Steroidal Affinity Labels of the Estrogen Receptor. 1. 17α-(Bromoacetoxy)alkyl/alkynylestradiols

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To develop steroidal affinity labels for the estrogen receptor, we prepared five electrophilic estradiol derivatives bearing the 17α-propyl, 17α-(1'-butynyl), or 17α-(1'-octynyl) chain, with either a terminal epoxy function (for the 17α-propyl substituent) or a terminal bromoacetoxy function (for all three 17α-substituent types). These compounds displayed low affinity for the lamb uterine estrogen receptor; with apparent relative affinity constants ranging from 0.02% to 0.24% that of estradiol. They were also rapidly transformed in cytosol, probably to the corresponding vicinal diols (epoxy compounds) or primary alcohols (bromoacetoxy compounds). Nevertheless, bromoacetates induced irreversible inactivation of the hormone-binding site but only with ligand-free binding sites. The effect of bromoacetates was prevented by treatment of the cytosol with the thiol-specific reagent methyl methanethiosulfonate. Inactivation of the receptor at 0 °C was rapid (<1 h) and strongly dependent on both compound concentration and pH, with significant effects obtained at either >150 nM (at pH 9) or pH > 7.5 (at 5 μM). Regardless of the conditions used, the order of efficiency for bromoacetates was always: 17α-propyl derivative < 17α-butynyl derivative < 17α-octynyl derivative, with maximal inactivation of ~30% and ~70% of the hormone-binding sites obtained for the less active and more active compounds, respectively. Characteristics of the receptor inactivation suggest that (i) prepared bromoacetates are highly reactive affinity labels for the estrogen receptor, (ii) they react with similar (or even a single) nucleophilic amino acid residues located within or near the hormone-binding site of the receptor; these residues are probably the -SH of cysteines, and (iii) position 17α of steroidal ligands is suitable for introducing electrophilic substituents to develop efficient affinity labels for the receptor.

Introduction

The hormone-binding domain of estrogen receptors[1] includes ~250 amino acids[2,3] which are highly conserved.[3,4] Mutation analyses have indicated that the whole domain is important for hormone binding,[5] and hormone-binding properties are frequently altered due to substitution of single amino acids.[6] Presently, very little is known about the molecular interaction between the hormone and the receptor. Because of the small size of estrogens, probably only a very small number of the 250 amino acids directly binds to the steroid. However, amino acids of the hormone-binding site that directly bind the hormone should be defined since this interaction results in receptor activation, the key process in triggering hormone action.

Affinity labels of the receptor, that can specifically recognize the hormone-binding site then covalently react with a suitable neighboring amino acid of this binding site, are useful for identifying amino acids in contact with the hormone. A battery of affinity labels of the estrogen receptor could be used to map the estrogen-binding site of the receptor and probably enhances our knowledge on molecular mechanisms of receptor activation. This could permit new potent and highly selective estrogens or antiestrogens to be defined for instance. Several steroids, including 4-mercuriestradiol,[7] 16α-hydroxyestrone,[8] 17β-((4'-nitrophenyl)dithio)-17β-deoxyestradiol,[9] and organometallic derivatives of estradiol,[10,11] were postulated as being able to alkylate the estrogen receptor. One or more of the four cysteines of the receptor hormone-binding domain are probably involved in covalent attachment of 4-mercuriestradiol, 17β-((4'-nitrophenyl)dithio)-17β-deoxyestradiol, or organometallic derivatives of estradiol to the receptor. Katzenellenbogen and co-workers developed two series of nonsteroidal affinity labels of the estrogen receptor bearing an aziridine function. One series was related to the triphenylethylene antiestrogen tamoxifen[12,13] the other to the diphenylethane estrogen ketononestrol.[14] A single cysteine (cysteine 530 of the human estrogen receptor) was identified as the covalent attachment site of both estrogen and antiestrogen affinity labels.[15] This does not reflect differential activation of the estrogen receptor by estrogen and antiestrogen but might be explained by the structural characteristics of these nonsteroidal affinity labels. Binding to the receptor of these flexible and relatively symmetrical ligands could be less constrained and less specific than that of the highly rigid and asymmetric steroidal ligands. We thus undertook to develop three series of steroidal affinity labels of the receptor, including the estradiol molecule bearing either a 17α, 11β, or 7α aliphatic or aromatic chain with a terminal reactive function. The 17α-, 11β-, and 7α-substitution were chosen in light of (i) reports that mentioned high affinity for estradiol bearing such substitutions,[16-19] and (ii) the hypothesis that each series of compounds labels a different part of the hormone-binding site. In this paper, we report the preparation of 17α-alkyl or -alkynyl derivatives of estradiol bearing a terminal epoxy or bromoacetoxy function. We then demonstrate that the three synthesized bromoacetoxy derivatives irreversibly inactivated the hormone-binding site of the estrogen receptor. The characteristics of receptor inactivation strongly suggest that these compounds are efficient affinity labels

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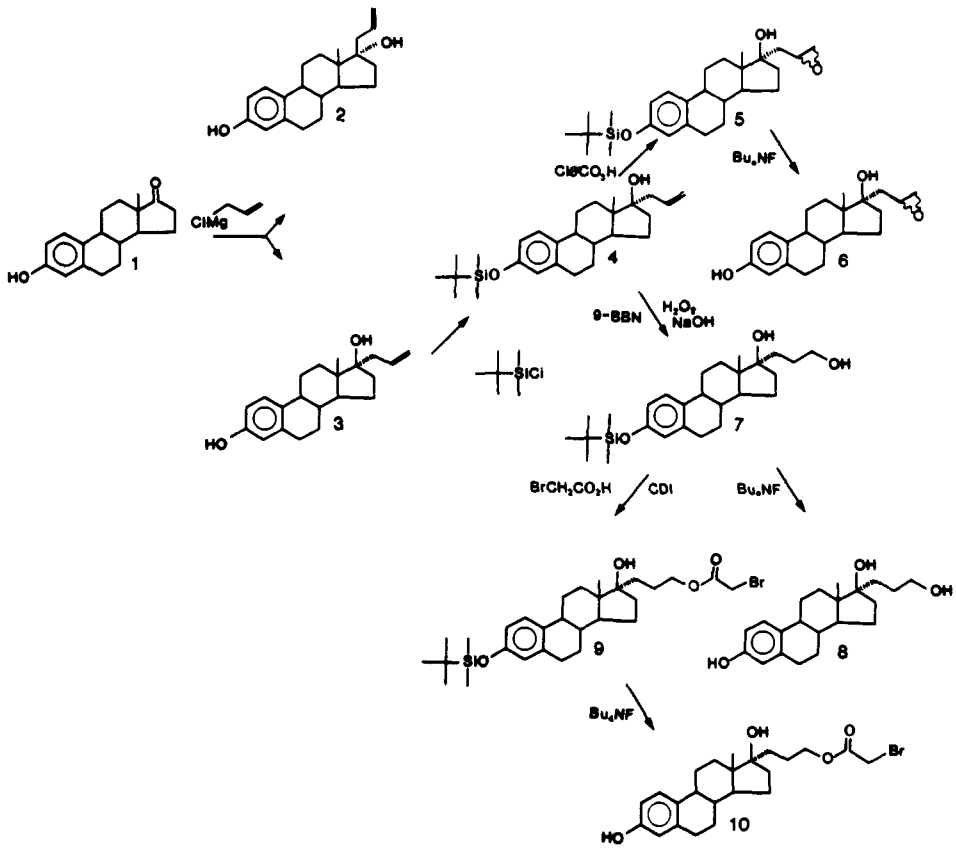
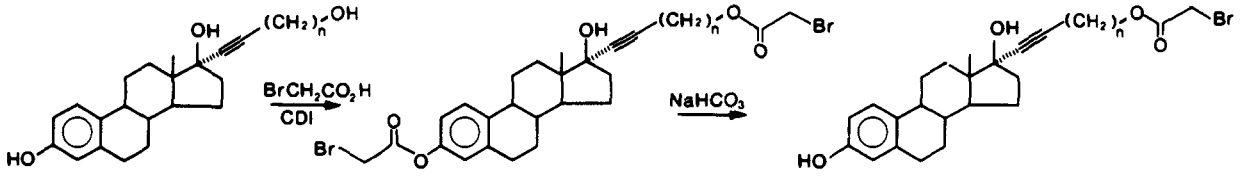
Scheme I



Scheme II



11 (n=2) 13 (n=2) 15 (n=2)
12 (n=6) 14 (n=6) 16 (n=6)

of the receptor and that their targets are basic nucleophiles of the hormone-binding site, probably the -SH of cysteines.

Results

I. Synthesis of Electrophilic 17α-Derivatives of Estradiol. Estrone was used as starting material for preparation of electrophilic derivatives of 17α-propylestradiol including bromoacetate **10** and epoxides **6a** and **6b** (Scheme I). Syntheses of 17α-allylestradiol **3**,[20] epoxides **6a** and **6b** and triol **8**[16] were already reported. We used a similar approach to obtain **6a**, **6b**, and bromoacetate **10**. Briefly, addition of the Grignard of allyl chloride to the ketone function of estrone afforded mixture of diastereoisomers: 17α-hydroxy-17β-(2′-propenyl)estradiol **2** (<5%) and 17α-(2′-propenyl)-17β-hydroxyestradiol **3** (>95%), which were separated by chromatography. The phenolic function of **3** was protected as TBDMS ether to give **4**; then epoxidation of the terminal alkene function by m-CPBA gave a mixture of the two diastereoisomers **5a** and **5b** (75/25), which were separated by chromatography. Removal of the TBDMS group from **5a** and **5b** by tetrabutylammonium fluoride afforded 17α-(2′,3′-epoxypropyl)estradiol **6a** and **6b**, respectively. The terminal alkene function of **4** was submitted to hydroboranation–oxidation conditions (9-BBN and basic hydrogen peroxide) to afford the primary alcohol **7**. Esterification of the dihydroxy compound **7** by bromoacetic acid in the presence of 1-ethyl-3-(3-(dimethylamino)propyl)carbodiimide (CDI) afforded monoester **9**. The sterically hindered tertiary 17β-hydroxy function was not esterified, even with excess of bromoacetic acid and CDI. Phenol deprotection from **9** gave 17α-(3′-(bromoacetoxy)propyl)estradiol **10**. For comparative purposes, triol **8** was prepared from **7** by phenol deprotection.

17α-(ε′-(Bromoacetoxy)alkynyl) derivatives of estradiol **15** and **16** were prepared from the corresponding triols **11** and **12** in two steps (Scheme II). Action of bromoacetatic acid on **11** and **12** in the presence of CDI gave diesters **13** and **14**, respectively. Selective saponification of the phenolic esters with $NaHCO_3$ afforded 17α-(4′-(bromoacetoxy)butynyl)estradiol **15** and 17α-(8′-(bromoacetoxy)-octynyl)estradiol **16**, respectively.

II. Estrogen Receptor Binding Affinity. Competition experiments using the cytosolic estrogen receptor were performed at pH 7 to determine the apparent relative affinity constant (RAC) of epoxides **6a** and **6b**, primary

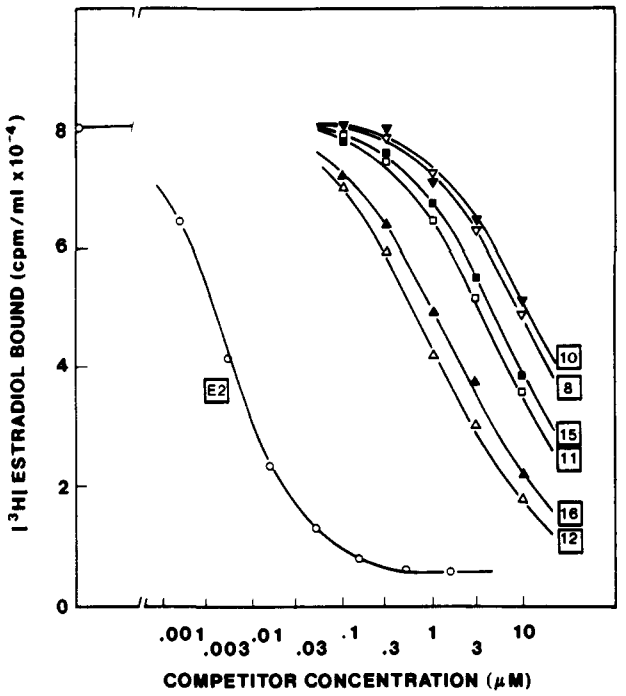


Figure 1. Inhibition of estradiol binding to the estrogen receptor by estradiol 17α-derivatives. Cytosol (2 mg protein/mL, pH 7.0) was incubated with 5 nM [^{3}H]estradiol and increasing concentrations of either nonradioactive estradiol (E_2, ○), 17α-(bromoacetoxy)alkyl/alkynylestradiols (10▼, 15■, and 16▲) or corresponding primary alcohols (8▽, 11□, and 12△) for 20 h at 20 °C. Binding of [^{3}H]estradiol in cytosol measured by charcoal assay is plotted against the competitor concentration. Experimental variation between duplicate determinations was less than 10%.

Table I. Apparent Relative Affinity Constants of Estradiol 17α-Derivatives[a]

compound		apparent RAC 4 h, 0 °C	apparent RAC 28 h, 0 °C	apparent RAC 20 h, 20 °C	RAC ratio (4 h, 0 °C/20 h, 20 °C)
estradiol		100	100	100	1
epoxide	6a	12	1.3	0.044	273
	6b	3.9	0.20	<0.017	>229
bromoacetate	10	0.94	0.21	0.021	45
	15	2.0	0.79	0.066	30
	16	2.4	1.5	0.24	10
alcohol	8	1.1	0.28	0.024	46
	11	3.9	1.0	0.070	56
	12	6.5	3.4	0.40	16

[a] Binding curves similar to those shown in Figure 1, corresponding to competition experiments, were used to determine the concentration of competing ligands which inhibited 50% of the specific binding of [^{3}H]estradiol in cytosol (2 mg protein/mL, pH 7.0) after 4 h–0 °C, 28 h–0 °C, or 20 h–20 °C competition. From these values, the apparent relative affinity constants (RACs) of competitors (relative to that of estradiol) were calculated for the various competition conditions as described in the Experimental Section, with the RAC of estradiol = 100. The ratio of the RAC determined for 4 h–0 °C incubation to the RAC determined for 20 h–20 °C incubation was then calculated. Data given for the apparent RACs correspond to means of duplicate determinations. Experimental variation was less than 10%.

alcohols 8, 11, and 12, and bromoacetates 10, 15, and 16. At equilibrium, competitive binding assays allow determination of the RAC of reversibly binding ligands of the receptor. An apparent RAC can be calculated for (i) reversibly binding ligands under nonequilibrium conditions or (ii) reactive ligands able either to irreversibly bind to the receptor and cytosolic proteins or to be modified during incubation. At 0 °C, the displacement curves of [^{3}H]estradiol by the competitors moved to the right over the time course. They were not stabilized even after a 72-h incubation (not shown), indicating that equilibrium was considerably delayed at 0 °C. At 20 °C, this process was much more rapid since the competition curves were stabilized after a 20-h incubation (Figure 1). All compounds displayed low apparent RAC for the receptor, ranging from <0.02 to ~0.4 (with the RAC of estradiol = 100). The apparent RAC for epoxides dropped considerably from 4 h-0 °C competition to 20 h-20 °C competition; whereas variation in the RAC of the other compounds was much less pronounced (Table 1). For each of the three bromoacetate/related alcohol combinations, we observed a progressive time- and temperature-dependent decrease in the distance between the competition curves. In fact, these effects were due to instability of epoxides and bromoacetates in the cytosol. TLC analyses performed after the compound incubations showed that epoxides were stable when incubated at 0 or 20 °C in buffer. In cytosol they were transformed into more polar compounds displaying the same R_f as that of the corresponding low affinity vicinal diols (~80–100% conversion for 16 h-0 °C or 4 h-20 °C incubation). Bromoacetates were also fairly stable when incubated in buffer alone; in cytosol they were rapidly transformed (even at 0 °C) to form compounds displaying the same R_f as that of the corresponding alcohols (>90% conversion for 1 h-0 °C incubation). We were unable to protect bromoacetates from hydrolysis using various esterase inhibitors (diisopropyl fluoro phosphate, diethyl *p*-nitrophenyl phosphate, and phenylmethylsulfonyl fluoride).

III. Inhibition of Ligand Binding to the Estrogen Receptor by Bromoacetates. Specific alkylation of a receptor by an affinity label normally hinders the receptor from further binding common ligands due to permanent occupancy of the ligand-binding site by the covalently bound affinity label. An irreversible decrease in the specific estradiol-binding capacity of the uterine cytosol following its incubation with a potential affinity label of the receptor is thus a strong indication that part of the estrogen receptor molecules were specifically alkylated by the reactive ligand. We applied this principle to evaluate the estrogen receptor-alkylating ability of synthesized estradiol electrophilic derivatives. Samples of uterine cytosol were first exposed to electrophilic derivatives, at various derivative concentrations, temperatures, times, and pH. Control samples were incubated under identical conditions, without steroid or with chemically inert steroids such as primary alcohols 8, 11, and 12. Thereafter all samples were treated with charcoal to remove unbound or loosely bound steroids. The concentration of remaining specific binding sites for estradiol (corresponding to unfilled and/or reversibly steroid-filled estrogen receptor) was then determined by incubating, under exchange conditions, aliquots with excess [^{3}H]estradiol either alone, to measure total [^{3}H]estradiol binding, or with a large amount of nonradioactive estradiol, to measure nonspecific [^{3}H]estradiol binding.

Figure 2 shows the total (specific plus nonspecific) and nonspecific binding of [^{3}H]estradiol measured in uterine cytosol following a 1-h incubation at 0 °C, pH 8, without steroid or with 5 μM of the various electrophiles or the primary alcohol 12 (the highest affinity compound of the three primary alcohols). Only bromoacetates induced significant decreases in specific binding of [^{3}H]estradiol in cytosol, with ~20, 30, and 50% decreases for compounds

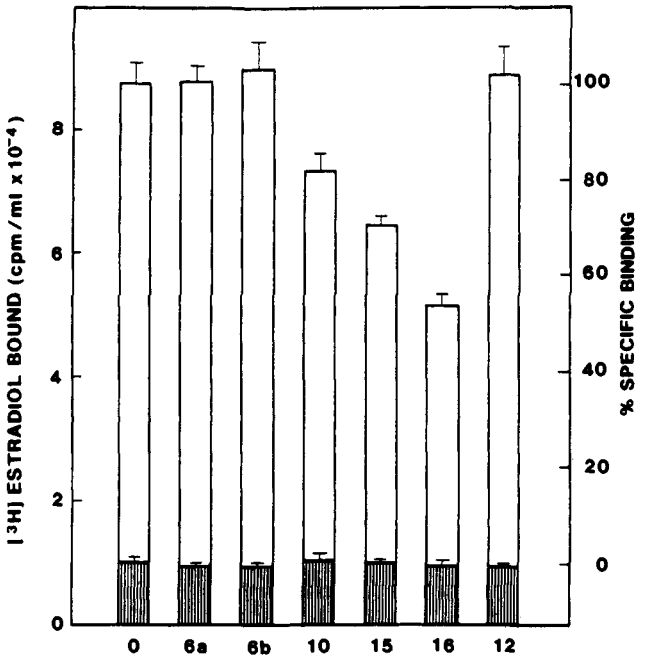


Figure 2. Irreversible inhibition of estradiol binding after exposure of uterine cytosol to estradiol 17α-derivatives. Cytosol (pH 8.0) was incubated for 1 h at 0 °C either without steroid or with 5 μM epoxide **6a** or **6b**, bromoacetate **10**, **15**, or **16** or primary alcohol **12** which is the homologue of **16**. After treatment with an equal volume of charcoal suspension and centrifugation, concentrations of total (unfilled bars) and nonspecific (filled bars) bound [^{3}H]estradiol in the supernatant were determined, after 16 h - 20 °C incubation with 20 nM [^{3}H]estradiol, by charcoal assay. Values are means of triplicate determinations; error bars indicate standard deviations.

10, **15**, and **16**, respectively. Similar quantitative results were obtained when cytosol incubation with steroids was performed at 20 °C instead 0 °C or when the duration of incubation at 0 °C was changed from 1 to 0.25 or 25 h (not shown). However, the effect varied greatly depending on the steroid concentration or the pH in the cytosol (cf. parts VI and VII). Identical results were obtained when 4-hydroxy[^{3}H]tamoxifen, a high affinity triphenylethylene antiestrogen whose characteristics of interaction with the estrogen receptor differ from those of estrogens,[21-23] was used instead of [^{3}H]estradiol to measure the hormone-binding site concentration after incubation of the cytosol with or without steroids (not shown). Finally, the effect was specific to the prepared bromoacetates and was not observed with cytosol exposed to 5 μM of acetates homologous to **10**, **15**, and **16**, respectively, to 5 μM of 11β-(bromoacetoxy)-17α-ethynylestradiol or 17β-(*O*-bromoacetyl)testosterone. Bromoacetic acid and ethyl bromoacetate did not alter [^{3}H]estradiol specific binding in cytosol even at concentrations as high as 100 μM (not shown).

IV. Evidence of Irreversible Inactivation of the Estrogen Receptor Hormone-Binding Site by Bromoacetates. Two kinds of experiments were undertaken to examine whether the observed decrease in [^{3}H]estradiol specific binding of cytosol induced by bromoacetates actually resulted from irreversible inactivation of the hormone-binding site of the receptor.

(A) The Saturable Binding of [^{3}H]Estradiol in Cytosol Measured After Exchange, Reflects Formation of the Estrogen Receptor: [^{3}H]Estradiol Complex. Following prolonged incubation of cytosol with [^{3}H]estradiol under exchange conditions and treatment by charcoal, we analyzed the [^{3}H]estradiol-binding species of cytosol in high salt sucrose gradients. Figure 3 shows the total and nonspecific binding of [^{3}H]estradiol in gradients, from cytosol first exposed or not to 5 μM steroids for 1 h at 0 °C, pH 9. For cytosol not exposed to steroid (Figure 3A), saturable binding of estradiol appeared to mainly involve a 4.5 S species, with minor species in the 8–10 S region. This saturable 4.5 S peak corresponded to the classical salt-dissociated estrogen receptor:[^{3}H]estradiol complex since it shifted to the right of the gradient when serum from mouse immunized with purified preparations of lamb uterine estrogen receptor[23] was added to cytosol before gradient analysis (not shown). The 8–10 S species probably represented aggregated forms of the receptor, resulting from 20 °C-20 h incubation of cytosol with [^{3}H]estradiol. Exposition of cytosol to the primary alcohol **12** (Figure 3C) did not significally change the results observed with the control cytosol. However, exposition of cytosol to bromoacetate **16** resulted in a quantitative change which similarly affected the 4.5 S and the 8–10 S species. Finally, for all samples there was very close agreement between the specific binding of [^{3}H]estradiol measured by the charcoal assay and obtained by integration of specific binding in the sucrose gradient fractions. We conclude that saturable binding of [^{3}H]estradiol in uterine cytosol, measured by charcoal assay after incubation under exchange conditions, actually corresponded to the estrogen receptor:[^{3}H]estradiol complex.

(B) Bromoacetates Irreversibly Inactivate the Estrogen Receptor Hormone-Binding Site. The decrease in [^{3}H]estradiol specific binding in cytosol observed following its exposure to bromoacetates could have resulted from permanent occupancy of the hormone-binding site of a corresponding portion of receptor molecules by covalently linked bromoacetate. However, another possibility would be that this decrease resulted from affinity of [^{3}H]estradiol for the receptor, lower for bromoacetate-treated cytosol than for control cytosol and with a similar specific [^{3}H]estradiol-binding capacity for both types of cytosol, since we used a relatively high but single concentration of [^{3}H]estradiol to measure the concentration of accessible hormone-binding sites. To assess these two possibilities, we performed saturation experiments of control cytosol and bromoacetate-treated cytosol with increasing concentrations of [^{3}H]estradiol. Binding data obtained from cytosol incubated for 1 h at 0 °C, pH 9, either without steroid or with 5 μM bromoacetate **16** or alcohol **12**, are shown in Figure 4A. From the Scatchard plots of saturable binding (Figure 4B), calculated binding site concentration and the apparent equilibrium affinity constant of estradiol were N = 1.03 nM and K_A = 4.83 × 10^9 M^{-1} respectively, for the control cytosol. Binding data obtained for cytosol incubated with **12** reflected competitive inhibition of [^{3}H]estradiol binding, since a similar binding site concentration (N = 0.977 nM) but an ~2.5-fold lower affinity constant (K_A = 1.94 × 10^9 M^{-1}) than for control cytosol were determined. The presence of a low residual concentration of compound **12** in the cytosol after charcoal treatment could account for such competitive inhibition, since the efficiency of charcoal in removing

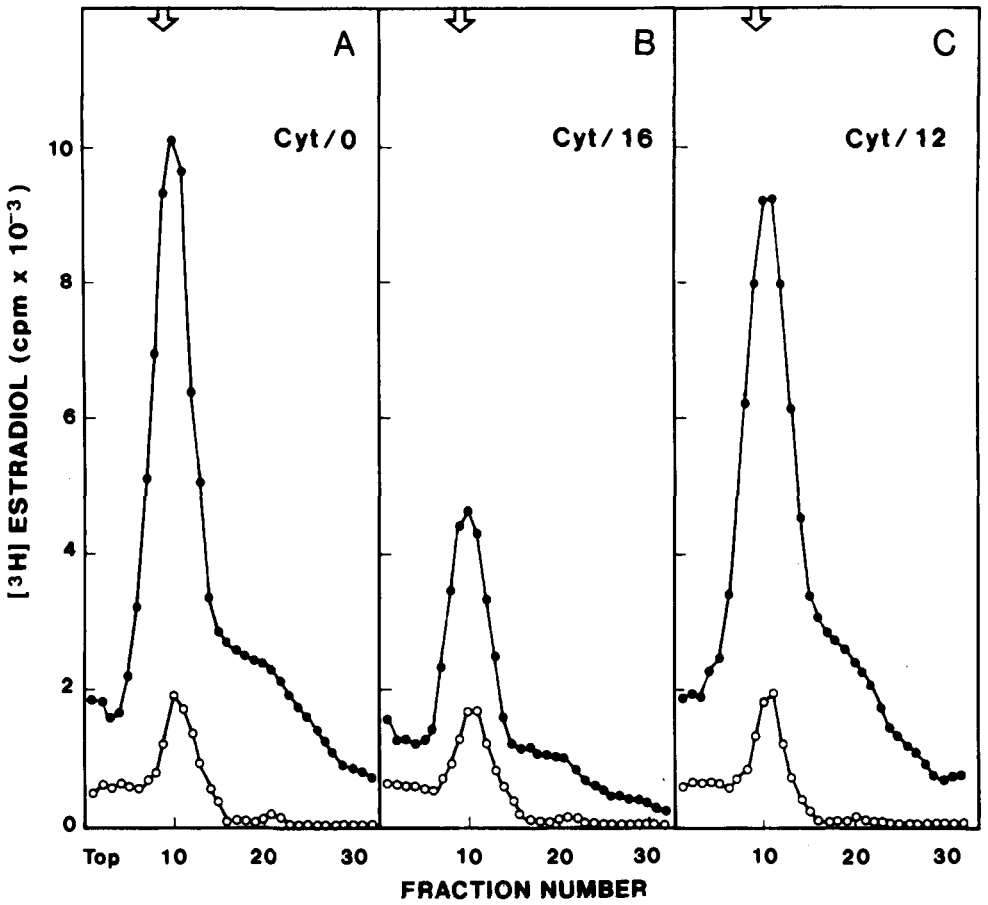


Figure 3. Sedimentation profile in high salt sucrose gradients of estradiol binding in cytosol following incubation under exchange conditions. Cytosol (pH 9.0) was incubated for 1 h at 0 °C either without steroid (A) with 5 μM bromoacetate **16** (B) or corresponding alcohol **12** (C). After treatment with an equal volume of charcoal suspension and centrifugation, aliquots of the supernatant were incubated under exchange conditions with [^{3}H]estradiol in the absence or presence of nonradioactive estradiol to measure total and nonspecific [^{3}H]estradiol binding, respectively. Following a second charcoal treatment, residual [^{3}H]estradiol binding in aliquots was analyzed by centrifugation on the sucrose gradients. Total (●) and nonspecific (○) bound [^{3}H]estradiol in gradient fractions are represented. The arrow indicates the sedimentation position of ovalbumin (3.7 S).

steroids from cytosol was never total (in these conditions, the efficiency of charcoal in removing [^{3}H]estradiol was ~95–97%). For cytosol incubated with bromoacetate **16**, there was a strong decrease in the capacity of cytosol to specifically bind estradiol (N = 0.408 nM), whereas the apparent affinity constant ($K_A = 2.57 \times 10^9$ M^{-1}) was ~2-fold lower than that measured with control cytosol. In this case, inhibition of [^{3}H]estradiol binding resulting from incubation of cytosol with **16** was not overcome by increasing the [^{3}H]estradiol concentration. This sharply contrasted with the situation concerning the related compound **12**, in spite of (i) an affinity of **12** for the receptor higher than that of **16** and (ii) the fact that much of **16** was rapidly converted to **12** in cytosol. We conclude that the decreased binding site concentration induced by **16** was due to irreversible inactivation of the hormone-binding site of a corresponding portion of the receptor molecules. Compared to the control cytosol, the ~2-fold decrease in the apparent affinity constant probably resulted, as stated for cytosol incubated with **12**, from the presence of residual steroids (mainly **12**, due to the instability of **16** in cytosol) in charcoal-treated cytosol.

V. Bromoacetate Inactivation of the Hormone-Binding Site Requires Unfilled Binding Site. To examine the specificity of the bromoacetate effect on inactivation of the estrogen-binding site, we determined the effect of bromoacetates when the hormone-binding site of the estrogen receptor was occupied with either estradiol or 4-hydroxytamoxifen. As shown in Figure 5, the concentration of hormone-binding sites from cytosol not preexposed to estradiol or 4-hydroxytamoxifen, decreased by ~70% after a 1-h incubation of cytosol at 0 °C, pH 9, with 5 μM **16**. This effect was totally abolished for cytosol preexposed to either 50 nM estradiol or 4-hydroxytamoxifen before the addition of **16**. In these latter two cases, the concentration of hormone-binding sites titered with [^{3}H]estradiol was identical to that obtained for the control cytosol (incubated without steroid or with alcohol **12**). Concentrations of hormone-binding sites measured with [^{3}H]estradiol in all samples preexposed to either estradiol or 4-hydroxytamoxifen were, respectively ~30% and ~40% lower than that measured in control cytosol not preexposed to the ligands, due to incomplete removal of these high affinity ligands by charcoal (mainly because of estrogen receptor-bound ligands which dissociated very slowly from the receptor at 0 °C). Hence, there was incomplete labeling of hormone-binding sites by [^{3}H]estradiol.

VI. Bromoacetate Inactivation of the Hormone-Binding Site Is Concentration-Dependent. Since bromoacetates **10**, **15**, and **16** are low affinity ligands of the estrogen receptor with low stability in cytosol, their efficiency for inactivating the hormone-binding site might be highly concentration-dependent. We therefore compared the abilities of increasing concentrations of the three bromoacetates to inactivate the binding site, with control

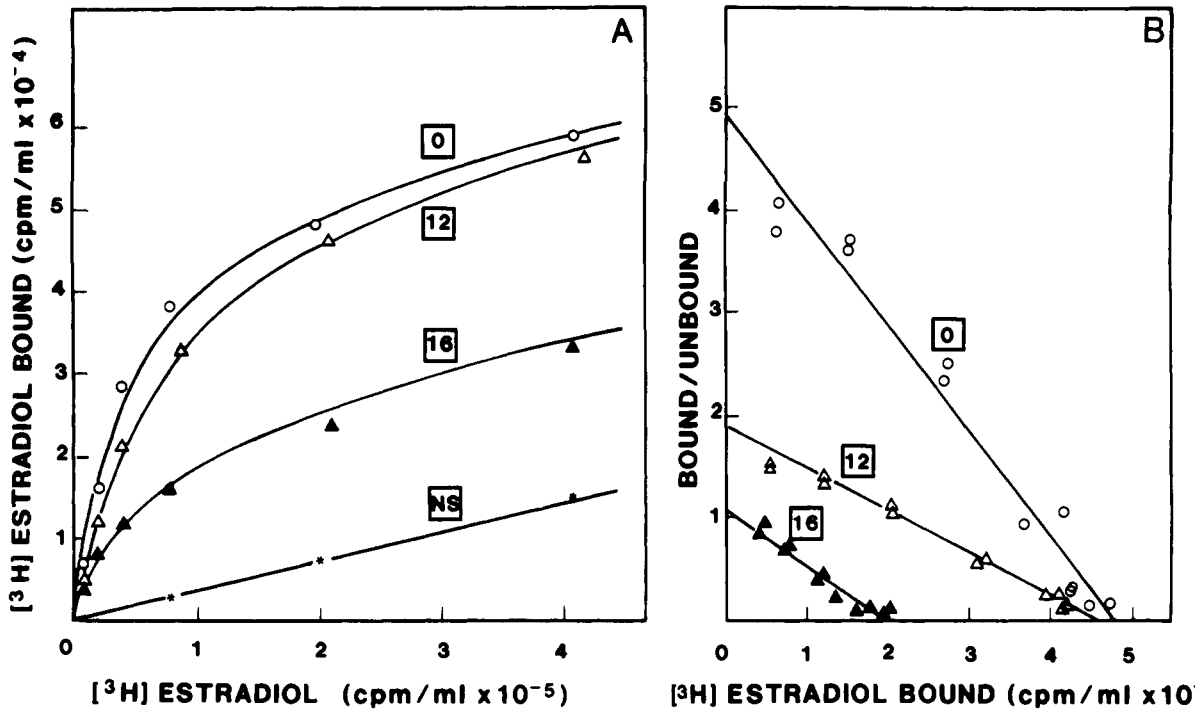


Figure 4. Saturation analysis of estradiol binding after cytosol exposure to alcohol 12 or bromoacetate 16. Cytosol (pH 9.0) was incubated for 1 h at 0 °C either without steroid (●), with 5 μM primary alcohol 12 (Δ), or corresponding bromoacetate 16 (▲). After charcoal treatment, and centrifugation, supernatant aliquots were incubated for 16 h at 20 °C with increasing concentrations (0.2 to 10 nM) of [^{3}H]estradiol in the absence or presence of 5 μM nonradioactive estradiol. Binding of [^{3}H]estradiol was measured by charcoal assay. (A) Mean values of duplicate determinations for concentrations of total (○, Δ, ▲) and nonspecific (*) bound [^{3}H]estradiol are plotted against the total concentration of [^{3}H]estradiol; experimental variation was less than 10%. (B) Scatchard plots of individual specific [^{3}H]estradiol binding data, considering specifically bound [^{3}H]estradiol, as being the difference between the total bound and the nonspecific bound [^{3}H]estradiol and unbound [^{3}H]estradiol, as being the difference between the incubated and the total bound [^{3}H]estradiol. Regression lines were determined respectively from plots relative to control cytosol ($K_A = 4.83 \times 10^9$ M^{-1}, $N = 1.03$ nM, correlation coefficient 0.981), cytosol exposed to alcohol 12 ($K_A = 1.94 \times 10^9$ M^{-1}, $N = 0.977$ nM, correlation coefficient 0.997), and cytosol exposed to bromoacetate 16 ($K_A = 2.57 \times 10^9$ M^{-1}, $N = 0.408$ nM, correlation coefficient 0.967).

incubations performed using increasing concentrations of alcohol 12. The results in Figure 6 illustrate the decreased concentration of accessible estradiol-binding sites, resulting from incubation of cytosol for 1 h at 0 °C, pH 9, with 15 nM to 5 μM of bromoacetates. Inactivation of the binding site by bromoacetates was practically undetectable until 50 nM, slight but significant receptor inactivation occurred at 150 nM; with an ~5–10% decrease in the concentration of accessible binding sites, depending on the bromoacetate used. These effects were progressively more pronounced as the bromoacetate concentration increased. The strongest effects, observed at the highest bromoacetate concentration, corresponded to inactivation of 25, 45, and 60% of binding sites by 10, 15, and 16, respectively. In the 150 nM to 5 μM concentration range, the same order of efficiency (10 < 15 < 16) was observed for the three bromoacetates. Control compound 12 (up to 5 μM) did not significantly inhibit [^{3}H]estradiol binding to the estrogen receptor. However, at higher concentrations (>15 μM) there was a slight inhibition of [^{3}H]estradiol binding, probably resulting from competition of the residual concentration of 12 in cytosol, which was not adsorbed by charcoal. We did not test concentrations of compounds higher than 5 μM so as to avoid such competition interference with bromoacetates.

VII. Bromoacetate Inactivation of the Hormone-Binding Site Is pH-Dependent. Since reactivity of most nucleophilic amino acids is strongly influenced by pH, we studied the extent of inactivation of hormone-binding sites by bromoacetates, relative to pH. We used the 7–9 pH-range, to preserve both the hormone-binding property of the receptor and the chemical integrity of bromoacetates. Figure 7 shows that the hormone-binding capacity of the control cytosol, incubated for 1 h at 0 °C without or with 5 μM 12, decreased slightly above pH 8. Inactivation of the hormone-binding sites by 5 μM bromoacetates, was either slight (~15% for bromoacetate 16) or practically undetectable (≤5% for bromoacetates 10 and 15) at pH 7. The proportion of inactivated binding sites greatly increased with increasing pH, reaching ~20, 50, and 60% for 10, 15, and 16, respectively, at pH 9. As already observed when investigating the effect of the bromoacetate concentration on hormone-binding site inactivation, we observed the same order of efficiency (10 < 15 < 16) at any pH from 7 to 9.

VIII. Treatment of Cytosol with Methyl Methanethiosulfonate Prevents Inactivation of the Hormone-Binding Site by Bromoacetates. To examine the possible involvement of cysteine residues in the inactivation of the hormone-binding site, we studied the effect of the sterically small thiol-specific reagent methyl methanethiosulfonate (MMTS) on bromoacetate-induced inactivation of the hormone-binding site. This reagent, which converts thiol groups to -SSCH$_3$ groups, was previously used to study the involvement of thiols in binding of glucocorticoids to their receptors[24] and to evince the reaction of cysteine residues of wild-type[15] and mutant[25] estrogen receptors with nonsteroidal affinity labels. In agreement with previous reports,[15,25] we observed that preexposure of cytosol to MMTS did not impair the ability of the receptor to bind estradiol (not shown). However, the reagent progressively prevented inactivation

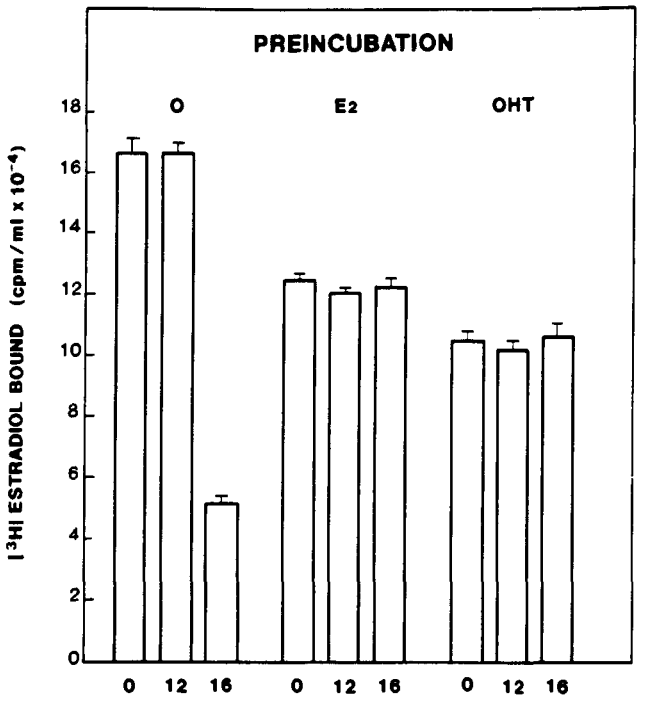


Figure 5. Inability of bromoacetate 16 to inactivate estrogen- or antiestrogen-filled hormone-binding site of the estrogen receptor. Cytosol (pH 9.0) was incubated for 4 h at 0 °C without ligand (O) with 50 nM estradiol (E_2) or 4-hydroxytamoxifen (OHT). Aliquots of the various samples were then incubated for 1 h at 0 °C, either without steroid (O) with 5 μM alcohol 12 or bromoacetate 16. After charcoal treatment and centrifugation, the supernatant was incubated with [^{3}H]estradiol under exchange conditions to determine total and nonspecific [^{3}H]estradiol binding. Mean specific [^{3}H]estradiol binding from triplicate determinations are represented; error bars indicate standard deviations.

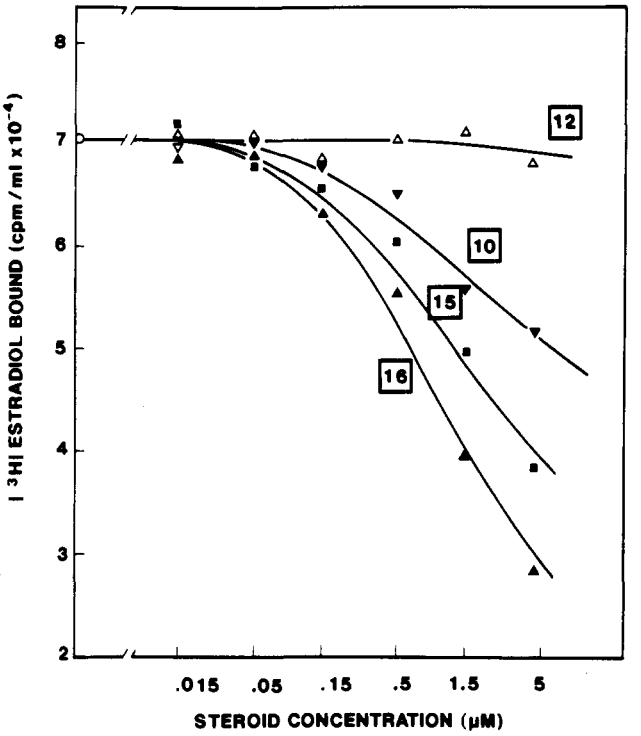


Figure 6. Concentration-dependent effects of 17α-(bromoacetoxy)alkyl/alkynylestradiols on the inactivation of specific estradiol-binding sites. Cytosol (pH 9.0) was incubated for 1 h at 0 °C with increasing concentrations (0 to 5 μM) of bromoacetate 10 (▼), 15 (■), or 16 (▲) or alcohol 12 (Δ). After charcoal treatment and centrifugation, supernatant aliquots were incubated with [^{3}H]estradiol under exchange conditions. Binding of [^{3}H]estradiol was measured by charcoal assay. The specific binding of [^{3}H]estradiol is plotted against the steroid concentration. Experimental variation between duplicate determinations was less than 10%.

of the hormone-binding site by bromoacetates (Figure 8). The effect was detectable from 0.03 mM and practically complete at a reagent concentration equal to that of the total -SH group in cytosol. Finally, the IC_{50}s of MMTS were very similar (0.1–0.2 mM) for all three bromoacetates.

Discussion

I. 17α-(Bromoacetoxy)alkyl/alkynylestradiols Are Estrogen Receptor Affinity Labels. In this paper, we describe the preparation and characterization of five 17α-derivatives (two epoxides and three bromoacetates) of estradiol bearing an electrophilic group at the extremity of the 17α-chain. Evaluation of these electrophiles revealed that all three bromoacetates irreversibly inactivated the hormone-binding site of the lamb uterine estrogen receptor in a concentration- and pH-dependent manner. The inactivation was due to the alkylating properties of these compounds since it did not occur with closely related but unreactive molecules, such as alcohol and acetate homologues of the three bromoacetates. Moreover this inactivation was a specific property of these compounds since other bromoacetoxy derivatives including nonsteroidal compounds (ethyl bromoacetate and bromoacetic acid) and steroidal compounds (17β-(*O*-bromoacetyl)testosterone and 11β-(bromoacetoxy)-17α-ethynylestradiol) did not induce such effects. We cannot exclude that the observed inactivation of the hormone-binding site could have been due to alkylation of the estrogen receptor remote from the hormone-binding site, which would indirectly affect the hormone-binding properties of the receptor. However, this is less probable than receptor alkylation at or near the hormone-binding site since occupancy of the binding site by estrogen or antiestrogen totally prevented the bromoacetate effects. We therefore conclude that the three prepared bromoacetates are probably affinity labels of the estrogen receptor. Specific alkylation of a receptor by a reactive ligand normally occurs in two steps. As a conventional ligand the reactive molecule first reversibly associates with the receptor, and then a reaction takes place between the reactive function of the ligand and an appropriate amino acid of the ligand-binding site. Stability of the reversible receptor:reactive ligand complex is thus a crucial parameter which directly influences both the efficiency and specificity of the alkylation process, the higher the stability of the complex, the higher the probability that alkylation of the hormone-binding site occurs. Considering the ability of bromoacetates **10**, **15** and **16**, to specifically alkylate the receptor, two characteristics appear to be unfavorable.

(i) The low stability of compounds in cytosol: These bromoacetates are rapidly hydrolyzed to the corresponding primary alcohols. This process not only decreases the concentration of the compound able to alkylate the receptor but also generates a non-alkylating competitor whose affinity for the receptor is higher than that of the parent compound and which inhibits interaction of available receptors with intact bromoacetate. High concentrations of bromoacetates were thus required to permit alkylation of the hormone-binding site of receptors, since (1) to complex a given proportion of receptors, the higher the steroid concentration, the lower the duration of

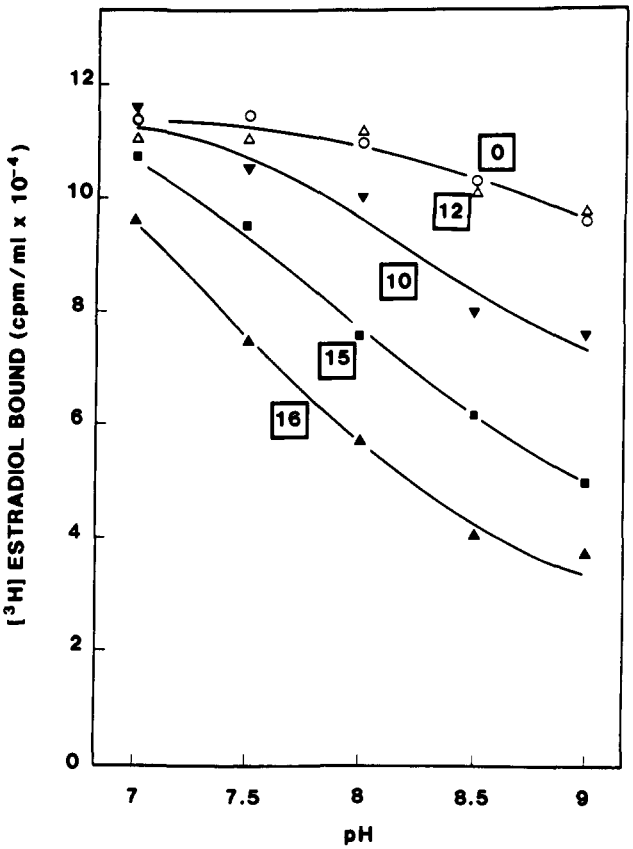


Figure 7. pH-dependent inactivation of specific estradiol binding sites by 17α-(bromoacetoxy)alkyl/alkynylestradiols. Cytosol (pH 7.0) was progressively alkalized by the addition of NaOH 0.5 M. Samples were removed at the following pH levels: 7.0, 7.5, 8.0, 8.5 and 9.0. Samples were incubated for 1 h at 0 °C either without steroid (●), with 5 μM bromoacetate 10 (▼), 15 (■), or 16 (▲) or alcohol 12 (Δ). After charcoal treatment and centrifugation, supernatant aliquots were incubated with [^{3}H]estradiol under exchange conditions. The specific binding of [^{3}H]estradiol is plotted against pH. Experimental variation between duplicate determinations was less than 15%.

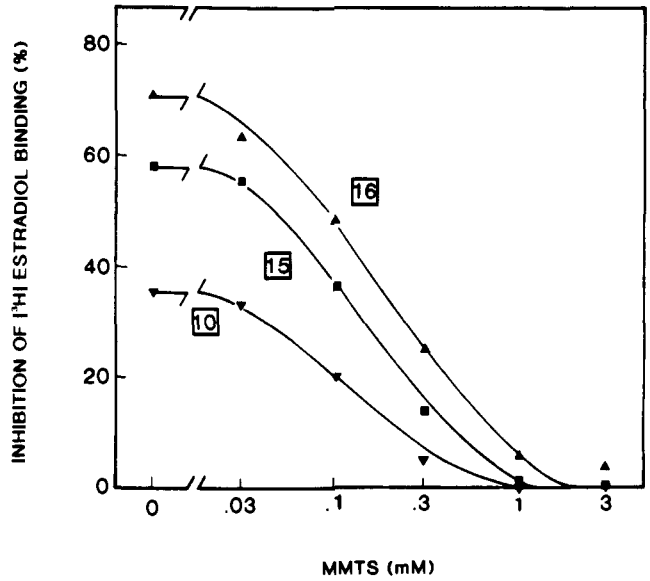


Figure 8. Effect of methyl methanethiosulfonate on the inactivation of specific estradiol-binding sites by bromoacetates. Cytosol (pH 9.0) was incubated for 2 h at 0 °C with increasing concentrations (0–3 mM) of methyl methanethiosulfonate (MMTS). Aliquots of the various samples were then incubated for 1 h at 0 °C with 5 μM bromoacetate 10 (▼), 15 (■), or 16 (▲) or alcohol 12 as a control. After charcoal treatment and centrifugation, supernatant aliquots were incubated with [^{3}H]estradiol under exchange conditions. The specific binding of [^{3}H]estradiol in cytosol exposed to each of the bromoacetates, expressed as a percent of the specific binding of [^{3}H]estradiol in cytosol exposed to alcohol 12, is plotted against the MMTS concentration. Experimental variation between duplicate determinations was less than 10%. The 100% specific binding of [^{3}H]estradiol in the absence or presence of MMTS was 62 300 cpm/mL; the total -SH group concentration was ~1 mM.

exposure of receptors to steroid (relative to the steroid concentration, formation of the complex is a kinetic first-order process) and (2) the lower the duration of exposure, the higher the ratio of the bromoacetate concentration to that of the formed alcohol (at the beginning this ratio is roughly inversely proportional to time) and then the higher the ratio of the receptor:bromoacetate complex concentration to that of the receptor:alcohol complex.

(ii) The low stability of the receptor/bromoacetate complexes: The apparent RAC calculated for bromoacetates ranged from ~0.02% (compound **10**) to ~0.24% (compound **16**) to that of estradiol. Assuming, as usually observed, that affinity mainly reflects the dissociation rate of the complex, then from the half-dissociation times reported for ligands such as estradiol[26] and from the apparent affinity constants determined for bromoacetates, one can obtain a rough estimate of the half-lives of the receptor:bromoacetate complexes at 0 °C: <5 min with **10**, <15 min with **15**, and <60 min with **16**.

When comparing the ability of bromoacetates to alkylate receptor molecules, according to either the compound concentration or pH, in both cases we observed strong differences between the three compounds, with the same efficiency order: **10** < **15** < **16**. These differences might partly reflect different intrinsic properties of the common electrophilic group in the three compounds, such as chemical reactivity and/or location relative to those of nucleophiles in the hormone-binding site. For instance, the degree of freedom of the reactive terminal carbon is considerably higher in compound **16** than in compound **15** or **10**. However, the more trivial previously described properties, i.e., the relative affinity and relative stability of compounds in cytosol, certainly played a major role in the compound efficiencies. It should be noted that there was a good correlation between the apparent relative affinity of bromoacetates and their ability to alkylate the hormone-binding site of the receptor.

II. A Single Cysteine Residue Is Probably the Target of All Three Bromoacetates. The marked pH-dependence of the alkylation processes, with very weak effects at pH 7 and then increasing effects up to pH 9, observed with all three bromoacetates, suggests that similar (or even a single) basic nucleophiles of the receptor hormone-binding site react with the three bromoacetates. It is unlikely that methionines or histidines are bromoacetate targets since these amino acids are usually reactive at neutral pH. Among the amino acids which contain a reactive side chain with a basic pK_a, cysteine (pK_a ~8.3) and lysine (pK_a ~9.2) are more probable candidates than tyrosine (pK_a ~10.1) and arginine (pK_a ~12.5).

Alkylation of the receptor by the three bromoacetates required intact cysteine residues since the cysteine-specific reagent MMTS totally prevented the effect of the compounds. One of the following two possibilities could therefore be considered: (i) cysteines are the actual targets of bromoacetates, and (ii) MMTS-modification of cysteine residues by steric hindrance impairs the reaction of

neighboring nucleophiles with bromoacetates. The second hypothesis appears unlikely since (1) the size of the functional group which is transferred by MMTS to cysteine residues is relatively small and (2) the fact that each bromoacetate displayed, for MMTS-modified receptor, a relative affinity which was equal to that of untreated receptor suggests that modification of the cysteine residues of the receptor does not significantly impair bromoacetates/receptor interactions. Therefore, cysteine residues of the receptor are probably bromoacetate targets. Moreover, the fact that the concentration-dependent blockade of the bromoacetate effects by MMTS was very similar for the three compounds with practically identical IC_{50} values strongly suggests that the same cysteine residue is the target of the three bromoacetates. pH-dependent alkylation of the receptor by bromoacetates was in sharp contrast to that displayed by the two nonsteroidal affinity labels: the antiestrogen tamoxifen aziridine and the estrogen ketononestrol aziridine which, at neutral pH, both efficiently react with the same cysteine residue of the hormone-binding site.[15] This contrast could result from the low affinity and low stability of bromoacetates which, for efficient alkylation of the receptor would require a highly reactive negatively charged cysteine residue. Finally, the suspected cysteine target of bromoacetates might differ from the lamb homologue of cysteine 530 of the human estrogen receptor since construction of steroidal analogues of tamoxifen aziridine and ketononestrol aziridine led to mean positions of the aziridine function nearer to the C11–C12 region of the C ring than to the C17 region of the D ring of the steroid. Another possibility could involve the difference in the chemical nature of the two types of electrophiles, since aziridine groups, which show very little reactivity in the uncharged form, become highly reactive after protonation.[13] This suggests that aziridine compounds alkylate the receptor at neutral pH on cysteine 530, which could act as a proton donor.

In conclusion, estradiol derivatives bearing a 17α, C3–C8-alkyl or alkynyl chain, with a terminal bromoacetate function, are highly reactive affinity labels of the estrogen receptor. At least one basic nucleophile, probably the -SH of a cysteine, appears to react with the electrophilic carbon of bromoacetates. Preparation of homologous compounds bearing similar electrophilic groups linked to the estradiol molecule by means of a nonhydrolyzable 17α-chain is in progress in our laboratory. Such compounds will probably be reactive affinity labels useful for identifying target amino acids in the hormone-binding site of the estrogen receptor.

Experimental Section

Synthesis and Characterization of Estradiol Derivatives. Estrone was purchased from Fluka, and 17α-(4'-hydroxy-1'-butynyl)estradiol and 17α-(8'-hydroxy-1'-octynyl)estradiol were donated by Dr. Poirier (Centre Hospitalier de l'Université Laval, Québec, Canada). Solvents and reagents were purchased from the following commercial sources: Aldrich, Carlo Erba, Fluka, Merck, and Sigma. Analytical TLC was performed with Merck aluminium sheet-baked silical gel (F-254, 0.2 mm). Column chromatography was performed with silica gel (0.063–0.2 mm). Nuclear magnetic resonance spectra were recorded on a WM 360 WB spectrometer at 360 MHz. Chemical shifts are reported downfield of tetramethylsilane internal standard (δ scale). High-resolution, electron-impact mass spectra were obtained on a LKB 2091 spectrometer. Elemental analyses were performed by the microanalytical service of CNRS (Vernaison, France).

Except when otherwise stated, a standard procedure was used for product isolation. This involved quenching of the reaction mixture in water or aqueous solution, followed by exhaustive extraction with ether or ethyl acetate, washing of extracts with aqueous solutions when necessary, drying of organic extracts over Na_2SO_4, filtration, and evaporation of the solvent under reduced pressure. The solvents and the aqueous solutions used for quenching the reaction and washing are mentioned in parentheses after "product isolation".

17α-(2'-Propen-1'-yl)estra-1,3,5(10)-trien-3,17β-diol (3). Ten milliliters (120 mmol) of allyl chloride was added to 2.43 g (100 mmol) of magnesium turning under nitrogen. After starting of the reaction, dry THF (20 ml) was added, and the mixture was agitated until complete reaction of magnesium. A solution of 4.05 g of estrone (15 mmol) in 150 mL of dry THF and 10 mL of allyl chloride was added dropwise to the Grignard solution. After the addition of estrone was completed, the reaction mixture was heated to reflux for 2 h, and stirring was maintained for 15 h at 20 °C. The mixture was then pourred into a saturated NH_4Cl solution. Product isolation (Et_2O, $NaHCO_3$, Na_2SO_4) afforded mixture of diastereoisomers **2** and **3** (**2**/**3** < 1/20): chromatography (Et_2O/heptane 1/4) gave 4.1 g of **3** (88%); ^1H NMR ($CDCl_3$) δ 0.92 (s, 3H, 18-CH_3), 5.19 (m, 2H, 3'-CH_2), 6.00 (m, 1H, 2'-CH), 6.55 (d, 1H, J = 2.7 Hz, 4-CH), 6.61 (q, 1H, J_1 = 2.7 Hz, J_2 = 8.3 Hz, 2-CH), 7.13 (d, 1H, J = 8.3 Hz, 1-CH); MS (70 eV), m/z (rel intensity) 312 (M^+, 8), 294 (9), 272 (11), 43 (100). Anal. ($C_{21}H_{28}O_2$) C, H.

3-(*tert*-Butyldimethylsiloxy)-17α-(2'-propen-1'-yl)estra-1,3,5(10)-trien-17β-ol (4). A mixture of 1.68 g (11.1 mmol) of TBDMS chloride and 1.58 g (23.2 mmol) of imidazole in 7 mL of DMF was added to 2.9 g (9.3 mmol) of **3**. After the mixture was stirred for 1 h at 35 °C, product isolation (Et_2O, $NaHCO_3$, Na_2SO_4) and then chromatography (Et_2O/heptane 1/9) gave 3.48 g of **4** (88%): ^1H NMR ($CDCl_3$) δ 0.17 (s, 6H, $Si(CH_3)_2$), 0.92 (s, 3H, 18-CH_3), 0.96 (s, 9H, $SiC(CH_3)_3$), 5.19 (m, 2H, 3'-CH_2), 6.00 (m, 1H, 2'-CH), 6.55 (d, 1H, J = 2.7 Hz, 4-CH), 6.61 (q, 1H, J_1 = 2.7 Hz, J_2 = 8.3 Hz, 2-CH),7.13 (d, 1H, J = 8.3 Hz, 1-CH); MS (70 eV) m/z (rel intensity) 426 (M^+, 43), 370 (21), 327 (27), 163 (12), 97 (100).

3-(*tert*-Butyldimethylsiloxy)-17α-(2',3'-epoxyprop-1'-yl)estra-1,3,5(10)-trien-17β-ol (5a) and (5b). m-CPBA (549 mg, 3.18 mmol) was added to a solution of alkene **4** (680 mg, 1.59 mmol) in 15 mL of CH_2Cl_2. The mixture was stirred for 17 h at 20 °C and then poured into 80 mL of CH_2Cl_2. Production isolation (KI 2%, $Na_2S_2O_3$ 10%, H_2O, Na_2SO_4) and then chromatography (Et_2O/heptane 4/6) afforded to 423 mg (60%) and 125 mg (18%) of less polar (**5a**) and more polar (**5b**) diastereoisomers, respectively.

Diastereoisomer 5a: ^1H NMR ($CDCl_3$) δ 0.17 (s, 6H, $Si(CH_3)_2$), 0.91 (s, 3H, 18-CH_3), 0.96 (s, 9H, $SiC(CH_3)_3$), 2.50 (q, 1H, J_1 = 2.7 Hz, J_2 = 5.0 Hz, 3'-CH_A), 2.79 (q, 1H, J_1 = 4.1 Hz, J_2 = 5.0 Hz, 3'-CH_B), 3.27 (m, 1H, 2'-CH), 6.53 (d, 1H, J = 2.7 Hz, 4-CH), 6.58 (q, 1H, J_1 = 2.7 Hz, J_2 = 8.3 Hz, 2-CH), 7.08 (d, 1H, J = 8.3 Hz, 1-CH); MS (70 eV), m/z (rel intensity) 442 (M^+, 63), 385 (56), 327 (33), 272 (13), 270 (12), 41 (100). Anal. ($C_{27}H_{42}O_3Si$) C, H.

Diastereoisomer 5b: ^1H NMR ($CDCl_3$) δ 0.17 (s, 6H, $Si(CH_3)_2$), 0.91 (s, 3H, 18-CH_3),0.96 (s, 9H, $SiC(CH_3)_3$), 2.53 (q, 1H, J_1 = 2.7 Hz, J_2 = 5.0 Hz, 3'-CH_A), 2.85 (q, 1H, J_1 = 4.1 Hz, J_2 = 5.0 Hz, 3'-CH_B), 3.26 (m, 1H, 2'-CH), 6.53 (d, 1H, J = 2.7 Hz, 4-CH), 6.60 (q, 1H, J_1 = 2.7 Hz, J_2 = 8.3 Hz, 2-CH), 7.09 (d, 1H, J = 8.3 Hz, 1-CH); MS (70 eV), m/z (rel intensity) 442 (M^+, 35), 385 (29), 41 (100). Anal. ($C_{27}H_{42}O_3Si$) C, H.

17α-(2',3'-Epoxyprop-1'-yl)estra-1,3,5(10)-trien-3,17β-diol (6a). Tetrabutylammonium fluoride (90 mg, 0.34 mmol) was added to a solution of 100 mg (0.23 mmol) of diastereoisomer **5a** in 1 mL of THF at 0 °C. After stirring for 5 min at 0 °C and then 15 min at 20 °C, product isolation ($NaHCO_3$, AcOEt, Na_2SO_4) then chromatography (Et_2O) afforded to 54 mg (73%) of (**6a**); ^1H NMR ($CDCl_3$) δ 0.91 (s, 3H, 18-CH_3), 2.50 (q, 1H, J_1 = 2.7 Hz, J_2 = 5.0 Hz, 3'-CH_A), 2.80 (q, 1H, J_1 = 4.1 Hz, J_2 = 5.0 Hz, 3'-CH_B), 3.24 (m, 1H , 2'CH_B), 6.54 (d, 1H, J = 2.7 Hz, 4-CH), 6.60 (q, 1H, J_1 = 2.7 Hz, J_2 = 8.3 Hz, 2-CH), 7.10 (d, 1H, J = 8.3 Hz, 1-CH); MS (70 eV), m/z (rel intensity) 328 (M^+, 8), 310 (7), 270 (4), 43 (100). Anal. ($C_{21}H_{28}O_3$) C, H.

17α-(2',3'-Epoxyprop-1'-yl)estra-1,3,5(10)-trien-3,17β-diol (6b). Diastereoisomer **6b** was prepared from the corresponding protected compound (diastereoisomer **5b**) in 82% yield by the procedure described for compound **6a**: ^1H NMR ($CDCl_3$)

δ 0.91 (s, 3H, 18-CH_3), 2.52 (q, 1H, J_1 = 2.7 Hz, J_2 = 5.0 Hz, 3'-CH_A), 2.85 (q, 1H, J_1 = 4.1 Hz, J_2 = 5.0 Hz, 3'-CH_B), 3.24 (m, 1H, 2'-CH), 6.54 (d, 1H, J = 2.7 Hz, 4-CH), 6.60 (q, 1H, J_1 = 2.7 Hz, J_2 = 8.3 Hz, 2-CH), 7.10 (d, 1H, J = 8.3 Hz, 1-CH); MS (70 eV), m/z (rel intensity) 328 (M^+, 39), 310 (13), 270 (10), 213 (100). Anal. ($C_{21}H_{28}O_3$) C, H.

3-(*tert*-Butyldimethylsiloxy)-17α-(3'-hydroxyprop-1'-yl)estra-1,3,5(10)-trien-17β-ol (7). 9-BBN (28.14 mmol, 0.5 M solution in THF) under nitrogen was added dropwise to a solution of 2 g (4.69 mmol) of alkene 4 in 15 mL of THF at 60 °C. After 40 min, the solution was cooled to 0 °C, 9 mL of water was added and stirring was continued for 5 min. Sodium hydroxide (9 mL, 3M) was added, and after an additional 5 min, 9 mL of hydrogen peroxide (30%) was added dropwise. After 30 min product isolation ($NaHCO_3$, Et_2O, Na_2SO_4) and then chromatography ($CHCl_3$) afforded to 1.77 g (85%) of the primary alcohol 7: ^1H NMR ($CDCl_3$) δ 0.16 (s, 6H, $Si(CH_3)_2$), 0.91 (s, 3H, 18-CH_3), 0.96 (s, 9H, $SiC(CH_3)_3$), 3.70 (m, 2H, 3'-CH_2), 6.53 (d, 1H, J = 2.7 Hz, 4-CH) 6.60 (q, 1H, J_1 = 2.7 Hz, J_2 = 8.3 Hz, 2-CH), 7.10 (d, 1H, J = 8.3 Hz, 1-CH) ; MS (70 eV), m/z (rel intensity) 444 (M^+, 56), 426 (50), 41 (100). Anal ($C_{27}H_{44}O_3Si$) C, H.

17α-(3'-Hydroxyprop-1'-yl)estra-1,3,5(10)-trien-3,17β-diol (8). Triol 8 was prepared from 7 in 86% yield, by the procedure described for compound **6a**. It was purified by chromatography ($Et_2O/CHCl_3$ 1/4): ^1H, NMR (DMSO) δ 0.79 (s, 1H, 18-CH_3), 3.41 (m, 2H, 3'-CH_2), 6.42 (d, 1H, J = 2.7 Hz, 4-CH), 6.50 (q, 1H, J_1 = 2.7 Hz, J_2 = 8.3 Hz, 2-CH), 7.03 (d, 1H, J = 8.3 Hz, 1-CH); MS (70 eV) m/z (rel intensity) 330 (M^+, 12), 312 (61), 271 (9), 270 (7), 28 (100).

3-(*tert*-Butyldimethylsiloxy)-17α-(3'-bromoacetoxyprop-1'-yl)estra-1,3,5(10)-trien-17β-ol (9). Bromoacetic acid (561 mg, 4.04 mmol), 775 mg (4.04 mmol) of 1-ethyl-3-(3-(dimethylamino)propyl)carbodiimide hydrochloride, and 0.245 mL (3.03 mmol) of pyridine were added successively to a solution of 900 mg (2.02 mmol) of primary alcohol 7 in 20 mL of THF. The mixture was stirred for 2 h at 25 °C and then poured into Et_2O. Insoluble material was removed by filtration, product isolation ($NaHCO_3$, H_2O, and Na_2SO_4) and then chromatography (Et_2O/heptane 1/1) afforded to 917 mg (80%) of bromoacetate 9: ^1H NMR ($CDCl_3$) δ 0.16 (s, 6H, $Si(CH_3)_2$), 0.89 (s, 3H, 18-CH_3), 0.96 (s, 9H, $SiC(CH_3)_3$), 4.07 (s, 2H, CH_2Br), 4.23 (t, 2H, J_1 = J_2 = 6.6 Hz, 3'-CH_2), 6.53 (d, 1H, J = 2.7 Hz, 4-CH), 6.59 (q, 1H, J_1 = 2.7 Hz, J_2 = 8.3 Hz, 2-CH), 7.10 (d, 1H, J = 8.3 Hz, 1-CH); MS (70 eV) m/z (rel intensity) 567 (M^+, 5), 565 (M^+, 8), 426 (8), 408 (3), 272 (17), 97 (94), 73 (100).

17α-(3'-Bromoacetoxy)prop-1'-yl)estra-1,3,5(10)-trien-3,17β-diol (10). Deprotected bromoacetate 10 was prepared from 9 in 67% yield by the procedure described for compound **6a**. It was purified by chromatography (Et_2O/heptane 3/7): ^1H NMR ($CDCl_3$) δ 0.90 (s, 3H, 18-CH_3), 4.03 (s, 2H, CH_2Br), 4.25 (t, 2H, J_1 = J_2 = 6.6 Hz, 3'-CH_2), 6.54 (d, 1H, J = 2.7 Hz, 4-CH), 6.61 (q, 1H, J_1 = 2.7 Hz, J_2 = 8.3 Hz, 2-CH), 7.12 (d, 1H, J = 8.3 Hz, 1-CH); MS (70 eV), m/z (rel intensity) 452 (M^+, 0.5), 450 (M^+, 0.4), 408 (17), 406 (22), 390 (8), 388 (14), 312 (35), 294 (4), 213 (100).

17α-(4'-(Bromoacetoxy)butyn-1'-yl)estra-1,3,5(10)-trien-3,17β-diol (15). Bromoacetic acid (55.6 mg, 0.4 mmol), 76.6 mg (0.4 mmol) of 1-ethyl-3-(3-(dimethylamino)propyl)carbodiimide hydrochloride, and 24 μL (0.3 mmol) of pyridine were added successively to a solution of 34 mg (0.10 mmol) of triol 11 in 2 mL of THF. The mixture was stirred for 2 h at 25 °C and then poured into Et_2O. Insoluble material was removed by filtration, product isolation ($NaHCO_3$, H_2O, and Na_2SO_4) gave ~60 mg of crude dibromoacetate 13. The compound was solubilized in 2 mL of THF after cooling to 0 °C, a solution of 12.6 mg $NaHCO_3$ (0.15 mmol) in 0.5 mL CH_3OH/H_2O (1/1) was added and the mixture was stirred for 15 h at 0 °C. Product isolation (Et_2O, H_2O, and Na_2SO_4) and then chromatography (Et_2O/heptane 3/7) afforded to 14 mg (30%) of monobromoacetate 15: ^1H NMR ($CDCl_3$) δ 0.85 (s, 3H, 18-CH_3), 2.63 (t, 2H, J_1 = J_2 = 6.7 Hz, 3'-CH_2), 4.05 (s, 2H, CH_2Br), 4.30 (t, 2H, J_1 = J_2 = 6.7 Hz, 4'-CH_2), 6.54 (d, 1H, J = 2.7 Hz, 4-CH), 6.60 (q, 1H, J_1 = 2.7 Hz, J_2 = 8.3 Hz, 2-CH), 7.14 (d, 1H, J = 8.3 Hz, 1-CH); MS (20 eV), m/z (rel intensity) 462 (M^+, 0.6), 460 (M^+, 0.5), 307 (15), 160 (100).

17α-(8'-(Bromoacetoxy)octyn-1'-yl)estra-1,3,5(10)-trien-3,17β-diol (16). The monobromoacetate 16 was prepared from triol 12 by the procedure described for compound 15. Compound 16 (47%) was purified by chromatography (Et_2O/heptane 1/3): ^1H NMR ($CDCl_3$) δ 0.85 (s, 3H, 18-CH_3), 2.24 (t, 2H, J_1 = J_2 = 6.7 Hz, 3'-CH_2), 3.99 (s, 2H, CH_2Br), 4.17 (t, 2H, J_1 = J_2 = 6.77 Hz, 8'-CH_2), 6.54 (d, 1H, J = 8.3 Hz, 4-CH), 6.60 (q, 1H, J_1 = 2.7 Hz, J_2 = 8.3 Hz, 2-CH), 7.14 (d, 1H, J = 8.3 Hz, 1-CH); MS (20 eV), m/z (rel intensity) 518 (M^+, 1), 516 (M^+,1), 474 (10), 472 (10), 160 (100).

Estrogen Receptor Ligands. [6,7-^3H]Estradiol (specific activity 70 Ci/mmol, radiochemical purity > 97%) was purchased from the Commissariat à l'Energie Atomique (Gif-Sur-Yvette, France). 4-Hydroxy[*N*-*methyl*-^3H]tamoxifen (specific activity 83 Ci/mmol, radiochemical purity >99%) was from Amersham International (Amersham, England). Estradiol, 4-hydroxytamoxifen, and all the 17α-derivatives of estradiol used for binding studies were solubilized in absolute ethanol. Solutions were stored at −20 °C in the dark. Purity of solubilized compounds was checked before use by TLC.

Preparation of Cytosol. Immature lamb uteri were stripped from connective tissue, placed in liquid nitrogen, and then stored at −80 °C until use. The uteri were defrosted and then immediately homogenized in five volumes of chilled 20 mM Tris-HCl buffer, pH 7.0 (T_{20}), using an ultraturrax. The homogenate was centrifuged at $10^5 \times g$ for 45 min. The protein concentration determined according to Layne[27] was then adjusted to 4 mg/mL. Depending on the experiments, the cytosol was used either at pH 7.0 or after alkalinization with NaOH 0.5 M to reach pH values between 7.0 and 9.0.

Competitive Binding Assay: Apparent Relative Affinity Constants. Cytosol (pH 7.0) was diluted to 2 mg of protein/mL with T_{20}. It was then incubated with 5 nM [^3H]estradiol and increasing concentrations of either nonradioactive estradiol (1.67 nM to 1.5 μM) or estradiol 17α-derivatives (0.1 to 10 μM) either at 0 or 20 °C. Binding of [^3H]estradiol in samples was determined after a 4-, 20-, or 72-h incubation. Aliquots were treated with an equal volume of charcoal suspension (0.5% charcoal, 0.05% dextran T_{70} in T_{20}) for 30 min at 0 °C; charcoal was pelleted by 5-min centrifugation at $10^3 \times g$ and then radioactivity in the supernatant was measured. Apparent relative affinity constants (RACs) of competitors relative to that of estradiol were calculated from the concentration of unlabeled estradiol (E) and competitor (C) which inhibited 50% of the specific binding of [^3H]estradiol, according to Korenman:[28]

$$\mathrm{RAC} = \frac{\dfrac{E_t^* - E_b^*}{E_{sb}^*} \cdot \dfrac{E}{C}}{\dfrac{E_t^* - E_b^*}{E_{sb}^*} + 1 - \dfrac{E}{C}}$$

where E_t^* is the total concentration of [^3H]estradiol, E_b^* and E_{sb}^* are the respective concentrations of bound and specifically bound [^3H]estradiol at 50% inhibition; $E_t^* - E_b^*$ was assumed to be the unbound [^3H]estradiol concentration at 50% inhibition.

Standard Irreversible Binding Assay. Cytosol was first incubated for 1 h at 0 °C with estradiol 17α-derivatives or without steroid. Samples were then treated with an equal volume of charcoal suspension for 30 min at 0 °C. Thereafter charcoal was removed by centrifugation, and supernatant aliquots were incubated under "exchange conditions" (16 h at 20 °C) with 20 nM [^3H]estradiol either in the absence (to measure total [^3H]estradiol binding) or presence (to measure nonspecific [^3H]estradiol binding) of 5 μM nonradioactive estradiol. Binding of [^3H]estradiol was determined by a 30 min-0 °C charcoal assay (cf "competitive binding assay").

Sucrose Gradient Analyses. Cytosol aliquots (pH 9.0), incubated with or without steroids, treated with charcoal, then incubated with [^3H]estradiol for 16 h at 20 °C, and finally submitted to a second charcoal treatment (cf "irreversible binding assay") were added with ^{14}C-labeled ovalbumin. Then 200 μL of the mixture was layered onto 10–30% sucrose gradient (4.8 mL) prepared in T_{20} and containing 400 mM KCl. Gradients were centrifuged in a Beckman SW 50.1 rotor for 14 h at 216 000 g_{av} (48 000 rpm). Thirty-two successive fractions were collected from

the bottom of the tube, and their radioactivity measured. In some experiments, before centrifugation aliquots were incubated for 3 h at 0 °C with 1% control serum from nonimmunized mouse or with serum from mouse immunized with purified preparations of lamb uterine estrogen receptor.[23]

Radioactivity Determinations. The radioactivity of gradients fractions (~160 μL), and of the various cytosol samples (100 or 200 μL) was counted in 4 mL of Emulsifier Safe (Packard).

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