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Biosynthesis of Long-Chain Hydrocarbons. II. Studies on the Biosynthetic Pathway in Tobacco*

Toshi Kaneda

ABSTRACT: Two classes of long-chain hydrocarbons (C_{25}–C_{35}), branched (mainly iso and anteiso) and normal, occur in tobacco in almost equal proportions. The biosynthetic pathway for hydrocarbons has been studied through measurements of the incorporation of radioactive fatty acid substrates into the branched-chain hydrocarbons by the excised leaf. Acetate is the best precursor and its incorporation into the alkane fraction is independent of light. Carboxyl-labeled butyrate, valerate, caproate, and caprylate are all incorporated to the extent of 0.01–0.05% of the added activity into long-chain hydrocarbons. The amount incorporated into branched hydrocarbons was 40–90% of that incorporated into normal hydrocarbons. Furthermore, about 80% of the radioactivity of the branched-chain hydrocarbons isolated from the experiment with [8-^{14}C]caprylate is found in the methyl carbon. These results, together with previous findings that the terminal branched portions of the branched-chain hydrocarbons are derived from the amino acids valine, leucine, and isoleucine, suggest that the hydrocarbons very likely are produced from precursors resulting from a condensation of two long-chain fatty acids, at least one being a normal fatty acid. This mechanism is consistent with the observed relative distributions of long-chain fatty acids and of hydrocarbons in tobacco.

The wide occurrence of long-chain hydrocarbons (C_{25}–C_{35}) in the plant kingdom, particularly in higher plants, is a well-known fact. They occur mostly in the cuticle waxes of leaves and stems. More recently similar hydrocarbons have been found in the animal kingdom, wool wax (Downing *et al.*, 1960), beeswax (Downing *et al.*, 1961), and even in beef brain (Nicholas and Bombaugh, 1965). Consequently the occurrence of long-chain hydrocarbons is of wide generality in nature and the mechanisms of their biosynthesis are of general interest.

Biosynthesis of long-chain hydrocarbons has not been studied to any significant extent; the incorporation of acetate into long-chain hydrocarbons has been reported (Matsuda, 1962; Kolattukudy (1965) and very recently Kolattukudy (1966) has presented evidence showing that *n*-nonacosane, the only major hydrocarbon in *Brassica oleracea*, may be produced by elongation of the common fatty acids.

The present investigation deals with the biosynthetic mechanism of the long-chain hydrocarbons which occur most commonly in higher plants, *viz.*, C_{25}–C_{35}. Studies on the precursors of branched portions of iso and anteiso hydrocarbons have been published in the first paper (Kaneda, 1967). This paper, dealing with the biosynthesis of branched-chain hydrocarbons in tobacco, presents evidence supporting the "condensation mechanism" for the hydrocarbon synthesis as contrasted with the elongation mechanism referred to in the previous paragraph.

Experimental Procedure

Tobacco Plants. Nicotiana tabacum var. "yellow gold" (a "Bright" type) (4–5-months old) (Kaneda, 1967), was used throughout the present work. Unless specified, leaves approximately half-way up the stem of the plant, weighing 10–20 g (1.0–2.0 g dry wt) and with dimensions of about 20 × 30 cm, were excised at a position 1–2 cm from the junction with the stem and used immediately for experiments.

Chemicals. Standard hydrocarbons (branched and normal series) were the same preparations synthesized previously (Kaneda, 1967). Three additional branched-chain hydrocarbons, 3,22-dimethyltetracosane, 3,24-di-

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methylpentacosane, and 2,25-dimethylhexacosane, were prepared by electrolysis of a mixture of 11-methyltridecanoic and 13-methyltetradecanoic acids followed by gas–liquid partition chromatographic purification. The starting fatty acids were synthesized as before (Kaneda, 1963a). Other chemicals used were the best grade commercially available.

Radioactive Substrate. [1-^{14}C]Caprylate and -decanoate were purchased from New England Nuclear Corp., Boston, Mass.; [8-^{14}C]caprylate was from Schwarz Biochemical Inc., Orangebury, N. Y.; [1-^{14}C]-acetate was from Merck Sharp and Dohme of Canada, Ltd., Montreal, Quebec; and [1-^{14}C]butyrate,-valerate, -caproate were from Calbiochem, Los Angeles, Calif.

Chemical Degradation of Hydrocarbons. Chromic acid oxidation (Eisenfraum *et al.*, 1954) was used to obtain the terminal two carbon atoms of the hydrocarbon sample as acetic acid and the acetic acid produced was isolated as before (Kaneda, 1963b).

Administration of Radioactive Substrate to Tobacco Leaf. The excised tobacco leaf was inserted into a 5-ml culture bottle containing 1 ml of an aqueous solution of a radioactive substrate. Usually, the solution was taken up within 20 min, at which time 1 ml of water or, in some cases, acetate solution as described later, was added to the bottle in order to keep the excised portion of the leaf immersed in water, and repeated additions were made throughout the administration period. During this time the excised leaf was illuminated by two 40-W fluorescent lamps, cool white (Westinghouse), unless otherwise specified.

Isolation and Fractionation of Tobacco Hydrocarbons. At the end of the administration period, the leaf was removed from the culture bottle and dipped into *ca.* 400 ml of chloroform in a beaker for 3–5 min to extract the surface wax, then hung up overnight to dry completely at room temperature. The dried leaf thus obtained was broken into small pieces and treated with a portion of 70 ml of chloroform, and then two portions of 50 ml of the same solvent to extract the internal lipids. These extracts were combined, freed from the chloroform, and then fractionated by thin-layer chromatography on silica gel with either *n*-hexane or benzene or both, as developer. Visualization of compounds on the thin-layer chromatograms was achieved by either 0.002% Rhodamine 6G or α-cyclodextrine (Mangold *et al.*, 1955). The alkane fraction (usually 1–2 mg) located at the solvent front was eluted with chloroform and further purified by recrystallization from acetone before measuring the amount by gas–liquid partition chromatography and the radioactivity with a Geiger-Müller counter. Gas–liquid partition chromatographic systems used were identical with those used in the previous work (Kaneda, 1967). Separation of branched-chain hydrocarbons from straight-chain hydrocarbons was carried out with Molecular Sieve 5A as described before and the separation was confirmed by gas–liquid partition chromatography.

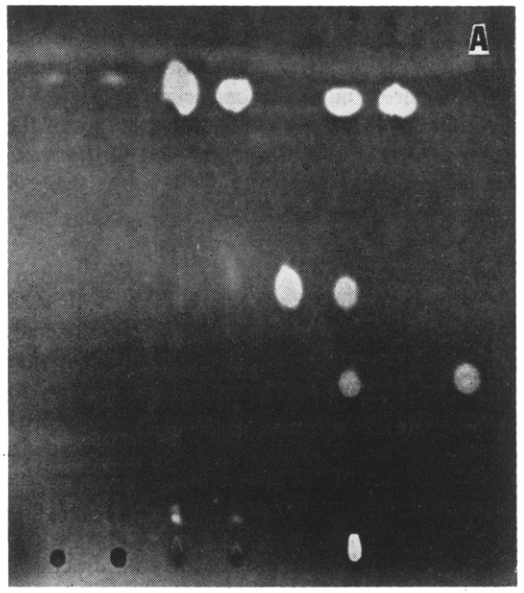
Determination of Tobacco Hydrocarbons and Their Specific Activity. The amount of hydrocarbon was determined by gas–liquid partition chromatography. The response of standard hydrocarbons (n-C_{21}–n-C_{35}) in the hydrogen flame detector, was found to be proportional to the number of carbon atoms and the gas chromatographic peaks were evaluated relative to n-C_{32} synthesized by Kolbe electrolysis which was used as an internal standard. The total alkane fraction from the thin-layer chromatography, in an amount less than 0.1 mg, was plated on an aluminum planchet with chloroform as solvent, and radioactivity was measured with a gas-flow Geiger-Müller counter. No correction for self-absorption was made. The average specific activity of the individual hydrocarbons was calculated on the basis of these two measurements.

Results

Constituents of Surface Wax and Internal Lipid. Components of the leaf wax and internal lipids were analyzed by thin-layer chromatography. Figure 1 shows that the surface wax (Figure 1A) was resolved into one major hydrocarbon component (designated as the alkane fraction) and two minor components, one having a similar R_F value to that of palmitone and showing a characteristic strong absorption at 1700 cm^{-1}, and the other staying at the origin. The former is designated as the ketone fraction and the latter as the polar fraction. The internal lipid (Figure 1B) is far more complex, being resolved into 13 components. Most of these are complex lipids as judged by the dark color of the spots when the α-cyclodextrin–iodine vapor method is used, in which aliphatic long-chain compounds give white spots and complex lipids give dark spots. When the internal lipid sample was treated with a methanolic BF_3 solution prior to thin-layer chromatography, only one spot located at the same position as methyl palmitate was detected (Figure 1C). This suggests that complex lipids may be derivatives of the common fatty acids. Alkanes were definitely present in the internal lipid but the amount was very small. No attempt has been made to identify the other components of the internal lipid. These results thus show that the surface wax and the internal lipid are entirely different in their components, and that the hydrocarbons are located mainly on the surface of the leaf.

Effect of Light on Incorporation of [1-^{14}C]Acetate into Lipid Fractions. [1-^{14}C]Acetate has been found to incorporate equally well into both branched and normal hydrocarbon fractions by the growing tobacco plant (Kaneda, 1967). The effect of light on the incorporation was studied with the excised leaf. Data presented in Table I show that the incorporation of [1-^{14}C]acetate into the total lipids was about 1% in light and 0.3% in dark. The incorporation of [1-^{14}C]acetate into the surface wax is not affected by light. The incorporation into the internal lipid, however, is greatly increased when light is used during the administration of the substrate to the excised leaf.

This result shows that the synthesis of hydrocarbons (which are the major component of the surface wax) from acetate is independent of light, whereas the synthesis of complex lipids is closely related to the light energy yielding system in the excised tobacco leaf. The biosynthesis of long-chain fatty acids has been reported to



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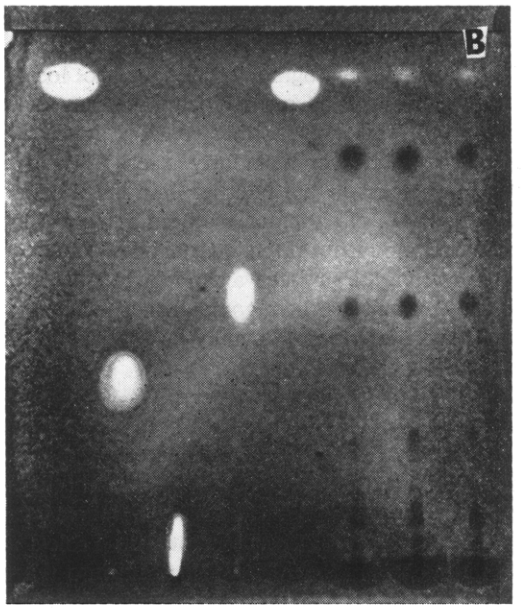

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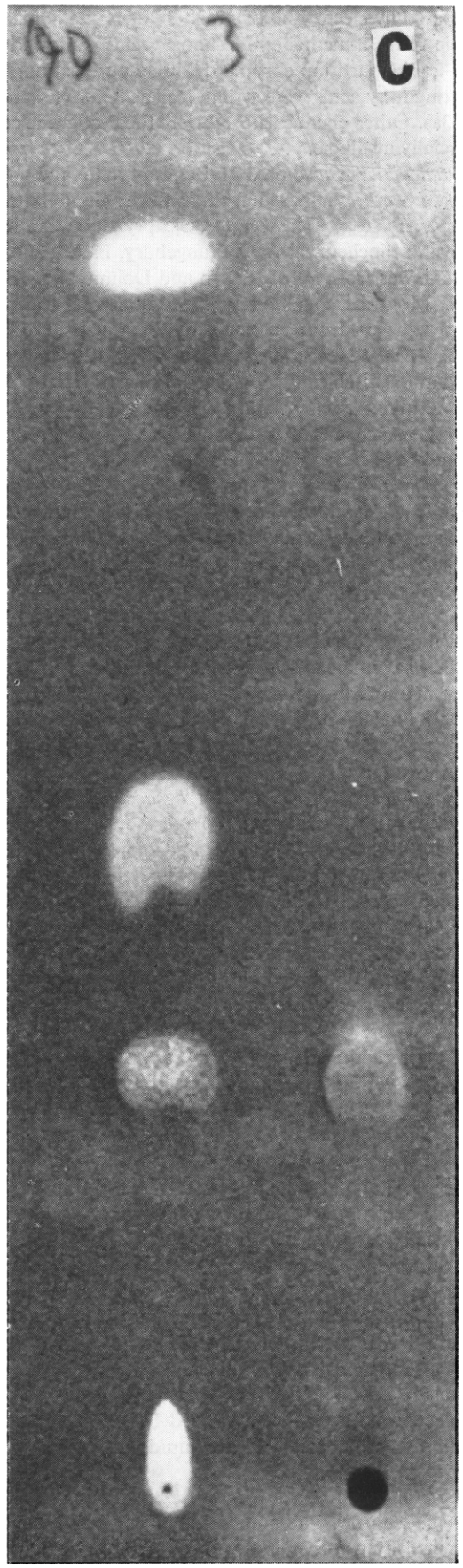

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FIGURE 1: Thin-layer chromatography of tobacco lipids on silica gel G. The plate was developed first with benzene and then with *n*-hexane. Spot was visualized by spraying an α-cyclodextrine solution followed by exposure to iodine vapor. The symbols in the figure are: a, surface wax; b, internal lipid; c, bulk internal lipid; d, after c was treated with methanolic BF_3; 1, *n*-C_{32} hydrocarbon; 2, palmitone; 3, methyl palmitate; 4, palmitic acid; and 5, a mixture of 1–4.

TABLE I: Effect of Light on Lipid Synthesis.[a]

Condition	Leaf Dry Wt (g)	Incorporation of [1-^{14}C]Acetate[b] Surface Wax	Internal Lipid
In light	2.2	2.5	86.5
	1.3	3.1	59.0
In dark	1.8	5.0	16.3
	1.7	1.8	12.7

[a] The leaf was incubated with a [1-^{14}C]acetate solution (5.1 μmoles, 30 μCi in 3 ml) for 30 min with or without light as described in the text. Portions of the extracted surface wax and internal lipids (see text) were plated on aluminum planchets using chloroform as solvent and counted for radioactivity after standing overnight at room temperature to permit evaporation of volatile radioactive materials. When leaves excised from the same plant, or plants seeded at the same time and grown under identical conditions, were used, activities of the [1-^{14}C]acetate incorporation into the surface wax or into the internal lipid among the leaves normally agreed within 30% of the incorporation. [b] Cpm $\times 10^{-3}$ per g dry wt of leaf.

take place mainly in choroplasts and to depend largely on light (Stumpf and James, 1963). This may be related to the present finding, *i.e.*, the synthesis of internal lipids from acetate by the excised tobacco leaf is largely light dependent.

Time Course of the Incorporation of [1-^{14}C]Acetate into Lipid Fractions. An aqueous solution of [1-^{14}C]acetate was administered constantly to the excised tobacco leaf in the presence of light. After 5-min administration, half of the leaf was cut off and the incorporation of the acetate into the surface wax and the internal wax was determined. Administration of the substrate to the remaining half of the leaf was continued for a further 5 or 25 min.

TABLE II: Incorporation of [1-^{14}C]Acetate into Lipid Fractions.[a]

Time (min)	Leaf Dry Wt (g)	Incorporation of [1-^{14}C]-Acetate (cpm $\times 10^{-3}$/ g dry wt of leaf) Surface Wax	Internal Lipid
5	0.8	0.2	1.6
10	1.0	0.5	8.6
30	1.2	1.2	57.4

[a] The incubation conditions (in light) were the same as those described in Table I.

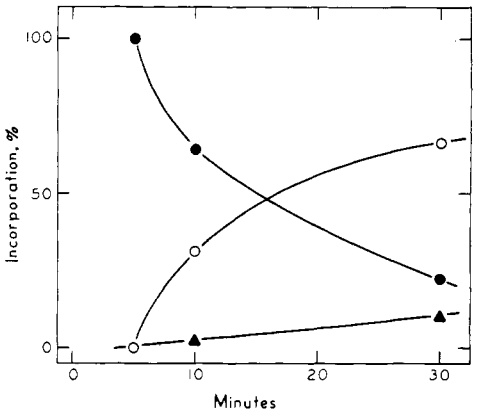

FIGURE 2: Incorporation of [1-^{14}C]acetate into the three major fractions of surface wax. The incorporation was expressed by per cent of a total incorporation in each fraction of the surface wax. (●) The polar fraction; (○) the alkane fraction; (▲) the ketone fraction.

Half of another leaf was cut off after 10-min administration and the amount of incorporation was determined. Administration was continued on the remaining half of this leaf for 20 min, to make a total of 30-min administration. A total of three leaves was used to cover the three administration times of 5, 10, and 30 min, in duplicate, for the series of experiments.

Table II shows that the incorporation of [1-^{14}C]-acetate into the surface wax increases nearly linearly with time of administration, whereas the incorporation into the internal lipid increased exponentially. The ratio between the incorporation into the surface wax to that into the internal lipid was 1:0.5 after 5 min. This ratio, however, changed to 1:2 and 1:5 after 10- and 30-min administration, respectively.

Time Course of [1-^{14}C]Acetate Incorporation into Various Fractions of the Surface Wax. A portion of the radioactive surface wax described in Table II was fractionated by thin-layer chromatography, and each spot visualized by the α-cyclodextrin method was scraped and extracted with chloroform. Radioautography was also used to confirm that no additional radioactive spot was produced. The chloroform extract, thus obtained, was plated on an aluminum planchet and counted for radioactivity by a gas-flow G-M counter. Figure 2 illustrates the incorporation of [1-^{14}C]acetate into each fraction. After 5-min administration, almost all the activity was located in the polar fraction which did not move in the thin-layer chromatogram. As the administration and incubation continued, the relative proportion of the activity going into this fraction decreased sharply, whereas that in the alkane fraction increased steadily. The ketone fraction remained as a minor product throughout the administration period. This result suggests that a compound or compounds, which do not move during development with benzene and *n*-hexane, may be precursors of hydrocarbons synthesized in the tobacco leaf, and that ketones, at least in free form, are probably not precursors.

Incorporation of Various 1-^{14}C Short-Chain Fatty Acid Substrates into the Long-Chain Hydrocarbons. The 1-^{14}C short-chain fatty acids, acetate, butyrate, valerate,

TABLE III: Incorporation of Carboxyl-Labeled Short-Chain Fatty Acid Substrates into Long-Chain Hydrocarbons.[a]

Substrate	Amount Added (μmole)	Amount Incorpd into Long-Chain Hydrocarbons (mμmoles)[b]	% Incorp	Long-Chain Hydrocarbons (cpm × 10^{-2}/mg of hydrocarbon) Total	Branched	Normal[c]
Acetate	3.4	7.5	0.22	202	165	239
Butyrate	10.2	4.8	0.05	32	21	43
Valerate	22.7	2.3	0.01	6	6	6
Caproate	4.0	1.0	0.03	17	14	20
Caprylate	10.2	3.1	0.03	11	9	13
Decanoate	4.1	0.2	0.005	1.4	0.8	2.0

[a] Leaves ranging 1–1.2 g dry wt were used to incubate with one of the substrates (20 μCi) in 1 ml of water described above, for 3 hr in light. The long-chain hydrocarbons (1 to ~2.7 mg) were isolated by thin-layer chromatography and recrystallized from acetone. This total alkane sample was fractionated by Molecular Sieve 5A into the branched and the normal series as described in the text. [b] This was calculated based on the radioactivity incorporated in the long-chain hydrocarbons. [c] This was calculated based on the specific activities of the total alkanes and of the branched series.

TABLE IV: Incorporation of [1-^{14}C]- and [8-^{14}C]Caprylate into Long-Chain Hydrocarbons.[a]

Substrate	Active Substrate Added (μmole)	Substrate Incorpd into Long-Chain Hydrocarbons (mμmoles)[b]	% Incorp	Long-Chain Hydrocarbons (cpm × 10^{-2}/mg of hydrocarbon) Total	Branched	Normal[c]
[1-^{14}C]Caprylate	10.2	2.3	0.02	10.9	10.4	11.4
[1-^{14}C]Caprylate + acetate	10.2	1.0	0.01	4.1	4.4	3.8
[8-^{14}C]Caprylate	3.8	0.21	0.006	2.1	0.6	3.9
[8-^{14}C]Caprylate + acetate	3.8	0.23	0.006	2.4	0.7	4.4

[a] Leaves ranging 1.5–1.7 g dry wt were used to incubate with one of the substrates (20 μCi) in 1 ml of water or of 20 mM acetate solution described above for 3 hr in light. The long-chain hydrocarbons (1.8 to ~2.3 mg) were isolated and fractionated into the branched series and normal series as before (Table III). [b,c] See in Table III.

caproate, and caprylate, all were incorporated to the extent of 0.01–0.22% of the added activity, into hydrocarbons of the surface wax (Table III). This small incorporation would not be unexpected since the radioactive fatty acid substrate would be mixed with large amounts of common fatty acids or their biologically active forms present in the leaf during its incorporation into the alkane fraction and the occurrence of the total alkanes is very small, to the extent of 0.1% of the dry leaf. The incorporation of the acetate was at least four times that of the other substrates. The incorporation of the butyrate was a little better than that of the four remaining substrates. When [1-^{14}C]laurate was administered, the excised leaf was dehydrated within 10 min and it was no longer able to stand by itself. The surface wax isolated after 4-hr incubation did not contain any significant radioactivity. The radioautogram of the leaf showed that all the radioactivity was located at the bottom portion of the leaf, within 4 mm of the point of excision. This suggests that when the laurate (as sodium salt) is taken up to the capillaries of the leaf, it is acidified immediately with the cell sap and forms the free acid which plugs the capillaries, resulting in dehydration of the leaf. Both [1-^{14}C]myristate and -palmitate show the same behavior. [1-^{14}C]Decanoate also was not incorporated well, being one-tenth of the incorporation of [1-^{14}C]-caprylate but here no signs of dehydration of the excised leaf were noted.

The total alkane fraction, isolated from the experiments in which various 1-^{14}C short-chain fatty acids were used, was treated with Molecular Sieve 5A in isooctane to remove the normal series of hydrocarbons and the radioactivity that remained in the solution was measured. As can be seen in Table III, all the short-chain

TABLE V: Distribution of Radioactivity Derived from [8-^{14}C]Caprylate in the Branched-Chain Hydrocarbons.[a]

Total Act. of Branched-Chain Hydrocarbons (cpm)	Activity of Acetic Acid from Chemical Degradation (cpm)	Assumed Distribn in Branched-Chain Hydrocarbons (cpm)		
		CH_3	CH_2[b]	R[c]
168	135	134	1.1	33

[a] The radioactive branched-chain hydrocarbons isolated from the experiment shown in Table IV were used in this experiment. An aliquot of this sample was mixed with a carrier hydrocarbon, *n*-C_{32} (1.15 mg), and then oxidized with CrO_3 to acetic acid and CO_2 (Kaneda, 1963b). Yield of the acetic acid isolated by steam distillation was found to be quantitative. [b] This was calculated from the activity in the R portion (33 cpm) divided by the average carbon atoms of R, 29. [c] R represents $-(CH_2)_nCH(CH_3)_2$ and $-(CH_2)_mCH(CH_3)C_2H_5$.

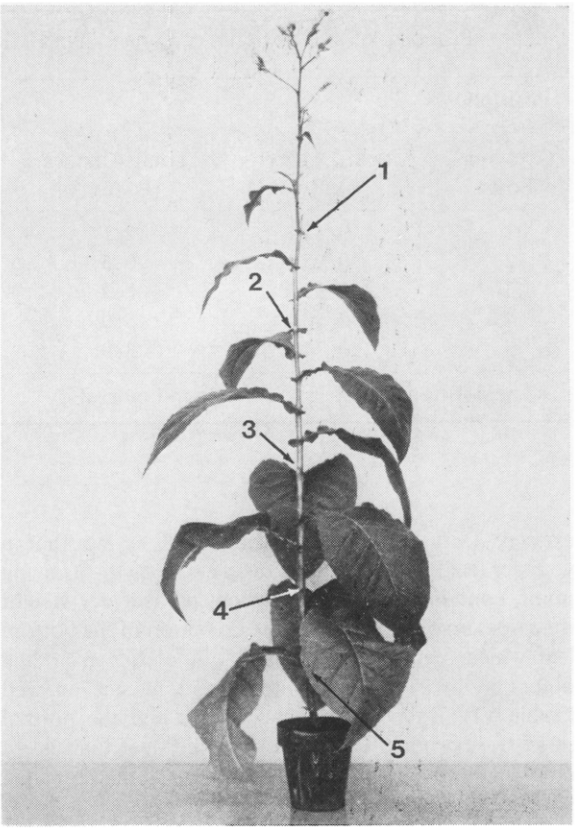

FIGURE 3: Location of leaf samples on the tobacco plant.

substrates appear to be incorporated into the branched series as well as into the normal series and with specific activities at least 40% of those for the normal hydrocarbons.

Incorporation of [1-^{14}C]- and [8-^{14}C]Caprylate Substrates into Long-Chain Hydrocarbons. It is possible that the 1-^{14}C short-chain fatty acid substrate administered might be degraded to [1-^{14}C]acetate by a system such as the β-oxidation scheme and consequently that the radioactivity found in the long-chain hydrocarbons might be a result of the incorporation of this acetate. This possibility was examined through a study of the effect of acetate on the incorporation; a large excess of nonactive acetate should in such cases, virtually eliminate the incorporation of radioactivity from the active substrate.

A large excess of added acetate decreased the incorporation of the radioactivity of [1-^{14}C]caprylate into the alkane fraction to about one-half of the control value (Table IV). This indicates that up to half of the radioactivity in the alkane fraction enters *via* acetate or a form which is biologically equivalent to acetate. The remainder must enter by some other pathway. On the other hand, excess acetate made no change in the incorporation of the radioactivity of [8-^{14}C]caprylate. This suggests that the ω-carbon of caprylate is incorporated into the long-chain hydrocarbons without going through the acetate stage, presumably by incorporation of the molecule as a whole, and also that the degradation of caprylate, possibly by the β-oxidation scheme, does not proceed completely to give acetate from the terminal two carbon atoms.

To examine the strong possibility of incorporation of caprylate into the long-chain hydrocarbon as a whole, the branched-chain hydrocarbons isolated from the experiment with [8-^{14}C]caprylate were degraded with chromic acid to acetic acid and CO_2 and the activity in the acetic acid (representing the methyl and adjacent carbons) was determined. The results (Table V) showed that 80% of the total radioactivity of the branched-chain hydrocarbons was located at the methyl carbon and/or the carbon adjacent to the methyl carbon. It is hardly expected to incorporate the ω-carbon of caprylate into the terminal two carbons of the branched portion of the hydrocarbons with such high degree of specificity (80%). In addition, the terminal branched C_4 and C_5 portions of the branched-chain hydrocarbons have been shown to be derived from the respective amino acids, valine, leucine, and isoleucine (Kaneda, 1967). Hence the caprylate must be specifically incorporated as a whole into the straight portion of the branched-chain hydrocarbons. The detailed pathway for the incorporation of caprylate will be discussed in a subsequent section.

The results with [8-^{14}C]caprylate differ significantly from those with [1-^{14}C]caprylate in that the relative and absolute incorporations of activity into the branched-chain hydrocarbons were much lower for the 8-^{14}C compound. This may be due to the participation of a third pathway in the incorporation of caprylate into the long-chain hydrocarbons, in addition to incorporation of caprylate molecule *via* acetate and as a whole. This pathway, however, remains to be elucidated.

Leaf Hydrocarbons in Relation to Leaf Position. Comparison was made among leaves for hydrocarbon contents in relation to their relative position. Figure 3 illustrates the location from which the leaf samples were

TABLE VI: Proportions of Two Series of Long-Chain Hydrocarbons in Relation to Leaf Position.[a]

Position from Growing Point	Leaf Dry Wt (A) (g)	Total Alkanes (B) (mg)	Hydrocarbon Contents in Leaf (mg/g dry wt of leaf)			
			B/A	Branched Series	Normal Series	Branched/ Normal
1	0.45	2.1	4.7	2.9	1.8	1.57
2	0.95	3.5	3.7	2.3	1.4	1.67
3	2.12	8.2	3.9	2.3	1.6	1.38
4	2.40	5.0	2.1	1.0	1.1	0.91
5	0.74	3.9	5.3	2.5	2.8	0.92

[a] The position of each leaf is shown in Figure 2.

excised. Data presented in Table VII shows that a younger leaf, *i.e.*, a leaf positioned closer to the growing point, contains more hydrocarbons per leaf dry weight than does an older leaf, with the exception of the bottom leaf which seems to be entirely out of line from the others in hydrocarbon contents and in size as well (Table VI). Both the branched series and the normal series were produced more in a younger leaf than in an older leaf and a greater relative amount of the branched series content occurred in the younger leaves.

L-Valine, L-isoleucine, and L-leucine have been found to be incorporated into the specific branched-chain hydrocarbons in tobacco (Kaneda, 1967). A higher content of the branched series in a younger leaf may be related to the availability of these precursor amino acids which are higher in a younger leaf than in an older leaf.

TABLE VII: Relative Proportions of Total Fatty Acids and Hydrocarbons in Tobacco Leaf.

Homologous Series	Fatty Acid	%	Hydrocarbon[a]	%
Anteiso	C_{15}	0.2	C_{30}	5.3
	C_{17}	2.5	C_{32}	11.6
	C_{19}	0.7	C_{34}	1.0
Iso	C_{14}	~0	C_{29}	3.0
	C_{15}	0.2	C_{30}	
	C_{16}	0.4	C_{31}	18.5
	C_{18}	1.1	C_{33}	11.7
	C_{19}	~0	C_{35}	0.1
Normal, even	C_{12}	~0	C_{27}	2.6
	C_{14}	0.8	C_{29}	3.3
	C_{16}	70.7	C_{31}	18.8
	C_{18}	13.6	C_{33}	15.8
	C_{20}	2.2	C_{35}	0.4
Normal, odd	C_{13}	~0	C_{28}	0.2
	C_{15}	1.1	C_{30}	1.1
	C_{17}	~0	C_{32}	5.7
	C_{19}	~0	C_{34}	0.3

[a] A hydrocarbon which is expected to derive from the fatty acid by the condensation mechanism described in Figure 4 is placed on the same line as the fatty acid.

Fatty Acids in the Leaf. Fatty acids as either free or combined forms are mostly present in the internal lipid but not in the surface wax (Figure 1). Relative proportions of the saturated total fatty acids are shown in Table VII. Proportions of the common fatty acids (myristic, palmitic, and stearic) are extremely high and they make up 87% of the total saturated fatty acids. Odd-numbered normal fatty acids were also found but the proportion was much smaller, 2.9%. Branched-chain fatty acids were present with proportions similar to those of the odd-numbered fatty acids, iso and anteiso fatty acids being 1.7 and 3.4% respectively. The relative amounts of fatty acids in tobacco lipids has been previously reported by Mold *et al.* (1966).

Search for Biterminally Methyl-Branched Hydrocarbons. Chemically synthesized samples of 3,22-dimethyltetracosane (anteiso, anteiso-C_{26}), 3,24-dimethylpentacosane (anteiso, iso-C_{27}), and 2,25-dimethylhexacosane (iso, iso-C_{28}) were chromatographed on an SE 30 column, and their relative carbon numbers in relation to straight-chain hydrocarbons were determined from retention times by a standard method. They are 25.46, 26.39, and 27.25, respectively, being 0.54, 0.61, and 0.75 smaller than the carbon numbers of the corresponding normal hydrocarbons. Since anteiso and iso hydrocarbons on the same column are 0.2 and 0.35 smaller than the carbon numbers of the corresponding normal hydrocarbons, members of the biterminally methyl-branched series should easily be distinguished from both anteiso- and isoalkanes by chromatography. Within the estimated detection limits of the chromatographic system (corresponding approximately to 0.05% of the total hydrocarbon sample) biterminally methyl-branched hydrocarbons in the carbon number range 21–35 were absent.

Discussion

The metabolic pathway shown in Figure 4 is postulated for the biosynthesis of long-chain hydrocarbons in

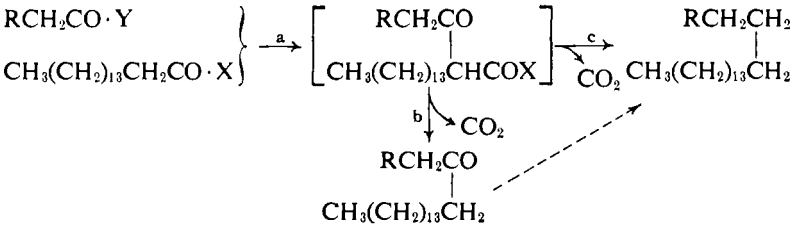

FIGURE 4: Biosynthetic pathway for long-chain hydrocarbons in tobacco.

tobacco. Step a in the figure is adopted from the microbial synthesis of corynomycolic acid from two entire molecules of palmitic acid *via* the β-keto acid (Gastambide-Odier and Lederer, 1959). Two separate pathways (b and c) are postulated for the further transformation of the β-keto acid according to the observations described in this paper.

First consider step a. This is assumed to be a condensation involving primarily a biologically active form of palmitic acid, $CH_3(CH_2)_{13}CH_2COX$, the predominant fatty acid (over 70% of the total fatty acids in tobacco) and a molecule of one of the other saturated fatty acids either branched or normal in the range C_{14}–C_{20}, represented as RCH_2COY.

The principal evidence favoring the condensation mechanism comes from the finding that 1-^{14}C short-chain fatty acids administered to the excised leaf are incorporated into both series of hydrocarbons, normal and branched, with almost equal efficiency, and that ten times or more acetate added does not reduce greatly the efficiency of incorporation. If the hydrocarbons C_{25}–C_{35} were produced from the fatty acids with carbon atoms of 26–36 which are produced by elongation of the fatty acids, C_{12}–C_{20}, in tobacco, a 1-^{14}C short-chain fatty acid administered would be expected to incorporate into only the normal series but not into the branched series. Consequently, it is concluded that the elongation mechanism does not satisfactorily account for the synthesis in tobacco of long-chain hydrocarbons of the surface wax. This is supported by the finding that [8-^{14}C]caprylate is incorporated into the branched series as well as the normal series and that the major activity in the branched series is located within the terminal two carbon atoms, presumably the methyl carbon.

Two possible routes for the further reaction of the condensation product of step a are considered in steps b and c. Step b represents a decarboxylation to give a ketone while step c is a complete decarboxylation and reduction to the hydrocarbon. The ketone might reasonably be expected to be an intermediate in the formation of hydrocarbon. If so, in a time-course experiment, the proportion of the ketone fraction relative to the total radioactivity of the surface wax incorporated should pass through a maximum and then decrease while the radioactivity in the hydrocarbon increased. This is not what was found (Figure 2); both the ketone and the hydrocarbon showed a steady increase in radioactivity, the hydrocarbon being several times more than the ketone. Hence paths b and c are considered to be alternatives with a strong preference for path c.

According to the mechanism proposed above, anteiso fatty acids, C_{15}, C_{17}, C_{19}, present in tobacco plant would likely condense with one of the major fatty acids, palmitic (over 80% of the total fatty acids) or stearic (15%), to produce anteiso hydrocarbons, C_{30}, C_{32}, and C_{34}, respectively. All these expected hydrocarbons are, indeed, found in tobacco (Table VII). Similarly, iso fatty acids, C_{16} and C_{18}, and odd-numbered normal fatty acids, C_{15} and C_{17}, present are expected to be converted into iso hydrocarbons, C_{31} and C_{33}, and even-numbered hydrocarbons, C_{30} and C_{32}, respectively. As expected, all these hydrocarbons are found. Within any homologous series, relative abundances of the fatty acids reflect reasonably well the relative abundances of the corresponding hydrocarbons. The experimental results between different homologous series, however, seem to indicate this is not so. Although the branched-chain fatty acids present are only 5.1% of the total fatty acids, the proportion of the branched-chain hydrocarbons produced is unexpectedly high, about a half of the total long-chain hydrocarbons. This apparent high degree of selectivity may be due to the specificity of the hydrocarbon-synthesizing enzyme system being much more favorable for the branched-chain precursors than for the straight-chain precursors, and further studies are essential to clarify this point.

Branched-chain hydrocarbons having branching at both ends are not found in tobacco, although such compounds could theoretically be produced if two branched-chain fatty acids, iso and/or anteiso series, were condensed according to the mechanism postulated. Considering the low relative abundance of the branched-chain fatty acids, the proportion of biterminally methyl-branched hydrocarbons that would be expected is probably below the limits of detection.

Eglinton and Hamilton (1963) have generalized the distribution of long-chain hydrocarbons in leaf waxes as follows: alkanes are mostly *n*-C_{25}–*n*-C_{35} with a clear odd-number preference, *n*-C_{27}, *n*-C_{31}, and *n*-C_{33} being the major constituents. The condensation mechanism proposed here for the biosynthesis of long-chain hydrocarbons agrees very well with this generalization. The fatty acids present in higher plants, as in other organisms, are mostly *n*-C_{14}, *n*-C_{16}, and *n*-C_{18}, and these fatty acids would be converted by the proposed mechanism into the odd-numbered hydrocarbons primarily in the range of *n*-C_{27}–*n*-C_{33} as observed. More conclusive evidence for the condensation mechanism should be obtainable from enzyme experiments with tobacco, and work along these lines is in progress.

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The Catalytic Versatility of Erythrocyte Carbonic Anhydrase. IV. Kinetic Studies of Enzyme-Catalyzed Hydrolyses of *p*-Nitrophenyl Esters*

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ABSTRACT: Kinetic studies on the esterase activity of bovine carbonic anhydrase with *p*-nitrophenyl propionate, *n*-butyrate, isobutyrate, *n*-valerate, isovalerate, *n*-caproate, and trimethyl acetate as substrates are reported. Esterase activity rises continuously but non-uniformly with increasing pH between 4.0 and 10.9. Below pH 9.0 the profiles for propionate, *n*-butyrate, isobutyrate, and isovalerate are quite similar to that previously obtained for *p*-nitrophenyl acetate: they are sigmoidal with an inflection occurring around pH 7.3. In contrast, the two longer substrates, *p*-nitrophenyl *n*-valerate and *p*-nitrophenyl *n*-caproate, exhibit an "abnormal" behavior. (i) Their rate profiles show inflections at pH 6.2 and 5.7, respectively, rather than at 7.3, and (ii) their K_i values for the specific inhibitor acetazolamide are 10^3 times larger than those found with *p*-nitrophenyl acetate and propionate as substrates. The sterically hindered trimethyl acetate ester is subject to little or no enzymatic catalysis below pH 8.5. The enzymatic hydrolyses of all the esters reported in this paper exhibit a second inflection, of much greater magnitude around pH 10.5; this dramatic increase in enzymatic activity at high pH has not been reported in any previous kinetic study pertaining to carbonic anhydrase.

A formal Michaelis–Menten treatment of these enzyme-catalyzed hydrolyses shows that both k_2 and K_m vary with pH. The variation of K_m with pH is shown to be dictated by the pH dependency of the turnover number (k_2) while the apparent binding constant (k_1/k_{-1}) appears to be pH independent below 9. A comparison of the various esters reveals that binding increases with the size of the ester. The relative increase in free energy of binding associated with the larger esters is shown to be well accounted for by a parallel increase in the free energy of hydrophobic interactions. D_2O studies indicate that k_2 in D_2O is twice as large as it is in H_2O.

Recent investigations conducted in these laboratories have demonstrated that carbonic anhydrase catalyzes the reversible hydration of various aldehydes (Pocker and Meany, 1965a,b, 1967a,b; Pocker *et al.*, 1965) and the hydrolysis of *p*-nitrophenyl acetate (Pocker and Stone, 1965, 1967). Kinetic studies on propionaldehyde, isobutyraldehyde, and pivalaldehyde hydrations revealed that the catalytic efficiency of bovine carbonic

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