The Paraffin Hydrocarbons of Tobacco; *Normal*, *Iso*-, and *Anteiso*-Homologs

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The presence of homologous series of *normal*, *iso* (2-methyl), and *anteiso* (3-methyl) paraffin hydrocarbons in several types of tobacco has been established. The homologs with odd numbers of carbon atoms predominate for the *normal* and *iso* series. The $C_{31}H_{64}$ compound is the most abundant component of each of these series. For the *anteiso* series, the homologs with even numbers of carbon atoms are present in major amounts, with the $C_{32}H_{66}$ compound present in largest amount.

Early studies on the composition of tobacco leaf wax indicated that the tobacco paraffins consisted of a relatively simple mixture of *normal* homologs, with hentriacontane ($C_{31}H_{64}$) predominating. Recently, several reports have appeared which indicate the much greater complexity of the tobacco paraffin mixture. Utilizing x-ray diffraction techniques as well as mass spectrometry on several column chromatographic fractions, Kosak and Swinehart (1960) reported the presence in cigarette smoke of two homologous series of tobacco paraffins. One of these series consisted of the fifteen *normal* compounds (C_{25}–C_{39}) present to the extent of 63% of the total mixture. The compounds with odd numbers of carbon atoms were predominant, with *n*-hentriacontane the major constituent. The other series was believed to consist of branched paraffins (C_{21}–C_{33}), with the compounds with even numbers of carbon atoms predominant. The major constituents were thought to be the $C_{30}H_{62}$ and $C_{28}H_{58}$ compounds.

Concurrently with this work, Carruthers and Johnstone (1959) reported the results of a study of the paraffin wax composition of green tobacco leaf, fermented tobacco, and cigarette smoke. These workers utilized gas-liquid chromatographic techniques as well as mass spectrometric analysis of the mixed paraffins to identify the components of the wax samples. They reported the presence of a homologous series of *normal* paraffins (C_{25}–C_{33}), with the compounds containing odd numbers of carbon atoms predominant and with *n*-hentriacontane the major component. A series of *iso* paraffins (2-methyl compounds of C_{27}–C_{33}) was also found. Unlike Kosak and Swinehart, Carruthers and Johnstone found the compounds with odd numbers of carbon atoms to be predominant for the *iso* paraffins as well as for the *normal* compounds. *Iso*-hentriacontane was believed to be the major component. Similar patterns for distribution were found for the green tobacco leaf, the fermented tobacco, and cigarette smoke. The *normal* series was estimated to make up 50–60% of the total paraffins.

At the time that the reports by Kosak and Swinehart and by Carruthers and Johnstone appeared there had been no reports in the literature that other leaf waxes contained significant amounts of branched paraffins. Since then, Waldron *et al.* (1961) have reinvestigated some of the plant waxes by mass spectrometry of the mixed paraffins and have found that rose petal wax also contains small amounts of *iso* paraffins in addition to the *normal* series. Eglinton *et al.* (1962), utilizing gas-liquid chromatography, have reported that the surface wax of leaves of certain species of the genera *Monanthes*, *Greenovia*, *Aichryson*, and *Aeonium* of the subfamily Sempervivoideae (Crassulaceae) contain significant amounts of the homologous *iso* paraffins, those with odd numbers of carbon atoms predominating. In some of the materials studied the *iso* paraffins were present in greater amount than the *normal* series.

Results

In view of the rather fragmentary reports concerning branched paraffins in plant waxes and the conflicting results of Kosak and Swinehart and of Carruthers and Johnstone, we felt it was desirable to reinvestigate the paraffin wax of tobacco. There seemed to be certain shortcomings inherent in all of the methods previously used, and the availability of some of the newer techniques afforded an opportunity to carry out a more definitive study of these materials. A commercial blend of aged Bright, Burley, Turkish, and Maryland cigarette tobaccos was used for the major portion of our studies, and these results were compared with results for an aged Bright tobacco blend, an aged Burley tobacco blend, and an aged Turkish tobacco blend. It was assumed that, owing to the relatively inert character of the paraffins, curing and aging of the tobaccos would not markedly alter the types of compounds present. This assumption was also fortified by the similar results obtained by Carruthers and Johnstone for green leaf, fermented (aged) tobacco, and cigarette smoke.

The paraffin hydrocarbons were obtained from tobacco leaf wax by chromatography on alumina followed by crystallization from acetone. The total paraffin fraction constituted 0.20–0.28% of the dry weight of tobacco. By use of a molecular sieve (5 A, Linde) it was possible to achieve a rather clean separation of the branched paraffins from the *normal* paraffins. This was necessary since otherwise considerable overlapping of the branched and *normal* isomers occurred on gas-liquid chromatography. The gas-liquid chromatography curves for the *normal* and branched paraffins subsequent to the molecular sieve treatment are presented in Figures 1 and 2.

Samples of individual components were collected from several gas-liquid chromatographies and rechromatographed by gas-liquid chromatography and on alumina to remove traces of silicone. Identifications were made principally on the basis of the mass spectra and infrared spectra. A series of isomeric tetratriacontanes, generously provided by Prof. E. Stenaghen (Göteborg University), was used to obtain retention times, mass spectra, melting points, and infrared spectra for reference purposes.

The selective fragmentation in the mass spectrometer of branched paraffins at the bonds attached to the carbon bearing the substituent affords ready recognition of the type of isomer, provided the sample is relatively pure (Beynon, 1960). For example, the higher intensities of the 31-carbon and 33-carbon ion fragments for 2-methyltritriacontane as compared to *n*-tetratriacontane (Table I) indicates a preferential cleavage adjacent to the carbon bearing the methyl group.

TABLE I

RELATIVE ION INTENSITIES FOR THE MASS SPECTRA OF AUTHENTIC ISOMERIC TETRATRIACONTANES[a,b]

Ion Carbon Number	*n*-Tetratriacontane (1-methyltritriacontane)	2-Methyltritriacontane	3-Methyltritriacontane	4-Methyltritriacontane	5-Methyltritriacontane	5% 2-Methyltritriacontane, 95% 3-Methyltritriacontane	5% 3-Methyltritriacontane, 95% 2-Methyltritriacontane	10% 3-Methyltritriacontane, 90% *n*-Tetratriacontane
25	4	4	4	2	4	3	8	6
26	4	5	3	2	4	6	5	5
27	4	4	2	2	3	4	4	6
28	4	4	2	2	21	2	5	5
29	4	4	2	18	6	3	4	4
30	3	5	13	2	100	12	5	5
31	3	38	2	100	8	12	34	2
32	2	5	100	10	8	100	15	25
33	0.6	20	6	8	8	14	15	1
34	100	100	26	30	21	52	100	100

[a] The intensities are calculated relative to the highest peak in the portion of the spectrum presented. Only the heavy fragment portions of the mass spectra are tabulated. The relative ion intensities are presented as functions of the carbon number of the ion. The peak height used is the highest for the unresolved cluster of peaks at that carbon number. [b] The *n*-tetratriacontane was obtained in 99% purity from Applied Science Laboratories, State College, Pa. The branched compounds were synthesized by Prof. E. Stenhagen, Göteborg University, and were further purified by gas-liquid chromatography.

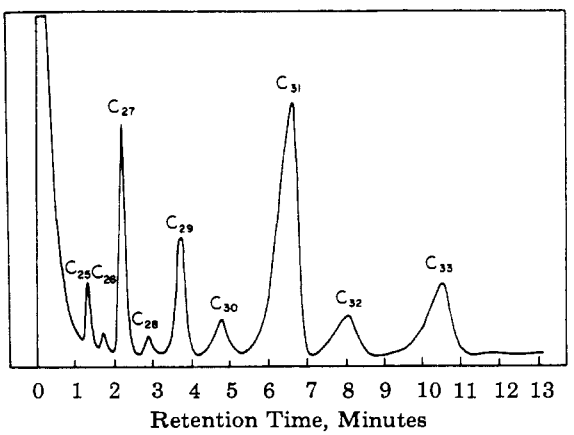

FIG. 1.—Gas-liquid chromatogram of the normal paraffin fractions from a commercial blend of Bright, Burley, Turkish, and Maryland tobaccos. Conditions used: Sample size, 500 μg; 0.25 in. OD × 1 meter copper column containing 80–100 mesh Gas Chrom P coated with 3% SE-30; helium flow rate, 46 ml/min; column temperature, 253°; detector and inlet temperature, 300°; 1 mv full span recording potentiometer; chart speed, 1 in./min.

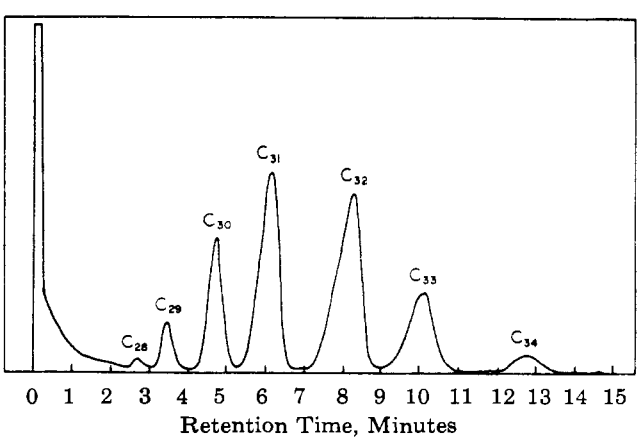

FIG. 2.—Gas-liquid chromatogram of the branched paraffin fraction from a commercial blend of Bright, Burley, Turkish, and Maryland tobaccos (conditions were similar to those described in Figure 1).

The identification of the principal component of each of the fractions from gas-liquid chromatography (Table II) was readily accomplished. The spectra for several of the isolated tobacco paraffins indicated the presence of small amounts of the other isomer. Estimation of the amount of the contaminating isomer was made possible by utilization of the spectra for the two pure components and of a known mixture containing both (Table I). The unknown sensitivity factors cancel out in the mathematical treatment of the data.[1] Since only the isomers for the tetratriacontanes were available, in order to estimate the concentrations of isomeric minor constituents in the unknown mixtures, it was necessary to assume that the ratio of the sensitivity factors for two isomeric tetratriacontanes in the heavy ion region is the same as the ratio for the corresponding two isomers of a homolog differing by one to five carbon atoms. Because of the normal multiplier voltage instabilities, the greater statistical fluctuations of the ion intensities for the low concentrations of the contaminant species, and the possible deviations from the assumed steady-state conditions in the ion source, these minor component concentrations cannot be calculated with high precision. The estimates obtained have been included in footnote *b* of Table III.

[1] The usual quantitative analysis of a mixture by the mass spectrometric method requires a knowledge of the nature of the constituents, the mass spectra for the individual pure components, and the sensitivity of each component to the ionization process in terms of the peak height per unit of sample partial pressure or equivalent units (Beynon, 1960). Such pressure measurements are not obtained when the sample is evaporated from a hot filament into the high vacuum system of the Bendix instrument. Indeed, the sample vapor is not at thermal equilibrium with the walls of the chamber but exists to a large extent as a broad, diffuse molecular beam, which leaves the hot filament, traverses the ionizing region, and is partly trapped upon the cooler surfaces that it strikes. The extent of the trapping depends on a number of variables. The fact that the total pressure recorded on the ionization gauge is very little related to the sample molecule population in this "molecular beam" means that the standard sample component sensitivities cannot be determined in the usual manner. The details of the mathematical method used for the present calculations may be obtained by writing to John M. Ruth, Mass Spectrometry Laboratory, Liggett and Myers Tobacco Company, Durham, N. C.

TABLE II
MASS SPECTRA OF TOBACCO WAX PARAFFINS

Ion Carbon Number	Relative Ion Intensities[a]								
	Branched Isomers						*Normal* Isomers		
	C_{29}	C_{28}	C_{31}	C_{32}	C_{33}	C_{34}	C_{28}	C_{30}	C_{31}
25	7	4	5	3	4	7	6	7	7
26	42	11	5	2	4	6	5	18	7
27	10	4	8	2	4	4	4	7	6
28	26	100	45	13	4	4	100	38	6
29	100	7	20	6	5	4		2	5
30		33	47	100	35	13		100	2
31			100	8	28	8			100
32				38	22	100			
33					100	8			
34						33			

[a] See footnote *a*, Table I.

TABLE III
RELATIVE PER CENT COMPOSITION OF THE TOBACCO HYDROCARBONS ON THE BASIS OF GAS-LIQUID CHROMATOGRAPHIC INFORMATION[a,b]

Paraffin Carbon Numbers	Commercial Blend Tobacco			Bright Tobacco			Burley Tobacco			Turkish Tobacco		
	n	*i*	*a*	*n*	*i*	*a*	*n*	*i*	*a*	*n*	*i*	*a*
25	1.71			2.04			1.05			1.37		
26	0.83			1.02			0.49			0.69		
27	7.73			5.71			4.78			8.60		
28	0.89		0.13	1.38		0.43	1.05		0.36	1.78		0.18
29	6.72	1.24		5.87	3.06		5.43	2.54		7.93	1.83	
30	3.16		5.65	3.06		6.71	2.92		6.75	5.46		5.31
31	26.3	10.92		24.49	14.26		27.51	12.6		23.24	6.69	
32	4.88		13.02	4.24		11.30	5.60		11.73	7.39		8.94
33	10.77	5.62		7.19	6.35		8.09	6.45		12.85	4.89	
34			1.15			2.88			2.58			2.34

[a] *n, normal; i, iso; a, anteiso.* [b] Mass spectrometric evaluation of the individual branched compounds for the wax from the commercial blend of tobaccos indicated the presence of the *anteiso* isomers to the extent of 2–3% of the C_{28}, 5–7% of the C_{31}, and 10–12% of the C_{32} homologs. Similarly, the *iso* compounds were present to the extent of 2–3% of the C_{32} and 6–7% of the C_{34} homologs. Small additional amounts of each branched compound would be expected in the corresponding *normal* isomer. The presence of the branched C_{25} homologs to the extent of 0.16% in the paraffins from the commercial blend of tobacco and 0.42%, 0.28% and 0.10% for the Bright, Burley and Turkish tobaccos was demonstrated in a study carried out subsequent to submitting this manuscript. A Mikrotek GC2500R instrument with dual H_2 flame detection was utilized.

With the exception of the doublet at 1368 and 1383 cm^{-1}, indicative of the 2-methyl branching, only the relatively weak bands in the "fingerprint" region of the infrared differ for the isomeric hydrocarbons. Since these bands are weak, it is desirable to expand the ordinate for this region. The spectra, expanded five times, for selected compounds representing each isomeric series are compared in Figure 3 with spectra for purified samples of synthetic hydrocarbons.

Although the melting point is not highly definitive for this type of compound it does offer confirmation of structure if the samples are highly purified. Melting points for several of the homologs obtained in larger amount are compared in Figure 4 with several values for related compounds.

Utilizing the techniques described we have been able to show that the tobacco wax paraffin consists principally of three homologous series, *normal*, *iso* (2-methyl), and *anteiso* (3-methyl). The branched isomers constituted 30–46% of the total paraffin mixture, with the *iso* and *anteiso* series present in nearly equal amounts. Our findings for the *normal* series, with regard to the relative amounts and carbon numbers, are in general agreement with those of Carruthers and Johnstone. We find the compounds with odd numbers of carbon atoms to be predominant for the *iso* series as well as for the *normal* series. The *anteiso* series, which has not been previously reported, consists principally of homologs with even numbers of carbon atoms. Smaller amounts of the alternate series are present in each instance. The results obtained for the Bright, Burley, and Turkish tobaccos were similar with the exception that relatively smaller amounts of the branched paraffins were present in the Turkish tobaccos. The relative amounts of individual hydrocarbons estimated to be present in these waxes are presented in Table III. Paraffins of carbon number less than C_{25} were present in trace amounts. They would likely account for less than 0.5% of the mixture.

Although the molecular sieve treatment gave a rather complete removal of the *normal* from the branched paraffins, small amounts of the latter compounds were adsorbed by the sieve and remained with the *normal* series. This would not be expected to exceed a few per cent of the total branched isomers. The C_{28} *normal* compound gave no evidence for the presence of branched isomers. This would establish with certainty that small amounts of *normal* paraffins with even numbers of carbon atoms are present in tobacco wax.

DISCUSSION

Previous attempts to identify the individual components of natural plant paraffins have failed because of the inadequate resolution of homologous mixtures

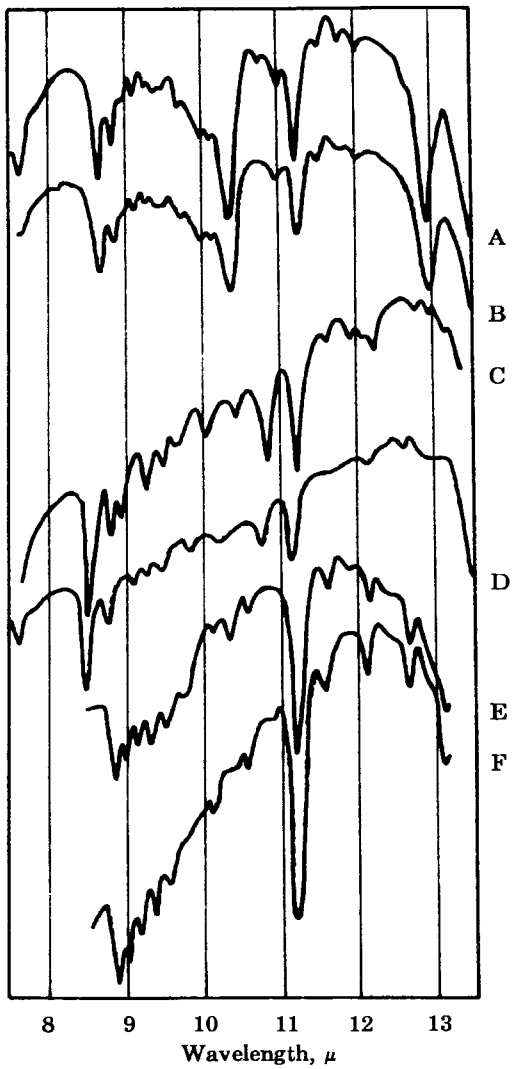

FIG. 3.—Infrared absorption spectra for selected paraffin isomers from tobacco wax compared with spectra for several authentic samples of isomeric paraffins. *A*, authentic 3-methyltritriacontane; *B*, C_{34} branched paraffin from tobacco wax; *C*, authentic 2-methyltritriacontane; *D*, C_{33} branched paraffin from tobacco wax; *E*, authentic hentriacontane; *F*, C_{31} normal paraffin from tobacco wax.

prior to the application of various methods of identification. X-ray diffraction or mass spectral analysis can, of course, be applied successfully to evaluate mixtures. However, it is necessary to be aware of the isomeric types possible if a mixture is to be correctly analyzed without prior separation of the components.

The present technique, which utilizes separation by a molecular sieve of the *normal* from the branched paraffins, permits the homologs of the *normal* and of the branched series to be well resolved by gas chromatography. However, gas chromatographic retention times do not appear to be sufficiently selective for the *iso* series *versus* the *anteiso* series to permit definitive identifications on this basis alone. Examination of the infrared and mass spectral data for the leading portion and the trailing portion of the gas-liquid chromatography eluate representing the C_{31} branched homolog indicated that no significant resolution of these two isomers had been achieved.

By use of packed columns coated with a low per cent of silicone rubber good separation of the paraffin homologs has been possible in samples of as much as 6 mg, thus making available samples large enough for various spectrophotometric and other measurements. While

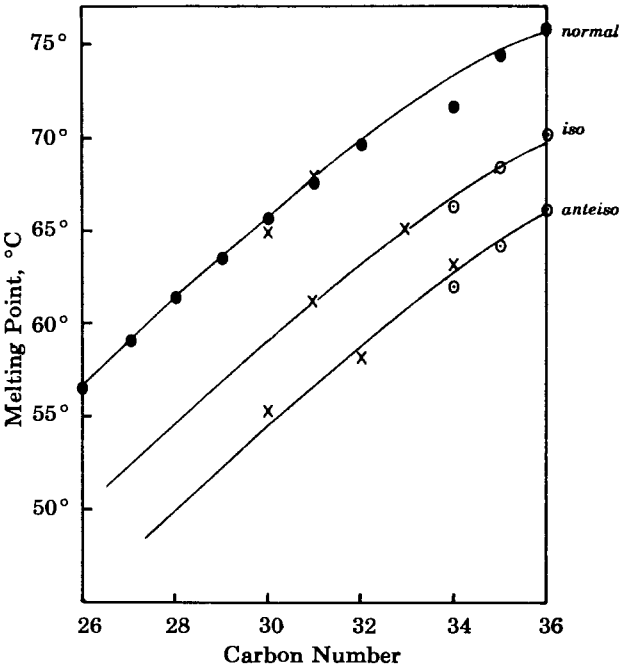

FIG. 4.—Melting points for *normal*, *iso*, and *anteiso* paraffins. ●, Piper *et al.* (1931); ⊙, Ställberg-Stenhagen and Stenhagen (1948); ×, components of tobacco wax.

the 2-methyl and 3-methyl isomers are not sufficiently well resolved to allow complete separation, the ability to collect a sample containing only isomeric compounds of the same carbon number presents fewer variables in evaluation by mass spectrometry or other physical means.

The *anteiso* series of paraffins has not previously been reported in plant waxes. However, the presence of *anteiso*, *iso*, and *normal* paraffins in wool wax has been recently reported by Downing *et al.* (1960). These workers found that the homologs with odd numbers of carbon atoms predominated for the *anteiso* paraffins and the homologs with even numbers predominated for the *iso* paraffins. These findings are the reverse of ours for tobacco wax. The only basis given by Downing *et al.* for the identification of the isomeric wool wax paraffins was the gas-liquid chromatography retention times compared to those for a mixture of homologous paraffins prepared from the isomeric wool wax alcohols. We feel that the slight differences in retention times observed for the isomeric branched paraffins make this criterion of identification highly subject to error. We are in the process of applying our techniques to a sample of wool wax paraffins to clarify this issue. Preliminary results indicate that these paraffins do not consist of as simple a mixture as we have found for the tobacco wax paraffins but include compounds with multiple branching.

EXPERIMENTAL

Separation of Paraffin Hydrocarbons from Tobacco.—The samples of shredded tobacco were dried to 4.5–7% moisture content and were extracted for 24 hours with methylene chloride and for 24 hours with absolute methanol in Soxhlet extractors. The methylene chloride was evaporated and the residue combined with the methanol extract. The methanol extracts were diluted to 67% with water and extracted four times with one-third volumes of hexane. The combined hexane extracts were concentrated somewhat *in vacuo* and then

evaporated to dryness on a portion of activated alumina, final drying being accomplished in a vacuum desiccator. This portion of alumina was added to the top of a column of fresh alumina (Merck, acid-washed, 100–200 mesh) which had been activated by heating at 110° overnight. An amount of alumina was used which was approximately equivalent in weight to the original weight of the tobacco sample. The wax hydrocarbons were eluted with hexane and collected in a volume equivalent to less than 2.5 ml per g of adsorbent. Yields of 0.34, 0.41, 0.36, and 0.36%, respectively, were obtained for the samples of aged Bright, Burley, Turkish, and commercial blended tobaccos. These values are in agreement with previous reports (Stedman and Rusaniwskyj, 1959). Presence of olefins with conjugated unsaturation (predominantly neophytadiene) was indicated by ultraviolet and infrared spectrometry. There was no evidence for the presence of carbonyl compounds. To remove the olefinic compounds the hydrocarbon fractions were crystallized from acetone at +5°. Only negligible amounts of the saturated paraffins were left in the filtrates from this crystallization. The paraffinic hydrocarbons were obtained from the Bright, Burley, Turkish, and commercially blended tobaccos to the extent of 0.20, 0.28, 0.28 and 0.24%, respectively.

Separation of Branched from Normal Paraffins.—Separation of the branched paraffins from the normal paraffins was accomplished by treatment of the mixture with a molecular sieve. Twenty-two grams of Linde molecular sieve 5 A ($^1/_{16}$ in. pellets) was washed with purified isooctane. One gram of the paraffin mixture from the commercial blend of tobaccos, in 50 ml of isooctane, was shaken with the sieve for several hours and allowed to stand overnight in contact with the sieve. The suspension was filtered and the sieve washed twice with isooctane. Thirty-eight per cent of the weight was recovered in the filtrates. The treatment was repeated on the filtrates with a second 25-g portion of the sieve. Only 1% additional material was retained by the sieve, and the branched paraffins, totaling 37% of the original mixture, were left in the filtrates. The relative amounts of branched paraffins for the aged Bright, Burley, and Turkish tobaccos were 45, 43 and 30%, respectively. The melting point of the mixed branched paraffins from the commerical blend of tobaccos was 57.7–58.3°.

The normal paraffins were removed from the molecular sieve by allowing it to stand with *n*-hexane for several days. A 60–70% yield was obtained (O'Connor *et al.*, 1962). Nearly quantitative recoveries were obtained with longer standing or repetition of the elution procedure. In calculating total amounts of individual hydrocarbons, losses at this step were assumed to be due to low recovery of proportionate amounts of the *normal* hydrocarbons. The melting point of the mixed *normal* paraffins from the commercial blend of tobaccos was 65.5–66.3°.

Separation of Homologs by Gas-Liquid Chromatography.—The instrument utilized for the gas-liquid chromatography was a Mikrotek GC-2500 (Mikrotek Instruments, Inc., Oak Villa Blvd., Baton Rouge, La.). This instrument was equipped with dual columns and hot-wire detectors. For the preparative runs the temperature was maintained at 258° for 5 minutes and then was programmed to increase at a rate of 8° per minute for 4 minutes. It was then held at 290° until completion of the run. The column packing found to be most suitable was 3% silicone rubber, SE-30 (General Electric Co.) on 80–100 mesh Gas Chrom P (Applied Science Laboratories). No. 316 stainless steel columns of $^5/_8$ in. O.D. by 3 ft. were used with helium as a carrier gas at a flow rate of 120 cc per minute. The inlet, detector, and exit were maintained at 300° throughout the run. The detector filament current was 500 ma. Columns were preconditioned for 48 hours prior to use at 275° with a continuous flow of nitrogen.

Gas-liquid chromatograms for the branched paraffins and the *normal* paraffins from the commercial blend of tobaccos are presented in Figures 1 and 2. Retention times for the unknown paraffins were compared with internal standards of authentic *n*-eicosane, *n*-octacosane, *n*-hentriacontane, *n*-dotriacontane, and the *n*-, *iso*-, and *anteiso*-tetratriacontanes.

Samples of mixed paraffins up to 6 mg in 10 μl of cyclohexane were successfully chromatographed on $^5/_8$-in. diameter columns. The resolution of components was comparable to that obtained with $^1/_4$ in. × 7 ft. copper columns. Individual components from the gas-liquid chromatograms were collected in traps cooled in liquid air (for the design of the trap see Stevens and Mold, 1963). Several runs were usually necessary to obtain sufficient amounts for the characterization studies. Each component was rechromatographed by gas-liquid chromatography and then passed through a small column of active alumina in hexane to remove silicone contaminants from the gas-liquid chromatography column.

Identification of Individual Components.—The melting points for components obtained in sufficient quantity are presented in Figure 4.

Infrared spectra were determined with a Perkin-Elmer Model 21 spectrophotometer with use of smears on KBr pellets. Expansion of the ordinate scale fivefold was necessary to enhance the weak "fingerprint" bands. Representative spectra for several of the isolates are compared with spectra for authentic samples in Figure 3.

Mass spectra were obtained with the Model 14-101 Bendix Time-of-Flight mass spectrometer, equipped with an S-14-105 ion source. The samples were introduced by means of a modified Bendix hot filament sample probe. This modification was essentially that developed in the laboratory of K. Biemann (Department of Chemistry, Massachusetts Institute of Technology, Cambridge, Mass.). Spectra for the tobacco paraffins are given in Table II. Spectra for synthetic reference compounds and mixtures are given in Table I.

Reference Samples of Authentic Hydrocarbons.—The authentic samples of 2-methyl-, 3-methyl-, 4-methyl-, and 5-methyltritriacontane, which had been synthesized in Dr. E. Stenhagen's laboratory, were further purified by gas-liquid chromatography to remove small amounts of impurities prior to determination of their spectral properties. Hentriacontane was synthesized from palmitic acid *via* palmitone which was reduced with hydrazine by the Wolff-Kishner reaction to yield the hydrocarbon.

Acknowledgments

The authors are most grateful to Prof. E. Stenhagen of Göteborg University for generously providing synthetic samples of 2-methyl-, 3-methyl-, 4-methyl-, and 5-methyltritriacontanes and thereby considerably facilitating this work. The hentriacontane was synthesized by Mr. T. P. Chen. The cooperation of E. Sunas in obtaining infrared spectra and of Dr. John F. Williams and Mr. R. Lax in preliminary gas-liquid chromatography studies is also gratefully acknowledged.

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Metabolism of Fluorene-9-C^{14} in Rats, Guinea Pigs, and Rabbits*

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The metabolism of fluorene-9-C^{14} in several animal species is reported. The urine and feces contained 57 and 16, 82 and 6, and 39 and 1% of the isotope 48 hours after intraperitoneal injection of 100 mg/kg of fluorene-9-C^{14} into rats, guinea pigs, and rabbits, respectively. The soluble and insoluble liver proteins at 48 hours bound isotope to 155 and 97, 35 and 24, and 67 and 33 mμmoles of compound per gram of dry proteins in the rats, guinea pigs, and rabbits, respectively. Radioactivity was found in all organs analyzed. The liver and kidneys had a relatively high isotopic content. The respired carbon dioxide was void of radioactivity. The urinary radioactivity was composed of 5.6, 1, and 1% free compounds; 39, 17, and 23% sulfuric acid ester fraction; and 48, 79, and 64% glucosiduronic acid fraction in the rat, guinea pig, and rabbit, respectively. The chief metabolites were, in rats, 9-fluorenol glucuronide and 2-fluorenol sulfate; in guinea pigs, 9-fluorenol glucuronide and the 2-fluorenol glucuronide and sulfate; in rabbits, 2- and 9-fluorenol glucuronide and 2-fluorenol sulfate. The 2,9-difluorenol and conjugates were also present in the urine of the three species.

Fluorene is the parent hydrocarbon from which the important carcinogen *N*-2-fluorenylacetamide (2-acetylaminofluorene) is derived. We have performed studies on the fate of fluorene in various animal species to serve as a model and to gain a better understanding of the metabolism of the more complex carcinogen molecule.

The fate of fluorene in the rabbit has been investigated previously by Neish (1948), who reported that 2-fluorenol and the glucuonide of 2-fluorenol were present in the urine. He also observed that little, if any, fluorene or metabolites were eliminated in the feces. The present studies extended the investigation to rats and guinea pigs as well as to rabbits in view of the species differences known to exist in the metabolism of the acetylamino derivative (Enomoto *et al.*, 1962; Irving, 1962; Miller *et al.*, 1960; Weisburger *et al.*, 1959b), as well as in other polynuclear aromatic hydrocarbons (Williams, 1959).

Fluorene-9-C^{14} was synthesized and used in the metabolism experiments reported here. The isotopic label afforded a more detailed and complete picture of the excretion and distribution of fluorene and metabolites in the animals. The use of this sensitive tool permitted the identification of metabolites which might not have been detected by isolation and color reaction techniques.

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MATERIALS AND METHODS

Synthesis of Fluorene-9-C^{14}.—Fluorene-9-C^{14} was synthesized in 75% yield from $BaC^{14}O_3$ (25.07 mc/mmole) by the procedure of Weisburger and Weisburger (1958). The specific activity of the product was 22 × 10^6 cpm/mg as determined by the wet combustion technique.

From the above material, two products with specific activities of 1.36 × 10^6 and 6.4 × 10^5 cpm/mg were prepared by dilution with pure unlabeled fluorene in a crystallization step. They were used for the biochemical experiments.

Reference Compounds.—2-Fluorenol, 4-fluorenol, 9-fluorenol, and 2,9-difluorenol were supplied by Dr. E. K. Weisburger. The glucuronide of 9-fluorenol was kindly furnished by Dr. W. J. P. Neish, University of Sheffield.

Radioactivity Determinations.—Radioactivity measurements were performed on a windowless gas-flow counter with an efficiency of 46% for C^{14}. The wet combustion technique of Weisburger *et al.* (1952) was used for the conversion of solid samples, feces, tissues, and proteins to barium carbonate, which was plated and the radioactivity determined. The urine and other liquid samples were plated directly in infinitely thin layers. A suitable correction factor was determined for adjusting the counts obtained by the two methods.

Treatment of Animals.—A. Rats. Six 3-month-old female Buffalo-strain rats weighing 150–160 g were injected intraperitoneally with 1 ml of a gum acacia suspension of fluorene-9-C^{14} (1.36 × 10^6 cpm/mg) at a dose level of 100 mg per kilogram body weight. The