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CHEMICAL GENETICS OF WAX FORMATION ON LEAVES OF *BRASSICA OLERACEA*

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Abstract—Analysis of wax from the leaf surfaces of normal and "glossy" mutant *Brassica oleracea* shows that genes at at least two different loci responsible for the "glossy" character, result in a severe reduction of the C_{29} compounds of the wax, nonacosane, 15-nonacosanone, and 15-nonacosanol. Aldehydes, primary alcohols and esters are more prominent components of the wax in "glossy" mutants. In the free acid fraction of the wax the normal glaucous plant possesses ten times or more of pentadecanoic (C_{15}) acid than palmitic(C_{16}) whereas in the mutant types this ratio is approximately reversed. This indicates that the fate of fatty acids in this range is connected with the formation of C_{29} compounds. Although in one mutant the content of C_{30} acid was reduced in parallel with the reduction in C_{29} compounds, this was not true for the mutant gl_4 and the common factor in both glossy mutants was a reduction in the quantity of C_{15} acid present. It is suggested that in normal glaucous *Brassica* the pattern of free acid distribution in the surface wax is caused by an α-oxidation system which is inactivated in the mutants. Possibly explanations of the correlation between the occurrence of C_{15} acid and C_{29} compounds are discussed. At present there is insufficient evidence to decide on the role of pentadecanoate in wax metabolism.

INTRODUCTION

THE CUTICLE wax of *Brassica oleracea* has been the subject of studies by Chibnall and his co-workers.[1] More recently, Purdy and Truter[2] gave a detailed account of the chemistry of the wax. Normal *B. oleracea* wax consists predominantly of nonacosane and its derivatives 15-nonacosanone and 15-nonacosanol. Horn *et al.*[3] found the ketone to consist of 15-nonacosanone, with traces of other ketones, but Purdy and Truter[2] found a mixture of 15-nonacosanone and 10-nonacosanone.

Electron microscope studies of the surface wax by Hall *et al.*[4] revealed that part of it takes the form of well-defined crystallites which form the visible "bloom" on the glaucous leaf surface. The glossy mutant (gl_5) present in the cauliflower variety, "Blightproof", lacks the crystallite structure and is a dark green without bloom. The paraffin fraction of the glaucous form was characterized by a high (95 per cent) proportion of nonacosane, while in the mutant the proportion was about 50 per cent. Also the C_{29} ketone was found to be reduced from 30 to 5 per cent of the total wax.

Biosynthesis of wax in *Brassica* has been studied by Kolattukudy.[5–8] Evidence was adduced that the carbonyl group in the C_{29} ketone was not derived from the carboxyl group of acetate but from the methyl group. To explain this, α-oxidation of palmitate was postulated[5] to give rise to pentadecanoate, which then undergoes a condensation reaction according to

[1] A. C. CHIBNALL and S. H. PIPER, *Biochem. J.* **28**, 2209 (1934).
[2] S. J. PURDY and E. V. TRUTER, *Proc. R. Soc. B* **158**, 538 (1963).
[3] D. H. S. HORN, Z. H. KRANZ and J. A. LAMBERTON, *Australian J. Chem.* **27**, 464 (1964).
[4] D. M. HALL, A. I. MATUS, J. A. LAMBERTON and H. N. BARBER, *Australian J. Biol. Sci.* **18**, 323 (1965).
[5] P. E. KOLATTUKUDY, *Biochemistry* **4**, 1844 (1965).
[6] P. E. KOLATTUKUDY, *Biochemistry* **5**, 2265 (1966).
[7] P. E. KOLATTUKUDY, *Phytochem.* **6**, 963 (1967).
[8] P. E. KOLATTUKUDY, *Science* **159**, 498 (1968).

the scheme of Channon and Chibnall[9] to form a ketone which is then reduced via secondary alcohol to C_{29} paraffin. Subsequent evidence,[6,7] recently summarized,[8] is against this hypothesis and Kolattukudy has concluded[8] that C_{29} compounds are formed in *Brassica* by elongation from palmitate or stearate with subsequent decarboxylation of tricosanoic acid. The origin of the functional group in C_{29} ketones and secondary alcohols was still not satisfactorily explained.

The hypothesis[9] of long-chain fatty acid condensation receives some indirect support from the work of Gastambide-Odier and Lederer[10] who observed that corynomycolic acid was formed in *Corynebacterium diphtheriae* via palmitate condensation. This pathway could also lead to palmitone (16-hentriacontanone) by decarboxylation and oxidation. In *B. oleracea* the apparent absence of pentadecanoic acid[1] has been cited as evidence against the condensation pathway.

To obtain further information we have compared the chemical composition of normal and mutant wax types in *B. oleracea*. Wax mutants in this species have been numbered arbitrarily

TABLE 1. WAXY MUTANTS OF *Brassica oleracea*

Symbol allotted	Variety	Reference
gl_1	Sprouting broccoli	Anstey and Moore[11]
gl_2	Brussels sprout	North and Priestly[12]
gl_3	Marrow stem kale	Thompson[13]
gl_4	Marrow stem kale	No reference
gl_5	Cauliflower	Hall *et al.*[4]

Single gene mutants listed in order of discovery. Only gl_2–gl_5 were used in the present work. Genetic evidence (K. F. Thompson and own work, unpublished) indicates that $gl_3 = gl_5$. From chemical evidence, gl_2 is possibly also in this group. gl_1 is not allelic with gl_5 and gl_4 is not allelic with gl_5.

gl_1 to gl_5. All are single recessive genes which segregate in a simple Mendelian manner (Table 1). It is clear that there are at least three distinct loci involved, viz. gl_1, gl_4, whilst gl_3 and gl_5 are allelic, probably representing the same mutation for the wild-type gene. It is possible that gl_2 is also allelic to gl_3 and gl_5. Full genetic details will be published later.

In this paper, chemical comparisons have been made between wax samples from plants segregating in the F_2 generation for the glaucous-glossy difference influenced by the four lines gl_2 to gl_5. The results appear to have some bearing on the possible mechanisms of wax formation and indicate fruitful areas for future investigations.

RESULTS AND DISCUSSION

(i) *General*

Table 2 shows the approximate per cent composition of wax from a normal glaucous type (cauliflower) and from the four glossy lines examined. Esters and aldehydes were not

[9] H. J. CHANNON and A. C. CHIBNALL, *Biochem. J.* **23**, 168 (1929).
[10] M. GASTAMBIDE-ODIER and E. LEDERER, *Nature* **184**, 1563 (1959).
E. LEDERER, 6th Congress Biochem. N.Y. Proceedings of Plenary Sessions, p. 63 (1964).
[11] T. H. ANSTEY and J. F. MOORE, *J. Heredity* **45**, 39 (1954).
[12] C. NORTH and W. G. PRIESTLY, *Hort. Res.* **1**, 95 (1962).
[13] K. F. THOMPSON, *Nature* **198**, 209 (1963).

completely separated from one another, although pure samples could be prepared from mixed fractions by preparative TLC. In all glossy lines, the composition was shifted away from the predominance of paraffin, ketone, and secondary alcohol. Instead, esters, aldehydes, and primary alcohols are the main components.

TABLE 2. PER CENT COMPOSITION OF NORMAL MUTANT TYPES OF *Brassica* WAXES

	% Composition of				
	Normal	gl_2	gl_3	gl_4	gl_5
Paraffin	33	10	6	5	7
Ester+aldehyde	19	43	51	50	25
Ketone	20	<2	<2	<2	<2
Secondary alcohol	8	<2	<2	<2	<2
Primary alcohol	12	42	35	28	50
Free acid	8	5	8	14	8

Figures are estimates from column separations and from thin-layer separations of bulked column fractions.

(ii) *Paraffins*

The carbon numbers of the paraffins in phenotypically normal and mutant plants are compared in Table 3. In addition to the reduction in overall per cent of paraffins apparent from Table 2 the mutant plants had a different carbon number distribution, with much less of the C_{29} component and more of the C_{25} and C_{27} components. This observation agrees with that of Hall *et al.*[4]

TABLE 3. PER CENT COMPOSITION OF PARAFFINS OF NORMAL AND MUTANT *Brassica*

* Symbols as for Table 6

Carbon No.	Normal	gl_2	gl_3	gl_4	gl_5
C_{21}	*	0·2	0·6	0·5	tr
C_{22}	*	0·2	1·1	0·4	tr
C_{23}	*	0·4	2·4	0·8	0·2
C_{24}	*	0·7	2·5	1·1	0·4
C_{25}	*	4·2	24·5	3·1	7·0
C_{26}	1·2	1·5	3·5	3·4	0·8
C_{27}	0·8	44·0	37·5	15·8	26·2
C_{28}	0·8	2·4	3·2	7·8	2·6
C_{29}	90·3	33·7	23·8	51·2	60·7
C_{30}	2·2	2·5	0·9	1·4	0·3
C_{31}	4·7	10·2	tr	14·5	1·8

Table 4 shows the comparison between yields of C_{29} and C_{27} paraffins in normal and mutant forms. These yields are based on amounts obtained in one petrol ether extraction and subsequent separations by chromatography.

TABLE 4. C_{27} AND C_{29} PARAFFINS IN NORMAL AND MUTANT FORMS OF *Brassica*

Paraffin	mg/kg Fresh wt.				
	Glaucous	gl_2	gl_3	gl_4	gl_5
C_{27}	1·3	1·3	1·9	3·7	1·0
C_{29}	153	1·0	1·2	8·5	3·0

These figures are based on total yield of wax in the first extraction only. Figures for the glaucous form are based on wax from all glaucous plants examined amongst which there was little variation.

(iii) *Secondary Alcohols and Ketones*

The composition of the secondary alcohol and ketone from the normal glaucous *Brassica* was of some interest, since previous results conflicted.[2,4] The C_{29} ketoximes were made from a sample of the ketone purified by preparative TLC and also from the ketone prepared by oxidation of the secondary alcohol. The per cent composition of the fatty acids formed by hydrolysis of the derived amide are shown in Table 5. Pentadecanoate (C_{15}) was the major acid formed, but 20 per cent of $C_{14}+C_{16}$ and traces of $C_{13}+C_{17}$ and $C_{12}+C_{18}$ were found. Thus about 16 per cent of the C_{29} ketone and 39 per cent of the secondary alcohol was substituted at the 14-position.

TABLE 5. PER CENT COMPOSITION OF ACIDS DERIVED FROM C_{29} KETOXIMES DERIVED IN TURN FROM NORMAL *Brassica oleracea* C_{29} COMPOUNDS

Carbon number of acid derived	From secondary alcohol	From ketone
C_{13}	2	1
C_{14}	22	10
C_{15}	52	78
C_{16}	17	6
C_{17}	1	1
$C_{12}+C_{18}$	6	4

This observation does not support the idea that the ketone is the biogenetic precursor of the secondary alcohol. If anything the secondary alcohol seems a more likely precursor. This conclusion is supported by the constitution of the C_{31} compounds of *Pisum*.[14] Here the symmetrical 16-hentriacontanol is present in the wax but the corresponding ketone is absent.

Secondary alcohols and ketones are present in the mutant plants, though in much reduced quantities. Secondary alcohol was isolated from gl_5 by TLC. After oxidation with ethereal chromic acid, this component behaved as a ketone in TLC. GLC of this component on

[14] M. J. K. MACEY, Ph.D. Thesis, "The Chemistry and Genetics of Plant Cuticle Waxes", University of N.S.W. (1967).

S.E.30 before and after oxidation with chromic acid showed two main peaks with a series of subsidiary ones, all peaks falling on a logarithmic series of retention times and probably representing a homologous series. The C_{27} component, barely detectable in the glaucous form, now formed 45 per cent of the total, C_{29} formed 50 per cent and the rest was made up mainly of peaks corresponding to C_{28} and C_{26} components, with detectable traces of lower homologues. The retention time of all components was slightly decreased following oxidation and the retention time of the component having the greatest carbon number was then identical with a sample of C_{29} ketone.* Thus the mutant still contained some C_{29} secondary alcohol but had an increased proportion of C_{27} and other components. Ketones from the mutant type gl_5 were also isolated and contained traces of shorter-chain components, which were not detected in the ketones from the normal glaucous form.

It is clear that all the lines gl_{2-5} have a block in the formation of C_{29} compounds. Since the mutant wax still contains some C_{29} compounds, however, the blockage is incomplete. Secondary alcohols and ketones were detected in all mutants, but the amounts present were too low to analyse except in gl_5.

(iv) *Free Acids*

Evidence concerning the cause of the changes observed in the C_{29} compounds was sought in the free acid fraction of the wax. *Brassica* wax,[2] as found here, contains 5–10 per cent free acid which is not esterified. These acids possibly represent part of a pool of compounds from which other wax compounds are derived. It has been reported[6] that the very long chain acids in *Brassica* are all esterified in glycerides or phospholipids. Although this is probably true of internal lipid, the free acids in the surface wax are an exception. The composition of the free acids in the wax of *Brassica* (Table 6) shows that free acids of carbon numbers C_{12}–C_{20} differ greatly between mutants and corresponding normal plants. All mutants show the usual sequence of even-numbered components while the glaucous type shows a predominance of C_{14} and C_{15} components. What is unusual in natural products is the large C_{15}/C_{16} ratio of the order of 10.

Table 6 also shows changes in the C_{24}–C_{30} range of carbon numbers. In gl_4 there was an increase in C_{30} acid, paralleled by an increase in C_{30} aldehyde (Table 8). On the other hand, in $gl_{2,3,5}$ the C_{30} acid content was reduced in comparison with the glaucous form. Since in all mutants there was a decrease in C_{29} compounds, there was no overall correlation between the content of C_{30} acid and the C_{29} compounds.

(v) *Primary Alcohols*

Changes parallel to those observed in the free acids were also found in free primary alcohols. Table 7 lists the per cent composition of primary alcohols C_{12}–C_{20} in glaucous kale and the mutant gl_4. The similarity to the free acid distributions, including the marked predominance of C_{15} alcohol in the normal form, suggests a close relationship between wax-free acids and alcohols. The free primary alcohols of greater carbon number formed a complex mixture. As checked against the reduced mixture of wool wax acids described by Downing *et al.*,[15] the main components were tentatively identified as n-C_{26}, ante-iso C_{27}, iso-C_{28} and n-C_{28}. These components did not appear to be significantly affected by the mutations, and neither were the esterified alcohols.

* Obtained from Dr. J. A. Lamberton, C.S.I.R.O., Melbourne.

[15] D. T. Downing, Z. H. Kranz and K. E. Murray, *Australian J. Chem.* **13**, 1 (1960).

TABLE 6. PER CENT COMPOSITION OF FREE ACID FRACTIONS OF NORMAL AND MUTANT *Brassica* WAXES

Carbon No.	Glaucous kale Gl_3	gl_3	Glaucous kale Gl_4	gl_4	Glaucous caulifl. Gl_5	gl_5	gl_2
12	*	*	0·7	0·7	*	*	tr
13	0·3	*	0·8	tr	*	*	tr
14	3·6	tr	8·8	1·0	7·1	0·1	tr
15	4·1	tr	10·6	0·1	5·9	0·3	tr
16	1·2	3·6	1·7	5·0	1·3	0·7	0·6
17	tr	tr	2·0	0·3	tr	0·1	tr
18	0·5	3·0	0·7	2·2	1·1	0·6	0·4
19	tr	tr	tr	0·2	tr	0·2	tr
20	tr	1·3	tr	0·9	tr	0·3	0·2
21	tr	tr	tr	0·5	tr	tr	tr
22	0·6	1·8	1·0	1·8	tr	0·4	0·8
23	0·4	1·0	0·2	1·1	*	0·2	0·3
24	2·0	8·6	1·5	3·7	2·2	1·6	2·9
25	2·4	5·1	1·5	1·3	1·0	2·8	2·4
26	31·2	58·3	8·2	14·8	13·6	57·0	50·5
27	3·2	3·7	4·4	2·4	3·4	5·8	7·9
28	23·3	13·3	26·0	15·1	25·7	28·0	33·8
29	3·4	*	9·4	8·5	5·3	0·4	tr
30	10·6	*	21·5	31·8	33·0	1·3	tr
31	*	*	0·5	3·2	*	*	*
32	*	*	0·5	4·9	*	*	*

Only normal straight-chain acids are included in calculations, except for kale ex gl_3, where iso-C_{28} formed 13·8 per cent of the total. Components less than 0·1 per cent shown as "tr". * Not detected.

The first three pairs of columns refer to comparisons within a segregating F_2 generation. Hence "Kale Gl_3" means the glaucous phenotype segregating in the same F_2 as the mutant gl_3. Dominance of the glaucous phenotype was complete; no distinction was made between heterozygous and homozygous dominant glaucous plants. For gl_2 no glaucous form was available for comparison within the line.

TABLE 7. PER CENT DISTRIBUTION OF CARBON NUMBERS IN STRAIGHT-CHAIN PRIMARY ALCOHOLS FOUND FREE IN THE WAX OF GLAUCOUS *Brassica* AND gl_4

Carbon No.	gl_4	Glaucous Gl_4
C_{12}	24·4	1·9
C_{13}	25·7	1·9
C_{14}	26·8	67·0
C_{15}	20·8	0·7
C_{16}	2·3	15·2
C_{17}	*	3·8
C_{18}	*	3·8
C_{19}	*	*
C_{20}	*	5·7

"*"=not detected. Only carbon numbers C_{12}–C_{20} have been included. For remarks about higher carbon numbers, see text.

(vi) *Aldehydes*

The presence of long-chain alphatic aldehydes in the wax was shown by the following:

(*a*) aldehydes isolated from sugar cane wax by Lamberton[16, 19] had the same R_f in TLC as the *Brassica component;*

(b) both the main component of sugar cane wax and the *Brassica* component reacted to produce a red colour with Schiff's reagent;

(c) during GLC, components from sugar cane wax aldehydes agreed in retention times with two components from *Brassica* wax;

(d) mixtures of ester and presumed aldehyde eluted from the column had two distinct peaks in the carbonyl region of the i.r. spectrum, showing bands at 1745 cm^{-1} (ester) and 1725 cm^{-1} (aldehyde).

The mutant plants possessed wax in which the aldehydes were much more prominent and thus more easily detected. However, glaucous plants also showed traces of aldehyde, the amount varying considerably with the variety used for analysis. The compositions of aldehydes from normal and mutant plants are shown in Table 8. The distribution between C_{26}, C_{28}, and C_{30} differed as between normal, gl_2 and gl_3, and gl_4. In gl_2 and gl_3 there was a reduction and in gl_4 an increase in C_{30} aldehyde in comparison with normal wax. These observations suggest interference with chain elongation in gl_2 and gl_3 but not in gl_4. There have been no previous reports of aldehydes in *Brassica*, but they are well-known components of other plant waxes.[16–19] The unidentified component described by Kolattukudy[5, 8] is probably aldehyde.

TABLE 8. PER CENT COMPOSITION OF *Brassica* ALDEHYDES INCLUDING CARBON NUMBERS OF C_{26} AND UPWARD ONLY

Carbon No.	Normal	gl_2	gl_3	gl_4
C_{26}	10·2	29·5	69·0	1·2
C_{27}	*	5·7	8·5	tr
Unknown	*	tr	tr	3·1
C_{28}	49·3	61·0	22·5	7·7
C_{29}	4·4	1·6	*	4·6
C_{30}	36·1	2·2	*	75·4
C_{31}	*	*	*	0·8
C_{32}	*	*	*	7·2

Traces only (<0·1 per cent) of unidentified components were present with carbon numbers $< C_{26}$.

(vii) *Esters*

Esterified primary alcohols were similar to the free alcohols in the C_{24}–C_{28} range. The C_{12}–C_{20} range consisted mainly of traces of the even-numbered components in both glaucous and glossy plants, mainly C_{16} and C_{18}.

Esterified acids contained much abnormal, apparently branched-chain, acids in the range

[16] J. A. LAMBERTON, *Australian J. Chem.* **18**, 911 (1965).
[17] F. RADLER, *Australian J. Biol. Sci.* **18**, 1045 (1965).
[18] F. RADLER and D. H. S. HORN, *Australian J. Chem.* 7, 1059 (1965).
[19] J. A. LAMBERTON and A. H. REDCLIFFE, *Australian J. Chem.* **13**, 261 (1960).

C_{12}–C_{20}. The *Brassica* distributions were similar to those of wool wax and the relative retentions of the various non-normal components are in accordance with those in the wool wax acids designated as iso- and ante-iso by Downing *et al.*[15] Although the content of C_{15} acid is very low in both glaucous and glossy plants, the glaucous normal clearly has more n-C_{15} acid than the green. Ester acids of the C_{24}–C_{30} range were similar to the free acids of the normal glaucous type but showed no observable trends as a result of the mutations.

DISCUSSION

The assumption that *Brassica oleracea* contains no C_{15} acid[1–9] does not apply to the wax free acids. This component is characteristic of the common glaucous form, and is severely reduced in mutant forms which also have a marked reduction in C_{29} compounds.

The characteristic free-acid distribution in the wax of the glaucous plants can best be explained by assuming an active α-oxidation system to be genetically inactivated in the leaves of the glossy mutants. The inactivation can occur by mutation at at least two different loci.

Our observation of the presence of free pentadecanoate in glaucous *Brassica* wax, together with its absence in two genetically different non-glaucous mutants, is in apparent conflict with Kolattukudy's final conclusion.[8] The simplest explanation for the presence of the C_{15} acid in glaucous wax is to assume that it is the precursor of C_{29} ketone, according to the hypothesis of Channon and Chibnall,[9] and that it is made by a process of α-oxidation, as originally assumed by Kolattukudy.[5]

Analysis of the ketone has revealed that it contains about 20 per cent of 14-nonacosanone and also traces of 13-nonacosanone. This finding is consistent with that of Kolattukudy,[8] who reports that a $C_{15}H_{31}\cdot CO$ group in the ketone, which could arise from 14-nonacosanone, became labelled after incubation of tissue with palmitate-2-^{14}C. The presence of 14-nonacosanone can also be explained by assuming the condensation process to occur between C_{16} and C_{14} acids, the hypothetical condensing enzyme lacking complete specificity with respect to carbon number. However, experiments designed to detect loss of CO_2 from the 1-position of fatty acids during incorporation into C_{29} compounds have yielded results which indicate incorporation of the intact fatty acid without loss of CO_2.[8]

The "mycolic acid" condensation for the production of corynomycolic acid is established in the case of *Corynebacterium*,[10] and this reaction probably leads to the production of palmitone which is also present in that organism. *Pisum*[14] contains the secondary alcohol 16-hentriacontanol together with large quantities of hentriacontane but the corresponding ketone (palmitone) is entirely absent from the wax. Pea-wax has the usual even-numbered sequence of carbon atoms in the free acids, there being no detectable pentadecanoate. If the 16-hentriacontanol were made by a condensation mechanism similar to that for mycolic acid condensation[10] the precursor would be palmitate, in agreement with the distribution of free acids.

It is possible that our results in *Brassica* could be reconciled with the elongation-decarboxylation pathway[8] by assuming that α-hydroxypalmitate, shown to be an intermediate of α-oxidation,[20] is the substrate which is elongated to C_{30} and subsequently decarboxylated. The pentadecanoate found in the free-acid fraction could on this view be considered as a by-product of α-hydroxy acid production, the latter acid being a specific substrate for an elongation system concerned with production of C_{29} compounds. If a special elongation system is invoked to explain the production of C_{29} components it is necessary to postulate as well a

[20] C. HITCHCOCK and A. T. JAMES, *Biochem. Biophys. Acta* **116**, 413 (1966).

more conventional elongation system producing not only acids, primary alcohols and aldehydes, but a series of paraffins also, including nonacosane. Kolattukudy[5] found that TCA selectively inhibited C_{29} biosynthesis compared with other wax components or "internal" lipids.

Our results clearly support the idea of two distinct biosynthetic systems. First the mutations appear to affect specifically C_{29} compounds. Second, the mutations fell into two groups, those which increased C_{30} acid (gl_4), and those which reduced it ($gl_{2,3}$) relative to other acid components. Macey[14] has reported a similar result for emerald mutants in *Pisum*. The action of the mutants is apparently complex, reducing both C_{15} and C_{30} acids ($gl_{2,3,5}$) or only C_{15} (gl_4) with increases in C_{30}. If C_{15} acid change is ignored, gl_4 could inhibit simultaneously hydroxylation and decarboxylation of C_{30} acid, and the other group, elongation.

In a recent publication, Kolattukudy *et al.*[21] have found that when stearate-1-^{14}C or stearate-U-^{14}C are fed to *Brassica* leaf discs, label could be found only in the C_{15} acid after degradation of the C_{29} ketone formed. This result was interpreted to mean that α-oxidation of stearate + elongation could not have been a mechanism of C_{29} ketone formation. Together with previous results this observation appears to rule out anything but elongation from stearate, with specific oxygenation at some subsequent stage. Moreover, in that work it was shown that breakdown and resynthesis could not account for the results. However, the function of such a specific oxygenation reaction is obscure. Our genetical studies show that the biosyntheses of C_{29} compounds are linked, probably by a common precursor. The only way, apart from C_{15} acid condensation or obligatory α-oxidation of precursors, in which we can imagine the insertion of a functional group to play some part in the production of nonacosane is to assume that it somehow influences decarboxylation of the long-chain acid. On chemical grounds this seems very unlikely.

The results of the present analysis are relevant to another phase of wax metabolism. Kolattukudy[22] has reported that isoleucine, and isobutyrate-1-^{14}C, are incorporated largely into the non-C_{29} components of the surface wax of *Brassica*. His results show especially that incorporation into esters and primary alcohols occurs. From our analysis branched-chain components form an especially high proportion of the esterified acids, while in the free acids the proportion is much lower. Also the free primary alcohols contain branched-chain components in the C_{26}–C_{30} range. In *B. oleracea* it appears that branched-chain acids are partially compartmentalized from straight-chain components and are preferentially incorporated into esters. Other species, such as *Pisum sativum*,[15] do not contain non-normal components.

EXPERIMENTAL

(*a*) *Wax Extraction*

Whole unbroken leaves were immersed for 20 sec in a dish containing petrol. ether (b.p. 60–80°) at 55–60°. 1 l. of solvent was required for 1 kg (fresh wt.) of leaves. The solvent was evaporated and the remaining traces of solvent removed *in vacuo*. Glaucous plants yielded 600–900 mg wax/kg fresh wt. and glossy plants 100–400 mg, depending on mutant. For any one analysis about 1 g wax was used. More wax could be extracted using additional solvent dips, but subsequent extractions yielded increasing amounts of internal pigments and internal lipid and were not used for the wax analyses. Separation of wax components was achieved using a combination of column chromatography and TLC.

(*b*) *Column Separations*

Fully activated Florisil (60–100 ǂ) was mixed with 5 per cent of its weight of water before use. Columns were packed with Florisil by settling in a column containing petrol. ether (b.p. 60°–80°). For 1 g wax, 50 g

[21] P. E. KOLATTUKUDY, R. H. JAEGER and R. ROBINSON, *Nature* **219**, 1038 (1968).
[22] P. E. KOLATTUKUDY, *Plant Physiol.* **43**, 1423 (1968).

Florisil were packed into a 2 × 30 cm column. The wax was dissolved in a minimal quantity of petrol. ether at room temp, placed on the column, and the latter then eluted with petrol at a flow rate of 3 ml/min, 50-ml fractions being collected. Under these conditions, paraffins were eluted from the column as a homogeneous fraction. The best solvent for the stepwise elution of esters and aldehydes was 5 per cent benzene in petrol. ether (60–80°). Esters were eluted slightly before aldehydes and samples of ester were obtained free of aldehydes. Ketones were eluted somewhat later than esters and aldehydes, and almost pure ketone could be obtained from column chromatography.

Fractions containing ester, ester+aldehyde, and +ketone were bulked separately. Amounts of ketone in the glaucous form were estimated by separation on preparative TLC following saponification. Traces of aldehyde were removed during saponification from possible contamination of the ketone because they were converted to condensation products, and esters were converted to primary alcohols and acids which were then easily separable from the ketone. Aldehyde and ester yields were calculated from ketone yield by subtraction.

When only small amounts of ketone and secondary alcohol were present as in all the glossy mutants, elution with 5 per cent benzene was continued until the small amounts of ketone and secondary alcohol were detected. The fractions containing these components (contaminated with traces of ester and aldehyde) were saponified and the ketones and secondary alcohol isolated by preparative TLC. Where large amounts of ketone and secondary alcohol were present, the per cent benzene was increased to 10 per cent in order to elute secondary alcohols in a second step after most of the ketone was eluted. Secondary alcohols were isolated from mixtures with the ketone following saponification to remove traces of ester and aldehyde.

Primary alcohols were eluted with benzene or benzene containing 10 per cent ether. In the green mutants with a large proportion of primary alcohol, benzene was normally used, but in the glaucous forms with much less, the addition of ether was often necessary. After primary alcohols were eluted, the column was washed with ether to remove traces of a variety of unknown compounds; together forming about 1–2 per cent of the total wax.

Free acids were eluted using 4 per cent acetic acid in ether, according to the method suggested by Carroll.[23] The free-acid fraction was contaminated with pigments and other material. The material was converted to the methyl esters and rechromatographed using benzene and a 10-cm Florisil column. Esters were eluted while more polar unesterified material remained on the column. Column fractions were checked using TLC and i.r. spectra (see below).

(*c*) *Thin-Layer Chromatography*

TLC was carried out according to the methods of Purdy and Truter.[2] In addition, aldehydes were detected by spraying with Schiff's reagent.

(*d*) *Infra-red Spectra*

I.r. spectra of column fractions and purified column fractions were taken using melted films, but bands were also checked using 10 per cent solutions in CCl_4. The correlations used for identifications were collated from the work of Bellamy.[24] Although the carbonyl absorptions obviously overlap, ketones, aldehydes and esters were distinguishable as separate bands, ketone at 1700 cm^{-1}, aldehyde at 1720 cm^{-1} and ester at 1745 cm^{-1} (melted film). Secondary alcohols had a series of bands at 1022, 1040, 1070, 1110, and 1130 cm^{-1}, also described by Purdy and Truter.[2]

(*e*) *Detection of Unsaturated Components*

Unsaturated components were detected in the glossy mutants but were a minor component of the ester-aldehyde fraction. Absorption in i.r. at 1620–1640 cm^{-1} was correlated with conjugated carbonyl absorption at 1680^{-1}. Other wax components were tested for the presence of unsaturated components by bromination,[25] and by TLC on Silica gel G with and without silver nitrate.[26]

(*f*) *Conversion of Ketone to Ketoxime and Secondary Amide*

The position of the functional group in the ketone, and in the secondary alcohol after conversion to ketone, was determined by conversion of the ketone to the ketoxime, followed by Beckmann re-arrangement to a secondary amide. The amide was hydrolysed and the acids recovered and analysed by GLC. The method used was modified from that described by Furukawa.[27] In order to catalyse the conversion of ketoxime to amide, ether saturated with PCl_5 was used instead of an acetic–sulphuric acid mixture.

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(*g*) *Gas Chromatography*

GLC was carried out using 5 ft × ⅛ in. 20 per cent S.E. 30 columns on Chromasorb W. The instrument used was an Aerograph Series 200 with dual columns and flame ionization detectors. Carrier gas was N^2, used at a flow rate of 30 ml/min.

(i) *Paraffins.* Paraffins were analysed at 270° using a series of reference standards supplied by Lamberton.* The carbon numbers analysed were confied to the $C_{23-33}C$ range, since only traces were found with carbon numbers less than C_{23}.

(ii) *Ketones and secondary alcohols.* These were analysed under the same conditions as the paraffins. Secondary alcohols were analysed by GLC before and after oxidation with ethereal chromic acid. Both secondary alcohols and ketones were checked using a sample of C_{29} ketone supplied by Lamberton. Carbon No. of the ketone received an indirect check from degradation studies ((*f*) above).

(iii) *Primary alcohols.* Very long chain alcohols C_{22-32} were analysed as such using a temperature of 270° and standards supplied by Dr. K. E. Murray.* C_{12}–C_{20} alcohols were analysed using a temperature of 200° and standard mixtures purchased from Polyscience Corpn. (Kenilworth, Illinois). The whole range was analysed by temperature programming from 190 to 290° at 6°/min.

(iv) *Aldehydes.* Aldehydes were isolated from sugar cane wax supplied by Dr. J. A. Lamberton and used as standards during GLC on S.E. 30 columns, following the technique of Radler and Horn.[12] Aldehydes were separated from the appropriate column fractions by preparative TLC.

(v) *Acids.* Methyl esters were prepared from the free acids and from acids obtained from saponifications of esters.[28] GLC was carried out for very long chain acids at 270°, for C_{12}–C_{20} at 200° and for analyses over the whole range by programming from 200 to 290° at 6°/min. Relative retentions at 200° were checked where possible from the list published by Burchfield and Storrs.[29] Direct checks were possible for the critical C_{12}–C_{20} range. The following list gives the average values found and those published (in brackets) for the normal straight-chain acids: C_{12} 0·244 (0·238); C_{13} 0·351 (0·340); C_{14} 0·492 (0·485); C_{15} 0·709 (0·699); C_{16} 1·0 (1·0); C_{17} 1·42 (1·41); C_{18} 2·01 (2·05); C_{20} 4·02 (4·12).

Iso- and ante-iso acids were tentatively identified using a mixture of the free acids of wool wax, supplied by Dr. D. T. Downing,† isolated from that source by column chromatography on Florisil. These acids have been characterized by Downing *et al.*[23] The relative retentions (relative to $C_{16:0}=1·0$) found for iso- and ante-iso acids from wool wax and which coincided with *Brassica* wax components were: iso-C_{15}, 0·652; iso-C_{16}, 0·896; ante-iso-C_{17}, 1·30; ante-iso-C_{19}, 2·60; ante-iso-C_{19}, 2·60; iso-C_{20}, 3·56.

Normal straight-chain methyl ester standards C_{16}–C_{32} and a C_{29} ketone were a gift from Dr. J. A. Lamberton.

(vi) *Quantitation.* Values are given in the tables as a per cent of the total peak area occupied by a particular component on GLC. Per cent composition values given in the tables are uncorrected.

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