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# Further Evidence for an Elongation-Decarboxylation Mechanism in the Biosynthesis of Paraffins in Leaves

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*Abstract.* In isolated tobacco leaves L-valine-U-$^{14}$C gave rise to labeled even-numbered *iso*branched fatty acids containing 16 to 26 carbon atoms and *iso* $C_{29}$, *iso* $C_{31}$, and *iso* $C_{33}$ paraffins. L-Isoleucine-U-$^{14}$C on the other hand produced labeled odd-numbered *anteiso* $C_{17}$ to $C_{27}$ fatty acids and *anteiso* $C_{30}$ and $C_{32}$ paraffins. Trichloroacetic acid inhibited the incorporation of isobutyrate into $C_{20}$ and higher fatty acids and paraffins without affecting the synthesis of the $C_{16}$ and $C_{18}$ fatty acids. Thus the very long branched fatty acids are biosynthetically related to the paraffins. In *Senecio odoris* leaves acetate-1-$^{14}$C was incorporated into the paraffins (mainly *n*-$C_{31}$) only in the epidermis although acetate was readily incorporated into fatty acids in the mesophyll tissue. Similarly only the epidermal tissue incorporated acetate into fatty acids longer than $C_{18}$ suggesting that the epidermis is the site of synthesis of both paraffins and the very long fatty acids. In broccoli leaves *n*-$C_{12}$ acid labeled with $^{14}$C in the carboxyl carbon and $^{3}$H in the methylene carbons was incorporated into $C_{29}$ paraffin without the loss of $^{14}$C relative to $^{3}$H. Since *n*-$C_{18}$ acid is known to be incorporated into the paraffin without loss of carboxyl carbon these results suggest that the condensation of $C_{12}$ acid with $C_{18}$ acid is not responsible for *n*-$C_{29}$ paraffin synthesis in this tissue. Thus all the experimental evidence thus far obtained strongly suggests that elongation of fatty acids followed by decarboxylation is the most likely pathway for paraffin biosynthesis in leaves.

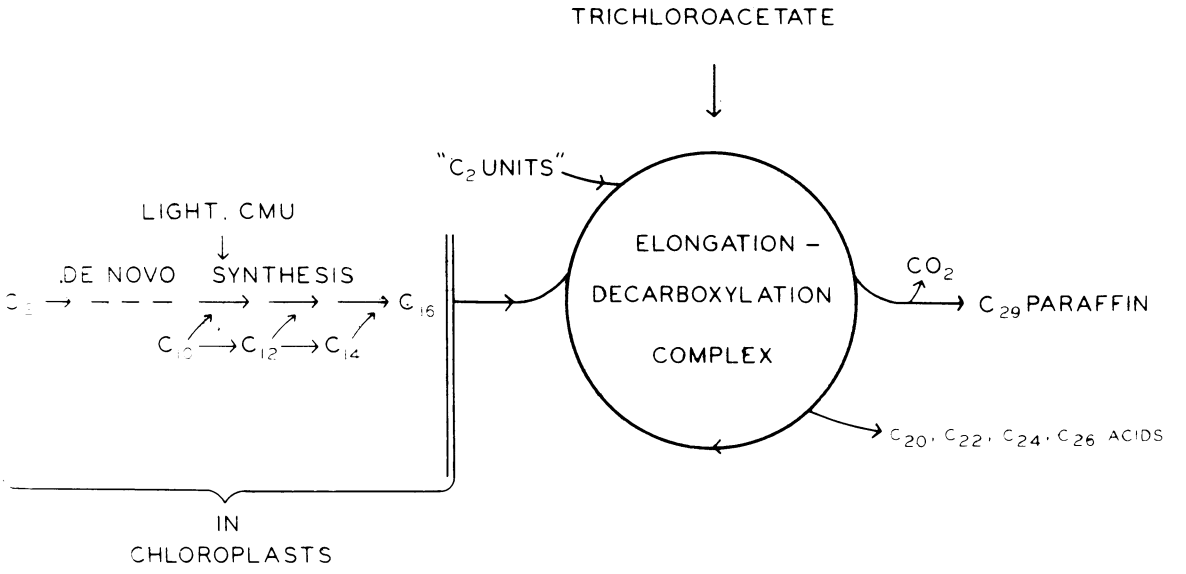
The epidermis of plants is covered with a thin layer of waxy chemicals. The chemistry of this surface wax has been reviewed thoroughly in the past few years (2, 3, 4). The 2 most common components of the surface wax are waxy esters and hydrocarbons. The waxy esters are synthesized in the leaf by the esterification of aliphatic primary alcohols with fatty acyl moieties from fatty acyl coenzyme A, phospholipid, or free fatty acid (12). The straight chain hydrocarbons in plants originate from acetate (9). Longer fatty acids such as $C_{16}$ and $C_{18}$ are incorporated into the *n*-$C_{29}$ hydrocarbon of *Brassica oleracea* more efficiently and without prior degradation of their carbon chains (10). In this tissue $C_{16}$ and $C_{18}$ fatty acids are also incorporated into very long fatty acids ($C_{20}$–$C_{28}$). The effects of light, 3-(4-chlorophenyl)-1,1-dimethyl urea (CMU), and trichloroacetate on the incorporation of labeled precursors into paraffins and very long fatty acids suggested that *n*-paraffins are synthesized by elongation of a common fatty acid ($C_{16}$) followed by decarboxylation as shown in Scheme I (11). Although this hypothesis was derived from work on the biosynthesis of *n*-$C_{29}$ paraffin in *B. oleracea*, it seemed likely that such a hypothesis would be of wider application. In order to test such a possibility *Senecio odoris*, a plant which has primarily *n*-$C_{31}$ paraffin and tobacco which has considerable proportions of 2-methyl (*iso*) and 3-methyl (*anteiso*) paraffins were used.

In the present paper experimental results are presented which suggest that the elongation-decarboxylation pathway holds good not only for *n*-$C_{29}$ hydrocarbon but also for the paraffins of *S. odoris* (mainly *n*-$C_{31}$) and branched paraffins of tobacco leaf. I have also found that the epidermis is the most probable site of synthesis of paraffins.

## Materials and Methods

*Plants.* Tobacco plants (*Nicotiana tabacum*, var Havana seed) *Senecio odoris* plants and broccoli [*Brassica oleracea* var italica (Plenck)] plants were grown in sand on a subirrigated bench in the greenhouse.

*Experiments with Tobacco Leaves.* Since the most rapidly expanding leaves of *Brassica oleracea* synthesized paraffins most rapidly, young tobacco leaves (third or fourth from the apex) were also used for incorporation studies. The excised leaves were kept in the dark with the petioles immersed in water for 1 to 2 hours in order to make them fully turgid. Radioactive substrates were dissolved in a small volume of water (1 ml or less) and placed in a small polyethylene cup fixed in a beaker. The petioles of the fully turgid leaves were transferred to this solution and about 2000 ft-c of light from tungsten lights illuminated the leaf. When the leaves had taken up the radioactive solution, 2 successive 0.5 ml portions of water were added to the cup. When the water was taken up the petioles




SCHEME I. A plausible pathway for paraffin synthesis in *Brassica oleracea*. The reactions which light, CMU and trichloroacetate are most likely to affect are indicated by the arrows.

were immersed in 100 ml of distilled water and the leaves were kept under about 1000 ft-c of light until the end of the experimental period. The leaves (but not the cut surface) were then immersed in chloroform for 30 seconds to isolate the surface lipids.

The paraffin fraction was isolated from the surface lipid by column chromatography on SilicAR cc-4 100 to 200 mesh (Mallinckrodt Chemical Works, St. Louis, Missouri) with hexane as the solvent (9). Further analysis of the paraffin fraction was done by radio gas-liquid chromatography, using 5 % silicone gum rubber (SE 30) on 80 to 100 mesh Anakrom SD (Analabs, Hamden, Connecticut).

The leaf, after being washed with chloroform, was chopped and the total lipids were extracted with a 2:1 mixture of chloroform and methanol. The lipid extract was washed (5) and evaporated to dryness under reduced pressure. The total lipid was then saponified by refluxing with 15 % alcoholic KOH for 2 hours under nitrogen and the fatty acids were isolated from the acidified solution by extraction with chloroform. The free fatty acids were purified by preparative thin layer chromatography on 0.5 mm silica gel G layers with hexane:ethyl ether:formic acid (40:10:1) as the solvent mixture (11). Methyl esters of the fatty acids were then prepared with $BF_3$-methanol reagent. The methyl esters were purified by thin-layer chromatography on 0.5 mm silica gel G layers with benzene as the solvent (10). This purified methyl ester fraction was then analyzed by radio gas-liquid chromatography with 5 % silicone gum rubber (SE 30) on 80 to 100 Anakrome SD.

The effect of trichloroacetate on the synthesis of branched very long fatty acids and paraffins was studied with chopped tobacco leaves. Each Warburg flask (75 ml) contained 2.2 g of chopped tobacco leaves in 8 ml of water containing about 25 $\mu$c sodium isobutyrate-1-$^{14}$C with or without 30 $\mu$moles of trichloroacetate. The flasks were incubated at 30° with shaking under 2000 ft-c of light for 7 hours. The contents of the flasks were then mixed with 250 ml of a 2:1 mixture of chloroform and methanol. After washing the extract (5) part of the lipid solution was subjected to column chromatography on silica gel to isolate the paraffins (9). The remaining lipid solution was saponified, and the fatty acids were isolated and analyzed as before.

*Experiments with* Senecio odoris *Leaves.* The stem of *S. odoris* has an abundance of white crystalline wax on the surface whereas on the leaves very little powdery wax can be seen. The wax from the stem when precipitated by cold acetone gave a broad melting point around 200°, whereas the corresponding wax from the leaf had a melting point of 78 to 80° which is comparable to other surface waxes. The high melting point of the stem wax resulted from the high concentration of a terpenoid material that could be eluted with benzene from an alumina or silica gel column. Column chromatography showed that both the stem and leaf surface waxes contained about 17 % paraffins. The more polar materials have not been fully identified.

Leaves of *S. odoris* plants were excised from the top part of the stem where the white powdery wax had not yet accumulated. Discs (1.5 × 1 cm) were cut with a razor blade and kept in water. Both the upper and lower epidermal layer of cells were carefully peeled off from 12 discs. The epidermis fraction contained some green tissue but the

mesophyll fraction was devoid of epidermis. The epidermis, the mesophyll tissue fraction, and 12 intact discs were placed in 3 Warburg vessels each containing 100 μc of sodium acetate-1-$^{14}$C (10 μmoles) in 3 ml of water. The flasks were incubated under 2000 ft-c of light with shaking (140 oscillations/min) at 30° for 3 hours. Then the total lipids were isolated with a 2:1 mixture of chloroform and methanol. The lipids were washed and the hydrocarbon fraction isolated as described for tobacco leaves. Hydrocarbons were also isolated by thin-layer chromatography on 0.3 mm silica gel G plates with hexane as the solvent (9). Isolation of fatty acids, methyl ester preparation and analysis by radio gas-liquid chromatography were all done as described for tobacco.

*Experiments with Broccoli Leaves.* Chopped young broccoli leaves (second and third from the apices) weighing 2 g were transferred into Warburg flasks (75 ml) containing 6 ml of distilled water in which the substrate and appropriate inhibitors were dissolved (10). The flasks were incubated under light at 30° as described before. Extraction of the total lipids and isolation of the paraffin fraction were also done as described above.

*Substrates.* Dodecanoic acid-1-$^{14}$C (21 mc/mmole), valine-U-$^{14}$C (110.0 mc/mmole), isoleucine-U-$^{14}$C (150 mc/mmole), isobutyrate-1-$^{14}$C (9.6 mc/mmole), and sodium acetate-1-$^{14}$C (44.4 mc/mmole) were purchased from Nuclear-Chicago Corporation, Chicago, Illinois. Tritiated dodecanoic acid was prepared by the Wilzbach method (30 mg lauric acid was exposed to 6 curies of $^{3}H_2$ for 2 wks) at New England Nuclear Corporation, Boston, Massachusetts. The fatty acid was purified by repeated thin layer chromatography after the exchangeable $^{3}$H was removed. A mixture of about 120 μc dodecanoic-1-$^{14}$C acid and 1500 μc dodecanoic acid-$^{3}$H was dissolved in 10 ml water with the aid of Triton X-100 (Rohm-Haas, Philadelphia, Pennsylvania) as described before (10). Tween 20 was avoided because this detergent might release its dodecanoyl residues in the leaf (12). Each reaction vessel contained 500 to 600 mμmoles of dodecanic acid with about 3 mg of Triton X-100.

*Determination of Radioactivity.* When only one isotope was present in the sample, radioactivity measurements were made as described before (10). In double labeling experiments $^{14}$C and $^{3}$H were assayed by the simultaneous equation method.

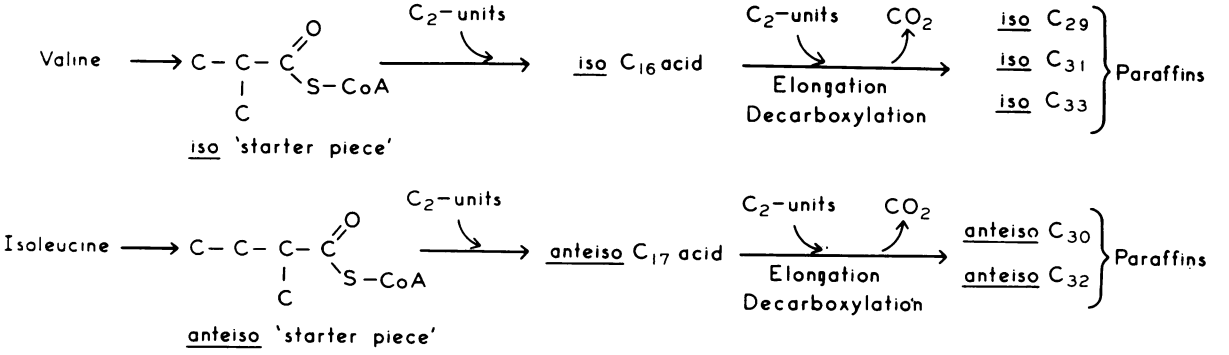
## Results and Discussion

*Incorporation of Branched Precursors into Branched Paraffins and Branched Very Long Fatty Acids in Tobacco Leaves.* Branched carbon chains found in fatty acids were suggested to be derived from branched amino acids such as valine and isoleucine (17). This suggestion has been well substantiated experimentally with labeled amino acids (6, 18). Branched chains of carbon atoms found in the paraffins and long chain fatty acids may also be derived from branched amino acids. Such a

Table I. *Incorporation of Labeled Isobutyrate, Valine, and Isoleucine into Hydrocarbons of Tobacco Leaf*

Isolated leaves received the appropriate amounts of labeled substrates shown through the petioles. After the period of metabolism in the light, surface lipids were extracted into chloroform and the hydrocarbons were isolated from this extract by chromatography.

| Substrate | Amount | Time | Incorporation into paraffins |
|---|---|---|---|
| | | *Hr* | % |
| Sodium isobutyrate-1-$^{14}$C | 5.2 μmoles-50 μc | 24 | 2.15 |
| L-Valine-U-$^{14}$C | 8.6 μmoles-25 μc | 16 | 2.8 |
| L-Isoleucine-U-$^{14}$C | 30 μmoles-50 μc | 24 | 2.5 |




SCHEME II. Proposed pathway for the biosynthesis of branched paraffins in tobacco leaves. The branched very long fatty acids ($C_{20}$-$C_{27}$) are biosynthetically related to the branched paraffins in a manner similar to that proposed for straight chain paraffins (11).

hypothesis is supported by the results summarized in table I which shows that the branched amino acids are readily incorporated into paraffins. In tobacco, *iso* fatty acids with an even number of carbon atoms and *iso* paraffins with an odd number of carbon atoms predominate whereas in the *anteiso* series fatty acids with an odd number of carbon atoms and paraffins with an even number of carbon atoms predominate (15, 16). Such a distribution would be expected if the *iso* $C_4$ fatty acid derived from valine and the *anteiso* $C_5$ fatty acid derived from isoleucine served as starters for the elongation-decarboxylation sequence of reactions that would lead to the formation of the corresponding paraffin (Scheme II).

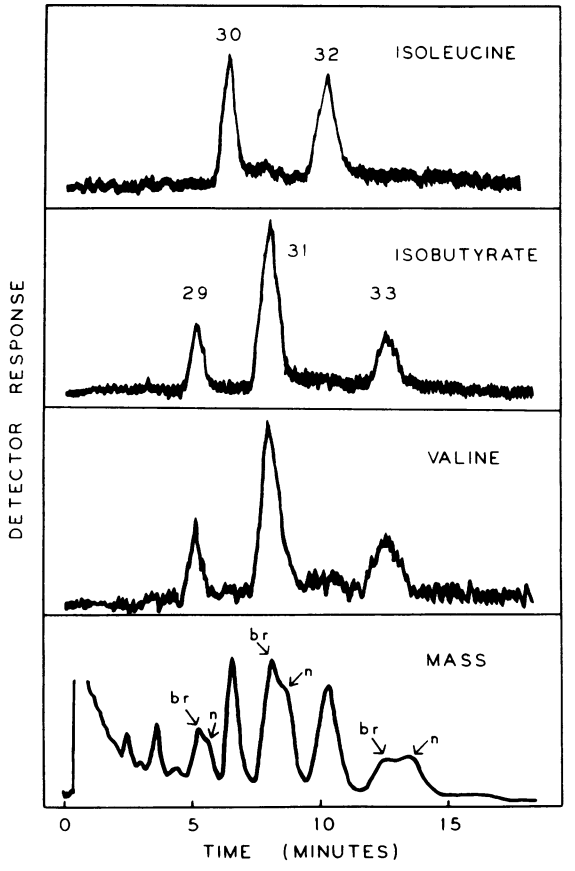



FIG 1. Radio gas-liquid chromatogram of hydrocarbons isolated from surface lipids of excised tobacco leaves which had metabolized the labeled substrates shown on each tracing for 16 to 24 hours. The flame ionization detector response shown in the bottom tracing was similar in all cases. Coiled copper column, (4 ft × 0.25 in OD) packed with 5 % silicone gum rubber (SE 30) on 90 to 100 mesh Anakrom SD was used for gas-liquid chromatography. Temperature of the column was 280° and the carrier gas, argon was at 75 ml/min. The radioactivity in the column effluent was continuously monitored with a Barber-Colman radioactivity monitor. The chain length is shown on each peak; br, branched; n, normal. Although *iso* and *anteiso* isomers are not separated even-numbered and odd-numbered branched hydrocarbons of tobacco are known to be almost exclusively *anteiso* and *iso* respectively (8, 16). Therefore the radioactivity peaks of branched $C_{30}$ and $C_{32}$ are assigned to the *anteiso* series and branched $C_{29}$, $C_{31}$, and $C_{33}$ are assigned to the *iso* series.

The paraffin fractions isolated from lipids obtained from tobacco leaves which metabolized labeled amino acids were analyzed by radio gas-liquid chromatography and the results are shown in figure 1.

It is clear from figure 1 that valine-U-$^{14}$C gave rise primarily to *iso* $C_{29}$, *iso* $C_{31}$, and *iso* $C_{33}$ paraffins. The $C_4$ acid (isobutyric acid) normally expected to be derived from valine was also incorporated specifically into the same paraffins. As shown in figure 1 isoleucine-U-$^{14}$C specifically labeled the *anteiso* $C_{30}$ and *anteiso* $C_{32}$ paraffins.

Very long fatty acids found in broccoli leaves appear to be closely related biosynthetically to straight chain paraffins (11) and if the branched paraffins of the tobacco plant are synthesized by a similar elongation-decarboxylation pathway the correspondingly branched very long fatty acids might be expected to be formed from the branched precursors. Therefore the total fatty acids isolated from tobacco leaves which had metabolized labeled branched amino acids were analyzed by radio gas-liquid chromatography and the results are shown in figure 2. As predicted, valine-U-$^{14}$C and isobutyrate-1-$^{14}$C gave rise to labeled even *iso* fatty acids $C_{16}$ to $C_{26}$. Similarly isoleucine-U-$^{14}$C produced labeled odd *anteiso* $C_{17}$ to $C_{27}$ fatty acids. The highly specific labeling patterns in figures 1 and 2 show that the carbon chains of administered branched precursors did not undergo extensive degradation and the results are in accordance with Scheme II.

Recently Kaneda (8) reported that 0.02 to 0.09 % of labeled valine, threonine, isoleucine, and leucine were incorporated into paraffins in intact tobacco plants. The much higher incorporation obtained in the present study is apparently because of the different experimental technique used. Kaneda administered the substrates to 3 feet tall tobacco plants through the stem by the 'wick method' and the substrates were allowed to be metabolized for a month. Such an experimental procedure also resulted in extensive degradation of the labeled molecules and the consequent non-specific incorporation as compared to the highly specific labeling reported here.

*Effect of Trichloroacetate on Incorporation of Isobutyrate into Branched Paraffins and Fatty Acids.* Trichloroacetate, at $10^{-5}$ to $10^{-4}$ M concentrations, inhibits paraffin synthesis in *B. oleracea* (9). It has been reported that application of trichloroacetate decreases cuticular wax formation considerably only in plants which show the powdery wax on their surface (1). However since experiments with labeled substrates indicated that trichloroacetate inhibited the elongation process re-

sponsible for paraffin synthesis this inhibitor should also inhibit paraffin synthesis in plants which do not have a powdery wax on the surface if the elongation route is of general occurrence. Results in table II show that trichloroacetate inhibited incorporation of isobutyrate-1-$^{14}$C into paraffins in tobacco leaves, a species which does not have a powdery wax. Just as in *B. oleracea* (9, 10, 11), trichloroacetate failed to inhibit incorporation of $^{14}$C from isobutyrate-1-$^{14}$C into total lipids.

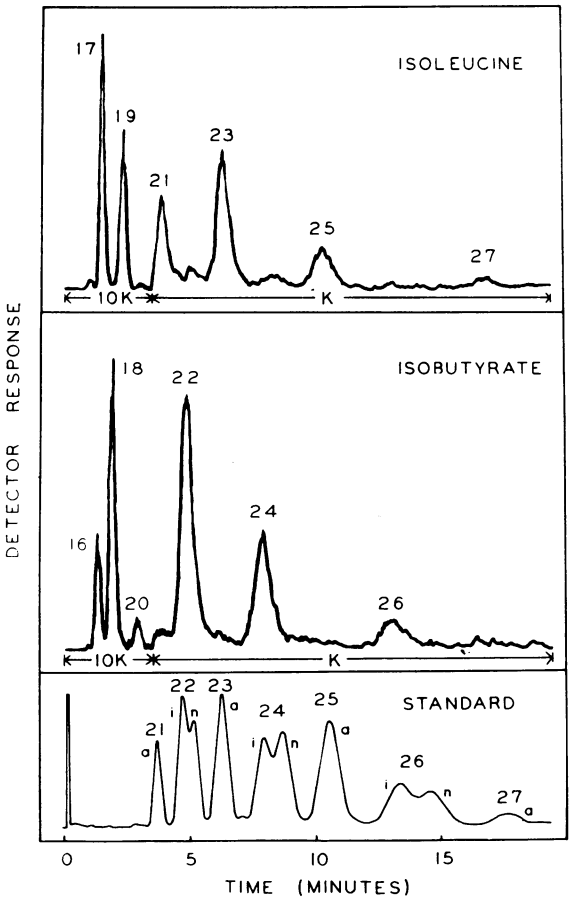



FIG. 2. Radio gas-liquid chromatogram of fatty acids (methyl esters) from the lipids of tobacco leaves which had metabolized the labeled substrates shown on each tracing for 16 to 24 hours. The flame ionization detector response shown in the bottom tracing was obtained from the methyl ester mixture of known composition injected with the tobacco fatty acids. Experimental conditions were the same as in figure 1 except the column temperature was 260°. The chain length is indicated on each peak: i, *iso*branched; a, *anteiso*-branched; n, normal. Although *iso* and *anteiso* isomers are not separated by gas-liquid chromatography branched even-numbered fatty acids and branched odd-numbered fatty acids of tobacco are known to be *iso* and *anteiso* respectively (15). Therefore the radioactivity peaks of the branched even-numbered acids are assigned to the *iso* series and branched odd-numbered acids are assigned to the *anteiso* series. When the unsaturated methyl esters were separated from the saturated by silver nitrate-silica gel G thin-layer chromatography, essentially all the $^{14}$C was found in the saturated methyl ester fraction. Radio gas-liquid chromatography of the saturated esters at 198° showed that small amounts of $^{14}$C was present in the normal $C_{16}$ acid in every case.

Table II. *Effect of Trichloroacetate on the Incorporation of Isobutyrate-1-$^{14}$C into the Paraffins and Fatty Acids of Tobacco Leaf*

Each reaction mixture contained 2.2 g chopped tobacco leaves, 8 ml water, 25 μc sodium isobutyrate-1-$^{14}$C, and 30 μmoles trichloroacetate where indicated. After 7 hours incubation at 30° under 2000 ft-c light the lipids were extracted and the paraffins and fatty acids were analyzed as described under Materials and Methods. In the presence of trichloroacetate measurable $^{14}$C could not be detected in $C_{24}$ and $C_{26}$.

| | Control | Trichloroacetate |
|---|---|---|
| % Incorporation into total lipids | 10 | 10.5 |
| % Incorporation into paraffins | 1.0 | 0.25 |
| % Distribution of $^{14}$C in branched fatty acids | | |
| $C_{16}$ | 16 | 15 |
| $C_{18}$ | 67 | 81 |
| $C_{20}$ | 6.6 | 2.6 |
| $C_{22}$ | 7.0 | 1.3 |
| $C_{24}$ | 2.2 | ... |
| $C_{26}$ | 1.5 | ... |

It was suggested that the very long fatty acids ($C_{20}$ and higher) found in *B. oleracea* are biosynthetically related to the paraffins (11). Correspondingly the branched very long fatty acids found in tobacco might be related to the branched paraffins. If so trichloroacetate, which inhibits the incorporation of labeled isobutyrate into paraffins should also inhibit the synthesis of branched very long fatty acids from isobutyrate. Typical experimental results given in table II clearly show that incorporation of isobutyrate into $C_{20}$ and higher fatty acids was inhibited by trichloroacetate, whereas synthesis of branched acids of common chain length ($C_{16}$–$C_{18}$) was not affected by the inhibitor. Thus it is very likely that the very long branched fatty acids in tobacco are biosynthetically related to the branched paraffins just as the very long *n*-fatty acids of *B. oleracea* are related to the *n*-paraffins.

Although no direct proof for the elongation-decarboxylation pathway of paraffin synthesis in tobacco leaves exists, the formation of the appropriately branched very long ($C_{20}$–$C_{27}$) fatty acids by the leaf that synthesized the branched paraffins and the suggested relationships between the branched very long acids and the branched paraffins are consistent with the elongation decarboxylation pathway depicted in Scheme II.

*Site of Synthesis of Paraffins and Very Long Fatty Acids.* The relation of very long fatty acids to paraffins is also suggested by their site of syn-

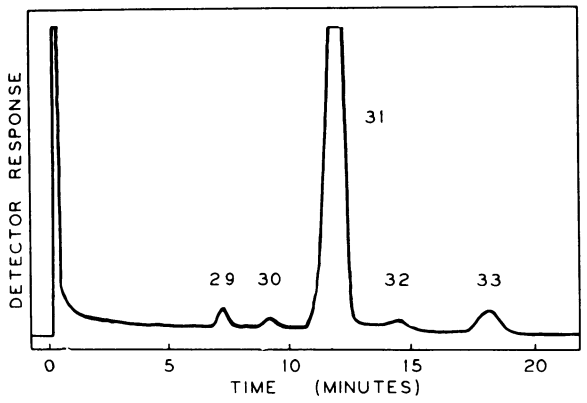



FIG. 3. Gas-liquid chromatogram of the paraffin fraction isolated from the surface lipids of *S. odoris*. The stem and leaf gave identical patterns. Experimental conditions: 6 foot coiled copper column (0.25 in OD), 3 % SE-30 on 80 to 100 mesh siliconized chromasorb W; temperatures of column, injector and detector 280, 360, and 310° respectively. Carrier gas He at 45 ml/min. Identification by comparison with a sample of cabbage paraffin and $n$-$C_{32}$ paraffin. The number on each peak represents the chain lengths.

thesis. The ease with which newly synthesized paraffins are excreted on to the surface of the leaf indicates that the site of synthesis is near the cuticle. This observation together with the lack of involvement of chloroplasts in paraffin synthesis (13) suggested that the epidermis of the leaf might be the site of paraffin synthesis. If the very long fatty acids are related to the paraffins as pictured in Scheme I the very long fatty acids must also be synthesized in the epidermis. Since the epidermal layer of the leaves of *S. odoris* can be easily removed (14) this plant was used to test this hypothesis.

About 17 % of the surface wax of the *S. odoris* leaves could be eluted from alumina and silica gel columns by hexane, and thin layer chromatography and infrared spectroscopy showed that this fraction contained only long chain paraffins. Gas-liquid chromatography (fig 3) of the hydrocarbon fraction shows that the major paraffin is $n$-$C_{31}$ with smaller amounts of $C_{29}$, $C_{30}$, $C_{32}$, and $C_{33}$. Mass spectral analysis confirmed this and showed no evidence for branching; neither did the NMR spectrum give any evidence for branching or unsaturation. Results summarized in table III show that the epidermis of the *S. odoris* leaves incorporated as much acetate-1-$^{14}$C into hydrocarbon as the entire discs. Furthermore the mesophyll tissue converted hardly any acetate into paraffins although this tissue readily incorporated acetate into fatty acids. These results strongly suggest that the epidermis is the site of paraffin synthesis.

Table III. *Incorporation of Acetate-1-$^{14}$C into Lipids of* S. odoris *Leaves*

Each large (75 ml) Warburg flask contained the appropriate tissue from 12 discs (1.5 × 1 cm) in 3 ml water containing 100 $\mu$c sodium acetate-1-$^{14}$C (10 $\mu$moles). After incubating at 30° for 3 hours under 2000 ft-c of light, total lipids were extracted and paraffins isolated as described in Materials and Methods.

| Tissue | Incorporation of $^{14}$C into Total lipids | Paraffins |
|---|---|---|
| | *cpm* × $10^{-6}$ | *cpm* × $10^{-4}$ |
| Intact discs | 22.5 | 2.85 |
| Mesophyll | 27.0 | 0.10 |
| Epidermis | 11.5 | 2.63 |

If an elongation-decarboxylation pathway synthesizes the paraffins in this tissue, the epidermis is likely to contain very long fatty acids ($C_{20}$ and higher) similar to those found in broccoli (10). Therefore the fatty acids synthesized by the epidermis, the mesophyll, and intact discs were analyzed by radio gas-liquid chromatography and the results are compared in figure 4. The distribution of radioactivity in the fatty acids synthesized by the mesophyll tissue was similar to that found in most leaf tissues (7, 10); namely $^{14}$C was mostly in $C_{16}$ acid and lesser amounts were present in $C_{18}$ acids. The $^{14}$C distribution in fatty acids synthesized by the epidermis however showed large proportions of $^{14}$C in fatty acids longer than $C_{16}$ and in particular $C_{20}$, $C_{22}$, and $C_{24}$. Radioactivity could be detected in $C_{26}$ and $C_{28}$ fatty acids also (not shown in the figure). Thus only the epidermal layer of cells, which is the site of the synthesis of paraffins, incorporated acetate-1-$^{14}$C into very long fatty acids. The mesophyll tissue, which was incapable of incorporating acetate-1-$^{14}$C into paraffins, was also unable to convert acetate-1-$^{14}$C into very long fatty acids. These observations support the contention that paraffins are synthesized by an elongation-decarboxylation mechanism and the very long fatty acids are biosynthetically related to paraffins (11, 13).

In the mesophyll tissue the amount of $^{14}$C in $C_{18}$ acid was very small when compared to that in $C_{16}$ acids. On the other hand, in the epidermis relatively large proportion of the $^{14}$C was in $C_{18}$ (fig 4); in fact $C_{18}$ acid contained almost as much $^{14}$C as in $C_{16}$ acid in this tissue. Thus the $C_{18}$ acid is synthesized in 2 compartments: one with the usual fatty acids that are made mostly in the chloroplasts, and the other in the epidermis where it presumably is the first product of elongation during the synthesis of paraffins and other very long chain waxy compounds. A study of the effect of trichloroacetate on fatty acid synthesis in broccoli leaves had previously lead to a similar conclusion with respect to the synthesis $C_{18}$ acid (11).

*Incorporation of Doubly Labeled Dodecanoic Acid into the Paraffins of Broccoli Leaves.* Since there is no direct proof that the elongation process involves $C_2$ units the possibility of a condensation of $C_{16}$ or $C_{18}$ acids with a preformed fatty acid remained open. For example, in *B. oleracea* $C_{18}$

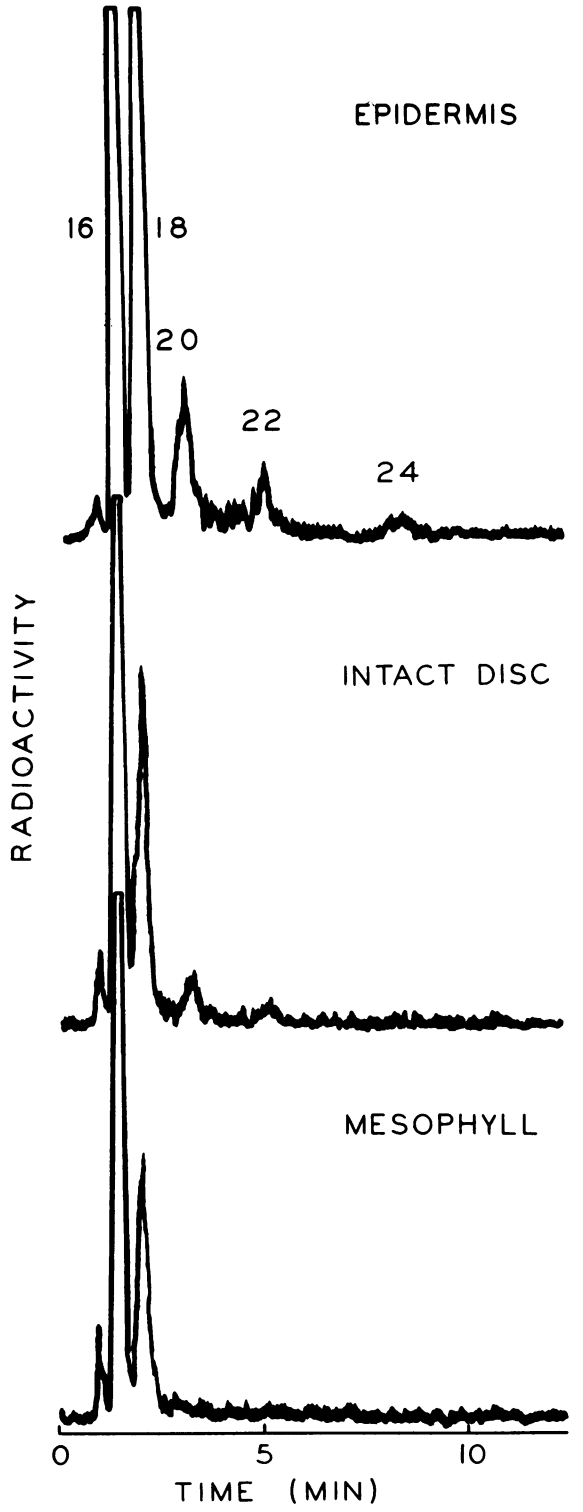



FIG. 4. Radio gas-liquid chromatogram of the fatty acids (methyl esters) isolated from the tissues shown on each tracing. The lipid samples were obtained from the experiment shown in table III. The same conditions of gas-liquid chromatography as in figure 2. With the methyl esters from epidermal tissue radioactive $C_{26}$ and $C_{28}$ could be detected but are not shown in this figure.

acid might be incorporated into the $C_{29}$ paraffin by condensation with a $C_{12}$ acid followed by decarboxylation as shown in Scheme III, instead of a stepwise addition of 6 $C_2$ units followed by decarboxylation. If such is the case, the carbon dioxide that is lost in the process must be derived specifically from the $C_{12}$, the 'donor' (13), and not from the $C_{18}$ acid, the 'acceptor', because the $C_{18}$ acid is known to be incorporated without loss of its carboxyl carbon atom (11). Therefore if $C_{12}$ acid labeled with $^{14}C$ in the carboxyl carbon and $^{3}H$ on the methylene carbon atoms participates in hydrocarbon synthesis by the condensation pathway, the $^{14}C$ would be lost but not the $^{3}H$. However some of the doubly labeled $C_{12}$ acid may reach the paraffin via $C_{18}$ acid into which $C_{12}$ acid is known to be incorporated in *B. oleracea* leaves (10), and any of the $C_{12}$ acid molecules thus incorporated into the paraffin would not lose its $^{14}C$. Even then if the condensation pathway plays a major role the $^{3}H$:$^{14}C$ ratio in the paraffin would be expected to be much higher than that in the $C_{12}$ acid supplied. On the other hand, if the $C_{12}$ acid is incorporated into the paraffin by the elongation-decarboxylation pathway the paraffin should have the same $^{3}H$:$^{14}C$ ratio as in the $C_{12}$ acid. The results summarized in figure 5 show that the $^{3}H$:$^{14}C$ ratio in the paraffin was not higher than that in the $C_{12}$ acid administered to the leaf at any of the experimental periods studied. Since the times used included 30 minutes, by which time the rate of incorporation had not yet reached a plateau, it is unlikely that the $^{3}H$:$^{14}C$ ratio in the paraffin was higher than that in the $C_{12}$ acid substrate at any time. Thus $C_{12}$

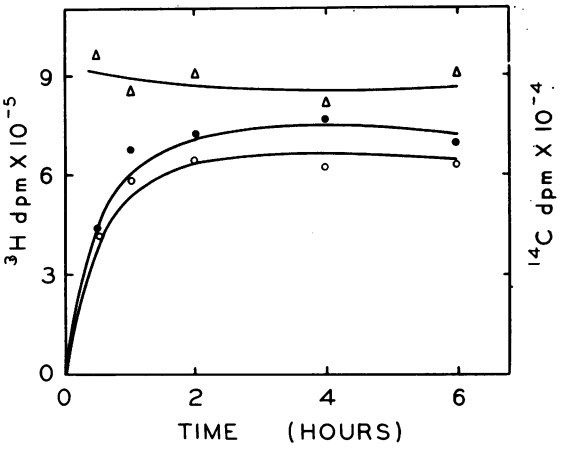
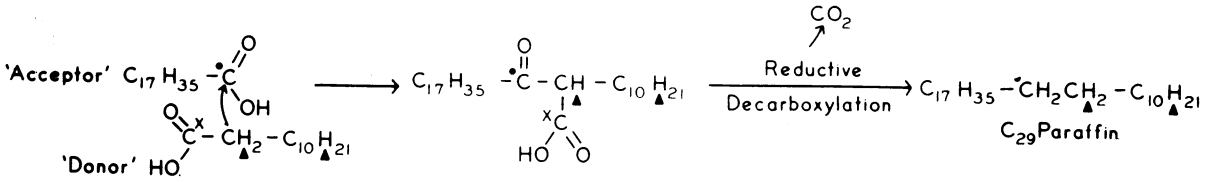



FIG. 5. Incorporation of $^{3}H$ and $^{14}C$ from doubly labeled dodecanoic acid into paraffins of broccoli leaves. Each Warburg flask contained 2 g chopped young broccoli leaves in 6 ml water containing a mixture of dodecanoic acid-U-$^{3}H$ and dodecanoic acid-1-$^{14}C$; the ratio $^{3}H$ : $^{14}C$ was 13. At the end of desired period of incubation at 30° and 2000 ft-c of light the total lipids were extracted and paraffins were isolated as described under Materials and Methods: —○—, $^{3}H$; —●—, $^{14}C$; △, $^{3}H$ : $^{14}C$.




SCHEME III. The hypothetical condensation between $C_{18}$ and $C_{12}$ acid to form $C_{29}$ paraffin. The symbols ●, x and ▲ indicate the fate of the carboxyl carbon of the $C_{18}$ acid, the carboxyl carbon of the $C_{12}$ acid, and the hydrogen atoms of the $C_{12}$ acid respectively.

Table IV. *Incorporation of Dodecanoic Acid*[1] *Labeled with* $^{14}C$ *and* $^{3}H$ *into Paraffins and other Lipids of Broccoli Leaf*

2.0 g Chopped broccoli leaves was incubated with 530 mμmoles of dodecanoic acid (about 75 μc $^{3}H$ and 6 μc $^{14}C$) and the appropriate inhibitors dissolved in a total volume of 7 ml water, at 30° under 2000 ft-c light for 1 hour.

| Experimental condition | Incorporation of radioactivity into paraffins[2] | $^{3}H$ : $^{14}C$ Ratio in Paraffins | Fatty acids |
|---|---|---|---|
| | % | | |
| Control | 100 | 10.2 | 13.9 |
| Trichloroacetate (20 μmoles) | 17 | 11.6 | 13.4 |
| Chlorophenyldimethylurea (0.5 μmole) | 76 | 10.4 | 13.8 |

[1] $^{3}H$ : $^{14}C$ Ratio in the substrate was 13.0.

[2] In the control experiment, 0.4 % of the administered radioactivity was incorporated into the paraffins.

acid did not participate in the condensation pathway shown in Scheme III to any appreciable extent, and these results instead are clearly in agreement with the elongation-decarboxylation pathway.

In order to test further whether the observed incorporation of $^{14}C$ and $^{3}H$ into paraffins represents incorporation of intact $C_{12}$ units the effect of CMU and trichloroacetate on incorporation of $^{3}H$ and $^{14}C$ from the doubly labeled $C_{12}$ acid into the paraffin was studied. As shown in table IV, trichloroacetate severely inhibited the incorporation of both $^{14}C$ and $^{3}H$ into the paraffins to the same extent, while CMU gave a smaller inhibition. In both experiments, however, the ratio of $^{3}H$:$^{14}C$ remained the same as in the $C_{12}$ acid supplied. These results are clearly consistent with the contention that the incorporation of $^{3}H$ and $^{14}C$ into the $C_{29}$ paraffin represents incorporation of intact doubly labeled $C_{12}$ acids. Since CMU is known to inhibit the incorporation of $C_{12}$ acid into $C_{18}$ acid (data not shown here), in the presence of this inhibitor there must be less likelihood of the doubly labeled $C_{12}$ acid reaching the paraffin via $C_{18}$ acid. Therefore if a direct condensation takes place the chances of observing the loss of $^{14}C$ from the $C_{12}$ acid and consequently higher $^{3}H$:$^{14}C$ ratio in the paraffin must be better in the presence of CMU. But even then the ratio of $^{3}H$:$^{14}C$ in the paraffin was not higher than that in the substrate $C_{12}$ acid.

The $^{3}H$:$^{14}C$ ratio in the paraffins was always slightly lower than that in the substrate acid. The reason for this small decrease is not clear at present but the most likely explanation appears to be that small amounts of $^{14}C$ from the degradation products of the substrate $C_{12}$ acid are incorporated into the paraffin. The $^{3}H$ has much less chance of being incorporated in this manner because of the great dilution by the tissue water. The degradation products are most likely to be formed outside the chloroplasts and therefore these products do not cause any decrease in $^{3}H$:$^{14}C$ ratio in the usual fatty acids synthesized within the chloroplasts (table IV). Individual fatty acids ($C_{12}$–$C_{18}$) reisolated from the tissue and separated by gas-liquid chromatography also showed the same $^{3}H$:$^{14}C$ ratio as the substrate $C_{12}$ acid.

The above results rule out the condensation pathway depicted in Scheme III, unless the administered $C_{12}$ acid for some reason failed to reach the hypothetical condensation site except via $C_{18}$ acid, or failed to participate as such in the condensation reaction because the pool of the 'active donor' may have a very slow turnover rate. Another way to circumvent this experimental evidence against the condensation pathway would be to suggest a different combination of fatty acids that does not include the pair of $C_{18}$ and $C_{12}$ acids. Although these possibilities cannot be ruled out with certainty, the elongation-decarboxylation pathway can best explain all the experimental results obtained with *n*-$C_{29}$ paraffin of *B. oleracea*, *n*-$C_{31}$ paraffin of *S. odoris*, and the branched paraffins of tobacco leaf.

It is not known whether the very long fatty acids found in tobacco and *S. odoris* leaves are intermediates in paraffin synthesis. From the experimental results it appears likely that these very long fatty acids are derived from the elongation-decarboxylation complex which synthesize paraffins. However, it is possible that the observed close relationship between the synthesis of paraffins and

very long fatty acids is merely because of their common site of synthesis, most probably the epidermis.

The factors that control the composition of the paraffins synthesized by a species are not known. Differences in specificity of the elongation-decarboxylation complex could explain the difference in carbon chain length. What controls the structural characteristics, such as unsaturation and branching, is even more intriguing. The availability of branched "starter" pieces which in turn may depend on the amino acid balance, may control the branchings found in the paraffins. If this be the case, exogenous branched starters may induce a plant such as *B. oleracea,* which does not normally synthesize branched paraffins, to make branched paraffins; however, if the innate specificity of the enzymes involved excludes branched molecules this may not be possible. In the case of unsaturation, it appears that the specificity of the enzymes excludes unsaturated molecules from being incorporated into paraffins of *B. oleracea* since $C_{18}$ acid was well incorporated into broccoli paraffins whereas the acid with the same chain length but containing one double bond failed to be incorporated (10).

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