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MAMMALIAN EXOCRINE SECRETIONS XI. CONSTITUENTS OF THE PREORBITAL SECRETION OF KLIPSPRINGER, *Oreotragus oreotragus*

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Abstract—The ketones 3-pentanone, 4-methyl-2-pentanone, 5-methyl-3-hexanone, 4-methyl-3-hexanone, and the esters ethyl propanoate, 2-methylpropyl acetate, ethyl 3-methylbutanoate, and 2-methylpropyl propanoate were identified as the only volatile organic constituents of the preorbital secretion of the klipspringer, *Oreotragus oreotragus*. These compounds are considerably more volatile than those found in the preorbital secretions of other antelopes belonging to the tribe Antelopini. More than 50% of the preorbital secretion consists of proteinaceous material, which probably acts as a controlled-release substance in retarding the evaporation of the volatile constituents of the secretion from territorial marks.

Key Words—*Oreotragus oreotragus*, klipspringer, mammalian semiochemicals, mammalian pheromones, exocrine secretion, preorbital secretion, controlled release, proteins.

INTRODUCTION

The considerable damage done to vineyards, orchards, and pine plantations in various parts of South Africa by several small antelope species, the important

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role the preorbital secretions of these animals play in their territorial behavior, and the possibility of using synthetic preorbital constituents to keep these animals out of cultivated areas were the main reasons for the development of a research program aimed at the chemical characterization of their preorbital secretions. In the previous paper in the series on mammalian exocrine secretions (Mo et al., 1995) it was explained that a response-guided strategy in this research met with little success. We therefore decided to compare the chemical composition of the exocrine secretions of animals belonging to different species and genera to gain insight into the function of the various compounds and compound types present in the secretions of these animals. Two groups of small antelope, in which the structures of the preorbital glands differ, were chosen for future study. One of these was the duikers, of which there are 18 species in Africa, belonging to three genera. In the duikers the preorbital secretion is emitted from a very prominent preorbital gland through a row of tiny pores. The second group of antelope is the Raphicerini, in which the secretion accumulates in a preorbital cavity or pouch not much bigger than the size of a pea. Three members from the tribe Raphicerini, the grysbok, *Raphicerus melanotis* (Burger et al., 1996), the steenbok, *R. campestris* (Burger et al., unpublished), and the oribi, *Ourebia ourebi* (Mo et al., 1995), have already been studied. The klipspringer, *Oreotragus oreotragus*, in which marking with the preorbital secretion appears to play an extraordinarily important role in the animal's territorial behavior, was chosen as the next suitable candidate in this research program.

The klipspringer is a small antelope weighing 8–18 kg, with a mean height of 43–51 cm. The habitat of the klipspringer is typically rocky outcrops or the steep rocky slopes of mountains and gorges, where they may be found up to an altitude of 4000 m (Dorst and Dandelot, 1970). The name klipspringer comes from the Afrikaans language and derives from the animal's ability to jump from rock to rock and to bound up steep rock faces.

Klipspringers are territorial animals, an adult male establishing and defending an area that varies in size according to the mean annual rainfall (Norton, 1980). Klipspringers do not spend very much time chasing intruders off their territories; instead there is evidence that they use dunging and scent-marking to demarcate their territories. Dung heaps are scattered more or less randomly throughout their territories but there is a tendency to place dung heaps at prominent sites and near territorial boundaries (Norton, 1980). Scent-marking with secretion from the preorbital glands is possibly the most important method used by the klipspringer to define territorial boundaries. In both sexes the preorbital gland is a large, thin-walled pocket anterior to the forward corner of the eyes, in which cornified epidermal cellular material from the sac wall, secretions from the orbit of the eye, and various particles of environmental debris accumulate (Albone, 1984). The preorbital glands are prominent in both sexes and open to the surface in a naked, black, forwardly sloping slit or cavity in front of the

eyes. Scent-marking is carried out by either partner but, if the female scent marks, the male usually follows and overmarks. This suggests that scent-marking plays an important role in the maintenance of the pair-bond, as well as serving to demarcate a territory. Territorial marking is carried out by the careful choice of a low, bare twig of a small bush, which, by maneuvering the head, is carefully inserted into the open preorbital cavity. This leaves a 4- to 6-mm smear of black, tarry glandular exudate on the twig (Smithers, 1983). The territorial marks have a shiny black, resinous appearance. Most of the twigs, grass stems, and some of the rocks throughout the more intensively used areas of a home range have deposits of facial secretions if they are at face level. A male klipspringer has even been observed biting off grass tips and inserting the stalks into his preorbital glands (Kingdon, 1982). In the present investigation it was found that a male klipspringer invariably licked the black deposits before and/or after adding fresh exudate to a territorial mark.

A very interesting observation is that adults of the African tick, *Ixodes matopi*, are attracted to and aggregate on the preorbital secretion marks that the klipspringer leaves on vegetation (Spickett et al., 1981; Colborne et al., 1981). In this way, the tick stands an excellent chance of making contact with its host when the scent mark is revisited. Rechav et al. (1978) found that if the solid secretion is extracted with different solvents, only the water-soluble fraction is active in attracting another *Ixodes* species, *I. neitzi*. This phenomenon appears to be another example of the pheromone of one species being utilized as a kairomone by another species to find its prey or a host animal.

The secretion of the klipspringer is unique among the preorbital secretions of members of the tribe of the Antelopini in that the bulk of the secretion consists of solid material that probably acts as a controlled-release carrier material for the volatile constituents of the secretion. If a klipspringer is housed in a small pen having only a few objects that can be marked, preorbital material deposited on, for example, the corner of a feeding trough accumulates to form rodlike structures that can reach a length of 5 cm with a thickness of about 1 cm. The preorbital secretion of the grysbok, *Raphicerus melanotis*, in contrast, contains only minute quantities of nonvolatile material, which, after evaporation of all volatile material, has a black powdery or chalklike appearance.

In this paper the identification of the volatile organic constituents of the preorbital gland secretion of the klipspringer and the partial characterization of the nonvolatile constituents of the secretion are reported.

METHODS AND MATERIALS

General. All Pyrex glassware used in the handling of biological material and extracts, as well as in the preparation of reference compounds, was heated to 500°C in an annealing oven to remove any traces of organic material. Di-

chloromethane (Merck, Residue Analysis Grade) was used for extraction purposes. Syringes, stainless-steel needles, and similar equipment were cleaned with this solvent.

Gas Chromatography. Gas chromatographic (GC) analyses were carried out with Carlo Erba 4200 and 5300 gas chromatographs equipped with flame ionization detectors, Grob split-splitless injectors, and glass columns coated with a 0.25-μm film of the apolar stationary phase PS-089, a silanol-terminated 95%-dimethyl-5%-diphenylsiloxane copolymer. Helium was used as carrier gas at a linear velocity of 28.6 cm/sec at 40°C. The flame ionization detector was operated at 280°C and the injector was normally used at 220°C. Samples were injected in the split mode, the analytes thermally focused on the column at ca. 30°C and analyzed using a temperature program of 2°C/min from 40°C to 260°C (hold). In headspace gas analyses and in analyses in which solventless sample introduction was used, cryotrapping with Dry Ice was employed. Quantification was done with Barspec software and a 386 personal computer.

Mass Spectrometry. Electron impact (EI) mass spectra were recorded at 70 eV on a Carlo Erba QMD 1000 gas chromatograph–mass spectrometer (GC-MS system), using the columns and conditions described above. An interface temperature of 250°C was used. The ion source temperature was set at 200°C, and the pressure in the source housing was ca. 2×10^{-5} torr at a column temperature of 40°C, decreasing to ca. 1×10^{-5} torr towards the end of the temperature program.

An electrospray mass spectrum of the nonvolatile fraction of the preorbital secretion was obtained by dissolving 9 mg of the core of a territorial mark in 1 ml of deionized H_2O, centrifuging the solution to precipitate dust particles and other insoluble material, and diluting a few microliters of the supernatant solution with water containing 0.01% of TFA for injection into the electrospray source of a VG Quattro Triplequad mass spectrometer.

Sample Collection. Preorbital secretion was collected from a tame male klipspringer kept in a pen (10 m × 10 m) at the Tygerberg Zoo. The animal was allowed to mark a tubular Teflon scoop with an inside diameter of 5 mm and furnished with a Teflon plunger with which the collected material could be ejected into a 300-μl or 1-ml Reacti-Vial. About 20–30 μl of secretion were collected per marking event.

Extraction of Volatile Organic Constituents. The collected secretion was stirred with a thin glass rod, and dichloromethane was added to produce a homogeneous suspension. The suspension was centrifuged at 2000–3000 rpm for 15 min or longer to precipitate solid material such as dust particles and to separate the solvent from water and mucus. Only very small quantities of dichloromethane extract could be removed with a 100-μl syringe from underneath the supernatant water layer and transferred to a Reacti-Vial. This extract was stored at −30°C for subsequent GC and GC-MS analyses.

Solventless Sample Introduction. Two solventless sample introduction techniques were used. To obtain the strongest possible total ion chromatograms, about 20 μl of fresh, unprocessed preorbital secretion was introduced into the GC-MS system by inserting a glass injector liner containing the secretion supported on a few glass-wool fibers, into the injector. The glass-wool fibers were used in the liner to prevent the secretion from flowing down into the bottom of the injector. This technique is not suitable for retention time comparison, and the analytes could also undergo oxidation in the injector, as the injector liner has to be introduced into the injector at atmospheric pressure. These shortcomings were circumvented by introducing the sample-containing liner into the injector at 40°C, purging the injector with the carrier gas, and heating the injector at a reasonably slow rate to allow volatile compounds to be desorbed from the liner at as low a temperature as possible. The volatile material desorbed from the injector was cryotrapped on the column. As the secretion consists largely of water, a section of 1.0-mm fused silica tubing was used at the inlet end of the column to prevent the column being blocked by ice formation. The liner was cleaned by washing it with distilled water and drying it at 250°C in a GC oven after each analysis.

Analyses were also carried out using a solventless sample introduction technique in which the sample is introduced using a capillary tube with an open-ended sample cavity of about 15 μl (Burger et al., 1990a). The sample introduction capillary was weighed, the sample (2–3 μl) injected into the sample cavity, and the loaded capillary weighed again and introduced into the injector without delay, with the split and septum-purge valves closed. Various injector temperatures were used and the volatile material, desorbed from the capillary, was cryotrapped on the column.

Analysis of Headspace Gas of Secretion. Solid-phase microextraction (SPME) (Arthur et al., 1992) was used to extract the volatile organic compounds from the headspace of 50- to 100-μl samples of secretion in a 1-ml Reacti-Vial. The extracted volatiles were desorbed from the SPME fiber for 5 min at an injector temperature of 220°C and were cryotrapped on the column. After the volatile material had been desorbed, cryotrapping was terminated and the program started. In a few analyses the SPME fiber was left in the injector for varying lengths of time to allow desorption of any heavy material that could have been trapped. SPME also was carried out on territorial marks by placing a territorial mark in a 50-ml glass bottle fitted with a screw cap and Teflon-lined septum and sampling the headspace for 3 hr.

Determination of Affinity of Preorbital Protein for Volatile Preorbital Compounds. Two experiments were designed to find out whether the nonvolatile part of the secretion could act as a controlled-release substance for the volatile organic compounds present in the secretion. In the first of these experiments the headspace gas of a territorial mark was analyzed by the following procedure

once every 24 hr for as long as the target compounds could be detected. A territorial mark (2.6 g) was collected from the pen of the male klipspringer and suspended on a thin stainles-steel wire to facilitate the handling of the material. The solid material was placed into the 50-ml glass bottle described in the previous section, the headspace atmosphere of the mark sampled by SPME for 3 hr at 22°C, and the collected volatile compounds quantitatively analyzed by gas chromatography. The territorial mark was removed from the bottle after completion of each SPME sampling period and was exposed to the atmosphere in a shaded place until the next analysis was carried out 21 hr later. The mean maximum and minimum temperatures during the time it took to complete the experiment were 21° and 9°C, respectively. The humidity was relatively high during the first 24-hr period, and on the second day it started raining. The territorial mark remained soft for about the first two days of the experiment, but became hard and brittle towards the end of the experiment, which was continued for six days.

In a second experiment the capability of the preorbital protein to adsorb and retain the volatile constituents of the preorbital secretion was compared to that of other proteins. Glass-fiber wicks were individually coated with the proteins and exposed to the vapor of some of the volatile constituents of the preorbital secretion, and the amount of the volatile organic material adsorbed by each of the proteins was determined gas chromatograhically. The device employed in this experiment has been described in a paper on the pheromone-dispensing proteinaceous secretion of certain dung beetle species (Burger et al., 1990b) and is depicted in Figure 1. In the present investigation 2.0 mg each of the solid preorbital secretion, bovine albumin, bovine pancreas trypsin, and chicken egg white lysozyme were dissolved separately in 100 μl of distilled water or, in the case of the preorbital protein, in 0.1% TFA. The wicks were coated with the proteins by allowing the protein solutions to be drawn up into the wicks by capillary action, and the wet glass-fiber wicks were suspended on thin glass rods to dry. The coated wicks were further dried under carrier gas flow in a glass injection liner at an injector temperature of 60°C and were then slipped onto the four outer prongs of the glass framework in Figure 1. The uncoated pheromone-dispensing wick was slipped onto the central prong and was treated with 1 μl of a mixture of 3-pentanone, 3-pentanol, isobutyl acetate, and ethyl isovalerate.

The framework was placed in a glass vial (28 mm OD × 140 mm), which was closed with a ground-glass stopper without using stopcock grease. The vial with its contents was kept in a cupboard, away from radiant light or heat at 22°C for 24 hr, and the wicks were immediately sealed individually in soda glass tubes (5 mm ID) with a small flame. the wicks were left in the glass tubes for at least 6 hr to allow equilibration of the volatiles with the glass surfaces before the volatile material retained on each wick was analyzed. These analyses

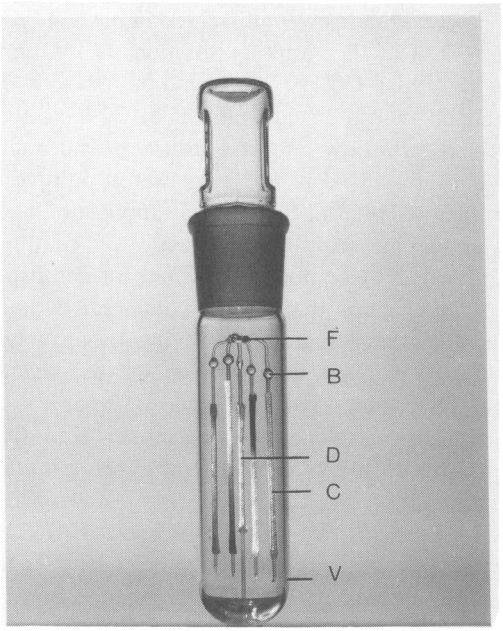

FIG. 1. Device for the determination of the affinity of various proteins for volatile organic compounds. V, glass vial (28 mm OD × 140 mm); F, five-pronged glass framework constructed from 1.0-mm glass rod and supported on the central prong; B, glass beads formed in the framework to keep the outer four prongs from touching the wall of the vial; D, dispensing glass-fiber wick treated with the volatile organic compound(s) under investigation; C, adsorbing wicks (collectors).

were done by inserting each wick into a wide-bore injector liner and desorbing the adsorbed compounds onto the capillary column at an injector temperature of, for example, 80°C for 30 min. After completion of an experiment, the wicks were stored in screw-capped glass tubes.

Electrophoresis. Polyacrylamide slab-gel electrophoresis in 20% acrylamide was performed in the presence of sodium dodecyl sulfate (SDS) (Laemmli, 1970). Protein (100 μg/100 μl) was dissolved in the β-mercaptoethanol-containing application buffer of which 20 μl was applied per lane. Gels were stained with Coomassie brilliant blue R250.

Amino Acid Analysis. Proteins were hydrolyzed by gas-phase hydrolysis at 110°C for 24 hr in 5.7 M HCl–0.5% phenol (Bidlingmeyr et al., 1984). Amino acids were quantified by ion exchange chromatography on a Waters amino acid HPLC system using the OPA in a postcolumn derivative method as specified by the manufacturers.

HPLC Purification. Proteins were dissolved in 0.1% TFA at concentrations of 1 mg/ml, and 100 μl of the resulting solution loaded on a 30- × 0.4-cm column packed with Vydac-C4 [10 μm, equilibrated with 0.1% (w/v) TFA (solvent A) and eluted with a linear gradient of 70% acetonitrile containing 0.1% TFA (solvent B) at a flow rate of 1 ml/min]. The fractions were freeze dried and subsequently dissolved in 100 μl of water. Samples of 10 and 20 μl were dissolved in equal volumes of SDS sample application buffer and subjected to electrophoresis and amino acid determination.

Reference Compounds. Compounds required for comparison with constituents of the preorbital secretion that are not commercially available were synthesized from authentic starting materials. 5-Methyl-3-hexanone was synthesized by condensation of isobutyl magnesium bromide with propanal to give 5-methyl-3-hexanol (51 g, 44%). Oxidation of 5-methyl-3-hexanol with pyridinium chromate (Singh et al., 1979), gave 5-methyl-3-hexanone. ^{1}H: δ ($CDCl_3$) 0.914 [6H, d, (CH_3)$_2$-CH-], 1.046 (3H, t, CH_3-CH_2-), 2.151 [1H, m, -CH-(CH_3)$_2$], 2.398 (2H, q, -CH_2-CH_3), 2.280 (2H, d, -CH_2-CH-); ^{13}C: δ ($CDCl_3$) 7.787 (C1), 22.621 (C6, C7), 24.713 (C5), 36.461 (C2), 51.492 (C4), 211.520 (C3).

4-Methyl-3-hexanone was synthesized from *sec*-butyl magnesium bromide and propanal. Column chromatography of the final product on silica gel produced a sample of the pure reference compound, bp 134°C/760 mm. ^{1}H: δ ($CDCl_3$) 0.869 (3H, t, CH_3-CH_2-CO-), 1.047 (3H, t, CH_3-CH_2-CH-), 1.065 (3H, d, CH_3-CH-), 2.464 (2H, m, -CH_2-CO-), 2.440 (2H, m, -CH_2-CH_3), 6.479 (1H, m, -CH-CO-); ^{13}C: δ ($CDCl_3$) 7.083 (C1), 11.724 (C6), 16.051 (C7), 26.100 (C5), 34.294 (C2), 47.664 (C4), 215.38 (C3).

RESULTS AND DISCUSSION

The preorbital glands of the klipspringer are remarkably productive. The experimental male animal available for this research was tame enough to be coaxed into marking the Teflon scoop, which greatly simplified collection of material. In one experiment the animal marked the scoop 12 times and produced 102.1 mg of secretion in 22 min. The animal lost interest in the scoop after a while and started overmarking existing marks in his pen.

The secretion consisted of 38% nonvolatile material after water and other volatiles were evaporated at 100–110°C from freshly collected secretion. The high production of secretion and its relatively high content of nonvolatile material accounts for the accumulation of conspicuous territorial marks on twigs and other objects marked by the animal.

Characterization of Volatile Organic Constituents. The secretion is reasonably viscous, flowing quite slowly down the walls of vials in which it had been collected. When rubbed between the fingers, it is sticky and has a pleasant

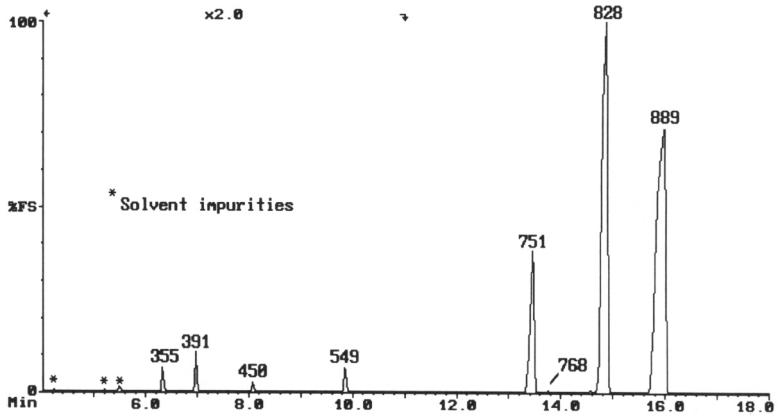

FIG. 2. Total ion chromatogram of volatile organic compounds extracted with dichloromethane from the preorbital secretion of a male klipspringer.

and distinctly fruity smell. In contrast to experience with other preorbital secretions, extraction of the volatile organic material from the secretion with dichloromethane was not very successful. An emulsion was formed and only a small percentage of the solvent was recovered when the emulsion was centrifuged. Nevertheless, using this extract, a satisfactory gas chromatogram was obtained (Figure 2). The extract contained only a few volatile constituents.

As the possibility existed that other, more volatile constituents could have been coeluted with the solvent, solventless sample introduction techniques were also employed. The neat secretion was, for example, injected onto glass wool in the injector liner and the volatile material desorbed from the glass wool by temperature programming the injector to allow the volatiles to be desorbed at the lowest possible temperature. Although the splitless introduction of large samples resulted in a total ion chromatogram with broad peaks and an unstable baseline, this method nevertheless produced results that confirmed the presence in the secretion of low concentrations of a few short-chain compounds.

The more elegant introduction of the neat secretion by a technique developed for the solventless introduction of biological material into the injectors of GC and GC-MS systems (Burger et al., 1990a) gave the total ion chromatogram shown in Figure 3. This method was used for qualitative as well as quantitative analyses.

Complications arising from the high water content of the secretion and the formation of ice in the column when applying cryotrapping were avoided by analyzing the headspace volatiles of the secretion after solid-phase microextraction (SPME) (Arthur et al., 1992). Total ion chromatograms exhibiting good

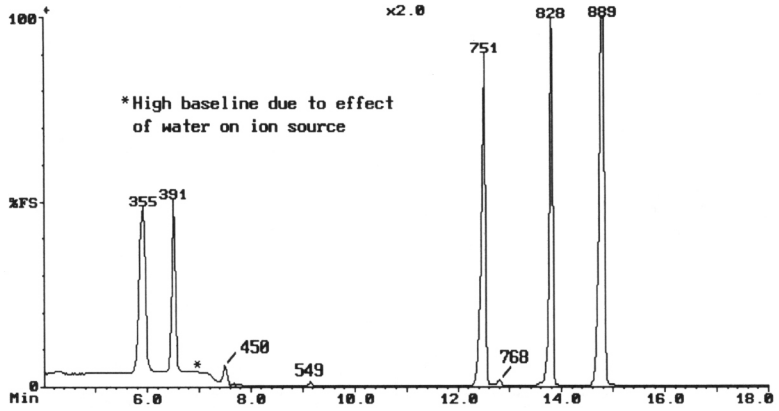

FIG. 3. Total ion chromatogram of volatile organic constituents of the preorbital secretion of a male klipspringer. The chromatogram was obtained by solventless introduction of the secretion into the injector of the GC-MS system.

capillary column resolution and an acceptable baseline were obtained. A typical total ion chromatogram obtained with this method is shown in Figure 4. Similar chromatograms were obtained when older material, deposited as territorial marks, was subjected to similar analyses (Figure 5). As expected, the more volatile constituents of the secretion were present in extremely low concentrations in the older territorial marks.

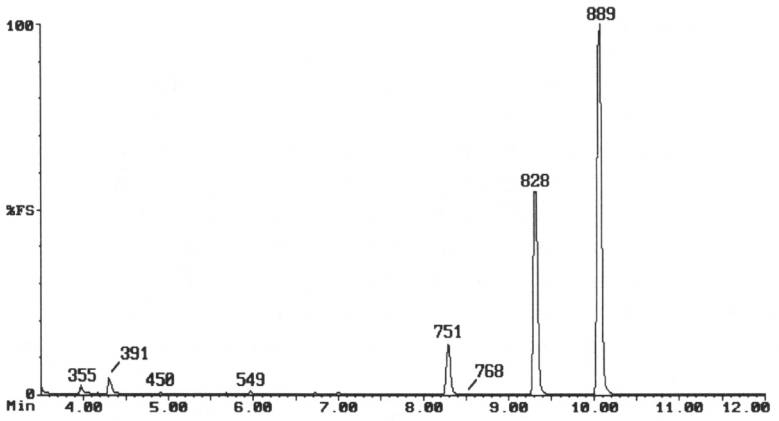

FIG. 4. Total ion chromatogram of the volatile organic constituents sampled by solid phase microextraction (SPME) from preorbital secretion, freshly collected from a male klipspringer.

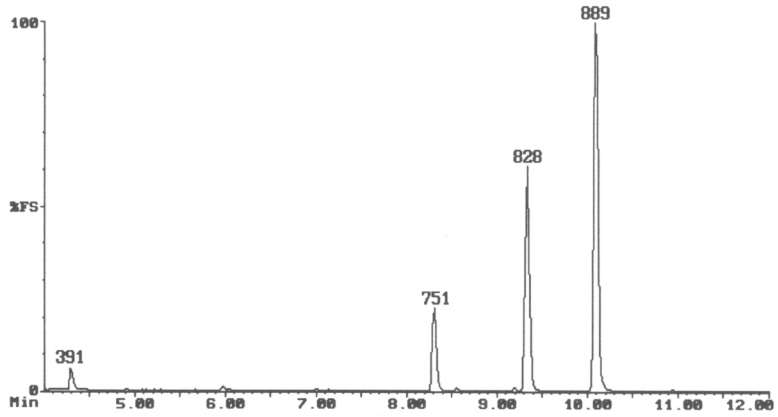

FIG. 5. Total ion chromatogram of volatile constituents sampled by SPME from a preorbital territorial mark deposited by a male klipspringer.

The volatile organic constituents identified in the preorbital secretion are listed in Table 1 together with the relevant mass spectral data and information on the quantitative composition of the secretion obtained in analyses with the synthetic substances as external standards.

Compared to the exocrine secretions from 12 other antelope species investigated in the Laboratory for Ecological Chemistry, the composition of the preorbital secretion of the klipspringer is extraordinarily simple. Whereas the other secretions typically contain at the very least about 80, and frequently more than 150 volatile organic constituents, only eight volatile organic compounds were consistently found in the fresh preorbital secretion of the klipspringer and in older territorial marks left in the field, regardless of the sample introduction technique employed. Five major constituents comprise about 98% of the total volatile organic fraction.

Characterization of Nonvolatile Fraction of Secretion. The fresh preorbital secretion has a tacky consistency when rubbed between the fingers. It was therefore assumed to contain a substantial amount of one or more polysaccharides or proteins.

Amino acid analysis after acid hydrolysis revealed that about 80% of the dry weight of the secretion consisted of protein. The results (Table 2) of the determination of the amino acid composition of the fresh (wet) secretion and old (dry) territorial mark gave practically identical results. Electrophoretic analysis (Figure 6) showed that both the fresh secretion and territorial marks consist of a relatively simple mixture of a few proteins ranging in molecular mass from 12 to 20 kDa. The similarity of fresh and old secretion is remarkable because

TABLE 1. COMPOUNDS IDENTIFIED IN PREORBITAL SECRETION OF KLIPSPRINGER

No. in Figure 2	Compound	Quantitative composition (μg/mg secretion)[a]	Mass spectra m/z (%)
355	3-Pentanone	0.020	86(30), 58(4), 57(100), 56(5), 55(4), 29(76), 27(40), 29(8)
391	Ethyl propanoate	0.032	102(14), 87(3), 75(14), 74(11), 73(7), 58(4), 57(100), 56(5), 45(12), 44(3), 43(6), 29(89), 27(39), 26(8)
450	4-Methyl-2-pentanone	0.0030	100(18), 85(15), 58(31), 57(20), 43(100), 42(14), 41(28), 39(26)
549	2-Methylpropyl acetate	0.0062	86(2), 73(12), 71(2), 61(2), 57(3), 56(20), 55(3), 44(3), 43(100), 42(11), 41(20), 39(17), 32(5), 29(11), 28(16), 27(14), 26(3)
751	5-Methyl-3-hexanone	0.083	114(20), 99(5), 85(40), 72(10), 58(7), 57(100), 56(5), 55(7), 43(31), 42(25), 41(56), 39(37), 32(19), 29(66), 28(49), 27(55)
768	4-Methyl-3-hexanone	0.0020	114(7), 86(5), 85(9), 58(4), 57(100), 56(8), 55(6), 53(3), 43(4), 42(10), 41(26), 40(4), 29(62), 28(39), 27(49), 26(9)
828	Ethyl 3-methylbutanoate	0.12	130(1), 115(10), 103(4), 88(100), 85(69), 70(19), 61(23), 60(50), 59(20), 57(84), 56(13), 55(12), 45(25), 43(58), 42(48), 41(94), 40(15), 39(79)
889	2-Methylpropyl propanoate	0.22	100(0.8), 86(7), 75(5), 57(100), 56(28), 55(6), 43(12), 41(27), 39(20), 32(6), 29(50), 28(27), 27(37), 26(7)

[a] A single collection from the two preorbital pouches of a klipspringer typically yielded 20–30 mg of secretion.

TABLE 2. AMINO ACID COMPOSITION OF PREORBITAL SECRETION OF KILPSPRINGER

Amino acid	Amount (mol %)[a]			
	Secretion		HPLC fractions	
	Dry	Fresh	F1	F2
Asp	9.3	9.4	2.1	13.8
Thr	11.2	11.7	14.5	5.7
Ser	14.5	15.7	24.6	6.0
Glu	11.6	11.6	1.6	19.1
Pro	nd	nd	16.8	nd
Gly	16.1	16.8	22.2	7.3
Ala	10.4	10.5	14.8	4.6
Val	3.3	3.4	0.1	5.9
Met	0.8	0.9	0.0	1.2
Ile	4.2	3.6	0.4	8.2
Leu	3.4	3.1	0.8	4.5
Tyr	3.1	2.9	0.3	4.7
Phe	3.6	2.1	0.8	7.7
Trp	nd	nd	0.0	nd
His	2.7	2.5	0.0	1.8
Lys	2.4	2.3	0.0	4.8
Arg	3.4	3.5	1.0	4.7

[a] Amounts are expressed in moles of amino acid per 100 moles of amino acid recovered. Dry and fresh refer to a dry territorial mark and freshly collected liquid sample, respectively. Amino acid analysis was done on 100 μg of each of these samples. F1 and F2 refer to fractions 1 and 2 in which the two major components were isolated from the freshly collected secretion by HPLC on a reverse-phase column.

territorial marks are subjected to harsh environmental conditions such as irradiation by sunlight and temperatures up to about 30°C. The frequent licking of the territorial marks, which brings the territorial marks into contact with certain enzymes, also does not seem to have any influence on the deposited material.

The molecular masses of three of the preorbital proteins were determined by electrospray mass spectrometry (ES-MS). The major protein has a molecular mass of 18,120.79 ± 15.05 Da and two of the minor proteins have molecular masses of 14,652.60 ± 19.02 and 17,631.34 ± 18.37 Da.

Further characterization of the proteins by reverse-phase chromatography on a Vydac C4 column revealed the presence of two fractions (Table 2, HPLC fractions 1 and 2) eluting at 20% and 40% acetonitrile concentration, respectively. Electrophoretic analysis of these fractions indicates that the more hydro-

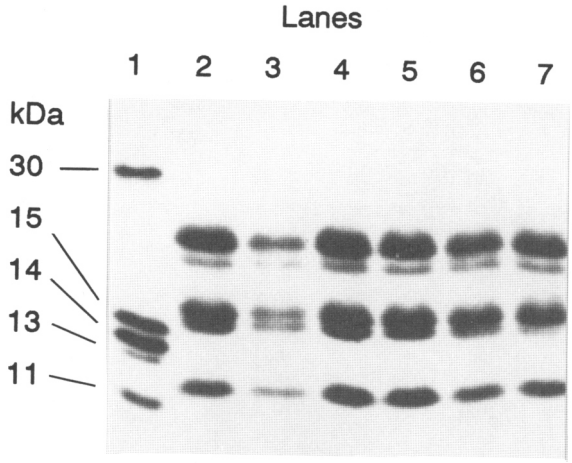

FIG. 6. SDS-PAGE of the proteinaceous fraction of the preorbital secretion of the klipspringer. Lane 1, chicken histones as mass marker proteins; lane 2, liquid secretion aged for several weeks at room temperature; lane 3, centrifuged liquid secretion (supernatant liquid); lane 4, fresh secretion; lane 5, centrifuged fresh secretion (supernatant liquid); lane 6, territorial mark dissolved/suspended in H_2O; lane 7, supernatant solution from a territorial mark dissolved in H_2O and centrifuged.

phobic fraction 2, comprising about 60% of the proteinaceous material, was heterogeneous and contained all the bands visible in the SDS-PAGE of the total secretion. Mass spectrometry (TOF-MALDI) revealed that fraction 2 contains proteins with molecular masses of about 18 kDa. The amino acid composition of this fraction is characteristic of a small globular albuminlike protein.

Fraction 1 has a very unusual composition in that it contained mainly five amino acids (Ser, Thr, Gly, Pro, and Ala). This, together with the fact that this fraction could not be stained by Coomassie blue protein stain, suggests the presence of a glycoprotein or mucoprotein (Hashimoto et al., 1964). TOF-MALDI mass spectrometry revealed that fraction 1 consisted of a broad peak at 25 kDa at various dilutions, indicating a fairly high degree of glycosylation. The anthrone test for carbohydrates showed that less than 3% of anthrone-positive sugars are present. The presence of other sugars cannot be excluded.

Preorbital Protein as Controlled-Release Medium. In order to find out whether the proteinaceous fraction of the klipspringer's preorbital secretion could possibly act as a controlled-release substance for the volatile organic constituents of the secretion, the desorption or evaporation of two of the major volatile constituents from the secretion was monitored using solid-phase microextraction (SPME). A large territorial mark that contained relatively old material was

selected for this experiment. The experiment was continued for six days, by which time the volatile components originally present in the mark were almost totally depleted and replaced by a large number of other compounds that were presumably extracted from the air. The results of this experiment are given in Figure 7. Arbitrary units are used, as absolute quantities have little significance in an experiment such as this, in which the territorial mark has been deposited over an unknown period and which is, furthermore, not homogeneous as far as the volatile content of its older parts and freshly deposited material are concerned. If it is taken into consideration that traces of the volatile constituents of the secretion will evaporate from a twig within a few hours if deposited in the absence of a controlled-release substance, it is clear that the proteinaceous material considerably prolongs the release of the volatile constituents of the secretion. However, it also has to be realized that this effect could be due merely to the bulk of material in which the volatiles are suspended and not necessarily to the protein being a particularly efficient controlled-release material.

An attempt was also made to find out whether the preorbital proteinaceous material has a higher affinity for the volatile organic constituents of the secretion than other proteins. The basic principle of an experiment devised for this purpose is the exposure of four glass-fiber wicks, coated with different proteins, to a source of the volatile material until equilibrium is reached. By determining the quantities of the volatile material retained by each of the proteins, a rough estimate of the affinity of the proteins for the volatiles can be obtained. Bovine albumin, bovine pancreas trypsin, and chicken egg white lysozyme were compared with the preorbital protein. Three of the volatile constituents of the preorbital secretion, 3-pentanone, isobutyl acetate, ethyl isovalerate, and another standard, 3-pentanol, were chosen as volatile organic compounds.

The experiment was repeated several times using different equilibration and

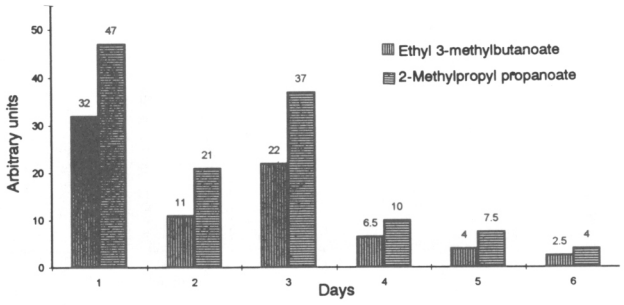

FIG. 7. Release of ethyl 3-methylbutanoate and 2-methylpropyl propanoate from a territorial mark. The headspace gas of the territorial mark (2.6 g) was sampled for 3 hr at 22°C once every 24 hr.

desorption parameters, starting with equilibration for 6 hr at 22°C and thermal desorption for 10 min at an injector temperature of 60°C. Although it was not attempted to determine the minimum time required to reach equilibrium, it was found that after 6 hr only very small quantities of the volatile compounds were left on the dispensing wick. With desorption of the volatiles from the protein-coated wicks for 15 min at an injector temperature of 60°C, the threshold conditions for complete desorption of the volatiles from the protein-coated wicks seemed to have been barely reached, as a faint smell of ethyl isovalerate could still be detected on removal of the wick from the injector before starting the analysis. Quantitative desorption was achieved in 15 min at an injector temperature of 80°C. Some of the results obtained in these experiments are summarized in Figure 8.

The affinity of the preorbital protein for organic volatiles of the secretion appears to be similar to that of albumin. Trypsin has a very low affinity for the volatiles, barely higher than the untreated glass fiber, whereas lysozyme appears to have a very high affinity, especially for the higher boiling esters. The contribution of the glass-fiber support to the retention of the volatiles is negligible. Apparently the klipspringer does not use a mixture of proteins having an excep-

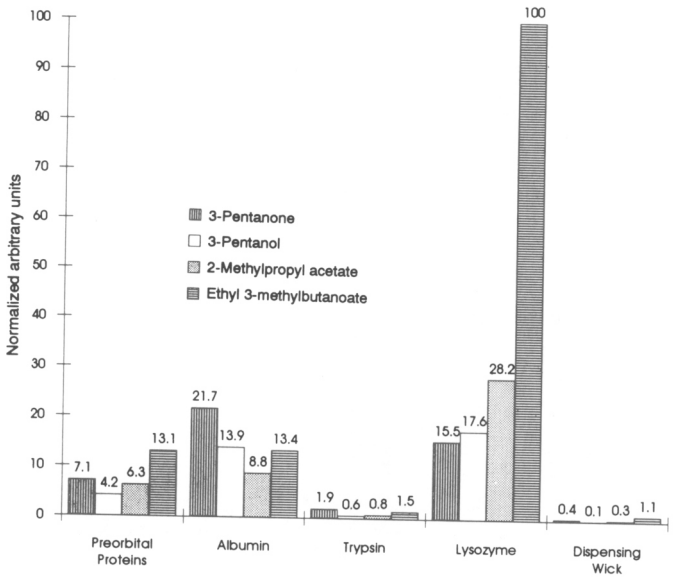

FIG. 8. Relative affinity of proteins for volatile organic compounds. Quantitative data were obtained by integrating the FID chromatograms of the volatiles desorbed (30 min at 80°C) from the dispensing wick and wicks coated with the proteins.

tionally high affinity for the volatiles in the secretion to control the release of these substances. However, the relatively large proportion of glycoprotein in the secretion results in an affinity for the hydrophobic volatile organic compounds approximately similar to the affinity of albumin. Lysozyme has an exceptionally high affinity for ethyl isovalerate. The large amounts of preorbital material deposited as territorial marks could possibly compensate for the lack of a highly specific affinity of the proteinaceous material for the volatile constituents of the secretion.

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