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Dynamics of excretion of urinary chemosignals in the house mouse (*Mus musculus*) during the natural estrous cycle

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Summary. The volatile fraction of urinary metabolites was investigated chromatographically at five different stages of the natural estrous cycle. A very substantial endocrine dependency has been noted for 11 compounds: 4 ketones, 2 acetate esters, 3 dihydrofuran isomers, dehydro-*exo*-brevicomin, and 2,5-dimethylpyrazine. The compounds were structurally verified through combined gas chromatography/mass spectrometry.
Key words. Estrous cycle; female mouse urine; urinary volatiles; capillary gas chromatography/mass spectrometry.

Introduction

In a rather complex sequence of olfactory communication processes that occur in mice [1–3], the urine of estrous animals may carry a variety of messages to both males and females. The physiological and behavioral phenomena that are readily observed in association with the estrus of rodents include an increase in activity and a phase advance in circadian rhythm[4] and a distinct change in the urination patterns[5,6]. Moreover, male mice are sexually attracted by estrous odors[7].

Occurrence and length of estrous cycle are known to be influenced by certain olfactory signals originated from other females in a social group. While the onset of the first estrus is delayed[8,9] by a signal from grouped adult females, the urine from estrus, pregnant, or lactating animals appears to accelerate the sexual development of young females, in addition to promoting estrus in adult conspecifics[10–12]. According to Drickamer, the chemosignals involved are influenced by population density[12] and dominance[13] of the donor females. The female-to-female puberty-accelerating signals have not been chemically characterized at present, but it is known that both the bladder and excreted urine are biologically active[14].

As a part of our on-going efforts to chemically characterize[15–20] and biologically test[18,19] the urinary volatile substances excreted by female mice, we have designed an experiment in which the variations of these volatiles were followed during the natural estrous cycle. Statistically significant alterations in the excretion of several volatile urinary constituents are reported here and correlated with the well-characterized stages of the female mouse ovarian cycle.

While the selected volatiles were measured quantitatively by the established chromatographic methodology, their structures were verified through mass spectra and chromatographic retention of authentic compounds.

Materials and methods

All mice used in this experiment were from a randomly-bred, closed colony of ICR/Alb (*Mus musculus*) purchased from Ward's Natural Science Establishment, Inc., Rochester, New York. Females were individually housed in plastic cages (12 × 28 × 27 cm), maintained at 21 ± 0.2 °C, 50–70% humidity, and a 12-h-light/12-h-dark schedule (lights on at 06.00 h). Unlimited amounts of Purina Mouse Chow (Ralston Purina Corp., St. Louis, Missouri) and water were supplied throughout the whole experimental period. Bedding was changed weekly.

Virgin ICR/Alb females, 2–3 months of age, were used as urine donors. They were maintained in olfactory isolation from males for the duration of the entire experiment. Vaginal smears were taken daily from each animal between 09.00 and 10.00 h in the morning throughout a two-week period before urine collections were started. Only animals (n = 14) with a regular 5–6-day cycle were used as the urine donors. Smears were examined microscopically at 60 × magnification, and classified as to the stage of the cycle according to the criteria by Rugh[21] and Vandenbergh[22]. Since 86% of estrous cycles investigated in 14 regularly cycling females exhibited a two-day estrus, the urines of the first day of estrus (e) were collected separately from the second-day samples (e II). Urine from diestrus (d) was collected regardless of the length of this stage. However, more than 60% of the investigated females showed one-day long diestrus. Proestrus (P) and metestrus (mt) in all cases lasted one day.

Urine samples were collected from 10.00 to 16.00 h, using metabolic cages; during collection, the collection vessels were placed in dry ice.

Immediately after the collection, the samples were stored at −20 °C until analyzed. To collect 1 ml of urine from the same female in one of the well-established stages of the estrous cycle, the collection had to be conducted for 2 or 3 consecutive estrous cycles of hers. 3–8 1-ml samples of urine were collected from the same estrus stage of different females. Thus, for each stage of the estrous cycle urines were analyzed 3–8 times.

Urine samples (1 ml) were analyzed by capillary gas chromatography, using glass capillary columns (60 m × 0.25 mm i.d.) coated with UCON-HB-2000[15,16]. Prior to gas chromatography, the urinary volatiles were concentrated on a precolumn of Tenax GC, as previously described[20,23]. A gas chromatograph Varian Model 1400 (Walnut Creek, California) equipped with a flame ionization detector was employed. Quantitative comparisons of chromatograms were facilitated through peak integration routines (Sigma 10 Data Station, Perkin-Elmer, Norwalk, Connecticut), while identification of the individual profile constituents was established through a combined gas chromatograph/mass spectrometer (Hewlett-Packard 5982 dodecapole instrument) using electron impact ionization at 70 eV. Whenever feasible, authentic samples were either purchased or synthesized in our laboratory to verify agreement of spectral information and gas-chromatographic retention time.

Statistical comparisons of the levels of excreted volatiles were made using one-way analysis of the variance (ANOVA) with Duncan's new multiple range test[24]. The probability level for statistical significance was set at $p < 0.02$.

Results and discussion

Design of the experiment reported here was based on a series of pilot observations indicating that concentrations of some urinary volatiles were changed in response to different gonadotropin levels during the reproductive cycle. The biological importance of urinary volatile metabolites recognized in our previous work on puberty delay[18] and the variations seen

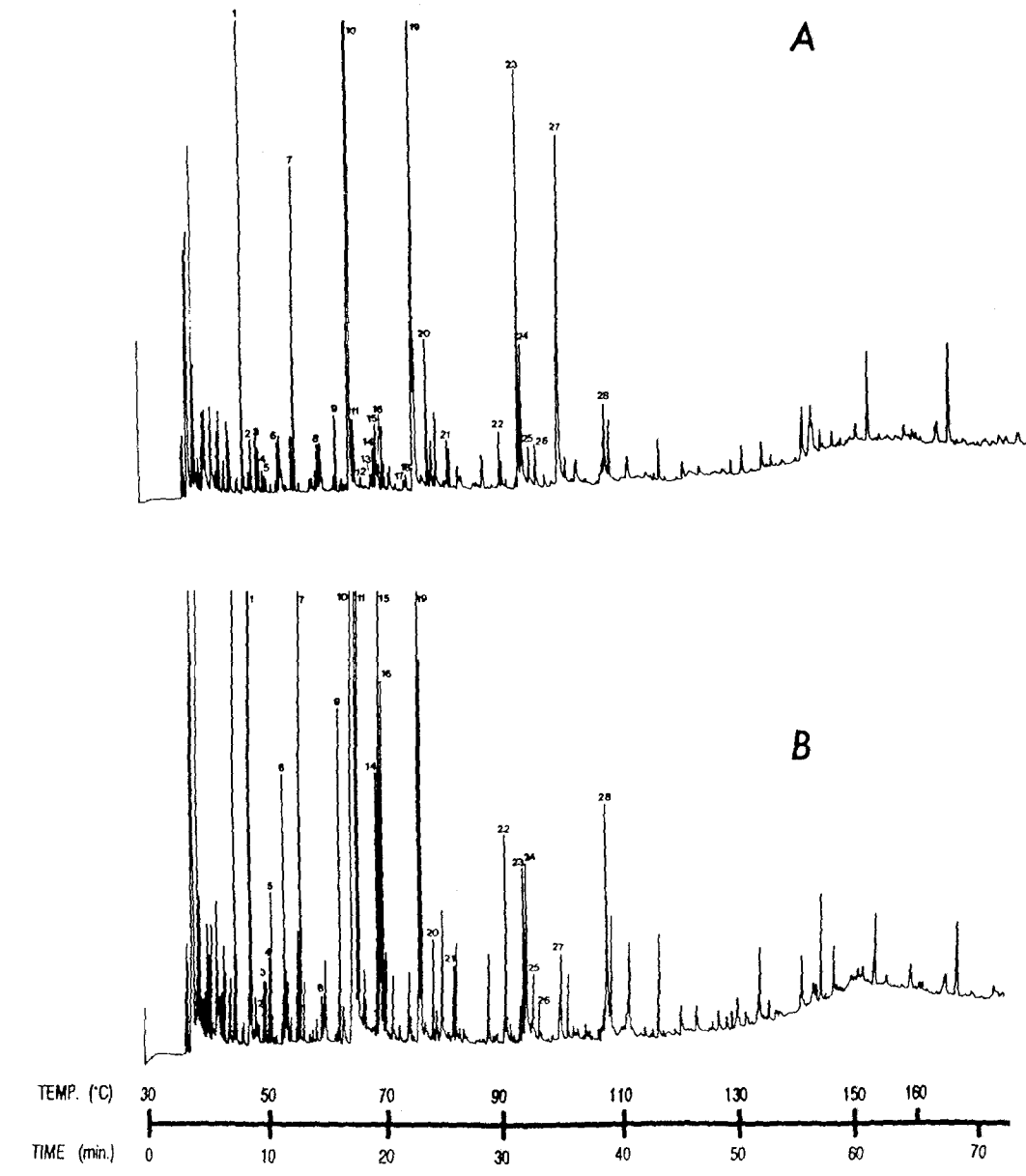

Figure 1. Gas chromatographic profiles of urinary volatile compounds from urine of ICR/Alb female mice in *A* proestrus; *B* second day of estrus.

in the excretion of similar compounds during different periods of pregnancy and lactation[20] have naturally led to the present investigations of urinary volatiles during the natural estrous cycle.

The capillary gas chromatograms, shown in figure 1, are quite representative of the substance profiles obtained from proestrus (A), and the second day of estrus (B) urine of ICR/Alb female mice. The list of compounds identified in these profiles is provided in the table. A visual comparison of numerous volatile profiles further permitted selection of substances that deviated quantitatively in different stages of the estrous cycle. Out of 28 followed substances, 11 volatiles exhibited statistically significant ($p < 0.02$) changes in concentration. They have been subsequently identified as various ketones (peaks 6, 10, 15 and 16), two esters (peaks 11 and 14), dihydrofurans (peaks 1, 7 and 9), dehydro-*exo*-brevicomin (peak 22), and 2,5-dimethylpyrazine (peak 19).

As seen in figure 2, concentrations of these ketones change significantly during the estrous cycle. The levels of 2-heptanone, trans-5-hepten-2-one, and trans-4-hepten-2-one rise gradually from proestrus to the second day of estrus. The fourth ketone, 2-heptanone, exhibited a significant increase that began in the first day of estrus and endured well into the second day. In metestrus, the concentrations of all ketones dropped down to the levels which were not significantly different from those found in diestrous and proestrous animals. There are no significant differences between metestrus, diestrus, and proestrus levels.

Figure 3 contains the graphs recorded for the remaining volatiles with a distinct endocrine dependency: two esters, three dihydrofurans, dehydro-*exo*-brevicomin, and 2,5-dimethylpyrazine. Once again, similar trends of increase during the first and second day of estrus are exhibited by the two esters and three dihydrofurans. The levels of these compounds recorded for metestrus, diestrus, and proestrus were quite similar. Dehydro-*exo*-brevicomin also showed a rising trend in concentration which was similar to that observed for 2-heptanone. For all investigated compounds, the pyrazine

The volatile constituents of female mouse urine identified in the chromatograms shown in figure 1

Peak numbers	Structure	Changes in concentration during the estrous cycle
1	MW 126 dihydrofuran*	+
2	4-ethylcyclohexene	–
3	3-methyl-1-buten-3-ol	–
4	2-ethyl-5-methyl-furan	–
5	3-hexanone	–
6	2-hexanone	+
7	MW 126 dihydrofuran*	+
8	4-heptanone	–
9	MW 126 dihydrofuran*	+
10	2-heptanone	+
11	n-pentyl acetate	+
12	methyl hexanoate	–
13	6-hepten-2-one	–
14	2-penten-1-yl acetate	+
15	trans-5-hepten-2-one	+
16	trans-4-hepten-2-one	+
17	4-penten-1-ol	–
18	unidentified ester	–
19	2,5-dimethylpyrazine	+
20	6-metyhl-6-hepten-3-one	–
21	6-methyl-5-hepten-3-one	–
22	dehydro-*exo*-brevicomin	+
23	2-nonanone	–
24	benzaldehyde	–
25	unidentified	–
26	1-octen-3-ol	–
27	furfuryl alcohol	–
28	acetophenone	–

* Presumed isomeric dihydrofurans unique to the mouse[17]. +, significant changes during the estrous cycle; –, changes not significant at $p < 0.02$.

derivative is the only substance that exhibited a trend opposite to the others, i.e., higher levels during metestrus, diestrus, and proestrus, as compared to both days of estrus.

The natural cycle is representative of various hormonal changes that occur in the plasma patterns of gonadotropins and ovarian steroids. Bronson and Stetson (30) reported that the plasma level of estradiol rises from the late metestrus to the early proestrus, and decreases before the peak concentration of LH, FHS, and progesterone is reached. The cornified cells observed in ICR/Alb females during the two days of estrus, and the gradual increases in concentration of certain volatiles during the estrogen-dominant period (proestrus-estrus stage) suggest dependence of these volatiles on the biosynthesis of estrogens. In some respects, variations in the excretion levels in naturally cycling females reported here appear to parallel qualitatively the changes seen in the animals implanted with estrogen pellets[15] in a previous experiment, where the rises of certain volatile ketones and esters were also observed.

The appearance and levels of these volatile compounds during the reproductive cycle of female mice should now be viewed in relation to the previous chemical and biological findings of this laboratory. Adrenalectomized females, that lack the puberty-delay factors, exhibited depressed levels of the three ketones, both esters and 2,5-dimethylpyrazine[18]. The synthetic mixture of all six compounds or any esters with 2,5-dimethylpyrazine at the concentration mimicking their natural levels in the urine resulted in full recovery of biological activity. However, the ketones themselves were inactive[18, 19].

According to the biological results of Drickamer and coworkers, the urine from pregnant/lactating[10, 25] and from estrus[12, 14] females accelerates puberty. In the present and

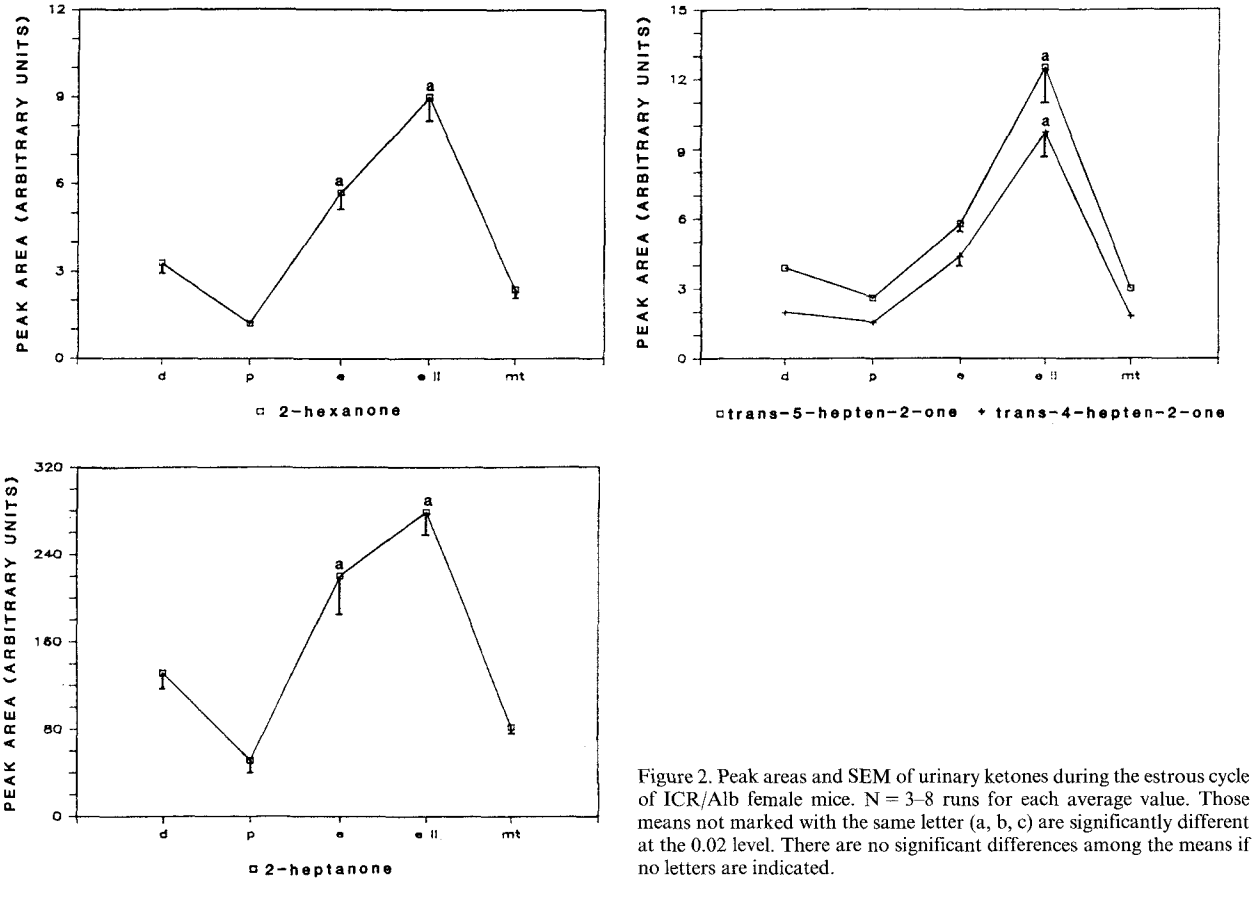

Figure 2. Peak areas and SEM of urinary ketones during the estrous cycle of ICR/Alb female mice. N = 3–8 runs for each average value. Those means not marked with the same letter (a, b, c) are significantly different at the 0.02 level. There are no significant differences among the means if no letters are indicated.

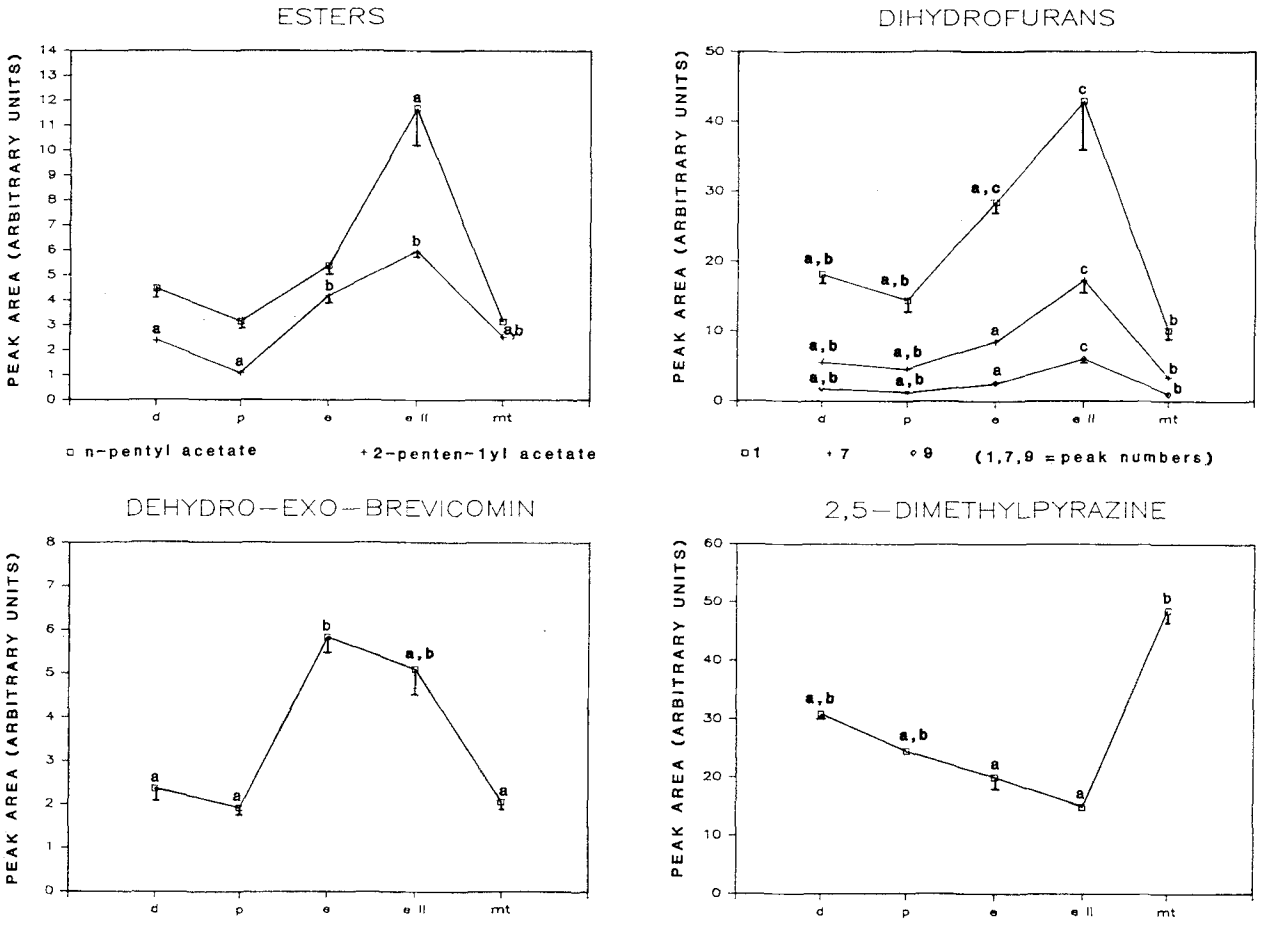

Figure 3. Peak areas and SEM of urinary esters, dihydrofurans, dehydro-*exo*-brevicomin, and 2,5-dimethylpyrazine during the estrous cycle. N = 3–8 runs for each average value. Those means not marked with the same letter (a, b, c) are significantly different at the 0.02 level. There are no significant differences among the means if no letters are indicated.

previous analytical studies, animals in the middle period of pregnancy/lactation[20] or in the estrous cycle showed significant elevations of the ketones and esters. The inhibitory substance, 2,5 dimethylpyrazine[18], was absent during pregnancy/lactation[20], and significantly reduced in the estrogen-dominant period (estrus stage).

Increases of the three dihydrofurans, the 'mouse-specific' compounds, during the estrogen-dominant period are also interesting. The structures of isomeric dihydrofurans with molecular weight 126 were structurally postulated in a previous publication[17] and they presented elevated concentrations also during pregnancy/lactation.

The rise of dehydro-*exo*-brevicomin during the estrogen-phase was most surprising. Until this investigation, this compound was not observed in the female urine in a significant quantity. In fact, an androgen dependency of dehydro-*exo*-brevicomin was clearly established in our previous reports[26,27]. An enhanced level of this compound in estrus females is still considerably lower than the concentrations excreted by normal males[28,29]. Its biological role in the female urine is open to speculation.

Whether the volatile substances investigated in this study serve as chemosignals, or are just secondary, coincidental metabolic products, has not been assessed at this time. Biological tests are underway to study the potential roles of the synthetic analogs, both singularly and in mixtures, in male and female mice.

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Enhancement of fluorescence of pyrene-containing lipids by polar media, detergents and phospholipids

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Summary. The fluorescence intensities of a medium-chain fatty acid and of several amphiphilic lipids, each containing pyrene in covalent linkage, were enhanced considerably by: 1) Dissolving in mixtures of a polar solvent (e.g. methanol, ethanol, tetrahydrofuran or dimethylsulfoxide) and water; for each individual compound, a certain ratio of solvent to water provided maximal fluorescence intensity. 2) Incorporating into micelles of reduced Triton X-100; an excess of detergent was used so that, statistically, only one molecule of lipid resided in one micelle of the Triton X-100. 3) Incorporating into liposomes of egg phosphatidylcholine; maximal fluorescence was observed using a large excess of phosphatidylcholine. When related to the fluorescence intensities in chloroform/methanol (2:1, by vol.) or water, the enhancement of fluorescence in the above three systems was about 2–6-fold or up to 60-fold, respectively.
Key words. Fluorescent lipids; pyrene-labeled lipids; environment (effect on fluorescence).

Introduction

Pyrene, a polycyclic hydrocarbon, exhibits an intense fluorescence in the ultraviolet region[1]. It has been linked covalently to short, medium and long-chain fatty acids[2–5], and these subsequently bound to neutral[6–9], phospho[3, 10–14] and glycolipids[15]. Use of the pyrene fatty acids and their phospholipid derivatives to measure lateral diffusion, lipid exchange between different vesicles, translocation, phase separation phenomena and lipid-protein interactions has been reviewed[3]. In our previous studies, pyrene-linked lipids were used as substrates for lipolytic enzymes, e.g. sphingomyelinase and lipase[9, 13, 14]. The fluorescence intensities of the products were used as a measure of the activity of the respective enzymes. In other studies, pyrene fatty acids have been employed for following their transport across the plasma membrane of cultured cells[4, 16]. These acids, as well as several pyrene-containing lipids, have also been used for following continuously their insertion into liposomes[17] and uptake into cultured cells derived from normal individuals or patients affected with lipid storage diseases[14, 18, 19]. For quantitation, the products of enzymatic or cellular metabolic reactions were extracted with organic solvents, employing the procedures of Folch et al.[20] or Dole[21] and their fluorescence intensities recorded in mixtures of chloroform and methanol or in a heptane-rich phase, respectively.

While developing these procedures, we encountered systems in which the fluorescence intensities of the pyrene-lipids exceeded several-fold those recorded in the above-mentioned solvents. Using the latter could potentially increase the sensitivity of analysis in enzymatic or metabolic reaction studies. In this paper, three such systems are described for enhancing the fluorescence of pyrene derivatives of fatty acids and several amphiphilic lipids. For this purpose, the fatty acid or lipid was dissolved in mixtures of a polar solvent and water, or incorporated into micelles of a nonionic detergent (reduced Triton X-100) or liposomes of an unsaturated phospholipid (egg phosphatidylcholine). The latter two procedures are of special interest since they permit high-sensitivity spectrofluorometric measurement of the fluorescence intensities of lipids in aqueous media devoid of organic solvents, thereby making them especially useful for laboratories not fully versed in techniques of lipidology.

Materials and methods

Chemicals. 10-(1-pyrene)-decanoic acid (P10), 12-(1-pyrene)-dodecanoic acid (P12) and 1-pyrenesulfonyl chloride were purchased from Molecular Probes (Junction City, OR, USA). 12-(1-pyrenesulfonyl)-aminododecanoic acid (PSA12) was synthesized from 1-pyrenesulfonyl chloride and 12-aminododecanoic acid (Aldrich Chemical Corp., Milwaukee, WI, USA) following the procedure of Goldberg et al.[22] modified in that the 1-pyrenesulfonyl chloride was dissolved in dichloromethane. 1-Ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride was obtained from Story Chemical Corp. (Muskegon, MI, USA); N,N'-dicyclohexylcarbodiimide and reduced Triton X-100 from Sigma (St. Louis, MO, USA); silica gel 60 H from Merck (Darmstadt, FRG); sodium taurocholate from Calbiochem (La Jolla, CA, USA) and sodium dodecylsulfate from Bio-Rad (Richmond, CA, USA). Sphingomyelin and sulfatide were purified from bovine brain[23, 24], glucosylceramide from the spleen of a patient with type I Gaucher disease[25] and phosphatidylcholine from egg yolk. All solvents were of analytical grade. Fluorescence measurements were recorded on a Perkin-Elmer LS-5 spectrofluorometer.

Synthesis of fluorescent derivatives of lipids. The following lipids were deacylated as previously described: sphingomyelin, by acid hydrolysis[23, 26]; glucosylceramide and sulfatide,