Species Specificity in the Biosynthesis of Branched Paraffins in Leaves

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Abstract. Isobutyrate-1-14C and L-isoleucine-U-14C fed through the petiole labeled the surface lipids of broccoli leaves, but the incorporation was much less than from straight chain precursors. Not more than one-third of the 14C incorporated into the surface lipids was found in the C29 paraffin and derivatives, whereas more than two-thirds of the 14C from straight chain precursors are usually found in these compounds. The small amount of 14C incorporated into the paraffin fraction was found in the s-C29 paraffin rather than branched paraffins showing that the 14C in the paraffin must have come from degradation products. Radio gas-liquid chromatography of the saturated fatty acids showed that, in addition to the s-C15 acid which was formed from both branched precursors, isoleucine-U-14C gave rise to branched C15, C17, and C19 fatty acids, and isobutyrate-1-14C gave rise to branched C16 and C18 acids. Thus the reason for the failure of broccoli leaf to incorporate branched precursors into branched paraffins is not the unavailability of branched fatty acids, but the absolute specificity of the system that synthesizes paraffins, probably the elongation-decarboxylation enzyme complex. Consistent with this view, no labeled branched fatty acids longer than C16 could be found in the broccoli leaf. The branched fatty acids were also found in the surface lipids indicating that the epidermal layer of cells did have access to branched chains. Thus the paraffin synthesizing enzyme system is specific for straight chains in broccoli, but the fatty acid synthetase is not.

Hydrocarbons are widely distributed in plants, animals, and microorganisms (3, 17). Although certain organisms have very complicated mixtures of hydrocarbons (2, 13, 15) many contain relatively simple mixtures with one or a few hydrocarbons predominating (3, 10, 20). Normal saturated hydrocarbons, branched saturated hydrocarbons and unsaturated normal hydrocarbons have all been found in living organisms. Some organisms, such as the American cockroach, can synthesize all 3 kinds of hydrocarbons (1) whereas others, such as tobacco leaves, synthesize only saturated normal and branched hydrocarbons (5, 15) while still others such as Brassica oleracea leaves synthesize only normal saturated hydrocarbons (6, 20).

Normal paraffins are synthesized from normal saturated fatty acids (7, 8, 10), unsaturated paraffins from unsaturated fatty acids (10), and branched hydrocarbons from branched fatty acids which in turn originate from branched starter pieces derived from branched-chain amino acids (5, 10, 11). What controls the structural characteristic, such as branching and unsaturation, in the paraffins synthesized by a particular organism is not known. Either the availability of the precursors or the innate specificity of the enzyme systems which synthesize the paraffins could determine the nature of the paraffin synthesized.

When Brassica oleracea, a plant that normally does not synthesize unsaturated hydrocarbons, was fed radioactive oleic acid, no formation of unsaturated hydrocarbons could be detected (7). This result suggests that the innate specificity rather than the availability of the precursor determines the nature of the hydrocarbons. In the present paper further evidence for such a hypothesis concerning branched paraffins is presented. Preliminary results of these experiments have been reported (12).

Materials and Methods

Plants. Broccoli [Brassica oleracea, var. italica (Plenck)] and tobacco plants [Nicotiana tabacum, var. Havana seed] were grown in sand on a sub-irrigated bench in the greenhouse (6).

Chemicals. All of the radiochemicals were purchased from Nuclear-Chicago Corporation. Methyl esters of branched chain fatty acids used as standards were obtained from Applied Science Laboratories, State College, Pennsylvania, straight chain fatty acids and materials for gas chromatography from Analabs, Hamden, Connecticut. Straight chain hydrocarbons used as standards were isolated from Brassica oleracea and Senecio odorus as described earlier (6, 11) or were purchased from Humphrey Chemical Company, North Haven, Connecticut. Branched paraffins used as standards were isolated from tobacco surface lipids by column and thin-layer chromatography (11).
Incorporation of Labeled Substrates. Labeled compounds were administered to excised leaves as described before (6). Each leaf (about 5 g fr wt) received 50 μC of L-isoleucine-U-14C (290 μc/mmole) or 50 μC of isobutyrate-1-14C (2.0 μc/mmole). At the end of the experimental period (18 hrs for isoleucine and 24 hrs for isobutyrate, both under about 1000 ft-c of light), the surface lipids were isolated by dipping the leaf (excluding the cut surface) in 400 ml chloroform for 30 seconds. The lipids remaining in the leaf (internal lipids) were extracted after cutting up the tissue, with a mixture (2:1 v/v) of chloroform and methanol (11).

Experiments with chopped leaves were done as described before (7). At the end of the incubation period, the material was transferred to a sintered glass funnel and washed with 100 ml of distilled water. Then 100 ml of a mixture (2:1) of chloroform and methanol was added to the funnel, the suspension was stirred for 30 seconds and the solvent mixture was then quickly withdrawn by suction. This solution contained all of the surface lipids (10) which were recovered by the method of Folch et al. (4). The lipids remaining in the leaf residue (internal lipids) were extracted with a mixture (2:1) of chloroform and methanol as described before (7).

Isolation of the Products. The surface lipids were fractionated by thin-layer chromatography on silica gel G with benzene as the developing solvent (6). Hydrocarbons were isolated by column chromatography on SilicaAR cc-4 100 to 200 mesh (Mallinckrodt Chemical Works, St. Louis, Missouri) with hexane as the eluant (6).

The internal lipids were saponified by refluxing them under nitrogen with 12% KOH in 95% ethanol for 2 hours. After acidification, lipids were extracted with chloroform, and the fatty acids were isolated by thin-layer chromatography on 0.5 mm silica gel G plates with hexane:ethyl ether:formic acid (40:10:1) as the solvent system (8). At this stage some radioactive alcohols and hydroxy fatty acids were detected, but these were not further analyzed. Methyl esters of fatty acids were prepared with boron trifluoride-methanol reagent. The saturated esters were separated from the unsaturated by thin-layer chromatography on silver nitrate-silica gel G with 10% ethyl ether in hexane as the developing solvent (16). The surface lipids were saponified by refluxing them under nitrogen with 12% KOH in 95% ethanol for 8 hours. The methyl esters of saturated fatty acids were then prepared and isolated as described above.

Gas-liquid chromatography was carried out on a Perkin-Elmer gas chromatograph equipped with a flame ionization detector and an effluent splitter. Silicone gum rubber (SE-30) was the liquid phase for the analysis of methyl esters and hydrocarbons. Further details of chromatographic conditions are shown in the legends to the figures.

Determination of Radioactivity. Radioactivity in samples of lipid solutions and thin-layer chromatograms was determined as previously described (6,7). An internal standard of toluene-14C was always used in order to correct for quenching which occurred especially in the colored internal lipid samples. The counting was usually done with 60% efficiency. The standard deviation was less than 3%. Radioactivity in the effluent from the gas chromatograph was continuously monitored with a Barber-Colman radioactivity monitor.

Results and Discussion

Branched amino acids are undoubtedly present in all organisms. However, some organisms such as tobacco leaves, synthesize branched chain surface lipids while others (e.g., broccoli) make only straight chain surface lipids. In order to understand what factors determine whether or not an organism can synthesize branched surface lipids, the incorporation of 14C labeled branched precursors into the surface lipids of broccoli leaves was studied. Radioactivity from isobutyrate-1-14C and L-isoleucine-U-14C was incorporated into both surface lipids and internal lipids of broccoli leaves (table 1). However the incorporation was much less in comparison to that of other substrates such as acetate. Although these results are not strictly comparable with those obtained

| Table 1. Incorporation of Isobutyrate-1-14C and L-Isoleucine-U-14C Into the Lipids of Broccoli Leaves |
|---|---|---|---|
| | Time | Incorporation of administered |
| | | internal lipids | surface lipids |
| Exp | Substrate | hr | % | % |
| 1 | Sodium isobutyrate-1-14C | 6 | 6.5 | 0.8 |
| 2 | Sodium isobutyrate-1-14C | 24 | 6.7 | 0.8 |
| 3 | L-Isoleucine-U-14C | 18 | 5.0 | 1.9 |

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with tobacco leaves (11) the incorporation of branched precursors into surface lipids of broccoli was much smaller than the corresponding incorporation in tobacco.

Surface lipids of *Brassica oleracea* contain many classes of compounds (18, 19, 20). They fall into 2 groups as far as their biosynthesis is concerned—the C29 compounds, which include nonacosane, nonacosan-15-one, and nonacosan-15-ol, and the non-C29 compounds which include free fatty acids, fatty alcohols and waxy esters (6, 7). Therefore it was necessary to analyze the labeled surface lipids so that possible incorporation of branched precursors into the various classes of compounds could be determined. The thin-layer chromatographic analysis of surface lipids isolated from broccoli leaves that metabolized isoleucine-U-14C is shown in figure 1a. The major radioactive components were waxy esters and primary alcohols with much less radioactivity in the hydrocarbons, ketones, and secondary alcohols. Similarly, most of the 14C incorporated into the surface lipids of broccoli from isobutyrate-1-14C was in the non-C29 compounds (fig 1b). Comparable results were obtained when leaf slices were used in short-term (few hours) experiments as well as when whole excised leaves were used in relatively long term (24 hr) experiments (figs 1b and c). When leaf slices were fed radioactive substrates the surface lipids were extracted by washing the slices for 30 seconds with a 2:1 mixture of chloroform and methanol which extracted a small quantity of polar lipids in addition to the relatively non-polar surface lipids (fig 1c). In any case about two-thirds of the 14C in the surface lipids was in non-C29 compounds and this distribution of 14C is clearly in contrast to the distribution usually obtained with straight chain precursors (such as C18 fatty acids) because these straight chain substrates were incorporated mostly (at least two-thirds) into the C29 compounds (6, 7). The present results could be explained if the branched precursors are essentially excluded from participating in paraffin synthesis, but not from fatty acid synthesis. Furthermore the long branched fatty acids synthesized from the administered branched starter pieces can undergo reduction to the corresponding alcohols and subsequently be esterified.

Tobacco leaf incorporated 14C from L-valine-U-14C and isobutyrate-1-14C into branched C29, C31, and C33 paraffins. Similarly L-isoleucine-U-14C
labeled branched C₃₀ and C₂₀ paraffins of tobacco leaves (5, 10, 11). The reason for the absence of branched paraffins in some plants such as broccoli may be that either the amino acid balance is such that the branched amino acids do not provide their catabolic products for lipid synthesis or that the paraffin-synthesizing system specifically excludes branched chains. The considerable incorporation of ¹⁴C from branched precursors into the lipids of broccoli (table I) indicates that the branched chain starters are available for lipid synthesis. Hence the small amount of radioactivity found in the hydrocarbons represents either the ability of the hydrocarbon-synthesizing system to utilize limited amounts of available branched chains, or may merely be the result of degradation of a small proportion of the substrate which could provide ¹⁴C to the paraffins via unbranched catabolic products. If the first possibility is correct then the radioactivity in the hydrocarbons should be found in branched molecules while, according to the second hypothesis, the small amount of ¹⁴C found in the paraffin fraction should be present in the normal C₂₀ paraffin. Figure 2 shows that the radioactivity in the paraffin fraction of surface lipids derived from l-isoleucine-¹⁴C was in n-C₂₀ paraffin. Identical results were obtained with the paraffin fraction derived from isobutyrate-¹⁴C. If both the possibilities discussed above occurred, radioactivity in the paraffin fraction would be expected in both normal and branched paraffins. Although a small proportion of radioactive branched C₂₀ paraffin would have been difficult to detect with the major C₂₀ peak of radioactivity in the case of the isobutyrate experiments, in the case of isoleucine the expected branched paraffins would be C₃₂ or C₃₀ which would be clearly separated from C₂₀ and therefore would have been easily detected if present. Since no branched radioactive paraffin could be found from either precursor, it is clear that the small amount of ¹⁴C incorporated into the paraffins of broccoli from the branched precursors represents only the incorporation of degradation products and not intact branched pieces.

![Radio gas-liquid chromatogram of paraffins isolated from surface lipids from broccoli leaves which metabolized l-isoleucine-¹⁴C (expt 3 of table I).](image)

**Fig. 2.** Radio gas-liquid chromatogram of paraffins isolated from surface lipids from broccoli leaves which metabolized l-isoleucine-¹⁴C (expt 3 of table I). Chain length is indicated by the number on each peak. The top tracing shows the radioactivity monitor response and the bottom tracing the flame ionization detector response. 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 the gas-liquid chromatography. Temperature of the column and injector were 260° and 360° respectively and the carrier gas argon was supplied at 80 ml/min.

![Radio gas-liquid chromatogram of saturated fatty acids from the internal lipids of broccoli leaves which metabolized sodium isobutyrate-¹⁴C (expt 2 of table I).](image)

**Fig. 3.** Radio gas-liquid chromatogram of saturated fatty acids from the internal lipids of broccoli leaves which metabolized sodium isobutyrate-¹⁴C (expt 2 of table I). Since significant amounts of branched fatty acids were not found in this tissue, a standard methyl ester mixture of normal and branched fatty acids was added for identification purposes (top tracing). The column length is indicated by the number on each peak: br-branched, n-normal. The column and conditions used for gas-liquid chromatography were the same as in figure 2 but the temperature of the column was 195°.

The inability of broccoli leaves to synthesize branched paraffins from exogenously supplied branched precursors could be explained because the fatty acid synthesizing system of broccoli is so specific for straight chains that the branched starter pieces, such as isobutyrate, are not incorporated into branched fatty acids. If so, then the incorporation of ¹⁴C into lipids from the branched precursors should merely represent incorporation of the degradation products: therefore the radioactivity of the fatty...
acids must be only in the usual straight chain molecules. Alternatively, the absolute specificity for straight chains may reside in the enzyme system which synthesizes paraffins and not in the fatty acid synthetase. In that case a substantial part of the radioactivity in the lipids isolated from the broccoli leaf that metabolized branched precursors would be in the branched fatty acids. In order to distinguish between the 2 possibilities, the saturated fatty acids were isolated from the internal lipids by silver nitrate-silica gel chromatography and then subjected to gas-liquid chromatography (fig 3 and 4). Clearly isobutyrate-$\text{C}^{14}$ labeled branched $\text{C}_{16}$ and $\text{C}_{18}$ fatty acids (fig 3) and L-isoleucine-$\text{U}^{14}$C labeled branched $\text{C}_{19}$, $\text{C}_{27}$, and $\text{C}_{34}$ fatty acids (fig 4). In fact the incorporation pattern of branched precursors into fatty acids of broccoli was similar to that observed with tobacco (fig 5). These results clearly show that the fatty acid synthesizing system of broccoli is not specific for straight chains, and that it can synthesize branched fatty acids when the branched substrates are available.

Another possibility considered was that the epidermal cells, where paraffin synthesis takes place (11), for some reason do not have access to the branched starter pieces that can be incorporated into the branched fatty acids. However, analysis of the fatty acids from the surface lipids clearly showed (fig 4) the presence of branched fatty acids on the surface. Hence it is likely that the epidermis did have access to branched chains but that it was unable to synthesize branched paraffins. Thus the paraffin synthesizing system of broccoli, unlike that of tobacco, must be specific for straight chains.

In all cases, some radioactivity was found in the n-$\text{C}_{16}$ acid; this $\text{U}^{14}$C must have come from degradation products of the radioactive branched acids fed to the leaves. In tobacco leaves very little radioactivity was incorporated into the unsaturated fatty acids from branched precursors whereas a considerable amount of $\text{U}^{14}$C could be detected in the unsaturated fatty acids of broccoli. Apparently degradation of the branched precursors proceeds to a larger extent in broccoli than in tobacco. However, in spite of the fairly large incorporation of intact branched pieces into fatty acids, no branched paraffins were found in broccoli leaves. So the degradation of the branched acids fed to broccoli leaves undoubtedly is not the reason for the lack of synthesis of branched paraffins.

The incorporation pattern of branched precursors into fatty acids of broccoli differed from that observed with tobacco (fig 5) in the proportion of $\text{U}^{14}$C found in the very long ($\text{C}_{20}$-$\text{C}_{27}$) fatty acids. In tobacco, substantial amounts of the branched precursors were incorporated into the appropriately branched very long ($\text{C}_{20}$-$\text{C}_{27}$) fatty acids that are apparently biosynthetically related to the branched paraffins (10,11). Since the branched precursors were not incorporated into branched paraffins in...
broccoli, no incorporation into very long branched acids should be found if the elongation system responsible for the synthesis of both paraffins and very long fatty acids specifically exclude branched chains. In fact no measurable $^{14}$C could be detected in the very long fatty acids in broccoli under the present experimental conditions. Thus it appears that the elongation system responsible for the synthesis of very long fatty acids and paraffins excluded branched chains.

Another difference between the $^{14}$C distribution in the fatty acids of tobacco and broccoli was that the former showed a higher proportion of $^{14}$C in the branched C$_{18}$ acid than the latter. Branched C$_{18}$ acid is apparently synthesized by the chloroplast system and also during the elongation associated with the synthesis of surface lipid. In tobacco the branched C$_{18}$ acid is synthesized by both of these systems whereas in broccoli, where the elongation is specific for straight chains, only the chloroplast system synthesizes this acid. From inhibition studies with trichloroacetic acid on broccoli leaf (8) and studies on fatty acid synthesis in various parts of Senecio odoris leaf (11), dual sites of synthesis for straight chain C$_{18}$ acid had been previously postulated. The present work indicates that this may also apply to branched C$_{18}$ acid.

The absolute specificity for straight chains suggests that the hypothetical elongation-decarboxylation enzyme complex can reject molecules when there is a deviation in structure even at the end far removed from the functional end where chain elongation takes place. Although the elongation decarboxylation mechanism can best explain the results of biosynthetic experiments with labeled fatty acids, it is possible that a multienzyme complex, which is also capable of accepting long chain fatty acids, carries out the entire paraffin synthesis starting with acetate or branched starter pieces. If such is the case, the enzyme complex of B. oleracea must utilize only acetate and other straight chain starter pieces whereas that of tobacco can use straight chain as well as branched chain starter pieces.

In plants such as tobacco, which is capable of synthesizing branched paraffins, the relative amounts of these substances synthesized may depend on the availability of branched precursors which in turn is controlled by the metabolic balance of the branched amino acids. Although exogenous branched amino acids gave rise to branched fatty acids in broccoli leaves, this tissue normally does not synthesize significant amounts of branched fatty acids. The reason for this is not understood at present: compartmentation could be a possible explanation.

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Literature Cited


