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FEMALE-TO-MALE SEX PHEROMONES OF LOW VOLATILITY IN THE ASIAN ELEPHANT, *Elephas maximus*

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Abstract—In their natural ecosystems, the sexes of Asian elephants, *Elephas maximus*, live separately. For several weeks prior to ovulation, the urine and cervical mucus of female Asian elephants contain extractable chemical agents of low volatility that elicit a high frequency of flehmen responses from bull elephants as an integral part of mating. Subsequent to flehmen responses, male sexual arousal occurs and, if the female is available, mating results. During the course of our project to determine the agent(s) and describe the responses associated with female to male sexual communication, we have identified an unusual compound. This compound, apparently the sole component of the active fraction, was identified by mass, proton nuclear magnetic resonance, ultraviolet/visible, and infrared spectrometries as indolo-[2,1-b]quinazoline-6,12-dione (tryptanthrine). Exhaustive and repetitive bioassays established that pure authentic (synthetic) tryptanthrine was not the compound responsible for the bioresponse. Rather a coeluting minor component, also of low volatility, elicited the male bioresponse.

Key Words—*Elephas maximus*, Asian elephant, preovulatory pheromone, tryptanthrine indolo[2,1-b]-quinazoline-6,12-dione, novel substance response, estrus.

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INTRODUCTION

Urine from preovulatory female Asian elephants elicits a high frequency of flehmen responses, nonhabituating in nature, from Asian bull elephants (Figure 1) that are an integral part of the mating sequence. The presence of a sex pheromone is suggested by sexual arousal behaviors observed in the males including erection, mounting, and copulation. Initial extraction and fractionation procedures suggested the feasibility of purifying an active pheromone from the preovulatory urine (Rasmussen et al., 1982). However, extensive studies demonstrated that the active principal(s) was either nonvolatile or not detected by capillary column gas chromatography or gas chromatography–mass spectrometry (Rasmussen et al., 1986). Rather, under slightly acidic conditions (pH 4–6) organic solvent extracts of the preovulatory urine exhibited high biological activity (Rasmussen et al., 1982, 1986). Separation procedures were coupled to a quantitative and standardized bioassay using this high frequency (3–35/hr) of flehmen responses by a free-roaming bull elephant who detected randomly placed samples during a 1-hr test session (Rasmussen et al., 1982, 1986). By a series of normal-phase high-performance liquid chromatography (HPLC) fractionations, an active fraction was obtained that exhibited a single HPLC band. This bioactive sample was examined by mass spectrometry (MS), nuclear magnetic resonance spectrometry (NMR), ultraviolet/visible (UV/VIS) spectrometry and Fourier transform infrared spectrometry (FTIR) (Rasmussen and Lee, 1991), and the principal component was identified.

METHODS AND MATERIALS

Separation and Purification Methods. Urine was collected from eight mature female Asian elephants during the appropriate preovulatory days as determined by measurement of serum progesterone concentrations, by assessment of cervical mucus, and by monitoring daily the responses of bulls to cows (Hess et al., 1983).

The active sample was separated and purified from the pre-ovulatory urine as outlined in Figure 2. Preovulatory urine was extracted with dichloromethane using four 5-liter capacity liquid–liquid extractors. The 1000-fold concentrated organic solvent extract (100 liters to 100 ml) was then fractionated by flash chromatography using EM silica gel 60, particle size 0.040–0.063 mm (Rasmussen et al., 1982; Still et al., 1978); the activity was localized in a blue-colored fraction. Further separation by reverse-phase HPLC resulted in the loss of the majority of the bioactivity. Normal-phase HPLC (Figure 2) has proved to be a more reliable technique for fractionation. We used an as-yet-unidentified blue compound present in the urine as an effective marker to standardize fractions because of the variability encountered in retention times during normal-

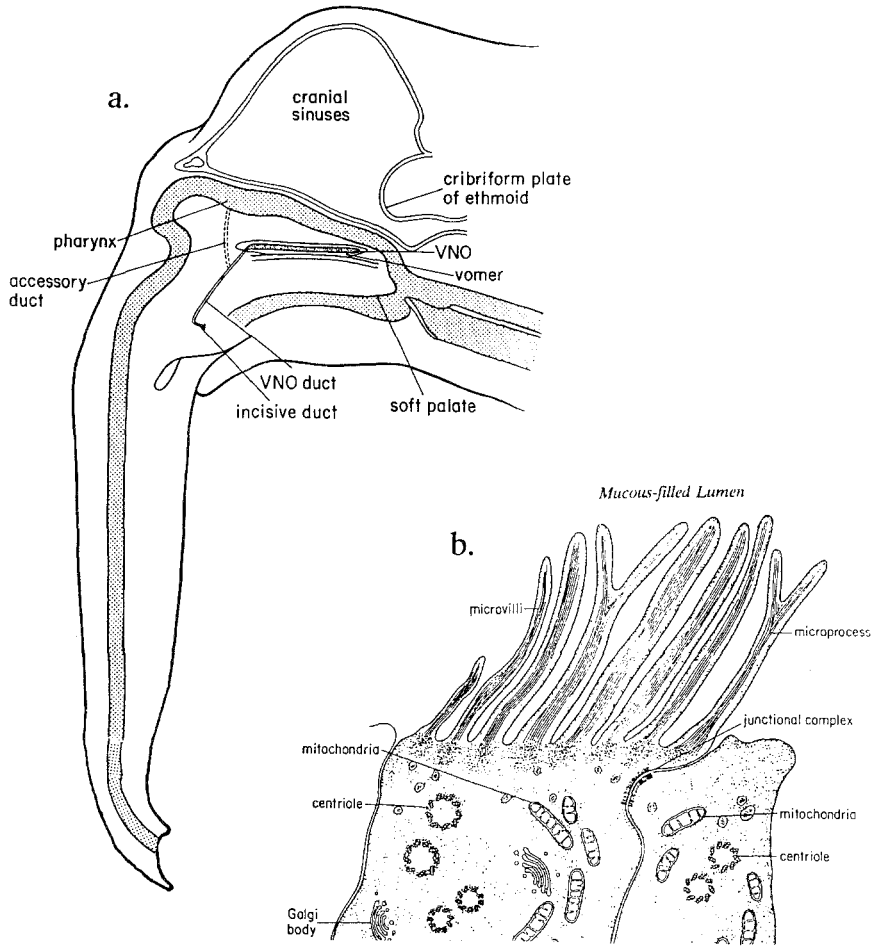

FIG. 1. The flehmen response: (a) Initial olfactory detection is accompanied by audible aspirations and exhalations, followed by placement of the dorsal trunk tip finger in the sample odorant. At the trunk tip mucus from the trunk is mixed either with urine that contains mucus and perhaps pheromones, or with samples. The superficial dermis of the tip contains many free nerve endings and unusual multiinnervated corpuscles (Rasmussen and Munger, 1990). The wetted tip is precisely placed for 1–10 sec on the paired openings of the vomeronasal organ ducts. This is termed a flehmen response. (b) The pheromones apparently reach the central mucus-filled lumen by transiting through the mucus-filled vomeronasal organ ducts, contacting microprocesses of receptor cells where signal transduction is apparently initiated.

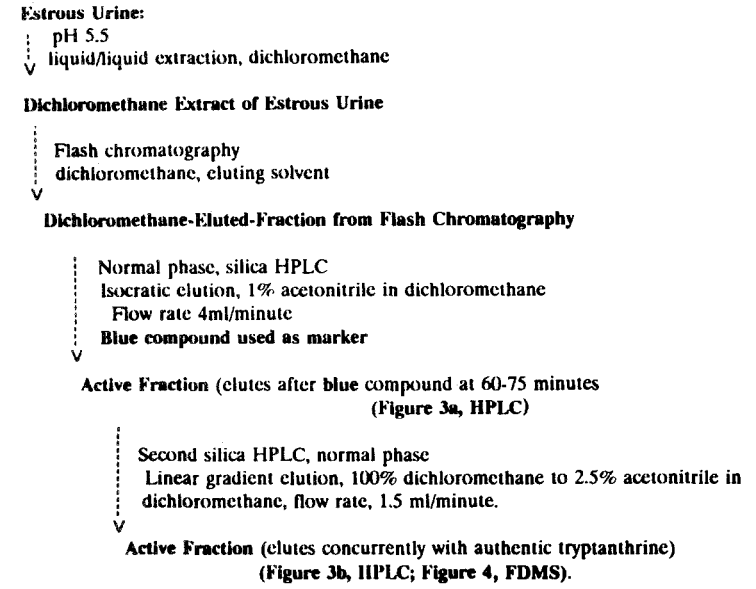

FIG. 2. The extraction, fractionation, and purification methodology is outlined.

phase HPLC. Marker use has also allowed reduction in the number of steps for purification, allowing minimal exposure to oxidants and reducing the potential for contamination associated with the use of large solvent volumes.

The first of two successive normal-phase HPLC separations was carried out on a Whatman microparticulate silica gel, Partisil 10 (Magnum 9) normal-phase 25-cm × 2.5-cm column employing isocratic elution using 1% acetonitrile in dichloromethane. The flow rate, 4 ml/min, was maintained using Waters model 501 dual-piston pumps. A dual-wavelength absorbance detector (Waters 440) set at either 225 or 280 and 340 nm, and a variable wavelength absorbance detector (ISCO) were utilized. The second HPLC separation, using a Partisil 10 analytical column 25 × 1 cm, employed a linear gradient system from 100% dichloromethane to 2.5% acetonitrile in dichloromethane at 1.5 ml/min, programmed by the Waters Data Module 740.

Structural Characterization. The active fraction obtained from the chromatography depicted in Figure 3b was analyzed by infrared, ultraviolet, ^{1}H-proton nuclear magnetic resonance, and mass spectrometries. Both field desorption (FD) and electron ionization (EI) mass spectra were obtained using a Jeol HX100HF mass spectrometer operating at 5-kV acceleration potential and a nominal resolving power of 500 for FD and 5000 for EI spectra. For FD spectra, the sample was dissolved in methanol and the sample loaded onto activated carbon emitters. Spectra were collected over the range of m/z 0–1000 as the

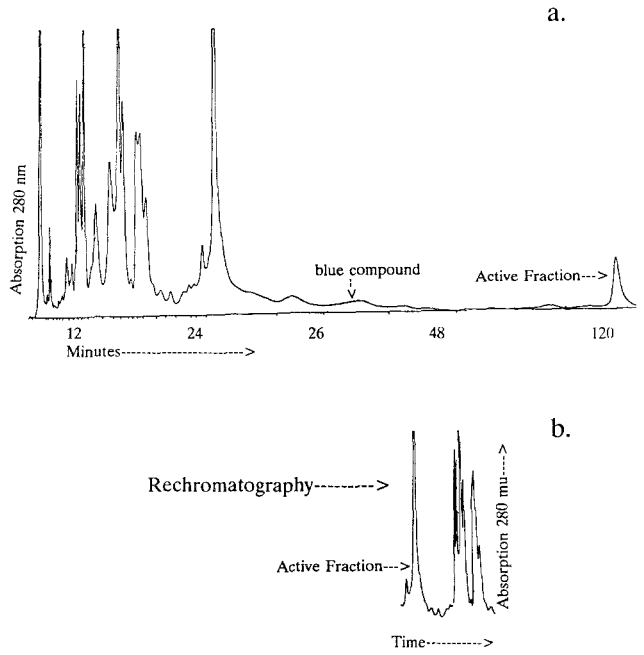

FIG. 3. (a) Initial high-performance liquid chromatography fractionation. Isocratic elution with 1% acetonitrile in dichloromethane. The active fraction eluted at 120 min (x axis) subsequent to a blue marker compound. Absorption at 280 nm is indicated on y axis. (b) Rechromatography by HPLC using gradient elution system, 100% dichloromethane to 2.5% acetonitrile in dichloromethane. The active fraction elutes in the yellow fraction prior to several inactive components. The ordinate represents absorbance at 280 nm.

emitter heating current was ramped from 0 to 25 μamp. For EI spectra, the sample was placed on the outside of a short melting point capillary and inserted into the 150°C ion source using the direct insertion probe. A 70-eV electron beam was used for sample ionization. The reference compound PFA (Pierce) was used as the reference standard for the exact mass measurements.

Infrared spectra were obtained using a Perkin-Elmer 1800 Fourier transform infrared spectrophotometer. Samples dissolved in dichloromethane were spread on a 25-mm-diameter, 4-mm-thick sodium chloride crystal window and dried before scanning from 650 cm^{-1} to 4000 cm^{-1}. Appropriate blanks and controls were scanned.

Ultraviolet/visible (UV/VIS) spectra were obtained using a Perkin-Elmer Lambda-9 spectrophotometer, scanning from 180 nm (UV) through 900 nm (VIS). Samples from HPLC fractionation were dried and dissolved in methanol

or acetonitrile. Appropriate solvent blanks and adjacent chromatographic fractions were scanned as controls. Samples were scanned at several dilutions.

One-dimensional and two-dimensional ^1H NMR spectra were obtained with a Bruker AM-500 spectrometer with a deuterochloroform solution using tetramethylsilane as the internal reference.

Bioassay. The flehmen response (a motor pattern exhibited specifically only during the sensory evaluation of chemicals) by the Asian bull elephant meets the specificity and quantitative requirements for a reliable bioassay. It clearly is a discrete, all-or-none response that can be quantified. The bull elephant apparently utilizes this response not only to detect the presence of a cow elephant and to determine her estrous state, but definitive sexual behaviors occur after the high frequency of flehmen. This high frequency of male flehmen responses has, for a number of years, served as a guide to the fractionation procedures during the purification of a bioactive component (Rasmussen et al., 1986). By testing separated fractions, the bioresponsive region was located and the entire fractionation sequence monitored by bioassays. The distinguishable response involves the vomeronasal organ (Rasmussen and Hultgren, 1990) whose chemoreceptive cells apparently respond to substances of low volatility (Figure 1). The nonhabituating nature of the bull's response in successive bioassays made this flehmen response an invaluable bioassay tool, especially during the long-term purification protocol.

Standard bioassay units were developed (Rasmussen et al., 1986). One bioassay unit was set at a 500-ml equivalent of preovulatory urine demonstrated to elicit seven flehmen responses per hour. The utilization of these standard units during each bioassay step ensured a definitive bioassay result to guide the continued purification.

The procedures for bioassay at the test site, the Washington Park Zoo (WPZ), Portland, Oregon, and the elephants employed have been described in detail in Rasmussen et al. (1982, 1986). Four bulls (between 8 and 30 years old) were available for bioassays several times a week on a random rotation basis. (Three were experienced breeders, but no differences in response frequency was observed among the four bulls.) Samples were presented in 250 ml of appropriate buffered solutions at approximately physiological amounts, estimated from the bioactivity of starting urine and based on calculated bioassay units. The focal-animal sampling technique (Altman, 1974) was employed during a 60-min observation period.

A wide variety of appropriate controls have been tested over the years (Rasmussen et al., 1982, 1986). For the present report, during every bioassay, control urine and the actual extracting solvents were tested. When relevant, other synthetics or combinations of synthetics were also bioassayed. The bioassay had a built-in control; the bulls were routinely let into the exercise yard without any bioassay samples being placed.

Flehmen responses of moderately high frequency (between three and six responses per hour) in the initial bioassay, which decrease to zero with subsequent tests (by test 3 or 4), have been termed novel substance responses (Rasmussen et al., 1986). These responses are generally observed when an elephant is tested with compounds or mixtures that have not been previously encountered either singly or in a particular combination; these components may or may not be naturally occurring compounds in elephant secretions or exudates. Because of this phenomenon, it is necessary to conduct repetitive testing (three to five trials) so that false positives that could result from novel substance responses can be reliably distinguished from sustained positive responses elicited by compounds with apparent biological meaning. Negatives may also require several bioassays to be certain the negativity has not resulted from insufficient concentration (Rasmussen et al., 1986). The standardized bioassay unit utilized was significantly higher than either control, baseline, or novel substance response levels.

Bioassay of Authentic Tryptanthrine. When tryptanthrine was identified as the principal component of the active elephant preparation, we carried out a systematic series of bioassays to determine whether tryptanthrine was responsible for the observed behavioral response. Authentic tryptanthrine (obtained from Aldrich; tested both as received and repurified) and tryptanthrine isolated from elephant preovulatory urine were assayed in replicate tests. Commercially available tryptanthrine was bioassayed at concentrations ranging from 1 ng to 100 mg/250 ml of bioassay media. A wide variety of conditions were employed during bioassays, both singly and in combinations. These included variations in concentration, the use of diverse solubilizing agents including dichloromethane, methanol, or acetone (necessary because of solubility characteristics of tryptanthrine), and the employment of buffers or control (non-estrous) urine. Various temperatures (ranging from 20°C to 60°C), pHs, and mixing effects such as stirring and sonification were also tested.

Preparation and Bioassays of Controls. Nonestrous female urine (100 liters), male urine (50 liters), and the three major food substances, timothy hay, carrots, and grain, were extracted and separated by the same methodology employed for the purification of the active component from estrous urine. We examined nonestrous urine and male urine for tryptanthrine and for artifacts of extraction. The food sources were examined as possible sources of tryptanthrine. In one experiment, 10 liters of estrous and nonestrous urine and a day's collection of cervical mucus were isolated and compared. We extracted the amount of carrots and grain equivalent to a one-day food ration and the amount of hay equivalent to one fourth of a daily ration. Aliquots of these preparations were "spiked" with authentic tryptanthrine to ensure the precise localization of the tryptanthrine fraction. All extracts and fractions were examined for tryptanthrine using analytically HPLC, TLC, FD-MS and UV spectrometries.

Cervical Mucus. Cervical mucus was obtained from the urogenital tract anterior–dorsal to the ureter opening using a specially modified colonscope.

RESULTS

Purification of Active Fractions by HPLC. Our methodology of extraction, flash chromatography, and successive HPLCs, has yielded active fractions with good run-to-run reproducibility and reasonable recovery rates (Table 1).

The activity was localized in a blue fraction obtained from flash chromatography. In the first normal-phase HPLC separation (Figure 2), using 1% acetonitrile in dichloromethane as the eluting solvent, the majority of inactive material eluted first, followed by the inactive blue compound, and finally a late-eluting discrete band containing the bioactivity (Figure 3a). The area of this band was 1–3% of the material (based on UV spectral data) fractionated during this chromatographic separation. The second HPLC separation, using a gradient system from 100% dichloromethane to 2.5% acetonitrile in dichloromethane (Figure 2), resulted in the localization of the bioactivity in a discrete band, yellow in color (Figure 3b). The active fraction was a single peak by HPLC (Figure 3b), a single band by TLC, and gave a single prominent ion by field desorption mass spectrometry (Figure 4).

TABLE 1. PROGRESSIVE PURIFICATION

Fraction	Solutes in bioactive fraction/liter urine	Bioresponse retained (%)
Whole urine	50 g	
Solvent extract	41 mg	90
Flash chromatography	6 mg	80
First HPLC	600 μg	65
Second HPLC	50 μg	50

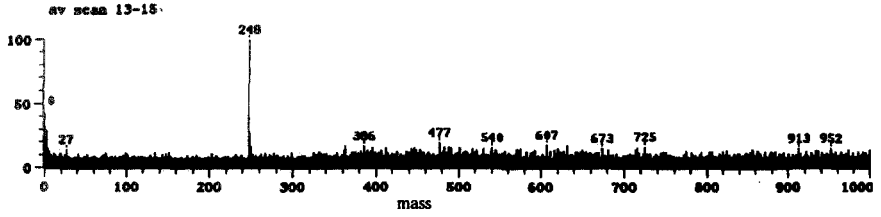

FIG. 4. Field desorption mass spectrum of the active peak indicated in Figure 3b, scans 13–15. Intensity is indicated on *y* axis and mass on the *x* axis.

Assays demonstrated that the active material was effective at microgram or lower levels, but large starting volumes (100 liters) of the chemically complex preovulatory urine were required to provide sufficient material to support the chemical characterization. One hundred liters of urine, in a scaled-up extraction scheme, were required to obtain 2 mg of tryptanthrine.

Identification of Tryptanthrine in Purified Active Fraction. High-resolution EI-MS of the principal component yielded an exact mass for the molecular ion of 248.056, corresponding to a molecular composition of $C_{15}H_8N_2O_2$. Ions at *m/z* 220 and 192 in the spectrum (Figure 5) suggested the sequential loss of two carbonyl groups. The FTIR spectra also indicated two different carbonyls at 1726 cm^{-1} and 1691 cm^{-1}.

Definitive ^1H NMR spectrometry indicated eight aromatic hydrogens (Fig-

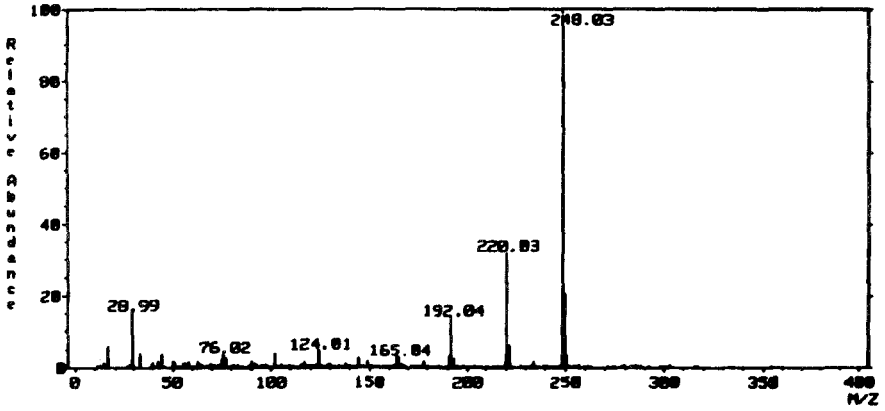

FIG. 5. Electron ionization mass spectrum of the active peak indicated in Figure 3b. Relative abundance is indicated on the ordinate and *m/z* on the abscissa.

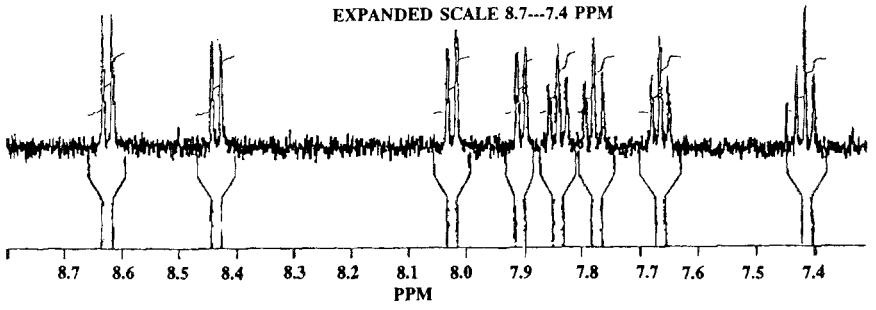

FIG. 6. ^1H nuclear magnetic resonance spectrum of the active peak indicated in Figure 3b. Eight aromatic hydrogens are indicated.

ure 6), assigned on the basis of coupling observed in the 2-D spectrum as hydrogens of two ortho-disubstituted aromatic rings. These spectral data and corresponding infrared and ultraviolet spectra suggested the structure indolo [2,1-b]-quinazoline-6,12-dione (tryptanthrine) (Figure 7), or a closely related isomer. Comparison of the spectral data obtained from the isolated material with the spectra of an authentic sample established unambiguously that the elephant urine isolate is tryptanthrine. Authentic tryptanthrine and tryptanthrine purified from the female urine coeluted when mixed together and fractionated by analytical HPLC.

Bioactivity of Purified Active Elephant Preparations and Authentic Tryptanthrine. We have assayed more than 10,000 samples of preovulatory urine or its fractions during 1527 bioassay sessions. A mean of 14.5 ± 0.7 flehmen/hr was recorded during 310 tests of 1 liter of preovulatory urine and 0.6 ± 0.5 flehmen/hr during 850 tests of anestrous urine. A illustrative bioassay pattern observed during the purification, representative of 20 different preparations, is delineated in Table 2. At each step in purification, 1 liter-equivalent (2 units) was used for bioassay; the other 9 liter-equivalents were used for the chemical purification. Therefore half the starting material was used for bioassays during the chemical isolation procedures.

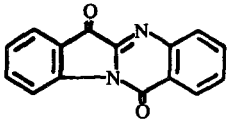

FIG. 7. Structure of tryptanthrine, indolo[2,1-b]-quinazoline-6,12-dione, isolated from preovulatory elephant urine.

TABLE 2. BIOASSAY EFFECTIVENESS DURING FRACTIONATION FROM TEN LITERS OF URINE

Fraction	Units used for bioassay[a]	Flehmen responses/hr
Preovulatory urine		
10 liters	2[b]	14
Solvent extract	2	12.5
Flash chromatography	2	11
First HPLC	2	9
Second HPLC	2	6

[a] One bioassay unit = 7 flehmen responses/hr.
[b] An average liter of active estrous urine contains 2 bioassay units based on bioassays of 500 ml of urine.

When tryptanthrine was identified as the component that was dominant in the active "pure" elephant preparation, we carried out a systematic series of bioassays to determine if indeed the synthetic, authentic tryptanthrine was the responsible pheromone. Flehmen responses by Asian bull elephants could be categorized into five quantitative divisions (Table 3). The negative category included compounds such as 4-*n*-propylphenol, a natural component of elephant urine (Rasmussen et al., 1986); successive tests over months invariably were negative. The elephant fraction was positive during repetitive bioassays. Novel substances, such as compounds isolated from estrous urine (2-*n*-propylphenol) or new mixtures, elicited low, moderate, or high frequency responses on the first one to three bioassays and then diminished to zero. Of the hundreds of compounds bioassayed, only acetic acid showed a consistent pattern of low-level responses. Responses to tryptanthrine, after the third presentation, revealed a lower (one to three) frequency of flehmen responses per hour compared to those observed during tests 1–3 (Table 3). The low-level response to tryptanthrine persisted over several months. After six months, subsequent tests at monthly intervals failed to elicit any bioresponses from any of the WPZ bulls.

A comparison was made between the flehmen responses to equal amounts of elephant preparation and authentic tryptanthrine during 60 bioassay sessions (5–10 samples per session). These tests involved four bulls at Washington Park Zoo and one bull at Taronga Zoo (Sydney, Australia). Tested aliquots of the elephant preparation (containing tryptanthrine) resulted in a dose response dependent on concentration (Table 4). Such results were not obtained for synthetic tryptanthrine. The synthetic only elicited responses during the first several trials and the frequency was not affected by concentration.

TABLE 3. FLEHMEN RESPONSES PER HOUR[a]

Category	Trial 1–3	Trial 4–10	Trial 10–30 (3–4 months)	Trial 30–40 (6 months)
Negative, 4-*n*-propylphenol	0	0	0	0
Positive, elephant fraction (one unit)	5–7	5–7	5–7	5–7
Novel substance, 2-*n*-propylphenol	4	0	0	0
Constant low level, acetic acid	2	2	2	2
Prolonged, tryptanthrine	4	1–3	1	0

[a] Four bulls were tested in 30–40 trials each. Except for the ranges indicated, the results were identical for these bulls.

Possible Sources of Tryptanthrine. We were not able to detect tryptanthrine in 100 liters of nonestrous urine or in male urine or in the three principal food sources. All HPLC peaks in the near-tryptanthrine eluting regions were monitored by UV spectrometry. No ultraviolet/visible spectra characteristic of tryptanthrine were observed.

Bioactivity in Peak Eluting prior to Tryptanthrine. Both the elephant urine preparations subsequent to two HPLC purifications and the tryptanthrine derived from elephant estrous urine were biologically active. During the past three years these preparations consistently always elicited more than five flehmen responses per hour. In contrast, in tests involving synthetic tryptanthrine the response eventually diminished to zero. Subsequently, we were able to obtain repurified, elephant-prepared tryptanthrine that no longer elicited bioresponses, whereas a newly separated, pretryptanthrine eluting peak elicited multiple flehmen responses (Figure 8). This substance has a characteristic ultraviolet absorption spectrum. As assessed by its absorption maxima at several wavelengths, its

TABLE 4. DOSE RESPONSE[a]

Absorption at 251 nm	Flehmen responses/hr
0.5	5
1.0	8
2.5	13

[a] One aliquot of HPLC-purified elephant preparation per milliliter; sample bioassayed was 10 aliquots.

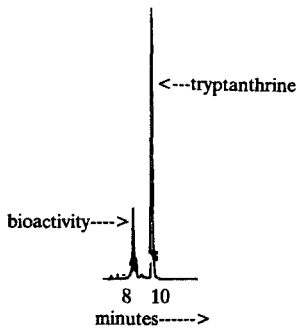

FIG. 8. Rechromatography of tryptanthrine purified from elephant estrous urine (active peak, in Figure 3b). The eluting solvent, 10% hexane in dichloromethane, was employed in the isocratic mode. Tryptanthrine is the large peak on the right. The smaller peak on the left contains the bioactivity. Absorbance at 220 nm is on the *y* axis; time is indicated on *x* axis.

concentration may be only about 1/100th that of tryptanthrine. We are diligently obtaining sufficient quantities of this low-volatile, active material for chemical and structural identification.

DISCUSSION

There is a paucity of chemical knowledge about specific pheromones operational prior to or during mating in animals of high intelligence. We have isolated from the chemically complex urine of elephants a highly bioactive fraction. Such isolation was accomplished using a highly reliable bioassay to guide the chemical fractionation.

The principal constituent of the highly concentrated active fraction was a component, indolo[2,1-b]-quinazoline-6,12-dione (tryptanthrine), lacking bioactivity. Interestingly, we were unable to detect tryptanthrine in nonestrous female urine or male urine. Tryptanthrine has previously been found only in plants such as the cannonball tree *Couroupita guaianensis*) (Nelson and Wheeler, 1937) and yeasts *Candida* sp. (Bergman et al., 1977; Bird, 1963; Friedlander and Roschestwensky, 1915). We did not find tryptanthrine in the three major food items of the elephants, although it could be present at very low levels. To our knowledge, this is the first time this compound has been reported in any animal.

The bioassay of synthetic tryptanthrine, the apparently sole component of the active purified elephant preparation, and its negativity in bioassays after its low-level positivity demonstrated the necessity of extensively testing authentic compounds after their identification in an apparently homogeneous active fraction. The prolonged novel substance response we have observed is intriguing. Perhaps during the experiments to isolate the active pheromone, which was in the same fraction as tryptanthrine, the bull learned to associate tryptanthrine with the pheromone, or with other novel compounds, or possibly tryptanthrine is part of an active sex pheromone set.

Our results indicate that: (1) tryptanthrine is the dominant molecule in the active, low-volatile fraction from the preovulatory urine, (2) tryptanthrine is inactive in bioassay and is not a singly active pheromone, and (3) the bioactive, tryptanthrine-containing elephant preparation contains a minor, coeluting substance that is bioactive.

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