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Isolation and Characterization of Alarm Pheromone From Electric Shock-Induced Earthworm Secretion

XIAN CHENG JIANG,* DALTON WANG* AND MIMI HALPERN†

*Departments of *Biochemistry and †Anatomy and Cell Biology*
SUNY Health Science Center at Brooklyn, 450 Clarkson Ave., Brooklyn, NY 11203

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JIANG, X. C., D. WANG AND M. HALPERN. *Isolation and characterization of alarm pheromone from electric shock-induced earthworm secretion.* PHARMACOL BIOCHEM BEHAV **34**(2) 213–221, 1989.—Electric stimulation of earthworms, *Lumbricus terrestris*, causes secretion of a yellow mucus which has alarm properties for conspecifics and chemoattractive properties for garter snakes. An alarm pheromone was isolated and purified to homogeneity from the mucus by means of permeation, thin-layer and high performance liquid chromatographies. The purified substance was highly active as an alarm chemosignal to earthworms (*L. terrestris*), but it did not elicit alarm responses from either sandworms (*Nereis virens*) or bloodworms (*Glycera debranciata*). It was not a snake chemoattractant. The alarm pheromone could not be retained with 1 kDa cut-off dialysis tubing, and it was eluted from a Bio-gel P2 column ahead of p-nitrophenol. These data suggest an apparent mass greater than 139 Da but less than 1 kDa. The order of solubility of this alarm pheromone is H_2O > DMSO > MeOH > 2-propanol > acetone. It was thermostable, and it fully retained activity after heating at 100°C for 1 hour. This alarm pheromone fluoresced under u.v. light, and it showed an optimal excitation wavelength of 420 nm and emission wavelength of 465 nm.

Lumbricus Alarm pheromone

EARTHWORMS secrete a variety of fluids. Several functions of such fluids have been suggested: they may act as lubricants when the animal moves through its burrow, as soil particle binders to prevent the burrow walls from collapsing (14), as buffer systems outside the body (10), or as sources for alarm signals to other earthworms (4, 8, 12, 14). In terms of chemical communication, it is the latter function that is most interesting.

During the last two decades, alarm pheromones have been reported in different species of earthworms (4, 7, 12, 14, 15). The electric shock-induced secretion of the earthworm, *Lumbricus terrestris*, appears to function as an alarm pheromone for other earthworms (14), i.e., an earthworm contacting such secretions escapes more rapidly from the area than from a clean area. Ressler *et al.* (14) suggested that this aversive property might have confounded earlier studies of earthworm learning that employed electric shock, an observation later confirmed and extended by Ratner and Boice (12). Rosenkoetter and Boice (15) reported that the pharyngeal fluids of *L. terrestris* and *Diplocardia riparia* and the coelomic secretions of *D. riparia* induced by electric shocks are repellents. However, the coelomic fluids of *L. terrestris* are attractants. Krishnamoorthy and Keshavamurthy (8) reported that shock-induced coelomic secretion of *Megascolex mauritii* are also repellents, but neither the pharyngeal fluid nor the mucus secretions of *M. mauritii* were aversive to conspecifics. On the other hand, dead earthworms do not produce electric shock-induced secretion (4).

Preliminary chemical analyses of several of these alarm substances have been reported (8,14). Ressler *et al.* (14), found that the alarm substance of *L. terrestris* was not readily soluble in cold water but was effective and stable when dried and when deposited on aluminum, stainless steel, paper, soil, glass as well as Plexiglas. Peanut oil extract of deproteinized coelomic fluid obtained by electrical stimulation *M. mauritii* elicited an aversive response from conspecifics (8). The alarm substance was more stable when extracted in oil than in methanol (8). The substance was thermolabile, fat soluble, and sensitive to ultraviolet or visible light. Dilution and ageing decreased its effectiveness. The difference in characteristics between the alarm substances obtained from *L. terrestris* and *M. mauritii* may be due to species differences, however, in neither case, has the pheromone been purified. We have now succeeded in isolating a potent alarm pheromone from electric shock-induced secretion of *L. terrestris*, and in this communication, we describe its purification and partial characterization.

METHOD

Materials

The following chemicals were commercial products: Ultragel AcA 44 from LKB Co.; Bio-gel P2 from Bio-Rad; Silica-gel G 60 (20 × 20 cm plastic, Cat. 5506 and 20 × 20 cm glass, Cat. 7400) and High Performance Silica-gel G 60 (10 × 10 cm glass, Cat.

5631) from Alltech Associates; HPLC apparatus (consisting of a 2150 HPLC pump, 2138 Uvicords, 2112 Redirac fraction collector, and 2210 recorder) from LKB Co.; RP-18 column from Pierce; Fluorescence Spectrophotometer (model 204-A) from Perkin-Elmer. All other reagents were the highest grade commercially available. Earthworms were purchased from Connecticut Valley Biological Supply, South Hampton, MA and sandworms (*Nereis virens*) and bloodworms (*Glycera debranceata*) from local bait and tackle stores.

Procedure

Preparation of electric shock-induced earthworm secretion. Electric shock-induced earthworm secretion was prepared according to procedures described previously (4,7), with modifications as follows. One hundred earthworms were freed from soil by a quick rinse with tap water. Excess water was removed before they were placed into the cone of an electric stimulator, a design similar to that of Krishnamoorthy and Keshavamurthy (7), but modified by Halpern *et al.* (4). An electric current from a 9-V battery was applied as 20 6-second bursts with 30-second intershock intervals. During this time, the earthworms secreted a viscous, cloudy, yellowish mucus-like fluid that drained and collected into a beaker placed below the cone. This procedure normally yielded 15–20 ml of secretion.

Preparation of aqueous earthworm wash. The procedures for the preparation of aqueous wash of earthworms were described previously (13) and modified by Wang *et al.* (18). Briefly, 45 g of earthworms were suspended in 150 ml of distilled water at 60°C for 1 min, the wash was removed by decantation and centrifuged at about 550 × g for 20 min at ambient temperature. The supernatant solutions were pooled and lyophilized. The lyophilized material was redissolved in about 8 ml of distilled water; it was then centrifuged to remove insoluble materials.

Preparation of plates containing secretions from nonstressed earthworm. Eight earthworms were gently washed and placed on clean glass plates identical to those used in earthworm bioassay (see the Earthworm Bioassay section below). The plates were placed in a cold room (4°C) for three hours. The worms were removed from the plates and the plates dried at 37°C.

Permeation chromatography. 1) Ultragel AcA 44 column chromatography: A 3-ml (about 3 mg protein) sample was loaded onto an AcA 44 column (1.45 × 100 cm), preequilibrated with distilled water and eluted with the same solvent at a flow rate of 20.76 ml/hr maintained with an ISCO metering pump (model 312) which was coupled to a Type 6 optical unit, a fraction collector (model 328), and a recorder (model UA-5) with an event marker. The eluate was monitored continuously at 280 nm, and fluorescence intensity of each fraction (5.2 ml) was determined manually with a fluorescence spectrophotometer using 420 and 465 nm for excitation and emission wavelength, respectively. The content of each peak was lyophilized and redissolved in 3 ml distilled water. Earthworm alarm bioassays and snake attractant bioassays were carried out on the material of each peak. 2) Bio-gel P2 column chromatography: A sample (about 3 ml) was loaded onto a Bio-gel P2 column (1.45 × 120 cm), preequilibrated with distilled water, and eluted with the same solvent at a flow rate of 19.6 ml/hr. Each collected fraction (5.0 ml) was monitored in a manner similar to that described above. The content of each peak was lyophilized and redissolved in 3 ml distilled water. Earthworm alarm bioassays were conducted on the material in each peak.

Thin-layer chromatography. 1) Analytical chromatography: Silica gel G 60 plates or high performance silica gel plates were prewashed ascendingly with a chosen organic solvent system determined empirically. The washed plates were dried, the sample was spotted onto the plate and developed with the selected solvent system. The various solvent systems used are listed in the Results section. 2) Semipreparative TLC: Pretreatment of the plates and separation of samples were carried out in a manner similar to those described above. In this case, the sample was applied to the plate as a streak (about 150 μl) instead of spots. The developed chromatogram was viewed under a u.v. lamp to detect both u.v. absorbing and fluorescent materials. Guide strips were sprayed with reagents for amino acids [ninhydrin (1)] and carbohydrates [aniline-diphenylamine (3)]. They were used to locate and recover, by elution, the corresponding compounds from the unsprayed plates. Earthworm alarm bioassays were conducted on material obtained from each recovery.

High performance liquid chromatography. The purity of the isolated alarm pheromone obtained from TLC was checked on a RP-18 column (Pierce) coupled to a LKB HPLC apparatus. The sample was eluted with a stepwise water:acetonitrile gradient. The eluant was continuously monitored at 280 nm. Fractions of 20 drops were collected. The content of each fraction was monitored manually for fluorescence with a Perkin-Elmer fluorescence spectrophotometer (model 204-A).

Protein determination. The protein concentration of samples was determined according to the method of Lowry *et al.* (11), using bovine serum albumin as the standard.

Solubility determination. Purified sample was lyophilized and redissolved in 2.5 ml distilled water. Following measurement of fluorescence intensity, it was divided into 5 equal portions and lyophilized. The lyophilized materials were each solubilized in 0.5 ml of a different selected solvent. The solutions were centrifuged and each supernatant was transferred into a clean tube and lyophilized. The lyophilized material in each tube was redissolved in 0.5 ml of distilled water, and its fluorescence intensity was then determined.

Snake bioassay. The snake, *Thamnophis sirtalis*, bioassay was performed according to the procedures described by Wang *et al.* (18). Briefly, the bioassay consists of placing 50 to 100 μl test samples or control samples on artificial worm bits (Creme Lure™) and observing whether the snake attacks the worm bit. Snakes almost never (<5%) attack the bits covered with control substances and almost always (>90%) attack bits covered with prey washes (18).

Earthworm bioassay. Earthworms tested in the bioassay were never subjected to shock and each worm was used in the bioassay only once.

The bioassay system consisted of a glass lantern slide (8 × 10 cm) on which 300 μl of a test or control solution were applied as uniformly as possible, and then dried on a hot plate at 37°C. Glass slides with dried distilled water or appropriate blank eluate were used as controls and randomly interspersed between test samples. The assay was performed in a cold room (4°C) under illumination provided by a 25-watt red light bulb placed one meter from the test plate. The earthworm was placed on the center of the slide in a detachable glass ring. Following removal of the glass ring, the behavior of the earthworm was observed and recorded. Earthworms in contact with shock-induced secretion consistently displayed the following behaviors: violent twisting, rolling, contraction, and secretion of a mucoid substance. When such responses were exhibited the test substance was considered to have yielded a positive response. Contraction alone, however, was not considered a positive response. Although we measured the latency to escape from the plate, timed from the moment the glass ring was removed, we did not consider that measure a valid measure of alarm, since earthworms reacting to a test material with violent twisting and rolling often remained on the slide for an interval equal to earthworms on control slides. At each step of alarm pheromone purification, the earthworm bioassay was performed at least three times.

TABLE 1

ALARM RESPONSES OF CONSPECIFICS TO MATERIALS DERIVED FROM EARTHWORM SECRETIONS

Material	Alarm Responses (positive/no. earthworms)
Earthworm electric shock-induced secretion	12/12*
Control (H_2O)	0/12
From Ultragel AcA 44	
Peak 1	2/7
Peak 2	0/7
Peak 3	7/7*
Control‡ (H_2O)	0/7
From Bio-gel P2	
Peak 1	2/12
Peak 2	0/12
Peak 3	6/12†
Peak 4	12/12*
Control‡ (H_2O)	0/12
Aqueous earthworm wash	0/7
Control (H_2O)	0/7
Nonstressed earthworm secretion	0/8
Control (H_2O)	0/8

*Chi-square test: $p<0.001$.

†Chi-square test: $p<0.05$.

‡Controls were carried out separately with each test peak material. The numbers given in controls represent averages of the total number of determinations for each set of peak materials.

RESULTS

Earthworm Response to Electric Shock-Induced Secretion

Earthworm secretions produced by electrical stimulation consistently evoked alarm response from conspecifics (Table 1). This observation is consistent with previous reports (4, 8, 12, 14). Aqueous earthworm wash and nonstressed earthworm secretion showed no earthworm alarm activity (Table 1).

Analysis of Earthworm Electric Shock-Induced Secretion on Ultragel AcA 44 Column

When the secretion was chromatographed on an AcA 44 column with distilled water as eluting solvent, it resolved into three u.v. absorbing and one fluorescent peaks as shown in Fig. 1. The alarm activity was found to reside in peak 3 (Table 1); this peak corresponded to the fluorescent peak. It should be noted that when 0.15 M NaCl was used as the eluting solvent, the elution pattern was similar, but the materials recovered from each peak contained sufficient salt to make the bioassay impossible. This finding is in agreement with an earlier report made by Laverack (9) that 0.1 M and higher concentrations of NaCl were aversive to the worms. On the other hand, removal of salt by dialysis using 1 kDa cut-off dialysis tubing, from samples containing alarm properties, resulted in a loss of all alarm activity. This lost activity could readily be recovered from the dialysate (Table 2). These results suggest that the bioactive compound(s) is(are) relatively small in size and its (their) molecular mass(es) is(are) probably less than 1 kDa.

It is noteworthy that during drying on the slides the salt in the sample tended to accumulate and aggregate in a few small spots, the resulting solid salt appeared to irritate the earthworms. When

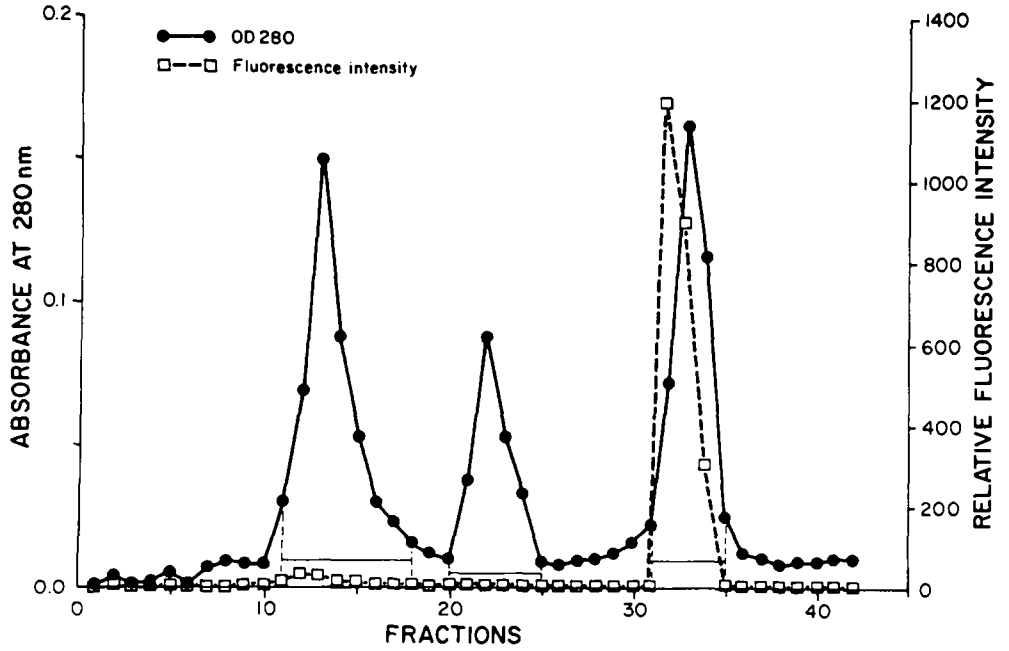

FIG. 1. Elution profile of earthworm secretion from Ultragel AcA 44 column. The AcA 44 column (1.45 × 100 cm) was preequilibrated with distilled H_2O; 0.5 ml sample (2.5 mg protein) was loaded on the column and eluted with H_2O at 21 ml/hr, fractions (5.2 ml/fraction) were collected. The fractions under each peak were pooled as indicated by the bar.

TABLE 2

ALARM RESPONSES OF CONSPECIFICS TO DIALYZED MATERIALS DERIVED FROM ELECTRIC SHOCK-INDUCED EARTHWORM SECRETION

Materials	Alarm Responses (positive/no. earthworms)
Earthworm secretion	
Undialyzed	10/10*
Dialyzed	0/10
Control (H_2O)†	0/10
Fluorescent material recovered from AcA 44	
Dialyzed	0/10
Dialysate‡	10/10*
Control (H_2O)†	0/10

*Chi-square test: $p<0.001$.

†Controls were carried out separately with each test material. The numbers given in controls represent averages of the total number of determinations for each test material.

‡A 10-ml sample recovered from AcA 44 (peak 3) was dialyzed against 100 ml of distilled water for 14 hours. Ninety-five percent of fluorescent intensity was found in dialysates. The dialysate was concentrated by lyophilization and redissolved in 10 ml distilled water. The fluorescent intensity of dialysate was three active units (3700/300 μl) for bioassay.

the same amount of salt was mixed with a small amount of bovine serum albumin and then dried on a test plate, it dried uniformly as a film, and no irritation was observed.

Analysis of Bioactive Material From AcA 44 on Bio-gel P2 Column

When the material derived from the active peak from the AcA 44 column was chromatographed on a Bio-gel P2 column, it resolved into 4 major u.v. absorbing peaks and one fluorescent peak (Fig. 2). The bioactivity of the material recovered from each peak (Table 1) indicated that the alarm compound(s) was(were) located mainly in peak 4, which corresponded to the fluorescent peak. Although some activity was also detected in peak 3, we decided to concentrate our effort on the material in peak 4. This fluorescent peak was eluted ahead of the p-nitrophenol, suggesting that the molecular weight of the alarm pheromone was greater than 139 Da.

Purification of the Bioactive Material by Thin-Layer Chromatography

In addition to alarm pheromone, the material derived from the fluorescent peak of the Bio-gel P2 column undoubtedly contained a large number of low molecular weight contaminants, such as amino acids, sugars, etc. The alarm pheromone was further purified by means of semipreparative thin layer chromatography. Guide strips were used to reveal ninhydrin positive compounds or aniline-diphenylamine positive compounds (data not shown). The gel containing the corresponding bands on the unsprayed portion of the plate were scraped off, collected, eluted, and assayed for earthworm alarm activity. None of the ninhydrin or aniline-diphenylamine positive compounds showed any earthworm alarm activity.

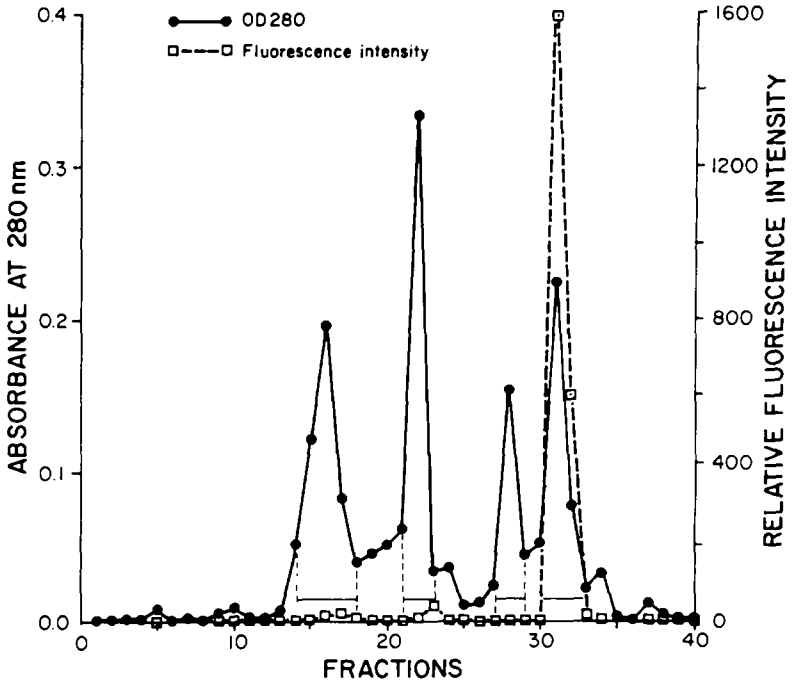

FIG. 2. Elution profile from Bio-gel P2 column of active fraction derived from Ultragel AcA 44 column. The Bio-gel column (1.45 × 120 cm) was preequilibrated with distilled H_2O; 1 ml sample (derived from the third peak of Fig. 1 and concentrated by lyophilization) was loaded on the column and eluted with H_2O at 18.6 ml/hr, fractions (5 ml/fraction) were collected. The fractions under each peak were pooled as indicated by the bar.

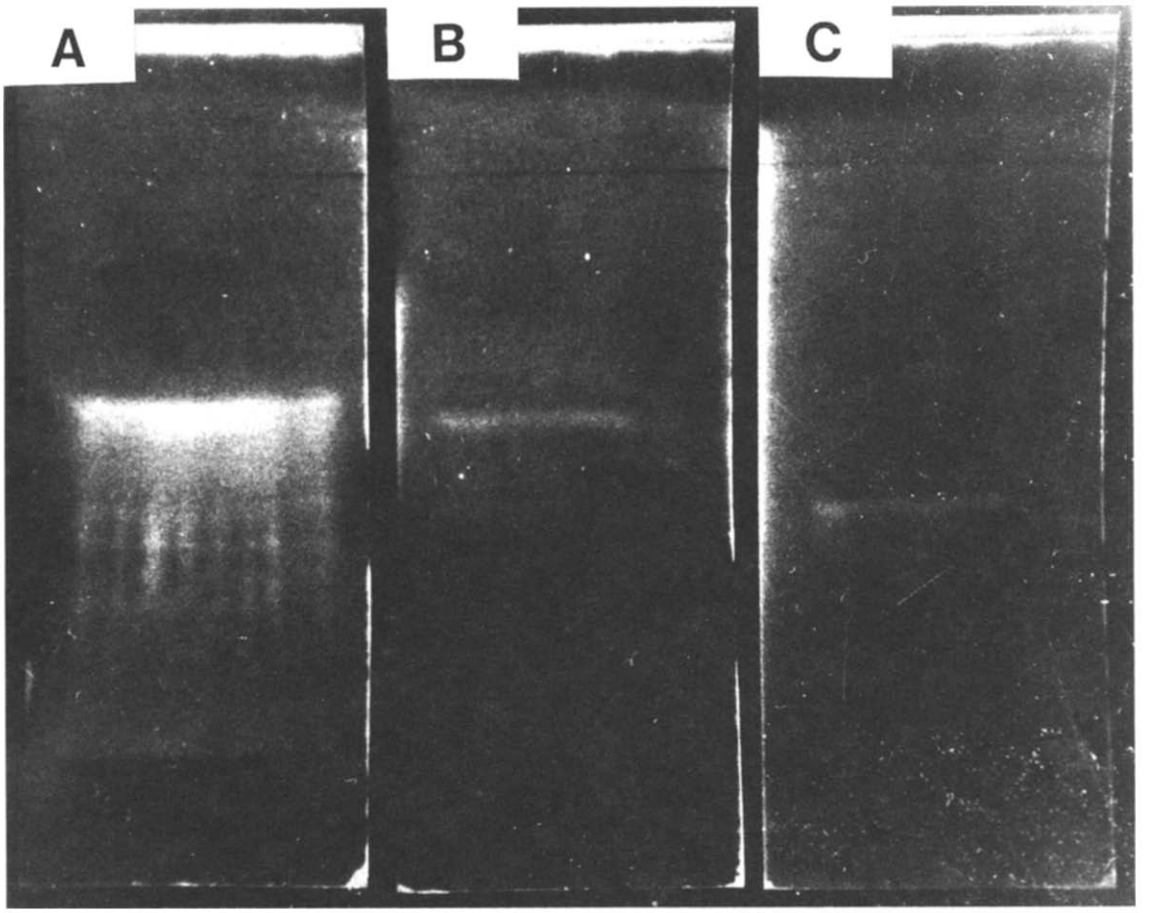

FIG. 3. Purification of alarm pheromone on silica gel G 60 thin layer. (A) Sample (300 μl, derived from peak 4 of Fig. 2) developed with n-butanol:ethanol:0.2 M HAc (2:1:1, v/v). (B) Sample (200 μl, derived from the alarm active fluorescent band in A) developed with n-butanol:ethanol:H_2O (2:1:1, v/v). (C) Sample (200 μl, derived from the alarm active fluorescent band in B) developed with n-butanol:ethanol:0.2 M NH_4OH (2:1:1, v/v).

When the unsprayed plate was viewed under u.v. illumination, several u.v. absorbing and fluorescent bands were detected. Among these a major fluorescent band showed earthworm alarm activity and gave a negative response to snake bioassay (data not included).

A number of solvent systems were explored. These included acetone:2-propanol:0.2 M lactic acid (2:1:1, v/v); acetone:2-propanol:0.2 M acetic acid (1:1:1, v/v); acetone:n-butanol:H_2O (8:1:1, v/v); acetone:2-propanol:0.2 M citric acid (1:1:1, v/v); ethylacetate:methanol:2-propanol:n-butanol:H_2O (8:1:1:1:1, v/v); n-butanol:ethanol:0.2 M acetic acid (2:1:1, v/v); n-butanol:ethanol:H_2O (2:1:1, v/v); and n-butanol:ethanol:0.2 M NH_4OH (2:1:1, v/v). We adopted a three-solvent systems (an acid, a neutral and a basic) in a sequential manner for purification of the alarm pheromone. That is, the fluorescent material recovered from the first system was rechromatographed with the second solvent system, and so on. The three solvent systems were: (a) n-butanol:ethanol:0.2 M acetic acid (2:1:1, v/v), (b) n-butanol:ethanol:H_2O (2:1:1, v/v), and (c) n-butanol:ethanol:0.2 M NH_4OH (2:1:1, v/v). Both distilled water and gel eluates from the corresponding area of a blank thin layer plate served as controls. The results of each purification and bioassay are shown in Fig. 3 and Table 3, respectively. At each step the test sample was aversive to earthworms and the controls yielded no responses.

TABLE 3

ALARM RESPONSES OF CONSPECIFICS TO PHEROMONE ISOLATED BY THIN LAYER CHROMATOGRAPHY

Alarm Pheromone (recovered from)	Alarm Responses (positive/no. earthworms)
Acidic system*	7/7†
Control (blank gel eluate)	0/7
Neutral system*	5/7‡
Control (blank gel eluate)	0/7
Basic system*	6/7§
Control (blank gel eluate)	0/7

*Two active units (2480/300 μl) per assay was used. The alarm pheromone was purified by acidic, neutral and basic system in a sequential manner.

†Chi-square test: $p<0.001$.

‡Chi-square test: $p<0.05$.

§Chi-square test: $p<0.02$.

TABLE 4

YIELD OF ALARM PHEROMONE AT EACH STEP OF PURIFICATION

Purification Step	Fluorescence Intensity	Active Units	Yield (%)
Bio-gel P2			
last peak*	22050	17.78	100
First TLC			
Acidic system†	11100	8.95	50.3
Second TLC			
Neutral system†	9200	7.72	41.7
Third TLC			
Basic system†	6000	4.84	27.2

*The material was derived from 3 ml of secretion placed on the column. The recovered material was concentrated to a final volume of 3 ml. An aliquot of 0.5 ml was used for fluorescence intensity determination.

†The final volume of the recovered material from each system was 3 ml.

We have attempted to quantitate the recovery of this alarm pheromone at each step of purification. Because it is likely that fluorescence was quenched in the crude secretion, it was not feasible to estimate accurately the exact amount of the alarm pheromone present in a given preparation of secretion. We, therefore, used the relative fluorescence in material from the Bio-gel P2 column to represent the total amount of alarm pheromone present in the crude secretion. The recovery of alarm pheromone at each step of purification is given in Table 4. One unit activity is defined as the minimum of fluorescence intensity, obtained by a series of dilutions, still yielding a positive response from earthworms. We found that 300 μl of a test solution having a relative fluorescence intensity of 1240 constituted one active unit.

Purity Determination of the Isolated Alarm Pheromone

After three steps of TLC purification, the homogeneity of the isolated alarm pheromone was checked by means of high performance thin layer chromatography with a developing solvent system of n-butanol:ethanol:0.2 M NH_4OH (2:1:1, v/v). A single fluorescent band (Fig. 4) was observed with no detectable contaminants, judged by the absence of u.v. absorbance and by negative ninhydrin and aniline-diphenylamine reactions.

High performance liquid chromatography was also employed to check the purity of the isolated product using a stepwise acetonitrile gradient in water as the developing solvent. The eluate was continuously monitored at 280 nm for u.v. absorbing compounds, while the fluorescent material was measured manually on the collected fractions. A typical composite of elution patterns is shown in Fig. 5. Only a single symmetrical fluorescent peak was evident suggesting that the purified fluorescent compound was homogeneous. No u.v. absorbing material that overlapped with the fluorescent peak could be detected. The alarm pheromone so obtained was presumed rather pure.

Response of Sandworms and Bloodworms to Earthworm Alarm Pheromone

Earthworm alarm secretion did not produce an alarm response in either sandworms (*N. virens*) or bloodworms (*G. debranciata*) (Table 5), suggesting that the alarm properties are species specific.

Chemical and Physical Properties of the Purified Alarm Pheromone

We have determined the following properties of this purified alarm pheromone: 1) it fluoresces and shows an optimal excitation at 420 nm and emission at 465 nm (Fig. 6), 2) the order of the solubility is H_2O > DMSO > MeOH > 2-propanol > acetone (Fig. 7), 3) it gives negative reactions with ninhydrin and aniline-diphenylamine (data not shown), and 4) it retains its full alarming activity after heated in boiling water for 1 hour (Table 6).

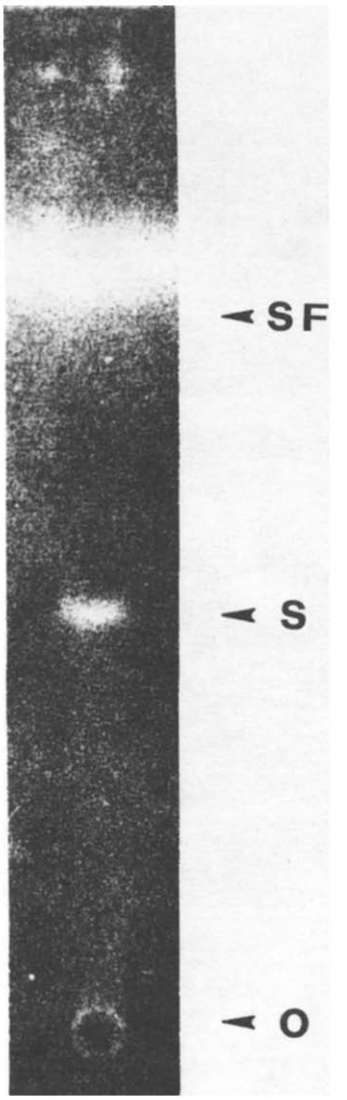

FIG. 4. High performance thin layer chromatography of purified alarm pheromone. The sample, a final product from TLC (Fig. 2C), was analyzed on HPTLC silica gel G, with developing solvent of n-butanol: ethanol:0.2 M NH_2OH (2:1:1, v/v). O, origin; S, sample; SF, solvent front. No U.V. absorbing, ninhydrin or aniline-diphenylamine positive material could be detected.

DISCUSSION

It has been known that invertebrates as well as vertebrates, under stressful environmental conditions, release chemicals serving as chemosignals. These chemicals are known generically as alarm chemosignals or alarm pheromones which may cause conspecifics to explore and investigate (17), to hesitate and induce physical signs of frustration (5), to become immobilized or hide

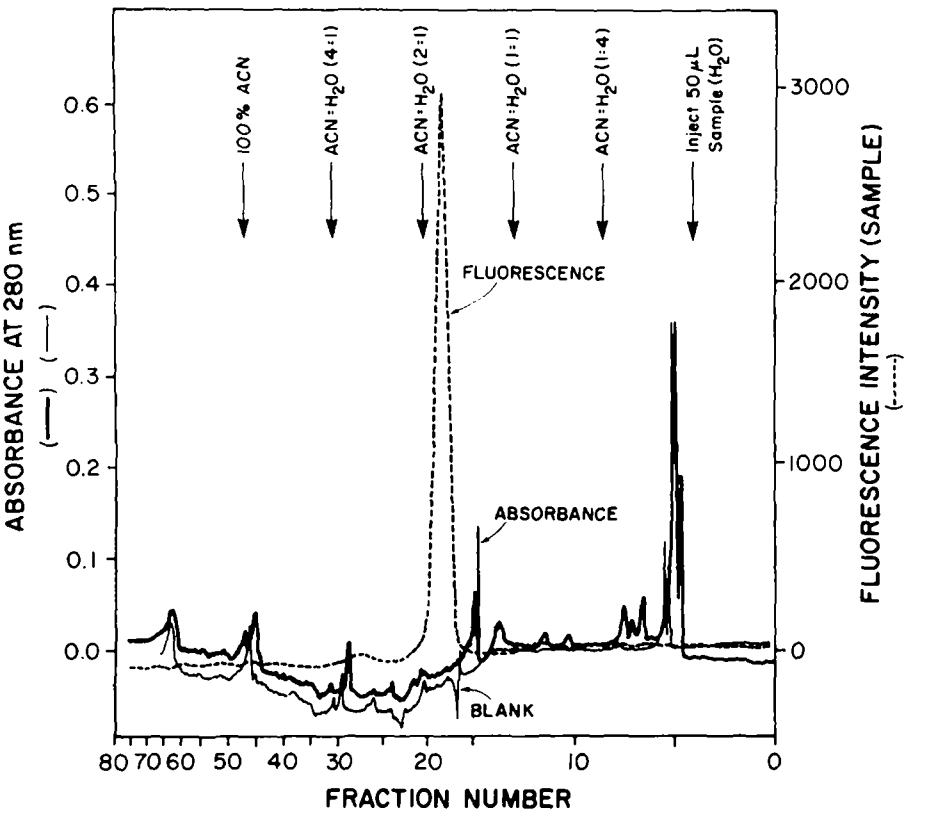

FIG. 5. High performance liquid chromatography of purified alarm pheromone. A TLC purified sample (50 µl) was analyzed on a RP-18 column using stepwise gradients of acetonitrile and water. Changes of solvents were indicated by arrows and the duration of elution with each solvent was 10 min. It was continuously monitored at 280 nm for u.v. absorbing material, while fluorescence was determined manually on the fractions collected.

(16), to flight and disperse (14), or to mobilize socially and attack (2). The environmental factors which provoke the release of such chemicals are diverse, but usually involve stimuli of potential danger, such as novelty, social intrusion, or physical insult. In the case of vertebrates few compounds have been isolated and identified. Recently, a fish alarm pheromone has been demonstrated and shown to be a pterin-like compound (6).

In 1986, Halpern *et al.* (4) suggested that the alarm pheromone in the secretion from earthworm, *L. terrestris*, might also function as a chemoattractant for garter snakes. We now provide evidence for the existence of two distinct chemical entities: a nonproteinaceous compound which possesses strong conspecific alarming properties and a protein which exhibits chemoattractive properties to garter snakes (this will be discussed elsewhere). The alarm

TABLE 5

ALARM RESPONSES OF EARTHWORM, SANDWORM AND BLOODWORM TO ELECTRIC SHOCK-INDUCED *L. TERRESTIS* ALARM PHEROMONE DERIVED FROM BIO-GEL P2 COLUMN

Tested Worms	Alarm Responses (positive/no. worms)
Earthworms	10/10*
Sandworms	0/10
Bloodworm	0/10
Earthworms (controls)†	0/10

*Chi-square test: $p<0.001$ for earthworms. Two active units (2480/300 µl) per assay were used.

†Distilled water was used instead of electric shock-induced alarm pheromone derived from Bio-gel P2 column.

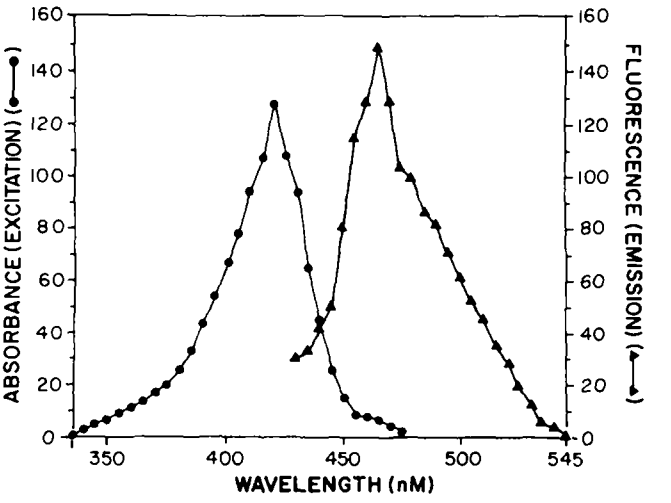

FIG. 6. The excitation and emission spectra of purified alarm pheromone.

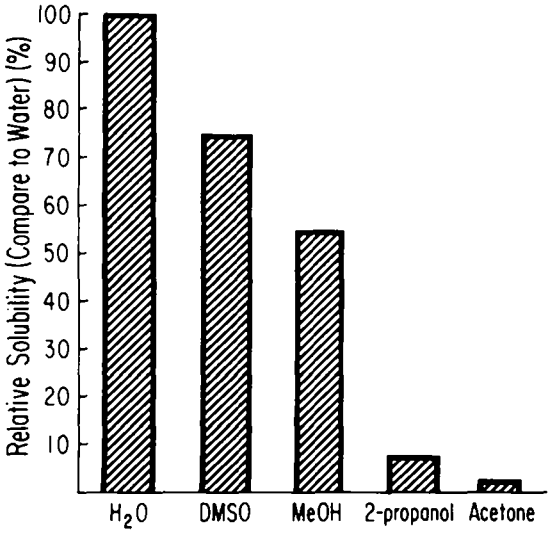

FIG. 7. The relative solubility of the alarm pheromone.

pheromone reported here is evidently very different from the one isolated from the earthworm, *M. mauritii* (8). The bioactive substance reported by Krishnamoorthy and Keshavamurthy (8) was fat extractable, sensitive to ultraviolet or visible light, thermolabile, and insoluble in water. In contrast, the alarm pheromone from *L. terrestris* was water soluble, rather stable to either ultraviolet or visible light (i.e., exposure to u.v. light could not destroy its activity), and thermostable. Furthermore, it retained its bioactivity for several months. Such differences in properties of these alarm pheromones produced by *M. mauritii* and *L. terrestris* suggests species specificity. The specific nature of our isolated alarm pheromone was shown by the negative responses obtained from both sandworm and bloodworm. The solubility of the alarm pheromone in water and the stability on aging concur with the findings reported by Ressler *et al.* (14), although exact comparison can not be easily made, because the alarm pheromone isolated by Ressler *et al.* was not in a pure state.

The nonstressed earthworms secretion did not give positive bioassay suggesting the alarm pheromone is secreted from the body during or after electric shock. The source of the alarm pheromone as well as its route of secretion remain unknown.

TABLE 6

RESPONSES OF CONSPECIFICS TO ALARM PHEROMONE HEATED IN BOILING WATER BATH FOR 1 HOUR

Materials	Heat Treatment	Alarm Responses (postive/no. earthworms)
Alarm pheromone	No	7/7*
Alarm pheromone	Yes	7/7*
dH_2O		0/7

*Chi-square test: $p<0.001$. Two active units (2480/300 μl) per assay were used.

However, Rosenkoetter and Boice have reported that *L. terrestris* and *D. riparia* when shocked with an electric current secrete pharyngeal fluids which act as repellents to respective conspecifics. Therefore, it is possible that the alarm pheromone separated here may be secreted from pharynx under stress from electric shock. The absence of alarm pheromone in the earthworm wash is probably due to the rapid death of the worms preventing them from secreting the pheromone. On the other hand, the concentration of the pheromone in the earthworm wash, if any, may be too low to be detected by the worms.

Since the alarm pheromone gave negative results when reacted with ninhydrin or aniline-diphenylamine, we suggest that it is not an amino acid, amine or sugar. The purified product must contain strong polar groups, as it was soluble in distilled water and dimethylsulfoxide. It fluoresces under u.v. illumination and shows optimal excitation and emission wavelengths of 420 and 465 nm, respectively. The molecular structural identity of this pheromone remains to be elucidated. Based on the available evidence, we would predict that this alarm pheromone is likely aromatic in nature with strong polar groups. Despite the lack of its structural identity at the moment, the availability of such a purified alarm pheromone will allow the investigation of a host of biochemical, cellular as well as molecular biological problems such as the interaction with its receptor, message transduction and transmission.

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