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Factors affecting the pheromone composition of voided boar saliva

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Summary. The pheromone binding protein 'pheromaxein' which binds the pheromonal 16-androstene steroids in the saliva of the male pig (boar), was degraded and lost its binding activity in saliva incubated in air for 72 h at 21°C and 37°C. However, pheromaxein and its binding activity were retained in saliva incubated for 168 h at 4°C. When the ^{3}H-labelled pheromones 5α-androst-16-en-3α-ol (3α-androstenol), 5α-androst-16-en-3-one (5α-androstenone) and 5α-androst-16-en-3β-ol (3β-androstenol) were incubated with boar saliva for 168 h at 21°C, 3α-androstenol was primarily converted to 5α-androstenone and 5α-androstenone to 3β-adrostenol; 3β-androstenol was unchanged. Evidence was obtained for microorganisms being responsible for these steroid transformations.

Introduction

When a mature boar is aroused by the presence of oestrous pigs or unfamiliar boars, he champs copious amounts of frothy saliva. Frequently the saliva foam falls to the ground or is deposited on objects in the environment by a rubbing action of the boar's snout. The purpose of the excessive salivation is to provide a medium for the release of large amounts of musk-smelling 16-androstene steroids into the environment. These odorous steroids, in particular 5α-androst-16-en-3α-ol (3α-androstenol) and 5α-androst-16-en-3-one (5α-androstenone), act as signalling pheromones by facilitating the adoption of the mating stance in oestrous pigs (Melrose *et al.*, 1971; Reed *et al.*, 1974; Perry *et al.*, 1980) and probably indicate to other boars that their status is being challenged (Booth & Baldwin, 1980). There is also evidence suggesting that 3α-androstenol has a primer pheromone role in the 'boar effect' on puberty acceleration in female pigs (Kirkwood *et al.*, 1983; Booth, 1984a).

The pheromonal steroids are primarily produced in the boar's testes (Booth, 1982) and transported in the blood to the submaxillary salivary glands where they are concentrated by association with a specific binding protein called 'pheromaxein' (Booth, 1984b); the steroid–protein complex is then secreted into the saliva. It is highly likely that pheromaxein is vital for the transportation of the pheromonal steroids in the aqueous medium of saliva, since these steroids are not only very lipophilic, causing boar taint in the fat of mature boars (Booth, 1982; Bonneau, 1982), but occur in very high concentrations in the submaxillary glands and saliva of these animals (Booth, 1980). Since both the signalling and primer pheromone effects of 3α-androstenol, and the signalling pheromone effect of 5α-androstenone, are mediated by the free steroid (Melrose *et al.*, 1971; Reed *et al.*, 1974), this suggests that the pheromonal steroids dissociate from their binding protein in voided saliva and become volatile as air-born pheromones. Indeed, preliminary observations on the relative amounts of free and bound pheromone in boar saliva had shown that about 10% of the pheromones are in the free form in freshly voided saliva at 4°C, increasing to 30% at 21 and 37°C (W. D. Booth, unpublished).

The question arises as to the stability of the pheromonal steroids and pheromaxein in voided saliva, since changes in the nature of these factors due to decomposition are likely to affect the

pheromone potency of saliva deposited in the environment. Experiments were therefore carried out to examine some factors which are likely to influence the fate of the pheromonal steroids in voided boar saliva.

Materials and Methods

Saliva samples. Saliva was collected into beakers as it dripped from the mouths of two aroused domestic boars while they were mounting a dummy to provide semen samples. The saliva was centrifuged to precipitate debris and the supernatants stored frozen at −70°C.

The stability of pheromaxein. Saliva was thawed and samples (0·2 ml) were incubated at 4, 21 and 37°C, for 24, 72, and 168 h at each selected temperature. After each of these times the saliva samples were diluted to 0·5 ml with 10 mmol Tris–HCl (pH 7·4)/l containing EDTA (1 mmol/l) and 1% propanediol, and incubated for 1 h at 4°C with 300 000 c.p.m. [5α,6α-^{3}H]5α-androstenone (sp. act. 25 Ci/mmol: Isocommerz, Dresden, East Germany). The samples were then subjected to non-denaturing polyacrylamide gel electrophoresis (PAGE) using 7% acrylamide specially purified for electrophoresis (BDH Ltd, Poole, Dorset, U.K.). A detailed account of the method has been described (Booth, 1984b). The distribution of ^{3}H-labelled 16-androstene steroid on the gel, and its association with pheromaxein, were determined by scintillation counting of 2 mm gel slices.

The stability of pheromonal steroids. Saliva (2–3 ml) was filtered through a Millex-GS, non-pyrogenic sterile filter (0·22 μm) (Millipore, Harrow, U.K.). Samples (0·2 ml) were taken from the filtrate and also from unfiltered saliva before dilution with 0·3 ml of 10 mmol Tris–HCl (pH 7·4)/l containing EDTA (1 mmol/l). Some samples received buffer containing 1·2 mg of the antibiotic kanamycin sulphate (Sigma (London) Poole, Dorset, U.K.). The saliva samples and control (buffer only) were then added to loosely screw-capped culture tubes containing 300 000 c.p.m. of [^{3}H]5α-androstenone, [^{3}H]3α-androstenol or [^{3}H]3β-androstenol (the last two ^{3}H-labelled steroids were formed by enzymic reduction of [^{3}H]5α-androstenone as described by Booth (1984b)), and incubated in air for 168 h at 21°C. Steroid metabolites and unreacted substrates were extracted with diethyl ether (peroxide free) from May & Baker (Dagenham, Essex, U.K.) after the addition of carrier steroids, and purified by thin-layer and radio–gas liquid chromatography (see Booth, 1984b).

Results

Pheromaxein stability

Figure 1 shows the binding profiles for [^{3}H]16-androstene steroid to the two α- and β-charge isomers of pheromaxein which occurred in the saliva of one boar. Binding of steroid to pheromaxein in saliva incubated at 4°C was essentially unchanged after 168 h; only the β-isomer showed some reduced binding with time. Binding at zero time (not shown) was the same as that after 24 h at 4°C. However, the saliva samples incubated at 21 and 37°C had lost most binding activity by 72 h, and all by 168 h. Similar binding profiles were obtained for the other boar which had only the β-isomer. Binding profiles for 16-androstene steroid in saliva and submaxillary gland tissue preparations of several pigs have indicated that the presence or absence of one or other of the charge isomers of pheromaxein, is genetically determined (Booth, 1984b). Coomassie blue staining of non-denaturing and denaturing SDS gels (Booth, 1984b) showed that the bands corresponding to pheromaxein and other major salivary proteins had disappeared in saliva samples incubated for 168 h at 21 and 37°C; this finding was in keeping with the loss of pheromone binding.

Pheromone stability

The substrate remained unchanged in the incubation with buffer only or filtered saliva. However, in untreated saliva incubations, [^{3}H]3α-androstenol was primarily converted to [^{3}H]5α-androstenone (mean ± s.d. 2 boars, 29·4 ± 1·48%) with some [^{3}H]3β-androstenol (mean ± s.d. 2 boars, 11·9 ± 3·34%). Substrate [^{3}H]5α-androstenone was primarily converted to [^{3}H]3β-androstenol (mean ± s.d. 2 boars, 8·41 ± 0·70%) and [^{3}H]3α androstenol (mean ± s.d. 2 boars 1·71 ± 0·8%); no other metabolites were formed. Substrate [^{3}H]3β-androstenol remained unchanged in all incubations. The presence of kanamycin prevented or markedly reduced the metabolism of a particular substrate. The results of incubations with [^{3}H]3α-androstenol are summarized in Fig. 2.

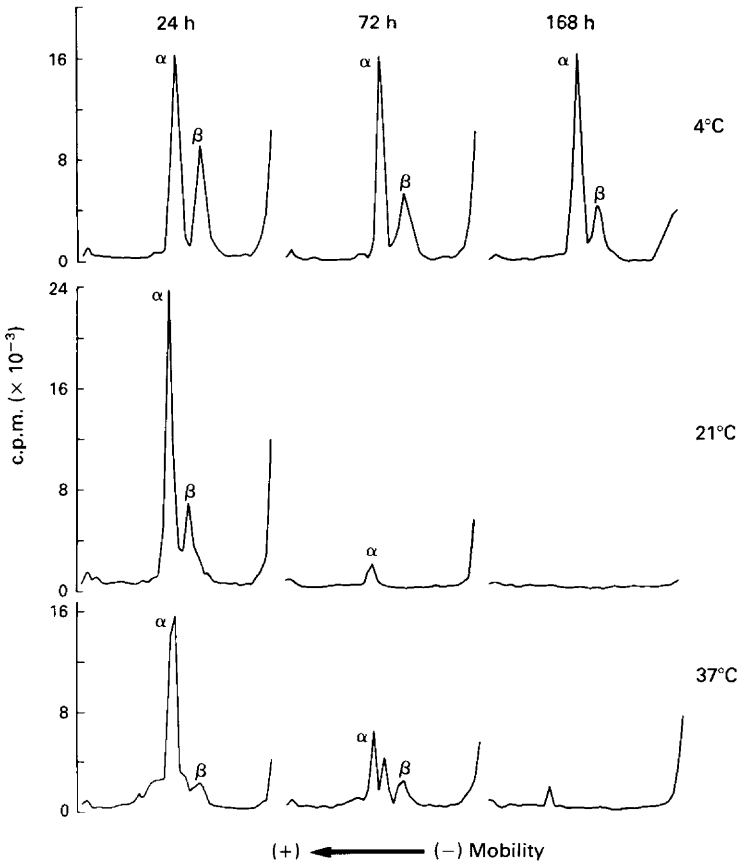

Fig. 1. Non-denaturing polyacrylamide gel (7%) electrophoresis of a boar saliva sample incubated with [^{3}H]5α-androstenone for different periods at different temperatures. The binding of the ^{3}H-labelled pheromone to its binding protein (pheromaxein existing as α- and β-charge isomers) was retained for 168 h at 4°C, but lost at 21 and 37°C due to decomposition of the binding protein.

Discussion

In the free-ranging pig, particularly the feral animal and wild boar, adult males and females are free to associate with each other at any time of the year, but the greatest degree of contact is during the breeding season. One can speculate that it is at this time when maximum amounts of saliva would be deposited in the environment with the boars being aroused during competitive encounters with each other, and by the presence of females coming into oestrus. The female pigs would therefore be exposed to high concentrations of the pheromonal steroids emanating directly from the saliva foam on the boars lips, and also from a secondary source, i.e. the saliva deposited by the boars into the environment. This 'environmental' source of pheromones might be particularly important to the wild boar since the main breeding season is during the coldest months of the year, i.e. late autumn and winter (Mauget, 1982). The retention of the pheromonal steroids in saliva deposited in the environment at this time of the year would be optimized, as indicated by the present study, with the degradation of pheromaxein being minimum at low temperatures. However as pheromaxein became slowly degraded, this would provide a background of free pheromone over a period of

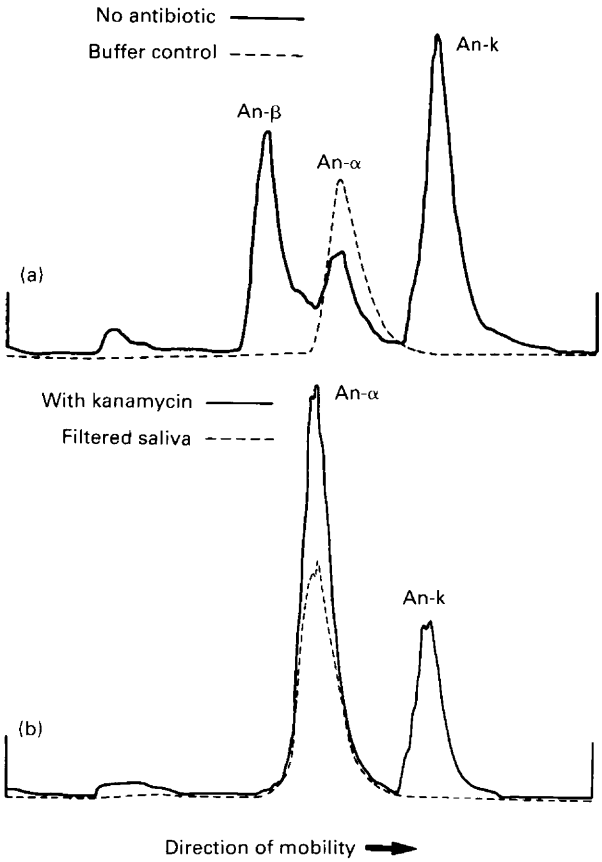

Fig. 2. Thin-layer chromatography (silica gel with solvent systems toluene:ethyl acetate, 9:1 (v/v), run twice; toluene:diethyl ether, 9:1 (v/v) run once) of diethyl ether extracts of boar saliva samples subjected to various treatments before incubation with [^{3}H]3α-androstenol for 168 h at 21°C. (a) Superimposed chromatograms of extracts arising from incubations of [^{3}H]3α-androstenol with buffer only and with untreated saliva, (b) superimposed chromatograms of extracts arising from incubations of [^{3}H]3α-androstenol with boar saliva samples containing kanamycin sulphate or filtered to remove microorganisms. An-α (3α-androstenol), An-β (3β-androstenol), An-k (5α-androstenone).

time. In nature this occurrence and the presence of boars would ensure a maximum pheromone stimulus to the female pig whereby oestrus and ovulation are facilitated (Melrose *et al.*, 1971; Reed *et al.*, 1974; Perry *et al.*, 1980; Kirkwood *et al.*, 1983; Booth, 1984a).

The present work has also shown that microorganisms of oral origin in boar saliva are likely to change the relative concentrations of the pheromonal steroids, once the saliva has been deposited in the environment. There is also the strong possibility that the metabolism of the pheromonal steroids would be affected by microorganisms already present in the environment. The results of the present study show that the metabolism of the pheromonal steroids which occurs in voided boar saliva, brought about by salivary microorganisms, proceeds from 3α-androstenol and 5α-androstenone with known priming and signalling properties, to the relatively inactive 3β-androstenol (Reed *et al.*, 1974). This pattern of metabolism parallels the sequence of reproductive events in the female pig, i.e. induction of ovarian activity, oestrous behaviour associated with ovulation and then an apparent refractory state to the pheromonal steroids in the post-ovulatory period.

Further evidence to support the finding that microorganisms were involved in the metabolism of pheromonal steroids in boar saliva, was obtained from short-term incubations of saliva from the same two boars with [^{3}H]3α-androstenol (W. D. Booth & S. H. Lambie, unpublished). In these incubations, pheromone metabolism increased with time using saliva which had been frozen and thawed without a cryoprotective agent to preserve the integrity of cells such as leucocytes. It is therefore unlikely that leucocytes were responsible for the steroid metabolism under these circumstances, although it has been demonstrated that these cells are responsible for steroid metabolism in fresh saliva collected from patients with gingivitis (Elattar, 1975). The class of steroid-transforming enzymes detected in boar saliva, i.e. 3-oxidoreductases, is commonly found in microorganisms (Charney & Herzog, 1967). Although the production of 5α-androstenone in human axillary sweat seems to be due to bacteria (Bird & Gower, 1982), it has yet to be determined which type of microorganism is responsibile for the metabolism of the pheromonal steroids in freshly voided boar saliva.

Preliminary evidence has also been obtained for the metabolism of a number of steroid hormones but excluding cortisol in incubations with boar saliva (W. D. Booth & S. H. Lambie, unpublished). This finding and those of Elattar (1975) suggest caution in studies in which steroids are measured in saliva as an alternative to blood in animals and man.

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