Volatile compounds associated with estrus in mouse urine: potential pheromones

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Summary. Female mice that had been made estrous through hormone implantation excreted in their urine significantly enhanced levels of n-pentyl acetate, *cis*-2-penten-1-yl acetate, p-toluidine, 2-heptanone, and 3 unsaturated ketones. The relationship of these volatiles to a signaling function of the estrous urine is postulated. Structural elucidations of these compounds were carried out through capillary gas chromatography/mass spectrometry and the synthesis of authentic samples.

Several signaling phenomena have been observed to be associated with female mouse urine, including a sex attractant[2], an aggression-reducing signal[3,4], and an ultrasound-eliciting signal[5]. The attractant signal appears to be related to the estrous state of the female, while the other two are not. Whitten[6] observed that male mice are more interested in sniffing tubes containing urine from estrous females than from diestrous females. Female mouse urine may also be the source of a primer pheromone responsible for the Lee-Boot effect[7] that reflects the shortened periods of estrus and prolonged periods of diestrus in isolated females. Although the source and nature of this primer pheromone have not been deduced, once again, urine is suspected. Maruniak et al.[8] also observed that the females' marking behavior increases in the presence of males.

Materials and methods. General observations in various other mammals support the notion that females excrete substances cuing males to their endocrine status. The possibility that some estrus-related activities in *Mus musculus* may be caused by volatile substances present in the female urine led us to design the following experiment. Urine samples were collected from 5 immature female mice of 3 strains: BALB/C, ICR, and C57BL. The collections were performed over 24-h periods to avoid diurnal fluctuations; urines were immediately frozen in dry ice to preserve the samples for later analyses. At 9 weeks of age, the mice were implanted with 4-mm silastic tubing pellets packed with estradiol, according to the technique described by Maruniak et al.[9]. After allowing 1 week for recovery, urine was collected from these mice as described above. Estrous state was verified by obtaining vaginal swabs by lavage and inspection of the cells (stained with methylene blue) under magnification. The estrogen implants created a continuous estrous state in all cases, as noted by the preponderance of squamous epithelial cells in the swabs. This induced estrous state was maintained throughout urine collection.

Urine samples were analyzed by capillary gas chromatography, using glass capillary columns coated with a polypropylene glycol stationary phase. Prior to gas chromatography, the urinary volatiles were concentrated on a precolumn of the porous polymer, Tenax GC. The technical aspects of this sampling procedure were previously described[10]. Areas of the resolved chromatographic peaks were computed through integration performed by a commercial data system. Quantitative comparisons of the chromatograms showed consistently marked elevations in 7 urinary constitutents; an example is demonstrated in the figure where the urinary volatile profiles are compared between the immature animals and those with an induced estrus condition.

Results and discussion. The mixture components responding drastically to the estrogen treatment were identified through capillary gas chromatography/mass spectrometry and co-injection of authentic chemicals with the urinary volatiles. The identified compounds are 2-heptanone, n-pentyl acetate, p-toluidine, *trans*-5-hepten-2-one, *trans*-4-hepten-2-one, 3-hepten-2-one, and *cis*-2-penten-1-yl-acetate. Because of their commercial unavailability, the last 4 compounds were synthesized for structural verification. Mass spectral data for these compounds are shown in table 1.

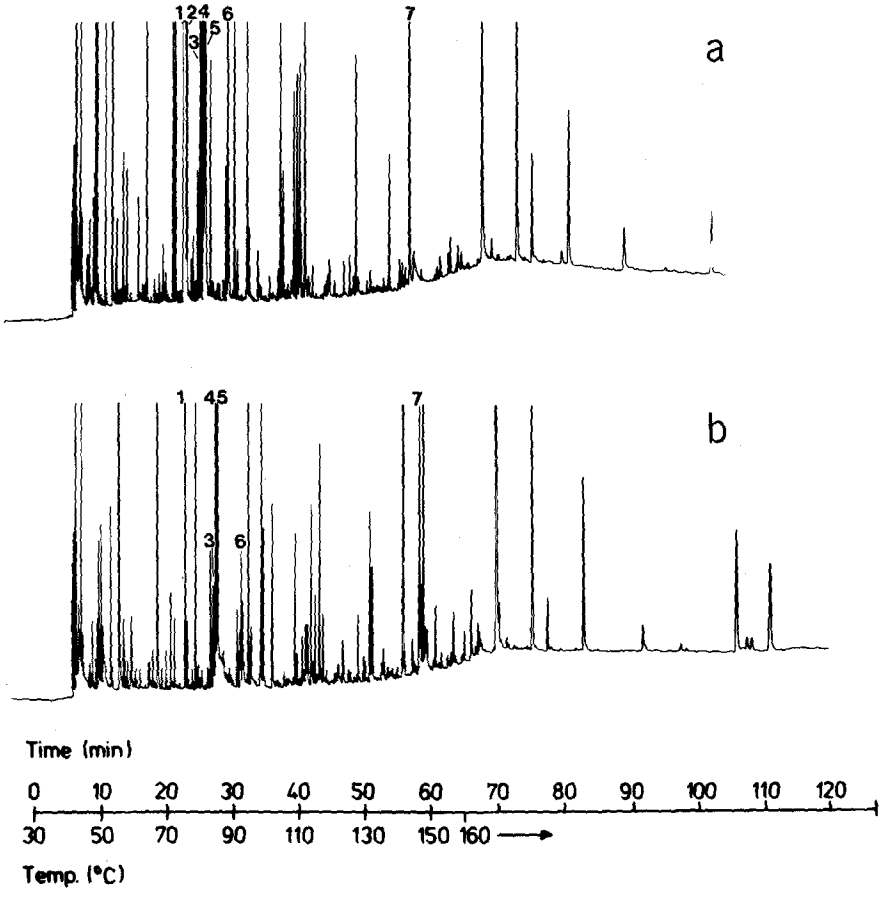
Quantitative results obtained for the females of 3 different strains are shown in table 2. Although sensitivity of different strains to the estrogen treatment varies, the magnitude of changes is universally evident. Preliminary efforts[11] of our laboratory to compare female urinary volatiles dependent on endocrine status involved normally cycling females; urine was obtained from these mice by bladder palpitation. A preliminary implication of 2-heptanone as being elevated in the estrous urines has now been corroborated in the estrogen-implanted animals. Interestingly, 2-heptanone and n-pentyl acetate are frequently observed secondary metabolites associated with fruit maturation. Both of these compounds have also demonstrated pheromonal activity in insects; 2-heptanone is an alarm pheromone for ants[12] and honeybees[13], while n-pentyl acetate has been evaluated as a honeybee repellent[14].

N-Pentyl acetate is known to stimulate olfactory receptors in rats[15], and is frequently used as a model stimulus source in olfaction experiments. Long-chain alkyl acetates have been reported in the preputial gland of the mouse[16], and appear to be under testosterone control[17]. Gawienowski and Stacewicz-Saputzakis[18] have reported the occurrence and attractive properties of n-pentyl acetate for the rat[19]. The occurrence of these 2 compounds in the urine of estrous female mice is likely due to estrogen effects on the metabolism of fatty acids in the liver[20]. We speculate that both compounds may be derived from a common precursor: β-ketooctanoic acid. In vitro, pentyl acetate can be made by a Bayer-Villiger oxidation from 2-heptanone, the decarboxylation product of β-ketooctanoic acid. Such elaboration of an initial secondary metabolite present in excess, due to an induced alteration of metabolism, is in agreement with the theory of secondary metabolite formation proposed by Bu'Lock and Powell[21], and is a reasonable explanation for the observed increases of 2-heptanone and n-pentyl acetate.

Three unsaturated ketones were observed to increase upon

Table 1. Mass spectral data

No.	Compound	m/e (relative intensity)
1	2-Heptanone	114 (9), 99 (7), 85 (7), 71 (26), 58 (80), 43 (100)
2	n-Pentyl acetate	73 (13), 70 (31), 61 (25), 55 (24), 43 (100)
3	*cis*-2-Penten-1-yl acetate	128 (3), 113 (3), 99 (6), 86 (30), 85 (13), 68 (46), 67 (45), 57 (23), 53 (20), 43 (100)
4	*trans*-5-Hepten-2-one	112 (9), 97 (8), 94 (12), 69 (13), 43 (100), 41 (29), 39 (15)
5	*trans*-4-Hepten-2-one	112 (4), 97 (6), 94 (8), 69 (9), 43 (100), 41 (26), 39 (18)
6	*cis*-3-Hepten-2-one	112 (13), 97 (37), 69 (16), 55 (100), 53 (10), 43 (54), 39 (24)
7	p-Toluidine	107 (80), 106 (100), 79 (28), 77 (32), 51 (20), 39 (16)



Chromatograms of the urinary volatiles from C57BL. 10 female mice: *a* induced estrus, *b* immature. Numbers refer to list of compounds in table 1.

induction of estrus. These compounds are unusual, because the location of the carbon-carbon double bond does not correspond to any of the naturally occurring unsaturated lipid classes. 4-hepten-2-one was reported as a component of the steam distillate of corn kernels by Buttery et al.[22]. The assignment of geometric configuration was assumed to be *cis* by these workers; retention and mass spectral properties of *cis* and *trans* isomers were assumed identical, and only the *trans* isomer could be synthesized. *Cis* configuration was assumed, because it is the most likely configuration in naturally occurring lipids. We have tentatively assigned the *trans* configuration, because retention times and spectra matched for the authentic sample and the volatile component, and because our polar capillary columns demonstrated the general ability to separate *cis* and *trans* isomers. Confidence in retention time comparisons was further enhanced by co-injection analysis which was performed in all cases.

The occurrence of *trans*-3-hepten-2-one in male mouse urine has been reported by Mayashita and Robinson to be correlated with the age of mice[23]. In estrous females, we are reporting 3-hepten-2-one, but we are uncertain of the geometric assignment. Mass spectral evidence seems to rule out possibilities other than 3-hepten-2-one, but the *trans* isomer does not co-elute. *Cis*-3-hepten-2-one is unstable with respect to isomerization and would not be an expected natural product in the absence of the *trans* isomer. Other researchers have identified 3-hepten-2-one in natural products without assignment of geometric configuration: as a component of roasted filbert volatiles[24], and of the essential oil from deertongue *(Carphephorus odoratissimus)*[25].

It appears that 5-hepten-2-one has never before been reported as a natural product. We are assigning the *trans* configuration on the basis of co-elution with the authentic *trans* isomer. Another acetate, *cis*-2-penten-1-yl acetate, is reported here for the first time as a natural product. Both the *cis* and *trans* isomers of this acetate were synthesized, but only the *cis* compound co-eluted with the volatile component.

Para-toluidine has not been reported as a natural product

Table 2. Urinary volatiles which increase upon induction of estrus

No.	Compound	Integrated peak areas (arbitrary units)					
		C57BL/10		ICR		Balb/C	
		Immature	Estrous	Immature	Estrous	Immature	Estrous
1	2-Heptanone	16.1	216.5	13.6	239.9	5.6	49.9
2	n-Pentyl acetate	0.6	13.1	0.3	15.8	0.2	7.8
3	*cis*-2-Penten-1-yl acetate	5.5	10.9	5.1	15.3	4.7	5.8
4	*trans*-5-Hepten-2-one	6.6	20.4	3.9	10.1	9.9	3.1
5	*trans*-4-Hepten-2-one	26.9	64.7	4.5	45.1	5.4	22.8
6	*cis*-3-Hepten-2-one	2.4	7.3	1.1	3.7	0.7	2.2
7	p-Toluidine	18.9	45.6	1.6	28.6	1.6	4.0

except as a decomposition product of some pesticides[26]. We observed both *ortho* and *para* isomers in mouse urine[27], but only the *para* compound appears to demonstrate a strong correlation to estrogen treatment as performed in this experiment for all strains tested.

While probably not possessing specific pheromonal activity by themselves, the compounds reported here, combined in specific ratios and in the presence of other unique volatiles recently discovered by us in mouse urine, may confer any of the releaser pheromonal effects associated with estrous urine. The physiological and behavioral properties of these compounds are currently being investigated[27].

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Respective roles of circulating T_4 and T_3 in control of TSH secretion in severely iodide-deficient rats[1,2]

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Summary. After a 6-month iodide deficiency, Wistar male rats were submitted to a normal iodine diet (20 and 50 µg of ^{127}I daily). Plasma T_3, T_4 and TSH were determined by RIA from 0 to 140 days of iodide refeeding. A highly significant correlation was found between plasma TSH and T_4 concentrations, but not between plasma TSH and T_3 levels. These data suggest that an increase in plasma T_3 alone, up to the normal value, is not able to inhibit TSH secretion. It is only when a certain plasma T_4 concentration is also reached, resulting in further T_3 formation through deiodination, that TSH secretion is inhibited.

In the thyrotrope cells, thyroid hormones modulate the thyrotropin (TSH) secretion[5-7]. However, the respective roles of circulating thyroxine (T_4) and triiodothyronine (T_3) levels in this feedback mechanism are still controversal. The particular experimental conditions of iodide refeeding in severely iodide-deficient rats allow us to study this process. Indeed, during the first days of iodide refeeding, only T_3 is newly synthesized; T_4 appears later. The present results show that in severely iodide-deficient rats under conditions of iodide refeeding, when a normal level of plasma T_3 is reached it is not able by itself to inhibit TSH secretion. The role of T_3 should not be neglected, but TSH secretion probably starts to decrease only when the T_4 concentration also increases to reach a crucial level, which after T_4 deiodination will provide a further T_3 concentration in the thyrotrope cells.

Materials and methods. Wistar male rats received a low iodide diet during 6 months (LID Remington and distilled water). During the last 2 months of LID, propylthiouracil (PTU) was added. At the end of this treatment, i.e., on day 0, a normal iodide diet was reestablished with 2 doses of iodide in drinking water: 0.9 and 2.27 µg ^{127}I/ml, i.e., approximately 20 or 50 µg ^{127}I/day/rat, and the PTU treatment was stopped. The rats were sacrificed on days 0, 1, 2, 4, 8, 12, 16, 30, 45, 80 and 140 of normal iodide diet. Two control groups received a Remington diet without PTU, and drinking water containing 0.9 or 2.27 µg ^{127}I/ml during 8 months. After sacrifice, blood was collected and centrifuged, and plasma was frozen at −20 °C. Plasma T_3, T_4 and TSH concentrations were determined by radioimmunoassay as previously described[8]. The results were analyzed by Student's t-test.

Results. For the first 4 days of iodide refeeding (50 µg daily) (fig. 1), T_3 and T_4 concentrations remained as low as on day 0. Between days 4 and 8, plasma T_4 started to increase slightly but not significantly, whereas T_3 rapidly reached its control value. From days 8 to 12, plasma T_3 remained at its normal level, whereas T_4 was higher than on