Estrus-Inducing Pheromone of Male Mice: Transport by Movement of Air

Abstract. *The proportion of female SJL/J mice exhibiting estrus when placed 2 meters downwind (6 meters per minute) from a group of 15 hybrid males was not significantly less than that of females placed directly under the males and exposed to their urine. The proportion of mice showing estrus when placed 2 meters upwind was significantly less than that of mice downwind or of mice below males, but not different from that of females remote from males. These findings show that the pheromone from male mice is volatile and further support the concept that it acts through olfactory receptors.*

Pheromones are substances produced by some animals to induce one or more specific responses within members of the same species (*1*). For example, male mice produce a pheromone that both induces and accelerates the attainment of estrus, so that estrus is synchronized when the pheromone acts simultaneously on a group of female mice (*2*). The estrous synchrony of SJL/J female mice has been quantified for detection of the male pheromone in biological fluids (*3*).

It has been clearly demonstrated that the male pheromone is produced to the exterior, because the estrus-synchronization response occurs when male urine is delivered to the females' cage or onto their noses (*3, 4*). The active substance in the urine has not been characterized, and so far an unequivocal response has been obtained only in females having direct access to male urine. Nevertheless the evidence suggests that the pheromone acts through olfactory receptors (*2*), the implication being that the substance is volatile and, like odors, may be transported by movement of air; the effective range, and perhaps also the ecological significance of the pheromone, will depend on this property. Our object was to determine whether the compound is airborne.

A chamber having a controlled flow of air was constructed to test for transfer of the pheromone by air currents. A test group of females was placed 2 m downwind and thus exposed to any volatile substances, while the control group was situated 2 m upwind, protected from the flow of such compounds. Both groups had equal opportunity for visual and vocal communication with the males, and other environmental conditions were uniform except for a negligible difference in humidity; no temperature gradient was demonstrable. Another control group was placed directly below the males and exposed to their urine for determination of maximum response in the test apparatus.

The chamber (Fig. 1) was 30 by 30 cm in cross section and 6 m long; it was of transparent plastic sheeting over a wooden frame. It was made in five 1.2-m modules so as to permit access to the animals during the experiment; these sections were sealed together with masking tape. A fan at one end drew air through the apparatus at between 6 and 11 m/min (0.23 and 0.41 mile/hour) from a large hose at the other end, which in turn opened to fresh air outside the building and was remote from exhaust vents. The experiment coincided with equitable temperatures between July and September 1967.

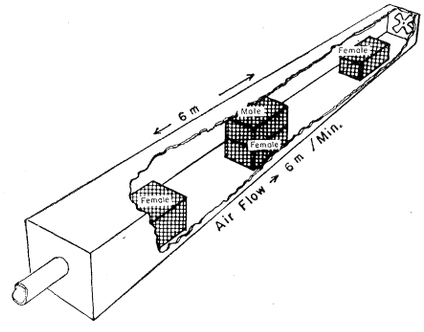

Fig. 1. Diagram of wind tunnel.

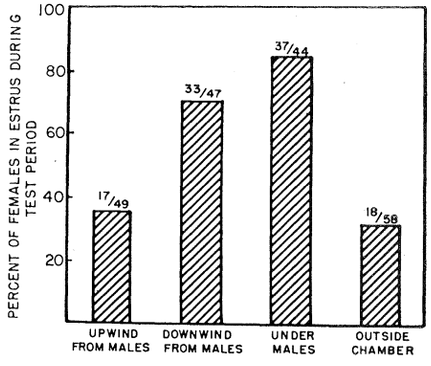

Fig. 2. Histograms of the percentages of females in estrus at three positions in the wind tunnel.

A group of 15 males, hybrids from eight inbred strains, was used as the source of pheromone. Five replicate groups, each of eight to ten SJL/J virgin females, were placed in the three positions; their responses were evaluated by examination of vaginal smears taken 48, 72, and 96 hours after their introduction to the tunnel.

The proportion of animals in the downwind groups that exhibited estrus during the experimental periods was significantly greater ($P < .001$) than of those upwind (Fig. 2). No significant difference regarding estrus was observed between the downwind group and the group maintained under the males. No significant heterogeneity between the data obtained from different replicates was detected. The proportion of females in estrus in the upwind controls was consistent with a mean estrous cycle of just over 7 days, which is common among females of this strain when remote from males (*5*). The downwind group showed synchronization of estrus, characteristic of response to the pheromone (*3*).

From these results we conclude that the pheromone is volatile and that it acts almost certainly through the olfactory receptors. The only previous pertinent reports were that expected patterns of mating were altered if males were housed in the same room (*6*); unpublished data indicated that no vision, hearing, or touch was necessary for elicitation of response by the female to the presence of a male (*2*).

Under natural conditions the effective range of the pheromone will depend on rate of production and diffusion, its chemical stability, the rate of flow of air in the animals' microhabitat, and the mobility of both sexes (*7*). In our experiment the decrease from 84 to 70 percent, while not statistically significant, does suggest some degree of attenuation over the 2-m interval. The ecological significance of this 2-m interval in the normal social environment of house mice, however, can only be conjectured. It is possible that the pheromone may be detected as an odor at a concentration below the threshold required for induction of the estrous response, and thus attract females to within effective range. No doubt other pheromones, or other means of com-

munication between the sexes, could also produce the same effect. One further implication of our demonstration that the pheromone is volatile is that pathways other than renal excretion may be important—for example, excretion from the lungs.

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References and Notes

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8. Supported by PHS grants HD-00473 and HD-00767, and NSF grant GY 2550. The principles of laboratory animal care as promulgated by the National Society of Medical Research, the American Psychological Association, and the Council of the American Physiological Society are observed in our laboratory.

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Zona Pellucida Dissolution Enzymes of the Rabbit Sperm Head

Abstract. *Enzymes which dissolve the zona pellucida have been extracted from the rabbit sperm head and characterized as having the enzymic properties of hyaluronidase and trypsin. Their combined actions produce a rapid and complete dissolution of the zona pellucida, but the vitellus and its membrane are unaffected by prolonged exposure to this enzyme complex. Inhibition of the proteolytic component with lima bean or soybean inhibitors of trypsin prevents dissolution of the zona pellucida by the extract.*

Penetration of the zona pellucida of the ovum by a spermatozoon was observed as early as 1875 (*1*), but the mechanism of the process has remained obscure. The zona pellucida, a thick, transparent membrane, is best developed in the ova of placental mammals, but is also recognizable in those of marsupials, monotremes, and even of reptiles. The matrix of the zona pellucida appears essentially homogeneous, even when observed with electron microscopy, and consists of neutral or weakly acidic mucoprotein. Initially, the zona pellucida lies in close apposition to the vitellus, but it becomes separated by the fluid extruded from the vitellus when the first polar body is emitted. At the time of ovulation the zona pellucida is surrounded by the cumulus oophorus and corona radiata, but both of these cell masses are rapidly loosened and dispersed after ovulation (*2*). Thus, the principal barrier to fusion of a sperm with an ovum is the zona pellucida. Recently Srivastava *et al.* (*3*) have obtained occasional dissolution of the zona pellucida with extracts of rabbit sperm acrosomes. Obviously, the process of zona penetration is enzymic in nature, but no enzymes for zona dissolution have been characterized or identified in extracts of sperm.

We have examined extracts of washed rabbit epididymal spermatozoa disrupted by sonic oscillation for hyaluronidase and proteolytic activity, which might hydrolyze this mucoprotein layer. These extracts displayed high hyaluronidase activity against bovine vitreous humor hyaluronic acid, and high proteolytic activity with denatured bovine hemoglobin. The synthetic substrates *p*-toluenesulfonyl-L-arginine methyl ester (TAME) and benzoyl arginine ethyl ester (BAEE) were also hydrolyzed by the extracts. Elastin, L-leucinamide, and hippuryl-L-phenylalanine were not hydrolyzed.

Since the spermatozoon penetrates obliquely and head first through the zona pellucida, the enzymes for zona dissolution must be localized in the head of the spermatozoon, and probably within the acrosomal cap. Using a new procedure of sucrose density-gradient centrifugation (*4*) we have isolated from the disrupted rabbit epididymal sperm a homogeneous head fraction which contains most of the hyaluronidase and proteolytic activity. Prolonged sonic disruption of this purified head fraction results in solubilization of the hyaluronidase and proteolytic activities, which sediment as a single molecule with a molecular weight of 59,000, as determined by the procedure of Martin and Ames (*5*). This procedure does not visibly damage the dense nucleus of the sperm, but most of the fragile acrosomes are disrupted and dislodged by the sonic oscillation. It would appear, therefore, that this enzymic particle is being extracted from the acrosomal cap.

Since hydrolysis of BAEE, TAME, and denatured hemoglobin is characteristic of the trypsin type of specificity, the sperm head extract was further compared with purified bovine pancreatic trypsin. The *p*H optimum for

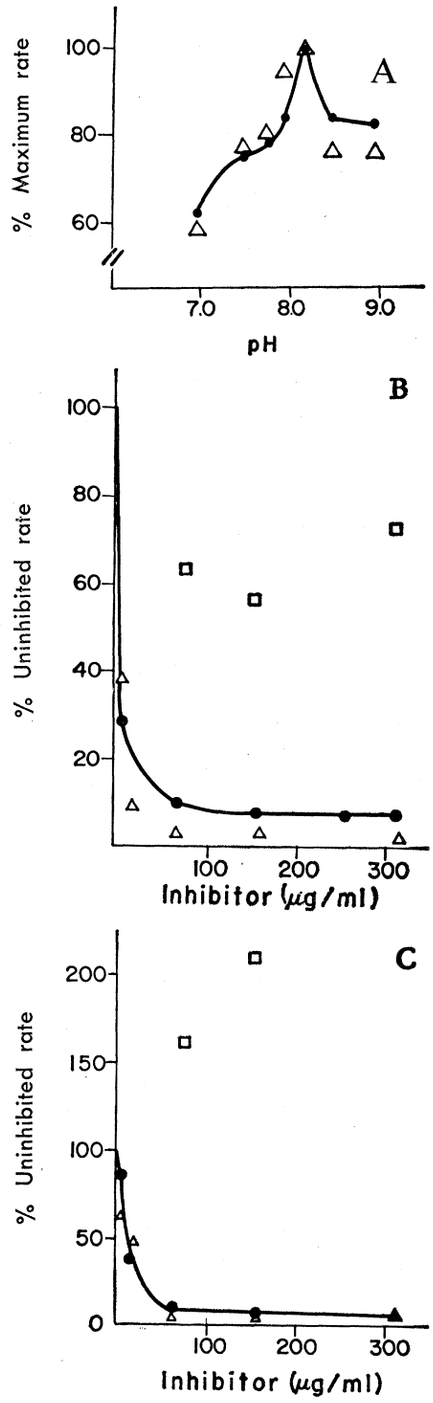

Fig. 1. (A) Effect of *p*H on BAEE hydrolysis by sperm head extract (●) and by crystalline bovine trypsin (△). (B) Inhibition of BAEE hydrolysis with soybean trypsin inhibitor of sperm head extract (●), crystalline bovine trypsin (△), and crystalline papain (□). (C) Inhibition of BAEE hydrolysis with lima bean trypsin inhibitor of sperm head extract (●), crystalline bovine trypsin (△), and crystalline papain (□).