Chemical isolation of a female urinary chemosignal which elicits ultrasonic vocalizations in male mice (mus musculus)

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CHEMICAL ISOLATION OF A FEMALE URINARY CHEMOSIGNAL WHICH ELICITS ULTRASONIC VOCALIZATIONS IN MALE MICE (MUS MUSCULUS)

by

Jason Diamond

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# Table of Contents

Chemical Isolation of a Female Urinary Chemosignal Which Elicits
Ultrasonic Vocalizations in Male Mice (*Mus musculus*)

<table>
<thead>
<tr>
<th>Abstract</th>
<th>1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chapter I: Introduction</td>
<td>2</td>
</tr>
<tr>
<td>Previous Attempts to Isolate Priming Pheromones</td>
<td>6</td>
</tr>
<tr>
<td>Puberty-accelerating Male Urinary Chemosignal</td>
<td>7</td>
</tr>
<tr>
<td>Pubert-delays Female Urinary Chemosignal</td>
<td>12</td>
</tr>
<tr>
<td>Pregnancy-blocking Male Urinary Chemosignal</td>
<td>14</td>
</tr>
<tr>
<td>Estrus-synchronizing Male Urinary Chemosignal</td>
<td>17</td>
</tr>
<tr>
<td>Gonadotrophin-secretion Female Urinary Chemosignal</td>
<td>19</td>
</tr>
<tr>
<td>Similarities in the Nature of Priming Pheromones</td>
<td>21</td>
</tr>
<tr>
<td>Previous Attempts to Isolate Releasing Pheromones</td>
<td>22</td>
</tr>
<tr>
<td>Aggression-promoting Male Urinary Chemosignal</td>
<td>22</td>
</tr>
<tr>
<td>Aggression-reducing Female Urinary Chemosignal</td>
<td>26</td>
</tr>
<tr>
<td>Ultrasound-eliciting Female Urinary Chemosignal</td>
<td>28</td>
</tr>
</tbody>
</table>

Chapter II: Methods and Results

Isolation of the Ultrasound Eliciting Female Urinary Chemosignal

General Methods | 31
Animals | 31
Apparatus | 32
Procedure | 32
Social Experience | 33
Behavioral Screening | 33
Stimulus Collection | 34
Chromatographic Procedure | 34
Abstract

A series of experiments were conducted to isolate and characterize a urinary pheromone produced by female house mice (*Mus musculus*) that elicits ultrasonic vocalizations from males. Results obtained from Sephadex G-10 chromatography suggested that the active component of the pheromone had a molecular weight of near 700 daltons, while results from Sephadex G-15 chromatography indicated a molecular weight of less than 1500 daltons. Thus, the pheromone would appear to have a molecular weight in the range of 700 to 1500 daltons. Such a molecule would be large enough to be nonvolatile, but small enough not to be a major urinary protein (MUP). However, MUP's may play a role in the pheromonal elicitation of ultrasounds.

Comparison between my findings and those obtained in attempts to isolate the aggression-reducing pheromone and the pheromone causing luteinizing hormone release are consistent with the hypothesis that both these other pheromones contain the same or similar active molecule(s) as the pheromone that elicits ultrasonic vocalizations.
Chapter I: Introduction

Chemical Isolation of a Female Urinary Chemosignal Which Elicits Ultrasonic Vocalizations in Male Mice (*Mus musculus*)

Successful means of communication between the sexes is important for successful cohabitation and reproduction. Communication has been defined as "involving the giving off by one individual of some chemical or physical sign, that, on being received by another, influences its behavior" (Frings and Frings, 1964). This type of communication occurs throughout the Plant & Animal Kingdoms. From molds to humans, the communication systems become more elaborate as the species becomes more complicated.

One method of chemical communication involves the use of pheromones. The term "pheromone" was originally proposed for exogenous chemosignals; "secreted to the outside by an individual and received by a second individual of the same species, in which they released a specific reaction, for example, a definite behavior or a developmental process" (Karlson & Luscher, 1959). Pheromones are known to occur in animals ranging from single-celled organisms to certain primate species.

The initial investigations of pheromones utilized insects (Karlson & Luscher, 1959). From this research, pheromonal communication became associated with the stimulation of the fixed action patterns observed in insects. However, pheromonal studies in mammals have shown that pheromone stimulated behaviors are more complicated than originally thought. In fact, the behavioral responses of mammals to pheromones
appear quite variable and are often susceptible to classical conditioning (Bronson, 1968).

Odor (pheromonal) communication guides and influences almost every mammalian behavior. There are numerous advantages associated with chemical communication. First of all, odors can be used in situations where other senses can not be used due to environmental and/or organismal constraints. Secondly, odors provide information long after an animal has deposited the chemical signal. Thus the odor provides safety to the signaling animal in addition to providing information to other organisms present in the area. Lastly, animals do not need to be in close proximity to communicate. Proximity becomes relatively more important if the animals use either visual or auditory communication.

A number of schemes have been proposed to classify pheromones based upon the types of behaviors they elicit. The classification scheme that has gained the greatest acceptance classifies pheromones as either releasing or priming. Chemical signals which elicit immediate and specific behavioral responses are designated releasing pheromones. In contrast, priming pheromones induce gradual physiological changes which may or may not elicit overt behavioral changes. This scheme is broken down even further according to the type of behavioral and/or physiological response elicited.

However, a recurrent theme for both types of pheromones (releasing and priming) is the coordination of reproductive functioning. Numerous priming and releasing pheromones have been identified in the house mouse (Mus musculus). Priming pheromones cause the Bruce, the Vandenbergh, the Lee-Boot, and the Whitten effects. The Bruce effect occurs when a female, exposed to the urine produced by "strange" males
terminates her pregnancy (Bruce, 1959). The Vandenbergh effect involves the acceleration of female puberty in the presence of male or male odors (Vandenbergh, 1983). The Lee-Boot effect is the disruption of the normal estrus cycle resulting in anesterous or in the development of pseudopregnancies in the absence of males or male odors (Lee & Boot, 1955). The Whitten effect involves the promotion of regular four or five day estrus cycles in female mice in response to male urine (Whitten, 1956). Releasing pheromones in house mice elicit attraction and courtship (Bronson, 1971), (Whitney et al., 1974), (Nyby et al., 1979), aggression (Mugford & Nowell, 1970), and territorial behavior (Jones & Nowell, 1973).

Although the influences of male produced pheromones on female physiology and behavior have been well documented, the effects of female pheromones on males are less studied. In mice, urinary odors from females elicit male ultrasonic mating vocalizations (Nyby, Wysocki, Whitney, & Dizinno, 1977), promote male sexual behavior (Dixon & Mackintosh, 1975), and induce surges in both the luteinizing hormone (LH) (Clancy et al., 1984) and testosterone (T) (Bronson, 1982).

Early experience is important in determining adult responses to different chemosignals in rodents. Early experience is a contributing factor in marking behavior (Clancy et al., 1984; Maruniak et al. 1986), ultrasonic vocalizations (Nyby et al., 1978), and in adult preference for certain odors (Fillion & Blass, 1986).

The volatility of a chemosignal determines which chemoreception system will play the important role in perception. Since the main olfactory system is primarily responsible for the detection of volatile stimuli and the vomeronasal system for nonvolatile stimuli, the two different chemoreception systems appear to serve different functions. Nyby (1983)
has postulated that long range airborne attractants produced by females are perceived by the main olfactory system. However, once the male reaches the source, relatively non-volatile water-soluble components can be perceived by the vomeronasal system which elicit specific behavioral and physiological responses. Thus, responses to mammalian chemosignals can be mediated by either the main olfactory system, the vomeronasal/accessory olfactory system, or by an interaction of the two.

The vomeronasal organ has been suggested to be responsible for the perception of many pheromones. The vomeronasal system is functionally and anatomically distinct from the olfactory system and has direct neural projections into reproductive areas of the brain. Therefore, it should not be surprising that it's inputs influence reproductive behavior and physiology.

The male and female vomeronasal organ is clearly important in maintaining many behaviors influenced by chemosignal perception. Vomeronasal organ removal eliminates the Vandenbergh effect (Kaneko et al., 1980), prevents the pregnancy block effect (Bruce effect) (Bellringer et al., 1980), reduces the latency for virgin female mice to show maternal responses to pups (Fleming et al., 1979), disrupts mating behaviors in male hamsters (Powers and Winans, 1973), decreases aggressive behavior (Clancy et al., 1984), and disrupts male 70 kHz vocalizations to females (Bean, 1982).

To better understand pheromones, numerous investigators have attempted to chemically isolate them. Research examining insect pheromones has advanced greatly since the isolation and chemical identification of bombykol, the silkworm attractant and the first pheromone to be characterized (Butenandt, 1963). Today over 800 different insect pheromones have been identified and isolated. Aided by the evolution of
microchemical techniques, such as spectrometric and chromatographic techniques, the chemical investigations of mammalian pheromones also have advanced, although at a considerably slower pace.

The isolation techniques used most often are extraction via organic solvents, gas-liquid chromatography, high performance liquid chromatography, thin-layer chromatography, dialysis filtration, and sephadex chromatography. Once molecules have been isolated, spectrographic techniques such as mass spectroscopy, nuclear magnetic resonance spectroscopy, infrared spectroscopy, and ultraviolet spectroscopy can be used to correctly identify the biologically active components.

**Previous Attempts to Isolate Priming Pheromones**

Attempts to isolate priming pheromones of mice have been reported throughout the literature. Due to the ease of collection and quantification, urine has been the most studied source of pheromonal signals. Priming pheromones that have been partially isolated are;

1) the components of male mouse urine responsible for the puberty accelerating effect on female mice [the Vandenbergh Effect] (Vandenbergh, 1976),
2) components of female mouse urine responsible for the puberty delaying effect on other females (Drickamer, 1977),
3) the components of male mouse urine responsible for inducing estrous cyclicity in female mice [the Whitten Effect] (Jemiolo, Harvey, and Novotny, 1986),
4) the components of male mouse urine responsible for blocking pregnancy in recently inseminated females [the Bruce Effect] (Marchlewksa-Koj, 1977, 1981), and
5) the components of female mouse urine responsible for stimulating gonadotrophin secretion in males (Singer, Clancy, Macrides, Agosta, and Bronson, 1988).
As a basis for later comparison with my results I will describe the known chemical characteristics of these pheromones.

Puberty-Accelerating Male Chemosignal

Puberty is not an instantaneous event, it is a gradual change in morphology, physiology, neuroendocrinology, and behavior which leads to sexual maturity. The age at which puberty begins in female mice is affected by the environment. Young female mice placed in environments devoid of males show delayed puberty (Stiff et al., 1974). On the other hand, Vandenberg (1967) and Castro (1967) both found that when juvenile females are placed in an environment where a male is present, the females undergo an accelerated prepubertal period. Such females show earlier vaginal openings, earlier first estrus, and earlier matings than females housed in all female colonies. However, physical contact with adult males is not necessary for puberty acceleration. Initiation of puberty acceleration can be produced by the soiled bedding of males (Vandenberg, 1969), or by their urine (Colby and Vandenberg, 1974).

Partial Purification of Puberty-Accelerating Male Chemosignal

Numerous attempts have been made to identify the puberty accelerating pheromone present in male urine. Many properties of the chemosignal have been characterized: a) it is effective in extremely small quantities (.0001 ml/day) (Drickamer, 1982a), b) it is nonvolatile (Vandenberg, 1976), c) it is perceived by the vomeronasal/accessory olfactory system (Meredith, 1983), and d) its effects are blocked when mixed with urine from grouped females.
Initial steps in isolating the pheromone responsible for accelerating puberty in females indicated an association with the "pre-albumin" proteins present in male urine (Vandenbergh, 1975). These proteins comprise the major urinary proteins (MUP) in mice and have molecular weights of around 17,500 daltons (Finlayson & Morris, 1965). For example, the largely protein containing nondialysable fractions of male urine and urinary fractions obtained through ammonium sulfate precipitation retained puberty accelerating properties. Further evidence that the pheromone may be a protein or associated with proteins was the finding, that when male urine was treated with a proteolytic enzyme (Pronase), male urine lost its proficiency in accelerating puberty (Vandenbergh, Whitsett, & Lombardi, 1975).

Dialysis, sequential ultrafiltration, and sephadex chromatography were used to further investigate the protein hypothesis (Vandenbergh et al., 1976). Male urine was collected and subjected to dialysis from which a retentate and a dialysate were obtained. Both the retentate and the dialysate accelerated puberty when compared to control substances.

To further examine molecular weight, male urine was filtered by means of an Amicon 202 stirred ultrafiltration cell equipped with Amicon series UM ultrafiltration membranes. Urine was initially filtered through a UM10 membrane, general retentivity greater than 10,000 daltons. The filtrate was then collected and filtered through a UM2 membrane, general retentivity greater than 1,000 daltons. The filtrate was again collected and filtered through a UM05 membrane, general retentivity greater than 500 daltons. Vandenbergh found that the filtrate through all the membranes retained the activity. Vandenbergh (1976) proposed two hypotheses to explain the pheromone's presence in filtrates containing both high and low
molecular weights; 1) two or more compounds with differing molecular weights could possess the activity and 2) the active substance could be bound to, or be part of a protein, and could be continuously released so that it would be detected in all the urinary filtrates tested. Vandenberghe based his subsequent attempts at isolation on the latter hypothesis.

Vandenbergh's subsequent attempts included the further separation of the filtrate using Sephadex G-15 chromatography. This procedure produced only one fraction which exhibited pheromonal activity. Using paper chromatography, six compounds were found to reside in the active fraction. Using elution profiles of standards, the active components were estimated to have a molecular weight of approximately 860 daltons. The active components are therefore not steroids nor steroidal metabolites which have molecular weights in the range of 260 - 300 daltons.

Novotny (1980) replicated and expanded Vandenbergh's (1976) isolation work. Sephadex G-15 separations by Novotny (1980) yielded an active fraction which corresponded to the 860 dalton component isolated by Vandenbergh et al. (1976). Novotny further subjected this active fraction to chromatography on a DEAE-Sephadex anion exchanger. Two subfractions produced the most activity, however an even higher activity was obtained when these two fractions were combined. Novotny suggested that this might indicate the presence of two active interacting components. High-voltage paper electrophoresis revealed that the active fractions were a mixture of several substances. This procedure was limited in that only substances with primary amino-groups were detectable.

Novotny (1980) then tested the possibility that the pheromone was a peptide as suggested by Vandenbergh et al. (1976). Active fractions were subjected to treatment with a non-specific peptidase and then re-
chromatographed on Sephadex G-15 column to remove the enzyme. The subsequent bioassay data indicated that the enzyme had no effect on pheromonal activity. However, Novotny went on to report that the enzyme did not remove all traces of peptides. So, if the pheromone was a peptide with activity at low concentrations, then the enzyme may not have significantly diminished pheromonal activity.

A characteristic of the puberty accelerating pheromone was that it appeared to be relatively nonvolatile. This characteristic was supported by the fact that soiled bedding (Vandenbergh, 1969), urine aged up to seven days (Drickamer, 1986), and repeated purified and lyophilized urine (Novotny, 1980), were all capable of accelerating puberty. In addition, its size (860 daltons) indicated that it most likely was not airborne (Vandenbergh et al., 1976). Novotny (1980) proposed the possibility of a pheromone-carrier model. This model suggests that active molecules are bound to a larger "carrier" molecule. This model implies that a number of components could be responsible for the capacity of male mouse urine to accelerate puberty in female mice. Consistent with this hypothesis, evidence is presented below that the chemosignal is composed not only of non-volatile components, but also volatile ones.

Gas chromatography and mass spectrometry methods identified two volatile amines, isobutylamine and isomethyhimine, in male mouse urine (Nishimura et al., 1989). These amines were effective in accelerating puberty in female mice.

Until exact molecules have been completely isolated one can only speculate about the precise nature of the pheromone. The presence of several molecules which can accelerate puberty suggests that some sort of molecular synergy might exist. For example, the volatiles might serve to
attract the female to the source of the male chemosignal, and the non-volatile component might be responsible for inducing accelerated puberty in females.

**Puberty-Delaying Female Chemosignal**

As already noted, male and female urinary chemosignals can accelerate puberty in young females. However, urinary chemosignals produced by adult females kept in groups of four or more, and isolated from male contact, delays puberty in young females (Drickamer, 1977). In contrast, urine from females individually housed and isolated from males does not delay puberty. However, individually housed females exposed to urine from grouped housed females will produce urine which delays puberty (Drickamer, 1982b). The puberty delay signal also is found in the bladder urine of all female mice, regardless of housing condition (McIntosh & Drickamer, 1977). Therefore, the puberty delay signal must either be deactivated or its release is halted in isolated females. In attempting to discover the biological source of the pheromone, Drickamer and McIntosh (1980) adrenalectomized female mice and found that their urine lost its capacity to delay puberty.

Drickamer (1974) has suggested that urinary chemosignals are released at times of high population density and that the delay in puberty helps maintain a manageable population size. In order to better investigate the relationship of population biology to reproductive behavior, Drickamer (1982a) combined urine from different treatment sources and observed the ensuing pubertal effects. Drickamer (1982b) mixed male urine, urine from grouped housed females, and urine from lactating and pregnant females, in two and three-way combinations. Urine from group housed females took
precedence over all others and delayed puberty. This experiment helps to answer the question of what happens to the potency of puberty-accelerating male urine when presented at the same time as puberty-delaying chemosignals. This experiment also simulates the interaction of female and male chemosignals that occur in natural populations. Similar to the puberty-accelerating phenomenon the puberty-delaying phenomenon is another chemosensory phenomenon that maximizes environmental and reproductive resources.

Partial Purification of Puberty-Delaying Pheromone

Coppola and Vandenbergh (1985) first suggested that the puberty-delaying chemosignal was relatively volatile after finding that the puberty-delaying activity in the urine was lost after exposure to air at room temperatures. The subsequent literature on the isolation of the puberty-delaying pheromone was relatively recent with much of it from Novotny and his coworkers. Novotny et al. (1986) took urine samples from grouped housed females and compared them to adrenalectomized females. Using head space techniques, quantitative chemical differences were uncovered. The gas chromatographs of the volatiles revealed differences in six substances which belong to three structurally distinct classes: 1) acetate esters (n-pentyl acetate and cis-2-penten-1-yl acetate), 2) ketones (2-heptanone, trans-5-hepten-2-one, and trans-4-hepten-2-one), and 3) pyrazine (2,5-dimethylpyrazine). When these laboratory-synthesized chemicals were added to either the urine of adrenalectomized females or to water, the resulting mixture was found to delay puberty. In addition, adding pyrazine (2,5-dimethylpyrazine) alone to the adrenalectomized urine was sufficient to restore the puberty-delaying activity (Novotny et al., 1986).
Chemical characterization of volatile profiles of urine samples collected during different stages of pregnancy, lactation, and the natural estrus cycle also were compared (Jemiolo et al., 1989). During the middle parts of pregnancy/lactation, there was a marked increase in the levels of ketones, while 2,5-dimethylpyrazine levels, a proven puberty-d delaying substance, were depressed (Jemiolo et al., 1989). 2,5-dimethylpyrazine levels were also depressed during the estrogen-dominated period while other volatiles increased (Jemiolo et al., 1989).

Jemiolo et al. (1989) took volatile urinary components and prepared them in different mixtures to observe their effects on the puberty of female mice. Results showed that high concentrations of urinary ketones were necessary for accelerating puberty and for extending estrus in young female mice. In addition, the ketones present in certain concentrations were able to override the known puberty-d delaying effects of \( n \)-pentyl acetate, \( cis \)-2-penten-1-yl acetate, and 2,5-dimethylpyrazine. These results contrast with results by Drickamer (1982b) who reported that the puberty-d delaying effect of grouped housed female urine overrode the puberty-accelerating effect of urine obtained from male, pregnant, and lactating females. However, Jemiolo's experiments used concentration levels that were higher than those used by Drickamer.

The results of Drickamer (1982a), Jemiolo et al. (1989) and Novotny et al. (1989) studies show that the ratio of components present in the urine determines the pheromone's ability to exert its effects. The ratio of the urinary components that occur in the natural environment are determined by the physiology of its inhabitants.
Pregnancy Blocking Male Urinary Chemosignal

Bruce (1959) reported that a novel male mouse or his odors are known to terminate the pregnancy of a recently impregnated female and return her to estrus. The pregnancy block is greatly enhanced if the male is placed with the female within 24 hours of mating and is left with the female for a period of 7 - 10 days. In addition, urinary odors from a novel male (male belonging to a different genetic strain than the female’s mate (stud)) are sufficient to cause the termination of the pregnancy, while the urinary odors from the stud male do not. (Bruce, 1960).

As is the case with other primer pheromones, direct contact with the strange male is not necessary. Contact with male urine (Dominic, 1964) or soiled bedding (Parkes and Bruce, 1961) is sufficient to prevent blastocyte implantation. The Bruce effect is affected by both the duration and latency of the pregnancy blocking stimulus. The sooner after the pregnancy, the better chance the stimulus has of blocking the pregnancy. In addition, the number of novel males introduced to the pregnant female does not increase the likelihood of the pregnancy block.

The pregnancy-blocking pheromone is androgen dependent. Dominic (1964) demonstrated that the pregnancy block was caused only by mature males, and that the pheromone is inhibited by castration and restored by testosterone treatment. In addition, female mice injected with testosterone were themselves able to cause Bruce effects in other females.

The ecological significance of the pregnancy block has been debated. A question yet unanswered is whether the Bruce effect is a laboratory artifact or whether it somehow confers an adaptive advantage in nature. Recent data shows that the pregnancy block does occur in natural populations of wild rodents, however the data were only observational.
Partial Purification of the Pregnancy-Blocking Male Urinary Chemosignal

There are conflicting reports regarding the volatility of the pregnancy-blocking chemosignal. The chemosignal was originally considered volatile, since pregnancy was blocked even if physical contact between the alien male and pregnant male was precluded (Bruce, 1960). In addition, early chemical analyses were based upon the Bruce effect pheromone being androgen dependent. Early speculations were that the pheromone was either androgen itself or androgen metabolites.

Using gas chromatography, Hoppe (1975) analyzed volatile fractions of male urine that were either effective or ineffective in eliciting the pregnancy block. Hoppe found that ineffective fractions lacked low molecular weight compounds. In addition, testosterone treatment restored not only the ability of castrated male urine to block pregnancies, but also the appearance of the low molecular weight components. Volatiles obtained by bubbling a stream of nitrogen through male urine and condensing the vapor were also shown to retain the pregnancy-blocking activity. The activity of this volatile fraction of urine was lost after it was lyophilized. Hoppe (1975), suggested that the pregnancy-blocking phenomenon was a low-molecular-weight, androgen metabolite.

Further research by Monder, Lee, Donovick, & Burright (1978) showed the pheromone to be volatile. Pregnant female mice were precluded from contact with odor stimuli by placing odor samples on cotton swabs inside Girton drinking tubes that were placed inside the cages of pregnant females. Despite the lack of contact, these samples from alien males were still able to elicit the Bruce effect.

In contrast, numerous other studies have concluded that the pheromone responsible for the Bruce effect is non-volatile. Rajendren &
Dominic (1984) demonstrated the importance of allowing pregnant females to contact urine stimuli inorder to allow the pregnancy block to occur. Further indication of the nonvolatile nature of the pregnancy-blocking chemosignal is that the presence of the pheromonal activity remained after repeated lyophilization, thin layer electrophoresis, and evaporation in a vacuum indicating that at least some molecules possessing the pregnancy-blocking activity are not volatile (Marchlewksa-Koj, 1977, 1981, 1983).

Further research by Marchlewksa-Koj (1977) showed that proteins from the urine of male mice precipitated out of solution with ammonium sulfate were sufficient to elicit the pregnancy block. This finding is similarity to that of Vandenbergh's (1975) which showed that the pheromone responsible for accelerating puberty also is associated with the protein portion of male urine. Proteins from the urine of females and castrated males were not effective in eliciting the Bruce effect. However, testosterone treatment was able to restore the ability of protein from castrated male and female urine to block pregnancies.

To further investigate the protein nature of the pheromone, Marchlewksa-Koj (1981) subjected urine to fractionation on a Sephadex G-75 column followed by thin-layer electrophoresis. Pregnancy block was associated with the fraction containing low-molecular weight peptides. The peptide fraction was subjected to further separation using thin-layer electrophoresis on Sephadex G-75 gel column. This analysis found that the pregnancy block was observed only to the peptide fractions. However, these peptides are different from the higher molecular weight major urinary proteins (MUP) which do not block pregnancy (Marchlewksa-Koj 1977).

In order to further characterize this pheromone, Marchlewksa-Koj (1983) injected males with radiolabeled testosterone. Their urine was
subsequently fractionated on a Sephadex G-75 column and tested for activity and radioactivity. The greatest pheromone activity was found in the fraction containing low molecular weight proteins. These fractions also contained the greatest amount of radioactivity. These results led to the hypothesis that the pheromone responsible for the Bruce effect was a product of testosterone metabolism bound to a peptide and excreted in this form.

_Estrus Synchronizing Male Chemosignal_

Odors from the environment have the ability to exert differential influences on the estrous cycles of female mice. Female mice experience complete suppression of their estrous cycle (anestrus) when housed in large female colonies (30 females) (Whitten, 1956). Contact with the soiled bedding of grouped housed females is also sufficient to cause anestrus, therefore, the responsible chemosignal is suspected to be present in the urine (Champlin, 1971).

Male mice can influence the duration and frequency of the female's estrous cycle. In contrast, the estrous cycles of grouped-housed, anestrus females can be restarted in a synchronized fashion following exposure to a male [Whitten Effect] (Whitten, 1956). Such females come into estrus three days after the introduction of a male (Whitten, 1958).

Contact with the male himself, is not necessary for the Whitten effect to occur. Soiled male bedding (Whitten, 1956), male urine (Marsden & Bronson, 1965), and air from a male's cage (Whitten et al., 1956) are all sufficient stimuli to initiate the Whitten effect. However, the effects are not as robust as when the male himself serves as the stimuli, indicating that
the male may provide additional contextual cues that accentuate the effects of the pheromone.

Partial Purification of the Estrus Synchronizing Male Chemosignal

Early studies demonstrating the Whitten effect used wind tunnels to present the pheromone from a distance (e.g. Whitten et al., 1968) indicating that the pheromone is volatile. Monder et al. (1978) were the first to attempt to identify the chemical characteristics of this pheromone. Female mice were exposed to a series of stimuli placed in Girton drinking tubes so that the animals could not tactually contact the stimulus. Female mice were grouped housed and anestrous. Thereafter, only exposure to male urine or to a dichloromethane extract of male urine shortened the female's estrous cycles. Because of the method of stimulus presentation, the odor could be perceived only via olfaction. Thus, the pheromone responsible for the Whitten effect appeared volatile in this study as well.

Examination of urinary volatile components led to the discovery of a testosterone-dependent, volatile constituent unique to male mouse urine. The compound was identified as 7-exo-ethyl-5-methyl-6,8-dioxabicyclo-3-octene also called dehydro-exo-brevicomin (Novotny et al., 1984). In another study, Schwende et al., (1986) replicated this finding and identified yet another androgen dependent volatile component in male mouse urine, 2-(sec-butyl)-4,5-dihydrothiazole. These two compounds stimulate both intermale aggression (Novotny et al., 1985), and female attraction (Jemiolo et al., 1985). These volatile components were then tested for their ability to induce estrus in female mice grouped housed at different densities (1, 4, or 8 per cage) (Jemiolo et al., 1986). Results show that the synthetic analogs when added to castrated male urine and water, in concentrations equal to those in
normal intact urine, were sufficient to induce the Whitten effect. However, the effects of these synthetic analogs were not as efficacious when presented to individually housed females grouped one per cage.

These results combined with those of Monder et al. (1978) and Whitten et al. (1968) provide evidence that the urinary chemosignal responsible for the Whitten effect is indeed volatile and can be accounted for in large part, by 2-(sec-butyl)-4,5-dihydrothiazole and dehydro-exobrevicomin. The interesting efficacy of the synthetic pheromone analogs for females housed under conditions of high population density supports the Marchlewksa-Koj (1983) notion that same chemical messengers can exert different effects based upon the hormonal status of the female. Probably the most significant finding of Jemiolo et al. (1986) was that synthetic analogs can act as primer pheromones even when present in a "non-urinary" context such as water.

Gonadotrophin Secretion Stimulating Female Urinary Chemosignal

In male mice, luteinizing hormone (LH) is continually released by the male's pituitary glands at relatively low levels. Usually occurring every few hours are episodic surges of LH release. These episodic LH surges in turn cause episodic testosterone surges by the testis. Exposure to a female mouse will stimulate surges of LH and testosterone mimicking the typical endogenous episodic surges (Bronson, 1982). LH levels quickly return to normal following exposure to a female, but exposure to a novel female stimulates yet another LH surge (Coquelin and Bronson, 1979).

The reproductive function of the female-initiated LH surge remains unknown. Bronson and Desjardin (1982) performed a series of experiments demonstrating that LH release is a side-effect of sexual arousal. In
addition, they concluded that a primer pheromone was responsible this
female-initiated surge.

Clancy et al., (1988) performed a series of experiments to examine the
importance of sexual experience on LH release in response to different
stimuli (female urine, male urine, saline, or a mixture of these stimuli).
These experiments showed that copulatory experience is not necessary for
LH release. Both sexually naive and sexually experienced males had
significantly larger LH surges to female urine than to male urine. In
addition, when male and female urine was mixed, the male urine
suppressed the effects of the female urine. However, LH surges were
evident when the female urine was mixed with saline. Castrated male
urine had just as suppressive an effect as intact male urine. Therefore, the
suppressive activity in male urine is not androgen-dependent (Clancy et al,
1988).

Partial Purification of the Gonadotrophin Secretion Stimulating Female
Urinary Chemosignal

Singer et al., (1988) performed a series of experiments to characterize
the chemical properties of the gonadotrophin stimulating pheromone.
Female urine was fractionated by absorption chromatography and dialysis
(6000-8000 MW cutoff). The retentate (high molecular weight fraction) from
the dialysis significantly elevated LH levels in male mice when compared to
the effect of the dialysate or saline (control). This finding led the
experimenters to believe that the pheromone was a protein or associated
with proteins, particularly MUP's.

In contrast, after fractionation of female urine on Sephadex G-15 gel
permeation column, the active component was found in the fraction
containing low-molecular weight substances. To fully examine the role of proteins in pheromone activity, the urine was incubated with pronase (a broad-based protease). The protein-depleted urine was then chromatographed on a Sephadex G-15 gel permeation column and tested for activity. The low molecular weight fraction of protein-depleted urine was just as potent as the undepleted urine. Therefore, the chemosignal is not dependent upon MUP's, either directly or as part of a ligand-protein complex, and the chemosignal is not a peptide, unless it is an unusually small peptide resistant to degradation by enzymes (Singer et al., 1988). The chemical properties of this pheromone are very similar to the other non-volatile primer pheromones discussed so far.

Similarities in the Chemical Nature of Priming Pheromones

The four male priming effects which exert their influences on females which have been discussed in this paper, 1) the acceleration of puberty (Vandenbergh, 1967), 2) the delay of puberty (Drickamer, 1977), 3) the blockage of pregnancy (Bruce, 1959), and 4) the acceleration of the estrous cycle (Whitten, 1956) possess many of the same characteristics suggesting that these pheromones may be similar. Along these lines Marchlewksa-Koj (1983) suggested that the same chemical messenger can exert different effects depending upon the hormonal status of the responding female. According to the literature reviewed thus far, we know that primer pheromones can be either large (non-volatile) or small (volatile) molecular weight molecules. Unfortunately, there is still some discrepancy as to whether some pheromones are volatile or non-volatile.
Previous Attempts to Isolate Releasing Pheromones

The relatively large literature examining the isolation of priming pheromones stands in contrast to the small literature on attempts to isolate releasing pheromones. Isolation of releasing pheromones have centered mainly on two pheromones: 1) the aggression-promoting chemosignal in bladder urine (Jones and Nowell, 1973; Ingersol, 1986), voided urine (Lee & Ingersol, 1983; Lee, Lutkon, Bobotas, & Ingersoll, 1980) and preputial excretions (Jones and Nowell, 1973; Ingersol, Morley, Benvenga, & Hands, 1986) of adult male mice, and 2) the aversion and aggression-inhibiting chemosignal present in the urine of female mice (Evans, Macintosh, Kennedy, & Robertson, 1978).

Aggression-promoting Male Urinary Chemosignal

Intermale aggression in house mice is governed by urinary chemosignals. Blind mice behave exactly as sighted males in initiating aggressive behavior (Uhrich, 1938). The importance of olfaction is further indicated by the finding that bilateral olfactory bulbectomy eliminates aggressive behavior, even if the male was a trained fighter prior to bulbectomy (Da Vanzo, 1983).

This chemosignal is androgen dependent. For example, males display more aggression to intact males. Further evidence is that testosterone propionate administrated to castrated males restores aggressive-promoting properties to their urine (Mugford & Nowell, 1970).

The source of the urinary aggression-promoting also has been investigated. Ingersol, Morley, Benvenga, & Hands (1986) found after removing the coagulating gland, Cowpers gland, prostate gland, or
preputial gland, that only the preputial gland was necessary for production of the aggression-promoting signal.

*Previous Attempts to Isolate the Aggression-promoting Male Chemosignal*

Using various isolation techniques (i.e. infrared spectrometry, thin-layer chromatography (TLC), gas liquid chromatography, and mass spectrometry), Lee (1976) provided the most detailed chemical analysis of the aggression-promoting pheromone. In order to assess the chemosignal's volatility, urine was passed through a vacuum distillation apparatus in which volatile and nonvolatile fractions were collected and tested. The volatile components resembled whole urine in their ability to promote aggression, while the non-volatile components were inactive.

Lee (1976) also used dichloromethane (DCM) to assess other chemical properties of the pheromone. After separation, the DCM fraction (lipophilic constituents) and the aqueous fraction (polar, hydrophilic constituents) were tested for the ability to elicit aggression. The DCM fraction, but not the aqueous fraction, retained the aggression-promoting properties of whole unfractionated urine. These results have since been replicated by Lee, Lukton, Bobotas, & Ingersoll (1980) who demonstrated that volatile components of the DCM extract were able to initiate aggression among pairs of mice.

Lee (1976) further investigated the aggression-promoting chemosignal's androgen dependency. Lee (1976) injected males with radiolabeled testosterone, collected their urine, and subjected it to thin layer chromatography (TLC). The biologically active fraction was also found to contain the most radioactivity. The radioactive spots were then compared to the TLC profile of twelve different steroid hormones. However, the profile of
these steroids did not produce a spot which matched the biologically active spot. Therefore, Lee (1976) concluded that the substance(s) responsible for the aggression-promoting chemosignal is composed of an androgen component but is not a common androgen metabolite, because they did not correspond to the TLC profile of the twelve steroid hormones.

Ingersol et al., (1982) compared the capacity of voided urine to initiate aggression with that of intact male bladder urine. Voided urine elicited significantly more aggression than the bladder urine. Using repetitive extractions with DCM, Ingersol et al. (1982) determined that the aggression-promoting chemosignal in bladder urine was readily soluble in DCM. However, the extract elicited less agonistic behavior than voided urine suggesting that the aqueous layer of male bladder urine may contain a portion of the chemosignal in an inactive form (Ingersol et al. 1982). Ingersol et al. (1982) findings suggest that β-glucuronidase may transform the inactive pheromone into the active form. Preputial tissues have been shown to contain β-glucuronidase, the enzyme responsible for cleaving the steroid-glucuronic acid bond (Levvy, 1966, c.f. Ingersoll et al., 1982). Ingersol et al. (1982) were able to restore the aggression-promoting properties to the aqueous layer of male bladder urine by incubating with β-glucuronidase.

β-glucuronidase activity is androgen dependent and is significantly decreased by castration (Conchie, 1959). The lack of an aggression-promoting chemosignal in the urine of castrated mice could be accounted for by the decrease in β-glucuronidase activity (Ingersol et al., 1982). Even though incubation of bladder urine with β-glucuronidase increased antagonistic behaviors, its activity was significantly lower than that of voided urine. Ingersoll et al. (1982) suggested that the difference in the potency of the voided urine and of the bladder urine may be due to exposure
of the voided urine to secretions of the preputial gland which may supply additional chemosignals and/or activate the bladder urine conjugate by β-glucuronidase activity.

Ingersoll (1986) further investigated the aggression-promoting properties of the bladder urine of male mice. Using different quantities of bladder urine and voided urine, Ingersoll (1986) further confirmed that bladder urine contains a weaker chemosignal. Ingersoll (1986) postulated that this "weaker" chemosignal becomes more powerful by releasing the prochemosignal(s). Specifically, Ingersoll believes that the inactive of "weaker" chemosignal is a conjugated steroid and that the active form was a result of the enzymatic cleavage of the conjugated form (prochemosignal). In order to understand how this latent prochemosignal(s) in the bladder urine is activated, Ingersoll (1986) found that heating bladder urine enhanced its aggression-promoting property, but not to the level of voided urine. To account for the bladder urine's low potency, Ingersoll (1986) suggested two possible mechanisms of action: 1) bladder urine may contain prochemosignals which are not acted on by exposure to heat, or 2) voided urine may contain chemicals added after the urine has left the bladder (i.e. preputial contributions). Ingersoll (1986) further speculated that the preputial gland may add β-glucuronidase to the urine to activate the prochemosignal.

Although Lee (1976) suggested that the aggression-promoting chemosignal was not an androgen or androgen metabolite and therefore androgen dependent, other reports have found contradictory evidence (Novotny, Harvey, Jemiolo, & Alberts, 1985; Ingersoll & Laundry, 1986). Male urine contains two structurally unique androgen dependent volatile compounds (2-sec-butyl-dihydrothiazole and dehydro-exo-brevicomin).
(Novotny et al., 1984; Schwende et al., 1986). These compounds added to castrated urine or water accelerate puberty in females (Jemiolo et al., 1986) and attract females (Jemiolo et al., 1985). Novotny et al. (1985) performed the same experiment using aggression as the dependent variable. These compounds were able to restore the aggression-promoting ability of castrated urine (Novotny, Harvey, Jemiolo, & Alberts, 1985).

Ingersoll & Laundry (1986) also found that the addition of an androgen-dependent steroid, 5a-androst-16-ene-3-one, to castrated urine restored its aggression-promoting capabilities. The steroid was not able to stimulate agonistic behaviors when presented in water, but it did promote aggression when presented in conjunction with urine. This steroid, isolated from boars, is the principle chemosignal responsible for initiating mating in sows (Patterson, 1968, c.f. Ingersol & Laundry, 1986).

Novotny et al. (1985) and Ingersol et al. (1986) have shown evidence contradicting Lee's (1976) finding that the aggression-promoting factor in male mouse urine is not androgen dependent. Therefore, the prospect that androgens or androgen metabolites may act as aggression-promoting chemosignals can not be discounted. According to these more recent studies the aggression-promoting factor in male mouse urine appears to be an androgen or androgen metabolite which Lee (1976) did not test for.

Aggression-reducing Female Chemosignal

Dixon & Mackintosh (1971) carried out a series of experiments examining an aggression-reducing chemosignal in female mouse urine. When males were rubbed with urine from adult females they elicited less attack from other males than when rubbed with water. Therefore, the female's impunity to attack from males seems to be due to the protection
afforded them from aggression-reducing chemosignals present in their urine (Dixon & Mackintosh, 1970). Mugford & Nowell (1971) reported that ovarian hormones are necessary for chemosignal production. However, more recent reports have demonstrated that urine from ovariectomized females is just as potent in reducing aggression as is that from intact females (Dixon & Macintosh, 1975).

**Previous Attempts to Isolate the Aggression-reducing Chemosignal**

Only a few attempts have been made to characterize the aggression-reducing chemosignal. Unfortunately, the evidence is inconsistent.

Evans et al. (1978) found the presence of a behaviorally active chemosignal in both bladder urine and voided urine. The chemosignal was resistant to oxidative and bacterial breakdown and to changes in pH. Following extraction with diethylether, the aqueous residue retained the aggression-reducing activity. Following extraction with dichloromethane (DCM), both the aqueous and dichloromethane extracts possessed aggression-reducing activity. Further DCM extractions (x16) of the aqueous fractions found that both the resulting aqueous residue and precipitate remained active. Therefore, Evans et al. (1978) concluded that the aggression-reducing compounds are relatively hydrophilic.

Distillation procedures showed the aggression-reducing chemosignal to be non-volatile. Since the pheromone is an olfactory chemosignal, it was expected that it was associated with a degree of volatility and that it could be successfully distilled. However, Evans et al. found that the distillate did not decrease aggressive behavior. The aqueous residue, however, reduced aggression.
Evans et al. (1978) further investigated the properties of the pheromone utilizing organic extraction procedures. Evans et al. (1978) added ethanol to the urine to precipitate the proteins from solution and then tested the precipitate and the residue. The residue was significantly more effective at reducing aggression than the precipitate. The precipitate was comparable to water in activity. This suggests that the active components are associated with the protein or other macromolecules. To further examine molecular weight, a sample of fresh urine was dialysed against water (2 X 100 ml) for two days at 40 degrees C. The dialysis retentate containing the relatively large molecules did not reduce aggression among male mice, whereas the diffusate containing smaller molecules was active. Unfortunately, the molecular weight cut-off of the membrane was not reported.

Ultrasonic Eliciting Female Chemosignal

Male mice emit 70 kHz ultrasonic vocalizations when courting and copulating with females (Sales, 1972). Vocalizations tend to be most intense shortly after the male first encounters the female. Vocalizations are also present when the male is engaged in sniffing and mounting the female and they occasionally coincide with pelvic thrusts (Nyby, 1983). In contrast, few vocalizations are monitored when the male is not sniffing or mounting the female. The vocalizations cease following ejaculation but resume prior to the start of another mounting sequence (Nyby, 1983).

Male mice also emit 70 kHz courtship vocalizations to anesthetized females suggesting that vocalizations are not associated with active behaviors on part of the female (Nyby et al., 1977). In addition, male mice emit vocalizations to female cage shavings, and to odors from different
parts of the female body (Nyby et al., 1977). Disrupting the chemical senses either by vomeronasal removal or olfactory bulbectomy in the male severely depresses ultrasonic vocalizations (Wysocki, Nyby, Whitney, Beauchamp, & Katz, 1982). Thus, male vocalizations are mediated by a female chemosignal.

The chemicals responsible for eliciting the vocalizations are present in many female excretions and secretions, including vaginal fluids and urine. However, due to the ease of collection and quantification, urine has been the most studied source of the ultrasound-eliciting pheromone. Female urine is a very robust stimuli for eliciting male mouse courtship vocalizations (Nyby, Dizinno, & Whitney, 1977) and can be used as a bioassay for the presence of the female pheromone.

Previous Attempts to Isolate the Ultrasound Eliciting Female Chemosignal

Nyby and Kerchner (Kerchner, 1988) were the first to attempt to isolate the ultrasound eliciting pheromone. Using simple distillation experiments, Kerchner confirmed the pheromone's non-volatility. Kerchner subsequently found that neither acidification or alkalynization significantly reduced pheromone activity. Kerchner later found that activity was retained in the aqueous polar fractions of organic solvent extractions, indicating that the pheromone is polar and water soluble. Finally, using an ultrafiltration procedure, Kerchner found the active molecules to have a molecular weight less than 6,000 daltons. Nyby and Simon (1988, pilot study) who used G-10 Sephadex chromatography in order to assess the molecular weight of the pheromone, found that the pheromonal activity resided in a nonvolatile, polar molecule with a molecular weight slightly less than 700 daltons, and that the molecule was
not a urinary protein. The research described in this thesis was an attempt to further examine the pheromone structure through the use of Sephadex chromatography.
Chapter II: Methods and Results

Methods that have been employed by other investigators to isolate urinary pheromones are based upon molecular polarity, weight or size, and volatility. I chose to use gel chromatography which separate molecules based upon size. By repeatedly fractionating urine through Sephadex gel columns I have attempted to more precisely identify the molecular size (and weight) of the pheromone.

General Methods

The procedures common to all experiments are described in this section. Descriptions of procedures used in specific experiments are provided in subsequent sections.

In attempting to isolate the urinary pheromone, I fractionated biologically active urine samples. These fractions were then tested for pheromonal activity. Ideally, only one active fraction would be found in each experiment and then that fraction could be further fractionated. Once the chemosignal has been sufficiently purified, various techniques exist to identify it. The ultimate goal was to chemically isolate and characterize the chemical, or group of chemicals, responsible for eliciting the vocalizations.

Animals:

All animals were adult (>55 days) male and female CK (F1 offspring of AKR/J male x C57BL/6J female) house mice (Mus domesticus). Separate groups of mice served as subjects, social experience animals, and stimulus donors. Subjects were male mice from whom ultrasonic vocalizations were
measured in response to various stimuli and chemical fractions. Social experience animals were male and female mice that were systematically introduced into each subject’s home cage prior to the start of behavioral testing. Stimulus donors were female mice that provided the urine samples which served as stimuli. Subjects were individually housed after weaning, whereas social experience animals and stimulus donors were grouped housed by gender.

**Apparatus:**

All animals were housed in opaque plastic cages (12.5 x 17.0 x 28.0 cm) containing wood shavings as bedding. Animals were provided with tap water and Purina Mouse Chow *ad libitum*. Subject’s home cages served as testing chambers.

Stimulus urine was collected in metabolic cages (Maryland Plastics E110 Metabolism Units). Stimuli were placed on 6.0 in cotton tipped applicators (swabs) using 1.0 cc disposable tuberculin syringes. Swabs containing stimuli were handled in 2.0 x 17.5 cm pyrex test tubes prior to presentation to subjects.

Ultrasounds were monitored using a QMC (Model S100) bat detector tuned to a frequency of 70 kHz. The bat detector transduces the ultrasonic vocalizations of mice into audible sounds which can be heard by a human observer. A stop watch was used to measure time.

**Procedure:**

All animals were maintained on a 12/12 light/dark cycle (lights on: 0630-1830hrs.). All experiments consisted of five phases: a) social
experience, b) behavioral screening, c) stimulus collection, d) chromatographic separation, and e) behavioral testing.

*Social Experience.* Heterosexual experience facilitates male ultrasonic vocalizations to females and their odors (Nyby, 1983). Therefore, subjects were provided with a social experience regimen consisting of daily sequential 3-min presentations of a single male and single female conspecific into the subject's home cage for 8 consecutive days. The order of presentation of the male and female social experience animals was counterbalanced across subjects and reversed each day. Male social experience animals were made nonaggressive through olfactory bulbectomy in order to prevent possible suppressive effects upon vocalizations of the subject (Nyby et al., 1977). Social experience animals were removed at the first sign of aggressive behavior to prevent an injury to the animal.

*Behavioral Screening.* Twenty-four hours after the completion of the social experience regime, subjects were screened for ultrasonic vocalizations to social experience females. Vocalizations were quantified in a 3-min test period divided into 36 five-second intervals. An investigator recorded the number of intervals in which the subjects emitted vocalizations in response to a female mouse placed into the subject's home cage. Scores ranged from 0-36. If any animals scored fifteen or less they were omitted from subsequent behavioral testing. However, no animals scored below fifteen.
Stimulus Collection. Urine was collected from stimulus donors by group housing them the night proceeding the chromatographic separation (approximately 12 hrs.). Five ml of urine were drawn from the pooled urine and stored in a syringe until chromatographed. During the behavioral tests, vocalizations were monitored in response to the chromatic fractions of female urine and untreated urine.

Chromatographic Procedure. In gel-permeation chromatography, the components of a sample are separated according to molecular size. The gel columns operate in a fashion similar to a sieve. The column is made up of two phases: a non-mobile phase and a mobile phase. The urine is placed in the buffered solution (mobile phase) and is passed through the column containing the dextran matrix which contains hollow dextran beads with pores. The fluid filled space inside the dextran serves as the nonmobile phase. The matrix consists of cross-linked polysaccharide (dextran) and is available commercially in a wide range of pore sizes. This diversity in pore size makes Sephadex suitable for the fractionation of molecules from 500 to more than 5x10^6 daltons in molecular weight. Therefore, the smallest molecules enter the pores of the dextran beads while excluding molecules larger than the diameter of the pores. Thus, the larger molecules elute off the column first, while the smallest molecules will be last. Any molecules larger than the molecular cut-off of a specific gel will be found in the void volume (Vo). The void volume was determined by the use of Blue Dextran, a high molecular weight compound that exceeds the molecular weight cut-off of the gel and is easily visualized.

In experiments 1-5, 5 ml urine was collected from stimulus donors as described under the stimulus collection. In experiments 1 and 2, the urine
was applied to a G-10 Sephadex column packed with (Phararamacia Fine Chemicals) (void volume = 67.5 ml, total column volume = 180), 0.0002 M NH₄HCO₃ (ammonium bicarbonate). Samples of 4.5 ml were collected.

In experiments 3-5, the urine was fractionated with G-15 Sephadex (Phararamacia Fine Chemicals) (void volume = 41 ml, total column volume = 98), fractionation range of 0 to 1500 daltons, also equilibrated with 0.0002 M NH₄HCO₃ (ammonium bicarbonate). Samples of 1.0 ml were collected.

Both columns were used at room temperature. A flow rate of 1.0 ml/min was achieved by use of a peristaltic pump. The fractions were pooled, lyophilized, reconstituted to half the original volume with distilled water, and tested for pheromonal activity. This doubling of fraction concentration was to compensate for losses during the procedure.

Behavioral Testing. Forty eight hours after screening, 20 subjects were tested for ultrasonic vocalizations to different fractions. Ultrasound quantification was identical to that used in the screening process with the exception that immediately preceeding the 3-min trial the subject was habituated for 1 min in the testing apparatus. This habituation was to ensure that the subject was not vocalizing to incidental aspects of the test situation. If vocalizations were monitored during the habituation period, then two minutes without a vocalization were required to elapse before testing could begin.

The test period began when the stimulus (urine or fractionated urine) which was injected onto a cotton swab was dropped into the subject's home cage. All subjects were presented with every stimulus, but only one stimulus was presented per day. Stimuli were presented in a counterbalanced fashion and the order of their presentation was
determined randomly. Testing days were separated by 48 hours to minimize carryover effects. The observer recording ultrasounds was "blind" to stimulus type.

Data Analysis. Data were summarized in terms of the mean number of 5-sec blocks containing ultrasonic vocalizations plus or minus the standard error. Differences in vocalizations were analyzed with a repeated measure analysis of variance (ANOVA) using CLR-ANOVA(v.1.12) statistical package for the Apple Macintosh Computer. Tests for both planned and unplanned comparisons also utilized the CLR-ANOVA (v.1.12) statistical package.

The experiments which comprise this thesis are described in the following sections. These experiments were of two general types; 1) Sephadex G-10 and 2) Sephadex G-15.

Experiments 1-2; Sephadex G-10 Separations

Previous investigations (Nyby and Simon, 1988, unpublished) indicated that pheromonal activity from female mouse urine was located in the fractions immediately following the void volume of the Sephadex G-10 column. This section describes two additional experiments to further examine this finding.
**Experiment 1: Sephadex G-10 Separation**

This experiment tested whether any of the three fractions covering the fractionation range of Sephadex G-10 gel chromatography column elicited courtship vocalizations.

*Animals.* Subjects were 19 hybrid male mice between the ages of 60 and 67 days at the beginning of behavioral tests. Urine samples were obtained from 18 hybrid female mice, 160 days of age when behavioral tests began. Social-experience animals were ten female and ten male hybrid mice, 160 days of age at the start of the social experience regimen.

*Results.* Figure 1 summarizes the various fractions tested in experiment 1. Fraction 1 contained samples 12-15 (includes the void volume), fraction 2 contained samples 16-23 and fraction 3 contained samples 24-30 (Figure 1, Experiment 1). These ranges were selected based on the previous work of Nyby and Simon (1988, unpublished).

![Sample No. (4.5 ml/sample) Exper. 1 Fraction # Void Volume (Vo) Total Volume (Vt) 12 15 16 23 24 30 40 60](image)

*Figure 1.* The fractions were prepared by pooling the samples shown above.

The bioassay results of experiment #1 are presented in Figure 2.
**Figure 2.** Experiment #1. Ultrasounds in response to various urinary fractions from a Sephadex G-10 column. The fraction are defined in figure 1.

The within-subjects ANOVA indicated that differences existed among the four stimuli, $F(3, 54) = 8.176, p < .05$. Subsequent post hoc contrasts indicated that fraction 2 elicited significantly more ultrasonic vocalizations than fractions 1 and 3, $F(1, 18) = 21.899, p < .05$. Fraction 1 and 3 did not significantly differ in the number of ultrasounds elicited, $F(1, 18) = 7.318, p > .05$. Additionally, fraction 2 and the unaltered urine did not significantly differ in activity, $F(1, 18) = 0.6795, p > .05$. Thus, these results indicate that the active chemosignal resides in the elutant emerging immediately after the void volume.
Experiment 2: Sephadex G-10 Separation

This experiment attempted to further isolate the chemosignal activity identified in experiment 1. This time three different fractions were tested for their ability to elicit courtship vocalizations.

Animals. Subjects were 20 hybrid male mice between the ages of 85 and 92 days, at the beginning of behavioral tests. Urine samples were obtained from 18 hybrid female mice, 190 days of age when behavioral tests began. Social-experience animals were ten female and ten male hybrid mice, 180 days of age at the start of the social experience regimen.

Results. Figure 3 summarizes the various fractions tested in experiment 2. Fraction 1 contained samples 12-17 (includes the void volume), fraction 2 contained samples 18-21 and fraction 3 contained samples 22-25 (Figure 3, Experiment 2). These fractions were chosen by dividing the fractions that were found to be active in the previous experiment.

![Figure 3](image)

Figure 3. The fractions were prepared by pooling the samples shown above.
The bioassay results of experiment #2 are presented in Figure 7.

Figure 4. Experiment #2. Ultrasounds in response to various urinary fractions from a Sephadex G-10 column. The fractions are defined in figure 1.

The within-subjects ANOVA indicated that differences existed among the four stimuli, F(3, 57) = 6.764, p < .05. Fraction 1 and the unaltered urine did not significantly differ in activity, F(1, 19) = 0.135, p > .05. Additionally, fraction 2 and fraction 3 did not significantly differ in the number of ultrasounds they elicited, F(1, 19) = 0.114, p > .05. Subsequent post hoc contrasts indicated that fraction 1 elicited significantly more ultrasonic vocalizations than fractions 2 and 3, F(1, 19) = 13.274, p < .05. These data provide additional evidence that the ultrasound eliciting chemosignal resides either in the void volume or in the upper end of the fractionation range.
Experiments 3-5; Sephadex G-15 Chromatography of Female Mouse Urine

Experiment 3: Sephadex G-15 Separation

Following experiments 1 and 2 a decision was made to use a Sephadex G-15 gel because of its higher molecular weight fractionation range. The larger fractionation range of the G-15 column allows for a quantitative way to test whether the activity was residing in the void volume of the G-10. In addition, this larger fractionation range allows for a greater separation of the active chemosignal from the non-active molecules. This experiment tested whether four fractions obtained from gel chromatography on a Sephadex G-15 elicited courtship vocalizations.

Animals. Subjects were 15 hybrid male mice between the ages of 65 and 70 days, at the beginning of behavioral tests. Urine samples were obtained from 10 hybrid female mice, 100 days of age when behavioral tests began. Social-experience animals were fifteen female and fifteen male hybrid mice, 100 days of age at the start of the social experience regimen.

Results. Figure 5 summarizes the various fractions tested in experiment 3. Fraction 1 contained sample 41 (the void volume), fraction 2 contained samples 42-72, fraction 3 contained samples 73-89, and fraction 4 contained samples 90-98 (Figure 5, Experiment 3). These fractions were chosen by dividing the selectivity range of the column into three fractions while also including the void volume. Unaltered urine was left out of the experiment so that a fourth fraction could be tested.
Figure 5. The fractions were prepared by pooling the samples shown above.

The bioassay results of experiment #3 are presented in Figure 5.

Figure 6. Experiment #3. Ultrasounds in response to various urinary fractions from a Sephadex G-10 column. The fractions are defined in figure 5.
The within-subjects ANOVA indicated that differences existed among the four stimuli, \( F(3, 42) = 7.72, p<.05 \). Fraction 3 and fraction 4 did not significantly differ in the number of ultrasounds elicited, \( F(1, 14) = 5.335, p>.05 \). Subsequent post hoc contrasts indicated that fraction 2 elicited significantly more ultrasonic vocalizations than fractions 1, 3 and 4 \( F(1, 14) = 9.432, p<.05 \). Thus, these results indicate that the active chemosignal resides within the selectivity range of the column. Therefore, the active chemosignal has a molecular weight less than 1500 daltons.

**Experiment 4: Sephadex G-15 Separation**

This experiment attempted to further isolate the activity found in experiment 3. Here three fractions were obtained from gel chromatography on a Sephadex G-15 and tested for the elicitation of courtship vocalizations.

**Animals.** Subjects were 11 hybrid male mice between the ages of 85 and 92 days at the beginning of behavioral tests. Urine samples were obtained from 18 hybrid female mice, 90 days of age when behavioral tests began. Social-experience animals were ten female and ten male hybrid mice, 190 days of age at the start of the social experience regimen.

**Results.** Figure 7 summarizes the various fractions tested in experiment 4. Fraction 1 contained the samples 42-55, fraction 2 contained samples 56-69 and fraction 3 contained samples 70-75 (Figure 7, Experiment 4). These ranges were chosen by diving the fractions that were previously found to be active in the previous experiment.
Figure 7. The fractions were prepared by pooling the samples shown above.

The bioassay results of experiment #4 are presented in Figure 8.

Figure 8  Experiment #4. Ultrasounds in response to various urinary fractions from a Sephadex G-15 column. The fractions are defined in figure 4.

The within-subjects ANOVA indicated that differences existed among three stimuli, $F(2, 20) = 10.35$, $p<.05$. Subsequent orthogonal
contrasts indicated that fraction 1 elicited significantly more ultrasonic vocalizations than fractions 2 and 3, $F(1, 10) = 10.42, p<.05$. Fraction 2 and 3 did not significantly differ in the number of ultrasounds they elicited, $F(1, 10) = 1.440, p>.05$. These results indicate that the pheromone was successfully isolated in one fraction.

**Experiment 5: Sephadex G-15 Separation**

This experiment attempted to further isolate the activity found in experiment 4. Again three fractions were obtained from gel chromatography on a Sephadex G-15 and tested for the elicitation of courtship vocalizations.

**Animals.** Subjects were 15 hybrid male mice between the ages of 88 and 94 days at the beginning of behavioral tests. Urine samples were obtained from 19 hybrid female mice, 97 days of age when behavioral tests began. Social-experience animals were ten female and ten male hybrid mice, 190 days at the start of the social experience regimen.

**Results.** Figure 9 summarizes the various fractions tested in experiment 5.
Figure 9. The fractions were prepared by pooling the samples shown above.

The bioassay resulted in no significant activity in any of the fractions. Consequently, at the end of the testing session, the remaining stimuli from all three fractions were combined and retested. The resulting combination did appear to elicit ultrasounds, although insufficient data due to the small number of animals participating do not allow for any statistical conclusions. However, theoretical conclusions will be discussed further in the discussion section.
Chapter III: Discussion

The experiments of this thesis provide further information concerning the chemical and physical properties of the chemosignal in female urine that elicits vocalizations from males.

Volatility

Previous research indicated that the pheromone which elicits ultrasonic vocalizations was nonvolatile (Kerchner, 1988, unpublished). My findings support this conclusion. For example the finding that the substance can be repeatedly chromatographed and lyophilized without losing activity provides further support. Therefore, it is likely that the male must first contact female urine to be stimulated, but contact need not take place immediately after urine deposition.

The ultrasound eliciting pheromone does share its non-volatile characteristic with the aggression-inhibiting and gonadotrophin-stimulating pheromones. Evans et al. (1978) using numerous isolation techniques showed that the aggression-inhibiting pheromone was also non-volatile. Singer et al. (1988) similarly showed that ability of the gonadotrophin-stimulating pheromone did not diminish after it was repeatedly lyophilized and chromatographed.

Protein Characteristics

Kerchner (1987) suggested that the ultrasound eliciting pheromone is somehow associated with the urinary protein found in the urine of female mice. Seeberger (1988, unpublished) measured the emergence of urinary proteins from a Sephadex G-10 column using a colorimetric assay. He
found that the proteins emerged with the void volume. Seeberger (1988, unpublished) also found that the active components of the pheromone emerged after the void volume. This would indicate that the active components of the pheromone are not urinary proteins. The data from experiments 1 and 2 in this study indicate that the pheromonal activity was found in the sephadex fraction immediately following the void volume fraction (See figure 10).

![Figure 10. Fractions tested in experiments 1 and 2.](image)

These findings suggest that the pheromone is not a protein. In addition, experiments three and four show that the pheromone resides within the fractionation range of the Sephadex G-15 and thus, has a molecular weight of less than 15,000 daltons (See figure 11).
This finding shows that the pheromone is smaller than the proteins which comprise the MUP complex (molecular weights of around 17,500 daltons). However, the major urinary proteins (MUPs) could still play some role in mediating successful pheromonal elicitation of ultrasounds. For example, MUPs may allow the pheromone to remain water-solubility. Water-soluble chemical cues are generally non-volatile and are thought to be perceived via the accessory olfactory system (VNO) (Wysocki et al., 1982).

The ultrasound-eliciting pheromone may share its mechanism of water-solubility with the other female pheromones. Evans et al. (1978) found that the precipitate obtained following ethanol extraction did not reduce aggression. The precipitate would have contained denatured proteins. Therefore, the aggression-inhibiting pheromone does not appear to be associated with macromolecular (protein) components of urine. These results were interpreted as indications that the aggression-inhibiting chemosignal is hydrophilic. To support this conclusion, Evans et al. (1978) found that the retentate from fresh urine dialyzed against water for two days also did not elicit aggression. This latter finding provides further
evidence that the aggression-inhibiting pheromone is not a protein. Clancy et al. (1988) reported that the female pheromone which stimulates LH secretion in mice was not a urinary protein, but a low molecular weight compound. However, Clancy (1988) hypothesized that the pheromone may be weakly bound to urinary proteins and that binding to the MUP may be important for maintaining water solubility of the female mouse pheromone and its retention in urine.

**Molecular Weight**

Experiments 1 and 2 indicate that the molecular weight of the pheromone appeared to be near 700 daltons. Experiments 3 and 4 show that the pheromone emerged within the selectivity range of the Sephadex G-15 indicating that the molecular weight is less than 1500 daltons (See figures 10 and 11). The finding that the activity was in the upper range of the G-15 suggests that some activity must have resided in the void volume of the G-10 column.

Vandenbergh et al. (1976) using marker proteins, reported the upper limit of the Sephadex G-15 gel activity to be 1200 daltons. Therefore, based upon my data and that of Vandenbergh et al.'s (1976) I conclude that the molecular weight of the pheromone is somewhere between 700 and 1200 daltons. Such a molecular weight further supports that the pheromone being non-volatile.

As already mentioned, the ethanol precipitate in Evans et al.'s (1978) experiment did not inhibit aggression signifying that the pheromone is not associated with the macromolecular weight compound. Unfortunately, no estimate of molecular weight was given. However, the results of my experiment are in accordance with Evans et al. (1978) findings and suggest
that the two pheromones may be the same or that they at least contain similar chemical and physical characteristics. Clancy et al. (1988) found that the female pheromone that stimulates LH secretion in mice was a low molecular weight compound. The fraction in my study containing the pheromonal activity corresponded to a fraction in Clancy’s (1988) study which was deemed behaviorally inert. Thus, the ultrasound eliciting pheromone does not appear identical to that which stimulates LH release. However, they may share some of the same molecular components and/or chemical characteristics (i.e. non-volatile and non protein like characteristics).

In experiment five, the active fraction was broken into three separate fractions. None of these fractions contained any pheromonal activity. At the completion of the last testing session I combined the fractions and the resulting combination elicited ultrasounds. Unfortunately, there were not enough stimuli left to amass valid data to draw firm conclusions. This tentative finding suggests that the pheromone may be composed of more than one active component. Therefore, for successful pheromone elicitation all the components or some "critical" number of components need to be present. This fractionation could have resulted in the separation of the active components resulting in the loss of activity. Therefore, when the three stimuli were combined the active components, or some "critical" number of active components were recombined resulting in restored activity. This possibility will be tested further in the future.

Vandenbergh et al. (1976) similarly found that in attempting to break down a pheromonally active fraction of urine none of the resulting fractions elicited any behavior. Furthermore, when the fractions were recombined, the resulting mixture did not elicit any behavior. Vandenbergh et al. (1976)
postulated that for pheromonal activity, the active components must exist in the proper proportions to each other.

In my experiments, and in numerous other reports, repeated difficulty has occurred in isolating pheromones into purely active fractions. This difficulty was experienced by earlier investigators attempting to isolate the female pheromone (Wellington, personal communication with Kerchner; Kerchner, unpublished pilot data, c.f. Kerchner, 1988). This difficulty could result from the chemosignal being comprised of multiple chemical constituents whose interaction results in the biologically active olfactory melange (Kerchner, 1988, unpublished). Therefore, gel fractionation would separate the components contained in the olfactory melange according to their different molecular weights. However, in experiments 1-4, I was able to get varied levels of activity in some fractions which suggests that there are many components to the signal and these components were spread out among these active fractions. It is possible that there could be a correlation between the number of components to the amount the animal ultrasounded. However, there is no current evidence to support this hypothesis. The fact that the components which did elicit ultrasounds were adjacent to each other indicates that the different components are of similar molecular weight. Yet, when I further broke down the active fraction in experiment 5, I did not get any activity until I recombined the resulting fractions. The outcome could be explained by the notion that a "critical" number of constituents are needed for pheromonal activity and this "critical" number was not obtained until the different components were recombined. This implies that ultrasound eliciting pheromone may be an olfactory melange.
In the future, the olfactory melange theory will be tested using the odor-image approach. The odor-image approach assumes that no single component of a complex odor is effective at eliciting a response as the complex odor itself. Novotny et al. (1986) applied the odor-image approach to identify the volatile components of urine from female housed mice which delay puberty in grouped housed females. Novotny compared the gas chromatograph profiles of urine from intact females with urine from adrenalectomized females whose urine does not delay puberty. He found that there were six components of the urine that were produced in lower concentration by adrenalectomized females than by normal females. Novotny then added these components to urine from adrenalectomized females. Puberty was delayed in females exposed to urine from adrenalectomized female that were treated with three of these components. Therefore, this theory postulates that the receiver responds to the pattern/interaction of the components of the complex odor. This strategy has also been used to identify the volatile components of urine from males which initiates aggression (Ingersoll & Laundry, 1986; Novotny, 1985), produces the Whitten effect (Jemiolo et al., 1986) and female attraction (Jemiolo et al., 1985).

In the future, fractions which prove to be behaviorally inactive when presented alone should be added in combination to urine which does not normally elicit vocalizations, e.g., hypophysectomized females, castrated males, or normal males mice. The odor-image strategy is best at identifying chemosignals comprised of multiple components (olfactory melange). If these combinations exhibit the ultrasound eliciting property there would be support of the olfactory melange.
To more accurately identify the female pheromone which elicits ultrasounds, standard compounds used to estimate molecular weight need to be run through the column. Marker compounds, whose molecular weights are known, will allow more accurate assessments of the molecular weight of the active fraction. Additionally, high performance liquid chromatography will also provide accurate estimates of molecular weight.

To further prove the pheromone’s protein independence, unfractionated female urine needs to be incubated with a protein enzyme (Pronase). Then, the resulting protein-depleted urine would be fractionated via gel chromatography and tested for behavioral activity. If the resulting fractions prove behaviorally active, protein independence can be further supported.
References


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