Characterization and distribution of the hydrocarbons found in diapausing pupae tissues of the tobacco hornworm, *Manduca sexta* (L.)[1]

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Abstract The cuticular hydrocarbons of diapausing pupae of the tobacco hornworm, *Manduca sexta* (L.), are composed of two types of hydrocarbons: *n*-alkanes (3%) and unsaturated hydrocarbons (97%). The integument, fat body, muscle, and hemolymph are composed of three types of hydrocarbons: *n*-alkanes (9, 6, 9, and 2%, respectively), branched alkanes (75, 85, 80, and 56%, respectively), and unsaturated hydrocarbons (16, 9, 11, and 42%, respectively). The absence of branched alkanes on the cuticular surface indicated that hydrocarbons are selectively synthesized or deposited according to the presence or absence of methyl branches in the molecule. The hydrocarbons consisted of homologous series of *n*-alkanes from 21 to 41 carbon atoms, monomethylalkanes from 23 to 39 carbon atoms, dimethylalkanes from 25 to 43 carbon atoms, and trimethylalkanes from 33 to 37 carbon atoms. All branched components had methyl groups near the center of the molecule, and the di- and trimethyl branched components had three methylene units between the branch points. Straight chain alkenes, alkadienes, and alkatrienes from 23 to 44 carbon atoms were partially characterized. The percentage composition of each homologous series varied from tissue to tissue.—**Coudron, T. A., and D. R. Nelson.** Characterization and distribution of the hydrocarbons found in diapausing pupae tissues of the tobacco hornworm, *Manduca sexta* (L.). *J. Lipid Res.* 1981. **22:** 103–112.

Supplementary key words *n*-alkanes · branched alkanes · gas–liquid chromatography–mass spectrometry

Hydrocarbons comprise one of the lipid components of the hydrophobic layer on the cuticular surface of insects and are also found in internal tissues of the insect. Recently a number of reviews have been written on various aspects of insect hydrocarbons (1–3). Surface hydrocarbons aid in the protection of the insect throughout its life and are important in chemical communication processes. Several hydrocarbons have been reported to function as sex pheromones, mating pheromones, kairomones, and alarm-defense substances. The function of the hydrocarbons located within the insect tissues, however, remains unknown.

Insect hydrocarbons are unique with respect to the chain length, degree and type of branching, and the degree and position of unsaturation. Few 2-methylalkanes containing an odd number of carbon atoms in the molecule have been reported but many 3-methylalkanes with an even number of carbon atoms have been isolated. Internally branched monomethylalkanes and dimethylalkanes have frequently been isolated from insects. Internally branched trimethylalkanes are less common. All alkenes that have been characterized in insects have a (Z) configuration, from one to three double bonds, and no methyl branching.

In an attempt to understand better the role of hydrocarbons located within insect tissues and to determine structural differences that may occur between hydrocarbons found in various locations, we analyzed different tissues of diapausing pupae of the tobacco hornworm for their hydrocarbon content. Since the amount of surface lipid deposited by the diapausing pupae increased with time after ecdysis (4, 8), we also investigated the amount of hydrocarbon present during the diapause stage in insect development.

Abbreviations: GLC–MS, gas–liquid chromatography–mass spectrometry; ECL, equivalent chain length; TLC, thin-layer chromatography.

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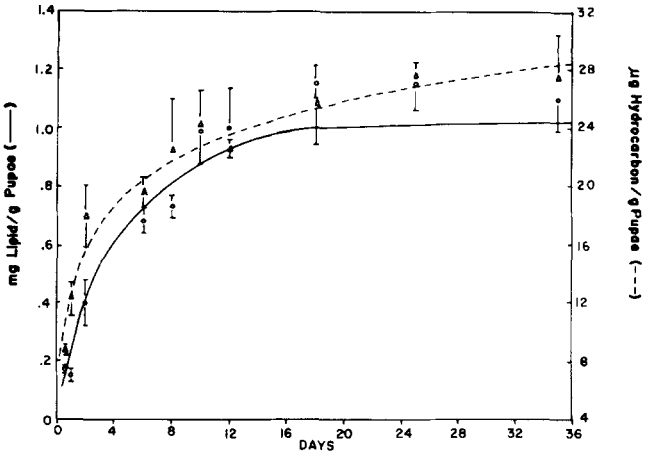

Fig. 1. Formation of total surface lipids and surface hydrocarbons on the pupal cuticle of the tobacco hornworm, *Manduca sexta* following larval-pupal ecdysis. Lipid weight (○); hydrocarbon weight (△).

MATERIALS AND METHODS[3]

The tobacco hornworms used for these studies were from a colony that originated from eggs collected in North Carolina and had been maintained for ten years at Fargo, North Dakota (5).

Diapausing pupae were removed from the growth blocks, cleaned of cast larval skins, weighed, and separated by sex 20 hr after larval-pupal ecdysis. The pupae were held at 26°C and 40% relative humidity under a 12:12 photoperiod. The surface lipids were obtained by extracting groups of seven to ten pupae with three 20-sec immersions in chloroform. An internal standard of eicosane in methanol was placed on the pupae before extraction and was used to quantitate the total amount of hydrocarbon present in the lipid extract by gas–liquid chromatography.

Pupae were chilled at 6°C before the hemolymph was collected. The proboscis was clipped and the hemolymph was allowed to flow into a centrifuge tube that was maintained at 0°C to reduce clotting of the hemolymph. The hemolymph was immediately centrifuged at 2000 *g* to remove any cells and debris.

Internal tissues were dissected by making a pair of lateral incisions from the abdomen to the head. The halves of the pupal cuticle were separated by cutting the rows of muscles associated with the integument. Care was taken to prevent puncturing the gut. Integument was obtained from both the abdominal area, where removal of fat body and muscle was necessary, and from the wing pad area, where the integument usually pulled free of any fat body or muscle. When the hydrocarbons of the integument were to be extracted, the pupae were first rinsed in chloroform to remove all surface hydrocarbons. Fat body was collected from the abdominal region, as far from the integument as possible and free of hemolymph. Muscle was excised from the abdominal wall. The integument, fat body, and muscle were rinsed in a saline solution after dissection and then weighed.

An internal standard of octadecane in methanol was added to all the tissues before extraction. Each tissue was homogenized in chloroform–methanol 2:1, using a glass homogenizer. The homogenate was partitioned against an equal volume of *Manduca* saline solution (6) and centrifuged at 2000 *g* for 10 min to separate the solution into two phases with tissue residue and denatured protein at the interphase. The organic layer was removed and the partitioning procedure was repeated three times.

Lipid extracts were taken to dryness, resuspended in 0.5 ml of hexane, and placed on a small silicic acid column (7). The column was eluted with 3 ml of hexane in excess of the void volume to obtain the hydrocarbon fraction. The purity of the hydrocarbon fractions was verified by thin-layer chromatography on silica gel G using hexane as the developing solvent. Alkenes were detected by chromatographing the hydrocarbon fraction on silver nitrate-impregnated (20% by weight) silica gel G plates and comparing the R_f values with those for (Z)-9-octadecene, (Z,Z)-9,12-octadecadiene, and 1-eicosene standards by using hexane–diethyl ether 99:1 as the developing solvent. Branched chain alkanes were separated from *n*-alkanes with molecular sieve (7). The internal standard served as a measure of the molecular sieve efficiency.

The hydrocarbons were analyzed on a Varian 3700 gas chromatograph equipped with stainless steel columns (2.2 m × 3 mm o.d.) packed with 3.5% OV-101 on 100–120 mesh Gas Chrom Q. The column temperature was programmed from 125° to 310°C at 4° per minute unless otherwise stated. The on-column injection port was maintained at 250°C and the flame ionization detector was held at 350°C. The carrier gas was helium at a flow rate of 30 ml/min. The data were recorded by a Hewlett-Packard 3380A recording integrator and were corrected for changes in detector response with chain length.

The equivalent chain length measurements were

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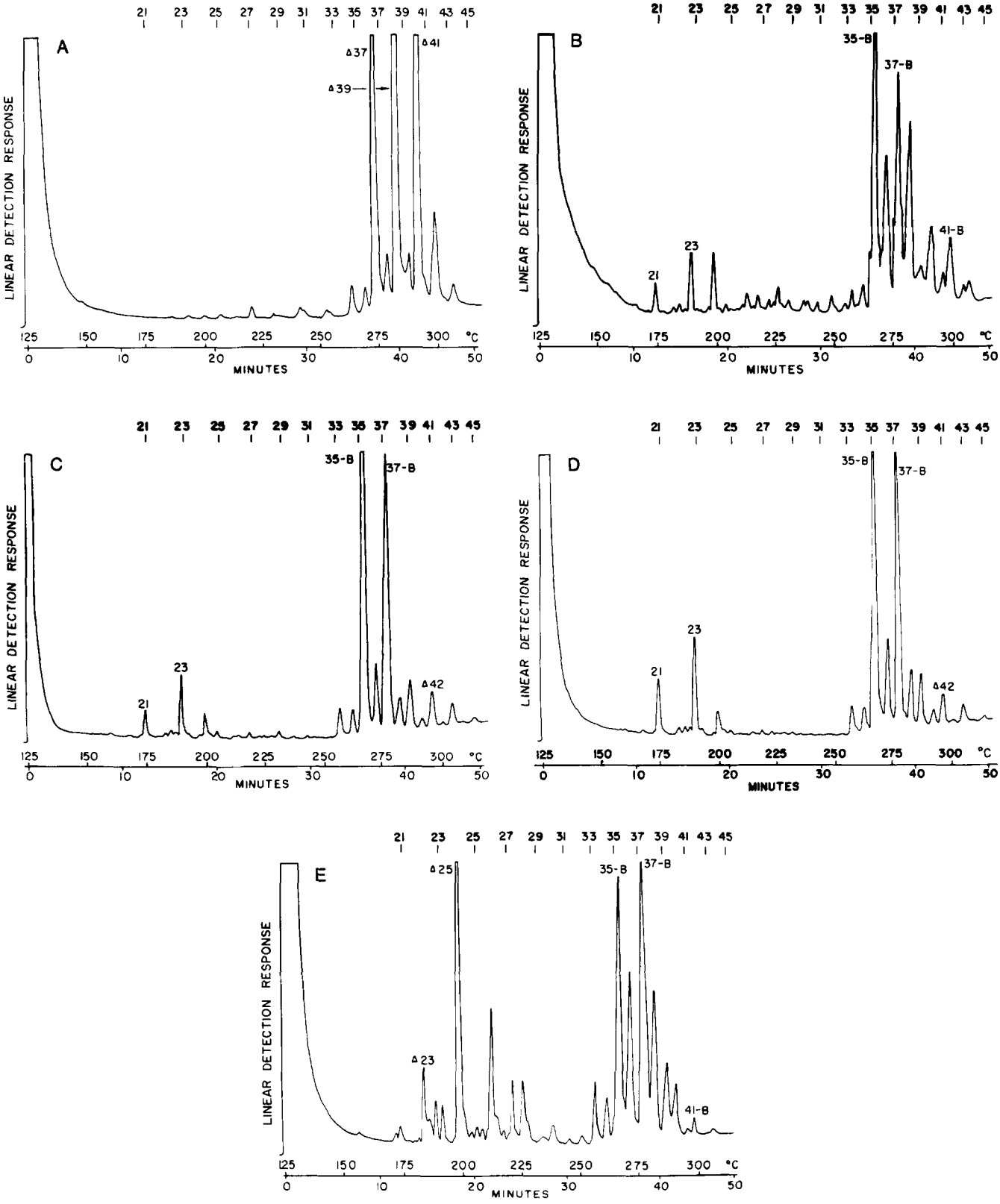

Fig. 2. Gas–liquid chromatographic elution profile of hydrocarbons in: A, cuticular surface lipids; B, integument; C, fat body; D, muscle; and E, hemolymph from diapausing pupae of the tobacco hornworm, *Manduca sexta*.

TABLE 1. Identification and percentage composition of hydrocarbons from cuticular surface lipids of diapausing pupae of the tobacco hornworm, *Manduca sexta*

GLC Peak No.[a]	ECL[b]	Carbon No.[c]	Composition[d] (%)	Hydrocarbon[e]
23	23	23	T	*n*-tricosane
24	24	24	T	*n*-tetracosane
25	25	25	T	*n*-pentacosane
27	27	27	T	*n*-heptacosane
31:3	30.2	31	T	hentriacontatriene
31:2	30.4	31		hentriacontadiene
33:3	32.2		T	
33:2	32.4			
35:3	34.2			
35:2	34.4	35	1.4	pentatriacontadiene
35:1	34.6	35		pentatriacontene
36:3	35.2			
36:2	35.4			
36:1	35.6	36	1.3	hexatriacontene
37:3	36.2	37		heptatriacontatriene
37:2	36.4	37	18.1	heptatriacontadiene
37:1	36.6	37		heptatriacontene
38:3	37.2			
38:2	37.4		2.9	
38:1	37.6			
39:3	38.2	39		nonatriacontatriene
39:2	38.4	39	37.4	nonatriacontadiene
39:1	38.6	39		nonatriacontene
40:3	39.2			
40:2	39.4		2.5	
40:1	39.6			
41:3	40.2	41		hentetracontatriene
41:2	40.4	41	23.3	hentetracontadiene
41:1	40.6	41		hentetracontene
42:3	41.2			
42:2	41.4		6.1	
42:1	41.6			
44:3	43.2			
44:2	43.4		1.8	
44:1	43.5			

[a] Identification of homologous series of hydrocarbons by GLC and GLC–MS. Unsaturated hydrocarbons are designated by :1, :2, :3 to indicate alkenes, alkadienes, or alkatrienes, respectively. Brackets indicate peaks included in percentage composition value.

[b] ECL: equivalent chain length. Average of values obtained from gas–liquid chromatographic elution profiles using temperature-programmed runs (linear plots) and isothermal runs (log-linear plots) and from continuous scanning GLC–MS (linear plots).

[c] Total mass was determined from parent ion peak when present in the mass spectra scan.

[d] Percentage composition was obtained by GLC. Values calculated by a Hewlett-Packard 3380A integrator. T indicates an amount less than 1%. Percentage composition of an unresolved mixture is given opposite that component appearing to be the most intense component in the mixture.

[e] Structure was identified from mass spectral fragmentation patterns.

determined from graphs of the logarithms of retention times versus carbon numbers of standards for isothermal analyses and graphs of the retention times versus carbon numbers of standards for temperature-programmed analyses.

Mass spectral data were obtained by gas–liquid chromatography–mass spectrometry (GLC–MS) on a Varian 200 gas chromatograph interfaced to a Varian/MAT CH5-DF mass spectrometer and on a Hewlett-Packard 5992A gas chromatograph-mass spectrometer. The gas chromatographs were equipped with 3.3 m × 2 mm i.d. glass columns packed with 3% OV-101 on 100–120 mesh Gas Chrom Q.

RESULTS AND DISCUSSION

Quantitation of the hydrocarbon fraction found in the cuticular surface lipid on diapausing pupae revealed an increase in the total amount of hydrocarbon with the age of the pupae (**Fig. 1**). An earlier report (8) had shown an increase in the amount of cuticular lipid with the age of the pupae and had also demonstrated a greater lipid production in diapausing pupae than in non-diapausing pupae. The results of this study show a 4 to 5-fold parallel increase in the amount of hydrocarbons and the amount of lipids found on the cuticle of diapausing pupae (Fig. 1). This suggests that during diapause the increase in hydrocarbon production parallels the total cuticular surface lipid production.

The presence of both saturated and unsaturated hydrocarbons in the surface lipids was demonstrated by TLC and GLC–MS. The hydrocarbon fraction accounted for about 4% of the surface lipid and consisted mainly of a mixture of alkenes, alkadienes, and alkatrienes of 37, 39, and 41 carbon atoms (**Fig. 2A,** Table 1). *n*-Alkanes accounted for only about 3% of the hydrocarbon fraction while alkenes accounted for 97%. No branched alkanes were identified in the surface hydrocarbons using molecular sieve and GLC–MS.

No significant differences were found in the percentage composition of the hydrocarbon components between sexes or with the age of the pupae. Therefore, hydrocarbons isolated from the surface lipids of pupae 1 day after larval-pupal ecdysis were used for further studies.

Mass spectral analysis indicated that the hydrocarbon fraction was made up of four homologous series: *n*-alkanes, and *n*-alkenes, *n*-alkadienes, and *n*-alkatrienes. The ECL and percentage composition of

TABLE 2. Identification and percentage composition of hydrocarbons in the integument, fat body, muscle, and hemolymph of diapausing pupae of the tobacco hornworm, *Manduca sexta*

GLC Peak No.[a]	ECL[b]	Carbon No.[c]	Percentage Composition[d]				Hydrocarbon[e]
			Integument	Fat body	Muscle	Hemolymph	
21	21	21	1.0	1.6	2.7	T	*n*-heneicosane
22	22	22	T	T	T	T	*n*-docosane
23:3	22.2	23				2.8	tricosatriene
23:2	22.4	23					tricosadiene
23	23	23	4.4	3.8	4.8	1.1	*n*-tricosane
24:3	23.2	24					tetracosatriene
23-A	23.3	24	T	T	T	1.2	11-methyltricosane
24	24	24	T	T	T	T	*n*-tetracosane
25:3	24.2	25				17.5	pentacosatriene
24-A	24.3	25	2.8	1.9	1.6		11-methyltetracosane
25:2	24.4	25					pentacosadiene
25	25	25	T	T	T	T	*n*-pentacosane
26:3	25.2	26				T	hexacosatriene
25-A	25.3	26	T	T	T		9-, 11-, and 13-methylpentacosanes
25-B	25.5	27	T	T	T	T	9,13-dimethylpentacosane
26	26	26	T	T	T		*n*-hexacosane
27:3	26.2	27				5.9	heptacosatriene
26-B	26.5	28	T	T	T		
27:1	26.6	27					heptacosene
27	27	27	1.0	T	T	T	*n*-heptacosane
27-B	27.5	29	T	T	T	2.5	9,13-dimethylheptacosane
28	28	28	T	T	T	T	*n*-octacosane
29:3	28.2	29				2.9	nonacosatriene
29:2	28.4	29					nonacosadiene
29:1	28.6	29					nonacosene
29	29	29	1.6	T	T	T	*n*-nonacosane
31:3	30.2	31				1.0	hentriacontatriene
31:2	30.4	31					hentriacontadiene
31:1	30.6	31					hentriacontene
31	31	31	T	T		T	*n*-hentriacontane
31-A	31.3	32				T	
31-B	31.5	33	T	T		T	13,17-dimethylhentriacontane
33-A	33.3	34					13-, 15-, and 17-methyltritriacontanes
33-B	33.5	35	1.1	2.4	2.1	2.7	13,17- and 15,19-dimethyltritriacontanes
33-C	33.7	36					11,15,19-trimethyltritriacontane
35:3	34.4	35					pentatriacontatriene
34-B	34.5	36	2.0	2.0	1.9	2.5	13,17-, 14,18- and 15,19-dimethyltetratriacontanes
35:1	34.6	35					pentatriacontene
35	35	35					*n*-pentatriacontane
35-A	35.3	36	3.2	T	9.1	11.2	13-, 15-, and 17-methylpentatriacontanes
35-B	35.5	37	13.5	35.2	24.1	1.2	13,17- and 15,19-dimethylpentatriacontanes
36	36	36					*n*-hexatriacontane
36-A	36.3	37	1.3	T	T	T	13-, 15-, and 17-methylhexatriacontanes

TABLE 2. (*Continued*)

GLC Peak No.[a]	ECL[b]	Carbon No.[c]	Percentage Composition[d] Integument	Fat body	Muscle	Hemolymph	Hydrocarbon[e]
37:2	36.4	37				9.3	heptatriacontadiene
36-B	36.5	38	9.0	6.2	6.9		13,17-, 14,18-, 15,19-, and 16,20-dimethylhexatriacontanes
37:1	36.6	37					heptatriacontene
36-C	36.7	39	T				14,18,22-trimethylhexatriacontane
37	37	37					*n*-heptatriacontane
37-A	37.3	38	4.4	T	T	T	13-, 15-, 17-, and 19-methylheptatriacontanes
37-B	37.5	39	10.4	25.8	24.2	10.7	13,17-, 15,19-, and 17,21-dimethylheptatriacontanes
37-C	37.7	40				4.8	13,17,21- and 15,19,23-trimethylheptatriacontanes
39:3	38.2	39					nonatriacontatriene
38-A	38.3	39					15-, 17-, 18-, and 19-methyloctatriacontanes
39:2	38.4	39	12.5	3.2	4.4	8.1	nonatriacontadiene
38-B	38.5	40					12,16-, 13,17-, and 14,18-dimethyloctatriacontanes
39:1	38.6	39					nonatriacontene
39	39	39					*n*-nonatriacontane
39-A	39.3	40	1.3				13-, 15-, and 17-methylnonatriacontanes
39-B	39.5	41	2.9	4.8	4.7	4.5	13,17-, 15,19-, and 17,21-dimethylnonatriacontanes
41:3	40.2	41					hentetracontatriene
41:2	40.4	41	T	T	T	3.1	hentetracontadiene
41:1	40.6	41					hentetracontene
41	41	41	7.3	1.0	1.7		*n*-hentetracontane
42:3	41.2	42	2.4	3.0	2.7	T	
41-B	41.5	43	2.4	T	T	1.0	13,17-dimethylhentetracontane
44:3	42.2	44	1.5	2.1	1.9	T	
43-B	43.5	45	4.8	T	T	T	13,17-dimethyltritetracontane

[a] Identification of homologous series of hydrocarbons by GLC and GLC–MS. Internally branched hydrocarbons are designated by adding A, B, or C to indicate mono-, di-, or trimethyl internal branch points, respectively. Unsaturated hydrocarbons are designated by adding :1, :2, or :3 to indicate alkenes, alkadienes, or alkatrienes, respectively. Brackets indicate peaks included in the percentage composition value.

[b] ECL: equivalent chain length. Average values obtained from gas–liquid chromatographic elution profiles using temperature-programmed runs (linear plots) and isothermal runs (log-linear plots) and from continuous scanning GLC–MS (linear plots).

[c] Total mass was determined from parent ion peak when present in the mass spectra scan.

[d] Percentage composition was obtained by GLC. Values were calculated by a Hewlett-Packard 3380A integrator. T indicates an amount less than 1%. The percentage composition of an unresolved mixture is given opposite that component appearing to be the most intense component in the mixture.

[e] The structure was identified from mass spectral fragmentation patterns.

these components are listed in **Table 1.** The mass spectrum of GLC peak 37 had parent ions at 518, 516, and 514, indicating the presence of a mixture of heptatriacontene, heptatriacontadiene, and heptatriacontatriene, respectively. The other two major GLC peaks in Fig. 2A, designated by Δ39 and Δ41, were shown by GLC–MS to represent mixtures of alkenes, alkadienes, and alkatrienes of 39 and 41 carbon atoms in length. Hydrogenation of the unsaturated hydrocarbon mixture resulted in the corresponding *n*-alkanes.

There was approximately 0.5, 650, and 1 mg lipid per gram dry tissue weight for the integument, fat body, and muscle tissues, respectively. The hemolymph supernatant contained about 5.4 mg lipid per ml. The hydrocarbon fractions of the total lipid extracts from the integument (free of surface lipids), fat body, muscle, and hemolymph comprised 0.8, 0.2, 0.9, and 4%, respectively, of the total lipid weight. The GLC elution patterns (Fig. 2B-E) of the hydrocarbons isolated from the four pupal tissues showed no significant differences in the percentage composition of the hydrocarbon components between sexes or with the age of the pupae. Therefore, hydrocarbons isolated from the four tissues of pupae 1 day after larval-pupal ecdysis were used for subsequent studies.

There were more hydrocarbon components in the pupal tissues than on the cuticular surface, and they consisted of seven homologous series: *n*-alkanes, alkenes, alkadienes, alkatrienes, and internally branched monomethyl-, dimethyl-, and trimethylalkanes (**Table**

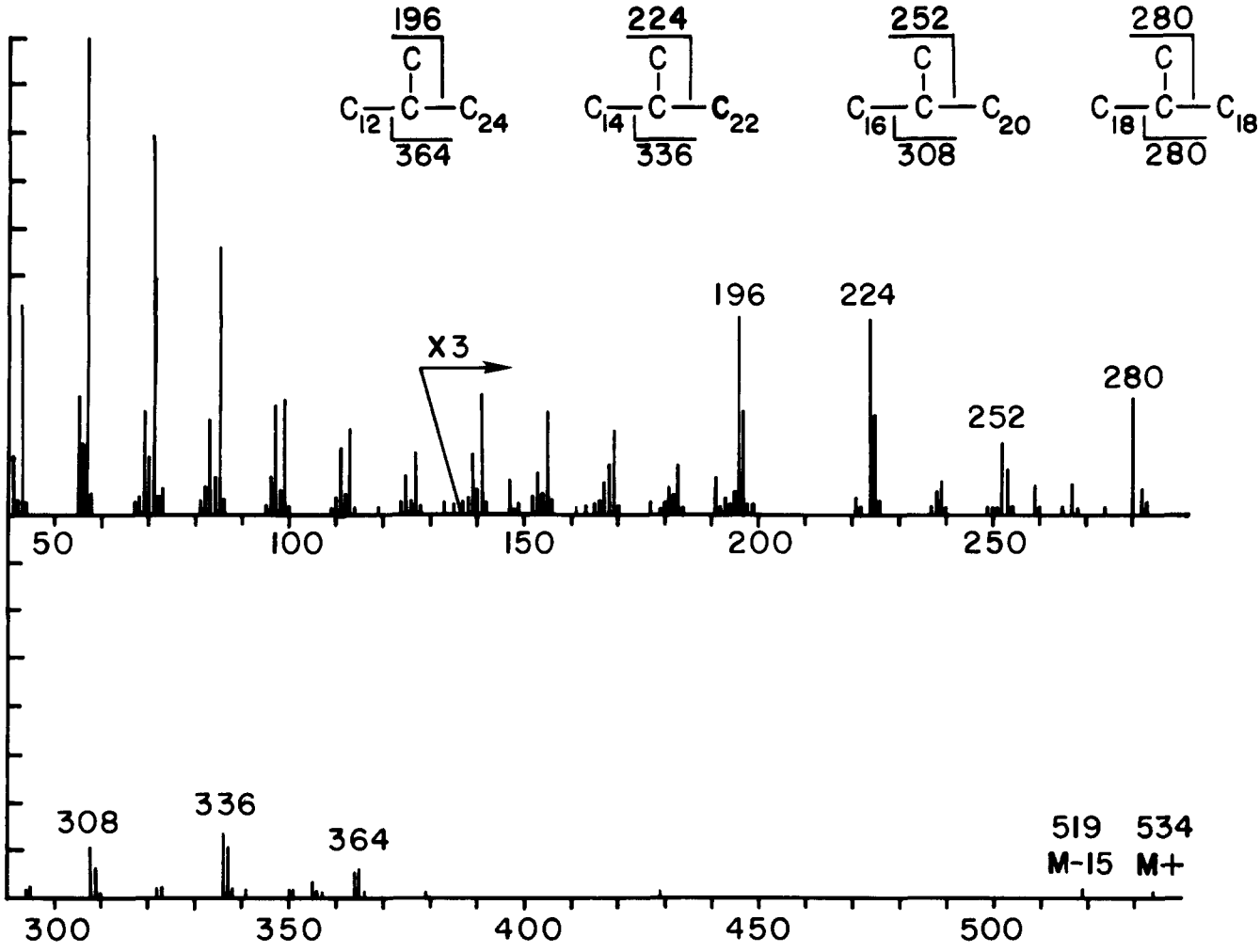

Fig. 3. Mass spectrum of GLC peak 37A, 13-, 15-, 17-, and 19-methylheptatriacontanes.

2). All four tissues contained the seven homologous series of hydrocarbons. However, the percentage composition of each homologous series varied from tissue to tissue. This variation was due, in part, to the close elution of the internally branched alkanes and the unsaturated hydrocarbons. The presence of the different GLC components that eluted at the same time were detected by analyzing sequential GLC–MS scans taken continuously during the elution of the components. However, after the removal of the unsaturated components with silver nitrate TLC and the *n*-alkanes with molecular sieve, it was possible to determine that the integument (free of surface lipids), fat body, muscle, and hemolymph hydrocarbons consisted of *n*-alkanes at 9, 6, 9, and 2%, respectively, branched alkanes at 75, 85, 80, and 56%, respectively, and unsaturated hydrocarbons at 16, 9, 11, and 42%, respectively. Dimethylalkanes were the major components of the hydrocarbon fraction from the three internal tissues.

The hemolymph contains a greater percentage of unsaturated hydrocarbons and lower molecular weight alkenes than the other three tissues. The gas–liquid chromatographic traces of the hydrocarbon extracts from pupal hemolymph centrifuge precipitate, which consisted of cells, clotted material, and cellular debris, contained the same components as the pupal hemolymph supernatant after centrifugation. This suggests that hydrocarbons may be associated with the cellular material as well as with the fluid of the hemolymph. The possibility that some hydrocarbons may have been trapped in the centrifuge precipitate cannot be eliminated.

The major *n*-alkanes from all tissues were of odd-numbered chain lengths although trace amounts of almost every *n*-alkane from 21 to 41 carbon atoms in length were present. Internally branched monomethylalkanes isolated from the pupal tissues occurred as isomeric mixtures with the methyl branch located near the center of the molecule. The methyl

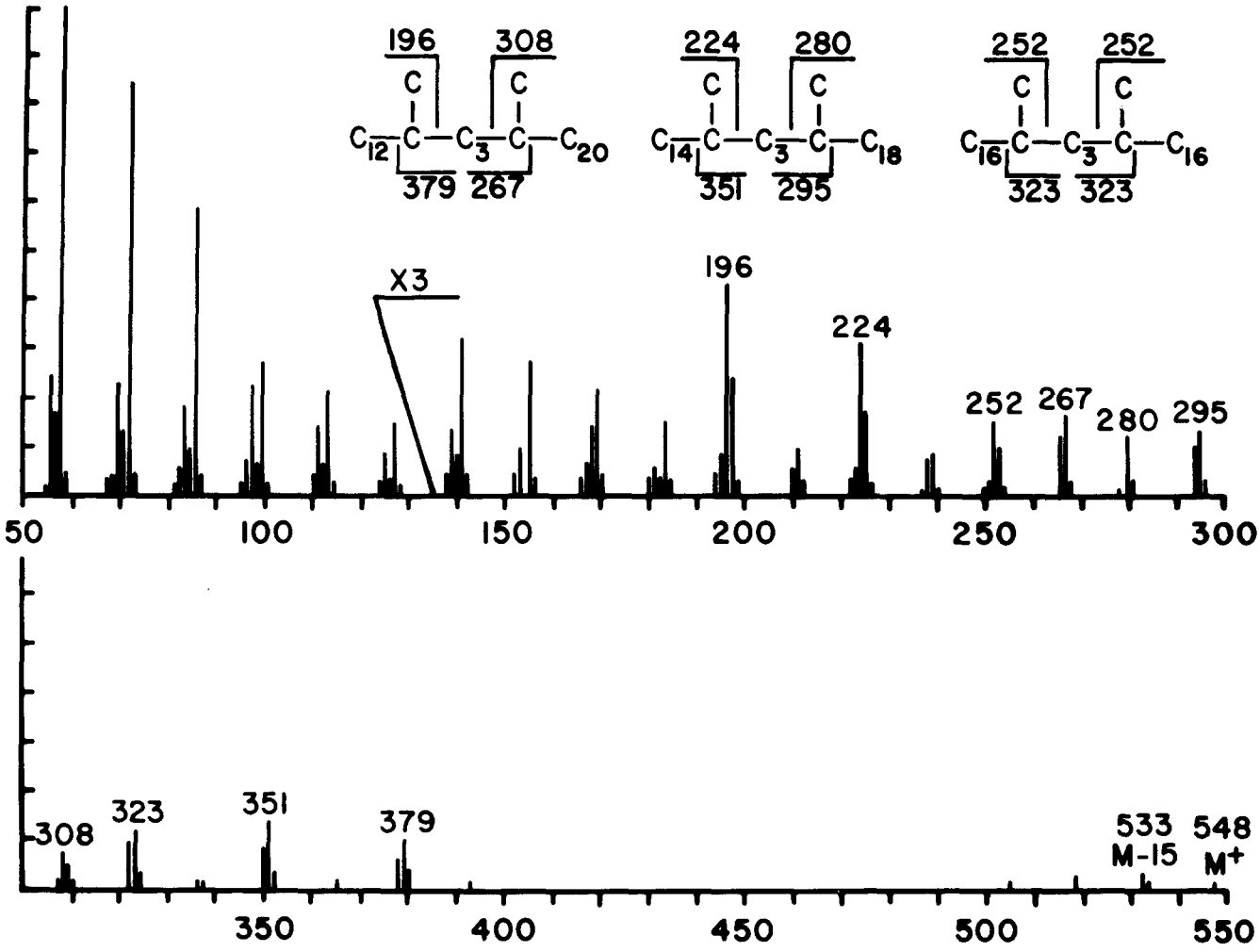

Fig. 4. Mass spectrum of GLC peak 37B, 13,17-, 15,19-, and 17,21-dimethylheptatriacontanes.

branch group was located on an odd-numbered carbon atom of those components that had an odd-numbered carbon backbone, and the methyl branch group was located on both odd- and even-numbered carbon atoms on those components with an even-carbon backbone. **Fig. 3** shows the mass spectrum of GLC peak 37A, 13-, 15-, 17-, and 19-methylheptatriacontanes, and demonstrates the type of fragmentation that occurred for the monomethylalkanes. This interpretation of the mass spectral fragmentation patterns of methyl branched alkanes has been described previously (3, 7, 9, 10).

The internally branched dimethylalkanes occurred as mixtures of isomers with methyl branch points located on odd- or even-numbered carbon atoms in the same fashion as the monomethylalkanes. **Fig. 4** shows the mass spectrum of GLC peak 37B, 13,17-, 15,19-, and 17,21-dimethylheptatriacontanes, and illustrates the fragmentation pattern of the dimethylalkanes. The methyl branch points of all the dimethylalkanes were located near the center of the molecule and had three methylene units between the branch points. **Fig. 5** shows the mass spectrum of GLC peak 37C, 13,17,21- and 15,19,23-trimethylheptatriacontanes. As with the dimethylalkanes, the trimethylalkanes also have three methylene units between the branch points.

Unsaturated hydrocarbons isolated from the four pupal tissues ranged from 23 to 44 carbon atoms in length (Table 2). The degree of unsaturation was determined by GLC–MS. Complete characterization of these hydrocarbons is in progress at the present time.

It was not possible to confirm the exact identification of the components with the ECL values of 26.5, 31.3, 41.2, and 42.2 (Table 2) because of the small amounts of these components present. However, ECL and molecular weight data were sufficient to assign each to a particular homologous series.

These results demonstrate a difference in the type

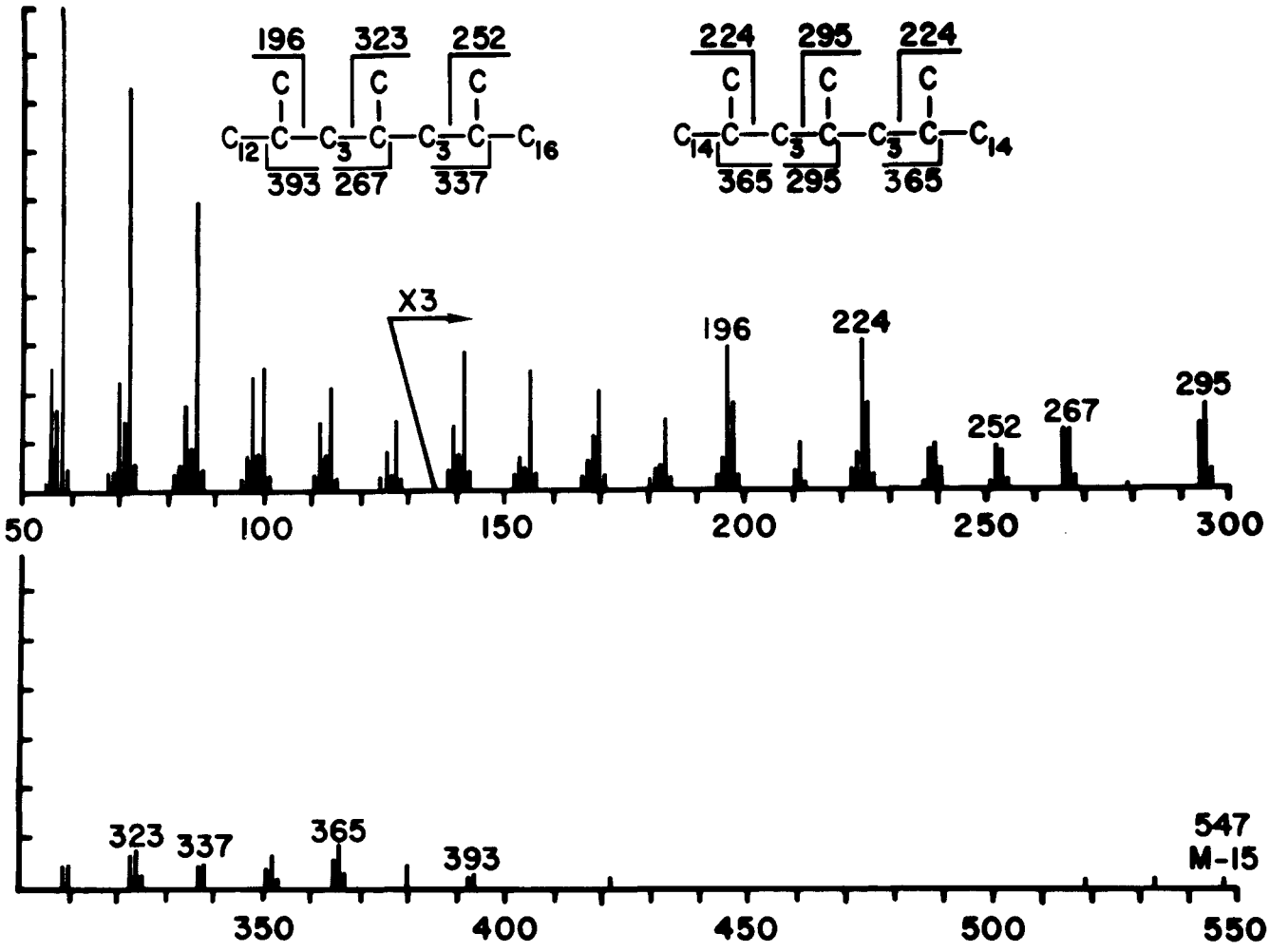

Fig. 5. Mass spectrum of GLC peak 37C, 13,17,21- and 15,19,23-trimethylheptatriacontanes.

of hydrocarbons found on the cuticular surface of the pupae and in the pupal integument tissue, which is thought to be the site of cuticular hydrocarbon biosynthesis. The same *n*-alkane and unsaturated hydrocarbon components appeared on the surface and in the tissues of the pupae. The branched components, however, appeared only in the tissues. This suggests either that a transport system exists that selectively carries almost exclusively unsaturated components to the cuticle surface of the diapausing pupae, or that separate biosynthetic pathways exist for cuticular and tissue hydrocarbons. The restricted location of branched alkanes implies that hydrocarbons serve a unique function in tissues of the insect.

Four homologous series of alkanes (*n*-alkanes and internally branched mono-, di-, and trimethylalkanes) were previously identified in the hydrocarbon fraction from tobacco hornworm eggs (9, 10). The structures characterized in the present study are in agreement with earlier reports although we were able to characterize more components in each homologous series, and in some cases were able to resolve more individual components within an isomeric mixture. This is most likely the result of using more sensitive techniques rather than a chemical difference between egg hydrocarbons and pupal hydrocarbons. The presence of both straight chain and branched hydrocarbons was previously demonstrated in the integument, fat body, and hemolymph of fifth-instar larvae of the tobacco hornworm (11). To our knowledge, this is the first report of unsaturated hydrocarbons isolated from the tobacco hornworm. These results seem to indicate that unsaturated hydrocarbons are unique to the pupal stage of development. However, since the unsaturated hydrocarbons have ECL values close to those of the internally branched hydrocarbons, it is also possible that the presence of unsaturated components was not detected during the earlier studies.

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