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**STUDIES ON AGGRESSION PROMOTING CHEMOSIGNALS OF THE
MOUSE**

City University of New York

PH.D. 1982

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STUDIES ON AGGRESSION PROMOTING CHEMOSIGNALS
OF THE MOUSE

by

GEORGE BOBOTAS

A dissertation submitted to the Graduate Faculty
in Biochemistry in partial fulfillment of the
requirements for the degree of Doctor of Philosophy,
The City University of New York.

1982

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GEORGE BOBOTAS

1982

This manuscript has been read and accepted for the Graduate Faculty in Biochemistry in satisfaction of the dissertation requirement for the degree of Doctor of Philosophy.

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Abstract

STUDIES ON AGGRESSION PROMOTING CHEMOSIGNALS OF THE MOUSE

by

George Bobotas

Adviser: Professor Aaron Lukton

The studies performed in this work confirm and extend the results of previous studies on the aggression-promoting cues of mice. The results of behavioral studies performed in Experiment 1 confirmed previous reports of the androgen dependency of the aggression-promoting cues of male mice. In Experiment 3, a lipophilic aggression-promoting cue was detected in extracts prepared from bladder urine of male mice. At the same time, a series of experiments performed on the preputial gland of male mice have established that at least two aggression-promoting cues are present in this sebaceous gland. Finally, it has been shown that a wax ester, tetradecyl decanoate, can function as an aggression-promoting chemosignal for male mice under the conditions of the behavioral paradigm employed in this work.

To my wife and daughter,
Marie and Demetra

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INTRODUCTION

The ability of an organism to sense and react to external chemical stimuli is widely distributed throughout the animal kingdom. Chemoreception in primitive organisms plays a major role in the detection and movement toward nutrients as demonstrated for the bacterial system of chemotaxis (Adler, 1975; Koshland, 1980). Similarly, in more complex organisms, chemoreception in the form of olfaction aids in the orientation to sources of food (Alberts, 1976). In addition, ethological studies have shown that social interactions between organisms are mediated by chemical signals. To describe those chemical substances with communicative functions, the term semiochemical was proposed by Law and Regnier (1971). These semiochemicals may be further subdivided based on whether the sender and receiver of the chemical message belong to the same species.

If the semiochemical is involved in communication between individuals of two different species then the terms allelochemic (Whittaker, 1970) or xenomone (Chernin, 1970) are used. Such interspecific chemical communication would include the floral scents which mediate the symbiotic relationship between plants and pollinating insects (Henry, 1966). On the other hand, the term pheromone was proposed (Karlson and Luscher, 1959; Karlson and Butenandt, 1959) to describe those semiochemicals involved in an intraspecific

communication. Earlier, the term ectohormone was proposed by Beth (1932) which has the same meaning but has not found wide usage.

Recently, ethological and physiological studies have shown that many mammalian conspecific interactions are mediated by airborne chemical signals. In particular, the role of olfactory communication in house mice has been studied intensively as a model of animal social interactions. The present discussion will deal with the olfactory cues of small rodents, with particular attention being paid to those cues mediating agonistic interactions of conspecific males. For a discussion of olfactory communication in other mammals the reader is referred to several recent reviews on the subject (Birch, 1974; Bronson, 1976, 1979; Cheal and Sprott, 1971; Doty, 1976; Eppler, 1974a, b; Montagna and Sadler, 1974; Ralls, 1971; Steiner and Ganchrow, 1982; Whitten and Bronson, 1970).

It should be kept in mind that the value of the pheromone concept used in earlier studies of mammalian olfactory communication has recently been questioned (Beauchamp et al., 1976; Bronson, 1976). Pheromones or ectohormones were originally defined for the insect model of chemosensory communication (Karlson and Luscher, 1959; Karlson and Butenandt, 1969), in which a unique chemical compound is secreted which releases a stereotyped pattern of behavior or endocrinological change in a conspecific. On the other hand, mammalian communication systems employ

a variety of interacting sensory modalities which can modify the effect of an olfactory cue depending on the context in which the message is received (Bronson, 1971). Moreover, the effect of a mammalian semiochemical can vary depending on the emotional or endocrinological status of the conspecific recipient. The latter phenomena is illustrated by the ability of male murine odors to induce estrous in one conspecific female (Whitten, 1956a) while blocking her pregnancy if she is recently inseminated by another male (Bruce, 1965).

Another criticism of the use of the pheromone concept in mammals has been that the complexity of mammalian secretions or excretions imply that, unlike the classical pheromone, a complex mixture will constitute a mammalian chemosignal. As a result of these and other criticisms, the use of pheromone concept in mammalian studies has been severely restricted. Terms such as olfactory cue or chemosignal are now used to represent mammalian pheromones.

However, this usage is problematic since these new terms are actually synonymous with semiochemicals. One may correctly argue that the former terms are unable to distinguish between inter- and intraspecific functions of chemical communication. One solution to this problem is to continue the use of the pheromone concept in mammalogy but with a restricted definition of pheromone in mind. In this case a pheromone would only imply that a semiochemical is used in the communication between conspecifics. This restricted definition may also be useful in the study of

insect chemoreception since many of the aforementioned criticisms of mammalian pheromones can also be applied to insect pheromones. For example, it has been demonstrated that many insect pheromonal responses are not initiated by single compounds but instead require complex mixtures of semiochemicals for full activity (Silverstein, 1979). Moreover, it has been stated that prior experience with a cue should not be required for pheromonal responses (Beauchamp, 1976), however, it has been shown that learning is necessary for the expression of several pheromonal responses in insects (Hendry et al., 1975; Roitberg and Prokopy, 1981).

Rodent Priming Cues

The chemosignals of rodents were classified by Bronson (1968, 1971) as having either priming or signalling functions. The primary responses are implicated when the recipient's endocrine state is modified sometime after perceiving the odor. Various studies have shown that conspecific odors can influence reproductive processes in rodents. For example, housing female mice in groups tends to suppress estrous cyclicity by prolonging the length of their cycles (Lamond, 1959). Depending on the size of the group, one can observe a concomitant increased occurrence of anestrus (Whitten, 1959) and pseudopregnancies (Van der Lee and Boot, 1955). These effects acting to reduce the incidence of estrus persist after optic nerve section, ear drum rupture or prevention of physical contact

among the group-housed females (Whitten, 1959). The results of this study provided indirect evidence for the involvement of an olfactory cue in this process.

A more direct approach to the question of olfactory mediation of estrous suppression in group-housed female mice would be to employ subjects which are unable to smell. These anosmic females could be produced by lesions of the central or peripheral neural structures involved in olfactory function (Murphy, 1976). Unfortunately, both of these procedures can produce severe changes in the estrous cyclicity of the mouse (Vandenbergh, 1973; Whitten, 1956b).

The estrous suppressive effects of group housing female mice can be eliminated by their exposure to an adult male mouse (Whitten, 1956a). Direct exposure is not necessary, since this estrous-inducing male cue has been shown to be airborne (Whitten et al., 1968). In fact, the mere presence of an adult male mouse in the same room with group-housed female was sufficient for the induction of normal estrous activity (Chipman and Fox, 1966). In addition, sensory deprivation experiments (Whitten, 1966) eliminated the possibility that visual, auditory or tactile cues participate in this phenomena. This male chemosignal appears to be androgen-dependent in that castrated male mice are not effective stimuli for estrous induction (Whitten and Bronson, 1970). Further evidence for the biochemical source of this cue was provided by an earlier study (Marsden and Bronson, 1964) which showed that an estrous inducing cue to be present

in male mouse urine.

In addition to its estrous inducing effects, urine of conspecific male has been shown to accelerate sexual development in both immature female house mice (Cowley and Wise, 1972; Colby and Vandenberg, 1974) and prairie deer mice (Teague and Bradley, 1978). In contrast, the presence of an adult male appears to delay the onset of puberty in immature male mice (Vandenberg, 1971). On the other hand, the presence of an adult female mouse will accelerate the onset of puberty in male while delaying it in immature females (Drickamer, 1974; Fox, 1968; Vandenberg et al., 1972).

Other priming effects of male mouse urine include the elevation of serum levels of luteinizing hormone and estradiol in conspecific females (Bronson and Desjardins, 1974; Bronson, 1981), as well as the pregnancy-block phenomena in recently inseminated females (Bruce, 1965; Dominic, 1965). For a further discussion of the effects of primary cues on reproductive processes of rodents, the reader is referred to several current reviews on the subject (Aron, 1979; Bronson and Coquelin, 1980; Vandenberg, 1980).

Rodent Signalling Cues

Signalling cues have been shown to mediate a variety of social interactions in rodents. A semiochemical is presumed to serve as a signalling cue if the recipients behavior is modified immediately upon sensing the olfactory

cue (Bronson, 1968). The chemosignals emitted by female rodents include male attractants (Lydell and Doty, 1972; Hayashi and Kimura, 1974; Singer, et al., 1976), aggression inhibitors (Mayford and Nowell, 1971c; Dixon and Mackintosh, 1971), and elicitors of ultrasonic vocalizations in male mice (Nyby et al., 1977; Nyby and Whitney, 1980). In addition, the attraction of preweanling pups for maternal odors has been demonstrated in rats (Leon and Moltz, 1971) and house mice (Breen and Leshner, 1977).

Unlike the stereotyped behavior of insects or the physiological responses to mammalian priming cues, the behavioral response to these signalling cues may be altered by the experience of the recipient. For example, the ultrasonic response of male mice to female conspecific urine has been shown to be dependent on both neonatal imprinting and postpubertal learning (Dizinno et al., 1978; Nyby et al., 1978). Similarly, the preferences of adult male rats for odors of estrous over non-estrous female conspecifics is increased by prior sexual experience on the part of the male.

Other factors besides experience can also affect the signalling value of female urine. Recent evidence (Yamazaki et al., 1976, 1979a) from studies of mating preferences in congenic mice indicates a genetic basis for the male discrimination of conspecific females. By congenic strains, it is meant that they are genetically identical except for allelic differences at a particular genetic locus. The locus in question is the major histocompatibility complex

MHC) of the mouse, also known as the H-2 region (Hood et al., 1978). Male mice differing only at this locus were shown to exhibit significant differences in mating preferences (Andrews and Boyse, 1978). Moreover, the strain preference of males was found to favor females of dissimilar H-2 types, although a small number of significant preferences for a similar H-2 type occurred.

The chemosensory nature of these signals was established by use of a Y-maze in which male mice of different H-2 types were tested for their ability to discriminate between two air streams scented by females of different H-2 types (Yamazaki et al., 1979b). The results of this study indicated that both male and female mice could be trained to discriminate between the differentially scented arms of the Y-maze, presumably on the basis of H-2 differences. This result would appear to favor a chemosensory model for perception of H-2 haplotypes.

However, there are several problems with the interpretation of these experimental results in terms of an olfactory model. First, an experiment should have been conducted employing subjects rendered anosmic by either ZnSO_4 irrigation of the olfactory mucosa and or lesioning of the vomeronasal organ (Johns, 1980). Moreover, it is possible that the air streams may have transported signal-containing aerosols as well as volatile signals from the stimulus to the subject animals. Thus, it is premature to identify these MHC-linked chemosignals as only olfactory, in view of

the growing role of the vomeronasal organ in murine chemoreception (Ingersoll and Lee, 1979). This matter of which nasal structure is involved in the reception of an olfactory cue, will be later examined in more detail with respect to aggression-promoting cues.

One may also ask, what is the biological relevance or adaptiveness of these MHC-linked mating preferences in the mouse? Moreover, can the effects of these MHC-linked chemosignals be observed in feral populations of *Mus musculus*? It has been proposed (Yamazaki et al., 1979b) that this chemocommunicative system may function to both increase and preserve the observed polymorphism of the H-2 locus in a given population of house mice. Moreover, these mating preferences should drastically increase the heterozygosity of individuals with respect to this genetic locus.

There are also several studies which indicate that male as well as female chemosignals are involved in murine mate selection. These studies (Mainardi et al., 1965; Kennedy and Brown, 1970) have shown that juvenile female mice prefer to mate with males of a different strain. However, in contrast to the mating preferences of congenic mice, male mice did not appear to choose to mate with females of a different strain. In addition, it was found that olfaction played a major role in the discrimination of potential mates by female mice.

In another study, (Gilder and Slater, 1978), the preferences of adult female mice for odors from males of

the same strain was examined. It was found that the female mice preferred bedding soiled by males who were not their brothers over the bedding of their brothers. These findings together with the results obtained with congenic mice would imply that both male and female chemosignals are involved in the mating preferences of mice.

Male rodents are not always attracted to the olfactory cues produced by a conspecific. For example, male mice when tested in an open-arena will avoid areas marked with the urine of a singly-housed male conspecific (Jones and Nowell, 1973a). The isolation of the urine donor is particularly significant since urine collected from group-housed males has been shown to lack aversive properties (Jones and Nowell, 1974). In a related study (Jones and Nowell, 1974b), the latency of approach to a food pellet by male mice increased in the presence of bedding soiled by a foreign male. The olfactory nature of these aversive cues was demonstrated by the use of zinc sulfate-induced anosmia to eliminate the avoidance response of male mice (Jones and Nowell, 1976).

The production of this urinary cue is thought to be androgen-dependent since male mice will not avoid an area marked with the urine of gonadectomized male conspecifics (Jones and Nowell, 1974c; Sawyer, 1978). Androgens have also been shown to influence the responsiveness of male mice to these aversive cues. Castration eliminates the aversive response of a male subject to the urine of a foreign male (Sawyer, 1980). In the same study, the administration of

testosterone to castrates was shown to effect a restoration of their avoidance response. The failure of castrated mice to display an aversive response is not due to a specific anosmia, since such mice can be trained to discriminate between the urinary odors of intact and castrated male conspecifics (Sawyer, 1981). Moreover, castrated male mice will exhibit an aversive reaction if exposed to the urine of severely-stressed castrated donors (Sawyer, 1980).

It has been suggested that aversive cues of male mice function by increasing the fearfulness of the recipient since exposure to male urinary odors can increase the emotionality of male subjects (Jones and Nowell, 1977). Such cues are thought to serve as territorial markers for mice (Egan, Royce and Poley, 1972). These cues could function in a feral population to limit agonistic encounters between conspecific males occupying adjacent territories.

Aggression-Promoting Cues

The aggressive reactions of rodents have been classified into several distinct classes, each of which has a particular physiological and neural basis (Moyer, 1968, 1976). These classes which also may be differentiated on the basis of eliciting stimuli include predatory, fear-induced, territorial, instrumental, irritable, inter-male and maternal aggression. It should also be noted that a variety of behavioral patterns are included under the rubrics of agonistic behavior such as attack, submission, dominance, defense, flight and pursuit (Scott and Fredericson, 1951).

The present section will examine the role of olfactory cues in the agonistic interactions of conspecific male mice. Generally, in most species of rodents, inter-male aggression is elicited by the presence of a strange male to which the attacker has not been habituated. In contrast, male rats and mice will rarely attack a female conspecific (Barnett, 1963; Mackintosh, 1970). Female mice, in turn will usually not attack a strange conspecific of either sex. This passivity of female mice cannot be generalized to other species of rodents since female hamsters and gerbils have been shown to exhibit levels of aggression toward unfamiliar conspecifics (Floody and Pfaff, 1977; Swanson, 1974). The only time when female mice may display equally high levels of aggression occurs when they are lactating during the postpartum period (Svare et al., 1981). Apart from such instances of maternal aggression, male and female mice will

not engage in agonistic encounters.

In mice, olfactory cues play a major role in the regulation of aggressive reaction to strange conspecifics. This has been demonstrated in a number of ways. First, it has been observed that agonistic encounters in mice are usually preceded by a period of ano-genital sniffing (Lee and Brake, 1971). Moreover, visual cues are not required for the discrimination between conspecifics during murine agonistic encounters, since trained fighters with genetically impaired vision vigorously attack other males but not females (Lee and Brake, 1971). In addition, the aggression of a male mouse towards a male conspecific can be greatly reduced if the latter's odor is masked by perfume (Ropartz, 1968) or deodorant (Lee and Brake, 1971).

Further evidence for the involvement of chemosignals in murine agonistic behavior can be found in studies employing urine-coated castrates as stimulus animals. For example, Mugford and Nowell (1970a) have demonstrated that female mouse urine coated upon the fur of castrated male mice significantly reduced the incidence of attacks from trained fighters. Furthermore, male castrates coated with water elicit fewer attacks from blind fighters than are elicited by castrates coated with intact male urine (Crumb, 1975). The results of these studies suggest that male mice produce olfactory cues which promote intermale aggression, whereas female mice produce olfactory cues which deter such agonistic behavior.

If a functional nasal sensory system is required for the perception of aggression-promoting chemosignals then any form of olfactory impairment should reduce the agonistic responsiveness of male mice towards male conspecifics. Such an approach can provide additional evidence for the role of chemosignals in murine inter-male aggression. For example, removal of the central neural structures mediating olfactory perception, namely the olfactory bulbs, has been shown to result in a severe reduction of inter-male aggression in mice (Ropartz, 1968; Rowe and Edwards, 1971). At the time, the decrement in agonistic behavior was interpreted as being a direct consequence of the impairment of olfaction. However, this interpretation has been challenged on the basis of recent work indicating that the olfactory bulbs can perform certain nonsensory functions in rodents (Alberts, 1974; Edwards, 1974; Murphy, 1976).

Another method of producing olfactory deficits involves destruction of the peripheral olfactory receptor neurons by intranasal application of zinc sulfate solution. Such treatment has also been shown to produce degenerative changes in the olfactory bulbs of mice (Margolis et al., 1974). If olfaction is necessary for the mediation of murine agonistic behavior one would expect peripheral and central olfactory lesions to have similar behavioral effects. However, when a study of the effects of peripheral olfactory lesions on inter-male aggression was performed, it was found that a majority of the male mice rendered anosmic by zinc sulfate treatment, would still engage in attack behaviors (Edwards,

Thompson and Burge, 1972). Thus, it would appear that a functional olfactory system is not a requirement for aggressive responsiveness in mice. On the other hand, it may be possible that the zinc sulfate was improperly applied so that the olfactory mucosa was only partially lesioned.

It should be kept in mind that, in addition to the primary olfactory mucosa, four other chemosensory structures are present in the nasal fossae of most mammals. Namely: (1) vomeronasal organ of Jacobson, (2) trigeminal nerve, (3) septal organ of Masera and (4) nervus terminalis. Recently, the role of the vomeronasal organ in the chemoreception of odorants have been firmly established (Ingersoll, 1980; Wysocki, 1979; Scalia and Winans, 1976). Moreover, it has been shown that the peripheral receptor cells of the vomeronasal organ are unaffected by the zinc sulfate treatment used to produce peripheral anosmia (Margolis et al., 1974). This is not surprising in view of the cul de sac nature of this organ which in turn limits the access of liquids to the sensory receptors lining its inner surface (Negus, 1958). Thus, it may be possible that the persistence of agonistic behavior following zinc sulfate-induced anosmia was due to a functional vomeronasal organ.

The question of a possible role for the vomeronasal organ in murine agonistic behavior has been recently examined by Ingersoll (1980) in a study employing genetically blind mice. Using naive male mice as subjects, he found that vomeronasal deafferentation produced an abolition of attack behavior and significantly reduced the occurrence of

ano-genital sniffing behavior. If male mice were subjected to joint olfactory and vomeronasal lesioning then all forms of agonistic behavior were abolished. On the other hand, if trained fighters were used instead of naive males then a small but significant amount of aggressive behavior was found to persist after the joint deafferentation procedure. These findings indicate that the vomeronasal organ is involved in the perception of aggression-promoting cues. Of interest is the finding that trained fighters retain a greater degree of aggressive-responsiveness after joint lesioning treatment as compared to naive male mice. It appears that the experience of fighter training, in addition to reducing variability in aggressive responsiveness of male mice, can also alter the importance of nonolfactory cues as aggression-promoting stimuli.

This interpretation is supported by the results of a recent study of the role of submissiveness in agonistic interactions of mice (Grimm, 1980). These findings indicated that submissive postures of defeated male mice can serve as visual cues to limit further attacks by the victor. Thus, agonistic interactions of male mice can be affected by the use of visual signals. One should note that most studies of olfactory communication in house mice have not considered the possible confound produced by the use of vision during agonistic encounters in mice. Only in the studies of Lee and coworkers (e.g. Lee, 1976) have genetically blind mice of the SJL/j strain been used in order to abolish the transfer

of visual signals during inter-male aggression.

The production of aggression-promoting olfactory cues in mice appears to be androgen-dependent (Lee and Brake, 1972; Mugford and Nowell, 1970b). A similar conclusion has been reached by Jones and Nowell (1973a, 1977) for the aversive effect of male mouse urine on the investigatory behavior of male conspecifics. Moreover, the priming effects of male urine on reproductive processes in female mice such as estrous-induction (Bronson and Whitten, 1968), and termination of pregnancy (Bruce, 1965; Dominic, 1965) have also been shown to be mediated by androgens. Whether the olfactory cues which elicit aggression or aversion also influence the endocrine physiology of the female mouse is unknown. However, it has been shown that dichloromethane extracts of male mouse urine promote aggression when assayed behaviorally and also accelerate estrous in female mice (Lee, 1976; Monder et al., 1978).

The administration of androgens to female mice can also modify the olfactory information emitted. Testosterone propionate administered to adult intact and ovariectomized female mice increases the number of attacks by fighter mice toward these females as compared with oil-injected controls (Mugford and Nowell, 1970b). The urine of spayed females injected with androgens was also shown to elicit aggression when coated on castrated males who then were used as stimulus animals (Mugford and Nowell, 1972). Moreover, it has been shown that neonatally androgenized female mice released

aggression-promoting urinary cues (Lee and Griffio, 1973). Additionally, Dominic (1969) has demonstrated that the urine of early androgenized female mice is effective in blocking the pregnancy of inseminated females.

Androgens administered neonatally to female mice have been shown to be effective in producing agonistic behavior toward conspecifics when these females are treated with testosterone propionate in adulthood (Bronson and Desjardins, 1968; Edwards, 1968). However, such studies fail to distinguish between the direct increase in aggressive behavior and the concurrent induction of aggression-promoting cues in androgenized females. A similar confound also is operating in previous reports of the induction of intermale aggression in castrated male mice by androgen treatment (Bronson and Desjardins, 1968; Edwards, 1970). Such experimental confounded variables can be resolved by separately assessing the stimulus and behavioral effects of androgens.

Sources of Olfactory Signals

There is evidence that the preputial glands are a possible source for the production of urinary chemosignals. First, McKinney and Christian (1970) found that preputial-ectomy of a member of a pair of mice resulted in an increase of intermale aggression, a result that was not replicated by Mugford (1973). Mugford and Nowell (1971b) have further shown that the preputial glands of mice exposed to an intense

electric shock appear to release an aggression-promoting cue. However, a more natural stressor, an intense fight did not induce preputial production of these signals (Mugford, 1973), nor did the concurrent administration of corticotrophin and epinephrine (Mugford and Nowell, 1971b). Nevertheless, it is known that subordination in male mice is correlated with a hypotrophic effect on the preputial gland (Christian, Lloyd and Davis, 1965; Lloyd, 1971). Jones and Nowell (1973b) have demonstrated that either the secretions of the preputial gland or bladder urine from male mice can elicit aggression. Moreover, a combination of the two substances appeared to be more aggression-promoting than bladder urine alone. Thus it would appear that there may be at least two tissue sources of aggression-promoting cues in the male mouse.

The preputial glands of the male mouse have also been postulated to release arousing and attracting chemosignals (Bronson and Caroom, 1971). Caroom and Bronson (1971) found that saline homogenates of preputial glands from male mice are investigated more often than control substances by both sexually experienced females and estrogenized sexually naive females. It had been previously reported that a sexual attractant from the preputial of male mice was localized in the free fatty acid fraction (Gaunt, 1968). In the rat, preputial gland homogenates were shown to be attractive to the opposite sex (Orsulak and Gawienowski, 1972). Interestingly, a later study showed unlike the mouse, the sexual

attractant in the rat preputial was not associated with the free fatty acid fraction of the volatile lipids that were isolated. Curtis et al. (1971) has suggested that the sexual attractant of estrous rhesus monkeys consists of a mixture of short-chain aliphatic acids.

The metabolism of sebaceous glands is known to be under the influence of steroidal hormones (Ebling, 1972). The mouse preputial gland, a sebaceous gland, has been shown to be responsive to administration of exogenous androgens in a number of studies. Mugford and Nowell (1972) reported a dose-dependent response to treatment with testosterone propionate in the hypertrophy of female preputial glands. Administration of testosterone propionate to female mice, prepubertal male mice or castrated male mice produced a significant increase in the synthesis and storage of preputial lipids (Sansone-Bazzano et al., 1972). In the rat, Drucker (1977) has reported that preputial glands of male castrates increase in weight after treatment with either testosterone or dehydroepiandrosterone (DHA). The finding that DHA, an adrenal androgen, can stimulate preputial gland growth raises the question of the physiological role of adrenal androgens in the production of olfactory cues, particularly in terms of intense stress (e.g., Mugford and Nowell, 1971b). Moreover, Hodgins and Hay (1973) have observed that the preputial glands of rats, actively metabolize DHA but without the formation of testosterone or dihydrotestosterone.

Information exists which suggests the possible involve-

ment of other tissues in the biosynthesis of aggression-promoting cues. The report of the presence of olfactory chemosignals in the bladder urine from male mice by Jones and Nowell (1973b) has been mentioned. In the same study they found that the coagulating glands may produce an aggression-inhibiting cue. In a study (Yee, 1977) of the aggression-promoting chemosignals of various organ extracts, homogenates were prepared by the method of Caroom and Bronson (1971) and extracted with dichloromethane. It was found, as previously mentioned, that dichloromethane extracts of intact male mouse urine appear to contain aggression-promoting chemosignals. The results of these tissue studies demonstrated an aggression-promoting property in a kidney extract. This would appear to support the behavioral activity observed in bladder urine by Jones and Nowell (1973b). The preputial extract did not elicit an aggressive response. The discrepancy between this finding on the preputial and the results of Caroom and Bronson (1971) may be due to a number of factors. First, it is possible that the concentration of the preparation was not high enough for behavioral assays since Caroom and Bronson (1971) injected the organ donors with testosterone propionate. Additionally, it is possible that extraction of preputial homogenates with dichloromethane is ineffective for this tissue or that other chemosignals are released by this procedure.

The extracts of seminal vesicles, prostate glands, testes, and submaxillary glands did not show positive results.

These tissues may be a logical source of urinary chemosignals in terms of the well known action of androgens on primary and accessory sexual structures (Williams-Ashman and Reddi, 1971; Brandes, 1974). Moreover, it has been reported (Melrose et al., 1971) that the submaxillary glands of boars are the source of chemosignals stimulating sexual receptivity in the sow. Recently, it has been found that the saliva of male mice appears to contain an androgen dependent chemosignal that is aggression-promoting when behaviorally assayed (Lee and Ingersoll, 1979).

Another possible source of aggression-promoting cues in mouse urine may be volatile androgen metabolites. The results of Jones and Nowell (1973b) on bladder urine and the work with kidney extracts suggest a hepatic metabolite. It is known that the liver actively metabolizes androgens (Makin, 1975). Also, two volatile androgen metabolites, 5 α -androst-16-en-3-one and its related alcohol, have been identified as the active substances produced in the submaxillary gland of boars that induce lordosis in the sow (Melrose et al., 1971; Signoret, 1970). Moreover, preliminary studies in our laboratory suggest that hydrolysis by β -glucuronidase of the water fraction from a dichloromethane extraction of urine increases the aggression-promoting quality of the treated water extract. The hepatic conjugation of androgens to the corresponding glucuronide or sulphate (Bernstein and Solomon, 1970) increases the solubility of steroids in water while enzymatic hydrolysis of glucuronides

or sulphatase preparations of the conjugated steroid species liberates the lipophilic aglycone. This parallels the latency of the aggression-promoting cue in hydrolyzed water extracts, in that the β -glucuronidase treatment releases a lipophilic chemosignal that can be isolated in a second extraction with dichloromethane (Yee, 1977). It should be noted, however, that this study used a mixture of bladder and voided urine of male mice as a chemosignal source for the extraction procedure. Thus, the possibility exists that the latency of the chemosignal was due to a product of the accessory sex glands.

Chemical Analysis of the Olfactory Cues

Wilson and Bossert (1963) proposed, on the basis of a volatility argument, that chemical substances suitable for chemocommunication would have between 5 to 20 carbon atoms. Most of the mammalian chemosignals presented in Table 1 appear to have molecular weights which are consistent with the notion of Wilson and Bossert (1963). The volatility of these chemosignals is the only common chemical or physical property with which to characterize them. In addition, no functional group is common to all of these cues, although an ester linkage can be found in three of the compounds.

Although olfactory communication in house mice has been widely studied, relatively little is known regarding the compounds which serve as olfactory cues in mice. One reason for the slow progress in this area is the fact that at each stage

Table 1
Mammalian Chemosignals

<u>Source</u>	<u>Chemical</u>	<u>Behavior Elicited</u>
Boar, Submaxillary gland ^a	16-androstenol	lordosis
Black-tailed Deer, Tarsal gland ^b	cis-4-hydroxydodec-6-enoic acid lactone	sniffing
Dog, Vaginal secretion ^c	methyl-p-hydroxybenzoate	sexual behavior in female
Gerbil, Male ventral gland ^d	phenylacetic acid	investigation in males
Hamster, Vaginal secretion ^e	dimethyl disulfide	attraction in males
Marmoset monkey, Male circumgenital gland ^f	wax esters of butyric acid	sniffing, preference
Rhesus monkey, Vaginal secretions ^g	short-chain fatty acids	male sexual activity

^aReed, Melrose and Patterson (1974)

^bBrownlee et al. (1960)

^cGoodwin, Gooding and Regnier (1979)

^dThiessen et al. (1974)

^eSinger et al. (1976)

^fEpplé, Golob and Smith (1979)

^gCurtis et al. (1971)

during the purification of a chemosignal, a large number of behavioral assays must be performed for each fraction that is isolated. In addition, a chemosignal may be present in extremely low amounts in a tissue or natural fluid so that a large amount of tissue must often be accumulated in order to perform chemical analyses. Moreover, many of the chemosignals used by mice are found in urine, a situation which can hamper subsequent chemical identification of these cues due to the complex nature of this natural fluid. For a discussion of these and other problems common to the isolation of mammalian chemosignals, the reader is referred to several recent reviews on the subject (Claesson and Silverstein, 1977; Mykytowycz, 1979; Thiessen, 1977).

Several chemical studies of murine chemosignals have found that many of the priming cues are associated with the peptide fraction of male mouse urine. Using gel filtration chromatography, Vandenberg et al. (1976) were able to show that puberty-accelerating pheromonal activity is found in a fraction with a peak molecular weight of 860 daltons. Although this fraction appeared to contain only urinary peptides, one cannot conclude that a peptide is responsible for the behavioral activity of this fraction. It is also possible that a smaller, volatile molecule bound to one of these peptides was the actual puberty-accelerating cue.

In a study of pregnancy block pheromone, Marchlewska-Koj (1977) was able to isolate a behaviorally-active fraction which also appeared to contain urinary peptides. Since this

priming cue is androgen-dependent, the possibility existed that androgens were also present in this fraction. When the isolation procedure was repeated with urine of male mice administered ^3H -testosterone, most of the radioactivity was found in the behaviorally-active peptide fraction. This finding is consistent with the notion that smaller molecules, possibly androgen metabolites, could be present in these peptide fractions. On the other hand, not all of the murine priming cues have been found to be associated with urinary peptides. For example, chemical studies of estrous inducing cues have shown that these cues can be isolated in an organic layer following the extraction of male urine with methylene chloride (Monder et al., 1978).

Most of the aforementioned studies of murine priming cues have used a classical approach to this problem, namely chemical separation followed by behavioral testing of the resulting fractions. On the other hand, a more direct approach can be used to determine which compounds are promising candidates for behavioral testing. The logic behind this approach rests on the finding that many olfactory cues while being present in intact male urine are absent from female or castrate male urine. If one could compare the analytical profiles of these three types of urine, it should then be possible to determine what compounds are present only in the intact male sample. One or more of these uniquely male compounds could then be selected for subsequent behavior.

This approach was utilized by Novotny et al. (1977) for their combined gas chromatography-mass spectrometry study of mouse urinary volatiles. They were able to identify two heterocyclic compounds, namely 2-isopropyl-4,5-dihydro-1,3-thiazole and 2-secbutyl-4,5-dihydro-1,3-thiazole, which appeared only in the gas chromatogram of male urinary volatiles. Unfortunately, when these compounds were later tested in puberty acceleration bioassays, no significant effect was found. It may be that these thiazoles are involved in mediating some other type of chemocommunication. On the other hand, these compounds may not be utilized as signals. It should also be noted that most of the compounds present in the gas chromatogram of mouse urinary volatiles contained ten or fewer carbons. If a murine chemosignal contained more than ten carbons it would not be isolated from mouse urine by this headspace distillation technique.

In another study (Lee, 1976), the volatile fraction of male mouse urine was examined for the presence of aggression-promoting activity. Intact male mouse urine was subjected to vacuum distillation at room temperature for about 40 minutes, and volatile compounds were trapped in a vessel cooled in liquid nitrogen and dry ice. This volatile fraction and the residue were assayed behaviorally. Since the volatile fraction was more aggression promoting than the residue, this would indicate that the active substances are volatile. Whitten et al. (1968) also demonstrated that the active substances in male urine for estrus induction was volatile.

Lee (1976) also reported extracting male mouse urine using dichloromethane. After separation of the layers, the CH_2Cl_2 extract was evaporated in vacuo without heat. The residue was taken up in a small amount of water and then assayed behaviorally. The bioassay indicated the aggression-promoting substances were successfully extracted by dichloromethane. They then separated the extract by silica gel TLC. When antimony pentachloride, a steroid-specific reagent, was sprayed on the TLC plate which had been spotted with urine extracts, a spot with an R_f value of about 0.80 was detected. Subsequent behavioral testing, indicated that when this spot was eluted from the TLC plate and reconstituted in a small amount of water, its behavioral activity was retained.

To further check the androgen dependency of these cues, 20 male castrates were injected with radiolabeled testosterone, and their urine was collected, extracted, and run in TLC. Different zones of the TLC plate were then removed and their radioactivities were determined by tritium counting in a liquid scintillation counter. The highest radio-activity was found in the biologically active spot, that migrated with an R_f of 0.80. Another procedure for gaining insight into the chemistry of the olfactory cues was to compare the TLC profile of various steroids with urine extracts. The biologically active substance of the urine was found to have a different R_f from the tested androgens, whose R_f values ranged from 0.60 to 0.9. Thus the urinary cues may be an androgen related substances but do not appear to be common androgens or steroids.

Statement of Purpose

The goal of the present investigation is to identify the olfactory cues influencing inter-male aggression in house mice. This will involve a systematic determination of the optimum conditions for isolating these chemosignals by either extraction or head-space distillation of male mouse urine. The isolation procedure will then proceed to the use of high-pressure liquid chromatography or thin-layer chromatography to further fractionate the urinary compounds. At each step of the isolation procedure behavioral assays will be used to determine the presence of aggression-promoting chemosignals. The final purification of the chemosignals will be accomplished by preparative gas chromatography. The fractions produced by this step should contain a relatively small number of compounds per fraction. Identification of the compounds will be done through the use of mass spectrometry. These cues will then be synthesized for use in subsequent behavioral testing in order to confirm the initial structural assignments for these cues. Additional experiments will be conducted in order to determine the possible source of these aggression-promoting cues.

The success in chemically identifying the urinary chemosignals would equip students of animal behavior with a precise tool to study chemocommunication in mice.

MATERIALS AND METHODS

Materials

All solvents and chemicals used were of reagent grade. Solvents were obtained from Burdick and Jackson (Michigan) and Fisher Scientific Co. (New Jersey) and were redistilled in an all-glass fractionating system before use. Lauryl laurate was purchased from Nu-Chek Prep (Minnesota) and was used without further purification. Wax ester standards were also synthesized from fatty acyl chlorides and fatty alcohols in the presence of pyridine using anhydrous diethyl ether as a solvent (Iyengar and Schlenk, 1968). They were recrystallized and their purity checked by gas chromatography. The structures of all wax esters were checked by infrared and mass spectrometry.

All glassware used for extraction of lipids was silanized before use with 10% (v/v) dimethyldichlorosilane in toluene in order to minimize sample adsorption. Deionized organic-free water was supplied by a Milli-Q water purification system (Millipore Corp., Massachusetts).

Subjects

All SJL/J male mice were randomly chosen from our colony stock, originally obtained from Jackson Laboratories (Maine). The animals served as either urine donors, trained fighters or stimulus animals. In addition, C57/10J male mice served as dangles in the aggression training of SJL/J fighters. Animals were weaned at 30 days of age and ranged from 90 to

160 days of age at the start of testing or urine collection. Animals were housed in opaque polypropylene cages (33x20x12.7 cm high) which contained pine shavings and food (Wayne Lab Blox) and water ad lib. The colony room was maintained on a 12 hour light-dark cycle at $20.5^{\circ}\text{C} \pm 1^{\circ}$, with lights on at 0800 hours.

Surgery

For use as castrate-male urine donors, a group of SJL/J males were bilaterally gonadectomized. Also, prior to each behavioral experiment, 20 C57/10J males were bilaterally gonadectomized and served as dangles during the training of prospective fighters. At 65-70 days of age, a male was anesthetized (i.p) with Equi-thesin (Jen-Sal, Missouri) (0.1 ml/25 g body weight), had both testicles and vas deferens removed, and was sutured with a single autoclips. These males were given a minimum of 14 days to recover from surgery.

Urine Collection

Voided urine was obtained from two groups of SJL/J urine donors: intact and castrated males. On a given day, between 0800-01000 hours, a male was placed in a clean polypropylene cage, and induced to urinate by gentle massage of the abdomen. Excreted urine was withdrawn with a polypropylene syringe and transferred into a 10 ml glass vial; urine contaminated by foreign matter (e.g. feces) was not collected. During urine-collecting sessions, both vial and

syringe were immersed in ice water. At all other times, urine or its components were kept frozen at -10°C. This storage procedure minimized chemical or bacterial activity (Lee and Griffio, 1974; Albone et al., 1977). Separate sets of urine-collecting equipment were used for the intact and castrated donor animals.

For the collection of bladder urine, intact SJL/J males were anesthetized with chloroform. A 5 mm long incision in the lower abdomen was made to expose the bladder, and its contents were withdrawn by a polypropylene syringe equipped with a 26 gauge needle. The animals were then sacrificed by cervical dislocation.

Fighter Training

Adult intact SJL/J males were trained to fight via the dangling method (Scott, 1966; Lee and Ingersoll, 1981). On three different days a castrated C57/10J male was held just over an SJL/J male in a neutral cage, inciting the latter to display aggression. On the third and final day, an SJL/J male had to attack a castrated C57/10J stimulus animal within three minutes of cage introduction in order to be used as a trained fighter.

Behavioral Testing

The behavioral assay for each chemosignal condition consisted of pairing a fighter (n=12/condition) with a stimulus animal (n=24/condition) on two occasions, with a

different stimulus animal per session. Each test session was 15 minutes in duration, commencing after the first attack. Test sessions were conducted at 48-hour intervals. Each fighter was given 15 minutes of cage-adaptation prior to testing. Aggression tests were conducted in a 36.8x31.1x16.5 cm high polypropylene cage, the floor of which was covered with fresh pine shavings.

Prior to each session, the appropriate test substance(s) was thawed at room temperature. With a polypropylene syringe, 0.3 ml of fluid was withdrawn from a vial and transferred to a Q-tip cotton swab. It should be noted that this quantity of sample has been shown by a dose response study (Lee and Ingersoll, 1981) to correspond to the minimum quantity of urine which can reliably elicit aggression in the present paradigm. Stimulus material was then brushed on the urogenital area of a stimulus animal prior to testing. The stimulus material was labeled so that the observer could not know which urinary stimulus material was used in each session.

As shown in Figure 1, the two test sessions served to expose a fighter to two types of stimulus cues: urinary substances and distilled water as control substance. Water was chosen as a control in order to determine the base level of aggressions for each fighter. Six fighters were initially paired with stimulus animals coated with water followed by a second test with urine-coated stimulus animals. The remaining six fighters received the reverse stimulus presen-

Figure 1. Test paradigm for assaying a single test substance (TS). For a particular group, six fighters received sequence 1 of distilled water (W) followed by its presentations. The remaining six fighters received sequence 2.

	Test 1	Test 2
Sequence 1	W	TS
Sequence 2	TS	W

tation sequences. Each session was initiated by placement of a stimulus male into a corner of the apparatus most distant from the fighter.

During each test session the following measures of fighters attack and urogenital sniffing behaviors were recorded on electromagnetic counters and Hunter timers (model 120X): (1) cumulative frequency and duration of each behavior and (2) time to first occurrence of each behavior--i.e., latency. An attack was defined as any sort of thrust towards the castrate by the fighter. Similarly, a sniff was registered whenever the fighters nose was observed to be within one cm of the castrates urogenital area.

Data Analysis

Within-group comparisons were analyzed via correlated t-tests ($df=11$), performed on the original scores. In contrast, between-group comparisons were performed on difference scores (D)-treatment minus control scores per subject; $D>0$ represents an increase in occurrence of a behavior for the treatment condition, and $D<0$ represents a corresponding decrease. For example, a negative latency score would indicate that a treatment produced a lower latency score than did the corresponding water condition. This data transformation was necessary, since typically there were significant differences between the control groups. For between-group comparisons, these difference scores were analyzed either by independent t-tests ($df=22$) or by F-tests supplemented by

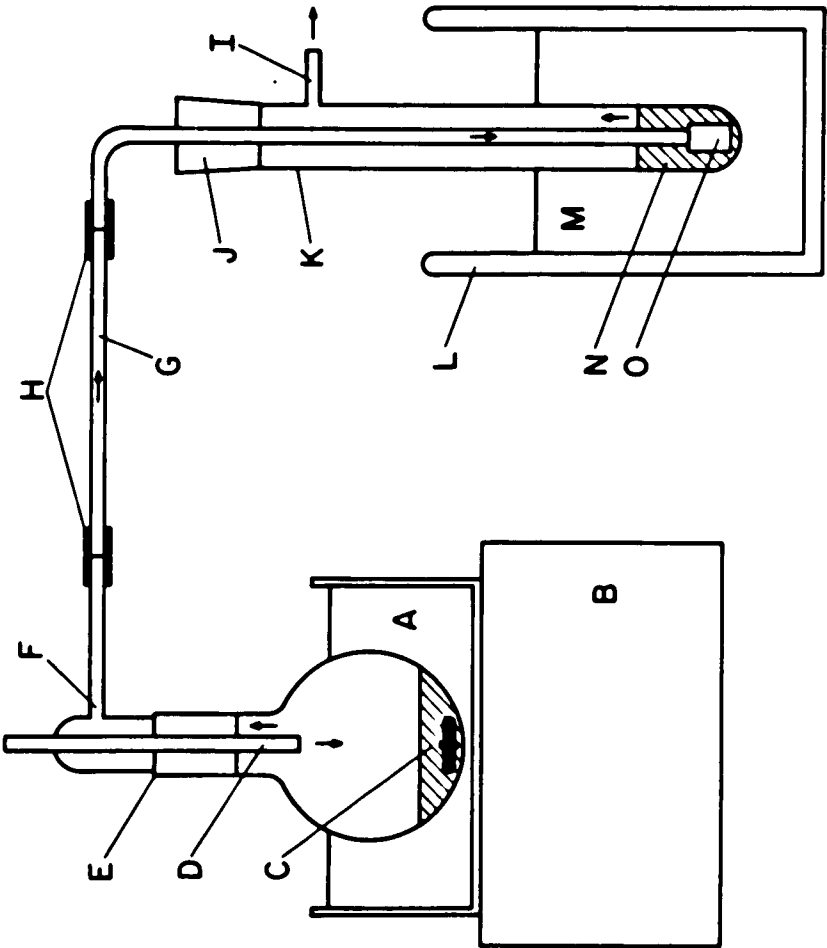
scheffe analyses (df=22). The scheffe analysis, a test of significance for selected contrasts between more than two means was performed as described by Wilcox et al. (1979).

Chemical Procedures

Head-space distillation

The apparatus used in the head-space distillation of male mouse urine (either castrate or intact) is shown in Figure 2. Twelve ml of the appropriate urine and a magnetic stirring bar were transferred to a 250 ml round-bottom flask and 1.6 ml of HCl was added to produce a final pH of 1. Ten ml of CH_2Cl_2 was added to the collection trap and cooled to -78°C by a dry ice-acetone slurry. Heat-shrinkable Teflon tubing (Alltech Associates, Arlington Heights, Ill.) was used to connect the various components of the apparatus. The distillation was started by introducing a flow (15 ml/min) of oxygen-free nitrogen over the stirred acidified urine, which was maintained at a temperature of 40°C by a thermostatically controlled water bath. The gas flow was maintained for one hour, after which the fritted-glass bubbler was allowed to thaw (because ice had formed) and was rinsed with 2 ml of CH_2Cl_2 . These washings were combined with the original 10 ml of CH_2Cl_2 from the trap and dried over 1 gm of anhydrous Na_2SO_4 . This volatile fraction was then transferred to a vial and evaporated to dryness at room temperature with a stream of nitrogen. In the past, this method of concentration has been shown to be effective for the recovery of aggression-

Figure 2. Headspace sampling apparatus. A = water bath;
B = heater-stirrer; C = acidified urine;
D = nitrogen inlet tube (40); E = 500 ml flask;
F = outlet tube, 40 x 9 mm O.D.; G = connecting
tubes, 80 x 9 mm O.D.; H = Tygon tubing;
I = Vent; J = one-holed rubber stopper;
K = glass trap, 28 x 2.8 cm O.D.; L = Dewar;
M = dry ice-acetone slurry; N = methylene chloride,
20 ml; O = fritted-glass bubbler.



promoting cues (Lee, 1976; Yee, 1977). On the other hand, this procedure may result in the loss of urinary volatiles. In order to facilitate a comparison with past work (Lee, 1976; Yee, 1977) water was used as a dissolution medium for behavioral testing. To produce the test substance, 4 ml of water was added to the residue.

For the preparation of a test substance from the nonvolatile fraction, 10 ml of the nonvolatile residue remaining in the distilling flask was transferred to a 30 ml beaker and titrated to a final pH of 7.0, with a 50% (w/v) NaOH solution. Neutralization of this fraction was done in order to eliminate the possibility that the differences in pH values of the volatile and nonvolatile test substance was the basis for any behavioral differences of these substances. This was necessary since the pH of a solution can affect the degree of ionization of a solute which may be significant if proteins or other ionizable substances are present. The neutralized residue was then stored at -10°C until behavioral testing. A similar procedure was followed for the preparation of a castrate residue test substance.

There was a possibility of behavioral effects from the solvent dichloromethane, CH_2Cl_2 , used throughout these isolation procedures. Consequently, CH_2Cl_2 , was tested against water for possible effects on attack and urogenital sniffing behaviors. Twelve milliliters of CH_2Cl_2 was dried over 1 gm of anhydrous sodium sulfate, Na_2SO_4 , and evaporated to dryness with a stream of oxygen-free nitrogen. Four milli-

liters of water was added to the residue, and the test substance was stored frozen at -10°C until needed.

High-Pressure Liquid Chromatography (HPLC)

Separations by HPLC were conducted with a commercial apparatus (Waters Associates, Massachusetts) consisting of a Model M6000A solvent-delivery system, Model U6K injector, and a Model 440 absorbance detector operated at 254 nm. Solvents were filtered through a $0.5\mu\text{m}$ (av pore size) filter and then degassed under reduced pressure before use in HPLC purifications.

A. HPLC of intact male mouse urine extract

Five ml of urine was subjected to two extractions with two 5.0 ml portions of CH_2Cl_2 . The two organic layers were pooled and dried over 1 g of anhydrous Na_2SO_4 . Prior to HPLC, the dried organic layers were passed through a silica Sep-Pak cartridge (Waters Associates) and thereafter evaporated by a stream of oxygen-free nitrogen to a final volume of 1.0 ml. The purification of the sample with a silica cartridge was implemented in order to prevent the irreversible adsorption of any polar compounds on the HPLC column. Since the chemosignals are not tightly bound to silica, one would not expect a loss of behavioral activity under these conditions.

The HPLC separation was obtained using a μ Porasil-packed stainless steel column (Waters Associates) measuring 30cm x 3.9mm I.D., and eluted with CH_2Cl_2 at a flow rate of

1.0 ml/min. A guard column (7.6cm x 3.0mm I.D.) packed with Porasil was used to protect the main column. The aforementioned CH_2Cl_2 -urinary extract was applied in 100 μl aliquots to the HPLC apparatus and fractions collected for use in behavioral testing. Fraction boundaries were selected at points where two peaks were adequately resolved as indicated by the HPLC profile of UV absorbance.

The four fractions from ten HPLC analyses were separately pooled and dried over 2 g of Na_2SO_4 . In order to produce a test substance for behavioral analysis, each water-free fraction was evaporated to near dryness using a stream of oxygen-free nitrogen. Four ml of distilled water was added to each residue and the test substances were stored frozen at -10°C until behavioral testing.

B. HPLC of the volatile distillate of intact male mouse urine

Prior to HPLC analysis, a 12 ml sample of distillate prepared as previously described was dried over 1 g of Na_2SO_4 and concentrated by evaporation with a stream of nitrogen to approximately 0.8 ml. Sufficient CH_2Cl_2 was then added to bring the final volume to 1.0 ml. The sample was then applied in 50 μL aliquots to the HPLC apparatus, from which three fractions were collected. This process was repeated until all of the sample had been purified. Two Corasil (Waters Associates) columns (61cm x 2mm I.D.) connected in series and eluted isocratically with CH_2Cl_2 at a flow rate of 1 ml/min were used to obtain the separation. After

collection of the appropriate fractions, CH_2Cl_2 was removed and 4 ml of water added as previously described.

Thin Layer Chromatography (TLC)

TLC was performed using glass plates (20x20cm) with a 0.25 mm layer of silica gel 60 (EM Reagents, New Jersey). To aid in the visualization of compounds, analytical separations were also carried out using plates coated with silica gel containing a fluorescent indicator (Brinkman Instruments, New York). Before use, plates were prewashed in redistilled methanol by ascending chromatography. After air-drying, a plate was activated by heating at 100°C for 15 min. After sample application, the TLC plates were transferred to the developing tank and pre-equilibrated with the vapors of the mobile phase for 15 min before development. The solvent front was permitted to migrate to a distance of 15 cm from the origin, at which time the plates were removed and air-dried.

Where applicable, the separated bands on each plate were first visualized by their quenching effect on the fluorescence of the incorporated indicator. Afterwards, in order to characterize the separated bands, the following spray reagents were typically used on different plates: (a) 25% (w/v) SbCl_5 in CCl_4 , (b) phosphomolybdate, (c) Folin-Ciocalteu reagent and (d) iodine vapors. The first two can specifically detect the presence of steroids (Lisboa, 1968). The third reagent is used for the detection

of phenolic steroids. The final test, visualizes most lipophilic compounds.

A. TLC analysis of HPLC-purified and untreated extracts of intact and castrate male mouse urine

Both untreated extracted urines were prepared and dried, and the eluates corresponding to a behaviorally active HPLC (Corasil) fraction were obtained as previously described. Each CH_2Cl_2 extract was prepared from a 5.0 ml initial volume of urine. Prior to TLC, both HPLC eluates and crude CH_2Cl_2 extracts were concentrated to a volume of 0.5 ml. Five TLC plates were used and 100 μl of each sample was applied in a 3.5 cm long streak to each plate. Following solvent development in a 1:1 (v/v) mixture of cyclohexane and ethyl acetate, one of the five plates was treated with SbCl_5 in order to visualize the steroidal bands. The remaining plates were examined under UV light to facilitate a comparison of their R_f values with those of the SbCl_5 -treated plate. A zone of the unsprayed plates, 1 cm on either side of an R_f value of 0.70 was separately scraped for each sample. The scrapings from four plates were combined for each sample and extracted two times with 5.0 ml portions of CH_2Cl_2 . These CH_2Cl_2 washings from each sample were then evaporated to dryness and converted to final test substances by the addition of 4.0 ml of water. Thus, each test substance contained the urinary compound present in a region of the TLC plate corresponding to the SbCl_5 -positive spot.

Gas Chromatography (GC)

GC separations were conducted on a Beckman Model GC-65 gas chromatograph equipped with a flame detector (FID). After amplification, the FID response was displayed on a Beckman Model 1005 chart recorder and the peak area determined by use of a Infotronics Model CRS-2008 digital integrator. For use as a carrier gas, helium was purified by use of molecular sieve 5Å trap and an oxy-trap (Alltech Associates, Illinois) for the removal of trace water and oxygen, respectively.

GC columns were prepared from either 2 mm or 4 mm I.D. glass tubing which was silanized with a 10% (v/v) solution of dimethylchlorosilane (Analabs, Connecticut) in toluene and packed with Gas Chrom-Q (100-120 mesh) coated with 3% OV-17 (Supelco, Pennsylvania). The ends of each column were then plugged with silanized and acid-washed glass wool (Alltech Associates, Illinois). Following an initial 30 minute purge with carrier gas at ambient temperatures, each column was conditioned at an oven temperature of 300°C for eight hours.

Preparative GC of urinary substances was carried out using an inert outlet system fashioned from glass lined 0.5 mm O.D. stainless tubing (Scientific Glass Engineering, Texas). A stainless steel "T" configuration micro needle valve (Scientific Glass Engineering, Texas) installed between the column and FID was used as a variable outlet splitter. Fractions were collected either in 15 cm long coils of 1/16" O.D. teflon tubing (Regis Chemical, Illinois) packed

with silanized glass wool or in 10 cm long glass capillary tubes. The condensed sample was then eluted using anhydrous CH_2Cl_2 and concentrated using oxygen-free nitrogen. Saponification of purified GC fractions was conducted by refluxing with 10% (w/v) KOH in ethanol-water (9:1, v/v) in an inert atmosphere according to the method of Sansone and Hamilton (1968).

Mass Spectrometry

Gas Chromatography-Mass Spectrometry (GC-MS) was performed using a Series 104 gas chromatograph (Pye Unicom Ltd., England) interfaced to a Model MS-50L Mass Spectrometer (Kratos Scientific Instruments, New Jersey). Operating conditions were as follows:

electron energy of ion source	70 eV
accelerating voltage	6000 KV
ion source temperature	150°C
interface temperature	230-300°C
operating pressure	1×10^{-7} Torr
scan rate	1 sec/decade
emission current	500 μA

GC-MS separations were obtained using a 10 m long x 0.5 mm I.D. open tubular glass column coated with SE-30. Prior to operation the instrument was calibrated using perfluorokerosene.

Direct probe electron impact mass spectra were obtained using a Model CH-7 single focusing mass spectrometer

(Finnigan MAT, California) operated at an electron energy of 70 eV and an ion source temperature of 150°C. Chemical ionization mass spectra (CI-MS) using the direct probe mode were obtained by use of a Biospect series mass spectrometer (Searle Analytic, Illinois). All such spectra were obtained using methane at 1.0 torr as the reagent gas and at a ion source temperature of 130°C. To observe the cleavage of ester linkages during CI-MS runs, a crystal of NaOH was transferred to the probe-tip according to the method of Prakash and Bose (1982).

β-Glucuronidase Hydrolysis of Urinary Fractions

Eight ml of male mouse bladder urine was extracted three times with 4 ml aliquots of CH₂Cl₂. The final aqueous layer was divided into two equal portions (3.7 ml) which were then diluted by the addition of distilled water to a final volume of 20 ml. The solution was then titrated to a pH value of 4.8 by the use of 5 M hydrochloric acid. Four mg of streptomycin sulfate (749 µg/mg) and 2 mg of penicillin G (sodium salt; 1650 Units/mg), in 3 ml of 0.5 M sodium acetate buffer of pH 4.8, were added to each sample. To one sample 28,500 Fishman units of bovine liver β-glucuronidase (380 Fishman Units/mg; Worthington Biochemicals, New Jersey) in 2 ml of acetate buffer were added. The control sample, however, received enzyme which had been denatured by heating at 100°C for 15 min. The sealed tubes were incubated for 70 hr at 38°C with constant agitation and their contents

separately received a fourth extraction with 25 ml of CH_2Cl_2 . Thus, both samples received a total of 4 extractions. Each organic layer was dried and concentrated as described previously. To each residue, 4 ml of distilled water was added.

Isolation and Purification of Preputial Lipids

The preputial glands of 35 adult male SJL/J male mice were removed, and placed in a chilled beaker. Extraction of total lipid was carried out using a procedure of Folch et al. (1957) which was modified by substituting CH_2Cl_2 for the CHCl_3 of the original method. The extract was dried with Na_2SO_4 and excess solvent evaporated with oxygen-free nitrogen to yield 1 g of crude lipid. The residue was redissolved in 2 ml hexane and applied to silic acid column (1.2 cm x 45 cm). The column was first washed with 100 ml of hexane, during which time 2 100 ml fractions were collected. This was followed by 200 ml of 5% (v/v) diethyl ether in hexane with the collection of 4 50 ml fractions. All six fractions were separately concentrated under a stream of nitrogen to a final volume of 1 ml. A ten μl aliquot of each fraction was examined by TLC on silica gel G plates using CH_2Cl_2 as the developing solvent.

RESULTS AND DISCUSSION

Experiment 1: Androgen-Dependence of Aggression-Promoting Cue

The first experiment was designed to investigate the aggression-promoting properties of intact male mouse urine and its dependence on endogenous androgens. Consequently, a group of fighters was exposed to intact urine-coated castrates and a second group of fighters was tested with castrate urine.

For each test condition 3.6 ml of urine was collected and a volume of 0.3 ml was coated on each stimulus animal at the appropriate time.

Within-group comparisons showed significantly more cumulative attacks initiated by fighters presented with intact urine than with water ($t=5.97$, $P<0.01$). However, there was no evidence for the presence of aggression-promoting cues in castrate urine relative to base level behavior of water presentations ($t=0.54$, $P>0.5$). Analyses of difference scores indicated that significantly ($P<0.01$) more attacks were initiated by intact urine-exposed fighters ($M=10.2$) than by fighters presented with castrate urine ($M=0.7$).

The cumulative time and latency to first attack mirrored that of its frequency measure and are presented in Table 2.

Intact urine-coated stimulus animals significantly increased the frequency of urogenital sniffs, relative to water-coated animals ($t=4.37$, $P<0.01$). In contrast, stimulus animals coated with castrate urine produced urogenital sniffing

Table 2

Summary of Experiment 1: Attack Behavior

Index	Castrate Urine				Intact Urine			
	Water		Urinary Cues		Water		Urinary Cues	
	$\bar{X}$	SD	$\bar{X}$	SD	$\bar{X}$	SD	$\bar{X}$	SD
ATTACKS								
Cumulative Frequency ^a	10.1	4.7	10.8	5.9	11.7	4.0	21.8*	6.1
Cumulative Duration ¹	7.2	3.2	8.3	4.5	12.2	5.3	30.9*	8.1
Latency ¹	56.7	87.5	53.0	85.8	21.6	10.1	15.1 ⁺	8.8

¹ Measured in seconds.

*p<.01

⁺p<.05

^aEach frequency unit represents one attack.

Table 3

Summary of Experiment 1: Uro-genital Sniff Behavior

Index	Castrate Urine				Intact Urine			
	Water		Urinary Cues		Water		Urinary Cues	
	$\bar{X}$	SD	$\bar{X}$	SD	$\bar{X}$	SD	$\bar{X}$	SD
URO-GENITAL SNIFFS								
Cumulative Frequency ^a	7.0	3.6	7.7	4.1	10.3	5.6	17.7*	8.6
Cumulative Duration ¹	6.7	3.9	6.2	3.8	11.4	7.1	24.5*	9.9
Latency ¹	50.0	63.9	56.3	78.8	20.8	10.4	11.2 ⁺	6.2

¹ Measured in seconds.

*p<.01

⁺p<.05

^a Each frequency unit represents one sniff.

sniffing behavior not significantly different from that of water stimulus presentations ($t=0.74$, $P>0.5$). Between-group comparisons indicated significantly more urogenital sniffing obtained under intact urine than castrate urine exposure ($t=3.52$, $P<0.01$).

The cumulative duration and latency measures of sniffing behavior paralleled that of the frequency measure for both within-group (see Table 3) and between-group analyses.

The demonstration of aggression-promoting properties in intact male voided urine replicates the findings of previous reports (Lee and Brake, 1972; Mackintosh and Grant, 1966; Mugford and Nowell, 1970a) and further supports the use of water as a within-subject control.

Experiment 2: Aggression-Promoting Cues in Mouse Bladder Urine

The previous experiment demonstrated that castrated stimulus animals coated with intact male urine can incite more attack behavior than similar animals coated with castrate male urine. It has also been shown that testosterone propionate administered to castrated males can restore the attack-promoting properties of their urine (Lee and Brake, 1972; Mugford, 1974). As has been shown in the pig (Melrose et al., 1971), it may be that some metabolic endproducts of steroid catabolism (e.g. androst-16-en-3-one) in the mouse have chemosignal activity.

In most mammalian species (Jayle and Pasqualini, 1966) excreted urinary steroid metabolites are distributed as

(a) free lipophilic aglycones, (b) polar glucuronides and (c) polar sulfates. Unpublished work (Yee, 1977) indicated that an extracted aqueous layer of voided urine is not behaviorally active unless incubated with β -glucuronidase and a previous report (Lee, 1976) has shown that the chemosignal is lipophilic. Thus, previous work is consistent with the aqueous layer containing a conjugated chemosignal which was activated by treatment with β -glucuronidase.

Since our goal is the isolation and identification of the aggression-promoting chemosignal, it is of interest to determine whether it is present in urine as a glucuronide. The liver is the major site of steroid glucuronide synthesis (Samuels and Eik-Nes, 1968). However, the murine kidney, epididymal and preputial tissues have also been shown to contain β -glucuronidase, the enzyme responsible for the cleavage of the steroid-glucuronic acid bond (Levy and Conchie, 1966). Thus, one may expect to find a greater portion as a conjugate in bladder urine than in voided urine. The preputial gland in rodents has been shown to contain substances which possess chemosignalling activity (Gawienowski et al., 1975; Jones and Nowell, 1973). Consequently, to avoid potential preputial chemosignal contamination and β -glucuronidase activity, we have chosen to work with bladder urine as the source of chemosignal activity.

Previous work has demonstrated that castrated stimulus animals coated with intact male bladder urine elicit more

aggression than those coated with water (Jones and Nowell, 1973). Since our behavioral assay differs from the one used in the aforementioned study, it is of interest to confirm and extend these earlier findings. The present experiment compares the aggression-promoting properties of intact male bladder urine with those of voided urine collected separately from intact and castrated adult males. Thus three test substances were examined using a total of 36 fighters and 12 stimulus animals.

Fighters presented with voided ($t=16.61$, $P<.01$) or bladder urine ($t=2.97$, $P<.05$) from intact males significantly increased their number of attacks over that of their respective water base level scores. For castrate voided urine exposed fighters, no significant ($t=0.33$, $P>.50$) change in frequency of attacks was obtained across sessions (see Table 4).

Analyses of the difference scores showed significance ($F=98.06$, $P<.01$) and was subsequently found to be due to significant differences for comparisons between intact urine and other groups. As shown in Table 4, the bladder urinary chemosignal induced less aggression than did the voided intact urinary stimulus.

The findings of the attack duration index were similar to that of the frequency measure. For the attack latency measure, however, only the intact urine chemosignal induced significantly ($P<.05$) lower latency scores than both water and castrate stimuli.

Table 4

Summary of Experiments 2 and 3

Effects of Urinary Test Substances on the Attack Behavior of Trained Fighters*Exp. 2 Bladder Urine Aggression-Promoting Chemosensory Cues

	<u>Intact male voided urine</u>		<u>Intact male bladder urine</u>		<u>Castrated male voided urine</u>	
	<u>$\bar{X}$</u>	<u>SD</u>	<u>$\bar{X}$</u>	<u>SD</u>	<u>$\bar{X}$</u>	<u>SD</u>
Frequency ^a	20.5 ⁺	4.1	3.2 ⁺	3.5	0.3	3.3
Duration**	24.0 ⁺	13.1	8.8 ⁺	10.7	0.3	3.5
Latency**	-19.2 ⁺	16.8	2.9	18.1	-0.7	17.2

Exp. 3 Dichloromethane Extractions of Male Bladder Urine

	<u>Voided Urine</u>		<u>Initial CH₂Cl₂ Extracts</u>		<u>Final CH₂Cl₂ Extract</u>		<u>Final Aqueous Extract</u>	
	<u>$\bar{X}$</u>	<u>SD</u>	<u>$\bar{X}$</u>	<u>SD</u>	<u>$\bar{X}$</u>	<u>SD</u>	<u>$\bar{X}$</u>	<u>SD</u>
Frequency ^a	15.8 ⁺	3.0	5.8 ⁺	3.3	-1.6 ⁺	1.0	0.7	2.2
Duration**	24.6 ⁺	4.6	12.8 ⁺	5.7	-2.1 ⁺	1.6	-0.7	2.4
Latency**	-18.3 ⁺	11.7	-5.3	14.1	10.0	16.9	-6.4	12.1

*Values are mean difference scores (test substance minus water scores per subject).

**Measured in seconds.

^a Each frequency unit represents one attack.

⁺ $p < .05$, vs. H₂O

The presence of aggression-promoting cues in intact male voided urine and the lack of such a chemosignal in castrate urine replicates the findings of previous reports (Lee and Brake, 1972; Mugford and Nowell, 1970). Experiment 2 also supports the findings of Jones and Nowell (1972) that bladder urine from intact males contains aggression-promoting cues. However, the agonistic behaviors induced by bladder urinary cues were not equivalent to those induced by intact voided urinary stimuli. It may be that (a) a portion of the bladder urine total stimulus complex is in conjugated form and thereby behaviorally inactive, and/or (b) voided urine of intact males contains additional aggression-promoting cues supplied by secondary sexual glands.

Experiment 3: Extraction of Bladder Urinary Cues

The present experiment attempted to determine the lipophilicity of the aggression-promoting chemosignal in bladder urine from SJL male mice. Any nonpolar substances present in bladder urine were removed by a series of three repetitive extractions with dichloromethane (CH_2Cl_2). The organic layers from these extractions were then pooled prior to behavioral testing. In order to ascertain that all of the chemosignal was removed by extraction, the final aqueous layer was extracted with CH_2Cl_2 . The organic and aqueous layers from this fourth extraction were then each separately tested for their aggression-promoting properties. In addition, a fourth group of animals were exposed to stimulus animals

coated with untreated intact male voided urine.

Forty-eight mice were trained as fighters and 96 animals were bilaterally gonadectomized to serve as stimulus animals. Urine collection and behavioral testing were performed as previously described.

Both groups of intact voided urine ($t=17.70$, $p<.01$) and initial CH_2Cl_2 extracts ($t=5.74$, $p<.01$) chemosignal exposed fighters significantly increased their frequency of attacks over water base level scores. In contrast, the final CH_2Cl_2 extract induced a significant decrease ($t=5.06$, $p<.01$). No significant change ($t=1.02$, $p>.30$) in frequency of attacks was obtained for the final aqueous extract stimulus condition.

Between group analysis was significant ($F=102.98$, $p<.01$) and was subsequently found to be due to significant differences for five of the six group comparisons (t 's >4.71 , $p<.01$). No significant difference ($t=2.09$, $p>.01$) in frequency of attacks was found between the final CH_2Cl_2 and aqueous extract groups. The rank order from lowest to highest in mean frequency of attacks was: (a) final CH_2Cl_2 extract, (b) final aqueous extract, (c) initial CH_2Cl_2 extracts, (d) intact voided urine (see Table 4).

Since the final CH_2Cl_2 extract stimulus induced significantly ($t=3.10$, $p<.05$) fewer attacks than its own base level scores, it can be concluded that the final CH_2Cl_2 extract contains aggression-inhibiting properties, perhaps due to residual presence of the solvent CH_2Cl_2 . The finding that the initial CH_2Cl_2 extracts induced attacks significantly greater than the final aqueous extract ($t=4.71$, $p<.01$) and

significantly less than intact voided urine ($t=0.34$, $p<.01$) confirms the results of Experiment 2 that bladder urine contains aggression-promoting cues. Also, the lack of obtaining an aggression-promoting chemosignal effect with either the final CH_2Cl_2 extract or the final aqueous extract stimuli indicates that most if not all of the active chemosignal have been removed within the first 3 extraction procedures.

The findings of this experiment indicate that the behaviorally active form of the aggression-promoting chemosignal can be initially purified from intact male bladder urine by three CH_2Cl_2 extractions. Also, aggression-promoting cues were not uncovered in either the final CH_2Cl_2 extract or final aqueous extract stimuli. The lower level of agonistic behavior induced by the initial CH_2Cl_2 extracts of bladder urine relative to intact voided urine stimuli suggests that either the aqueous layer may contain a portion of the chemosignal material in a behaviorally inactive form or that intact voided urine contains cues from other sources.

Experiment 4: Conversion of Conjugated Chemosignal Into a Behaviorally Active Form

The following experiment assayed for the presence of a conjugated chemosignal in bladder urine. The lipophilicity of the aggression-promoting chemosignal suggests that it may be found in bladder urine as a glucuronide or sulfate. Most lipophilic molecules exhibit a low rate of renal excretion unless their polarity is increased by conjugation to an

endogenous substrate (Smith and Williams, 1966). If conjugation is important for the renal clearance of the chemosignal, then one would expect the aqueous layer after CH_2Cl_2 extraction to contain a significant amount of the lipophilic chemosignal in a conjugated form.

Two types of urine were collected from male donors: voided and bladder urine. Half of the aqueous layer following three CH_2Cl_2 extractions of bladder urine was incubated with β -glucuronidase, whereas the remaining portion was exposed to denatured enzyme. In order to suppress bacterial growth, both samples were treated with antibiotics.

Behavioral testing was performed on these two reconstituted extracts as previously described. At the same time, a third group of animals were tested with intact voided urine in order to facilitate a comparison with normal levels of chemosignal activity. Thus, three test substances were examined using 36 trained and 72 stimulus animals.

Fighters exposed to either intact urine ($t=13.66$, $p<.01$) or enzyme-treated sample ($t=6.76$, $p<.01$) significantly increased their frequency of attacks over control test scores, whereas the incubation control induced no significant ($t=1.51$, $p>.10$) change in behavior. In addition, all between group comparisons were significantly different ($F=41.91$, $p<.01$). As shown in Table 5, the enzyme-treated stimulus material induced intermediate attack scores between that of intact urine ($t=5.24$, $p<.01$) and the denatured incubation control ($t=3.88$, $p<.01$). Also, the latter, two stimulus conditions

Table 5

Summary of Experiment 4

Effects of Urinary Test Substances on the Attack Behavior of Trained Fighters*Incubation of the Extracted Aqueous Layer of Male Bladder Urine With β -glucuronidase

	<u>Voided Urine</u>		<u>Enzyme Treated</u>		<u>Control</u>	
	<u>$\bar{X}$</u>	<u>SD</u>	<u>$\bar{X}$</u>	<u>SD</u>	<u>$\bar{X}$</u>	<u>SD</u>
Frequency ^a	18.7 ⁺	4.5	9.0 ⁺	4.4	1.8	4.0
Duration**	28.9 ⁺	6.9	14.0 ⁺	6.0	1.8	3.4
Latency**	-16.0 ⁺	11.2	-11.2	18.0	-5.3	17.4

*Values are mean difference scores (test substance minus water scores per subject).

**Measured in seconds.

^aEach frequency unit represents one attack.

⁺p<.05, vs. H₂O.

were significantly different ($t=0.12$, $p<.01$).

The findings of the cumulative duration of attack behavior mirrored that of the frequency index (see Table 5). Latency to attack indicated that only fighters exposed to intact voided urine significantly ($t=4.73$, $p<.01$) decreased the time to first attack over base level scores.

The findings of Experiment 4 indicate that male bladder urine contains a chemosignal in conjugate form. This glucuronide may be a "pro-chemosignal" having latent chemo-communicative properties when exposed to β -glucuronidase.

Experiment 5: Behavioral Effects of Dichloromethane

Of concern are possible behavioral effects of dichloromethane, the solvent used in the isolation of the aggression-promoting chemosignal. Although an evaporation procedure was implemented, odor traces of this substance were noticeable to us. Thus, CH_2Cl_2 was tested against water for possible effects on attack and urogenital sniffing behaviors.

Results

Fighters significantly ($t=2.93$, $p<0.01$) decreased their frequency of attacks when exposed to CH_2Cl_2 ($M=4.7$) relative to water ($M=6.4$) exposure. No significant findings were obtained for either the cumulative duration or latency measures.

Sniffing behavior occurred with lower frequency and for less time when stimulus material was CH_2Cl_2 than when it was

water ($t=2.77$, $p<0.01$). No significant latency effect was obtained.

Thus, contamination of the test substance by CH_2Cl_2 should have counteracting behavioral effects. In order to avoid statistical exaggeration of the effects of aggression-promoting cues, we continued to use water as a chemosignal control stimulus in the following remaining experiments.

Experiment 6: Head-Space Distillation of Aggression-Promoting Cues from Male Mouse Urine

In the present study the partial purification by head-space distillation of aggression-promoting urinary cues is described. The volatile distillates of intact male mouse urine and castrate urine were assayed for the aggression-promoting chemosignal. Since the results of Experiment 5 indicate that a portion of the chemosignal is conjugated, acidification was employed for the hydrolysis of these conjugated cues. On the other hand, acidification may present a problem since it could hydrolyze any chemosignals containing an ester linkage. Moreover, it is difficult to estimate the efficacy of this acidification procedure.

Fighters exposed to the volatile components of intact urine displayed significantly more frequency of attacks than when presented with water ($t=6.27$, $p<0.01$). However, the volatile fraction of castrate urine produced no significant difference in attack scores from that of water ($t=0.62$, $p>0.5$). Between-group comparisons showed significantly ($p<0.05$) more attacks for intact urine than castrate urine-exposed fighters.

The index measuring cumulative duration of attacks was similar to that of its frequency measure, and both indices are presented in Table 6.

For the latency measure of attack behavior, both groups of fighters initiated attacks more quickly when presented with urinary cues than with water ($t=2.23$, $p<0.05$). Also analysis of between-group differences did not approach significance.

The cumulative frequency of urogenital sniffs was significantly greater in fighters exposed to volatilized intact urine than in those exposed to water ($t=8.89$, $p<0.01$). No reliable findings were obtained for the castrate urine condition ($t=0.30$, $p>0.05$). Analysis of difference scores indicated significantly ($p<0.01$) more attacks emitted by intact urine exposed fighters.

The cumulative duration of urogenital sniffs parallel its frequency measure (see Table 6). However, analysis of the latency measure showed that both volatilized intact urine and castrate urine induced less time to first sniff than water control ($t>1.84$, $p<0.05$). Between-group analysis showed no significant effect for the latency measure.

Attacks were initiated with greater frequency and duration when fighters were presented with the volatile fraction of intact urine. In concordance with Experiment 1, no differential effects of frequency of attacks and urogenital sniffs were obtained for volatilized castrate urine. However, the reduced latency scores for castrate urine may be indicative

Table 6

Summary of Experiment 6: Head-Space Distillation

<u>Index</u>	<u>Volitalized Castrate Urine</u>				<u>Volitalized Intact Urine</u>			
	<u>Water</u>		<u>Urinary Cues</u>		<u>Water</u>		<u>Urinary Cues</u>	
	<u>$\bar{X}$</u>	<u>SD</u>	<u>$\bar{X}$</u>	<u>SD</u>	<u>$\bar{X}$</u>	<u>SD</u>	<u>$\bar{X}$</u>	<u>SD</u>
<u>Attacks</u>								
Cumulative frequency ¹	6.7	2.1	6.3	1.5	14.2	6.7	26.8*	11.8
Cumulative duration ^a	4.7	2.1	4.8	1.7	15.5	8.8	35.0*	13.3
Latency ^a	38.1	23.1	25.5+	13.2	20.9	13.4	7.4*	12.0
<u>Uro-genital sniffs</u>								
Cumulative frequency ²	4.5	1.2	4.8	0.9	11.4	7.5	22.3*	11.3
Cumulative duration ^a	3.5	0.8	3.7	1.1	13.1	8.1	31.9*	13.0
Latency ^a	33.1	21.3	22.3+	8.7	18.7	11.1	9.3*	6.9

^aMeasured in seconds.

*P<0.01

+P<0.05

¹Each frequency unit represents one attack.²Each frequency unit represents one sniff.

of a quantitative rather than a qualitative difference in volatilized intact urine and castrate urine. Moreover, it is possible that acidification or concentration of castrate urine produced an attractive cue, as untreated castrate urine does not produce the same result.

Of interest is the behavioral demonstration of aggression-promoting cues in the volatilized fraction of intact urine. This finding has been replicated in our laboratories.

The final study of this experiment investigated the aggression-promoting properties of the nonvolatile components of either intact or castrate urine. It may be that the volatile distillate procedure does not remove all of the chemosignal in the urine. Thus, the urine residue may contain aggression-promoting cues. Two groups of fighters were used for the behavioral assays, with each animal exposed to 0.3 ml of stimulus substance per test.

No significant findings were obtained for any of the attack indices ($p > 0.05$). Only fighters exposed to the intact urine residue ($M=6.7$) significantly ($t=2.94$, $p < 0.01$) increased frequency of sniffing behavior over water-exposed animals ($M=4.0$). Similarly, stimulus animals coated with intact urine residue ($M=6.5$ seconds) were sniffed for longer ($p < 0.05$) durations than stimulus animals coated with water ($M=3.2$ seconds). No significant latency effects were obtained. Also, between-group analyses showed no significant effects.

Since no differential attack behavior was obtained

between intact urine and water, it is proposed that most of the aggression-promoting chemosignal has been captured in the volatile distillate. However, the increased sniffing of intact urine residue may represent either residual chemosignal or presence of an attractive odoriferous substance.

The findings of the present investigation demonstrated the usefulness of head-space distillation for the preliminary purification of aggression-promoting chemosignals from male mouse urine. These cues appear to be relatively stable at 40°C in an inert atmosphere of oxygen-free nitrogen. Their presence in the intact urine distillate suggests that they are fairly volatile. However, it is possible that a nonvolatile cue was transported as an aerosol. The absence of behavioral activity in the distillate prepared from castrate urine suggests that the behavioral effects of intact urine are due to a natural chemosignal rather than being an artifact produced by the action of low pH on a non-androgen-dependent metabolic end product.

Experiment 7: High-Pressure Liquid Chromatography of the Volatile Fraction from Intact Male Mouse Urine

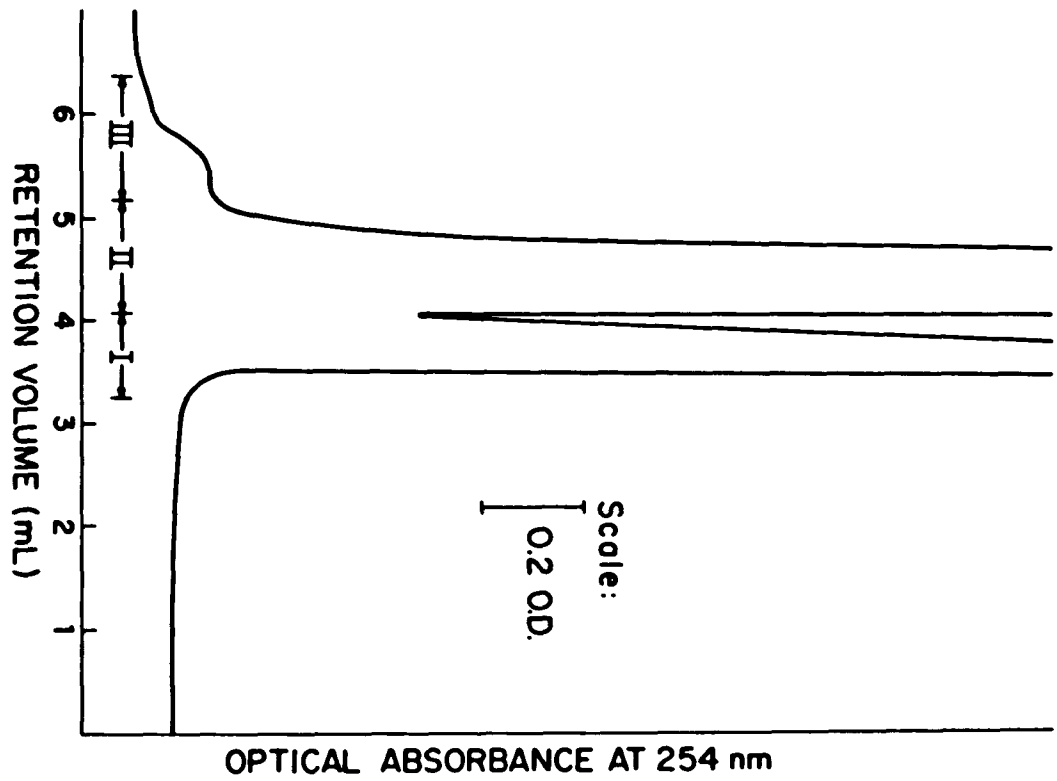
The lipophilic nature of the aggression-promoting chemosignal in the distillate of intact male mouse urine indicates that further purification should be possible by use of normal-phase high-pressure liquid chromatography (HPLC) (Snyder and Kirkland, 1974). Therefore, the behaviorally active distillate has been further purified by HPLC on a pellicular silica-based Corasil column eluted isocratically

with CH_2Cl_2 . This solvent was chosen for use on the basis of its past effectiveness in separations of these chemosignals (Lee, 1976) and its suitability for use in behavioral testing as shown by the results of Experiment 5. Three major peaks were detected by their absorbance in the ultra-violet at 254 nm and fractions were collected as indicated in Figure 3. This method of detection was chosen on the basis of equipment availability. In addition, the lack of baseline resolution for the peaks in Figure 3 indicates that cross contamination of the fractions will occur. However, this will be a major problem only if two adjacent fractions prove to be aggression-promoting. After collection of the appropriate fractions, solvent was removed and water added as previously described.

Examination of the fractions by thin-layer chromatography (Lee, 1976) showed that only Fraction II contained a compound with a R_f similar to that of the aggression-promoting cue. Therefore, Fractions I and III (1,3) were pooled and were tested as a single source of chemosignal substance, just as Fraction II (F2). Thus, the experiment consisted of two groups of fighters, each exposed to a different fraction--F1,3 and F2--of the HPLC eluate.

The F2 fraction significantly increased frequency of attack ($t=4.87$, $p<.01$) over that of water (see Table 7). In contrast, differences between the F1,3 fraction and water were nonsignificant ($t=0.23$, $p>.5$). Between group analyses showed that F2 exposed fighters had significantly ($p<.01$)

Figure 3. Separation of volatile fraction on intact male mouse urine on two 61cm x 2mm I.D. stainless steel columns packed with Corasil and eluted isocratically with CH_2Cl_2 at a flow rate of 1ml/min. Injection volume: 50 μL . Fractions collected for behavioral testing are indicated by roman numerals.



greater frequencies and durations of attacks and shorter latencies ($p < .01$) to first attack than F1,3 exposed fighters. As shown in Table 7, the indices of cumulative duration and latency of attack were similar to that of their frequency measure.

Stimulus animals coated with either fraction produced a greater number of uro-genital sniffs than water coated animals ($t > 2.41$, $p < .05$). However, as depicted in Table 7, only F2 presentations induced significantly more time sniffing, relative to water ($t = 4.37$, $p < .01$) and only F2 presentation induced significantly shorter latencies to first sniff ($t = 2.62$, $p < .05$).

The findings of Experiment 7 clearly indicate that F2 contains the aggression-promoting chemosignal. Although the increased frequency of uro-genital sniffs produced by F1,3 may indicate the presence of a different chemosignal, but may also be viewed as F2 contamination. In addition, for all attack indices, nonsignificant differences were obtained between F1,3 and water conditions.

Experiment 8: High-Pressure Liquid Chromatographic
Purification of an Extract of Intact Male
Mouse Urine

The present experiment was designed to further purify a CH_2Cl_2 extract of voided urine from intact male mice using a modified HPLC procedure. A $\mu\text{Porasil}$ column replaced a previously used Corasil column with the former chosen on the basis of its greater sample capacity and resolution capabilities. Five ml of intact urine was extracted with CH_2Cl_2 and

Table 7

High Pressure Liquid Chromatographic Purification of
Intact Male Urinary Distillate

Index	Fraction I and III				Fraction II			
	Water		Urinary Cues		Water		Urinary Cues	
	<u>$\bar{X}$</u>	<u>SD</u>	<u>$\bar{X}$</u>	<u>SD</u>	<u>$\bar{X}$</u>	<u>SD</u>	<u>$\bar{X}$</u>	<u>SD</u>
Attacks								
Cumulative frequency ^a	19.8	11.6	19.0	11.2	13.3	5.4	32.3*	15.2
Cumulative duration ¹	15.7	22.2	14.6	21.5	7.6	6.0	47.5*	43.8
Latency ¹	29.5	16.1	22.4	29.5	56.0	29.0	18.2*	10.1
Uro-Genital Sniffs								
Cumulative frequency ^b	9.2	3.1	12.6*	2.3	8.4	3.5	14.9 ⁺	8.0
Cumulative duration ¹	24.3	12.5	34.3	10.1	14.1	10.0	36.6*	28.2
Latency ¹	20.8	11.2	16.0	9.0	35.1	19.9	17.2*	9.9

¹Measured in seconds.

*P<.01

⁺P<.05

^aEach frequency unit represents one attack.

^bEach frequency unit represents one sniff.

the extract concentrated to a final volume of 1.0 ml as previously described in Materials and Methods. An extract of urine was chosen for use rather than fraction F2 of Experiment 7, since the HPLC separation of the previous experiment was impractical with respect to the time needed to purify a given amount of sample. This concentrate was applied in 100 μ l to the HPLC apparatus and fractions were collected as indicated in Figure 4. The four fractions were separately pooled and tested for aggression-promoting cues by four groups of fighters.

As shown in Table 8, only the stimulus material from fraction II (F2) induced a significant increase in attack behavior over that of the control test condition for both frequency ($t = 8.48$, $p < .01$) and duration ($t = 10.50$, $p < .01$) indices. Also F2 stimulated significantly more attacks ($t's > 6.42$, $p < .01$) than any of the remaining fractions. The latter stimulus conditions were not significantly different (Fr: $t's > 1.57$, $p < .50$; Du: $t's > 1.93$, $p < .30$). For only F2 exposed fighters, latency to first attack significantly decreased ($t = 2.69$, $p < .05$) over that of base level scores.

Only stimulus animals coated with F2 induced significantly more episodes ($t = 7.62$, $p < .01$) and durations ($t = 9.63$, $p < .01$) of fighter sniffing behavior, over the control condition. Also, significantly more bouts ($t's > 4.88$, $p < .01$) and time spent sniffing ($t's > 5.16$, $p < .01$) were produced under F2 cues than the remaining test substances, i.e., F1, F3 and F4 (see Table 8). The latter fractions were not significantly different (Fr: $t's < 1.85$; Du: $t's > 2.13$, $p > .20$).

Figure 4. HPLC separation of intact male mouse urinary extract on a stainless steel column (30 cm x 3.9 mm I.D.) packed with μ Porasil preceded by a guard column (7.6 cm x 3.9 mm I.D.) packed with Porasil. Sample volume: 100 μ l. Fractions collected for behavioral testing are indicated by roman numerals.

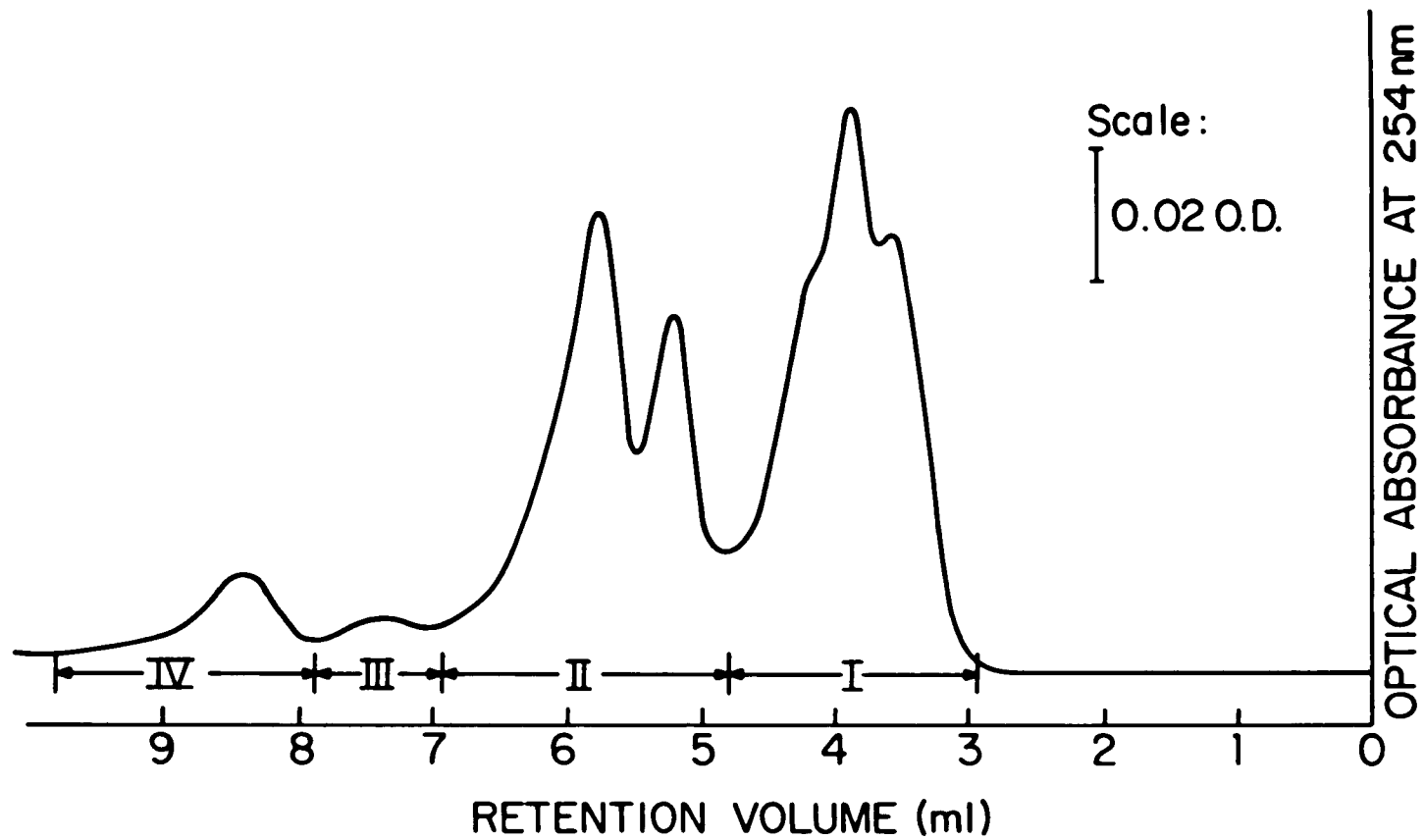


Table 8

High Pressure Liquid Chromatographic Purification
of Intact Male Voided Urinary Extract

	<u>Source of Stimulus Material</u>							
	<u>Fraction #1</u>		<u>Fraction #2</u>		<u>Fraction #3</u>		<u>Fraction #4</u>	
	<u>X̄</u>	<u>SD</u>	<u>X̄</u>	<u>SD</u>	<u>X̄</u>	<u>SD</u>	<u>X̄</u>	<u>SD</u>
	<u>Attacks⁺</u>							
Frequency ¹	1.7	3.5	12.4 ^a	4.9	-0.2	2.3	-0.3	2.4
Duration*	3.2	5.9	19.7 ^a	6.2	-0.6	2.9	-0.3	3.0
Latency*	-3.2	11.1	-10.4	12.8	1.2	9.8	4.4	12.0
	<u>Uro-genital sniffs⁺</u>							
Frequency ²	1.8	4.1	11.8 ^a	5.1	-0.9	2.2	0.1	3.1
Duration*	3.9	6.5	17.8 ^a	6.1	-0.9	3.5	-0.4	3.3
Latency*	-3.5	13.4	-13.0	14.0	2.8	10.4	1.1	9.1

⁺ Difference scores.

* Measured in seconds.

^ap<.05, vs. H₂O

¹ Each frequency unit represents one attack.

² Each frequency unit represents one sniff.

Occurrence of first sniff was significantly hastened for only F2 exposed fighters over both water cues ($t=3.10$, $p<.01$) and the F3 stimulus condition ($t=3.02$, $p<.05$). No other comparisons approached significance.

The findings of Experiment 8 clearly indicated the presence of aggression-promoting cues in fraction II. Substances contained in this fraction stimulated agonistic behavior above that of base level scores and above that of the remaining test substance fractions, i.e., F1, F3 and F4. Moreover, the latter sources of test substances induced agonistic behavior equivalent to that of water cues. Thus, the chemosignal has been shown to be restricted to within the boundaries of F2.

The present study demonstrated that an aggression-promoting chemosignal in a CH_2Cl_2 extract of intact urine can be partially purified by HPLC on a $\mu\text{Porasil}$ column. This finding suggests previous work in which HPLC on a Corasil column was used to purify an aggression-promoting cue from a distillate of intact urine. In both cases, only a single HPLC fraction, namely fraction II, was found to contain the chemosensory cue. Since the retention characteristics of $\mu\text{Porasil}$ and Corasil columns are similar, it may be inferred that both a CH_2Cl_2 extract and a head-space distillate of intact urine contain the same aggression-promoting chemosignal.

Experiment 9: Thin-Layer Chromatography of HPLC Fractions
from Intact Male Mouse Urine

Previous work (Lee, 1976), has shown that following thin-layer chromatography (TLC) of a CH_2Cl_2 extract prepared from intact male mouse urine, steroid compounds can be detected in the behaviorally active region of the plate. This steroid(s) may be the source of aggression-promoting chemosensory activity. Since the findings of Experiment 8 indicated that aggression-promoting cues are found in F2, it is of interest to determine the presence of steroids within this HPLC fraction. Using TLC on silica plates and a mobile phase of 1:1 (v/v) cyclohexane and ethyl acetate (Lee, 1976), the present experiment investigated each HPLC fraction, shown in Figure 4, for the presence of steroidal material. The separated bands were examined for the presence of steroids by the use of antimony pentachloride and phosphomolybdate spray reagents (Lisboa, 1968) on different plates. In addition, Folin-ciocalteau reagent was used for the detection of phenolic compounds. Finally, a fourth plate exposed to iodine vapor was used for the nonspecific detection of lipophilic compounds.

Use of the antimony pentachloride (AP) reagent following TLC showed that antimony pentachloride positive compounds were present in F2. These compounds visualized in a tan-colored spot, migrated with an $R_f=0.70$ and represented the only detectible compounds in F2. None of the three other fractions tested contained these compounds.

Both the Folin-Ciocalteau and phosphomolybdate reagents

visualized compounds at $R_f=0.70$. The positive reaction of the latter reagent confirms the results obtained with antimony pentachloride since both can specifically detect steroids. In addition, the blue spot produced by the Folin-ciocalteau reagent in this region indicates that phenolic steroids may be present. These phenolic steroids cochromatographed with the only lipophilic compound detected in F2 by use of the iodine reagent. Of interest, is the difference in R_f values obtained for the antimony pentachloride spot between the present (0.70) and previous (0.80) TLC studies (Lee, 1976). This lack of agreement between these studies may be due to possible differences in either the technique or silica gel.

Experiment 10: Behavioral Testing of Male Mouse Urinary
Substances Prepared by Thin Layer Chromatography

Previous work (Lee, 1976) has demonstrated that the antimony pentachloride zone from TLC of an extract contains steroidal material. The results of a TLC separation performed in Experiment 9 indicated that this same steroid-containing zone is present in F2. Since the antimony pentachloride zone of the intact urine extract was previously shown to contain an aggression-promoting cue, it is possible that this same cue is also present in the antimony pentachloride zone of F2. The present experiment investigated this possibility by behaviorally assaying test substances prepared from the antimony pentachloride zones of an intact urine extract and an HPLC-F2 eluate.

Since this chemosignal has been shown to be absent in

untreated castrate urine (Expt. 1), its distillate (Expt. 6), and its extract (Yee, 1977), the antimony pentachloride zones of two corresponding castrate urine samples were also obtained and tested.

Substances tested were HPLC fraction II and untreated extracted urine of both intact and castrate male mice. Thus a 2 x 2 factorial design was implemented in which four samples were subjected to further TLC purification as described in Experiment 9. Both untreated extracted urines were prepared and dried, and the eluates corresponding to HPLC fraction II were obtained as described in Experiment 8. Each test substance contained the urinary compounds present in a region of the TLC plate corresponding to the antimony pentachloride positive spot. Four groups of fighters were used for behavioral testing of the four TLC samples. As shown in Table 9, antimony pentachloride spot chemosignal activity was demonstrated only for the two intact urine stimulus conditions: untreated intact urine (I-IU) and HPLC fraction II of intact urine (F2-IU). For these two stimuli, both indices of attack behavior (Fr; $t's > 8.21$, $p < .01$; Du: $t's > 9.28$, $p < .01$) showed significant increased over water control cues. In addition, U-IU and F2-IU were nonsignificantly different (Fr; $t = 0.40$, $p > .50$) and both induced significantly more attack behavior (Fr: $t's > 8.16$, $p < .01$; Du: $t's > 9.20$, $p < .01$) than either of the two castrate urine stimuli. Under either intact urine condition, initiation of first attack was significantly hastened ($t's > 3.34$, $p < .01$)

Table 9

Thin Layer Chromatographic Purification of HPLC
Fraction II and Extracted Urine Stimulus

Source of Stimulus Material

	Extracted Urine				HPLC Fraction II			
	Intact-male		Castrated-male		Intact-male		Castrated-male	
	$\bar{X}$	SD	$\bar{X}$	SD	$\bar{X}$	SD	$\bar{X}$	SD
Attacks ⁺								
Frequency ¹	12.2 ^a	5.2	-1.0	2.2	13.1 ^a	5.1	-0.8	2.1
Duration*	21.3 ^a	7.3	-0.9	1.9	22.9 ^a	8.5	-0.5	2.1
Latency*	-14.6 ^a	14.3	0.7	11.5	-17.6 ^a	9.8	-1.7	10.3
Uro-genital sniffs ⁺								
Frequency ²	12.1 ^a	5.5	-0.1	2.5	13.3 ^a	6.6	-0.8	3.4
Duration*	19.6 ^a	7.2	-0.7	2.6	24.4 ^a	9.5	-0.8	3.5
Latency*	-14.7 ^a	14.9	2.6	9.4	-16.5 ^a	10.5	0.4	8.8

⁺Difference scores.

*Measured in seconds.

^ap<.05, vs. H₂O.

¹Each frequency unit represents one attack.

²Each frequency unit represents one sniff.

over that of water cues. Also, F2-IU induced significantly shorter latencies than either of the two castrate urine conditions ($t > 3.89$, $p < .01$).

The two intact urine stimuli demonstrated presence of aggression-promoting cues. Both U-IU and F2-IU stimulated significantly more sniffing behavior over that of water cues for both indices (Fr: $t's > 6.99$, $p < .01$; Du: $t's > 8.92$, $p < .01$). Also, fighters exposed to these stimuli elicited significantly more sniffing behavior than either castrate urine stimulus material (Fr: $t's > 6.56$, $p < .01$; Du: $t's > 8.65$, $p < .01$). The two castrate urine stimuli induced equivalent sniffing behavior ($P > .50$) and were nonsignificantly different ($P > .40$) from water chemosignal properties. Occurrence to first sniff was significantly hastened under either U-IU ($t = 3.28$, $p < .01$) or F2-IU ($t = 5.44$, $p < .01$) over that of water. Also significantly shorter latencies were obtained for both intact urinary stimuli than either castrate urine conditions ($t's > 2.93$, $p < .05$).

The findings of this experiment clearly indicate that the aggression-promoting cue can be purified by TLC. Also, this chemosignal was taken from a region on the plate that contained steroid molecules. The finding that the antimony pentachloride spot from the HPLC-F2 sample was behaviorally active indicates that F2 may contain behaviorally active steroid compounds.

These results indicate that a region of the TLC plate corresponding to an antimony pentachloride spot was aggression-

promoting for both intact urine samples. However, it is possible that additional aggression-promoting cues were present in these samples and were located in a region of the plate other than the antimony pentachloride spot.

The present experiment tested for chemosensory activity from the unscraped TLC zones of each sample from the initial study of this experiment. These scrapings from the four plates were separately combined for each sample and eluted with DCM as described previously. Thus, four groups of fighters were used to assay the four TLC test substances.

Neither attack nor uro-genital sniffing activities approached significance ($P > .10$). Thus, it is concluded that the underlining compound(s) of the aggression-promoting chemosignal are restricted to within the antimony pentachloride staining spot. This finding, however, does not imply that subjecting HPLC-F2 to TLC treatment necessarily leads to further purification of the chemosignal. It may be that all compounds from the F2 eluate co-migrate to the antimony pentachloride spot.

Experiment 11: Effects of Preparative Gas Chromatography on Behavioral Activity of Aggression-Promoting Cues from Male Mouse Urine

The results of Experiments 8 and 9 indicated that both HPLC and TLC can be used for the partial purification of an aggression-promoting cue(s) from male mouse urine. However, neither approach was able to achieve a high degree of

separation which may be required for the complete isolation of these urinary cues. In the case of the separation by HPLC, this was due to limitations imposed by equipment availability.

On the other hand, one of the most common separation methods for isolation of components from complex mixtures of natural products is preparative gas chromatography (GC). This technique has been repeatedly used for purification of lipophilic compounds, such as steroids and lipids, from a variety of natural sources (Vandenheuvel and Kuron, 1971).

Successful application of preparative GC to an isolation problem, requires that the sample in question meet the criteria of volatility, moderate polarity and thermal stability. In the present case, the results of previous experiments indicate that most of these criteria can be satisfied. The volatility of the aggression-promoting cue(s), was demonstrated in Experiment 6 by the presence of behavioral activity in the distillate of intact male mouse urine. In addition, the high mobility of these cues during normal-phase chromatography would indicate that they are, by HPLC and TLC, of moderate polarity. Thus, one would not anticipate a major problem with respect to sample adsorption during the GC process.

On the other hand, very little is known regarding the thermal stability of these urinary cues at the relatively

higher temperatures employed in preparative GC. The present experiment was designed to investigate this question by comparing the aggression-promoting properties of these urinary cues before and after purification by GC. As a source of chemosignal, a fraction corresponding to a behaviorally active TLC zone was prepared from 30 ml of urine and then divided into two equal portions. One portion was used to prepare a test substance equivalent to the aggression-promoting fraction of Experiment 10.

The other portion was then subjected to preparative GC and the total GC eluate collected as previously described under Materials and Methods. The collected sample was eluted from the trap with CH_2Cl_2 and a test substance prepared. Behavioral testing was performed on these two samples as previously described. At the same time, a third group of fighters were tested with intact male mouse urine. Thus, three test substances were examined using 36 trained fighters and 72 stimulus animals.

The results of this experiment are shown in Table 10. Within-group comparisons show significantly more cumulative attacks initiated by fighters presented with any of the three test substances than with water ($t's > 9.75$, $P < .001$). The findings of within-group comparisons of both attack duration ($t's > 11.60$, $P < .001$) and latency ($t's > 5.86$, $P < .001$) mirrored that of the frequency index. Similar results were obtained for all three measures of uro-genital sniffing activity.

Table 10
Effects of Gas Chromatography on Behavioral Activity
of Aggression-Promoting Cues

	<u>Source of Stimulus Material</u>					
	<u>Intact Male</u> <u>Urine</u>		<u>TLC</u> <u>Fraction</u>		<u>Total GC</u> <u>Eluate</u>	
	<u>$\bar{X}$</u>	<u>SD</u>	<u>$\bar{X}$</u>	<u>SD</u>	<u>$\bar{X}$</u>	<u>SD</u>
	<u>Attacks⁺</u>					
Frequency ¹	15.1 ^a	5.1	14.8 ^a	5.0	14.8	4.4
Duration*	24.4 ^a	7.0	23.8 ^a	6.0	22.1	5.6
Latency*	-20.2 ^a	11.4	-21.2 ^a	5.2	-16.0	5.9
	<u>Uro-genital sniffs⁺</u>					
Frequency ²	15.5 ^a	6.8	19.4 ^a	5.7	14.3	5.3
Duration*	23.2 ^a	7.6	28.5 ^a	5.8	23.5	6.9
Latency*	-27.2 ^a	8.6	-24.4 ^a	6.9	-20.0	11.3

⁺Difference scores.

* Measured in seconds.

^a $p < .05$, vs. H₂O.

¹Each frequency unit represents one attack.

²Each frequency unit represents one sniff.

In contrast, between-group comparisons of difference scores did not approach significance ($P > .10$) for all measures of attack and uro-genital sniffing behaviors. These findings indicate that exposure of murine aggression-promoting cues, from a 15 ml urine sample, to the high temperatures of preparative GC does not produce a significant change in their behavioral activity. This finding, however, does not imply that the chemosignal is completely stable under these conditions. Since a four-fold excess of chemosignal was used, it follows that a 25% or greater yield after GC treatment would still produce a behaviorally active sample.

Experiment 12: Preparative Gas Chromatography (GC) of the Aggression-Promoting Chemosignal from Intact Male Mouse Urine

The present experiment was designed to further purify the aggression-promoting chemosignal from behaviorally inactive urinary compounds by means of preparative GC. In order to obtain a sample for GC analysis, these cues were first partially purified by TLC from 15 ml of intact male mouse urine as previously described in Materials and Methods. Preparative GC separations were conducted on glass columns (4 m x 2 mm I.D.) packed with a 3% loading of OV-17, a phenyl methyl silicone polymer of moderate polarity. The effluent from the column, passing thru an inert glass-lined outlet system, was split into two streams, so that 10% of the total flow was diverted to a flame ionization detector (FID). For the collection of fractions, the remaining portion

of the column effluent was passed thru the silica-packed tubes which were then washed with CH_2Cl_2 . The test substances were prepared as previously described. In order to monitor the presence of the chemosignal, behavioral assays were performed on the fractions isolated at each stage of the purification.

A typical GC-FID profile of the behaviorally-active TLC fraction of intact male mouse urine is shown in Figure 5. Even after partial purification, this sample produces a complex profile as indicated by the presence of six major and over twenty five minor peaks. The complexity of this chromatogram indicates that the TLC purification was only partially effective so that further purification of the chemosignal was required. As indicated in Figure 5, the sample was separated into three fractions, A, B and C, by use of preparative GC. Test substances prepared from each of these fractions were then examined for the presence of aggression-promoting cues.

As shown in Table 11, between-group of difference scores indicated fighters exposed to fraction B initiated significantly more attacks than either of the two other test substances ($t's > 10.72$, $P < .001$). Moreover, the duration of attacks produced by fraction B were significantly higher than those of A or C ($t's > 13.22$, $P < .001$). In contrast, the latencies to first attack of fighters exposed to either fraction A or B, were not significantly different ($P > .1$). However, fraction B did produce a significantly lower latency

Figure 5. Preparative GC purification of intact male mouse urine-TLC fraction. Column (1.5 m x 2 mm I.D.) packed with 3% OV-17 on Gas Chrom-Q (100-120 mesh); He carrier, 27.5 cc/min; FID, 1×10^{-9} AFS. Sample size, 10 μ L. Fractions were collected as indicated.

INTACT MALE MOUSE URINE-
TLC FRACTION ON OV-17

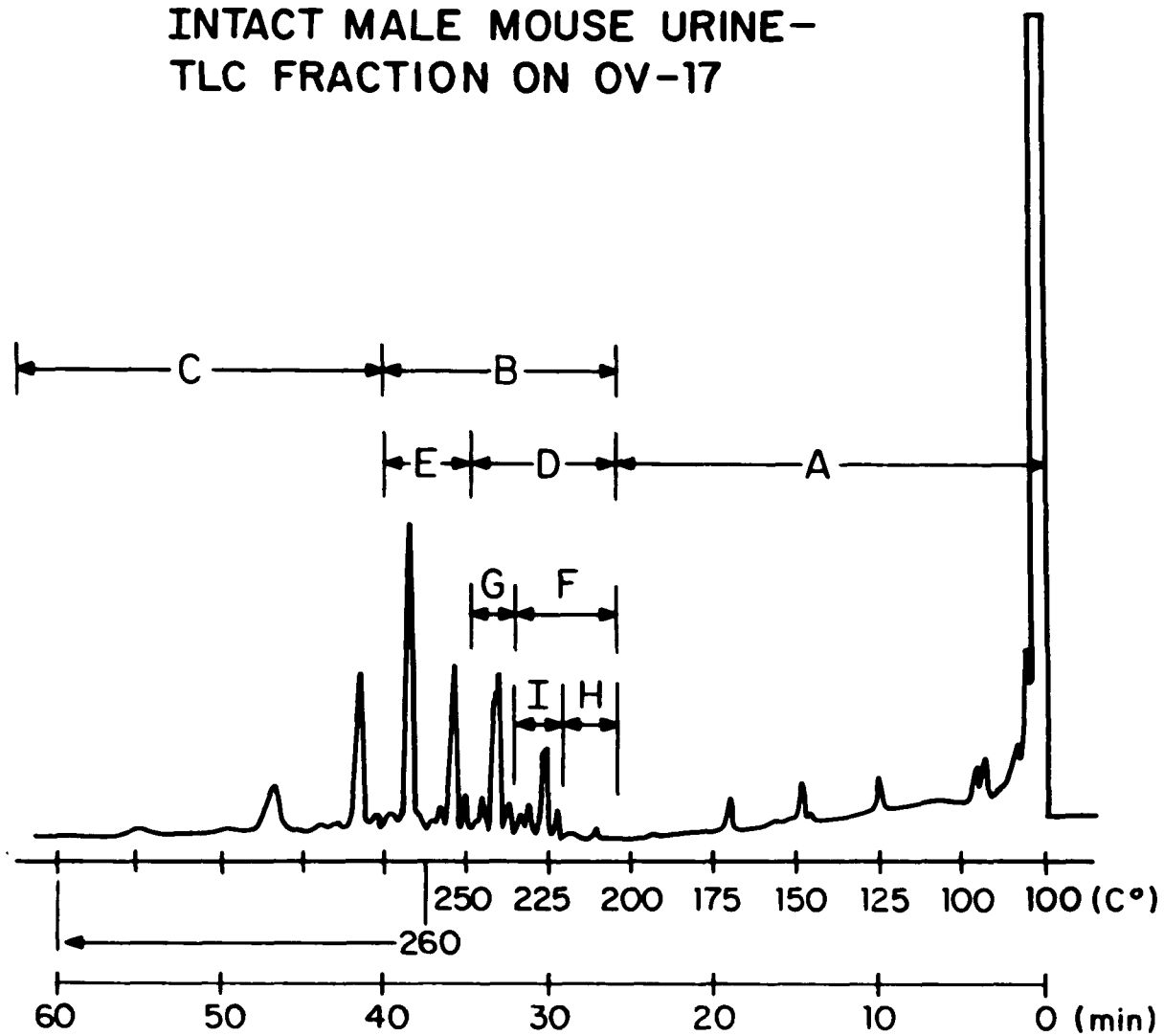


Table 11

Attack Difference Scores of Fighters Exposed to GC Fractions

<u>Sample</u>	<u>Freq + SD</u>		<u>Duration + SD</u>		<u>Latency + SD</u>	
A	0.8	+ 2.2	1.3	+ 2.7	- 3.6	+ 10.8
B	13.6	+ 3.3	22.3	+ 4.5	-12.7	+ 10.6
C	0.9	+ 1.3	1.3	+ 1.7	- 1.3	+ 9.6
D	13.5	3.0	20.4	4.2	-11.3	8.4
E	1.2	1.7	1.5	2.0	- 3.2	6.1
F	11.5	2.8	18.4	4.1	-11.6	7.4
G	2.3	2.4	3.0	3.6	- 4.3	10.3
H	4.3	3.0	6.3	3.8	- 8.7	12.2
I	9.8	3.5	13.6	5.5	- 4.5	9.9
J	5.7	3.7	9.2	6.6	-13.3	15.3
K	7.3	2.5	12.1	4.2	-11.5	9.7
L	4.2	2.4	7.0	4.3	- 5.2	8.9
M	6.0	1.9	10.4	4.0	- 8.3	12.3

score when compared with fraction C ($t > 2.66$, $p < .04$).

Although the mean latency score of fraction B appeared to be much lower than that of fraction A, this difference was not found to be significant. This was due to the relatively high standard deviations associated with these measurements.

These findings of differentiated attack behavior were paralleled by the analyses of uro-genital sniffing presented in Table 12. Both frequency ($t's > 8.23$, $p < .001$) and duration ($t's > 11.75$, $p < .001$) of uro-genital sniffing were found to be significantly higher for fraction B-exposed fighters than for the other groups. In addition, between-group analyses of the latency measure only showed significant differences between groups B and C ($t > 4.99$, $p > .001$).

The findings of the behavioral testing performed on this initial group of GC fraction, clearly indicates that most of the chemosignal has been isolated in fraction B. This is somewhat surprising in view of the relatively high temperature required for the elution of the compounds present in this fraction. The high retention time at a temperature above 200°C suggests that the olfactory cues are relatively nonvolatile. In contrast, the results of the head-space distillation performed in Experiment 6 indicated that the chemosignal(s) was fairly volatile. It may have been possible that the non-volatile cues, present in fraction B, were transferred as an aerosol to the distillate during head-space distillation. On the other hand, the present information may not be sufficient.

Although most aggression-promoting activity was found in fraction B, there was evidence for a much lower but significant

Table 12

Uro-genital Sniff Difference Scores of Fighters Exposed to GC Fractions

<u>Sample</u>	<u>Freq $\pm$ SD</u>		<u>Duration $\pm$ SD</u>		<u>Latency $\pm$ SD</u>	
A	1.5	$\pm$ 2.3	1.9	$\pm$ 2.6	- 1.2	$\pm$ 18.3
B	17.4	6.0	27.6	6.8	-17.2	9.9
C	0.6	2.1	1.0	2.3	8.0	13.5
D	14.3	4.2	23.2	5.3	-12.8	13.7
E	2.0	2.5	2.4	2.4	- 4.7	7.5
F	12.4	2.4	18.8	4.8	-16.0	8.0
G	1.9	3.1	3.3	4.7	- 7.4	11.1
H	5.3	3.2	8.3	5.6	- 9.7	11.3
I	8.2	5.0	12.8	6.9	-11.0	11.4
J	7.1	4.1	9.7	7.5	-15.7	16.7
K	8.7	3.4	12.6	4.4	-13.2	12.6
L	3.3	3.0	5.4	5.0	- 9.3	11.9
M	5.3	1.9	10.6	3.0	-11.8	11.9

amount of behavioral activity in fractions A and C relative to control scores. This trace activity may be a small part of a partially resolved chemosignal, the major portion of which is found in fraction B. It should be noted that of the three fractions tested, only fraction B was found to be significantly different from control scores for all six measures of agonistic behavior.

The next step in the isolation procedure involved the division of fraction B into two fractions, D and E, as shown in Figure 5. To prepare these fractions, a fresh 15 ml sample of urine was employed. Test substances were isolated from these fractions for subsequent behavioral testing.

Fighters presented with fractions D ($t=14.72$, $P<.001$) or E ($t=2.24$, $P<.05$) initiated significantly more attacks than the water base level scores. Between-group comparisons of attack difference scores were found to be significant ($F=136.9$, $P<.001$). As shown in Table 11, the fraction D-exposed fighters initiated significantly more attacks than fighters presented with fraction E.

The cumulative duration index of attack behavior paralleled the findings of the frequency index, for both within-group and between-group comparisons. For the attack latency measure, however, only fraction D produced significantly lower latency scores than water ($t=4.49$, $P<.001$). In addition, between-group comparisons indicated significantly lower latency difference scores under fraction D exposure than for fraction E ($t=2.60$, $P<.02$).

Within-group comparisons of three measures of uro-genital sniffing presented in Table 12, were all found to be qualitatively similar to those of attack behavior produced by fractions D and E. In addition, between-group comparisons of difference scores showed that fraction D induced significantly more sniffs ($t=8.33$, $P<.001$) and of longer duration ($t=11.86$, $P<.001$) than did fraction E. In contrast, no significant difference ($P>.1$) between the sniff latency measures for these two fractions was found.

These findings clearly indicate that fraction D contains significantly more aggression-promoting activity than does fraction E. Comparing the attack behavior of fighters exposed to fraction B or D, as shown in Table 11, it is evident that these two fractions initiate equivalent amounts of behavioral activity. Thus, it appears that fraction D retained the major portion of the chemosignal(s) originally present in fraction B.

Although fraction E appeared to be more active than water, between-group comparisons showed fraction D to contain a significantly higher amount of chemosignal than fraction E. This unequal distribution of chemosignal was observed for five of the six measures of agonistic behavior. The only comparison which was not found to be significantly different was that of sniff latency.

Fraction D was then subjected to further purification by preparative GC. Using a fresh urine sample, two new fractions F and G were prepared for subsequent behavioral testing. Each

of these fractions, pictured in the chromatogram of Figure 5, contained one major peak together with five or six smaller peaks.

Within-group comparisons showed fraction F to be significantly more active than water for all six measures of attack and uro-genital sniffing behaviors presented in Tables 11 and 12 (P 's<.001). In contrast, fraction G initiated agonistic behavior significantly different from that of water in only four of six measures of this behavior (P 's<.05).

Analyses of between-group differences showed both the frequency ($t=9.24$, $P<.001$) and duration (9.29, $P<.001$) of attacks to be greater in fighters exposed to fraction F than in those exposed to fraction G. For the latency measure of attack behavior, however, no significant difference was found between the two groups ($P>.07$). In addition, all three measures of uro-genital sniffing indicated fraction F to be significantly more active than fraction G (P 's<.05).

These findings demonstrate that further purification of the chemosignal has been achieved through recovery of the major portion of the chemosignal in fraction F. Indeed, it appears that with less than 10% of original GC eluate remaining, we still have over 80% of the original chemosignal present. This latter estimate of behavioral activity is based on a comparison of both the frequencies and durations of attacks initiated by fraction F and the total GC eluate group of Experiment 11.

In order to identify those peaks present in fraction F which contained aggression-promoting cues, further purification was necessary. Therefore, fraction F was divided into two smaller fractions, H and I, using the same methods employed in the previous fractionation steps. Thus, two test substances were examined using 24 trained fighters and stimulus animals.

Fighters presented with fractions H ($t=4.78$, $p<.001$) or I ($t=9.30$, $p<.001$) significantly increased their number of attacks over that of their respective water control scores (see Table 11). Analyses of the difference scores for sniff behavior presented in Table 12, showed significant differences between each of the two fractions and water ($p's<.02$). In contrast, analyses of between-group differences did not approach significance for any index of sniff behavior. Thus, it appears that the two fractions initiate equivalent amounts of uro-genital sniffing.

Analyses of the attack frequency and duration measures indicate that of the two fractions assayed, fraction I is the more aggression-promoting. In contrast, the attack latency index and all three measures of uro-genital sniffing indicate no significant differences between the behavior initiated by these fractions. At the same time, both of these fractions initiated significantly more sniff behavior than water.

These findings indicate that chemosignal activity was distributed between the two fractions. In the case of fraction H, this activity may be due either to a unique

component or to a chemosignal present in both fractions. However, since the major portion of the aggression-promoting cue(s) is located in fraction I, any behavioral activity in fraction H is of secondary importance. Therefore, further purification was only directed to the components of fraction I.

The next step in the purification was the division of fraction I into two smaller fractions J and K by preparative GC. As shown in Figure 6, these fractions contained at least three and six components, respectively. Test substances were then prepared from these fractions for use in behavioral testing.

Within-group comparisons showed each fraction to be more active than water across all six measures of attack and uro-genital (p 's < .02). In contrast to the between-group comparisons of the difference scores, shown in Tables 11 and 12, approached significance (p 's > .24) for these two fractions. These findings demonstrated that the two fractions contained behavioral equivalent levels of chemosignal activity.

The question remained whether or not these nonsignificant difference scores were due to the distribution of a single chemosignal among the two fractions. Therefore, aliquots from each fraction were separately examined by GC using the same OV-17 column employed for their isolation. The GC-FID profiles, shown in Figures 7 and 8, clearly indicate that these two fractions have been completely separated from each other, so that cross-contamination was not a likely possibility. Thus, assuming the concentrations of these cues were suffi-

Figure 6. Portion of GC chromatogram showing the production of fractions I-M by preparative GC of intact male mouse urine-TLC fraction. Column and carrier gas as given in Figure 5, FID, 1×10^{-11} AFS; sample size, 5 μ L.

INTACT MALE MOUSE URINE -
TLC FRACTION ON OV-17

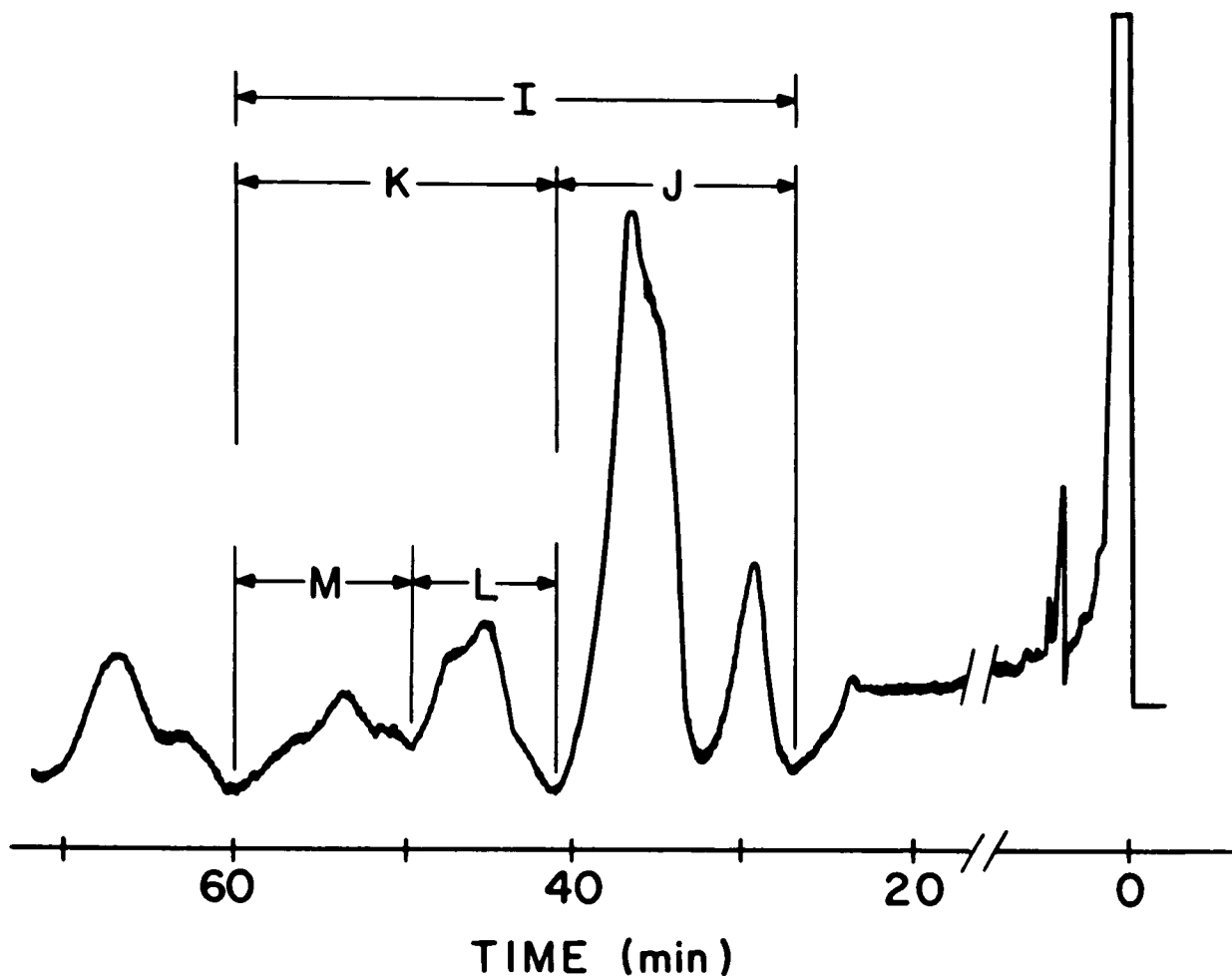


Figure 7. GC chromatogram of fraction J from intact male mouse urine. Glass column (1.5 m x 2 mm I.D.) packed with 3% OV-17 on Gas Chrom-Q; 200°C; He carrier, 32.5 cc/min; FID, 1×10^{-11} AFS. Sample size, 5 μ L.

Fraction J
on OV-17

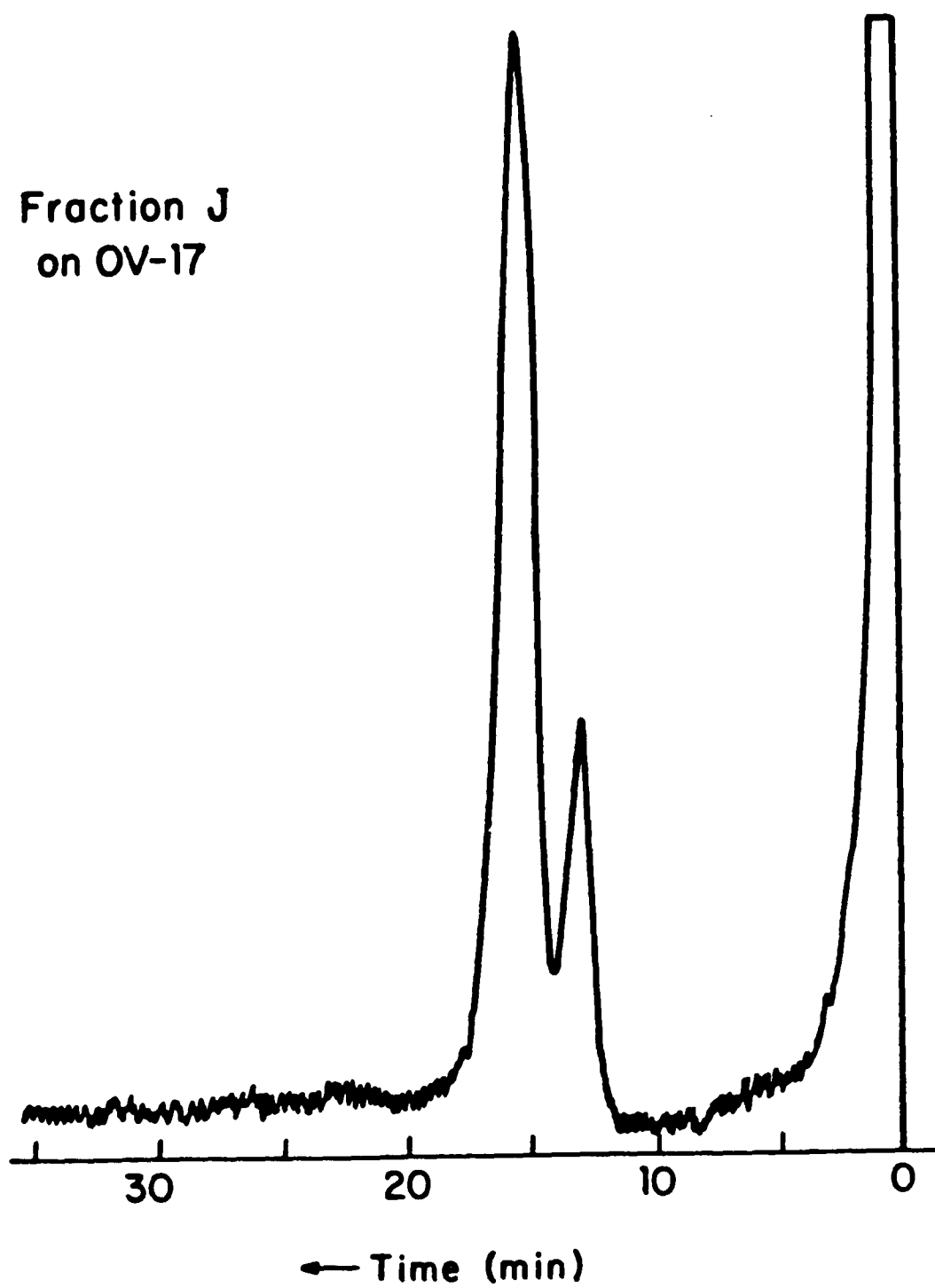
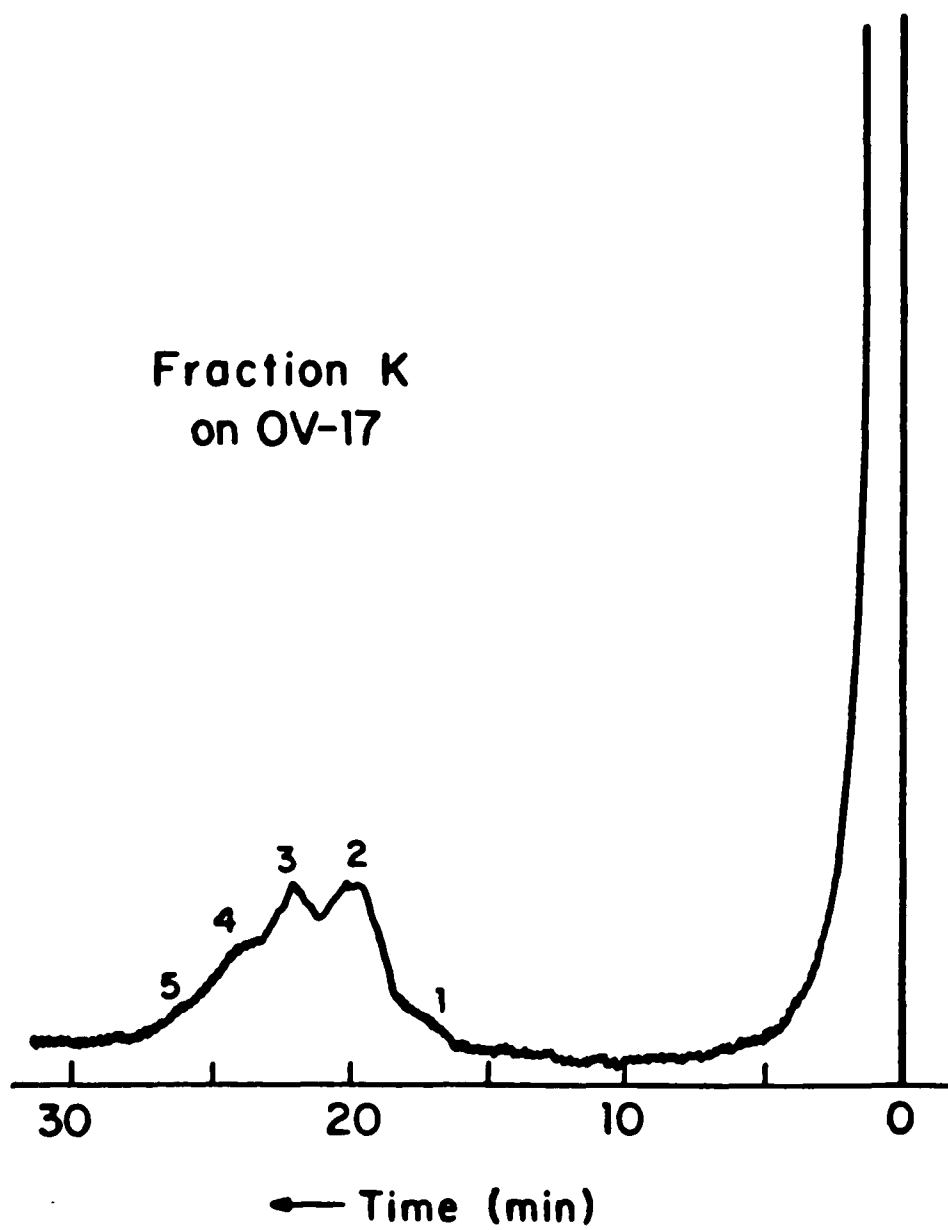


Figure 8. GC chromatogram of fraction K from intact male mouse urine. Chromatographic conditions given in Figure 7.

Fraction K
on OV-17



ciently high to allow their detection by FID, it must be concluded that at least two distinct aggression-promoting cues were present in fraction I. In addition, their similar retention times suggests that they may be homologs of each other.

Before chemical identification of these compounds commenced, one final purification by preparative GC of fraction K was attempted. As shown in Figure 6, two smaller fractions, L and M, were isolated for subsequent behavioral testing. Although both of these fractions proved to be more behaviorally active than water ($p's < .03$), between-group comparisons of difference scores (see Tables 11 and 12), were not found to be significant. Moreover, when these two fractions were examined by GC, considerable overlap of components among the two fractions was evident. Thus, it appears that any further purification of these cues must be achieved either through a method of separation whose resolution is higher than that of the present one or a method using a different property of these compounds for their separation.

In summary, the effectiveness of preparative GC for the purification of aggression-promoting cues from male mouse urine has been demonstrated. By use of this method, evidence has been obtained for the presence of at least two aggression-promoting chemosignals in male mouse urine. In addition, the chemical identification of these cues has been greatly simplified by the removal of several behaviorally inactive components of urine.

Experiment 13: Preliminary Examination of Aggression-Promoting
Chemosignals by Gas Chromatography-Mass
Spectrometry (GC-MS)

The findings of the previous experiment demonstrated that substances associated with the aggression-promoting activity of male mouse urine could be isolated by preparative GC. It was not possible, however, to achieve the total purification of these cues by the GC techniques used. Instead, a fraction was obtained containing nine components, two of which appeared to be aggression-promoting. Further purification of the cues was attempted but was unsuccessful due to incomplete resolution of the compounds of interest.

For many GC separations, increased resolution can be obtained using open tubular or capillary columns (Jennings, 1980). The effluent from these columns can be introduced through a suitable interface, into a mass spectrometer (MS), for the identification of individual components in a complex mixture (McFadden, 1973). Combined GC-MS, coupling the high resolution of capillary GC to the high sensitivity and specificity of MS, provides a powerful method for structure elucidation of natural products.

The present experiment describes the use of this method for the examination of an aggression-promoting fraction prepared from intact male mouse urine. This fraction was purified by TLC and preparative GC as described in Experiment 12 and was equivalent to fraction I of that experiment. The earlier procedures were modified by increasing the initial volume of urine to 50 ml. At the time, this amount of urine

appeared to be of sufficient size to provide the minimum quantity of sample required by the GC-MS procedure. However, when the GC fraction prepared from this sample was analyzed by GC-MS, no mass spectra were obtained apart from those of instrumental background. Consequently, this procedure was repeated several times using progressively higher initial volumes of urine.

At the same time, changes were made in the GC-MS procedure in order to maximize the amount of sample delivered to the ionization stage of the MS instrument. These changes included the use of a split-less injection mode and the bypassing of the GC-MS interface so that all of the GC effluent passed into the ionization chamber of the instrument. In addition, the length of the 0.5 mm I.D. glass SE-30 capillary column used for this separation was shortened to ten meters in order to minimize sample hold-up by this wide-bore column. For the instrumental conditions employed during the GC-MS procedure the reader is referred to the accompanying figures and to the Materials and Methods section.

These procedural changes when combined with the use of an initial urine sample of 160 ml allowed the reconstructed chromatogram of Figure 9 to be obtained. The GC-MS profile of the total ion current can be compared to the chromatogram obtained using a 2 meter x 2 mm I.D. OV-17 column, shown in Figure 10. The relatively low resolution exhibited by the capillary GC-MS chromatogram was most likely due to the relatively high sample volume (4.5 μ l) which was applied to the GC-MS instrument.

Figure 9. Computer reconstruction of total ion chromatogram of GC-MS analysis of fraction I. Column (10 m x 0.5 mm I.D.) coated with SE-30. Initial column temperature of 200°C was increased at a rate of 10°C/min to a final temperature of 257°C which was maintained until completion of the run.

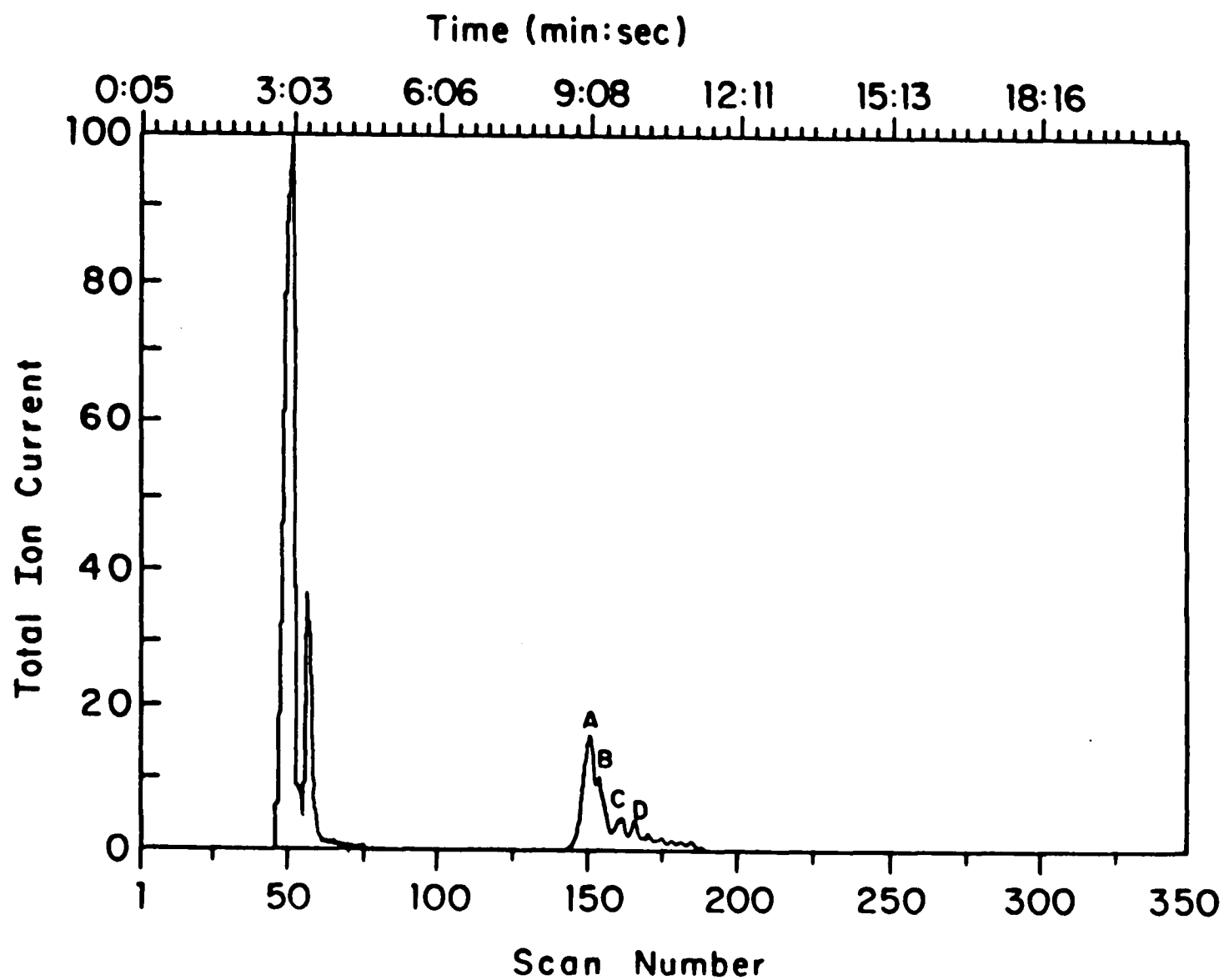
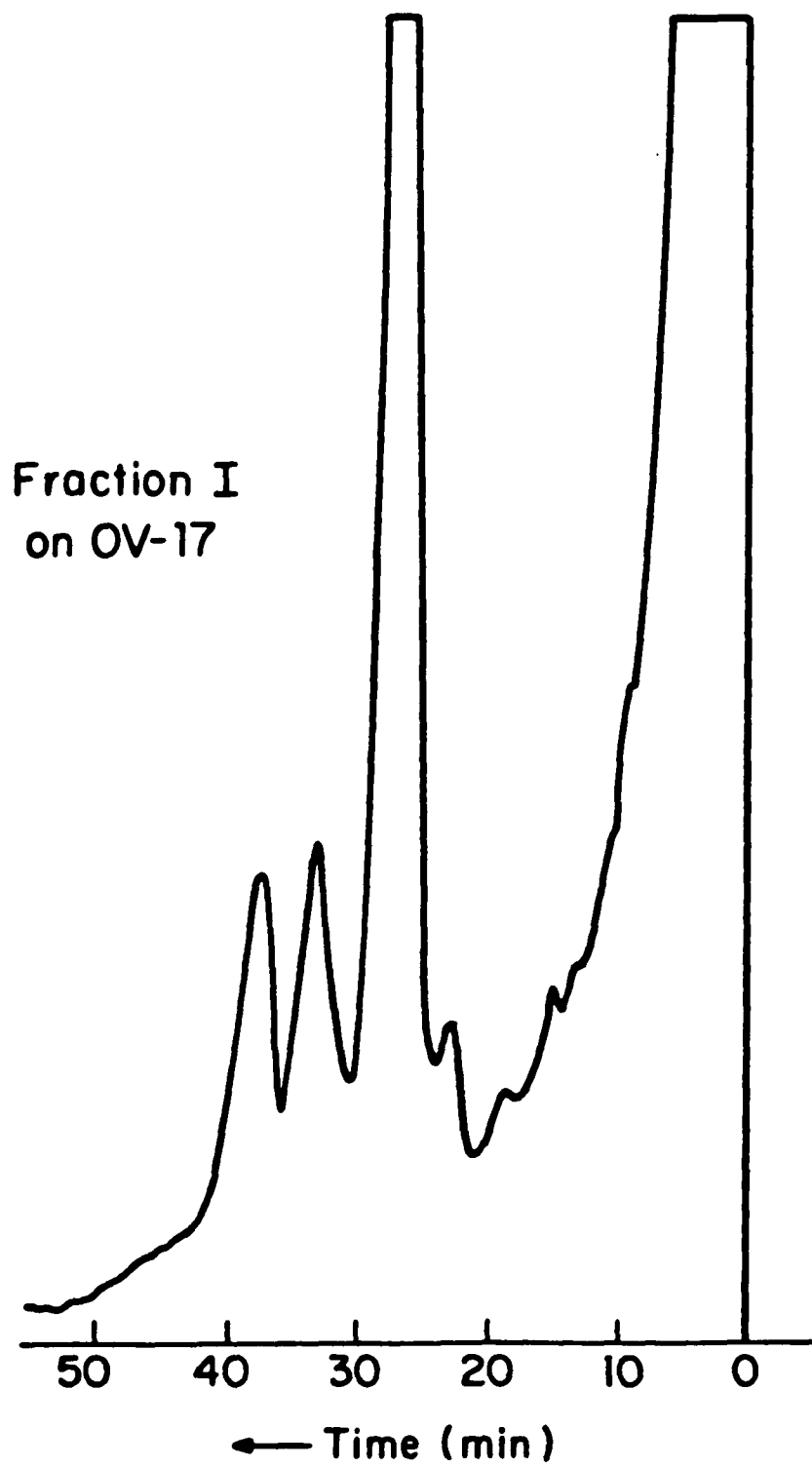


Figure 10. GC chromatogram of urinary fraction I. Column (1.5 m x 2 mm I.D.) packed with 3% OV-17 on Gas Chrom-Q; 200°C; He carrier, 30 cc/min; FID, 1×10^{-11} AFS. Sample size, 5 μ L; total volume of fraction, 400 μ L.



Although not all of the peaks were separated in this GC-MS run, some useful structural information was obtained regarding peaks A and B of Figure 9. The mass spectra of the peaks are shown in Figures 11 and 12. To produce each of these spectra, the mass spectrum at the minimum point between the two peaks was subtracted from the spectrum at the peak maximum. This difference technique was necessary in order to minimize the interference by each of the partially resolved peaks on the mass spectrum of its partner.

A comparison of the spectra with previously published spectra (Odham and Stenhagen, 1972; Jennings and Shibamoto, 1980) indicated that peaks A and B may contain esters of fatty acids with fatty alcohols. At the same time, the molecular ions of peaks A and B were tentatively assigned to ions of m/e values, 364 and 368, respectively. The assignments of peaks A and B as long-chain wax esters, although tentative, were supported by the presence of several significant homologous series of ion in their mass spectra. Both of these spectra exhibit series of ions (e.g. 43, 57, 71, 85,...) separated by CH_2 groups, which indicates that they possibly arose from compounds with long alkyl chains. In addition, the possible presence of an ester group was supported by the appearance of a series of several ions in these spectra which may have arisen from McLafferty type ester of rearrangements (McLafferty, 1959). Whereby the fragments shown in Table 13 are produced by the characteristic cleavage of the ester group and proton migration.

Figure 11. Electron impact mass spectrum of peak A
representing scan 150-scan 152. Electron
energy of ion source, 70eV.

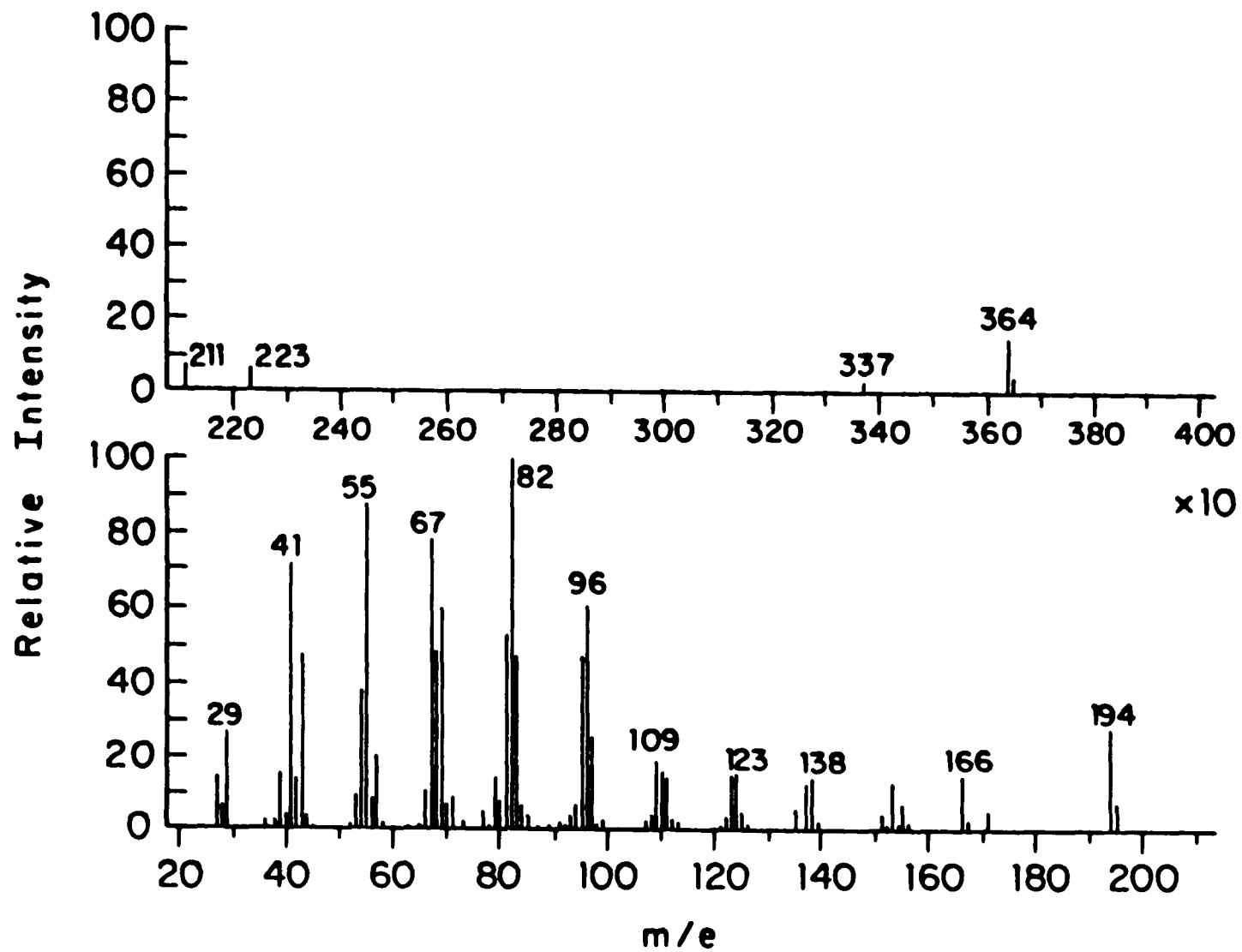
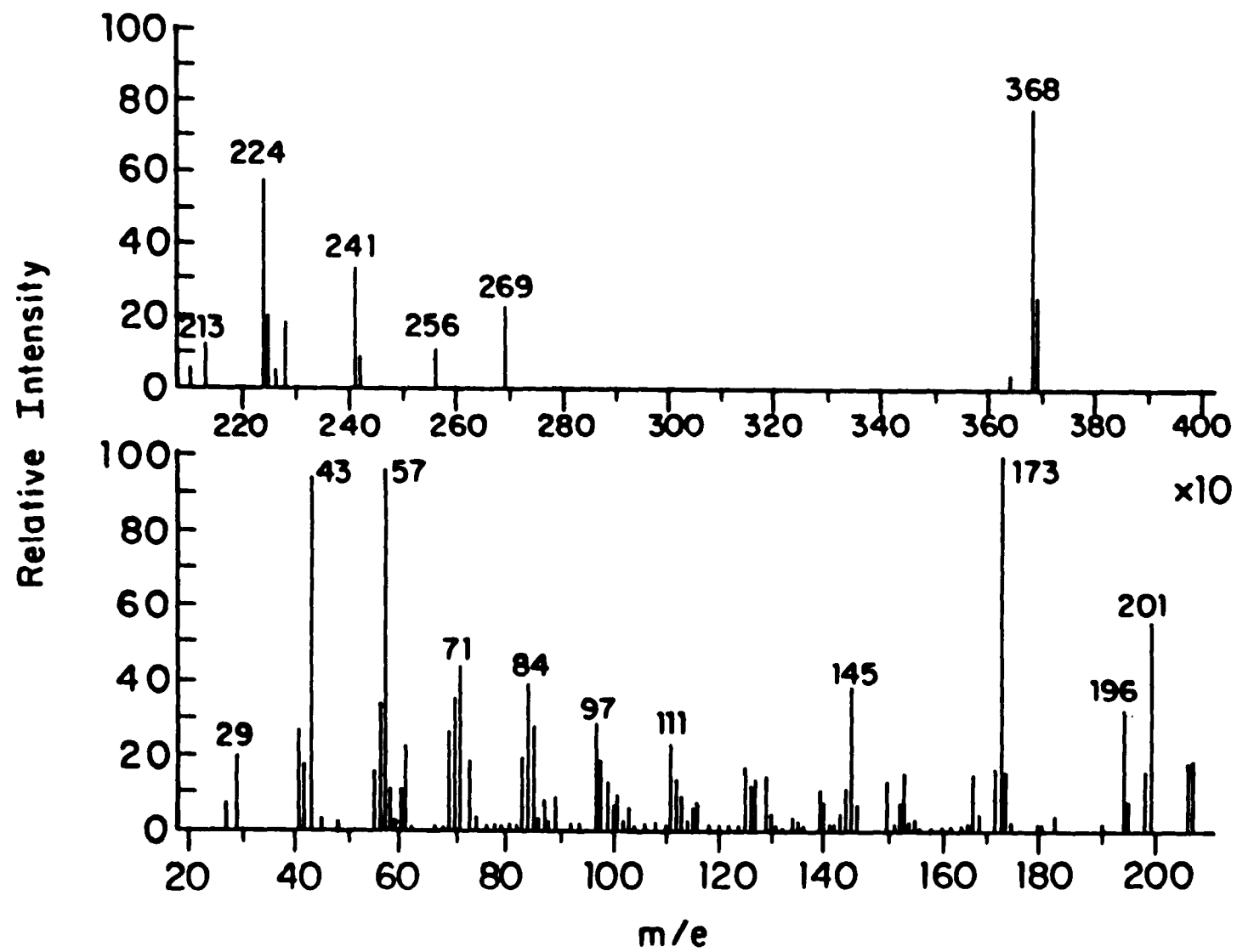


Figure 12. Electron impact mass spectrum of peak B
representing scan 154-scan 152. Ion source,
70eV.



The assignment of these rearrangements together with other characteristic fragments for the mass spectrum of peak B are shown in Table 13. The series of ions at m/e 140, 168, 196 and 224 are assigned to the cleavage of an alkyl-oxygen bond with the concomitant loss of one hydrogen atom to produce the fragment $[R-1]^+$ from an ester, $R-C(=O)OR$ (Ryhage and Stenhagen, 1959). A corresponding series of fragments produced by a McLafferty type of rearrangement occurs at m/e 200, 228 and 256. Another series of fragments occurs at m/e 185, 213, 241 and 269 indicating the formation of an alkoxy carbonyl ion from the alcohol portion of the ester. In a similar manner, one can also determine the chain length of the fatty acid portion of the ester, using the acylium ion $[R-C=O]^+$, as shown in Table 13.

The presence of these characteristic ion series suggests that the molecular ion at m/e 368 was produced by a mixture of several saturated 24-carbon wax esters. If these assignments are correct then the fatty alcohol chain would range from 10 to 16 carbons while the lengths of the fatty acids would range from 8 to 14 carbons.

In the case of peak A, whose molecular ion (m/e 364) is four a.m.u. lower than that of B (m/e 368), one might expect to find a fragmentation pattern which is characteristic of a diene wax ester. These two double bonds could be either equally or unequally distributed between the acid and alcohol components of the wax ester. If they are equally distributed so that both the acid and alcohol are unsaturated then one

Table 13

Assignment of the Main Fragments in the Mass Spectrum
of Peak A'

Fragments	<u>Fatty Alcohol Portion</u>		
	$R'-1^+$	$R'-O-\overset{O}{C}^+$	$R'-O-\overset{OH}{C}=\text{CH}_2^+$
R' Carbon No.	m/e		
16	224	269	
14	196	241	256
12	168	213	228
10	140	185	200

Fragments	<u>Fatty Acid Portion</u>	
	$R-\overset{O}{C}-O+2H^+$	$R-\overset{O}{C}=O^+$
R Carbon No.	m/e	
13		211
11	201	183
9	173	155
7	145	127

' As an ester, $R-\overset{O}{C}-OR'$

would expect to find fragments in the mass spectrum of peak A (Figure 11) which are shifted by -2 a.m.u. from the corresponding fragments of B shown in Table 13. A comparison of the ions shown in Figure 11 with those of Table 13 reveals that such a shift is observed in most but not all cases. In addition, a shift of -4 a.m.u. is observed for some minor fragments which may indicate the presence of a diene alcohol or acid.

It must be remembered, however, that these postulated structures are tentative and must be confirmed. In view of the low resolution exhibited by the capillary column under these conditions, even the purity of these peaks must be established. Ideally, one could investigate this matter, by use of chemical ionization-mass spectrometry, in which only the molecular ion ($M+1$) of a species is formed with appreciable abundance. If only one such ion is apparent in the mass spectrum of a compound, then its purity is confirmed. Unfortunately, due to limitations in instrumentation such capabilities were not available at the time.

Experiment 14: Chromatographic Analysis of Preputial Gland Extracts

The findings of mass spectral analysis performed in Experiment 13 indicated that long-chain esters may be present in an aggression-promoting fraction prepared from male mouse urine. Since such compounds are not typically found in urine as a result of hepatic metabolism, a search was made for other murine sources of wax esters. Of interest, was the

report (Sansone, 1968) that preputial glands of male mice contain high levels of wax esters. Moreover, previous work has shown that preputial glands are a source of aggression-promoting odors in mice (Mugford and Nowell, 1971). Since male mouse urine contains preputial secretions, it may be possible that the ester compounds in the behaviorally-active GC fraction were also secreted by the preputial gland.

The present experiment was designed to investigate this possibility by examining a preputial gland extract for the presence of any compounds which might be similar to those in the behaviorally-active GC fraction (I). This comparison was based on TLC and GC analyses performed on each sample. A preputial extract was prepared and purified using a procedure adapted from Sansone (1968).

The minced preputial glands from 35 adult male mice were extracted with CH_2Cl_2 in the presence of CH_3OH to yield 1 g of crude lipids. These lipids were then separated on a silicic acid column (1.2 cm x 45 cm) which was washed with 200 ml hexane followed by 200 ml of 5% (v/v) ether in hexane. Fractions of the eluent were collected and then separately concentrated as previously described in Materials and Methods.

Aliquots from each of these fractions were then examined by TLC on silica gel G plates using CH_2Cl_2 as the developing solvent. At the same time, a sample from an extract of male mouse urine was included for TLC analysis in order to establish the location of the behaviorally-active TLC zone under these conditions. The resulting chromatogram, developed by exposure to I_2 vapors, is shown in Figure 13. Of interest

Figure 13. TLC chromatogram of male mouse urine extract, U, crude preputial extract, P, and corresponding fractions from silic acid chromatography of preputial extract. An arrow indicates the position of the aggression-promoting zone.



is a band ($R_f=.64$) of preputial fraction IV which appears to comigrate with the aggression-promoting zone of the urinary sample. The differences in R_f values for this zone as compared to earlier work may have been due to small differences in either the mobile phase or in the activity of the silica.

The compounds present in preputial fraction IV were then subjected to GC analysis using an OV-17 column. The resulting GC profile is shown in Figure 14. On inspection, this chromatogram bears some resemblance to those obtained from GC analysis of the aggression-promoting TLC zone. In order to facilitate a comparison of these two GC profiles, a sample of the behaviorally-active TLC zone of male mouse urine was subjected to GC analysis at this time under conditions identical to those used for the preputial sample. The two GC-FID profiles which are presented together in Figure 15 appear to be almost identical on the basis of the retention time and relative amount of each of their constituent peaks. Moreover, the preputial profile clearly contains four peaks matching each of the peaks in the region of the urinary profile corresponding to an aggression-promoting GC fraction (I) of Experiment 12.

The next step in this study was to determine whether or not these four peaks of interest in the preputial GC-FID profile would comigrate with the behaviorally-active TLC zone of male mouse urinary extract. Since preputial fraction IV produced three TLC bands which did not comigrate with the aggression-promoting urinary zone, it was possible

Figure 14. GC analyses of preputial fraction IV. Column (1.5 m x 2 mm) packed with 3% OV-17 on Gas Chrom-Q (100-120 mesh); 200°C; He carrier, 27.5 cc/min; FID, 1.6×10^{-10} AFS. Sample size, 2 μ L.

INTACT MALE MOUSE
PREPUTIAL EXTRACT—
SILICA FRACTION IV
ON OV-17

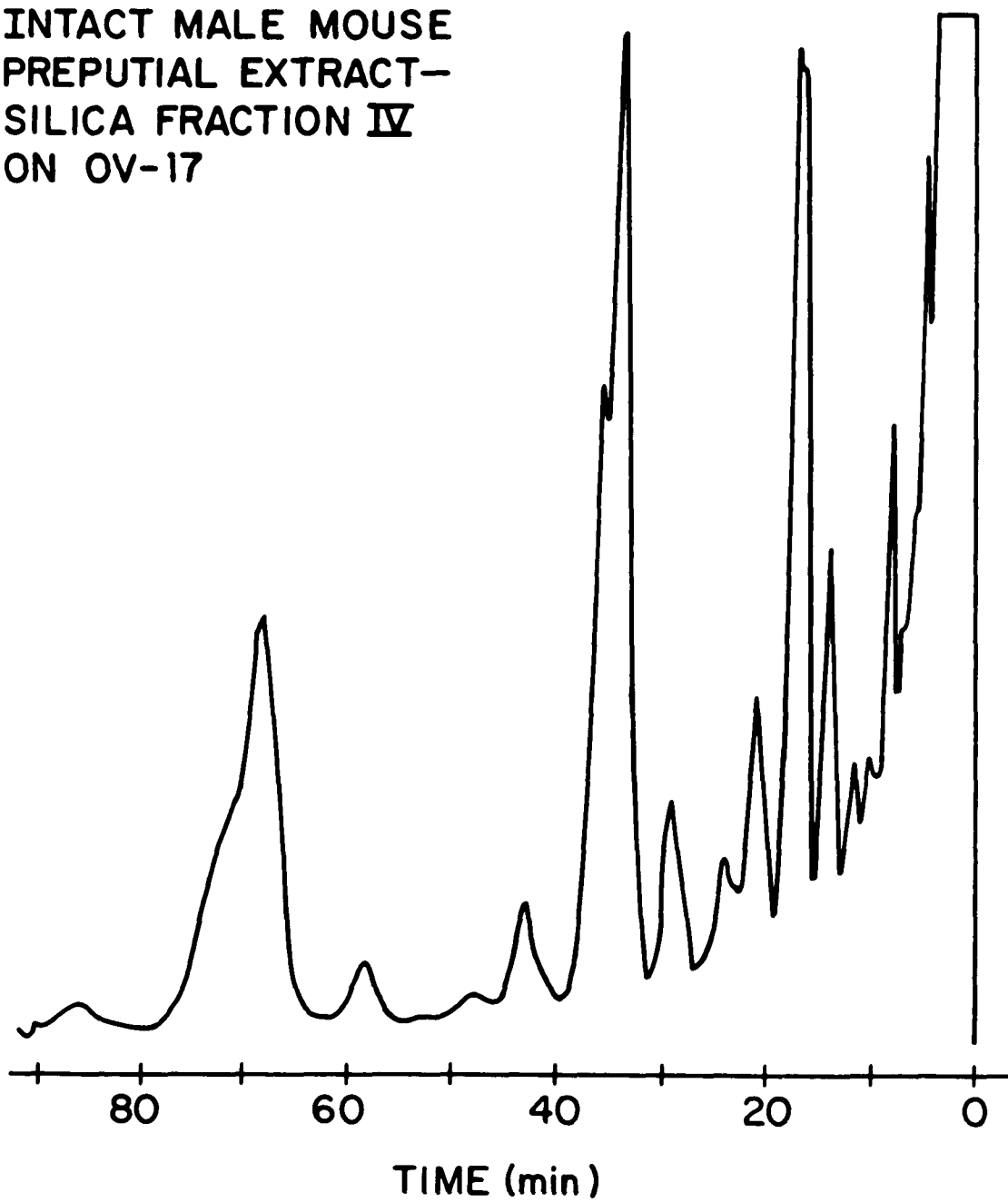
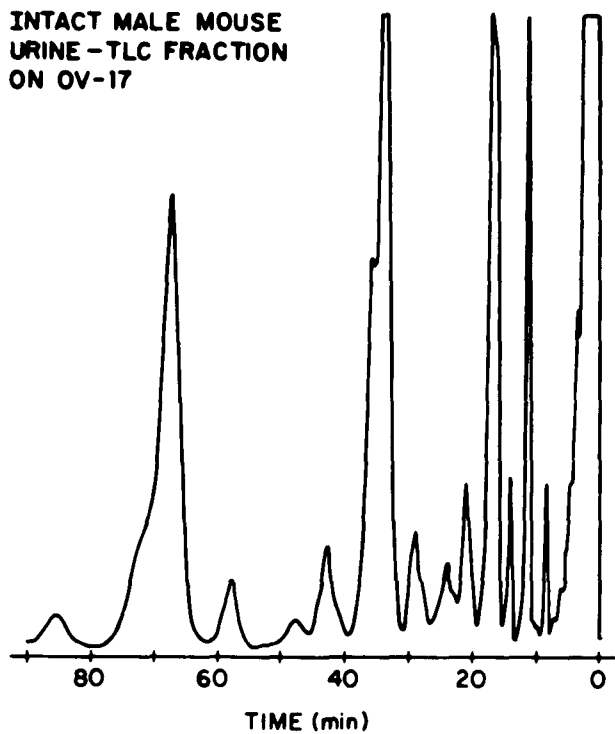
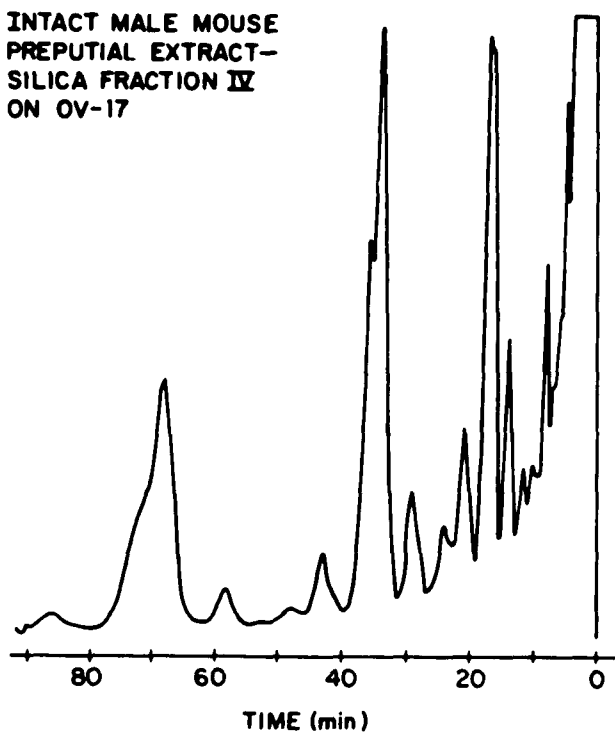


Figure 15. GC profile of intact male mouse urine-TLC
fraction (top) and preputial fraction IV (bottom).
Chromatographic conditions given in Figure 14.

INTACT MALE MOUSE
URINE - TLC FRACTION
ON OV-17



INTACT MALE MOUSE
PREPUTIAL EXTRACT -
SILICA FRACTION IV
ON OV-17

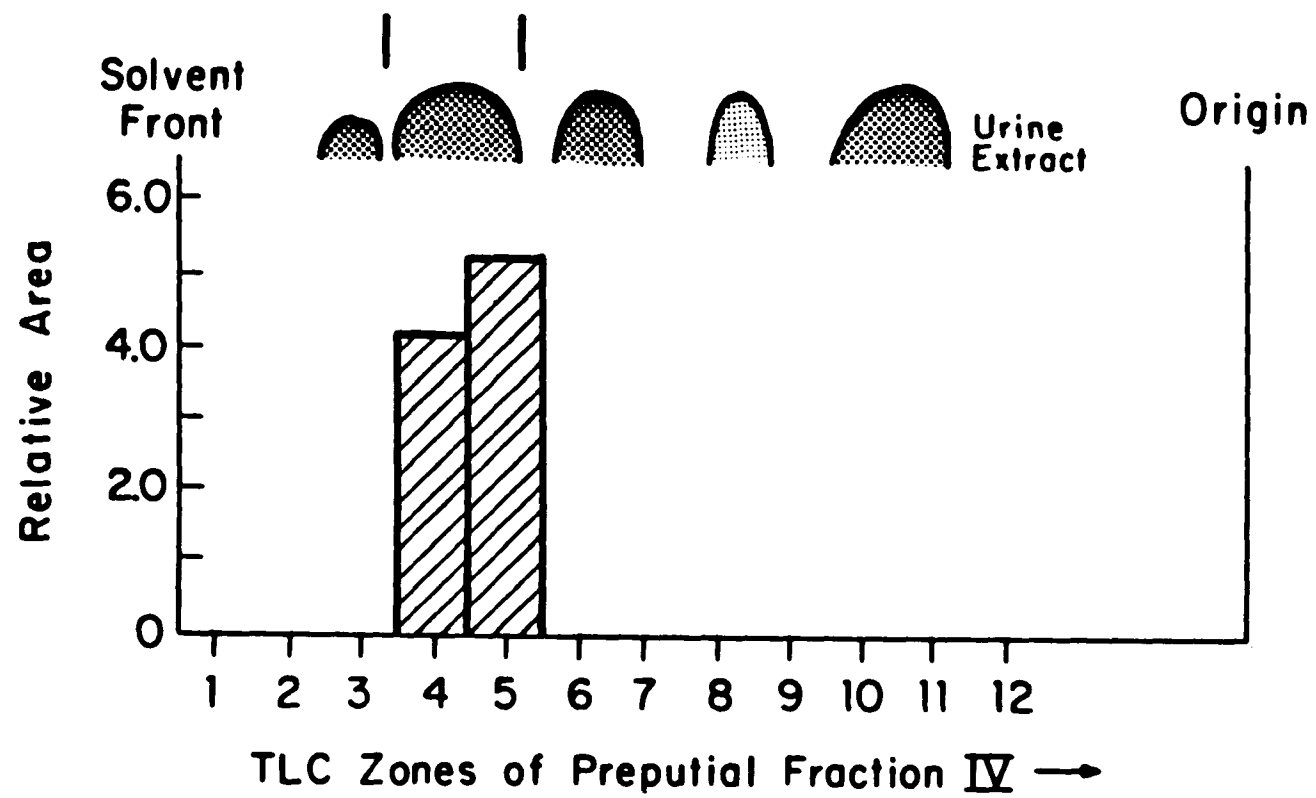


that the four GC peaks of interest were associated with one of the three TLC bands.

In order to examine this question, an aliquot from preputial fraction IV was separated by TLC together with a sample of male mouse urinary extract. Zones corresponding to the region of the preputial sample were separately scraped and eluted with CH_2Cl_2 to produce samples for GC analysis. The remainder of the plate was then exposed to I_2 vapors in order to visualize the bands of the urinary sample.

The results of this study are shown in Figure 16, where the total relative area of preputial GC peaks with retention times corresponding to urinary GC fraction I are plotted for each of the preputial TLC zones. At the same time, the locations of the urinary TLC bands including the aggression-promoting zone are shown for reference. The results of this combined TLC-GC study clearly indicate that the four preputial GC peaks of interest comigrate with the aggression-promoting zone of male mouse urine. Moreover, it appears that preputial fraction IV shares at least four compounds in common with an aggression-promoting GC fraction of mouse urine. It should be noted, however, that this assignment was tentative and required further examination by a more specific technique such as mass spectrometry.

Figure 16. TLC chromatogram of intact male mouse extract where vertical lines indicate the position of the aggression-promoting zone. Bottom: Plot of total area of preputial peaks coeluting with urinary GC-fraction I versus zone used to prepare GC sample from TLC chromatogram of preputial fraction IV.



Experiment 15: Gas Chromatographic and Mass Spectral
Analyses of Putative Aggression-Promoting
Cues in Preputial Extraction

The results of the previous experiment indicated that preputial glands of male mice contain several compounds which also appeared in an aggression-promoting GC fraction of male mouse urine. These compounds are of interest since one or more of them may represent an aggression-promoting chemosignal for male mice. If their structures were known then one could establish their chemosignalling function by synthesis of suitable reference compounds for subsequent behavioral testing.

Chemical identification of these compounds, however, has not proved to be an easy task. In male mouse urine, these compounds are present in minute quantities (approx. 1-10 ng/ml) so that after purification only a limited amount of sample is available for structural analysis. If these compounds could be easily purified then their structures could be investigated by use of mass spectrometry.

The total purification of these compounds, however, could not be achieved by packed-column GC methods as shown by the partial nature of the purification obtained in Experiment 12. In a later experiment, a fraction prepared by this method was examined using capillary GC-MS. This sample contained only trace amounts of the compounds of interest so that only a limited amount of structural information was obtained. If a larger amount of sample amount had been available then it would have been possible to

achieve both the purification and identification of these compounds by GC-MS.

At that time, the only known source of these compounds was the urine of male mice. Since these compounds are present in relatively low amounts in this fluid, a large volume of urine was required in order to produce a sample of sufficient size for subsequent structural analysis. Unfortunately, the collection of large amounts of male mouse urine is a slow and laborious process which can limit the final amount of sample to be used for chemical identification. In Experiment 13, for example, over 200 man-hours were required over a twelve week period in order to accumulate sufficient amounts of urine for a single GC-MS analysis. This amount of sample did not permit the capillary GC separation to be optimized so that only a partial purification of these compounds was achieved.

Clearly, an alternative source of the compounds was needed, one in which they are present in much higher amounts than can be found in male mouse urine. Fortunately, the results of the previous experiment indicated that an alternative source for the isolation of the compounds can be found in the preputial glands of male mice. Although that experiment was not designed to compare the amounts of compounds present in each sample, still the results of GC analyses indicated that much higher amounts were present in the preputial samples than in the urinary sample. Thus, if a preputial extract was used as a source then it should be

possible to obtain much larger amounts for analysis by MS. In addition, a larger amount of sample would allow the separation of the compounds by capillary GC to be improved so that total purification could be achieved during a GC-MS analysis.

The present experiment was designed to identify the preputial compounds representing possible aggression-promoting cues by use of mass spectrometry. As a source of the preputial compounds, fraction IV from Experiment 14 was subjected to further purification using the preparative GC procedure of Experiment 12. To accomodate larger amounts of sample, a 4 mm I.D. column was substituted for the 2 mm I.D. glass column which had been previously used.

A typical GC-FID profile, obtained during the purification of these compounds is shown in Figure 17. This process yielded nine fractions, with fractions 4 through 9 containing the compounds of interest as components of peaks a through d. The purity of these fractions was examined by GC analysis on an OV-17 analytical column (2 mm x 1.5 m). The results of the analyses performed on fractions containing the compounds are shown in Figure 18 through 22. The first chromatogram shows that peak a was the only major component present in fraction 4. Similar results were obtained for the recovery of peak b in fraction 5 as shown by Figure 19. In both cases, the use of preparative GC resulted in the enrichment of the desired component in a single fraction.

Peaks c and d, on the other hand, were found to be

Figure 17. Preparative GC purification of preputial fraction IV. Column (1.5 m x 4 mm I.D.) packed with 3% OV-17 on 100-120-mesh Gas Chrom-Q; 190°C; Eluent split 1:6; FID, 4×10^{-11} AFS. Vertical lines indicate the boundaries between fractions. Peaks a-d contain the compounds of interest. Sample size, 5 μ l.

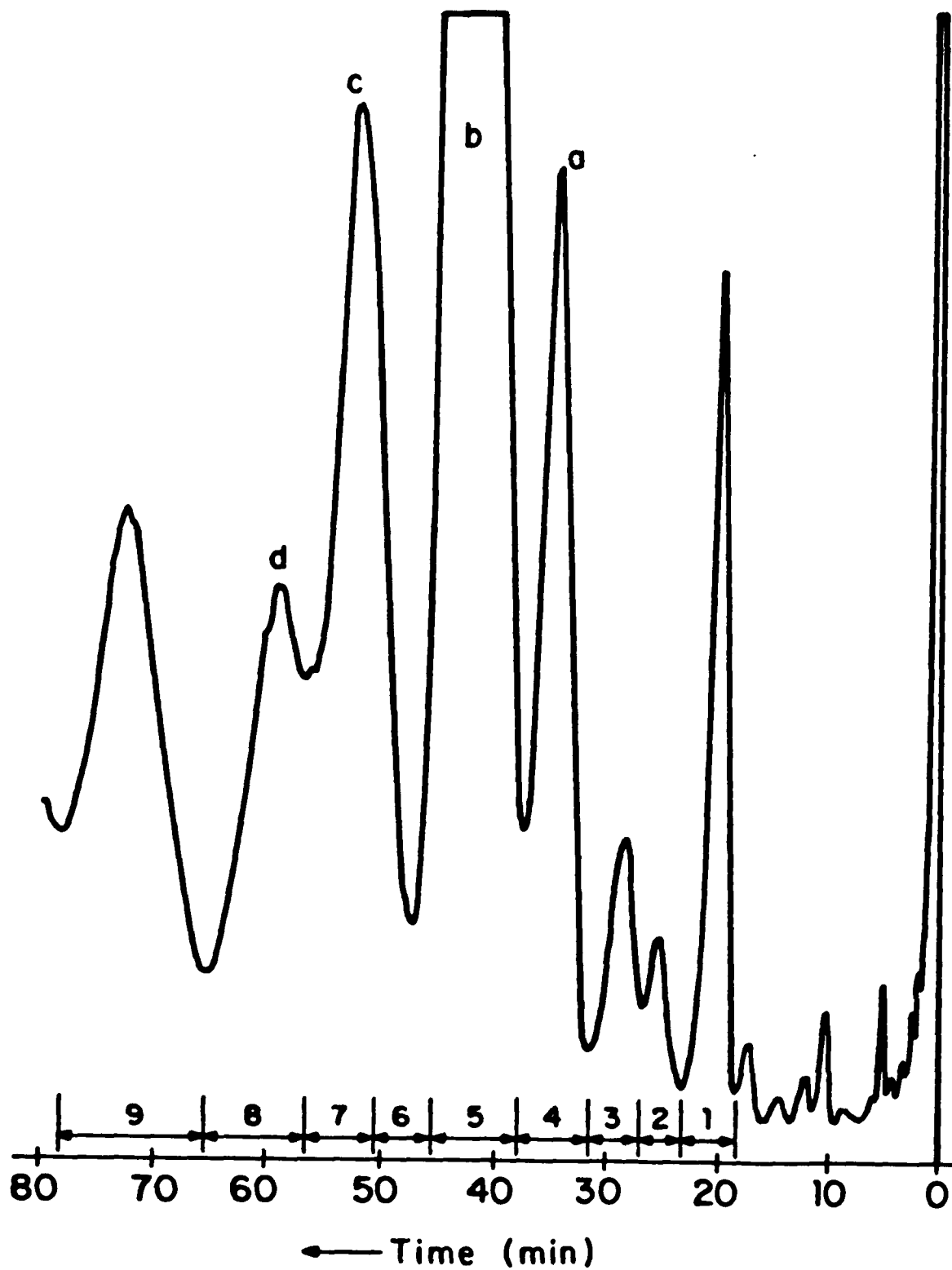
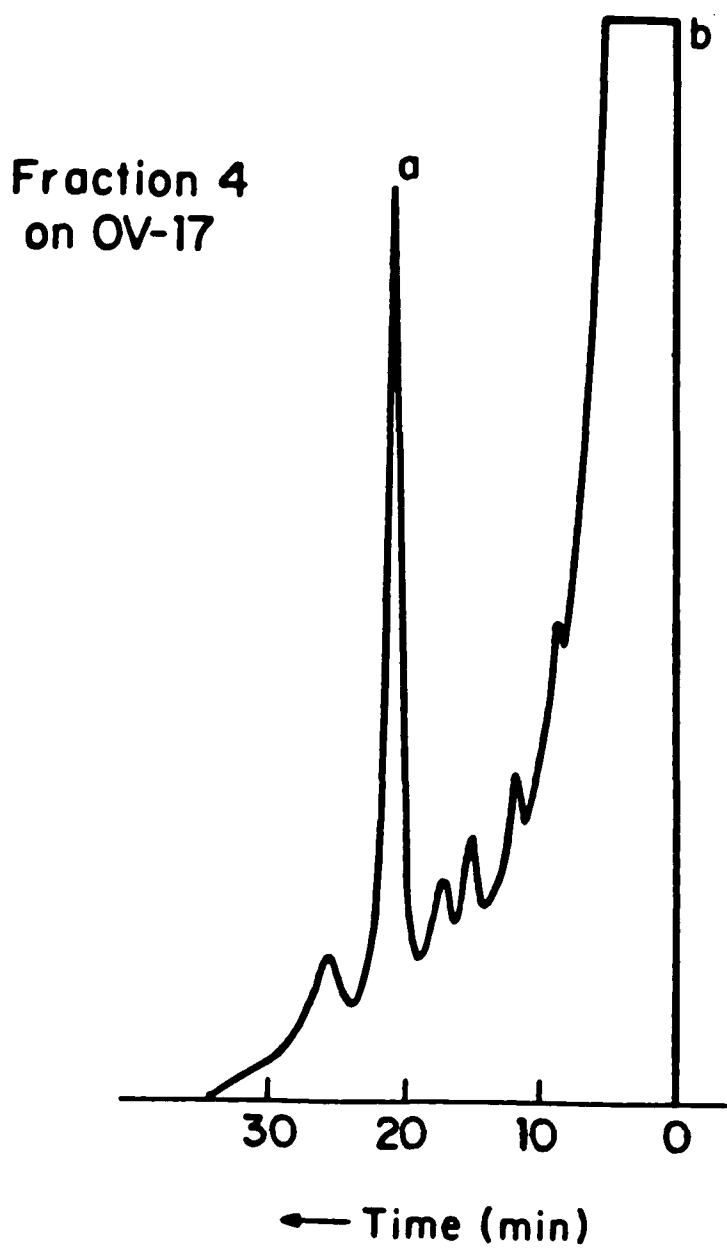
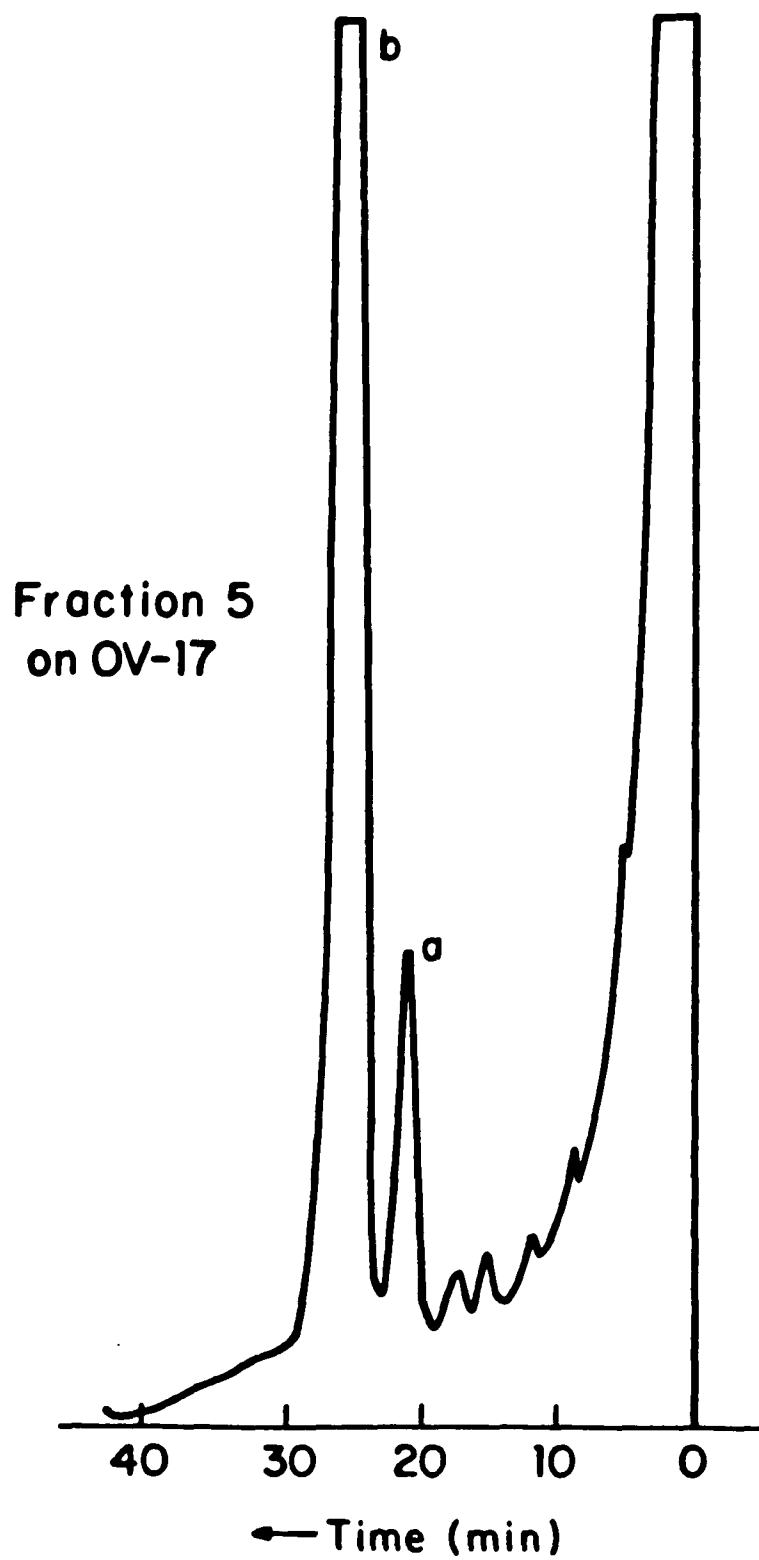
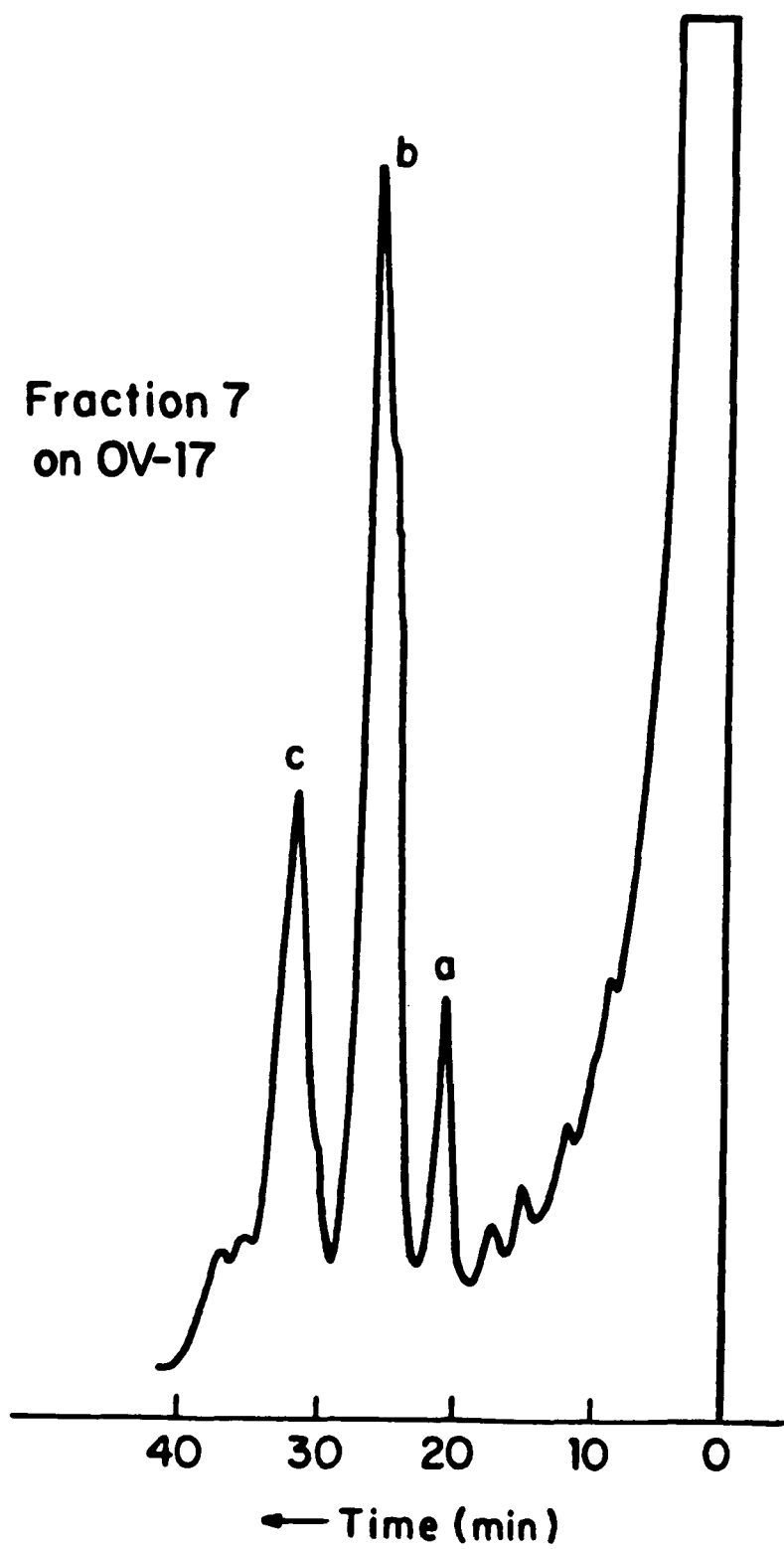


Figure 18-22. GC analyses of preputial preparative GC fractions 4, 5, 7, 8 and 9. Column (1.5 m x 2 mm I.D.) packed with 3% OV-17 on Gas Chrom-Q; 200°C; He carrier, 27.5 cc/min. Detection by FID, 2×10^{-11} AFS. Peaks designated a-d correspond to those shown in Figure 17. Sample size, 1 μ l.

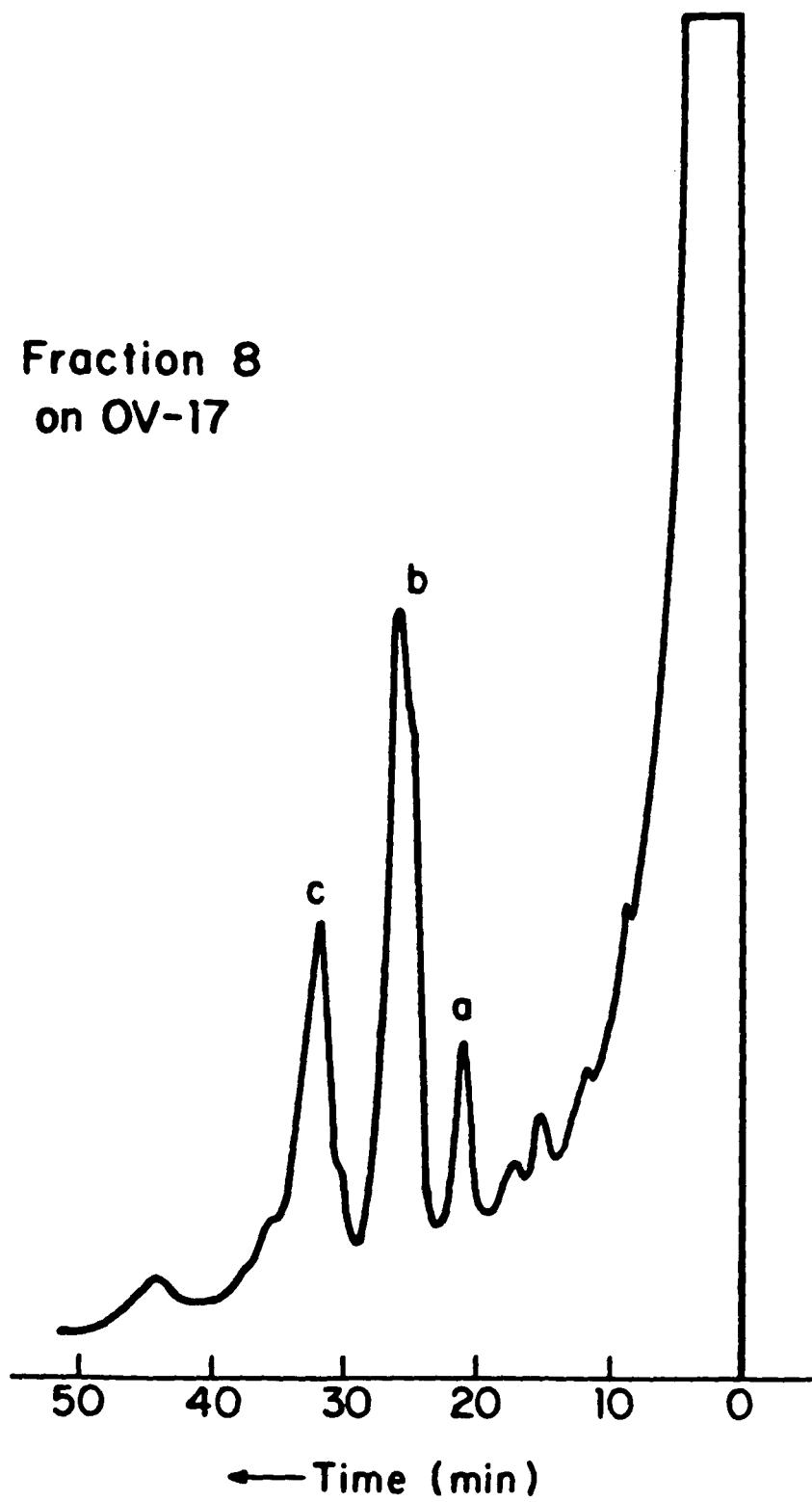




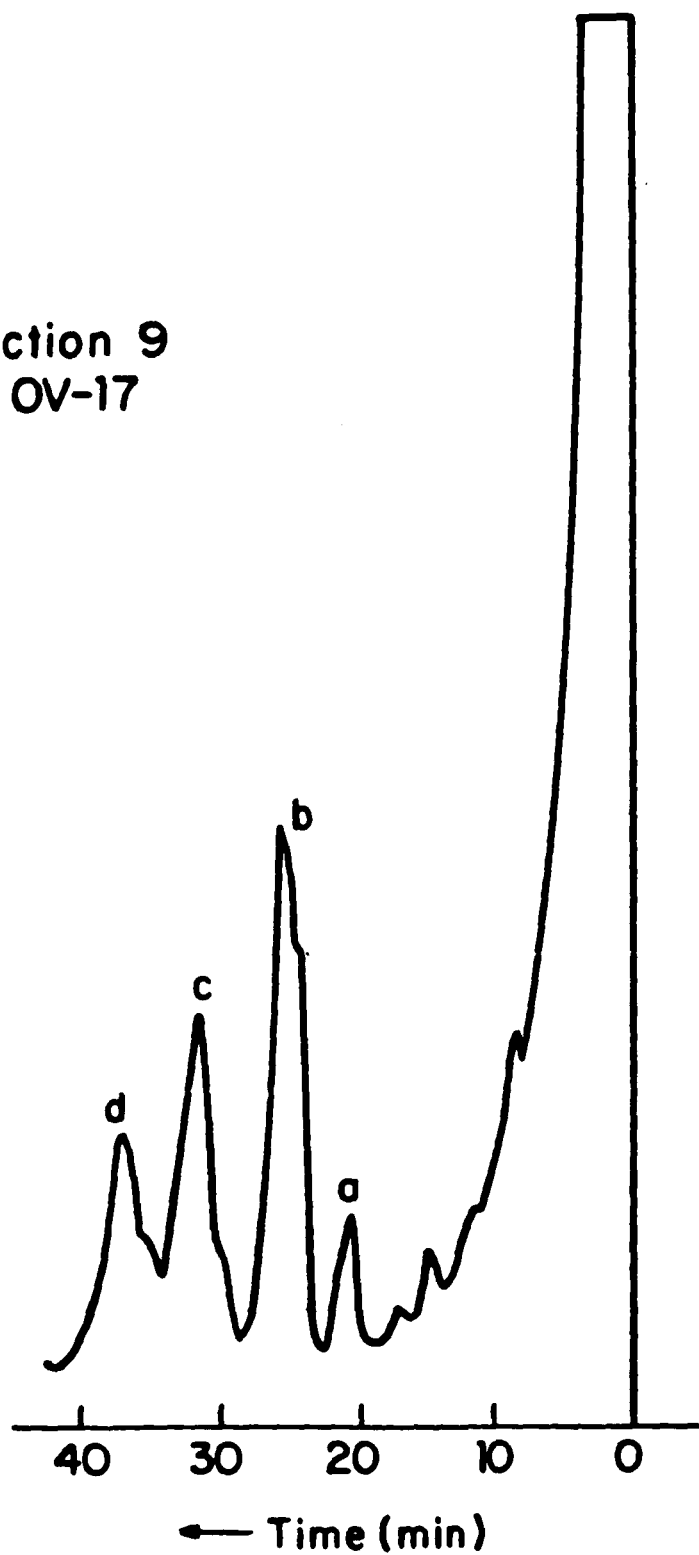
Fraction 7
on OV-17



Fraction 8
on OV-17



Fraction 9
on OV-17

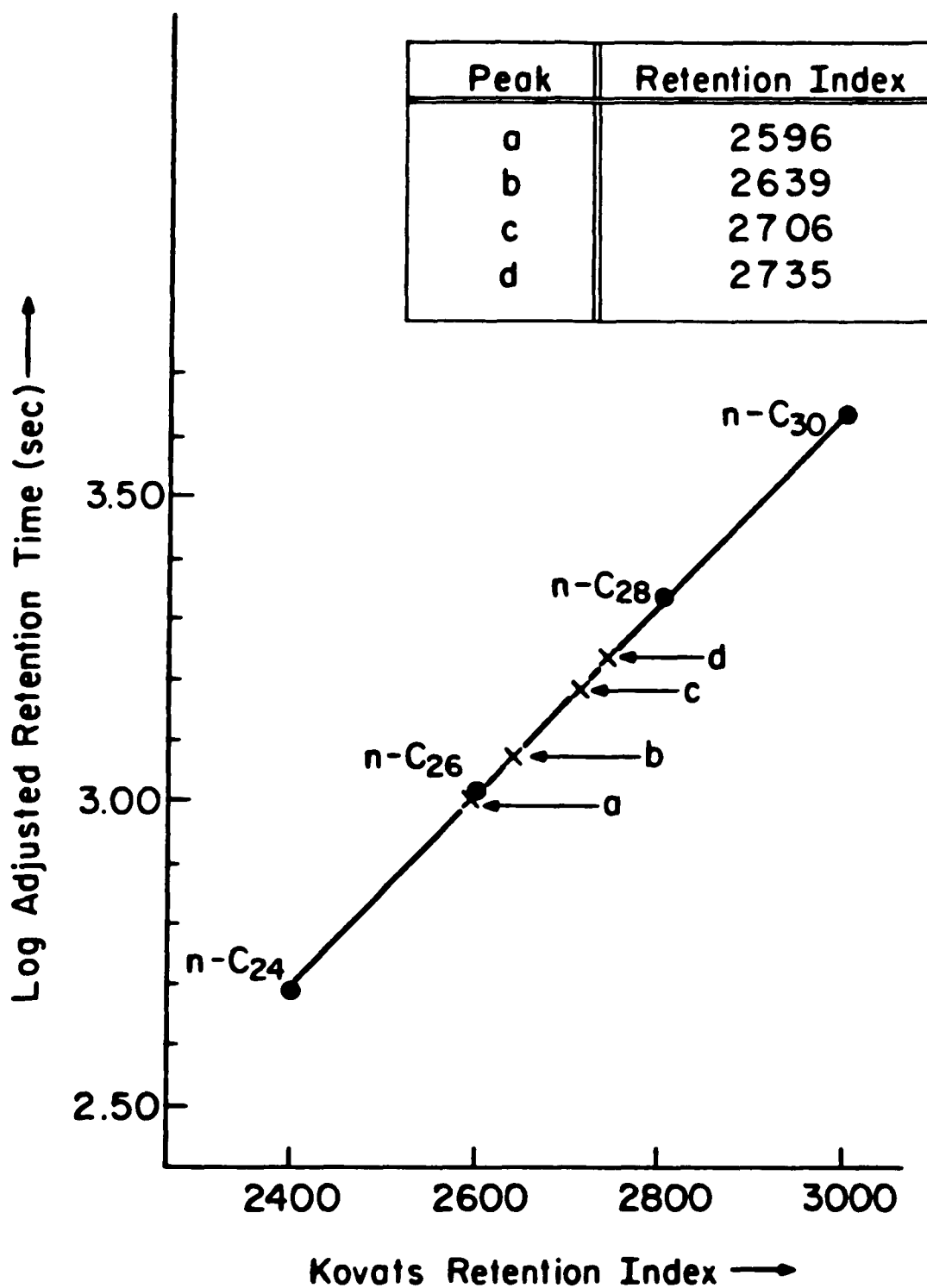


distributed among fractions 7, 8 and 9, together with significant amounts of peaks a and b. The presence of these two earlier peaks, a and b, indicates that trailing of these peaks occurred. This may have been caused by poor injection technique or by the partial adsorption of these high-boiling components in some part of the outlet system. These three fractions were then pooled together in order to produce a sample for further analyses.

To aid in the identification of these compounds, a calculation was made of their Kovats retention indices by comparing their retention behavior with those of a reference mixture of n-hydrocarbons (C_{24} , C_{26} , C_{28} , C_{30}). These results shown in Figure 23 indicated that the retention indices of these compounds ranged from 2596 for peak a up to 2735 for peak d. Although these retention indices are valid only under the conditions used in their determination, they can still be used to estimate the retention times to be expected for these compounds during the chromatographic phase of subsequent GC-MS analyses.

One problem encountered during the capillary GC-MS analyses described in Experiment 13 was the limited quantity of sample which could be applied to the inlet of the capillary column. On the other hand, use of a direct probe inlet for MS sample introduction allows an increased quantity of sample to be used. For this and other reasons subsequent MS analyses of preputial GC fractions were conducted using a direct probe technique. In addition, sample ionization by

Figure 23. Calculation of retention indices for preputial peaks a-d on a 1.5 m x 2 mm I.D. glass column packed with 3% OV-17 on Gas Chrom-Q (100-120-mesh). Column temperature, 200°C; flow rate of He carrier, 27.5 cc/min.



electron impact as used in Experiment 13 was abandoned in favor of a chemical ionization-mass spectrometric (CI-MS) method. The latter technique, by reducing sample fragmentation, can simplify the task of spectral interpretation. With this technique, however, one is unable to obtain any detailed structural information. In CI-MS, if methane is used as a reagent gas, the m/e value of the molecule is increased by one m/e unit by means of proton transfer from the reagent gas. In most cases, this $(M + 1)^+$ ion is also the most abundant ion in the spectrum.

At this time, a decision was made to confine any further analyses to just two of the GC fractions prepared from mouse preputial gland. The samples chosen for further analysis by CI-MS were fractions 4 and 5 which contained relatively large amounts of the compounds of interest. These fractions were examined by use of CI-MS for the presence of the esters which had been identified in a urinary GC fraction as described in Experiment 13.

It should be noted that the molecular weights of the urinary esters of Experiment 13 were 364 and 368 daltons. If they are also present in preputial fractions 4 and 5 then one should observe their respective $(M + 1)^+$ ions at m/e values of 365 and 368. In the CI-MS spectra of both fractions, major ions were found at m/e 173, 195, 220, 365 and 381. In addition, a small peak was found at m/e 369 in the mass spectrum of fraction 5. In either case, the ion at m/e 365 was also the most abundant. The existence of

the ion at m/e 365 is consistent with the possible presence of one of the esters from Experiment 13. Of interest is the relatively abundance of the ion at m/e 369 which would correspond to the second ester.

If any of the compounds in these preputial GC fractions correspond to esters than they should be subject to the action of reagents which can cleave the ester linkage. A technique using one such reagent has been developed by A.K. Bose (personal communication) for the in situ cleavage of esters within the ionization chamber during CI-MS studies. This technique simply consists of the placement of a small crystal of NaOH on the tip of the sample probe prior to its introduction into the ion chamber. The addition of NaOH can result in the cleavage of esters, lactones and amides.

Such a combined CI-MS-NaOH degradation study was performed on fraction 4. As expected, the abundance of the major ion at m/e 365 decreased to half its original value with a corresponding increase in the abundance in the possible fragment ions at m/e 173 and 194. This could correspond to the cleavage of the ester into its constituent acid and alcohol. However, this interpretation must be confirmed through the use of capillary GC-MS or another suitable technique. Of interest, was the appearance of a new ion at m/e 409 during the treatment of fraction 4 with NaOH. The ion could have been produced by the addition of water to the ion present in the original CI-MS spectromat m/e 381. This interpretation is consistent with the notion

of an intramolecular ester or lactone being present at m/e 381 so that its hydrolysis results in a new ion at an increased m/e value. The possible presence of esters and lactones in these fractions is a question that deserves more attention in the future. It should be noted that no one to date has reported the presence of lactones in the preputial glands of mice.

These findings indicate that at least one of the esters present in a behaviorally-active urinary GC fraction may also be present in the corresponding GC fraction from preputial glands.

Experiment 16: Aggression-Promoting Properties of a Wax Ester

The present experiment was designed to investigate the aggression-promoting activity of a synthetic wax ester. This ester, tetradecyl decanoate was shown in Experiment 13 to be a major constituent of a behaviorally-active GC fraction prepared from male mouse urine. At the same time, a comparison of this urinary GC fraction with a corresponding preputial sample, performed in Experiment 14, had found the two fractions to be quite similar on the basis of their identical TLC and GC analyses. In Experiment 15, the results of a CI-MS study of the same preputial fraction indicated the presence of wax esters similar to those found in the behaviorally-active urinary fraction.

In order to determine if the preputial fraction also contains aggression-promoting cues, a preputial GC fraction

equivalent to GC fraction 5 of Experiment 15 was prepared and tested for behavioral activity. At the same time, a third test substance was prepared from a saponified preputial sample as described in Materials and Methods. Since esters and lactones are hydrolyzed by this saponification procedure, one could compare the aggression-promoting activity of this sample with that of the untreated preputial fraction. If an ester or lactone is behaviorally active then one would expect that saponification would eliminate the aggression-promoting activity of a preputial sample. In order to facilitate a comparison of the activity of the three test substances with intact urine a fourth group of animals was exposed to castrates coated with intact male mouse urine.

Tetradecyl decanoate was synthesized from the fatty alcohol and acyl chloride as described in Materials and Methods. The structure of the synthetic ester was confirmed by infra-red and mass spectrometry. The purity of the ester was at least 99.8% as determined by gas chromatography on a OV-17 column. Prior to testing, the final concentration of the ester and peak of the untreated preputial fraction 5 were adjusted to a level equivalent to that of intact urinary fraction on the basis of the detector response. Analysis of the behavioral assay data was performed by the Tukey method which was used at a level of significance of .05.

The results of behavioral testing are shown in Table 14. Within-group comparisons of the synthetic ester, tetradecyl

Table 14
Behavioral Testing of GC Fractions

	<u>Saponified</u> <u>Preputial</u>		<u>Preputial</u>		<u>Voided</u> <u>Urine</u>		<u>Tetradecyl-</u> <u>decanoate</u>	
	$\bar{X}$	SD	$\bar{X}$	SD	$\bar{X}$	SD	$\bar{X}$	SD
Attacks								
Frequency ¹	0.8	2.2	13.8 ^a	2.8	14.7 ^a	2.3	2.8 ^a	2.1
Duration*	0.3	2.1	22.0 ^a	4.2	21.8 ^a	3.0	6.9 ^a	3.8
Latency*	8.3	31.5	-29.1 ^a	4.0	-28.7 ^a	2.4	-15.9 ^a	8.5
+ Uro-genital sniffs								
Frequency ²	0.1	2.4	14.7 ^a	3.2	15.3 ^a	2.8	2.9 ^a	2.2
Duration*	-0.1	2.6	22.3 ^a	4.4	23.9 ^a	4.2	7.3 ^a	3.9
Latency*	9.4	22.0	-24.4 ^a	4.3	-30.8 ^a	1.9	-16.7 ^a	6.1

⁺difference scores.

*measured in seconds.

^ap<.05, vs. H₂O.

¹Each frequency unit represents one attack.

²Each frequency unit represents one sniff.

decanoate, showed it to be significantly ($p < .05$) more aggression-promoting than water on the basis of significant differences for all six observed measures of agonistic behavior. In addition, both untreated preputial and urinary fractions elicited significantly more attack and sniffing behavior in trained fighters than did water as shown by the significant differences obtained for the frequency, duration and latency of each behavior. On the other hand, no significant differences were found between the saponified test substance from mouse preputial and water.

Between-group comparisons were performed on the difference scores and were found to indicate that the preputial GC fraction was behaviorally equivalent to the urinary GC fraction. This suggests that in the male mouse, the preputial gland is a major source of the chemosignals associated with the urinary GC fraction in question. However, this does not mean that murine aggression-promoting urinary cues are only associated with the male preputial gland. The results of Experiments 2 and 3 suggest that additional nonpreputial aggression-promoting cues are found in male mouse urinary extracts. The identification of these nonpreputial cues could be attempted by applying the methodology developed in Experiment 12 on a sample of male bladder urine.

In addition, both the urinary and untreated preputial test substances were found to be significantly more aggression-promoting than the ester sample with respect to the

frequency and duration of each observed behavior. In contrast, a comparison of latency scores between the three groups produced only one significant difference. That occurred in the comparison of sniff behavior between the ester and the urinary fraction.

These three fractions were each also found to be more aggression-promoting than the saponified preputial sample as shown by significant differences ($p < .05$) in all six measures of agonistic behavior. Of interest, is the activity of the ester fraction which was less than the untreated fractions but more than the saponified fractions.

These results clearly demonstrate that a wax ester, tetradecyl decanoate, can function as an aggression-promoting chemosignal for male mice under the conditions of the present behavioral paradigm. In addition, the presence of additional chemosignals in GC fraction 5 was indicated by the increased behavioral activity of the two untreated test substances as compared to the ester test substance. These unidentified cues may represent either esters or lactones since the saponification procedure which eliminates behavioral activity also degrades these two types of compounds.

It should be noted that the wax esters of the male mouse preputial exhibit a pattern of androgen-dependence (Sansone, 1968). This pattern matches that found for the aggression-promoting cues (Lee, 1976). The level of preputial wax esters was found to be very low in juvenile and castrate males. In addition, postpubertal levels of wax

esters were found to increase dramatically as compared to prepubertal levels. Moreover, the normally low levels of wax esters in the preputials of female mice can be increased by the administration of androgens. All of these findings resemble those of the androgen-dependence of the aggression-promoting cues. This further supports the designation of a wax ester as a chemosignal.

One surprising finding of this experiment was the significant decrease in latency scores produced by these cues. Since these cues operate at a distance, it was expected that a smaller molecule would be involved. However, it may be possible that this long-chain ester was hydrolyzed on the skin surface of the castrate to form a smaller alcohol and acid. This may have occurred from bacterial action (Albone et al., 1977). In order to distinguish between these two alternatives, one could determine how many stimulus molecules are needed to produce a behavioral response and compare the behavioral activity of the ester and its hydrolysis products over a range of concentrations.

CONCLUSION

The studies performed in this work confirm and extend the results of previous studies on the aggression-promoting cues of mice. The results of behavioral studies performed in Experiment 1 confirmed previous reports of the androgen-dependency of the aggression-promoting cues of male mice. At the same time, this study served as a validation of the behavioral methodology employed in subsequent studies.

In Experiment 3, a lipophilic aggression-promoting cue was detected in extracts prepared from bladder urine of male mice. At the same time, a series of experiments performed on the preputial gland of male mice have established that at least two aggression-promoting cues are present in this sebaceous gland. Thus, it appears that there are at least three agonistic urinary cues arising from at least two separate tissues. One from the bladder urine, appears to be present in two forms, free and conjugated. The other two cues which were found in GC fractions, prepared from mouse preputial glands, appear to be relatively nonvolatile.

There are several questions which remain to be answered with regards to these bladder and preputial chemosignals. While evidence exists to indicate that one of the preputial cues is a wax ester, relatively little is known regarding the chemical nature of the bladder chemosignal.

The existence of this bladder cue raises some interesting questions regarding the findings of Experiment 12. In that

experiment, a behaviorally active GC fraction was prepared from voided male mouse urine. The compounds present in this fraction appeared to have a preputial origin on the basis of subsequent chemical studies. However, voided male urine should also contain bladder urinary cues, so that the activity of the fraction may have been due to a bladder cue. If not, then where do the bladder cues appear in the chromatograms presented in Experiment 12? Are they among the unidentified compounds present in the behaviorally active region of the chromatogram?

One solution to this problem involves the study of a sample of male bladder urine by the methodology developed in Experiment 12. If the bladder cue(s) is stable at the temperatures encountered in the GC purification, then it should be possible to recover sufficient material for behavioral testing. Eventually, this approach should lead to the identification of these aggression-promoting cues.

Still other questions are raised with respect to the head-space distillation performed in Experiment 6. Since the wax esters of the preputial gland are relatively nonvolatile one would expect them to appear in the residue fraction which later was found to be inactive. It is possible that the nonvolatile preputial cues were transported as an aerosol during the head-space experiment. In addition, the use of an acidified urine sample raises the possibility that these wax esters were subject to hydrolysis. At the same time, one must not forget the bladder chemosignals which

may have been present in the active distillate fractions. Finally, a combination of these factors may have been responsible for the outcome of the head-space experiment.

The demonstration of aggression-promoting activity for a synthetic wax ester represents an important step forward in the field of mammalian chemocommunication. Firstly, it indicates that further effort should be directed towards the chemical identification of these cues. In particular, the results of Experiment 16 suggest that one or more additional cues remain to be identified in the GC fractions eliciting behavioral changes. These may include other C₂₄ esters with either two or no double bonds based on the observed retention indices of Experiment 14 and the results of mass spectrometry performed in Experiment 13. In addition, the results of Experiment 15 suggest that one or more lactones may also be present in these fractions.

It should be noted that a series of saturated and unsaturated wax esters have also been shown to act as chemosignals in the marmoset monkey (Epple et al., 1979). The fact that these relatively non-volatile lipids can function as chemosignals raises several questions which should be considered in other chemocommunication studies in mammals. First, should we continue to use the term olfactory cue for chemosignals which appear to be mediated by vomeronasal perception? Secondly, should we continue to emphasize techniques for the isolation of cues which are primarily volatile. Techniques such as head-space distillation which

might collect sufficient volatile material for behavioral studies would not be useful for less volatile material such as that of the present investigation.

Further work is needed with respect to the quantitation of these murine chemosignals. In addition, future studies should be directed toward establishing the minimum number of chemosignal molecules required for behavioral activity. It should be noted, however, that these studies can be performed only after the identity and relative amounts of the remaining cues are known. Of interest, is the nature of the glucuronide "prochemosignal" of Experiment 4. Although it was thought that this cue may be a steroid, there is no firm evidence to indicate that this is so. In fact, the whole question of steroid aggression-promoting cues in mice remains unanswered.

It is hoped that these studies will provide a new direction for future studies of murine chemocommunication.

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