Mammalian Pheromone: Identification of Active Component in the Subauricular Scent of the Male Pronghorn

Abstract. *Male pronghorn* (Antilocapra americana) *mark vegetation by rubbing with their subauricular glands. Males respond to scent marks by sniffing, licking, thrashing, and marking. Hand-raised males were confronted with artificial scent marks consisting of whole extract of the subauricular gland or synthesized compounds corresponding to those identified from the extract. Of these, isovaleric acid released the strongest responses.*

Male pronghorn (*Antilocapra americana*) respond as strongly to one component (isovaleric acid) of the secretion of their subauricular glands as to the total secretion. The pronghorn has four different scent glands (*1*). These are the ischiadic glands in the "rump patches" and the interdigital glands on all four feet in both sexes, and a pair of subauricular glands and a single dorsal gland in the male only. These glands function in several social contexts (*2–4*). We now report the responses to some components of the secretion of the subauricular gland. After sniffing, males rub their subauricular gland areas on vegetation and leave a scent mark readily detectable by the human nose. Other males may sniff, lick, and thrash the scent marks with their horns and then mark, in turn, the same site. Females sniff, lick, and thrash with their forehead and small horns. Although sagebrush (*Artemisia*) is marked, some preference is shown for such taller plants as mullein (*Verbascum*) or thistles (*Cirsium*).

Single bucks maintain territories and mark there frequently (*3, 5, 6*). They undertake regular marking journeys during which they renew old scent marks around the territory and do not engage in any other activities (*5*). Females gather in some territories, and breeding takes place in September and October (*2, 3, 5–7*). Marking with the subauricular secretion also occurs during direct encounters between two neighbor bucks (*7*). Then marking serves as a threat. Marking with the subauricular gland also occurs frequently in "bachelor groups" of 1- to 2-year-old males. Our captive bucks mark in response to strange persons near or in the pen and follow up with a direct attack. The subauricular gland itself is also sniffed and licked by females (*4*).

Males can distinguish their own subauricular scent from that of others, as shown by the differing intensities of spontaneous responses to scent marks stemming from different known individuals (*4, 8*). Factors intensifying the marking responses are strangeness of odor, novelty of environment, absence of dominant male in a bachelor herd, and return to the bachelor group (*8*).

We report the consequences of confronting hand-raised, captive males with artificial scent marks, consisting of whole extract of the subauricular gland or synthetic compounds corresponding to those identified from the extract.

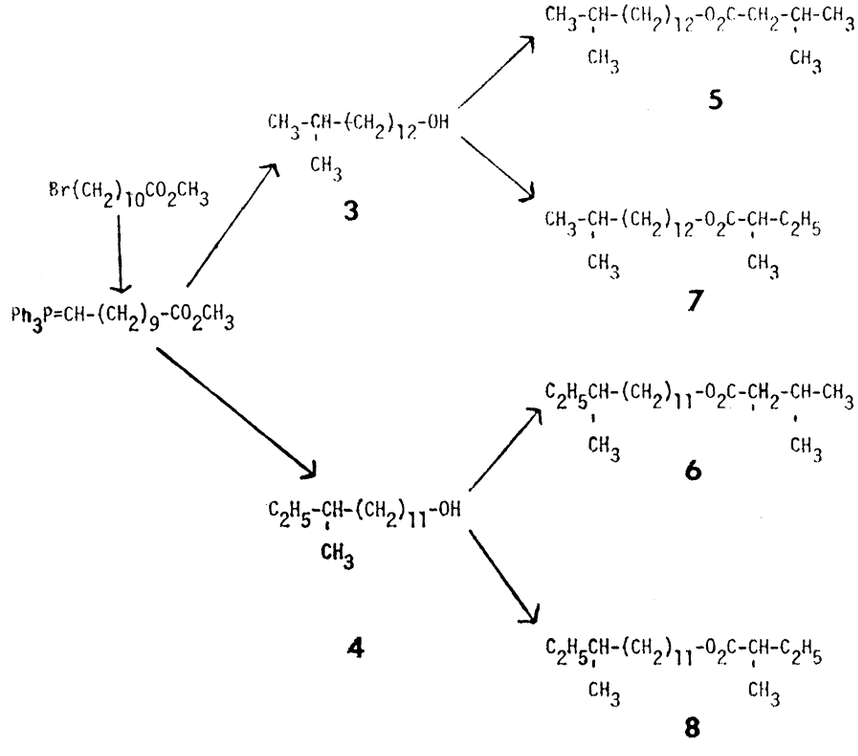
Subauricular glands were extracted with pentane; the extract was concentrated by distillation through a column at reduced pressure, and the volatile compounds were separated from the concentrated extract by short-path distillation. The distillate was fractionated by gas chromatography. Eight major components were identified.

Two compounds were shown to be 2-methylbutyric acid (**1**) and isovaleric acid (**2**) by gas chromatography and mass spectrometry of their methyl esters; about 10 μg of each of these acids were isolated from one gland. Two alcohols (about 30 μg each per gland) were identified as 13-methyl-1-tetradecanol (**3**) and 12-methyl-1-tedradecanol (**4**) from their mass, infrared, and nuclear magnetic resonance spectra and from the mass spectra of the hydrocarbons obtained on hydrogenolysis. The remaining four compounds (70 to 100 μg of each was obtained from one subauricular gland), when treated with a mixture of boron trifluoride and methanol, gave the alcohols **3** and **4** and the methyl esters of the acids **1** and **2**. Thus these compounds are 13-methyltetradecyl 3-methylbutyrate (**5**), 12-methyltetradecyl 3-methylbutyrate (**6**), 13-methyltetradecyl 2-methylbutyrate (**7**), and 12-methyltetradecyl 2-methylbutyrate (**8**).

The identity of each of these compounds was confirmed by comparison of their gas chromatographic retentions and mass spectra with those of the synthetic compounds prepared by the scheme shown.

These six compounds and the two commercially available acids were further purified by gas chromatography for the bioassay experiments.

Six nylon rods (15 cm long and 1 cm in diameter) were placed horizontally in a row 50 cm apart and 80 cm above the ground, and the array was placed in a 24-m^2 triangular pen. The horizontal position was chosen to obviate the tendency to mark untreated vertical rods. Two of the rods were scented with one compound, or a mixture of compounds and two with another compound or mixture. The compounds were dissolved in 0.25 ml of



petroleum ether and applied with a syringe from which the needle was removed. The two remaining rods served as controls (blanks); one was treated with solvent, the other was untreated. Since the responses to both blanks were identical, these responses were combined. Whole subauricular scent was also applied on the horizontal rods at intervals of several days to determine the momentary level of response to a natural stimulus. The total subauricular scent was transferred to the nylon rods by wiping each gland of a mature male three times with the rod. Finally, there were three vertical wooden marking posts in the pen which were never exchanged or cleaned, and were frequently marked by the pronghorn bucks. Thus there were three different controls: blank (untreated and solvent treated), subauricular scent on horizontal nylon rods, and subauricular scent on the wooden permanent posts.

During the 8 months of experiments (July 1972 to February 1973), three adult males were used; one was 3 years old, and two were 2 years old at the beginning of the period. They had been taken in the Upper Pahsimeroi, Idaho, when a few hours old, and hand-raised in 1969 and 1970. They marked all year round, with a low in December and a high during August and September.

For each substance tested, each male was introduced separately into the testing pen for 15 minutes. For each male, a new set of rods was scented. Since two rods carried the same stimulus, the three males were exposed to a total of six rods containing the same stimulus. When tests were repeated, the total number of rods was usually a multiple of 6. In most cases a male would visit and react to all experimental rods during the first minutes. If a male did not encounter all rods within 5 minutes, he was led, by a person holding a bunch of grass, to those missed. One clean rod and one treated with petroleum ether only were always presented simultaneously with test samples. Sample positions were not disclosed to the recording observer.

The eight identified compounds were tested individually at 0.1, 1.0, 10, and 100 μg to cover the possible range from partial deposition of glandular products to repeated deposition at the same site (Fig. 1). To test the effects of combinations of the identified compounds, we added individual compounds in order of increasing and decreasing boiling points: ascending series (not shown), and descending series (Fig. 2). The compounds were combined in the relative amounts of acids, alcohols, esters (1 : 3 : 10) corresponding to the proportion in the scent on the hair overlying the subauricular gland.

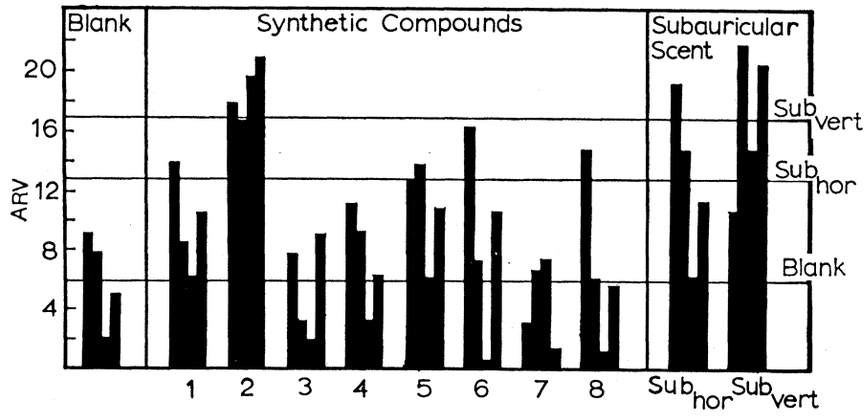


Fig. 1. Intensities of responses (*ARV*; ordinate) to single synthetic compounds of subauricular scent. Each bar of a group of four represents the reactions to one concentration (from left to right: 0.1, 1, 10, and 100 μg). The blank is on the left, the total subauricular scent on the horizontal rod (Sub_{hor}) and on the vertical post (Sub_{vert}) are on right, with single components between. From left to right: 2-methylbutyric acid (**1**), isovaleric acid (**2**), alcohols (**3** and **4**), and esters (**5** to **8**). The horizontal lines mark the average levels of responses to total subauricular scent on vertical posts (Sub_{vert}; top), total subauricular scent on horizontal rods (Sub_{hor}; center), and blanks (bottom). The number of rods (*N*) for each compound at each level and for Sub_{hor} is 6; *N* for blanks from left to right: 24, 30, 18, and 24; *N* for Sub_{vert}: 48, 60, 48, and 48.

In the tests of the individual compounds (Fig. 1), isovaleric acid is the most active. Again, in the tests of mixtures (Fig. 2), isovaleric acid is the dominant compound. It is possible that the esters present in relatively large amounts are the precursors for the acids, and that the high boiling esters and alcohols function as diluents and releasing regulators for the more volatile acids. Other components or different ratios of components may account for recognition of marks of individual animals. Some of the components may serve as priming pheromones.

In Figs. 1 and 2, the average levels of the response values (*9*) to blanks, to total subauricular scent on horizontal rods, and to total subauricular scent on vertical posts are indicated by horizontal lines. More single values reach or surpass the level of total subauricular scent on horizontal rods if isovaleric acid is present in a stimulus (8 of 11, or 72.7 percent), than if not (6 of 49, or 12.3 percent). The same holds true for values reaching the average level of total subauricular scent on vertical posts (36.4

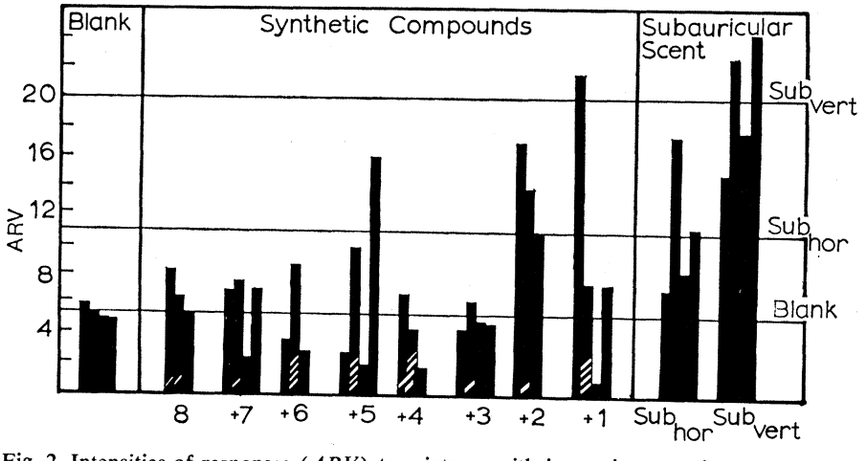


Fig. 2. Intensities of responses (*ARV*) to mixtures with increasing completeness, starting with components with high boiling points (100-μg samples were used only in increments of two components: 8 to 7, 8 to 5, and so on). One stripe at base of bar indicates $N = 9$; four stripes indicates $N = 18$; no stripe indicates $N = 6$; *N* for blanks: 36, 72, 24, and 12; *N* for Sub_{hor}: 12, 12, 12, and 4; *N* for Sub_{vert}: 72, 132, 48, and 24.

percent when isovaleric acid was present in stimulus; 0 percent when absent). The differences between the percentages is significant at $P < .001$ in both cases.

The variations usually encountered in field experiments (Figs. 1 and 2) are in part due to known causes, such as diurnal, seasonal, and meteorological factors, even though the pronghorn were tested at the same time of day. Second, physiological short-term periodicities influence the readiness to scent marking: an animal will be less inclined to mark if it is ready to recline and chew its cud, whereas it will be more likely to mark after a period of rest. This has been demonstrated for black-tailed deer (*10*). Third, sudden noises of vehicles, airplanes, gunshots, and the like often cause the animals to assume the alert posture and change their response to odor stimuli. In view of these possible sources of variation, the results obtained are as consistent as they can be under the circumstances. No response value was discarded. We are aware of no other comparable study on chemical communication in any mammal under similar outdoor conditions.

DIETLAND MÜLLER-SCHWARZE*
CHRISTINE MÜLLER-SCHWARZE
Ecology Center and
Department of Wildlife Science, Utah State University, Logan 84322
ALAN G. SINGER
ROBERT M. SILVERSTEIN
Department of Chemistry, College of Environmental Science and Forestry, State University of New York, Syracuse 13210

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9. The average response value (ARV) for each tested stimulus was determined by adding points assigned to each of the four behavior patterns (sniffing, licking, marking, and thrashing) and dividing by N, the number of nylon rods. The points were based on a large number of observations wherein sniffing was 1 to 3 times more frequent than licking, 4 to 20 times more frequent than marking, and 5 to 30 times more frequent than thrashing. Points assigned, therefore, were 1 for sniffing, 1 to 3 for licking, 4 to 20 for marking, and 5 to 30 for thrashing. The exact number of points for each behavior was determined by the response to blanks that were presented with the samples of a particular test series. For example, in one particular series, in response to a total of 24 blanks, the animals sniffed 52 times, licked 27 times, marked 14 times, and thrashed 4 times; points given, therefore, were 1 for sniffing, 2 for licking, 4 for marking, and 13 for thrashing. In this series when isovaleric acid was presented at 0.1 μg on 6 rods, the animals sniffed 16 times, licked 9 times, marked 5 times, and thrashed 4 times. The total number of points, thus, was $(1 \times 16) + (2 \times 9) + (4 \times 5) + (13 \times 4)$, for a total of 106. The ARV for isovaleric acid was $106/6 = 17.7$ (see Fig. 1, left bar of compound **2**).
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11. Supported by NSF grant GB 26640 to D.M.-S. The spectra were obtained by H. Jennison on a Hitachi RMU-6E mass spectrometer at 70 ev, which was obtained through an NSF grant.
* Present address: Department of Zoology, College of Environmental Science and Forestry, State University of New York, Syracuse 13210.

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Brain Hyaluronidase: Changes in Activity during Chick Development

Abstract. *Development of embryonic chick brain is characterized by high levels of hyaluronidase activity and of hyaluronate, both of which decrease rapidly soon after the chick hatches. By analogy to other systems, it is proposed that the sequence of hyaluronate production and its enzymatic removal may have a developmental role in brain formation.*

Recent work in this laboratory has indicated that hyaluronate production and its subsequent enzymatic removal may be involved in the temporal control of cell migration and cell differentiation in a variety of embryonic and regenerating tissues. Studies of the regenerating newt limb (*1*) and of the developing chick cornea (*2*), limb bud, and axial skeleton (*3*) have led to the postulate that hyaluronate inhibits certain cell interactions necessary for tissue formation (*4*) and that enzymatic or hormonal influences, or both, release this inhibition at the appropriate time (*5, 6*). The possibility that hyaluronate turnover may be linked to the numerous waves of neuronal migration and differentiation during embryonic brain development is now being explored. We report here that relatively high levels of hyaluronidase activity and of hyaluronate are present during chick embryonic brain formation and that they diminish soon after hatching.

Previously, other investigators showed that mucopolysaccharides including hyaluronate are present in brain tissue of several species (*7*) and that they are major components of the extracellular microenvironment of brain cells (*8*). Levels of hyaluronate are known to vary considerably during development (*9*), and Margolis *et al.* (*10*) recently reported the presence of a hyaluronidase in the brains of newborn and adult rats and cows.

Hyaluronidase activities were determined by measurement of the terminal *N*-acetylhexosamine in oligosaccharides released during incubation of hyaluronate with homogenized chick brains from which endogenous substrate had been removed. Homogenization of chick brain in ten volumes of buffer (0.05*M* sodium acetate and 0.05*M* sodium chloride, *p*H 4.5) and centrifugation at 25,000*g* for 20 minutes resulted in the separation of brain hyaluronidase (localized in the precipitate) from brain endogenous substrate (localized in the supernatant). The precipitate containing the enzyme was resuspended with 0.1 percent Triton-X detergent in either 0.05*M* sodium acetate and 0.05*M* sodium chloride or 0.1*M* sodium formate and 0.15*M* sodium chloride buffers for 6 hours. All procedures were performed at 0°C. A portion (0.25 ml) of the Triton-X suspension (approximately 30 mg of protein per milliter) was pipetted into each tube with 200 μg of hyaluronate (Sigma Type IIIP) and incubated at 37°C for varying times up to 8 hours. Enzymatic activity was terminated by boiling at 100°C for 1 minute. Release of terminal reducing *N*-acetylhexosamine as a result of enzymatic action was measured according to Reissig *et al.* (*11*).

That brain hyaluronidase activities are higher in the embryo than in the hatched chick may be seen in a semiquantitative fashion in Fig. 1, which shows a determination of the enzymatic *p*H maximum in acetate and formate buffer systems. A comparison of these curves measuring the amount of terminal *N*-acetylhexosamine released after 8 hours of incubation shows that the embryo had approximately three to four times the enzymatic activity of the hatched chick when equal amounts and concentrations of protein were used for the range of *p*H values examined. Furthermore, a broad *p*H maximum, centered around *p*H 4.5, was ob-