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THE HIGHER FATTY ACIDS OF FLUE-CURED TOBACCO

METHYL AND CYCLOHEXYL BRANCHED ACIDS

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Abstract—The principal free and combined higher fatty acids of flue-cured (Bright) tobacco have been shown to contain sixteen or eighteen carbon atoms. Seventy-six per cent of the total acids were found to be relatively non-polar saturated or olefinic types with palmitic acid comprising 20·7%, stearic acid (2·1%), oleic acid (3·5%), linoleic acid (5·8%) and linolenic acid (26·2%). Ninety per cent of the total acids were shown to have unbranched structures of 10–34 carbon atoms. Only 4·1% were homologous monomethyl or cyclohexyl substituted compounds and 5·9% had more complex branched structures. The saturated hydrocarbons derived from the simply branched acids proved to be homologs of 2-methyl and 3-methyl isomers with 15–26 carbons atoms and 1-cyclohexyl isomers with 22–25 carbon atoms.

PREVIOUS reports concerning the higher fatty acids of tobacco have included only the identification of the major saturated and unsaturated compounds of the *normal* series, although it was speculated that branched higher fatty acids are also present in small amounts.[1] Significant quantities of the branched lower fatty acids are known to be present in tobacco and to contribute markedly to its aroma.[2]

We have noted the presence of considerable amounts of 2-methyl and 3-methyl paraffins in the wax of Bright, Burley and Turkish tobaccos.[3] These homologous series of branched paraffins constituted 30–46 per cent of the total paraffin mixture. In view of the relative abundance of these branched paraffins, we felt it to be important to establish the quantity and nature of the branched higher fatty acids of tobacco.

Our approach to this problem was to convert the total fatty acids from tobacco into the corresponding saturated hydrocarbons of the same skeletal arrangement. By this procedure, evidence regarding the nature of the branching could be obtained without complications due to the presence of unsaturated or hydroxy acids. The procedure used for this conversion involved the catalytic reduction of the unsaturated methyl esters with hydrogen over Pt, reduction of the saturated esters to alcohols with $LiAlH_4$, conversion of these alcohols to iodides, and subsequent reduction of the iodides with $LiAlH_4$ to yield the saturated hydrocarbons.[4]

The mixture of hydrocarbons was resolved by procedures similar to those previously described for the separation of tobacco paraffins.[3] *Normal* hydrocarbons were removed by inclusion into 5A molecular sieve. The branched isomers were separated into two groups by

[1] A. P. SWAIN and R. L. STEDMAN, *J. Assoc. Offic. Agr. Chemists* **45**, 536 (1962).
[2] I. ONISHI and K. YAMASAKI, *Bull. Agr. Chem. Soc. Japan* **21**, 82 (1957); Y. KABURAKI and Y. SATO, *Nippon Nôgei kagaku Kaishi* **36**, 865 (1962); I. SCHEMLTZ, R. L. MILLER and R. L. STEDMAN, *J. Assoc. Offic. Agr. Chemists* **46**, 779 (1963).
[3] J. D. MOLD, R. K. STEVENS, R. E. MEANS and J. M. RUTH, *Biochemistry* **2**, 605 (1963).
[4] D. T. DOWNING, Z. H. KRANZ and K. E. MURRAY, *Australian J. Chem.* **13**, 80 (1960).

treatment with urea under conditions for complex formation. Gas–liquid chromatography of these fractions then allowed the separation and quantitative estimation of the individual homologues. Structural identifications for the branched compounds were based on gas–liquid chromatographic and mass spectrometric measurements.

RESULTS AND DISCUSSION

The total amount of free and bound higher fatty acids recovered from a typical blend of aged Bright (flue-cured) cigarette tobaccos was found to be 1·43 per cent. of the total dry wt. of the tobacco extracted. The gas–liquid chromatogram for the methyl esters of this fatty acid mixture is given in Fig. 1.

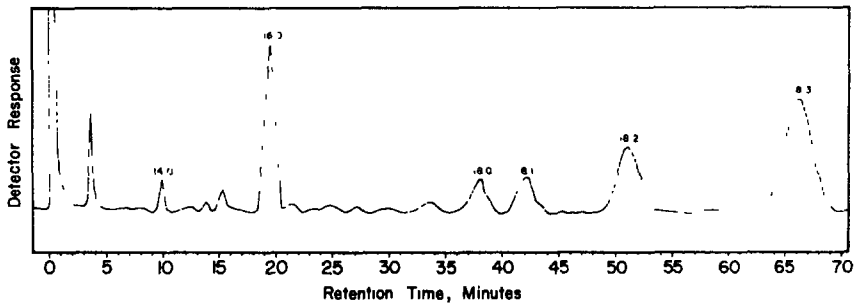

FIG. 1. GAS–LIQUID CHROMATOGRAM OF THE METHYL ESTERS OF THE HIGHER FATTY ACIDS OF BRIGHT TOBACCO.

Conditions used: A MicroTek 2500-DPFF instrument with dual hydrogen flame ionization detectors was used for the chromatographic separations (MikroTek Instruments, Inc., Oak Villa Blvd., Baton Rouge, La.); sample size 270 μg; $\frac{3}{16}$ in i.d. × 10 ft dual stainless steel columns containing 80–100 mesh Gas Chrom P coated with 20% butanediol succinate; helium pressure 38 lb/in.^{2}; flow rate 90 ml/min; column temperature 170° (isothermal); detector temperature 240°; inlet and exit temperatures 270°.

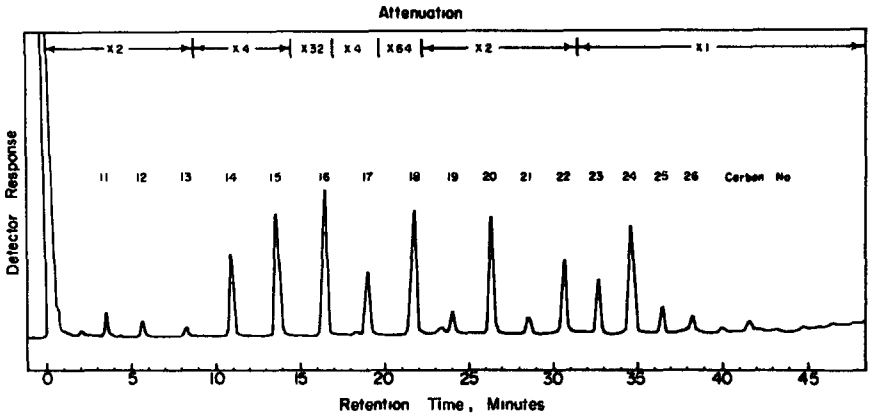

FIG. 2. GAS–LIQUID CHROMATOGRAM OF THE SATURATED NORMAL HYDROCARBONS DERIVED FROM THE HIGHER FATTY ACIDS OF BRIGHT TOBACCO.

Conditions used: MicroTek 2500-DPFF with dual hydrogen flame ionization detectors; sample size 176 μg; $\frac{1}{8}$ in i.d. × 6 ft dual stainless steel columns containing 80–100 mesh Gas Chrom P coated with 6% SE-30; helium pressure 35 lb/in.^{2}; flow rate, 60 ml/min; column temperature initially 100° C then programmed to 280° at 4°/min; detector temperature 260°; inlet and exit temperature 280°.

The less polar, saturated or olefinic esters were separated from the more polar compounds, including those having i.r. spectra indicative of hydroxy functions, by chromatography on silica gel. Seventy-six per cent of the esters recovered from the chromatogram were of the less polar types with the principal components being palmitic acid (20·7%), stearic acid (2·1%), oleic acid (3·5%), linoleic acid (5·8%) and linolenic acid (26·2%). These values are the percentages of the total isolated fatty acids of tobacco. Gas–liquid chromatography of this

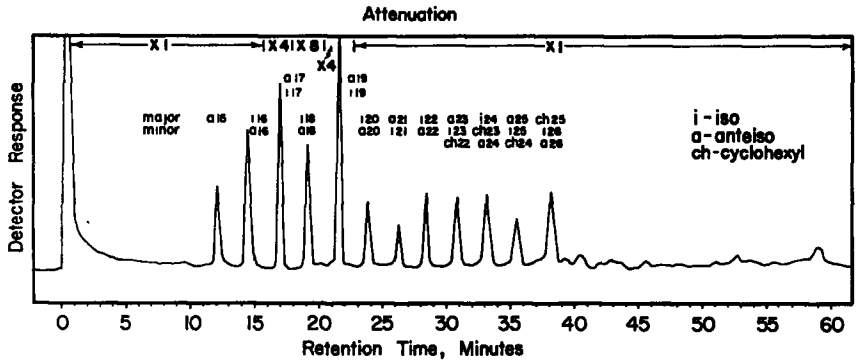

FIG. 3. GAS–LIQUID CHROMATOGRAM OF THE SIMPLY BRANCHED SATURATED HYDROCARBONS DERIVED FROM THE HIGHER FATTY ACIDS OF BRIGHT TOBACCO.

Conditions used as in Fig. 2.

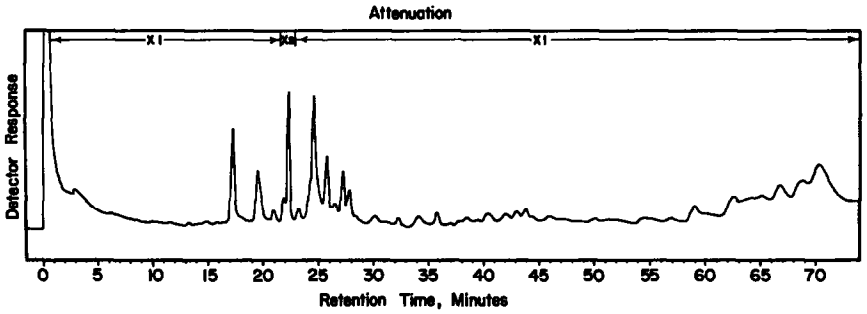

FIG. 4. GAS–LIQUID CHROMATOGRAM OF THE MORE HIGHLY BRANCHED SATURATED HYDROCARBONS DERIVED FROM THE HIGHER FATTY ACIDS OF BRIGHT TOBACCO.

Conditions used as in Fig. 2.

fraction following catalytic hydrogenation demonstrated that of the total non-hydroxy acids 1·5% were tetradecanoic acids, 2·3% were pentadecanoic, 27·6% hexadecanoic, 1·0% heptadecanoic, 56·5% octadecanoic, with less than 1% each of C-19 to C-22 acids.

Ninety per cent of the saturated hydrocarbons recovered from the mixture of higher fatty acids by the conversion procedure described were unbranched, *normal* paraffins (Fig. 2). Only 4·1 per cent were simply-branched compounds which formed urea complexes (Fig. 3) while the remaining 5·9 per cent appeared to be more highly branched or cyclic compounds (Fig. 4). The structures of these latter compounds have not been determined.

The urea-complexed compounds were shown to be a mixture of homologs of 2-methyl-, 3-methyl- and cycloalkyl-alkanes. These structural assignments were based on a comparison

of gas–liquid chromatographic retention values, i.r. and mass spectra with those for compounds of known, similar structures.[3] The mass spectral fragmentation patterns for several of the homologs present in greater amounts are given in Fig. 5. The very intense peaks representing ion fragments of *m/e* 82 and 83, coupled with the absence of any significant increase in the intensity of the *m/e* 68 and 69 peaks over those noted for the spectrum of the reference cyclohexyl alkane, demonstrated that the cycloalkyl alkanes derived from the tobacco fatty acids were cyclohexyl compounds with no observable quantity of cyclopentyl alkanes present. Confirmation of this assignment is given by the evidence for loss of a six-carbon fragment from the parent molecule. Comparison of our mass spectra with those of several isomeric cyclohexyl-eicosanes[5] indicates that these hydrocarbons are 1-cyclohexyl alkanes. Confirmatory evidence for the identity of cyclohexylalkanes was given by a comparison of the i.r. spectrum for known 1-cyclo-hexyleicosane with that for the suspected cyclohexylalkane. Absorption bands characteristic of the mono-substituted cyclohexyl group were observed at 888 cm^{-1} (CH_2 rocking) and at 843 cm^{-1} (C—C deformation).

Approximate compositions for the mixed fractions were calculated using the intensities for the $(M-43)^+$ peak as a measure of the 2-methyl compounds, the $(M-29)^+$ peak as a measure of the 3-methyl compounds, and the M^+ peak as a measure of the cyclohexyl compounds (Table 1). *n*-Hexadecane and *n*-octadecane comprised 27·1 and 53·4 per cent of the total

TABLE 1. RELATIVE PERCENTAGE COMPOSITION OF THE HYDROCARBONS DERIVED FROM FREE AND BOUND HIGHER FATTY ACIDS OF BRIGHT TOBACCO

Paraffin carbon numbers	Percentage of total derived hydrocarbons			
	Normal	2-Methyl	3-Methyl	1-Cyclohexyl
10	0·02			
11	0·18			
12	0·09			
13	0·05			
14	1·84			
15	2·61	0·02	0·09	
16	27·10	0·12	0·02	
17	1·35	0·10	0·54	
18	53·40	0·87	0 03	
19	0 27	0 06	0·83	
20	1·35	0·08	0 01	
21	1·35	0·02	0·04	
22	0·81	0 05	0 01	0·01
23	0·27	0·03	0·08	0 03
24	0·63	0·06	0 03	0 01
25	0·11	0·03	0·05	0 08
26	0·05	0 04	0·03	
27	0·02			
28	0·04			
29	trace			
30	trace			
31	trace			
32	0·08			
33	trace			
34	0·14			

[5] Mass Spectral Data, American Petroleum Institute Research Project 44, Petroleum Research Laboratory, Carnegie Institute of Technology, Pittsburgh, Pennsylvania.

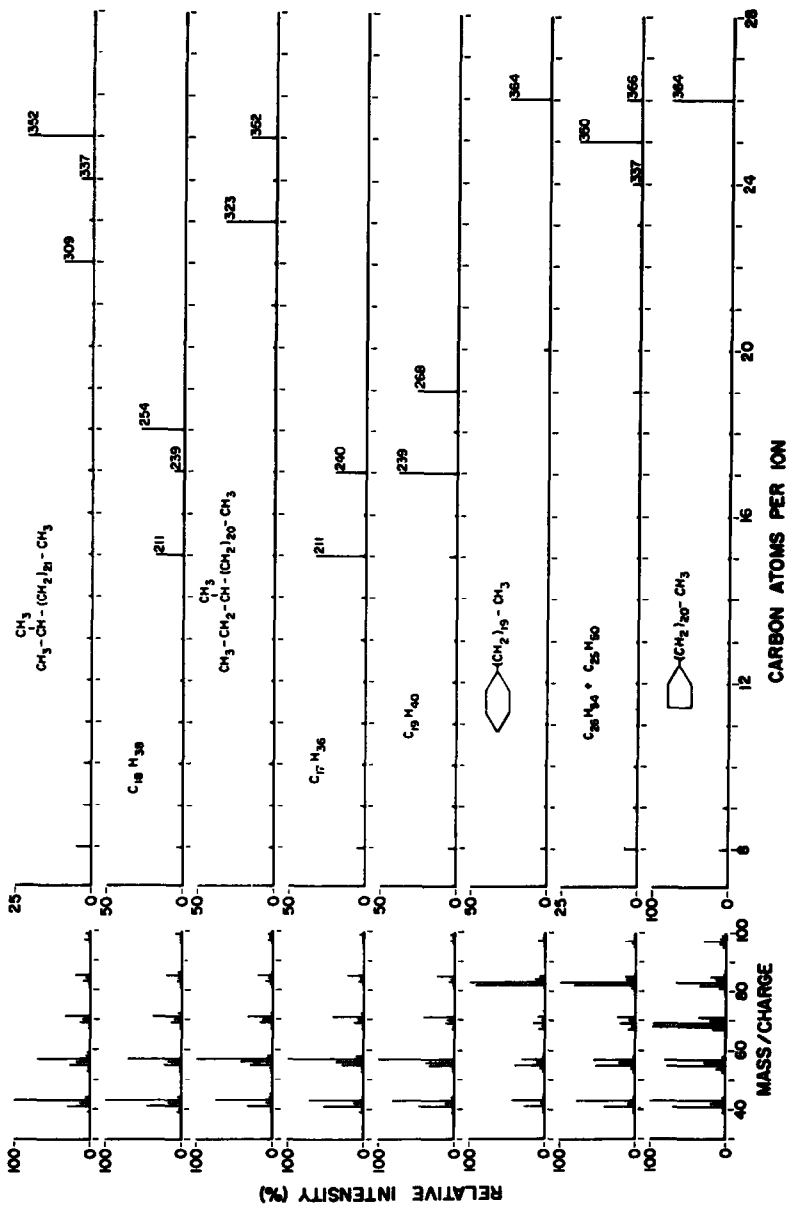

FIG. 5. MASS SPECTRA FOR SEVERAL REPRESENTATIVE COMPONENTS OF THE SIMPLY BRANCHED SATURATED HYDROCARBONS DERIVED FROM THE HIGHER FATTY ACIDS OF BRIGHT TOBACCO AND FOR SEVERAL MODEL COMPOUNDS.

Spectra were obtained with the Model 14-101 Bendix Time-of-Flight Mass spectrometer, equipped with with an S-14-105 ion source. The samples were introduced by means of a modified Bendix hot filament sample probe. The spectra of the model compounds are labelled with structural formulas, and those of the tobacco acid derivatives with empirical formulas.

derived hydrocarbons. For this to be the case, the major components of the more polar, hydroxy acids must also be hexadecanoic and octadecanoic acids. No clear-cut pattern of relative abundance was noted for the minor homologs of the *normal* hydrocarbons. Each carbon number was represented from C_{10} through to C_{34} in amounts from a trace to 2·6 per cent of the total mixture. The presence of homologs with less than ten carbon atoms was precluded by their volatility since no attempt was made to recover these.

For the branched isomers, the homologs with even numbers of carbon atoms were found to consist predominantly of the 2-methyl isomers, while those with odd numbers of carbon atoms were principally 3-methyl isomers. This is the reverse of what we have found for the tobacco paraffins.[3] It is, however, consistent with what has been reported for the branched fatty acids from other natural sources. The presence in wool wax of homologous series of *iso* fatty acids with even numbers of carbon atoms and of *anteiso* fatty acids with odd numbers of carbon atoms was first established by Weitkamp.[6] Other reports have noted the presence of these two series of acids in small amounts in butter fat,[7] ewe milk fat,[8] human sebum,[9] human milk[10] and human blood.[11] *Iso* and *anteiso* acids with 15–18 carbon atoms have been shown present in bacteria.[12]

There have been no definitive reports which have established the presence of these higher fatty acid isomers in plants. We have also been unable to find reports of cyclohexyl fatty acids in *any* natural fats and we did not observe any cyclic substituted alkanes in our investigation of tobacco paraffins. The present report concerns tobaccos which had been flue-cured and aged. However, since this treatment does not involve exposure to flue gases, the materials found in the cured tobacco are likely to have either been present in the green leaf or to have been formed by heat from some precursor in the leaf.

The 2-methyl and 3-methyl isomers are relatively minor constituents of the hydrocarbons derived from the tobacco higher fatty acids. It is of interest to note, however, that the major 2-methyl isomer is of identical carbon number to the C_{18} or major *normal* component while the major 3-methyl isomers have one more carbon atom than the major *normal* components C_{18} and C_{16}.

It should perhaps be reiterated that the present studies only define the carbon structure of these acids, since the conversion to hydrocarbons would be expected to replace functional groups and saturate any unsaturated bonds with hydrogen.

EXPERIMENTAL

Isolation of the Free and Combined Higher Fatty Acids from Flue-cured Tobacco

A blend of aged flue-cured tobaccos typical of those used as a component of American cigarettes was finely ground in a Wiley mill and extracted exhaustively in a Soxhlet extractor with methylene chloride and methanol. The methylene chloride extract from 412 g of tobacco was evaporated and the residue combined with the methanol extract. After adding an equal vol of water, the solution was extracted four times with equal vols of hexane. The

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[7] R. P. HANSEN and F. B. SHORLAND, *Biochem. J.* **50**, 207 (1951).
[8] T. GERSON, F. B. SHORLAND and C. R. BARNICOAT, *Biochem. J.* **68**, 644 (1958).
[9] A. T. JAMES and V. R. WHEATLEY, *Biochem. J.* **63**, 269 (1956).
[10] W. INSULL, JR. and E. H. AHRENS, JR., *Biochem. J.* **72**, 27 (1959).
[11] A. T. JAMES, J. F. LOVELOCK and J. P. W. WEBB, *Biochem. J.* **73**, 106 (1959).
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hexane extracts (2 l.) were washed three times with 1 l. portions of 1 N HCl. The hexane was concentrated to 100 ml.

To liberate the bound fatty acids this mixture was diluted with 105 ml benzene and refluxed for 30 min with 5·5 g of NaOH in 105 ml of 95% ethanol.[13] After cooling, the alcohol layer was diluted with two vols of water and the hexane: benzene layer was separated. The aqueous alcohol layer was further extracted five times with equal vols of hexane.

After acidification with 0·05 N HCl, the organic acids were extracted from the aqueous layer with three vols of hexane and three vols of ethyl ether. The ether and hexane extracts of crude higher fatty acids were evaporated to dyness (12 g, 2·9%) redissolved in wet ether, and applied to a column containing 400 ml of 100–200 mesh Dowex-1 (Dow Chemical Co.), in (OH^- form).[14] The resin was equilibrated with ethanol and with ether prior to use. After rinsing with ether, the organic acids were eluted with a solvent mixture composed of 120 ml 95% ethanol, 60 ml of 12 M HCl, and 280 ml of ethyl ether. The weight of acids recovered was 5·9 g, (1·43 per cent). The mixture of higher fatty acids was esterified with methanol using BF_3 as a catalyst.[15] The G.L.C. of the methyl esters is shown in Fig. 1.

Separation of the Methyl Esters of the Higher Fatty Acids into Classes by Chromatography on Silica Gel

A 762-mg portion of the methyl esters of tobacco higher fatty acids was applied to a column containing 26 g of 100–200 mesh air-dried silica gel which had been suspended in hexane: ether (85:15).[16] The chromatogram was developed with 120 ml of the same solvent mixture, to give the non-polar fraction (518 mg; 68%) and the column was then extensively washed with acetone and methanol to give the more polar hydroxy acid esters (160 mg; 21%).

Catalytic Reduction of the Methyl Esters of the Non-hydroxy Higher Fatty Acids

A 63·7 mg portion of the saturated and olefinic methyl esters separated as above was catalytically hydrogenated with Adam's Pt catalyst in acetic acid: cyclopentane (1:4). The i.r. spectrum showed that complete saturation of the olefinic bonds had been achieved. This product was evaluated by g.l.c. to determine the relative amounts of major components.

Conversion of the Fatty Acids to Structurally Equivalent Hydrocarbons

A portion of the purified acids from the ion exchange separation was methylated using BF_3-methanol.[15] Hydrogenation of the methyl esters was accomplished with Adam's Pt catalyst in acetic acid at 25° under a presure of a one atmos of H_2. Infra red indicated saturation of the olefinic bonds was complete.

The saturated esters were converted to alcohols by reduction with $LiAlH_4$ in dry ether.[17] (The i.r. spectrum confirmed the loss of the ester function.)

Conversion of the alcohols to iodides was accomplished by heating at 100° for 6 hr in a sealed glass tube with a two-fold excess of I_2 and red P (20 per cent of the wt. of the I_2 used).[4] The product showed no i.r. evidence for unsaturation, carbonyl or hydroxyl groups.

The alkyl iodides were converted to hydrocarbons by reduction with a three-fold excess

[13] C. S. Barnes, R. G. Curtis and H. H. Hatt, *Australian J. Appl. Sci.* **3**, 88 (1952).
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[17] R. F. Nystrom and W. G. Brown, *J. Am. Chem. Soc.* **69**, 1197 (1947).

of $LiAlH_4$ in dry ether.[4] After 18 hr, the unreacted hydride was destroyed with ethyl acetate, the solution washed with dil. H_2SO_4 and the solvent evaporated. The residue was refluxed for 30 min with 0·5 N ethanolic KOH, to hydrolyze traces of unchanged iodides, and the recovered product was chromatographed on alumina. Catalytic hydrogenation with Adam's Pt catalyst converted small amounts of terminally unsaturated olefins to the paraffins. The overall recovery of derived hydrocarbon by this procedure was 54 per cent. Conversion of methyl stearate to octadecane by this procedure gave 48 per cent over-all recovery.

Separation of the Derived Hydrocarbons into Normal and Branched Compounds

A 1·58-g portion of the hydrocarbons derived from higher fatty acids of tobacco were dissolved in cyclopentane and shaken for three hours with 110 g of 5A molecular sieve (1·6 mm pellets, Linde Division of Union Carbide) and allowed to remain in contact with the sieve overnight. The mixture was filtered and rinsed with cyclopentane to yield 150 mg (9·45%) of branched paraffins in the filtrates.

Sixty-eight per cent of the *normal* hydrocarbons, retained by the sieve, were recovered by stirring with hexane for 3 hr and allowing the suspension to remain in contact with the sieve overnight.

The *normal* paraffins were analysed by gas–liquid chromatography (Fig. 1 and Table 1).

Separation of the Derived Branched Hydrocarbons by Complex Formation with Urea

A 50-mg portion of the branched hydrocarbons derived from the tobacco fatty acids was dissolved with 160 mg of urea in 4 ml of boiling *n*-propyl alcohol. This solution was sealed, to prevent evaporation, placed in a preheated Dewar flask and allowed to stand without disturbance for 18 hr. At this time the temperature had decreased to 25° and needles of the urea-hydrocarbon complex, as well as rod-shaped crystals of urea, had separated. The crystals were filtered and rinsed with *n*-propyl alcohol. This precipitation was repeated a second time with the filtrates. The precipitated urea-hydrocarbon complex was mixed with water and the hydrocarbon was extracted several times with hexane to yield 19 mg. This is equivalent to 4·1 per cent of the total hydrocarbons derived from the fatty acids. The 25 mg (5·4 per cent of the total derived hydrocarbons) of hydrocarbons which were not complexed with urea was also recovered by dissolving the urea in water and extracting with hexane. Each of these fractions was examined by means of gas–liquid chromatography (Figs. 2 and 3 and Table 1).

Reference Samples of Authentic Compounds

Compounds which were used as reference materials included: 1-cyclohexyleicosane supplied by Dr. Joseph A. Dixon (API Research Project 42 at Pennsylvania State University); 2-methylheptadecane, 2-methyltetracosane, and 2-methyltriacontane prepared by Mr. T. P. Chen (Liggett and Myers Research Laboratory, Durham, N.C.); methyl myristate and methyl oleate, (Eastman Organic Chemicals); *n*-nonadecane (Humphrey-Wilkinson, Inc.); and *n*-tetratriacontane (Applied Science Laboratories).

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