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A CHEMISTRY OF MAMMALIAN PHEROMONES

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Summary—Many mammalian social odors do not elicit an observable specific response in the recipient and therefore strictly cannot be considered to be pheromones. The pheromones now known in mammals are mostly transferred by contact and detected by accessory olfaction, which further indicates that pheromones in mammals should not be considered to be even a subclass of social odors. Aphrodisin, a female hamster pheromone that elicits sexual behavior in male hamsters, is a member of the lipocalycin family of 20 kDa extracellular proteins, and it is most closely related to rat odorant binding protein. Homologous proteins occur in the urine and scent glands of mice, rats and possibly voles, where they may serve as pheromone binding proteins. A 20 kDa protein, pheromaxein, binds the known pheromones androstenol and related steroids in boar saliva, and uncharacterized small proteins have been found in monkey and human skin gland secretions. Thus it appears that proteins may generally be associated with mammalian pheromones.

Early research on the chemistry of mammalian pheromones was inspired by the very successful chemical investigation of insect pheromones, which used the newly available microanalytical techniques of gas chromatography and spectrometry to identify the small amounts of volatile compounds emanating from female insects to attract males from a distance and initiate copulation. The chemical identification of bombykol, the sex pheromone of the silk moth [1], coincided with the original definition of the term pheromone, which was based on the hormone concept of regulation at a distance [2]. According to this definition a pheromone is a substance emanating from one member of a population and eliciting a specific behavioral or physiological response in other members of the same population. The early chemistry of insect sex attractants demonstrated that pheromonal effects can be elicited by single volatile compounds or simple mixtures detected at a distance by olfaction, although later results suggested that the chemistry might not be as simple as it first appeared [3]. Nevertheless, these early results continue to have a major influence on assumptions about the chemical nature of mammalian pheromones and social odors, and consequently the procedures chosen to characterize them [4].

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Another major body of research that is basic to chemical investigations consisted of the numerous studies designed to elucidate the functions of mammalian social odors. Scent marking and olfactory investigation of scents are obvious components of the social interactions within most mammalian populations [5], but often the function of a mammalian social odor such as a scent mark can be obscure. The bulk of the speculation holds that smelling these odors enables an animal to identify the sex, the physiological or emotional state, or possibly even the identity of the individual from which the scent emanates. Obviously such a communication system could be useful in the integration of a population of mammals, but this use of social odors in mammals differs from those described for insects, especially the use of odors by mammals for individual identification. This difference creates both conceptual and technical problems for the application of the pheromone concept to mammalian social odors. The conceptual difficulty was noted in the caveat of Bronson [6]:

> "it is doubtful that the term pheromone can realistically have merit when referring to the melange of odors probably used in individual identification".

The technical difficulties were already apparent in the early investigations of the chemistry of mammalian social odors. Foremost among these difficulties was the complex composition of the sources, such as castoreum from the castor sacs of the beaver [7]. This complexity is

consistent with the view that these scents enable mammals to make perceptual discrimination of populations or individuals or their inner states based on odor alone, and suggested that it might be difficult or impossible to chemically characterize pheromones in mammals because the observed biological activity is based on a response to the complex mixture as a whole, or a response to a perceived chemical image [4, 8]. It is possible that the effects of a complex scent gland secretion such as castoreum might be reproduced by a few of the components, but investigations guided by this hypothesis faced the further technical difficulty that the behavioral effects of such secretions on the recipients are not characteristic of the stimulus.

As anyone who has walked a dog knows, a mammal can appear to be very interested in the investigation of the scent marks of another member of its population, but this behavior is not reliably followed by any other specific behavioral or physiological effect. The response may depend upon uncontrollable elements of context or upon the prior experience of the subject animal, or it may be that the purpose is simply to gather information with no immediate behavioral consequences. When there are no other consistent responses to a social odor, attempts to identify behaviorally active compounds in scent gland secretions or urine have frequently relied on sniffing, that is on measures of the intensity of the olfactory investigation of a purified chemical component. The result is that the compound identified elicits sniffing from a subject test animal, but it has no further apparent effects. A specific pheromonal function for a compound identified by a sniffing bioassay can then be inferred only from more or less indirect arguments [9–11]. When sniffing is the only observable response, it is at least arguable that the compound identified as an "attractant" may not be a pheromone at all, because the response is not specific to the compound.

This is not to say that there is no use for a sniffing bioassay. A bioassay based on the time of olfactory investigation or preference does demonstrate that the subject animal is capable of detecting the stimulus or of making a discrimination between two stimuli. Such a bioassay is necessary in the elucidation of the chemical basis for olfactory discrimination. A bioassay based on sniffing however, may not be useful, even as a preliminary guide in the isolation of a pheromone, because for a pheromonal effect it is not necessary that the subject animal be aware of an odor, only that the stimulus have a specific effect on the subject. It is possible that compounds with potent pheromonal effects have no particular odor. As a consequence of this reasoning, pheromones in mammals should not necessarily be considered as a subclass of social odors. They may be more usefully considered to constitute a separate class of biologically active compounds with a function of regulation, rather than transfer of information.

Strict adherence to the pheromone concept focuses attention on a group of very interesting responses in which a specific effect is apparent and a specific bioassay may in principle be designed. Some of the most specific responses to pheromones were among those first recognized in mammals [12], for example the primer effects in which the pheromone may not have an immediately apparent behavioral effect, but typically has characteristic endocrinological effects [13]. When we look for mammalian behavioral and physiological responses to pheromones in which the responses appear to be specific, we find that a remarkable number of them are mediated by accessory olfactory organs such as the vomeronasal organ rather than by primary olfaction, and that many of the pheromones are transferred by contact [14]. Since the stimuli are not necessarily olfactory, we have to consider the possibility that they are nonvolatile, that the pheromones are not necessarily airborne, but may be transferred by direct contact of the responding animal with the stimulus source. With the removal of the restriction to volatile compounds, even macromolecules such as proteins could function as pheromones, which is what we have found in our identification of an aphrodisiac pheromone in hamsters.

The female golden hamster produces a substance which is emitted in vaginal discharge around the time of oestrus and stimulates sexual behavior in male hamsters. This pheromonal effect may be demonstrated in a bioassay using an anesthetized male as a surrogate female, which is placed in the cage of a normal male [15]. If estrous vaginal discharge is applied to the hindquarters of the surrogate female, the male will typically make several intromission attempts consisting of distinct bouts of pelvic thrusting directed at the surrogate female. The number of these bouts can be used as a measure of the activity of the pheromone in vaginal

discharge, as demonstrated by a dose–response relation [16].

The major protein aphrodisin, when isolated from vaginal discharge, elicits copulatory behavior from males in levels comparable to those elicited by the unfractionated vaginal discharge [17]. The behavioral response to high molecular weight fractions of the vaginal discharge containing aphrodisin, occurs only if the male can make contact with the stimulus source, and the response is mediated entirely by the vomeronasal organ [18, 19]. The proteinaceous nature of the vomeronasal stimulus aphrodisin has been confirmed by the loss of behavioral activity in the surrogate female bioassay after the protein was degraded with proteolytic enzymes or heat [17]. Other proteins in the vaginal discharge have no activity in the behavioral assay [20].

The amino acid sequence of aphrodisin is 40% identical with the sequence of rat odorant binding protein and 31% identical with the sequence of rat probasin (Fig. 1). These 20 kDa extracellular proteins are members of the lipocalycin family of proteins, which was named for the ability of some of its members to bind relatively small hydrophobic molecules in a interior fold between two sheets of antiparallel beta strands [21], as demonstrated by the X-ray crystal structures of serum retinol binding protein [22], and insect bilin binding protein from two species [23, 24]. Some of the other lipocalycins, for example the odorant or pyrazine binding proteins [25, 26], α-1-acid glycoprotein [27], and apolipoprotein D [28] have been shown to bind a variety of relatively small lipophilic molecules, such as odorants, retinoids, steroids and bile pigments *in vitro*, although ligands have not been found in the isolated proteins. Another lipocalycin, prostaglandin D_2 synthetase, catalyzes the specific isomerization of prostaglandin H_2 to prostaglandin D_2 and therefore presumably binds these prostaglandinds [29]. In general these proteins appear to bind with low specificity a variety of lipophilic molecules having about ten to twenty carbon atoms. In spite of the name however, only three of the twenty odd proteins in this family actually have lipophilic ligands that have been detected in the purified protein. It is therefore by no means certain that chemically purified aphrodisin should have a low molecular weight ligand, solely by virtue of its membership in the lipocalycins.

The early evidence from the ultraviolet and fluorescence emission spectra (unpublished), as well as the retention of activity with the protein on dialysis or gel filtration in aqueous media, suggested that the purified, behaviorally active vaginal discharge protein did not include a ligand [17]. More recently however, gel filtration experiments in aqueous acetonitrile buffers have indicated that a mixture of ligands may be present in the purified protein (unpublished). Activity is absent in the high molecular weight fraction, which appears to be chemically unaltered, native aphrodisin, but we have not yet been able to restore its activity by recombining it with low molecular weight fractions. If aphrodisin does have a ligand essential for activity, then this ligand must be tightly bound to survive repeated dialysis and gel permeation chromatography in aqueous buffers. The ligand itself apparently has no activity or it may decompose when it is separated from the protein. In either case it is clear that the female hamster pheromone requires the protein, and it probably requires a ligand, for biological activity.

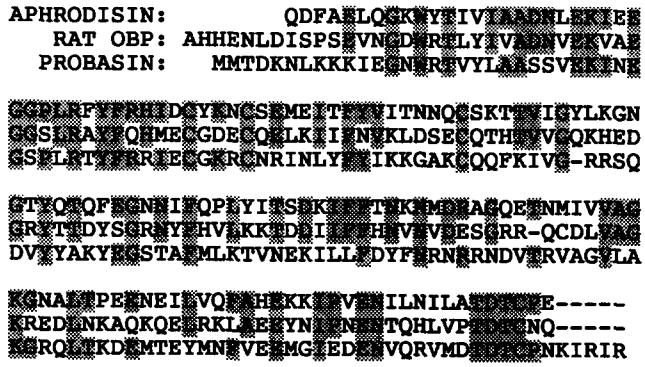

Fig. 1. An alignment of the amino acid sequence of hamster aphrodisin [53] with the sequences of rat odorant binding protein (OBP) [54] and rat probasin [55] produced by the software package CLUSTAL [56] on the sequences of the mature proteins. Amino acid residues are designated by the conventional one letter code and residues identical to those in aphrodisin are emphasized by shading.

In the house mouse a similar female pheromone that stimulates a rapid increase in circulating levels of LH in males is not absolutely dependent on protein. Like the female hamster pheromone, the mouse pheromone is probably transmitted by contact with the stimulus source and the response is mediated by the vomeronasal organ [30, 31]. Female mice excrete a high concentration (5 mg/ml) of major urinary proteins in their urine [32]. Since these proteins are lipocalycins with about 25% identity to aphrodisin, it seemed probable that the pheromonal activity would prove to be associated with this protein. Dialysis experiments indicated that the activity was associated with protein, but in contrast to the hamster results, the activity was partly dissociated from the protein by dialysis, and it could be completely dissociated from the protein by gel filtration in aqueous solvent. Most significantly, the activity was not destroyed by enzymatic degradation of the protein [33].

Apparently the protein is not necessary for the activity of the female mouse pheromone, but it is possible that it enhances the biological activity. This remains to be experimentally demonstrated by dose–response measurements comparing the responses to various doses of ligand and protein–ligand complex. Recalling that the activity of aphrodisin was destroyed by proteolytic enzymes, and in as much as sequence similarity suggests homologous function in the two proteins, we can speculate that the ligand of aphrodisin is more tightly bound to the protein and is more unstable than the mouse ligand when it is separated from the protein. Some earlier chemical work on the pheromones responsible for the primer effects of male mouse urine suggested that the major urinary protein had pheromonal activity [34, 35], but as we observed for the female mouse pheromone, bioassay of the products of further chemical fractionations indicated that relatively lower molecular weight stimuli are also active [36, 37].

The common occurrence of lipocalycins and other uncharacterized extracellular 20 kDa proteins in urine, saliva, and scent gland secretions associated with pheromonal effects does fuel speculation that these proteins are pheromones or pheromone binding proteins in mammals [38, 39]. The major urinary proteins have been extensively investigated as favorable subjects in which to elucidate the mechanisms of hormonal regulation of protein synthesis. In the course of the investigation of this and related questions, several results have emerged incidentally that are consistent with a role for these proteins in pheromonal communication.

The major urinary protein in the rat and the mouse consists of several closely related variants encoded by a family of 20 to 30 genes [38, 40–42]. The various sequences are differentially regulated by multiple hormones, and they occur in developmentally and sexually specific combinations in a number of skin glands producing external secretions, as well as in urine [43–45]. Histological studies have found close association between major urinary protein and lipids in preputial, meibomian and perianal glands, and have suggested that these proteins are combined with lipid in peroxisomes before they are secreted [39]. Their propensity for combination with hydrophobic molecules apparently is involved in a nephropathy specific to male rats resulting from exposure to a component of unleaded gasoline and other chemicals, such as limonene, that are bound to the protein [46]. And finally, the physiological potency of this protein is indicated by its ablity to stimulate the pituitary–testicular axis in estrogenized male rats [47], but in spite of all this and much more work on the major urinary proteins, their function is not yet established.

Aphrodisin and the major urinary proteins are the only known lipocalycins yet demonstrated to be associated with pheromones in mammals, but there are some examples of proteins that occur in skin gland secretions or urine with known or potential pheromonal function. These uncharacterized proteins have some of the salient properties of the lipocalycins, that is they are extracellular, abundant proteins with a molecular mass of about 20 kDa and a relatively high negative charge. An abundant, androgen dependent protein with the same molecular weight as the major urinary protein occurs in bank vole urine [48]. Other minimally characterized 20 kDa proteins are found in the secretions of perianal glands of a South American primate [49], and in human armpit secretions [50].

A partially characterized, abundant, negatively charged (pI: 4.78, 5.35), extracellular protein, pheromaxein binds the known pheromone androstenol and related steroids in boar submaxillary gland saliva [51, 52]. This is the only pheromone binding protein in mammals for which the ligand is known, and this ligand is a stable, readily available compound. It therefore would be of considerable interest to know whether this protein is a lipocalycin and what

is its effect on the pheromonal action of androstenol on female pigs. If the effect of the protein on pheromonal activity could be measured in a reliable bioassay, and if the protein were characterized, it would then be possible to design experiments to determine the importance of the various potential interactions between transport protein, ligand and nasal chemoreceptors.

This is what we hope to do with aphrodisin in the hamster when we have characterized the putative ligand. There are many questions that remain unanswered about the occurrence and function of these proteins and their ligands in mammalian pheromonal responses, but the available evidence at least demonstrates that application of the original pheromone concept requiring a specific response in a bioassay can lead to a coherent chemistry of mammalian pheromones.

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