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ON THE NATURE OF CHEMICAL FOOD SIGN STIMULI FOR NEWBORN SNAKES

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ABSTRACT

The nature of substances that elicit prey attack behavior in garter snakes (Thamnophis sirtalis) was investigated. Warm water extracts of nightcrawlers (Lumbricus terrestris) would release attack when presented on a cotton swab to a hungry snake. The attack eliciting substances in these extracts were found to be non-volatile, to be stable under a wide variety of conditions and were characterized as large molecules (molecular weight of over 5000) by gel filtration and dialysis. It is proposed that these molecules are transferred from the surface of the prey to Jacobson's organ by direct contact via the tongue and that recognition of these molecules initiates the attack. Direct observation and use of an electronic counting device support this interpretation.

INTRODUCTION

The release of characteristic prey-attack behavior in newborn snakes bypresentation of surface extracts of organisms preferred by the species has been discussed by Burghardt (1967). In typical experiments with garter snakes (Thamnophis sirtalis) the food material (for example, earthworms, fish, frogs) was extracted with warm water and the clarified extract was presented on a cotton swab. The snake began flicking its tongue and often followed with an open-mouthed lunge at the swab. Newborn young of different species attacked different extracts. These differences were well correlated with the normal feeding patterns shown in the field and in captivity.

Although vision can play a role in the orientation of an attack response, it is not involved in the elicitation of attack in the naive snake (Burghardt, 1966; Burghardt and Hess, 1968). Of the sense organs available to snakes, it has been shown that Jacobson's organ, which opens into the anterior roof of the mouth, is most critically involved in the consummatory feeding act (Wilde, 1938; Naulleau, 1966). The tongue aids in the transfer of chemical cues from the environment to this organ.

In this paper we are concerned with a preliminary analysis of the chemical cues by which the snake recognizes its prey.

Subjects

The eastern garter snake (Thamnophis sirtalis) was used throughout the experiments. Usually young or adults of the subspecies (T. sirtalis semifasciata) found in the Chicago area were utilized. Young animals (under six months) were the preferred subjects but adults were used in the bioassay before the young became available. The young snakes were born to females captured in the field. All the snakes were housed individually; details are recorded by Burghardt (1967, 1968). Six adults and 40 young were involved in the experiments. Normally, a given test extract had to be presented only a few times to responsive snakes to determine whether it would elicit prey-attack behavior.

Methods and Results

We decided to concentrate our attention upon the active components in the surface substances of the nightcrawler (Lumbricus terrestris). This worm is readily eaten by garter snakes, is relatively large, and is available in large quantities from commerical dealers.

In order to follow active substances a semi-quantitative assay procedure based upon attack behavior was used. Materials to be tested were dissolved or dispersed in aqueous media and presented to the snakes on cotton swabs. The swab was held 2 centimeters in front of the animal's snout. If no attack occurred by the end of one minute the swab was removed and a negative response recorded. If an attack did occur its latency was noted.

Crude extracts were used as a standard of comparison for extracts treated in various ways. Distilled water controls were interspersed into a random series of test extracts at about every three trials. Water never elicits a prey-attack response in inexperienced snakes and only very rarely does so in experienced snakes (less than one percent of the time).

For the preparation of the extract, nightcrawlers were washed with cold water, blotted, placed in warm distilled water (300 grams of worms per liter of water) for 90 seconds and then removed. The extract was filtered and the clear filtrate used. To determine the optimal water temperature for maximum extract effectiveness, the following experiment was performed.

A series of eight extracts was prepared at ten degree intervals between 20 and 90 degrees centigrade. The subjects were 21 seven-day old unfed T.s. parietalis born to a snake captured near Iowa City, Iowa. The eight extracts plus a water control were presented once to each snake utilizing random orders. Each swab was presented a maximum of 30 seconds and approximately 20 minutes elapsed between tests for each snake. If no attack occurred after 15 seconds, the swab was touched gently to the snout, and then held in front of the snake for another 15 seconds or until an attack occurred. Figure 1 shows that the maximum number of attacks was given to the extract prepared at 60 degrees centigrade. Utilizing tongue flick rates in addition to the attack data in the manner described elsewhere (Burghardt, 1967) gave essentially the same result.

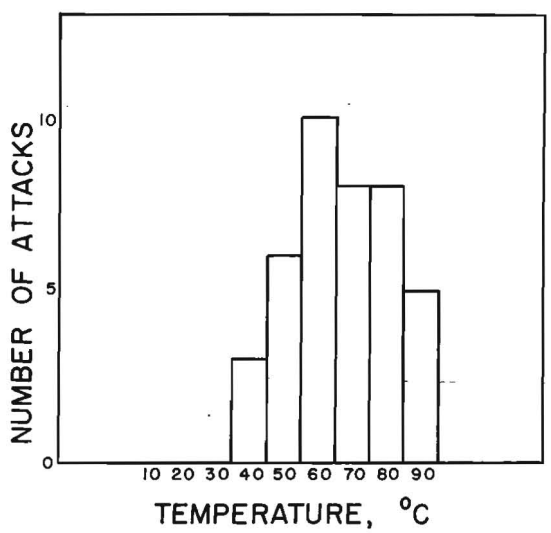

Figure 1. Profile of effectiveness of nightcrawler extract prepared at different temperatures.

The refrigerated extract retains its effectiveness for at least three days (Burghardt and Hess, 1968) but shortly thereafter it developed a most unpleasant odor and miscroscopic examination revealed the presence of innumerable bacteria. To carry out detailed examination of the active materials, storage of the extracts for considerable periods was desirable and it was found that frozen extracts retained activity for many months.

It was also found that nightcrawler extract could be concentrated to dryness by freeze-drying without appreciable loss of activity and this resulted in a more convenient storage method. A typical preparation yielded 300-600 milligrams of dry powder per liter of extract (dependent upon temperature of preparation). Reconstitution in water gave a solution which readily provoked attack by newborn as well as adult snakes. In this way, greatly concentrated solutions of the active materials could be obtained. These were often turbid, but they could be clarified by centrifugation with much active material remaining in the clear supernatant solution.

We now present the experiments performed to elucidate the nature of the chemical cues eliciting the pre-attack response in garter snakes. Since our main concern was with the chemical manipulations and because the bioassay employed gave basically a 'yes-no' answer, detailed presentation of the behavioral results is omitted. In all cases, a test solution was considered effective only if several snakes repeatedly responded to it.

We first investigated the possibility that volatile chemical compounds emanated from the prey and that these vapors were transferred by the snake's tongue to the sensing organ. If volatile substances were involved, then it should have been possible to distill them out of the active solutions or to extract them with solvents.

No active material could be removed from the active extract with petroleum ether or ethyl ether. After extraction, the aqueous phase retained full activity. In another experiment lyophilized powder was dispersed in a small volume of water and 10 volumes of methanol were added. The mixture was centrifuged and the supernatant solution was decanted and reduced to a small volume. The supernatant solution was found to have only very slight activity, while the insoluble precipitate could be dispersed in water and was shown to be active.

We attempted to distill out an active material in the following way. Lyophilized powder was placed in the bottom of a sublimation apparatus and a condenser surface was placed one centimeter above it. The condenser was cooled with solid carbon dioxide, while the powder was heated to 100 degrees centigrade. The apparatus was connected to a vacuum pump and the pressure was lowered to approximately 1 millimeter for three hours. At the end of this time the apparatus was opened and the materials washed from the surface of the condenser as well as the powder were tested. No activity was found in the condensate, but the powder remained fully active.

We began to doubt that the active material was a small, volatile material and we devised experiments to examine other possibilities.

When 260 milligrams dry powder were dispersed into 20 milliliters of water and placed in a dialysis sack, this solution could be dialyzed against 750 milliliters of water 48 hours without the release of any detectable active material into the dialysate. The material in the sack remained fully active. This indicated that the material was either a small molecule very tightly bound to a macromolecule or that the active material was macromolecular.

We reasoned that if the former were the case, it should be possible to dissociate the small molecule under appropriate pH conditions. For example, at high or low pH values protein molecules would be denatured or destroyed, allowing small bound molecules to dissociate. Other

charged macromolecules might behave similarly.

Extracts adjusted to pH 2 or 11 were found to be fully active. Furthermore, extracts made 0.01 molar in hydrochloric acid, or those made 0.01 or 0.1 molar in sodium hydroxide, allowed to stand for 1 hour at room temperature and returned to pH 7 were fully active. After treatment with acid or base, no active material could be extracted into organic solvents such as ether or pentane. In other experiments lyophilized powder was suspended in either 0.01 molar or 0.05 molar sodium hydroxide, the suspension was reduced to dryness, and the residue was subjected to heating under vacuum, as before, No active material could be trapped on a chilled condenser surface.

At this point it seemed possible that the active material might itself be macromolecular in nature, and experiments with gel filtration columns tended to confirm this notion. Sephadex gels (Pharmacia Fine Chemicals, Piscataway, New Jersey) were used in accordance with instructions supplied by the manufacturer. After swelling, the gels were de-gassed under vacuum and packed into colums 2.5 x 38 centimeters (bed volume, about 200 milliliters). In the first experiment, 180 milligrams of lyophilized powder were dispersed into 5 milliliters of water, centrifuged at 1000 x g for 15 minutes and blue dextran (Pharmacia Fine Chemicals) - a dyed high molecular weight dextran - was added to the supernatant solution for a marker. A portion of this sample (3.5 milliliters) was applied to Sephadex G-25 and the column was eluted with water. Fractions (10 milliliters) were collected and tested. Only fractions 6, 7, 8 and 9 were active and these all emerged with the blue dextran marker. This indicated a molecular weight in excess of 5000.

In a more refined experiment, dry powder was dispersed in water (10 to 15 milligrams per milliliter), using a Branson Sonified (Branson Sonic Power, Danbury, Connecticut). The dispersion was centrifuged at 100,000 x g for two hours. Both blue dextran and vitamin B-12 (red color - low molecular weight) were added for markers. This mixture was applied to a column of Sephadex G-200; the column was eluted with 0.1 molar sodium chloride and 5 milliliter fractions were collected automatically. Fractions 9-12 were blue, 29-34 were red. Active fractions were 10, 11, 19-31 and 33. Since some proteins are known to be adsorbed to blue dextran (Kass, Brock and Bloch, 1967), the experiment was repeated omitting this component. The elution profile of active material was similar. Because the sonifier treatment might have fragmented large molecules, another sample was dispersed without sonification, centrifuged and chromatographed. Again a similar elution profile was obtained.

We were now faced with the following paradox. When active materials were presented, snakes showed interest and after a variable number of tongue flicks they would attack. But how were they perceiving essentially non-volatile macromolecules at a distance. The attack-eliciting cues were not visual or olfactory since little difference in numbers of attacks was observed when the eyes, nostrils or both of inexperienced newborn snakes were covered with a carbon-black and collodian solution (Burghardt and Hess, 1968). We repeated this experiment twice with experienced young snakes with similar results. Although it might be thought that the tongue flick rate, as contrasted with prey-attack frequency, would be controlled by vision and olfaction, our swab technique showed no significant differences in either tongue flick rates or latency until the first flick. The comparison of fresh dissolved lyophilized powder yielded no sensory or behaviorally specific differences.

There was the possibility, however, that the snakes actually touched the swab (or prey) before the characteristic attack behavior was observed. In thousands of tests utilizing snakes of many species and extracts from many organisms, an attack was always observed to be preceded by at least one tongue flick. Samples were always within reach of the long tongue and in many

cases one could observe that the tongue appeared to touch the swab before an attack occurred. However, the tips of a snake's forked tongue are very fine (especially in a recently born individual) and beyond the resolution of a human eye in our testing situation. What had seemed to be part of a single pattern of released behavior could be considered as two separate phenomena - exploration of the environment and actual tongue contact with the prey. The first of these, involving frequent tongue flicking and orientation might be released by a number of stimuli, including visual, chemical, thermal and mechanical. The hungry snake explores whenever anything interesting happens. The second phase - open mouth prey-attack - would only be released when the proper food cue was transferred from the surface of the prey (or swab) to Jacobson's organ by the tongue. If actual tongue contact is necessary for cueing the attack, then the active material need not be a volatile small molecule; a macromolecule could serve as well.

In order to test this hypothesis we needed a method for determining if actual tongue contact usually or invariably preceded attack. Close observation, even with a magnifying glass was not completely convincing. Analysis of motion picture film taken at 24 frames per second was not satisfactory due to the lack of resolution and the quickness of the tongue flicks. The film did demonstrate, however, that the duration of the tongue's contact with an object could be less than 40 milliseconds. Rather than further perfect the film technique we employed a simple electronic device which allowed us to determine tongue contact through a completed electrical circuit. This apparatus was originally used for counting drops with a fraction collector. The completion of a circuit between two electrodes when a drop of solution entered the space between them was registered on a counter. The completed circuit caused an audible click. We connected one electrode to damp filter paper under the snake, and the other electrode connected to a lead embedded in the wet cotton swab on which the sample was presented. Resistors were used to adjust the current so that the snake would not receive a detectable shock. If an individual did receive a shock he would often cease responding to the swab. Repeated testing of many young garter snakes showed that invariably a tongue contact preceded an attack. We also used actual pieces of worms attached to the end of the lead with similar results. We inserted the thin copper wire lead through a piece of worm or wrapped the wire around the outside of the worm.

We are convinced that the chemical cues extracted from earthworms are for the most part non-dializable macromolecules which are physically transferred from the skin of the prey to Jacobson's organ via the snake's tongue. We have attempted further purification of these materials by use of ion exchange materials. For example, when applied to carboxymethyl Sephadex (Pharmacia - a cation exchanger) in 0.05 molar phosphate buffer pH 5.75, or to diethylaminoethyl Sephadex (an anion exchanger) in 0.05 molar phosphate buffer pH 7.5, the active materials did not bind to the exchanger. The active materials were also not bound to Dowex 1 (Dow Chemical Company, Midland, Michigan - an anion exchanger), but were retained by Dowex 50 (a strong cation exchanger). Active material could be eluted from Dowex 50 with 0.5 molar ammonium hydroxide solution. It seems possible that the active materials may bear net negative charges, but it is not possible to define their nature further at this time.

Discussion

It appears from our work that the substances which initiate attack behavior in garter snakes are non-volatile macromolecules on the surface of the earthworm. These macromolecules are readily removed by the snake's tongue from the moist skin of the earthworm and are passed into solution in warm water. Cool water does not free these materials from the mucus, and at high temperatures surface proteins would probably be denatured trapping the active materials in an insoluble matrix. This would explain the temperature profile for extracting active substances.

We do not exclude the possibility that there are volatile components which may have been lost during the extraction procedure (and have remained undetected). There may be a volatile material which stimulates exploratory behavior but this is a phase of the entire feeding behavior sequence which we have not investigated.

Feeding behavior in this species, then, appears to have at least three distinct but interrelated phases: 1) exploration of environment characterized by alert movements and tongue-flicking, 2) tongue contact with suspected prey (and Jacobson's organ) and 3) open-mouthed attack.

We certainly do not mean to imply that all snakes must touch all prey before attacking. Indeed, we know this is not true (e.g., Naulleau, 1966). Even the species tested here feeds upon frogs in nature and tongue contact would not always be feasible with such alert and quick moving prey. However, we have observed that newborn garter snakes always investigate their first food objects with the tongue. It is possible that conditioning might occur during ontogeny to allow 'stimulus substitution' of the classical type. More detailed hypotheses about how this might occur are reported elsewhere (Burghardt, 1968). Nonetheless, the dependency of even adult snakes upon the chemical senses is very great.

BIBLIOGRAPHY

Burghardt, G.M. Stimulus control of the prey attack response in naive garter snakes. Psychonomic Science, 4, 37-38, 1966.

Burghardt, G.M. Chemical-cue preferences of inexperienced snakes: comparative aspects. Science, 157, 718-721, 1967.

Burghardt, G.M. Comparative prey-attack studies in newborn snakes of the genus Thamnophis. Behaviour (in press).

Burghardt, G.M. and Hess, E.H. Factors influencing the chemical release of prey-attack in newborn snakes. J. Comp. Physiol. Psychol. (in press).

Kass, L.R., Brock, D.J.H. and Bloch, K. β -Hydroxydecanoyl thioester dehydrase. I. Purification and properties. J. Biol. Chem., 242, 4418-4431, 1967.

Naulleau, G. La biologie et le comportement predateur de Vipera aspis au laboratoire et dans la nature. These, P. Fanlac, Paris, 1966.

Wilde, W.S. The role of Jacobson's organ in the feeding reaction of the common garter snake, Thamnophis sirtalis sirtalis Lin. J. Exp. Zool., 77, 445-465, 1938.

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