HYDROCARBONS IN HAEMOLYMPH FROM HEALTHY
AND DISEASED JAPANESE BEETLE LARVAE

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Abstract—The hydrocarbons in extractable lipids of haemolymph from healthy
and diseased larvae of the Japanese beetle (Popillia japonica) have been char­
cacterized. Haemolymph contains at least 21 saturated hydrocarbons having
from 21 to 27 carbon atoms. Normal, monomethyl-branched, and dimethyl­
branched alkanes with even and odd carbon numbers were identified. Trico­
sane (12%), 11-methyltricosane (19%), 9,13-dimethyltricosane (27%), and
11-methylpentacosane (11%) are the major hydrocarbons. Haemolymph from
third instar larvae contains at least six minor hydrocarbons that could not be
obtained in sufficient quantities for structure identification. The total amount
of hydrocarbons per ml is reduced in haemolymph from larvae infected with
Bacillus popilliae; however, the relative concentration of each hydrocarbon is
not significantly changed as a result of infection.

INTRODUCTION

To ELUCIDATE the complete chemical environment conducive for growth and
sporulation of the milky disease organism, Bacillus popilliae (Dutky), we are study­
ing the chemistry of haemolymph from healthy and diseased Japanese beetle larvae,
Popillia japonica (Newman). Larval haemolymph (in vivo) is the only known
efficient medium for sporulation of B. popilliae. Recent studies on the lipids in
haemolymph from healthy and diseased larvae have established that hydro­
carbons are a major component of haemolymph lipids (BENNETT and SHOTWELL,
1971). Probably the most extensively studied insect hydrocarbons are those in the
surface lipids of cockroaches. Hydrocarbon constituents vary qualitatively and
quantitatively among insect species (HUTCHINS and MARTIN, 1968; AGARWAL and
RAO, 1969; CAVILL et al., 1970). Both qualitative and quantitative differences also
exist in hydrocarbons of several genera of the same insect (BAKER et al., 1963;

Although the primary physiological rôle of hydrocarbons in insects is the
regulation of transpiration, their presence in haemolymph may influence the
pattern of proliferation of an invading pathogen, such as B. popilliae. This paper
reports the structures and relative concentrations of hydrocarbons in haemolymph
from healthy and diseased P. japonica larvae.

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MATERIALS AND METHODS

Isolation of haemolymph hydrocarbons

Haemolymph was obtained from healthy and diseased third instar larvae as previously described (SHOTWELL et al., 1963). Lipids were extracted from 2 or 4 ml haemolymph with chloroform-methanol (2:1) (FOLCH et al., 1957) as outlined by BENNETT and SHOTWELL (1971). Hydrocarbons were isolated from total lipid extracts by silicic acid column chromatography. Columns (1.2 x 37 cm) of silicic acid (Bio Sil HA-minus 325 mesh, Biorad Corporation, Richmond, Calif.) were poured as hexane slurries and washed with spectroquality hexane by gravity flow overnight. Total lipid extracts were dissolved in hexane-anhydrous diethyl ether (1:1) and quantitatively transferred to the columns. Hydrocarbons were eluted with 6% spectroquality benzene in hexane (50 ml) under nitrogen pressure (90 mm Hg). After evaporation of excess solvent under nitrogen, hydrocarbon samples were desiccated with phosphorous pentoxide and the dry weights determined. Samples were dissolved in hexane for analytical gas-liquid chromatography (GLC) and gas-liquid chromatography-mass spectroscopy (GLC-MS).

General analytical procedures

Haemolymph hydrocarbons were analysed for unsaturation by thin-layer chromatography (TLC) on 20 x 20 cm plates coated with Adsorbosil-5-ADN (5% silver nitrate, Applied Science Laboratories, State College, Pa.). After the plates were developed in hexane, they were air-dried and sprayed with 50% sulphuric acid.

Normal and branched-chain hydrocarbons were separated by treatment with molecular sieves (Hylar M 80/100 molecular sieve 5A, Supelco, Bellefonte, Pa., and molecular sieve 5A, % in. pellets, Baker, Chicago, Ill.). Hydrocarbons were dissolved in 2,2,4-trimethyl pentane (30 ml) and refluxed with freshly activated molecular sieve 5A for 3 to 4 hr. Efficiency of n-alkane adsorption was determined by GLC. Hydrogenation of haemolymph hydrocarbons was carried out at room temperature and atmospheric pressure in an ethanolic solution containing palladium catalyst (10%) on powdered charcoal. After 2 hr the reaction mixture was filtered and the products were examined by GLC. A Model 810 F&M gas chromatograph equipped with flame ionization detectors was used to determine the results of column separation, hydrogenation, and molecular sieve 5A treatment of hydrocarbons. Dual 6 ft x % in. columns of 5% SE-30 (Hi-Pak, Hewlett-Packard Corp., Skokie, Ill.) were operated isothermally at 200°C or temperature programmed from 180 to 225°C at 4 deg/min. Operating parameters were: carrier gas (helium), 30 ml/min; hydrogen, 60 ml/min; purge air, 240 ml/min; injection ports and detectors, 250°C.

Tentative identification of normal alkanes was made by comparing their retention times on SE-30 columns to authentic standards of n-C20 to n-C32 (Supelco, Bellefonte, Pa.). Normal alkanes were also identified by their absence from GLC patterns of samples after molecular sieve 5A treatment.
Gas-liquid chromatography-mass spectroscopy analyses

Mass spectral analyses of individual hydrocarbons were obtained after they were separated on a Packard Model 7401 chromatograph equipped with a 50 ft x ½ in. column of Apiezon L (Hi-Pak, Hewlett-Packard Corp., Skokie, Ill.) and operated in tandem with a Dupont (CEC) 21-492 mass spectrograph. Operating parameters were: column temperature, 180°C; injection ports and detector temperature, 200°C; carrier gas (helium) inlet pressure, 80 psi. The transfer line to the mass spectrograph was held at 250°C; source temperature, 220°C; helium separator, 200°C; source voltage and current, 70 eV and 50 mA; and source pressure, $4 \times 10^{-6}$ torr. Separate GLC analyses were run for quantitation of individual hydrocarbons, and peak areas were integrated with an Infotronics CRS-40 integrating system.

RESULTS

Hydrocarbons isolated from haemolymph of healthy and diseased larvae are compared in Fig. 1. Both samples gave similar chromatograms. Compounds 1 to 15 are present in both samples; however, the total quantity of hydrocarbons decreases as a result of milky disease. No significant change in relative concentration was detected in any particular hydrocarbon species. Table 1 gives the identity and relative concentrations of hydrocarbons in haemolymph from healthy and diseased larvae. Compounds not detected or adequately separated on the 6 ft SE-30 column were identified on a 50 ft Apiezon L column. Equivalent chain

![Gas chromatograms of hydrocarbons from haemolymph of healthy and diseased larvae. Hi-Pak SE-30 columns (6 ft x ½ in.) were operated isothermally at 200°C. Peaks 1 to 15 identified in Table 1.](image-url)
lengths (ECL) were calculated by plotting log retention time vs. carbon number (Miwa, 1963).

### Table 1—Identity and Relative Concentrations of Hydrocarbons in Haemolymph from Healthy and Diseased Japanese Beetle Larvae

<table>
<thead>
<tr>
<th>Peak</th>
<th>ECL*</th>
<th>Hydrocarbon</th>
<th>% of total hydrocarbons</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>21·0</td>
<td>n-Uncosane</td>
<td>0·3</td>
</tr>
<tr>
<td>1a</td>
<td>21·6</td>
<td>Unidentified</td>
<td>0·3</td>
</tr>
<tr>
<td>2</td>
<td>22·0</td>
<td>n-Docosane</td>
<td>0·9</td>
</tr>
<tr>
<td>3</td>
<td>22·3</td>
<td>11-Methyldocosane</td>
<td>0·9</td>
</tr>
<tr>
<td>4</td>
<td>22·7</td>
<td>9,11-Dimethyldocosane</td>
<td>3·2</td>
</tr>
<tr>
<td>4a</td>
<td>22·8</td>
<td>Unidentified</td>
<td>0·4</td>
</tr>
<tr>
<td>5</td>
<td>23·0</td>
<td>n-Tricosane</td>
<td>12·6</td>
</tr>
<tr>
<td>6</td>
<td>23·2</td>
<td>11-Methylintricosane</td>
<td>19·7</td>
</tr>
<tr>
<td>7</td>
<td>23·6</td>
<td>9,13-Dimethylintricosane</td>
<td>27·7</td>
</tr>
<tr>
<td>8</td>
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<td>X-Methylintricosane</td>
<td>1·1</td>
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<tr>
<td>9</td>
<td>23·8</td>
<td>X-Methylintricosane</td>
<td>1·1</td>
</tr>
<tr>
<td>10</td>
<td>24·0</td>
<td>n-Tetracosane</td>
<td>1·6</td>
</tr>
<tr>
<td>11</td>
<td>24·3</td>
<td>12-Methyltetracosane</td>
<td>3·4</td>
</tr>
<tr>
<td>12</td>
<td>24·5</td>
<td>9,11-Dimethyltetracosane</td>
<td>2·7</td>
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<tr>
<td>12a</td>
<td>24·6</td>
<td>Unidentified</td>
<td>0·9</td>
</tr>
<tr>
<td>12b</td>
<td>24·8</td>
<td>Unidentified</td>
<td>0·7</td>
</tr>
<tr>
<td>13</td>
<td>25·0</td>
<td>n-Pentacosane</td>
<td>5·1</td>
</tr>
<tr>
<td>14</td>
<td>25·3</td>
<td>11-Methylpentacosane</td>
<td>11·1</td>
</tr>
<tr>
<td>15</td>
<td>25·5</td>
<td>11,15-Dimethylpentacosane</td>
<td>4·5</td>
</tr>
<tr>
<td>15a</td>
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<td>0·8</td>
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<tr>
<td>16</td>
<td>26·0</td>
<td>12-Methylhexacosane</td>
<td>3·3</td>
</tr>
<tr>
<td>17</td>
<td>26·3</td>
<td>12,14-Dimethylhexacosane</td>
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<tr>
<td>17a</td>
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</tr>
<tr>
<td>17b</td>
<td>26·7</td>
<td>Unidentified</td>
<td>0·8</td>
</tr>
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</table>

* ECL = equivalent chain length.

Treatment of haemolymph hydrocarbons with molecular sieves removed peaks 1, 2, 5, and 13 from the GLC patterns in Fig. 1. These peaks correspond to n-alkanes of 21, 22, 23, and 25 carbon atoms. Since peak 9, 10 is reduced in size, the presence of a C\(_{24}\) n-alkane is indicated. Co-chromatography of hydrocarbons and n-alkane standards confirmed the identity of peaks 1, 2, 5, 10, and 13 as n-alkanes. Both TLC on silver nitrate plates and GLC of hydrogenated samples indicate that all the hydrocarbons shown in Fig. 1 are saturated compounds. Five n-alkanes were detected in haemolymph hydrocarbons and constitute 21 per cent of the total hydrocarbons. Odd numbered n-alkanes (n-C\(_{21}\), n-C\(_{23}\), n-C\(_{25}\)) make up 87 per cent of the straight-chain hydrocarbons.

Fig. 2 is the mass spectrum of peak 6, a branched hydrocarbon (11-methyltricosane). The parent peak at 338 and the prominent peaks at 168 (C\(_{12}\)H\(_{24}\)) and 196 (C\(_{14}\)H\(_{28}\)) show that this compound has one methyl branch at carbon atom number 11. Cleavage of the parent ion on either side of the methyl group results...
in two C₁₂H₂₅ fragments or C₁₆H₃₄ and C₁₄H₂₉ fragments. Analogous mass spectra were obtained for GLC peaks numbered 3, 11, and 14. These compounds are singly branched hydrocarbons with 23, 25, and 26 carbon atoms, respectively.

Peak 11 (Fig. 1) appears to be a mixture of at least two isomers. This GLC peak is primarily 12-methyltetradecane with a parent ion of 352 and major peaks at 183 (C₁₄H₂₅) and 197 (C₁₄H₂₅). Mass spectra peaks at 225 (C₁₆H₃₄), 211 (C₁₅H₃₁), 155 (C₁₁H₂₃), and 127 (C₉H₁₉) suggest the presence of 10-methyltetradecane. The absence of peaks at 169 (C₁₂H₂₅) and 141 (C₁₀H₂₁) rules out the presence of an 11-methyl compound. Hydrocarbons with a single methyl branch make up 35 per cent of the total hydrocarbons. GLC peaks 8 and 9 are present in minor amounts (1 per cent) and did not give a clearly distinguishable mass spectra. They are tentatively identified as internally branched hydrocarbons of 25 carbon atoms.

Hydrocarbons that contain two methyl branches constitute 44 per cent of haemolymph hydrocarbons. Fig. 3 is the mass spectrum of peak 7 of Fig. 1. This major haemolymph hydrocarbon is identified as 9,13-dimethyltetradecane with a parent ion at 352. Prominent peaks at 141 (C₁₀H₂₁), 168 (C₁₂H₂₅), 211 (C₁₅H₃₁), and 239 (C₁₇H₃₅) suggest two methyl branches separated by three —CH₂— groups. Compounds 4, 12, and 15 also contain two methyl branches on internal carbons. Peak 4 is assigned the structures 9,11-dimethylhexadecane and 5,7-dimethylhexadecane on the basis of peaks at 141 (C₁₀H₂₁), 183 (C₁₃H₂₇), 211 (C₁₅H₃₁), and 225 (C₁₆H₃₅). Peak 12 is a 26 carbon atom hydrocarbon whose mass spectrum fits either a 9,11- or a 8,10-dimethylhexadecane structure. Major peaks at 169 (C₁₂H₂₅), 183 (C₁₃H₂₇), and an intense peak at 225 (C₁₆H₃₅) suggest a mixture of the 8,10- and 9,11-dimethylisomers. Peak 15, with intense peaks at 169 (C₁₂H₂₅) and 239 (C₁₇H₃₅)
and a parent ion at 380, indicates a hydrocarbon of 27 carbon atoms with branches at 11 and 15 carbon atoms. This compound is similar to compound 6, which also has three methyl groups separating the branch points. Rupture of the molecule on either side of the two branch points gives rise to \( \text{C}_{12} \) and \( \text{C}_{17} \) fragments since both terminal ends contain 10 carbon atoms.

**Fig. 3.** Mass spectrum of a double-branched hydrocarbon, 9,13-dimethyltricosane (peak 7 of Fig. 1).

The unidentified peaks in Table 1 are trace components and are not present in sufficient quantities for identification. These compounds could not be separated on the 6 ft SE-30 column.

**DISCUSSION**

Monomethyl- and dimethyl-branched alkanes represent 79 per cent of the hydrocarbons in third instar larvae haemolymph whether diseased or healthy. Hydrocarbons with even and odd carbon numbers were identified. The predominate hydrocarbon contains 25 carbon atoms and makes up 27 per cent of the total. Little is known about the biosynthesis of these compounds, except that they are synthesized in the integument of some insects (NELSON, 1969). Their physiological rôle in the haemolymph probably relates to formation of epicuticular lipids, and haemolymph hydrocarbons may undergo changes as larvae progress through morphological stages of development. In the cockroach family, fluctuations in hydrocarbons in haemolymph occur with age, as well as with sex (ACREE et al., 1965; JACKSON, 1970). Although \( ^{14} \text{C} \) from \( 1-^{14} \text{C} \) acetate was incorporated into the hydrocarbons of *Musca domestica* L. (ROBBINS et al., 1960) and *Apis mellifera* (PIEK, 1964), the pathways of hydrocarbon biosynthesis remain unclear. CONRAD and JACKSON (1971) demonstrated the incorporation of several labelled substrates
(acetate to linoleate) into surface hydrocarbons and internal lipids of Periplaneta americana. These data show that optimum hydrocarbon biosynthesis occurs during the 1- to 4-week period of life and no extensive synthesis occurs during the adult life of this insect.

The relative concentrations of the different hydrocarbons in P. japonica are not significantly altered as a result of milky disease; therefore, it is unlikely that the invading organism, B. popilliae, is utilizing these branched compounds for synthesis of the branched-chain fatty acids that are characteristic of this organism (Bulla et al., 1970). Since the total quantity of hydrocarbons is reduced as a result of milky disease, as is the total lipid content (Bennett and Shotwell, 1971), it appears that the lipid synthesis mechanisms of the larvae are impaired or blocked. The biosynthetic route of these hydrocarbons is probably independent of haemolymph fatty-acid synthesis. If the synthetic routes were related, one would expect to find branched-chain fatty-acid precursors. These have not been detected in P. japonica and are not common to insects.

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REFERENCES


