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CHEMICAL GENETICS OF WAX FORMATION ON LEAVES OF *PISUM SATIVUM*

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Abstract—The wax on the surface of stems and leaves of four genetically different mutants of *Pisum sativum* has been analysed and compared with wax from the normal glaucous plants. In the latter over 50 per cent of the wax consists of the normal paraffin, hentriacontane. Associated with this component is a mixture of secondary alcohols also with 31 carbon atoms and consisting mainly of 16-hentriacontanol and appreciable amounts of 15-hentriacontanol together with traces of other secondary alcohols. All four mutants possess a wax containing much less hentriacontane and hentriacontanol, the total paraffin content being reduced from over 50 per cent to less than 10 per cent of the total wax. At the same time the ester, aldehyde, and primary alcohol content of the wax is increased. The paraffins of the mutants show a greater spread of carbon numbers. In the normal glaucous wax over 98 per cent of the paraffin is hentriacontane. In *wa*, the predominant paraffin is nonacosane (62 per cent) instead of hentriacontane. In other mutants less drastic but significant changes are apparent in the distribution of carbon numbers of the paraffins. In the free acids, reduction in the amount of compounds with 31 carbon atoms is accompanied by reduction in dotriacontanoic acid in mutants *wa* and in *wb* but not in mutants *was* or *wsp*. The changes in the various wax fractions are discussed in relation to the results of analyses of *Brassica* mutants and the various hypotheses of wax metabolism.

INTRODUCTION

ALTHOUGH the wax from *Brassica oleracea* has been thoroughly investigated chemically[1,2] there have been few analyses of other plants having a visually similar kind of wax. Hall *et al.*[3] observed that the glaucous surfaces of "wild-type" pea plants consist of a mass of crystallites as seen in electron microscopy, somewhat similar to the glaucous surface of *B. oleracea*.[3] "Glossy" mutants in *Brassica* or "emerald" in peas have a modified structure showing either a reduction in number of crystallites and a tendency towards a smoother surface, or with broader flat scales predominating over the rods characteristic of the glaucous wild-type.

Juniper[4] observed that the development of crystallites on the leaf surface of peas is dependent on the presence of light. He also has shown that the contact angle of water droplets is reduced as a result of uptake of trichloracetic acid (TCA), this effect again being due to a partial failure in the formation of wax-crystallites on the leaf surface. These observations indicate a special mechanism for the production of the crystallite structure which is dependent on light, inhibited by TCA and by certain mutations. No parallel chemical studies have yet been reported.

This paper describes the chemical analysis of the surface wax from glaucous peas and the variations in the composition caused by mutation at four distinct genetic loci. Lamprecht[5]

[1] A. C. CHIBNALL and S. H. PIPER, *Biochem. J.* **28**, 2209 (1934).
[2] S. J. PURDY and E. V. TRUTER, *Proc. R. Soc.* **B158**, 536 (1963).
[3] D. M. HALL, A. I. MATUS, J. A. LAMBERTON and H. N. BARBER, *Australian J. Biol. Sci.* **18**, 323 (1965).
[4] B. E. JUNIPER, *Endeavour* **69**, 20 (1959).
[5] H. LAMPRECHT. Report from the Plant Breeding Institute, Weibullsholm, Landskrona Tryckeri, A.B. (1961).

has summarized the genetic relationships of the various "emerald" mutants in peas. The lines and phenotypes used are given in Tables 1a and 1b. The mutants occur on the following chromosomes: *wa* and *wb* on chromosome II (83 map units apart); *was*, IV; *wsp*, VII; *wlo*, VI. No chemical analysis was made of *wlo*.

TABLE 1a. OCCURRENCE OF BLOOM AND CAPACITY FOR WATER REPELLANCY IN MUTANT PHENOTYPES

Plant part	*wa* and *was*	*wb*	*wsp*	*wlo*
Leaflets, adax.	+	+	+	−
Leaflets, abax.	−	−	−	+
Peticles	+	+	−	+
Stipules	−	−	−	+
Stem	+	+	−	+

Different parts of the mature pea plants were scored as to the presence (+) of bloom or absence (−) of bloom, which was correlated with the capacity for water repellancy.

TABLE 1b. LINES OF *Pisum* USED AND THE ASSOCIATED GENES

Lines	Wax gene	Marker genes in crosses
6	*wa*	*le*
29	*wa*	*Le*
32	*wb*	—
33	*was*	—
35*	*wsp*	*Le, tl, St*
36*	*wsp*	*Le, Tl*
38	*wsp*	*le, Tl*
31	*wb, wlo*	*st*

* Indicates containing originally unknown mutants but characterized by breeding tests as *wsp*.

RESULTS

(*a*) *Overall Composition of Different Waxes*

The composition of various normal and mutant waxes is shown in Table 2. The glaucous form contains 50 per cent paraffins and all mutants contain much less. There is variation in the extent to which the different mutants reduce the paraffin content of the wax. However, this variation in chemical composition is not well correlated with the changes in the pattern of glaucousness shown in Table 1. For example *wa* has a wax distribution similar to *wb*, but its paraffin content and composition is quite different (Tables 2 and 3).

The other change of note is the much reduced amount of secondary alcohol in the emerald mutants. This change is obvious in TLC of whole waxes. It is apparent from this and other data that *Pisum* wax contains a secondary alcohol, but no ketone. Esters, aldehydes and primary alcohols are present. The proportion of the latter three components is increased by the presence of an "emerald" gene, owing mainly to the great reduction of C_{31} paraffin.

TABLE 2. PER CENT COMPOSITION OF NORMAL AND MUTANT WAX TYPES IN *Pisum*

Component	Normal	*wa*	*wb*	*wsp*	*was*
Paraffin	50	10	15	24	28
Ester+aldehyde	18	41	44	36	36
Secondary alcohol	7	—	—	—	—
Primary alcohol	19	45	37	29	26
Free acid	6	4	4	11	10

"—" indicates accurate figures not available; but less than 1 per cent. Except in normal *Pisum*, with appreciable amounts of secondary alcohol, figures for the latter do not contribute significantly to the total.

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

Carbon No.	Normal	*wa*	*wb*	*was*	*wsp*
$C_{23}+<C_{23}$	*	5·2	0·5	13·0	9·5
C_{24}	*	tr	0·1	1·1	*
C_{25}	0·2	12·4	3·8	3·8	3·3
C_{26}	*	1·5	0·2	0·6	0·5
C_{27}	0·1	13·6	1·6	4·2	2·4
C_{28}	*	1·0	tr	0·7	0·3
C_{29}	1·2	62·0	10·0	4·3	2·0
C_{30}	*	0·5	1·7	1·6	0·5
C_{31}	98·4	3·8	82·4	68·5	71·9
C_{32}	*	*	*	2·2	2·0
C_{33}	*	*	*	0·2	7·4

Figures were obtained from the peak areas on GLC using a flame ionization detector. *=not detected.

(*b*) *Paraffin*

The composition of the paraffin is shown in Table 3. The paraffins from normal *Pisum* are 98 per cent n-hentriaceontane (C_{31}). In *wa* the content of hentracontane is reduced to 3·8 per cent with nonacosane (62 per cent) becoming the main component. In other mutants, although a general reduction of total paraffin content occurs (Table 2), hentriacontane remains at least a major component, but in reduced proportion. The mutant *wsp* shows appreciable amounts of tritriacontane (C_{33}) not detectable in the normal glaucous plants.

The paraffins of etiolated plants were examined from *normal* and *wa*. Juniper[4] has shown that pea leaf surfaces only develop a clearly defined crystallite structure in the light. The wax from etiolated plants does not contain any secondary alcohol detectable by TLC, in contrast to the light-grown plants. Table 4 shows the paraffin compositions of wax from normal and *wa*. The first and second and the fourth and fifth columns show that normal and *wa* differ even in the dark. The difference is in the direction expected from analysis of light-grown plants; *wa* possesses only 13·3 per cent hentriacontane, while the *normal* plants contain 65·7 per cent. This indicates that *wa* can act even in the dark, and in the absence of any effect on secondary alcohols, the level of which remained undetectable in both *wa* and *normal*. These observations leave room for doubt that secondary alcohols are obligatorily involved in the synthesis of paraffins with 31 carbon atoms. Table 4 shows that, in the light, the composition of the wax of light-grown plants can be approximately computed from the

composition of the dark-grown plants by assuming a tenfold increase in nonacosane for *wa* and hentriacontane for normal glaucous wax. In the latter case, however, there is a concomitant increase in secondary alcohol while in the former, this does not occur.

TABLE 4. PER CENT COMPOSITION OF PARAFFINS ISOLATED FROM DARK-GROWN NORMAL AND *wa Pisum* PLANTS

Carbon no.	Normal *Pisum*			*wa*		
	Dark	Light	Derived figures	Dark	Light	Derived figures
C_{23}	1·3	*	tr	11·0	*?	5·2
C_{23}	4·7	*	0·7	5·2	5·2	2·4
C_{24}	1·8	*	0·3	3·0	*?	1·4
C_{25}	11·3	*	1·6	26·9	12·4	12·6
C_{26}	1·1	*	0·1	1·9	1·5	0·9
C_{27}	5·3	0·2	0·8	21·0	13·6	9·8
C_{28}	0·7	0·1	tr	4·4	1·0	2·1
C_{29}	5·2	1·2	0·8	12·5	62·0	58·8
C_{30}	2·8	0·1	0·4	0·8	0·5	0·4
C_{31}	65·7	98·4	95·0	13·3	3·8	6·3

The figures in columns 3 and 6 have been computed from 1 and 4 by assuming a tenfold increase in C_{31} in the light in normal *Pisum* and a tenfold increase in C_{29} in *wa*.
* Indicates "not detected".

(*c*) *Secondary Alcohols*

Secondary alcohols were isolated by TLC from the appropriate column fractions and analysed by GLC and i.r. before degrading. The component has an i.r. spectrum corresponding to that of the secondary alcohol isolated from *Brassica oleracea*.[2,6] On GLC together with a sample of *Brassica* secondary alcohol, the main pea wax component falls on the logarithmic series of retention times with the minor (C_{27}?) and major components (C_{29}) of *Brassica* secondary alcohols, in a position which would be assumed for a C_{31} component. There were also traces of a component having the same retention time as C_{29} secondary alcohol. Thus, the main component of the *Pisum* secondary alcohol is probably a C_{31} compound. This conclusion is supported by the degradation analysis (see below) and, by analogy with *Brassica*, by the presence of large amounts of hentriacontane (99 per cent) in the paraffins instead of the nonacosane found in *Brassica*.[6]

The secondary alcohol from the normal glaucous form was oxidized to the corresponding ketone, and then degraded using the method described for analysis of the *Brassica* ketone.[6] The composition of the mixture of acids, derived from hydrolysis of the secondary amide resulting from the Beckmann re-arrangement, was as follows: C_{14}, 4 per cent; C_{15}, 19 per cent; C_{16}, 58 per cent; C_{17}, 14 per cent; C_{18}, 5 per cent. These figures indicate that the main component is 16-hentriacontanol, but that an appreciable amount of a component having a substituent group at the 15-position (33 per cent) is also present together with traces of other secondary alcohols (9 per cent). Traces of components having a chain length different from C_{31} are probably present in the secondary alcohol fraction isolated. However, these do not amount to 33 per cent, being more of the order of 2–3 per cent. Hence, some 15-hentriacon-

[6] M. J. K. MACEY and H. N. BARBER, *Phytochem.* **9**, 13 (1970). M. J. K. MACEY, Ph.D. Thesis, University of N.S.W. (1967).

tanol is present together with the main component. It is interesting to compare these results with those for the degradation of the *Brassica* secondary alcohol,[6] which are essentially similar except that the fatty acid degradation products had a distribution centring on the C_{15} component.

When the secondary alcohol fraction from *wa* was examined, the chain-length distribution appears to be C_{27}, C_{29} and C_{31} with about equal amounts of these three components. After oxidation, the C_{29} component has a retention time of a standard C_{29} ketone, and the other two components from a homologous series with it. Unfortunately, there was not enough material available to ascertain the position of the functional group in these components of the mutant form.

In parallel with similar observations in *Brassica*[6] therefore, mutations to the "emerald" character in *Pisum* lead to the great reduction in the amount of the predominant paraffin and its associated secondary alcohol of the same chain length. In contrast with *B. oleracea*, however, wax from *P. sativum* does not contain measurable amounts of long-chain ketones.

(*d*) *Primary Alcohols and Aldehydes*

GLC analysis shows that the free primary alcohols of normal glaucous plants are a mixture of the following composition: C_{25}, 1·2 per cent; C_{26}, 61·0 per cent; C_{27}, 0·5 per cent; C_{28}, 37·3 per cent. All the mutants examined have a similar composition, with slight quantitative variations. Esterified alcohols are essentially similar with appreciable amounts of components in the range C_{14}–C_{20} (5–10 per cent), which were not detected in the free primary alcohols.

TABLE 5. PER CENT COMPOSITION OF ALDEHYDES OF NORMAL AND MUTANT *Pisum* INCLUDING CARBON NUMBERS OF C_{26} AND UPWARD ONLY

Carbon no.	Normal	*wa*	*wb*	*was*	*wsp*
C_{26}	30·2	36·8	71·5	30·8	20·6
C_{27}	6·6	2·8	1·6	25·1	7·7
C_{28}	45·4	53·3	24·6	25·3	22·4
C_{29}	4·2	*	tr	18·8	10·8
C_{30}	8·2	7·1	2·3	*	3·9
C_{31}	1·7	*	*	*	3·8
C_{32}	3·7	*	*	*	30·8

In all cases shorter chain lengths accounted for < 3 per cent of the total.
* = not detected. tr = < 0·1 per cent.

Aldehydes in normal *Pisum* follow a pattern in most respects similar to the free primary alcohols. Table 5 shows the per cent composition of aldehydes from various mutants and the normal form. Variations in the mutants occur. In *wb* there is significantly less C_{28} than C_{26}, a reversal of the normal distribution. In *wsp* a large increase in the proportion of C_{32} aldehyde is evident. These changes are reflected in the composition of the free acid (see below), indicating a close relationship between aldehydes and acids. It is possible that some of these changes are not really due to the genes under investigation since the different lines of peas used had differing genetic backgrounds. However, preliminary observations show that though the C_{26}/C_{28} ratio can vary considerably between different normal forms, the content of C_{32} aldehyde does not increase in the manner of *wsp*. Hence, this latter effect is probably

a direct effect of *wsp*. Further investigations involving breeding further stocks are necessary to resolve this doubt.

(*e*) *Free Acids*

The free acids of the wax were examined by techniques described previously.[6] The per cent compositions (as calculated from peak areas only) are recorded in Table 6. A comparison of Tables 5 and 6 shows that the carbon number distributions of aldehydes and free acids are closely correlated. Again, some caution is needed in interpretation of the results owing to varying genetic backgrounds of the lines used. However, certain trends are apparent. The mutant *wa* was available in two different lines with dissimilar genetic backgrounds. Both lines give a distribution like that listed in Table 6. In comparison with the normal form, the per

TABLE 6. PER CENT COMPOSITION OF FREE ACID FRACTIONS FROM NORMAL AND MUTANT *Pisum* WAX

Carbon no.	Normal	*wa*	*wb*	*was*	*wsp*
16	0·5	0·5	4·1	2·4	0·7
17	*	0·1	*	*	*
18	0·6	1·3	2·7	1·8	1·4
19	tr	0·1	*	*	*
20	0·5	0·9	1·6	0·6	0·7
21	0·2	0·3	0·4	tr	0·2
22	0·3	6·4	14·3	5·9	6·5
23	0·5	2·3	0·6	0·7	0·3
24	2·1	6·1	4·4	1·3	1·2
25	5·1	5·4	2·5	9·3	1·0
26	25·7	19·9	45·2	55·1	21·5
27	5·0	1·4	1·2	2·6	1·5
28	29·9	33·6	15·4	10·4	16·0
29	2·4	1·0	tr	0·9	1·0
30	17·4	20·5	6·6	3·2	9·4
31	1·4	*	*	0·4	3·0
32	8·0	*	*	6·0	35·6

Carbon numbers below C_{16} were not detected. *=not detected. tr=present <0·1 per cent of total.

cent of C_{32} acid found in *wa* is much less than in the normal and cannot be detected. The same is true of the mutant *wb*. However, *was* and *wsp* do not exhibit this feature; in *wsp* the quantity of C_{32} acid is markedly increased compared to the normal form. Thus we have a situation analogous to that found in *B. oleracea*.[6] This observation suggests that a genetic block in the formation of C_{31} compounds can occur in two ways. One of these (in *wa* and *wb*) involves the reduction of the amount of C_{32} acid. The other (in *wsp*) involves an accumulation of C_{32} acid, and possibly diversion to production of extra aldehyde, with which this increase is correlated. In *wa* and *wb* this could imply a block in the elongation mechanism. It is possible to envisage a condensation mechanism involving a long-chain acid as the product, but then the very unreactive ω-methyl group would be involved in the mechanism. It is of some interest that the mutant gl_4 in *B. oleracea* exhibits the same pattern of changes in the C_{30} component as *wsp* does in the C_{32} component. In gl_4 however, there is evidence that diversion to C_{32} acid is not the only effect of the mutation, since the content of C_{15} acid is in that case, very much

reduced. The corresponding change in *P. sativum*, which would be a change in C_{16} acid, was not observed.

Analysis of free acids at the other end of the carbon number range (C_{12}–C_{20}) indicated only traces of C_{16} and C_{18} fatty acids. No significant changes could be detected in these components between the various mutant types and the normal glaucous form.

The same observations applied to the esterified acids with respect to the lower carbon number range. The upper ranges were not examined in detail, but in general the content of very long chain acids ($> C_{18}$) was less in the esterified acids. Unlike *B. oleracea*[6] there was no evidence for the existence of branched chain acids in the esters of the wax or in any other component.

As observed for sugar cane wax and *Brassica*[6–8] the very long chain aldehydes of cuticle waxes have been found to be even-numbered. There seems little doubt from the above correlations of aldehyde–free acid changes that the aldehydes are closely related to the free acids of the wax.

CONCLUSIONS

Pisum sativum possesses a paraffin-rich cuticle wax which in many respects is similar to that of *Brassica oleracea*. It differs, however, from *B. oleracea* in that (i) the principal paraffin present is a C_{31} component, not C_{29}; and (ii) although there is a secondary alcohol present corresponding in chain length with the C_{31} paraffin, there is no detectable ketone of the same chain length. The secondary alcohol is about 60 per cent 16-hentriacontanol, but the 15-ol, and possibly the 14-ol, are also present. In *Brassica*,[5] 15-nonacosanol was present together with 14-nonacosanol, and traces of the 13-ol. Whatever the mechanism of introducing the functional group, the latter varies about a median position in the molecule, in both *B. oleracea* and *P. sativum*.

In *wb* and *wa*, there was some correlation between a reduction in amount of C_{31} component and C_{32} fatty acid, the latter being undetectable in the mutant forms. This suggests that the elongation mechanism proposed by Kolattukudy[9] might be correct. On the other hand *wsp* and *was* had no reduction in C_{32} acid while also suffering reduction in C_{31} paraffin and secondary alcohol.

The analysis of wax from etiolated *Pisum* showed that it contained no detectable C_{31} secondary alcohol. The paraffins lost the predominance of the C_{31} component, though it was still formed. The dependence of crystallite formation by light as observed by Jupiner[4] has obviously some connexion with this observation. The etiolated plant is similar to the glossy mutant in having a modified wax crystallite structure.[3] In both cases the modification of crystallites is correlated with the reduction of the C_{31} paraffin. The fact that C_{31} paraffin synthesis is not abolished in the dark indicates again that there is more than one pathway open to the formation of paraffins, one of which is not correlated with the formation of secondary alcohol, and is not dependent on light.

The absence of the C_{31} ketone in *Pisum* indicates that the ketone may be an end product of metabolism rather than an intermediate stage in the formation of the paraffin. This conclusion

[7] Z. I. KRANZ, J. A. LAMBERTON, K. E. MURRAY and A. J. REDCLIFFE, *Australian J. Chem.* **13** (4) 498 (1960).
[8] F. RADLER and D. H. S. HORN, *Australian J. Chem.* **7**, 1059 (1965); F. RADLER, *Australian J. Biol. Sci.* **18**, 1045 (1965).
[9] P. E. KOLATTUKUDY, *Biochemistry* **5**, 2265 (1966).
[10] T. KANEDA, *Biochemistry* **7**, 1194 (1968).
[11] P. E. KOLATTUKUDY, *Science* **159**, 498 (1968).

is supported by the fact that there is greater variety in substituent position in secondary alcohol than in the ketone of *Brassica*,[6] which suggests derivation of ketone from secondary alcohol but not the reverse. Alternatively, the two components may be derived from a common precursor, with a structure similar to the corynomycolic acid found in *Corynebacterium* sp. by Gastambide-Odier and Lederer.[12]

The variety in substituent position found in secondary alcohols and ketones indicates, in terms of the condensation hypothesis, that the enzyme system responsible is not specific for chain length but can condense C_{15} and C_{17} as well as C_{16} acids.

Although the components were not identified by further analysis other than TLC, GLC, and i.r., the emerald mutants appeared to possess C_{29} and C_{27} secondary alcohols as well as the C_{31} component. This observation would imply that a pool of mixed fatty acids could condense together to give a variety of products. Unfortunately, there is no evidence as to the position of the functional groups in these components. The presence of secondary alcohols differing in carbon number by two could imply that at least some secondary alcohols are formed by an elongation system.

As previously suggested,[6] an alternative to the long-chain fatty acid condensation hypothesis is that the functional group be introduced by abortive α-oxidation of palmitate, and that the resulting α-hydroxy acid becomes the substrate of elongation reactions. If this idea is extended to *Pisum* it is necessary to assume β-hydroxystearate as the substrate, and that the main secondary alcohol is produced by subsequent elongation from this substrate. The necessity of making this additional assumption undoubtedly weakens the hypothesis.

Recently, Kolattukudy[13] has carried out experiments with pea leaves similar to those reported before, using *Brassica*.[9] These experiments are consistent with the elongation-decarboxylation pathway for the production of hentriacontane. However, the presence of 16-hentriacontanol in *Pisum* and of other secondary alcohols of the same chain length, should be emphasized. In *Pisum*, as in *Brassica*, the production of paraffins with one chain length predominating is correlated, through various mutants and differing physiological conditions, with production of a secondary alcohol of the same chain length. While it is clear that peas can form hentriacontane *without* forming secondary alcohol, the production of large quantities of hentriacontane in normal light-grown plants is always correlated with production of secondary alcohol. The elongation-decarboxylation hypothesis, in itself, does not explain this correlation, and appears to require further investigation.

EXPERIMENTAL

Techniques used for separation and identification were as previously described.[6] The lines of peas used were kindly donated by I. C. Murfet.*

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[12] M. GASTAMBIDE-ODIER and E. LEDERER, *Nature* **184**, 1563 (1959).
[13] P. E. KOLATTUKUDY, *Plant Physiol.* **43**, 1466–70 (1968).