THE EFFECTS OF INHIBITORS ON THE BIOSYNTHESIS OF THE LONG CHAIN LIPIDS WITH EVEN CARBON NUMBERS IN BARLEY SPIKE EPICUTICULAR WAX

by

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The effects of inhibitors on the incorporation of label from [2-¹⁴C]-acetate into the epicuticular wax classes with even carbon numbers have been determined. They include the free fatty acids, the primary alcohols, the aldehydes, the ester acids and a newly identified wax class on barley spikes, the short chain esters. Dithiothreitol, which has no effect on β -diketone synthesis, inhibits decarboxylation of very long fatty acyl chains which are precursors of the alkanes. Mercaptoethanol, which greatly stimulates wax synthesis at low concentrations and has no effect on β -diketone synthesis, blocks further elongation of fatty acyl precursors having 20 carbons at higher concentrations. A relatively small inhibition of decarboxylation was also observed with mercaptoethanol. Arsenite, which inhibits β -diketone synthesis presumably by blocking the entrance of C₁₆ precursors into the β -diketone elongation mechanism, was also found to prevent the C₂₀-C₂₂ elongation step in the sequence leading to the alkanes. Cyanide, which blocks β -diketone synthesis by an unknown mechanism, greatly stimulated synthesis of the other wax classes. At higher concentrations a block in the elongation sequence leading to the alkanes was also apparent between C₂₆-C₂₈. These results are incorporated into a composite figure illustrating the biosynthetic relationships among the wax classes. All identified chemical and genetic blocks have been included. The conclusion is reached that at least three elongation systems must be involved.

1. INTRODUCTION

Epicuticular wax on barley spikes consists primarily of β -diketones, hydroxy- β -diketones, esters, aldehydes, primary alcohols, free fatty acids and on certain genotypes also of secondary alcohols. Barley spikes have already proven to be a suitable system to investigate the biosynthetic relationships among the wax classes (37, 47, 51). For example, the elongation system leading to the β -diketones was found to be a different one from that giving rise to the other wax classes (37). The term system is used to include both biochemical and compartmentalization facets of the elongation. The origin of the other wax classes is believed to be as follows: With the aid of an elongation system(s), palmitate or longer fatty acids are elongated by malonyl-CoA to appropriate long chain precursors, which (i) serve as substrates for the decarboxylases leading to alkanes, secondary alcohols and ketones, (ii) serve as substrates for reductases giving rise to aldehydes, free and esterified alkan-1-ols or, (iii) are detached from the elongation system(s) as free fatty acids (23, 26, 31, 48).

Of great interest at present is the question of whether the elongation to specific chain lengths referred to in the previous sentence involves only one elongation system or more than one. Thus, different elongation systems could conceivably give rise to the fatty acyl precursors of i, ii and iii. Or perhaps, a sequence of elongation systems exists, in which the first elongation is from C_{16} - C_X , the second from C_X - C_Y and the third from C_Y - C_Z . In such a situation the various enzymes utilizing the elongated precursors would be restricted to those from only one or perhaps two of the sequential elongation systems. Results from genetic and physiological studies as well as preliminary investigations with thiocarbamates suggest that very likely several elongation systems are functioning. Since the use of inhibitors in conjunction with labelled precursors have proved an effective method to dissect

various parts of the wax biosynthetic mechanism(s) (5, 9, 10, 14-17, 19, 21, 22, 29, 30, 36, 43), this technique might also help resolve the questions of both the number and type of elongation systems involved.

The present communication presents the effects of inhibitors on the incorporation of [2- 14 C]-acetate into wax classes with even chain lengths. These were isolated in the course of the same experiments with *cer-u*⁶⁹ in which the biosynthetic relationships of the β -diketones and alkanes were investigated (37). The observed differential sensitivity of some elongation steps to the inhibitors – mercaptoethanol (ME), dithiothreitol (DTT), arsenite and cyanide – clearly demonstrates that more than two elongation systems are involved in wax biosynthesis on barley spikes.

2. MATERIAL AND METHODS

2.1. General

Sources of chemicals, methods of incorporation of [2- 14 C]-acetate into whole spikes, techniques of plant culture, location of the radioactive lipids on the thin layer plates (radio-TLC), and determination of the radioactivity in the various lipids by liquid scintillation are described elsewhere (37). The results for the amount of label in the lipid classes separated by thin layer chromatography are the average of at least two independently carried out incorporation experiments. The results for the distribution of radioactivity among the chain lengths of a given wax class are the mean of five injections into the radio gas chromatograph. Identification of the chain lengths within the wax classes was done using internal standards. No significant difference between injections was found using a χ^2 -test; in all cases P was greater than 80%.

Abbreviations: DTE = 1,4-dithioerythritol; DTT = 1,4-dithiothreitol; GLC = gas liquid chromatography; HEA = hexane; diethylether; acetic acid; ME = 2-mercaptoethanol; TCA = sodium trichloroacetate; TLC = thin layer chromatography; u-1 = unknown lipid 1; u-2 = unknown lipid 2.

Figure 1. Initial separation by radio-TLC in benzene of surface lipids isolated from whole barley spikes which had been fed 60 μ moles of mercaptoethanol followed by 1.44 μ moles (83.3 μ Ci) of [2- 14 C]-acetate. The lipid classes were divided into three groups.

2.2. Thin layer chromatography (TLC)

An initial separation of the total epicuticular wax was carried out by preparative TLC using 20 $\times$ 20 cm plates coated with silica gel H type 60 (Merck) and benzene as the developing solvent (Fig. 1). The following divisions were made: hydrocarbons; group one lipids consisting of β -diketones, aldehydes and esters; and group two lipids consisting of free fatty acids, primary alcohols, polar lipids and minor unknowns. Considerable label was also present in an unknown lipid class (u-1) having a retention time in benzene slightly less than the aldehydes after treatment with 60 μ moles ME (Fig. 1). The hydrocarbons, u-1 and the lipids of group one were eluted from the silica gel using chloroform. To obtain 100% recovery of the more polar lipids in group two, hot acidic methanol: chloroform 1:1 (v/v) plus a few drops of HCl was used for the extraction. During this procedure the free fatty acids were converted to methylesters (42).

2.2.1. Separation of lipid classes in group one

The separation of the three lipid classes in group one using »hybrid« copper acetate TLC plates is described elsewhere (37). Radio-TLC of the aldehydes in benzene and HEA (hexane: diethylether:acetic acid, 70:30:2, v/v/v; 33) showed only one peak (Fig. 2A). Because initial radio-gas liquid chromatography (radio-GLC) of the aldehydes revealed the presence of more than one homologous series, however, the aldehydes from selected treatments were treated as follows. They were reduced to primary alcohols using NaBH₄ in 96% ethanol at 35°C for 1 hour, a procedure adapted from ALLEBONE et al. (1) and KOLATTUKUDY (27), and separated from an unreduced fraction by TLC with benzene as shown in Fig. 2B. This unreduced fraction was resistant to repeated exposure to NaBH₄ and co-chromatographed with standard aldehydes isolated from sugar cane wax (32) both in benzene and HEA. When aliquots of the unreduced fraction and also u-1

Figure 2. Separation of the aldehyde into two lipid classes by reduction with NaBH_4 . A, before treatment with NaBH_4 ; B, after treatment with NaBH_4 . In both cases silica gel H plates were used. The true aldehydes were reduced to primary alcohols. The non-reducible fraction has been identified as short chain esters. When estimating the proportion of aldehydes to short chain esters, the radioactivity present in the shoulder of the non-reducible peak was included with that of the main peak. The aldehydes were isolated from wax labeled with $[2\text{-}^{14}\text{C}]\text{-acetate}$ after treatment with 6 μmoles arsenite.

Figure 3. Procedure used to demonstrate that the unknown-1 (u-1) of Figure 1 consists of short chain esters. (A) Fatty acids at the origin contain almost all the label subsequent to base hydrolysis of u-1. (B) Label is present in methyl esters after $\text{BF}_3/\text{methanol}$ treatment of the fatty acids. In both cases the separation was carried out on silica gel H plates using benzene as developing solvent.

were subjected to base hydrolysis (46) all but a trace of the radioactivity was recovered as free fatty acids (Fig. 3A). U-1 and the unreduced aldehyde fraction when converted to methyl esters and subjected to radio-GLC gave identical traces. These results suggest that the unreduced fraction and u-1 are in fact esters. The u-1 lipid class was apparently resolved in the initial TLC experiments from the group one lipids only in the case of the 60 μ moles ME treatment. This resolution probably depends not only on the relative amounts of label present in these esters and aldehydes, but also on the proportion of the label in the various chain lengths within these two lipid classes. Having established that the non-reducible fraction of the aldehydes were esters, it was more practical in the experiments reported here to carry out a transmethylation reaction to form the methyl esters of the acid moieties for injection into the radio-GLC. The procedure which was adapted from that of BUCKNER and KOLATTUKUDY (5) consisted of a 2 hour treatment of the non-reducible fraction at 80°C with BF_3 /methanol reagent. Since so little activity was present in the alcohol moieties no attempt was made to identify them. As these esters (i) co-chromatograph with the aldehydes which acetate esters are known to do (1), (ii) have a lower R_f than the well characterized long chain esters and (iii) have fatty acid moieties of similar chain lengths as the long chain esters suggests that the alcohol moieties of the »aldehyde esters« are shorter than those of the long chain esters. Thus, the aldehyde ester fraction will, hereafter, be referred to as short chain esters.

2.2.2. Separation of lipid classes in group two

The lipid classes in group two were first subdivided into free fatty acid methyl esters, primary alcohols plus unknown-two (u-2) and polar lipids plus minor unknowns by TLC using HEA as developing solvent (Fig. 4). The primary alcohols plus u-2 from selected treatments were extracted using chloroform, applied to another silica gel H plate and treated with acetyl chloride (46). This converted the primary alcohols to acetates which allowed them to be

Figure 4. Separation of group two lipids (Fig. 1) by radio-TLC in HEA. The free fatty acids were converted to methyl esters during the extraction of the group two lipids from the silica gel after preliminary separation of wax into groups as shown in Fig. 1.

separated from u-2 by using benzene as the developing solvent (R_f primary alcohols = 0.06-0.09; primary alcohol acetates = 0.34-0.39; u-2 = 0.11-0.14).

2.3. Radio-gas liquid chromatography (radio-GLC)

Radio-GLC was carried out using a Varian Aerograph 202-1 B series gas chromatograph and a Nuclear Chicago proportional counter (40). Calibration of the instrument, which was carried out as described in (40), allows one to determine the specific activities to two significant figures. All parameters except the temperature program which went from 150 to 250°C at a rate of 2°C per minute have been detailed elsewhere (37).

3. RESULTS

3.1. Incorporation of label into the various epicuticular wax fractions

Intact barley spikes readily incorporate [^{14}C]-acetate into their epicuticular waxes. Separation of the surface lipids by TLC, followed by a quantitative determination of the label present in each lipid class, revealed that

incorporation was highest in the hydrocarbons 24%, followed by aldehydes plus short chain esters 22%, primary alcohols plus unknown 16%, β -diketones 14%, polar lipids plus minor unknowns 10%, free fatty acids 9% and esters 4%. This distribution is quite different from that of the mass (hydrocarbons 15.9%, esters 10.8%, aldehydes 3.7%, β -diketones 50%, primary alcohols 2.6%, free fatty acids 7% and some unknowns 9.9%; 50, 51 and P. VON WETTSTEIN-KNOWLES personal commun.). This indicates that even though an *in vivo* system has been utilized for the incorporation, the results are not a true facsimile of the actual synthesis of the epicuticular waxes by the plant. The effects of various concentrations of DTT, ME, arsenite, cyanide and TCA on the radioactive distribution of incorporated [2- 14 C]-acetate among the esters, aldehydes plus short chain esters, primary alcohols plus u-2, free fatty acids and polar lipids are shown in Tables I to III. In the control experiment 62% of the label is present in these five wax fractions. With each 10-fold increase in inhibitor concentration, the proportion of the label present in these five lipid fractions is enhanced to 74-95% of the total label incorporated into the epicuticular waxes. Simultaneously the label in the hydrocarbons and β -diketones decreases. Since the effects of the five investigated inhibitors on the synthesis of the latter two wax classes are dealt with in detail elsewhere (37), they will not be further mentioned here.

3.1.1. Effect of dithiothreitol (DTT)

Only at the highest concentration of DTT investigated (60 μ moles) was the total incorporation of label into the wax significantly inhibited (47%). Examination of the data in Tables I and II reveals that of the five wax fractions of concern the reduction in incorporation of label is most marked in the two components of the aldehyde fraction, that is, true aldehydes and short chain esters. Synthesis of the latter was already affected at 36 μ moles at which concentration aldehyde synthesis was stimulated by 59%. Some inhibition (48%) of label incorporation into the primary alcohol plus u-2 fraction also occurs at 60 μ moles, but it is not possible from the present data to say whether

only one or both types of lipid are affected. At 36 μ moles, however, synthesis of both are increased as was that of the aldehydes (Tables I and III). The stimulatory effect of DTT at this concentration occurred also for the free fatty acids although the total amount of label in the wax remained essentially unchanged.

3.1.2. Effect of mercaptoethanol (ME)

Feeding the spikes 0.6 or 6 μ moles of mercaptoethanol resulted in a one-fold increase in the synthesis of all the wax fractions except the free fatty acids which showed only a small increase (28-46%) in label accumulation (Table I). The initial inhibitory effects of mercaptoethanol on total wax synthesis were found at a concentration of 60 μ moles. The most sensitive wax classes are the primary alcohols followed by u-2 showing 60 and 39% inhibition, respectively (Tables I and III). At the other extreme are the short chain esters whose synthesis is stimulated by 96% (Tables I and II).

3.1.3. Effect of arsenite

In contrast to the results with ME, low concentrations (0.6 and 6.0 μ moles) of arsenite have little effect on total wax synthesis or on the individual wax fractions. Interestingly, however, 6 μ moles of arsenite did significantly inhibit accumulation of label in the aldehydes and markedly increased it in the short chain esters (Tables I and II). With 60 μ moles total wax synthesis was reduced to 21% of the control values. The data in Table I indicate that formation of all wax classes was severely inhibited, the least affected being the aldehyde fraction (47% inhibition). On the basis of the results with 6 μ moles, it is believed that the majority of the label in the aldehyde fraction after treatment with 60 μ moles is in the short chain esters. The latter is a wax class that is synthesized by the spikes on barley plants in only trace amounts relative to the other wax lipids.

3.1.4. Effect of cyanide

Low concentrations of cyanide (0.6 and 6 μ moles) more than doubled the amount of label incorporated into the wax lipids (Table I). As observed with the stimulation by low concentrations of ME, the free fatty acids appear to be

Table I

Effects of pre-incubations with inhibitors on the incorporation of [2-¹⁴C]-acetate into five fractions of barley spike epicuticular wax which are separable by thin-layer chromatography (TLC)^a.

Experimental condition	(μmoles)	Total label in wax ^b (cpm x 10 ⁻³)	Wax fraction (cpm x 10 ⁻³)					% of total ^c
			Polar lipids + minor unknowns	Fatty acids	Primary alcohols + u-2	Aldehydes + short chain esters	Esters	
Control		1460	152	134	228 ^d	327 ^e	61	61.8
Dithiothreitol	(0.6)	1840	283	186	147	350	98	57.8
	(6.0)	1640	185	230	248	366	54	66.0
	(36.0)	1510	157	233	371 ^d	389 ^e	113	83.6
	(60.0)	770	179	122	119	56	146	80.8
Mercaptoethanol	(0.6)	3020	317	172	556	670	121	60.8
	(6.0)	2640	333	196	465	853	145	75.5
	(60.0)	1190	113	204	110 ^d	561 ^{e,f}	96	91.1
Arsenite	(0.6)	1210	110	87	241	278	30	61.7
	(6.0)	1190	133	124	186 ^d	514 ^e	45	84.2
	(60.0)	310	43	38	28	174	12	95.2
Cyanide	(0.6)	3230	433	210	394	611	171	56.3
	(6.0)	3360	319	192	450 ^d	867 ^e	188	60.0
	(60.0)	1470	182	79	178 ^d	453 ^e	66	65.2
	(360.0)	1050	115	86	191	399	36	78.8
TCA	(0.06)	1440	124	111	311	262	66	60.7
	(0.6)	660	103	56	165	90	17	65.3
	(6.0)	230	68	23	49	24	5	73.5

a Details of the TLC procedures used are given in the Materials and Methods. By themselves they are insufficient to resolve the various polar lipids plus minor unknowns, the unknown (u-2) from the primary alcohols or the short chain esters from the aldehydes.

b Total amount of label extracted by chloroform which is equivalent to total label in the epicuticular wax.

c Label in the 5 wax fractions/total label in wax (100).

d Relative proportions of primary alcohols and u-2 are given in Table III.

e Relative proportions of aldehydes and short chain esters are given in Table II.

f Also includes u-1; that is, a portion of the short chain esters which were partly resolved in the initial TLC in benzene (see Materials and Methods).

Table II

Percent distribution of label between the aldehydes and the short chain esters^a.

Experimental condition	(μmoles)	Aldehydes	Short chain esters
Control		72	28
Dithiothreitol	(36)	96	4
Mercaptoethanol	(60)	68	32
Arsenite	(6)	31	69
Cyanide	(6)	43	57
	(60)	34	66

a These two lipid classes were resolved by reducing the aldehydes to primary alcohols and separating them from the short chain esters by TLC in benzene.

Table III

Percent distribution of label between the primary alcohols and an unknown (u-2)^a.

Experimental condition	(μmoles)	Primary alcohols	U-2
Control		61	39
Dithiothreitol	(36)	66	34
Mercaptoethanol	(60)	51	49
Arsenite	(6)	56	44
Cyanide	(6)	77	23
	(60)	71	29

a These two lipid classes were resolved by converting the primary alcohols to acetates and separating them from u-2 by TLC in benzene.

the least affected of the wax fractions. Separation of the primary alcohols and u-2 after treatment with 6 μmoles of cyanide (Table III), however, reveals that the stimulating effect is primarily a result of label increase in the primary alcohols. In the aldehyde fraction the short chain esters are preferentially stimulated, amounting to 57% of the total (Table II). Feeding 60 μmoles of cyanide resulted in incorporation of label to the same extent as in the control. The distribution of label among the wax classes, however, was altered in that synthesis of the short chain esters has been stimulated more than two-fold at the expense of the aldehydes, free fatty acids and u-2. With 360

μmoles of cyanide, total wax synthesis starts to decrease. The aldehyde fraction alone is not inhibited and this is believed to be due to a stimulatory effect on short chain ester synthesis combined with an inhibition of aldehyde synthesis (see analogous results under arsenite).

3.1.5. Effect of trichloroacetate (TCA)

Total surface lipid biosynthesis is essentially unaffected in the presence of 0.06 μmoles TCA. The distribution of label, however, is slightly altered with an increased proportion in the primary alcohol plus u-2 fraction and decreased amounts in the aldehyde fraction and possibly the fatty acids and polar lipids. With increasing concentrations of TCA, a profound inhibition occurs that effects all wax fractions. Thus, unlike the other four investigated inhibitors, TCA does not show marked differences in its action on the synthesis of the individual wax lipids.

3.2. Distribution of label among the members of individual wax classes

To examine more closely the mode of action of the different inhibitors of the biosynthesis of the epicuticular wax classes, the distribution of label among the various chain lengths composing given wax classes was determined by radio-GLC. The analyses were carried out on the lipid classes isolated from selected inhibitor treatments. Selection of the given inhibitor concentrations examined was based on their effect on total wax synthesis as well as on the β-diketones and/or hydrocarbons (37).

3.2.1. Aldehydes

The aldehydes, which had been reduced to primary alcohols, were analysed by radio-GLC as their acetates. The observed mass distribution is very similar to the radioactivity distribution of the aldehydes obtained in the control experiment (Table IV). In the presence of DTT, the radioactive distribution of the aldehydes was unchanged although synthesis of all chain lengths was increased. Treatment with the other three inhibitors, however, resulted in rather striking changes in the distribution of label among the aldehydes. Firstly, label in the shorter chain lengths C₁₆-C₂₄ increased from 2%

in the control to 15-38% after ME, cyanide and arsenite treatments. Whereas in the control the label was present only in the C₂₀ chain length, in the inhibitor treatments the C₁₈, C₂₂ and C₂₄ aldehydes were also labelled. Most interestingly the C₁₆ chain length also accumulated label but only after treatment with ME. Secondly, a marked inhibition ($\cong$ 58%) of the synthesis of the C₃₀ and C₃₂ chain lengths occurred after treatment with 60 μ moles of cyanide and 6 μ moles of arsenite. Treatment with ME, however, had little effect on the synthesis of these long aldehydes.

3.2.2. Free fatty acids

The radioactivity distribution of the free fatty acids is surprisingly similar to that of the mass (Table V), and in both cases there is a suggestion of a two headed curve. Treatment with the inhibitors resulted in several marked changes in the pattern of incorporation of ¹⁴C. In the presence of DTT and ME, the synthesis of the long chain fatty acids C₂₈-C₃₂ was inhibited while a four-fold increase of label occurred in the short chain fatty acids C₁₆-C₂₀. The shortest of these is the C₁₆ acid in which no label was found in the control or after the

cyanide or arsenite treatments. Both of the latter inhibitors, however, stimulated incorporation of label into C₁₈ and C₂₀ acids. After feeding the spikes 6 μ moles of cyanide or arsenite, label accumulated to the same extent in the C₃₂ acid as it did in the control experiment although synthesis of the other long acids was somewhat reduced. Increasing the cyanide concentration to 60 μ moles also resulted in the inhibition of the synthesis of the C₃₂ acid. The amount of label present in the fatty acids of medium chain length (C₂₂-C₂₆) was also affected by the inhibitor treatments, but no consistent pattern of change is visible except in the arsenite treatment which reduced the amount of label in all three.

3.2.3. Primary alcohols

The radioactivity and mass distributions (49) of the primary alcohols are quite different (Table VI). The C₃₂ alcohol which has 40% of the label represents only 3% of the mass while the C₂₆ alcohol has only 25% of the label, but forms 58% of the mass. Feeding the inhibitors brought about some interesting changes in the incorporation pattern. DTT slightly inhibited synthesis of the C₂₆ chain, and stimulated syn-

Table IV

Effects of pre-incubations with inhibitors on the incorporation of [2-¹⁴C]-acetate into the aldehydes of barley spike epicuticular wax^a.

Experimental condition	(μ moles)	16	18	20	Chain length						Total
					22	24	26	28	30	32	
					(weight %) ^b						
Control		2.2	1.4	0.6	0.3	0.4	5.4	4.5	15.5	67.9	98.2
					(radio %)						
				2.1			2.0	2.7	12.7	80.5	100
					(cpm $\times 10^{-3}$)						
		—	—	5	—	—	5	6	30	190	236
Dithiothreitol	(36)	—	—	9	—	—	13	11	36	304	373
Mercaptoethanol	(60)	11	8	11	11	16	16	11	21	277	382
Cyanide	(6)	—	18	27	9	16	18	19	47	219	373
	(60)	—	7	11	6	13	14	12	23	69	155
Arsenite	(6)	—	12	34	8	6	6	3	7	84	160

a Analysed as alcohol acetates.

b Since the other peaks present in the mass trace amounting to approximately 1.8 % of the total aldehydes were not radioactive, they are not listed separately here.

— = Not detected.

Tabel V

Effects of pre-incubations with inhibitors on the incorporation of [2-¹⁴C]- acetate into the free fatty acids of barley spike epicuticular wax^a.

Experimental condition	(μmoles)	16	18	20	Chain length						Total
Control											
		1.4	2.6	11.9	20.2	21.4	14.4	13.6	8.2	3.8	97.5
					(radio %)						
		–	2.4	18	24	9.5	11.1	14.7	11.1	9.2	100
					(cpm x 10 ⁻³)						
Dithiothreitol	(36)	–	3	24	32	13	15	20	15	12	134
		21	20	90	42	19	17	15	5	4	233
Mercaptoethanol	(60)	13	56	73	26	12	8	9	4	3	204
Cyanide	(6)	–	33	63	27	24	11	10	8	16	192
	(60)	–	15	27	10	11	5	4	3	4	79
Arsenite	(6)	–	23	44	13	8	7	5	7	17	124

a Analysed as methyl esters.

b Data from (49). Since the other peaks present in the mass trace amounting to approximately 2.5 % of the total free acids were not radioactive, they are not listed separately here.

– = Not detected.

Table VI

Effects of pre-incubations with inhibitors on the incorporation of [2-¹⁴C] -acetate into the primary alcohols of barley spike epicuticular wax^a.

Experimental conditions	(μmoles)	Chain length							Total
		20	22	24	26	28	30	32	
Control				(weight %) ^b					
		2.2	2.6	9.7	57.5	14.7	5.5	3	95.2
				(radio %)					
				3.9	25.1	12.6	18.0	40.4	100
				(cpm x 10 ⁻³)					
Dithiothreitol		–	–	5	35	18	25	56	139
	(36)	–	–	9	22	17	48	149	245
Mercaptoethanol	(60)	3	–	4	11	6	6	26	56
Cyanide	(6)	–	–	8	119	63	43	113	346
	(60)	–	–	4	59	24	15	24	126
Arsenite	(6)	3	–	4	27	11	8	51	104

a Analysed as alcohol acetates.

b Data from (49). Since the other peaks present in the mass trace amounting to approximately 4.8 % of the total primary alcohols were not radioactive, they are not listed separately here.

– = Not detected.

thesis of the C₃₀ and C₃₂ alcohols by 92 and 166%, respectively. ME blocked incorporation of label into all the different chain lengths, but did not significantly alter the distribution of label. By contrast 6 µmoles of cyanide stimulated synthesis of them all. When the cyanide concentration was increased to 60 µmoles, only C₂₆ formation was still elevated (71%), while that of the C₃₀ and C₃₂ alcohols was reduced (51%). This is the opposite effect of treatment with DTT. At the concentration of arsenite investigated, no marked changes were found in the distribution of radioactivity among the different alcohols.

3.2.4. Short chain ester fatty acids

Among the acid moieties of the short chain esters, the C₂₀ acid is by far the most heavily labelled (48%) while the C₃₂ acid has 16% of the label (Table VII). After the inhibitor treatments label was never found in the C₃₂ or C₃₀ acids, but accumulated instead in the shorter homologues. In all but the ME experiment in which the C₁₆ acid was labelled, C₁₈ was the shortest chain length with ¹⁴C. The largest accumulation of label in the C₁₈-C₂₆ acids (a six-fold increase) occurred after treatment with 6 µmoles of cyanide. Increasing the amount of cyanide to 60 µmoles, resulted in a significant decrease of label to four times the amount in

the control experiment. This decrease was selective, in that only the C₁₈-C₂₂ acids were affected.

No mass trace was obtained during the radio-GLC of these fatty acid methyl esters. At the sensitivity at which the apparatus was being used (40), as little as 3.7 nmoles of palmitic acid methyl ester could have been seen. The obtaining of mass traces under these conditions for all other wax classes investigated, implies that these fatty acid moieties have the highest specific activity of all the wax lipids investigated to date.

3.2.5. Esters

The radioactive distribution of the ester acids is similar to that of the mass in that C₂₀ is the dominant chain length in both (Table VIII). The two distributions differ, however, in that no label is present in the C₁₆ chain which accounts for 12% of the mass. Treatment with ME and DTT did not change the radioactive distribution drastically, although the C₁₆ and C₁₈ chains became more prominent. The similarity between the distribution of label in the acids of the short chain esters (Table VII) and the esters (Table VIII) is very clear after treatment with ME.

Table VII

The effects of pre-incubations with inhibitors on the incorporation of [2-¹⁴C]-acetate into the fatty acid moieties from the short chain esters of barley spike epicuticular wax^a.

Experimental condition	(µmoles)	16	18	20	Chain length						Total
					22	24	26	28	30	32	
Control ^b					(radio %)						
			6.7	47.8	10.1	8.2	5.5	1.9	4.3	15.5	100
					(cpm x 10 ⁻³)						
Mercaptoethanol	(60)	12	6	44	9	8	5	1	4	14	91
Cyanide	(6)	-	132	79	24	10	3	3	-	-	180
	(60)	-	50	176	68	83	34	-	-	-	493
	(60)	-	50	83	43	83	39	-	-	-	298
Arsenite	(6)	-	77	199	45	34	-	-	-	-	355

a Since the short chain esters contained only 16 x 10⁻³ cpm in the presence of 36 µmoles of dithiothreitol, their fatty acid moieties were not subjected to radio-GLC. Analysed as methyl esters.

b Mass peaks were not present in the radio-GLC traces, see text.

- = Not detected.

Table VIII

The effects of pre-incubations with inhibitors on the incorporation of [2-¹⁴C] - acetate into the esters of barley spike epicuticular wax.^a

Experimental condition	(μmoles)	Ester acids ^b Chain length					Ester alcohols	
		16	18	20	22	24	Total	Total
		(weight %) ^c						
		12.4	13.4	50.8	19.4	2.0	98	
Control		(radio %)						
		–	6.8	69.5	20.4	3.3	100	
		(cpm x 10 ⁻³)						
		–	3	33	10	2	48	13
Dithiothreitol	(36)	3	10	41	16	3	73	40
Mercaptoethanol	(60)	3	21	36	14	2	76	20

a The esters were transesterified and the resulting fatty acid methyl esters and alcohols were separated on silica gel H plates using benzene. Estimation of the label present was carried out as described in (37). results based on a single determination.

b Analysed as methyl esters. Results based on single injections into the radio-GLC.

c Data taken from (49).

– = Not detected.

4. DISCUSSION

When an enzyme in a metabolic pathway is blocked by a chemical inhibitor or mutant gene, it would be predicted that the concentration of its substrate tends to increase. Although numerous examples of such substrate increase have been reported, inhibition of an enzyme in a metabolic sequence is not invariably followed by an accumulation of the substrate for this enzyme. Earlier intermediates may also accumulate, sometimes in larger amounts than the immediate precursor. In addition, alternate pathways for the metabolism of any of the preceding intermediates may exist so that the block will result in a shift of the precursors into another pathway. Thus in trying to determine where a gene or inhibitor exerts its effects in a biosynthetic pathway, an examination of all the preceding intermediates as well as of compounds in possible branching pathways should be made. This type of analysis may reveal not only the point at which the block occurs but may often lead to an increased knowledge of the biosynthetic relationships of a given group of compounds.

The present study was concerned with the biosynthesis of the long chain epicuticular lipids, in which the various lipid classes consist of a number of chain lengths with either the odd or even chains predominating. Fig. 5 which presents the biosynthetic relationships of the wax classes is a summary of the information available in the literature and the present results (2, 5, 9, 10, 12-17, 19, 21, 22, 29-31, 34-38, 43, 47, 48, 51). At least two independent elongation systems exist, one leading to the β-diketones, hydroxy-β-diketones and esterified alkan-2-ols and the other giving rise to the hydrocarbons, secondary alcohols, ketones and perhaps also to the aldehydes, primary alcohols and free fatty acids. In looking for the site(s) of action of the investigated inhibitors, ME, DTT, cyanide and arsenite, the amount of label present in each chain length from four wax classes dominated by even chain lengths was first summed. These results which are presented in Table IX together with the knowledge of the effect of these inhibitors on the odd chain lengths (37) form the basis of the conclusions drawn.

Figure 5. Summary of effects of chemicals, genes and 35° C (HT) on the biosynthesis of the epicuticular wax classes. The general biosynthetic relationships among the wax classes, which are based upon the schemes presented in references 31 og 51, have been modified to include the present results. The elongation pathway leading to the alkanes etc. has been indicated as a single elongation mechanism in order not to complicate the figure. Whether or not more than one elongation mechanism is involved is not yet known. The most common chain lengths of the wax classes are indicated. Hypothetical intermediates are indicated by the use of brackets. Solid lines indicate primary pathways; dotted line possible alternate biosynthetic pathway. The point(s) of action of the chemicals (DTT, dithiothreitol; DTE, dithioerythritol; ME, mercaptoethanol; CAR, thiocarbamates, specifically: dichloroallyl diisopropylthiocarbamate, S-ethylidipropylthiocarbamate and α-chloroallyl diethylidithiocarbamate; TCA, trichloroacetate; AS, arsenite; CY, cyanide; FL, fluoride) and the genes (see Table X) are indicated by red arrows. In the text references are given which support the points of action indicated for the chemicals and HT. Those for the genetic blocks are given in Table X.

4.1. Identification of new elongation blocks

4.1.1. Mercaptoethanol

Treatment with ME resulted in a 2.4 fold increase in the synthesis of C_{18} and C_{20} chains, a small increase in the synthesis of C_{22} and C_{24} chains, but had no marked effect on the synthesis of the C_{32} chains when compared to the control (Table IX). On the basis of these results, the primary effect of ME on wax synthesis is to block the elongation of C_{20} to C_{22} chain lengths (Fig. 5). BUCKNER and KOLATTUKUDY (5) had earlier deduced from their studies using tissue slices of pea leaves, however, that neither ME nor dithioerythritol (DTE) inhibited the elongation process. The data which they present tends to support their contention with respect to DTE. That is, a marked accumulation of label occurred in the longest chain of interest, specifically in the C_{32} aldehyde. No accumulation of label, however, occurred in the C_{32} chains of the other lipid classes. Their experimental results with ME are quite different. Compared to the DTE experiments, relatively little label was found in the C_{32} aldehyde and none was reported in the C_{32} chains of the other lipid classes. A dramatic accumulation of label in the C_{22} to C_{32} chains of the aldehydes and acyl moieties of the short chain esters is the most obvious effect of their ME treatment. Whether elevated amounts of label were present in the C_{24} - C_{28} acyl moieties of the polar lipids, as they imply, is highly questionable (see their Fig. 2). When combined with the observation that no label was detected in these two lipid classes for C_{20} and shorter

chains, BUCKNER and KOLATTUKUDY's results (5) certainly do not appear to support the hypothesis that ME causes a block in elongation between C_{20} and C_{22} .

In seeking an explanation for the inconsistencies between the action of ME on the synthesis of the epicuticular waxes of pea leaves and barley spikes it may be significant that while only one elongation system draws on the pool of wax precursors in peas, two elongating systems do so in barley (Fig. 5). On pea leaves the alkanes and secondary alcohols compose 47-57% of the wax (34, 25), while on barley spikes the alkanes amount to only 16% (50) and the β -diketone lipid classes to 50% (51). Thus since a far greater proportion of the acyl precursors on barley spikes enter the β -diketone elongating system than that leading to the alkanes, it might be reasonable to expect that the pool of long chain endogenous precursors available for the synthesis of the alkanes is much smaller in barley spikes than in pea leaves. Upon introduction of ME this pool of acyl chains with C_{22} - C_{28} carbons can still undergo further elongation and/or be channeled into other wax classes. The final result is to mask the effect of ME on the C_{20} - C_{22} elongation step. That a pool of endogenous long chain acyl intermediates is available for elongation is suggested by Kolattukudy and Buckner's experiments with a 27,000 x g supernatant from extracts of pea epidermis which was shown to be capable of the addition of single C_2 -units to endogenous fatty acids having as many as 16-22 carbon atoms (28). The above hypothesis can

Table IX

The effects of pre-incubations with inhibitors on the incorporation of $[2-^{14}C]$ -acetate into various chain lengths of the free fatty acids, primary alcohols, aldehydes and acid moieties of the short chain esters.

Experimental condition	(μmoles)	Chain length (cpm x 10 ⁻³)									Total
		16	18	20	22	24	26	28	30	32	
Control		—	9	73	41	26	60	45	74	272	600
Dithiothreitol	(36)	21	20	99	42	28	52	43	89	457	851
Mercaptoethanol	(60)	36	113	166	61	42	38	29	31	306	822
Cyanide	(6)	—	183	266	104	131	182	92	98	348	1404
	(60)	—	72	121	59	111	117	40	41	97	658
Arsenite	(6)	—	112	280	66	52	40	19	22	152	743

be tested. Firstly, similar experiments can be made using tissue slices of seedling leaves from barley or maize (3). Since the β -diketone elongation system is not operating in either of these systems, the possibility exists that a larger pool of endogenous long chain precursors will be present that will aid in obscuring the C_{20} - C_{22} block caused by ME. Secondly, an analysis of the free fatty acids in the pea system should be made as this was one of the wax classes in the present investigations in which the accumulation of label in the C_{20} and shorter chains was most marked. Unfortunately, BUCKNER and KOLATTUKUDY (5) did not separate the small amount of free fatty acids present in the wax from the large amount of internal lipids which together composed their polar lipid fraction.

4.1.2. Arsenite

The primary effect of arsenite is to block the elongation sequence leading to the β -diketones (37). Analysis of the distribution of label within the β -diketone molecule after arsenite treatment indicated that this effect was most likely closely related to the established ability of arsenite to block elongation of C_{16} - C_{18} (9, 10, 15-17). The marked accumulation of label in chains longer than C_{16} in the present analysis was unexpected. The overall distribution of label among the even chains after treatment with arsenite is very similar to that after ME treatment, except that only half as much label is present in the C_{32} chains (Table IX). These results infer that arsenite blocks the C_{20} - C_{22} elongation step in an even more effective manner than does ME (Fig. 5).

4.1.3. Significance of a C_{20} - C_{22} elongation block

If the overall distribution of label among the C_{16} - C_{24} chains after treatment with ME or arsenite is examined, a resemblance to both the mass and radioactivity distribution of the ester fatty acid moieties is evident (Tables VIII and IX). For this reason the ester acid data has not been included in Table IX. The ester acids also contained a relatively small amount of label (max 10%) compared to the other four wax classes. Furthermore, treatment with ME has little effect on the ^{14}C distribution except that

somewhat more counts are found in the C_{16} and C_{18} chains (Table VIII). These results imply that the blocking action of ME and arsenite results in a channeling of acyl moieties into the pathways normally leading to the ester fatty acids. This has been indicated in Fig. 5 by the use of separate brackets to show departure of the C_{16} - C_{24} and the C_{22} - C_{32} fatty acyl chains from the elongation sequence. Whether or not the action of these inhibitors is (i) to interrupt a single elongation sequence, (ii) to have no effect on the elongation sequence leading to the ester acids but to block that leading to the alkanes and other lipid classes or (iii) to prevent the entrance of the C_{20} acyl chains into a new elongation sequence leading to the alkanes etc. cannot be determined from the presently available data. By examining the distribution of label among the ester fatty acid moieties (5, 29) and noting that an accumulation of label occurs in the C_{22} chains of the free and ester bound acids after treatment with thiocarbamates (29), the analogous block in peas appears to occur between C_{22} and C_{24} . From his results NETTING (38) has suggested that the final elongation leading to the long chain aldehydes in *B. oleracea* starts from an acyl chain having 22 carbons.

4.1.4. Cyanide

Although synthesis of all chain lengths is stimulated at the lower concentration of cyanide, the magnitude of the increase is relatively small for the three longest chains compared to the shorter ones. This sharp boundary between C_{26} and C_{28} is even more evident at the higher concentration of cyanide which actually markedly depressed synthesis of C_{28} - C_{32} while that of the C_{26} and shorter chains remained elevated (Table IX). These results suggest that at higher concentrations cyanide may inhibit the C_{26} - C_{28} elongation step (Fig. 5). How this happens is unknown. The same possibilities mentioned above for the C_{20} - C_{22} block are also applicable in this case. At the lower concentration of cyanide this inhibition of C_{26} - C_{28} either does not occur or the effect is masked by the marked stimulation that cyanide has on the synthesis of all wax classes except the β -diketones (37). The magnitude of the in-

crease is far in excess of what might be expected if only a re-channeling of the acyl precursors that normally enter the β -diketone elongation system were occurring. The major effect of cyanids is an inhibition of β -diketone synthesis (37). This is also depicted in Fig. 5. The position of the block is shown before the β -keto fatty acids (37, 51). Whether the block also influences those β -keto fatty acids leading to the alkan-2-ols remains to be confirmed.

4.2. Stimulation of wax biosynthesis by low concentrations of inhibitors

With the lowest two concentrations of cyanide employed, a very substantial increase of label occurred in all wax classes except the β -diketones (37). Accumulation of label in the latter was only slightly elevated by 0.6 μ moles and was somewhat depressed by 6 μ moles (37). The following sequence of events may be responsible for this stimulation. The production of NADPH by non-cyclic electron flow in the chloroplasts is not sensitive to cyanide at low concentrations (4). The electron transport chain which links the reduced nicotinamide nucleotides to oxygen, however, is sensitive to low cyanide concentrations (45). Thus a high level of NADPH is produced which can be consumed in lipid biosynthesis. NADPH is required not only in the *de novo* synthesis of fatty acids (11), but also in the formation of very long chain fatty acids (28) and in the synthesis of primary alcohols (27). In addition, NADH is probably required in the synthesis of aldehydes (27). A large stimulation of the overall fatty acid synthesis would, in turn, provide a substantial increase in the acyl precursors available for the wax elongation systems. The results obtained by OUITRAKUL *et al.* (41) and COHEN *et al.* (6) indicated that the non-cyclic electron flow is sensitive to high cyanide concentrations. This would lower the amount of available NADPH and consequently wax biosynthesis would be inhibited at 360 μ moles. This hypothesis assumes that the physiological concentrations of cyanide within the cells of the intact barley spike system are in the same range as those giving the effects noted above.

A marked stimulation of the synthesis of all the wax lipids occurred with low concentrations

of ME (Table I). A similar effect was reported for imidazol when 1-50 μ moles were used per leaf (20). Preliminary experiments with intact barley spikes indicated an analogous stimulation (170%) when 60 μ moles imidazole per ten spikes were employed. The mechanisms by which ME and imidazole enhance the biosynthesis of the epicuticular wax classes will not be speculated upon at present.

4.3. Biosynthetic relationship of the short chain esters to the other lipid classes

The short chain esters have not been reported before in barley waxes and, even in the present study, were only detected because of their very high specific activity. Their synthesis was stimulated by ME, arsenite and cyanide but repressed by DTT. A short chain ester has also been reported in pea waxes but it was found only after ME treatment (5). Whether or not the same alcohol moiety is involved in both the barley spikes and pea leaves is unknown. Since the distribution of chain lengths among the acid moieties of the short chain esters is quite different from that of the ester acids in barley, the two esterification systems involved differ in more than their choice of alcohol moiety. The short chain esters are not included in Fig. 5 as they do not form a significant part of the wax in terms of mass. The labeling pattern of their acid and their alcohol moieties in the control and after the various inhibitor treatments suggests however, that they originate from the elongation sequence leading to the alkanes.

4.4. Previously identified blocks in the elongation system(s) leading to even chain wax classes

4.4.1. Inhibitors

TCA was the first of the chemicals investigated and subsequently shown to inhibit elongation in a number of plant systems; *B. oleracea* (19, 21, 36), *Nicotiana tabacum* (22), *P. sativum* (5) and *Vicia faba* (30). A single elongation block has not been identified, instead the severity of the inhibition increased as the chain length increased. Therefore in Fig. 5 a blocking action by TCA is indicated between C_{28} and C_{30} and also between C_{30} and C_{32} .

Three thiocarbamates were found to inhibit

the synthesis of C_{31} hydrocarbons in *P. sativum* leaves at a concentration that did not affect the synthesis of C_{26} and C_{28} primary alcohols (29). This was interpreted as evidence that thiocarbamates inhibit the elongation mechanism at the C_{28} and/or C_{30} steps as indicated in Fig. 5. At higher concentrations synthesis of the C_{26} and C_{28} primary alcohols was also inhibited and label accumulated in the C_{22} fatty acids. This result indicates that a second block brought about by thiocarbamates exists between C_{22} and C_{24} .

Fluoride ions and a temperature of 35°C were found to inhibit the formation of C_{20} from C_{18} in germinating peas and safflower seeds (9). Since the effects on these two agents on the epicuticular wax lipids were not investigated, a tentative block between C_{18} and C_{20} is indicated in Fig. 5.

4.4.2. Genetic blocks

A comparison between the composition of the wax present on wild type and on *wa* leaves of *P. sativum* (13, 34) demonstrated that this gene is involved in the elongation of C_{30} - C_{32} in the sequence leading to the alkanes (Fig. 5). The first study of the *wb* gene in *P. sativum* intimated a role for *wb* in the elongation from C_{26} -

C_{28} (34). Recent results do not support this (13), and therefore no site of action for *wb* is indicated in Fig. 5. A similar analysis of *B. oleracea* waxes (2, 35) led to the conclusion that (i) the action of *gl₃S* is to inhibit elongation from C_{26} - C_{28} and more strongly that from C_{28} - C_{30} , while (ii) *gl₁S* acts only on the elongation of C_{28} - C_{30} (Fig. 5, Table X). In addition to compositional analyses, the roles of the *gl₁K* and *gl₁C* (also from *B. oleracea*, see Table X) were investigated with the aid of radioactive precursors (36, 38). The identified elongation blocks occurring between C_{26} and C_{30} are also included in Fig. 5.

4.5. Decarboxylation blocks

4.5.1. Inhibitors

The results of the present analysis with DTT (see also 37) substantiate BUCKNER and KOLATTUKUDY's (5) identification of DTT as a decarboxylation inhibitor. Their detailed analysis was actually carried out using DTE. It is possible to roughly estimate, from the data they present, the increase of label in the C_{32} chains of the wax and the concurrent decrease in the C_{31} chains. The ratio of increase in C_{32} to decrease in C_{31} was only 0.23, indicating that not all of the label prevented from entering the

Table X

Identification of gene symbols used in Fig. 5^a

Plant species	Variety ^b	Gene ^b	References ^c
Brassica oleracea	Marrow stem Kale	<i>gl₃K</i>	(35, 38)
Brassica oleracea	Marrow stem Kale	<i>gl₄K</i>	(35, 36, 38)
Brassica oleracea	Cauliflower	<i>gl₃C</i>	(35, 36)
Brassica oleracea	Brussel Sprout	<i>gl₁S</i>	(2)
Brassica oleracea	Brussel Sprout	<i>gl₂S</i>	(2)
Brassica oleracea	Brussel Sprout	<i>gl₃S</i>	(2)
Brassica napus	Rape (Rigo)	<i>gl₁R</i>	(12)
Pisum sativum		<i>wa</i>	(13, 34)
Pisum sativum		<i>wsp</i>	(13, 34)
Hordeum vulgare	Bonus	<i>cer-q</i>	(47, 51)
Hordeum vulgare	Bonus	<i>cer-c</i>	(47, 51)
Hordeum vulgare	Bonus	<i>cer-u</i>	(47, 51)

a Only those genes whose site of action on the wax biosynthetic apparatus have been clearly pinpointed are included.

b Since the same symbol (e.g. *gl₁*) has been used for mutants induced in more than one variety of Brassica, a letter indicating the variety has been attached to the gene symbols to avoid confusion.

c Reference number (s) is (are) to the paper(s) leading to the conclusion as to the site(s) of action shown in Fig. 5.

C_{31} chains by DTE accumulated in the C_{32} chains. In the present study with DTT (see also 37), the same ratio equals 0.69, a value somewhat closer to 1, which is the expected in an ideal situation. The higher ratio in barley is in part due to the fact that data for more of the wax classes is available than it is for the pea system (5). If a similar calculation is made for the ME experiments the following ratios are obtained: barley 0.12 and pea 0.06. That the ratio for ME is 5.8 times less than for DTT in barley and 3.8 times less than for DTE in peas is strong evidence that the primary effect of ME is not on decarboxylation. Instead as discussed in section 4.1.1., the primary effect of ME on wax biosynthesis is on elongation and the decarboxylation effect is of relatively minor importance (Fig. 5).

4.5.2. Genetic blocks

Compositional analyses of the waxes from *P. sativum* and its mutant *wsp* as well as of those from the wild type Brassicas and the mutants *gl₄K*, *gl₂S* and *gl₇R* (Table X) led to the conclusion (shown in Fig. 5) that the primary effect of these genes was on the decarboxylation reaction(s) of the elongated fatty acyl precursors of the alkanes. In addition to compositional analyses, the role of the *gl₄K* gene was investigated using radioactive precursors with a similar result (see Fig. 5).

4.6. Biosynthesis of the primary alcohols

Two alternative routes to the primary alcohols are shown in Fig. 5. Tracer studies with barley leaves indicated that the C_{26} aldehyde was an intermediate in the synthesis of the C_{26} alcohol (8). The mechanism involved is probably similar to that shown to occur in *Mycobacterium tuberculosis* (44), *Clostridium butyricum* (7) or *B. oleracea* (27) where the aldehydes are true intermediates of the alcohols. In the present study the stimulation of C_{32} aldehyde synthesis after treatment with the decarboxylation inhibitor DTT was accompanied by a large accumulation of label in the C_{32} primary alcohols. Treatment with the elongation inhibitors ME, arsenite and the

higher concentration of cyanide did not, however, affect the amount of label present in the C_{32} aldehyde and alcohol in any parallel manner. In addition to the formation of primary alcohols via an aldehyde, as mentioned above, they have also been shown to come directly from a fatty acyl-CoA intermediate in *Euglena gracilis* (18, 24). This pathway is indicated by the dotted line in Fig. 5. In some *B. oleracea* waxes branched ester fatty acids as well as free and esterified alcohols have been identified. No branched components were present in the aldehydes, however, (12, 39) indicating that in *B. oleracea* at least two pathways lead to the primary alcohols. The situation in barley remains to be investigated.

4.7. Biosynthesis of β -diketones and esterified alkan-2-ols

While this paper has dealt with the effect of chemical inhibitors on the synthesis of the even chained wax lipids of barley spikes, the predominant wax classes are the β -diketones with 31 carbon atoms. As shown in Fig. 5 the elongation sequence leading to the β -diketones is a different one from that leading to the alkanes (37, 51). From a study of the effects of inhibitors on β -diketone synthesis (see sections 4.1.2. and 4.1.4.), a C_{18} β -keto acid has been suggested as the starting point for the elongation to the final carbon chain of the β -diketones (37, 51). This keto acid as well as the C_{16} and C_{14} keto acids which arise from C_{16} , C_{14} and C_{12} acids by the addition of one C_2 -unit, respectively, are also thought to serve as precursors for the esterified alkan-2-ols (see 37). The latter wax class was found to be more closely related biosynthetically to the β -diketones than to any of the other wax classes. This deduction was based on a compositional analysis of the waxes of a number of *eceriferum* (*cer*) mutants (51). The blocks caused by three of these mutants, *cer-q*, *-c* and *-u* are shown in Fig. 5 (51). These three genes do not affect the chain length distributions of any of the wax classes arising via the elongation sequence leading to the alkanes (*P. von WETTSTEIN-KNOWLES*, pers. commun.).

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