Improved Delivery through Biological Membranes. 32.[1] Synthesis and Biological Activity of Brain-Targeted Delivery Systems for Various Estradiol Derivatives

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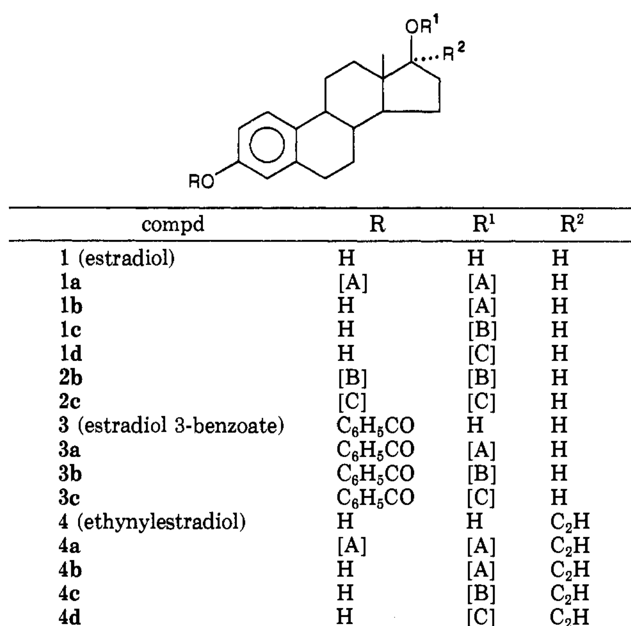
Brain-targeted delivery systems based on the dihydropyridine ⇄ pyridinium salt redox interconversion were synthesized for estradiol, estradiol 3-benzoate, and ethynylestradiol. Initial biological evaluation indicated that while all four compounds synthesized exerted central estrogenic activity as measured by serum LH suppression, only the delivery systems based on the 17-substituted estradiol and ethynylestradiol demonstrated prolonged action (>12 days). The 17-(1-methyl-1,4-dihydronicotinic acid ester) of ethynylestradiol behaved in a similar manner to the previously described estradiol analogue in various assays. Tissue distribution studies in rats showed that administration of the ethynylestradiol derivative resulted in high sustained levels of the corresponding pyridinium salt in the central nervous system (CNS) while blood levels of the oxidized metabolite rapidly fell. The sustained brain levels were associated with a prolonged release of ethynylestradiol. By 24 h, posttreatment, no ethynylestradiol was found by HPLC in the blood while levels of over 20 ng/g of tissue were detected in the CNS. This enhanced central delivery gave a dose- and time-dependent LH suppression, which indicated a three- to fivefold increased potency compared with the corresponding estradiol derivative.

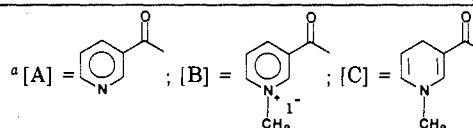
A specific method for delivering estrogens to the brain would not only reduce peripheral dose-related toxicities associated with these sex steroids but would also increase the central potency of the estrogen by diverting a larger portion of the administered dose to the central nervous system (CNS).[2-4] Recently, a dihydropyridine ⇄ pyridinium type chemical delivery system (CDS) was applied to estradiol.[3] The delivery form of estradiol is a 1-methyl-1,4-dihydronicotinic acid ester located in the 17-position. This relatively lipophilic ($\log P = 4.5$) compound widely distributes in the body of rats after iv administration.[3] With time, the enzymatically labile dihydropyridine oxidizes, yielding a quaternary salt. This hydrophilic salt ($\log P = -0.14$) is easily lost from the visceral organs and systemic circulation. The $t_{1/2}$ of efflux from liver, lung, and kidney was estimated to be 46 min, 5.5 h, and 8 h, respectively. In the CNS, the blood–brain barrier (BBB) prevents rapid loss of this polar species, which results in sustained levels of this compound ($t_{1/2} = 23$ h) in the brain.[3] This "locked-in" salt slowly hydrolyzes to release the parent, active drug, which permits activation of estrogen receptors by estradiol. Various studies have shown that 17-esters of estradiol are, in and of themselves, inactive since they do not bind to estrogen receptors.[5,6] Therefore, both the oxidized and reduced 17-carrier esters should not be directly estrogenic. These processes are summarized in Scheme I.

The slow selective release of estradiol in the CNS is associated with a number of prolonged and extended biological effects. A single dose of the delivery system for estradiol **1d** (Table I) causes sustained serum luteinizing hormone (LH) suppression in castrate rats (for at least 24 days), which cannot be attributed to circulating estrogen levels.[4,7] Equimolar doses of estradiol or other depot forms of estradiol including estradiol 17-valerate or estradiol 3-benzoate are far less potent than **1d** and are active for only a fraction of the time. Compound **1d** is also a potent reactivator of copulatory behavior in castrated male rats[8] and causes a decrease in the rate of weight gain by rats.[9] The present study was designed to look at various *estradiol derivatives* as possible estrogenic delivery forms. The delivery form for estradiol **1d** is derivatized at the 17-position. The effect of 3,17-bisacylation was studied. The first compound considered, which was previously synthesized but not tested,[3] was the 3,17-bis(dihydro-

Table I. Chemical Delivery Systems, Precursor Quaternary Salts, and Synthetic Intermediates for Various Estrogens[a]



compd	R	R¹	R²
1 (estradiol)	H	H	H
1a	[A]	[A]	H
1b	H	[A]	H
1c	H	[B]	H
1d	H	[C]	H
2b	[B]	[B]	H
2c	[C]	[C]	H
3 (estradiol 3-benzoate)	C_6H_5CO	H	H
3a	C_6H_5CO	[A]	H
3b	C_6H_5CO	[B]	H
3c	C_6H_5CO	[C]	H
4 (ethynylestradiol)	H	H	C_2H
4a	[A]	[A]	C_2H
4b	H	[A]	C_2H
4c	H	[B]	C_2H
4d	H	[C]	C_2H



trigonellinate) (compound **2c**). A second carrier moiety is attached in this compound. The oxidative and hydro-

(1) Part 31 of this series "Water Soluble Complexes of Brain-Targeted Drug Delivery Systems". Brewster, M.; Loftsson, T.; Estes, K.; Mullersman, G.; Derendorf, H. and Bodor, N. in press.
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(6) Larner, J. M.; Hochberg, R. B. *Endocrinology (Baltimore)* **1985**, *117*, 1209.
(7) Estes, K.; Brewster, M.; Simpkins, J.; Bodor, N. *Life Sci.* **1987**, *40*, 1327.
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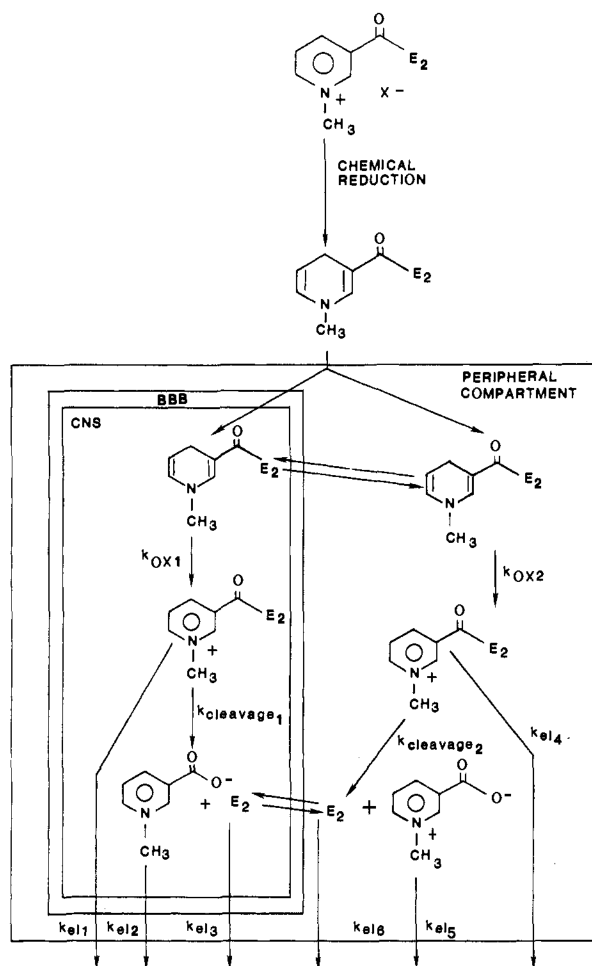
Scheme I. A Chemical Method Allowing for Increased Retention of Estradiol (E_2) in the CNS[a]



[a] Estradiol is condensed with nicotinic acid, quaternized to form the 17-trigonellinate iodide, and reduced, giving the 17-(1,4-dihydrotrigonellinate) (**1d**). Systemic administration of this dihydropyridine results in extensive distribution. In all locations, the labile dihydropyridine is converted to the quaternary salt (k_{ox1} and k_{ox2}). This more polar species is rapidly eliminated from the periphery but retained in the CNS ($k_{el1} \ll k_{el4}$) because of its charge and size and its inability to readily back-diffuse through the BBB. In the CNS, the "locked-in" quaternary salt is slowly hydrolyzed ($k_{cleavage\ 1}$), liberating estradiol and the carrier salt. The E_2 can then interact with estrogen receptors while the nontoxic salt is actively eliminated from the CNS (k_{el2}).

lytic stability of this second carrier will differ from the 17-derivative since it involves a more labile phenyl ester. The 3-benzoate of estradiol was studied as well. These two compounds may, depending on their stability, reduce the rate of 3-sulfation or glucuronidation and therefore provide a more prolonged effect. The third compound is a delivery form for ethynylestradiol. The initial step in the deactivation of estradiol is 17-oxidation to form estrone. Placement of an ethynyl group in this position prevents this metabolism and greatly increases the potency of the steroid.[10] In the delivery system, the stabilizing effect of the alkynyl group should be small initially but should become important after the compound is delivered. Importantly, the toxic peripheral side effects of ethynylestradiol should be diminished by decreasing peripheral concentrations of the drug. The three new estrogen delivery systems were tested and their potency compared with the original derivative, **1d**. Active compounds were further studied.

Results and Dicussion

Chemistry. The delivery systems for estradiol based either on the 17-carrier or on the steroid disubstituted in both the 3- and 17-positions were prepared according to previously published procedures.[3] This involved 3,17-bisacylation of estradiol with nicotinoyl chloride in pyridine to produce **1a**. Preferential hydrolysis of the phenolic ester in methanolic potassium hydrogen carbonate yielded the 17-nicotinate **1b**, which was quaternized with methyl iodide, giving the trigonellinate ester **1c**. Reduction of the pyridinium salt in basic aqueous *tert*-butyl alcohol with sodium dithionite gave the dihydropyridine **1d** in good yield.

The synthesis of the bis-substituted estradiol delivery system (**2c**) involved quaternization of the 3,17-dinicotinate **1a** with methyl iodide to give the bis salt **2b**. Reduction of this species could not be performed in aqueous media as hydrolysis of the phenolic ester invariably occurred. The desired compound **2c** was obtained by reduction of **2b** in ice-cold acetonitrile with the activated hydride transferring reagent, 1-benzyl-1,2-dihydroisonicotinamide.[3]

The 3-benzoate system was obtained by acylation of commercially available estradiol 3-benzoate (compound **3**) with nicotinic anhydride in pyridine in the presence of 4-(dimethylamino)pyridine (DMAP). The resulting nicotinate, **3a**, was quaternized with methyl iodide, giving **3b**, and reduced under conditions similar to those used for the reduction of **1c** to give **3c**.

The delivery system based on ethynylestradiol was synthesized by reaction of the parent steroid with nicotinic anhydride in the presence of DMAP. The resulting diester **4a** was hydrolyzed in methanolic potassium hydrogen carbonate to give the 17-nicotinate **4b**. The ester was alkylated with methyl iodide, giving **4c**, and reduced in basic aqueous *tert*-butyl alcohol with sodium dithionite to give **4d**. In all of the above reductions, aqueous *tert*-butyl alcohol was the only solvent mixture that gave adequate yields. In aqueous methanol, ethanol, propanol, 2-isopropanol, or butanol or in water, the rate of reduction was exceedingly slow.

Initial Screen. In this study, as in previous cases,[4,7] the suppression of circulating levels of luteinizing hormone (LH) by the delivery systems without concomitant elevation of serum estrogens or peripheral estrogenic effects was used as an indication of their central estrogenic potency and latency. The dihydropyridine estrogen derivatives (**2c**, **3c**, and **4d**) were tested, and the results were compared with those obtained from **1d**. The study was designed to identify compounds with similar or improved potency (compared with **1d**) for further, more detailed examination. Ovariectomized rats were injected with 0.5 mg/kg **1d** or equimolar **2c**, **3c**, **4d**, estradiol valerate (EV), or ethynylestradiol (**4**) iv. The last two compounds were included as controls. Estradiol valerate is a lipophilic 17-ester prodrug of estradiol. LH was measured at 12 and 18 days postinjection and expressed (in Figure 1) in terms of the LH-RP-2 standard as ng/mL. While previous studies showed that LH was transiently suppressed by estradiol, serum LH levels were not suppressed on day 12 and 18 following injection of ethynylestradiol or estradiol valerate. The values obtained were well within the range of vehicle control values obtained from other studies. At day 12, all four delivery systems significantly decreased LH levels. Serum LH levels were suppressed 76%, 72%, 53%, and 80% on day 12 following treatment with **1d**, **2c**, **3c**, and

(9) Estes, K.; Brewster, M.; Bodor, N. Endocr. Soc. Abstr., Anaheim, CA. 1986, p. 288.
(10) Bolt, H. *Pharmacol. Ther.* **1979**, *4*, 155.

Table II. Lipophilicity of Various Dihydropyridine Estrogen Derivatives

compd	R_m	r^a	log P^b
1d	6.06	0.99993	4.50
2c	8.03	0.992	
3c	7.01	0.999	
4d	6.098	0.999	4.53

[a] Regression coefficient for 100% H_2O extrapolated R_m value. [b] Values from ref 3 and from unpublished work.

Table III. In Vitro Oxidation Studies of 1d and 4d in Various Biological Matrices

media	compd	rate const (k), $min^{-1} \times 10^{-2}$	$t_{1/2}$, min
brain homogenate	**1d**	5.37	12.9
	4d	5.67	12.4
liver homogenate	**1d**	2.83	30.5
	4d	2.27	24.5
whole rat blood	**1d**	1.75	39.6
	4d	1.39	49.9

4d, respectively. In contrast, LH levels were lower but not significantly different from controls on day 18 posttreatment in the case of **2c** and **3c** but remained more than 80% decreased compared to controls in rats treated with **1d** and **4d**. Uterine and pituitary weights, which give an indication of peripheral estrogenic activity, were not significantly different in rats showing suppressed or nonsuppressed LH values at necropsy (day 18). The shorter duration of action elicited by **2c** and **3c** may be attributed to a lower delivery of the compounds to the CNS. This may result from a metabolic effect (i.e., the rapid oxidation of the dihydrotrigonellinate in the 3-position of **2c** or be due to precipitation after injection or other physicochemical problems. The high lipophilicity (Table II) and associated poor water solubility of **2c** and **3c** compared with **1d** and **4d** are consistent with this latter hypothesis.

The described delivery scheme requires facile oxidation of the dihydropyridine carrier to the corresponding quaternary salt. This reaction serves the dual purpose of accelerating the peripheral loss of the drug conjugate and of causing the retention of the same complex in the CNS. The rate of oxidation of **4d** in various biological matrices was studied, and the data were compared to that for **1d**. As summarized in Table III, the rates of disappearance of both **1d** and **4d** are similar and provide empirical information, based on the previous study, that **4d** is acting in a manner not unlike **1d** in vivo.

Distribution Studies. A distribution study was next performed. Equimolar ethynylestradiol (4) or **4d** was administered to rats and the brain/blood distribution examined. As illustrated in Figure 2 and Table IV, the parent steroid readily penetrated the BBB but was also rapidly lost. The brain level at 1 h was only one-fourth of that at 15 min. In contrast, the quaternary salt **4c**, which was delivered by **4d** was locked in the CNS with an estimated half-life of 2.8 days. In addition, blood levels

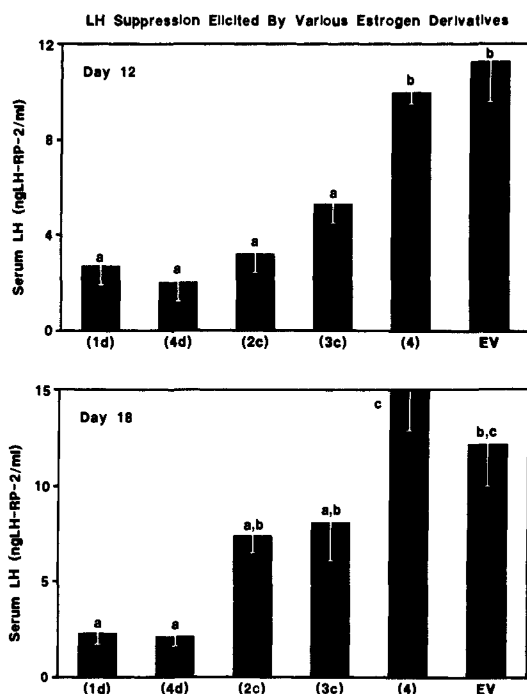


Figure 1. Serum LH levels in ovariectomized rats 12 days (upper panel) and 18 days (lower panel) following a single 1.27 μmol/kg iv dose of four estrogen derivatives, ethynyl estradiol (4), or estradiol-17-valerate (EV). Bars represent SEM values. Different superscripts designate significantly different mean values (p < 0.5).

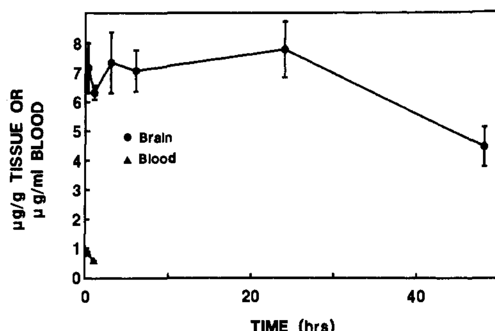


Figure 2. Brain and blood concentrations of 4c after iv administration of 7.5 mg/kg of 4d.

of **4c** were low relative to brain concentrations (brain/blood ratio = 8.7 at 15 min and 11.4 at 1 h) and fell rapidly. At 3 h, no **4c** could be detected in blood. These sustained brain levels of **4c** were associated with a small but prolonged release of the parent steroid. The low levels produced required that samples be pooled and concentrated prior to HPLC analysis. In the brain, 22.0 ng/g of 4 was found at 6 h, 18.5 ng/g at 24 h, and 7.3 ng/g at 48 h. In the blood, no 4 was detected at 24 or 48 h by this technique. The limit of detection was approximately 4 ng/g.

Table IV. Brain and Blood Concentration of **4c** after Administration of **4d** or of 4[a]

	administration of 4d		administration of 4	
time, h	brain concn ± SEM	blood concn ± SEM	brain concn ± SEM	blood concn ± SEM
0.25	7.16 ± 0.85	0.822 ± 0.069	8.30 ± 0.60	0.73 ± 0.06
1	6.32 ± 0.24	0.552 ± 0.028	2.18 ± 0.45	0.43 ± 0.05
3	7.35 ± 1.04	b	c	c
6	7.07 ± 0.97	b	c	c
24	7.79 ± 0.95	b	c	c
48	4.45 ± 0.67	b	c	c

[a] The dose was 7.5 mg/kg **4d** or equimolar 4. [b] Below detection limit. [c] Not determined.

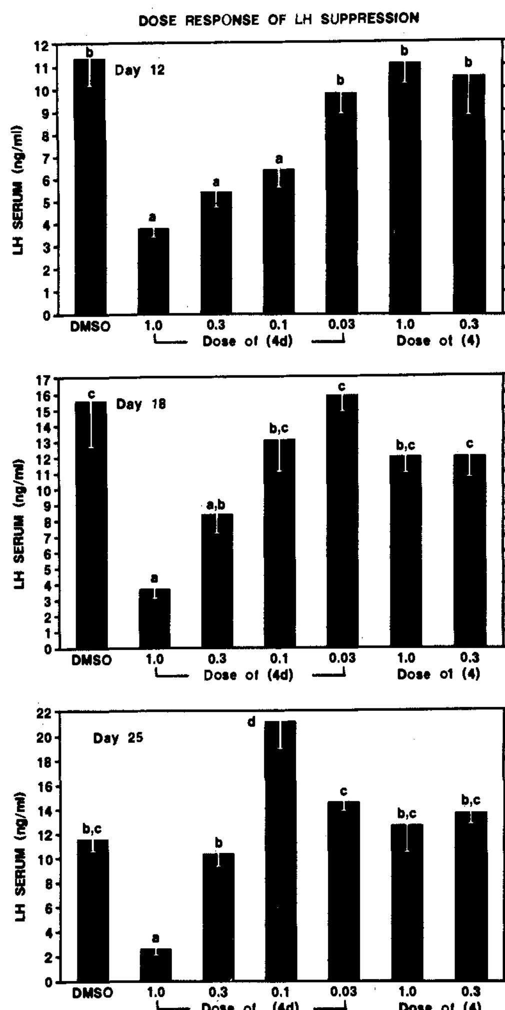


Figure 3. Dose-response of serum LH suppression to **4d** in ovariectomized rats on days 12, 18, and 25 posttreatment. Rats ovariectomized for 2 weeks were treated with 1.0, 0.3, 0.1, or 0.03 mg/kg of **4d** or two doses of 4 equimolar to 1.0 and 0.3 mg/kg of **4d** or DMSO vehicle (0.5 mL/kg). Bars represent SEM values. Different superscripts designate significantly different mean values ($p < 0.5$).

The absence of detectable levels of ethynylestradiol (4) in blood at 24 h and later indicates that 4 found in the CNS is derived from "locked-in" **4c** and not from peripheral sources.

Pharmacologic Studies. The last experimental series was designed to look at the dose response and the duration of action of **4d**. Rats were given vehicle, **4d** in doses of 1.0, 0.3, 0.1, or 0.03 mg/kg, and ethynylestradiol equimolar to the two highest doses of **4d**. Serum LH was measured on day 12, 18, and at sacrifice on day 25. As illustrated in Figure 3, neither dose of the parent steroid had any effect at any of the time points measured. In contrast, **4d** suppressed LH in doses as low as 0.1 mg/kg on day 12, 0.3 mg on day 18, and the 1 mg/kg dose continued to significantly dampen LH secretion at day 25. These data, when compared to those previously reported, indicate that **4d** is 3–5 times more potent than **1d**.[7] Several factors may contribute to this increase in potency. First, the delivery system for 4 is more effective in the deposition of **4c** and presumably ethynylestradiol in the brain. Figure 4 shows the brain levels of **4c** corrected for dose compared to those obtained for **1c** after administration of the corresponding delivery form. As can be seen, **4c** is locked in the brain at levels 4 times higher than those of **1c**. Second, the compound released is far more stable metabolically in the case of **4c**. The decreased metabolic inactivation of 4 may act to improve the potency of **4d**.[10,11]

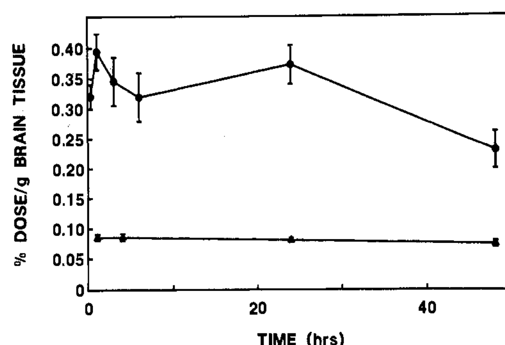


Figure 4. Brain levels of **1c** (▲) and **4c** (●) after iv administration of **1d** and **4d**, respectively.

Interestingly, while the potency of ethynylestradiol is much higher than estradiol in vivo, the difference is much smaller for the corresponding delivery systems. The increased stability of 4 is associated with the 17-ethynyl group, which prevents 17-oxidation mediated by 17-hydroxysteroid dehydrogenase.[11] In the derivatives studied, this stabilization is inconsequential until the parent compound is released since the 17-alcohol is protected by the relatively stable carrier ester. The observation that **1d** and **4d** do not vary in potency by orders of magnitude is contributing, through indirect evidence, for the specificity of the delivery. If these compounds were acting peripherally and simply providing a low sustained level of steroid via the systemic circulation to the CNS, one would expect **4d** to exert a similar improvement in potency as that seen between 1 and 4. The results obtained clearly do not support this view. The undetectable steroid levels in the periphery and the relatively high levels measured in the CNS provide more direct evidence for the brain enhanced delivery. Also the similarity in the in vitro data do not support the thesis that **4d** is operating in some manner fundamentally different from **1d**.

In conclusion, a series of brain delivery systems for estrogens were developed. Selective delivery of these compounds to the CNS would be useful as neuroendocrine probes and could prove beneficial in treating menopausal hot flushes, and prostatic cancer and as a contraceptive. The compounds synthesized were tested in a castrate rat model. While all four compounds tested showed some sustained activity in terms of dampening LH secretion compared to 4 alone, only **1d** and **4d** demonstrated prolonged (>12 days) activity. The novel system **4d** was shown to be similar to **1d** in several in vitro assays but was three- to fivefold more potent in vivo.

Experimental Section

Chemistry. Microcombustion analyses of compounds synthesized were performed by Atlantic Microlabs, Atlanta, GA. Uncorrected melting points (mp) were determined with an Electrothermal melting point apparatus. Ultraviolet spectra (UV) were obtained with either a Hewlett-Packard 8451A diode array or a Shimadzu UV-160 rapid scan spectrophotometer. For kinetic analyses, a dedicated HP-85 microprocessor equipped with a kinetic software package was used. Infrared spectra (IR) were recorded on a Beckman Microlab 620MX spectrophotometer. Samples were analyzed as potassium bromide pellets. Nuclear magnetic resonance spectra (NMR) were recorded on either a Varian EM360 or a EM390 spectrometer. The samples were dissolved in an appropriate deuteriated solvent and chemical shifts (δ) reported relative to an internal standard (tetramethylsilane,

(11) Longcope, C.; Williams, K. *J. Clin. Endocrinol. Metab.* 1974, *38*, 602.

TMS). Thin-layer chromatography was performed on EM reagents DC-aluminum foil plates coated to a thickness of 0.2 mm with indicated silica gel 60. Steroids were obtained from Sigma Chemical Co. Pyridine was routinely distilled over CaH_2 before use. The synthesis of **1d** and **2c** has been previously described.[3] These descriptions are summarized below.

3,17β-Bis[(3-pyridinylcarbonyl)oxy]estra-1,3,5(10)-triene (1a). To nicotinoyl chloride (5.3 g, 0.03 mol) in 30 mL of dry pyridine at 0 °C was added 2 g (0.0073 mol) of β-estradiol. After being refluxed for 1 h, the mixture was poured over 100 mL of ice water, and the resulting precipitate was collected by filtration. The precipitate was dried over P_2O_5 in vacuo, yield 90% (3.18 g): mp 148–150 °C; UV (MeOH) 222, 262 nm. IR (KBr) ν 1750, 1725 (2 C=O stretch); NMR ($CDCl_3$) δ 9.2–9.0 (br s, 2 H, C-2,2′ pyridine protons), 8.7–8.3 (m, 2 H, C-6,6′ pyridine protons), 8.4–8.0 (m, 2 H, C-4,4′ pyridine protons), 7.5–7.1 (m, 3 H, C-5,5′ pyridine protons + C-1 E_2 proton), 6.9–6.7 (m, 2 H, C-2,4 E_2 protons), 5.0–4.7 (t, 1 H, C-17α E_2 proton), 3.2–1.3 (m, 15, skeletal E_2 protons), 1.0–0.9 (s, 3 H, C-18 E_2 protons). Anal. $C_{30}H_{31}N_2O_4$: C, H, N.

3-Hydroxy-17β-[(3-pyridinylcarbonyl)oxy]estra-1,3,5-(10)-triene (1b). To 1a (0.5 g, 0.0010 mol) was added 60 mL of a 0.5% $KHCO_3$ solution in 95% aqueous methanol. The suspension was allowed to stir overnight at room temperature. After addition of 60 mL of H_2O, the suspension was extracted repeatedly with chloroform. The organic layer was separated, dried over $MgSO_4$, and removed in vacuo. The resulting pinkish solid was suspended in cold methanol and filtered to give 0.36 g (94%) of the desired product: mp 216–217 °C; UV (MeOH) 222, 264 nm; IR (KBr) ν 3500–3000 (phenolic OH stretch), 1735–1725 (C=O stretch); NMR ($CDCl_3$) δ 9.1–8.9 (d, 1 H, C-2 pyridine proton), 8.7–8.5 (dd, 1 H, C-6 pyridine proton), 8.2–8.1 (dt, 1 H, C-4 pyridine proton), 7.4–6.9 (m, 3 H, C-5 pyridine proton + C-1 E_2 proton + phenol OH (exchangeable with D_2O)), 6.7–6.4 (m, 2 H, C-2,3 E_2 protons), 5.1–4.7 (m, 1 H, C-17α E_2 protons), 3.0–1.2 (m, 15, E_2 protons), 1.0–0.9 (s, 3 H, C-18 E_2 proton). Anal. $C_{24}H_{27}NO_3$: C, H, N.

1-Methyl-3-[[[3-hydroxyestra-1,3,5(10)trien-17β-yl]oxy]carbonyl]pyridinium Iodide (1c). To 1b (2.095 g, 0.006 mol) in 200 mL of acetone was added 2 mL (0.032 mol) of methyl iodide. The solution refluxed overnight, yielding a yellow solid, which was collected by filtration, washed, and dried in vacuo. The yield was 83% (2.42 g): mp 255–261 °C dec; UV (MeOH) 222, 268 nm; IR ν (KBr) 3600–3150 (phenolic OH stretch), 1740 (C=O stretch); NMR (DMSO-d_6) δ 9.2–9.0 (s, 1 H, C-2 pyridinium proton), 9.0–8.5 (m, 3 H, C-4,6 pyridinium protons + phenolic OH (exchangeable)), 8.2–7.8 (m, 1 H, C-5 pyridinium proton), 7.0–6.7 (m, 1 H, C-1 E_2 proton), 6.5–6.2 (m, 2 H, C-2,4 E_2 proton), 5.0–4.7 (t, 1 H, C-17α E_2 proton), 4.5–4.3 (s, 3 H, N-CH_3), 2.9–1.2 (m, 15 H, E_2 skeletal protons), 1.0–0.9 (s, 3 H, C-18 E_2 protons). Anal. $C_{25}H_{29}NO_3I$: C, H, N.

3-Hydroxy-17β-[[(1-methyl-1,4-dihydropyridin-3-yl)carbonyl]oxy]estra-1,3,5(10)-triene (1d). To 1c (1.09 g, 0.0021 mol) in 150 mL of 50:50 *tert*-butyl alcohol/H_2O were added 1.06 g (0.0126 mol) of $NaHCO_3$ and 1.46 g (0.0084 mol) of $Na_2S_2O_4$. The mixture stirred at 0 °C under N_2 for 1 h. The solution was then extracted with $CHCl_3$. The organic layer was dried over $MgSO_4$ and reduced in vacuo, yielding a yellow foam, yield 64% (0.24 g): mp 115–130 °C dec; UV (MeOH) 222, 220, 356 nm; IR (KBr) ν 3600–3150 (phenolic OH stretch), 1700 (C=O stretch); NMR ($CDCl_3$) δ 7.0–6.8 (m, 2 H, C-1 E_2 proton + C-2 pyridine proton), 6.7–6.4 (m, 2 H, C-2, 4 E_2 protons), 5.7–5.4 (br d, 1 H, C-6 pyridine proton), 5.0–4.5 (m, 3 H, C-17α E_2 proton + C-5 pyridine proton + phenolic OH (exchangeable)), 3.2–3.0 (m, 2 H, C-4 pyridine protons), 3.0–2.9 (s, 3 H, N-CH_3), 2.8–1.1 (m, 15 H, E_2 skeletal protons), 1.0–0.9 (s, 3 H, C-18 E_2 protons). Anal. $C_{25}H_{30}NO_3 \cdot {}^1/_2H_2O$: C, H, N.

1,1′-Dimethyl-3,3′-[[(estra-1,3,5(10)-trien-3,17β-diyl)dioxy]dicarbonyl]dipyridinium Diiodide (2b). Two grams of 1a (0.004 mol) was added to 50 mL of acetone and 2 mL (0.032 mol) of methyl iodide. The solution was heated at reflux overnight. The resulting precipitate was filtered, washed with acetone, and dried. The yield was 88% (2.75 g): mp 251–252 °C; UV (MeOH) 222, 268 nm; NMR ($CDCl_3$ + DMSO-d_6) δ 9.87–9.73 (br s, 1 H, C-2′ pyridinium proton), 9.6–8.90 (m, 5 H, C-2,6,6′,4,4′ pyridinium protons), 8.57–8.17 (m, 2 H, C-5,5′ pyridinium protons), 7.60–7.0 (m, 3 H, C-1,2,4 E_2 protons), 5.23–4.8 (br t, 1 H, C-17α E_2 proton), 4.73–4.4 (br s, 6 H, 2 N^+CH_3), 3.10–1.10 (m, 15 H, E_2 skeletal protons), 1.10–0.97 (s, 3 H, C-18 E_2 protons). Anal. $C_{32}H_{39}N_2O_4I_2$: C, H, N, I.

3,17β-Bis[[(1-methyl-1,4-dihydropyridin-3-yl)carbonyl]oxy]estra-1,3,5(10)-triene (2c). One gram (1.31 mmol) of **2b** was dissolved in 100 mL of dry acetonitrile. To this solution, which was flushed with N_2, was added 0.28 g (2.62 mmol) of 1-(phenylmethyl)-4-(aminocarbonyl)-1,2-dihydropyridine, and the reaction mixture was stirred at 0 °C for 1 h. The solvent was then removed under reduced pressure, leaving a solid. The solid was suspended in methylene chloride and filtered, and the filtrate was chromatographed several times on a neutral alumina column prepared with methylene chloride. A solid foam was obtained after purification and evaporation of the solvent in vacuo: UV (MeOH) 216, 364 nm; NMR ($CDCl_3$) δ 7.23–7.10 (br s, 2 H, C-2,2′ pyridine protons), 6.97–6.73 (m, 3 H, C-1,2,4 E_2 protons), 5.77–5.53 (dt, 2 H, C-6,6′ pyridine protons), 4.93–4.57 (m, 3 H, C-5,5′ pyridine protons and C-17α E_2 protons), 3.27–3.03 (br s, 4 H, C-4,4′ protons), 3.00–2.73 (d, 6 H, 2 N CH_3), 2.53–1.13 (m, 15 H, E_2 skeletal protons), 0.97–0.77 (s, 3 H, C-18, E_2 protons). Anal. $C_{32}H_{38}N_2O_4$: C, H, N.

3-[(Phenylcarbonyl)oxy]-17β-[(3-pyridinylcarbonyl)oxy]estra-1,3,5(10)-triene (3a). Estradiol 3-benzoate (3; 2.5 g, 6.6 mmol) was dissolved in 50 mL of dry pyridine. To this were then added 1.66 g of nicotinic anhydride and a catalytic amount of DMAP. After 5 days of stirring at room temperature, the pyridine solution was poured into ice water. The solid produced was collected by filtration, yielding 3.01 g (94%) of a white powder after drying in vacuo: mp 151–154 °C; UV (MeOH) nm 204, 224; NMR ($CDCl_3$) δ 1.0 (s, 3 H, angular methyl group), 1.10–3.13 (m, 15 H, skeletal protons), 4.77–5.17 (t, 1 H, 17α proton), 6.85–7.15 (m, 2 H, E_2-2,4 protons), 7.18–7.73 (m, 5 H, E_2-1 + phenyl C-2,3,4 + pyridine C-5 protons), 8.02–8.47 (m, 3 H, pyridine C-4 + phenyl C-1,6 protons), 8.53–8.97 (br s, 1 H, pyridine C-6 proton), 9.00–9.60 (br s, 1 H, pyridine C-2 proton); TLC, R_f 0.61 (60:40 hexane/ethyl acetate). Anal. $C_{31}H_{31}NO_4$: C, H, N.

1-Methyl-3-[[[3-[(phenylcarbonyl)oxy]estra-1,3,5(10)-trien-17β-yl]oxy]carbonyl]pyridinium Iodide (3b). Compound **3a** (1.5 g, 3.1 mmol) was suspended in 2.5 mL of acetone. To this mixture was added 2 mL of methyl iodide, and the system was refluxed over night. The yellow solid (1.8 g, 93%) was collected by filtration and dried in vacuo: UV (MeOH) nm 204, 222, 268; NMR (DMSO-d_6 + pyridine-d_5) δ 1.00 (s, 3 H, angular methyl group), 0.83–3.17 (m, 15 H, skeletal protons), 4.92 (s, 3 H, N^+CH_3), 4.83–5.33 (t, 1 H, 17α proton), 7.00–7.87 (m, 8 H, phenyl C-2,3,4,5,6 + E_2-1,2,4 protons), 8.27–8.83 (m, 1 H, pyridine C-5), 9.10–9.43 (m, 1 H, pyridine C-4 proton), 9.73–10.07 (m, 2 H, pyridine C-2,6 protons). Anal. $C_{32}H_{34}NO_4I$: C, H, N, I.

3-[(Phenyloxy)carbonyl]-17β-[[(1-methyl-1,4-dihydropyridin-3-yl)carbonyl]oxy]estra-1,3,5(10)-triene (3c). Compound **3b** (1.2 g, 1.93 mmol) was suspended in 100 mL of 50:50 *tert*-butyl alcohol/water. To this were added 0.81 g of $NaHCO_3$ and 1.0 g of $Na_2S_2O_4$. After 1.5 h, the solution was extracted with CH_2Cl_2, and the organic phase was dried over $MgSO_4$ and removed in vacuo, leaving 650 mg of a yellow foam: UV (MeOH); NMR ($CDCl_3$) δ 0.83 (s, 3 H, angular methyl group), 0.80–2.83 (m, 15 H, skeletal protons), 2.88 (s, 3 H, NCH_3), 3.00–3.25 (m, 2 H, pyridine C-4), 4.53–4.90 (m, 2 H, pyridine C-5 + E_2-17α protons), 5.47–5.73 (m, 1 H, pyridine C-6 proton), 6.80–7.10 (m, 3 H, E_2-2,4 + pyridine C-2 protons), 7.10–7.70 (m, 4 H, E_2-1 + phenyl C-3,4,5 protons), 8.03–8.33 (m, 2 H, phenyl C-2,6 protons). Anal. $C_{32}H_{35}NO_4$: C, H, N.

3,17β-Bis[(3-pyridinylcarbonyl)oxy]-19-nor-17α-pregna-1,3,5(10)-trien-20-yne (4a). Two grams (6.7 mmol) of ethynylestradiol (4) was added to 50 mL of dry pyridine. To this were added 6.16 g (0.027 mol) of nicotinic anhydride and a catalytic amount of 4-(dimethylamino)pyridine (DMAP). The solution was gently warmed (50 °C) to effect solution. After 2 weeks, the pyridine solution was poured over ice, and the solid produced was collected by filtration. The solid was dried over P_2O_5 in vacuo to give 3 g (85%) of an off-white powder: UV (MeOH) nm 220, 266; NMR (DMSO-d_6) δ 1.12 (s, 3 H, angular methyl group), 1.25–3.15 (m, 15 H, skeletal protons), 2.78 (s, 1 H, alkynyl proton), 6.92–7.17 (m, 2 H, E_2-2,4 protons), 7.28–7.67 (m, 3 H, E_2-1 + pyridine C-5 protons), 8.20–8.63 (m, 2 H, pyridine C-4 protons),

8.73–9.02 (m, 2 H, pyridine C-6 protons), 9.18–9.50 (m, 2 H, pyridine C-2 protons); TLC R_f 0.41 (80:20 $CHCl_3$/acetone). Anal. $C_{32}H_{31}N_2O_4 \cdot {}^1/_4H_2O$: C, H, N.

3-Hydroxy-17β-[(3-pyridinylcarbonyl)oxy]-19-nor-17α-pregna-1,3,5(10)-trien-20-yne (4b). Two grams (3.9 mmol) of **4a** was added to 200 mL of 0.5% methanolic $KHCO_3$. After 6 h, the slurry was diluted with 200 mL of water, and the mixture was extracted with chloroform. The combined organic layers were dried over $MgSO_4$ and removed in vacuo. The resulting oil was triturated with hexane. A white solid (1.48 g, 94% yield) was obtained: UV (MeOH) nm 222, 266; NMR ($CDCl_3$) δ 1.03 (s, 3 H, angular methyl group), 0.73–3.30 (m, 15 H, skeletal protons), 2.73 (s, 1 H, alkynyl proton), 6.52–6.90 (m, 2 H, E_2-2,4 protons), 7.02–7.65 (m, 2 H, E_2-1 + pyridine C-5 protons), 8.03–8.53 (m, 2 H, pyridine C-4 + phenolic protons), 8.70–8.93 (m, 1 H, pyridine C-6 proton), 9.18–9.43 (m, 1 H, pyridine C-2 proton); TLC, R_f 0.21 (60:40 hexane/ethyl acetate). Anal. $C_{26}H_{27}NO_3 \cdot {}^1/_2H_2O$: C, H, N.

1-Methyl-3-[[(19-nor-17α-pregna-1,3,5(10)-trien-20-yn-17β-yl)oxy]carbonyl]pyridinium Iodide (4c). One gram (2.5 mmol) of **4d** was added to 50 mL of acetone. Methyl iodide (2 mL) was added, and the reaction mixture was refluxed for 12 h. The solid produced was collected by filtration, yielding 1.15 g (85%) of the yellow iodide salt: UV (MeOH) nm 220, 268; NMR (DMSO-d_6 δ 1.12 (s, 3 H, angular methyl group), 0.93–3.17 (m, 15 H, skeletal protons + alkynyl proton), 4.58 (s, 3H, N^+CH_3), 6.40–6.80 (m, 2 H, E_2-2,4 protons), 6.98–7.07 (m, 1 H, E_2-1 proton), 8.17–8.63 (m, 1 H, pyridine C-5 proton), 8.93–9.73 (m, 4 H, pyridine C-4,6,2 protons + phenolic proton). Anal. $C_{27}H_{30}NO_3I$: C, H, N, I.

3-Hydroxy-17β-[[(1-methyl-1,4-dihydropyridin-3-yl)-carbonyl]oxy]-19-nor-17α-pregna-1,3,5(10)-trien-20-yne (4d). One gram (1.8 mmol) of **4c** was suspended in 50:50 water/*tert*-butyl alcohol (100 mL). To this cooled solution were added 0.77 g of $NaHCO_3$ and 0.96 g of $Na_2S_2O_4$. After 1 h of stirring at 0 °C, the reaction was stopped, and the reaction mixture was extracted twice with 100 mL of CH_2Cl_2. The combined and dried ($MgSO_4$) organic solvents were removed under reduced pressure to give 520 mg (69%) of a yellow foam: UV (MeOH) nm 212, 282, 360; NMR ($CDCl_3$) δ 1.26 (s, 3 H, angular methyl group), 0.87–3.00 (m, 15 H, skeletal protons), 2.63 (s, 1 H, alkynyl proton), 2.93 (s, 3 H, N-CH_3), 3.00–3.23 (m, 2 H, pyridine C-4 protons), 4.57–4.90 (m, 1 H, pyridine C-5 proton), 5.50–5.78 (m, 1 H, pyridine C-6 proton), 6.50–6.73 (m, 2 H, E_2-2,4 protons), 6.92–7.23 (m, 2 H, E_2-1 + pyridine C-2 protons); TLC R_f 0.55 (80:20 $CHCl_3$/acetone). Anal. $C_{27}H_{31}NO_3$: C, H, N.

Analytical Methodology. Several high-pressure liquid chromatographic (HPLC) systems were developed to separate, detect, and quantitate the CDS and their metabolites. The structurally similar dihydropyridines **1d** and **4d** were analyzed with a Toya Soda ODS-120T 5-μm particle size, 25 cm × 4.6 mm i.d., C18 reversed-phase analytical column fitted with a guard column. A mobile phase consisting of 90:10 acetonitrile/water eluted **1d** at 5.2 min and **4d** at 5.8 min. The compounds were detected at 360 nm. For the more polar components, a mobile phase consisting of 55:45 acetonitrile/0.05 M KH_2PO_4 buffer was used. Under these conditions, both the quaternary salts and parent steroids could be conveniently eluted in the same chromatographic run. The retention time for **4c** was 5.01 min, for **1c** 5.2 min, for **4** 7.60 min, and for **1** 7.70 min. The compounds in this series were detected at 220 nm. Ethisterone (retention time 8.8 min) served as an internal standard. In all cases, the flow rate was 1 mL/min, and the determinations were made at ambient temperature. A Perkin-Elmer Series 4 pump, a Kratos Spectroflow 757 variable-wavelength detector, a Perkin-Elmer LC1-100 integrator, and a Perkin-Elmer ISS-100 autosampler were used in the analysis.

In Vitro Studies. Lipophilicity Studies. The lipophilicity of the various derivatives was determined using an R_m method. Solutions of **1d**, **2c**, **3c**, or **4d** in methylene chloride were spotted on C18 reversed-phase TLC plates (Baker $SIC_{18}F19C$) and developed with various mixtures of methanol and water. R_m was determined by the expression

$$R_m = \log (1/R_f - 1)$$

where R_f is the distance traveled by the compound divided by the distance traveled by the solvent front. Extrapolation of the calculated R_m values to 100% water gave the reported values. In all cases, the line generated gave an $r > 0.99$.

Organ Homogenate and Blood Stability. Fresly obtained rat brain, liver, and blood were used in this study. The organs were homogenized in phosphate-buffered saline, giving a final homogenate concentration of 20% w/v. Rat whole blood was used. The dihydropyridines **1d** and **4d** were dissolved in dimethyl sulfoxide (DMSO) and added to either of the three biological matrices, all of which were maintained at 37 °C. At various times after addition of the CDS, 100 μL of the homogenate or blood were removed, rapidly mixed with 400 μL of cold acetonitrile, and centrifuged at 13000*x* (Beckman Microfuge 12). The supernatant was then analyzed by HPLC. The disappearance of the dihydropyridines was first order and the rate constant was obtained from a plot of the log [peak height] versus time.

In Vivo Studies. Distribution Studies. A dose of 18 μmol/kg of **4d**, **4**, or 0.5 mL/kg vehicle was administered iv (tail vein) into conscious, restrained Sprague–Dawley rats (body weight = 180–230 g). Animals were sacrificed 15 min, 1, 3, 6, 24, or 48 h posttreatment in the case of **4d** and 15 min or 1 h posttreatment in the case of **4**. For each time point and for each drug, six animals were used. Animals were decapitated, and trunk blood was collected into heparinized tubes. Brains were removed, weighed, and placed on dry ice within 90 s of death. In the preparation of brains for analysis, each organ was homogenized in 1 mL of water. To this were then added 4 mL of ice-cold acetonitrile containing 5×10^{-6} M internal standard (ethisterone) and 1 mL of saturated sodium chloride. The mixture was then vortexed, centrifuged, and cooled to −15 °C. The organic layer was removed, filtered through 0.45 μm polyvinylidene difluoride membranes, and analyzed by HPLC. One milliliter of blood was similarly treated. Standard curves were constructed for brain homogenate and blood, and peaks corresponding to either **4c** or **4** were measured and divided by the peak height of the internal standard. The standard curves were linear ($r > 0.999$). In determining the concentration of **4** in the brain after 6 h, the low levels required that blood and brain samples be pooled, evaporated, and assayed. Standard curves were prepared by treating known concentrations of **4** in a manner similar to the unknowns. In this procedure, **4c** did not significantly hydrolyze during the sample preparation.

LH Studies. Female Sprague–Dawley rats (Charles River Laboratories, Wilmington, MD) were anesthetized and ovariectomized after 1 week of acclimatization in our temperature (22 ± 2 °C) and light (lights on 0600–2000 h) controlled animal facility. Two weeks later, 0.5 mg/kg of **1d** or equimolar doses of **2c**, **3c**, or **4d** were administered iv to conscious, restrained animals (n = 8/group). The vehicle was DMSO. At day 12 posttreatment, a blood sample was taken by cardiac puncture from ether anesthetized rats, and at day 18, animals were sacrificed by decapitation, trunk blood was collected, and rats were necropsied. Organs (including adrenals, anterior pituitaries, and uteri) were weighed. Sera were separated by centrifugation from the blood samples and frozen at −15 °C until analyzed. Serum LH was measured by radioimmunoassay with kit materials and directions provided by NIADDK. Values are expressed in terms of LH-RP-2 reference standard.

The effect of dose of **4d** on LH suppression and its length of effect were studied. Two weeks postovariectomy, rats were treated with 1.0, 0.3, 0.1, or 0.03 mg/kg **4d**, **4**, or vehicle (DMSO, 0.5 mL/kg). Blood samples were taken on day 12 and 18 posttreatment via heart puncture, and trunk blood was collected at sacrifice on day 25. Serum LH levels were determined as previously described.

Data Analysis. The significance of differences among mean values was determined by analysis of variance (ANOVA) and Student–Newman–Keuls tests. The level of probability for all tests was set at $p < 0.05$.

Acknowledgment. We thank Dr. J. W. Simpkins for providing materials and facilities to assay LH and Rebecca Hamilton for technical assistance with this assay. These studies were supported financially in part by Gynex, Inc. and by a grant from the NIH (GM 27167).