Biosynthesis of Wax in *Brassica oleracea*. Relation of Fatty Acids to Wax*

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ABSTRACT: According to the literature nonacosane and its derivatives, the major components of the surface wax of the leaves of *Brassica oleracea*, are supposed to be synthesized by the condensation of two molecules of pentadecanoic acid which in turn may be produced from palmitic acid by α oxidation. If this hypothesis is true the carboxyl carbon of palmitic acid would be lost during the incorporation of palmitic acid into the C_{29} compounds. However, equal amounts of radioactivity were incorporated into *n*-nonacosane of broccoli leaves from [1-^{14}C]palmitic acid, [U-^{14}C]palmitic acid, and [9,10-^{3}H]palmitic acid. [1-^{14}C]Palmitic acid was as efficient as [U-^{14}C]palmitic acid in labeling the other C_{29} compounds of broccoli leaf, nonacosanone, and nonacosanol.

These results strongly suggest that palmitic acid is incorporated as a unit into the C_{29} compounds and, therefore, these compounds are not synthesized primarily by condensation of two molecules of pentadecanoic acid. Fatty acids of chain length C_{10}, C_{12}, C_{14}, C_{16}, and C_{18} served as precursors of wax components, the longer fatty acids being much more efficient; incorporation of administered stearic acid into wax was as high as 50% in 4 hr. These results indicate that the C_{29} compounds are synthesized by elongation of a preformed fatty acid with subsequent decarboxylation. In fact broccoli leaves incorporated fatty acids such as C_{16} and C_{18} into fatty acids of length up to at least C_{26}, longer substrates being converted into these very long fatty acids much more efficiently than the shorter ones. However it is yet to be ascertained whether this elongation is biosynthetically related to the C_{29} wax components. Incorporation of [1-^{14}C]acetate and [^{14}C]-palmitic acid into the paraffin was independent of light, whereas under the same conditions acetate incorporation into lipids was inhibited up to 60% by the absence of light, indicating that, unlike fatty acid synthesis, paraffin biosynthesis is not tightly coupled to photosynthetic reactions. Acetate incorporation into paraffins was less sensitive to 3-(4-chlorophenyl)-1,1-dimethylurea than into fatty acids, palmitate incorporation into paraffins was insensitive to this inhibitor indicating that the elongation system is different from the fatty acid synthesizing systems.

The surfaces of animals (Nicolaides, 1965) and insects (Baker *et al.*, 1963; Bowers and Thompson, 1965), and the epidermis of plants (Silva Fernandes *et al.*, 1964), are usual y covered by a mixture of compounds with long carbon chains. This mixture, generally called wax, contains pr marily hydrocarbons, esters, ketones, alcohols, and acids sometimes mixed with terpenes and aldehydes (Radler and Horn, 1965). Of all the wax components, hydrocarbons have the widest distribution in nature, being found even in human artery walls (Gazzarrini and Nagy, 1966) and beef brain (Nicholas *et al.*, 1955). However hydrocarbons found in animals may be derived at least in part from the diet, especially since the dietary hydrocarbons are known to be incorporated into various organs of animals (McCarthy, 1964; Kolattukudy and Hankin, 1966). Orally administered [1-^{14}C]octadecane was not transported into the lipid of the surface of rat skin (Nicolaides and Kellum, 1966).

Possible biological origin of petroleum hydrocarbons has been the subject of extensive studies, and hypothetical mechanisms by which hydrocarbons may be derived from fatty acids have been postulated (Cooper and Bray, 1963). However the biosynthesis of the long-chain paraffins that are found in various organisms has not been studied save for a few attempts to incorporate acetate into paraffins. Although attempts to incorporate acetate into paraffins of apple (Mazliak, 1964) and human skin (Nicolaides *et al.*, 1955) were unsuccessful, acetate units were incorporated into *n*-heptane in *Pinus jeffreyi* (Sanderman *et al.*, 1960), long-chain paraffins of beeswax (Piek, 1964), and *n*-nonacosane in *Brassica oleracea* (Kolattukudy, 1965).

The cuticular wax of *B. oleracea* leaves consists primarily of nonacosane and its derivatives (Purdy and Truter, 1963). It has been suggested that these C_{29}[1] compounds are synthesized by the condensation of two molecules of pentadecanoic acid accompanied by de-

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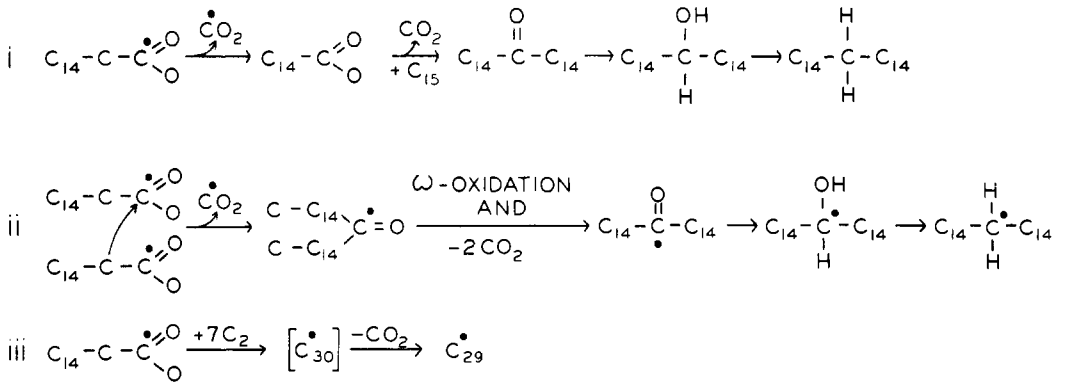
[1] Abbreviations used: CMU, 3-(4-chlorophenyl-1,1-dimethylurea; ATP, adenosine triphosphate; TPNH, reduced triphosphopyridine nucleotide; C_{29} compounds, *n*-nonacosane and its derivatives that are found in *Brassica* wax; fatty acids are represented by the number of carbon atoms followed by the number of double bonds.



SCHEME I

carboxylation (Channon and Chibnall, 1929). Chemical degradation of 15-nonacosanone derived from specifically labeled acetate showed that the carbonyl carbon of the ketone arose from the methyl carbon of acetate (Kolattukudy, 1965). This observation, although it ruled out pathway ii in Scheme I and the possibility of the direct incorporation of a C_3 unit[2] (Peeters and Lauryssens, 1963) in the synthesis of the C_{29} compounds, is in agreement with the other two hypothetical pathways (Scheme I): (i) condensation of two molecules of pentadecanoic acid accompanied by decarboxylation; (ii) condensation o two molecules of palmitic acid with subsequent removal of the two methyl carbons by ω oxidation and decarboxylation (Kreger, 1948); (iii) synthesis of a C_{30} unit by elongation of a fatty acid with subsequent decarboxylation. This paper presents experimental evidence that rules out the first two mechanisms and supports the third for the biosynthesis of *n*-nonacosane and its derivatives. It is further suggested that the other long-chain compounds of the wax are synthesized also by elongation.

Experimental Procedure

Plants. Young leaves (second and third from the apices) from broccoli plants grown in the greenhouse as described earlier (Kolattukudy, 1965) were used in all experiments because wax biosynthesis seems to be most rapid at this stage of growth. After removing the midribs, the leaves were chopped with a razor blade into strips of about 1–2-mm in. width and 8–12 mm in. length, which were mixed well before weighing 2.2 g into an appropriate experimental medium. Disks 1.6 cm in diameter were cut from young leaves under distilled water with a sharp punch, and were mixed well before being sampled.

Experiments with Chopped Leaf. Chopped leaves weighing 2.2 g were transferred into a large Warburg flask (75 ml) containing 6 ml of distilled water in which the substrate and the appropriate inhibitors were dissolved. The contents of the flask were well m xed and the leaf fragments were then spread out at the bottom of the flask. The flask was incubated in a water bath at 30° with shaking (140 oscillations/min), under approximately 2000 foot-candles of light. At the end of the incubation period the flask contents were shaken with 175 ml of a mixture (2:1) of chloroform and methanol and left overnight. The white leaf residue was filtered, and the extract was washed by the method of Folch *et al.* (1957). The lipid solution was dried with anhydrous sodium sulfate and a sample was analyzed for paraffins by column chromatography. Another portion of the lipid solution was evaporated to dryness in a stream of N_2 and saponified under N_2 with 15 ml of 15% alcoholic KOH for 3 hr. Mos of the unsaponifiable lipids were removed by extracting the alkaline solution with chloroform; then fatty acids were recovered by extracting the acidified solution with chloroform. The fatty acid solution was dried with anhydrous sodium sulfate and methyl esters were prepared with BF_3 as catalyst. The esters were purified by thin layer chromatography on silica gel G with benzene as the de velop ng solvent. At this stage the remaining unsaponifiable lipids were eliminated. The methyl esters were analyzed by gas–liquid partition chromatography and the radioactivity was followed with a Barber-Colman radioactivity monitor.

Experiments with Leaf Disks. In experiments where disks were used, 20 disks (1.6 cm in diameter, 1.1-g fresh weight) were spread out in a Petri dish in which 1.0 ml of appropriate substrate solution was placed as a thin layer. At the beginning of the incubation both sides of the disks were moistened with the substrate solution and as the solution evaporated small amounts of distilled water were added. About 800–1000 foot-candles of light fell on the disks. At the end of the incubation period, the disks were immersed in a chloroform–methanol mixture (2:1) for 30 sec by which time almost all of the wax and only a little green-yellow had dissolved in the solvent. The disks were then immersed in 60 ml of $CHCl_3$–MeOH (2:1) for several hours to extract the total internal lipids. The white disks were filtered and the extract was processed as described above. The wax solution obtained by the brief extraction with $CHCl_3$–MeOH was washed by the method of Folch *et al.* (1957), and the lipid solution was dried with anhydrous sodium sulfate and fractionated by column and thin layer chromatography. Since this solution did not contain significant amounts of internal lipids or

[2] Experiments with [1-^{14}C]propionate also support this view (P. E. Kolattukudy, unpublished results).

pigments, thin layer chromatography could be used to separate most of the wax components. The short wash with $CHCl_3$–MeOH was found to remove 98–99% of the paraffins.

Chromatography. Silica gel for column chromatography was prepared essentially as described by Bulen *et al.* (1952); before the activation step the dry silica gel was washed three times with a $CHCl_3$–MeOH mixture in order to remove the residual lipid contaminants. A portion of the hexane solution of the total lipids was placed on a silica gel column (30 × 1 cm) and paraffins were eluted with hexane. Eluent was collected in 8–10-ml portions and the paraffins were located by assaying for radioactivity; almost all of the paraffins were in the third tube. The other components of the wax cannot be easily eluted in pure form from this column.

Wax isolated by washing the disks for 30 sec with $CHCl_3$–MeOH was fractionated by thin layer chromatography on silica gel G with benzene as the solvent (Kolattukudy, 1965). The methyl esters were purified on thin layers (0.5 mm thickness) of silica gel G with benzene as the solvent, 2,7-dichlorofluoresceine being used to locate the lipids on the thin layer plates. The methyl esters migrated between the ketones and the secondary alcohols. The gas–liquid partition chromatography was carried out on a Perkin-Elmer gas chromatograph equipped with a flame ionization detector and an effluent splitter. The paraffins were separated on a 5% silicone gum rubber (S.E. 30) and the methyl esters of fatty acids on 15% diethylene glycol succinate and 12% Apiezon L columns. Details of the experimental conditions are shown in the legends.

Authentic samples were always used as standards in thin layer and gas chromatography. In gas chromatography when a trace component contained significant radioactivity the sample was rechromatographed after being mixed with standards to make sure that the radioactivity peak coincided with the mass peak.

Determination of Radioactivity. Radioactivity in samples of lipid solutions and thin layer chromatograms was determined as previously described (Kolattukudy, 1965). Internal standards of [^{14}C]toluene and [^{3}H]toluene were always used; the efficiency in colorless samples was 60% for ^{14}C and 8% for ^{3}H. In colored total lipid extracts the efficiency was much lower, 15–25% for ^{14}C and 0.8–1.5% for ^{3}H. Radioactivity in the gas chromatographic fractions was measured with a Barber–Colman radioactivity monitor. Specific activities of the wax components were measured as described earlier (Kolattukudy, 1965).

Substrates and Inhibitors. [1-^{14}C]Palmitic acid (sp act. 26 mc/mm) and [9,10-^{3}H]palmitic acid (sp act. 188 mc/mmole) were purchased from New England Nuclear Corp. [U-^{14}C]Palmitic acid (sp act. 93 mc/mmole), [U-^{14}C]stearic acid (sp act. 93 mc/mmole), [U-^{14}C]-oleic acid (sp act. 108 mc/mmole), [1-^{14}C]myristic acid (sp act. 15.4 mc/mmole), [1-^{14}C]lauric acid (sp act. 21.0 mc/mmole), and [1-^{14}C]decanoic acid (sp act. 3.5 mc/mmole) were purchased from Nuclear Chicago Corp. Sodium [1-^{14}C]acetate (sp act. 10 mc/mmole) was purchased from Volk Radiochemical Co. Radioactive fatty acid (usually 50 μ) was dissolved in a small amount of ether, 2 small drops of Tween-20 were added, and the ether was evaporated with a stream of N_2. Then 0.1 M phosphate buffer, pH 6.8, was slowly added with shaking to give 10 ml of an almost clear solution, and appropriate amounts of this solution were used in the incubation. 3-(4-Chlorophenyl-1,1-dimethylurea) (CMU) was dissolved in a minimal amount of hot ethanol and then mixed with enough water to give 5×10^{-4} M concentration. Appropriate amounts of this solution were added to the incubation medium to give the desired final concentration. Trichloroacetic acid was neutralized to pH 7.0 and an appropriate amount of this concentrated solution was added to the incubation medium to give the desired final concentration.

Results and Discussion

Although the absence of pentadecanoic acid in plants prompted Chibnall to discard (Chibnall and Piper, 1934) his original suggestion (Channon and Chibnall, 1929) of a pentadecanoic acid pathway for C_{29} biosynthesis, the discovery of α oxidation in plants (Martin and Stumpf, 1959), especially in young leaves, where there is no known function for this enzyme system (Hitchcock and James, 1964, 1965), raised the interesting possibility that it may provide the pentadecanoic acid for the biosynthesis of C_{29} compounds. The fact that the methyl carbon and not the carboxyl carbon of acetate becomes the carbonyl carbon of 15-nonacosanone supported this hypothesis (Kolattukudy, 1965). Therefore attempts were made to obtain more direct evidence for the pentadecanoic acid pathway.

Precursor–Product Relationship among the C_{29} Compounds. First of all the specific activity of the three C_{29} compounds nonacosane, 15-nonacosanol, and 15-nonacosanone was measured at different time intervals after administering [1-^{14}C]acetate. According to the pentadecanoic acid pathway hypothesis, 15-nonacosanone would be expected to have the highest specific activity and nonacosane the least, during the initial phase of incorporation. The specific activity of 15-nonacosanol measured after 30 min and 2 and 4 hr of metabolism of [1-^{14}C]acetate was 2.45×10^4, 3.3×10^4, and 4.7×10^4 cpm/mg, respectively. The specific activity of the paraffins at corresponding intervals was at least 2–3 times as high, as reported earlier, the specific activity of the ketone being closer to the paraffin (Kolattukudy, 1965); at no time was the specific activity of the ketone or the secondary alcohol greater than that of the hydrocarbon. These results are not in agreement with the pentadecanoic acid pathway hypothesis, especially the involvement of 15-nonacosanol as the intermediate. Comparison of the specific activity of the three C_{29} compounds and the fact that the specific activity of the three compounds increased for at least 4 days argues in favor of the suggestion by Chibnall and Piper (1934) that the ketone is an end product rather than intermediate.

Another approach was made to test whether the ketone is an intermediate or an end product. Pure 15-nonacosanone was isolated by repeated thin layer chro-

matography from a leaf which had metabolized 1 mc of [^{14}C]acetate for 2 days. A fine suspension of this ketone, made with the aid of a drop of Triton X-100, was painted on a healthy, fully turgid young broccoli leaf. Even after 4 days, during which time the leaf appeared healthy, all of the radioactivity remained in the ketone washed from the leaf surface. Similar attempts to detect conversion of the ketone into 15-nonacosanol or nonacosane in leaf disks, peeled epidermis of cabbage leaves, and homogenates of cabbage and broccoli leaves failed, again arguing against the pentadecanoic acid pathway and indicating that there may not be any precursor–product relationship among the three C_{29} compounds. The results mentioned above also indicate that the surface lipids of Brassica undergo little or no interconversion.

Effect of Imidazole on Acetate Incorporation into Wax. If α oxidation of palmitic acid provides the pentadecanoic acid for the C_{29} biosynthesis, imidazole, which is known to be an inhibitor of α oxidation in plants (Martin and Stumpf, 1959) and in leaves in particular (Hitchcock and James, 1965), would be expected to inhibit the C_{29} biosynthesis. However imidazole when administered with [1-^{14}C]acetate into isolated broccoli leaves not only failed to inhibit acetate incorporation into C_{29} compounds but stimulated it. For example, 1–50 μmoles of imidazole/leaf (approximately 5-g fresh weight) stimulated incorporation of acetate 30–100%. In spite of the fact that imidazole caused some wilting of the leaves it did not inhibit acetate incorporation into C_{29} compounds. These observations also argue against the pentadecanoic acid pathway.

Incorporation of Specifically Labeled Palmitic Acid and Pentadecanoic Acid into Wax. If the C_{29} compounds were synthesized primarily *via* pentadecanoic acid, [1-^{14}C]-pentadecanoic acid incorporation might be faster than that of a similar fatty acid such as [U-^{14}C]palmitic acid unless palmitic acid is converted into pentadecanoic acid by α oxidation at least as fast as the incorporation of pentadecanoic acid into the C_{29} compounds. If the latter possibility is the case [1-^{14}C]palmitic acid would not contribute its ^{14}C into the C_{29} compounds because the carboxyl carbon would be lost during α oxidation. In preliminary experiments it was found that [1-^{14}C]-pentadecanoic acid was incorporated into C_{29} compounds less rapidly than [U-^{14}C]palmitic acid or [1-^{14}C]-palmitic acid. For example, after 3 hr of incubation with about 2.5×10^6 cpm each of [1-^{14}C]pentadecanoic acid, [1-^{14}C]palmitic acid, and [U-^{14}C]palmitic acid, 2.4×10^4, 10×10^4, and 13×10^4 cpm, respectively, were found in paraffins isolated from the unsaponifiable material. Thus [1-^{14}C]palmitic acid and [U-^{14}C]palmitic acid are equally efficient in contributing their ^{14}C to the C_{29} compounds. These results also cast serious doubt on the validity of pentadecanoic acid pathway for the biosynthesis of C_{29} compounds.

The results mentioned above can be most eaily explained as an incorporation of palmitic acid as a unit into the C_{29} compounds. However, an alternative mechanism by which palmitic acid is first converted into acetate units, which then form the C_{29} compounds, cannot be ruled out, although absence of radioactivity in fatty acids smaller than C_{16} and unsaturated fatty acid argues against such a view. However if [9,10-^{3}H]palmitic acid participates in the latter pathway most of the ^{3}H would be lost during the process of conversion into acetate units and the synthesis of C_{29} compounds from these units. The results of experiments to examine this possibility are summarized in Table I. The paraffin fractions were analyzed by gas chromatography to establish that

TABLE I: Incorporation of [1-^{14}C]Palmitic Acid, [U-^{14}C]-Palmitic Acid, and [9,10-^{3}H]Palmitic Acid into Paraffins of Broccoli Leaves.[a]

Expt	Position of Label in Substrate	Exptl Condn	Radioact. in Paraffin (% of Total Lipid)
1	1-^{14}C	Chopped leaf	12.9
		Disks	13.8
	U-^{14}C	Chopped leaf	12.8
		Disks	13.6
2	1-^{14}C	Chopped leaf	10.4
	1-^{14}C	Chopped leaf + 2 μmoles of trichloroacetic acid	7.1
	U-^{14}C	Chopped leaf	10.0
	U-^{14}C	Chopped leaf + 2 μmoles of trichloroacetic acid	7.6
3	U-^{14}C	Chopped leaf	12.0
	U-^{14}C	Chopped leaf + 2 μmoles of trichloroacetic acid	7.9
	9,10-^{3}H	Chopped leaf	12.1
	9,10-^{3}H	Chopped leaf + 2 μmoles of trichloroacetic acid	7.4
4	U-^{14}C	Chopped leaf	10.0
	9,10-^{3}H	Chopped leaf	11.0
5	1-^{14}C	Chopped leaf	6.4
	9,10-^{3}H	Chopped leaf	6.7

[a] Chopped leaves (2.2-g fresh weight) or 20 disks 1.6 cm in diameter (1.1-g fresh weight) received about 2.5 μc of ^{14}C or 15 μc of ^{3}H; chopped leaves in 6 ml of water containing the inhibitor and substrate for 4 hr and the disks over a thin layer of substrate solution (1 ml) for 8 hr; all incubations under light at 30°. Paraffins were separated from total lipid extract by column chromatography. About 80% of the administered radioactivity was recovered in the total lipids. Although [1-^{14}C]palmitic acid was 3–4 times as efficient as [U-^{14}C]palmitic acid in contributing ^{14}C to CO_2, only a small fraction of a per cent of the administered radioactivity was converted to CO_2. In expt 5, the temperature of the bath rose 3–4° and remained at this temperature for about 0.5 hr, thus leading to lower values; however, both ^{3}H and ^{14}C were similarly affected.

the major radioactive paraffin is nonacosane (see Figure 1). It is clear that the carboxyl carbon of palmitic acid is not lost during the incorporation of palmitic acid into the paraffin. Neither was the tritium lost from palmitic acid, the ratio $^{3}H:^{14}C$ being 1.0, 0.95, and 1.0 in the substrate, lipid, and paraffin, respectively. Under the experimental conditions only about 5% of the administered ^{3}H appeared in the water and a fraction of a per cent of the administered ^{14}C appeared in CO_2, thus supporting the contention that palmitic acid undergoes very little catabolic breakdown. Low concentrations of trichloroacetate inhibited to the same extent the incorporation of radioactivity into the paraffin from palmitic acid irrespective of the position or kind of isotopic labeling in the substrate. This argues against the possibility that identical incorporation of label from the specifically labeled substrate may have been the result of the incorporation of the isotopes by different means. These results are clearly in contradiction to the pentadecanoic pathway for the biosynthesis of nonacosane, and hence the paraffins are suggested to be synthesized by elongation of preformed fatty acids.

In order to find out whether this is true for other C_{29} compounds such as 15-nonacosanone and 15-nonacosanol, radioactive palmitic acid was administered to leaf disks and the wax formed was isolated and fractionated as described in the previous section (see Table II). [1-^{14}C]Palmitic acid was as efficient as [U-^{14}C]-palmitic acid in contributing ^{14}C to 15-nonacosanone and 15-nonacosanol. These results clearly indicate that the carboxyl carbon of palmitic acid is not lost during the incorporation of palmitate into these C_{29} components also. Hence the pentadecanoic acid pathway does not seem to be involved significantly in their biosynthesis; instead an elongation pathway is suggested

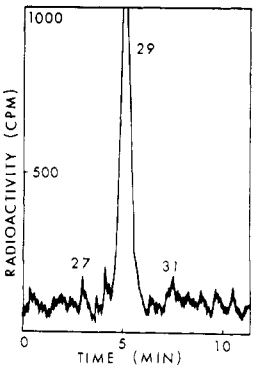


FIGURE 1: Radioactivity monitor tracing of the paraffin fraction obtained from broccoli leaves metabolizing [1-^{14}C]palmitic acid. The pattern was the same for all substrates described in the text; identification was made by superimposing the mass detector tracing and the radioactivity tracing. The number on the peak represents the chain length. Experimental conditions: 4-ft coiled-copper column (0.25 o.d.), 5% SE-30 on 80–100 mesh siliconized Chromosorb W, temperatures of the column and injector 290 and 360°, respectively. Carrier gas argon at about 70 cc/min.

TABLE II: Incorporation of [1-^{14}C]Palmitic Acid and [U-^{14}C]Palmitic Acid into the Surface Wax Components of Broccoli Leaves.[a]

Wax Fraction	Incorp % of Total in Surface Wax: [1-^{14}C]-Palmitic Acid	[U-^{14}C]-Palmitic Acid
Hydrocarbon	48	49
Ester	10.4	11.6
Ketone	26	25.4
Secondary alcohol	9.5	9.5
Primary alcohol	6	4.3

[a] Leaf disks (20) 1.6 cm in diameter received about 2.5 μc of palmitic acid in 1.0 ml of solution and the wax was isolated after 8 hr of metabolism under light, by immersing the disks in $CHCl_3$–MeOH (2:1) and fractionated by thin layer chromatography. About 20% of the administered radioactivity was converted into wax compounds from both substrates, and percentages are calculated excluding the free acids since this fraction includes the remaining substrates.

TABLE III: Incorporation of Fatty Acids into Paraffins and CO_2 in Broccoli Leaves.[a]

Substrate	Radioact. as % Administered: Total Lipids	Paraffins	CO_2
[U-^{14}C]Stearic acid	92	21	0.018
[U-^{14}C]Stearic acid + 8×10^{-5} M CMU	92	20	0.17
[U-^{14}C]Palmitic acid	80	9.6	0.024
[1-^{14}C]Myristic acid	61	4.0	0.13
[1-^{14}C]Lauric acid	44	2.8	0.26
[1-^{14}C]Decanoic acid	34	1.6	0.37
[1-^{14}C]Decanoic acid + 8×10^{-5} M CMU	20	1.1	8.1

[a] Each substrate (2.5 μc) was used in 6 ml of medium and the samples were incubated under light for 4 hr at 30°. The paraffins were isolated from the total lipids by column chromatography. CO_2 was collected at the end of the experiment by injecting 5 N KOH into the side arm; when the vessels contained CMU only the CO_2 liberated during the last half of the incubation period was collected. The values for palmitic acid are taken from an experiment with a different batch of leaves.

TABLE IV: Incorporation of Fatty Acids into the Wax Components in Broccoli Leaf Disks.[a]

	Substrate					
	C_{10}	C_{12}	C_{14}	C_{16}	C_{18}	$C_{18:1}$
Administered cpm $\times 10^{-5}$	29.5	36.5	36.0	33.8	34.0	25.4
Remaining in the medium cpm $\times 10^{-5}$	0.89	0.30	0.40	0.54	7.0	1.46
Washed into $CHCl_3$–MeOH in 30 sec cpm $\times 10^{-5}$	1.77	2.4	3.81	7.55	16.1	1.28
Wax fractions	Radioactivity (% of Total Administered)					
Hydrocarbon	1.57	2.2	2.9	6.46	14.0	0.12
Esters	0.31	0.57	1.31	2.35	2.56	0.12
Ketone	0.40	0.76	1.15	3.0	6.25	0.11
Unknown 1	0.64	0.80	1.22	3.0	5.6	0.07
Secondary alcohol	0.55	0.48	0.68	1.34	2.7	0.027
Unknown 2	0.37	0.13	0.095	0.16	0.23	0.024
Primary alcohol	0.33	0.41	0.62	0.94	1.75	0.46
Acid	1.84	1.25	2.65	5.14	14.35	4.4

[a] Disks (20) 1.6 cm in diameter (1.1-g fresh weight) were used in each case and were incubated under light for 6 hr. The wax isolated from the surface by immersing the disks for 30 sec in $CHCl_3$–MeOH was fractionated by thin layer chromatography. Substrate fatty acids are represented by their chain length and $C_{18:1}$ represents oleic acid. The experiment with oleic acid was done with a different batch of leaves; however, along with it a control experiment was done with stearic acid, and the values for stearic acid were almost identical with the values given in this table. Hence the values given for stearic acid and oleic acid may be directly compared.

for the biosynthesis of these C_{29} compounds. From the results in Table II, an elongation pathway seems to hold good for the non-C_{29} compounds of the wax as well.

Effect of Chain Length of the Precursor on Incorporation into Wax. In the experiments described thus far, palmitic acid is clearly shown to be a good precursor for the hypothetical elongation system which synthesizes the various wax components, especially the paraffins. However other long-chain fatty acids may also serve as precursors of paraffins if the elongation system is not specific for palmitic acid. Results of experiments to study this possibility are summarized in Table III.

As is clear from the table, longer fatty acids were more efficient as precursors of paraffins. The major radioactive hydrocarbon formed in all cases was nonacosane (see Figure 1). Shorter fatty acids contributed less to lipids in general and paraffins in particular, whereas conversion into respiratory CO_2 increased as chain length decreased. Most of the respiratory CO_2 is probably refixed because the experiments were done under light. This is clear from the fact that, when photosynthetic CO_2 fixation was inhibited by CMU, the $^{14}CO_2$ release was greatly increased (see Table III). Thus although the values given for respiratory $^{14}CO_2$ do not represent absolute values it is justifiable to use them as an indication of the relative amounts of fatty acids that served as respiratory substrate. When water-soluble products were examined for radioactivity, it was found that shorter fatty acids gave more water-soluble products, an observation in agreement with the conclusion that shorter fatty acids contribute more to the respiratory CO_2 than longer fatty acids.

Since the chain length of the precursor fatty acid was found to have a significant effect on incorporation into paraffins, it is important to find out whether such a relationship exists for other components of the wax. The results of experiments conducted for this purpose are summarized in Table IV. The fatty acid fraction shown includes the administered substrate adhering to the disks and thus the radioactivity in this fraction cannot be taken as fatty acid components of the wax. In fact most of the radioactivity of the acid fraction was found to be in the substrate and therefore radioactivity in this fraction together with the radioactivity remaining in the medium reflects uptake of the substrate. Thus shorter fatty acids were taken up into the leaf better than the longer ones. Although <10% of the palmitic and shorter fatty acids were left outside the leaf, one-third of the stearic acid failed to get in. This factor evidently complicates the interpretation of the data. However, since longer fatty acids are better precursors in spite of the smaller uptake, the longer fatty acids may be even more efficient precursors than the data indicate. This conclusion appears to hold good for all wax components. It is remarkable that almost half of the administered stearic acid is converted into wax under the experimental conditions. The most significant aspect of the incorporation pattern is that the increase in incorporation with increasing chain length

TABLE V: Distribution of Radioactivity in Fatty Acids Isolated from Leaves Metabolizing Various Fatty Acids (Per Cent of Total Radioactivity in Recovered Fatty Acids).[a]

Substrate	Leaf Fatty Acids										
	C_{10}	C_{12}	C_{14}	C_{16}	C_{18}	$C_{18:1}$	$C_{18:2}$	$C_{18:3}$	C_{20}	C_{22}	C_{24}
[1-^{14}C]Decanoic acid	?	2.6	Tr	46	7.7	19	19.2	5.2	...	...	...
[1-^{14}C]Lauric acid		27.5	12.4	27	7.4	8.8	11.5	3.8	1.6	...	...
[1-^{14}C]Myristic acid	0	0	44	37	8.1	3.6	4.7	...	2.5	...	...
[U-^{14}C]Palmitic acid	0	0	0	87	8.3	0	0	0	2.3	1.3	1.3
[U-^{14}C]Stearic acid	0	0	0	4.8	76.2	0	0	0	10.2	3.4	3.4

[a] Chopped leaf (2.2) metabolized about 2.5 μc of each substrate for 4 hr under light at 30°. Total fatty acids were analyzed by gas–liquid partition chromatography; the radioactivity was measured by integration of the radioactivity monitor tracing by the triangulation method; significant amounts of C_{10} might have been lost during evaporation with streams of N_2, etc. Measurable amounts of radioactivity could also be found in C_{17} especially when C_{16} or C_{18} was the substrate, 0.13% from C_{16} and 1.9% from C_{18}. The very long chain fatty acids could be detected even from decanoic acid if large amounts of radioactivity were subjected to radio gas chromatography; however, in regular assays it was difficult to measure. No detectable impurities could be found in the substrate. Fatty acids are represented by their chain length and the number of double bonds represented by the number after the colon.

of the substrate seems to be much more marked in the case of the C_{29} compounds than the esters which contain shorter alkyl chains (Purdy and Truter, 1963) and thus require less elongation. This distinction between C_{29} compounds and the non-C_{29} compounds such as esters is reminiscent of the previously reported inhibition by trichloroacetate of acetate incorporation into these two classes of compounds where the C_{29} compounds were much more severely inhibited than the esters (Kolattukudy, 1965). Thus it is suggested that the trichloroacetic acid inhibition may be exerted at the elongation stage, which could explain why this substance does not inhibit fatty acid synthesis. However the precise point at which inhibition by trichloroacetate occurs can be determined only when the cell-free system for the biosynthesis of C_{29} compounds is worked out. When the per cent incorporation is plotted against chain length of the precursor (see Figure 2) there is a distinct lag in the increase of incorporation, as demonstrated by the sudden change in the slope of the line near C_{16}. The fact that the lag is predominant only in the case of C_{29} compounds and not esters is significant. This lag suggests that fatty acids shorter than C_{16} must first be elongated to C_{16} (or C_{18}) before they can serve as substrates for the elongation system which synthesizes the C_{29} compounds. The elongation of medium chain length fatty acids to C_{16} and C_{18} has been well documented (James, 1963), and in the broccoli leaves used in these experiments C_{16} was the major radioactive product formed from C_{10}, C_{12}, and C_{14} fatty acids (Table V).

In view of the fact that the broccoli wax contains no detectable olefinic compounds, unsaturated fatty acids would not be expected to undergo the elongation process which is responsible for C_{29} biosynthesis. In fact, unlike stearic acid, oleic acid was not incorporated into wax although it was well taken up by the leaf disks (see Table IV). Thus the elongation system excludes

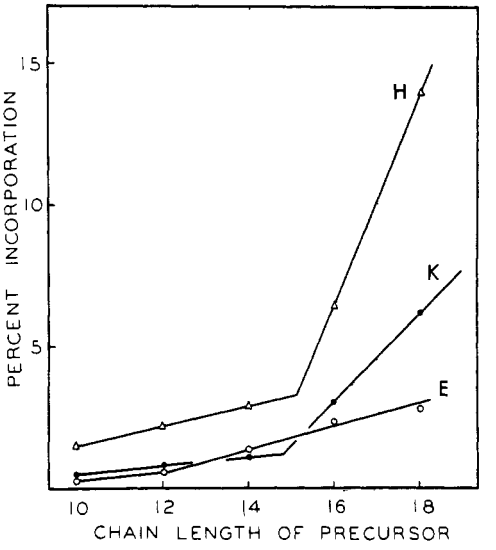


FIGURE 2: Effect of chain length of the precursor fatty acid on the incorporation into hydrocarbons (H), ketones (K), and esters (E). Experimental conditions are given under Table IV.

the unsaturated fatty acids and the wax is free from unsaturated compounds. This observation is consistent with the elongation hypothesis for C_{29} biosynthesis.

In order to solve the difficulty arising from the apparent absence of pentadecanoic acid in plants, Kreger (1948) proposed a modification of the pentadecanoic acid pathway. According to this, two molecules of palmitic acid would condense with the loss of CO_2 to give palmitone which would subsequently lose two methyl carbons by ω oxidation and decarboxylation

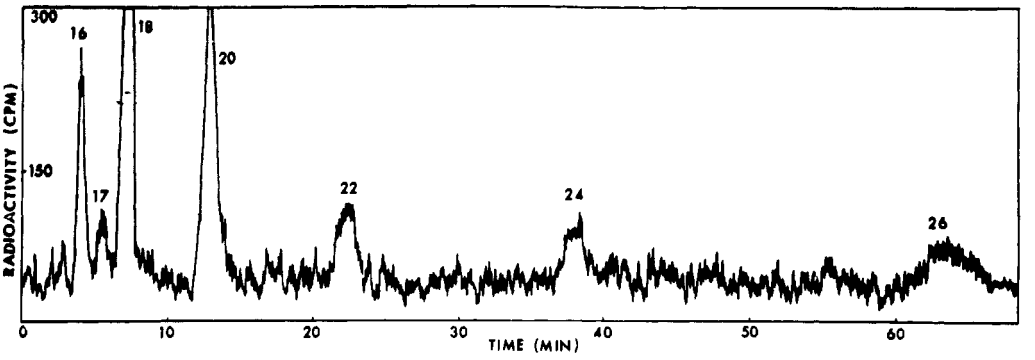


FIGURE 3: Radio gas chromatography pattern of methyl esters of fatty acids isolated from broccoli leaf disks which metabolized [U-^{14}C]stearic acid for 6 hr. The pattern represent total fatty acids from within the leaf; total lipids including surface lipids gave a similar pattern. Number on each peak represents chain length; the identification was confirmed by analysis on 12% Apiezon L column. No radioactive impurities could be detected in the substrate even when the total radioactivity injected was 10–20 times as much as the amount shown in this figure. Experimental conditions: 6-ft (0.25 o.d.) coiled-copper column, 15% stabilized diethylene glycol succinate on 60–70 mesh Anakrom AB, temperatures of column and injector 200 and 350°, respectively. Carrier gas argon at 70 cc/min. The relative proportion of C_{16} and C_{17} showed variation; often C_{17} contained more radioactivity than C_{16}. With a column temperature of 270°, measureable amounts of C_{28} also were found.

(see Scheme I). The position of ^{14}C in the 15-nonacosanone derived from specifically labeled acetate (Kolattukudy, 1965) does not agree with this hypothesis. In addition according to this pathway [1-^{14}C]palmitic acid would be only 55% as efficient as [U-^{14}C]palmitic acid in incorporating ^{14}C into the C_{29} compounds, because [1-^{14}C]palmitic acid would lose 50% of its ^{14}C and [U-^{14}C]palmitic acid would lose only 9% of its ^{14}C during the condensation and decarboxylation. Nevertheless, the data presented here show that [1-^{14}C]-palmitic acid is 100% as efficient as [U-^{14}C]palmitic acid in contributing ^{14}C to the C_{29} compounds. Furthermore, on the basis of Kreger's hypothesis it is difficult to explain why stearic acid is at least twice as good a precursor as palmitic acid.

Elongation of Medium Chain Length Fatty Acids into Long Fatty Acids. In view of the evidence shown above for an elongation pathway it is of interest to examine the fatty acids produced by the leaf from the various precursors. Decanoic, dodecanoic, and tetradecanoic acids gave rise to higher fatty acids both saturated and unsaturated, generally in agreement with the results of James (1963), the major radioactive product in all cases being palmitic acid (see Table V). Palmitic and stearic acids did not produce significantly radioactive unsaturated acids. As the chain length of the precursor increased, relative incorporation into unsaturated fatty acid decreased. Small amounts of radioactivity could be detected in fatty acids with an odd number of carbon atoms, particularly in C_{17} when C_{16} and C_{18} acids were used as the substrates. α Oxidation of the C_{18} acid (Hitchcock and James, 1965) is probably responsible for this. The most significant part of the fatty acid pattern is the conversion of stearic acid into a C_{20} acid which contained >10% of the radioactivity of stearic acid, with lesser amounts of ^{14}C in C_{22}, C_{24}, and C_{26}. With the experimental procedure used, fatty acids longer than C_{26} would be hard to detect if present in small quantities. Conversion of palmitic acid into long-chain fatty acids such as C_{20} and higher can be demonstrated (Table V) although it seems to be a less efficient precursor than stearic acid. The shorter fatty acids also gave rise to very long fatty acids but much less efficiently than C_{16} or C_{18}. These observations demonstrate that there is indeed an elongation system in broccoli leaves and that this system uses longer fatty acids as substrates more efficiently than shorter ones.

This elongation system may be related to the C_{29} biosynthesis; it is possible that these very long fatty acids are intermediates in the biosynthesis of C_{29} compounds, although the existence of such free intermediates would seem to make the system inefficient. It seems more likely that the long-chain fatty acids (C_{20} and up) are biosynthetically related to other wax components such as the esters, alcohols and acids. To gather some information as to the location of these acids in the leaves, the wax was washed off by a short immersion (30 sec) in $CHCl_3$–MeOH. If we assume that all wax components behave like the paraffins, as far as extraction into the $CHCl_3$–MeOH is concerned, almost all of them must have been removed by this procedure. The free acids removed did not contain any C_{20} or higher fatty acid although C_{18} could be detected in the wax acid obtained from disks which received C_{16} as the substrate. These results indicate that the very long chain fatty acids (C_{20} and up) must have been within the leaf tissue rather than on the surface. In fact when the fatty acids obtained from within the leaf disks were analyzed, the pattern in Figure 3 was obtained, showing that the very long fatty acids are inside the leaf. Thus it is not possible at the present time to decide whether this elongation system is biosynthetically related to the

TABLE VI: Effect of Light on Incorporation of [1-^{14}C]Acetate and [1-^{14}C]Palmitic Acid into Lipids and Paraffins.[a]

Expt	Substrate	Exptl Condn	Lipid (cpm × 10^{-6})	% Inhib in Darkness	Paraffin (cpm × 10^{-5})
1	[1-^{14}C]Acetate	Water, light	2.75	..	1.35
		Water, dark	1.72	37	1.65
2	[1-^{14}C]Acetate	Water, light	1.36		1.5
		Water, dark	0.87	36	2.10
		Buffer, light	1.04		1.1
		Buffer, dark	0.51	51	0.95
3	[1-^{14}C]Acetate	Water, light	1.57		1.15
		Water, dark[b]	0.52	61	1.35
4	[U-^{14}C]Palmitic acid	Light			2.40
		Dark			2.72
5	[1-^{14}C]Palmitic acid	Light			2.55
		Dark			2.85

[a] Chopped leaf (2.2 g) received 5.5 μmoles (5 μc) of sodium acetate in 6 ml of water, or 0.2 M phosphate buffer, pH 7.4, or 3 μc of palmitic acid in 6 ml of H_2O; all incubations were done at 30° for 4 hr. In expt 1, 0.5 μmole of acetate (5 μc) was used. About 25% of the administered radioactivity was incorporated under light into lipids during the 4-hr experimental period. [b] In expt 3, the leaves in the dark sample were incubated in the dark for 1 hr before substrate was administered; this treatment did not have any effect on the incorporation of palmitic acid. Lipid fraction includes all lipids of the leaf (cytoplasm and surface) except paraffins.

elongation system that synthesizes the wax. However the fact that such elongation can take place in leaf tissue is important because a similar elongation pathway is suggested for the biosynthesis of the C_{29} and other components of the wax. Elongation of fatty acids has been observed in animal tissues (Wakil, 1963) as well as in certain plant tissues (Hawke and Stumpf, 1965). Although Hawke and Stumpf (1965) found that acetate was incorporated into C_{20} and longer fatty acids in barley seedlings, other substrates such as fatty acids (C_8–C_{18}) were not incorporated, whereas in broccoli leaves the longer fatty acids are better precursors than shorter fatty acids or acetate.

Effect of Light on Wax Biosynthesis. In the experiments with isolated whole leaves (Kolattukudy, 1965), the substrates were administered through the petiole. Hence uptake by the leaf and consequently entry of the substrate into the cells were influenced by transpiration which is greatly enhanced by light. Thus the effect of light on acetate incorporation into the wax was difficult to study. However with chopped leaf preparations this difficulty could be overcome, and the results of experiments to examine the effect of light on paraffin synthesis are summarized in Table VI.

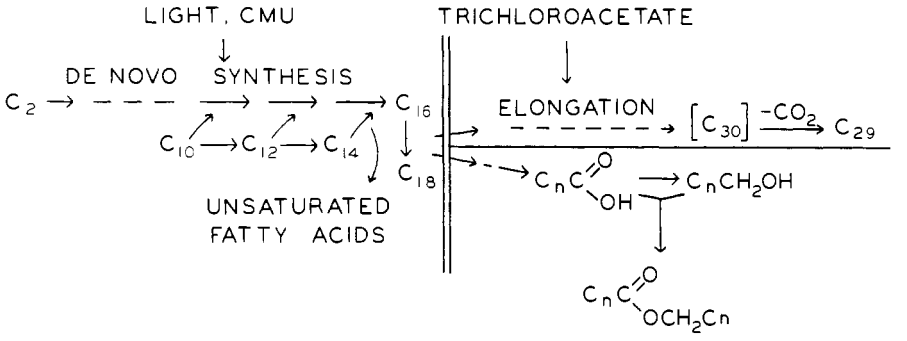
Incorporation of acetate or palmitate into the paraffin is not inhibited by the absence of light but is slightly stimulated, whereas incorporation of acetate into other lipids is inhibited by the absence of light; the slight stimulation of incorporation into the wax in the dark may be due to the inhibition of the competing pathways. The radioactivity in the lipids includes all lipids except paraffins, and thus some C_{29} compounds and other wax components are also included in this fraction. So the true inhibition of internal lipid synthesis by the absence of light may be greater than is indicated by Table VI. Furthermore inhibition of lipid synthesis could be increased from 40 to 60% by incubation of the leaf tissue in the dark before administering radioactive substrate, whereas this treatment had no effect on paraffin synthesis. Moderate (Hawke and Stumpf, 1965) to severe (Stumpf *et al.*, 1963; Stumpf and James, 1963) inhibition of acetate incorporation into lipids has been previously reported in chloroplast preparations. Since acetate and palmitate incorporation into the paraffin is independent of light under conditions which severely inhibit acetate incorporation into lipids, the paraffin biosynthesis apparently is not tightly coupled to photosynthetic reactions such as the production of ATP and TPNH. However from the known cofactor requirements for chain elongation in other systems (Wakil, 1963; Stumpf, 1964), the palmitate and acetate incorporation should require some energy source, probably ATP and reducing agents such as reduced pyridine nucleotides. So the photodependence of fatty acid synthesis and photoindependence of paraffin biosynthesis may be explained if the site of paraffin biosynthesis differs from that of general fatty acid biosynthesis. A mechanism may exist by means of which energy and reducing agents available in the dark are utilized more efficiently for paraffin biosynthesis than for fatty acid synthesis.

Effect of 3-(4-Chlorophenyl)-1,1-dimethylurea (CMU) on Wax Biosynthesis. Acetate incorporation was reported to be severely inhibited by CMU, a potent inhibitor of photophosphorylation (Stumpf and James, 1963). If paraffin biosynthesis is not tightly coupled to photosynthetic reactions such as photophosphorylation, it may be insensitive to CMU. Results of experiments

TABLE VII: Effect of CMU on the Incorporation of [1-^{14}C]Acetate and [1-^{14}C]Palmitic Acid into Lipids of Broccol Leaf.[a]

Expt	Substrate	Concn of CMU (M)	Radioact. in Lipids (cpm × 10^{-6})	% Inhib	Radioact. in Paraffins (cpm × 10^{-5})	% Inhib
1	[1-^{14}C]Acetate	0	2.13	..	1.35	..
		8×10^{-5}	0.37	83	0.5	63
2	[1-^{14}C]Acetate	0	2.3	..	1.45	..
		8×10^{-6}	1.1	52	0.95	34
		1×10^{-6}	2.0	13	1.45	0
3	[1-^{14}C]Acetate	0	1.6	..	1.13	..
		4×10^{-6}	1.34	16	1.3	0
4	[1-^{14}C]Palmitic acid	0			0.90	
		2×10^{-6}			1.15	
		8×10^{-6}			0.95	
5	[1-^{14}C]Palmitic acid	0			1.65	
		1.6×10^{-5}			1.80	
		8×10^{-5}			1.75	

[a] Chopped broccoli leaves (2.2 g) in 6 ml of water containing the inhibitor received either 5 μc (5 μmoles) of sodium acetate or 2 μc of palmitic acid. Incubation under light for 4 hr at 30°. In expt 4 the bath temperature rose 3–4° and remained at this temperature for about 0.5 hr and hence the lower values. Radioactivity in lipids includes all lipids except paraffins.



SCHEME II

to examine this possibility are summarized in Table VII. Evidently acetate incorporation into paraffin is not entirely independent of CMU at concentrations that are required for severe inhibition of fatty acid synthesis. CMU at such concentrations may inhibit some other reaction in the pathway for paraffin biosynthesis. It is more likely that inhibition of fatty acid synthesis from acetate may indirectly inhibit the acetate incorporation into paraffin by limiting the amount of radioactive fatty acids available for elongation. Attempts to reverse the inhibition of acetate incorporation into paraffins by exogenous palmitic acid failed. However this might be expected according to the above hypothesis. Acetate incorporation into the paraffin occurs in two phases, incorporation into fatty acid and subsequent elongation into a C_{30} unit which is then decarboxylated to give nonacosane. This would explain why acetate incorporation into the paraffin is less sensitive to CMU than acetate incorporation into fatty acids. According to the hypothesis offered above, incorporation of palmitic acid into the paraffin should be insensitive to CMU. The results summarized in Table VII clearly show that CMU, at a concentration which is adequate to inhibit acetate incorporation into fatty acids almost completely (80%), does not affect incorporation of palmitic acid into the paraffins. Incorporation of stearic acid into paraffins was also insensitive to CMU whereas incorporation of decanoic acid was inhibited by about 30%. Decanoic acid has to be first incorporated into C_{16} before it becomes the substrate for the elongation system which synthesizes paraffins. The enzyme system that incorporates decanoic acid into C_{16} being the same as or very similar to the enzyme systems that synthesize fatty acids, it is sensitive to CMU and thus shows a partial inhibition. Thus the elongation process is distinctly different from fatty acid synthesis.

On the basis of experimental evidence discussed thus far a working hypothesis for the biosynthesis of lipids in *B. oleracea* may be diagrammatically represented as shown in Scheme II. Although the C_{16} is shown in the

diagram to be supplied by the *de novo* synthesis, there may be a light-independent fatty acid synthesis which would provide substrates for the elongation. The possibility of less specific and unknown effects of CMU on this system remains open. At present one can only speculate concerning the biosynthetic relationships among the three C_{29} compounds, the ketones, the secondary alcohol, and the paraffin. However the alcohol is most likely not an intermediate of paraffin biosynthesis, although the ketone may be the precursor of secondary alcohol. If 15-nonacosanone is formed by elongation of palmitic and stearic acids as suggested by the results discussed here, the conversion of a specific methylene group of the substrate into a carbonyl group (in the ketone) would be an interesting problem. Work is in progress to further elucidate the biosynthetic pathway for the C_{29} compounds.

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