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LIGANDS OF URINARY LIPOCALINS FROM THE MOUSE: UPTAKE OF ENVIRONMENTALLY DERIVED CHEMICALS

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Abstract—Mouse urine contains large quantities of proteins (major urinary proteins, MUPs) that are thought to function by binding lipophilic and volatile semiochemicals in a central calyx of the MUP. Two notable semiochemicals are 2-*sec*-butyl-4,5-dihydrothiazole and a brevicomin (3,4-dehydro-*exo*-brevicomin). MUPs derived from deposits of urine from wild caught mice contain neither of these ligands, but are replete with menadione. The menadione is probably incorporated in vitro from the environment, although some incorporation in vivo can also be demonstrated. These data show that the calyx of MUPs can bind other hydrophobic molecules derived from the environment, which may influence longevity of signal and deposition patterns of urinary scent marks. The ability to displace, rapidly and completely, the natural ligands by menadione also provides a new tool in the analysis of MUP function.

Key Words—Major urinary protein, menadione, olfactory communication, scent marking, mouse.

INTRODUCTION

Mouse urine contains large quantities of major urinary proteins (MUPs) that are members of the lipocalin family (Flower, 1996) and are products of a multigene

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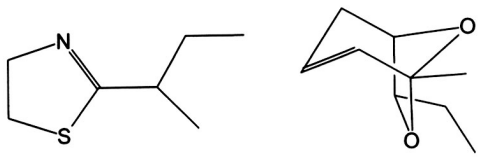
family highly expressed in liver (Finlayson et al., 1965; Knopf et al., 1983; Hastie and Held, 1978). The MUPs then enter the circulation, are efficiently filtered by the kidney, and are released in the urine. In urine from two different inbred mouse strains, there are at least 14 different MUPs. Full or partial cDNA sequence and mass spectral data from wild-caught individuals suggest the existence of more than 40 MUPs, but some of these have not been identified in urine. The total number of allellomorphs of these proteins must be large. Sequence variation is present in about 40% of residues, the majority of which are located at surface exposed residues.

MUPs derived from laboratory and wild house mice bind two ligands (Figure 1), a thiazole (2-*sec*-butyl-4,5-dihydrothiazole) and a brevicomin (3,4-dehydro-*exo*-brevicomin) (Bacchini et al., 1992; Robertson et al., 1993). X-ray electron density in the central calyx region is compatible with substantial occupation of the calyx with the thiazole (Bocskei et al., 1992). These ligands convey signals of male dominance (Novotny et al., 1985), and MUP binding may protect semiochemicals or facilitate a slow release of these otherwise volatile molecules. However, the precise role of the MUPs is untested, and current hypotheses are limited by the need for correlative biochemical and behavioral experiments.

In natural environments, mice deposit urine as scent marks in continuous streaks, small spots, or sometimes repeatedly in the same place, such that discrete posts are built (Hurst, 1987). These signals are used by males to advertise dominance over their territories (Gosling and McKay, 1990; Hurst, 1993). The purpose of the different deposition patterns is unclear but may be dictated by the need to replenish, in strategic sites, volatile molecules that are otherwise lost by evaporation. As part of a study of MUP heterogeneity and ligand binding in wild mice, we have examined the chemical composition of posts deposited by wild mice (*Mus domesticus*) in natural or seminatural environments. In this



I II

FIG. 1. Natural ligands bound to MUPs. The two ligands that have been observed to be bound to MUPs are I, 2-*sec*-butyl-4,5-dihydrothiazole, and II, 3,4-dehydro-*exo*-brevicomin.

manuscript, we report that MUPs derived from posts contain ligands derived from the environment that may modulate the signaling capabilities of these proteins or influence deposition patterns. Moreover, the ability to displace natural ligands opens up new opportunities for experiments to assess the structure–function relationships of this class of proteins.

METHODS AND MATERIALS

Recovery of Urine Post Material. Urine posts were collected from single wild male mice maintained in large enclosures. Portions (1 g) of the post material were homogenized in 5 ml of deionized water. The homogenate was clarified by centrifugation at 12,500*g* for 5 min and the supernatant was removed. MUPs (typically 100–200 μl) were recovered from the supernatant by size exclusion chromatography (SEC) on spun columns of Sephadex G25. MUPs were recovered from the fresh urine of wild mice and BALB/C inbred mice in the same way.

SDS-PAGE. SDS-PAGE was performed as described in Laemmli (1970). All samples were run under reducing conditions in 17.5 (w/v) acrylamide gels. Gels were run for 1 hr at 100 V, and the proteins were stained with Coomassie blue. Separated proteins were electroblotted to a nitrocellulose membrane. In this case, the gel was equilibrated in transfer buffer [25 mM Tris, 192 mM glycine, 20% (v/v) methanol, pH 8.3] for 30 min and subsequently overlaid with the nitrocellulose membrane in a blotting cassette. Electroblotting was subsequently conducted at 30 V overnight. The membrane was then probed with a 1 : 1000 dilution of polyclonal antibody to MUPs, purified from inbred BALB/C mice, in PBS–Tween (10 mM sodium phosphate, 0.15 M sodium chloride, 0.05% Tween 20, pH 7.4).

Electrospray Mass Spectrometry. The MUPs isolated from urine posts by SEC were further analyzed by electrospray ionization mass spectrometry (ESI-MS). The desalted urine post extract was diluted 1 : 10 with an aqueous solution of 50% (v/v) acetonitrile, 0.1% (v/v) formic acid. This solution was introduced into the mass spectrometer as a continuous infusion at 5 μl/min. The instrument used for this analysis was a VG-Quattro I, triple quadrupole mass spectrometer, upgraded to Quattro-II specifications, and fitted with an electrospray ionization source. Raw data were acquired between m/z 900 and 1600. These data were transformed to a true mass scale and refined with Maximum Entropy software. The Maximum Entropy refinement was conducted between masses 18,500 and 19,000 Da at 1 Da/channel with a peak width parameter of 0.75 Da. The instrument was calibrated with a 2 pmol/μl solution of horse heart myoglobin (Sigma Chemicals), the spectrum for which was acquired immediately after the urine post sample under identical conditions. The calibration spectrum also was

used to determine the peak width parameter used during Maximum Entropy processing (Micromass, Altringham, UK).

Volatile Ligand Analysis. The ligands associated with MUP fractions from urine posts and from freshly collected urine samples were analyzed by chloroform extraction and gas chromatography–mass spectrometry (GC-MS). Three volumes (typically 300 μl) of desalted MUPs were added to two volumes of chloroform and vortexed for 10 sec. The vortexed solution was allowed to stand at room temperature for 60 min prior to removal of the chloroform. A 2-μl aliquot of this extract was injected onto a Carbowax 20 M column in an HP 5890 gas chromatograph equipped with an HP 5971A mass selective detector and an on-column injector. The column head was attached to 45 cm of deactivated silica retention gap precolumn, and the column was run in a stream of pure helium at a head pressure of 10 psi. The oven temperature at the time of injection was 60°C, which was maintained for 7 min after injection. The temperature was then increased to 200°C at 10°/min and maintained at 200°C for 5 min. The mass selective detector was run in scan mode, between *m/z* 50 and 550. Menadione was identified by comparison of its sample mass spectrum to the Wiley library of mass spectra and to that of authentic menadione (Sigma Chemicals).

Menadione Incorporation Studies. Stock solution of 20, 10, 4, 2, 1, and 0.4 mg/ml menadione were made in absolute ethanol. A 10-μl aliquot of each solution was added to 190 μl of desalted MUPs isolated from BALB/C mice maintained on standard laboratory diet (dietary content of menadione = 30 mg/kg). This resulted in the MUPs being exposed to 1, 0.5, 0.2, 0.1, 0.05, and 0.02 mg/ml concentrations of menadione in 5% (v/v) ethanol. The MUPs were then recovered by SEC and the bound menadione and thiazole were extracted into chloroform as described above. The chloroform extract was then analyzed by GC-MS in selected ion monitoring (SIM) mode, where the selected ions were *m/z* 60 (thiazole) and *m/z* 172 (menadione).

Male BALB/C mice (N = 8) were injected subcutaneously with 100 μl of 50% (v/v) ethanol containing 0.2 mg of menadione or with solvent alone. At time intervals, urine samples were obtained from the animals by gentle bladder massage, and the MUPs were isolated by SEC. Bound menadione, thiazole, and brevicomin were analyzed by chloroform extraction and GC-MS with selected ion monitoring as described above.

Molecular Modeling. All structural analyses and simulations were performed with the X-ray crystal structure of MUP (PDB code: 1MUP) determined by Bocskei et al. (1992). The cavity of the MUPs was analyzed with the MSP program suite of Connolly (1993) to determine cavity size and volume. Docking simulations involving the menadione ligand and water molecules in the MUP cavity were performed with the Biased Probability Monte Carlo method of the

ICM program (Abagyan and Totrov, 1994; Abagyan et al., 1994). Simulations were propagated for 100,000 steps by using the standard ICM force field with the menadione ligand placed in the central cavity along with either 0, 1, 2, or 3 water molecules (all assigned initial random positions) at a temperature of 300°K. The ICM force field contains specific terms for van der Waals forces, electrostatic interactions, torsion angles, hydrogen bonds and atomic solvation. For each simulation, the menadione ligand and water molecules were assigned a random conformation within the cavity boundary and were unconstrained (except for a weak restraining term to keep them within the vicinity of the cavity). All atoms in the protein were fixed except for side chain moieties of amino acids forming the cavity surface. The lowest energy conformation from each simulation was further considered.

RESULTS AND DISCUSSION

Urine posts contained solid fecal matter and food residues, in addition to substantial quantities of protein (38 ± 6 mg/g wet weight post material, mean ± SEM, $N = 4$). The protein was predominantly an 18-kDa species that is strongly immunoreactive to a MUP antiserum and therefore highly likely to be MUP (Figure 2). Electrospray mass spectrometry confirmed that the MUPs were of the masses predicted from cDNA sequences (Robertson et al., 1996) and were thus largely intact in the posts. This confirms the belief that the posts are formed by urine marking (Hurst, 1987). The samples from wild-caught animals contained MUPs of masses 18,647 ± 2 Da, 18,691 ± 2 Da, and 18,707 ± 2 Da that had been observed previously (Robertson et al., 1996, 1997), and additional proteins, notably those of 18,808 Da, 18,971 Da, and 19,032 Da that might represent yet further alleles of this multigene family (Pes et al., 1998). The total number of MUPs is unknown, but from our observations on inbred and wild strains there must be in excess of 20 allelic variants that are expressed. Whether the different alleles exert subtly different functional characteristics is unknown.

The proteins recovered from urine posts were also analyzed by GC-MS for their associated volatiles. In contrast to MUPs recovered from fresh urine samples derived from either inbred mice or wild mice, we were unable to observe bound components with GC retention times or mass spectra characteristic of the thiazole and the brevicomin. Instead, less volatile compounds were evident. In particular, one component ($R_t = 22.99$ min) was abundant and gave a discrete mass spectrum that was consistent with that of a quinone. Library searches revealed that the best match was to menadione (vitamin K_3); the identification was substantiated by GC-MS analysis of the authentic compound (Figure 3).

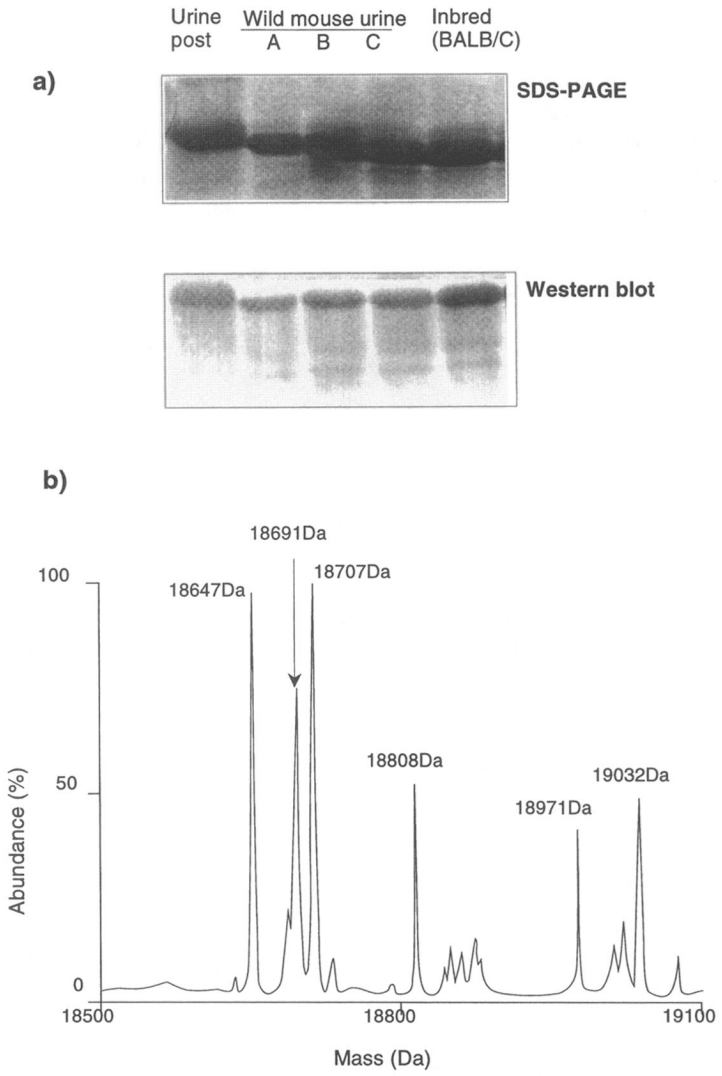


FIG. 2. Protein components in urine posts. (a) The SDS-PAGE analysis and western blot of proteins derived from the urine post and fresh urine samples from wild and inbred mice; (b) electrospray ionization mass spectrometry analysis of the proteins recovered in urine posts.

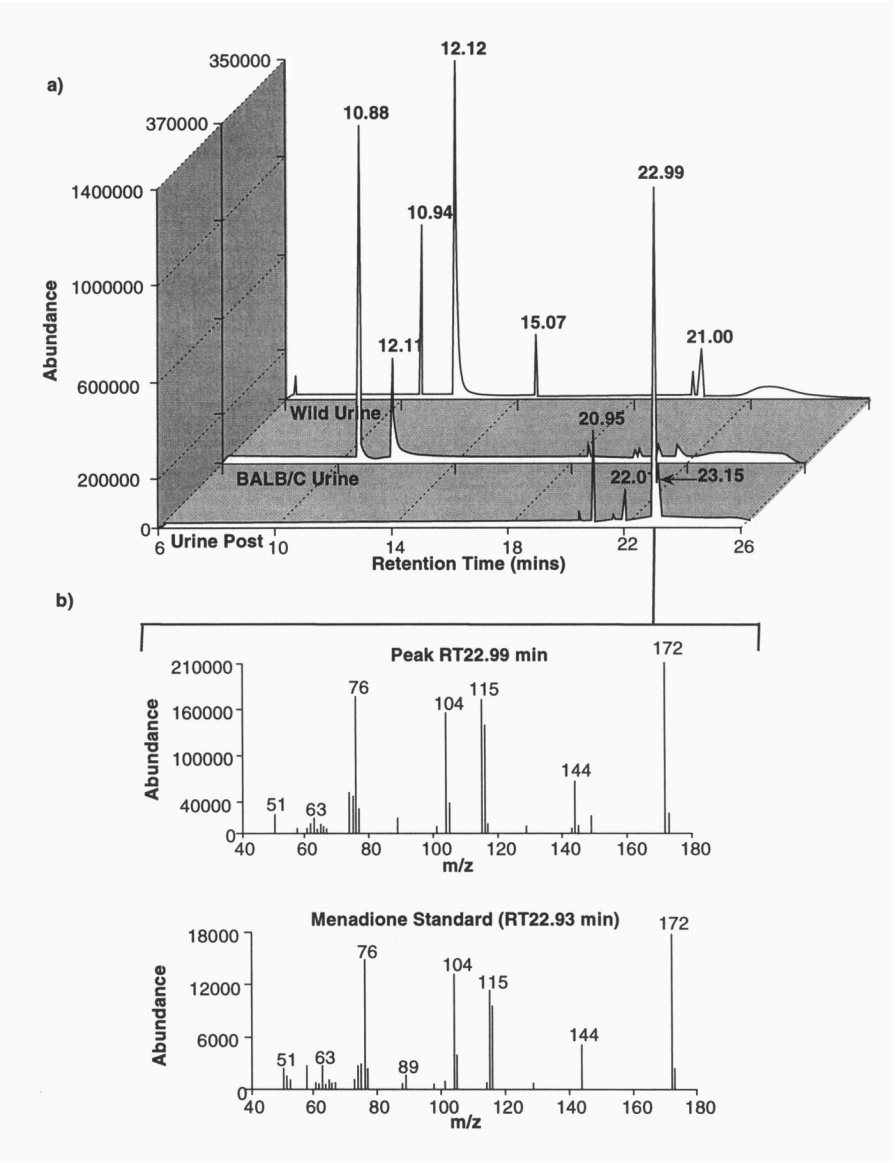


FIG. 3. Identification of ligands in urine samples and posts from wild-caught mice. (a) Gas chromatograms (GC) of bound ligands from urine posts, fresh urine from an inbred male mouse (BALB/C), and fresh urine from a wild male mouse maintained in captive conditions, (b) electron impact mass spectrum of the component with a GC retention time of 22.99 min from urine posts is compared with the mass spectrum of authentic menadione, which showed the same retention time.

The data are consistent with occupancy of the calyx by menadione in place of the thiazole and brevicomin. Upon inspection, trace amounts of menadione were also associated with MUPs in urine samples freshly collected from mice that had been fed laboratory diet (results not shown).

For menadione to displace MUPs, the central calyx of the protein must be able to accommodate the larger quinoid molecule. The overall orientation of MUP and the central cavity are illustrated in Figure 4A. Structural analysis of the ligand binding calyx of the crystal structure of MUP (Bocskei et al., 1992) revealed a large hydrophobic void (475 $Å^3$) into which the small thiazole molecule (volume 136 $Å^3$) was a loose fit, with a further two putative water molecules assigned within the cavity (Figure 4B). The slightly bulkier menadione (volume 151 $Å^3$) was also readily accommodated in the cavity (Figure 4C), and the optimal binding and orientation were analyzed by molecular dynamics with the ICM program. The lowest energy conformer from computer docking analyses repeatedly oriented the menadione in the same position in the cavity, regardless of the starting orientation and the solvent occupancy in the simulation. No unfavorable geometric or spatial constraints between the ligand and the MUP calyx were observed. Furthermore, menadione makes favorable interactions with surrounding hydrophobic protein side chains including a hydrogen bond, via a water molecule, to one of the quinoid oxygens in the otherwise unsatisfied hydroxyl group of Tyr_{124} (Figure 5). The molecular interactions of menadione and the binding pocket are consistent with a putative ligand and are at least as favorable as those made by thiazole in the crystal structure. The calyx is able to accommodate this ligand with ease. It is unclear whether the displacement is a consequence of tighter binding of menadione, or whether this reflects a much higher concentration of the displacer molecule (i.e., menadione) than the natural ligands. The insolubility of menadione and the lack of ligand-free protein preclude direct measurement of the dissociation constants for the individual ligands.

Menadione was identified at high levels only in urine post samples and not in freshly collected urine samples, whether from wild or inbred animals. This raises the issue of the mechanism of incorporation of menadione into the calyx. It was likely that the menadione was bound from a source in the environment, and the most likely source was the diet upon which these animals were maintained, since the artificial laboratory diet contained 150 mg/kg vitamin K_3. Because the posts contained solid food residues, the menadione could have become incorporated into the protein calyx after the urine was deposited. To test this idea, we isolated MUPs from inbred animals and incubated them with menadione (Figure 6). Menadione was able to bind rapidly (within 60 sec, data not shown) to MUPs in a saturable fashion, and the binding was coincident with virtually complete displacement of the natural ligands from the protein calyx in vitro. Because menadione is largely insoluble in water, it is not possible to estimate the binding constant for this ligand. However, the proteins are approx-

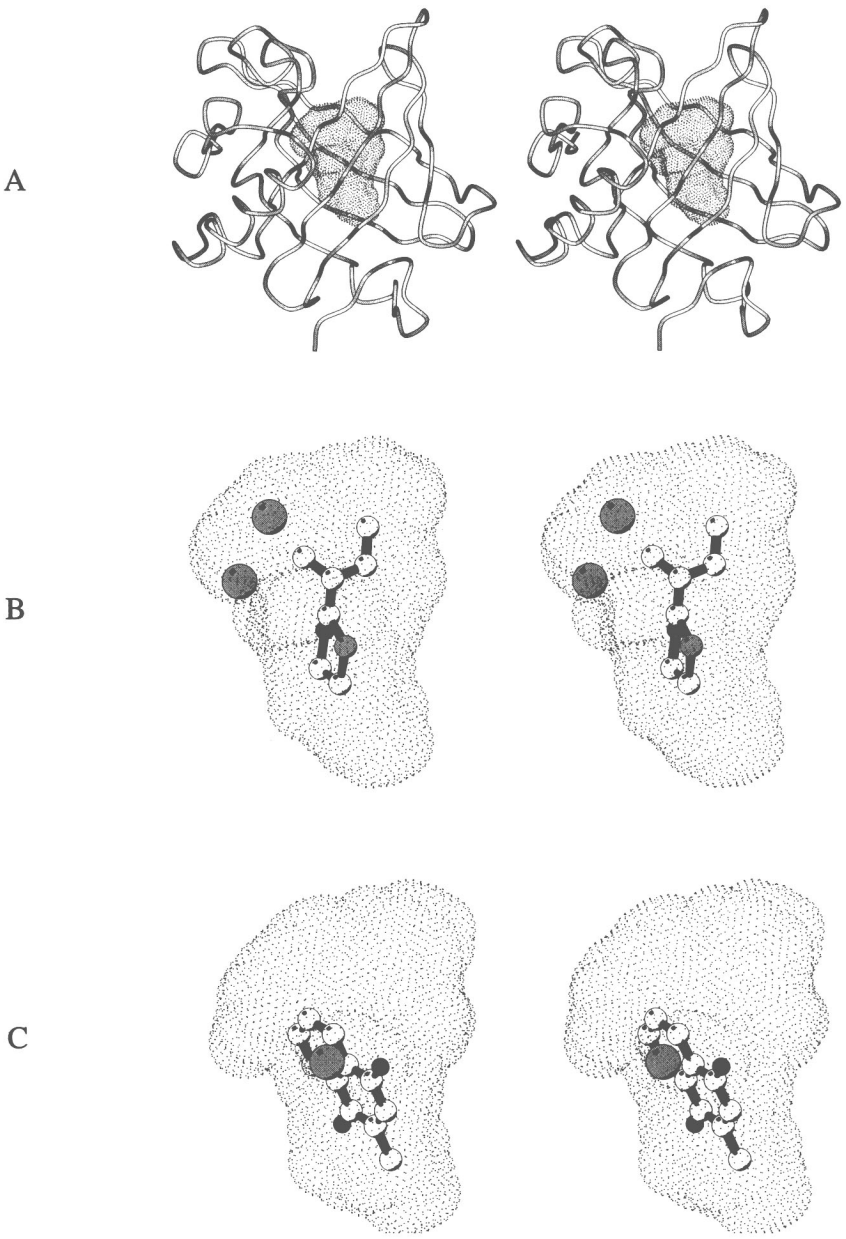


FIG. 4. Thiazole and menadione binding to MUPs. (A) The MUP ribbon structure encloses the binding cavity (dotted area); (B) a close-up of the cavity shows the relatively loose fit of the thiazole ligand into the cavity in a ball-and-stick representation, with the two solvent molecules depicted as grey spheres, (C) the lowest energy conformation of a number of docking simulations involving the menadione ligand and a single water molecule in the MUP cavity.

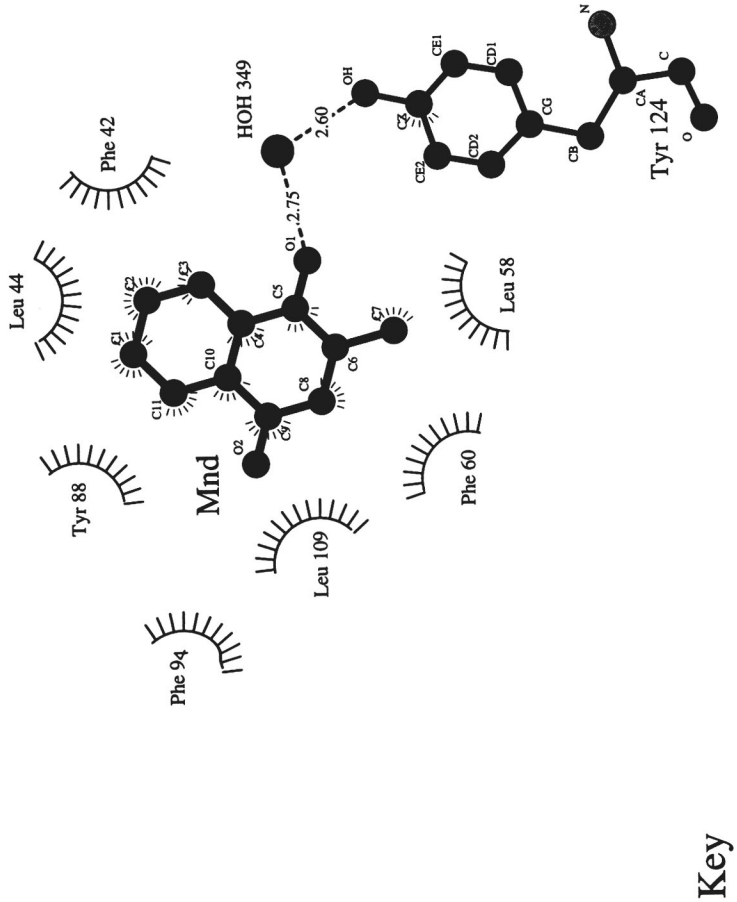


FIG. 5. Putative interactions between MUP and menadione. A LIGPLOT diagram (Wallace et al., 1995) of the modeled MUP–menadione complex indicates that the interactions are predominantly hydrophobic but that a stabilizing hydrogen bond can be formed between one of the quinoid oxygen atoms and the side chain of Tyr 124 via a water molecule.

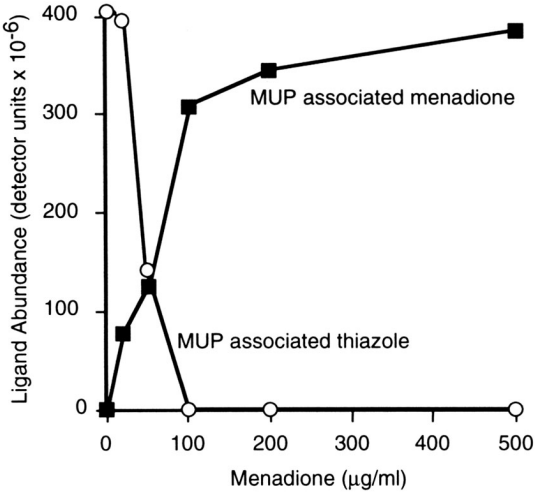


FIG. 6. Binding of menadione to MUPs in vitro. MUPs were isolated by Sephadex G25 size exclusion chromatography from BALB/C mice maintained on standard laboratory diet (menadione = 30 mg/kg). The MUPs were exposed to increasing quantities of menadione before being recovered for analysis of bound menadione and thiazole by GC-MS with selected ion monitoring.

imately 50% saturated at nominal ligand concentrations of 100 μg/ml. At 100 μg/ml menadione, thiazole was totally displaced from the ligand–protein complex.

Some menadione, absorbed across the gut, might have been incorporated into the MUPs during biosynthesis and secretion. Such a mechanism would allow for the variation of ligand content in MUPs, perhaps associated with changed metabolic or physiological status (for example, changes attendant upon diet, infection, or after puberty). To assess the possibility of incorporation in vivo, inbred mice were injected with menadione, and MUPs were monitored for protein-bound menadione over the next four days (Figure 7). Within 4 hr, the released MUPs contained menadione, although the thiazole and brevicomin signals were not appreciably reduced. After 24 hr, the menadione signal had returned to zero, attesting to the rapidity of association of menadione with MUPs and the apparently high turnover of these proteins. Although incorporation in vivo is demonstrable, it seems unlikely that it is responsible for the high degree of occupancy seen in the urine posts.

MUPs are probably capable of binding a broad range of lipophilic molecules and may modulate complex olfactory signals through a wide repertoire of volatiles. The rat equivalent, α-2u proteins, are known to bind a range of xeno-

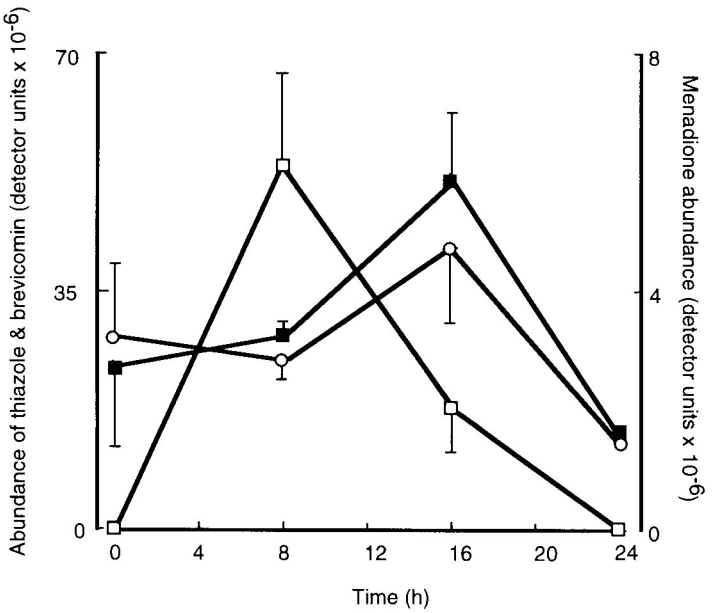


FIG. 7. Binding of menadione to MUPs in vivo. Male BALB/C mice were injected subcutaneously with 100 μl of 50% (v/v) ethanol containing 0.2 mg of menadione or with solvent alone. At time intervals, urine samples were recovered, the urinary MUPs were isolated by SEC, and bound menadione, thiazole, and brevicomin were analyzed by chloroform extraction and GC-MS with selected ion monitoring. The data are presented as mean $\pm$ SEM (N = 3–8, depending upon availability of urine samples from individual mice at each time point) (open squares, menadione; filled squares, brevicomin; open circles, thiazole).

biotics (Borghoff et al., 1991; Lehman-Keeman and Caudhill, 1992). Since association may occur in vivo or in vitro, these volatiles may derive from the diet, or from other lipophilic molecules present in the environment and accumulated after the MUPs have been released. It is likely that most of the menadione in urine post MUPs was incorporated in vitro, as the posts contained material (including food particles) derived from the environment and held in place by the sticky, semi-dry, protein-rich mass.

The consequence of menadione binding to deposited MUPs is the attendant loss of natural ligands. If the rate of loss of these ligands is greater than would occur by natural dissociation and evaporation, then repeated urine marking, leading to the formation of urine posts, may reflect the need to replenish natural signals because of accelerated displacement of natural semiochemicals by lipophilic molecules from the environment. At present, we have no evidence for a

specific or natural signaling or displacer role of menadione; its incorporation into MUPs in vitro might simply reflect its abundance in the single food source to which these animals have access. This is consistent with our inability to observe the thiazole and brevicomin ligands in urine posts, whether derived from wild or seminatural environments.

MUPs are expressed as a multigene family (Hastie and Held, 1978), and there are at least 10 allelic variants of MUPs in inbred mice (Robertson et al., 1996) and many more in wild-caught mice (Pes et al., 1998). Differences in the structure of the various allelomorphs may modulate specificity of ligand binding or kinetics of release of volatile molecules and thus add a new dimension to the subtleties of chemical signaling. The demonstrable ability of MUPs to bind other nonpolar molecules also suggests that they may find utility in the slow release or protection of a broad range of lipophilic semiochemicals, which in turn may be of value in biological control of mice and other species. Finally, displacement of natural ligands by lipophilic competitors such as menadione offers new possibilities for exploration of the role of the urinary proteins in semiochemical expression. Indeed, the utility of this tool is apparent in our recent work and has provided direct evidence, for the first time, in support of the hypothesis that MUPs act as a slow release mechanism for their associated volatiles (Hurst et al., 1998).

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REFERENCES

ABAGYAN, R. A., and TOTROV, M. M. 1994. Biased probability Monte Carlo conformational searches and electrostatic calculations for peptides and proteins. *J. Mol. Biol.* 235:983–1002.

ABAGYAN, R. A., TOTROV, M. M., and KUZNETSOV, D. N. 1994. ICM—a new method for protein modelling and design. Applications to docking and structure prediction from the distorted native conformation. *J. Comp. Chem.* 15:488–506.

BACCHINI, A., GAETANI, E., and CAVAGGIONI, A. 1992. Pheromone binding proteins of the mouse *Mus musculus*. *Experientia* 48:419–421.

BOCSKEI, Z., GROOM, C. R., FLOWER, D. R., WRIGHT, C. E., PHILLIPS, S. E. V., CAVAGGIONI, A., FINDLAY, J. B. C., and NORTH, A. C. T. 1992. Ligand binding of two rodent pheromone transport proteins revealed by X-ray crystallography. *Nature* 360:186–188.

BORGHOFF, S. J., MILLER, A. B., BOWEN, J. P., and SWEENBERG, J. A. 1991. Characteristics of chemical binding to alpha 2u-globulin in vitro—evaluating structure—activity relationships. *Toxicol. App. Pharmacol.* 107:228–238.

CONNOLLY, M. L. 1993. The molecular surface package. *J. Mol. Graphics* 11:139–141.

FINLAYSON, J. S., ASOFSKY, R., POTTER, M., and RUNNER, C. C. 1965. Major urinary protein complex of mice: Origin. *Science* 149:981–982.

FLOWER, D. R. 1996. The lipocalin protein family: structure and function. *Biochem. J.* 318:1–14.

GOSLING, L. M., and MCKAY, H. V. 1990. Competitor assessment by scent-matching: An experimental test. *Behav. Ecol. Sociobiol.* 26:415–420.

HASTIE, N. D., and HELD, W. A. 1978. Analysis of mRNA populations by cDNA-mRNA hybrid mediated inhibition of cell free protein synthesis. *Proc. Natl. Acad. Sci. U.S.A.* 75:414–417.

HURST, J. L. 1987. The functions of urine marking in a free-living population of house mice, *Mus domesticus* Rutty. *Anim. Behav.* 35:1433–1442.

HURST, J. L. 1993. The priming effects of urine substrate marks on interactions between male house mice, *Mus musculus domesticus* Schwarz & Schwarz. Anim. Behav. 45:55–81.

HURST, J. L., ROBERTSON, D. H. L., TOLLADAY, U., and BEYNON, R. J. 1998. Proteins in urine scent marks of male house mice extend the longevity of olfactory signals. *Anim. Behav.* 55:1289–1297.

KNOPF, J. L., GALLAGHER, J. A., and HELD, W. A. 1983. Differential multihormonal regulation of the mouse major urinary protein gene family in the liver. *Mol. Cell Biol.* 3:2232–2241.

LEHMAN-KEEMAN, L. D., and CAUDHILL, D. 1992. Alpha-2u-globulin is the only member of the lipocalin protein superfamily that binds to hyaline droplet inducing agents. *Toxicol. Appl. Pharmacol.* 116:170–176.

LAEMMLI, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T_4. *Nature* 227:680.

NOVOTNY, M., HARVEY, S., JEMIOLO, B., and ALBERTS, J. 1985. Synthetic pheromones that promote inter-male aggression in mice. *Proc. Natl. Acad. Sci. U.S.A.* 82:2059–2061.

PES, D., ROBERTSON, D. H. L., HURST, J. L., and BEYNON, R. J. 1998. Molecular heterogeneity of MUPs from wild mouse populations, *in* R. Johnston (ed.). Chemical Signals in Vertebrates. Plenum Press, New York. In press.

ROBERTSON, D. H. L., BEYNON, R. J., and EVERSHED, R. P. 1993. Extraction, characterization, and binding analysis of two pheromonally active ligands associated with major urinary protein of house mouse (*Mus musculus*). *J. Chem. Ecol.* 19:1405–1416.

ROBERTSON, D. H. L., COX, K. A., GASKELL, S. J., EVERSHED, R. P., and BEYNON, R. J. 1996. Molecular heterogeneity in the major urinary proteins of the house mouse *Mus musculus*. *Biochem. J.* 316:265–272.

ROBERTSON, D. H. L., HURST, J. L., BOLGAR, M. S., GASKELL, S. J., and BEYNON, R. J. 1997. Molecular heterogeneity of urinary proteins in wild house mice populations. *Rapid Commun. Mass Spectrom.* 11:786–790.

WALLACE, A. C., LASKOWSKI, R. A., and THORNTON, J. M. 1995. A program to generate schematic diagrams of protein:ligand interactions. *Protein Eng.* 2:127–134.