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Characterization of Vomeronasally-Mediated Response-Eliciting Components of Earthworm Wash-II[1]

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HALPERN, M., N. SCHULMAN, L. SCRIBANI AND D. M. KIRSCHENBAUM. *Characterization of vomeronasally-mediated response-eliciting components of earthworm wash-II.* PHARMACOL BIOCHEM BEHAV **21**(4) 655–662, 1984.—Garter snakes reliably spend more time and tongue flick more frequently at a dish containing surface washings of earthworm (EW) than at a control dish containing water or sodium chloride. In a series of experiments EW was examined to further characterize and purify the snake-attracting component(s). Earthworm wash boiled for up to two hours did not lose its snake-attracting activity, but EW boiled for four hours was not discriminated from a control substance. The active components of EW were completely precipitated by 90 and 100% saturated ammonium sulfate solutions. The molecular weight of the active components, as determined by dialysis, is larger than 67,000 daltons. Earthworm wash was separated into two peaks on either G-75 or AcA 44. The larger peak (F_2) contains snake-attracting material of molecular weight greater than 67K. The smaller peak material (F_4) is inactive as a snake-attractant and contains material of approximate molecular weight of 3K. Biological activity of EW covaried with Lowry (protein) assay and Dubois (carbohydrate) assay values and 280 nm absorbance. The response eliciting components of EW are not extractable from earthworms at temperatures below 60°C, but can be extracted at 60°C (for one minute) from the worms or the soil that forms the bedding for the earthworms.

Characterization Isolation Earthworm (*Lumbricus terrestris*) wash Snake (*Thamnophis sirtalis*) Vomeronasal system Snake-attractant Chemoattractant

NUMEROUS investigators have previously demonstrated that garter snakes (*Thamnophis sirtalis*) respond differentially to surface washings of earthworms (EW) with increased tongue flicking, approach and attack [5, 6, 11–14, 24]. These discriminative responses appear to be dependent upon a functional vomeronasal system but not on a functional olfactory system [6, 10, 15, 18, 24].

Balzarek [2] reported that a water wash of 1000 earthworms contained material which was undialyzable and reacted as protein. Sheffield and his colleagues [22] demonstrated that the attack-eliciting substances in earthworm wash were non-volatile, highly stable, large molecules. In a prior paper [18] preliminary attempts to characterize the response-eliciting components of earthworm wash were reported. These components are water soluble, retain their biological activity following boiling at 100°C for 15 minutes, following lyophilization, and following treatment with strong acid (pH 2) or strong base (pH 11) for 15 minutes at room temperature. They do not dissolve in a water immiscible solvent such as chloroform. Furthermore, biological activity and Bradford and Lowry assays covary [18].

The studies presented here are directed at a more complete characterization of the response-eliciting components in earthworm wash. Several lines of investigation were pursued in parallel. These included testing the limits of heating EW to determine when it would lose its biological activity; ammonium sulfate precipitation to produce a purer fraction of EW; gel filtration with Sephadex G-75 and AcA 44; and dialysis to obtain an estimate of molecular weight of the active fraction of EW. We also investigated the effect of water temperature during production of EW and the extractability of attractive substances from the earthworm bedding.

GENERAL METHOD

ANIMALS

The subjects were 18 male and female adult garter snakes (*Thamnophis sirtalis*) obtained from various animal suppliers. Snakes ranged in snout-vent length from 40 cm to 55 cm and in weight from 16 g to 45 g. All animals had been in captivity at least three months prior to the beginning of an experiment and were fed earthworms bi-weekly. During experiments snakes were fed only during testing. Snakes were selected from the laboratory stock on the basis of responding (attack) to a cotton swab dipped in EW.

[1]The first paper of this series is [17]. Parts of this paper were presented at AChemS V in Sarasota, FL, April, 1983.

The snakes were individually housed in clear plastic cages (30×18×15 cm), two sides of which were covered with paper towels to restrict external visual cues from adjacent cages. Each cage contained a water dish, a rock and a cardboard shelter and its floor was covered with paper toweling. The room in which the cages were kept was maintained on a twelve hour photo period and ranged in temperature from 20–24°C.

PROCEDURE

The testing procedure has been described in detail previously [18]. Briefly, the contents of each animal's cage were removed one hour prior to the first trial of each day of testing. Snakes were tested five days a week for two or three trials per day. A trial consisted of the simultaneous presentation of an EW sample (see below) and a double distilled water (dH_2O) sample on coplin jar covers placed in a presentation tray for two minutes from the time the snake first approached the presentation tray (see [18]). The side of EW or dH_2O presentation was determined by use of a random numbers table. A fresh swab was used on each trial to spread the EW or dH_2O sample on the coplin jar covers.

The number of tongue flicks directed to each side of the presentation tray was recorded with hand talleys and the time spent in the vicinity of each side of the presentation tray was timed with electric timers connected to foot switches. Instances of attack or biting of the dish were also noted, however since these occur rarely they have not been presented in the results reported here. For each trial a tongue flick interest score (tongue flicks multiplied by the time (in seconds) spent at the sample as a proportion of total time spent at the tray) was determined for the EW and dH_2O sample. At the end of a trial the snake was offered a small earthworm bit in the EW dish.

Test samples were judged to be discriminable from the control sample (distilled H_2O or other control substance) if the tongue flick interest score for the test sample was significantly higher than the tongue flick interest score for the control sample, using a T test for matched samples. A 5 percent level of confidence was used throughout.

Chemical Assays

1. Protein assay according to the method of Lowry *et al.* [16] read at 650 nm on a Bausch & Lomb Spectronic 20. 2. Carbohydrate assays: (a) Phenol-Sulfuric acid method of Dubois *et al.* [8] read at 595 on a Bausch & Lomb Spectronic 20. (b) Badin *et al.* [1] method for polysaccharides read at 520 nm on a Bausch & Lomb Spectronic 20.

Materials

1. Sephadex G-75 purchased from Pharmacia (Piscataway, NJ). 2. Ultragel Aca 44 purchased from LKB, (Gaithersburg, MD). 3. Dialysis Tubing purchased from A. H. Thomas, Co: or Spectrophor from Spectrum Medical Industries Inc. treated according to the directions of the manufacturer.

Chemicals

Ammonium Sulfate, Baker Analyzed Reagent, Granular (J. T. Baker Chemical Co., Phillipsburg, NJ). Molecular Weight Standards: Albumin (Bovine) MW 67,000; Myoglobin, MW 17,800; Ovalbumin, MW 45,000 (Schwarz/Mann Division of Becton Dickinson & Co., Orangeburg, NY); Blue Dextran 2000 MW 2×10^6 (Pharmacia, Piscataway, NJ); Dinitrophenyl-Alanine, MW 255 (Mann Fine Chemicals, New York, NY).

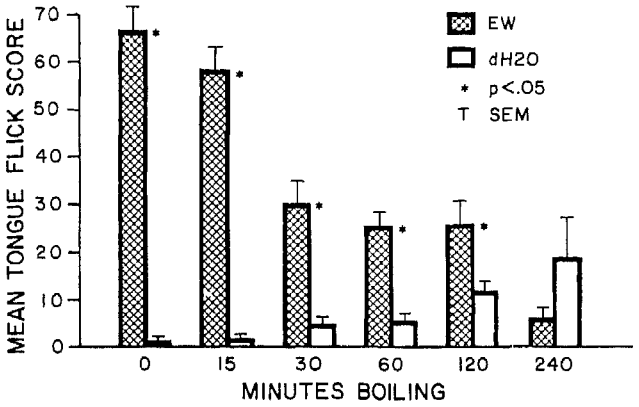

FIG. 1. Mean tongue flick interest scores of snakes presented with EW boiled for 0, 15, 30, 60, 120 or 240 minutes. Asterisk indicates that tongue flick interest scores to EW were significantly different from the tongue flick interest scores to dH_2O at the .05 level of confidence using a paired T test. SEM=standard error of the mean.

Lyophilization

Specimens were shell dried with a dry ice—acetone mixture and placed on Virtis Freeze-Mobile, Virtis Co., Inc. Gardner, NY.

EXPERIMENT 1

The purpose of this experiment was to determine whether prolonged heating of EW would cause a significant loss in biological activity.

METHOD

Preparation of Earthworm Wash

Earthworm wash (EW) was prepared [5,24] at a concentration of 6 g earthworm (*Lumbricus terrestris*, purchased from Connecticut Valley Biological, Southhampton, MA) per 20 ml dH_2O and was centrifuged at 2000 RPM for 20 minutes at room temperature. The clarified EW was distributed equally among four to six test tubes (depending on the test block) and large marbles were placed on all the tube tops to prevent evaporation. Equal amounts of dH_2O were placed in four to six additional test tubes which were also capped with marbles. One additional tube containing the thermometer immersed in water was added as the temperature indicator. All tubes were placed in a water bath maintained at 100°C making sure that the water level in the bath was above the water level in the test tubes. Tubes containing EW and dH_2O were removed at 0, 15, 30, 60, 120 and 240 minutes. For use in later experiments, aliquots of boiled EW were lyophilized.

Testing

Ten snakes were used to test the discriminability of EW heated for 0 to 240 minutes. During a testing block each snake was tested once with EW heated at each time interval. The order of testing was determined by a balanced Latin

TABLE 1
RESULTS OF PROTEIN AND CARBOHYDRATE ASSAYS ON BOILED EW

Sample (hours boiled)	Lowry Protein Assay* mean mg/ml	Carbohydrate Assay† mean mg/ml
0	1.10	0.088
0.5	1.13	0.088
1.0	1.18	0.085
2.0	1.15	0.085
4.0	1.14	0.057

*As mg albumin/ml.
†As mg glucose/ml.

square such that no heated EW sample was presented in the same ordinal position twice. Three test blocks were run: the first tested extracts heated 0, 15, 30, 60, 120 min, the second tested extracts heated 0, 15, 30, 60, 120, 240 min, and the third tested extracts heated 0, 60, 120, 240 min.

The EW of the third run was assayed by the method of Lowry [16].

RESULTS

Earthworm wash boiled for up to 2 hours was discriminated by snakes, but EW boiled for 4 hours was not discriminated by the snakes (Fig. 1). Mean tongue flick interest scores declined monotonically as a function of boiling time. Boiling of EW produced a significant loss in the biological activity of EW, $F(5,60)=28.40$, $p<0.001$. Using a Scheffé test [23] to compare the mean tongue flick interest scores at each boiling time we determined that 0 and 15 minutes did not differ significantly from each other, but 30, 60, 120 and 240 minutes differed significantly from 0 minutes boiling (all at $p<0.001$).

Although carbohydrate content assays [8] showed a consistent decline which was particularly marked after four hours of boiling, Lowry assays showed no consistent differences in protein content over the period 0–240 minutes (Table 1). Furthermore, whereas lyophilized EW is normally difficult to reconstitute with dH_2O, lyophilized boiled EW was readily soluble in dH_2O.

EXPERIMENT 2

The purpose of this experiment was to determine if we could produce a purer fraction of EW by ammonium sulfate precipitation.

METHOD

Ammonium Sulfate Fractionation of Earthworm Wash

Earthworm wash was prepared as described in Experiment 1. The EW was distributed among several centrifuge tubes. At room temperature sufficient solid ammonium sulfate was added to each tube to produce 50–100% saturation [9]. The contents of each tube was slowly stirred using a magnetic stirrer during the addition of the ammonium sulfate and for a subsequent 30 minutes. After the removal of the stirring bar the precipitate in each tube was separated by centrifugation at 2300 RPM for 20 minutes. The supernatant was removed with a disposable syringe and the test tube containing the precipitate inverted to drain excess supernatant. The precipitate was dissolved in dH_2O to a volume equal to the original sample volume.

Initially we tested the animals with the dissolved pellets and supernatants obtained by the method described above, but found that the residual ammonium sulfate was an attractant. In subsequent tests, using 3500 mw cutoff dialysis tubing, we dialyzed the supernatants and dissolved pellets against water at room temperature overnight. This procedure removed any ammonium sulfate residues.

Testing

Fourteen snakes were used to test the discriminability of the dialyzed supernatants (S) and dialyzed solution of the pelleted material (P) of ammonium sulfate treated EW. Six of the animals were tested with the S and P resulting from 50%, 100% and 50 to 100% saturation; five partial or complete replications were performed. Eight of the animals were tested with S and P resulting from 50, 60, 70, 80, 90 and 100% saturation with two complete replications. Since the latter two replications include samples similar to the first series we report only the results of the complete 50–100% series. The S and P were tested against dH_2O.

Sample testing was split into two sequences. During the first sequence the 8 snakes were tested with S and P resulting from the 50, 70, and 90% saturation procedures and with untreated EW. The order of testing was determined by an 8×8 balanced Latin square such that no sample was presented in the same ordinal position more than twice. During the second sequence the 8 snakes were tested with S and P resulting from the 60, 80 and 100% saturation procedures and with untreated EW. The order of testing was determined as described above for the first sequence. The entire series was repeated on the same animals a second time.

RESULTS

The solutions of the precipitates and supernatants of EW subjected to 50, 60, 70 and 80% ammonium sulfate precipitation were discriminated by snakes from dH_2O in both replications. However, only the dialyzed solution of the pelleted material of EW subjected to 90 and 100% ammonium sulfate precipitation were discriminated by snakes from dH_2O in both replicatons. The dialyzed supernatants resulting from 90 and 100% ammonium sulfate precipitation were not dis-

criminated by the snakes in either replication. The data resulting from the two replications were combined and are graphically presented in Fig. 2.

EXPERIMENT 3

This experiment was designed to obtain an approximation of the molecular weight of the active component or components of EW by use of dialysis.

METHOD

Dialysis of Earthworm Wash

Earthworm wash was prepared as described in Experiment 1. Aliquots of EW were placed into clean, washed dialysis bags with molecular weight cutoffs at 3500 or 50,000 and dialyzed against water at room temperature overnight.

Testing

Six snakes were used to test the discriminability of two samples each of EW dialyzed overnight in 3500 and 50,000 MW cutoff dialysis bags. Each snake was tested twice with each of the retentates and once with undialyzed EW. The order of sample presentation was balanced such that each retentate and the undialyzed EW was presented an approximately equal number of times in each position. All the retentates and the EW were tested against distilled water.

RESULTS

Earthworm wash was discriminable from dH_2O following overnight dialysis in tubing with MW cut-offs of 3500 and 50,000 (Table 2).

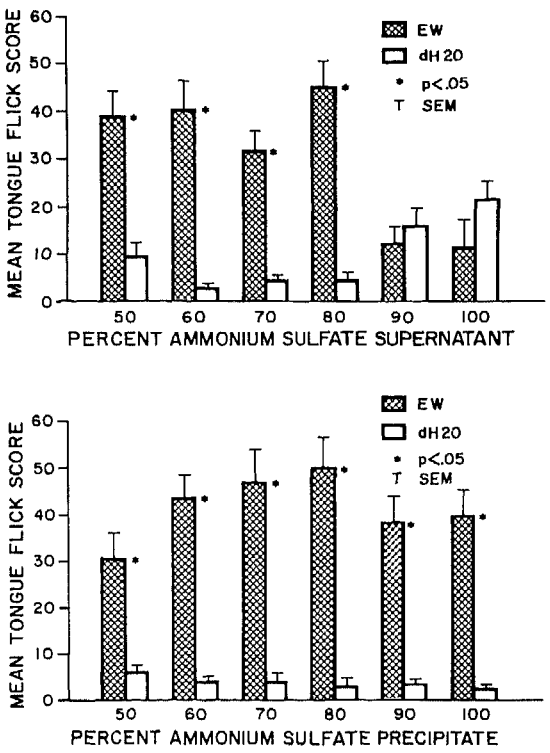

FIG. 2. Mean tongue flick interest scores of snakes presented with supernatants or resolubilized precipates obtained from 50, 60, 70, 80, 90 and 100% saturated ammonium sulfate EW. See Fig. 1 for other abbreviations.

EXPERIMENT 4

This experiment was designed to obtain a purified sample of EW and a better estimate of the approximate weight of the molecule(s) that constitute the active component(s) of EW.

METHOD

Gel Chromatography of Concentrated Earthworm Wash

Earthworm wash was prepared as described in Experiment 1. The EW was lyophilized, and the resulting dry, off-white powder dissolved with stirring with some difficulty in 0.15 M saline to approximately 5 times (5×) its normal concentration, i.e., 10 ml of EW was lyophilized and reconstituted to two ml. A 50.8×1.6 cm column of Sephadex G-75 was packed in a 60×1.6 cm glass chromatographic column (Glenco). This column was calibrated with Blue Dextran, bovine serum albumin and myoglobin. A sample of EW containing 16 mg in 2 ml 0.15 M saline (5×), clarified by centrifugation, was separated on the column. Fractions, 2 ml, were collected automatically (RSCo automatic fraction collector, Model 1205 in Time Mode, Research Specialties Co.). The optical density at 280 nm and Lowry assays were performed on all fractions. The fractions were combined into "superfractions;" the material eluting before the major peak constituted superfraction 1 (F_1), the peak material constituted superfraction 2 (F_2), and the material eluting following the peak divided into two superfractions, 3 and 4 (F_3 and F_4). This gel filtration experiment was repeated six times.

Another glass column, 60×1.6 cm (Glenco) was packed with AcA 44 (44×1.6 cm column), calibrated and EW applied as described for the G-75 column above. The eluent was collected as 2 ml fractions and assayed by measuring the optical density at 280 nm, by the Lowry method [16] and for carbohydrates [8]. Suitable fractions were combined as described for the G-75 column. This gel separation experiment was repeated five times.

Testing

Five snakes were tested with the four superfractions derived from the G-75 column and with EW. The control sample for the superfractions was 0.15 M saline and the control for EW was dH_2O. Order of testing of samples was controlled as in the previous experiments (5×5 Latin square) and at least 3 replications were performed with fractions obtained from the G-75 column.

Six snakes were tested with superfractions derived from the AcA 44 column. The F_1, F_3, and F_4 fractions were combined to form one sample type. Superfractions $F_{1,3,4}$ and F_2 were tested against 0.15 M NaCl and EW was tested against dH_2O. Order of testing was controlled as in the previous experiments (6×6 Latin square) and two replications were performed with fractions obtained from the AcA 44 column.

RESULTS

Gel filtration using Sephadex G-75 and 0.15 M saline as the eluent resulted in one major peak (Fig. 3). This peak overlapped the albumin marker position and was well in front of the ovalbumin marker position suggesting that the molecular weight of the substance(s) in the peak is larger than 45K daltons but equal to or more than 65K daltons if the

TABLE 2

MEAN TONGUE FLICK INTEREST SCORE TO UNDIALYZED EW AND TO EW DIALYZED IN 3500 AND 50,000 MW CUT-OFF DIALYSIS SACS

	EW Sample		dH_2O		
Dialysis Membrane	Mean	SEM	Mean	SEM	t
3500	24.90	2.80	1.73	0.56	3.7*
3500†	30.25	3.80	2.0	0.47	3.3*
50,000	24.71	2.25	1.38	0.43	4.7*
50,000†	22.72	3.05	1.93	0.56	2.9*
Undialyzed	27.35	2.58	0.98	0.40	4.7*

*$p<0.05$.
†Duplicate runs.

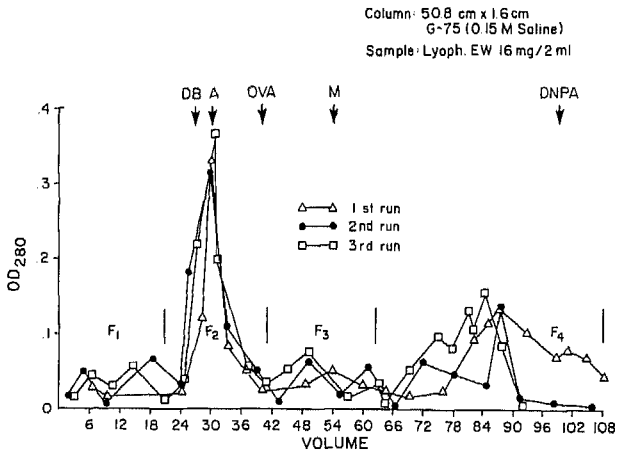

FIG. 3. Gel chromatography of lyophilized and reconstituted EW, 2 ml sample. Material was eluted from a 50.8×1.6 cm column of Sephadex G-75 at 0.15–0.43 ml/min with 0.15 M saline, 1.8–2 ml fractions collected. DB=Blue Dextran (MW 2×10^6); A=bovine serum albumin (MW 67,000); OVA=ovalbumin (MW 45,000); M=myoglobin (MW 17,800), and DNPA=DNP-alanine (MW 255). F1–F4 are the combined fractions used to test the biological activity of the eluates.

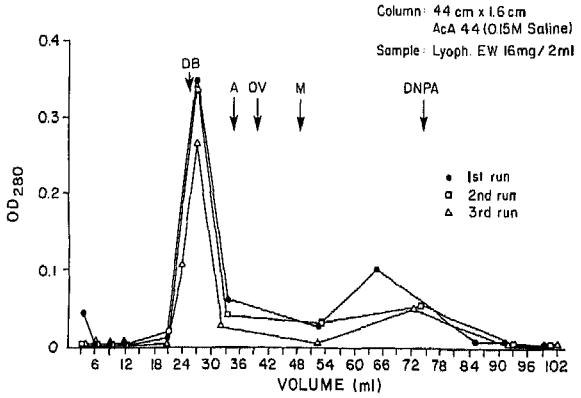

FIG. 4. Gel chromatography of lyophilized and reconstituted EW, 2 ml sample. Material was eluted from a 44×1.6 cm column of AcA 44 at 1 ml/min with 0.15 M saline, fraction size 2 ml. DB=Blue Dextran (MW 2×10^6); A=bovine serum albumin; OV=ovalbumin (MW 45,000); M=myoglobin (MW 17,800); DNPA=DNP alanine (MW 255).

substance(s) is (are) proteins. In addition we have consistently seen a minor peak which appears in a position close to or overlapping the DNP-alanine marker. This indicates that the molecular weight of the material is less than 17,800 daltons and larger than 3,000 daltons which is the weight below which no separation occurs on G-75.

The elution pattern obtained using AcA 44 and 0.15 M saline as eluent resulted in one major peak which appeared earlier than the albumin marker (Fig. 4). This indicated a molecular weight greater than 67,000. As in the G-75 column we consistently observed a minor peak which eluted at or in front of the DNP-alanine marker.

Snakes discriminated the G-75-derived F_2 superfraction from saline in each of the three replications but were unable to discriminate the F_1, F_3, or F_4 superfractions from saline (Fig. 5). The Lowry assay covaried with the behavioral activity of the superfractions (Fig. 5).

Snakes discriminated the AcA 44-derived F_2 superfraction from saline in each of two replications but were unable to discriminate the $F_{1,3,4}$ supefraction from saline (Fig. 6). The Lowry [16] assay and the assays for carbohydrate [1,5] covaried with the behavioral activity of the superfractions (Table 3).

EXPERIMENT 5

Since garter snakes feed normally on earthworms, and earthworms, to survive must be kept at low (4.4°C) temperatures, we investigated the effects of temperature of the "soaking" bath during production of earthworm wash. In addition, we examined the possibility that the soil in which the earthworms are maintained could be a source of attractant.

Our previous studies had indicated that the discriminability and attractiveness of EW covaried with protein content as measured by Bradford [4] and Lowry [16] assays. We therefore also tested the discriminability and attractiveness of a protein that is used as a standard in those two assays, bovine serum albumin.

METHOD

Preparation of Earthworm and Soil Washes

Earthworm wash was prepared as described in Experiment 1. The dH_2O used in preparation of EW was either at a temperature of 4°C (cold room temperature), 20–27°C (lab-

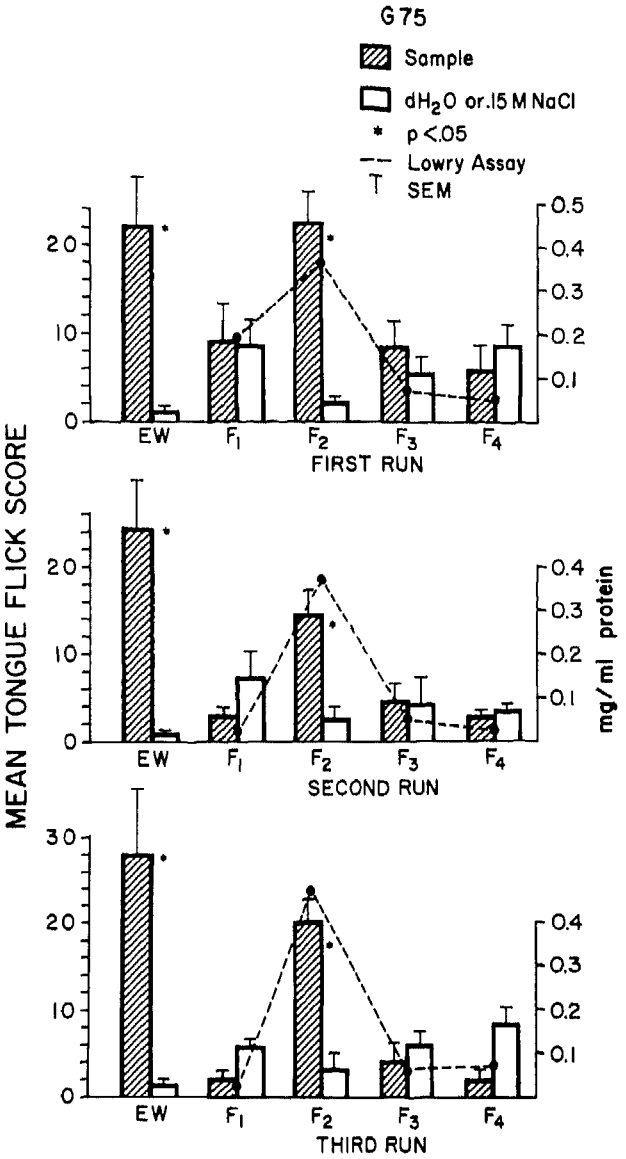

FIG. 5. Mean tongue flick interest scores of snakes presented with EW and the F1–F4 superfractions from the Sephadex G-75 column. The EW was tested against dH_2O, the F1–F4 fractions against 0.15 M saline. Results of the Lowry assay on the F1–F4 fractions are indicated by the dashed line. Other abbreviations as in Fig. 1.

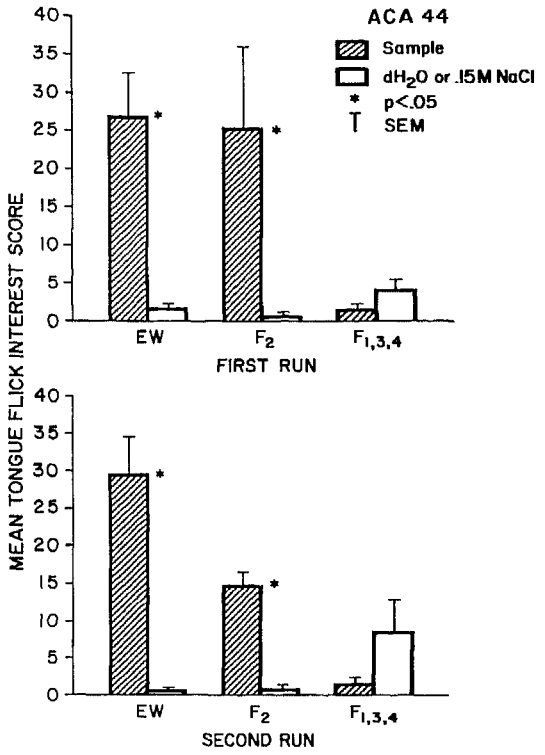

FIG. 6. Mean tongue flick interest scores of snakes presented with EW and the F2 (peak and adjacent fractions) and F1, 3, 4 (all other fractions) from the AcA 44 column. The EW was tested against dH_2O, the F_1–F_4 fractions against 0.15 M saline. Other abbreviations as in Fig. 1.

TABLE 3

RESULTS OF PROTEIN AND CARBOHYDRATE ASSAYS OF "SUPERFRACTIONS" OBTAINED FROM THE AcA 44 COLUMN

	Protein* mg/ml	Carbohydrate† mg/ml	Carbohydrate‡ mg/ml
F_2	0.52	0.036	0.040
$F_{1,3,4}$	0.06	0.008	0.010
EW (1×)	1.50	0.120	0.112

*Lowry *et al.* [16] assay as mg albumin/ml.
†Dubois *et al.* [8] as mg glucose/ml.
‡Badin *et al.* [1] as mg glucose/ml.

oratory room temperature), or 60°C (normal bath temperature for preparation of EW). In addition an albumin sample (bovine serum albumin, 2 mg/ml dH_2O) was prepared at room temperature.

The soil wash was prepared at a concentration of 6 g of soil to 20 ml of dH_2O. The soil (earthworm bedding) was obtained from a container in which earthworms had lived in excess of one month and in which they were still living. The soil was placed in dH_2O at 4°C, 27°C or 60°C for one minute. The suspensions were filtered through glass wool and centrifuged at 2000 rpm for 20 minutes. Only the supernatant was used for testing on the snakes.

Some of the soil extracts prepared at 4°C and 27°C were heated to 60°C after preparation to determine if heating after preparation would release an active component.

Testing

To determine if EW made up at different temperatures were active and to determine if albumin was discriminable, five snakes were tested for five trials each with EW made up at 4°C, 21°C and 60°C and albumin. The order of presentation was balanced so that no sample was presented in the same ordinal position more than twice and no animal was tested with a particular sample more than twice.

To determine if soil washes (SW) made up at different temperatures were discriminable, six snakes were tested for six trials each with SW made up at 4°C, 21°C and 60°C. Order of presentation was balanced as described above. Snakes were tested in three complete replications (runs).

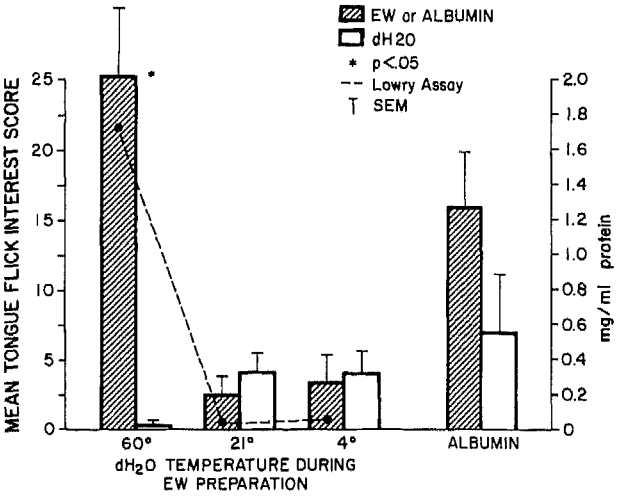

FIG. 7. Mean tongue flick interest scores of snakes presented with EW prepared at 60°, 21° or 4°C and albumin. Results of the Lowry assay of EW are indicated by the dashed line. Other abbreviations as in Fig. 1.

To determine if an active component in SW made up at 4°C or 21°C could be released by heating the SW for one minute at 60°C, we tested six snakes for six trials each with SW made up at 4°C and 21°C and not heated, SW made up at 4°C and 21°C and heated, and EW made up as usual at 60°C. Snakes were tested in two complete replications (runs).

RESULTS

Earthworm wash made up at room temperature (21°C) or cold room temperature (4°C) were not discriminated from dH_2O by the snakes nor was bovine serum albumin (Fig. 7).

Soil wash prepared at 60°C, the same temperature at which EW is normally prepared, was discriminated from dH_2O by the snakes, but SW prepared at room temperature (27°C or 20°C) or cold room temperature (4°C) was not (Fig. 8). The discriminability and attractiveness of the SW covaried with Lowry assay. Heating (for one minute at 60°C) SW prepared at 4°C or 21°C did not enhance its discriminability, nor did the Lowry assay results change.

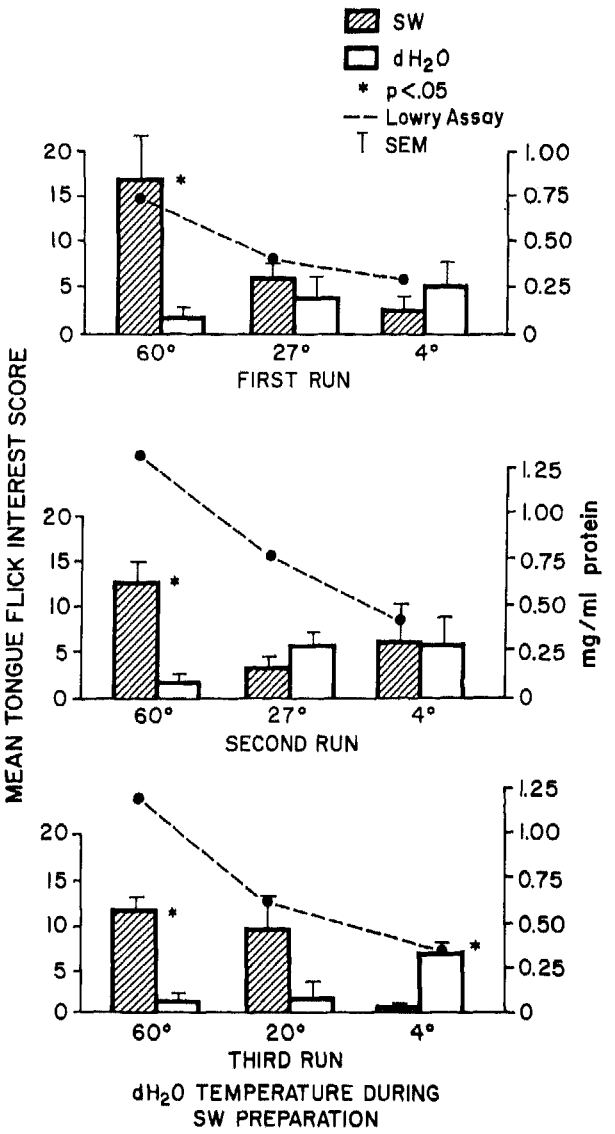

FIG. 8. Mean tongue flick interest scores of snakes presented with soil wash (SW) prepared at 60°, 21° or 4°C. Abbreviations as in Fig. 1.

GENERAL DISCUSSION

The experiments described here have demonstrated that the response-eliciting components of earthworm wash are sensitive to prolonged heating, can be fully precipitated by 90–100% saturation with ammonium sulfate, and have a molecular weight as determined by gel chromatography and dialysis greater than 67,000 daltons if considered as a globular protein. Furthermore, the response eliciting components of EW are not extractable from earthworms at temperatures well below 60°C, but can be extracted at 60°C from the worms or the soil that forms the bedding for the earthworms.

The heating data suggest that a two-stage process of change is occurring during prolonged exposure to high temperature. The first significant drop in activity occurs between 15 and 30 minutes of boiling. However some of the active components clearly are still present since EW boiled for 1 hour or 2 hours is still discriminated from dH_2O by the snakes. Although Lowry assay values did not change significantly with increased boiling time, the carbohydrate assay values decreased markedly after more than 2 hours boiling. These findings further suggest that the carbohydrate assay may be a better indicator of the presence of the active components of EW than the Lowry assay. The Lowry assay is traditionally considered a protein assay however it is sensitive also to the carbohydrate content of substances and may thus give a positive value even in the absence of significant protein [3].

The active components of EW are precipitated by ammonium sulfate. At 90 and 100 percent saturation all of the active components are precipitated. Thus ammonium sulfate precipitation represents a useful first step in extracting the active components from EW. Ammonium sulfate precipitation has been used in the preparation of proteins [9] and polysaccharides [17].

The data obtained from the dialysis experiment suggests that the major active component in EW is a molecule with a

molecular weight greater than 50,000 daltons. The gel filtration experiments confirm the estimates of the molecular weight of the active components of EW based on dialysis. However it is important to note that it is difficult to assign a precise molecular weight to a glycoprotein by gel chromatography ([21] p. 16). Furthermore, the gel filtration results indicate that we can obtain a purified sample of the active component of EW using Sephadex G-75 or AcA 44. Again, the highest Lowry assay values and carbohydrate assay values are obtained from those fractions with high biological activity and high 280 nm absorption.

It is interesting that soaking baths cooler than 60°C do not produce an active EW. Clearly, snakes are capable of responding to the surface substances of earthworms at temperatures well below 60°C since feeding occurs at cool temperatures consistent with earthworm survival. Our findings suggest that these substances are not readily released into a water bath at "normal" environmental temperatures, but require an extremely warm, perhaps shocking, solvent to be released [19]. Our observations that the soil in which the earthworms live contains response-eliciting substances suggests that these substances may be removed from the surface of the earthworm by abrasion as the worms burrow through the soil. These substances, although present in the soil, cannot be eluted from the soil at low ambient temperatures (4°C or 21°C), but can be extracted at 60°C. We have not excluded the possibility that the response-eliciting components derive from secretions of the coelomic cavity of earthworms, however since snakes respond to whole earthworms by tongue flicking their surfaces followed by attack, we feel confident that the response eliciting substances, regardless of their origins, are present on the external surface of the earthworms.

At the present time we can summarize our findings as follows. The garter snake attractant obtained from a wash of the surface of earthworms is a material of large molecular weight, larger than 67,000 daltons, giving positive assays for protein [4,16] and for carbohydrate [1,8], and 280 nm absorption. This material is water soluble, ammonium sulfate precipitable, chloroform insoluble, and separable on a cross-linked carbohydrate matrix, Sephadex G-75, and on a mixed matrix of agarose and polyacrylamide, AcA 44. It is remarkably resistant to heat, requiring boiling of a neutral aqueous solution for four hours to destroy its snake-attracting ability. From the properties cited above it would appear that our peak material contains glycoprotein-like substances [20], perhaps a mucin-like material [7].

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