The Aggregation Pheromone of the German Cockroach,
Blattella germanica (L.) (Dictyoptera: Blattellidae):
Isolation and Identification of the Attractant
Components of the Pheromone

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Attractant components of the aggregation pheromone of the German cockroach, *Blattella germanica* (L.) were isolated from frass-contaminated filter paper. Successive purification steps were monitored by olfactometer assay, and each attractant component was eventually identified by GC-MS and the assay on each preparative GC fraction. The attractant comprised several volatile amines. Amine evaporation was restricted by salt formation with acid(s) in the excreta. All the identified ammonia and 12 amines exerted both chemotaxis and anemotaxis on *B. germanica* nymphs. Typical excretory materials such as ammonia, methylamine, dimethylamine, and trimethylamine were the major components. The most active component was 1-dimethylamino-2-methyl-2-propanol (ED_{50} =ca. 10 pmol /l air), which was 50 to 1,000 times more active than the others.

INTRODUCTION

Blattella germanica (L.) excretes an aggregation pheromone (ISHII and KUWAHARA, 1967) with its frass and marks its harbouring place with it. Though several attempts have been made to identify the chemical(s) (BLOCK and MCCHESENEY, 1974; RITTER and PERSOONS, 1975; NAKAJIMA et al., 1974), no conclusive results has been attained. The complexity of this study may be ascribed to the ambiguity of the vertical paper assay (ISHII and KUWAHARA, 1967), which does not independently evaluate behavioural components (SAKUMA and FUKAMI, 1985). The vertical paper assay can be categorized as a typical 'alternative chamber' (KENNEDY, 1977), which leaves the relative contributions of chemokinesis and chemotaxis unmeasured. In addition the direct contact of a test animal with both pheromone source and other animals allows it to perceive the pheromone through contact chemo-reception other than olfaction. We focused our attention on the volatile chemicals which induce distant olfactory behaviours and investigated the activity with an olfactometer assay (SAKUMA and FUKAMI, 1985, 1990). This article deals with the isolation and identification of the attractant components of the aggregation pheromone.

MATERIALS AND METHODS

Animals. *Blattella germanica* (L.) were reared on insect diets (Oriental Yeast Co.,

Tokyo) at $29 \pm 1^\circ\text{C}$, $50 \pm 10\%$ RH and on a 14L-10D regime (SAKUMA and FUKAMI, 1985). Polyethylene boxes ($65 \times 35 \times 40$ cm) were used as rearing containers. Corrugated and flat sheets of filter paper (Toyo No. 2, 40×20 cm and 20×20 cm respectively) were layered alternately in a basket and placed in the containers for shelter. Test animals were the mixture of first and second instar nymphs isolated from the colony at one to 6 days post-eclosion.

Behavioural assay. The attractiveness of the successive purification steps were monitored by linear track olfactometer assay, which evaluates each instance of chemotaxis and odour-modulated anemotaxis of the animals (SAKUMA and FUKAMI, 1985, 1990).

A sample was applied to the olfactometer in one of the 2 following methods. A disc application method was used principally for each fraction in the preliminary purification processes in which the attractant amines formed salt with acids. Ten μl of a methanol dilution was dispensed onto a hooked 1.0 cm^2 stainless steel disc. After solvent evaporation, the disc was suspended in the air intake tube of the olfactometer. The other is a continuous application method that was used for each fraction of the ion exchange resin treatment and of the subsequent purification processes. Aqueous solution of amine hydrochloride was neutralized by mixing with 2 N-potassium hydroxide (KOH) in a syringe (500 μl). The syringe was then charged on a servo-controlled plunger pump and the solution was continuously forced out (20 $\mu\text{l}/\text{min}$) onto a piece of cotton gauze covering the air intake of the olfactometer, so that free amine was successively supplied.

Test animals were given free access to water before assays to exclude any hygrotaxis behaviour. Assays were carried out during the day, at $25 \pm 0.1^\circ\text{C}$, $50 \pm 6\%$ RH.

Statistics. Each of the olfactory responses was expressed in terms of excess proportion index, i.e. the proportion of the difference between each of both trap catches to the sum (SAKUMA and FUKAMI, 1985). Doses were expressed as gram equivalent of the original methanol extract of the frass-contaminated paper (g.f.e.). Dose/response relation between a series of decimal dilutions and excess proportion index was subjected to probit analysis. Parallel line assay on a set of preparations estimated median effective doses (ED_{50}) and relative activities (R) (SAKUMA and FUKAMI, 1990). The activity of each fraction was expressed as R% to the origin. The specific activity of each compound was expressed as ED_{50} in nmol/l air.

Extraction of the attractant. The attractant was extracted from the frass-contaminated filter papers (0.92 m^2 in total/container), which had been used as shelter strata in the mass culture. Each container housed ca. 10,000 animals. Filter papers were contaminated with the excreta for 3 months. These papers were immediately soaked in methanol and stored for several weeks in the ambient temperature.

Preliminary purification of the crude extract. The crude methanol extract was concentrated in a flash evaporator, washed with *n*-hexane 5 times, and then concentrated to viscous paste *in vacuo*. The paste was suspended in aqueous *n*-butanol, and then partitioned with *n*-butanol and water. The aqueous layer was decolorized by being passed through a polystyrene gel column (Diaion CHP-20P; Mitsubishi Chem. Ind. Ltd., Tokyo, 15×3.8 cm ID) with a labo-pump, and the column adsorbate was then recovered with methanol. The aqueous effluent was concentrated to dryness with Celite 545 (1 kg) in a rotary evaporator. The attractant was extracted from the Celite powder with methyl acetate in a Soxhlet apparatus for 12 hr. The residual Celite was then rinsed with water. These processes are outlined in Fig. 1.

Steam distillation of the volatile amines from the methyl acetate extract. After solvent evaporation, the methyl acetate extract (300 g.f.e., 21.6 g) was dissolved in water. Each 1/3 aliquot was then applied to an anion exchange resin column (Dowex 1 $\times$ 8, 100–200 mesh, OH form, 50 $\times$ 2 cm ID). The effluent was directly introduced with a labo-pump into a distillation flask through a Teflon catheter and then steam-distilled into a receiver flask containing 1.5 N-hydrochloric acid (100 ml). The distillate was concentrated *in vacuo* to dryness to remove excess hydrochloric acid.

Separation of the steam-distilled amines into primary, secondary, and tertiary amines by Hinsberg reaction (SHRINER et al., 1980). Benzenesulfonyl chloride (100 mmol, 17.7 g) and aqueous KOH (125 mmol/62.5 ml) were added to the distillate (as hydrochloride, 250 g.f.e., 943 mg/25 ml) in a stoppered flask, stirred at 0°C for one hr and then at 80°C. The mixture was then extracted with chloroform. Tertiary amines remaining in the aqueous layer were recovered by steam distillation from the aqueous layer. Excess benzenesulfonyl chloride in the chloroform layer was completely decomposed by agitating with 2N-KOH (62.5 ml). During this treatment, sulfonamides of primary amines were transferred into the aqueous alkaline layer, while those of secondary amines remained in the chloroform layer. Both the sulfonamides (50 g.f.e., 239 and 97.5 mg respectively) were hydrolyzed by refluxing with 8N-hydrochloric acid (10 ml) for 24 hr. After the mixture was made alkaline with KOH (50 mmol), the regenerated amines were steam-distilled and recovered as hydrochlorides.

Polystyrene gel liquid chromatography of tertiary amines. Tertiary amines (180 g.f.e.) were chromatographed with the following HPLC system: a stainless steel column (30 $\times$ 1 cm ID) was loaded with Diaion CHP-3C (particle size 10–20 μ m); eluent 33% (v/v) methanol in 0.1N-NaOH aqueous solution, flow rate at 2 ml/min. Aqueous tertiary amine hydrochlorides (50 g.f.e./200 μ l) mixed with 10N-NaOH aqueous solution (50 μ l) in a 500 μ l syringe was subjected to HPLC. Each 4 ml of the eluate was collected and analysed by GC. The separated amine was recovered as its hydrochloride after micro-scale steam distillation using a 50 ml distillation flask.

Analytical and preparative gas chromatography of amine components. Each separated group of amines, as well as the original steam distillate, was gaschromatographed on a Yanaco (Kyoto) model G-80F instrument equipped with an FID. Aqueous amine salts (1 g.f.e./100 μ l) were injected directly on a heated (250°C) pre-column (10 cm $\times$ 5 mm ID) packed with 20% KOH on Chromosorb W (60–80 mesh, acid washed). Emerging free amines were successively chromatographed with a 3 m $\times$ 3 mm ID stainless steel column packed with Chromosorb 103 (100–120 mesh) or the same size column with ASC-H (20% FFAP + 5% KOH on Neopak 2A, 80–100 mesh; Nishio Ind. Co., Tokyo). Helium was used as carrier gas (20 ml/min); temperature was programmed from 100 to 200°C at 4°C/min for Chromosorb 103, from 80 to 150°C at 2°C/min for ASC-H. The amount of each amine was evaluated by comparing a peak area with that of a known amount of the authentic material, using a Hewlett Packard 3390A reporting integrator.

Preparative GC was also conducted on the same instrument with the additional attachment and devices shown in Fig. 4. The effluent was divided by a splitter; ca. 1/100 portion was led to FID for monitoring, while the other major portion was introduced to a replaceable Porapak Q column impregnated with 6N-hydrochloric acid (ca. 0.4 ml). Each trapped component was recovered from the Porapak Q column by methanol extraction and then subjected to the behavioural assay.

Spectral analysis. Mass spectra were taken on a Hitachi M-80 GC-MS with a

M-003 data processing system, using the same GC-columns and conditions mentioned above. Samples were generally measured at low resolution mode (EI at 70 eV), but some at high resolution and GC-FI mode. The mass spectra were compared with those from commercially available authentic samples (Tokyo Chem. Ind. Co. Ltd., Tokyo; Nacalai Tesque, Kyoto) except for 1-dimethylamino-2-methyl-2-propanol (1-DMAM-2-P) which was prepared in the laboratory. The amount of ammonia, for which detection is impossible by FID, was evaluated by a quantitative operation of the mass chromatogram on m/z 16 peak. PMR spectra of the natural and synthetic 1-DMAM-2-P and their hydrochlorides were taken on a JEOL FX90-Q, 90 MHz spectrometer.

Synthesis of 1-dimethylamino-2-methyl-2-propanol (1-DMAM-2-P). 1-DMAM-2-P (CAS [14123-48-9], CAMPBELL and CAMPBELL, 1938) was synthesized by hydrogenation of acetone cyanohydrin (SATO and SUZUKI, 1969) and subsequent di-methylation of the amino group by Eschweiler-Clarke reaction. Acetone cyanohydrin (8.5 g, 0.1 mol) and cobalt dichloride (anhyd. 25 g, 0.19 mol) were dissolved in methanol (0.5 l) and NaBH_4 (18.8 g, 0.5 mol) was added in portions within 2 min under vigorous stirring with cooling. 12N-Hydrochloric acid (54 ml, 0.66 mol) was then added after 20 min and the generated hydrogen cyanide was ventilated. Then the mixture was basified with KOH (60 g, 0.88 mol) and the precipitated borax and cobalt dioxide were removed by filtration. The supernatant contained 1-amino-2-methyl-2-propanol and methylamine. The latter was removed during the concentration *in vacuo*.

Formic acid (85%, 67.6 g, 1.25 mol) and aqueous formaldehyde (35%, 55.8 g, 0.65 mol) were added to the supernatant containing 1-amino-2-methyl-2-propanol (0.25 mol equivalent) and the mixture was refluxed at 80°C for 18 hr with stirring. After addition of 12N-hydrochloric acid (83.3 ml, 1 mol), the mixture was concentrated *in vacuo*. After being basified with aqueous KOH (0.75 mol/200 ml), the product was continuously extracted with ethylether for 18 hr and desiccated with 3A molecular sieves. The product of 3 replications (0.75 mol equivalent) was fractionally distilled with a micro-spinning band distillation column, model NF-105 (Nester Faust Corp., Wilmington, DE, U.S.A.), at atmospheric pressure and 1-DMAM-2-P (22.2 g), distilled out at 126–128°C, and was collected (25% overall yield). GC r.t. and MS of the synthesized 1-DMAM-2-P were completely identical to those of the natural product (Peak No. 13 in Table 2). PMR (90 MHz, CD_3OD): 1.18 ppm (6H, s), 2.29 (2H, s), 2.31 (6H, s). PMR of hydrochloride (50 μmol /500 μl solution): 1.33 (6H, s), 2.97 (6H, s), 3.18 (2H, s).

RESULTS

Extraction of the attractant

The solvent suitable for the extraction of the attractant was preliminarily explored. The frass-contaminated filter paper (20 × 40 cm) was extracted with each of the various solvents (6 times, 1 l in total) and the extract was concentrated *in vacuo*. One fourth of the extract was applied on clean filter paper (5 × 40 cm). After air blown solvent evaporation, the treated paper was subjected to olfactometer assay (SAKUMA and FUKAMI, 1985). The attractiveness of the treated paper was compared with that of the original filter paper remaining after the extraction (residual paper), which was dried and cut into 5 × 40 cm pieces. The results in Table 1 indicate that solely polar solvents, such as methanol and water, completely extracted the attractant. Table 1 presents only chemotaxis activity, for almost the same results were obtained for anemotaxis.

Table 1. The attractiveness of solvent extracts and residues from excrement-soiled filter paper

Solvent	Wt. % ^a	Extract ^b <i>I</i> ^c (fiducial limits)	Residual paper <i>I</i> (fiducial limits)
<i>n</i> -Hexane	14.5	0.04 (−0.10, 0.18)	0.87 (0.78, 0.93)
Chloroform	20.0	0.36 (0.22, 0.49)	0.81 (0.71, 0.88)
Ethyl ether	16.2	−0.01 (−0.15, 0.13)	0.84 (0.75, 0.91)
Acetone	16.4	0.33 (0.19, 0.46)	0.74 (0.63, 0.83)
<i>n</i> -Butanol	13.6	0.22 (0.08, 0.36)	0.75 (0.64, 0.83)
Isopropanol	34.6	0.61 (0.49, 0.72)	0.35 (0.21, 0.48)
Aqueous <i>n</i> -butanol	42.5	0.54 (0.41, 0.65)	0.22 (0.08, 0.36)
Methanol	51.0	0.81 (0.71, 0.88)	−0.05 (−0.19, 0.09)
Water	83.7	0.88 (0.80, 0.94)	−0.13 (−0.27, 0.01)

Only chemotaxis is shown. The results for anemotaxis were almost the same.

^a Relative weight (%) of the solvent extract to the total weight of the extractable materials, calculated by the sum of the weight of the solvent extract and each extract from the residual paper with *n*-hexane, methanol and water.

^b The extract was applied on a piece of clean filter paper of the same size as the original paper.

^c Excess proportion index with 95% fiducial limits in parentheses. e.g. *I*=1: complete attraction, *I*=0: no response.

We chose methanol as the extracting solvent because of the convenience of handling. A total of 120 m² of the paper yielded 585 g of dark tan, unpleasantly smelling syrup. Extraction with methanol, however, resulted in contamination by a large amount of various waste materials such as fatty acids (McFARLANE, 1984; McFARLANE and ALLI, 1986; RITTER and PERSOONS, 1975), ammonia and other nitrogenous metabolites (COCHRAN, 1975), as well as waxes from the body surface. Thus some effective preliminary purification processes were required.

Isolation of the attractant components

Schemes of chemical processes, together with weight, chemotaxis, and anemotaxis activity of each fraction relative to the origin, are illustrated in Figs. 1 and 3. Details of each procedure are described in the materials and methods section.

Preliminary purification of the crude extract. The methanol extract of the frass-contaminated paper was purified as shown in Fig. 1. Two steps of solvent partition and decolorization with polystyrene gel column removed inactive, less polar materials, and gave active aqueous effluent. After adsorption of the aqueous effluent on Celite, extraction with methyl acetate from the Celite gave 42 g of clear yellowish paste containing active materials. The water eluate from the residual Celite, which contained a large amount of inorganic ammonium salts, showed almost no activity.

Treatment of the methyl acetate extract with anion- and cation-exchange resin. The aquaphilic nature and other properties of the methyl acetate extract suggested that the attractant might be forming salt, so we tried to investigate through ion-exchange resin treatment whether the attractant was acidic or basic. A scheme of treatments and the activity of materials under either disc or continuous sample application are shown in Fig. 2. Activities were expressed relative to the maximum record (100%) of the corresponding application mode. The methyl acetate extract was dissolved in water and

Fig. 1. Preliminary purification processes. Figures in each box show the weight, chemotaxis (R chemotaxis), and anemotaxis activity (R anemotaxis) of each fraction relative to the origin (100%). The original material is the methanol extract of the frass-contaminated filter paper. Sample materials were provided by disc application. Anemotaxis was observed in the air flow at 4 cm/sec.

Fig. 2. Treatment on the methyl acetate extract with anion and cation exchange resins. 'Disc' and 'Continuous' denote sample application mode. Figures in each box show the relative chemotaxis (R chemotaxis) and anemotaxis activity (R anemotaxis) of both modes.

Fig. 3. Purification processes leading to the isolation of attractant amine components. The original material is the product of the preliminary purification processes, i.e. methyl acetate extract of the aqueous effluent from the polystyrene gel column. The sample materials were provided by continuous application of free amine solution in the assay. See Fig. 1.

its aliquots were applied to anion- (Dowex-1) and cation-(Dowex-50w) exchange resin columns. Neither of the effluents exerted any activity when they were applied with disc application. Ammoniacal odour, emanating from the Dowex-1 effluent, suggested that the attractant(s) was a volatile amine lost during the handling. We developed a sample application method and applied the intact effluent continuously onto a piece of gauze covering the air intake of the olfactometer. As expected, only the Dowex-1 effluent exhibited potent activities by continuous application. In addition, the re-combined mixture of the Dowex-1 and Dowex-50w effluents regenerated the activities by disc application. This indicated that the attractant(s) was a volatile amine compound(s), with a release rate controlled by salt formation with acid(s) in the original extract.

Isolation and identification of attractant amine components of the methyl acetate extract. The methyl acetate extract was fractionated as shown in Fig. 3, where the relative weight and activities of each fraction were calculated based on those of the methyl acetate extract (100%). Samples were assayed with continuous application. Amines were separated from the methyl acetate extract by passing through an anion-exchange resin (Dowex-1) column, and then immediately steam-distilled. The distillate was intro-

Fig. 4. Preparative GC apparatus for amines. Aqueous solution of amine salt was injected onto a KOH pre-column. Liberated amines were successively chromatographed and fractionally collected with replaceable Porapak Q columns. Effluent was divided by a splitter; ca. 1/100 portion was led to FID, while the other major portion was recovered by a Porapak column impregnated with hydrochloric acid solution. The knurled nut on the collection vent port eliminates leaks in the Porapak column. Each trapped component was eluted with methanol from the Porapak column.

Fig. 5. Gas chromatogram of the steam distillate and the activity of each fraction. Numbers on peaks refer to compound identifications in Table 2. Peaks with asterisked numbers were also found in the tertiary amine fraction. Note that the peak height was multiplied by 40 times after r.t. 10 min. GC condition: Chromosorb 103, 100–120 mesh, 3 m × 3 mm ID; He, 20 ml/min; 4°C/min from 100°C to 200°C; FID.

duced into a hydrochloric acid solution and amines were collected as hydrochloride (2.2 g). The distillate, as well as the Dowex-1 effluent, preserved all the activities.

The distillate was analysed by GC-MS to identify ammonia and 11 amines, i.e.

Table 2. Mass spectral data and compound identifications of the steam distillate

Peak ^a No.	Compound ^b	Significant <i>m/z</i> (relative intensity)	GC r.t. ^c (min)	Amount (μ mol/g.f.e.)
1	Ammonia	17 (100), 16 (81)	1.6	24
2	Methylamine	31 (69), 30 (100), 18 (7)	3.2	3.6
3	Dimethylamine	45 (81), 44 (100), 43 (18), 42 (23), 30 (8), 18 (8)	5.3	22
4	Trimethylamine	59 (51), 58 (100), 57 (8), 43 (8), 42 (14), 30 (15)	7.1	5.3
5	Diethylamine	73 (30), 72 (16), 58 (100), 44 (24), 30 (64), 29 (12)	15.1	0.077
6	Isobutylamine	73 (10), 56 (3), 42 (3), 41 (8), 39 (4), 30 (100)	17.2	0.11
7	<i>n</i> -Butylamine	73 (9), 30 (100)	18.7	0.031
8	Pyrrolidine	71 (47), 70 (63), 43 (100), 42 (19), 41 (27)	20.8	0.16
9	Triethylamine	101 (28), 86 (100), 58 (25), 44 (8), 42 (9), 30 (19)	22.3	0.0069
10	Isoamylamine	87 (5), 70 (6), 55 (3), 42 (4), 41 (5), 39 (4), 30 (100)	23.0	0.46
11	Piperidine	85 (51), 84 (100), 57 (32), 56 (32), 43 (15), 42 (17) 30 (17), 29 (21)	25.3	0.074
12	2-Dimethylamino- ethanol (DMAE)	89 (8), 58 (100), 44 (11), 42 (19), 30 (8)	27.0	0.18
13	1-Dimethylamino- 2-methyl-2-propanol (1-DMAM-2-P)	117 (8), 102 (14), 100 (3), 58 (100), 44 (11), 42 (9)	29.7	0.046
—	2-Dimethylamino- 2-methyl-1-propanol (2-DMAM-1-P) ^d	117 (1), 102 (4), 87 (7), 86 (100), 72 (8), 70 (5), 58 (10), 56 (11), 44 (10), 42 (8)	41.6	

Also refer to the gas chromatogram in Fig. 5.

^a Each number refers to each GC peak.

^b All the components were verified by coelution and mass spectra with authentic samples. 1-DMAM-2-P was synthesized in the laboratory. Other compounds were commercially available.

^c GC condition: Chromosorb 103, 100–120 mesh, 3 m $\times$ 3 mm ID; He, 20 ml/min; 4°C/min from 100°C to 200°C.

^d Although 2-DMAM-1-P was not a component, analytical data is given for comparison with 1-DMAM-2-P.

methylamine, dimethylamine, trimethylamine, diethylamine, isobutylamine, *n*-butylamine, pyrrolidine, triethylamine, isoamylamine, piperidine, and 2-dimethylaminoethanol. Table 2 shows identification, GC-MS analytical data, and amount of each component. The same distillate was further fractionated with a preparative GC system, and then each fraction was subjected to the olfactometer assay. A model experiment using the mixture of authentic amines guaranteed at least 50–70% recovery on the preparative GC technique, so that the activity of each fraction was expected to reflect an activity spectrum of the original amine mixture.

The gas chromatogram and relative activity of each fraction to the original distillate are shown in Fig. 5. Only chemotaxis activity is given since the results for anemotaxis were almost the same. Ammonia and amines with short retention times such as methylamine, dimethylamine, and trimethylamine were the major components and contributed considerably to the activity of the distillate. Various alkylamines such as isobutylamine, isoamylamine, pyrrolidine, etc., were also found, but their activity and amounts were negligible. The most potent activity was marked by the fraction of a

Table 3. The attractiveness of the component amines for *B. germanica* nymphs

Compound	Chemotaxis				Anemotaxis ^a			
	<i>a</i>	$Y=a+1.390X^b$ ED ₅₀ (fiducial limits) ^c (nmol/0.5 l air)			<i>a</i>	$Y=a+1.460X$ ED ₅₀ (fiducial limits) (nmol/l air)		
Ammonia	-1.214	7.5	(6.0 - 9.3)		-2.207	33	(26 - 40)	
Methylamine	-0.092	1.2	(0.95 - 1.4)		-0.038	1.1	(0.86 - 1.3)	
Dimethylamine	0.252	0.66	(0.54 - 0.81)		0.153	0.79	(0.64 - 0.96)	
Trimethylamine	0.418	0.50	(0.40 - 0.62)		0.165	0.77	(0.63 - 0.94)	
Diethylamine	0.356	0.55	(0.45 - 0.69)		-0.246	1.5	(1.2 - 1.8)	
Isobutylamine	-0.316	1.7	(1.4 - 2.1)		-0.639	2.7	(2.2 - 3.4)	
<i>n</i> -Butylamine	-0.520	2.4	(1.9 - 2.9)		-0.689	3.0	(2.4 - 3.6)	
Pyrrolidine	-0.673	3.1	(2.5 - 3.8)		-0.610	2.6	(2.1 - 3.2)	
Triethylamine	0.022	0.96	(0.78 - 1.2)		0.184	0.75	(0.62 - 0.91)	
Isoamylamine	-0.319	1.7	(1.4 - 2.1)		-0.494	2.2	(1.8 - 2.7)	
Piperidine	-0.646	2.9	(2.3 - 3.6)		-0.524	2.3	(1.9 - 2.8)	
DMAE	-0.882	4.3	(3.5 - 5.3)		-0.885	4.0	(3.4 - 4.8)	
1-DMAM-2-P	2.934	0.0077	(0.0063- 0.0095)		2.899	0.010	(0.0087- 0.012)	
2-DMAM-1-P ^d	-0.884	4.3	(3.5 - 5.3)		-0.930	4.3	(3.6 - 5.2)	

Analysis of χ^2 for Chemotaxis

	d.f.	sum of squares	mean square	<i>p</i>
Parallelism	13	22.7	1.74	0.05
Heterogeneity	58	108.5	1.87	<0.001
Total	71	130.8		

ANOVA $F_0=1.091 < F(58, 13; 0.05)$ Analysis of χ^2 for Anemotaxis

	d.f.	sum of squares	mean square	<i>p</i>
Parallelism	13	22.3	1.72	0.05
Heterogeneity	43	53.5	1.24	0.13
Total	56	76.2		

ANOVA $F_0=0.713 < F(43, 13; 0.05)$ ^a Anemotaxis was observed in the air flow at 4 cm/sec.^b Regression formula for the parallel line assay results.^c Median effective dose with 95% fiducial limits in parentheses. Variance was multiplied by heterogeneity factor.^d Though 2-DMAM-1-P was not a component, its attractiveness is given for comparison with 1-DMAM-2-P.

small peak at r.t. 30 min, which was difficult to identify with only the low resolution mass spectrum.

Amines of the distillate were separated by Hinsberg reaction into primary, secondary, and tertiary amines (Fig. 3). By comparing the relative weight and activities of each fraction, we discovered that tertiary amines dominantly contribute to the total activity of the attractant. GC analysis of each group of amines revealed that the 30 min peak was found solely in tertiary amines. Tertiary amines were further chromatographed with HPLC on a polystyrene gel (Diaion CHP-3C) column to separate into 2-dimethylaminoethanol, trimethylamine, and the component of the 30 min peak; 0.6 mg of the material was obtained from 120 mg (180 g.f.e.) of tertiary amines.

Identification of 1-DMAM-2-P

A high resolution GC-MS spectrum of the 30 min peak gave $M^+=117.1150$ indicating the molecular formula as $C_6H_{15}O_1N_1$. An $m/z=117$ peak of the GC-FI spectrum

Fig. 6. Dose/response pattern in chemotaxis (●) and anemotaxis (○) of *B. germanica* nymphs for dimethylamine, trimethylamine, and 1-DMAM-2-P. Vertical bars indicate 95% fiducial intervals.

confirmed it to be the molecular ion peak. $M - OH^+ (C_6H_{14}N_1 = 100.1116)$ suggested the presence of $-OH$. $CH_3 \cdot$ would be lost ($C_5H_{12}O_1N_1 = 102.0931$) from a hetero carbon atom. The base peak ($C_3H_6N_1 = 58.0621$) immonium ion should be $(CH_3)_2N^+ = CH_2$, for this is a tertiary amine. If these moieties, $CH_3-\overset{|}{C}-OH$ and $(CH_3)_2NCH_2-$, were combined together, only CH_3 -remained, so a structure of $(CH_3)_2NCH_2C(CH_3)_2OH$ can be proposed for the candidate compound.

The PMR spectrum (in CD_3OD) of the compound showed no coupling peaks, i.e. 1.33 (6H, s), 2.97 (6H, s), 3.17 (2H, s), that agreed with the above structure as well as each chemical shift of $(CH_3)_2\overset{|}{C}O-$, $(CH_3)_2N-$, and $-NCH_2-$ respectively. There remained, however, another possible structure: $(CH_3)_2NC(CH_3)_2CH_2OH$. For the final identification we synthesized the former compound, 1-dimethylamino-2-methyl-2-propanol (1-DMAM-2-P), and compared the analytical data with those of the latter, commercial 2-dimethylamino-2-methyl-1-propanol (2-DMAM-1-P), and the natural product. As was expected, the synthesized 1-DMAM-2-P was identical to the natural product with respect to GC retention time, mass (Table 2) and PMR spectrum.

Activities of the attractant amines

The authentic material of each component of the distillate was subjected to behavioural assay. Table 3 shows that each chemical by itself exerted both chemotaxis and anemotaxis almost at the same dosage. The most active material was 1-DMAM-2-P showing ca. 50–1,000 times higher activity than the others. The ED_{50} value of other amines were in a close range, between those of ammonia and trimethylamine.

The attractiveness of each chemical was evaluated in terms of the increasing sigmoid of the response curve. Overall, both chemotaxis or anemotaxis responses showed an optimal dose range and decreased at higher doses (Fig. 6). It could be generalized that anemotaxis has a narrower optimal dose range than chemotaxis, though both response curves overlapped at the sigmoidal section. The width of the optimal dose range for chemotaxis varied with the chemical. Dimethylamine is representative for most of the alkylamines examined. Trimethylamine has an exceptionally wide optimal range and 1-DMAM-2-P shows a wide range of neutral response at higher doses.

DISCUSSION

The attractant components of the *B. germanica* aggregation pheromone were found to be alkylamines. This contrasts with the general view that frass-originated cockroach aggregation pheromones include fatty acids and related compounds (RITTER and PERSOONS, 1975; BROSSUT, 1975; McFARLANE, 1984). This difference may be the result of behavioural assay methods mentioned before, but olfactory behaviours could be exerted by contaminant amines in the acids. The attractant amines were also different from the aggregation pheromone of *Blaberus craniifer* (Burmeister) (Dictyoptera: Blaberidae) comprising volatile alkanes (BROSSUT et al., 1974), with an adult-biased activity (BROSSUT, 1975) indicating sex pheromonal character.

The olfactometer assays on the amines revealed that all of them exerted both chemotaxis and anemotaxis at the same dosage. This confirmed the suggestion that both olfactory behaviours are exerted simultaneously (rather than independently) as a component of an integrated mechanism (SAKUMA and FUKAMI, 1990). The prominent potency of 1-DMAM-2-P suggests that it is a special component and has a different origin from other general alkylamines. Despite its low content in the natural mixture, it was as significant as trimethylamine or dimethylamine.

The activities based on ED₅₀ values of free amines, however, do not reflect directly the contribution of each chemical in the natural mixture. Each amine has its own overall dose/response pattern, so the natural mixture represents an integrated result. Moreover the amines were spotted on the substrate by the animal not as free materials but as salts; thus the rate of evaporation and the dose/response relation should be examined again with the amine salts on a dry surface. These assays should be conducted on the salts of coupling acid(s) in the natural mixture. An ion chromatographic analysis revealed that hydrochloric acid was the major constituent. Details and the activity of amine salts will be described in future works.

The effect of salt formation was reflected on the dose/response pattern. All the examined amines represented a decrease in chemotaxis at higher doses, contrasting with a previous result on crude material in which chemotaxis retained maximal response and never declined (SAKUMA and FUKAMI, 1990). The patternal difference derived from the difference in the sample status. The crude material was given as the original salt condition on a disc dispenser, whereas the authentic samples were continuously applied as free amines. At higher doses salts may keep the amine odour concentration below the threshold to initiate the response decrease, but free amine solution provided much higher concentration, enough to exert even a negative chemotaxis response (or preference to low odour concentration.) In contrast, negative anemotaxis (or downwind preference) was never observed. Excessive doses appeared to terminate the modulation on positive anemotaxis.

Although olfactory behaviours of animals in the field should be simulated without the movement constraints of the olfactometer, this study showed that these amines clearly functioned as 'attractants' and worked at the first step of the aggregation behaviour. ISHII and KUWAHARA (1967) pointed out that 'the aggregation of the german cockroaches was clearly initiated by olfactory response to this pheromone serving as an attractant'. However, salts of these amines, as well as the crude methyl acetate extract, induced no aggregation activity by their vertical paper assay. This implies the presence of another chemical cue functioning as an 'arrestant(s)'. McFARLANE and ALLI (1986) suggested

that *l*-lactic acid found in the frass-contaminated paper could be this cue. It may be difficult to attribute the entire arrestant process to any single compound, since a multiple component system has been indicated with the same vertical paper assay (NAKAJIMA et al., 1974; RITTER and PERSOONS, 1975). The arrestants should also be systematically isolated from the natural mixture.

Among the attractant amines, 1-DMAM-2-P appeared to be a special component. The origin of this chemical will be the next issue: what organ or organism produces it? The rest of the amines however, can be categorized as typical waste materials. An exception among terrestrial insects, cockroaches excrete ammonia instead of uric acid (MULLINS and COCHRAN, 1976). COCHRAN (1985), in a discussion on the voiding of ammonia and other volatile nitrogenous materials, asked 'Does this process represent an alternative method of voiding excess nitrogen in the absence of uric acid excretion? Does it have other significance?'. Our results give an answer on the function of these volatile nitrogenous excreta with respect to the behavioural control of the animal themselves. Their recruitment for aggregation can be deduced from their physiological status.

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