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NASONOV PHEROMONE OF THE HONEY BEE, *Apis mellifera* L. (HYMENOPTERA: APIDAE) Part I. Chemical Characterization

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Abstract—Composition of the Nasonov pheromone of the honey bee has been reexamined using new procedures, including analysis of pheromone from single live insects by capillary column GC-MS. Two new components have been identified, nerol and (*E,E*)-farnesol, and the presence of components proposed previously has been confirmed. Absolute amounts or relative proportions of components in the pheromonal secretion have been determined.

Key Words—Honey bee, *Apis mellifera*, pheromone, Nasonov pheromone, extraction technique, GC-MS, [13C]-NMR, terpenoids.

INTRODUCTION

The Nasonov gland of the worker honey bee secretes into a groove in the seventh dorsal abdominal tergite (Zoubarev, 1883; McIndoo, 1914). On exposure the secretion releases a volatile pheromone that attracts other workers. Workers expose the groove on finding the entrance to their hive (Sladen, 1901; Butler et al., 1970) or when foraging, either at a source of water (Free and Williams, 1970) or artificial food, such as a dish of sugar syrup (von Frisch, 1923; Free and Williams, 1972). During swarming, workers expose the groove while flying (Morse and Boch, 1971; Avitabile et al., 1975), on the swarm cluster, or near a queen lost from the cluster (Mautz et al., 1972; Boch and Morse, 1974).

The pheromone has been reported to comprise (*Z*)- and (*E*)-citral, geraniol, nerolic acid, and geranic acid (Boch and Shearer, 1962, 1964;

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Weaver et al., 1964; Butler and Calam, 1969); the latter two compounds were isolated as methyl ester derivatives. However, presence of the citrals was not established with certainty. Shearer and Boch (1966) reported that they were initially absent and were formed only during aging of the glandular extract. However, Butler and Calam (1969) found the citrals in samples analyzed within 4 min of extraction. These apparently conflicting results remained unresolved, except for a suggestion by Blum (1971) that transformation of terpenes could be taking place in the Nasonov gland by enzymic oxidation. Presence of geranic acid was also uncertain as it could have been formed from nerolic acid during isolation which involved extraction into alkaline solution and subsequent derivatization (Boch and Shearer, 1964). Furthermore, although the identified components have been bioassayed for their attractiveness to foraging honey bees, the full biological activity of the natural secretion was not accounted for by the chemicals so far reported. Values for absolute amounts and relative proportions of some of the components have been proposed. Boch and Shearer (1963, 1964) found 0–1.5 μg geraniol/bee depending on age. The ratio of citrals to geraniol was estimated at 1:7 by Weaver et al. (1964) and at 1:33 by Butler and Calam (1969). However, a synthetic mixture based on these figures has not been tested. The most active mixture tested consisted of citral, with unspecified isomeric composition, and geraniol (Butler and Calam, 1969), but with proportionally more citral than was reported for the natural secretion.

Honey bees are important pollinators of many crops (Free, 1970; McGregor, 1976) and a synthetic Nasonov pheromone could possibly be used to attract them to crops requiring pollination. A synthetic Nasonov pheromone may also prove useful to attract swarms and migrating colonies to empty hives, and a suitable bioassay for investigating the importance of the pheromone and its components in cluster stabilization has recently been devised (Ferguson et al., 1979). However, in order to exploit these possibilities, it seems desirable to define the Nasonov pheromone unequivocally.

This paper describes the identification and measurement of volatile components of the Nasonov gland. In subsequent papers, results of testing these chemicals, singly and together in their natural proportions, by means of a bioassay using foraging honey bees will be presented (Williams et al., 1981) and investigations into the composition of pheromone released into the air and mechanisms regulating pheromone production and composition described (Pickett et al., 1981).

METHODS AND MATERIALS

Collection of Nasonov Secretion from Honey Bees. Worker honey bees were collected individually at their hive entrance as they were leaving their hive to forage.

To obtain a sample of the Nasonov gland secretion for analysis a live honey bee was held between forefinger and thumb, with its abdomen lying over the thumbnail and with the Nasonov groove unexposed. The tip of the blunted needle of a 10-μl syringe (Scientific Glass Engineering) containing hexane (2 μl) was placed between the 6th and 7th dorsal abdominal tergites. The hexane was expelled from the syringe onto the Nasonov gland groove and immediately withdrawn into the syringe. About 0.4 μl of hexane solution was recovered and immediately injected onto a gas chromatography (GC) column.

For determining the absolute amounts of components, the honey bee was killed by crushing its thorax, the sting apparatus and gut pulled from the abdomen, and the 6th and 7th abdominal tergites, with the Nasonov gland, dissected out. The excised glands were extracted and extracts analyzed immediately, as described below.

Identification of Components and Determination of Relative Proportions of Citrals, Nerol, and Geraniol. Two capillary columns were employed for GC: 50 m $\times$ 0.25 mm glass, wall-coated with heat-treated Carbowax 20 M (PhaseSep); 50 m $\times$ 0.25 mm glass, wall-coated with Ucon 5100 (PhaseSep). A splitless injection system, consisting of silanized glass-lined steel tubing fitted into modified injection ports, was used with the carrier gas, helium, at 1 ml/min, and a temperature programme of 40–140°C at 4°/min. The GC effluent was monitored by flame ionization (Pye 104) or by mass spectrometry (MS) (Pye 204-VG Micromass 70-70F). For GC-MS the GC column was linked directly to the glass jet leading to the ion source by silanized glass-lined steel tubing. MS was by electron impact at 70 eV with a source temperature of 200°C; the chromatogram was obtained by single ion monitoring at m/z 69.

Components were tentatively identified from mass spectra obtained by GC-MS. These spectra were then compared with those of authentic compounds and were either similar to published spectra (Mass Spectrometry Data Centre, 1975) or are given below. Identity was confirmed by peak enhancement in GC or GC-MS with authentic compounds on the two capillary columns, the test compound being dissolved in the hexane injected into the gland when obtaining a sample of the secretion.

Relative proportions of (*Z*)-citral, (*E*)-citral, and nerol to geraniol were estimated from the flame ionization response in six replicated GC analyses, on the basis of responses obtained with standard solutions of the synthetic compounds.

Quantitative Analysis of Secretion for Absolute Amounts of Geraniol and (E,E)-*Farnesol.* Absolute amounts of geraniol and (*E,E*)-farnesol were determined in each of 11 excised glands.

Calibration equations were first determined, by regression analysis, for standard solutions of geraniol and (*E,E*)-farnesol. Six solutions containing geraniol and (*E,E*)-farnesol at concentrations from 0.06 and 0.04 to 0.16 and 0.11 mg/ml, respectively, were made up in hexane containing the internal

standard, camphor, at 0.04 mg/ml. Duplicate samples (2 μl) from each solution were chromatographed on a Perkin-Elmer F11 gas chromatograph with a flame ionization detector using a glass column, 6 ft $\times$ 1/4 in., 2.5% Carbowax 20 M on Chromosorb G-HP 80-100 mesh, 50–200°C at 10°/min. Heights of geraniol peaks and areas (i.e., height $\times$ width at half-height) of (*E,E*)-farnesol peaks were normalized against camphor peak heights and the mean for each duplicate analysis obtained. Calibration equations, for geraniol and (*E,E*)-farnesol, using normalized peak heights or areas, were then calculated.

Immediately after dissection, a single Nasonov gland was placed in a small tapered glass tube, made from a Pasteur pipette, with the camphor solution (20 μl). The gland was then quickly pulverized with a fine glass rod to aid extraction and a sample of the hexane solution (2 μl) chromatographed. Amounts of geraniol and (*E,E*)-farnesol were then calculated by reference to the calibration equations.

Quantitative Analysis of Secretion for Nerolic and Geranic Acids. Absolute amounts of nerolic acid and geranic acid were estimated in two batches of 13 excised glands.

Calibration equations were first determined for standard solutions of nerolic acid. Five solutions containing nerolic acid at concentrations of 3.4–33.6 μg/ml were made up in ether containing the internal standard, nonanoic acid, at 15 μg/ml. Samples of each solution (1 ml) were methylated by treatment with diazomethane by the method of Schlenk and Gellerman (1960) and evaporated to ca. 100 μl. The concentrated solutions (0.2-μl sample) were chromatographed on the Pye 104 gas chromatograph using the Ucon 5100 capillary column described above, at 100°C. The peak heights from methyl nerolate were normalized against corresponding methyl nonanoate peak heights and a calibration equation obtained.

Thirteen Nasonov glands were dissected out as above and quickly pulverized with the nonanoic acid solution (1.3 ml). The resultant slurry was filtered through a cotton wool plug in a Pasteur pipette. A portion of the filtrate (1 ml) was methylated and analyzed as before. The amount of nerolic acid per honey bee (i.e., mean value from 13 honey bees) was calculated using the calibration equation. The analysis was repeated on a second batch of 13 glands and the mean from the two analyses calculated.

The amount of geranic acid per honey bee was estimated in the same way using the calibration equation obtained with nerolic acid.

Chemicals. Chemicals were obtained pure or purified as described below (97–99+% by GC). Geraniol was obtained commercially (Koch Light). (*Z*)-citral, (*E*)-citral, nerol, and (*E,E*)-farnesol were isolated from respective commercial samples: ca. 1:1 (*Z*)- and (*E*)-citral (citral, Koch Light), ca. 1:1 nerol and geraniol (nerol, Pfaltz and Bauer), 42% (*E,E*)-farnesol (farnesol,

Aldrich) by chromatography on silica gel impregnated with 20% $AgNO_3$ (Hi-Flosil-Ag, Field Instruments) eluting with hexane containing a gradually increasing proportion of ethyl acetate. The eluent was monitored by GC. (*Z*)-Citral and (*E*)-citral were obtained for large-scale field trials by oxidation of nerol and geraniol with activated manganese dioxide at 18-fold molar excess in cold pentane, the reaction being monitored by GC.

Nerolic Acid. Citral (Koch Light) (2.8 g) was dissolved in absolute ethanol (50 ml) and solutions of $AgNO_3$ (6.8 g) in water (10 ml) and NaOH (3.2 g) in water (10 ml) were added dropwise with stirring over 15 min. Stirring was maintained for a further 15 min. The mixture was then filtered, the residue washed with ethanol, and the combined filtrate partitioned by addition of water (200 ml) and hexane (50 ml). The aqueous phase was separated, brought to neutral pH with acetic acid, and extracted with hexane (100 ml). The hexane layer was washed with water, dried ($MgSO_4 \cdot H_2O$) and evaporated to give a straw-colored oil (2.5 g) containing 25% nerolic acid and 75% geranic acid by GC (2.5% Carbowax 20 M on Chromosorb G-HP, 80-100 mesh; 6 ft × 1/4 in. OD glass column at 200°C).

The product was chromatographed 3× on silicic acid 100 mesh (A.R. 2847 Mallinckrodt) (100 g, 2 × 30 g) eluting with hexane followed by 2.5% acetone in hexane, collecting fractions containing, respectively, >1:1 (0.75 g), >4:1 (0.30 g) of nerolic and geranic acids, and finally only nerolic acid as a straw-colored oil after evaporation (0.12 g): MS (by GC-MS as above) *m/z* 168 (M^+, 4), 150 (2), 125 (4), 123 (6), 100 (18), 82 (10), 69 (100).

Geranic acid was obtained by oxidation of (*E*)-citral (1.9 g), under similar conditions, as a straw-colored oil, (0.9 g): MS (by GC-MS as above) *m/z* 168 (M^+, 3), 150 (2), 125 (5), 123 (8), 100 (15), 82 (7), 69 (100).

The structure and purity of each compound was confirmed by [^{13}C]-nuclear magnetic resonance (NMR) spectroscopy (Joel-PFT-100), in $CDCl_3$, Me_4Si as standard ($\delta = 0.00$) (Table 1). Assignments for NMR signals were made after Bohlmann et al. (1976) for the citrals, nerol, and geraniol and on the basis of off-resonance decoupling studies. Samples were sealed under vacuum or nitrogen in glass ampoules and stored at −20°C ready for bioassay.

RESULTS AND DISCUSSION

Compounds identified in the Nasonov secretion are listed in Table 2 together with mean relative proportion or mean amount per honey bee. Nerol and (*E,E*)-farnesol have not previously been identified as components, and the presence of (*Z*)- and (*E*)-citral is confirmed. Nerolic acid and geranic acid were also identified as free acids without extraction into base and derivatization.

Chromatograms from the Nasonov secretion contained many peaks; a

TABLE 1. [^{13}C]NMR ANALYSIS OF SYNTHETIC NASONOV PHEROMONE COMPOUNDS (SHIFT, PPM FROM Me_4Si STANDARD)

	Carbon									
Compound	1	2	3	3 Me	4	5	6	7	7 Me(cis)	8
(*Z*)-Citral	190.5	128.7	163.4	25.0	32.6	27.2	122.4	133.6	17.7	25.6
(*E*)-Citral	190.9	127.5	163.3	17.7	40.6	25.8	122.7	132.8	17.7	25.8
Nerol	59.2	125.2	139.5	23.7	32.5	27.1	124.3	132.4	18.0	26.0
Geraniol	59.1	124.1	138.9	16.3	39.7	26.5	123.7	131.5	17.7	25.7
Nerolic acid	172.1	123.6	163.3	25.7	33.8	26.9	116.0	132.4	17.5	25.7
Geranic acid	172.3	122.9	162.7	19.1	41.3	26.1	115.5	132.6	17.7	25.7
(*E*,*E*)-Farnesol	59.5	124.4	139.8	16.0	39.6	26.4	123.8	135.4		
Carbon (cont.)				7 Me	8	9	10	11	11 Me(cis)	12
(*E*,*E*)-Farnesol (cont.)				16.3	39.6	26.8	123.5	131.3	17.7	25.7

TABLE 2. COMPONENTS OF HONEY BEE NASONOV PHEROMONE

	Relative proportions	Absolute amount per honey bee (μg)	Peak
(*Z*)-Citral	0.6		A
(*E*)-Citral	1.1		B
Nerol	0.4		C
Geraniol	100.0	1.8	D
Nerolic acid		1.4	E
Geranic acid		0.2	
(*E,E*)-Farnesol		0.8	F

typical chromatogram is given in Figure 1 with assignments in Table 2. Only peaks found consistently in the GC traces were identified. Other peaks were not likely to be from important components of the Nasonov pheromone and were mostly derived from the cuticle or sting apparatus. This was established by analysis of samples from between dorsal abdominal tergites other than the 6th and 7th and by analysis of headspace and samples extracted from the sting

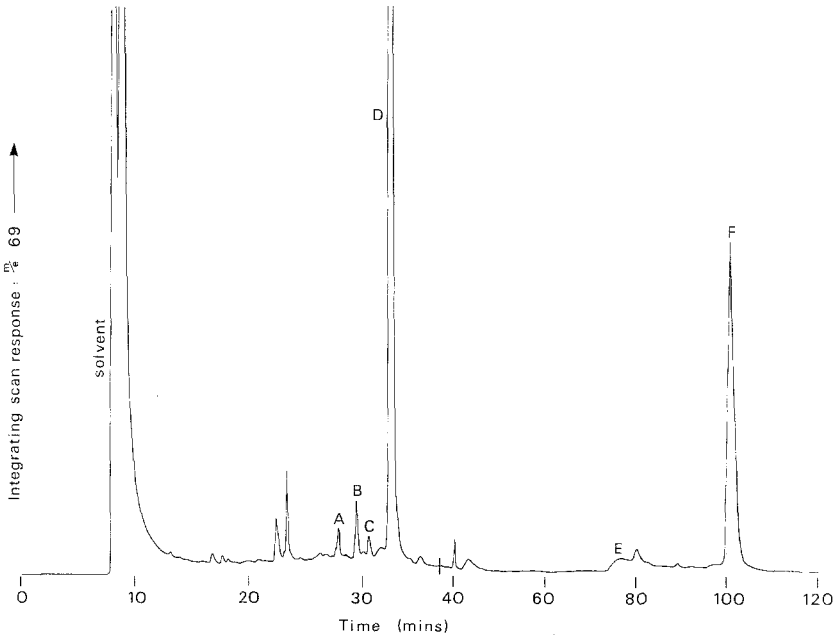

FIG. 1. GC-MS of Nasonov secretion from single honey bee (Ucon 5100 capillary column).

apparatus. Single ion monitoring at m/z 69 is highly sensitive for compounds containing the dimethylallyl group such as many terpenoids. However, no other peaks were found consistently in GC with the flame ionization detector which does not discriminate according to structure.

As samples for GC are taken from the closed gland of honey bees about to leave the hive, it is likely that the components identified are those present when pheromone release commences. Chemical alteration before analysis is avoided by the extract being transferred immediately from the honey bees to the gas chromatograph; the entire extraction procedure takes only a few seconds. Chemical interconversion of components during analysis was shown not to occur by individual GC of the pure synthetic compounds, which gave single major peaks consistent with NMR results.

Although satisfactory mass spectra for nerolic and geranic acids were obtained by GC-MS of the Nasonov secretion, the GC peaks were distorted because of interaction with the column stationary phase (see peak E in Figure 1). The peak for geranic acid (R_t = 88 min on Ucon 5100) could only be discerned at high sensitivity. Nonetheless, presence of the two acids was confirmed by peak enhancement with the authentic acids using amounts closely similar to those in the natural extract.

The seven components identified in the Nasonov secretion are biosynthetically related terpenoids. The monoterpenoid sequences nerol, (*Z*)-citral and nerolic acid, and geraniol, (*E*)-citral, and geranic acid are presumably derived, respectively, from neryl and geranyl pyrophosphate, with increasing order of oxidation in the functional group from alcohol through aldehyde to carboxylic acid. The sesquiterpenoid, (*E,E*)-farnesol is structurally related to geraniol and is presumably derived from (*E,E*)-farnesyl pyrophosphate. However, other isomers and oxidized congeners of (*E,E*)-farnesol were not detected.

The method employed for analysis of the Nasonov secretions from live single honey bees does not extract all the secretion. It cannot therefore be used to estimate absolute amounts of glandular components. However, because of the need for rapid analysis of (*E*)-citral (see Part III, 1981), the technique was used to estimate proportions, relative to geraniol, for this compound and the other minor components (*Z*)-citral and nerol (Table 2). Large variations between honey bees [(*Z*)-citral, σ_{n-1} 15%; (*E*)-citral, 48%; nerol, 51%] were observed, but the relative proportions remained the same. The ratio of citrals to geraniol found was less than that determined by Weaver et al. (1964) and Butler and Calam (1969), who were not able to analyze extracts immediately. For quantitative estimation of geraniol and (*E,E*)-farnesol and of nerolic and geranic acids, total extracts of excised glands were made with appropriate internal standards. For analysis of the two acids the mixture was methylated with diazomethane but without extraction into alkaline solution. This

procedure was shown not to influence the proportion of nerolic and geranic acids and therefore gave further proof that they are present in the secretion. As the latter analysis was somewhat lengthy, batches of 13 excised glands were analyzed in duplicate rather than analysis of single glands. Amounts of geraniol and (*E,E*)-farnesol also showed considerable variation between honey bees [geraniol, $\sigma_{n-1} = 65\%$; (*E,E*)-farnesol, 70%] which did not arise from the method (geraniol, $\sigma_{n-1} = 2\%$; $n = 6$) but the mean amount of geraniol per honey bee was similar to that found by Boch and Shearer (1963). Although the nerolic and geranic acid figures were from batches of 13 glands, the two replicates indicated considerable variation between honey bees (nerolic acid, 13.5 and 21.8 μg; geranic acid, 1.3 and 3.8 μg). Again, however, the method including methylation had only a small standard deviation (nerolic acid, $\sigma_{n-1} = 8\%$, $n = 5$). Boch and Shearer (1964) found more geranic than nerolic acid by their method and seven times as much nerolic acid as geranic acid was found here. Although some components of the Nasonov gland are converted into other components under natural conditions, these conversions would be too slow to influence the quantitative estimations described here (see Part III, 1981).

Volatile components of the Nasonov gland secretions have thus been identified. Further proof that these compounds comprise the Nasonov pheromone will be reported in parts II and III of this series.

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