Pharmacology Biochemistry & Behavior, Vol. 18, pp. 247–254, 1983. Printed in the U.S.A.

Preliminary Characterization of Response-Eliciting Components of Earthworm Extract

LORRAINE S. REFORMATO, DONALD M. KIRSCHENBAUM AND MIMI HALPERN[1]

Departments of Anatomy and Cell Biology and Biochemistry
Downstate Medical Center, Brooklyn, NY 11203

Received 9 July 1982

REFORMATO, L. S., D. M. KIRSCHENBAUM AND M. HALPERN. *Preliminary characterization of response-eliciting components of earthworm extract.* PHARMAC BIOCHEM BEHAV **18**(2) 247–254, 1983.—Fifteen garter snakes (*Thamnophis sirtalis*) reliably spent more time and tongue flicked more frequently at a dish containing earthworm extract than a dish containing distilled water when these were presented simultaneously for two minute intervals. The discriminability of the earthworm extract was directly related to its concentration. Garter snakes with their vomeronasal ducts sutured closed did not respond differentially to earthworm extract and water under these test conditions. Thus their ability to discriminate earthworm extract from water in this bioassay was dependent upon a functional vomeronasal system. Earthworm extract retains its biological activity after boiling at 100°C for 15 minutes and after lyophilization. Its effectiveness is not altered by changes in pH. Snakes continued to respond differentially to extracts with pH 2, 5–6 or 11. Chloroform extractions of the acid, neutral and alkaline earthworm extract yielded activity primarily in the water layer. The small amount of activity in the chloroform layer was removed by use of a drying agent. Bradford dye-binding tests indicated the presence of protein in the active fractions.

Snakes (*Thamnophis sirtalis*) Earthworm extract Vomeronasal system Characterization Olfaction

EARTHWORMS form a major portion of the diet of many garter snake species [20]. Prior to encountering their first earthworm meal, infant garter snakes respond to surface extracts of earthworms with increased tongue flicking and prey attack [2]. Similar responsiveness to aqueous extracts of earthworms has been demonstrated in adult garter snakes [5, 8, 19]. In addition, Kubie and Halpern [11–14] have demonstrated that garter snakes will accurately follow an earthworm extract trail in a 2-choice or 4-choice maze and that the accuracy of trail following and tongue flick rate during trailing increase as a function of increasing earthworm extract concentration.

A functional tongue-vomeronasal system appears to be necessary to demonstrate differential responding to earthworm extract by infant [5] and adult garter snakes [8] or accurate earthworm extract trail following [14]. Deafferentation of the olfactory system does not disturb this differential responding to earthworm extract [4,8] or accurate earthworm extract trailing [14].

Since the tongue-vomeronasal system and not the olfactory system appears to be involved in differential responding to earthworm extracts, identification and characterization of the active components in earthworm extract could contribute to an understanding of the essential differences in stimulus requirements for the vomeronasal and olfactory systems.

Previously Sheffield and his colleagues [16], using newborn garter snakes, demonstrated that the attack eliciting substances in earthworm extract were non-volatile, highly stable large molecules. To further these investigations we have developed another, sensitive assay to test components of earthworm extract. We describe in this paper the assay, its sensitivity, its reliance on a functional vomeronasal system and results of preliminary fractionation experiments.

GENERAL METHOD

Animals

The subjects were 15 male and female adult garter snakes (*Thamnophis sirtalis*) obtained from various animal suppliers. Snakes ranged in snout-vent length from 40 cm to 65 cm and in weight from 23 g to 60 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 the study snakes were fed only during testing. Snakes were selected from the laboratory stock on the basis of positive responding (attack) to a cotton swab dipped in earthworm wash (EW).

[1]Requests for reprints should be addressed to Mimi Halpern, Department of Anatomy and Cell Biology, Downstate Medical Center, 450 Clarkson Avenue, Brooklyn, NY 11203.

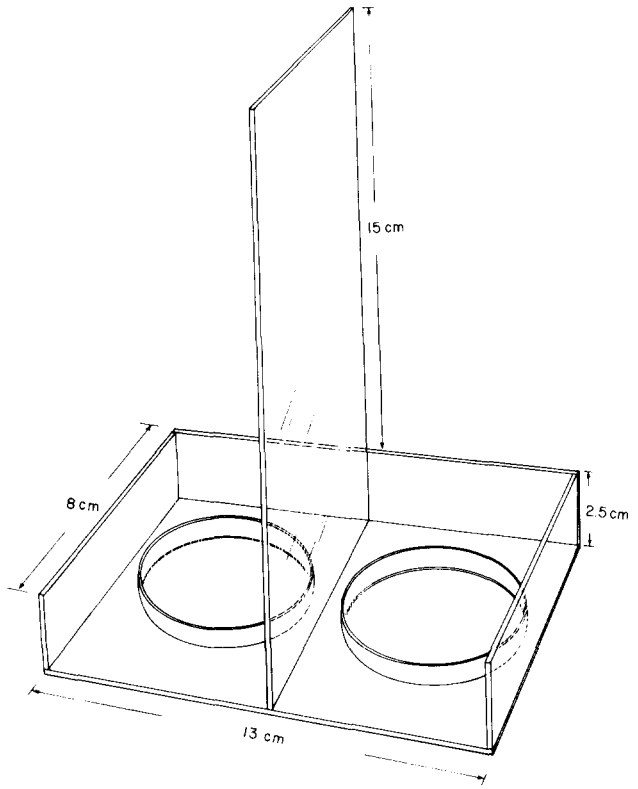

FIG. 1. Presentation tray used in simultaneous discrimination.

The snakes were individually housed in clear plastic cages (30×18×15 cm) with sliding tops. Two sides of the cages were covered with paper towels to restrict external visual cues from adjacent cages. The floor of each cage was covered with paper toweling and each cage contained a water dish, a rock and a cardboard shelter. 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 rock, water dish, cardboard box and paper towel lining were removed from each animal's cage 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; at midmorning, early afternoon and late afternoon. When the spectacle of a snake's eye became opaque prior to shedding, testing was discontinued until the day after the snake shed.

A trial consisted of the simultaneous presentation of an earthworm wash sample (see below) and double distilled water (dH_2O) sample on coplin jar covers placed in a presentation tray. The presentation tray was constructed of 1/4 in. Plexiglas and fit into the end of the animal's home cage (Fig. 1). The coplin jar covers, placed on either side of the tray were covered with an EW sample or dH_2O by spreading the sample over the surface of one coplin jar cover with a sterile cotton swab. The side of EW or dH_2O presentation was determined by use of a random numbers table. A fresh swab was used for each sample on each trial.

At the beginning of each trial the snake's cage top was removed and the presentation tray was placed at one end of the cage. A trial began when the snake's head was within the boundries of the presentation tray and continued for the following two minutes. 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. For each trial a tongue flick interest score was determined for the EW and dH_2O sample using the following formula:

$$\text{Tongue Flick Interest score} = \frac{\text{TF to Sample} \times \text{Time Spent at Sample}}{\text{Total Time Spent at the Tray}}$$

Thus the tongue flick interest score for EW on a given trial would be determined by multiplying the amount of time the animal spent at the EW dish by the number of tongue flicks emitted to the EW dish and the product divided by the total amount of time spent at the two dishes on the presentation tray.

At the end of a trial the snake was offered a small earthworm bit in the EW dish. The snake's attack and ingestion of the earthworm bit were noted by the experimenter.

EXPERIMENT 1

The purpose of this experiment was (1) to determine if the two choice presentation method was effective in eliciting discriminatory responses between EW and dH_2O and (2) to determine whether responsiveness to EW was related to the concentration of the EW using this bioassay.

METHOD

Preparation of Prey Extract

Earthworm wash was prepared by the methods of Wilde [19] and Burghardt [2] at a concentration of 6 g of earthworm per 20 ml of dH_2O (1×) and was centrifuged at 2000 RPM for 20 minutes. The EW was stored in a refrigerator; each morning it was removed and allowed to reach room temperature.

Dilutions of EW were made from 1× EW by serial dilution of 1:2 with distilled water 6× to make extract concentrations of 1/3, 1/9, 1/27, 1/81, 1/243 and 1/729 the original.

Testing

Eight snakes were tested for a minimum of ten trials with 1× EW sample and dH_2O in the presentation tray as described above. Following this testing four of the snakes were tested with the first five dilutions of 1/3×, 1/9×, 1/27×, 1/81× and 1/243× vs dH_2O in two ten trial series. In addition six snakes were tested for four trials with 1/9×, 1/81× and 1/729× dilutions.

RESULTS

All eight snakes spent significantly more time at the side of the presentation tray with the EW sample than at the side with dH_2O and their tongue flick interest scores were significantly higher to the EW sample (Fig. 2).

We found that in tests with diluted extracts all snakes tested could discriminate between EW and dH_2O at concentrations of 1/3×, 1/9×, 1/27×, 1/81×, and 1/243×. Snakes could not discriminate EW samples diluted to 1/729 their original strength, i.e., their tongue flick interest scores for the 1/729 dilution were not significantly different from interest scores for dH_2O (Fig. 3, top). Higher concentrations of EW were discriminated better than lower concentrations of

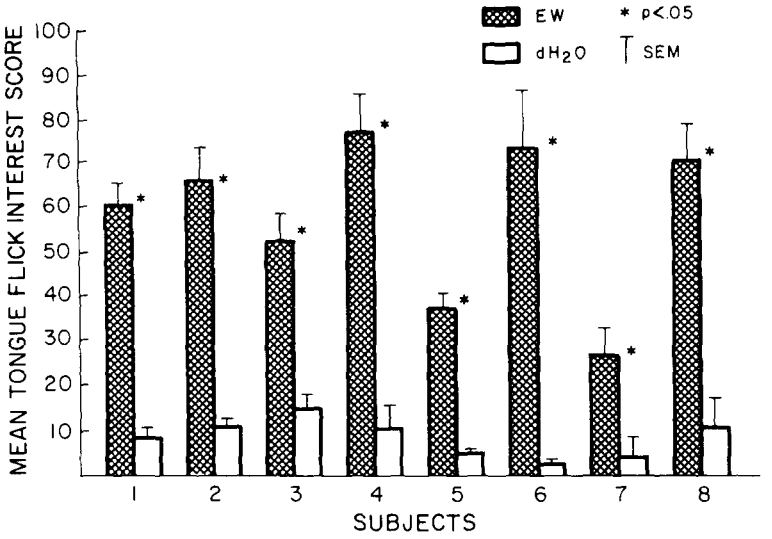

FIG. 2. Mean tongue flick interest scores of eight snakes presented with earthworm wash (EW) and distilled water (dH_2O). All snakes discriminated EW from dH_2O as determined by a *t*-test for paired comparisons. SEM=Standard error of the mean.

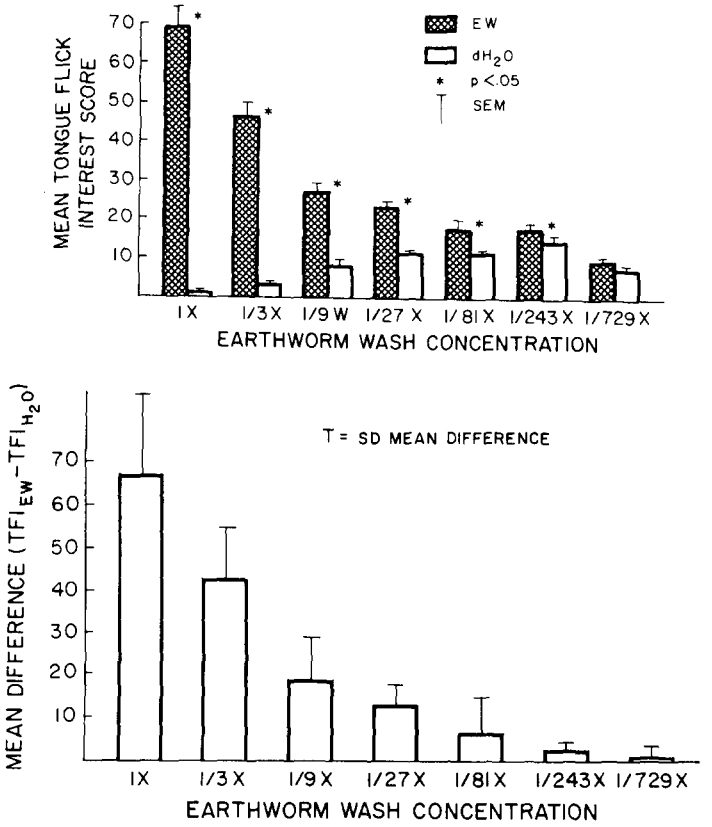

FIG. 3. (Top) Mean tongue flick interest score for snakes tested on seven concentrations of earthworm wash. (Bottom) Mean difference in tongue flick interest score on EW and dH_2O samples at seven concentrations of EW.

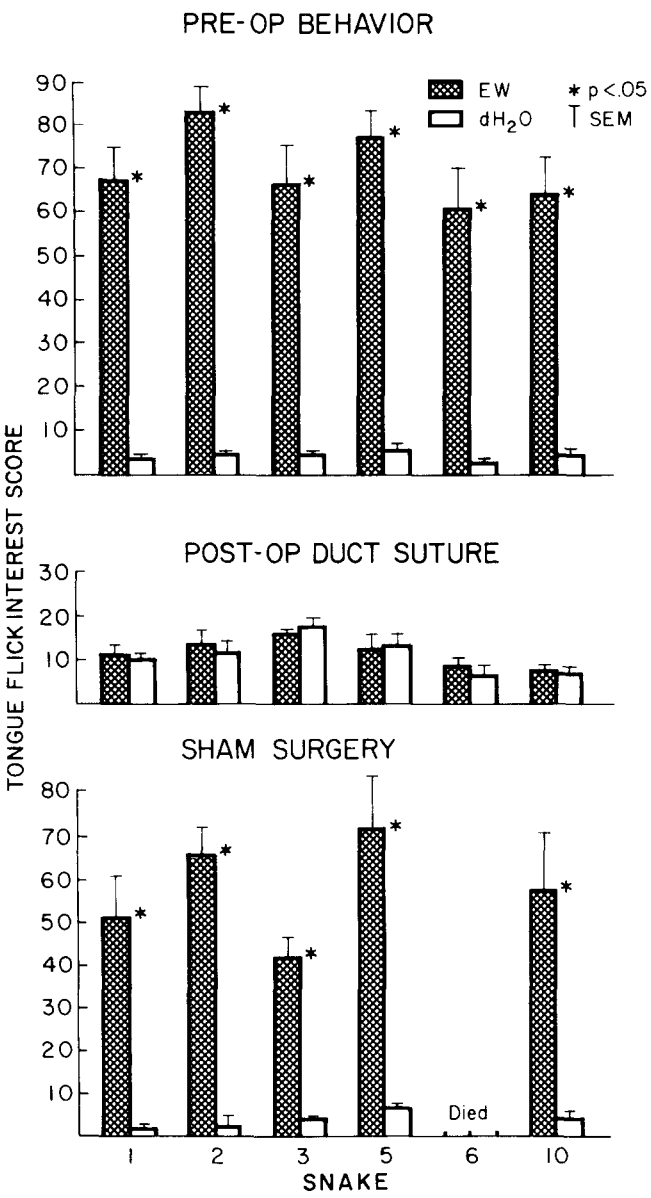

FIG. 4. Mean tongue flick interest scores of six snakes prior to duct suture (top), with duct sutures in place (middle) or during trials with sham sutures in place (bottom).

EW samples (Fig. 3, bottom); mean tongue flick interest scores increasing as a function of extract concentration, $F(6,61)=52.4$, $p<0.01$.

EXPERIMENT 2

This experiment was designed to determine if snakes would continue to discriminate EW from dH_2O when deprived of a functional vomeronasal system.

METHOD

Surgery

In preparation for surgery snakes were anesthetized with Sodium Brevital (15 mg/kg body weight, injected in a solution of 0.5% Brevital in saline; [18]). Surgery was performed with the animals restrained on their backs. A wooden rod was placed in the snake's mouth to keep it open. Using fine suture material (9-0 (35 μ) Ethilon; Ethicon Inc., Somerville, NJ) and delicate (#5) forceps; we inserted three or four individual sutures tying together the two lateral ridges of the fenestra vomeronasalis [14]. The procedure effectively closes the vomeronasal duct and prevents access of odorants to the vomeronasal organ [9,13]. Sham surgery consisted of placing four stitches, two on either ridge of the fenestra vomeronasalis. The suture material was not continuous across the midline, thus the vomeronasal duct remained open.

Testing

Eight snakes were tested preoperatively with 1× EW for eight trials in the days prior to surgery; four received duct sutures and four sham surgery. Two animals died, leaving three animals in each group. Postoperative trials began the day following surgery and consisted of eight trials with 1× EW for each animal. Five of the snakes were tested twice a day for four days and one snake was tested three times a day until the eight trial criterion had been met. Snake No. 3 pulled out its duct sutures on the first day of testing and had to have sutures replaced.

After the first postoperative series snakes with duct sutures had them removed and sham sutures placed as described above. The sham sutured animals of the first postoperative series had these removed and duct sutures sewn in place closing the vomeronasal duct. A second eight trial postoperative series was then run. One sham-operated animal died.

The tongue flick interest score was used to assess pre-and postoperative responsiveness to EW stimuli. At the completion of postoperative testing the snakes were visually checked to verify that the sutures had remained in place.

RESULTS

Preoperative Behavior

All eight animals of this study exhibited similar and consistent preoperative behavior; they discriminated between EW and dH_2O. Their tongue flick interest scores were significantly higher to EW (Fig. 4). Prior to surgery all animals ate earthworm bits offered at the end of a trial.

Postoperative Behavior

Sham surgery did not result in a significant change in tongue flick interest scores. All snakes discriminated between EW and dH_2O (Fig. 4).

Duct sutures significantly reduced the discriminatory ability of all snakes. Tongue flick interest scores for EW and dH_2O did not differ significantly for any of the six snakes (Fig. 4).

All snakes ate on all postoperative trials regardless of the type of surgery they had received.

EXPERIMENT 3

The purpose of the chemical studies was to determine the chemical nature of substances in the EW that stimulate the vomeronasal system. The new bioassay described in Experiment 1 was used to determine if a manipulation resulted in loss of activity in the EW sample.

METHOD

Chloroform Extractions

Freshly made EW was either acidified with concentrated sulfuric acid to a pH of 2, left neutral at a pH of 5–6, or made alkaline to a pH of 11 with one normal sodium hydroxide. The extraction procedure consisted of mixing 40 cc of chloroform with the acid, neutral or alkaline EW and subsequent separation of the mixture in a separatory funnel. Incomplete separation was remedied by centrifugation for 20 minutes at 2000 RPM. A distilled water control was run concurrently. Extraction of both the dH_2O control and the EW resulted in a heavier chloroform layer and a lighter water layer. The chloroform layers were air dried and later reconstituted with dH_2O. Samples were presented to snakes in the behavioral bioassay against a dH_2O control.

The acid chloroform extraction and the neutral chloroform extraction were performed twice; the alkaline chloroform extraction was performed once. For each of these five bioassays the samples consisted of the EW sample itself (acid, neutral or basic), a chloroform control, a water control (acid or basic), the chloroform layer of dH_2O, the chloroform layer of EW, and the water layer of EW. In each case a test sample was compared to dH_2O. Each bioassay was run for a minimum of eight trials and in all experiments at least six snakes were tested.

Heat Stability

Heat stability was tested with acid, neutral, and alkaline EW subjected to boiling at 100°C for 15 minutes; these three samples were then presented to snakes against a dH_2O control.

Boiling of acid, neutral and alkaline EW was performed once and resulted in three samples which were tested with six snakes for ten trials each.

Protein Determination

The Bradford test [1] of protein determination was used to compare the color developed using a typical protein—bovine serum albumin—with the color derived from EW. Albumin made up to 400γ was used to determine a standard curve against which the EW optical densities could be read. Bradford reagent (0.5 ml) was added to 0.1 ml of the sample and was read on a Bausch & Lomb Spectronic 20 at 595 nm. Bradfords were performed on the EW, the chloroform layer and the water layer of both acid extractions, both neutral extractions and the alkaline extraction.

Dry Weights

To obtain dry weights for the acid EW extraction three ml samples were taken of the EW, the acid EW, the water layer and the chloroform layer of the acid EW. These samples were placed in weighing boats and weighed on a Mettler balance. After drying for twelve days over a drying agent the samples were reweighed.

Drying of Chloroform Layer

Results of the dry weight determinations and Bradford tests indicated that most of the active material after chloroform extraction was present in the water layer. To determine if the biological activity we were obtaining in the chloroform layer was due to incomplete extraction, we dried the chloroform layer after extraction with 2 g of calcium chloride for 2 hr. The chloroform layers of extractions with acid and basic EW and acid and basic dH_2O were dried with the drying agent, reconstituted and presented with neutral EW to five snakes for eight trials each.

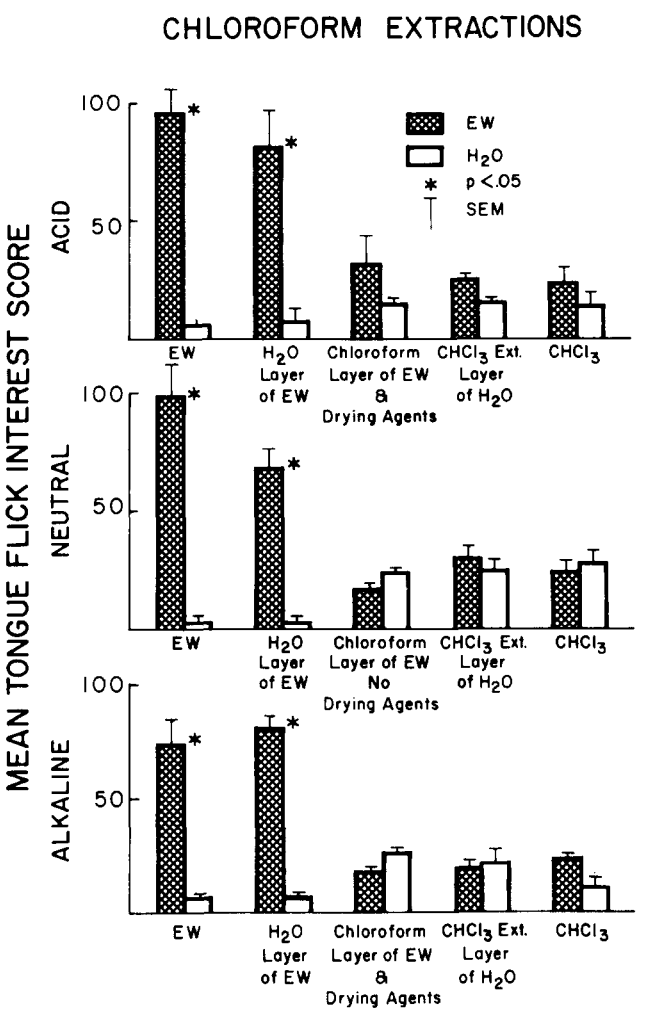

FIG. 5. Mean tongue flick interest scores of snakes presented with acid (top), neutral (middle) or alkaline (bottom) EW and results of chloroform extraction on attractivity of EW.

Lyophilization

Earthworm wash was frozen using dry ice and acetone and vacuum dried using a lyophilizer. The lyophilized powder was reconstituted to 1× concentration and tested for activity as follows: eight snakes were each tested with EW (1×) prior to lyophilization on one trial. This trial was followed by two trials with lyophilized EW (1×) for each snake. Each snake was presented with unlyophilized EW (1×) on a final trial.

RESULTS

Chloroform Extraction

Chloroform extraction of acid and alkaline EW led to partitioning of the material present in the aqueous EW. A small amount (ca. 10%) of material went into the chloroform layer with the major portion remaining in the water. The

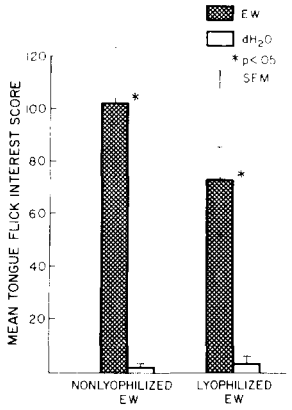

FIG. 6. Mean tongue flick interest scores of snakes presented with lyophilized and nonlyophilized EW.

active material was contained in the water layer and was also in the fine layer difficult to separate by pipetting.

Regardless of pH all snakes presented with EW and dH_2O samples spent a greater amount of time and tongue flicked significantly more often to the side of the presentation tray with EW (Fig. 5). This result was also true for the water layer of the EW (Fig. 5). The chloroform and dH_2O controls as well as the chloroform layer of dH_2O sample were not discriminated from dH_2O; the time spent and the number of tongue flicks to either side of the presentation tray were not statistically different (Fig. 5). In one case there was a significant decrease in the tongue flick interest score to the chloroform layer of the water sample compared to the dH_2O sample.

The time spent and the number of tongue flicks emitted to the chloroform layer of EW sample varied according to pH. We found that when EW was acidified or alkalinized (made alkaline) the snakes could discriminate between the sample and dH_2O. Snakes remained at the EW side of the presentation tray significantly more and tongue flicked to that side significantly more often than would be expected by chance; both layers of acid and alkaline extractions, chloroform and water, contained material which gave a positive bioassay. The chloroform layer of neutral EW did not produce this discrimination and the time spent and tongue flicks to either side of the presentation tray remained random (Fig. 5).

Drying of Chloroform Layer

Drying the chloroform layers of EW after chloroform extraction resulted in the loss of biological activity; the snakes no longer could discriminate the samples from dH_2O and their tongue flick interest scores to either side of the presentation tray were random (Fig. 5). Thus the activity previously observed in the chloroform layers of alkalinized and acidified extracts appears to have been due to "wetting" of those layers. We have no explanation for the absence of the "attractant" in the neutral chloroform layer. We appreciate the fact that the drying agent may be adsorbing the "attractant" in addition to drying out the chloroform.

Lyophilization

Snakes discriminated lyophilized EW from dH_2O (Fig. 6). Although tongue-flick interest scores to lyophilized extract were lower than tongue-flick interest scores to nonlyophilized extract, this difference was not significant, $F(1,11)=0.033$, $p>0.05$.

TABLE 1

BRADFORD PROTEIN DETERMINATION

	Sample/volume	OD	Equivalent
Standard Curve:			
	Albumin/0.2 ml	0.85	80γ
	Albumin/0.1 ml	0.45	40γ
	Albumin/0.05 ml	0.26	20γ
	Albumin/0.025 ml	0.12	10γ
Acid Chloroform Extraction:			
1st	Acid EW/0.1 ml	0.38	34γ
	H_2O layer/0.1 ml	0.39	36γ
	chloroform layer/0.1 ml	0.05	4γ
2nd	H_2O layer/0.1 ml	0.30	26γ
	chloroform layer/0.1 ml	0.04	3γ
Neutral Chloroform Extraction:			
1st	EW/0.1 ml	0.49	46γ
	H_2O layer/0.1 ml	0.49	46γ
	chloroform layer/0.1 ml	0	0γ
2nd	H_2O layer/0.1 ml	0.43	36γ
	chloroform layer/0.1 ml	0.02	2γ
Alkaline Chloroform Extraction:			
	EW/0.1 ml	0.25	18γ
	Alkaline EW/0.1 ml	0.37	32γ
	H_2O layer/0.1 ml	0.34	29γ
	chloroform layer/0.1 ml	0	0γ

TABLE 2

DRY WEIGHTS OF ACID CHLOROFORM EXTRACTION

Sample	Dry Wt g/3 ml EW
EW 1	0.0046
EW 2	0.0046
Acid EW 1	0.0089
Acid EW 2	0.0090
H_2O layer Acid EW 1	0.0086
H_2O layer Acid EW 2	0.0078
Chloroform layer 1	0.0005
Chloroform layer 2	0.0005

Protein Determination

There was protein present in the EW. In all chloroform extractions most of the protein was found in the water layer with little if any protein found in the chloroform layer (Table 1).

Dry Weights

Approximately 0.0046 g of solid material was present in 3 ml of neutral (pH 5–6) EW. Ninety-two percent of the solid material remained in the water layer after chloroform extraction with little extraction of material into the chloroform layer (Table 2).

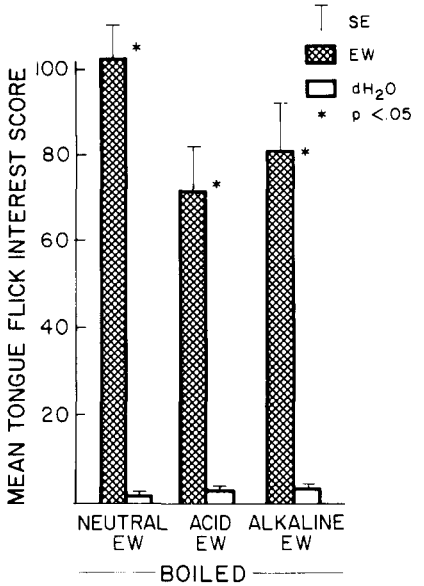

FIG. 7. Mean tongue flick interest score of snakes presented with neutral, acid or alkaline EW after 15 minutes of boiling at 100°C.

Heat Stability

Boiling acid, neutral, and alkaline EW was found to have no effect on EW. All snakes spent significantly more time and tongue flicked significantly more often to the side of the presentation tray containing sample than to dH_2O (Fig. 7).

GENERAL DISCUSSION

A two-choice simultaneous discrimination task can be used to assay earthworm wash for components that stimulate the garter snake's vomeronasal system. The two-choice discrimination is based on the natural tendency for garter snakes to respond to extracts of preferred prey objects with increased "attention" and tongue flicking [2,3]. The discrimination task is particularly useful as a bioassay because most animals perform it with little, less than a week, pretraining. This contrasts with extract trailing in a maze, a task we have used previously [11–13], which requires extensive training and careful preselection of animals. Another advantage of the task is that it does not involve active experimenter participation during stimulus presentation. Previous studies [3, 8, 16, 19] used tongue flick rates and attacks of swabs dipped in extracts to assay for responsiveness to prey substances. The swab attack task involves experimenter manipulation of the swab. Such manipulation could be a source of bias. Furthermore, since simultaneous presentation of the extract swab with a control swab is not possible, discriminability can only be inferred from successive presentations.

The simultaneous 2-choice discriminative task is sensitive to earthworm extract concentration. Snakes respond with increased tongue flick rates and time spent at dishes with higher earthworm wash concentrations than at dishes with lower earthworm wash concentrations. The present assay appears to be more sensitive at the lower concentration levels than the maze trailing task [13]. Whereas in maze trailing most snakes do not discriminate extracts less concentrated than 1/27×, the animals in the present experiment continued to discriminate extracts as low as 1/243×.

As with trailing and swab attack tasks, the 2-choice discrimination task is dependent on a functional vomeronasal system. With vomeronasal duct sutures in place snakes cannot discriminate between earthworm extract and water. Since control procedures do not affect this discrimination we can rule out ancillary effects of the surgery in causing the discrimination deficit. We have not determined whether the olfactory system is involved in this discrimination although it is clear that the olfactory system alone can not support EW discrimination.

The chemical analyses indicate that the active component of EW is heat stable, non-volatile and unaffected by large fluctuations in pH. The active component of EW appears to be a polar substance since it is found exclusively in the water layer after chloroform extraction when care is taken to avoid wetting the chloroform layer. Furthermore, the water layer contains 92% of the original EW by dry weight and most of the protein present in the original EW.

In general our results are in agreement with those of Sheffield *et al.* [16]. The Sheffield paper reports diminished responsiveness to extract prepared at 90°C but not to lyophilized powder heated to 100°C for 3 hr. We found no effect of prolonged heating of aqueous EW at 100°C. Our results are similar to the Sheffield group with respect to the efficacy of lyophilized extract although we never experienced difficulty in resolubilizing the lyophilized extract nor did the resolubilized extract ever develop the turbidity described by Sheffield *et al.* [16]. Our chloroform extraction yielded similar results to Sheffield's petroleum ether or ethyl ether extraction, i.e. the aqueous phase contained all the biologically active components provided no wetting of the organic solvent occurred. Interestingly, Sheffield *et al.* describe slight activity in the supernatant of a methanol extraction which may have resulted from wetting of the methanol layer. We have made no attempt in the present experiments to determine the size of the active ingredient in EW. Sheffield *et al.* report that following 48 hours of dialysis the earthworm extract retained full activity. Since the molecular weight cut off of the dialysis membrane is not reported this experiment does not add substantially to our knowledge of the size of the active molecule.

Using the known separation characteristics of the Sephadex gels used, Sheffield *et al.* were able to determine that the active ingredient is larger than 5,000 mol. wt. However, since active fractions eluted with a low molecular weight marker it is apparent that activity is not confined to a large molecular weight component. (In the separation using ion exchange material it is reported that the material has a large negative charge. However, this material was eluted from Dow X 50, a strong cation exchanger. The active material was eluted after retention on this cation exchanger. In order for this material to be retained it is necessary for it to be positively charged.)

These preliminary observations suggest that the component of EW that stimulates the vomeronasal organ may be a protein or protein complex. It is of some interest that this component is heat stable since commonly proteins denature upon prolonged heating. However, several recent reports [6, 10, 17] of naturally occurring chemical attractants indicate that they are also heat-stable. In addition, several heat resistant proteins have been described, among them amylase solution prepared from *Aspergillus oryzae* [7], dried renin [7] and Calpastatin [15].

ACKNOWLEDGEMENTS

This study was supported by National Institutes of Health Grant NS 11713. We thank Louise Scribani, Steve Heller, Barbara Mendelsohn for technical help and Rose Kraus for typing the manuscript.

REFERENCES

1. Bradford, M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* **72:** 248–254, 1976.
2. Burghardt, G. M. Stimulus control of the prey attack response in naive garter snakes. *Psychon Sci* **4:** 37–38, 1966.
3. Burghardt, G. M. Chemical perception in reptiles. In: *Communication by Chemical Signals*, edited by J. W. Johnston, Jr., D. G. Moulton and A. Turk. New York: Appleton-Century-Crofts, 1970, pp. 241–308.
4. Burghardt, G. M. and E. H. Hess. Factors influencing the chemical release of prey attack in newborn snakes. *J Comp Physiol Psychol* **66:** 289–295, 1968.
5. Burghardt, G. M. and C. H. Pruitt. Role of the tongue and senses in feeding of naive and experienced garter snakes. *Physiol Behav* **14:** 185–194, 1975.
6. Gleeson, R. A. and M. A. Adams. Chemical characterization of the sex pheromone of *Callinectes sapidus:* Initial results. *Book of Abstracts The Fourth Annual Meeting of the Association for Chemoreception Sciences*, 1982.
7. Haldane, J. B. S. *Enzymes*, Cambridge, Mass: MIT Press, 1965.
8. Halpern, M. and N. Frumin. Roles of the vomeronasal and olfactory systems in prey attack and feeding in adult garter snakes. *Physiol Behav* **22:** 1183–1189, 1979.
9. Halpern, M. and J. L. Kubie. Chemical access to the vomeronasal organs of garter snakes. *Physiol Behav* **24:** 367–371, 1980.
10. Kieber, D., D. Rittschoff and R. Shepherd. Effect of chemical and physical modification on the assay of an oyster drill chemotactic response. *Book of Abstracts The Fourth Annual Meeting of the Association for Chemoreception Sciences*, 1982.
11. Kubie, J. L. The role of the vomeronasal organ in garter snake prey trailing and courtship. Unpublished Ph.D. Dissertation, School of Graduate Studies. Downstate Medical Center, Brooklyn, New York, 1977.
12. Kubie, J. L. and M. Halpern. Laboratory observations of trailing behavior in garter snakes. *J Comp Physiol Psychol* **89:** 667–674, 1975.
13. Kubie, J. L. and M. Halpern. Garter snake trailing behavior: effects of varying prey extract concentration and mode of prey extract presentation. *J Comp Physiol Psychol* **92:** 362–373, 1978.
14. Kubie, J. L. and M. Halpern. The chemical senses involved in garter snake prey trailing. *J Comp Physiol Psychol* **93:** 648–667, 1979.
15. Murachi, T., K. Tanaka, M. Hatanaka and T. Murahami. Intracellular Ca^{2++}-dependent protease (Calpain) and its high molecular-weight inhibitor (Calpastatin). *Adv Enzyme Regul* **19:** 407–424, 1981.
16. Sheffield, L. P., J. M. Law and G. M. Burghardt. On the nature of chemical food sign stimuli for newborn garter snakes. *Commun Behav Biol* **2:** 7–12, 1968.
17. Sorensen, P. A chemical characterization of the fresh water attractants of the American eel. *Book of Abstracts The Fourth Annual Meeting of the Association for Chemoreception Sciences*, 1982.
18. Wang, R. T., J. L. Kubie and M. Halpern. Brevital sodium: An effective anesthetic agent for performing surgery on small reptiles. *Copeia* 738–743, 1977.
19. Wilde, W. S. The role of Jacobson's organ in the feeding reaction of the common garter snake, *Thamnophis sirtalis sirtalis* (Linn.). *J Exp Zool* **77:** 445–465, 1938.
20. Wright, A. H. and A. A. Wright. *Handbook of Snakes of the United States and Canada*. Ithaca, NY: Comstock Publishing Associates, 1957.