Normal and Branched Aliphatic Hydrocarbons from the Eggs of the Tobacco Hornworm*

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ABSTRACT: Gas–liquid chromatographic analysis of the hydrocarbons from the eggs of the tobacco hornworm, *Manduca sexta* (Johannson), demonstrated the presence of four homologous series of saturated hydrocarbons. One of the series was composed of the *n*-alkanes (50% of the total hydrocarbons); the other three were composed of branched alkanes. No iso- or anteisoalkanes were detected by mass spectrometry. Mass spectral analysis indicated that the branched hydrocarbons consist of a monomethyl-, a dimethyl-, and a trimethyl-substituted series, 8, 35, and 4%, respectively, of the total hydrocarbons, with the branch points near the center of the molecule and separated by three carbon atoms. The major *n*-alkanes were heptacosane and nonacosane, 16 and 10.9%, respectively. The major branched alkanes chromatographed in two groups of three with equivalent chain lengths for the first group of 35.2, 35.4, and 35.7 and for the second group of 37.2, 37.4, and 37.6. The components of the first group were identified by mass spectrometry as 15- and 17-methylpentatriacontane, 13,17- and 15,19-dimethylpentatriacontane, and 13,17,21-trimethylpentatriacontane, 1.3, 11.7, and 0.9% of the total hydrocarbons, respectively. Di- and trimethyl-substituted hydrocarbons with the substituents in the center of the molecule and separated by three carbon atoms (indicating possible isoprenoid-type biosynthesis) have not previously been reported in plants, higher animals, or insects. The discovery may indicate the existence of a new pathway for hydrocarbon biosynthesis. Alkanes with an odd number of carbons made up 79% of the total hydrocarbon fraction. In the *n*-alkane series, 89% were odd numbered; in the major branched alkane series (series B, dimethyl), 91% were odd numbered; and in the two minor branched alkane series (series A, monomethyl and C, trimethyl), 11 and 15% were odd numbered, respectively. When only the number of carbon atoms in the straight-chain portion of the molecule of the hydrocarbons of series A, B, and C was considered, 89, 91, and 85%, respectively, were odd numbered.

The cuticular lipids of insects are important because (1) they prevent desiccation of the insect when humidity is low; (2) they protect the insect from toxic inorganic chemicals; (3) they serve as a barrier to the penetration of microorganisms; and (4) they affect the absorption of agricultural chemicals used in insect control.

The hydrocarbons are an important component of the cuticular lipids of a number of insects and account for 48–58% of the cuticular lipids from the Mormon cricket, *Anabrus simplex* Haldeman (Baker *et al.*, 1960); 75% of the cuticular lipids from the American cockroach, *Periplaneta americana* (L.) (Gilby and Cox, 1963); 12% of the external lipids of the adult and 3% of the external lipids of the naiad of a stonefly *Pteronarcys californica* Newport (Armold *et al.*, 1969); and 59–66% of the external lipids of *Lucilia cuprina* (Wiedemann) (Goodrich, 1970).

Hutchins and Martin (1968) reported that the hydrocarbons of the house cricket, *Acheta domesticus* (L.), consisted of alkanes, 2-methylalkanes, *x*-monomethylalkanes, olefins, and a trace of unknown alkanes; the cuticular hydrocarbons of *L. cuprina* consisted of *n*-alkanes, a trace of *n*-alkenes, 3-methylalkanes, and *x*-monomethylalkanes (Armold *et al.*, 1969); the cuticular hydrocarbons of *Leucophaea maderae* (F.) and *Blatta orientalis* L. (Tartivita and Jackson, 1970) and of *P. australasiae* (F.), *P. brunnea* Burnmeister, and *P. fuliginosa* (Serville) (Jackson, 1970) consisted of *n*-alkane, *n*-alkene, 3-methylalkanes, and *x*-monomethylalkanes. However, to our knowledge, the presence of di- and trisubstituted hydrocarbons with all the branch points centrally located has not been reported for plants, higher animals, or insects. Multiple-branched alkanes with one of the methyl branches on the third or fourth carbon atom of the chain have been reported in an insect (Martin and MacConnell, 1970) and on the second carbon atom in shale (McCarthy and Calvin, 1967).

A gas–liquid chromatographic analysis of the hydrocarbons from the integument, fat body, and hemolymph of the tobacco hornworm, *Manduca sexta* (Johannson), demonstrated the presence of four homologous series of paraffinic hydrocarbons, an *n*-alkane series, and three series of branched alkanes; the major branched alkanes were absent from the diet, and the preliminary mass spectra indicated they were not iso- or anteisoalkanes (D. R. Nelson, unpublished data). Subsequent analysis of freshly oviposited eggs of the tobacco hornworm showed that their hydrocarbon composition was qualitatively the same as that of the integument, fat body, and hemolymph. Consequently, we selected the eggs of this insect as the source of hydrocarbons for further studies made to identify the individual hydrocarbon components.

Experimental Section[1]

Freshly oviposited tobacco hornworm eggs (or eggs stored frozen for not more than 2 days) were obtained from horn-

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worms reared as described by Yamamoto (1969). Eggs (20 g) were extracted with 400 ml of chloroform–methanol (2:1, v/v) in a Sorval Omni-mixer for 1 min at low speed (all solvents used were of Nanograde Mallinckrodt quality; the filter paper, glasswool, and glassware were rinsed with the appropriate solvent immediately before use to remove any contaminating lipids). The mixture was filtered through Whatman No. 1 filter paper that had previously been soaked in $CHCl_3$. This extraction procedure was repeated twice more, and all filtrates were combined and reduced in volume to about 100 ml on a rotating flash evaporator. The lipids were extracted into hexane by partitioning against three 50-ml portions of hexane, the portions of hexane were combined and washed with 50 ml of water saturated with sodium chloride, and the hexane was dried over anhydrous sodium sulfate.

The hydrocarbon components of the lipid extract were obtained by column chromatography. Florisil (25 g) was packed in hexane into a 1-cm diameter glass column and washed with 200 ml of hexane to remove the contaminating substances. The lipid sample, in a volume of about 1 ml, was then placed on the column and washed on with five 5-ml portions of hexane, and the hydrocarbons were eluted with 100 ml of hexane.

The hydrocarbon fraction obtained from the Florisil chromatography was further purified by thin-layer chromatography on silica gel G with hexane as the developing solvent. Polar material at the origin and two fractions migrating near the solvent front were visible with iodine vapors; the fastest migrating fraction corresponding to the paraffinic and olefinic hydrocarbons was collected and eluted by suspending the silica gel in 50 ml of hexane overnight. The hydrocarbon fraction recovered from the silica gel was 65% of the sample applied.

Branched hydrocarbons were separated from normal hydrocarbons with Linde Molecular Sieve 5A, 0.0625 in. pellets, as described by Hutchins and Martin (1968). The efficiency of the molecular sieve was checked with hexatriacontane, which was completely removed when the solvent was checked by gas–liquid chromatography 2.5 hr after the addition of the molecular sieve.

The hydrocarbon components were further separated by gas–liquid chromatography on a 6 ft × 0.25 in. stainless-steel column packed with 80–100 mesh Diatoport S coated with 5% of the liquid phase (OV-1 or OV-17). The column temperature was usually programmed from 210 to 320° in 32 min and was then held at 320°. The temperatures of the flash heater and detector were 250 and 350°, respectively. The carrier gas was helium.

Blanks were carried through the extraction and chromatographic procedures to make certain that none of the hydrocarbons were present as contaminants in the solvents or glassware. The presence or absence of unsaturated components in the hydrocarbon fraction was determined by bromination and hydrogenation of the total, straight-chain, and branched-chain fractions obtained after treatment with the molecular sieve.

Samples for infrared, nuclear magnetic resonance, and mass spectral analyses were collected from the gas chromatograph by placing a 10:1 stream splitter at the exit of the column and terminating it with 0.125-in. diameter stainless-steel tubing into which a 0.071-in. diameter glass tubing was inserted and held in place with a Barber-Colman thru-hole septum. The eluting hydrocarbons condensed in the glass tubing that was held at room temperature. Condensation of hydrocarbons with low molecular weight was facilitated by holding Dry Ice against the glass tubing. The sample was rinsed from the glass tubing with hexane, and a micropellet of potassium bromide was prepared for infrared analysis with a Perkin-Elmer 337 spectrophotometer. For nuclear magnetic resonance analyses, the tubing was rinsed with deuterated chloroform and spectra were obtained on a Varian A-60A instrument equipped with a computer for averaging transients (Fabri-Teck Instruments, Inc., Model 1062). Mass spectra were obtained with a Varian M-66 mass spectrometer by placing either the potassium bromide pellet from the infrared analysis or the portion of glass tubing that contained the condensed hydrocarbon in the solid sample probe.

Results

The 20 g of eggs (containing 34.1% water as determined by lyophilization) extracted to obtain the hydrocarbon fraction were equivalent to 14,100 eggs from which 0.839 g (59.5 μg/egg) of lipids was obtained. Column chromatography of the lipid extract on Florisil yielded a hydrocarbon fraction weighing 0.0435 g (3.1 μg/egg); further purification on silica gel G thin-layer plates yielded 0.0280 g (2.0 μg/egg) of hydrocarbons.

The total hydrocarbon fraction was a semisolid at room temperature, and the infrared spectra showed a single broad band at 720–730 cm^{-1}, a characteristic of methylene groups in long-chain compounds, which, in the case of a crystalline hydrocarbon, would be split into two bands at 710 and 730 cm^{-1}. The infrared spectra showed full-scale absorption for the characteristic methyl and methylene bands between 2825 and 2930 cm^{-1}, a strong methyl plus methylene band was present at 1455 cm^{-1}, and a medium methyl band was present at 1380 cm^{-1}. The ratio of the band at 1455 cm^{-1} to that at 1380 cm^{-1} for the total hydrocarbon fraction was 2.7, the ratio observed for *n*-octacosane was 4.4, and that for pristane was 1.6. Since the average chain length of the total hydrocarbon fraction was about 32 carbons (calculated from the molecular weight and the percentage of composition tabulated in Table I), the value of 2.7 for the ratio of the bands at 1455 and 1380 cm^{-1} indicated the presence of methyl branching. Bands characteristic of unsaturation or of cyclic, aromatic, amine, carbonyl, or hydroxyl groups were not present.

The total hydrocarbon fraction was analyzed by gas–liquid chromatography (Figure 1T) and found to be composed of over 60 components ranging in chain length from 16 to about 45 carbon atoms (based on their retention times). The numbers in the figure refer to the carbon numbers of the individual hydrocarbons and the vertical lines indicate the points of elution of an *n*-alkane of that particular carbon number.

Three peaks at equivalent chain lengths of about 41.3, 43.5, and 45.5 were present, but are not shown in Figure 1; in addition, there were traces of a component with an equivalent chain length of about 40.4 and sometimes of one with a chain length of about 42.4. The major hydrocarbon components corresponded to compounds with carbon numbers of 27 (heptacosane) and 29 (nonacosane); for the *n*-alkanes,

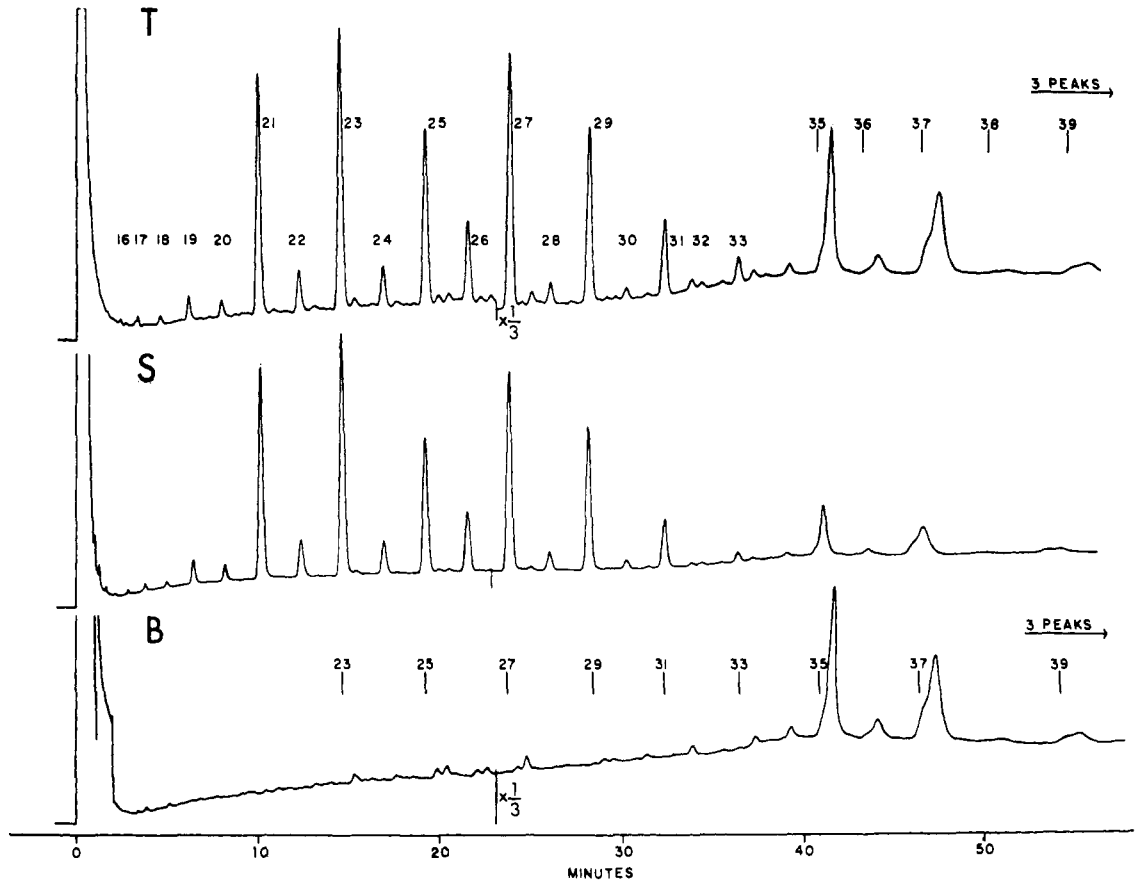

FIGURE 1: Gas–liquid chromatographic analysis of the total, T, straight-chain, S, and branched-chain, B, hydrocarbon fractions on a 6 ft × 0.25 in. column of 80–100 mesh Diatoport S coated with 5% OV-1, temperature programmed from 210 to 320° in 32 min and held at 320°. Three additional major peaks were eluted which are not shown on the traces; their equivalent chain lengths were about 41.3, 43.5, and 45.5, with traces of hydrocarbon peaks at 40.4 and 42.4. The straight-chain fraction S and the branched-chain fraction B were obtained by the use of molecular sieve 5A. The numbers on the traces refer to the carbon numbers of the corresponding *n*-alkanes; the vertical lines with the numbers above them indicate the position at which an *n*-alkane of that carbon number would elute.

the peak number, the carbon number, and the equivalent chain length are the same. Also, there were two multicomponent peaks, one that eluted with an equivalent chain length between 35 and 36 and the other with an equivalent chain length between 37 and 38. Also, many small peaks were present that had fractional equivalent chain lengths: three small peaks were present between each of the *n*-alkanes from 19 to 30; and at least one or more were present between the points of elution of the *n*-alkanes above 30. A fraction containing only branched alkanes was obtained by treating the total hydrocarbon fraction with Linde Molecular Sieve 5A; 34% of the sample was recovered in the straight-chain fraction and 42% in the branched-chain fraction. Analysis of the straight-chain fraction by gas–liquid chromatography (Figure 1S) showed that it contained branched hydrocarbons in addition to the *n*-alkanes; however, the branched-chain fraction (Figure 1B) contained only branched hydrocarbons. The absence in Figure 1B of the even- and odd-numbered peaks that are present in Figure 1T verifies the previous conclusion (based on gas chromatographic retention times) that peaks 16 through 33 are *n*-alkanes. The *n*-alkanes C-34, C-35, and C-36 may also be present in trace amounts, but their only positive identification is their gas chromatographic retention times. The branched-chain fraction contained the small peaks eluting between the *n*-alkanes and the two major peaks eluting with equivalent chain lengths between 35 and 36 and between 37 and 38; the long-chain hydrocarbons eluting with equivalent chain lengths of about 40.4, 41.3, 43.5, and 45.5 were also present in this fraction. Since the major peaks of the branched-chain fraction appeared to be composed of more than one component, this fraction was chromatographed on a 15-ft column of OV-1, and the temperature was programmed from 210 to 320° in 160 min (Figure 2). Both the major peaks in the branched-chain fraction were separated into three components as were all the other peaks that eluted with equivalent chain lengths larger than 35 although the peak that eluted at about 40.4 was not present in sufficient quantity to be certain that it consisted of more than one component. The components of the branched-chain fraction that eluted with equivalent chain lengths below 35 (Figure 1B) did not undergo any further separation. Because a maximum of three peaks eluted between the points of elution of any two adjacent *n*-alkanes (Figures 1T and 2), the three peaks were designated A, B, and C following the carbon number of the *n*-alkane after which they eluted. Thus, the three components that eluted with equivalent chain

TABLE I: Identification and Percentage Composition of the Tobacco Hornworm Egg Hydrocarbons.

Glc Peak No.	ECL[b]	Mass Spec Carbon No.[c]	Percentage Composition[a]			Alkane[d]
			Total	Straight Chain	Branched Chain	
16	16		*T*			
17	17		*T*			
18	18		*T*			
19	19		0.3	0.8		
-A	19.3		*T*			
-B	19.6		*T*			
-C	19.8		*T*			
20	20		0.2	0.6		
-A	20.3		*T*			
-B	20.6		*T*			
21	21		3.6	9.0		
-A	21.3		*T*			
-B	21.6		*T*			
22	22	22	0.7	1.8		*n*-Docosane
-A	22.3		*T*			
-B	22.6		*T*			
-C	22.7		*T*			
23	23	23	4.8	11.5		*n*-Tricosane
-A	23.3		0.2		0.4	
-B	23.6		*T*			
-C	23.7		*T*			
24	24	24	0.7	1.6		*n*-Tetracosane
-A	24.3		0.1		0.2	
-B	24.6		*T*			
-C	24.7		*T*			
25	25	25	3.2	6.4		*n*-Pentacosane
-A	25.3		0.1		0.3	
-B	25.6		0.2		0.4	
-C	25.8		*T*			
26	26	26	1.4	2.9		*n*-Hexacosane
-A	26.3		0.1		0.3	
-B	26.6		0.1		0.3	
-C	26.8		*T*			
27	27	27	16.0	30.4		*n*-Heptacosane
-A	27.3	28	0.2		0.6	
-B	27.6	29	0.7		1.8	9,13-Dimethylheptacosane
-C	27.7		*T*			
28	28		1.4	2.8		*n*-Octacosane
-A	28.3		*T*			
-B	28.5		0.1		0.2	
29	29	29	10.9	21.7		*n*-Nonacosane
-A	29.3		*T*			
-B	29.5		0.3		0.5	
-C	29.7		0.1		0.4	
30	30	30	0.6	1.3		*n*-Triacontane
-A	30.3		*T*			
-B	30.5		0.3		0.5	
31	31	31	4.8	7.5		*n*-Hentriacontane
-C	31.7		0.6		1.3	
32	32	32	0.3	0.4		*n*-Dotriacontane
-C	32.6		0.2		0.3	
33	33	33	1.5	1.5		*n*-Tritriacontane
-B	33.4	35	0.7	1.2		
34	34		*T*			
-B	34.4	36	0.7		1.3	
35			?			

TABLE I (*Continued*)

Glc Peak No.	ECL[b]	Mass Spec Carbon No.[c]	Percentage Composition[a] Total	Straight Chain	Branched Chain	Alkane[d]
-A	35.2	36	1.3		3.0	15- and 17-methylpentatriacontane
-B	35.4	37	11.7		26.2	13,17- and 15,19-dimethylpentatriacontane
-C	35.7	38	0.9		2.0	13,17,21-Trimethylpentatriacontane
36			?			
-A	36.2	37	0.5		1.1	
-B	36.4	38	1.7		4.1	
-C	36.7		0.2		0.4	
37						
-A	37.2	38	3.3		6.8	
-B	37.4	39	10.4		21.2	
-C	37.6	40	1.3		2.6	
38-A	38.3		0.2		0.4	
-B	38.5	40	0.4		0.9	
-C	38.6		0.2		0.3	
39-A	39.2	40	1.2		2.4	
-B	39.4	41	2.0		4.0	
-C	39.5		0.3		0.7	
40-B	40.4		*T*			
41-A	41.2		0.5		1.0	
-B	41.3	43	1.7		3.5	
-C	41.5		0.2		0.4	
43-A			0.2		0.4	
-B	43.5		2.6		5.1	
-C			*T*		*T*	
45-B	45.5		1.8		3.4	

[a] Values calculated by triangulation of the areas under the gas–liquid chromatographic peaks. Those components present in less than 0.1% are indicated by *T*. [b] Equivalent chain length. Averages of values obtained from isothermal gas–liquid chromatography at 230, 250, 280, and 320° (semilog plots) and from temperature programming from 210 to 320° in 160 min (linear plot). [c] Samples were collected from the gas chromatograph in glass tubing. Portion of tubing containing the sample was placed in the solid sample probe of a Varian M-66 mass spectrometer. The molecular ion of branched paraffins over 30 carbon atoms was not readily apparent. [d] Structure determined by mass spectrometry.

lengths greater than 35 but less than 36 were designated 35-A, 35-B, and 35-C, in order of increasing retention time.

The total, straight-, and branched-chain hydrocarbon fractions showed no significant change in gas–liquid chromatographic traces after bromination or hydrogenation, an indication of the absence of unsaturated hydrocarbons. The absence of unsaturation was also confirmed by the mass spectral fragmentation patterns (see Table I for those hydrocarbons analyzed by mass spectrometry).

The presence of four homologous series of hydrocarbons was demonstrated when the logarithm of the isothermal retention distances was plotted *vs.* the carbon number (Figure 3). The carbon numbers of the individual hydrocarbons of the *n*-alkane series were determined by a comparison of their retention distances with those of known standards; they were confirmed by trapping those individual peaks present in sufficient amounts from the gas chromatograph and obtaining their molecular weight by mass spectrometry. The molecular weights were also determined for those branched hydrocarbons present in sufficient amounts. The results of the gas–liquid chromatographic and mass spectral analyses are tabulated in Table I. The equivalent chain lengths of the *n*-alkane series ranged from 16 to 36 (*n*-alkanes with equivalent chain lengths of 34, 35, and 36, if present, were there only in trace amounts), those of the branched-chain A series ranged from 19.3 to between 43 and 43.5 (carbon numbers from 20 to 44), those of the branched-chain B series ranged from 19.6 to 45.5 (carbon numbers from 21 to 47), and those of the branched-chain C series ranged from 19.8 to between 43.5 and 44 (carbon numbers from 22 to 46). Hydrocarbons 43-A and 43-C are not plotted in Figure 3 because they were present in small amounts and formed broad peaks that overlapped with 43-B and made accurate determination of their equivalent chain length impossible. It was very probable that the peaks with equivalent chain lengths 40.4 (40-B) and 45.5 (45-B) had three components that would fall into the A, B, or C series, but the

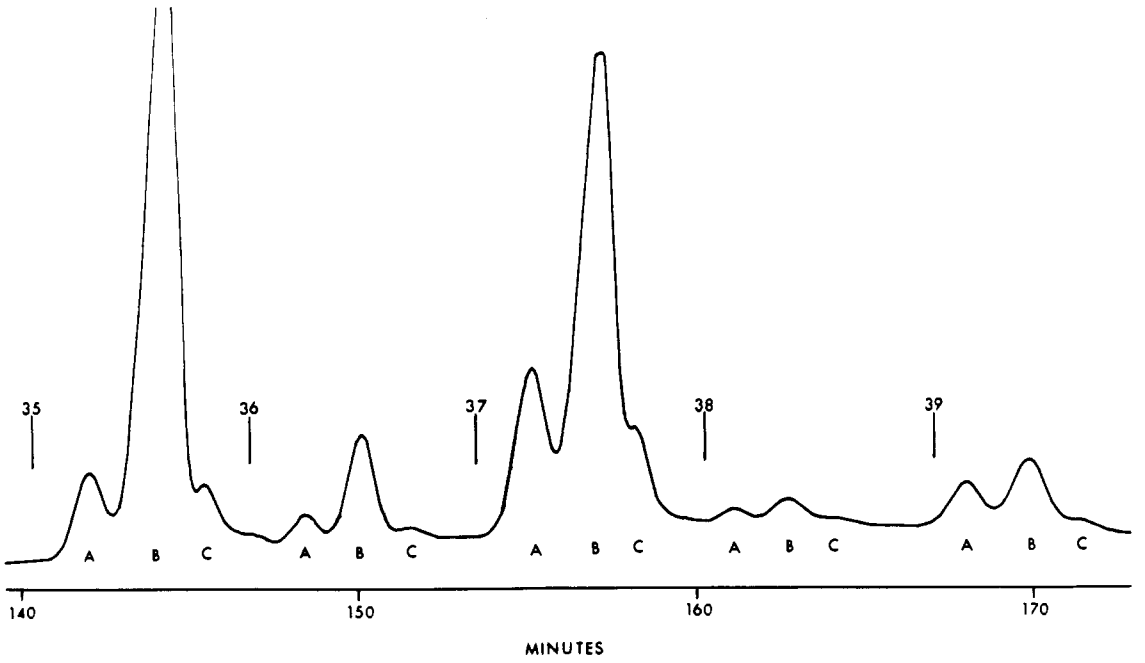

FIGURE 2: Gas–liquid chromatographic analysis of the branched-chain hydrocarbon fraction B, obtained by treatment of the total hydrocarbons with molecular sieve 5A, on a 15 × 0.25 in. column of 5% OV-1 on 80–100 mesh Diatoport S temperature programmed from 210 to 320° in 160 min. The vertical lines with the number above them refer to the point at which an *n*-alkane of that carbon number would elute. The letters A, B, and C designate the hydrocarbons which fall in the three homologous series A, B, and C of the branched-chain hydrocarbons

small amounts present and the broadening of the peaks made it difficult to determine whether the peaks had single, double, or triple components.

The major *n*-alkanes were heptacosane, C_{27}, 16%, and nonacosane, C_{29}, 10.9%, both with odd numbers of carbon atoms; the major even-numbered *n*-alkanes were hexacosane, C-26, 1.4%, and octacosane, C-28, 1.4% (Table I). The major branched hydrocarbons in the three branched-chain series were 35-A, -B, and -C, 1.3, 11.7, and 0.9%, respectively, and 37-A, -B, and -C, 3.3, 10.4, and 1.3%, respectively. The *n*-alkane series of hydrocarbons accounted for 50.4% of the total hydrocarbons; in the branched-chain series, the A series accounted for 7.9, the B series for 35.4, and the C series for 4.0% of the total hydrocarbons. In the total hydrocarbon fraction, about 79% by weight of the hydrocarbons had an odd number of carbon atoms, and 89% of the hydrocarbons of the *n*-alkane series had an odd number of carbon atoms. The major series of the branched-chain hydrocarbons, series B, had 91% of its hydrocarbons with an odd number of carbon atoms; the A series had only 11%; the C series had 15%.

The mass spectrum of a hydrocarbon peak trapped from the gas chromatograph for each of the branched-chain series of hydrocarbons is shown in Figure 4 along with the standard hydrocarbons 12-methylnonacosane and 16-methylhentriacontane.[2] The molecular ion was often absent or present to only a small extent in the mass spectrum of long-chain branched hydrocarbons. As a result, the molecular weight was taken to be 15 mass units greater than the last major peak at the high mass end of the spectrum, which is M − 15. The fragments of increasing intensity that began at M − 57 or M − 43 and continued through M − 29 to the fragment usually of greatest intensity in this region of the spectrum (M − 15) seem to be characteristic of long-chain, methyl-branched hydrocarbons with branching near the center of the molecule. They were not the result of the presence of methyl branching near the end of the carbon chain such as occurs in iso- and anteisoalkanes. With a methyl branch on carbon atom 2, a fragment peak would be expected at M − 43 of greater intensity than the fragment peak at M − 15, and the peak at M − 29 would be very small. With a methyl branch on carbon atom 3, a fragment peak would be expected at M−29 of greater intensity than the fragment peaks at M − 57 and M − 15, and the peak at M − 43 would be very small. Since these fragmentation patterns were not present in any of the mass spectra, the iso and anteiso structures are not present in the hydrocarbons from the tobacco hornworm. The absence of a doublet in the infrared spectra at about 1380 cm^{-1} for a mixture of the hydrocarbons from 37-A to 40-B also indicated that an iso structure was not present.

Since the evidence pointed to internally branched hydrocarbons, the mass spectra were interpreted as described by Mold *et al.* (1966) and Hutchins and Martin (1968). However, this led to immediate difficulties. When we interpreted 35-B with a carbon number of 37 as a mixture of monomethyl-branched hydrocarbons, we found that 36-A, also with a carbon number of 37, contained almost the same monomethyl-branched hydrocarbon mixture that was proposed for 35-B, but it eluted on the gas chromatograph as being one carbon longer than 35-B.

Textbooks concerned with the interpretation of mass spectra were of little or no help in interpreting the mass spectra of long-chain, methyl internally branched hydrocarbons. However, McCarthy *et al.* (1968) surveyed the mass spectra

[2] These hydrocarbons were kindly furnished by James Mold, Research Department, Liggett and Myers Tobacco Co., Durham, N. C.

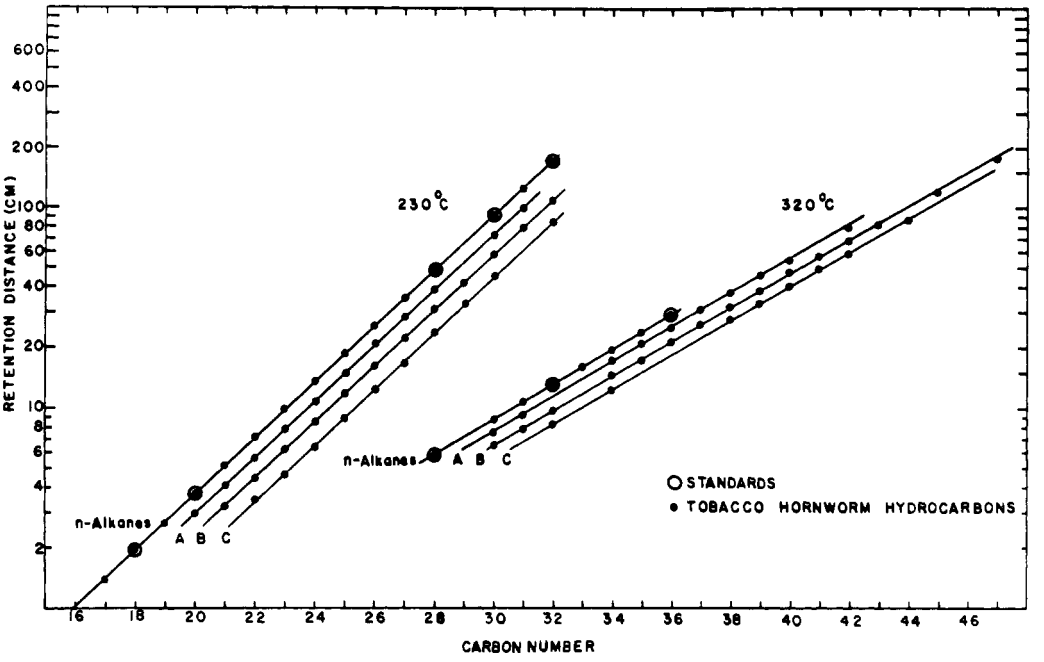

FIGURE 3: A semilog plot of the retention distance *vs.* the carbon number of the individual hydrocarbons in the homologous series of *n*-alkanes and the A, B, and C series of the branched-alkanes. The data were obtained by gas–liquid chromatography of the total hydrocarbons and of the straight-chain and branched-chain hydrocarbon fractions obtained after treatment with molecular sieve 5A. Chromatography was carried out isothermally at 230, 250, 280, and 320° on a 6 ft × 0.25 in. column of 5% OV-1 on 80–100 mesh Diatoport S and on a 15 ft × 0.25 in. column of OV-1 that was temperature programmed from 210 to 320° in 160 min. The values plotted for 230 and 320° are averages of the equivalent chain length values obtained from these chromatographic analyses.

published by the American Petroleum Institute (API) (1965) and determined the significance of characteristic even-mass fragmentation peaks of hydrocarbons. These authors concluded that the fragmentation of a saturated aliphatic hydrocarbon would cause a hydrogen transfer which would give rise to a C_nH_{2n} peak with an intensity greater than the intensity of the corresponding C_nH_{2n+1} peak when this fragment had a long straight-chain tail, provided that this fragment ion had seven or more carbons; when more than nine carbons are present, the ratio of the even to the odd peak begins to decrease, and when the fragment ion contains 18 or more carbon atoms, the odd peak will generally be more intense than the even peak. Moreover, when the fragment ion contains another branch, the even peak will not be dominant; also a dominant even peak is not present for dialkyl substituents on the same carbon or for iso- and anteisoalkanes.

We therefore made a search of the API mass spectra and hydrocarbon mass spectra [Biemann (1962), MacConnell (1969), Fehler and Light (1970), Gelpi *et al.* (1970), and Tartivita and Jackson (1970)] not covered by McCarthy and his group and concluded that the size of the neutral fragment formed has an effect on whether the fragment ion will lose a hydrogen and give a large, even mass peak in the mass spectrum. In 12-methylnonacosane, the ratio of the neutral fragment to the fragment ion (counting carbon atoms only) is 0.58, and the ratio of the intensity of the even to the odd peak at 266 and 267 is 1 (Figure 4A). However, if the methyl branch was on carbon 10, the neutral-to-fragment ion ratio would drop to 0.43, and the ratio of even to odd peaks would be less than 1. On the other hand, if the ratio of neutral fragment to fragment ion increases, as, for example, with 16-methylhentriacontane, to 0.88, the ratio of even to odd peaks would be greater than 1 (Figure 4B). In general, the greater the fragment ion mass, the higher must be the neutral-to-fragment ion ratio before the even peak will be more intense than the odd peak.

The mass spectrum of 12-methylnonacosane (Figure 4A) showed that cleavage on either side of the methyl branch gave rise to a fragment ion that had lost a hydrogen and resulted in an even mass peak in the spectrum. The preferred cleavage was on the side of the branch point which gave the largest neutral fragment; the charge remained on the fragment with the secondary carbon atom. Thus, cleavage resulted in a large fragment peak at 182 from the secondary fragment and a small peak at 238 from the straight-chain tail. The peak at 238 was expected to be about as large as or, as in many cases, slightly larger than, the peak at 239. The next point of cleavage was on the other side of the branch point which gave rise to a large peak at 266 and a smaller peak at 154. The peak at 266 was not larger than that at 265 because the neutral fragment was small compared to the fragment ion. The branched alkane 16-methylhentriacontane gave a secondary fragment ion at 238 and a straight-chain fragment ion at 210 (Figure 4B). In both 12-methylnonacosane and 16-methylhentriacontane, the formation of the secondary fragment ion and the straight-chain tail fragment ion involved a hydrogen transfer that gave even mass ions. Therefore, a monomethyl internally branched hydrocarbon does not ordinarily give a characteristic fragment peak with an odd mass that is significantly more intense than the even mass peak one mass unit less. Based on these interpretations of mass spectra, we concluded that the branched-chain A series is a series of monomethyl internally

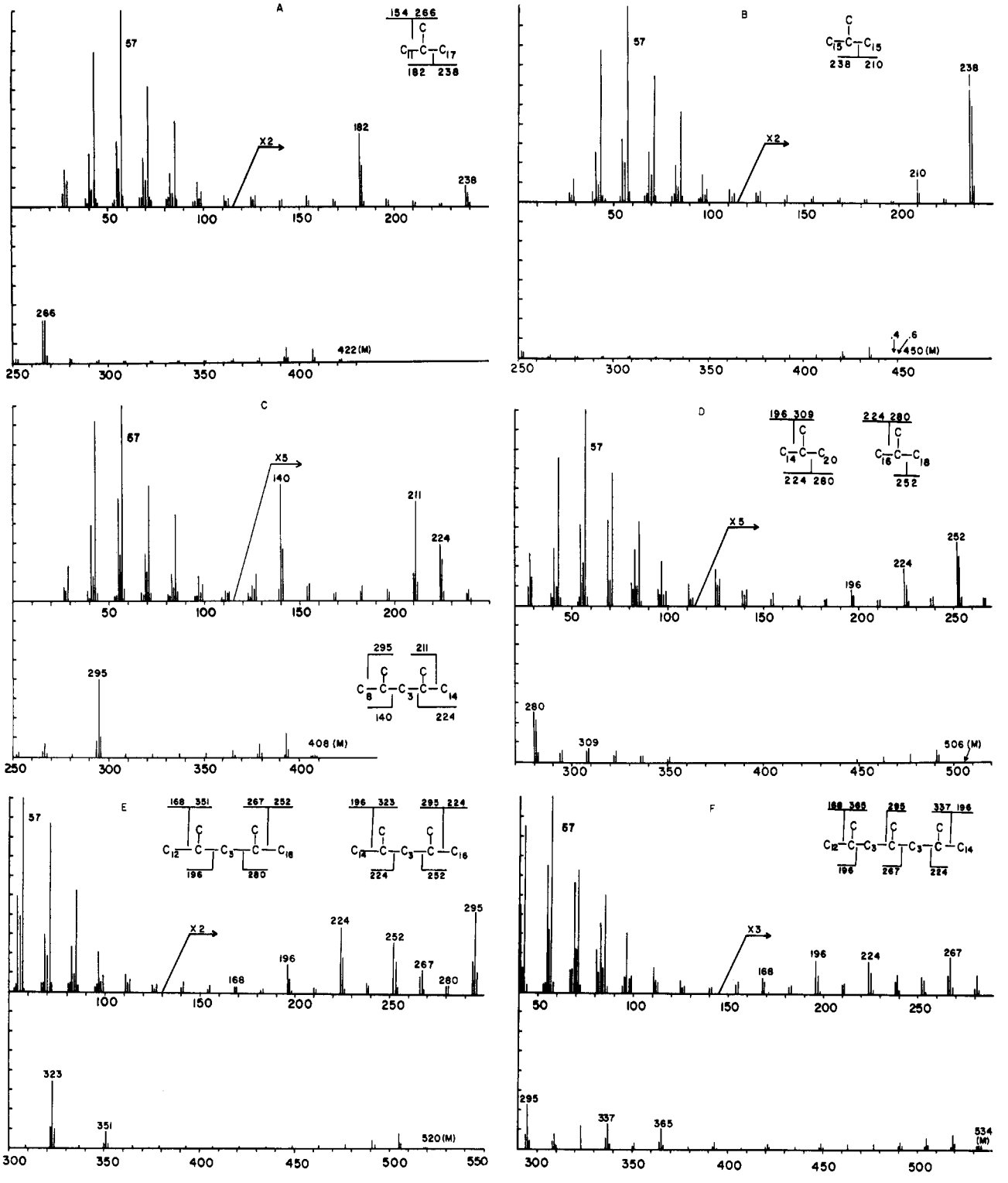

FIGURE 4: Mass spectra of standards and tobacco hornworm egg hydrocarbons trapped from the gas chromatograph and run on a Varian M-66 mass spectrometer: (A) 12-methylnonacosane; (B) 16-methylhentriacontane; (C) gas–liquid chromatographic peak 27-B; (D) gas–liquid chromatographic peak 35-A; (E) gas–liquid chromatographic peak 35-B; and (F) gas–liquid chromatographic peak 35-C.

branched hydrocarbons, the branched-chain B series is a series of dimethyl internally branched hydrocarbons, and the branched-chain C series is a series of trimethyl internally-branched hydrocarbons.

The first hydrocarbon we were able to identify was 27-B, which had 29 carbon atoms (Figure 4C). We first considered it to be a mixture of two monomethylalkanes, 9-methyloctacosane, and 14-methyloctacosane. Therefore, we expected the

9-methyl isomer would preferentially cleave to give a 19-carbon neutral fragment and a 10-carbon fragment ion with a large peak at 140; the 19-carbon tail would give a pair of small peaks at 266 and 267. The cleavage on the opposite side of the branch point would give a large peak at 295 and a pair of small peaks at 112 and 113. (In this cleavage, the characteristic peak was expected at 295 rather than at 294 because the neutral fragment is small compared with the fragment ion.)

The second component of the proposed mixture (the 14-methyl isomer) would be expected to give peaks of about equal intensity at 210 and 224 for the two fragment ions with the secondary carbon atom and small pairs of peaks at 182 and 183 and at 196 and 197 corresponding to the loss of the 13- and 14-carbon atom tails, respectively. However, in the mass spectrum obtained for 27-B, an intense peak was present at 211 instead of at 210; the peak at 224 was much smaller than the peak at 211; and the peak at 266 was small compared with the peak at 267. Therefore, the gas chromatographic hydrocarbon peak 27-B could not be a mixture of the two monomethylalkanes, 9-methyloctacosane, and 14-methyloctacosane.

We next considered 27-B as a dimethyl-branched alkane since the spectrum had intense, odd mass peaks at 211 and 295. Moreover, a survey of published spectra indicated that either a monomethyl hydrocarbon in which the methyl branch was not centrally located or a multi-branched hydrocarbon would give rise to intense odd mass peaks. A dimethyl-branched hydrocarbon was also indicated by the gas chromatographic retention time since Mold *et al.* (1966) showed that 12-methylnonacosane had an equivalent chain length of 0.62 carbon atom less than the corresponding *n*-alkane, and we found that 12-methylnonacosane had an equivalent chain length on our columns of 0.7 carbon atom less than the corresponding *n*-alkane. Thus, two methyl branches internally located might be expected to give an equivalent chain length for 27-B of 27.6 (29 − 2(0.7) = 27.6), which we observed (Table I).

The mass spectrum of 27-B (Figure 4C) was then interpreted as being that of 9,13-dimethylheptacosane. Cleavage at the first branch would give a fragment ion of mass 295 greater than 294 since the ion contained a second branch; cleavage at the other branch would give a fragment ion of 211 greater than 210. However, cleavage on the internal side of the first and second branch points would given even mass fragment ions of 140 and 224, respectively, since these ions have a straight-chain tail. The straight-chain tails of 8 and 14 carbon atoms would give a pair of peaks at 112 and 113 and at 196 and 197. Because all the expected peaks of the expected relative intensities were present in the mass spectrum, we concluded that 9,13-dimethylheptacosane was the hydrocarbon in the gas–liquid chromatographic peak 27-B.

The alternative to 9,13-dimethylheptacosane would be 9,14-dimethylheptacosane, which would give the same pairs of peaks in the mass spectrum. However, the relative intensities of the pairs of peaks at 210 and 211 and at 224 and 225 would change, and more importantly, the relative intensities of the peaks within each pair would change. The 9,14 isomer would be expected to give a pair of peaks at 224 and 225 more intense than those at 210 and 211. Also, 225 would be larger than 224 because this fragment ion would contain a second branch; the peak at 210 would be larger than the peak at 211 because this fragment ion would have a long straight-chain tail. Because the spectrum of 27-B did not have peaks at 210, 211, 224, and 225 with these relative intensities to each other, the 9,14 isomer was ruled out. The same approach was used to rule out alternative structures for the subsequent spectra.

The spectra of the three branched-alkane peaks 35-A, 35-B, and 35-C that eluted on the gas chromatograph with equivalent chain lengths between 35 and 36 are shown in Figure 4D, E, and F, respectively. The equivalent chain length of 35-A, 35.3, was 0.7 less than the carbon number 36, which indicated that 35-A might be an internally branched monomethylalkane. The first point considered in the spectrum of 35-A in Figure 4D was that no characteristic odd mass peaks were present which did not also have an even mass peak of about the same or greater intensity associated with them, an indication of the absence of a multisubstituted hydrocarbon. The spectrum was compatible with that expected for a mixture of 15-methylpentatriacontane and 17-methylpentatriacontane. Cleavage of the 15-methyl isomer would give a peak at 224 more intense than the peak at 308 (here again, the odd mass peak 309 was slightly greater because of the large dissymmetry of the molecule) and would give pairs of small peaks at 196 and 197 and at 280 and 281 from the 14 and 20 carbon straight-chain fragments, respectively. The peaks at 280 and 281 were larger than expected because of the contribution from the 17-methyl isomer. This isomer would cleave on either side of the branch point to give large peaks at 252 and at 280. The pairs of small peaks expected at 224 and 225 and at 252 and 253 for the 16 and 18 carbon straight-chain fragments, respectively, were not apparent because of the large contribution from other fragment ions of the same masses. Therefore, the spectrum indicated a mixture of 15-methylpentatriacontane and 17-methylpentatriacontane for the gas chromatographic peak 35-A.

The large odd mass peaks at 295, 323, and 351 in the spectrum of 35-B (Figure 4E) indicated that the hydrocarbon was not a monomethylalkane (unless the location of the branch made the molecule quite unsymmetrical and several isomers were present). Also, the previous interpretation of 27-B as a dimethylalkane of the B series and an equivalent chain length of 35.4 for 35-B made it quite likely that 35-B was composed of dimethyl alkane(s). The spectrum was therefore interpreted as being that of a mixture of 13,17-dimethylpentatriacontane and 15,19-dimethylpentatriacontane. The 13,17-dimethyl isomer would cleave external to the branch points and give rise to fragment ions containing a second branch with odd masses of 267 and 351; cleavage internal to the branch points would give fragment ions of even mass 196 and 280. The straight-chain 12-carbon tail would give a small pair of peaks at 168 and 169. The peaks for the 18-carbon straight-chain tail at 252 and 253 and for the 14- and 16-carbon straight-chain tails from the 15,19-dimethyl isomer at 196 and 197 and at 224 and 225, respectively, would also arise from other more preferred fragmentations so they were not considered further. The 15,19-dimethyl isomer would cleave external to the branch points to give peaks at 295 and 323 from fragment ions containing a second branch and would cleave internal to the branch points to give fragment ions with even masses at 224 and 252. Therefore, the proposed mixture of 13,17-dimethylpentatriacontane and 15,19-dimethylpentatriacontane was compatible with the mass spectrum, and a comparison of the relative intensities of the fragment peaks at 295 and 267 indicated that the 15,19 isomer was the major component.

The component of the C series of branched hydrocarbons

35-C contained 38 carbon atoms (molecular weight of 534, Figure 4F); then the equivalent chain length of 35.7 (Table I) would be observed if the molecule contained three methyl branches and if each branch decreased the retention time by an amount equivalent to 0.77 carbon atom. The mass spectrum (Figure 4F) contained both odd and even peaks, an indication of the presence of multiple internal branching. The expected mass spectrum of 13,17,21-trimethylpentatriacontane demonstrated a compatibility with the observed mass spectrum. Cleavage at the middle branch point would give rise to fragment ions of 295 and 267; cleavage external to the two outside branch points would give rise to fragment ions of 337 and 365. The even peaks at 196 and 224 would be formed by internal cleavage of the outside branch points, and the pair of peaks at 168 and 169 would arise from the 12-carbon straight-chain fragment. Therefore, the spectrum could be that of a triple methyl-branched hydrocarbon though 13,17,21-trimethylpentatriacontane was only a tentative structure because the intensity of the peaks at 252 and 323 and the inadequate resolution by gas chromatography (Figure 2) indicated that other components (35-B) might have been present in the sample.

A nuclear magnetic resonance spectrum was obtained from a mixture of 35-B and 35-C that had a methylene-to-methyl ratio of 4.4. The theoretical ratio for a dimethyl-branched hydrocarbon is about 5; that for a trimethyl-branched hydrocarbon is about 4. Therefore, the observed ratio of 4.4 was compatible with a mixture containing di- and trimethyl-branched hydrocarbons 35-B and 35-C, respectively.

Identification of the remaining hydrocarbons was difficult because of several factors: the small amount of many of the components, the inadequate gas chromatographic resolution, column bleed, and the presence of up to four isomers in a single peak. However, preliminary mass spectral data for 37-A, -B, -C, 39-A, and 41-B support the conclusion that the four series of hydrocarbons consist of *n*-alkanes, monomethylalkanes, dimethylalkanes, and trimethylalkanes, with the branch points located internally on the molecule.

Discussion

The presence of three homologous series of branched hydrocarbons in addition to the homologous series of *n*-alkanes in eggs of the tobacco hornworm emphasized the wide differences in the composition of the hydrocarbons from different insects (the structures of the hydrocarbons of the tobacco hornworm cuticle appear to be identical with those of the eggs) (D. R. Nelson, unpublished data). The tobacco hornworm hydrocarbons are saturated, and the branched-chain fraction consists of mono-, di-, and trimethyl homologous series of internally branched hydrocarbons. Homologous series of *n*-alkanes have been reported for a number of insects; however, Hutchins and Martin (1968) reported the presence of a series of internally branched monomethylalkanes in extracts of the house cricket, *A. domesticus*, in addition to 2-methylalkanes, olefins, and a trace amount of an unidentified series of alkanes. The major monomethyl-branched hydrocarbons of the cricket had 35 and 37 carbon atoms; in tobacco hornworm eggs, we found that the major monomethyl-branched hydrocarbons 35-A and 37-A had 36 and 38 carbon atoms, respectively. Armold *et al.* (1969) reported the presence of *n*-alkanes, anteisoalkanes, and internally branched monomethylalkanes in the surface lipids of *Pternarcys californica;* the major branched component contained 30 carbon atoms and was a mixture of four monomethyl isomers. The American cockroach had an unsaturated hydrocarbon, 6,9-heptacosadiene, as its major cuticular hydrocarbon, and lesser amounts of anteisopentacosane and pentacosane (Gilby and Cox, 1963, and Baker *et al.*, 1963). In several other species of cockroaches, the hydrocarbon fraction of the surface lipids consisted of *n*-alkanes, anteisoalkanes, and internally branched monomethylalkanes (Tartivita and Jackson, 1970, and Jackson, 1970); in some of the cockroaches, alkenes also were present. The only previous report of hydrocarbons with multiple internal methyl branches in insects was that of MacConnell (1969) who reported the presence of three series of hydrocarbons in an ant *Atta colombica* Guerin: the *n*-alkanes, 3,7,11-trimethylalkanes, and 4,8,12-trimethylalkanes. In both the branched-chain series, one of the methyl branches was near the end of the chain, and the branch points were separated from each other by three carbon atoms, *i.e.*, an isoprenoid-type structure. The mass spectra of the tobacco hornworm egg hydrocarbons showed that for the smallest triple-branched hydrocarbon analyzed (35-C), the nearest methyl branch was on carbon 13, and the branch points were separated by three carbon atoms. In addition, we found a monomethylalkane series and a dimethylalkane series that showed for the smallest double-branched hydrocarbon analyzed (27-B), the nearest methyl branch was on carbon 9, and the branch points were separated by three carbon atoms. Di- and trimethyl-substituted hydrocarbons with the substituents in the center of the molecule separated by three carbon atoms (an indication of an isoprenoid type of biosynthesis) have not been previously reported in plants, higher animals, or insects. The data may indicate the presence of a new pathway for hydrocarbon biosynthesis.

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Phosphorylation of Mouse Ascites Tumor Cell Lysine-Rich Histone*

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ABSTRACT: The lysine-rich histone of several mouse tissues has been compared by polyacrylamide electrophoresis to that of mouse Ehrlich ascites tumor cells. A much greater degree of heterogeneity was evidenced in the tumor histones. That a significant portion of this heterogeneity is caused by phosphorylation was demonstrated by labeling the lysine-rich histone with ^{32}P.

This observation was further supported by a reduction in heterogeneity upon removal of the phosphate with alkaline phosphatase.

Johns has shown that mammalian histones can be separated into five major groups by chemical fractionation procedures (Johns, 1964; Phillips and Johns, 1965). Using these separation procedures, a number of workers have shown that the lysine-rich histone fraction contains phosphate groups bound to histone in the form of serine phosphate (Kleinsmith *et al.*, 1966; Ord and Stocken, 1966; Stevely and Stocken, 1968a,b; Marushige *et al.*, 1969). What fraction of the lysine-rich histone is so phosphorylated is not clear, and there is some controversy as to whether the extent of phosphorylation of histone is negatively (Guttierrez and Hnilica, 1967) or positively (Stevely and Stocken, 1968; Buckingham and Stocken, 1969) correlated with the rate of cell division. However, most authors are agreed that it varies from tissue to tissue, and it seems likely that phosphorylation is not necessarily an all-or-none affair, thus opening the possibility for a histone and phosphorylated-histone heterogeneity. Recently, we have described an electrophoretic technique capable of resolving all five histone groups (Panyim and Chalkley, 1969a,b). Upon close examination of the bands, particularly after lengthy electrophoresis, it was shown that several of the bands were made up of multiple components. Further, it is likely that all members of a given electrophoretic group have similar chemistry since they are coisolated during the Johns chemical extraction procedures. A considerable heterogeneity within the lysine-rich histone group has also been documented by other workers (Bustin and Cole, 1969). This raised the possibility that the multiple electrophoretic bands within a group were, in fact, a demonstration of microheterogeneity imposed upon a parent histone molecule by minor modifications of the parent protein with attendant changes in electrophoretic mobility. The most rapidly moving arginine-rich histone is found as an equal-intensity electrophoretic doublet in calf thymus histone and almost exclusively (95%) as a single band for pea histone, which compares very well with the observation of DeLange *et al.* (1969) who found by direct amino acid sequence analysis that 50% of this histone was acetylated in calf thymus and only 6% acetylated in pea histone. In view of the reports of the phosphorylation of lysine-rich histones, it seemed likely that the multiplicity of bands observed in this region was due to varying degrees of phosphorylation of a lysine-rich histone molecule.

This paper presents the results of experiments designed to test this idea, arguing that it is, at least in part, correct. Further, a correlation between the replicative activity of the tissue and the extent of heterogeneity of the lysine-rich histone is described. Slowly replicating tissue, such as liver, contains lysine-rich histone primarily in the nonphosphorylated form, whereas a mouse ascites tumor cell line shows multiple phosphorylation.

Materials and Methods

The strain of Ehrlich ascites tumor cells was propagated in the peritoneal cavity of female mice, strain Swiss Webster. Tumor cells were withdrawn by syringe and washed in 0.9% NaCl, pH 8.0.

[^{32}P]Phosphoric acid (HCl-free) was purchased from New England Nuclear Corp. It was diluted to 1 mCi/ml with 0.9% NaCl–0.01 M Tris (pH 8.0).

^{32}P injections were always 200 μCi/mouse, *i.e.*, 0.2 ml and always intraperitoneal. Injections were given at zero time and 12 hr later. The mice were sacrificed 18 hr after the first injection and tissue was collected.

The isolation and electrophoresis of histone followed methods previously described (Panyim and Chalkley, 1969a).

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