Biosynthesis of Long-Chain Hydrocarbons. I. Incorporation of L-Valine, L-Threonine, L-Isoleucine, and L-Leucine into Specific Branched-Chain Hydrocarbons in Tobacco*

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ABSTRACT: The three major homologous series (normal, 2-methyl (iso), and 3-methyl (anteiso)) of long-chain hydrocarbons present in a "Bright" type of tobacco plant have been identified by mass spectrometry, melting point, X-ray diffraction analysis, and infrared spectroscopy in addition to gas–liquid partition chromatographic retention volume on a SE-30 column. The amounts of the branched-chain hydrocarbons (iso and anteiso) are almost equal to those of the straight-chain hydrocarbons (normal). Uniformly labeled but not carboxyl-labeled L-valine, L-isoleucine, or L-leucine are incorporated to an extent of 0.1% of the added activity into the hydrocarbon fraction by the growing tobacco plant. The radioactivity, in all cases, is found mainly in the branched-chain hydrocarbons. The distribution patterns of the radioactivity among the components of the branched-chain hydrocarbons are consistent with the hypothesis that the branched-short-chain acyl coenzyme A ester produced from the added branched-chain amino acid substrate is specifically incorporated into the corresponding branched-long-chain hydrocarbons. Uniformly labeled L-threonine is incorporated into the same hydrocarbons into which the isoleucine is incorporated. This suggests that the threonine is first deaminated to produce α-ketobutyrate which is further metabolized to yield the α-keto acid of L-isoleucine. Thus the threonine and the isoleucine are incorporated into the same hydrocarbons.

Aliphatic hydrocarbons occur very widely in both the plant and animal kingdoms. Particularly in higher plants they occur as a major constituent of the cuticle waxes of leaves and stems. In most cases, the normal series occurs more abundantly, but significant quantities of the branched series, mainly iso and anteiso, are also found in certain plants[1] (Waldren *et al.*, 1961; Mold *et al.*, 1963, 1964). The occurrence of branched-chain hydrocarbons in certain higher plants can be correlated to their taxonomic classification (Eglinton *et al.*, 1962a,b). Despite their chemotaxonomic importance, together with such wider occurrence in living organisms, little is known regarding the mechanism of biosynthesis of long-chain hydrocarbons. The present paper reports the results of investigations on precursors of the terminal branched-chain portions of iso and anteiso hydrocarbons in the tobacco plant.

Experimental Procedure

Tobacco Plants. Seeds of *Nicotiana tabacum* var. "yellow gold" (a "Bright" type) were kindly supplied by Dr. B. Povilaitis, Tobacco Research, Experimental Farm, Canada Department of Agriculture, Guelph, Ontario, Canada. Culture of the tobacco plants was carried out in a greenhouse, University of Alberta, Edmonton, Alberta. Plants (3–4-months old), height about 3 ft, were grown under two 40-w fluorescent lamps, watered once a day, and fertilized with a salt solution (Brown and Byerrum, 1952) twice a week, at which time radioactive substrates were administered.

Radioactive Substrates. Carboxyl- or uniformly labeled L-valine, L-threonine, L-isoleucine, and L-leucine, and [CH_3-^{14}C]acetate were purchased from New England Nuclear Corp., Boston, Mass., and used without further purification.

Administration of Radioactive Substrates to the Tobacco Plant. The wick method was used. About ten cotton threads were threaded through the plant at one-third of the height (about 1 ft) from the roots with one end at the center of the stem and the long end dipping into 1 ml of an aqueous solution of the radioactive substrate (50 μc) in a 2-ml centrifuge tube. The solution was taken up by the plant completely within a few hours. To assist the transportation of the radioactive substrate, three portions of 1 ml of water were subsequently administered to the plant by the same procedure. Degradation of the radioactive substrate by bacterial contamination during the administration may be negligible since the administration took only a short period (2–3 hr) and the solution contained only the single amino acid in a small quantity.

Isolation and Fractionation of Tobacco Hydrocarbons. The general procedure for the extraction and the iso-

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[1] The occurrence of isoalkanes in higher plants has been reported in other papers which are not referred to here. These, however, should be reexamined since the alkanes have been identified only by gas–liquid partition chromatographic retention volume. Usually in such cases iso refers to all branched species. Additional physical data would be desirable.

lation of the hydrocarbons from the tobacco plant was as follows. All the leaves were removed from the plant and the stem was cut into small pieces, which were dipped into 600 ml of chloroform in a beaker to extract the cuticle waxes. The chloroform was evaporated by a stream of nitrogen. The waxes thus isolated were dissolved in hexane, treated with concentrated H_2SO_4 to remove pigments and other polar components, and washed with water to neutral. The sample at this stage is designated as "total hydrocarbons" fraction. The white waxy materials isolated by evaporation of the washed extracts were put on an alumina column with *n*-hexane as solvent. The first eluent collected contained most of the hydrocarbons. The sample at this stage is designated as "long-chain hydrocarbon" fraction. Separation of the branched-chain hydrocarbons from normal hydrocarbons was achieved by means of Molecular Sieve 5A (O'Connor *et al.*, 1962). The branched series remained in isooctane solution. The Molecular Sieve 5A was treated with *n*-heptane overnight at room temperature to remove a small amount of the branched series nonspecifically absorbed, then refluxed with *n*-heptane overnight to recover the absorbed normal hydrocarbons. The recovery of the normal series from the sieve was at most 30% of the total amount absorbed.

Further fractionation of the branched series of hydrocarbons was achieved by gas–liquid partition chromatography on a preparative scale on a $^3/_8$ in. × 6 ft SE-30 (3%) column at 275° with a helium carrier gas-flow rate of 100–120 ml/min and with a thermal conductivity cell as detector. It was found that consecutive chromatography (preferably three times) was essential in order to obtain fractions giving single peaks on an analytical gas-liquid partition chromatogram ($^1/_8$ in. × 6 ft SE-30 (1%) column with a hydrogen-flame detector), programmed from 175 to 275° at 2°/min.

This chromatographic procedure is inadequate to fully resolve iso and anteiso hydrocarbons having the same number of carbon atoms. Attempts were made to use longer columns but these did not apparently improve the resolution and at the same time exposed the hydrocarbons to higher column temperatures for longer periods. The use of a column packed with a more polar liquid (a six-ring polyphenyl ether) showed no improvement in resolution in the C_{29}–C_{35} range. Mold *et al.* (1963), on the basis of mass spectrometric pattern, found iso–anteiso contamination in gas–liquid partition chromatographic fractions. Fortunately, in all experiments the branched hydrocarbon was predominantly either iso or anteiso with the other present only as a few per cent. It was found that this small amount of contaminant branched hydrocarbons could be completely removed by two recrystallizations of the gas–liquid partition chromatographic fraction from acetone. The purity of the hydrocarbon sample was checked by a modified procedure of Cason *et al.* (1959).

Standard Hydrocarbons. Standard normal and branched-chain hydrocarbons C_{23}–C_{37} were prepared by Kolbe synthesis as listed in Table I. The normal fatty acids used as starting materials were commercial products; the branched-chain fatty acids were prepared by Kolbe synthesis as before (Kaneda, 1963a). All products were purified by preparative gas–liquid partition chromatography.

TABLE I: Combinations of Starting Acids for Kolbe Synthesis of Hydrocarbons.

Normal Hydrocarbons	Branched-Chain Hydrocarbons
n-10[a], *n*-15 → *n*-C_{23}[c]	ante-*i*-6[a] di-10[b] → ante-*i*-14
n-12, *n*-14 → *n*-C_{24}	
n-12, *n*-15 → *n*-C_{25}	ante-*i*-14, *n*-18 → ante-*i*-C_{30}[c]
n-14, *n*-14 → *n*-C_{26}	
n-14, *n*-15 → *n*-C_{27}	
n-15, *n*-15 → *n*-C_{28}	*i*-7,[a] di-10 → *i*-15
n-15, *n*-16 → *n*-C_{29}	
n-16, *n*-16 → *n*-C_{30}	*i*-15, *n*-18 → *i*-C_{31}[c]
n-16, *n*-17 → *n*-C_{31}	
n-17, *n*-17 → *n*-C_{32}	
n-17, *n*-18 → *n*-C_{33}	
n-18, *n*-18 → *n*-C_{34}	
n-17, *n*-20 → *n*-C_{35}	
n-18, *n*-20 → *n*-C_{36}	
n-17, *n*-22 → *n*-C_{37}	

[a] *n*-10, decanoic acid; ante-*i*-6,3-methylpentanoic acid; *i*-7,5-methylhexanoic acid. [b] di-10, methylhydrogen sebacate. [c] *n*-C_{23}, tricosane; ante-*i*-C_{30}, 3-methylnonacosane; *i*-C_{31}, 2-methyltriacontane.

Mass Spectrometry. Mass spectrograms were made by a AEI-Model MS9 (Associated Electrical Industries Ltd., Manchester, England) at the Department of Chemistry, University of Alberta, Edmonton, Alberta, Canada.

Melting Point. A Fisher-Johns melting point apparatus (Fisher Scientific Co.) was used to measure melting point (uncorrected).

X-Ray Diffraction Analysis. The maximum spacings were measured as before (Kaneda, 1963a).

Infrared Spectroscopy. A Perkin-Elmer Model 221 was used and the micro KBr pellet sample was prepared as before (Kaneda, 1963a).

Optical activity of tobacco hydrocarbon samples was measured in *n*-heptane by a Rudolph no. 80 polarimeter.

Measurement of Radioactivity. Tobacco hydrocarbon samples were plated on an aluminum planchet with chloroform as solvent and the radioactivity counted by a gas-flow Geiger–Müller counter. The sample, in the range 0.1–0.2 mg/planchet, was weighed by a microbalance. No correction for self-absorption was made.

Results

Identification of Tobacco Hydrocarbons. Since the original tobacco wax extracts were chromatographed on an aluminum column after H_2SO_4 treatment, the first eluent from the column would be expected to contain

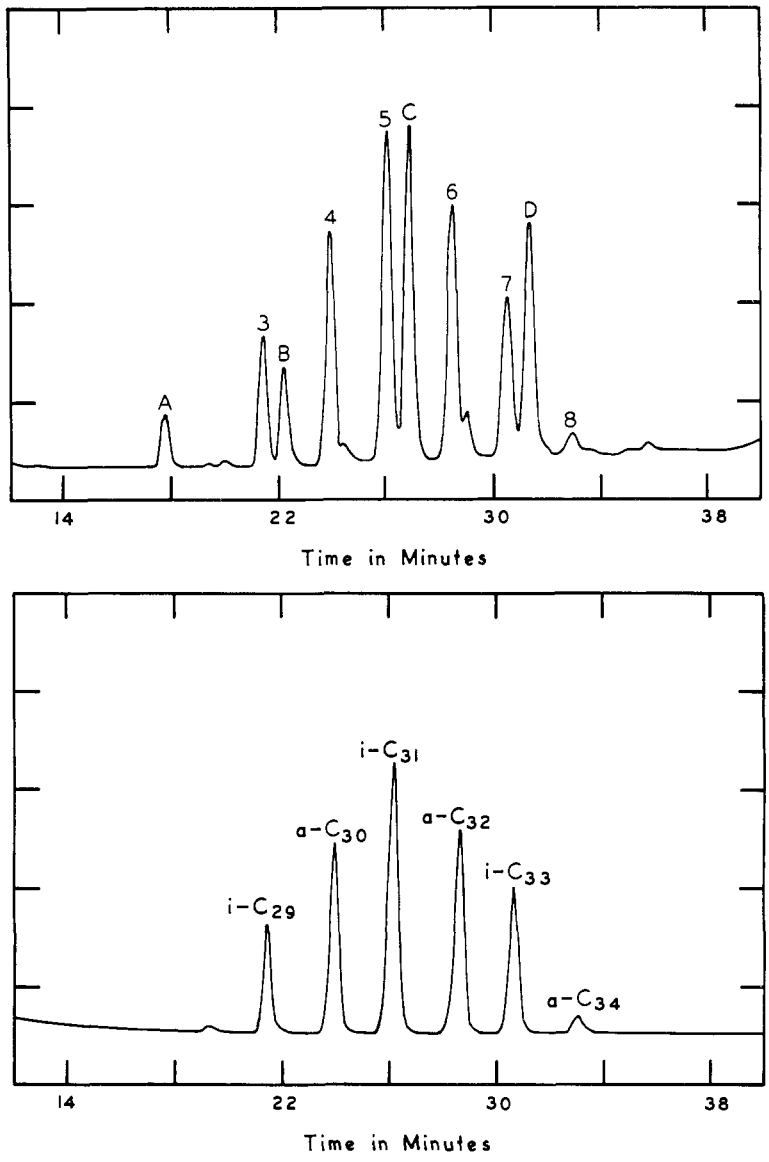
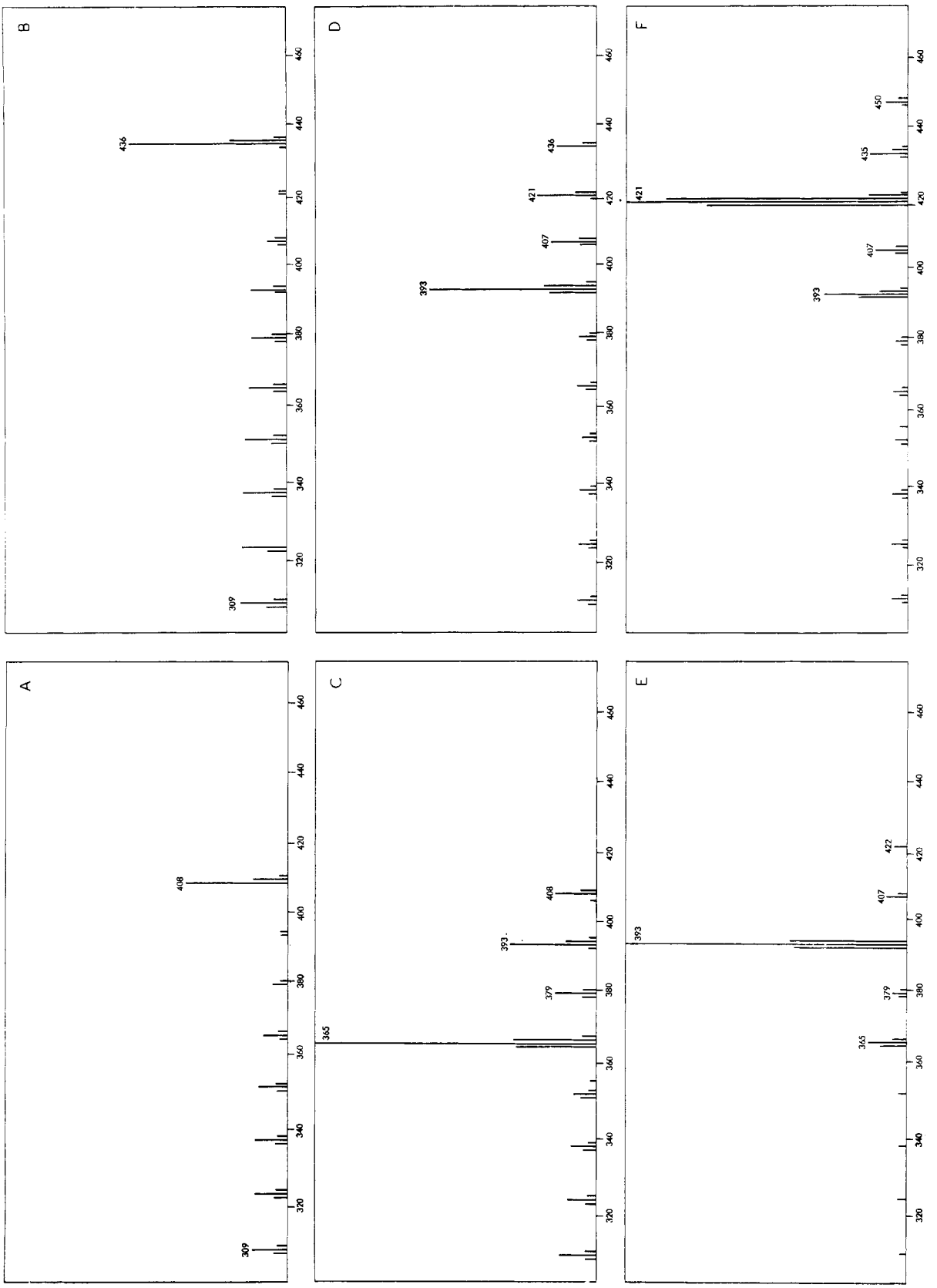


FIGURE 1: Gas–liquid partition chromatograms of tobacco long-chain hydrocarbons. A sample as indicated below in 2 μl of isooctane was injected through the heated pot, 300°. A 1% SE-30 column, $^1/_8$ in. × 6 ft. regular copper tube, was held at 175° for 2 min, then programmed to 275° with a rate of 2°/min, and held until all the expected peaks came off. Helium was used as carrier gas at a flow rate of 30 ml/min. (A) (top) A sample of 74 μg passed through alumina column only, then separated by gas–liquid partition chromatography. (B) (bottom) A sample of 18 μg passed through alumina column and Molecular Sieve 5A, then separated by gas–liquid partition chromatography.

only hydrocarbons or similar compounds. Figure 1A shows the gas–liquid partition chromatogram of the eluent thus obtained. The major peaks A–D had retention volumes identical with those of chemically synthesized *n*-C_{27}, *n*-C_{29}, *n*-C_{31}, and *n*-C_{33}, respectively. These components are completely removed when the eluent is treated with Molecular Sieve 5A (as shown in Figure 1B), further evidence that they are straight-chain hydrocarbons. The peaks retained in the solution after the Molecular Sieve 5A treatment are designated as 3–8 in the increasing order of the retention volume. The fraction eluted from the Molecular Sieve 5A and the fraction remaining in the solution without being absorbed which melted at 55°, were further fractionated into their components by preparative gas–liquid partition chromatography on a SE-30 column. The carbon skeleton of each component thus isolated was elucidated by mass spectroscopy and its further confirmation was carried out by melting point, X-ray diffraction analysis, and infrared spectroscopy.

The mass spectrograms of the fractions B and C (Figure 2A,B) show, in each case, a series of weak peaks with a decreasing intensity toward the more intense peak of mass number 408 and 436, respectively. This indicates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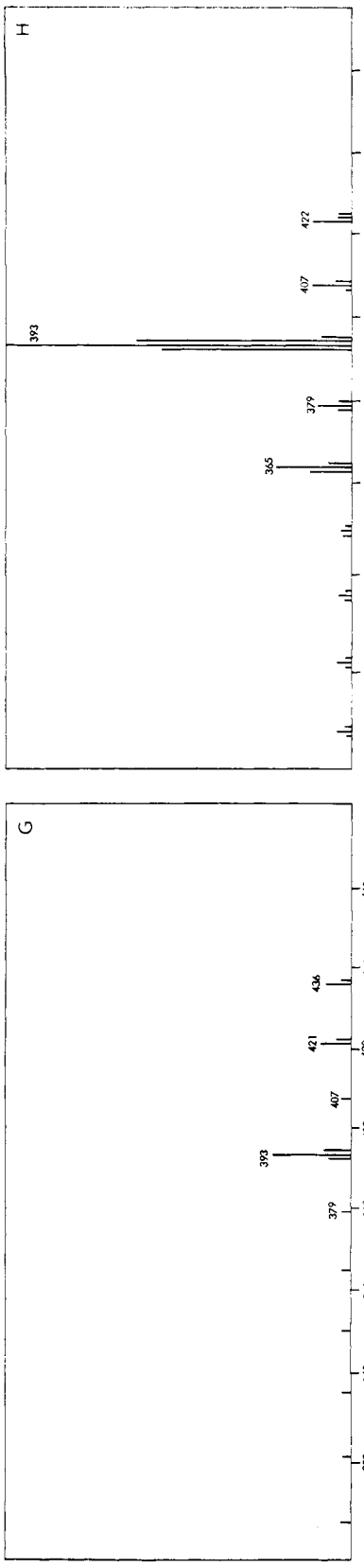


FIGURE 2: Characteristic portions of mass spectrometric patterns (MS 9,70V) for selected tobacco hydrocarbons compared with patterns for chemically synthesized samples of the corresponding hydrocarobns. Patterns A–E are fractionsB (n-C_{29}), C (n-C_{31}), 3 (i-C_{29}), 5 (i-C_{31}), 4 (ante i-C_{30}), and 6 (ante-i-C_{32}), respectively, shown in Figure 1A. Patterns G and H are chemically synthesized samples of 2-methyltriacontane (i-C_{31}) and 3-methylnonacosane (ante-i-C_{30}), respectively.

that they are n-C_{29} and n-C_{31} hydrocarbons. Similarly, the fractions A and D, were found to be n-C_{27} and n-C_{33} hydrocarbons. The mass spectrograms of the fractions 3 and 5 (Figure 2C,D) show a weak peak with the largest mass number of 408 and 436, respectively (the parent peaks), indicating that they are C_{29} and C_{31} hydrocarbons. A weak peak due to loss of one methyl group and an intensified peak due to a loss of a propyl group (presumably isopropyl group), in both cases, are indicative that they are 2-methylalkanes, namely, i-C_{29} and i-C_{31} hydrocarbons. Similarly, the mass spectrograms of fractions 4 and 6 (Figure 2E,F) indicate that they are C_{30} and C_{32} hydrocarbons, respectively. An intensified peak, owing to loss of an ethyl group from the parent molecule, in both cases, is indicative that they are 3-methylalkanes, namely, ante-i-C_{30} and ante-i-C_{32} hydrocarbons. The appearance of an intensified peak, owing to loss of a butyl group (presumably isobutyl group) and two weak peaks, owing to loss of methyl or propyl groups, with both samples are additional proof. The mass spectrograms of the chemically synthesized i-C_{31} hydrocarbon and ante-i-C_{30} hydrocarbon (Figure 2G,H) being identical with those of the tobacco samples, respectively, support further their identity.

Data on melting points from three different sources, the observed values of the tobacco sample and of the chemical sample, and the reported value are listed in Table II. There is no significant discrepancy among them.

Maximum spacings of the tobacco hydrocarbons are also listed in Table III. The data on the normal hydrocarbons, n-C_{27}, n-C_{29}, and n-C_{31}, isolated from the tobacco, are in good agreement with those of samples prepared chemically. The reported values are also in good agreement with the values mentioned above. One each of the chemically synthesized iso hydrocarbon and anteiso hydrocarbon (i-C_{31} and ante-i-C_{30}) was compared with the corresponding tobacco hydrocarbon samples. In the case of iso hydrocarbons, the maximum spacing of the tobacco i-C_{31} hydrocarbon was found to be in reasonably good agreement with that of the chemically synthesized i-C_{31} hydrocarbon. The tobacco i-C_{29} hydrocarbon gave 37.5 A, 2.4 A shorter than the homolog with two more methyl groups. This agrees well with a regular increment of 1.25 A/methylene group found among the normal hydrocarbon series (Clark, 1955). The difference between the maximum spacing of the tobacco ante-i-C_{30} and the ante-i-C_{32} is 2.8 A, also in fairly good agreement with the increment among the homologous series mentioned above. The chemically synthesized ante-i-C_{30} hydrocarbon, however, gave a much shorter spacing (40.1 A) in comparison with the corresponding tobacco sample. This may be due to the fact that the tobacco anteiso hydrocarbon, but not the chemical sample, is optically active; this was also shown to be the case with the anteiso fatty acids (Kaneda, 1963a).

The infrared spectra, in the range 800–1400 cm^{-1}, of three representative tobacco hydrocarbon samples, n-C_{31}, i-C_{31}, and ante-i-C_{30}, shown in Figure 3, are in good agreement with those of the corresponding chemically synthesized samples.

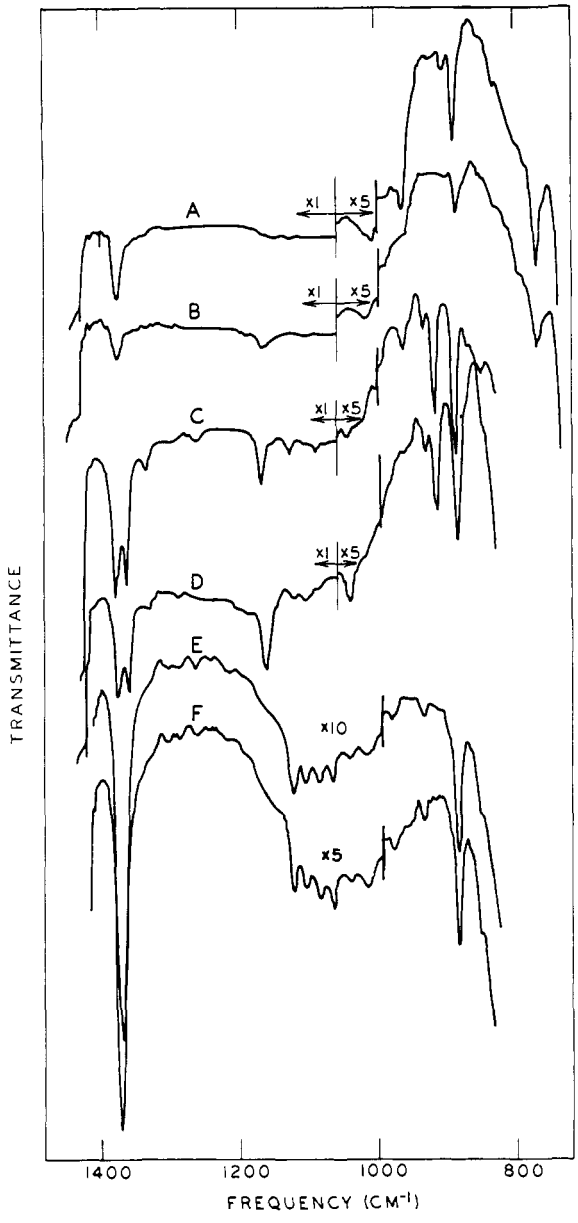


FIGURE 3. Characteristic portions of infrared spectra for selected tobacco hydrocarbons compared with spectra for chemically synthesized samples of the corresponding hydrocarbons. Spectra A, C, and E are fractions 4 (ante-*i*-C_{30}), 5 (*i*-C_{31}), and C (*n*-C_{31}), respectively, shown in Figure 1A. Spectra B, D, and F are chemically synthesized samples of 3-methylnonacosane (ante *i*-C_{30}), 2-methyltriacontane (*i*-C_{31}), and *n*-hentriacontane (*n*-C_{31}).

Thus all the data presented are consistent with the identifications given above, and are in agreement with the identification reported by Mold *et al.* (1963) for the tobacco hydrocarbons. Significant difference in the observed relative mass spectral abundances can be attributed to differences of the instruments; they used a time of flight mass spectrometer.[2] The hydrocarbons identified are *n*-C_{27} to *n*-C_{35} inclusive, *i*-C_{27}, *i*-C_{29}, *i*-C_{31}, *i*-C_{33}, *i*-C_{35}, ante-*i*-C_{28}, ante-*i*-C_{30}, ante-*i*-C_{32}, and ante-*i*-C_{34}. The total hydrocarbons were about 0.1% of the dry weight of the plant.

A mixture of the tobacco branched-chain hydrocarbons before fractionation but recrystallized twice from acetone gave a specific rotation of −1.9 in *n*-heptane. This sample was composed of anteiso series of hydrocarbons (43.7%) and iso series hydrocarbons (56.3%). Consequently the average specific activity of the anteiso series hydrocarbons is calculated at −4.4.

Incorporation of Branched-Chain Amino Acids and Acetate into Hydrocarbons by the Tobacco Plant. When L-valine, L-threonine, L-isoleucine, or L-leucine labeled at the carboxyl carbon were administered to the tobacco plant for 1 month, no radioactivity was detected in the long-chain hydrocarbon fraction. On the other hand, any one of these amino acids labeled uniformly, was incorporated into the long-chain hydrocarbon fraction to an extent of 0.1% of the total added radioactivity (Table IV). In all cases the specific activity of the branched series was higher than that of the normal series (Table V). The selectivity of the incorporation of these substrates into the specific branched series hydrocarbons was in increasing order, valine, isoleucine, threonine, and leucine. Attempts to minimize the nonspecific incorporation of uniformly labeled amino acids into the normal series without substantial reduction of incorporation into the branched series by shortening the time of administration have been unsuccessful.

Distribution of Radioactivity among Branched-Chain Hydrocarbons Derived from Amino Acid Substrates. The radioactive branched-chain hydrocarbons produced from the amino acid substrates, L-valine, L-threonine, L-isoleucine, or L-leucine, were fractionated by means of gas–liquid partition chromatography as already described. Table VI shows the specific activity of each fraction thus obtained.

The incorporation of L-valine into the iso series, *i*-C_{31} and *i*-C_{33}, was about twice as great as into the anteiso series, ante-*i*-C_{30} and ante-*i*-C_{32}. L-Threonine and L-isoleucine were both incorporated mainly into the anteiso series. Incorporation of L-leucine into certain iso series will be discussed in the following section.

Discussion

The metabolic pathways shown in Scheme I are postulated for the biosynthesis of iso and anteiso hydrocarbons. The three steps of the a, b, and c series, and steps d and e at the top of the scheme, are adapted from the

[2] By use of MS-9, the parent peak is found to be highest only when the normal series of hydrocarbons is analyzed. The iso- and the anteiso series, however, gave the highest peak corresponding to loss of C_3H_7 and C_2H_5 from the respective parent peaks. Accordingly, the mass spectrogram of the iso series obtained by MS-9, particularly, somewhat resembles that of the 5-methyl series obtained by a Bendix instrument (time of flight) (Mold *et al.*, 1963). The use of the chemically synthesized iso hydrocarbon in the present work has clearly eliminated this apparent confusion as shown in Figure 2.

TABLE II: Melting Points of Long-Chain Hydrocarbons.

	Melting Temperature of Peak Fraction						
	A	B	C	3	5	4	6
Hydrocarbon	*n*-C_{27}	*n*-C_{29}	*n*-C_{31}	*i*-C_{29}	*i*-C_{31}	ante-*i*-C_{30}	ante-*i*-C_{32}
Tobacco sample	59–60	64–65	67.5–68.6	52.5–54	58.5–59.5	52.5–53	56–56.5
Standard sample	59–60	64–65	67–68	–	59–60	55–56	–
Reported	59[a]	64[a]	68[a]	–	61[b]	55[b]	57[b]

[a] The values of the chemically synthesized samples, Piper *et al.* (1931). [b] The values of the tobacco samples, Mold *et al.* (1963).

TABLE III: Longest Interplanar Spacing of Long-Chain Hydrocarbons.

	Spacings in Angströms of Hydrocarbon Fraction						
	A	B	C	3	5	4	6
Hydrocarbon	*n*-C_{27}	*n*-C_{29}	*n*-C_{31}	*i*-C_{29}	*i*-C_{31}	ante-*i*-C_{30}	ante-*i*-C_{32}
Tobacco sample[a]	36.8	38.8	41.9	37.5	39.4	40.8	43.9
Standard sample[a]	36.4	39.2	41.3	–	39.9	40.1	–
Reported[b]	37.1	39.4	41.6	–	–	–	–

[a] These values were the average of second, third, and fourth diffractions, each of which agreed within ±0.2 A. [b] Müller and Saville (1925); Piper *et al.* (1925).

TABLE IV: Incorporation of Branched-Chain Amino Acids and Acetate into the Total Hydrocarbons.

Substrate[a]	μg	Plant (dry wt g)	Total Hydrocarbon[b] (mg)	Radioactivity Incorp (cpm × 10^{-3})	Incorp (%)
L-[$^{14}C_5$]Valine	29	242	197	8	0.02
L-[$^{14}C_4$]Threonine	37	342	234	22	0.06
L-[$^{14}C_6$]Isoleucine	26	434	419	18	0.05
L-[$^{14}C_6$]Leucine	29	385	273	34	0.09
[$^{14}CH_3$]Acetate	2500	288	309	180	0.50

[a] The radioactive substrate (50 μg) in the amount indicated, was dissolved in 1 ml of water and was administered to the growing tobacco plant. [b] The surface waxes extracted with chloroform were treated with concentrated H_2SO_4, and were washed with water to neutral.

scheme for the biosynthesis of branched-chain fatty acids presented in earlier papers (Kaneda, 1963b,c, 1966). Steps a_4, b_4, and c_4 are in accord with the generally accepted hypothesis that there is a close relationship between fatty acid and hydrocarbon metabolism, and that the common fatty acids, myristic, palmitic, and stearic, or biologically equivalent forms, are involved as precursors at a certain stage in the biosynthesis of long-chain hydrocarbons (C_{25}–C_{35}) (Channon and Chibnall, 1929; Chibnall and Piper, 1934; Wanless *et al.*, 1955). Similarly, branched-chain fatty acids with carbon numbers in the range 14–18 would be involved in the biosynthesis of branched-chain hydrocarbons. These fatty acids have been found in tobacco (Mold *et al.*, 1966). Evidence supporting each step is now considered in turn.

Initially, the two steps at the top, steps a_1, a_2, b_1, b_2, c_1, and c_2 are discussed. Any one of the uniformly labeled amino acid substrates, L-valine- L-leucine, or L-isoleucine when administered to the tobacco plant, is incorporated into the specific long-chain hydrocarbons which are structurally related to the amino acid substrate. On the other hand, none of the carboxyl-labeled amino acid

TABLE V: Specific Activities in the Long-Chain Hydrocarbons Derived from the Radioactive Substrates.

Substrate	Total Hydrocarbons[a] (cpm/mg)	Long-Chain Hydrocarbons[b] (cpm/mg)			
		Original	Branched	Normal	Branched: Normal
L-[$^{14}C_5$]Valine	42	36	75	47	1.6
L-[$^{14}C_4$]Threonine	93	116	215	38	5.7
L-[$^{14}C_6$]Isoleucine	43	66	264	47	5.6
L-[$^{14}C_6$]Leucine	125	91	120	14	8.6
[$^{14}CH_3$]Acetate	580	1014	1080	948	1.1

[a] The samples are the same as the ones described in Table IV. [b] The total hydrocarbons were chromatographed on an aluminum column to isolate the first eluent which was designated as the long-chain hydrocarbon fraction.

substrates mentioned above is incorporated into the long-chain hydrocarbons. These results are essentially identical with those observed in the incorporation of L-valine, L-leucine, or L-isoleucine into specific branched-chain fatty acids by *Bacillus subtilis* (Kaneda, 1963b,c, 1966), and *Micrococcus lysodeikticus* (Lennarz, 1961), *i.e.*, the amino acid substrate administered is first deaminated by steps a_1, b_1, or c_1 to form the α-keto acid which is oxidatively decarboxylated according to steps a_2, b_2, or c_2 to yield the corresponding branched-chain acyl-CoA[3] ester.

The elongation of the acyl-CoA ester, thus produced, by steps a_3, b_3, or c_3 is carried out by a mechanism similar to that demonstrated in the partially purified mammalian system, malonyl-CoA dependent, for palmitic acid synthesis (Horning *et al.*, 1961).

α-Ketobutyrate is known to be a precursor in the biosynthesis of L-isoleucine (Radhakrishnan and Snell, 1960). If this is also true in the tobacco plant, L-threonine, after conversion to α-keto-β-methylvalerate *via* α-ketobutyrate, should be incorporated into the same hydrocarbons into which L-isoleucine is incorporated. This hypothesis is supported by experiment. L-[$^{14}C_4$]-Threonine is incorporated mainly into ante-*i*-C_{30} and ante-*i*-C_{32} hydrocarbons with an efficiency similar to that of L-[$^{14}C_6$]isoleucine.

Similarly, α-ketoisovalerate, the keto acid corresponding to L-valine, is known to be a precursor in synthesis of L-leucine in organisms (Calvo *et al.*, 1962; Jungwirth *et al.*, 1961; Strassman and Ceci, 1962), and should be incorporated into the same hydrocarbons as L-leucine. In this case, however, L-[$^{14}C_5$]valine was incorporated into *i*-C_{31} and *i*-C_{33} hydrocarbons only, not into *i*-C_{30} and *i*-C_{32} hydrocarbons (Table VI). Since the radioactivity derived from L-[$^{14}C_5$]valine is found not only in the branched series but also in the normal series (Table V), considerable fragmentation of the amino acid during its administration over a period of a month must have occurred, with nonspecific incorporation of the fragments into the normal series. Consequently, little α-ketoisovalerate derived from the L-valine substrate would be available to the system of L-leucine synthesis.

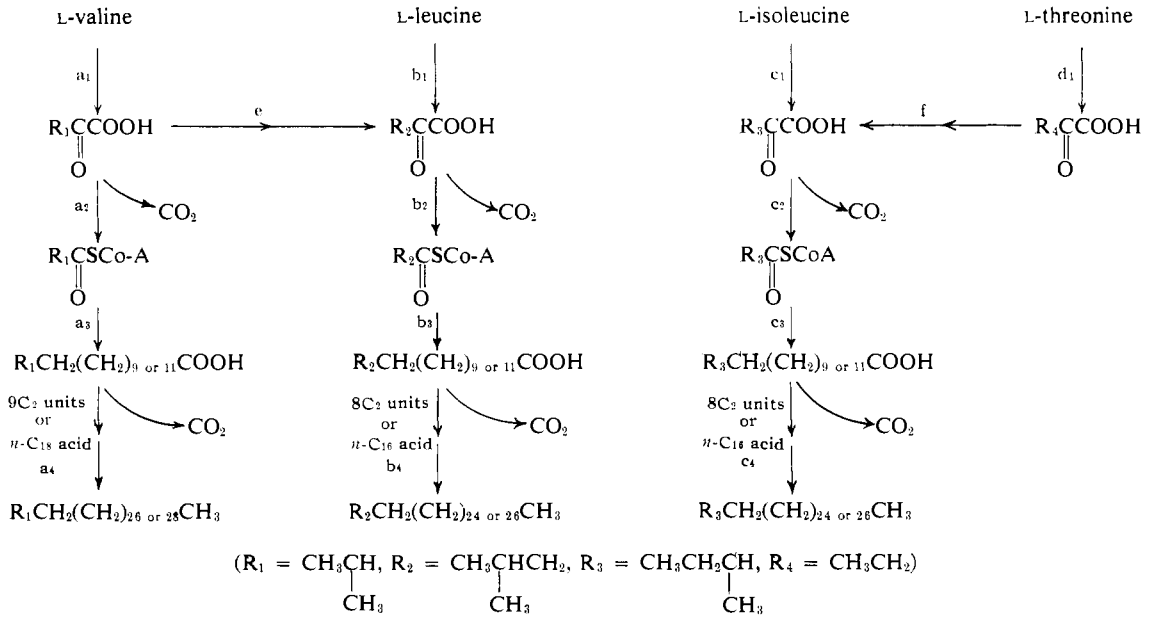
The experimental observation seems to indicate that L-leucine is nonspecifically incorporated into various fractions of the branched-chain hydrocarbons (Table VI). The slight incorporation of L-[$^{14}C_6$]leucine into ante-*i*-C_{30} and ante-*i*-C_{32} could be explained by assuming that the amino acid substrate is actually incorporated into *i*-C_{30} and *i*-C_{32} hydrocarbons which are present as contaminants of ante-*i*-C_{30} and ante-*i*-C_{32} fractions as

TABLE VI: Distribution of Radioactivity among Branched-Chain Hydrocarbons Isolated from L-[$^{14}C_5$]Valine, L-[$^{14}C_4$]Threonine, L-[$^{14}C_6$]Isoleucine, or L-[$^{14}C_6$]Leucine Experiments.

Added Substrate	Hydrocarbon Fraction[a] from Glpc (cpm/mg)			
	ante-*i*-C_{30}	*i*-C_{31}	ante-*i*-C_{32}	*i*-C_{33}
L-[$^{14}C_5$]Valine	81[b]	170	136	229
L-[$^{14}C_4$]Threonine	900[c]	119[c]	653[c]	144[c]
L-[$^{14}C_6$]Isoleucine	683	159	745[b]	189
L-[$^{14}C_6$]Leucine	160[b]	110[b]	170[b]	136[b]

[a] Abbreviations used: ante-*i*-C_{30}, 3-methylnonacosane; *i*-C_{31}, 2-methyltriacontane; ante-*i*-C_{32}, 3-methylhentriacontane; and *i*-C_{33}, 2 methyldotriacontane. Most samples are recrystallized from acetone. [b] These fractions, weighing less than 0.3 mg after three or four consecutive gas–liquid partition chromatographies (glpc), are not recrystallized. [c] These fractions (0.2–0.4 mg) were crystallized twice from acetone after addition of carrier hydrocarbons (1.0–1.4 mg). No significant change in specific activity is observed at each of three stages.

[3] Abbreviations used: acyl-CoA, acyl coenzyme A; malonyl-CoA, malonyl coenzyme A.



SCHEME I: Metabolic pathways for branched-chain hydrocarbon synthesis.

isolated. According to this hypothesis, the actual specific activity of *i*-C_{30} and *i*-C_{32} is more than ten times higher than the apparent values experimentally obtained for ante-*i*-C_{30} and ante-*i*-C_{32}.

The final step to the hydrocarbon synthesis (a_4, b_4, and c_4) involves either elongation by successive C_2 groups followed by decarboxylation or else condensation with a C_{16} or C_{18} straight-chain acid (the most abundant fatty acids), also with decarboxylation (see, for example, Eglington and Hamilton, 1963). The present data do not permit any differentiation between these two routes but additional experiments toward this end are in progress.

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5-Pregnene-3β,20α-diol-3-sulfate-20-(2′-acetamido-2′-deoxy-α-D-glucoside) and 5-Pregnene-3β,20α-diol-3,20-disulfate. Two Novel Urinary Conjugates*

Martha Arcos and Seymour Lieberman

ABSTRACT: Two new crystalline conjugates have been isolated from the urine of normal human subjects to whom pregnenolone was administered. One of the compounds was the 5-pregnene-3β,20α-diol-20-(2′-acetamido-2′-deoxy-α-D-glucoside). Its structure was proved by elemental analysis, infrared spectra, nuclear magnetic resonance (nmr) spectra, and the products formed by hydrolysis. Mild acid hydrolysis yielded 5-pregnene-3β,20α-diol and *N*-acetyl-D-glucosamine. Treatment with commercial beef glucuronidase yielded the known pregnenediol-3-monosulfate, thus locating both conjugating groups. Solvolysis gave the desulfated *N*-acetylhexosaminide from whose optical rotatory dispersion (ORD) curve the α configuration of the glycosidic bond was established. Thus, for the first time, a steroid metabolite conjugated with *N*-acetylglucosamine has been isolated from human urine. The results show that cleavage by crude glucuronidase preparations cannot itself offer proof of structure of conjugates. The second new metabolite of pregnenolone was the disulfate of pregnenediol. Of the pregnenolone produced *in vivo*, 8% is excreted as these two conjugates.

In an attempt to evaluate the influence that the secretion of pregnenolone and its sulfate would have upon the estimation of the secretory rate of progesterone, experiments were performed in which tracer doses of ^{3}H-labeled pregnenolone were administered intravenously to normal subjects (Arcos *et al.*, 1964). The experimental design required that the urinary metabolites be isolated as conjugates so that the sulfates could ultimately be separated from the glucuronides. For this purpose, the mixture of urinary conjugates was chromatographed on Celite using systems which previously (Siiteri *et al.*, 1963) had been shown capable of effecting this separation. In the course of these analyses, a ^{3}H-labeled conjugate was found in fractions of the chromatogram which were eluted much later than those containing the monoglucuronides. (The monosulfates are usually found in these chromatograms in fractions preceding those containing the monoglucuronides.) Our interest in this polar material was further aroused when preliminary experiments revealed that the steroid moiety of this conjugate could not be liberated in free form by either Ketodase hydrolysis or by solvolysis. The following results will serve as an illustration. Subsequent to the intravenous administration of a tracer dose of ^{3}H-labeled pregnenolone, a polar fraction representing 8% of the injected tritium was obtained by chromatography. Attempts to solvolyze this fraction (with $HClO_4$ in tetrahydrofuran) liberated only 8% of the ^{3}H as ether-soluble material. The aqueous-soluble conjugated fraction was then treated with Ketodase (incubation for 4 days) but this produced only an additional 28% of the radioactivity. Finally, the remaining conjugated material was hydrolyzed by boiling with HCl and this process converted about 40% of the radioactivity originally present into an ether-soluble form. Although this conjugate is not a glucuronide (*vide infra*), it is partially hydrolyzed by the commercial preparation of β-glucuronidase (Ketodase) and thus, it was evident that the bulk of the radioactivity found in this polar fraction (8% of the injected dose) was present as a conjugate possessing unusual hydrolytic properties. The present paper describes the isolation of this substance and the experiments which lead to the assignment of its structure as 5-pregnene-3β,20α-diol-3-sulfate-20-(2′-acetamido-2′-deoxy-α-D-glucoside) (II) (Figure 1).

Since pregnenolone is probably not produced by normal individuals in amounts that exceed a few

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