Coumestrol, NBD-Norhexestrol, and Dansyl-Norhexestrol, Fluorescent Probes of Estrogen-Binding Proteins†

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ABSTRACT: Coumestrol, a fluorescent plant estrogen, was used as a probe for the estrogen-binding sites of bovine serum albumin and the calf uterine estrogen receptor. In addition, 1-dansylamino-2,3-di(*p*-hydroxyphenyl)pentane, "dansyl-norhexestrol", and 1-(4-nitrobenzoxadiazole)amino-2,5-di(*p*-hydroxyphenyl)pentane, "NBD-norhexestrol", were synthesized for use as fluorescent probes. NBD-norhexestrol, dansyl-norhexestrol, and coumestrol competitively inhibit [^{3}H]estradiol-binding to the calf uterine estrogen receptor; their binding affinities, respectively, were 0.3, 1, and 20% of that of estradiol. Coumestrol is a highly fluorescent estrogen with an extinction coefficient of 24 800 M^{-1} cm^{-1} at 345 nm in ethanol; in buffered aqueous solution at pH 7.5, it has an emission maximum at 438 nm, a quantum yield of 0.20, and a fluorescence lifetime of 6 ns. The coumestrol–bovine serum albumin complex has an emission at 400 nm and a quantum yield of 0.74. Titration of bovine serum albumin with coumestrol was measured spectrofluorometrically; coumestrol has a dissociation constant of 1.4 (±0.02) × 10^{-7} M with a single binding site per molecule of albumin. The coumestrol–estrogen receptor complex detected spectrofluorometrically showed a blue shift from 438 to 410 nm and an increase in quantum yield. The effects of solvent polarity, pH, and energy-transfer properties of coumestrol were investigated. The spectroscopic properties and interactions of NBD-norhexestrol and dansyl-norhexestrol were also examined. The use of these fluorescent compounds provides a new probe to investigate the properties of the estrogen receptor.

The detection and quantification of the estrogen receptors were initially made possible by the synthesis of tritiated estradiol; radiolabeled estradiol subsequently led to the detection of the rat uterine estrogen receptor (Jensen and Jacobson, 1962; Gorski et al., 1968). Fluorescence spectroscopy, which has proven to be a valuable and sensitive technique in the study of ligand–protein interactions, protein structure, and protein interactions (Weber, 1952; Weber and Young, 1964; Brand and Witholt, 1967; Stryer, 1968), has not been applied to the study of the estrogen receptor or any other steroid hormone receptor. Quantitative and qualitative information not attainable using radiolabeled estrogens can be obtained using a fluorescent probe. An equilibrium analysis can be rapidly performed; i.e., the fraction of bound ligand can be measured without the necessity of separating the bound from the free ligand. Rapid kinetics in the millisecond range and measurements of the polarity and binding forces of the receptor binding can be realized. Receptor conformational changes can be determined by fluorescence polarization, fluorescence lifetime, or by energy-transfer techniques. Consequently, ligand-binding kinetics and receptor conformational changes can be studied with less receptor and without necessitating the greater purity required by other methods (e.g., equilibrium centrifugation analysis).

An effective fluorescent estrogenic probe must meet a number of criteria: (a) high affinity and specificity for the estrogen binding site of the receptor; (b) high fluorescence, i.e., a high extinction coefficient and quantum yield; (c) absorption and emission maxima that are well separated, i.e., a large Stokes shift; (d) absorption and emission maxima that are distinct from those of the aromatic residues of proteins; and (e) a change in its spectrofluorometric properties which distinguishes the receptor-bound ligand from the free ligand, either as a shift in wavelength, a change in quantum yield, or a change in fluorescence polarization. We report in this paper the properties of coumestrol,[1] dansyl-norhexestrol, and NBD-norhexestrol as specific fluorescent probes of the estrogen receptor.

Experimental Methods

Materials. Dansyl and NBD chlorides and crystallized bovine serum albumin (BSA), lot 83C-8090, were purchased from Sigma Chemical Co. Estradiol-17β and diethylstilbestrol were obtained from Steraloids, and [2,4,6,7-^{3}H]estradiol (109 Ci/mmol) was obtained from Amersham/Searle. The fluorescence standards quinine sulfate, β-naphthol, Rhodamine B, 3-aminophthalimide, and *m*-nitrodimethylaniline were obtained from Eastman Organic Co. or Fisher Chemical Co. All other solvents and reagents used were spectroscopic or reagent grades.

Synthesis of Fluorescent Estrogens. A pure crystalline derivative of hexestrol bearing a primary amino group, 1-amino-2,3-di(*p*-hydroxyphenyl)pentane, referred to as "aminonorhexestrol" (I),[2] was synthesized and then coupled to fluorescent reagents. The synthesis was initiated by condensing *p*-methoxybenzaldehyde with *p*-methoxyphenylacetonitrile in the presence of sodium ethoxide in dry ethanol (Wawzonek

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[1] Abbreviations used: aminonorhexestrol, 2,3-di(*p*-hydroxyphenyl)-1-aminopentane; BSA, bovine serum albumin; CLOM, clomiphene, 2-[*p*-(2-chloro-1,2-diphenylvinyl)phenoxy]triethylamine; CS, coumestrol, 3,9-dihydroxy-6*H*-benzofuro[3,2-*c*][1]benzopyran-6-one; dansyl, 1-dimethylaminonaphthalene-5-sulfonyl; Ans, 8-anilino-1-naphthalenesulfonate; HEX, hexestrol, *meso*-3,4-bis(*p*-hydroxyphenyl)hexane; NBD, 7-chloro-4-nitrobenz-2-oxa-1,3-diazole; RAC, ratio of association constants; Tris,tris(hydroxymethyl)aminomethane.

[2] Two diastereomeric forms of aminonorhexestrol are possible; the stereochemistry of the pure diastereomer of aminonorhexestrol prepared by the reaction sequence above has not been determined.

and Smolin, 1955) to give (*Z*)-α-cyano-4,4′-dimethoxystilbene, which on treatment with ethyl magnesium iodide at room temperature in ether gave 87% of the mixed diastereomers of the conjugate addition product, 2,3-di-*p*-anisylvaleronitrile, recrystallized from 95% ethanol to yield a major isomer, mp 130–132 °C. This compound upon treatment with pyridine hydrochloride at 210 °C gave 89% of the corresponding dihydroxy compound, mp 213–214 °C. Hydrogenation of the latter in ethanolic hydrochloric acid over Pd/charcoal, at 1 atm and room temperature, gave 1-amino-2,3-di(*p*-hydroxyphenyl)pentane as its hydrochloride, mp 280–281 °C. Anal. Calcd for $C_{17}H_{22}O_2NCl$: C, 66.45; H, 7.17. Found: C, 66.35; H, 7.13.

The coupling of dansyl chloride with aminonorhexestrol (I) was carried out in warm ethanol containing triethylamine to give 1-dansylamino-2,3-di(*p*-hydroxyphenyl)pentane (II), "dansyl-norhexestrol," mp 135 °C on recrystallization from methanol. Reaction of aminonorhexestrol (I) with NBD chloride (Ghosh and Whitehouse, 1968) in ethyl acetate with potassium carbonate at room temperature produced 1-NBD-amino-2,3-di(*p*-hydroxyphenyl)pentane (III), "NBD-norhexestrol," mp 200–201 °C. Anal. Calcd for $C_{23}H_{22}O_5N_4$: C, 63.58; H, 5.11; N, 12.90. Found: C, 63.43; H, 5.14; N, 12.76.

Coumestrol was purchased from Eastman Organic Co. To purify coumestrol, it was first acetylated by boiling with acetic anhydride and sodium acetate for 5 min. The coumestrol acetate was recrystallized twice from acetonitrile. Coumestrol was regenerated from the acetate and purified further by vacuum sublimation (Emerson and Bickhoff, 1958). Anal. Calcd for $C_{15}H_8O_5$: C, 67.17; H, 3.01. Found: C, 67.43; H, 3.03.

Coumestrol, dansyl-norhexestrol, and NBD-norhexestrol were analyzed by thin-layer chromatography on silica gel plates in a number of solvent systems. Each of the fluorescent estrogenic compounds migrated as a single component. The solvent systems used were benzene–ethanol (9:1), chloroform–ethanol (3:1), benzene–ethyl acetate–ether–methanol (6:4:3:1), and ethyl acetate–methanol (9:1).

Competitive Binding Assay. The partially purified estrogen receptor from calf uteri was used to estimate the relative binding affinity of the various fluorescent estrogenic compounds. Only calf uteri weighing less than 15 g were used; connective tissue was removed and the uteri were frozen in liquid nitrogen and stored at −80 °C for no longer than 1 month. The frozen uterine tissue was powdered with a steel mortar and pestle and cooled with liquid nitrogen. The powdered tissue was homogenized with a Polytron PT-10 in 5 mL of 40 mM Tris-Cl, 1 mM dithiothreitol, pH 7.5 (TD buffer), per g of tissue. The homogenate was centrifuged for 1 h at 220 000*g*. The cytosol fraction was then made 30% saturated with respect to ammonium sulfate. The ammonium sulfate precipitate was dissolved in TD buffer and used for the competitive binding assay. The ammonium sulfate step removed nonspecific binding protein(s), presumably serum albumin, thereby increasing the reliability of the assay.

Aliquots of the ammonium sulfate fraction (0.2 mL) were incubated with 2 nM [^{3}H]estradiol containing competitors from 10^{-10} to 10^{-5} M, and with 2 nM [^{3}H]estradiol without competitors. The aliquots were incubated for 18–20 h at 0–4 °C and then 0.1 mL of a 1% charcoal–0.01% dextran suspension was added. After a 5-min incubation, the aliquots were centrifuged and a 0.1-mL aliquot was removed; then the radioactivity was measured in a 5-mL toluene–Triton X-100 scintillation fluid at an efficiency of 40–45%.

The ratio of association constants (RAC) was estimated by the relationship described by Korenman (1970): RAC = (R × RBA)/(R + 1 − RBA), where RBA is the relative binding affinity or the ratio of the concentration of unlabeled competitor to unlabeled estradiol required to reduce the specifically bound [^{3}H]estradiol by 50%, observed from a plot of the percent bound [^{3}H]estradiol vs. log of the concentration of the competitor. R is the ratio of the free to bound [^{3}H]estradiol in the absence of a competitor. The RAC is expressed as percent (RAC × 100).

Spectral Analyses. Absorption spectra were recorded with a Cary 118 A spectrophotometer. A Perkin-Elmer MPF-3 spectrophotofluorometer was used to record uncorrected fluorescence spectra and fluorescence excitation spectra. Corrected fluorescence and excitation spectra were recorded with an Aminco corrected spectra spectrophotofluorometer or with the Perkin-Elmer MPF-3 using fluorescent standards to correct for the excitation source and the photomultiplier tube response using the method of Argauer and White (1964); both methods gave identical corrected spectra. Details for calculating the efficiency of energy transfer are given by Brand and Witholt (1967). The quantum yields of coumestrol were measured using quinine sulfate in 0.1 N H_2SO_4, quantum yield of 0.55, as a standard. All solutions used for quantum yield and fluorescence measurements had an absorbance of less than 0.02 at the excitation wavelength.

Binding Titration of Fluorescent Estrogens to BSA. Solutions of BSA in TD buffer, 5×10^{-5} M, were used to measure stoichiometric binding; 4.5×10^{-6} M BSA and 2×10^{-6} M BSA were used to measure equilibrium binding. The concentration of BSA was calculated from its absorbance using a molar extinction coefficient of 4.55×10^4 M^{-1} cm^{-1} at 280 nm; the molecular weight of BSA is 68 000. Increments of the fluorescent estrogens were added to 2 mL of a BSA solution or TD buffer in a 1-cm quartz cuvette at 20 °C, and the relative fluorescence was measured. The fluorescence of coumestrol was monitored at 400 nm and excited at 340 nm; the dansyl and NBD derivatives were excited with light at 340 nm and their emission was measured at 500 nm. The fraction of ligand bound (α) was calculated with the equation: $\alpha = (I - I_f)/(I_b - I_f)$, where I is the total fluorescence of the ligand BSA equilibrium mixture; I_f, the fluorescence of the unbound ligand in the TD buffer; and I_b, the fluorescence of the stoichiometrically bound ligand. The moles of ligand bound per mole of BSA (N) was calculated with the equation $N = \alpha L_0/P_0$, where α is the fraction of bound ligand, L_0 is the total ligand concentration, and P_0 is the total BSA concentration. The concentration of free ligand (F) is therefore: $F = L_0(1 - \alpha)$.

Results

The Binding Affinity of Fluorescence Labeled Estrogens for the Uterine Estrogen-Binding Protein. Tritiated estradiol can be displaced from the calf uterine estrogen-binding protein or receptor by a number of competing nonfluorescent and fluorescent estrogens (Figure 1). From the midpoint of each displacement curve the ratio of the association constants expressed as a percent (RAC × 100) of the affinity for estradiol was determined for the fluorescent estrogens (dansyl-norhexestrol (II), NBD-norhexestrol (III), and coumestrol (IV)). Coumestrol showed the highest affinity for binding among the fluorescent estrogens with an equilibrium association constant 20% of that of estradiol. The RAC × 100 of estrone was 45% of that of estradiol, while the affinity of estriol was 18% of that of estradiol. The binding affinity of dansyl-norhexestrol was much weaker than that of its parent com-

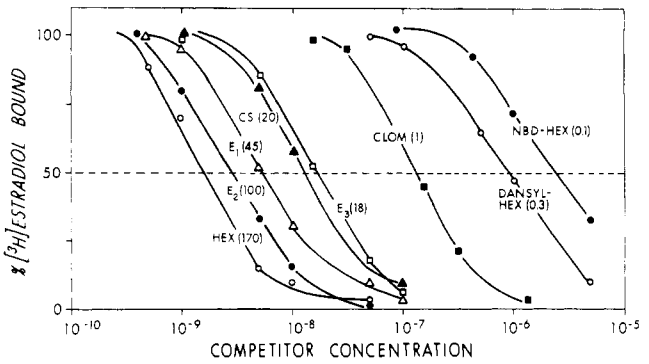


FIGURE 1: Competitive binding of fluorescent and nonfluorescent estrogens with [^{3}H]estradiol for the estrogen-binding proteins of the calf uterus. The abbreviations used are: HEX, hexestrol; E_1, estrone; E_2, estradiol; E_3, estriol; CS, coumestrol; CLOM, clomiphene; DANSYL-HEX, dansyl-norhexestrol; and NBD-HEX, NBD-norhexestrol. The values in parentheses are the RAC × 100.

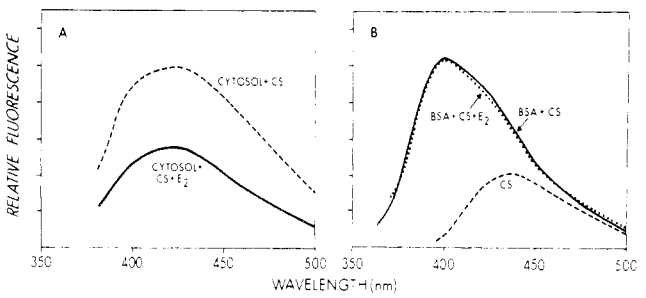
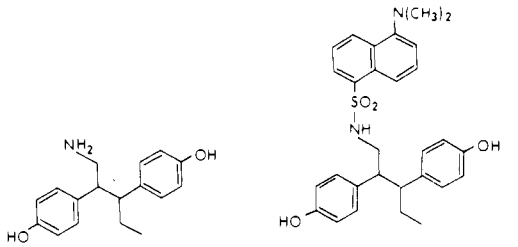


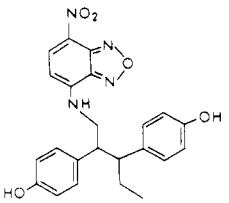
FIGURE 2: Emission spectra of uterine estrogen-binding protein and BSA–coumestrol complexes. (A) The 30% ammonium sulfate fraction of the calf uterine cytosol was preequilibrated with 10^{-8} M coumestrol for 1 h at 20 °C (- - -); then 10^{-7} M estradiol was added and incubated 1 h at 20 °C (—). (B) Bovine serum albumin, 10^{-6} M, was incubated with 10^{-8} M coumestrol for 1 h at 20 °C (—); then 10^{-6} M estradiol was added and incubated for 1 h at 20 °C (·····). Coumestrol, 10^{-8} M, in TD buffer (- - -). Excitation was at 340 nm with a 6-nm band width on both excitation and emission for all measurements.

pound hexestrol; competitive binding indicated that dansyl-norhexestrol and hexestrol had a binding affinity of 0.3 and 170% of that of estradiol, respectively. Coupling of the fluorescent moiety NBD to aminonorhexestrol reduced the hexestrol binding affinity to 0.1% of that of estradiol. The NBD- and dansyl-norhexestrol as employed are pure diastereomers, and their relatively weak inhibition of [^{3}H]estradiol-binding by the receptor may be due to the presence of both enantiomers. Aminonorhexestrol (I) even at 10^{-5} M did not compete with [^{3}H]estradiol for the estrogen receptor binding sites.

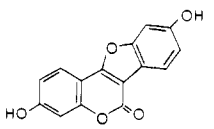


I AMINONORHEXESTROL

II DANSYL-NORHEXESTROL



III NBD-NORHEXESTROL



IV COUMESTROL

Fluorescence of Coumestrol Bound to the Estrogen Receptor. A coumestrol–estrogen receptor complex can be detected spectrofluorometrically. Calf uterine cytosol was made 30% saturated with respect to ammonium sulfate which precipitated the receptor but not the serum albumin, a major nonspecific binder of estrogenic compounds in tissue extracts. The partially purified receptor preparation contained a [^{3}H]estradiol-binding capacity of 8 nM. An aliquot of the partially purified receptor which had been preequilibrated with 10^{-8} M coumestrol showed an emission peak at approximately 410 nm (Figure 2A) that displayed a higher fluorescence yield and shorter wavelength maximum than free coumestrol in buffer (Figure 2B). The addition of 1×10^{-7} M estradiol was sufficient to displace the bound coumestrol from the receptor, suggesting that both ligands bind at an identical site (Figure 2A). Nevertheless, estradiol or diethylstilbestrol in concentrations as high as 10^{-5} M did not displace coumestrol from the BSA, implying that these ligands bind at different sites. The presence of estradiol or diethylstilbestrol did not influence the fluorescence of the unbound coumestrol in buffer.

Coumestrol was not degraded enzymatically by uterine cytosol or by long exposure to light. Coumestrol (10^{-6} M) was incubated with uterine cytosol at 37 °C for 2 h; reduction in fluorescence intensity was not observed, suggesting that coumestrol was not metabolized to a nonfluorescent compound. Thin-layer chromatography of the incubated uterine cytosol–coumestrol mixture showed a single fluorescent component with a migration identical with the coumestrol standard, indicating that it was not metabolized to a different fluorescent compound. Irradiation of coumestrol at its excitation maximum for 2–4 h in buffer did not decrease its fluorescence intensity with time, suggesting that it was not decomposed by light.

The detectability of coumestrol in dioxane was 1×10^{-9} M, slightly higher than in buffer, with a signal-to-noise ratio of 10 when using a S-19 type side-on photomultiplier tube. Thus, at a concentration of approximately 1×10^{-8} M of the estrogen receptor, the receptor-bound coumestrol can be observed with a standard laboratory spectrofluorometer. Although this level of detectability is sufficient for detecting the coumestrol–receptor complex, it is insufficient for accurate measurement of the coumestrol-binding kinetics of the receptor.

Fluorescence Titration of Bovine Serum Albumin with Coumestrol. In order to further assess the potential of fluorescence labeled estrogens as probes of estrogen receptor binding, the fluorescence properties and interactions of coumestrol with a model protein, bovine serum albumin (BSA), were studied. In the absence of BSA, coumestrol had an emission maximum at 438 nm; in the presence of a large excess of BSA (5×10^{-5} M), coumestrol (1×10^{-6} M) had an emission maximum at 400 nm. The fluorescence of the BSA–coumestrol complex showed a 38-nm shift of the emission peak toward the blue region of the spectrum, as well as a fourfold increase in intensity (Figure 2B and Table I). This allowed the fraction of coumestrol bound by BSA to be measured either by recording the intensity difference between the free and fully bound coumestrol at 400 nm or by measuring the wavelength maximum. The emission maximum was between 400 and 438 nm depending upon the fraction of coumestrol bound.

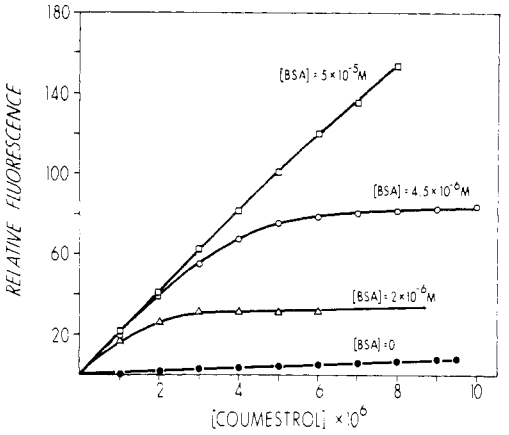


FIGURE 3: Fluorimetric titration of BSA with coumestrol. The fluorescence intensities of the coumestrol-BSA complexes were measured as a function of the total coumestrol concentration. Two milliliters of TD buffer solution containing 0.2, 4.5, or 50.0 × 10^{-6} M BSA was titrated with coumestrol at 20 °C. Excitation light was at 340 nm and emission was measured at 400 nm.

TABLE I: Spectroscopic Properties of Coumestrol.

Solvent	Absorption max (nm)	Excitation max (nm)	Fluorescence max (nm)	Quantum yield
TD buffer		374	438	0.20
Ethanol	346	345	412	0.70
Dioxane	340	340	388	0.91
BSA in TD buffer	349	348	400	0.74

The coumestrol binding curve shown in Figure 3 was obtained by measuring the increase in fluorescence intensity at 400 nm. At an extremely high BSA concentration (5 × 10^{-5} M), the fluorescence increased linearly as the concentration of coumestrol increased, indicating that coumestrol binding to BSA was stoichiometric. At lower concentrations of BSA (4.5 × 10^{-6} and 2 × 10^{-6} M), saturation of coumestrol binding was observed, indicating that all available binding sites on the BSA were occupied.

A Scatchard plot shows that coumestrol has a dissociation constant of 1.4 (±0.02) × 10^{-7} M and 0.94 ± 0.2 mol of coumestrol bound per mol of BSA, essentially, one binding site per molecule of BSA (data not shown). The dissociation constant of coumestrol for the calf estrogen receptor as estimated by the [^{3}H]estradiol-binding competition (Figure 1) was 20% of that of estradiol, or approximately 5 × 10^{-10} M, and 280-fold greater than that of BSA. The dissociation constant of the calf estrogen receptor for [^{3}H]estradiol was 1 × 10^{-10} M.

Absorption and Fluorescence of Coumestrol. Although coumestrol (IV) has polar functional groups, its solubility in water is low, a similarity to the steroidal estrogens that prevents recording absorption spectra in aqueous solutions. Absorption characteristics of coumestrol in different solvents are summarized in Table I; absorption at 345 nm was very high (E_{345} = 24 800 M^{-1} cm^{-1} ethanol) in contrast to steroidal estrogens. The absorption maximum of coumestrol shifted toward shorter wavelengths without changing the magnitude of absorption as the solvent polarity decreased.

As the solvent polarity decreased, the wavelength of maximum emission of coumestrol shifted toward the blue and the quantum yield increased (Table I). In dioxane a fivefold increase in quantum yield occurred and wavelength maximum

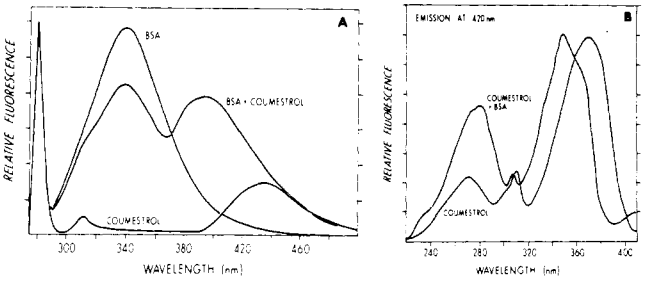


FIGURE 4: (A) Energy transfer from BSA to coumestrol. The BSA-coumestrol complex induced quenching of BSA at 340 nm and enhancement of coumestrol fluorescence at 440 nm. Coumestrol (5 × 10^{-7} M) and BSA (5 × 10^{-7} M) were in TD buffer; excitation light was at 280 nm with a 6-nm band width. (B) Excitation spectra of coumestrol free and bound to BSA. Coumestrol, 5 × 10^{-7} M, was in TD buffer or in solution with 5 × 10^{-7} M BSA in TD buffer. Emission was set at 420 nm and the band width was 6 nm.

was shifted 50 nm toward the blue. Increasing the fraction of ethanol in ethanol-water mixtures produced similar changes in fluorescence maximum and quantum yield. Interestingly, quantum yield and wavelength maximum of coumestrol fluorescence, when bound to BSA, were quite similar to those spectra in ethanol. This is an indication that the coumestrol binding site on BSA is moderately hydrophobic, comparable to the ethanolic environment, although not as hydrophobic as the dioxane environment. Coumestrol binding to BSA may also involve some hydrogen-bonding interaction of the kind expected in a coumestrol-ethanol system.

The fluorescence lifetime of coumestrol was measured using a nanosecond pulsed fluorescence apparatus capable of single photon counting. In organic solvents, mean fluorescence lifetime was 3–4 ns (3.1 ns in ethanol, 3.9 ns in dioxane) and 6 ns in Tris buffer, about 50% longer. The fluorescence lifetime of the coumestrol excited singlet state, from data on its absorption and fluorescence spectra, was 3.8 ns, calculated by the method of Strickler and Berg (1962), and is in agreement with our nanosecond fluorescence measurements.

Fluorescence Energy Transfer from BSA to Coumestrol. Coumestrol meets the spectroscopic properties necessary for energy-transfer studies that provide a potential method of receptor conformational analysis. When the emission spectrum of a donor, e.g., tryptophan or tyrosine of BSA, overlaps the absorption spectrum of the energy acceptor (i.e., coumestrol), excitation energy transfer can be transferred over a 10–100-Å distance by a resonance mechanism. The separation of absorption spectra of the donor and acceptor chromophores, donor excited-state lifetime, and high emission quantum yields are also necessary for successful energy transfer (Förster, 1959; Weber and Teale, 1959; Stryer and Haugland, 1967). The excitation of BSA at its maximal absorbance, 280 nm, produced a single BSA fluorescence peak at 340 nm which overlapped the wavelength maximum of coumestrol absorption. Excitation of coumestrol at 280 nm produced a minor peak at 438 nm in TD buffer. Energy transfer from BSA to coumestrol was observed (Figure 4A). The BSA fluorescence at 340 nm was quenched by the bound coumestrol while coumestrol fluorescence at 400 nm was concomitantly enhanced.

The efficiency of energy transfer depends upon the distance between the donor and acceptor chromophores as well as their orientation, thereby allowing detection of a conformational change in a protein. The efficiency of energy transfer was estimated from the excitation spectrum of the coumestrol fluorescence in the free and BSA bound state (Figure 4B) to be approximately 30% for 5 × 10^{-7} M concentrations of both

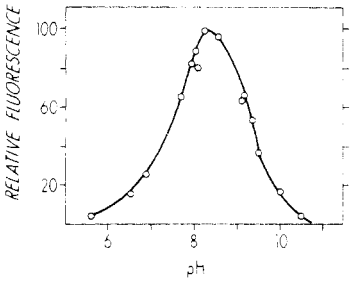


FIGURE 5: The variation in fluorescence intensity with pH. The buffers used were: 0.05 M phosphate, pH 5.5 to 6.5; 0.05 M Tris-maleate, pH 6.0 to 9.5; 0.05 M sodium borate, pH 9.0 to 10.5. Excitation light was 340 nm and emission was 430 nm.

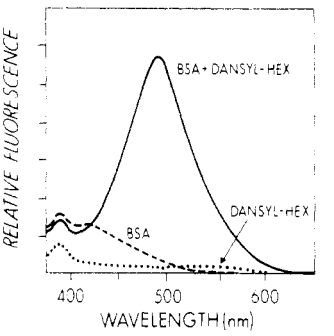


FIGURE 6: Fluorescence emission spectra of free and bound dansyl-norhexestrol. Dansyl-norhexestrol (10^{-6} M) in TD buffer (·····); background spectrum of BSA (5×10^{-5} M) without dansyl-norhexestrol (- - -); dansyl-norhexestrol (10^{-6} M) and BSA (5×10^{-5} M) in RD buffer (——). Excitation was at 340 nm with a 6-nm band width.

BSA and coumestrol (Weber and Teale, 1958; Stryer and Haugland, 1967). At this concentration the extinctions of BSA and coumestrol were very low and coumestrol binding was fractional, which eliminates errors due to inner filter effects or unbound coumestrol.

pH vs. Coumestrol Fluorescence. The relative fluorescence intensity of coumestrol was sensitive to pH change, showing maximum fluorescence efficiency at pH 8.0–8.5 (Figure 5). The pH optimum curve was assumed to consist of two sigmoidal curves; as the pH increased from 5 to 8.5 the fluorescence intensity increased with a midpoint at pH 7.5, while from pH 8.5 to pH 10.5 the fluorescence decreased with a midpoint at pH 9.4. The fluorescence–pH curve may reflect the two titratable hydroxy protons on coumestrol, one equivalent to the hydroxy group of 7-hydroxycoumarin, which has a pK_a of 7.4 while the other is similar to the phenol of the benzofuran moiety, which has a pK_a of 9.9 (Sober, 1970). The quenching of the fluorescence intensity at pH 5 or 11 could be reversed by readjusting the pH to 8.0, indicating that quenching was not caused by hydrolysis of the coumarin structure. Wavelength maximum of the coumestrol fluorescence or excitation remained unchanged from pH 5 through pH 10.5 when using buffers of phosphate, Tris, or borate. Thus, the use of coumestrol as a fluorescence probe requires the precise maintenance of pH.

Fluorescent Properties of Dansyl- and NBD-norhexestrol. Dansyl-norhexestrol (II) is nearly nonfluorescent in buffer, although in a solution of BSA the dansyl-norhexestrol showed a dramatic increase in fluorescence yield, about 70- fold, and a wavelength shift from 550 to 490 nm (Figure 6). This enabled the BSA bound dansyl-norhexestrol to be readily distinguished from the unbound species. The monitoring of BSA titration with dansyl-norhexestrol fluorescence indicated a complex nonsaturable kinetics, even at low BSA concentration (1×10^{-6} M), with about 5–6 mol of dansyl-norhexestrol binding per mol of BSA.

NBD-norhexestrol (III) served as a more suitable fluorescent probe than dansyl-norhexestrol because of its high extinction (E_{470} = 22 000 M^{-1} cm^{-1}, dioxane) compared with the dansyl derivative (E_{338} = 3300 M^{-1} cm^{-1}, dioxane) and its low affinity for BSA. Approximately 2–3 mol of NBD-norhexestrol were bound per mol of BSA with a dissociation constant of 1×10^{-6} M. NBD-norhexestrol exhibited spectral changes when bound to BSA which were similar to dansyl-norhexestrol; i.e., a 100-fold increase in fluorescence yield. However, no significant shift in the emission peak was observed.

Discussion

Among a number of known estrogens whose structure and spectroscopic properties were inspected for intrinsic fluorescence, coumestrol, a benzofuran derivative of hydroxycoumarin (Emerson and Bickoff, 1958), meets all the requirements for a specific fluorescent probe of the estrogen receptor. Coumestrol shows an increase in quantum yield and a blue shift in its emission maximum when bound to BSA or to the estrogen receptor from the calf uterus, and a relatively high affinity for the estrogen receptor, similar to that of estriol.

The changes in absorption and emission spectra of coumestrol and the increase in quantum efficiency with a decrease in solvent polarity imply (Weber and Laurence, 1954; Stryer, 1965) that the polarities of the estrogen-binding sites of BSA and of the receptor are hydrophobic. The blue shift of the coumestrol–receptor complex to 410 nm, compared with 400 nm for the coumestrol–BSA complex, may suggest that the estrogen-binding site of the receptor is less hydrophobic than that of BSA.

The excitation maximum of coumestrol at 340 to 370 nm is well removed from the usual interference of protein absorptivity at 280 nm and at the same time well situated for energy-transfer studies: the excitation of proteins at 280 nm produces protein fluorescence (i.e., tryptophan) emission at 340 nm. Since the efficiency of energy transfer depends upon the inverse of the sixth power of the distance between donor and acceptor chromophores (Brand and Witholt, 1967; Hillel and Wu, 1976), conformational changes of a protein can be measured as a function of the change in the distance or orientation between the protein chromophore, e.g., tryptophan, and the coumestrol-binding site.

Fluorescence polarization is another technique that can be used to measure ligand-binding and protein conformational changes, although the fluorescence probe must have a fluorescence lifetime comparable to the rotational relaxation time of the macromolecule (Weber, 1952). The fluorescence lifetime of coumestrol is approximately 3–6 ns, which indicates that it can be used to measure the fluorescence depolarization of proteins with a rotational relaxation of 1–13 ns. If one assumes a spherical protein, the most useful molecular weight range for proteins with a 1–13 ns rotational relaxation would be $10–120 \times 10^3$. Dansyl derivatives have a fluorescent lifetime of 12–14 ns, which allows accurate rotational depolarization studies of macromolecules with molecular weights up to 300 000 (Pesce et al., 1971). The molecular weight of the 4S monomer of the estrogen receptor is 70 000, while that of the 5S monomer is approximately double; the receptor, however, is very asymmetrical (Notides and Nielsen, 1974).

A different approach in the search for a fluorescent estrogenic probe couples an estrogen derivative, aminonorhexestrol, to fluorescent reagents. The fluorescence is extrinsic to the

estrogenic structure. Aminonorhexestrol at a 10 000-fold molar excess over that of estradiol did not compete for the estrogen-binding site of the receptor, presumably as a consequence of its very polar amino group. The addition of a bulky fluorescent reagent, dansyl or NBD, to the aminohexestrol resulted in a less polar reagent with a restored ability to bind the receptor. Dansyl- and NBD-hexestrol have a 1000-fold lower affinity for the receptor than has estradiol. The dissociation constants of the dansyl- and NBD-norhexestrol ($\sim 10^{-8}$ to 10^{-7} M) for the receptor are greater than those of many other fluorescent probes and were regarded as sufficient for binding analysis. The polarity-dependent fluorescent probe 8-anilino-1-naphthalenesulfonate (Ans) has a dissociation constant of about 10^{-5} M for luciferase (Tu and Hastings, 1975) or apomyoglobin (Stryer, 1965); propidium diiodide, a probe for acetylcholinesterase, has a dissociation constant of 3×10^{-7} M (Taylor and Lappi, 1975).

Dansyl- and NBD-norhexestrol have desirable spectroscopic properties as fluorescent probes: an excitation maximum removed from the absorption maximum of proteins, a large Stokes shift between the excitation maximum and emission maximum, and a relatively long lifetime. Unfortunately, the spectroscopic advantages are lost by the necessity of a high receptor concentration for analysis, due to low levels of detectability and low receptor affinity. Both these probes show complex binding kinetics and a relatively high affinity for serum albumin, the major nonspecific binder in tissue extracts. Thus, the addition of an extrinsic fluorescent group to a strong estrogen, hexestrol, results in a marked fall in binding activity and consequently a fluorescent probe inferior to coumestrol.

Acknowledgments

We thank Dr. Bruce Love for determination of the coumestrol fluorescence lifetime and Dr. Henry Auer for helpful discussions. We are grateful to Christen Giandomenico and Paul Naegely for their technical assistance.

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