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# EFFECTS OF LIGHT AND TEMPERATURE ON THE COMPOSITION OF EPICUTICULAR WAX OF BARLEY LEAVES

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**Key Word Index**—*Hordeum vulgare*; Gramineae; barley leaves; epicuticular wax formation; chemical composition; influence of light and temperature.

**Abstract**—The amount of wax/cm$^2$ on expanding primary leaves of Bonus barley depends on both the photo- and thermoperiods in which the seedlings are grown. With a temperature cycle of 15–10°, transfer of dark grown leaves to the light stopped leaf expansion and after 24 hr yielded 2·5 times more wax/cm$^2$ than is characteristic for light grown leaves. This demonstrates that wax synthesis and extrusion is not directly correlated with leaf expansion. The relative amounts of the wax classes formed by the decarboxylation pathways (< 1%), the reductive pathways (89%) or only by elongation (10%) are the same in light and dark. Within the reductive pathways, however, light stimulates aldehyde formation. Both environmental parameters can strongly influence the chain length composition of the wax classes. In the light one chain length or one group of chain lengths dominates a given wax class. In the dark two prominent chain lengths or groups thereof are found. The major chain length in these two groups differs by two or more carbons.

## INTRODUCTION

Earlier studies have yielded conflicting results on the influence of light on the formation of epicuticular wax on leaves. For example, development of wax structures as seen by electron microscopy on leaves was shown to be light dependent in the pea [1] but not in barley [2]. More than species differences are involved, however. Using labelled precursors, Kolattukudy [3,4] found that light had no affect on the synthesis of hydrocarbons in *Brassica*, whereas Macey [5] observed that light increased synthesis of hydrocarbons and secondary alcohols at the expense of aldehydes and esters. Formation of the ketones was almost light independent in the latter study. On the other hand, Netting [6], also working with *Brassica*, found that light stimulated synthesis of the ketones but not the hydrocarbons.

Temperature has been shown to affect wax production on leaves. For example, an electron microscopic study of three ecotypes of *Eucalyptus viminalis* Labill revealed a temperature dependent variation in the quantity of wax deposited on the leaves [7]. Marked differences not only in the amount of wax but also in the structure of the wax bodies occurred on leaves of *Brassica napus* grown under different temperatures [8].

In the present study the influence of light and temperature on the synthesis of epicuticular wax was investigated using intact seedling leaves of barley in order to provide a basis for labelling studies of wax biosynthesis.

## RESULTS

### *Amount of wax*

On leaves grown in the light at 15–10°, the amount of wax/cm$^2$ leaf remains constant (11 μg, s.d = 0·7 μg) from the 3rd to the 6th day after the leaf penetrates the coleoptile. During this time the leaves elongate rapidly. The amount of wax on the 4-day-old light grown leaves is similar to that

reported for much older leaves ranging from 2–8 weeks grown under similar conditions [9]. On leaves grown in the dark at 15–10° a significant increase in amount of wax/cm² leaf occurs between the 3rd and 4th days whereafter the value remains constant at 4·5 μg. This value is *ca* 2·5 times less than that for the light grown leaves. If the temperature in the dark is raised to 23° *ca* 40% more wax is present per cm² leaf than at the lower temperature. Thus both photo- and thermoperiods control the amount of wax found on primary leaves of Bonus barley. To investigate further the influence of light on the amount of wax synthesized and extruded onto the epidermis, 4-day-old seedlings grown in the dark at 15–10° were transferred to the light using the same temperature cycle. Light increases the amount of wax on the dark grown leaves so that after 24 hr the total amount of wax/cm² leaf is essentially the same as on the light grown leaves. This results from an increase in the rate of wax synthesis from 6 μg/cm²/24 hr in the dark to 46 μg/cm²/24 hr for the first 24 hr after transfer to the light. Thereafter the rate of synthesis is 15 μg/cm²/24 hr as it is on the leaves grown continuously in the light.

*Wax composition*

To determine whether light influences the relative amounts of the lipid classes composing the wax on primary leaves, wax from 4-day-old seedlings grown in the light and dark at 15–10° was separated by column chromatography. In both photoperiods the primary alcohols are the major constituent (83%) of the wax, with 10% free fatty acids, 0·5% hydrocarbons, *ca* 1% aldehydes and *ca* 5% esters (see Table 1). These values differ from those previously reported for barley waxes, where the primary alcohol content is given as 72% [9] and 15–20% [10] and the content of hydrocarbons as 5–7% [10]. It should be noted that in the first case [9] the results are similar if one considers that the wax came from leaves of older plants and was separated into lipid classes by preparative TLC. In the second report [10] the large deviation is most likely due to a pooling of the wax from all organs of the plant during isolation. From wax analyses [9,11,12] of individual organs, it is known that the proportion of the various classes in barley differ markedly from one organ to the next.

*Free and esterified fatty acids*

The major free fatty acids from light grown leaves are $C_{22}$, $C_{24}$ and $C_{26}$ which account for 79% of this lipid class (Table 2). For the dark grown leaves the same three acids dominate although to a lesser extent; namely, 48% if grown at 15–10° and 56% if grown at 23°. The decrease in the relative

Table 1. Light controlled synthesis of wax and its extrusion onto the surface of barley primary leaves

| Pathway* Wax class | Homologues present | Amount (ng/cm²) Light | Dark |
|---|---|---|---|
| Decarboxylation (<1%) | | | |
| Hydrocarbons | $C_{21,23,25}$ | 3 | 16 |
| | $C_{29,31,33}$ | 20 | 13 |
| Reductive (89%) | | | |
| Aldehydes† | $C_{20-25}$ | 9 | 4 |
| | $C_{26}$ | 175 | 8 |
| Alcohols | $C_{22}$ | 148 | 816 |
| | $C_{24}$ | 415 | 382 |
| | $C_{26}$ | 8152 | 2107 |
| Esters‡ | | | |
| Elongation (10%) | | | |
| Free fatty acids | $C_{22,24,26}$ | 902 | 204 |
| | $C_{34,36,38}$ | 7 | 131 |

* The figures in brackets represents the relative amount of wax formed by the given pathway.
† 1·7% of the wax in light; 0·3% of the wax in dark.
‡ 4·7% of the wax in light; 6·1% of the wax in dark.
The significant differences in wax composition found on primary leaves of barley grown with a thermoperiod of 15–10° (16–8 hr) in constant light (14000 lx) or dark are summarized in relationship to the biosynthetic wax pathways. Total ng wax/cm² area for light grown leaves amounted to 11100 and for dark grown leaves to 4200. The amount of epicuticular wax present on the surface appears to regulate via a feed-back system the amount of fatty acid wax precursors which enter the elongation system inside the epidermal cells.

Table 2. Per cent composition of free and esterified fatty acids in leaf wax of barley grown in four different environments

| | Free fatty acids | | | | | | Esterified fatty acids | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Days | 4 | 5 | 4 | 4 | 5 | 4 + 1 | 4 | 5 | 4 | 4 | 5 | 4 + 1 |
| Photoperiod (hr) | 24 | 24 | 0 | 0 | 0 | 0 + 24 | 24 | 24 | 0 | 0 | 0 | 0 + 24 |
| Thermoperiod (°C) | 15–10 | 15–10 | 15–10 | 23 | 23 | 23 + 15–10 | 15–10 | 15–10 | 15–10 | 23 | 23 | 23 + 15–10 |
| Carbon no. | | | | | | | | | | | | |
| 16 | 3·0 | 2·0 | 2·1 | 14·5 | 9·7 | 6·5 | 11·3 | 5·0 | 14·0 | 20·2 | 13·9 | 5·6 |
| 17 | | | | 0·7 | 4·2 | | | | | | | |
| X | 2·3 | | | 2·4 | 3·2 | | | | | 10·7 | 18·6 | 4·1 |
| 18 | 4·8 | 1·2 | 6·6 | 5·8 | 6·2 | 5·4 | 19·7 | 12·8 | 40·7* | 11·4 | 10·6 | 12·3 |
| 19 | | | | 0·2 | | | | | | | | |
| X | | | | | | | | | | | | 1·5 |
| 20 | 5·2 | 3·1 | 1·6 | 2·6 | 4·2 | 3·0 | 26·1 | 25·5 | 10·4 | 12·3 | 10·9 | 20·5 |
| 21 | | 0·9 | | 0·5 | 0·2 | | | | | | | 0·2 |
| 22 | 22·4 | 22·4 | 13·1 | 22·4 | 23·9 | 21·8 | 27·3 | 33·9 | 13·8 | 22·6 | 26·3 | 26·7 |
| X | | | | | | | | | | 4·0 | 2·0 | |
| 23 | 0·4 | 0·8 | 0·2 | 2·8 | 3·1 | 0·1 | | | | | | |
| 24 | 21·0 | 28·2 | 16·3 | 17·1 | 16·5 | 25·4 | 4·4 | 6·6 | 4·4 | 2·7 | 7·9 | 5·9 |
| 25 | 2·0 | 3·6 | | 3·0 | 1·0 | 1·4 | | | | | | 0·1 |
| 26 | 35·8 | 32·7 | 19·0 | 16·2 | 23·9 | 28·8 | 10·5 | 16·8 | 16·3 | 15·1 | 9·0 | 22·2 |
| 28 | 1·2 | 2·4 | 1·8 | 1·2 | 0·7 | 1·9 | | | | 1·2 | | 1·0 |
| 30 | 0·4 | 0·5 | 2·1 | 1·2 | 0·6 | 2·6 | | | | | | |
| 32 | 0·4 | 0·8 | 2·8 | 2·6 | 1·9 | 1·1 | | | | | | |
| 34 | 0·6 | 1·5 | 8·3 | 4·3 | 0·4 | 1·8 | | | | | | |
| 36 | | | 11·5 | 2·6 | | | | | | | | |
| 38 | | | 11·2 | 2·2 | | | | | | | | |
| 40 | | | 3·4 | | | | | | | | | |

Composition was obtained by GLC; peak areas were determined using an integrator. Compounds amounting to <0·1% not included in table.

X = unknowns, not belonging to a homologous series.

* Probably contaminated.

amounts of these three acids in the dark is compensated for in two different ways. At 15–10° $C_{34}$, $C_{36}$ and $C_{38}$ comprise 31% of the total wax acids, but at 23° these acids account for only 9%, while $C_{16}$ increases to 15%. If leaves grown in the dark at 23° are transferred to light at 15–10° for one day an increase of $C_{26}$ from 16 to 29% occurs, and the overall chain length distribution approaches that found for 5-day-old light grown leaves. This change does not result solely from the influence of light at 15–10°, but is partially an age effect. That is, for 5-day-old leaves grown in the dark at 23°, $C_{16}$ decreases to 10% and $C_{26}$ increases to 24% of the total free fatty acids. These percentage values lie in between those found for the 4-day-old leaves grown in the dark at 23° and the 5-day-old dark to light transferred leaves.

The distribution of the esterified fatty acids (Table 2) differs greatly from that of the free fatty acids in all environments. The short chain acids $C_{16}$, $C_{18}$ and $C_{20}$ are present in larger amounts while the $C_{24}$ and $C_{26}$ acids are present in smaller amounts than in the free fatty acid fraction. For the light grown leaves the chain length distribution is similar to that for leaves from older plants [9] except for some variation in the relative amounts of $C_{24}$ and $C_{26}$ acids. This relationship is characteristic for this lipid class from primary leaves in all environments. Four-day-old dark grown leaves were transferred to the light at 15–10° for 24 hr and resulted in a shift of the chain length distribution of the esters toward that found for 5-day-old light grown leaves. Unlike the free fatty acids, however, part of the change cannot be attributed to age since the difference between 4- and 5-day-old leaves grown in the dark at 23° involves no shift or shifts in the opposite direction for $C_{20}$, $C_{26}$ and the shortest unknown.

*Free and esterified primary alcohols*

Hexacosanol is the predominant free primary alcohol, forming 88% of this lipid class in the wax from light grown leaves and 60 and 55% in that from leaves grown in the dark at 15–10 and 23°, respectively (Table 3). The *ca* 30% decrease of this homologue in the dark is accompanied by increased amounts of the $C_{22}$ (20%) and $C_{24}$ (7%) homologues.

In contrast to the finding from the two fatty acid classes, the chain length distribution found for the esterified and the free primary alcohols from the same wax are similar. For leaves grown in the dark at 23° and in the light, however, the proportion of hexacosanol appears reduced, primarily because of

Table 3. Per cent composition of free and esterified primary alcohols in leaf wax of barley grown in four different environments

| | Free primary alcohols | | | | | | Esterified primary alcohols | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Days | 4 | 5 | 4 | 4 | 5 | 4 + 1 | 4 | 5 | 4 | 4 | 5 | 4 + 1 |
| Photoperiod (hr) | 24 | 24 | 0 | 0 | 0 | 0 + 24 | 24 | 24 | 0 | 0 | 0 | 0 + 24 |
| Thermoperiod (°C) | 15–10 | 15–10 | 15–10 | 23 | 23 | 23 + 15–10 | 15–10 | 15–10 | 15–10 | 23 | 23 | 23 + 15–10 |
| Carbon no. | | | | | | | | | | | | |
| 16 | | | | 0·1 | 0·1 | 0·1 | 2·0 | 0·2 | | 5·2 | 1·3 | 1·7 |
| 17 | | | | | | | 0·2 | | | | | |
| 18 | | 0·1 | | 0·1 | | 0·1 | | | 0·5 | 2·1 | 0·5 | 0·7 |
| 20 | 0·1 | | 4·1 | 2·1 | 2·9 | 2·4 | 0·5 | 0·2 | 3·4 | 2·0 | 2·5 | 2·9 |
| 21 | 0·1 | | | 0·4 | 0·2 | 0·2 | 0·2 | | 2·5 | | 1·1 | 1·5 |
| Y | 0·3 | 0·1 | | 1·4 | 0·6 | 1·2 | 8·2 | 2·4 | | 40·0 | 21·0 | 20·1 |
| 22 | 1·6 | 0·9 | 23·3 | 20·3 | 17·6 | 17·0 | 6·9 | 2·2 | 18·2 | 9·6 | 15·9 | 19·2 |
| 23 | | | | 0·3 | 0·2 | 0·1 | | | | | 0·3 | 0·8 |
| Y | | | | | 0·4 | | | | | | 8·4 | 8·1 |
| 24 | 4·5 | 4·5 | 10·9 | 12·7 | 10·0 | 10·2 | 5·0 | 4·9 | 14·7 | 5·2 | 5·6 | 5·7 |
| 25 | 3·3 | 0·3 | 0·1 | 1·7 | 0·5 | 0·4 | 0·3 | 0·5 | 0·2 | 0·9 | 3·4 | 3·5 |
| Y | | | | | | | | | | | 1·9 | 2·3 |
| 26 | 88·4 | 90·1 | 60·2 | 55·2 | 63·5 | 66·4 | 74·9 | 87·8 | 58·1 | 34·5 | 25·3 | 23·9 |
| X | | 0·7 | | | 0·6 | | | | | | 2·2 | 2·2 |
| 27 | | | | 0·6 | | | | | | | 1·0 | 1·6 |
| 28 | 1·4 | 2·2 | 1·0 | 2·3 | 2·2 | 1·3 | 1·5 | 1·8 | 1·1 | 0·6 | 3·8 | 2·7 |
| 29 | | | | | | | | | 1·4 | | 1·2 | 1·8 |
| 30 | 0·2 | 0·5 | 0·1 | 1·4 | 0·6 | 0·5 | | | | | 1·6 | 1·4 |
| 31 | 0·1 | 0·5 | | 0·7 | 0·3 | 0·2 | | | | | 2·2 | |
| 32 | 0·1 | 0·1 | 0·4 | 0·9 | 0·4 | 0·2 | | | | | | |

Composition was obtained by GLC; peak areas were determined using an integrator. Compounds amounting to <0·1% not included in table.

Y = belonging to a second unknown homologous series.

X = unknowns, not belonging to a homologous series.

an unknown that elutes from the SE-30 column shortly before $C_{22}$. Recalculation of the distributions excluding the unknown, yields values for the *n*-esterified alcohols similar to those of the free alcohols grown under the same two conditions. If the leaves are grown 5 days in the dark at 23°, then the unknown decreases to 21% and two longer members of the same homologous series as the unknown appear amounting to 10% of the lipid class. This indicates that, relative to the rate of synthesis of the *n*-esterified alcohols, the maximum rate of synthesis of this unknown series is reached by the 4th day and then decreases. The changes in composition of both the free and esterified alcohols observed when 4-day-old dark grown leaves are transferred to the light for one day can be attributed to an increase in age. In both cases the spectrum is practically identical to that found for 5-

Table 4. Per cent composition of aldehydes in leaf wax of barley grown in four different environments

| Days | 4 | 5 | 4 | 4 | 5 | 4 + 1 |
|---|---|---|---|---|---|---|
| Photoperiod (hr) | 24 | 24 | 0 | 0 | 0 | 0 + 24 |
| Thermoperiod (°C) | 15–10 | 15–10 | 15–10 | 23 | 23 | 23 + 15–10 |
| Carbon no. | | | | | | |
| <20 | 0·8 | | 0·7 | | 1·6 | 0·7 |
| 20 | | | 4·0 | 1·5 | | 0·6 |
| 21 | | | 8·9 | 0·5 | | 1·1 |
| 22 | 0·2 | | 3·1 | 6·2 | 7·9 | 3·6 |
| 23 | 0·3 | 0·1 | 3·4 | 1·3 | 3·8 | 7·6 |
| 24 | 3·4 | 1·7 | 8·7 | 15·3 | 15·0 | 9·1 |
| 25 | 1·3 | 0·9 | 2·9 | 1·4 | 3·4 | 2·7 |
| 26 | 93·2 | 92·5 | 65·3 | 68·1 | 52·6 | 57·3 |
| X | | | | | | 14·7 |
| 27 | | 4·4 | | 1·2 | 3·7 | |
| 28 | 0·8 | 0·6 | 3·0 | 4·0 | 6·7 | 2·7 |
| 30 | | | | 0·5 | 1·3 | |
| 32 | | | | | 3·9 | |

Composition was obtained by GLC; peak areas were determined using an integrator. Compounds amounting to <0·1% not included in table.

X = unknown.

day-old leaves grown in the dark at 23°. Thus in contrast to the acids, the synthesis of the primary alcohols with respect to their composition is not influenced by 24 hr light.

*Aldehydes*

Hexacosanal is the principal aldehyde (Table 4), and given the same growth conditions, occurs in the same relative amounts as does hexacosanol. The aldehydes differ from the alcohols, however, in that the *ca* 30% decrease of the $C_{26}$ homologue in the dark compared to light is not accompanied by increased amounts of an unknown and $C_{22}$. Instead in the dark at 15–10°, $C_{20}$ to $C_{24}$ and $C_{28}$ increase while at 23° $C_{22}$, $C_{24}$ and $C_{28}$ increase.

Exposing 4-day-old leaves to one day of light at 15–10° results in the appearance of an unknown amounting to 15% which elutes shortly before $C_{27}$ aldehyde on an SE-30 column. This unknown is not present in the wax of 5-day-old leaves grown either in the dark at 23° or in the light. Thus, the influence of light on the synthesis of the aldehydes with respect to their chain length depends on the age of the leaves when they are exposed to light.

*Hydrocarbons*

For light grown leaves the hydrocarbons on primary leaves fall into two groups (Table 5). The major group consists of the $C_{29}$, $C_{31}$ and $C_{33}$ homologues and comprises 62% of this lipid class, while the second group consists of $C_{16}$, $C_{17}$ and $C_{18}$ and amounts to 20%. No difference in the relative amounts occurred if the seedlings were left an additional day in the light. In contrast to the present observations, a different distribution was found on leaves of older plants [9]: $C_{33}$ = 66·9%, $C_{25}$ = 15·8% and $C_{31}$ = 5%. Combined, these data indicate that the composition of the hydrocarbons in the leaf wax depends on which leaves of the barley plant the wax is extracted from. The composition of hydrocarbons on wheat leaves has also been reported to vary for different leaves [13].

When leaves are grown in the dark a very different hydrocarbon composition is found in which a third new group consisting of $C_{21}$, $C_{23}$ and $C_{25}$ homologues forms 46% of this lipid class. In addition, in the dark at 23° a second homologous series and $C_{28}$ comprise 22% of the hydrocarbons, whereas at 15–10° $C_{33}$ is an important homologue

Table 5. Per cent composition of hydrocarbons in leaf wax of barley grown in four different environments

| Days | 4 | 5 | 4 | 4 | 5 | 4 + 1 |
|---|---|---|---|---|---|---|
| Photoperiod (hr) | 24 | 24 | 0 | 0 | 0 | 0 + 24 |
| Thermoperiod (°C) | 15–10 | 15–10 | 15–10 | 23 | 23 | 23 + 15–10 |
| Carbon no. | | | | | | |
| 16 | 8·1 | 7·8 | | 2·1 | 2·8 | 4·0 |
| X | | | | 1·1 | 1·5 | |
| 17 | 4·2 | 4·4 | | 1·4 | 1·9 | 4·0 |
| 18 | 7·5 | 5·1 | | 2·5 | 3·4 | 5·0 |
| 19 | 1·7 | 1·7 | 0·6 | 1·8 | 2·2 | 2·6 |
| 20 | 1·7 | 1·8 | 0·3 | 3·2 | 6·2 | 4·5 |
| Y | | | | 2·5 | | 5·5 |
| 21 | 1·0 | 1·1 | 4·7 | 10·0 | 3·5 | 7·3 |
| 22 | 0·7 | 0·9 | 3·1 | 2·9 | 1·3 | 4·0 |
| Y | | | | 2·4 | 1·7 | |
| 23 | 1·8 | 2·7 | 23·4 | 25·2 | 22·2 | 12·9 |
| 24 | 0·5 | 0·9 | 1·7 | 3·0 | 3·4 | 3·3 |
| Y | | | | 2·4 | 2·7 | 3·0 |
| 25 | 4·5 | 5·0 | 18·1 | 11·0 | 15·8 | 8·5 |
| X | | | | | | 4·1 |
| 26 | 0·2 | 0·4 | | 2·0 | 1·8 | 5·8 |
| Y | | | | 0·9 | 1·6 | |
| 27 | 3·5 | 4·0 | 5·7 | 2·0 | 4·9 | 4·4 |
| X | | | | 0·7 | | |
| 28 | 0·8 | 0·8 | 0·1 | 12·3 | 2·7 | 1·8 |
| Y | | | | 0·9 | 1·1 | |
| 29 | 9·6 | 10·4 | 8·9 | 2·6 | 5·0 | 6·7 |
| X | | | | 1·8 | 1·0 | |
| 30 | | | | 1·5 | 1·8 | 0·4 |
| Y | | | | 0·7 | 0·7 | |
| 31 | 11·8 | 12·1 | 9·4 | 1·0 | 2·5 | 2·4 |
| 32 | | | | 0·8 | 0·7 | 0·3 |
| 33 | 40·7 | 39·2 | 22·8 | 2·0 | 7·8 | 8·4 |
| 35 | 1·8 | 1·5 | 1·2 | | 0·6 | 0·4 |

Composition was obtained by GLC; peak areas were determined using an integrator. Compounds amounting to <0·1% not included in table.

Y = belonging to a second unknown homologous series.

X = unknowns, not belonging to a homologous series.

(23%) as it was in the leaves grown at 15–10° in the light (41%). This implies that a thermoperiod of 15–10° as well as light stimulates formation of the $C_{33}$ homologue.

The changes in chain length distribution resulting from transferring 4-day-old dark grown leaves to the light at 15–10° for 1 day are of two types. Firstly, marked shifts toward the composition of light grown leaves by the $C_{28}$ homologue which decreases to 2% as well as by the second and third groups of hydrocarbons. Secondly, a very small increase of the major group which can be attributed to age since the same small change also occurred on leaves left for an extra day in the dark at 23°. Thus, exposing 4-day-old seedlings to light at 15–10° stimulates synthesis of the short chain hydrocarbons but not of $C_{33}$ although when the leaves are younger formation of both is promoted.

*Incorporation of acetate-[1-$^{14}$C] into epidermal wax*

Excised 4-day-old light grown primary leaves took up 95% of the acetate-[1-$^{14}$C] containing solution through their cut ends in 2 hr. Only 1·3% of the acetate-[1-$^{14}$C] taken up was incorporated into the epidermal wax and the labelling of the wax increased continually, slightly more rapidly at the start than the end (Table 6). In addition, the distribution of label among the wax classes remained *ca* constant throughout the labelling time. In Table 6 a comparison is also made between the distribution of the masses of the lipid classes and the normalized distribution of the amount of their labelling. The primary alcohols, which are the dominant component of this wax, also show the highest incorporation. The free fatty acids contain less label than would be expected from the amount present in the total wax. A preferential labelling is apparent, on the other hand, for the aldehydes and esters. A small amount of label is found in the hydrocarbons after 1 and 2 hr of acetate incorporation. At the shorter labelling times, the incorporation was below the limit of detectability.

Preliminary investigations of the distribution of $^{14}$C in the various homologues were made for the free and esterified primary alcohols, the aldehydes and hydrocarbons. In the free and esterified primary alcohols as well as the aldehydes, $C_{26}$ was the most highly labelled chain length and was found in *ca* the same proportion as in the mass distribution. The relative sp. act. of the $C_{26}$ aldehyde was *ca* 6 times that of the $C_{26}$ alcohol. In the hydrocarbons $C_{33}$ was preferentially labelled (mass 41%, $^{14}$C 75%). No label was detected in the second important group of hydrocarbons consisting of $C_{16}$, $C_{17}$ and $C_{18}$ that are found in the wax of 4-day-old light grown primary leaves.

## DISCUSSION

Table 1 summarizes the significant differences in epicuticular wax composition found on primary

Table 6. Effect of time on the incorporation of $^{14}$C-label into barley leaf wax and its distribution among the wax classes

| | Total | Free fatty acids | Free primary alcohols | Aldehydes | Esters | Hydrocarbons |
|---|---|---|---|---|---|---|
| Incorporation time (min) (dpm × $10^2$/cm leaf) | | | | | | |
| 15 | 18·4 | 0·7 | 10·9 | 3·5 | 3·3 | 0·0 |
| 30 | 19·4 | 1·0 | 11·4 | 3·3 | 3·7 | 0·0 |
| 60 | 57·3 | 1·7 | 39·5 | 5·7 | 10·3 | 0·0 |
| 120 | 108·3 | 4·3 | 66·1 | 10·8 | 25·9 | 1·1 |
| 180 | 148·8 | 5·9 | 84·8 | 17·8 | 37·2 | 1·5 |
| Average distribution (%) | | | | | | |
| $C^{14}$ | | 4·0 | 61·0 | 13·6 | 20·8 | 0·6 |
| Mass | | 10·3 | 83·0 | 1·7 | 4·7 | 0·3 |

For incorporation the cut ends of 15 4-day-old light grown leaves were placed in vials containing 0·5 ml of an aqueous solution of 40 $\mu$Ci sodium acetate-[1-$^{14}$C], at 15° and 14000 lx. Wax was extracted and its radioactivity measured after different times. The lipid classes of the wax were separated from one another on TLC plates developed with $C_6H_6$, and the distribution of the radioactivity among the lipid classes determined with the aid of a TLC plate scanner and triangulation of the peak areas. A s.d. = 4·7% was determined for the average % $^{14}$C label in the primary alcohols.

leaves of Bonus barley grown in the light and dark at 15–10°. These data have been related to the prevailing ideas in the literature on the biosynthetic wax pathways [14,6]. The synthesis of the epicuticular wax components takes place in the epidermal cells [15]. First the fatty acid wax precursors are elongated by the addition of $C_2$ units. One or more pools of precursors and elongation systems therefrom may exist. Having attained given chain lengths, the elongated precursors either (a) enter the decarboxylation pathways leading to hydrocarbons, (b) enter the reductive pathways leading to aldehydes, primary alcohols and esters or (c) become detached from the elongation system giving rise to free fatty acids. Two alternative routes for formation of the primary alcohols have been suggested, one directly from the elongated fatty acid precursors [6] and the other via an aldehyde intermediate [14]. The latter appears to be the primary route in barley primary leaves since the relative sp. act. of the $C_{26}$ aldehyde was 6 times that of the $C_{26}$ alcohol. Subsequent to their synthesis the lipids are extruded through the cell wall and cuticle onto the surface of the leaves. For light grown leaves the chain lengths given in Table 1 represent 89% of the wax on a $cm^2$ of cuticle while in dark grown leaves they total 88%.

Under a given set of environmental conditions the amount of wax per unit surface area is constant. A 2·5-fold increase in amount of wax to that characteristic for light-grown leaves occurs when dark grown leaves are transferred to light. This intimates that the amount of wax/$cm^2$ on the cuticle regulates via a feed-back system the synthesis and extrusion of the wax lipids (Table 1). On the basis of investigations correlating the time of formation of wax structures and leaf expansion, the suggestion was made [16] that wax synthesis and extrusion stopped when cell expansion ceased, that is, during the hardening of the primary wall laid down during growth. The present results indicate that more than hardening of the primary wall is involved. Furthermore if such an indirect method of control is involved it is more likely correlated with the production of lipids present in the basic primary wall structure and not with those involved in a subsequent hardening thereof.

Light regulates wax biosynthesis and extrusion onto the surface of barley primary leaves in several ways (Table 1). Firstly, it determines the quantity of wax per unit surface area as discussed above. On the other hand, the relative amounts of the wax classes formed via the decarboxylation pathways (1%), the reductive pathways (89%), and elongation only (10%) are very nearly the same in wax isolated from light and dark grown leaves. This implies that light induced reactions are not involved in the mechanism regulating the relative amounts of the precursors entering these three sections of the wax synthesizing pathways.

A second point of control of the synthesis of barley epicuticular wax by light is its affect on the chain length distributions composing each wax class. In the wax classes arising from the decarboxylation and reductive pathways, the long chain lengths which predominate in the wax from light grown leaves are less prominent in the wax from dark grown leaves. For example, while the $C_{29}$, $C_{31}$ and $C_{33}$ homologues dominate the hydrocarbons in the light, the $C_{21}$, $C_{23}$ and $C_{25}$ group is proportionally much larger in the dark. In 9 of the 37 wax samples they analyzed, Stránský and Streibl [17] also observed two distinct maxima in the hydrocarbon chain length distributions. In the hydrocarbons, primary alcohols and free fatty acids from dark grown barley leaves, the two maxima characterizing the chain length distributions differ by two or more carbons. This intimates that the enzymes carrying out a given reaction in wax biosynthesis select specific chain lengths as substrates. In agreement with suggestion of von Wettstein-Knowles [18], two or more sets of enzymes with differing chain length specificities appear to be involved in formation of a wax class. For example, instead of a single fatty aldehyde reductase [19] able to utilize as a substrate both $C_{22}$ and $C_{26}$ aldehydes to form the respective alcohols, there may be two fatty aldehyde reductases one utilizing $C_{22}$ and the other utilizing $C_{26}$ aldehydes. In the wax from light grown leaves one chain length or group of chain lengths predominates. In terms of the above hypothesis this observation would suggest that the two or more sets of enzymes synthesizing the two groups of chain lengths within a wax class are differentially sensitive to light.

The temperatures studied also influenced the chain length composition of the hydrocarbons and free fatty acids, but had little influence on the composition of the aldehydes, free and esterified primary

alcohols. The differential response to temperature exhibited by the various homologues composing the hydrocarbons and fatty acids support the proposal that in wax biosynthesis there are at least two sets of enzymes with different chain length specificities.

In all wax classes a change in the chain length composition was brought about by transferring 4-day-old leaves grown in the dark at 23° to the light for 24 hr at 15–10°. Several different responses were observed. Firstly, a shift in the spectrum toward that characteristic for light grown leaves at 15–10° of the same age (free and esterified fatty acids, hydrocarbons). Secondly, a change in the chain length distribution that would have occurred even if the leaves had remained in the dark at 23° for an additional day (free and esterified alcohols). Thirdly, the appearance of a new spectrum, that is, one arising neither from a shift toward that characteristic for light grown leaves nor from a shift toward that of older dark grown leaves (aldehydes). These different responses indicate that the influence of light at 15–10° on the chain length distributions of the wax classes present on barley primary leaves as summarized in Table 1 depends in part on the age of the leaves when they are exposed to these given environmental parameters. Furthermore, since the wax classes responded differently more than one control mechanism must be involved.

The $C_{16}$, $C_{17}$ and $C_{18}$ group of hydrocarbons has not been included in Table 1. It resembles the major group ($C_{29}$, $C_{31}$ and $C_{33}$) in that its formation is stimulated by light. Unlike the long chain group, however, the odd chain length $C_{17}$ does not predominate and neither $C_{16}$, $C_{17}$ or $C_{18}$ is labelled with acetate-[1-$^{14}$C]. These results intimate a separate pathway for these short chain length hydrocarbons. Two different pathways for normal hydrocarbon synthesis have also been suggested by Kaneda [20] on the basis of his compositional analysis of hydrocarbons in the surface wax and internal lipids of spinach leaves.

## EXPERIMENTAL

*Materials and growth conditions.* Barley cultivar Svalöf's Bonus was used in all experiments, the most essential were repeated from 2 to 4 times. To overcome differences in germination time under various environmental conditions, seeds (20 g/box) were germinated in a dark room at 23°. When the primary leaves started to penetrate the *ca* 3 cm long coleoptile 3 days after planting, the seedlings were divided into 3 groups. The first group remained in the dark room at 23° and are referred to as dark leaves at 23°. The second group was transferred to the dark cabinet where the temp alternated between 15° for 16 hr and 10° for 8 hr (optimal growth conditions in the vegative phase for Bonus [21,22], and these leaves are designated dark leaves at 15–10°. The third group was placed in a growth chamber where the light intensity at vermiculite level was 14000 lx and the temp. conditions were as for the dark cabinet. These leaves are designated light leaves. Because the seedlings were only placed in the different environments 3 days after planting, their age is calculated from this time. Thus, 7 days after sowing the primary leaves are referred to as 4-day-old leaves. Two additional groups of leaves were established by taking 4-day-old dark leaves at 23° or 15–10° and transferring them to the growth chamber in the light for one day at 15–10°. These leaves are referred to as dark to light transferred leaves with the dark temperature being specified. In all experiments the humidity was 60–80%. For the analyses of the chain length distribution of the wax classes, light grown leaves were germinated in the lighted growth chamber and not in the dark at 23° as was all other material.

*Wax extraction and weighing.* Epicuticular wax for chemical analyses was extracted from that part of the 4 or 5-day-old primary leaves extending above the coleoptile using the method of von Wettstein-Knowles [9] except that two 15 sec dips were employed. To determine the $\mu g/cm^2$ leaf. wax was extracted by the same procedure from 3–6-day-old leaves grown in the different environments. Each sample comprised 40 leaves. The $CCl_4$ extract was evaporated to *ca* 0·5 ml, transferred to a weighing pan, heated until the remaining $CCl_4$ was evaporated (0·5–1 min) and then weighed on an electrobalance. No difference in wt was observed if the sample after being heated was dried over $P_2O_5$ in a desiccator for 12 hr.

*Leaf length and area measurements.* The amount of wax is expressed as $\mu g$ or ng wax/$cm^2$ leaf. Since the leaf length is much easier to measure than the leaf area, the ratio between these two parameters was established for 4-day-old primary leaves in 3 different environments. In each expt 19 leaves were cut above the coleoptile and the wax extracted as described above. Then the circumference of the leaves was traced on paper and the area determined using a planimeter. The ratio of leaf area in $cm^2$ is *ca* 0·9 × leaf length in cm for the leaves grown under all 3 conditions.

*Separation of lipid classes.* Separation of the wax components was performed in 2 ways. The first method combined column chromatography and TLC [9,18,23] and was used to rapidly obtain small amounts of the individual lipid classes for chain length analysis. The second method used only column chromatography and was employed to determine the % composition of the waxes. The free fatty acids were isolated from the other wax classes (97–100% recovery) by means of a florisil column. The latter consisting of 50–70 mg were separated from one another using the column chromatographic technique developed by Netting [24] with the following modifications. A 67 × 1·8 cm column was used with an almost constant flow rate of 0·2 ml/min. The volumes of solvents applied were: $CCl_4$, 1150 ml; $C_6H_6$, 300 ml; and EtoAc, 300 ml. Fractions (8–10 ml) were collected using a fraction collector. Recovery was 97–100%.

*GLC.* Analysis was performed on a dual FID instrument using two 152 cm × 0·22 cm stainless steel columns containing 7% SE-30 on Chromosorb W. Isotherm and temp programmed chromatograms were run using temps between 140 and 280° and a $N_2$ flow rate of *ca* 25 ml/min. Peak areas were determined using a digital electronic computer.

*Acetate-[1-$^{14}$C] incorporation.* Primary leaves from 4-day-old light grown seedlings were excised just above the coleoptile and placed cut end down in a 0·5 ml aq. soln containing 40 $\mu$Ci NaOAc–[1-$^{14}$C](61 mCi/mmol, Amersham, UK). 15 leaves were used for each expt which was carried out in a growth chamber under 14000 lx at 15°. At the end of the incorporation periods, which varied from 15 to 180 min, the amount of label not taken up by the leaves and that incorporated into the wax was determined using a scintillation counter. First, the remaining acetate-[1-$^{14}$C] soln was combined with a dist $H_2O$ rinse of the leaf bases to remove any adhering acetate and counted using Bray's soln[25]. Then aliquots of the wax, extracted as described above, were taken to dryness and counted using a scintillant consisting of 4 g 2,5 diphenyloxazole and 50 mg 1,4-*bis*-[2-(5-phenyloxazolyl)] $C_6H_6$ in 1 l toluene. Counting efficiencies were 80 and 84%, respectively; quenching corrections were performed by the external channel ratio method. Separation of the lipid classes was achieved using Si gel H TLC plates developed in $C_6H_6$. The relative amounts of label in the various wax classes were measured using a TLC Scanner, followed by triangulation of the resulting peak areas. The distribution of label within a lipid class was determined using GLC–RC. Programmed chromatographs were run on the same columns described above using column temps between 175–275° and a He flow rate of *ca* 50 ml/min. The % composition of the $^{14}$C containing compounds was obtained by triangulation of their peak areas.

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