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Biosynthesis of Surface Lipids

Biosynthesis of long-chain hydrocarbons and waxy esters is discussed.

P. E Kolattukudy

Lipids are responsible for the familiar water-repellent character of the surfaces of plants, animals, and insects. In chemical composition the surface lipids differ from the internal lipids. Collectively they are called waxes because of their peculiar physical properties, although in strict chemical terms, wax refers to esters of long-chain alcohols with long-chain acids. Identification of wax components has been progressing at a rapid rate because of modern analytical techniques. The biosynthesis of such compounds has been the subject of much conjecture, but only in the past few years have we gained any knowledge based on experimental evidence concerning the formation of surface lipids in nature.

Chemistry

In that several reviews on the chemistry of plant waxes (*1*) and animal surface lipids (*2*) have recently appeared, I shall discuss this aspect only briefly. The surface lipids usually contain hydrocarbons, waxy esters (long-chain alcohol esters of long-chain acids), primary alcohols, and fatty acids. Generally, the hydrocarbons are *n*-paraffins (10 to 50 percent in plants, 1 to 10 percent in animals, and up to 75 percent in insects) containing 21 to 35 carbon atoms in chains with an odd number of carbon atoms predominating. In most cases (especially in plants) one paraffin predominates; for example, in *Brassica oleracea* (broccoli, cabbage) more than 90 percent of the paraffin fraction is a C_{29} alkane, and in *Senecio odoris* it is a C_{31} alkane that predominates.

The waxy esters are usually made up of fatty acids and fatty alcohols with an even number of carbon atoms; the chain usually contains from 10 to 30 carbon atoms. Several dominant waxy esters together with a few minor components are usually found. The free alcohols and free fatty acids of the surface lipids also usually have an even number of carbon atoms and a chain length from C_{10} to C_{30}.

Many waxes are much more complex than this oversimplified picture. Some waxes contain branched hydrocarbons, primarily 2-methyl (*iso*) and 3-methyl (*anteiso*) (*3*), unsaturated hydrocarbons (*4, 5*), and even cycloparaffins (*6, 7*). Other classes of compounds such as ketones (*8, 9*), aldehydes (*10, 11*), diols (*12*), glycerides (*2, 13*), terpenes (*10, 14*), and flavones (*9, 15*) are also occasionally found in waxes.

Possible Significance

The surface lipids are of great importance in conserving water in the organism. In plants as well as in animals, because the surface lipid is the barrier between the organism and its environment, the nature of the surface lipid plays an important role in the interaction between the two. For example, in plants the cuticular wax controls the entry of agricultural chemicals and possibly pathogenic organisms (*16*). Furthermore, the following observations suggest that long-chain hydrocarbons may be of greater importance to organisms than is generally realized. The hydrocarbons usually found in waxes can be catabolized by microorganisms (*17*) and by animals (*18*). Hydrocarbons are found not only in the surface lipids of plants, insects, and animals but also in microorganisms (*19*). They are also found in a variety of animal tissues (other than skin), such as human arterial tissues and plaques (*20*), lymph nodes, spleen, portal triads of liver (*21*), and brain (*22*). Under certain pathological conditions, human spleen is reported to accumulate fat globules consisting mostly of long-chain (C_{25} to C_{28}) hydrocarbons (*23*). Livers of patients dying from malignant disorders have been reported to contain saturated hydrocarbons. Certain fungal infections in plants seem to

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change the hydrocarbon pattern of the plant (*24*).

In addition, some of the wax components in very low concentrations, especially the long-chain alcohols, have biological activity similar to that of androgens. For example, in chicks, comb growth induced by a minute amount of octacosanol (0.36 ng) was comparable to that produced by 11,000 times this amount of testosterone propionate (*25*). Very little is known about this aspect of wax biochemistry. It is possible that these very long-chain compounds have functional roles in metabolism at very low concentrations, but such important functions could have gone undetected because they are normally present in food stuffs.

Biosynthesis of Paraffins

Hydrocarbons constitute one of the most common components of surface lipids. Hence the biosynthesis of hydrocarbons has been studied more than that of other waxy compounds. Nevertheless, with the exception of squalene, which has been studied very extensively because of its connection with steroids (*26*), our understanding of biosynthesis of hydrocarbons is still scanty.

Animal surface lipids usually contain small amounts of paraffins (less than 10 percent). Hydrocarbons are present in the diet of animals (*18, 7*), and they are absorbed and transported into various tissues (*18, 27*). However, orally administered C_{18} hydrocarbon failed to reach the surface of the rat although metabolic products derived from the C_{18} hydrocarbon did reach the surface (*28*). These results suggest that dietary hydrocarbons are not readily transported into the surface lipids. Although exogenous petroleum products (*2*) are likely to contribute to the surface lipids of animals, it is premature to say that the surface paraffins originate entirely from such sources on the basis of the experimental results thus far obtained (*2, 21, 23, 29*). Acetate labeled with deuterium or ^{14}C and carbon atoms of glucose were incorporated into beeswax paraffins as well as other wax components (*30*).

Although paraffin synthesis also occurs in microorganisms, and *Debaromyces hansenii* was reported to incorporate carbon atoms of glucose into paraffins (*31*), most of the information we have about paraffin synthesis comes from studies on plants.

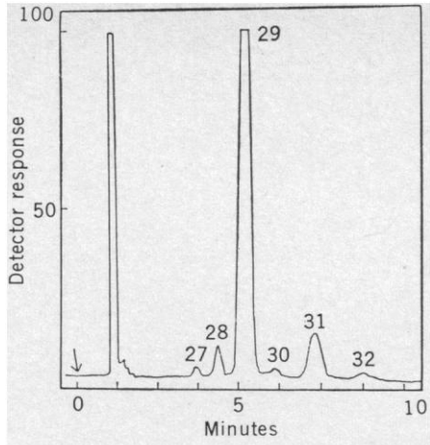


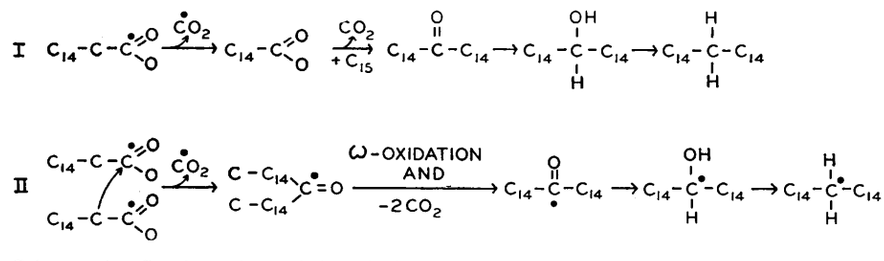
Fig. 1. (Left) Thin-layer chromatogram of leaf surface lipid of *Brassica oleracea* on silica gel G, with benzene as the solvent. *A*, fatty acids, *B*, primary alcohol, *C*, secondary alcohol, *D*, unknown, *E*, ketones, *F*, waxy esters, *G*, hydrocarbons. Spots were located by the dextrin-iodine method. (Above) Gas-liquid chromatogram of hydrocarbons eluted from the silica gel represented by spot *G* on the thin-layer chromatogram. The number of carbon atoms in each *n*-paraffin is shown on each peak.

The paraffin pattern of plant wax in general is much simpler than that of animals or insects, and therefore plants are ideally suited for study of the biosynthetic pathway for paraffins (Fig. 1). The volatile portion of terpentine from *Pinus jeffreyi* contains up to 98 percent *n*-heptane; this hydrocarbon is synthesized from acetate, and ^{14}C was located in alternate positions when acetate-1-^{14}C was the substrate (*32*). Although uniformly labeled acetate failed to be incorporated into *n*-nonacosane (C_{29}) in apple peel (*33*), cabbage and broccoli leaves readily incorporated acetate into the C_{29} paraffin (*34*). Both carbon atoms of acetate were equally well incorporated into the hydrocarbon, suggesting a polyacetate pathway (*34*).

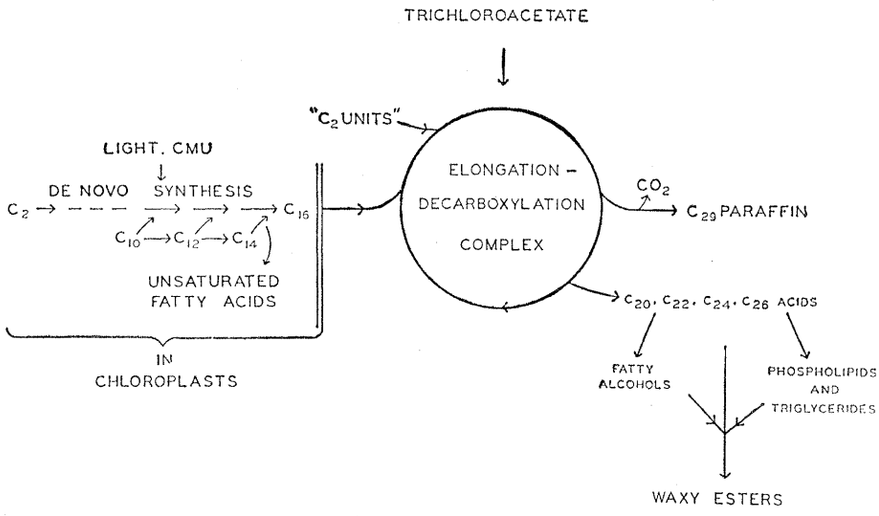
Condensation of Identical Fatty Acids

Chibnall and co-workers first suggested that condensation of two molecules of pentadecanoic acid (*n*-C_{15}) accompanied by decarboxylation could give rise to the 15-nonacosanone of cabbage wax which in turn could undergo reduction to give the paraffin (*8*). However, the absence of *n*-C_{15} acid in plants prompted them to discard this hypothesis (*35*). The recent demonstrations (*36*) of active α-oxidation in plants, especially in young leaves, suggested a possible source for *n*-C_{15} acid in the tissues, and therefore Chibnall's original suggestion was reexamined and was elaborated on (*34*), as shown in scheme 1, line I. The problem of the absence of fatty acids with an odd number of carbon atoms does not arise in the case of *n*-C_{27}, *n*-C_{31}, and *n*-C_{35} paraffins because the condensation pathway would in these cases involve *n*-C_{14}, *n*-C_{16}, and *n*-C_{18} fatty acids, known to be present in plant lipids. In order to explain how a condensation pathway could operate without having fatty acids with an odd number of carbon atoms, a double ω-oxidation and double decarboxylation has been suggested (*37*) (scheme 1, line II).

If the labeling pattern obtained in the C_{29} ketone of *Brassica oleracea* can be assumed to be the same as that in the C_{29} paraffin, the double ω-oxidation pathway (pathway II in scheme 1) can be ruled out because, if this pathway were followed, the carboxyl carbon of acetate would give rise to the carbonyl carbon of the ketone; but the methyl carbon of acetate was shown to give



Scheme 1: Condensation of identical fatty acids to form C_{29} paraffin in *Brassica oleracea* (*38*). Only the most pertinent structures are shown; the remaining part is represented by the number of carbon atoms—that is, C_{14} stands for a *n*-$C_{14}H_{29}$ group. The theoretical fate of the carboxyl carbon of C_{16} acid is represented by the solid circle.



Scheme 2: Hypothesis concerning the biosynthesis of surface lipids in *Brassica oleracea* leaves. The primary sites of action of light, chlorophenyldimethylurea, and trichloroacetate are indicated (adapted from *38, 41,* and *55*).

rise to the carbonyl carbon (*34*). This labeling pattern is in agreement with the condensation pathway suggested by Chibnall, provided that the *n*-C_{15} acid is produced by α-oxidation of C_{16} acid; if the *n*-C_{15} acid is produced from a C_3 starter and six acetate molecules, the carboxyl carbon of acetate would give rise to the carbonyl carbon of the ketone.

Further experimental evidence to test the above hypothesis was sought in the following manner. According to the condensation pathway (scheme 1, line I), *n*-C_{15} acid might be expected to be incorporated into the C_{29} paraffin at least as fast as C_{16} acid, which should be the precursor of C_{15} acid. However, in *Brassica oleracea* (*38*), C_{16} acid was more readily incorporated into C_{29} paraffin than C_{15} acid was. Furthermore, if the condensation pathway were followed, the carboxyl carbon of C_{16} acid would be lost during the α-oxidation process which precedes the condensation. Thus C_{16} acid labeled with ^{14}C only in the carboxyl carbon would not be expected to give rise to radioactive C_{29} ketone or paraffin. The fact that palmitic acid-1-^{14}C was as efficient as uniformly labeled palmitic acid in providing radioactivity to the paraffin (*38*) showed that the carboxyl carbon of C_{16} acid is not lost. Furthermore, palmitic acid-16-^{14}C and palmitic acid-1-^{14}C were equal in their incorporation of ^{14}C into the paraffin (*39*). Thus, the entire C_{16} acid molecule is incorporated into the paraffin. Such results indicate that the condensation pathway is not followed unless the administered C_{16} acid finds its way into the paraffin by way of acetate. The latter possibility could be ruled out by showing that palmitic acid-9,10-^3H did not lose the ^3H during its incorporation into the paraffin (*38*); indeed, if palmitate-9, 10-^3H undergoes β-oxidation to acetate, and these acetate molecules are rebuilt into the paraffin, most of the ^3H would be lost. These results also exclude the possibility of any other pathway (*40*) which involves loss of carboxyl carbon and incorporation of the rest of the molecule of palmitic acid.

The following observations, although not conclusive, also tend to invalidate this condensation hypothesis. Imidazole, which is an inhibitor of α-oxidation in leaves (*36*), failed to inhibit incorporation of acetate and palmitate into the C_{29} paraffin in excised leaves of broccoli (*38*); neither did it inhibit palmitate incorporation into paraffin by leaf discs, even when the inhibitor concentration was 0.045*M* (*39*). Measurements of the specific activity of the three C_{29} wax components of *Brassica oleracea* (nonacosane, 15-nonacosanone, and 15-nonacosanol) showed that the paraffin had the highest specific activity even with the shortest exposure to acetate-1-^{14}C (5 minutes), the ketone had nearly the same specific activity, but the secondary alcohol had a much lower one (*34*). These results suggest that the free secondary alcohol is not an intermediate in the paraffin synthesis. Attempts to find conversion of radioactive ketones into paraffins in the leaf failed (*38*).

Elongation-Decarboxylation Pathway

According to this hypothesis (scheme 2), the product of *de novo* fatty acid synthesis, namely C_{16} acid, becomes the substrate for an elongation-decarboxylation enzyme complex. This complex successively adds C_2 units until the chain length reaches the vicinity of C_{30}; then the fatty acid is decarboxylated, and the resulting paraffin is released. The chain length C_{30} is presumably preferred for decarboxylation in *Brassica oleracea* and many other plants where C_{29} is the major paraffin. Some decarboxylation must occur before C_{30} and after C_{30}, then giving rise to other paraffins. In other plants, where the major paraffin is C_{31} or C_{33}, a similar complex with a slightly different specificity may operate. At successive stages of elongation, minor amounts of fatty acids may be released from the enzyme complex and give rise to very long-chain fatty acids (C_{20} to C_{28}) which are immediately esterified into phospholipids and glycerides (*41*), a process making them unavailable to the elongation enzyme complex. All the experimental evidence thus far obtained supports this hypothesis.

Thus, incorporation of specifically labeled acetate into C_{29} compounds of *Brassica oleracea*, is in agreement with elongation pathway. As predicted by the hypothesis and indicated by the experimental evidence, the C_{16} palmitic acid is incorporated as a unit into the paraffin (earlier discussion).

Fatty acids of chain length from C_{10} to C_{18} served as precursors for wax in *Brassica oleracea*; as the chain length of the precursor increased from C_{10} to C_{14}, there was a relatively small increase in incorporation into the C_{29} compounds of the wax, but further increase in chain length of the precursor (up to C_{18}) caused a rather rapid increase in incorporation (*38*). Short-chain fatty acids (C_{10} to C_{14}) are elongated into C_{16} in leaf tissue (*38, 42*); this C_{16} acid apparently becomes the substrate for the elongation-decarboxylation system. The longer fatty acids (C_{16} and C_{18}) presumably can directly become the substrate for the enzyme system that synthesizes paraffins and therefore much more readily undergo conversion into paraffins.

Stearic acid (C_{18}) was incorporated at least twice as fast as C_{16} acid into the paraffins; about 40 percent of the administered stearic acid was converted into surface lipids, the rest being ester-

ified into phospholipids and glycerides (*41*). If the elongation pathway is the correct one, we may predict that the entire C_{18} chain would be incorporated into the paraffin, and this prediction has been shown to be true in the case of *Brassica oleracea* (*41*).

The effect of light, chlorophenyldimethylurea, and trichloroacetate on incorporation of labeled precursors into fatty acids and paraffins further illustrates the difference between *de novo* fatty acid synthesis and paraffin synthesis as depicted in scheme 2. Fatty acid synthesis is tightly coupled to photosynthetic reactions such as photophosphorylation and photosynthetic production of reduced nicotinamide adenine dinucleotide phosphate (NADPH) (*43*). Consequently absence of light greatly reduced acetate incorporation into fatty acids. The elongation process responsible for paraffin synthesis is not tightly coupled to photosynthetic reactions; therefore, in relatively short experimental periods (few hours) no inhibition of acetate incorporation into paraffins occurred in the dark (*38*).

Chlorophenyldimethylurea inhibits photosynthetic production of adenosine triphosphate and NADPH, and consequently it inhibits acetate incorporation into fatty acids (*43*). As the elongation process is not tightly coupled to photosynthetic reactions, acetate incorporation into paraffins should be less sensitive to chlorophenyldimethylurea than acetate incorporation into fatty acids is. Furthermore, according to the elongation-decarboxylation pathway (scheme 2), incorporation of exogenous C_{16} and C_{18} acids into paraffins should be insensitive to chlorophenyldimethylurea. Experimental results with *Brassica oleracea* leaves have shown that this is true (*38*).

Trichloroacetate at low concentrations (10^{-5} to $10^{-4}M$) strongly inhibits paraffin synthesis, but even at 100 times these concentrations it does not affect *de novo* synthesis of fatty acid in leaves (*34, 38, 41*). Therefore this inhibitor did not affect incorporation of labeled fatty acids shorter than C_{16} into C_{16} acid, but incorporations of acids shorter than C_{16} into the paraffins were inhibited equally. Incorporation of C_{18} acid into the paraffin was less sensitive to trichloroacetate, apparently because the C_{18} acid enters the reaction sequence at a point which is beyond part of the trichloroacetate-sensitive steps involved in the paraffin synthesis (*41*).

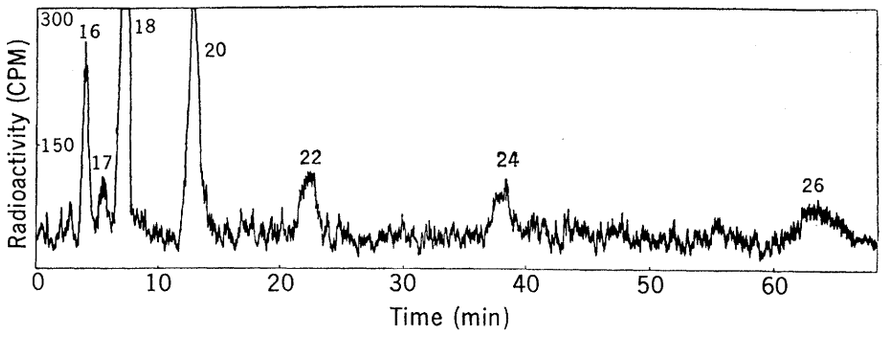


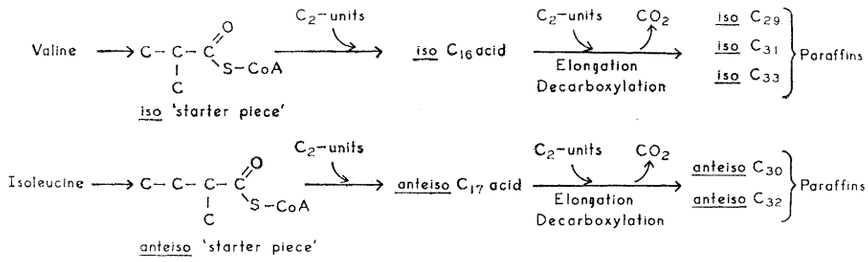
Fig. 2. Gas-liquid chromatography of labeled fatty acids (as methyl esters) isolated from *Brassica oleracea* leaves which had metabolized uniformly labeled ^{14}C-stearic acid. The radioactivity pattern shown here was obtained by continuously monitoring the effluent from the gas chromatograph with a Barber-Colman radioactivity monitor. The fatty acids are identified by the carbon number shown on each peak (*38*).

Further support for the elongation-decarboxylation pathway comes from the demonstration that the exogenous fatty acids, such as C_{16} and C_{18} are elongated into very long fatty acids C_{20} to C_{28} in *Brassica oleracea* (Fig. 2) (*38*). The following evidence suggests that these very long-chain fatty acids are biosynthetically related to the paraffins and that the synthesis of the very long-chain fatty acids is quite distinct from that of the common fatty acids (C_{16} to C_{18}). (i) Fatty acids such as C_{16} and C_{18} are much more efficiently converted into the very long-chain fatty acids, just as they are more readily converted into paraffins (*38*). (ii) Light and chlorophenyldimethylurea have relatively little effect on either synthesis of paraffins or of very long fatty acids (*41*), whereas the synthesis of the more common fatty acids (C_{16} to C_{18}) of the leaf is stimulated by light and inhibited by chlorophenyldimethylurea (*38*). (iii) Trichloroacetate, a potent inhibitor of paraffin synthesis, also inhibits the synthesis of very long-chain fatty acids (*41*). (iv) Anaerobic conditions inhibited the incorporation of stearic acid uniformly labeled with ^{14}C into paraffins (Fig. 3) and very long-chain fatty acids, and light partially reversed the inhibition in both cases (*39*). (v) Tobacco leaves which incorporated ^{14}C from branched-chain amino acids into branched-chain paraffins contained the appropriately branched-chain fatty acids up to at least C_{26} (*39*). (vi) Epidermal layer of cells removed from the leaves of *Senecio odoris* incorporated acetate into both paraffins and very long-chain fatty acids, whereas inside tissue incorporated acetate primarily into C_{16} acid (*39*).

A poor correlation between the abundance of fatty acids in the wax and the hydrocarbons which would be produced from the acids by decarboxylation has been taken as evidence against the decarboxylation hypothesis (*1*). However, there is no reason to expect a correlation to exist between the quantities of a precursor and its product in a biochemical system, and the abundance of components in the surface wax merely represents an accumulation in metabolically inert pools (*34, 38*).

The elongation-decarboxylation pathway probably holds good not only for the saturated normal paraffins which seem to predominate in most waxes, but also for the less common unsaturated and branched paraffins. In the American cockroach, for example, linoleic acid probably undergoes elongation and decarboxylation to give the 6,9-heptacosadiene, which is the major hydrocarbon in this organism (*5*).

The branched chains for the synthesis of branched-chain hydrocarbons, such as those in tobacco leaf and wool, originate primarily from three amino acids—valine, leucine, and isoleucine. Isoleucine would give rise to a C_5 segment of the *anteiso* type, and therefore addition of C_2 units to this piece would give homologous fatty acids with an odd number of carbon atoms. On elongation and decarboxylation these acids would produce *anteiso* paraffins with an even number of carbon atoms. Thus *anteiso* fatty acids with an odd number of carbon atoms and *anteiso* paraffins with an even number of carbon atoms would be expected to predominate. Valine would yield a C_4 piece of the *iso* type which would give homologous fatty acids with an even number of carbon atoms and *iso* paraffins with an odd number of carbon atoms. In fact, such a distribution of fatty acids and paraffins has been demon-



Scheme 3: Proposed pathway for the biosynthesis of branched-chain hydrocarbons from branched-chain amino acids.

strated both in tobacco and in wool (*3, 6, 44*).

The aforesaid hypothesis (scheme 3) concerning the biosynthesis of branched-chain paraffins has been supported by experiments with labeled amino acids. As predicted, labeled valine and isobutyrate when fed to excised tobacco leaves produced branched-chain C_{29}, C_{31}, and C_{33} paraffins; similarly isoleucine was incorporated into branched-chain C_{30} and C_{32} paraffins (*39*) (Fig. 4). When the fatty acids from these leaves were analyzed by gas-liquid chromatography, labeled and branched-chain fatty acids up to at least C_{26} were found. For example, labeled isobutyrate gave rise to labeled branched-chain fatty acids C_{16} to C_{26}, all tentatively identified as belonging to the *iso* series. Similarly labeled isoleucine produced labeled C_{17} to C_{25} (and possibly longer) *anteiso* fatty acids (*39*). Recently, very small amounts (0.02 to 0.09 percent) of ^{14}C from labeled valine, threonine, isoleucine, and leucine were shown to be incorporated into tobacco paraffins, largely in agreement with the predictions discussed above (*45*). However, in these experiments labeled substrates were administered through the stems of tall tobacco plants (0.9 m) for a month, and consequently extensive fragmentation of the carbon chain and subsequent nonspecific incorporation took place. The experimental results shown in Fig. 4, by contrast, were obtained from 16- to 24-hour experiments with isolated tobacco leaves (*34*). This technique resulted in higher (2 to 3 percent of administered) and more specific incorporation into the paraffins, with presumably little fragmentation as indicated by the highly specific labeling of the paraffins and the branched-chain, very long fatty acids.

Condensation of Nonidentical Fatty Acids

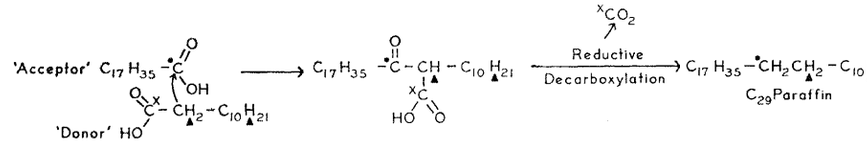
Since there is no direct proof that the elongation process involves C_2 units, the possible involvement of longer units cannot be ruled out completely. Thus the incorporation of C_{18} acid into the paraffin could also be represented as shown in scheme 4. Similarly, C_{16} acid could condense with C_{14} acid. Most of the ^{14}C in the ketone isolated from broccoli leaves that had metabolized palmitate-2-^{14}C was found to be in the carbonyl carbon of nonacosan-15-one, but some labeled $C_{15}H_{31}CO$ group could be detected (*39*). The presence of this $C_{15}H_{31}CO$ group in the ketone can be explained on the basis of the reaction sequence in scheme 4.

A similar reaction sequence has been suggested for the biosynthesis of paraffins in tobacco (*45*). Such a hypothesis appears to be based primarily on the observation that ^{14}C from carboxyl-labeled C_2 to C_8 fatty acids was incorporated not only into straight-chain paraffins, but also into branched-chain paraffins. From the elongation-decarboxylation pathway, one predicts incorporation of straight-chain acids only into straight-chain paraffins. However, if the label from the short-chain acids up to C_8, was incorporated into the paraffins via C_2 units in tobacco, then the results would be in accordance with the elongation-decarboxylation pathway.

In *Brassica oleracea*, relatively short-chain fatty acids (C_5 to C_{10}) could apparently provide C_2 units even when the experimental period was 4 to 24 hours (*34, 38*). If the results of incorporation of C_2 to C_8 acids into tobacco paraffins (*45*) were obtained under experimental conditions (such as 1-month period of incorporation) similar to those described for amino acid incorporation, extensive fragmentation of the administered C_2 to C_8 acids would have undoubtedly taken place.

If in *Brassica oleracea* the condensation of C_{12} or C_{14} acid with C_{18} or C_{16} acid results in the C_{29} paraffin (scheme 4) the carboxyl that is lost in the hypothetical reductive decarboxylation of the condensation product must be specifically that of the C_{12} acid, because intact C_{18} and C_{16} acid molecules have been shown to be incorporated into C_{29} paraffin (*41*). Also, the carboxyl carbon of linoleic acid (C_{18}) in the American cockroach does not appear to be lost during its incorporation into the heptacosadiene (*46*). Thus, the two condensing fatty acids must be dissimilar, and the condensation must be such that the carboxyl that is lost is specifically from one acid—from the "donor"—and not from the other—the "acceptor." The presence of branching on only one end of the paraffins from tobacco indicates that either the acceptor fatty acids or the donor may be branched; the other acid must be of *normal* series only. Unsaturation on only one end of the hydrocarbons from American cockroach indicates that the donor acid must be saturated and the acceptor unsaturated. Such specificities could be imagined, for example, if the condensing fatty acids are bound to proteins of the "acyl carrier" type; that is, one protein relatively nonspecific and capable of carrying branched-chain and unsaturated fatty acids (acceptor acid), and another capable of carrying only saturated straight-chain fatty acid (donor).

The incorporation of the *anteiso* C_5 segment from isoleucine exclusively into paraffins with an even number of carbon atoms (Fig. 4) suggests that the donor fatty acid must have an even number of carbon atoms. It is difficult to imagine how such a restriction could be imposed on the basis of the structural difference between an odd- and



Scheme 4: Condensation of nonidentical fatty acids. The fate of the carboxyl carbon of C_{18} acid is denoted by solid circles, and the fate of the carboxyl carbon of C_{12} acid and the tritium atoms attached to the rest of the C_{12} molecule are shown by the crosses and solid triangles, respectively.

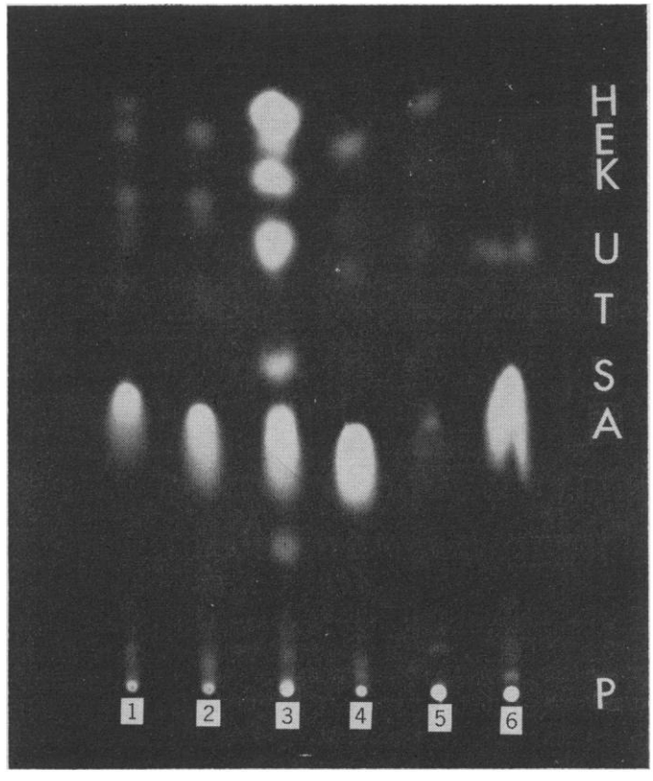
even-numbered chain, especially in view of the presumed availability of odd-numbered fatty acids which, according to the condensation pathway, would have to be involved in the synthesis of paraffins with an even number of carbon atoms (found in significant proportion in tobacco). Under conditions (such as presence of chlorophenyldimethylurea) which severely limit synthesis of the usual fatty acids (C_{10} to C_{18}), incorporation of exogenous labeled C_{16} and C_{18} acids into the paraffin was not inhibited (*38*). This observation can hardly be explained on the basis of condensations that involve C_{14} or C_{12} acids, unless the C_{14} and C_{12} are assumed to be synthesized in a manner quite unlike that of the common fatty acids, at least as far as their site of synthesis is concerned.

If C_{12} acid labeled with ^{14}C in the carboxyl carbon and ^{3}H on the methylene carbons condenses with a C_{18} acid (scheme 4), the resulting paraffin should contain no ^{14}C. Some doubly labeled C_{12} acid may reach the paraffin via C_{18} acid. Even then the ratio of ^{3}H to ^{14}C in the paraffin should be much higher than that in the substrate (C_{12} acid) if the condensation pathway plays a major role. But this ratio in paraffins isolated from broccoli leaves which had metabolized the doubly labeled C_{12} acid was the same as that in the C_{12} acid itself (*39*). Although other combinations of fatty acids could be suggested to circumvent such experimental evidence against the condensation pathway, the elongation-decarboxylation pathway can best explain all the experimental evidence yet obtained.

Waxy Esters

Waxy esters are almost invariably present in the surface lipid of animals, plants, and insects. Radioactive acetate is incorporated into this class of compounds in human skin (*29*), apple peel (*47*), *Brassica oleracea* leaves (*34*), and honey bees (*30*). Other fatty acids also are incorporated into waxy esters in *Brassica oleracea* (*38*). The esterification process has not been studied until very recently. In analogy to esterification of cholesterol in animals, several possible mechanisms for the biosynthesis of waxy esters can be suggested (scheme 5).

In rat-liver mitochondria and microsomes (*48*) and in adrenal microsomes (*49*) acyl coenzyme A participates in the esterification of cholesterol. A similar mechanism seems to be involved



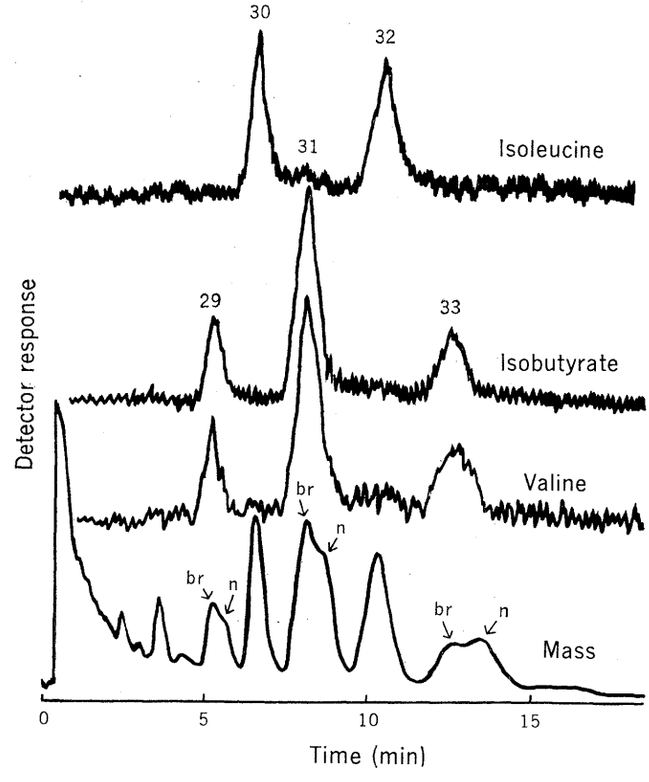


Fig. 3 (left). Autoradiogram of thin-layer chromatogram of lipids isolated from chopped leaves of *Brassica oleracea* which had metabolized uniformly labeled ^{14}C-stearic acid for 1 hour in the dark. 1,2-Lipids were removed from the incubation medium; 3,4-lipids were removed from the leaf slices by a 20-second immersion in a mixture of chloroform and methanol (2 : 1); 5,6-lipids remained in the leaf discs after they were rinsed for 20 seconds in a mixture of chloroform and methanol (2 : 1). The numbers 1, 3, and 5 represent incubation under aerobic conditions; numbers 2, 4, and 6 represent anaerobic conditions. *H*, hydrocarbons; *E*, esters; *K*, ketones; *U*, unknown; *T*, triglycerides; *S*, secondary alcohols; *A*, free fatty acids; *P*, polar lipids, mostly phospholipids. Esterification of the administered ^{14}C-stearic acid was hardly affected by anaerobic conditions, but the synthesis of the nonester part of the surface lipids was inhibited. ^{14}C-Stearic acid (uniformly labeled) which would have been incorporated into the C_{29} compounds of the wax under aerobic conditions remained as free fatty acids within the tissue under anaerobic conditions. Fig. 4 (right). Gas-liquid chromatogram of labeled hydrocarbons isolated from surface lipids of excised tobacco leaves which had metabolized the labeled substrates shown on each tracing for 16 to 24 hours. The mass flame-ionization detector response shown in the bottom tracing was similar in all cases. Coiled copper column, 1.2 m (6 mm outside diameter), packed with 5 percent silicone gum rubber (SE 30) on 90 to 100 mesh Anakrom-SD was used for gas-liquid chromatography. The temperature of the column was 280°C, and the carrier gas, argon, was passed through at 75 ml/min. The isolation and chromatographic techniques have been described (*34, 38*). The radioactivity in the column effluent was continuously monitored with a Barber-Colman radioactivity monitor. *Br*, branched; *n*, normal.

in the formation of certain volatile esters in yeast (*50*). A second mechanism would be analogous to esterification of plasma cholesterol, a mechanism which does not seem to involve coenzyme A derivatives; instead, an acyl group is transferred from lipoproteins to the cholesterol (*51*) (reaction II in scheme 5). A third mechanism would be the direct esterification of a free fatty acid with an alcohol, presumably catalyzed by an esterase (reaction III of scheme 5). Although many esterases are known in plants (*52*) and animals (*53*), very little is known about their ability to catalyze the formation of waxy esters. Pancreatic lipase has been shown to be capable of synthesizing oleyl palmitate, and the synthesis and hydrolysis of waxy esters apparently occurs in the intestine of the rats (*54*).

The waxy esters in the surface wax may be synthesized by any of these mechanisms. I have found that labeled stearyl alcohol administered to broccoli leaves is mostly esterified into wax esters (*55*). Aqueous suspension of a powdery residue obtained by homogenizing young broccoli leaves with cold acetone (acetone powder) also converted stearyl alcohol into wax esters, making use of endogenous acyl groups. The major endogenous acyl group that participated in esterification was C_{16}. Labeled free palmitic acid was also incorporated into esters presumably by the reversal of an esterase-type reaction.

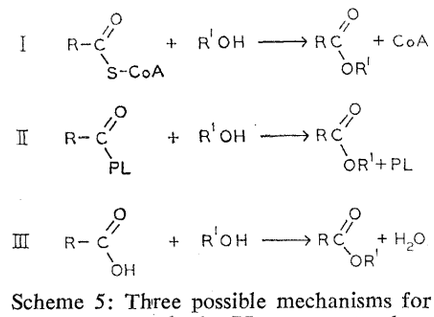
A radioactive palmityl group from phospholipid was incorporated into wax esters much more efficiently than would be expected on the basis of the free palmitic acid produced from it. This result suggests the occurrence of an acyl transfer from phospholipids to the hydroxyl group of the primary alcohol. At low concentrations of the alcohol, most of the ester synthesis catalyzed by the acetone powder prepared from the leaves was apparently mediated by this transesterification mechanism rather than by the direct esterification. Fractionation of the water-soluble constituents of the powder by ammonium sulphate precipitation and Sephadex G-100 gel filtration clearly demonstrated the presence of an acyl coenzyme A fatty alcohol transacylase. Waxy ester synthesis which is dependent on coenzyme A may have a major role in vivo because, from thermodynamic considerations, this reaction favors the synthesis of the ester.

Site of Synthesis

An obvious consequence of organized subcellular structures is the compartmentation of metabolites. Experimental results obtained with honey bees (*30*) and with leaves of *Brassica oleracea* (*34, 38*) suggested that compartmentation is involved in the biosynthesis of wax in these organisms. In the honey bee, acetate gave rise to strongly labeled hydrocarbons and free wax acids but only weakly labeled waxy esters. However, such differences in labeling were not found with labeled glucose or water. Therefore it was proposed that wax acids and hydrocarbons are synthesized in the oenocytes, but the waxy esters and their component acids and alcohols are synthesized in the fat cells. Both the compartments receive glucose, but fat cells are apparently impermeable to acetate. Such an explanation was apparently supported by microautoradiography. Similarly, there are thought to be several pools of lipids in human skin (*2*).

The following evidence suggests that paraffins are synthesized outside the chloroplast. (i) Trichloroacetate, which apparently interferes with coenzyme A synthesis (*34, 56*), does not inhibit the synthesis of common fatty acids (C_{16} and C_{18}), but it severely inhibits paraffin synthesis. Coenzyme A takes part in fatty acid synthesis and presumably in paraffin synthesis. Paraffin synthesis therefore may be outside the chloroplast, which presumably is impermeable to trichloroacetate (C_{16} and C_{18} acids are known to be synthesized within the chloroplasts).

An alternate explanation would be that decrease in pantothenate synthesis, brought about by trichloroacetate, affects the concentration of free coenzyme A, but not that of acyl carrier protein (*57*). Synthesis of common fatty acids in the chloroplast, being dependent more on acyl carrier protein than on free coenzyme A, would thus be relatively unaffected by trichloroacetate. Paraffin synthesis may be more dependent on free coenzyme A. (ii) Unlike fatty acid synthesis, paraffin synthesis is not tightly coupled to photosynthetic reactions as suggested by the effect of light and chlorophenyldimethylurea on paraffin synthesis discussed earlier. (iii) The chloroplast preparations capable of incorporating acetate into fatty acids did not incorporate acetate into the paraffins (*43*). (iv) The very long fatty acids that seem to be derived from the elongation complex which synthesizes paraffins could not be found in chloroplasts (*41*). (v) Hydrocarbons and waxy esters constituted up to 10 percent of the total lipids of whole spinach leaves, but these components were essentially absent in the chloroplast lipids of spinach (*58*).



Scheme 5: Three possible mechanisms for waxy ester synthesis. PL represents phospholipid; R and R^1 may or may not be identical alkyl groups (*55*).

There is substantial evidence that the surface lipids are synthesized in the epidermis of the leaf. It is known that the cuticle contains almost all the paraffins found in the leaf (*34, 59*). When radioactive acetate was administered through the petiole, almost all of the radioactive paraffins (that newly synthesized) could be rinsed out by dipping the leaf into chloroform in 10 to 30 seconds, even when incorporation time was as short as 5 minutes (*34*). Similarly after the chopped broccoli leaves were incubated with a solution of labeled stearic acid, a 10- to 20-second wash in a mixture of chloroform and methanol (2:1) removed almost all of the radioactive wax (Fig. 3). Paraffin therefore finds its way onto the cuticle as soon as it is formed. The epidermal layer of cells can in fact catalyze at least some of the reactions involved in wax synthesis. For example, epidermal layer peeled from *Senecio odoris* leaves readily converted added radioactive stearyl alcohol into waxy ester (*55*). Incorporation of acetate-1-^{14}C into paraffins by discs of *Senecio odoris*, from which the epidermal layer had been removed, was less than 10 percent of that by the whole discs, and the isolated epidermal layers incorporated as much acetate-1-^{14}C into paraffins as the whole discs. Incorporation of exogenous acetate into total lipids on the other hand was stimulated up to 100 percent by the removal of the epidermis.

presumably because of increased uptake (*39*). Large proportions (about 50 percent) of the labeled acetate incorporated into the fatty acids of the epidermis were found in C_{18} to C_{24} fatty acids, whereas most of the ^{14}C incorporated by the inside tissue was found in the C_{16} acid. These results suggest that wax is synthesized mostly, if not entirely, in the epidermal layer. The apparent lack of involvement of chloroplasts in wax synthesis is consistent with this hypothesis because the epidermal layer of cells contains very few chloroplasts.

How the wax components synthesized within the leaf reach the surface is not clear. Electron-microscopic techniques allow the plant surface to be studied in detail but these techniques have not given any unequivocal evidence for the mechanism of wax excretion. Those who apparently have evidence for the presence of pores in the cuticle (*60*) believe that wax is exuded through these pores. Others, who could find at best shallow pits but no pores (*61*), disagree and suggest that at the early stage of growth the wax penetrates the fragile cutin, but as the leaves mature the cutin layer thickens and wax excretion stops. Still another possibility has been suggested by the detection of lamellations in the cuticle which result in a series of anastomosing channels; wax may travel to the surface through these channels (*62*). Autoradiography in conjunction with electron microscopic studies may provide a more definitive answer to this problem.

Concluding Remarks

Biosynthesis of the unusually long carbon chains found in waxes has attracted little attention until recently, although such substances are widespread in nature. On the basis of the available experimental evidence, elongation of a common fatty acid such as palmitic to very long-chain fatty acids of appropriate chain length, followed by decarboxylation appears to be the most likely pathway for paraffin biosynthesis. The confirmation of this hypothesis must await isolation of the enzymes involved.

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