⁹* whole leaves and tissue slices incorporated 28 and 21 × 10⁻⁴ cpm, respectively. Why in these particular experiments the level of incorporation is so much less than in the experiments presented above is unknown. Nevertheless, these results imply that acetate is taken up primarily by intact epidermal cells and not just through the cut surfaces of the tissue slices. Support for this contention was derived from the appearance of the whole leaves during the incorporation. Occasional drops of liquid were observed on the upper surface of the floating light grown green leaves suggesting that the surface of this tissue repels the incubation medium. No drops were visible on the upper surface of the dark grown yellow leaves. Rather, the latter appeared to be completely submerged in the incubation medium. This clear difference in the light and dark grown leaves is lost when tissue slices are prepared.

No marked differences in the distribution of label among the wax classes isolated from tissue slices prepared from leaves of Bonus (Table I) or *cer-j⁵⁹* (Table III) exposed to the various il-

lumination conditions were observed. For Bonus the radioactive distributions are dominated by the primary alcohols, followed in order of decreasing amounts by the free acids, esters and aldehydes plus short esters. In this experiment although less than 1% of the label was present in the alkanes, it is the highest amount detected in this wax class. The overall distribution corresponds reasonably well with the ¹⁴C distribution among these lipid classes when Bonus seedling leaves were allowed to take up acetate through their cut ends (9). When compared with the mass distribution (9), neither the earlier nor the present ¹⁴C distributions have quite enough label in the primary alcohols. Furthermore, the labelled aldehyde or aldehyde plus short ester class is more prominent which is analogous to observations using tissue slices from spikes (22). In the latter paper the observation was made that while short esters are not detectable in vivo, they are often synthesized by tissue slices. Should aldehydes be present at all in the present experiments, it must be in trace amounts since no reaction with Schiff's reagent was obtained. It was outside the objective of the present study to pursue this question further.

In *cer-j⁵⁹* a different radioactive distribution from that found for Bonus was observed (Table III) in that the esters and free acids as well as the primary alcohols are highly labelled. No consis-

tent modification of the distribution was brought about by modifying the photoperiod. While a higher proportion of the label was found in the free acids of *cer-j⁵⁹* than Bonus tissue slices, the total cpm are somewhat greater in the latter than in the former. It is tempting to conclude from this difference between the mutant and the wild type that more chains are being elongated in *cer-j⁵⁹* than can be used by the enzymes of the associated reductive pathway. That no label was detectable in the alkanes of *cer-j⁵⁹* tissue slices was unexpected since alkanes form a larger percentage of this wax than Bonus wax (10). Why this should be is unknown.

3.2. Effect of light on the radioactive distribution among the primary alcohols isolated from Bonus and *cer-j⁵⁹* tissue slices.

In contrast to the above results, light has a marked effect on the distribution of label incorporated from [^{14}C]-acetate into primary alcohols of both genotypes (Tables IV and V). While the C_{26} chain length dominates the distribution obtained from wild type leaf tissue grown under all light conditions (Table IV), it represents only 65% of the alcohols from dark grown leaves and 80 to 93% of those from leaves exposed to six or more hours of light. The increased labelling of C_{26} alcohol is accompanied by decreases of the

Table IV

Effect of illumination on the incorporation of [^{14}C]-acetate into primary alcohols by tissue slices prepared from Bonus primary leaves^a.

Number of carbons	6 days dark grown + (hours of light)						6 days light grown
	0	3	6	9	12	18	
	(% cpm) ^b						(% cpm) ^b
20	3.0	1.4	—	—	—	—	—
22	18.8	10.7	2.1	6.3	1.9	3.2	—
24	10.9	11.1	4.1	8.7	4.7	10.1	5.4
26	64.6	73.5	93.8	80.3	93.4	83.0	90.5
28	2.6	3.3	—	4.7	—	3.7	4.1

^a Primary alcohols were analyzed by radio-GLC as described in section 2.4.

^b The reported distributions are based on 12 to 15 cpm $\times 10^{-3}$.

Table V

Effect of illumination on the incorporation of [^{14}C]-acetate into primary alcohols by tissue slices prepared from *cer-j⁵⁹* primary leaves^a.

Number of carbons	6 days dark grown + (hours of light)				6 days light grown
	0	6	12	18	
	(% cpm) ^b				(% cpm) ^b
20	6.0	3.7	3.2	—	2.9
22	43.2	26.8	22.8	20.2	16.8
24	30.4	34.2	47.6	42.8	45.3
26	20.4	32.2	26.4	37.0	35.0
28	—	3.1	—	—	—

^a Primary alcohols were analyzed by radio-GLC as described in section 2.4.

^b The reported distributions are based on 6 to 10 cpm $\times 10^{-3}$.

— = not detected.

shorter alcohols, especially those with 20 and 22 carbons. The ^{14}C distributions from both light and dark grown leaves are almost identical to the analogous earlier determined mass (wt %) distributions (9). Regardless of the photoperiod the C_{26} chain length does not dominate the alcohol spectra of any of the investigated *cer-j⁵⁹* waxes (Table V) but accounts for only 20% and 35% of this lipid class from dark and light grown leaves, respectively. In the wax from *cer-j⁵⁹* dark grown leaves as in the Bonus waxes, the C_{22} alcohol is better labelled than the C_{24} alcohol. Unlike Bonus however, *cer-j⁵⁹* leaves exposed to light incorporated 45% of the label into the alcohol with 24 carbons. Whereas the radioactive distribution from *cer-j⁵⁹* light grown leaves is similar to the earlier reported mass distribution (10), a discrepancy occurs between the analogous distributions from dark grown leaves. Compare the radioactive distribution (Table V) with the mass distribution of $\text{C}_{22} = 60\%$, $\text{C}_{24} = 19\%$, and $\text{C}_{26} = 5\%$ (10). Interestingly, the ^{14}C distribution for alcohols from dark grown leaves (Table V) is quite similar to the mass distribution of the alcohols isolated from *cer-j⁵⁹* leaves grown in the dark at 23 °C (10). Perhaps the fact that the present tissue slice incorporations were carried out at 25

°C has influenced the *cer-j⁵⁹* results. No difference was found between the composition of the alcohols isolated from Bonus leaves grown at 15/10 °C and 23 °C in the dark (9).

3.3. Effect of light on the incorporation of the long chain precursors into the primary alcohols of Bonus and *cer-j⁵⁹* epicuticular waxes.

Tissue slices from leaves of both genotypes were able to incorporate label from palmitic acid, stearic acid and the coenzyme-A derivatives of these two acids into the epicuticular wax lipids. Not only was the apparent total incorporation extremely variable (10-90%), but anywhere from 37-98% of the recovered label was present in the free fatty acid lipid class. While no attempt was made to investigate whether the C₁₆ and C₁₈ precursors had been elongated, probably only a small percentage at best of the label recovered in the free acids would have been present in C₂₀ and longer chains. Only when stearyl-CoA served as the precursor was a consistent effect of light observed, namely, a higher apparent incorporation took place when the tissue slices were prepared from light than from dark grown leaves (Bonus, 89 vs 60% and *cer-j⁵⁹*, 87 vs 37%, respectively). Since this observation is contrary to that obtained when acetate was used as a substrate,

Table VII

Percent distributions of radioactivity in the primary alcohols synthesized in the light from long chain coenzyme-A derivatives by tissue slices prepared from light and dark grown primary leaves of Bonus barley^a.

Number of Carbons	[1- ¹⁴ C]-palmitoyl-CoA		[1- ¹⁴ C]-stearyl-CoA	
	Dark	Light	Dark	Light
18	tr	—	9.2	—
20	12.8	—	3.8	1.4
22	47.4	6.5	16.4	4.5
24	12.0	11.9	17.4	10.5
26	27.8	81.6	53.2	83.6

^a The radioactive distributions are based upon 10 to 15 cpm × 10⁻³.

— = not detected.

tr = trace (<100 cpm).

however, it is more likely due to chance than to a real effect of photoperiod. Label was never recovered in the hydrocarbons and only trace amounts were detected in aldehydes. While sufficient radioactivity for further analyses was recovered in most cases in both the primary alcohols and esters, the distribution of label among the homologues of only the former lipid class was investigated.

Table VI

Percent distributions of radioactivity in the primary alcohols synthesized in the light from long chain fatty acids by tissue slices prepared from light and dark grown primary leaves of Bonus and *cer-j⁵⁹* barley^a.

Number of carbons	[1- ¹⁴ C]-palmitic acid				[1- ¹⁴ C]-stearic acid			
	Bonus		<i>cer-j⁵⁹</i>		Bonus		<i>cer-j⁵⁹</i>	
	Dark	Light	Dark	Light	Dark	Light	Dark	Light ^b
16	tr	—	1.6	—	—	—	—	—
18	tr	—	1.6	—	3.2	3.7	7.4	8.6
20	14.7	—	16.5	3.0	8.6	2.0	10.7	4.1
22	55.0	9.0	58.6	23.6	33.2	6.0	52.1	20.0
24	17.5	30.7	16.0	56.4	15.8	17.0	20.8	45.8
26	12.8	60.3	5.7	17.0	39.2	71.3	9.0	15.7

^a The radioactive distributions are based upon 10 to 15 cpm × 10⁻³.

^b 5.8% of the radioactivity was present in an unknown eluting between the C₁₈ and C₂₀ alcohols.

— = not detected

tr = trace (<100 cpm)

Regardless of which of the four long chain substrates was presented to tissue slices prepared from light grown Bonus leaves, the C₂₆ alcohol contained the majority (60-84%) of the label (Tables VI and VII). The distribution of radioactivity among the alcohols synthesized by tissue slices from dark grown Bonus leaves, however, depended upon the precursor used (Tables VI and VII). When palmitic acid and palmitoyl-CoA were used, the C₂₂ alcohol was best labelled (55 and 47%, respectively) which is in marked contrast to the results when acetate was used as a precursor (Table IV). In the latter case C₂₆ had 65% of the label and C₂₂ only 10%. By contrast stearic acid and stearoyl-CoA were less well incorporated into the C₂₂ alcohol (33 and 16%, respectively) while higher amounts of label (39 and 53%, respectively) were found in the C₂₆ alcohol. The latter distributions were similar to those obtained with acetate as the precursor. Whether the observed differences between the C₁₆ and C₁₈ precursors are significant can not be determined from the presently available data. In all cases when the dark grown Bonus seedlings were illuminated for 12 hours before carrying out the

incorporation experiment, the distribution of label among the primary alcohols resembled that obtained when using tissue slices from light grown leaves.

Tissue slices prepared from dark grown *cer-j⁵⁹* leaves incorporated both acids (Table VI) as well as their coenzyme-A derivatives predominantly into the C₂₂ alcohol (52-59%). When the tissue slices were prepared from light grown *cer-j⁵⁹* leaves, the C₂₄ alcohol was most heavily labelled (46-56%; data for palmitoyl-CoA not available). While these results appear in good agreement with the distribution of label found among the alcohols when acetate was the precursor (Table V), it should be noted that when the latter was used, relatively more label (roughly 15%) was present in the C₂₆ alcohol irrespective of the photoperiod in which the leaves were grown.

3.4. Effect of light on the incorporation of [1-¹⁴C]-acetate into intracellular lipids by tissue slices prepared from primary leaves of Bonus and *cer-j⁵⁹* barley

In marked contrast to the photoperiodic insensitivity of the amounts of acetate incorporated

Table VIII

Percent distributions of radioactivity incorporated from [1-¹⁴C]-acetate into the intracellular fatty acids by tissue slices from the primary leaves of Bonus and *cer-j⁵⁹* barley^a.

Genotype	Illumination		Total (cpm × 10 ⁻⁶)	Incorporation into fatty acids (% ^b)			
	1st 6 days	+ Hours light		Saturated	Monoenoic	Dienoic	Trienoic
Bonus	Dark	0	3.3	32.6	46.5	16.0	3.3
<i>cer-j⁵⁹</i>	"	0	1.8	33.3	46.6	17.0	3.1
Bonus	"	6	2.8	28.3	33.3	32.8	5.6
<i>cer-j⁵⁹</i>	"	6	5.4	26.4	39.2	30.2	4.2
Bonus	"	9	5.9	20.2	29.2	43.6	7.9
Bonus	"	12	5.2	23.3	30.9	40.5	5.4
<i>cer-j⁵⁹</i>	"	12	7.8	22.8	29.2	40.2	7.9
Bonus	"	18	12.9	24.0	29.1	41.0	5.7
<i>cer-j⁵⁹</i>	"	18	8.3	22.4	28.2	40.0	9.6
Bonus	"	21	10.4	18.8	30.3	44.6	6.4
Bonus	Light	0	11.0	24.2	43.4	29.7	2.9
<i>cer-j⁵⁹</i>	"	0	14.5	23.4	40.9	32.2	3.4

^a Procedures for extraction of the internal fatty acids subsequent to collection of the epicuticular lipids as well as preparation of the methyl ester derivatives and analyses thereof are detailed in section 2.

^b Saturated = C₁₆>C₁₈>C₂₀; Monoenoic = 18:1>16:1; Dienoic = 18:2; Trienoic = 18:3.

into the various lipid classes by tissue slices as described above, labelling of the intracellular fatty acids was highly dependent upon the photoperiod to which the leaves had been exposed (Table VIII). Tissue slices prepared from light grown Bonus seedlings incorporated several fold more label into intracellular fatty acids than those prepared from dark grown seedlings. The tissue slices from dark and light grown leaves incorporated approximately two and a half and ten times as much label, respectively, into the intracellular fatty acids as into the epicuticular lipids (Tables I and VIII). Six to nine hours after transferring dark grown seedlings to the light (Table VIII), the capacity of tissue slices prepared therefrom to synthesize intracellular fatty acids was doubled. The maximum synthetic capacity was found for tissue slices from leaves exposed to light for 15-18 hours. The described stimulatory effect of light on intracellular fatty acid formation confirms earlier results (2).

Not only the amount but also to some extent the type of fatty acids synthesized was influenced by the photoperiod to which the leaves had been exposed. As shown in Table VIII, when seedlings were grown in the dark only 16% of the label was present in dienoic acids. Growth of the seedlings in continuous light resulted in 30% of the label occurring in dienoic fatty acids at the expense of the saturated and monoenoic fatty acids. These small shifts in the labelling pattern which appear attributable to the photoperiod were expected since the reported (15) *in vivo* fatty acid compositions of the dark and light grown barley seedling leaves vary in a related manner (saturated 20, 17; monoenoic 6, 2; dienoic 19, 15; and trienoic 55, 66%, respectively). The only difference is that in the *in-vivo* comparison it is 18:3 instead of 18:2 that has been elevated by the light. On the other hand, that the tissue slices from neither the light nor dark grown leaves incorporated more than 3% instead of the majority of the label into the trienoic fatty acid (18:3) is a well established phenomenon. In this connection it is quite interesting that transferring dark grown seedlings to the light for some hours before preparation of tissue slices stimulated incorporation into dienoic and trienoic fatty acids. After six hours of exposure to light, the tissue slices incorporated into the dienoic acids as much label (ca. 30%) as did tissue

slices from light grown leaves. Exposure of the leaves for 9-18 hours increased the proportion of label in the dienoic acids to a minimum of 40% of the total. The amount of labelled trienoic acids synthesized by tissue slices from 9-18 hour light exposed leaves was two to three times greater than that synthesized by tissue slices from leaves grown continuously in either the dark or light. This stimulatory effect on the polyunsaturated fatty acids is not visible in the data presented in an earlier study, where the fatty acids of the "phospholipid, phospholipid + sulfolipid, digalactosyldiglyceride and monogalactosyldiglyceride"s are examined separately (2). A more recent study (27), however, gave results remarkably similar to ours when dark grown barley seedlings were exposed to 9 hours of low light and then returned for 15 hours to the dark before carrying out the incorporation for 5 hours.

The three unsaturated 18 carbon fatty acids were isolated via preparative radio- AgNO_3 -TLC. Hydrogenation followed by chemical α -oxidation of each alone showed unequivocally that they were all synthesized *de novo*, irrespective of the photoperiod to which the Bonus leaves had been exposed.

The ability of tissue slices from *cer-j*⁵⁹ to incorporate acetate into the intracellular fatty acids is shown by the data in Table VIII to be identical to that of the wild type. No significant differences in either total amount of label incorporated or distributions thereof among the various types of fatty acids occurred. Furthermore, we again found that the 18 carbon unsaturated fatty acids had been synthesized *de novo* in all cases. This supports the contention derived earlier from studying only epicuticular wax composition of leaves on plants of the two genotypes (10, 33) that the locus *cer-j* is blocked in the synthesis and use of the much longer C_{26} chains normally produced by the reductive pathway of epicuticular wax biosynthesis.

4. DISCUSSION

The critical role played by light in the development of the membrane systems of chloroplasts is a well established phenomenon. It is therefore not surprising that synthesis of the polyunsaturated fatty acids, localized mainly within chloroplast membranes where they are esterified primarily

with galactolipids, is also strongly influenced by the photoperiod. No generalization can be made at present as to the precise point(s) that light exerts its effect(s), except that many different ones have been identified in the numerous plant systems examined. Nevertheless it is clear that in barley light is not the primary factor in controlling 18:3 synthesis. When the abilities of tissue slices from light and dark grown leaves to synthesize intracellular fatty acids were compared, the major observed effect of light was to increase the total amount of these lipids. Only a slight shift in the proportion of the dienoic chains at the expense of the saturated and monoenoic chains occurred. This small difference was reasonable since the *in vivo* compositions are also quite similar (see section 3.4). That light does not markedly increase polyunsaturated fatty acid synthesis at the expense of the saturated and monoenoic chains leads us to the inference that when the membranes of the etioplasts are synthesized during elongation of the barley leaves in the dark, approximately the same proportions of the various fatty acids are needed therein as in the membranes of normally greened barley chloroplasts.

More intracellular fatty acids as well as epicuticular waxes are present in or on, respectively, light than dark grown barley leaves (9, 15). While this same relationship, as noted above, was observed with respect to the ability of tissue slices to synthesize the intracellular fatty acids, it does not hold for epicuticular wax synthesis. We will not detail here the effects of light on epicuticular wax composition, as this has been done recently (3, 35). Instead we would like to focus on the the just mentioned anomaly. Observations made while seeking an explanation thereof led us to the conclusion that acetate was being taken up primarily by the intact epidermal cells and presumably more efficiently by those of the dark than light grown tissue. In retrospect this might have been predicted from the available knowledge of firstly, the fewer, less complex wax bodies on dark versus light grown barley leaves (VON WETTSTEIN-KNOWLES, unpublished) plus the effect of wax body structure on the retention of water droplets by leaf surfaces (for example see 11, 26). Secondly, when a suspension of ^3H -labelled C_{30} fatty acid was applied to the surface of leaves on intact *Brassica oleracea* plants, some of the labelled

acid was converted into the epicuticular wax hydrocarbons (C_{29}) and ketones while a lesser amount was recovered among the internal lipids (17). Additional data supporting this movement of long chain fatty acids from the leaf surface into the leaf cells has been presented (6). In the latter study ^{14}C -labelled C_{18} and C_{24} fatty acids were applied to the surface of *Allium porrum* leaves.

On the other hand, the possibility exists that the structure of one or more of the cuticle subunits, not including the epicuticular wax, may also contribute to the observed apparent rates of wax synthesis by the tissue slices from the light and dark grown barley leaves. If this is true one might expect to find compositional differences. In the following we present a few selected examples of the latter which support this inference. Within the cuticle of plants one finds as the major structural component the relatively polar polyesters of hydroxy and epoxy fatty acids referred to as cutin. Therein are embedded less polar soluble lipids such as fatty acids and alcohols (4, 8), often referred to as intracuticular waxes, and perhaps other minor components (13). The locations and polarities of these cuticle subunits presumably influence which molecules and how many thereof can pass through. Although probed at increasingly finer levels of resolution during recent years, no universal model of the cuticle substructure has appeared. Rather one is struck by the enormous diversity (see 12). Furthermore, even in those instances where the substructure appears similar as in *Agave* and *Clivia*, agreement has not yet been reached as to what the substructures actually represent. The lamellae of the cuticular membrane serve as an illustration. A frequently mentioned idea is that a correspondence exists between the relatively electron-translucent layers and the soluble lipids vs the more electron-opaque layers and the cutin (see 12). While a very clear discussion of some of the difficulties in accepting the first part of the idea has been presented (31), the only alternative suggestion offered is that the more electron-translucent layers represent interstices. A quite different explanation, namely that the observed lamellation is attributable to layers of cutin differing in polarity has been put forth recently (21, 29).

More pertinent to the present question,

however, was the deduction in the latter study that the cutin from the studied young and mature *Clivia miniata* leaves differed chemically. This was based on a comparison of the permeability to water of chloroform-methanol extracted cuticles; those from young leaves being the more permeable. In *Vicia faba* an analysis of the positional isomers present in the cutin also revealed chemical differences (7). A dependence was found of the leaf cutin composition upon leaf age and of that from the stem upon the photoperiod. Very little is known about the soluble lipids of the cuticle. By analogy to our present understanding of epicuticular lipids, the intracuticular lipids are also thought to have a marked influence on cuticular permeability. By similar reasoning we predict that they will be present in larger amounts in the cuticle of light grown than in dark grown leaves. If they have similar chain lengths to the lipids of the epicuticular waxes, they may also differ in chain length composition. The latter proposal is based on the very likely marked similarity if not, in fact, identical biosynthetic mechanism being involved to that yielding the epicuticular lipids. The use of the term biosynthetic mechanism here does not include the aspect of the transport of the lipids from their site of synthesis to their final destination. In connection with the latter, we should like to mention that if the cuticles of the light and dark grown leaves do indeed vary in composition and/or structure such differences may also be influencing the ease with which the synthesized epicuticular lipids arrive on the surfaces of the tissue slices.

While the particular explanation presented above appears to adequately account for the higher than expected level of acetate incorporation into epicuticular waxes by tissue slices from dark grown leaves, it does not concur with the observed higher level of intracellular fatty acid synthesis by tissue slices derived from light versus dark grown leaves. Thus we must conclude that other factors such as the relative synthetic rates of the specified pathways, pool sizes and/or the ease by which the lipids concerned are transported to their destinations may be more important than the actual amounts of acetate entering the tissue in determining the observed rates of epicuticular wax synthesis. Since we have no data bearing on this question at present we defer a

discussion of the various possibilities until that time when such information is available.

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