Photoaffinity Labels for Estrogen Binding Proteins of Rat Uterus†

John A. Katzenellenbogen,* Howard J. Johnson, Jr., and Harvey N. Myers

ABSTRACT: A number of estrogen derivatives bearing a photosensitive functional group have been synthesized for use as photoaffinity labels for the high-affinity estrogen binding protein of rat uterus: diazoacetate derivatives of estradiol and estrone, 16-diazoestrone, and 3-diazo-2-ketopropyl ether and ortho-azide derivatives of estradiol, estrone, and hexestrol. All of these compounds are reasonably stable and have been carefully purified and characterized. The binding affinity of these compounds and their precursors for the rat uterine estrogen binding protein has been measured by a competitive binding assay. Introduction of substituents lowers the binding affinity in all cases, especially when one or both of the hydroxyl groups are blocked. Substituents at positions 2, 4, and 16 are reasonably well tolerated, and some preference for polar substituents is noted at these sites. Derivatives in which the A-ring hydroxyl is internally hydrogen bonded to a nitro group (2-nitro) have very low affinity, but the nitro group is well tolerated at position 4, where it does not hydrogen bond to the hydroxyl. The binding affinity of all the derivatives reflects the relative binding affinity of the parent ligands: hexestrol > estradiol > estrone. The requirements for selective affinity labeling of the estrogen binding protein from rat uterus are outlined. Based on the binding data, a number of the derivatives prepared appear to be promising candidates for covalent labeling of this protein in unpurified or only semipurified preparations.

High-affinity estrogen binding proteins from uterine tissue have been under intense study since the early 1960's (Jensen and DeSombre, 1972). However, purification and detailed characterization of these species have been hampered by their low state of purity and relative lability. Furthermore, identification of these proteins requires that they remain in or be revertible to a state capable of binding a labeled ligand.

We are interested in devising methods by which the estrogen binding protein from immature rat uterus can be labeled in a covalent fashion. With a "durable" label, this protein would be amenable to detailed study under a wide variety of conditions.

Several groups have reported the synthesis of steroid derivatives for the purpose of covalently labeling binding sites by the affinity-labeling method. In those cases in which pure proteins were investigated, selective labeling of binding site residues could be accomplished quite easily. Kallos and Shaw (1971) used bromoacetyldiethylstilbestrol to tag an allosteric binding site in glutamate dehydrogenase. Warren and coworkers (Chin and Warren, 1970, 1972; Ganguly and Warren, 1971; Sweet *et al.*, 1972) have successfully labeled the catalytic site of 20β-hydroxysteroid dehydrogenase using bromo and bromoacetyl derivatives of progesterone and cortisone. Selective reaction of 4-mercuriestradiol[1] with glutamate dehydrogenase has also been demonstrated (Chin and Warren, 1968).

In contrast, those investigations aimed at selective labeling of target tissue binding proteins in impure preparations have been much less successful. Liarakos and May (1969) were unable to observe any covalent attachment of a number of 3-*O*-bromoalkylestradiol derivatives to protein in immature rat uterine cytosol. Solo and Gardner (1968, 1971) prepared several halo ketone derivatives of progesterone as labels for the protein in the Clauberg assay, but again no covalent attachment could be demonstrated. Muldoon and Warren (1969) investigated the interaction between 4-mercuriestradiol and rat uterine cytosol. Although covalent attachment appeared to take place, the lability of the mercury–sulfur bond makes this derivative unsuitable as a reagent of general utility.

We have taken as our overall goal the development of labeling techniques that will allow the selective labeling of the rat uterine estrogen binding protein in the relatively impure preparations in which it is generally available. This has led us to choose the technique of photoaffinity labeling in preference to conventional affinity labeling based on alkylation and acylation (see Discussion). We have reported (Katzenellenbogen *et al.*, 1973) the preparation of a number of derivatives of estradiol, estrone, and hexestrol that embody photosensitive attaching functions (diazocarbonyl groups or azides) and are designed to act as photoaffinity labels. In this report we describe the measurement of the binding affinity of these derivatives for the estrogen binding protein of rat uterus by a competitive binding assay. Interesting features about the tolerance of different binding site regions to bulky, polar, or charged groups have been revealed by analysis of the effect that substitution at various positions on the estrogenic ligand has on the binding affinity. The binding affinity data can also be used to make a preliminary estimate of the degree to which each reagent will be capable of selective labeling of the binding protein in impure preparations. Studies on the solution photochemistry and the covalent attachment of these compounds to the estrogen binding site are currently in progress.

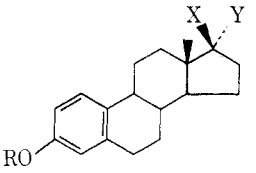
Materials and Methods

The following materials were obtained from the sources indicated: [^{3}H]estradiol ([6,7-^{3}H]-17β-estradiol, 46.6 Ci/mmol, New England Nuclear); dimethylformamide Baker

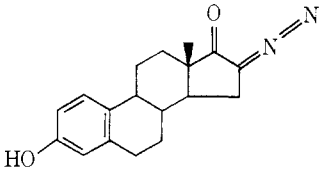
† From the Department of Chemistry, University of Illinois, Urbana, Illinois 61801. *Received April 16, 1973.* Support of this research through grants from the National Institutes of Health (AM 15556), the DuPont Co., the Eli Lilly Co., and the Ford Foundation (graduate fellowship to H. N. M.) is gratefully acknowledged.

[1] Common names used in this paper are: estradiol, 1,3,5(10)-estratriene-3,17β-diol; estrone, 3-hydroxy-1,3,5(10)-estratrien-17-one; hexestrol, *meso*-3,4-bis(4′-hydroxyphenyl)hexane.

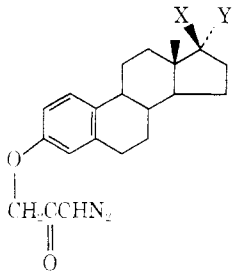
SCHEME I: Diazo Derivatives.



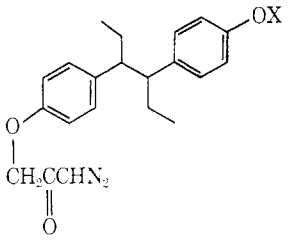
1, R = H; X = $OCOCHN_2$; Y = H [1.6][a]
2, R = $COCHN_2$; X, Y = O [1.8]



3, [0.40, 0.65]



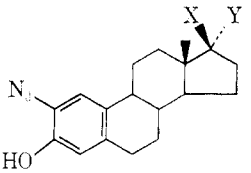
4, X, Y = O [0.02]
5a, X = OH; Y = H [1.4]
b, X = $OCOCH_3$; Y = H [0.06]



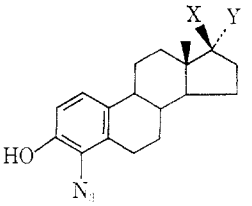
6a, X = H; [1.8]
b, X = $COCH_3$ [0.86]

[a] Numbers in square brackets are RAC × 100 values (see text).

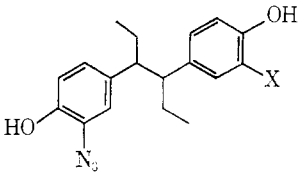
SCHEME II: Azide Derivatives.



7a, X,Y = O [0.08][a]
b, X = OH; Y = H [2.7, 3.3]



8a, X, Y = O [0.08]
b, X = OH; Y = H [0.8, 1.0]



9a, X = H [71, 67]
b, X = N_3 [12]

[a] Numbers in square brackets are RAC × 100 values (see text).

Analyzed Reagent (Baker Chemical); Norit A activated charcoal (Sigma); dextran grade C (Schwarz-Mann); polystyrene microtiter plates (Cooke Engineering, Scientific Products).

The Tris–EDTA–azide buffer (TEA buffer) used in all the binding experiments was 0.01 M Tris-HCl–0.0015 M EDTA–0.003 M (0.02%) sodium azide (pH 7.4 at 25°). The Norit A was washed successively (by centrifugation or vacuum filtration) with 1 N HCl, distilled water, dilute sodium bicarbonate solution, and finally with distilled water until neutral. The dextran–Norit suspension was prepared by dissolving 0.5 g of dextran C in 100 ml of TEA buffer and adding 5 g (dry weight equivalent) of washed Norit A.

Unless indicated otherwise, all volumetric additions were made with Eppendorf pipets. Incubations were conducted on disposable blood microtiter plates. Each plate has 96 wells which can accommodate the assay of four compounds run at twelve dilutions in duplicate. Because of their low water solubility, the steroid competitors were dissolved in 1:1 dimethylformamide–TEA buffer. The dimethylformamide concentration in the final assay incubation is 7%; this level readily solubilizes the steroid, and independent experiments have shown that it does not affect the competition assay. The polystyrene microtiter plates are unaffected by dimethylformamide concentrations below 50%.

Steroid Derivatives. The preparation of the steroid derivatives used in this study has been reported (Katzenellenbogen *et al.*, 1973). Because of the potential for serious error in the binding determinations of the low-affinity estrogen derivatives that would result from contamination with small amounts of their high-affinity parent steroid, the purity of all compounds tested was rigorously established. All samples used in the binding assay have demonstrated microanalytical purity, and were shown to be homogeneous and free from any detectable traces (generally 0.1%) of parent steroid by thin-layer chromatography under conditions where the two species were well resolved.

Derivatives **1**–**9** (Schemes I and II) are sensitive to ultraviolet irradiation and are stored as crystals in the dark at −20°. However, they may be handled for brief periods of time under normal laboratory illumination without any sign of decomposition.

Binding Assay. A modification of a procedure developed by Ellis (Ellis and Ringold, 1971; D. J. Ellis, personal communication[2]) was used. Immature Holtzman rats (21–25-day females) were decapitated and their uteri were collected in iced TEA buffer. After a rinse in fresh cold buffer, the uteri were homogenized at 4° in TEA buffer at 5 uteri/ml in a motor-driven all-glass conical tissue grinder (Kontes, Duall). The homogenate was adjusted to 2 uterine equiv/ml and centrifuged at 226,000*g* for 60 min. The supernatant (cytosol) was carefully pipetted from beneath the thin lipid layer.

An accurately weighed sample of nonradioactive competitor (4–5 mg) was dissolved in 10 ml of dimethylformamide. An appropriate volume of this solution was diluted to 2 ml with dimethylformamide and TEA buffer to give a stock solution 7×10^{-4} M in 1:1 dimethylformamide–TEA buffer. Serial dilutions with 1:1 dimethylformamide–TEA buffer were prepared to give concentrations 21×10^{-5} and 7×10^{-5} to 7×10^{-9} M. (With estradiol as competitor, a series tenfold lower in concentration was prepared.)

A microtiter plate was cooled on ice; 10 μl of a 7×10^{-8} M [^{3}H]estradiol solution in TEA buffer was added to each well (Hamilton repeating dispenser), followed by 10-μl aliquots of the competitor solutions (11 different concentrations, plus 1:1 dimethylformamide–TEA blank), and the plate was vortexed gently. After addition of 50 μl of cytosol to each well, the plate was again gently vortexed, covered with adhesive film, and placed on ice in the refrigerator. The final incubation conditions are: 10^{-8} M in [^{3}H]estradiol, from 10^{-4} to 10^{-9} M

[2] Ellis has determined the binding affinity of 140 steroid derivatives for a partially purified estrogen binding protein of mature rat uterus, using a competition assay with [^{3}H]estradiol and charcoal–dextran adsorption. These data are available from Dr. David J. Ellis, Institute of Biological Sciences, Syntex Research Center, Stanford Industrial Park, Palo Alto, Calif. 94304.

(11 concentrations) in competitor plus one blank, 1.43 uterine equiv/ml, and 7% in dimethylformamide.

After 20–24 hr, 10 μl of a well-mixed dextran–Norit suspension was added to each well, and the plate was vortexed vigorously (complete charcoal suspension) every 15 min over a 1-hr period. The plate was again covered with adhesive film and centrifuged at 800*g* for 20 min, and 50-μl aliquots were withdrawn carefully and counted in 5 ml of toluene-based scintillation fluid containing 0.54% 2,5-diphenyloxazole, 0.0036% *p*-bis[2-(5-phenyloxazolyl)]benzene, and 10% Biosolv BBS-3 (Beckman) in minivials at 43–48% efficiency.

A plot of bound [^{3}H]estradiol *vs.* log competitor concentration was prepared, and the concentration required to inhibit 50% of the high-affinity [^{3}H]estradiol binding was determined for each competitor (midpoint of the sigmoidal curve). The ratio of these concentrations, cold estradiol to competitor, is defined as the relative binding affinity (RBA) of the competitor. The equation derived by Korenman (1970) can be used to convert the relative binding affinity (RBA) to the ratio of association constants (RAC = $K_a^{\text{competitor}}/K_a^{\text{estradiol}}$; RAC = $(R \times \text{RBA})/(R + 1 - \text{RBA})$, where R is the ratio of free to bound [^{3}H]estradiol in absence of competitor). Under the conditions of our assay, R is 4–5; this corresponds to a binding site concentration of 2.0–2.5 $\times 10^{-9}$ M or a binding capacity of 1.4–1.7 pmol/uterine equivalent. The association constant for estradiol, measured under similar conditions, is 0.5–1.5 $\times 10^{10}$ M^{-1}. The RAC's are generally expressed as per cents (RAC $\times$ 100).

Because the parent ligands, estrone, estradiol, and hexestrol, all have different binding affinities, direct comparison of the effect that a particular substitution has on binding cannot be made when the binding is expressed relative to that of estradiol (RAC). Therefore, to facilitate discussion, the binding in most cases is expressed relative to that of the parent ligand (S or substituent value). S values are calculated by dividing the RAC of the derivative by that of the parent ligand (S = $\text{RAC}^{\text{derivative}}/\text{RAC}^{\text{parent ligand}}$) and are generally expressed as per cents ($S \times 100$). To make this conversion, the following values are used for the binding of the parent ligands (RAC $\times$ 100): estrone, 12, estradiol, 100, hexestrol, 300.

At several points in the discussion, reference is made to binding data obtained by Ellis (personal communication[2]). These data were obtained in the form of RBA's, and were converted to RAC and S values according to formulas given above, using a value of R = 5.7 given by Ellis.

Results

We have synthesized 14 derivatives of estrone, estradiol, and hexestrol that bear a photosensitive function, either diazocarbonyl group or aryl azide (Katzenellenbogen *et al.*, 1973). The diazo derivatives (Scheme I) are of three types. The simple diazoacetates **1** and **2** are easy to prepare, but are hydrolyzable, and thus may be of limited applicability. Diazoestrone **3** is hydrolytically stable, as are the diazoketopropyl ether derivatives **4–6**. The azide derivatives **7–9**, which all bear their substituent ortho to a phenolic hydroxyl, are shown in Scheme II.

Because most of these derivatives have not yet been prepared in radiolabeled form, their binding affinity for the estrogen binding protein of rat uterus has been measured by a competitive binding method, adapted from a procedure developed by Ellis (personal communication[2]), using [^{3}H]estradiol as a tracer and charcoal–dextran as an adsorbent for free steroid. The binding affinity of **1–9**, expressed relative to that of estradiol (ratio of association constants, RAC $\times$ 100), is given under the structures in Schemes I and II, and is summarized in Table V. Typical competition curves are shown in Figure 1. The affinities in all cases are less than that of estradiol, and where corresponding derivatives of estrone, estradiol and hexestrol have been prepared, the binding affinities reflect the different affinities of the parent ligand: estrone < estradiol < hexestrol. The implications of these binding data in terms of estimating labeling selectivity are covered in the Discussion.

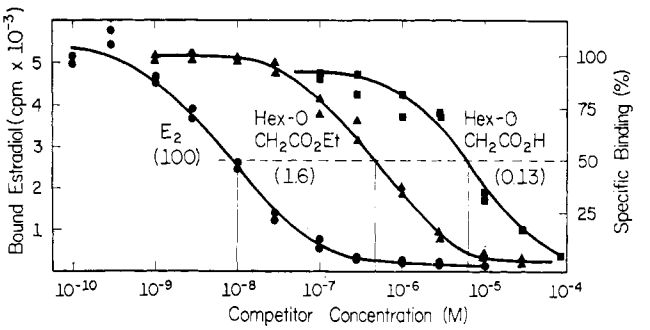


FIGURE 1: Binding affinity assay. Various concentrations of competitor, E_2 (estradiol), Hex-O-CH_2CO_2Et (4-*O*-(carbethoxymethyl)-hexestrol), and Hex-O-CH_2CO_2H (4-*O*-(carboxymethyl) hexestrol) were incubated with 10^{-8} M [^{3}H]estradiol in rat uterine cytosol (1.4 uterine equiv/ml). Charcoal–dextran was used to adsorb unbound steroid. RAC $\times$ 100 values given in parentheses (see Materials and Methods).

In addition to these fourteen derivatives, we have measured the binding affinity of all the precursors prepared in the course of their synthesis. These data are summarized in Tables I–IV.

Discussion

Affinity Labeling in Pure and Impure Systems: Selectivity and Ligand Affinity. The covalent labeling of binding sites is a powerful technique for studying the properties and function of proteins (Singer, 1967; Shaw, 1970). While the labeling is most often carried out on pure preparations of proteins, there are a number of particularly important instances in which the selective, covalent labeling of binding sites in impure preparations would be desirable. In investigations of physiological receptors for hormones and neurotransmitters, the only available preparations of binding proteins may be impure or only partially purified. Often the prospects for further purification are limited by the scarcity and low state of purity of the source material and the instability of the binding component. Furthermore, many studies are conducted most profitably either in whole tissue or whole cell preparations, which of necessity are "impure" with respect to the binding protein. In these instances it would be particularly useful to have a method for covalent labeling that would be sufficiently selective so that it could discriminate between the binding protein and the many other proteins present in the preparation.

The technique of affinity labeling, which has been widely used to study active sites of purified proteins (Singer, 1967; Shaw, 1970), appears to be the most promising one for labeling binding sites in impure preparations, because of the built-in selectivity that results from the ligand–binding site interaction. Furthermore, particular advantages in terms of selectivity have been ascribed to the photoaffinity labeling technique in some recent studies with impure binding preparations (Kiefer *et al.*, 1970; Winter and Goldstein, 1972).

The degree to which an affinity-labeling process will be

TABLE I: Effect of Substituents at Position 17β-O on Binding.

X	$S^a \times 100$
$-CH_3$	13.9
$-COCH_3$	22.0
$-COCHN_2$	**1.6**
2-Tetrahydropyranyl	0.11
4-Methoxy-4-tetrahydropyranyl	1.3

[a] S is substituent value ($S = RAC^{derivative}/RAC^{parent\ ligand}$). Binding of parent ligands are (RAC × 100): estrone-12, estradiol-100, and hexestrol-300 (see Materials and Methods). Data in boldface type are from this study; data in light face are from Ellis (personal communication[2]).

selective depends, of course, on the relative purity of the binding preparation that is being studied. But selectivity is also related to the affinity with which the labeling reagent is bound at the site to be labeled. This binding affinity–selectivity dependence is especially true for photoaffinity labeling, and can be appreciated by considering the situation with the estrogen binding protein.

Most studies on the estrogen binding protein have utilized the cytosol fraction of whole uteri. From this source, a 10,000- to 100,000-fold purification would be required to obtain the high-affinity binding protein in a homogeneous state.[3] Many of the other proteins that are present in the uterine cytosol preparations also are capable of binding estrogenic steroids. Although this secondary binding is of low affinity (generally termed "nonspecific" binding), it is of great importance in terms of selective labeling by the photoaffinity technique.

If, as is generally assumed (Kiefer *et al.*, 1970; Winter and Goldstein, 1972), binding site residues are labeled with reasonable efficiency by the photoaffinity technique, then the overall selectivity of the labeling process becomes a function of how the labeling reagent is distributed between the "specific," high-affinity sites (whose labeling is desired) and the nonspecific, low-affinity sites (whose labeling is to be avoided). It can be determined from the binding characteristics of rat uterine cytosol (Ellis and Ringold, 1971; our unpublished results) that, at low ligand concentrations, the amount of estradiol bound in the specific sites is 10–20 times greater than that bound in the nonspecific sites. This means that using a labeling reagent having the affinity and polarity of estradiol, the theoretical maximum labeling selectivity would be in the range of 90%.

The binding of estrogenic steroids to the high-affinity sites is known to be very stereospecific (D. J. Ellis, personal communication;[2] Korenman, 1969; Geynet *et al.*, 1972), while binding to the low-affinity, nonspecific sites is mainly a function of the overall polarity of the steroid, rather than its precise stereochemistry (Westphal, 1971). As a result, any alteration in the stereochemistry of the ligand that decreases its binding affinity for the specific, high-affinity sites, but does not alter its polarity, will cause a shift in the distribution of this reagent between the specific and nonspecific sites; a smaller fraction will be bound by the specific sites, and the situation for a selective labeling of these sites will be less favorable. In terms of designing a photoaffinity label, the implication is clear: the photoattaching function should be incorporated into the architecture of the ligand in a manner that will least disrupt the binding at the high-affinity sites.

A picture of the estrogen binding site has been provided by Ellis (personal communication[2]), who determined the binding affinity of some 140 estrogens for the rat uterine estrogen binding protein by competition assay. However, this study, together with less complete ones by Korenman (1969), Geynet *et al.* (1972), and Terenius (1972), do not provide sufficient information to allow one to make reliable, quantitative predictions as to the effect that a modification or substitution at a particular position in the ligand structure would have on the binding affinity. Because of this element of trial and error, we have chosen in this initial investigation to prepare a series of relatively simple estrogen derivatives, ones that bear one of the known photoattaching functions, diazocarbonyl or aryl azide groups (Katzenellenbogen *et al.*, 1973; Browne *et al.*, 1971; Knowles, 1972), and are functionalized at the sites that are chemically most accessible (2, 3-O, 4, 16, and 17β-O).

Binding Affinity of Estrogen Derivatives. Because of the close relationship between labeling selectivity and binding affinity, we were particularly interested in determining the binding affinity of our photosensitive estrogen derivatives. A knowledge of their binding affinity enables us to make a preliminary determination of their potential for selective labeling. Thus, at a relatively early stage, we can select the most promising compounds on which to continue our investigation.

However, from the binding affinity of these substituted estrogens, we obtain not only an estimate of their labeling selectivity, but also information about the nature of the region in the binding site in which the substituent is accommodated. Furthermore, in most cases the final derivative was prepared through a number of intermediates which are also functionalized at the same position. Since these substituents cover a range of size and polarity, their binding affinity can give valuable insight into how well the binding site is able to tolerate bulk, polarity, and charge.

The binding affinity of the photosensitive derivatives **1**–**9** has been presented in Schemes I and II, expressed as the ratio of their association constant with that of estradiol. However, because the parent estrogens (estrone, estradiol, and hexestrol) from which these compounds have been prepared differ considerably in their affinity, most meaningful comparison of the effect that a particular substituent has on the binding affinity of the ligand can be made only when the binding is expressed relative to that of the parent ligand. The substituent value S, obtained by dividing the RAC of the derivative by the RAC of the parent ligand (see Material and Methods), is a direct measure of the effect due to the substituent, regardless of the nature of the ligand. The effects that the various substituents at the five different positions have on binding affinity are presented in Tables I–IV. Comparison is made with some data of Ellis (personal communication[2]), and at each site some conclusions can be made about the nature of the region in the binding site that accommodates the substituent.

17β-O Region. The binding affinity of five derivatives of 17β-estradiol bearing substituents on the 17β oxygen is given in Table I. The binding displays a sensitivity primarily to the size of the substituent; large substituents, such as the 2-tetrahydropyranyl and the 4-(4-methoxypyranyl) ethers, are bound

[3] A cytosol preparation from an immature rat uterus contains *ca.* 1 mg of protein and 1.5 pmol of estrogen binding protein. This implies a 17,000:1 molar ratio, assuming an average molecular weight of 40,000, and one estrogen binding site per binding protein. Jensen and DeSombre (1972) estimate that a 20,000- to 100,000-fold purification is required to obtain homogeneous estrogen binding protein from immature calf uterus. For a discussion of some purification studies, see also DeSombre *et al.* (1969, 1971).

TABLE II: Effect of Substituents at Position 16 on Binding.

X	$S^a \times 100$	$RR_F{}^b \times 100$
=NOH	**41**	30
=CHOH	**11**	47
=N=N	**5.1**	72
(β)CH_3, H	19	
(α)OH, H	7.1	

[a] See footnote *a* Table I. [b] RR_F relative to estrone (0.56) in benzene–ethyl acetate–chloroform 5:1:5 on silica gel (Eastman Chromatosheet 6060).

with lower affinity than the less bulky methyl ether and acetate. Yet, the more polar acetate binds better than the smaller methyl ether, and the more highly oxygenated 4-(4-methoxypyranyl) ether better than the smaller 2-tetrahydropyranyl ether, revealing a preference of this region for polar groups. The diazoacetate, intermediate in size and polarity, is bound with intermediate affinity.

16 Region. The binding affinity of three estrone derivatives that bear a substituent doubly bonded to carbon 16 (Table II) follows an interesting progression. The appendage groups in all three cases are of comparable bulk; they differ in polarity, however, and their binding affinity decreases with the decreasing polar nature of the group. The 16 position, thus, seems able to tolerate a group of considerable bulk, particularly if it is reasonably polar. This behavior is in reasonable agreement with the nature of the neighboring 17β-O region.

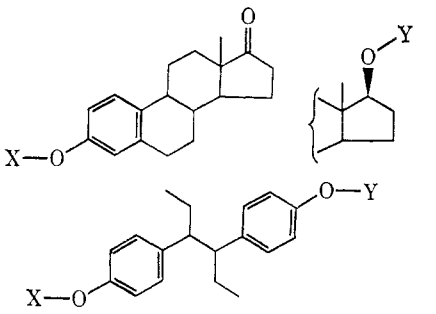
Ellis has investigated two estrone derivatives which have carbon-16 tetrahedrally hybridized. As the substituents in this case are directed either above (β) or below (α) the D-ring plane (rather than in the plane, as with the first three compounds), orientational differences in bulk and polarity tolerance are possible. The apolar methyl group, at 16β, is bound well, indicating that there is substantial tolerance to nonpolar bulk above the D ring near the region that accommodates the 18-methyl group. The isosteric, but polar, hydroxyl at 16α is bound less well, but it projects below the D-ring plane.

3-O Region. A number of studies have established that the phenolic hydroxyl group must be free for an estrogen to be biologically active (Korenman, 1969; Jensen *et al.*, 1966; Terenius, 1972). In terms of binding affinity, this group plays an important role as well. Table III summarizes the effect that various substituents on the phenolic hydroxyl group have on the binding affinity.

Most notable in this series is the unusually high affinity of the two ester derivatives (acetate and diazoacetate). Phenolic esters, however are readily hydrolyzable; so, it is very likely that with both of these esters, free steroid is being generated during the course of the binding assay. This hydrolysis would tend to raise the estimate of binding affinity in a competition assay with estradiol. In related studies (unpublished observations) we have found that [^{3}H]estradiol 3-acetate undergoes rapid hydrolysis when incubated with rat uterine cytosol at 0° for 1 hr.

The preference of the 3-O region for polar groups is reflected in the reasonably high affinity of the polar 2-hydroxyethyl ether and the low affinity of the nonpolar ethers, small (CH_3) or large (CH_2COCHN_2 and CH_2CO_2Et). It is interesting though that the estrone and estradiol carboxymethyl ether derivatives (CH_2CO_2H), which are very polar, but are also ionized in aqueous media, have a binding that is below the

TABLE III: Effect of Substituents at Positions 3-O and 17β-O on Binding.



Substituents		$S^a \times 100$		
X	Y	$E_1{}^b$	$E_2{}^b$	Hex^b
CH_3CO–		72		
N_2CHCO–		**15**		
CH_3–		0.035	0.06	
$HOCH_2CH_2$–		7.1		
$EtOCOCH_2$–		**bd**	**0.034**	**0.54**
$N_2CHCOCH_2$–		**0.18**	**1.4**	**0.60**
$HOCOCH_2$–		**bd**	**bd**	**0.044**
$N_2CHCOCH_2$–	$-COCH_3$		**0.059**	**0.29**
$HOCOCH_2$–	$-COCH_3$		**bd**	**0.02**

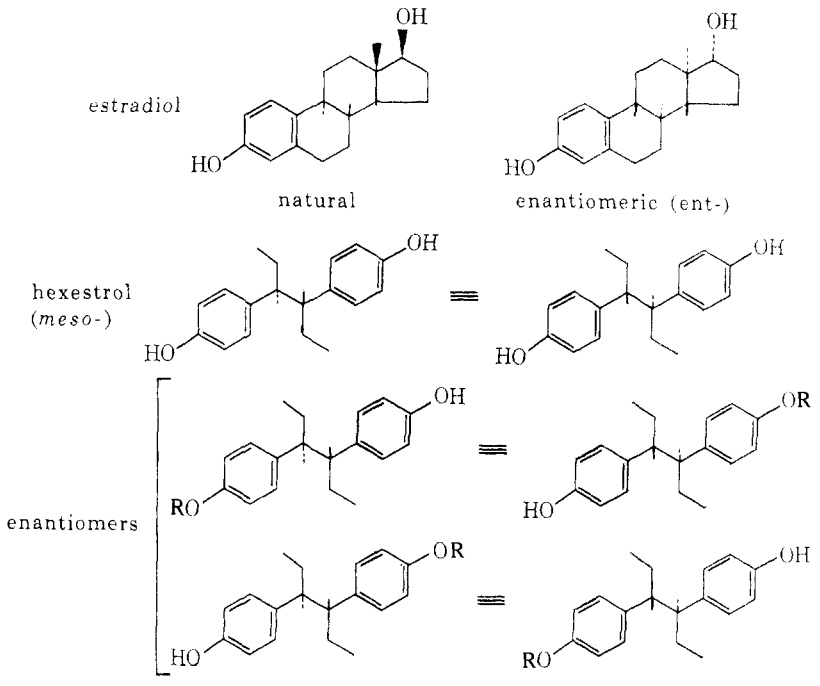
[a] See footnote *a*, Table I. [b] E_1 = estrone; E_2 = estradiol; Hex = hexestrol.

detection limit of the assay. The two estradiol derivatives functionalized on both 17β-O and 3-O have very low or undetectable binding.

Analysis of the binding affinity of the hexestrol derivatives has an inherent ambiguity. Although they are derived from an achiral molecule, *meso*-hexestrol, the hexestrol ether derivatives are chiral; hence they exist as racemic mixtures. The manner in which hexestrol and the enantiomeric monosubstituted hexestrol derivatives could bind is summarized in Scheme III. It is most likely that all these compounds will prefer to be bound in the "natural" configuration, as Ellis (personal communication[2]) has shown that *ent*-estrone is bound with only 2% the affinity of natural estrone, and studies by Edgren and Jones (1969) and Terenius (1968) have established that *ent*-estradiol is ineffective in inhibiting the uptake of [^{3}H]estradiol by rat uterus. The natural binding configuration places the substituent of one enantiomer in the region of the binding site that normally accommodates the 3-hydroxyl of estradiol. The substituent on the other enantiomer is bound near the D-ring region. If the two enantiomers are bound with different affinities, the binding affinity measured by competition assay will be somewhat greater than one-half the true binding affinity of the more strongly bound isomer. The factor of two error arises from the fact that the concentration of the individual enantiomers is only one-half the concentration of the racemate; competition by the more weakly bound isomer makes the measurement greater than one-half the true value of the higher affinity one.

With these ambiguities in mind, it is interesting to note that in the hexestrol series, the ethyl ester and diazoketone derivatives, which have similar steric size and comparable polarity (based on their chromatographic mobility), have nearly identical binding affinities, whereas the corresponding derivatives in

SCHEME III



the estrone and estradiol series have widely differing affinities. Such a pattern suggests that different enantiomers may be responsible for the predominant binding of the two hexestrol derivatives.

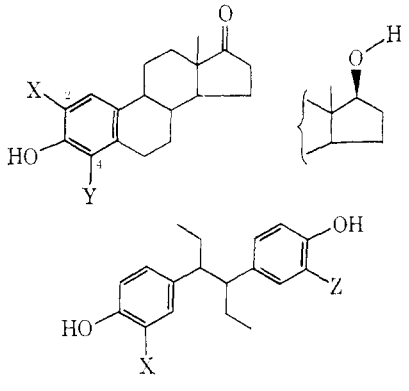
2 and 4 Region. The derivatives functionalized at positions **2** and **4** present an interesting picture of the nature of these binding regions (Table IV). All the estrone and estradiol derivatives we have prepared which are functionalized at C-4 bind with reasonable affinity. The azide group, which projects the greatest distance out from the ring, is bound considerably less well than the shorter, but wider, nitro group and the charged amino group.

Derivatives at C-2 of estrone and estradiol, on the other hand, show different binding behavior. The extended azide and charged amino groups are tolerated about as well as at C-4, but the nitro derivatives show no detectable binding. This clearly cannot be due to a lack of tolerance for bulk at this site, because of the reasonable affinity shown by the azides. Furthermore, Ellis has found that 2-methylestrone and estradiol bind well and that the very bulky methylthiomethyl group is actually better tolerated at C-2 than at C-4.

A possible explanation for the apparent anomalously low binding of the 2-nitro derivatives is intramolecular hydrogen bonding. It is well known from ultraviolet and infrared studies that *o*-nitrophenols are intramolecularly hydrogen bonded (Werbin and Holoway, 1956; Pickering and Werbin, 1958; Dearden and Forbes, 1960). When the nitro group is unhindered and can achieve complete coplanarity with the ring (as is the case in the 2-nitro estrogens) the *intramolecular* hydrogen bond is so strong that no *intermolecular* hydrogen bonding with hydroxylic solvents can be observed. Substituents adjacent to the nitro group (such as methylene-6 in the 4-nitro estrogens) tend to twist it out of the plane of the ring and weaken the intramolecular bond, so that hydrogen bonding with polar solvents takes place readily. Since a free hydroxyl at C-3 appears to be a prerequisite for high binding affinity, presumably due to a favorable hydrogen-bonding interaction with amino acid residues in the complementary region of the binding protein, it seems reasonable that those derivatives in which the hydroxyl group is bound exclusively in an intramolecular fashion should show low binding affinity.

Further support for this hypothesis comes from the binding affinity of the nitrohexestrol derivatives. As there are no groups comparable to C-6 in the hexestrol structure, strong intramolecular hydrogen bonding is in effect in both deriva-

TABLE IV: Effect of Substituents at Positions 2 and 4 on Binding.



	$S^a \times 100$ Position					
	X		Y		X	X, Z
Substituent	E_1[b]	E_2[b]	E_1	E_2	Hex[b]	Hex
$-NO_2$	**bd**	**bd**	**1.4**	**6.2**	**5**	**bd**
$-NH_2$	**0.3**	**9**	**2.3**	**5.1**	**13**	**2.4**
$-N_3$	**0.42**	**3**	**0.7**	**0.9**	**24**	**4.0**
$-CH_3$	3.5	6.5				
$-CH_2SCH_3$	0.33		0.013			
$-OCH_3$		0.06				

[a] See footnote *a* Table I. [b] See footnote **b** Table III.

tives. The reasonably high binding affinity of mononitrohexestrol can be rationalized by the fact that one enantiomer will bind with its unsubstituted phenol in the A-ring region. The dinitro derivative, however, must present a substituted, and hence internally hydrogen bonded, phenol in the A-ring site, and consequently shows no detectable binding.

Conclusions

The information presented in the preceding sections has given us a better knowledge of the regions in the binding site of the rat uterine protein that accommodate the A and D rings of estradiol. The fact that all the derivatives we have prepared have substantially lower binding than the parent ligand from which they were synthesized is a strong reaffirmation of the stereospecificity of this binding. However, the positions we have chosen to functionalize, because of their proximity to the hydroxyl groups, may prove to be ones particularly sensitive to bulky groups.

While comparison of the binding affinity of an estrogen derivative relative to the binding of the parent ligand (*S* value) is useful in discussions of the effect that a particular substituent has on the binding interaction, ultimately, it is the absolute binding affinity which is the important factor in estimating labeling selectivity. From this point of view, the derivatives of hexestrol appear to be the most promising reagents for selective labeling of binding sites in very impure preparations. As the derivatives functionalized at sites other than the two hydroxyl groups generally show higher binding affinity than those that have their groups blocked, it would be interesting to study the binding of molecules functionalized at sites more remote from 3-O and 17β-O, such as in the B and C rings of the steroids and the hexane positions in hexestrol. Both Ellis (personal communication[2]) and Hospital *et al.* (1972) have investigated the binding affinity of estrogens functionalized at positions 7α and 11β and have found that the substituents, particularly when apolar, are quite well tolerated.

The photosensitive estrogen derivatives we have prepared display a wide range of binding affinities for the estrogen binding protein of rat uterus. It is anticipated that some of these compounds might be utilized in photoaffinity labeling studies of other estrogen binding proteins, such as the plasma binding globulins (Westphal, 1971; Corvol *et al.*, 1971; Milgrom *et al.*, 1973), enzymes involved in steroidogenesis or metabolism (Westphal, 1971; Clayton, 1969), and certain enzymes that are affected allosterically by the estrogens (Douville and Warren, 1968). The relative binding affinity of the compounds for these other binding sites may well be different; however, since most of these proteins are either available in pure form or have been substantially purified, maintenance of high binding affinity of the labeling reagent would not be so crucial a requirement in such studies.

In terms of selective labeling of the estrogen binding protein from rat uterus, however, binding affinity is of great importance. Accordingly, the photosensitive derivatives can be divided into three categories (Table V): Derivatives that bind with an affinity less than 0.5% that of estradiol would require extensive purification of the uterine binding protein before selective labeling would become feasible. The ones whose affinity lies in the 0.5–5% range might be useful in labeling semipurified preparations of the protein, but those that bind with an affinity greater than 5% that of estradiol, when prepared in radiolabeled form, should be capable of selective labeling of the estrogen binding protein directly in the impure state in which it is found in crude uterine cytosol.

TABLE V: Binding Affinity of Photosensitive Estrogen Derivatives.[a]

Name	No.	RAC × 100
High (>5%)		
3-Azidohexestrol	**9a**	71, 67
3,3′-Diazidohexestrol	**9b**	12
Medium (0.5–5%)		
2-Azidoestradiol	**7b**	2.7, 3.3
3-*O*-(Diazoketopropyl)hexestrol	**6a**	1.8
Estrone diazoacetate	**2**	1.8
Estradiol 17β-diazoacetate	**1**	1.6
3-*O*-(Diazoketopropyl)estradiol	**5a**	1.4
4-Azidoestradiol	**8b**	0.80, 1.0
3-*O*-(Diazoketopropyl)hexestrol acetate	**6b**	0.86
16-Diazoestrone	**3**	0.40, 0.65
Low (<0.5%)		
2-Azidoestrone	**7a**	0.08
4-Azidoestrone	**8a**	0.08
3-*O*-(Diazoketopropyl)estradiol acetate	**5b**	0.06
3-*O*-(Diazoketopropyl)estrone	**4**	0.02

[a] The binding affinity of the parent compounds are (RAC × 100) estrone, 12, estradiol, 100, and hexestrol, 300.

Acknowledgments

Thanks are due to Mrs. Kathryn Carlson and Mr. Raymond Munaretto for their skillful assistance, to Dr. Thomas S. Ruh and Mr. Francisco A. Pinilla for their help during the early part of these studies, and to Drs. Jack Gorski, William H. Beers, David L. Williams, and Robin Leake for their encouragement and helpful suggestions. We are grateful to Dr. David J. Ellis for providing us with the results of his studies on the binding of estrogen derivatives.

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Studies on the Uterine, Cytoplasmic Estrogen Binding Protein. Thermal Stability and Ligand Dissociation Rate. An Assay of Empty and Filled Sites by Exchange†

John A. Katzenellenbogen,* Howard J. Johnson, Jr., and Kathryn E. Carlson

ABSTRACT: The thermal stability and the rate of estradiol dissociation from the cytoplasmic estrogen binding protein of rat uterus have been determined at 0, 25, 30, and 37°. Unfilled sites are very labile, but estrogen-filled sites do not undergo detectable degradation over a 24-hr period at temperatures up to 30°. At 37°, however, 50% of the sites are lost over an 8-hr period, while the remainder are stable for up to 24 hr. Dissociation of bound estradiol, as determined by exchange, is complete within 24 hr at 25° and within 8 hr at 30°. Comparison of freshly prepared and exchanged cytosol (24 hr, 25°) on high and low salt–sucrose density gradients shows that considerable aggregation of the binding activity occurs during the exchange period. A procedure for assaying soluble, cytoplasmic estrogen binding sites by exchange has been designed, using the optimum conditions for rapid exchange without degradation of binding capacity. The assay is convenient, quantitative, and linear up to 3 uterine equivalents/ml. Using this assay procedure, the concentration of estrogen binding sites can be determined, regardless of whether they are empty at the time of assay or filled with estradiol, a nonsteroidal estrogen (hexestrol), or an antiestrogen (dimethylstilbestrol, U 11,100A, or CI-628). By following the time course of exchange, the dissociation rate of an unlabeled ligand can be determined.

Numerous studies have demonstrated the presence of a binding protein with high affinity for estrogens in uterine tissue (Jensen and DeSombre, 1972). The binding of estradiol to this protein in the cytoplasm, and the subsequent translocation of the complex to the nucleus, are thought to be steps of fundamental importance in the action of estradiol on the uterus (Jensen and DeSombre, 1972).

Most of the methods for determining the concentration of these high affinity estrogen binding proteins involve a direct assay: addition of a saturating concentration of [^{3}H]estradiol is followed by separation of bound from free ligand; correction is usually made for binding due to sites of low affinity (nonspecific binding). However, this direct assay method, normally run at 0°, does not determine the total concentration of high-affinity binding protein, but only those sites that are unoccupied at the time of analysis; the rate of estradiol dissociation from the binding protein is so slow that only minimal exchange occurs during the time of assay. This limitation of the direct assay method is most serious in those situations in which a large fraction of the binding sites is filled with unlabeled ligand. In these cases, an assay which allows such sites to exchange with labeled estradiol would give a more accurate determination of the total concentration of high-affinity binding sites. Anderson *et al.* (1972) have

† From the Department of Chemistry, University of Illinois, Urbana, Illinois 61801. *Received May 4, 1973.* Support of this research through grants from the National Institutes of Health (AM 15556 and GM 722-12 (traineeship to H. J. J.)), the Ford Foundation (700-0333), and the DuPont Co. is gratefully acknowledged.