# Estrogenicity of Coumestrol in the Mouse: Fluorescence Detection of Interaction with Estrogen Receptors†

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ABSTRACT: The estrogenicity of coumestrol, a fluorescent phytoestrogen, has been examined in murine uteri. Coumestrol competed with 17β-[$^3$H]estradiol for binding to cytoplasmic estrogen receptors, caused cytoplasmic estrogen receptors to associate with chromatin in the nucleus, and induced progesterone receptors. By use of size-exclusion high-performance liquid chromatography (SEHPLC), the interaction of coumestrol with estrogen receptors was examined directly by monitoring the fluorescence associated with macromolecules having properties characteristic of estrogen receptors. These analyses were made possible by the addition of dimethylformamide to the elution buffer, at a concentration (7.5%) which improved recoveries but did not interfere with estrogen receptor binding. It was possible to detect fluorescent coumestrol at approximately 0.5 nM. All determinations were performed with preparations in which estrogen receptor activity was 3–10 nM. Exposure of these preparations to coumestrol (50 nM) resulted in the elution of increased fluorescent activity in the regions where estrogen receptors eluted during SEHPLC. This fluorescent activity was reduced when diethylstilbestrol, 17β-estradiol, hexestrol, or tamoxifen was present as a competitor (2 μM) but was unaffected by testosterone or progesterone. Diethylstilbestrol reduced fluorescence below endogenous base lines and thereby displayed a fluorescence quench property which was not observed with other ligands. When hepatic and renal estrogen receptor preparations were used, the injected receptor activity was observed to be the major limiting factor in detecting the interaction of coumestrol with estrogen receptors. These observations are relevant to attempts to visualize estrogen receptors in tumor cells and demonstrate that accepted biochemical criteria for ligand–receptor interaction can be satisfied when fluorescent ligands are examined.

Estrogenicity is a biological property which can be mediated by a variety of steroidal and nonsteroidal compounds (Hammond et al., 1979; Katzenellenbogen et al., 1979; Pavlik & Katzenellenbogen, 1980). Even though the chemical structure of different estrogens can appear quite diverse, these ligands share a structural similarity that permits them to interact with intracellular binding proteins which are referred to as estrogen "receptors" (Hammond et al., 1979; Pavlik & Katzenellenbogen, 1980). The estrogen–receptor protein complex, after being activated, becomes interactive with chromatin and thereby initiates certain events that control the rates of transcription, translation, and replication; these events ultimately account for estrogenic responses (Jensen & DeSombre, 1972, 1973; Katzenellenbogen & Gorski, 1975; Katzenellenbogen, 1980; O'Malley & Means, 1974; Yamamoto & Alberts, 1976; Sheridan et al., 1979; Martin & Sheridan, 1982).

Alfalfa and clover contain compounds that are estrogenic (i.e., phytoestrogens), the most potent of which is coumestrol[1] (Newsome & Kitts, 1977, 1980). Coumestrol is a fluorescent compound which in aqueous solution has demonstrated good quantum yields and has been reported to interact with estrogen receptors (Lee et al., 1977; Dandliker et al., 1978; Martin et al., 1978; Verdeal et al., 1980). To date, demonstrations of this interaction have been by indirect methods.

In the present report, we have examined directly the interaction of coumestrol with estrogen receptors by monitoring the fluorescence which elutes bound to macromolecules with properties characteristic of estrogen receptors. Thus, direct methods are described herein which demonstrate that ligand fluorescence remains detectable after binding by estrogen receptors.

Our observations are particularly relevant to clinical methodologies that propose to use fluorescent estrogens in order to visualize estrogen receptors in tumor cells which may be hormonally responsive. Visualization methodologies could be quite valuable for identifying receptor negative malignant cells in tumors that may demonstrate an overall receptor-positive status by biochemical analysis. To date, however, ligand visualization methodologies have generally failed to satisfy many of the biochemical criteria for ligand interaction with estrogen receptors (Mercer et al., 1980, 1981; McCarty et al., 1980; Nenci, 1981; Fisher, 1982).

The present report demonstrates that accepted biochemical criteria for receptor interaction with fluorescent ligands can be satisfied.

## Experimental Procedures

*Materials.* Radiolabeled steroids, 17β-[2,4,6,7-$^3$H$_4$]estradiol (105 Ci/mmol) and 17α-[*methyl*-$^3$H$_3$]R5020 (87 Ci/mmol), were obtained from New England Nuclear. The following biochemicals were used: coumestrol, diethylstilbestrol, 17β-estradiol, hexestrol, tamoxifen (Sigma Chemical Co.), and

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[1] Abbreviations: coumestrol, 3,9-dihydroxy-6*H*-benzofuro[3,2-*c*][1]-benzopyran-6-one; DES, diethylstilbestrol (α,α′-diethyl-4,4′-stilbenediol); DMF, dimethylformamide; 17β-estradiol, estra-1,3,5(10)-triene-3,17β-diol; HEX, hexestrol [*meso*-3,4-bis(4-hydroxyphenyl)hexane]; $Me_2SO$, dimethyl sulfoxide; $P_{200}$ buffer, $K_2HPO_4$ (200 mM), pH 7.4 (4 °C); TAM, tamoxifen [*trans*-1-[*p*-[(dimethylamino)ethoxy]phenyl]-1,2-diphenyl-1-butene]; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid; TEMG buffer, 10 mM Tris, 1.5 mM EDTA, 10 mM sodium molybdate, and 10% glycerol, pH 7.4 (4 °C); SEHPLC, size-exclusion high-performance liquid chromatography; CD assay, dextran-coated charcoal assay; DME, Dulbecco's modified Eagle medium; HAP, hydroxylapatite.

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sodium molybdate (Mallinkrodt). Hydroxylapatite (Bio-Gel HTP, Bio-Rad Laboratories) and dimethylformamide (Burdick & Jackson Chemicals) were also used. Immature CF-1 mice (Harlan Sprague-Dawley, Indianapolis, IN) were obtained at 19–23 days of age and sacrificed within 7 days.

*Preparation of Cytosols.* Freshly removed mouse uteri were freed of fat and mesentery and were then homogenized in $P_{200}$ buffer (10 uterine equiv/mL) with glass/glass mortar and pestle. After centrifugation at 2000*g* for 15 min to remove the nuclear-myofibrillar material, a high-speed supernatant was prepared by centrifuging at 105000*g* for 45 min. The supernatant was then made to 10 mM sodium molybdate.

*Receptor Determinations.* Estrogen receptor preparations were charged with [$^3$H]estradiol (10 nM). For determinations of nonspecific binding, parallel incubations contained radioinert DES as competitor (2 $\mu$M) (Williams & Gorski, 1973). Progesterone receptors were determined on preparations homogenized in TEMG buffer (1 uterine equiv/mL) after charging with [$^3$H]R5020 (10 nM) and using radioinert progesterone as competitor (2 $\mu$M) (Pavlik et al., 1982a). Receptor binding determinations were made by two methods. With the first method, dextran-coated charcoal (5 g of charcoal and 0.5 g of dextran C in 100 mL of 10 mM Tris buffer; DC slurry–cytosol, 1:9 v/v) was used to strip unbound ligand (Katzenellenbogen et al., 1973; Pavlik & Katzenellenbogen, 1980). Activity that remained soluble was regarded as "bound" radioactivity. With the second method, hydroxylapatite was used to adsorb steroid receptors, and radioactivity that was associated with hydroxylapatite after washing was regarded as bound radioactivity (Pavlik & Coulson, 1976). Differences between total binding measured with $^3$H steroid alone and unsaturable, nonspecific binding measured in the presence of excess competitor were used to determine the limited capacity, receptor specific binding (Williams & Gorski, 1973). Rates of association, dissociation, and equilibrium binding were determined as previously described (Katzenellenbogen et al., 1978, 1981; Pavlik & Rutledge, 1980). Liquid scintillation counting was performed in a Triton X-100–xylene fluor, and automatic correction for quench was made on each sample (Pavlik & Rutledge, 1980). Soluble protein concentrations were determined according to the method of Lowry et al. (1951).

*Size-Exclusion High-Performance Liquid Chromatography (SEHPLC).* All buffers were membrane filtered to remove particles. High-speed cytosols were membrane filtered with low protein binding filters (Millex GV, Millipore Corp.) to remove components that reduce column performance (less than 5% of the receptors are removed by Millex GV filters). Isocratic elution was employed by delivering buffer with a flow metered pump (Model 112, Beckman Instruments) to a Spherogel-TSK-G2000SW exclusion column (7.5 × 600 mm) which was fitted with a guard column (Spherogel-TSK precolumn 2000SW; 7.5 × 100 mm). Samples were applied with a syringe-loaded injector (Model 210, Beckman Instruments) which was fitted with a 250-$\mu$L sample loop (Pavlik et al., 1982a,b). Elution was with $P_{200}$ except where otherwise indicated. Ultraviolet absorbance at 280 nm was monitored with a fixed wavelength detector (ChemResearch Model 2020, Isco Co.). Fluorescence was determined with a filter fluorometer (FS950 Fluoromat, Kratos/Schoeffel Analytical Instruments). The fluorescence detector was equipped with an HPLC flow cell (20 $\mu$L illuminated volume) and FSA111 excitation lamp. Samples (1 mL/fraction) were collected with a rapid response programmable fraction collector (FOXY, Isco Co.). Fluorescence profiles were analyzed quantitatively after making light pen tracings on a semiautomatic image analysis system (Leitz A.S.M. System) in order to obtain integrated values. The HPLC system was maintained and operated at 2–5 °C in a refrigerated chromatography cabinet (Kelvinator). The column was routinely cleaned by washing with distilled water until all salts were removed (>2 h, 1 mL/min). Proteins that adhered to the column were then purged with 10% $Me_2SO$ in methanol (12–24 h, 0.1 mL/min). After the column was taken to 100% methanol in order to remove $Me_2SO$ (approximately 8 h, 0.1 mL/min), methylene chloride was pumped through to remove lipids (12–24 h, 0.1 mL/min). The column was then prepared for accepting aqueous buffers by using methanol (0.1 mL/min for approximately 6 h, followed by 1 mL/min, 4–8 h) and then distilled water (0.1 mL/min for first hour and then 1 mL/min).

## Results

*Estrogenicity of Coumestrol in the CF-1 Mouse Uterus.* Radioinert coumestrol competed with [$^3$H]estradiol for binding to cytoplasmic estrogen receptors with a relative binding affinity that was approximately 20% of estradiol (Figure 1A). Coumestrol (10 nM) caused cytoplasmic estrogen receptors to associate with nuclei but did so more slowly than estradiol (Figure 1B). Coumestrol also was able to induce progesterone receptors in the mouse uterus in vivo (Figure 1C). While estradiol or DES at 1 $\mu$g/mouse maximally induces progesterone receptors, more coumestrol (4–5 $\mu$g/mouse) was required for maximal induction.

*Demonstration of Increased Fluorescence Associated with Estrogen Receptors after Exposure to Coumestrol. (1) Fluorescent Signals and Detection.* Coumestrol in $P_{200}$ buffer (60 nM) demonstrated a maximum fluorescent emission at 435 mn after excitation at 300 or 340 nm (Figure 2A). The excitation source in the detector was chosen to match this with an upper wavelength excitation cutoff at 365 nm (Figure 2A). Fluorescent signals were limited with a 418-nm cutoff filter so that more than 90% of the coumestrol signal was measurable. Components in cytosol also overlapped the same wavelengths in which coumestrol fluorescence was observed (Figure 2B). Endogenous fluorescence had a maximum at 465 nm and could be reduced by treatment with dextran-coated charcoal (10 min, 0–4 °C). The sensitivity of detecting coumestrol with the HPLC fluorescence detector extended to approximately 0.5 pmol of coumestrol/mL of $P_{200}$ buffer (Figure 3). Increases in fluorescence were linear with coumestrol concentration, and it appeared that it would be practical to attempt to detect coumestrol binding to estrogen receptors in the 3–10 pmol/mL range.

*(2) Buffer Modification To Improve the Recovery of Binding Activity in SEHPLC Analysis.* Variations in the recovery of steroid receptors from SEHPLC analysis have been previously noted (Pavlik et al., 1982a,b) and have been corrected for on the basis of the recovery of $^3$H ligand. Thus, when radiolabeled ligands are used, parallel preparations (with and without competitor) can be adjusted to compensate for differences in eluted activity between the preparations. Since similar adjustments cannot be made for fluorescent ligands, it is essential to avoid the problems presented by differences in eluted (recovered) activity between preparations charged with coumestrol alone or those with various competitors. The addition of dimethylformamide to the elution buffer dramatically improved estrogen receptor recovery, even when column performance had deteriorated through extended use to the point where recoveries were less than 2% (Table I). Estrogen receptors were stable in low DMF concentrations (<20%) with only minor perturbations of total and nonspecific

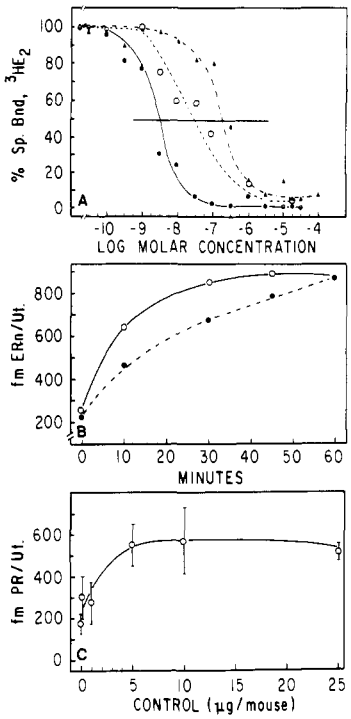


FIGURE 1: Estrogenicity of coumestrol in the CF-1 mouse uterus. Relative binding affinity of coumestrol, 17β-estradiol, and DES (A). Cytoplasmic preparations were charged overnight with 10 nM [$^3$H]estradiol with and without 2 μM DES as competitor in the presence of different concentrations of coumestrol (solid triangles), 17β-estradiol (open circles), or DES (solid circles). Estrogen receptor determinations were performed with the CD assay on duplicate experimental points, and mean values are expressed. Relative binding, which was determined as the concentration that inhibited 50% of estrogen receptor specific binding relative to 17β-estradiol, was 1 (estradiol), 6 (DES), and 0.2 (coumestrol). Nuclear association of estrogen receptors achieved with radioinert 17β-estradiol and coumestrol (B). Uteri were incubated in DME containing 17β-estradiol (10 nM) or coumestrol (10 nM) at 37 °C for the times indicated. Translocation was terminated and uteri homogenized. Crude nuclear pellets were washed with TE buffer (5×) and subjected to exchange assay conditions [20 h, 20 °C, 30 nM [$^3$H]estradiol (Anderson et al., 1972; Katzenellenbogen et al., 1975)] for the determination of translocated nuclear receptors. Replicates of four were run, and mean values are expressed. Induction of progesterone receptors by coumestrol (C). Coumestrol in isotonic saline was injected intraperitoneally (three mice per concentration). Animals were sacrificed after 3 days. Uteri were homogenized individually, and triplicate determinations of specific progesterone receptor binding were determined by adsorption to HAP after charging with [$^3$H]R5020 (10 nM) in the presence and absence of radioinert progesterone (2 μM) (Pavlik et al., 1982a).

Table I: Effect of Dimethylformamide on Estrogen Receptor Recovery[a]

| elution buffer composition | | | | |
|---|---|---|---|---|
| $P_{200}$ buffer | + | + | + | + |
| sodium molybdate, 10 mM | − | + | − | + |
| dimethylformamide, 7.5% | − | − | + | + |
| receptor activity injected, dpm (1)[b] | 226 850 | 231 600 | 226 845 | 279 371 |
| receptor activity recovered, dpm (2)[b] | 3409 | 615 | 182 406 | 246 471 |
| % receptor recovered | 1.5 | 0.3 | 80.4 | 88.2 |

[a] Estrogen receptors (approximately 4 nM) were charged and prepared for SEHPLC analysis. Just prior to injection, samples were treated with dextran-coated charcoal. Receptor activity refers to specific binding determined as the difference between activity in parallel preparations with and without radioinert competitor. [b] Receptor activity determined in 250-μL injection volume by CD assay (1) and activity eluting as receptor on column (2).

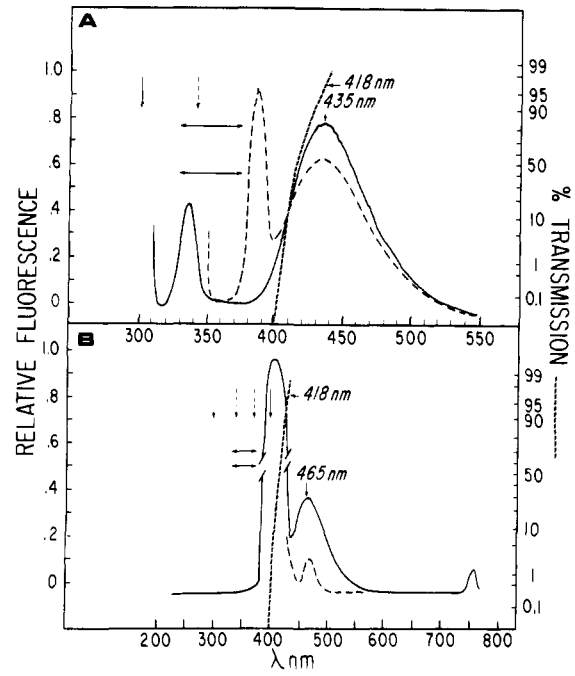


FIGURE 2: Spectral characteristics of coumestrol and cytosol: detection of fluorescent signals. Spectral profile of coumestrol after excitation at 300 (solid vertical arrow) and 340 nm (dashed vertical arrow) (A). Peaks occurring below 400 nm are scatter bands. The maximum intensity range of the excitation lamp in the HPLC fluorometer (solid horizontal arrows) was limited at the upper end by a 365-nm cutoff filter. Use of a 418-nm cutoff filter attenuated less than 10% of the coumestrol in $P_{200}$ buffer and is identified by the broken line. Spectral profile of cytosol after excitation at 300, 340, 375, and 410 nm (B). Absorption spectra were obtained on an Aminco corrected spectra spectrophotofluorometer. Vertical and horizontal arrows are as already defined in (B). The scatter peak lies to the left of the 418-nm cutoff filter. Cytosols before (solid line) and after (broken line) treatment with dextran-coated charcoal (10 min, 0–4 °C) are shown.

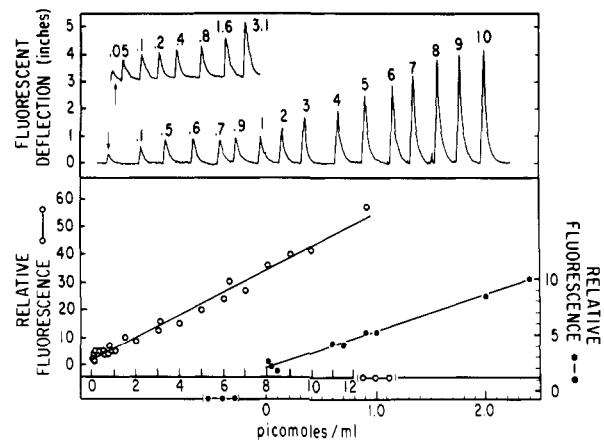


FIGURE 3: Fluorescence detection of coumestrol in the HPLC flow cell. Actual signals generated by different concentrations of coumestrol in $P_{200}$ buffer (top). Expanded range detection is inset for 0.05–3.125 pmol/mL. Linearity of fluorescence signal and coumestrol concentrations with regression analysis best fit lines (bottom). Open symbols: complete range (correlation coefficient, $r = 0.99$). Closed symbols: expanded range, $r = 0.97$. Injections (250 μL) were made directly to flow cell detector (20 μL illuminated volume, 1 mm i.d.). Vertical arrows indicate the signal detected from injection buffer alone.

binding activity (Figure 4). DMF (7.5%) had little effect on receptor binding parameters (Table II), although ligand dissociation appeared to be slightly more rapid when DMF was included in the buffer. No change in the elution behavior of either estrogen receptors or marker proteins was observed when elution was with $P_{200}$ buffer containing 7.5% DMF (data not shown).

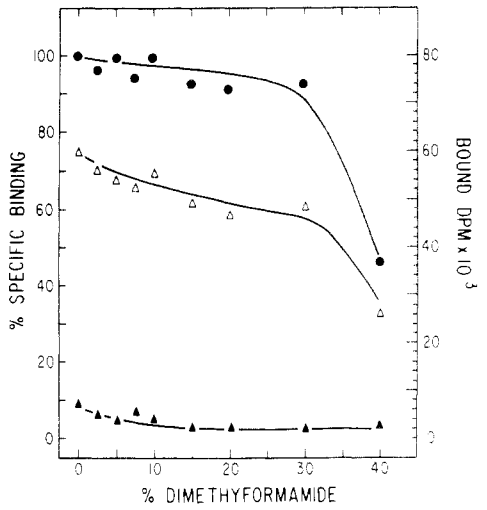


FIGURE 4: Stability of cytoplasmic estrogen receptors in dimethylformamide. Charged preparations were incubated with different concentrations of DMF for 60 min (0–4 °C). Receptor binding was determined by the HAP assay on duplicates. Mean values are expressed for total (open triangles) and nonspecific (solid triangles) binding on the right axis. Specific bound values (solid circles) are on the left vertical axis.

Table II: Effect of Dimethylformamide on Estrogen Receptor Binding[a]

| binding parameter | $P_{200}$ buffer | $P_{200}$ buffer, 7.5% DMF |
|---|---|---|
| association rate constant ($k_1$) ($M^{-1}$ $s^{-1}$) | 3.64 ± 0.86 | 3.14 ± 0.62 × $10^5$ |
| dissociation rate constant ($k_{-1}$) (a) ($s^{-1}$) | 3.17 ± 0.42 | 7.33 ± 3.77 × $10^{-6}$ |
| dissociation rate constant ($k_{-2}$) (b) ($s^{-1}$) | 59.3 ± 31.5 | 180.3 ± 31.7 × $10^{-6}$ |
| overall dissociation rate (c) ($s^{-1}$) | 5.17 ± 0.53 | 8.17 ± 5.23 × $10^{-6}$ |
| equilibrium association constant (d) (L $M^{-1}$) | 2.96 ± 0.62 | 1.22 ± 0.34 × $10^9$ |

[a] All values were obtained from linear regression analysis and are expressed ± standard error of the regression estimate. Major dissociating (slow) component (a), minor (fast) dissociating component (b), and regression analysis performed on all data points (inclusive of major and minor components) (c). Determined from equilibrium analysis of "bound/free" vs. "bound" (d). All determinations were performed at 2–5 °C. Association reactions were followed over a 0–15 min time course, while dissociation reactions were followed from 0 to 30 h. Equilibrium associations were determined over the 0.05–40 nM range after charging for 24 h.

*(3) Elution of Fluorescent Coumestrol during SEHPLC Analysis.* Estrogen receptors prepared from murine uteri eluted early (Fractions 10-15) as large macromolecules on the SEHPLC column (Figure 5A). Pretreatment with dextran-coated charcoal caused a minor reduction in bound 17$\beta$-[$^3$H]estradiol. Bound activity was greatly reduced by competition with DES. Free 17$\beta$-[$^3$H]estradiol eluted at the $V_T$ (fractions 22–24), but peaks of activity also eluted later (fractions 25–29 and 35–42), indicating that free [$^3$H]estradiol interacted with the column (Figure 5A). When the same preparation was charged with coumestrol (50 nM) (solid line), a fluorescent peak was observed to elute in the region where estrogen receptors eluted (i.e., minutes 11–14; Figure 5B). The fluorescence associated with this region was greatly reduced when 2 $\mu$M DES was included (broken line). The interaction of free coumestrol (Figure 5B) with the exclusion column was distinct from estradiol (Figure 5A) and resulted in even later elution (minutes 50–60, Figure 5B).

When ligands, which have a documented binding to estrogen receptors (Katzenellenbogen et al., 1973, 1975; Pavlik & Katzenellenbogen, 1980; Robertson et al., 1982), were used as competitive ligands, fluorescent activity in coumestrol-charged cytosols was reduced in the region where estrogen receptors were observed to elute (Figure 6A, fractions 11–14). Hexestrol was observed to reduce coumestrol-related fluorescence at minutes 11–14 to the endogenous level of fluorescence in untreated cytosol. In these determinations, free ligand interaction with the exclusion column occurred at two distinct elution times (i.e., minutes 41–44 and 51–64), indicating that variability in ligand–column interaction may result when coumestrol is combined with high concentrations of other hydrophobic ligands. In matched determinations (where untreated cytosols were compared to ligand-charged cytosols from the same preparation), charging with coumestrol (50 nM) resulted in almost a 300% average increase in fluorescence

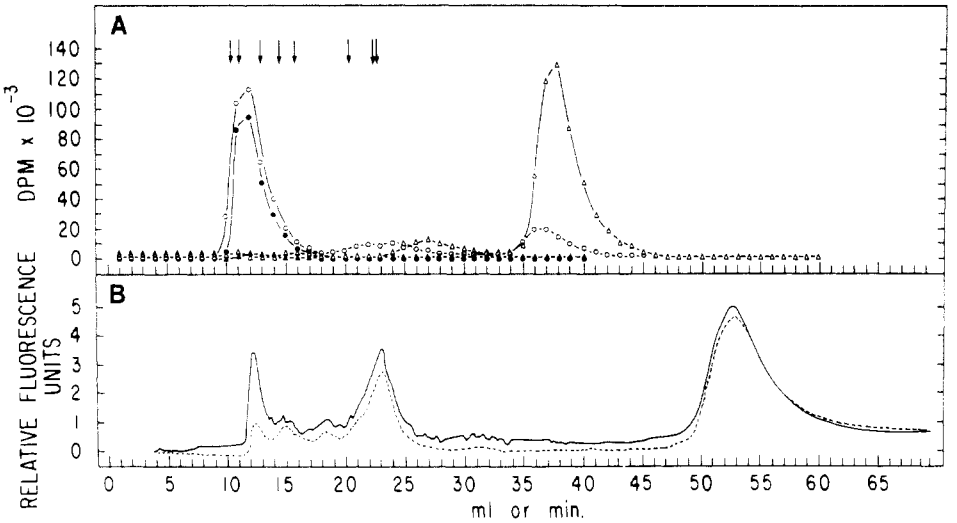


FIGURE 5: SEHPLC analysis of [$^3$H]estradiol and coumestrol binding: reduction of activity of early eluting forms by DES. Mouse uterine preparations charged with [$^3$H]estradiol (10 nM; circles) alone or which also included DES (2 $\mu$M); triangles) were either applied directly (open symbols) or treated with dextran-coated charcoal before injection (10 min, 0–4 °C; solid symbols) (A). Estrogen receptor concentration in these preparations was approximately 6 nM, and protein was approximately 4 mg/mL. Receptor recovery from the column was >90%. Vertical arrows designate the elution positions of blue dextran 2000, ferritin, $\gamma$-globulin, bovine serum albumin, ovalbumin, trypsin, trypsin inhibitor, and $Na_2MoO_4$ (from left to right). Same preparation charged with coumestrol (50 nM) (B). Elution was in $P_{200}$ buffer–7.5% DMF, pH 7.4 (1 mL $min^{-1}$).

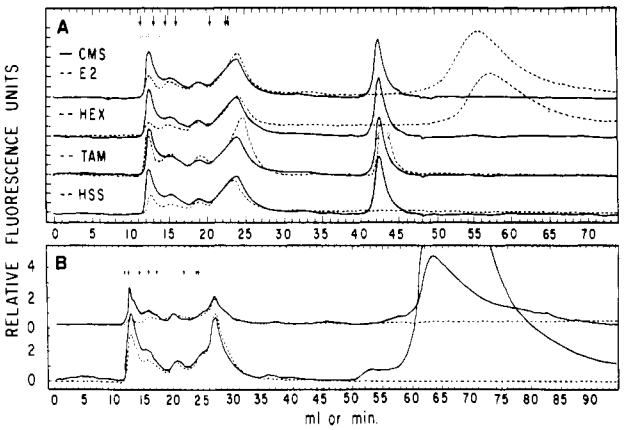


FIGURE 6: Competition and saturation of coumestrol fluorescent activity in the region where estrogen receptors elute. Conditions and designations are as described in Figure 5. Reduction of fluorescence by ligands which interact with estrogen receptors (A). The horizontal shaded bar identifies the region in which estrogen receptors eluted. Integration of this region was performed in order to determine fluorescent activity eluting in the same region as estrogen receptors. Fluorescence after charging with 50 nM coumestrol alone ("CMS", solid line, 100%) or when competitors were also present (2 $\mu$M, broken lines): estradiol ("$E_2$", 48%), hexestrol ("HEX", 31%), and tamoxifen ("TAM", 84%). Endogenous fluorescence of cytosol alone ("HSS", 32%). All determinations were made on the same preparation of cytosol, after charging with different ligands. Estrogen receptors were approximately 5 nM in the preparation. Cytosols that were charged with testosterone or progesterone (2 $\mu$M) in addition to coumestrol showed the same fluorescence as charging with coumestrol alone. Limited capacity binding after exposure to coumestrol (B). Fluorescent activity after exposure to coumestrol (solid line) at 50 nM (upper profile) or 2 $\mu$M (lower profile). The broken line shows the endogenous fluorescence of cytosol alone with each preparation adjusted to an estrogen receptor activity of 5 nM. Conditions and designations are as described in Figure 5.

integrated over minutes 11–14 (Table III). Fluorescence due to charging with other estrogen receptor interactive ligands was not observed in the region where estrogen receptors eluted. Charging the preparation with coumestrol had little effect on the total integrated fluorescence ($F_0$) of the elution profiles originating outside of the region in which estrogen receptors eluted (Table III). When preparations were exposed to DES alone or DES in combination with other ligands, the fluorescence integrated over minutes 11–14 averaged less than in untreated cytosol alone. Reductions in endogenous fluorescence due to DES were also observed in the elution profile exclusive to the region where estrogen receptors eluted. DES reduced coumestrol-related fluorescence that eluted in minutes 11–14 to levels less than the endogenous fluorescence integrated over this region. Thus, reductions in coumestrol fluorescence, observed when DES was included in the preparation, were not exclusively the result of classical competition for the binding of a fluorescent ligand but also resulted from a fluorescent quench property that was not seen with other ligands.

Preparations of uterine cytosol were exposed to high concentrations of coumestrol and demonstrated a limited capacity fluorescence in the region where estrogen receptors eluted (Figure 6B), as estimated by a limitation on fluorescence in minutes 11–14, despite a significant excess of free coumestrol (minutes 60–80).

Cytosols prepared from mouse liver and kidney demonstrated specific binding of 17$\beta$-[$^3$H]estradiol (Figure 7A,C). Fluorescence in the region where hepatic estrogen receptors eluted was high in untreated cytosols, was modestly increased by charging with coumestrol, and was quenched when DES was included (Figure 7B). After renal cytosols were charged

Table III: Effect of Different Estrogen Receptor Ligands on Mouse Uterine Cytosol Fluorescence[a]

| ligand (concentration) | $F_R$[b] | $F_0$[c] | $N$[d] |
|---|---|---|---|
| coumestrol (50 nM) | 286 ± 49 | 106 ± 12 | 9 |
| 17$\beta$-estradiol (50 nM and 2 $\mu$M) | 95 ± 8 | 133 ± 26 | 18 |
| hexestrol (50 nM and 2 $\mu$M) | 114 ± 8 | 110 ± 11 | 13 |
| tamoxifen (50 nM and 2 $\mu$M) | 98 ± 13 | 109 ± 17 | 8 |
| diethylstilbestrol (50 nM and 2 $\mu$M) | 62 ± 10 | 78 ± 5 | 7 |
| coumestrol and DES (50 nM/2 $\mu$M) | 60 ± 17 | 67 ± 15 | 6 |
| estradiol and DES (50 nM/2 $\mu$M) | 55 ± 11 | 112 ± 39 | 6 |

[a] Preparations were charged overnight (0–4 °C) and had estrogen receptor activity of 6–9 nM. All results are percentages expressed relative to matched control cytosols (i.e., 100%) which were prepared identically except for exposure to ligand. [b] Fluorescence eluting in receptor region (shaded bar, Figure 6) was integrated in both ligand-charged and control preparations and expressed as $F_R$ = [ligand-integrated fluorescence ($F_L$)/control integrated fluorescence ($F_C$)] × 100. For both ligand and control cytosol ($F_L$ and $F_C$) the area indicated by the shaded bar in Figure 6 was integrated. [c] All fluorescence other than $F_R$ was expressed as $F_0$ = [(total integrated fluorescence of ligand charged cytosol − $F_L$)/(total integrated fluorescence of control cytosol − $F_C$)] × 100. [d] The number of separate preparations that were made and which consisted of matched unexposed and charged cytosol. Data are expressed as mean percentages values ± the standard error of the mean. Results at different ligand concentrations have been grouped because no quantitative differences were observed.

with coumestrol, fluorescence did not change in the region where renal estrogen receptors eluted. Uterine cytosols which generally contained 4–6 mg/mL soluble protein and 5–10 nM estrogen receptors (1–3 pmol of receptor/mg of protein) had approximately 5 and 10 times more estrogen receptor activity in each injection than liver and kidney preparations (respectively). Receptor specific activity in uterine preparations was approximately 75 times higher than in renal preparations (33 fmol/mg of protein) and approximately 130 times higher than in hepatic preparations (19 fmol/mg of protein). The major limitation to the detection of coumestrol fluorescence in the region where estrogen receptor eluted appeared to be the injected receptor activity and not the receptor specific activity or the protein concentration of the preparation. This conclusion is based on the observation that kidney preparations, which had intermediate receptor specific activities and protein concentrations with respect to uterine and liver preparations, did not demonstrate fluorescence in the region where estrogen receptors eluted.

Endogenous fluorescence was only partially related to protein concentration since the major fluorescent peak occurred at the $V_T$ (approximately 27 min) where little protein was detected (Figure 8A). Endogenous fluorescence was barely detectable even when strong ($OD_{280}$ signals were recorded (Figure 8B). Treatment with dextran-coated charcoal reduced fluorescence and $OD_{280}$ activity eluting in the region of the $V_T$ (approximately 27 min) (Figure 8B). The effect of treatment with dextran-coated charcoal on protein concentration [determined according to Lowry et al. (1948)] was to eliminate detectable protein eluting in fractions 20 or later (approximately 25% of injected protein) and to reduce detectable protein in fractions 1–20 by approximately 30%. Although, after treatment with dextran-coated charcoal, elevated fluorescence was detected in the region where estrogen receptors eluted in coumestrol-charged preparations, there was no apparent advantage to this treatment. Moreover, because of coumestrol's reduced relative binding affinity, treatment with dextran-coated charcoal may strip some bound coumestrol and may allow for coumestrol to partially dissociate. In either event, such occurrences would further restrict the possibility

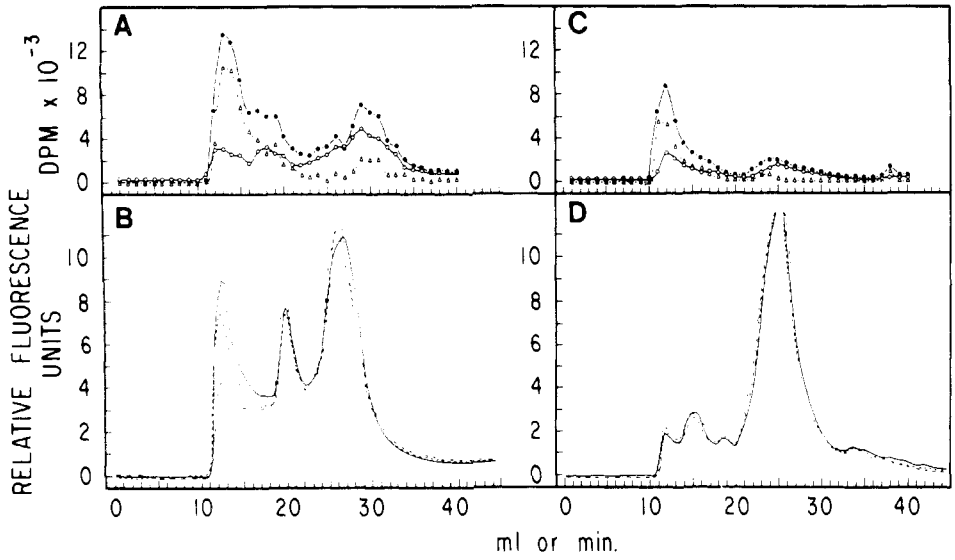


FIGURE 7: [$^3$H]Estradiol and coumestrol binding in murine liver and kidney cytosols. The liver preparation contained protein at a concentration of 48 mg/mL and estrogen receptors at 0.9 nM (A). Estrogen receptor recovery was 84%. Receptors were charged with 10 nM 17$\beta$-[$^3$H]estradiol alone (solid circles) or 17$\beta$-[$^3$H]estradiol in the presence of 2 $\mu$M DES (open circles). Specific binding is indicated by open triangles. Aliquots of the liver preparation were charged with 50 nM coumestrol alone (solid line) or with coumestrol in the presence of 2 $\mu$M DES (dot–dash line) (B). Fluorescence of cytosol alone (dashed line). The kidney preparation contained protein at a concentration of 12 mg/mL and estrogen receptors at 0.4 nM (C). Renal estrogen receptor recovery was 85%. Symbols in panels C and D are as already designated. Conditions for elution are as described in Figure 5.

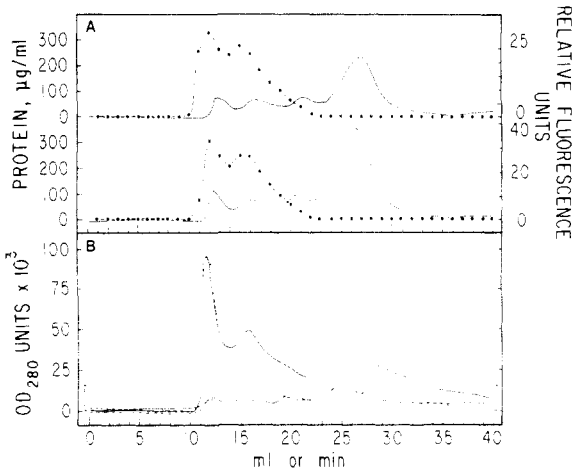


FIGURE 8: Relationships between protein concentration, endogenous fluorescence, and $OD_{280}$ absorbance. Protein concentration of mouse uterine cytosol was 6.5 mg/mL. Endogenous fluorescence (solid line). Protein (solid circles) in each fraction (1 mL) determined by the Lowry assay (Lowry et al., 1951) (A). Two different preparations were used without CD treatment. The effect of treatment with dextran-coated charcoal on endogenous fluorescence and UV absorbance at 280 nM (B). Solid lines: without CD treatment. Dashed lines: after CD treatment (10 min, 0–4 °C). Major profiles: $OD_{280}$ absorbance. Minor profiles: endogenous fluorescence. Left and right vertical markers indicate 10 relative fluorescence units.

of detecting a fluorescent signal that originates at an extremely low intensity.

## Discussion

The studies described herein show that the basis of coumestrol's estrogenicity in the CF-1 mouse uterus is the interaction of coumestrol with estrogen receptors. We have directly monitored the fluorescent signals that can be detected after exposing cytosols to coumestrol. First, increased fluorescence was observed to elute from an SEHPLC column in the same fractions that estrogen receptors eluted. Second, the increased fluorescence that resulted after exposing cytosols to coumestrol could be reduced by ligands which competitively inhibit the binding of 17$\beta$-estradiol to estrogen receptors. Third, the increased fluorescence appeared to result from a limited capacity binding of coumestrol. Fourth, the increased fluorescence with coumestrol was obtained at a charging concentration (50 nM) that could be predicted from competitive binding experiments (i.e., concentration required to saturate estrogen receptor with coumestrol = concentration required to saturate estrogen receptors with estradiol/relative binding affinity of coumestrol for estrogen receptors = 10 nM/0.2 = 50 nM). Taken together these observations provide *direct* evidence for the interaction of coumestrol with estrogen receptors. They also demonstrate that this fluorescent ligand can continue to generate fluorescent emissions after binding to estrogen receptor macromolecules. With the present detector we have been unable to determine if any quantum coupling [as indicated by Lee et al. (1977)], quenching, or spectral shifting occurs after ligand binding. We have used matched preparation comparisons and expressed the data relative to untreated cytosol because the output from the present detector cannot be adjusted to accurately compensate for day to day variation in spectral output of the source nor can it be calibrated to the fluorescence output of known fluorescent standards. As a consequence, precise quantitation is not possible with the present instrument. However, we have sought first to demonstrate that a fluorescent estrogen could be used to detect estrogen receptors and have done so in samples which we had prepared so that estrogen receptor concentration was quite high (5–10 nM).

The present fluorescence detector was unable to detect coumestrol binding to estrogen receptors in renal preparations which demonstrated low but measurable specific binding of [$^3$H]estradiol. Thus, the lower limits of detecting the interaction between coumestrol and estrogen receptors by SEHPLC occurred with preparations that had estrogen receptor concentrations of approximately 1 nM. Detection of lower concentrations of estrogen receptors will require photon counting instruments with increased sensitivity. The observations reported herein indicate that estimations of estrogen receptor activity with fluorescent ligands, where DES is used as a competitor, must also consider the quench properties of this ligand. With ligands having spectral properties that are similar

to those of coumestrol, hexestrol appears to be a more appropriate ligand to use for competitive inhibition of bound fluorescence.

A number of investigators have observed that coumestrol has an estrogenic potency in lambs, ewes, rat mammary tumors, and human breast cancer cells (Newsome & Kitts, 1977, 1980; Shutt & Cox, 1972; Verdeal et al., 1980; Martin et al., 1978). It has also been reported that coumestrol competed with [$^3$H]estradiol for binding to estrogen receptors, prepared from a variety of tissues and species (Shutt & Cox, 1972; Lee et al., 1977; Martin et al., 1978; Verdeal et al., 1980; Matheson & Kitts, 1980). Notides and collaborators have described the spectrofluorometric properties of calf uterine cytosol charged with coumestrol as compatible with receptor binding chiefly on the basis of the displacement of coumestrol fluorescence by estradiol (Lee et al., 1977). Finally, it has been reported that coumestrol can be used to visualize the cytoplasmic and nuclear estrogen receptors of human breast and prostatic cells (Pertschuk et al., 1980a); however, the strength of these observations was based upon a diminished fluorescence in the presence of DES, which, as shown here, could be due to quench rather than competition or displacement.

For the most part, interest in estrogens with fluorescent properties has focused on the utility these ligands might have for identifying the relative frequency of receptor negative neoplastic cells within a tumor that *biochemically* has been determined to be receptor positive (Mercer et al., 1980; Rao et al., 1980; Taylor et al., 1981). Even small subpopulations of receptor-negative tumor cells present a potential source of cells which eventually may be refractory to endocrine therapy. Second, because tumor samples may be partially composed of normal tissue regions which contain estrogen receptors, morphological determinations using estrogens with fluorescent properties might discriminate the extent to which receptor positive neoplastic cells predominate (Lee, 1980; McCarty et al., 1980; Pertschuk et al., 1980b; Taylor et al., 1981). With these end points in mind, methodologies involving 17$\beta$-estradiol and estrone conjugated to fluorescein (Dandliker et al., 1977, 1978; Rao et al., 1980; Witliff, 1980; Levison et al., 1976; Fisher et al., 1982), steroid–bovine serum albumin conjugates labeled with fluorescein isothiocyanate (Lee, 1978, 1980; Pertschuk et al., 1980a,b; McCarty et al., 1980), and fluorescent antibodies against steroid ligands (Pertschuk et al., 1978; Mercer et al., 1980, 1981) have been employed to visually identify steroid receptors. Validation of any of these fluorescence methodologies has focused on fluorescence determinations in cells and tissues, often coordinated with biochemical receptor determinations (Pertschuk et al., 1978, 1979, 1980a,b; McCarty et al., 1980; Mercer et al., 1980, 1981; Fisher et al., 1982). Previous efforts have not presented biochemical characterization of the *direct* interaction of fluorescent ligands with receptors, chiefly because low ligand–receptor affinity was coordinated with rapid dissociation of fluorescent estrogens and precluded any prolonged qualitative receptor analysis. Rapid analysis on SEHPLC overcomes this problem, as shown here. Moreover, this work establishes that the interaction of fluorescent estrogens with estrogen receptors can be characterized by direct methods which previously have been limited to use only with radiolabeled ligands. As a consequence, it should now be routinely possible to document and characterize the interaction of estrogen receptors with a variety of newly synthesized fluorescent ligands.

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**Registry No.** Coumestrol, 479-13-0; 17$\beta$-estradiol, 50-28-2; hexestrol, 84-16-2; tamoxifen, 10540-29-1; diethylstilbestrol, 56-53-1.

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# Estrogen-Induced Changes in High-Energy Phosphate Metabolism in Rat Uterus: $^{31}P$ NMR Studies†

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ABSTRACT: Changes in the concentrations of high-energy phosphate metabolites were measured by $^{31}P$ NMR spectroscopy of surviving rat uteri from 0–48 h following estrogen administration. Concentrations (millimoles per kilogram wet weight) of these metabolites in the untreated immature uterus, measured at 4 °C, were found to be the following: creatine phosphate (CP), 2.1 ± 0.2; nucleoside triphosphates, mainly adenosine 5′-triphosphate (ATP), 4.6 ± 0.4; phospho monoesters, primarily sugar phosphates (SP), 5.4 ± 0.7; and inorganic phosphate ($P_i$), 0.8 ± 0.4. Adenosine 5′-diphosphate (ADP) concentration was estimated to be approximately 40 μmol/kg wet weight from the assumed equilibrium of the creatine kinase reaction. The concentration of CP, and to lesser extent ATP and SP, declined within the first 1.5–3 h after injection of 17β-estradiol, returned to control values between 6 and 12 h, and then increased, reaching maximal concentrations at 24 h. From the fractions of the total soluble ATP in free and $Mg^{2+}$-bound forms, [free $Mg^{2+}$] in the untreated uterus was estimated to be 0.2–0.4 mmol/kg wet weight. An increase in [free $Mg^{2+}$] in the uterus was detected 1.5 h after estrogen injection. A subsequent parallel increase in the ratio of ATP to CP concentrations suggests that estrogen can also affect the apparent creatine kinase equilibrium by modulating [free $Mg^{2+}$].

Estrogen stimulates the immature or ovariectomized rat uterus in roughly two stages (Katzenellenbogen & Gorski, 1975); the first leads to an accumulation of RNA and protein and includes an early increase both in the rate of phosphate incorporation into phospholipids and in the wet weight, the latter due mainly to fluid imbibition (Astwood, 1938). In the second stage, the rate of DNA synthesis increases, and at approximately 24 h, cell division takes place in all uterine cell types (Kaye et al., 1972).

The discovery by Notides & Gorski (1966) of a uterine protein named the estrogen-induced protein (IP) provided a useful marker of estrogen activity due to (1) the rapidity of its induction both in vivo and in vitro (Barnea & Gorski, 1970; Katzenellenbogen & Gorski, 1972), (2) direct evidence for the rapid accumulation of translatable mRNA for IP (Walker & Kaye, 1981), and (3) the identification of IP as the BB isozyme of creatine kinase (Reiss & Kaye, 1981).

Creatine kinase (CK) regulates the intracellular concentration of adenosine 5′-triphosphate (ATP) by an energy "buffering" action (Jacobus & Lehninger, 1973). The high-energy utilization by uterine cells during the early response to estrogen could result in a reduction of energy stored as creatine phosphate (CP), or the increased glucose uptake and metabolism during this stage (Smith & Gorski, 1968) may provide an excess supply of energy, stored in the form of CP.

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