Identification of a Ventral Scent Marking Pheromone in the Male Mongolian Gerbil (Meriones unguiculatus)

Abstract. *Sebum from the ventral scent marking gland of the male Mongolian gerbil was fractionated and tested for its ability to elicit behavioral response in a conditioning task and in a stimulus preference situation. The active fraction was identified as phenylacetic acid; both it and a synthetic sample elicited the same behavioral response. Phenylacetic acid appears to be a major pheromone of the male Mongolian gerbil.*

Olfactory communication is a significant aspect of social organization in many mammalian species (*1*), and progress is being made in chemically identifying pheromones in several species (*2*). In only a few cases, however, has the isolation and identification of a mammalian pheromone led to the study of a behavioral response.

Both male and female Mongolian gerbils deposit a pheromone in their environment by rubbing an abdominal sebaceous scent gland on objects and each other. This marking response is regulated by homotypical gonadal hormones which, in the case of the male and probably the female, act on preoptic brain cells (*3*). Marking behavior in the male has been associated with exploration, social dominance, and territoriality; in the female it has been linked to exploration and maternal responsibilities (*4*).

The volatile components of the ventral gland sebum were obtained as follows. Charcoal-filtered air (50 ml/min) was passed over heated filter paper strips containing ventral gland sebum from 20 males for 18 to 24 hours. Volatile substances were swept into a condenser surrounded by Dry Ice and then rinsed from the condenser with 5 ml of ether. When the ether was evaporated the sample had the characteristic sebum odor.

Whole sebum from 90 to 100 males was collected onto filter paper and the paper was extracted with 10 ml of diethyl ether. The odor persisted for approximately 40 minutes after the solvents were evaporated. The extract retained its odor with freezing and immediately lost it with heating. The volatile material was fractionated by thin-layer chromatography (TLC). A 600-μl portion of the sample was placed on silica gel G plates (20 by 20 cm) and developed 10 cm with a benzene-acetone (80 : 20) solvent system. Discrete regions were eluted with 1 ml of diethyl ether. The ether was evaporated, and the residue was tested for odor. An odor comparable to the whole sebum odor was identified at an average R_F of 0.35. When the plates were sprayed with sulfuric acid and heated, ten bands appeared; however, none of these bands contained the odorous substance. The area containing the odor did not fluoresce under ultraviolet light and did not stain wth a wide range of indicators. Figure 1 illustrates the bands used in the behavioral tests and those which appeared with sulfuric acid sprays.

Volatiles trapped in the Dry Ice–cooled condensor (cold-trapped) were also separated by TLC. The odorous component appeared at an R_F of 0.35. When the critical fraction was neutralized by 0.1*N* NaOH or 5 percent $NaHCO_3$, the odor disappeared. If this solution was then treated with 0.1*N* H_2SO_4, the odor returned.

Gas chromatography (GC) of the odorous component esterified with diazomethane (column 183 by 0.64 cm; 3 percent Dexsil 300 on GC-Q; 138°C) yielded a methyl ester of molecular weight 150. The mass spectrum of this ester shows fragment ions at *m/e* 119 (mass 31), 91 (mass 59), 65, 51, and 39. The most likely structure for this ester was that of methyl phenylacetate (*5*). Comparison of the mass spectrum of synthetic methyl phenylacetate and the unknown indicate that the two compounds are identical. Identical behavior of the unknown and methyl phenylacetate in GC and TLC further confirms the identity of the ester.

Animals were trained to refrain from (suppression) bar pressing for food pellets in the presence of whole sebum odors; then they were tested for generalization of suppression to (i) cold-trapped volatiles, (ii) various fractions obtained by elution of the TLC plate, (iii) a sebum sample treated with NaOH, and (iv) phenylacetic acid.

The ventral scent glands were surgically excised from ten adult male gerbils so that gland odors would not interfere with conditioning or generalization testing. After recovery, the animals were deprived of food until their body weights stabilized at 80 to 85 percent of normal, and then training was begun. The training apparatus consisted of Skinner boxes (25 by 20 by 20 cm). Each box was ventilated through a chimney at the top and out an evacuation tunnel at the bottom. The floors of the boxes were metal bar grids through which a 1.1-ma shock could be delivered to the animals' feet.

The animals were trained to bar press until they stabilized at a fixed ratio of six presses (FR 6) to obtain a food pellet (Noyes, 45 mg); then differential conditioning was started. This consisted of introducing sebum odors (S) or control odors (C) into the air stream of the apparatus. Sebum was obtained from the ventral glands

Table 1. Bar pressing and latency to initial bar press with sebum fractions and phenylacetic acid.

Stimulus	*N*	Mean number of bar presses per minute			*P* values		Mean latency to initial bar press (sec)		*P* values
		Base level (A)	Stimulus (B)	Control (C)	A vs. B	B vs. C	Stimulus (A)	Control (B)	A vs. B
Cold trap vs. control	9	39.2 ± 2.96	22.2 ± 4.62	35.4 ± 3.79	<.01	<.02	58.1 ± 9.86	26.5 ± 8.77	<.05
Critical vs. control	9	41.1 ± 1.72	18.0 ± 4.82	35.0 ± 4.11	<.01	<.01	96.3 ± 23.69	34.4 ± 17.29	.01
Critical vs. remainder	9	39.7 ± 4.99	7.7 ± 3.1	28.3 ± 5.88	<.1	<.01	129.3 ± 18.31	42.6 ± 20.02	<.01
Whole vs. remainder	9	42.4 ± 4.85	19.1 ± 6.55	27.1 ± 4.92	<.01	.42	95.8 ± 24.72	39.2 ± 13.73	<.04
Whole vs. control	10	33.7 ± 2.86	19.5 ± 4.15	32.8 ± 4.05	<.02	<.03	70.0 ± 13.05	7.8 ± 2.15	<.01
NaOH fraction vs. control	10	33.6 ± 2.62	31.4 ± 1.24	32.7 ± 4.75	.71	.85	13.3 ± 7.33	12.3 ± 5.57	.90
Phenylacetic acid vs. control	8	45.4 ± 3.35	11.8 ± 4.43	42.7 ± 3.69	<.01	<.01	99.2 ± 21.01	9.1 ± 6.09	<.01

of 40 to 100 adult males and was shaken with ether and silica gel scrapings from an unused plate. Ether and silica gel served as a control. Immediately prior to a training session, approximately 60 μl of either S or C were evaporated on a watch glass which was then introduced into the air stream. One of the two stimuli was presented after 2 minutes and the other after 7 minutes of training. Both stimuli remained in the box for 3 minutes. The order of presentation of S and C was randomized on each day. If the animal pressed the bar during presentation of S, a shock was delivered.

When the discrimination between S and C had stabilized, generalization testing began. No shock was administered during the testing trial. Baseline presses, number of bar presses during each condition, and latency to the first bar press after introduction of a stimulus were recorded for each test.

Cold-trapped volatiles were evaporated on a watch glass; an evaporated sample of ether served as a control. These were tested as S and C, respectively. Sebum fractions from the TLC plate were tested in the same way. "Whole" and "critical" areas were each tested against "remainder" or "control" in a random order (Fig. 1). Whole sebum in ether in which the fatty acids had been neutralized by a 0.1*N* solution of sodium hydroxide was also tested. Finally, phenylacetic acid in ether (1 mg in 500 ml) was tested, with ether as a control.

Analyses of variance statistics, selected *t*-tests, and comparison among group means were carried out. Relative to base line and control levels of pressing, volatiles, any TLC fraction containing material from area B (Fig. 1), and phenylacetic acid decreased bar

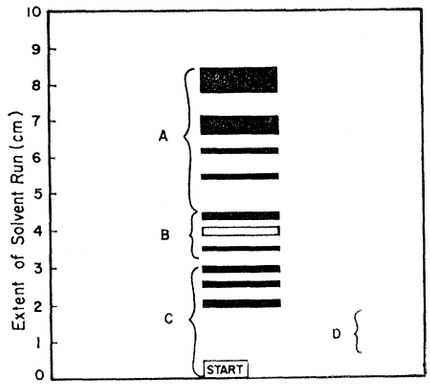

Fig. 1. Illustration of TLC separation of male gerbil sebum. Scrapings tested for behavioral reactivity were designated "whole" (fractions A + B + C), "critical" (fraction B), "remainder" (fractions A + C), and "control" (fraction D).

pressing frequency and increased the latency to the first bar press (Table 1). The sebum in which the acids were neutralized was not effective.

We devised a second test to assess exploratory interest in whole sebum, cold-trapped volatiles, acid and nonacid fractions of the sebum, and phenylacetic acid. The testing apparatus consisted of a rectangular chamber (97 by 30 by 30 cm) painted black with a clear Plexiglas insert running the length of the chamber so that an animal could be viewed at all times. Control or test stimuli were delivered into one end of the apparatus through a funnel and baffle system and drawn out the opposite end of the chamber by a small fan (*6*).

Test stimuli were cotton swabs saturated with sebum from two or four colony males, cold-trapped volatiles, a sebum sample with or without the fatty acids present (neutralized with 0.1*N* solution of sodium hydroxide), and samples of phenylacetic acid (2 mg/ml). When ether was used as a solvent and evaporated prior to testing, ether was used as a control. Otherwise, fresh air was the control.

Adult males were introduced singly into the test chamber and observed for 20 minutes under red light. During the first and third 5-minute periods the control was directed into the chamber, during the second and fourth 5-minute periods the stimulus was introduced. A response was recorded every time the animal approached the stimulus entry point, reached up, and sniffed. The scores were averaged for the two control periods and for the two stimulus periods and a *t*-test for correlated means was computed.

Investigative responses were significantly more frequent when the chamber was permeated with whole sebum from two or four males, cold-trapped volatiles, the fatty acid fraction, and phenylacetic acid. Neutralizing the acids eliminated the effect (see Table 2).

Our behavioral and biochemical analyses strongly suggest that phenylacetic acid is a major component of the marking pheromone in the male Mongolian gerbil (*7*). It may not be the only pheromone in the sebum. Glickman has indicated that individual gerbils can identify each other on the basis of sebum odors (*8*). Although we find little evidence for multiple pheromones in the sebum, other volatiles are present and could be used as signals under certain circumstances or in particular combinations.

Table 2. Direct interest displayed toward full sebum (swabs from ventral glands), cold-trapped volatiles, acidic and nonacidic extracts, and phenylacetic acid. Direct interest is shown by the mean number of responses during a 10-minute period.

Test stimulus	*N*	Mean number of responses		*t*
		Control	Stimulus	
Two swab 1	10	3.5 ± 0.75	9.0 ± 1.34	5.04†
Two swab 2	10	2.1 ± 0.72	3.6 ± 0.61	2.77*
Four swab	8	2.9 ± 0.69	7.8 ± 0.62	4.97†
Cold trap 1	10	1.5 ± 0.36	4.3 ± 0.83	3.37†
Cold trap 2	10	1.2 ± 0.41	4.5 ± 0.88	3.58†
Acid extract	10	0.6 ± 0.26	2.7 ± 0.47	4.88†
Nonacid extract	10	1.6 ± 0.45	1.0 ± 0.14	1.33
Phenylacetic acid 1	10	0.8 ± 0.29	3.8 ± 0.61	6.66†
Phenylacetic acid 2	10	0.4 ± 0.22	3.7 ± 0.54	4.78†
Phenylacetic acid 3	10	0.5 ± 0.16	3.6 ± 0.36	9.68†

* $P < .05$. † $P < .01$.

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6. The chamber was mounted on two Animex activity meters so that locomotion could be recorded on the sides toward or away from the odor source. The floor of the chamber was studded with 18 pegs (1.3 by 0.6 cm hex nuts) toward which animals direct their ventral marking.
7. Preliminary work suggests that the ventral gland secretion of females is little different from that of males. The TLC pattern appears identical, and the odorous component is found at the same R_F region. Both the female and male ventral gland responds to a wide range of gonadal hormones, including estrogen, progesterone, and testosterone.
8. S. Glickman, personal communication.
9. We wish to express appreciation to R. V. Coleman, R. Cocke, T. Mabry, and G. Vander Velde for valuable assistance during these studies. This research was supported by grants MH 14076-06 and NSF-USDP grant GU-1598 awarded to D.D.T. and NSF grant GB-30179X awarded to F.E.R.

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Prolactin Receptors in an Estrogen Receptor–Deficient Mammary Carcinoma

Abstract: *We demonstrate for the first time the presence of specific high-affinity receptors for prolactin in rat mammary carcinoma. There appear to be no significant differences between normal rat mammary tissue and the transplantable, estrogen receptor–deficient R3230AC tumor with regard to the number of binding sites, the affinity of the receptor for prolactin, or the specificity of binding.*

Although our understanding of hormone dependence in breast cancer is incomplete, prolactin and estrogen appear to be the principal hormones involved in breast tumor growth and regression (*1*). Recent efforts have focused on the presence of a cytoplasmic estradiol-binding protein (receptor), which correlates well with breast tumor regression following endocrine therapy (*2*). Nevertheless, it has been argued that prolactin might be the predominant hormone involved in mammary tumor growth and regression, and hence that the observations regarding estrogen receptors could be of secondary importance (*3*). Since techniques are now available for detecting specific membrane receptors for prolactin (*4*), we can now determine directly whether these prolactin receptors are present in mammary tumors and whether their presence is related to hormone dependence. Here we show that a rat mammary tumor which does not depend on either estrogen or prolactin for growth, and which has very little estrogen receptor, does possess prolactin receptors similar to those of the normal mammary gland.

Hilf *et al.* (*5*) originally described the transplantable rat mammary carcinoma R3230AC, which did not depend upon estrogen or prolactin for growth and yet would respond to these hormones with distinct alterations in certain enzymatic activities. Studies of this tumor revealed a marked deficiency in estrogen receptor content compared to hormone-dependent tumors or to normal estrogen target tissues (*6*) and suggested a possible causal relationship between the relative lack of estrogen receptor and the failure of the tumor to regress following ovariectomy (*7*). It thus became of interest to consider whether modified or deficient prolactin receptors might also be involved.

[^{125}I]Ovine prolactin was prepared by a soluble lactoperoxidase method (*8*). The iodinated prolactin was purified by diethylaminoethyl (DEAE) cellulose chromatography (*9*). Binding activity of this prolactin was checked independently with rabbit mammary gland particles according to Shiu *et al.* (*4*).

Tissue slices (1 mm thick, approximately 10 mg wet weight) were prepared from R3230AC rat mammary tumor grown in intact Fisher rats, or from freshly excised lactating rat mammary gland 18 hours after removal of pups. Five slices were placed in 1 ml of incubation medium [Medium 199 with 0.1 percent (wt/vol) bovine serum albumin, 5 m*M* $CaCl_2$, 5 m*M* Hepes buffer, *p*H 7.6] containing [^{125}I]-ovine prolactin (200,000 counts per minute at 55 to 77 μc/μg) and increasing quantities of nonradioactive prolactin (0 to 5000 ng). Experiments showed that both specific and nonspecific prolactin binding achieved a steady state within 2 hours of incubation when the slices were gently agitated at 22°C; a 3-hour incubation was routinely employed. The slices were then removed and washed four times with ice-cold Medium 199 containing 0.1 percent bovine serum albumin. Samples were assayed for radioactivity in a Nuclear-Chicago gamma counter, and the wet weight of each tissue slice was determined.

Figure 1 (left panel) is a representative Scatchard plot (*10*) of the binding data obtained by incubating normal rat mammary gland slices with increasing quantities of prolactin. The curved line represents the total prolactin binding. At low concentrations of prolactin, sites of high affinity and low capacity are revealed, whereas at progressively higher prolactin concentrations, binding of low affinity and high capacity predominates. We interpret the latter as nonspecific binding. Subtracting this

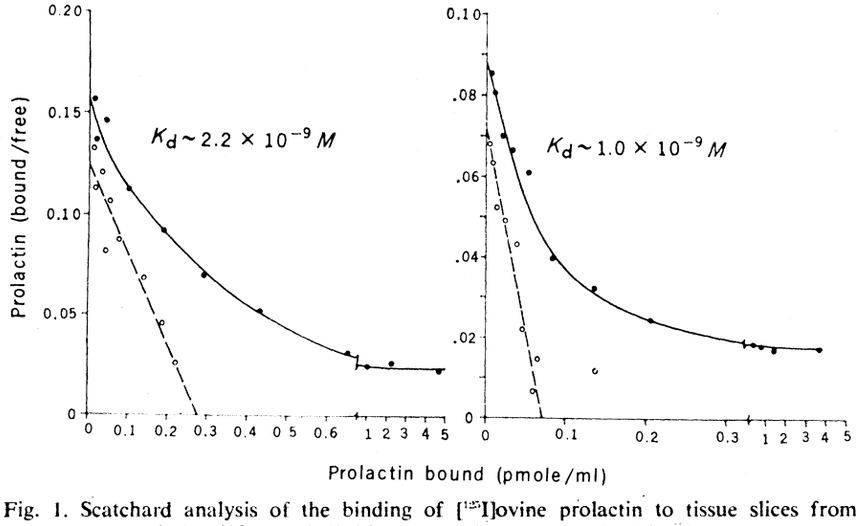

Fig. 1. Scatchard analysis of the binding of [^{125}I]ovine prolactin to tissue slices from rat mammary gland (left panel; Table 1, experiment 4) and R3230AC rat mammary carcinoma (right panel; Table 1, experiment 1). The high-affinity binding (dashed line) is derived from the total binding (solid line) as described in the text.