Hydrocarbons of the Cuticle, Sting Apparatus, and Sting Shaft of *Apis mellifera* L. Identification and Preliminary Evaluation as Chemotaxonomic Characters

by
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ABSTRACT

The hydrocarbon components of the cuticle, sting apparatus, and sting shaft of foragers of *Apis mellifera* L. have been identified and quantitated. Major qualitative and quantitative differences were found between the cuticular hydrocarbon components and those of the sting. The suitability of each of these hydrocarbon sources as possible chemotaxonomic characters is discussed. Radiochemical data is presented to show that the hydrocarbons of the sting apparatus and shaft are not biosynthesized on these structures, but rather originate elsewhere in the bee.

Apis mellifera L. is a bee of cosmopolitan distribution with a large number of recognized ecotypes, which differ phenotypically in varying amounts. One such ecotype, *Apis m. scutellata,* found in South Africa, and hybrids of this ecotype with European ecotypes in South America, the so-called Africanized honey bee, have received a large amount of attention because of their highly defensive natures. Unfortunately, stocks of *A. mellifera* that have varying degrees of *A. m. scutellata* genes in them are sometimes difficult to identify by conventional morphological attributes.

Hydrocarbons have been found to be ubiquitous in insects and to possess a remarkable range of physical and semiochemical properties (Howard and Blomquist 1982; Nelson 1978; Jackson and Blomquist 1976). They are also useful taxonomic characters at the species level, with some taxa possessing qualitatively different components (Termites, *Reticulitermes* spp.: Howard *et*

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al. 1978, 1982; Howard and Blomquist 1982; Ants, *Solenopsis* spp.: Lok *et al.* 1975; Nelson *et al.* 1980; Beetles, *Tenebrio* spp. Lockey 1980), while other taxa differ only quantitatively with respect to hydrocarbon components (Cockroaches, *Periplaneta* spp.; Jackson 1972; Baker *et al.* 1963; Sawflies, *Neodiprion* spp.; Howard, McDaniel, and Coppel, unpub.; Termites, *Nasutitermes* spp: Howard, McDaniel, Thorn, and Leving, unpubl.).

Hydrocarbons form one of the major groups of biochemicals produced and utilized by honey bees. A major portion of their cuticular lipids are hydrocarbons (Blomquist *et al.* 1980a; 1980b), and they incorporate large amounts of hydrocarbon into their beeswax (Tullock 1970; 1980). In addition, Blum et al. (1978), Gunnison and Morse (1968), and Pickett *et al.* (1982), all have reported that the sting apparatus of worker honey bees contains hydrocarbons.

As part of a general program of USDA-APHIS aimed at developing methodology for identifying introductions of *A. m. scutellata* into North America, we have initiated a research program aimed at evaluating the feasibility of utilizing hydrocarbons as phenotypic markers for various ecotypes of *A. mellifera.* This paper reports on the identification of the hydrocarbon components from the cuticle, sting apparatus,[6] and sting shaft of United States commercial stocks of *A. mellifera* (mixtures of European origin, i.e., *A. m. ligustica, A. m. carnica, A. m. causica, A. m. mellifera,* etc.), the relative abundance of individual components from each body source, the absolute abundance of hydrocarbons from each source, and an evaluation of the relative merits of each hydrocarbon source for taxonomic investigations. In addition, we provide radiochemical evidence that the sting hydrocarbons are not biosynthesized on the sting apparatus.

METHODS AND MATERIALS

Insects—Foragers (workers > 21 days old) from five colonies of United States commercial stock *Apis mellifera* were used for all experiments. Bees were collected in plastic bags as they exited the hive, and killed immediately by freezing.

Hydrocarbon Sources—A random sample of 25 bees from each of the five source colonies was used. The sting apparatus of each bee was removed from the body with forceps and placed by colony in pesticide grade methylene chloride (CH_2Cl_2) immediately. This procedure removes not only the sting shaft, but also the associated setose lobe and its various glands, which enfold

[6]In addition to the hydrocarbon components we also identified the same Δ 11-Eicosen-1-ol reported by Pickett *et al.* (1982). Our analytical methodology involved acetylation, methoxymercuration-demercuration, and infrared and mass spectral analysis. The identification of this alcohol was conducted in cooperation with M. S. Blum, University of Georgia, and H. Fales, National Institute of Health, Bethesda, Maryland.

the base of the sting shaft. An unknown amount of hemolymph contamination was also possibly present. Cuticular hydrocarbons were obtained from the same bees that were used for sting apparatus dissection. Immediately after removal of the sting apparatus, the bees were pooled by colony, immersed in CH_2Cl_2, and treated as described below. As an independent check on the assumption that the sting shaft itself possesses hydrocarbons, 50 foragers from each of three additional source colonies were obtained as described above. The stings were partially extruded from the bees' bodies, with particular care being taken not to rupture any internal connections, and snipped off using microdissection scissors. The sting shafts so obtained were grouped by colony and extracted in CH_2Cl_2.

Isolation of hydrocarbons—All samples were processed identically. Solvent was removed with a stream of nitrogen, the residue taken up in hexane, and chromatographed on a BioSil A mini-column in a Pasteur pipet. Hydrocarbons were eluted with *ca.* 3 ml hexane. Subsequent fractionation into saturated an unsaturated components was performed as previously described (Howard *et al.* 1978; Blomquist *et al.* 1980b).

Identification and Quantitation of Components—Components were identified using gas-liquid chromatography (GLC), gas chromatography-mass spectrometry (GC-MS), and infrared spectroscopy (IR). Electron impact mass spectrometry (EI-MS) was used for structural information; chemical ionization mass spectrometry (CI-MS) was used for unambiguous molecular weight assignments (Howard *et al.* 1980), and to corroborate structural assignments from EI-MS. Stereochemical assignments of alkenes were made using argentation thin layer chromatography Rf values and IR spectoscopy. Double bond locations were determined by methoxymercuration-demercuration (Blomquist *et al.* 1980b).

GLC analyses were performed using a Shimadzu GC-6AM gas chromatograph equipped with dual flame ionization detectors and dual stainless steel 1.8 m x 3 mm ID columns packed with 3% SP-2100 on 100/120 mesh Supelcoport. All analyses utilized temperature programming from 150-325°C at 8°/minute with a final five minute hold period.

GC-MS analyses were performed on a Hewlett Packard 5710A GC - 5982A Mass Spectrometer interfaced to a Hewlett Packard 5933 Data System. The GC was equipped with the dual glass 1.8 m x 2 mm ID columns packed with 3% OV-101 on 100/120 mesh chromosorb AWS. CI-MS were generated using ultrapure methane (Airco, Inc.) at a flow rate of 13 ml/minute as both carrier and ionizing gas, and at a source potential of 200 eV. Infrared spectra were obtained from neat films on KBr plates using a Perkin Elmer Model 337 Grating Infrared Spectrometer.

Percent composition of hydrocarbons was obtained by triangulation of GLC peaks. Total hydrocarbon was obtained by direct weighing of pooled samples from each colony. A variance estimate for mean variation across all

hydrocarbon components from a given source was obtained from the mean and standard deviation of the average co-efficient of variation for each source.

Radio-Incorporation Studies—Three groups of ten insects each were anesthesized with carbon dioxide and placed on ice. The sting apparatus of each bee was then immediately removed, pooled by colony, and placed in 100 μl of Graces' medium at 30°C. Ten μCi of [1-^{14}C] acetate (57 mCi/mmole, ICN, Irvine, California) were added and the samples were incubated for two hours. Lipids were extracted using the method of Bligh and Dyer (1959), the components isolated by thin layer chromatography, and radio-incorporation assayed by liquid scintillation counting for ten minutes at about 85% counting efficiency.

RESULTS

Major qualititive and quantitative differences in hydrocarbon composition were found between the cuticle and the sting. The cuticular composition was simplest, with components varying in carbon number between C23 and C36 (Table I). The complete sting apparatus and the isolated sting shaft possess the same mixture of C15 to C38 components (Table II), with only minor relative abundance differences, mainly involving the higher molecular weight components. Table III summarizes the percent composition of cuticular and sting components.

All sources examined contained similar homologous series of hydrocarbons, including n-alkanes, alkenes, alkadienes, and monomethyl alkanes. Interpretation of the EI and CI mass spectra of all components was straightforward, utilizing well-established criteria (Blomquist 1976; Howard *et al.* 1980; Jackson and Blomquist 1976; Nelson 1978). Note that the diagnostic EI-MS ion fragments listed in Tables I and II for the monoenes are for their monomethoxy derivatives. The dienes, some monoenes and some alkanes in Table II, were characterized only by CI-MS because of their low abundances. Monoenes up to C28 are predominately Z-9-, those between C29 and C31 are mixtures of Z-8-, Z-9-, and Z-10-, whereas monoenes of C33 and higher appear to be predominately Z-10.

Absolute abundance differences were also present among the three hydrocarbon sources. Cuticular hydrocarbons were present at *ca.* 236 ± 48 μg/Bee (X ± SD), whereas sting apparatus hydrocarbons were present at *ca.* 6 + 2 μg/Bee. Hydrocarbons from the excised sting shafts were present in insufficient quantities for direct weighing. A comparison of GLC peak intensities between sting apparatus and sting shaft analyses however, suggest that the shafts contain *ca.* 10% as much material as the entire sting apparatus.

No evidence was obtained from the radio-labeling experiment for the

direct biosynthesis of sting apparatus hydrocarbons by any of the tissues associated with the sting. Earlier studies by Blomquist *et al.* (1980a), using similar radiotracer methods, showed that *A. mellifera* cuticular hydrocarbons are biosynthesized by cuticle-related tissues.

DISCUSSION

A striking diversity of hydrocarbon components is produced by honey bee foragers. We earlier reported (Blomquist *et al.* 1980a) our preliminary findings on the identification of *Apis mellifera* cuticular hydrocarbon components. In this paper we provide a more detailed analysis and compare our results to a second hydrocarbon source in the honey bee. Hemolymph is a rich source of hydrocarbon in insects (Chino *et al.* 1981; Chino and Kitazawa 1981), but in our opinion it is not a practical hydrocarbon source for routine chemotaxonomic analyses, so we did not investigate it.

Although several authors (Blum *et al.* 1978; Gunnison and Morse 1968; and Pickett *et al.* 1982) have reported finding sting hydrocarbons, the chemicals in question were not identified, and questions were raised concerning the source of the sting hydrocarbons. In particular, Picket *et al.* (1982) suggested that they were sampling artifacts, introduced by contamination from cuticular material. Gunnison and Morse's (1968) morphological study, and Grandperrin and Cassier's (1983) ultrastructural study on the Koschewnikow's gland, which is associated with the sting apparatus, provided evidence that this gland could be the source of the sting apparatus hydrocarbons.

Our finding of both qualitative and quantitative differences between cuticular and sting hydrocarbons rules out the possibility that the sting hydrocarbons arise from cuticular contamination. Our finding of the absence of radiochemical incorporation of [1-^{14}C] acetate into the sting hydrocarbons also provides evidence against either the Koschewnikow gland or any of the other structures associated with the sting apparatus being involved in the biosynthesis of the sting hydrocarbons. The origin of these chemicals thus remains obscure.

Table IV provides a comparison of several factors that are important for deciding which source of hydrocarbons in the honey bee would be most suitable for chemotaxonomic purposes. Although cuticular hydrocarbons are the easiest to obtain and are present in greatest quantity, they provide only two-thirds as many individual components (characters) for analysis. They are also the most susceptible to contamination from beeswax and extraneous hydrocarbon sources such as pollen. Sting shaft hydrocarbons

are probably the least susceptible to contamination, but the low levels of chemical present per bee would probably preclude individual analyses. In addition, sample preparation would be rather time consuming. The entire sting apparatus appears to be a viable compromise. It is relatively free of external contamination, contains the maximum number of hydrocarbon components, and samples are readily obtained, even by unskilled personnel.

A large number of variables remain to be examined before hydrocarbons can be used to routinely identify ecotypes of *A. mellifera*. Among such variables, some important ones are likely to be age of bees, geographic source, and genotype. Interactions among these factors are likely, and sophisticated statistical analyses are likely to be required. Such is to be expected, however, since the problem involves identifying phenotypes along what is likely to be a continuous cline. Clearly, this is a problem requiring close cooperation between geneticists, entomologists, chemists, and statisticians. The requisite cooperation has been established, and further studies are now underway in our laboratories.

Table 1. Cuticular Hydrocarbons of *Apis mellifera* L. Foragers

Component	Carbon Number[a]	Diagnostic EI-MS Ions[b]
Z-9-C23:1	23	(157, 241, 171, 227)
n-C23	23	324
C24:1	24	---[a]
n-C24	24	338
Z-9-C25:1	25	(157, 269, 171, 255)
n-C25	25	352
Z-9-C26:1	26	(157, 283, 171, 269)
n-C26	26	366
Z-9-C27:1	27	(157, 297, 171, 283)
11-Me-, 13-Me C27	28	168/169; 238/239; 196/197; 210/211
n-C28	28	394
Z-8-, Z-9-, Z-10-C29:1	29	(143, 339, 157, 325, 171, 311, 185, 297)
11-Me; 13-Me; C28	29	168/169; 266/267; 196/197 238/239
n-C29	29	408
11-Me; 13-Me; 15-Me C29	30	168/169; 280/281; 196/197; 252/253; 210/211; 238/239
n-C30	30	422
Z-8-, Z-10-C31:1	31	(143, 353, 157, 339, 171, 325, 185, 311)

Table 1. Continued

n-C31	31	432
C32:1	32	448
11-Me-; 13-Me-; 15-Me C31	32	168/169; 294/295; 196/197; 266/267; 224/225; 238/239
n-C32	32	450
n-C33:2	33	---[a]
Z-10-C33:1	33	(171, 367, 185, 353)
n-C33	33	464
13-Me-, 15-Me-, 17-Me C33	34	194/195; 308/309; 224/225; 280/281; 238/239; 266/267
C34:2	34	---[a]
C34:1	34	476
C35:2	35	---[a]
C35:1	35	---[a]
13-Me; 15-Me; 17-Me C35	36	196/197; 336/337; 224/225; 308/309; 252/253; 280/281
$C37_1$	37	---[a]
$C37_2$	37	---[a]
C38	38	---[a]

a. Determined from CI-MS where $(M-1)^+$ is always the base peak.

b. Diagnostic ion fragments in parentheses are for the memomethoxy derivatives of the parent alkenes.

Table 2. Hydrocarbons of the Sting Apparatus and Sting Shaft of *Apis mellifera* L. Foragers

Components	Carbon Number[a]	Diagnostic EI-MS ions[b]
n-C15	15	212
n-C16	16	226
C17:1	17	238
n-C17	17	240
n-C18	18	254
C19:2	19	---[a]
Z-9-C19:1	19	(157, 185, 171)
n-C19	19	268
n-C20	20	282
Z-9-C21:1	21	(157, 213, 171, 199)
n-C21	21	296

Table 2. Continued

n-C22	22	310
Z-9-C23:1	23	(157, 241, 171, 227)
n-C23	23	324
11-MeC23	24	168/169; 196/197
Z-9-C24:1	24	(157, 255, 171, 241)
n-C24	24	338
Z-9-C25:1	25	(157, 269, 171, 255)
n-C25	25	352
11-MeC25	26	168/169; 224/225
Z-9-C26:1	26	(157, 283, 171, 269)
n-C26	26	366
Z-9-C27:1	27	(157, 297, 171, 283)
n-C27	27	380
11-Me; 13-M3C27	28	168/169; 252/253; 196/197; 224/225
n-C28	28	394
Z-8-; Z-9-; Z-10-C29:1	29	(143, 339, 157, 325, 171, 311,185, 297)
n-C29	29	408
11-Me-; 13-Me-; 15-MeC29	30	168/169; 308/309; 196/197; 280/281; 224/225; 252/253
n-C30	30	422
C31:2	31	432
Z-8-; Z-10-C31:1	31	(143, 353, 157, 339, 171, 325, 185, 311)
n-C31	31	436
11-Me-; 13-Me-; 15-Me-C31	32	168/169; 308/309; 196/197; 280/281; 224/225; 252/253
n-C32	32	450
C33:2	33	458
Z-10-C33:1	33	(171, 367, 185, 353)
n-C33	33	464
13-Me-; 15-Me-; 17-Me-C33	34	196/197; 308/309; 224/225; 280/281; 238/239; 266/267
C34:2	34	---[a]
C34:1	34	---[a]
C35:2	35	---[a]
C35:1	35	---[a]
C36	36	---[a]
$C37_1$	37	---[a]
$C37_2$	37	---[a]
C38	38	---[a]

Table 2. Continued

a. Determined from CI-MS where $(M-1)^+$ is always the base peak.

b. Diagnostic ion fragment in parentheses are for for monomethoxy derivatives of the parent alkenes.

Table 3. Mean Percent Composition[a] of the Hydrocarbons from the Cuticle and Sting Apparatus of Foragers of *Apis mellifera L.*

Component	Cuticle[b]	Sting Apparatus[b]
n-C15	---	TR
n-C16	---	TR
C17:1	---	TR
n-C17	---	TR
n-C18	---	TR
C19:2	---	TR
C19:1	---	4.3 ± 1.6
n-C19	---	0.7 ± 0.5
n-C20	---	TR
C21:1	---	TR
nC21	---	TR
nC22	---	TR
C23:1	0.6 ± 0.3	TR
nC23	3.0 ± 1.2	3.1 ± 1.2
MeC23	---	3.1 ± 1.2
C24:1	TR	TR
n-C24	TR	1.3 ± 1.4
C25:1	1.8 ± 0.5	1.3 ± 0.7
n-C25	11.1 ± 1.6	6.0 ± 1.4
MeC25	---	0.5 ± 0.4
C26:1	TR	TR
nC26	0.5 ± 0.5	1.9 ± 1.5
C27:1	1.8 ± 0.6	1.3 ± 0.6
nC27	19.5 ± 1.9	13.9 ± 1.3
MeC27	TR	1.4 ± 0.3
nC28	0.5 ± 0.3	1.8 ± 1.2
MeC28	0.8 ± 0.6	---
C29:1	2.7 ± 0.8	2.0 ± 0.8
nC29	14.2 ± 1.5	10.5 ± 1.4
MeC29	1.0 ± 0.6	1.1 ± 0.4
nC30	TR	0.9 ± 0.9

Table 3. Continued

C31:2	TR	TR
C31:1	13.0 ± 1.8	11.9 ± 1.8
nC31	10.1 ± 1.4	9.1 ± 1.5
MeC31	0.5 ± 0.3	0.9 ± 0.7
n-C32	TR	TR
C33:2	TR	TR
C33:1	15.8 ± 1.7	15.5 ± 2.9
n-C33	1.4 ± 0.5	4.1 ± 0.8
MeC33	TR	TR
C34:1	TR	TR
C35:2	TR	TR
C35:1	0.6 ± 0.4	TR
C36	TR	TR
$C37_1$	TR	TR
$C37_2$	TR	TR
C38	TR	TR

a. Mean ± SD; TR = Trace (less than 0.5%); --- = not detectable.

b. Means of 25 bees from each of 5 colonies; the sting apparatus and cuticle were from the same bees.

Table 4. Comparison of Attributes Used to Rank Suitability of Various *Apis mellifera* L. Hydrocarbon Sources for Chemotaxonomic Purposes

Attribute	Cuticle	Sting Apparatus	Sting Shaft
Quantity of hydrocarbon (ug/Bee)	236	6	0.6
Number of hydrocarbon components (by carbon number)	33	48	48
Ease of Sampling	greatest	medium	least
Likelihood of contamination from hydrocarbon sources	greatest	medium	least
Ability to run an analysis on single bees	yes	yes	doubtful
Between colony variability of of hydrocarbon composition*	$0.25 \pm 0.16^{+}$	$0.38 \pm 0.27^{+}$	---

*expressed as average co-efficient of variation $\pm$ SD for all components $\geq$ 1% abundance.

+means are not significantly different (= 0.05)

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