Lemieux, R. U. (1953), *Can. J. Chem. 31*, 396.
Royals, E. E. (1958), Advanced Organic Chemistry, Englewood Cliffs, N. J., Prentice-Hall.
Swern, D. (1948), *J. Am. Chem. Soc. 70*, 1235.
Swern, D. (1953), *Org. Reactions 7*, 378.
Tenny, K. S., Gupta, S. C., Nystrom, R. F., and Kummerrow, F. A. (1963), *J. Am. Oil Chemists' Soc. 40*, 172.
Weiss, B. (1957), *J. Am. Chem. Soc. 79*, 5553.
Weiss, B. (1963), *J. Biol. Chem. 238*, 1953.
Weiss, B. (1964), *Biochemistry 3*, 584.

The Paraffin Hydrocarbons of Wool Wax. Normal, Iso, Anteiso, and Other Branched Isomers

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The relative abundance of the homologs of the series of normal, iso (2-methyl), and anteiso (3-methyl) paraffin hydrocarbons has been determined for a sample of wool wax. The occurrence of pristane (2,6,10,14-tetramethylpentadecane) has been established for the first time in a wax not of marine origin. The presence of homologous cycloparaffins and at least two other homologous series of branched paraffins has been noted in wool wax.

Our discovery of the natural occurrence of the anteiso (3-methyl) series of paraffin hydrocarbons in a plant material, tobacco leaf wax (Mold *et al.*, 1963a) prompted an interest in a report of the presence of this class of compounds in wool wax (Downing *et al.*, 1960). A preliminary study of the hydrocarbon fraction of wool wax, utilizing the techniques which had been applied to tobacco leaf wax, indicated to us that the branched paraffins of wool wax did not consist solely of homologous series of iso and anteiso compounds but included other series of branched homologs. Furthermore, since we had observed that the iso paraffins of tobacco wax were predominantly homologs of odd numbers of carbon atoms and the anteiso paraffins were predominantly of even numbers of carbon atoms, it was of interest to establish whether a similar relationship existed for an animal wax. Downing *et al.* (1960) had reported that the reverse order of predominant homologs was true for wool wax, albeit on rather meager evidence.

Results

The paraffin hydrocarbons of wool wax, representing about 0.5% of the total wax, were separated into several groups of compounds by means of urea-adduct formation and treatment with a molecular sieve (5A, Linde). The normal compounds, which were complexed by the molecular sieve, were further separated by gas-liquid chromatography (Fig. 1) into the individual homologs. These compounds satisfied the requirement for a homologous series in that the logarithms of their retention times from isothermal chromatography gave a straight-line relationship when plotted against the number of carbon atoms. The series of normal hydrocarbons represented 16–20% of the total wool-wax paraffins. Small amounts of isomeric paraffins were carried along with the normal homologs into the sieve. This was corrected for in estimating the total amounts present. The relative amounts of the normal paraffins are given in Table I.

An interesting quantitative distribution of the normal homologs was observed. These compounds were grouped in two families. One of these included homologs in the range of about 13–25 carbon atoms. Each carbon number was represented in increasing amount up to the 20-carbon compound. The amounts then decreased regularly for the higher members. The second included homologs in the range of about 23–35 carbon atoms. These consisted predominantly of homologs of odd numbers of carbon atoms with the 29-carbon compound present in the largest amount.

TABLE I
AMOUNTS OF HOMOLOGOUS NORMAL PARAFFINS IN WOOL WAX[a]

Paraffin Carbon Number	Per Cent of Total Paraffins	Paraffin Carbon Number	Per Cent of Total Paraffins
12	Trace	24	0.34
13	0.08	25	0.50
14	0.11	26	0.17
15	0.14	27	1.04
16	0.29	28	0.43
17	0.65	29	2.45
18	1.05	30	Trace
19	1.55	31	1.55
20	1.55	32	Trace
21	1.18	33	0.88
22	0.76	34	Trace
23	0.71	35	Trace

[a] These values were calculated from the areas under the gas-liquid chromatograph. A correction was applied for low recovery from the molecular sieve but no correction was made for other losses in the isolation.

The branched paraffins, which formed urea adducts but were not removed from an isooctane solution by the molecular sieve, gave a gas-liquid chromatogram (Fig. 2) which indicated the presence of at least three homologous series with carbon contents ranging from 17 to 43 atoms per molecule. This group of compounds was present to the extent of about one-half that of the normal paraffins. The relative amounts of these components are given in Figure 3.

Individual homologs of these branched hydrocarbons were collected from gas-liquid chromatograms and identified by mass spectrometry. Examination of the infrared spectra for the total sample prior to gas-liquid chromatography and for several of the individual fractions collected from the chromatography indicated the absence of any olefinic impurities. The homologs of series III in the range from 17 to about 35 carbon atoms consisted largely of mixtures of 2-methyl and 3-methyl isomers. The relative intensities of the ions

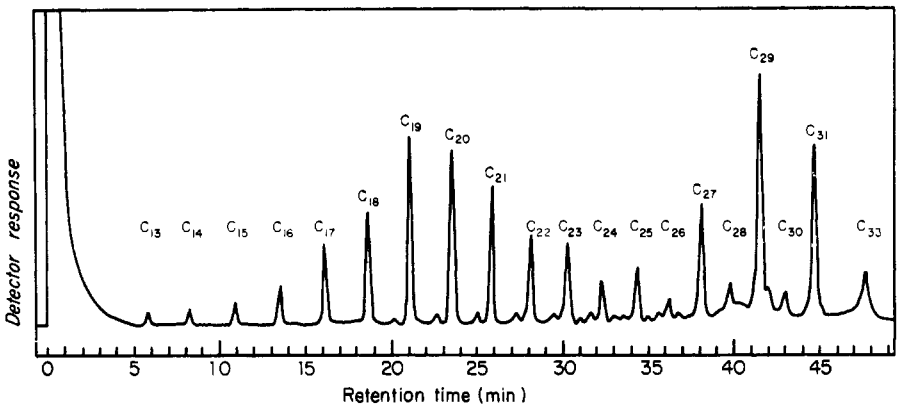

FIG. 1.—Gas-liquid chromatogram of the normal paraffins from wool wax. Conditions used: sample size, 50 μg; $^3/_{16}$ in. i.d. × 6 ft dual stainless steel columns containing 80–100 mesh Gas Chrom P coated with 6% SE-30; helium pressure, 40 psi; flow rate, 80 ml/min; column temperature, initially 128° for 4.5 minutes then programed to 270° at 4.0°/min; detector temperature, 270°; inlet and exit temperature, 300°.

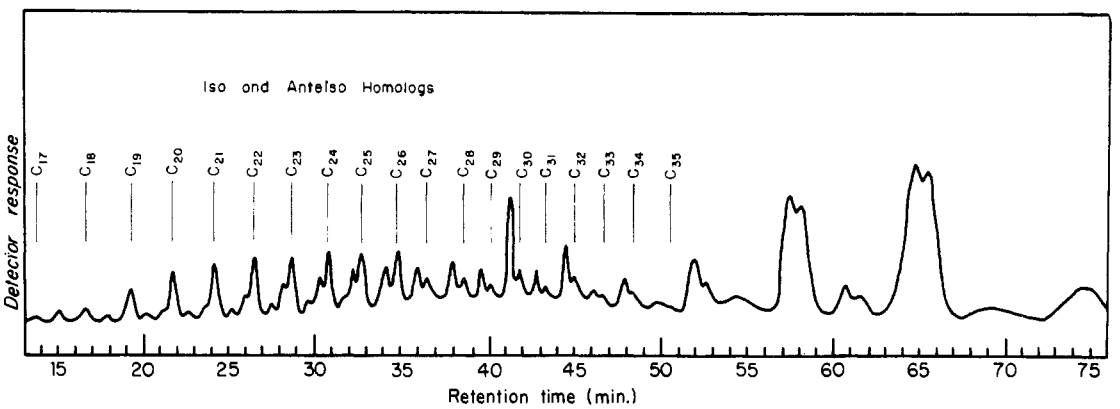

FIG. 2.—Gas-liquid chromatogram of the branched paraffins from wool wax which formed urea com plexes. Conditions used: sample size, 50 μg; $^3/_{16}$ in. i.d. × 6 ft dual stainless steel columns containing 80–100 mesh Gas Chrom P coated with 6% SE-30; helium pressure, 38 psi; flow rate, 80 ml/min; column temperature, initially 125° for 5.0 minutes then programed to 280° at 4.0°/min; detector temperature, 260°; inlet and exit temperature, 300°.

in the 5-carbon and 6-carbon groups and the presence of parent peaks of 16 units less than the iso and anteiso components with which these compounds were associated suggested that mixtures of cyclohexyl and cyclopentyl paraffins of one less carbon atom were present to the extent of 10–20% of each homolog mixture (Levy *et al.*, 1961). Insufficient quantities of most of the fractions collected from the chromatography were available for infrared evaluation; however, for those samples tested in this way, weak absorbance was noted at 844 cm^{-1} and 933 cm^{-1}, consistent with cyclohexyl and cyclopentyl paraffin structures. The intensity of these bands for the samples examined indicated the presence of about 10% of the former and 2% of the latter type compounds.

For the purpose of estimating the relative amounts of iso, anteiso, and cyclo paraffins in gas-liquid chromatographic fractions, the intensities of m/e for ions at parent(p)-16, p-29, and p-43 were taken to represent solely the cyclic, the anteiso, and iso components, respectively. Within the range of accuracy of these measurements, this appeared to be a valid assumption. The ratio of iso component to anteiso component is thus essentially independent of the identificaton and concentration of the cyclic component. The concentration found for the cyclic component depends, of course, upon its sensitivity factor, which in turn depends upon the ring size and its location in the alkane molecule. Since the cycloparaffins were present in too-small amount in our chromatographic fractions to identify them conclusively, for the purpose of the calculations, it was assumed that the parent peak intensity (p-16) was equal to that of a 1-cyclohexyl paraffin. An additional assumption involved in the estimations of these relative amounts is that the sensitivity factors for the three peaks used in the calculations are in the same ratio for each chromatographic fraction. This was a necessary assumption since only relatively few model compounds were available for standardization. Within the above limitations the values reported in Table II should represent reasonable estimates of the relative amounts of the iso and anteiso components and upper-limit estimates for the cycloparaffins.

A pattern similar to that which we had observed for the branched tobacco paraffins was noted for the 24–35 carbon homologs. That is, the 2-methyl isomers were present in greater amount for homologs of odd numbers of carbon atoms and the 3-methyl isomers were present in greater amount for homologs of even numbers of carbon atoms. This alternating pattern was also similar to that noted above for the normal paraffins in this range of carbon atoms.

The 2-methyl isomers were predominant for all branched homologs of less than 25 carbon atoms. This nonalternating pattern is also consistent with that observed for the normal paraffins in the 13–25 carbon

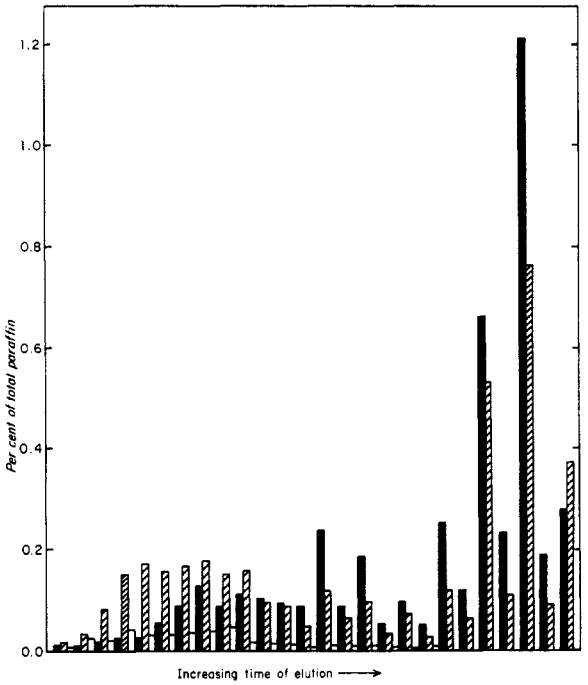

FIG. 3.—Relative amounts of branched homologous paraffins in wool wax. Series I □, series II ■, series III ▨.

TABLE II
AMOUNTS OF HOMOLOGOUS ISO, ANTEISO, AND CYCLO PARAFFINS IN WOOL WAX

Carbon Number		Per Cent of Total Paraffins			
Paraffin	Cyclo-paraffin	Iso	Anteiso	Cyclo	Total Mixture[a]
17	16				0.012
18	17				0.032
19	18	0.069	0.010	0.009	0.087
20	19	0.093	0.042	0.016	0.151
21	20	0.108	0.040	0.022	0.168
22	21	0.094	0.038	0.027	0.159
23	22	0.084	0.048	0.034	0.164
24	23	0.097	0.049	0.030	0.176
25	24	0.091	0.027	0.031	0.149
26	25	0.042	0.082	0.033	0.157
27	26	0.051	0.019	0.026	0.096
28	27	0.021	0.053	0.013	0.087
29	28	0.030	0.010	0.006	0.046
30	29		Major[b]		0.118
31	30	0.047	0.008	0.008	0.063
32	31		Major[b]		0.098
33		Major[b]			0.033
34			Major[b]		0.066
35		Major[b]			0.026

[a] The values for the relative abundance in wool wax of the total mixture of each homolog were calculated from the areas of triangles under the gas-liquid chromatograph peak for each component drawn to compensate for the overlap and related to the total area under the gas-liquid chromatograph. No corrections have been applied for losses in the isolation steps. [b] It was not possible to calculate an absolute amount for each constituent of these mixtures due to the presence of unknown dimethyl homologs; however the major constituent has been indicated.

atom range. Some overlapping of these two patterns seems to occur which makes it difficult to assign the most abundant homolog, but this appeared to be the 21-carbon compound for the 2-methyl compounds.

Although there were homologs of greater than 35-carbon atoms (series III) which appeared to form an extension of the straight line log R_T relationship for the iso-anteiso series, these compounds were in fact

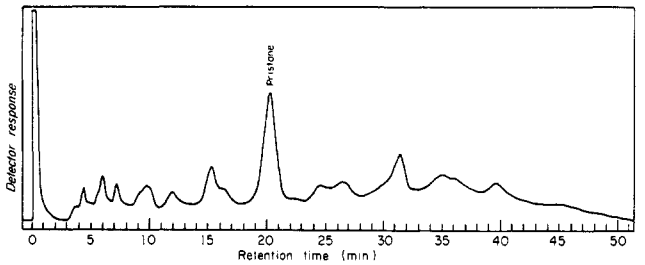

FIG. 4.—Gas-liquid chromatogram of the more volatile portions of the branched paraffins from wool wax which did not form urea complexes. Conditions used: sample size, 50 μg; $^3/_{16}$ in. i.d. × 6 ft dual stainless steel columns containing 80–100 mesh Gas Chrom P coated with 6% SE-30; helium pressure, 38 psi; flow rate 80 ml/min; column temperature, 123°; detector temperature, 210°; inlet temperature, 240°; exit temperature, 270°.

shown to represent a new series of branched paraffins (with probably two methyl branches) with retention times identical to the iso-anteiso isomers of one less carbon. Work is in progress to establish the structures of these homologs.

Members of series I were present in insufficient amount to obtain any useful information regarding their structures. Members of series II appear to be singly branched paraffins with a methyl group further down the chain. Studies are also in progress to establish the nature of these homologs.

The paraffins which did not form adducts with urea represented 68% of the total hydrocarbons recovered at this stage. A considerable portion of these failed to elute from gas-liquid chromatograms at temperatures below 270°. A short-path vacuum distillation was utilized to separate the more volatile materials from this fraction. A chromatogram for the materials collected up to 200° at 1–4 mm Hg is shown in Figure 4. The chromatogram for the higher-boiling residue was extremely complex with no major components evident.

A major constituent of the more volatile portion of this highly branched fraction of wool-wax paraffins was shown to be pristane (2,6,10,14-tetramethylpentadecane) (Mold *et al.*, 1963b). Its characterization was based on the identity of its gas-liquid chromatographic behavior, infrared (Fig. 5), and mass spectra (Table III) with those for an authentic sample of pristane. It was found to the extent of 1.1% of the total paraffins. Other materials present in this highly branched portion of wool-wax paraffins have not been characterized.

DISCUSSION

The presence of two different families of normal homologs in the paraffin fraction of wool wax, the lower-molecular-weight group consisting of compounds with even and odd numbers of carbon atoms in equivalent amounts and the higher-molecular-weight group consisting principally of compounds with odd numbers of carbon atoms, suggests that at least two general biochemical synthetic pathways are operative. Although Downing did not report the presence of the alternating series of normal hydrocarbons in wool wax, it is possible that these compounds were too high-boiling for efficient elution from his gas-liquid chromatogram. Tobacco paraffins (Mold *et al.*, 1963a) as well as other plant waxes (Waldron *et al.*, 1961) have been reported to contain only the latter type of alternating series with the major component differing for different species of plants.

The iso and anteiso series, which appear to be the major homologous branched paraffins in tobacco wax,

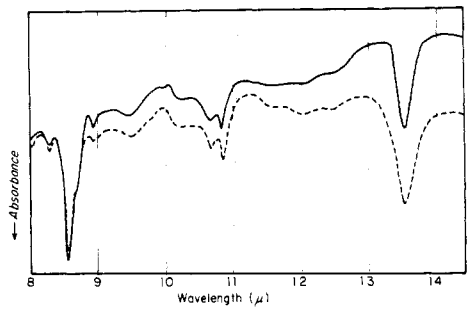

FIG. 5.—Infrared-absorption spectra for pristane, ———, isolated from wool wax, and - - - -, an authentic sample. The spectra were determined with a Perkin-Elmer Model 21 spectrophotometer with use of smears on KCl pellets. Expansion of the ordinate scale 5-fold was necessary to enhance the weak "fingerprint" bands.

TABLE III

RELATIVE INTENSITIES FOR THE PRINCIPAL ION FRAGMENTS IN THE MASS SPECTRUM OF PRISTANE

	Relative Intensity[a]	
m/e	Authentic Pristane	Pristane Isolated from Wool Wax
41	43	48
42	15	15
43	100	100
44	6.2	6.7
55	32	31
56	31	33
57	98	97
58	11	8.0
69	18	29
70	12	13
71	61	66
72	8.2	9.8
83	5.4	9.3
84	6.4	8.4
85	24	30
86	3.8	5.8
97	4.9	10
99	8.1	8.4
112	25	21
113	27	25
114	7.6	8.8
126	4.3	4.5
127	6.2	8.4
141	2.5	3.4
154	4.0	5.0
155	4.8	6.7
169	0.6	1.8
183	23	26
197	1.2	2.0
211	0.4	0.7
225	1.1	1.5
239	1.2	4.0
253	3.0	3.7
268	22	28

[a] For single scans of small samples, using a probe-type inlet, where the sample pressure in the ion source cannot be controlled during the scan, relative intensity variations of this magnitude are not unexpected.

are also present in wool wax. The occurrence of two different families of these branched compounds those of lower molecular weight having the 2-methyl compounds predominant for both odd and even numbers of carbon atoms and those of higher molecular weight, like tobacco wax, having the 2-methyl compounds predominant for homologs of odd numbers of carbon atoms and the 3-methyl compounds predominant for the homologs of even numbers of carbon atoms, suggests that there may be some interrelationship here with the two general modes of formation for the normal paraffins.

The much greater complexity of the branched paraffins of an animal wax as contrasted to those of plant wax is demonstrated by the presence of several new homologous series for wool wax. Pristane has been reported previously in marine organisms (Blumer *et al.*, 1963; Hallgren and Larsson, 1963; Lambertsen and Holman, 1963; see also Mold *et al.*, 1963b); petroleum (Bendoraitis *et al.*, 1962; Dean and Whitehead, 1961); or coal tar (Kochloefl *et al.*, 1963).

EXPERIMENTAL

Separation of the Paraffin Hydrocarbons from Wool Wax.—Centrifugal wool grease (385 g), generously provided by Mr. Clyde Rowntree (Wellman Combing Co., Johnsonville, S. C.), was dissolved in 6 liters of hexane and extracted three times with 1200-ml volumes of methanol-water (2:1). After washing the hexane solution thoroughly with water, it was dried over anhydrous Na_2SO_4 and filtered. The wax hydrocarbons were separated from the other components by chromatography of the hexane solution on acid-washed alumina. Merck chromatographic grade alumina was sieved to select 100–200 mesh particles; washed with hexane, acetone, methanol, and water, dried, and reactivated by heating at 110° for 16 hours. The major portion of the wax hydrocarbons, eluted with hexane, was free of carbonyl compounds. An additional portion was rechromatographed on alumina to provide a total of 2.06 g, 0.53% of the total wax. There was no evidence in the infrared spectrum of this material for the presence of carbonyl or olefinic functions. Succeeding fractions did contain olefinic materials.

Separation of Multiply Branched Compounds with Urea.—The 2.06-g sample of wax hydrocarbons and 20.88 g of urea were dissolved in 650 ml of boiling *n*-propyl alcohol. The solution was stoppered, placed in a preheated insulated Dewar flask, and allowed to stand without disturbance for 18 hours. At this time the temperature of the solution had decreased to 25° and needles of urea-hydrocarbon complex as well as rod-shaped crystals of urea had separated. The crystals were filtered and rinsed with *n*-propyl alcohol, and the urea was dissolved in water. The aqueous suspension of hydrocarbon was extracted several times with hexane to yield 488 mg of hydrocarbons. A second treatment of the filtrate from the urea complex was carried out in a similar manner by the addition of more urea. Only 6.5 mg of hydrocarbons was complexed. The noncomplexed materials (1.04 g) were recovered from the filtrates by diluting with water and extracting with hexane.

Separation of Normal Paraffins by Treatment with a Molecular Sieve.—The hydrocarbons recovered from the urea complex (494 mg) were dissolved in 35 ml of redistilled isooctane. This solution was shaken for 3 hours with 28.5 g of 5A molecular sieve (1.6-mm [1/16-in.] pellets, Linde Co., Div. of Union Carbide) and allowed to remain in contact with the sieve overnight. The mixture was filtered and the sieve was rinsed with fresh isooctane to yield 257 mg in the filtrates. A second treatment of the isooctane filtrates with fresh molecular sieve yielded 170 mg of branched compounds in the filtrates. The total branched paraffins separated by molecular-sieve treatment amounted to 34% of the materials which formed the urea complex.

Seventy-six per cent of the normal hydrocarbons retained in the sieve were recovered by stirring with

hexane for 3 hours and allowing the suspension to remain in contact with the sieve overnight. Further material was recovered by heating with hexane repeatedly. A sample of the normal paraffins recovered from the sieve was analyzed by gas-liquid chromatography as described in Figure 1. Minor amounts of branched compounds, carried along in the sieve treatment, were evident in addition to the homologous series of normal paraffins.

The instrument utilized for this and subsequent gas-liquid chromatography was a MicroTek 2500-DPFF equipped with dual columns and dual-flame ionization detectors (MicroTek Instruments, Inc., Baton Rouge, La.). The flames were operated at 15 psi pressure with hydrogen flow of 40 ml/min and a scavenger air flow of 120 ml/min. The detector sensitivity was 3.2×10^{-9} amp at full scale using a 1-mv recorder. A Brown 1-mv recorder, series 153078, with Model 201-B disk-chart integrator (Minneapolis Honeywell, Minneapolis, Minn.) was used. For the qualitative runs a stream-splitting device was used in which approximately 80% of the sample was directed through the detector.

Gas-Liquid Chromatographic Separation of the Branched Paraffins Which Formed Urea Adducts.—A portion of the branched paraffins which formed urea adducts was analyzed by gas-liquid chromatography as described in Figure 2. In order to facilitate the collection of individual components for structure identifications, the remainder of these branched paraffins were distilled at 1–5 mm Hg in a bent, sealed capillary tube at 145–155°, using an aluminum block placed on a hot plate as heat supply and cooling the other end of the tube in a Dry Ice–acetone bath. The distillate was then separated by gas-liquid chromatography using the conditions described in Figure 2. Eluates were collected in differential thermal traps (Stevens and Mold, 1963) cooled in Dry Ice and acetone. To further purify individual collected fractions, they were rechromatographed isothermally at temperatures 20–30° below the elution temperature observed for the initial temperature-programed separation. The residue from the vacuum short-path distillation was also fractionated by gas-liquid chromatography and the major eluates were collected as described above. The temperature of the column was programed from an initial temperature of 210° at a rate of 4.0°/min. Each eluate was then rerun isothermally as described above. Collected samples were chromatographed in hexane on alumina to remove contaminants introduced from the gas-liquid chromatographic substrate.

Mass spectra were obtained for selected fractions with the Bendix Model 14-101 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 probe (Mold *et al.*, 1963a).

Separation and Identification of Pristane.—A short-path distillation with 780 mg of the materials which did not form urea adducts (recovered to the extent of 50.6% of the wax hydrocarbons) was performed at 1–4 mm Hg pressure and 200°. Within 2 hours 50.4 mg of clear distillate was collected. The further fractionation of this distillate was accomplished by gas-liquid chromatography (Fig. 4). The major resolved peak in the chromatogram, with R_T approximately equivalent to a normal paraffin with 17 carbon atoms, was collected in a trap (Stevens and Mold, 1963) cooled in a Dry Ice–acetone bath. This compound was recovered to the extent of 1.1% of the total paraffin fraction of wool wax. Several runs were made in order to accumulate sufficient material for structural characterization. The collected fraction was chromatographed in hexane on alumina to remove contaminants introduced from the gas-liquid chromatographic substrate. The infrared spectrum (Fig. 5), mass spectrum (Table III), and R_T value for this material were identical to those for an authentic sample of 2,6,10,14-tetramethylpentadecane (pristane) and in agreement with spectra previously reported by others (Bendoraitis *et al.*, 1962).

Purification and Characterization of a Commercial Sample of Pristane.—A sample of pristane from whale-liver oil was obtained from Aldrich Chemical Co., Inc., Milwaukee 10, Wisc. This material gave no indication of impurities upon gas-liquid chromatography. The pure liquid exhibited no optical activity when observed at 25° in a 1-dm tube with a Zeiss Circle Polarimeter reading to 0.01°. Its density was 0.7838 at 20° and its refractive index was $n_D^{20} = 1.4390$. Sörensen and Sörensen (1949) reported d(20°/40) = 0.7833, $n_D^{20} = 1.4386$.

Anal. Calcd for $C_{19}H_{40}$: C, 84.98; H, 15.02. Found: C, 84.16, 84.28; H, 14.57, 14.75 (Schwarzkopf Microanalytical Laboratory, Woodside, N. Y.).

The gas-liquid chromatography eluate was used to obtain an infrared spectrum (Fig. 5) and a mass spectrum (Table III) which were consistent with the structure of this compound. The nuclear magnetic resonance spectrum measured at 60 and 100 mc in CCl_4 (Varian Associates, Spectroscopy Applications Laboratory, Palo Alto, Calif.) indicated only methyl and methyene groups. The appearance of the methyl resonances suggested that all the methyl groups were secondary. The intensity and sharpness of the lines indicated groups of four, i.e., two terminal isopropyl groups, and two methyls. Integration of the area under the methyl proton resonance showed eighteen methyl protons.

Model Compounds.—Compounds which were used as reference materials included: 1-cyclopentylheneicosane, 1-cyclohexyleicosane, 2-methyltricosane, and *n*-hexacosane supplied by Dr. Joseph A. Dixon (API Research Project 42 at Pennsylvania State University); 2-methylhexacosane, 2-methyloctacosane, 2,22-dimethyltricosane, 2-methyltetracosane, 3-methyltetracosane, 16-methylhentriacontane, *n*-pentacosane, and *n*-hentriacontane, which were prepared by Mr. T. P. Chen; *n*-nonadecane (Humphrey-Wilkinson, Inc.); *n*-eicosane (Matheson, Coleman and Bell); *n*-tetracosane, *n*-octacosane, and *n*-dotriacontane (Distillation Products Industries); *n*-heptacosane (Lachat Chemicals); and *n*-tetratriacontane (Applied Science Laboratories).

Acknowledgments

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References

Bendoraitis, J. G., Brown, B. L., and Hepner, L. S. (1962), *Anal. Chem. 34*, 49.

Blumer, M., Mullin, M. M., and Thomas, D. W. (1963), *Science 140*, 974.

Dean, R. A., and Whitehead, E. V. (1961), *Tetrahedron Letters 21*, 768.
Downing, D. T., Kranz, Z. H., and Murray, K. E. (1960), *Australian J. Chem. 13*, 80.
Hallgren, B., and Larsson, S. (1963), *Acta Chem. Scand. 17*, 543.
Kochloefl, K., Schneider, P., Rericha, R., Horak, M., and Bazant, V. (1963), *Chem. Ind. (London)*, 692.
Lambertsen, G., and Holman, R. T. (1963), *Acta Chem. Scand. 17*, 281.
Levy, E. J., Doyle, R. R., Brown, R. A., and Melpolder, F. W. (1961), *Anal. Chem. 33*, 698.
Mold, J. D., Stevens, R. K., Means, R. E., and Ruth, J. M. (1963a), *Biochemistry 2*, 605.
Mold, J. D., Stevens, R. K., Means, R. E., and Ruth, J. M. (1963b), *Nature 199*, 283.
Sörensen, J. S., and Sörensen, N. A. (1949), *Acta Chem. Scand. 3*, 939.
Stevens, R. K., and Mold, J. D. (1963), *J. Chromatog. 10*, 398.
Waldron, J. D., Gowers, D. C., Chibnall, A. C., and Piper, S. H. (1961), *Biochem. J. 78*, 435.

End-Product Inhibition of the Conversion of Cholesterol to Pregnenolone in an Adrenal Extract*

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An extract prepared from acetone-dried powder of mitochondria from bovine adrenal cortex was used to study the conversion of 7α-[^{3}H]cholesterol to [^{3}H]pregnenolone. This sequence of reactions is inhibited by pregnenolone at the stage of conversion of 7α-[^{3}H]cholesterol to 20α-hydroxy[^{3}H]cholesterol, and therefore represents an example of feedback or end-product inhibition. It is suggested that intramitochondrial levels of pregnenolone may play an important role in the regulation of steroid biosynthesis by the adrenal cortex.

It is at present thought (Lynn *et al.*, 1954; Solomon *et al.*, 1956; Shimizu *et al.*, 1960, 1961; Constantopoulos *et al.*, 1962; Hall and Koritz, 1964a) that the conversion of cholesterol to pregnenolone takes place in endocrine tissue by the following sequence of reactions:

$$\text{cholesterol} \xrightarrow{[I]} 20\alpha\text{-hydroxycholesterol} \xrightarrow{[II]} 20\alpha,22\xi\text{-dihydroxycholesterol} \xrightarrow{[III]} \text{pregnenolone}^1$$

These reactions and the enzyme systems associated with them will be referred to here as step [I], enzyme [I], and so forth, although it is realized that each step involves a number of enzymes and cofactors not shown.

During the course of experiments designed to study these reactions in bovine adrenal cortex it was observed that pregnenolone inhibited the conversion of 7α-[^{3}H]-cholesterol to [^{3}H]pregnenolone by acetone powder of "mitochondria."[2] Interest in this observation arose from two circumstances. First, the demonstration that in the adrenal cortex the overall reaction (steps I–III) is confined to the "mitochondria" (Halkerston *et al.*, 1961) suggests that the inhibition of these reactions by pregnenolone may constitute an important regulatory mechanism, since the rate of the overall reaction may be influenced by the rate of removal of pregnenolone from mitochondria. Second, it was noticed that inhibition by pregnenolone was not associated with the accumulation of either 20α-hydroxy-[^{3}H]cholesterol or 20α,22ξ-dihydroxy[^{3}H]cholesterol, suggesting that this inhibition was not simply an example of product inhibition.

The present report concerns experiments designed to examine the nature of the inhibition of the above sequence of reactions by pregnenolone. It appears that pregnenolone produces feedback or end-product inhibition.

Experimental Procedure

Preparation of Tissue.—Bovine adrenal glands were obtained fresh from a slaughterhouse, the cortex was separated from the medulla, and an acetone-dried powder of cortical "mitochondria" was prepared according to the procedure described by Halkerson *et al.* (1961). Before use in each of the present experiments a clear supernatant extract the powder was prepared in 0.07 M potassium phosphate buffer, *p*H 6.8 (20 mg powder/ml buffer), as described by the same authors. This extract will be referred to hereafter as adrenal extract. The following experiments were performed on six separate preparations of acetone-dried powder. The powder was stored at −18° and no significant loss of activity as measured by conversion of 7α-[^{3}H]-cholesterol to [^{3}H]pregnenolone was detected during the course of these studies. All six powders gave the same results in the various experiments to be described.

Incubation Procedure.—Incubation was performed in 20-ml beakers in air with constant agitation at 37.5° in a final volume of 2 ml for a period of 10 minutes unless otherwise stated. Additions were made in the following order: KCl, 0.15 M, to make a final volume of 2 ml; TPN, 5×10^{-4} M; glucose-6-phosphate, 5×10^{-3} M; glucose-6-phosphate dehydrogenase (approximately

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[1] The following abbreviations and trivial names are used: corticosterone, 11β,21-dihydroxy-4-pregnene-3,20-dione; cortisol, 11β,17α,21-trihydroxy-4-pregnene-3,20-dione; dehydroepiandrosterone, 3β-hydroxy-5-androsten-17-one; DOC, 21-hydroxy-4-pregnene-3,20-dione; 17α-hydroxypregnenolone, 3β,17α-dihydroxy-5-pregnen-20-one; 17α-hydroxyprogesterone, 17α-hydroxy-4-pregnene-3,20-dione; pregnenolone, 3β-hydroxy-5-pregnen-20-one; progesterone, 4-pregnene-3,20-dione; TPN, triphosphopyridine nucleotide; ACTH, adrenocorticotropic hormone.

[2] The expression "mitochondria" is used here to refer to large particles prepared by the procedure of Halkerston *et al.* (1961).