Phytochemistry, 1970, Vol. 9, pp. 757 to 761. Pergamon Press. Printed in England.

THE EFFECT OF LIGHT ON WAX SYNTHESIS IN LEAVES OF *BRASSICA OLERACEA*

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(*Received* 29 *August* 1969)

Abstract—The effect of light on wax synthesis in *Brassica oleracea* has been investigated. When using acetate-1-^{14}C as a substrate, light stimulated both total lipid synthesis and wax synthesis in parallel. When using palmitate-1-^{14}C, no effect of light on total wax synthesis was apparent. Light, however, altered the balance of synthesis of the various wax components using either acetate or palmitate. The synthesis of C_{29} compounds (paraffin, ketone and secondary alcohol) was increased in the light and the synthesis of aldehydes and esters decreased. After incubation with palmitate-1-^{14}C in the dark, the specific activity of the C_{29} paraffin, secondary alcohol and ketone was 3–5-fold less than in the light and that of C_{28} aldehyde 2–3-fold greater. Differences in specific activity between dark and light conditions were greater than the corresponding differences in total synthesis. After incubation with palmitate-1-^{14}C, fatty acids with a higher specific activity than any of the wax components were found mixed with the surface wax washed from the tissue by warm hexane, but were not detected in the internal lipid subsequently extracted by chloroform/methanol. The chain length of these fatty acids was C_{24}, C_{26}, C_{28}, and C_{30}. These components were found labelled after incubation with palmitate in the dark, but not in the light. Light does not appear to act by stimulating the elongation process, but rather by stimulating the utilization of elongation products in C_{29} production.

INTRODUCTION

JUNIPER[1] found that light promoted the formation of the wax crystallite structure in *Pisum sativum*. Macey and Barber[2] grew pea plants in the light and dark and in dim red light and found that the normal predominance of C_{31} paraffin was maintained only in the light. The C_{31} secondary alcohol was only formed in the light. Thus it seemed likely that in *Pisum*, the development of the typical glaucous structure typified by wax crystallite formation is dependent on the formation of large quantities of the C_{31} components.

Brassica oleracea contains wax which is somewhat similar to that of *Pisum*. The predominant paraffin is C_{29}, together with ketone and secondary alcohol of the same chain length. Macey and Barber[3] described mutants in this species which lacked C_{29} components and also described corresponding changes in free acids. The presence of comparatively large amounts of pentadecanoic acid in the normal forms and its absence in corresponding mutants indicated a role for this component in C_{29} synthesis. However, the evidence adduced by Kolattukudy[4–6] indicates that the C_{29} compounds can be formed by elongation from exogenously supplied palmitate or stearate and the role of the C_{15} acid remains uncertain. It has been suggested[7]

[1] B. E. JUNIPER, *Endeavour* **69**, 20 (1959); *J. Linn. Soc. (Bot.) London* **56**, 413 (1960).
[2] M. J. K. MACEY and H. N. BARBER, *Phytochem.* **9**, 13 (1970).
[3] M. J. K. MACEY and H. N. BARBER, *Phytochem.* **9**, 13 (1970).
[4] P. E. KOLATTUKUDY, *Biochemistry* **5**, 2265 (1966).
[5] P. E. KOLATTUKUDY, *Phytochem.* **6**, 963 (1967).
[6] P. E. KOLATTUKUDY, R. H. JAEGER and R. ROBINSON, *Nature* **219**, 1038 (1968).
[7] R. ROBINSON, unpublished communication.

that this product is in fact a possible breakdown product of the ketone, and this idea is certainly worth investigation. My recent work supports the elongation-decarboxylation hypothesis for the formation of C_{29} compounds in *Brassica* and in what follows I assume that this hypothesis is correct.

Kolattukudy[4,5] found that light did not affect the synthesis of C_{29} paraffin. This conclusion was based on the isolation of the paraffin fraction from total lipid extracts and its specific activity was not measured. *Brassica* contains long-chain aldehyde whose chain length is mainly C_{28}. In previous studies of wax biosynthesis, little account has been taken of this component, which in our experiments incorporates at least a fifth of the label in the wax when acetate or palmitate is used as a substrate. In order to judge the effect of light on wax synthesis, it was essential to include this component.

RESULTS AND DISCUSSION

Table 1 shows the overall pattern of uptake of palmitate and acetate into wax, chloroform-soluble components and CO_2 in light and dark. 6–8 per cent of the palmitate taken up by the tissue was incorporated into wax and light had no effect on total wax synthesis. The total uptake of palmitate (supplied as ammonium palmitate) was very efficient and was slightly depressed by light. Acetate incorporation was affected by light. Uptake was clearly promoted. Both total lipid synthesis and wax synthesis were increased in parallel in the light, but it is not possible to say whether this was a cause or a result of the increased uptake. Probably, acetate utilization was promoted in the presence of light, accounting for the increased uptake, but no assessment of acetate utilization was made. Light increased the evolution of CO_2 from acetate and this increase was well correlated with the increased uptake. Compensation did not occur in these experiments when using acetate or palmitate as substrates, due possibly to the depth of the solution in the flasks.

TABLE 1. INCORPORATION OF PALMITATE-1-^{14}C (140 mμmoles, $19{\cdot}0 \times 10^6$ dpm) AND ACETATE-1-^{14}C (120 mμmoles, $25{\cdot}1 \times 10^6$ dpm) INTO WAX AND TOTAL LIPID OF *Brassica oleracea* LEAVES

	Applied	Uptake	Wax	Other lipid	CO_2	Wax: Applied (%)	Wax: Uptake (%)	Wax: Other lipid (%)
Dark								
P (i)	19·00	17·52	1·24	7·58	0·14	6·52	7·07	16·35
P (ii)	19·00	17·59	1·17	8·33	0·12	6·18	6·59	13·90
A	25·10	4·52	0·08	0·65	0·15	0·34	1·88	13·11
Light								
P (i)	19·00	16·74	1·17	5·27	0·10	6·15	6·98	22·20
P (ii)	19·00	16·78	1·34	5·07	0·28	7·00	7·98	26·40
A	25·10	14·36	0·81	2·96	0·52	3·28	5·66	27·36

2 g fresh wt. of tissue was used in each incubation. Light was provided by sunlight at 2000–2500 lumens/ft^2. Uptake and incorporation given in dpm $\times 10^6$. (i) and (ii) are duplicates.

P = palmitate-1-^{14}C, A = acetate-1-^{14}C.

Table 2 shows the distribution of label amongst the different wax components in light and dark, using acetate and palmitate as substrates. This shows increased synthesis of C_{29} compounds in the light, at the expense of aldehydes and esters. The effect on esters is clear cut in Experiment 1, but results from a further experiment (Experiment 2) are listed and both aldehydes and esters are labelled less in the light.

TABLE 2. INCORPORATION (AS % OF RADIOACTIVITY IN TOTAL SAMPLE) IN VARIOUS WAX COMPONENTS AFTER INCUBATION WITH PALMITATE-1-^{14}C AND ACETATE-1-^{14}C IN THE LIGHT

	Experiment 1						Experiment 2	
	Dark			Light				
	P_I	P_{II}	A	P_I	P_{II}	A	Dark (P)	Light (P)
Origin	5·7	6·5	13·0	8·7	14·5	2·1	8·3	10·2
Primary alcohol	4·7	4·4	7·1	4·9	5·5	10·0	3·5	3·2
Secondary alcohol	6·7	5·8	8·8	13·4	13·7	11·7	8·8	15·7
Aldehyde	27·6	19·3	17·2	17·0	15·2	26·0	25·0	12·8
Ketone	6·8	9·7	10·3	7·5	7·1	4·4	8·9	10·5
Ester	26·9	34·0	23·7	11·3	9·7	8·0	20·4	8·0
Paraffin	21·9	20·3	19·9	37·1	34·2	37·6	24·9	39·5

P = palmitate, A = acetate. P_I and P_{II} are duplicates. Incubation conditions and total wax incorporation as for Table 1. Origin = origin of chromatogram, includes free acid of wax. Experiment 2 performed with leaves from plants 2 weeks older than Experiment 1.

TABLE 3. SPECIFIC ACTIVITIES (dpm/μg) OF VARIOUS WAX COMPONENTS AFTER INCUBATION OF LEAF TISSUE WITH PALMITATE-1-^{14}C IN LIGHT AND DARK

	Light	Dark
Paraffin (C_{29})	1211	478
Secondary alcohol (C_{29})	1210	251
Ketone (C_{29})	1000	410
Aldehyde (C_{28})	1800	4760
Free acid C_{24}	—	6100
C_{26}	—	5900
C_{28}	—	5200
C_{30}	—	6000

Chain length of component given as C_n. Components were isolated by preparative TLC from the mixtures of composition given in Table 2. —not detected.

Table 3 shows the specific activities of the paraffins, ketones, secondary alcohols, aldehydes and free acids C_{24}–C_{30} after feeding with palmitate in light and dark. The acetate figures are not included but were similar though uniformly lower than those for palmitate. The specific activities of the C_{29} compounds were increased in the light and that of the aldehydes decreased. A large amount of label was incorporated into aldehydes, which had the highest specific activity of all the wax components, apart from the acid. Aldehydes might be precursors of both primary alcohols and esters. In this experiment and all others that have

been done, the free primary alcohols did not take up very much of the label, whilst the amount of label entering the esters changed in parallel with that for aldehydes.

The chain length of the aldehyde (mainly C_{28} with some C_{26}) is such as to compete for acid precursors with the C_{29} compounds. Labelled C_{24}–C_{30} acids in the wax were only found after incubation in the dark, but not in the light (Table 3). The full meaning of this result is not clear without further work, but one possibility is that light promotes the formation of C_{29} compounds from a C_{30} precursor. The specific activities of the free acids of the wax were higher than for any other wax components (Table 3). This supports the hypothesis that these acids are the precursors of the other wax components. The increases in specific activities of the C_{29} compounds over the same in the dark are greater than the increases in total synthesis. The converse is true of the aldehydes. In the case of the ketone no increase in total synthesis was recorded in the light, but the specific activity was 2·5 times higher than in the dark. Such a result could imply that wax turnover is greater in the light than in the dark. Robinson's suggestion[7] concerning the possible origin of the C_{15} acid in *Brassica* by oxidative breakdown of the ketone would take on added significance if wax turnover could be shown to occur in developing leaves. In these experiments, however, no label has been found in the C_{15} acid.

TABLE 4. PERCENT RADIOACTIVITY IN FREE ACIDS OF THE SURFACE WAX AFTER INCUBATION OF TISSUE WITH PALMITATE-1-^{14}C. THE SPECIFIC ACTIVITIES FOUND ARE INDICATED IN TABLE 3

C_{16}	77·5
C_{18}	—
C_{20}	—
C_{22}	—
C_{24}	6·0
C_{26}	5·4
C_{28}	3·0
C_{30}	8·0

— = mass detected, but not labelled.

The very long chain fatty acids in the lipid extracted by chloroform/methanol from the interior of the tissue were not labelled. This could mean either that the internal fatty acid synthesizing system is not capable of elongating exogenously supplied palmitate, or that the elongation products are sent to the peripheral wax synthesizing system. Experience with other systems indicates that in barley[8] and in pea cotyledons[9] the fatty acid synthetase system does not use exogenous palmitate for elongation reactions. It is probably a special property of the wax synthesizing systems which need large quantities of long-chain components and which may obtain supplies of palmitate *in vivo* from the internal lipid synthesizing apparatus via the cell membrane system. The proportions of long-chain fatty acids to supplied substrate extracted from the free acid fraction of the wax are indicated in Table 4. Apart from the considerable proportion of very long chain product present and its high specific activity compared to the other wax components, the absence of C_{18}, C_{20}, and C_{22} fatty acids is also striking. The C_{24} acid is apparently the lowest chain-length observable as a product of the elongation system. Whether this acid, which cannot be used directly for more than traces of the wax

[8] J. C. HAWKE and P. K. STUMPF, *Plant Physiol.* **40**, 1023 (1965).
[9] M. J. K. MACEY and P. K. STUMPF, *Plant Physiol.* **43**, 1637 (1968).

component,[3] becomes the substrate of reactions introducing the functional group of secondary alcohols and ketones, or whether these are introduced after elongation is completed, remains to be elucidated.

Kolattukudy[6] partially separated epidermis from mesophyll tissue using *Senecio odoris* leaves. When supplied with labelled acetate, the epidermis synthesized more of the very long chain acids (C_{18}–C_{24}) than the mesophyll. The main paraffin of *S. odoris* is C_{31}. The apparent peripheral location of the C_{24}–C_{30} acids, their direct relation without further elongation to the known wax components and their high specific activity after labelling with palmitate-1-^{14}C, support the hypothesis of an epidermally located elongation system manufacturing wax, and the additional hypothesis that the wax free acids are direct precursors of wax components. The results also show that the nature of the products of the system is under environmental control. However, only light of the order of intensity of full sunlight had the described effect on wax synthesis, under the prevailing experimental conditions.

EXPERIMENTAL

Preparation and Incubation of Tissue

Tissue was cut into thin slices with a razor blade. Each flask contained 2 g (fresh wt) cut from very young leaves of 6-week-old cauliflower seedlings, together with 10 ml 0·05 M phosphate buffer, pH 7·6. Flasks were incubated at 30° for 4 hr wrapped in foil or without foil in sunlight (2–2500 lumens/ft^2). At the end of the incubation period, 1 ml 5 N H_2SO_4 was added to the flask, and 0·3 ml Hyamine 10X into a polythene cup attached through the cap of the flask. Radioactivity in CO_2 was estimated by subsequently counting the CO_2 trapped in the Hyamine, and that in the ambient solution by sampling the washings after the tissue was thoroughly washed with distilled water. After washing with water, the tissue was washed with 50 ml hexane warmed to 50°. The hexane wash contained most of the labelled wax and was not contaminated with internal lipid. The tissue was further treated with $CHCl_3$/MeOH to extract the internal lipid.

The wax fraction was separated into its components by TLC, the relevant areas scraped off and the activity in the various components estimated as a percentage of the total amounts recovered. The reagent of Snyder[10] was used for counting the Silicagel scrapings. Preparative TLC in benzene was used to separate the components before analysis by gas radiochromatography. The internal lipid was interesterified using BF_3 in methanol as described by Appelqvist *et al.*,[11] and the methyl esters purified by preparative TLC before analysing by GLC.

Gas radiochromatography was performed as described previously,[9] using a thermal conductivity detector and a continuous monitoring system. After standardization with known compounds, specific activities were estimated directly from the ratio of the mass to the radioactivity peaks. The free acid area of the thin-layer chromatograms was interesterified and the methyl esters examined without further purification. Other components were examined directly by GLC. The column used was 4% SE 30 on Diatoport S.

Acknowledgements—The financial assistance of the Australian Research Grants Council is acknowledged. My thanks are due to Mr. E. Leis for technical assistance.

[10] F. SNYDER, *Advances in Tracer Methodology*, Vol. 4, p. 81, Plenum Press, New York (1968).
[11] L. APPELQVIST, J. E. BOYNTON and P. K. STUMPF, *J. Lipid Res.* 9, 425 (1968).