Photoreactivity of Some Light-Sensitive Estrogen Derivatives. Use of an Exchange Assay to Determine Their Photointeraction with the Rat Uterine Estrogen Binding Protein†

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ABSTRACT: The photoreactivity of a number of photosensitive estrogen derivatives with the estrogen binding protein of rat uterus can be ascertained using a cytosol exchange assay. The competitive effect of estradiol localizes the reaction at the estrogen binding site and permits the assignment of a reaction efficiency for each compound—per cent of the binding capacity that is consumed upon photolysis. The kinetics of reaction of several azide and diazo derivatives is biphasic, the initial portion corresponding to the photolysis rate of the photosensitive function and the slower portion to the direct photolytic degradation of the binding protein. Reaction efficiencies of these compounds range from 0 to 21%. In certain cases there is a pronounced dependence on the orientation of the reactive function in the binding site. Investigation of a number of other chromophoric estrogens and antiestrogens shows some to be unreactive (benzil, *o*-iodophenol, *o*-nitrophenol, α-oximo ketone, stilbene, and styrene chromophores) and others to be highly reactive (20–51%; triarylethylene chromophores). As opposed to the biphasic kinetics of these derivatives, reaction of 6-oxoestradiol is cleanly first order. A reagent of this type is classified as "photoexcitable" (reversibly photoactivatable), while the others are "photolabile" (irreversibly photoactivatable).

Estrogen-sensitive target tissues contain a cytoplasmic protein with a high binding affinity for estrogens. This protein, often called the "estrogen receptor," is considered to be centrally involved in the mediation of estrogen action in these tissues. Numerous studies over the past several years have focused on its physical and biological characterization and have attempted to define its biochemical significance (Jensen and DeSombre, 1972); however, aggregation and the thermal lability of the binding activity have been impediments to detailed characterization studies.

We have undertaken a chemical approach to the characterization of the estrogen binding protein obtained from rat uterus, using the technique of affinity labeling. In previous reports, we have described the synthesis of several photosensitive derivatives of estradiol, estrone, and hexestrol (Katzenellenbogen *et al.*, 1973c) and the determination of their binding affinity relative to estradiol by a competitive binding assay (Katzenellenbogen *et al.*, 1973b). In addition, we have developed an assay procedure that permits the determination of the concentration of estrogen binding sites in uterine cytosol by exchange (Katzenellenbogen *et al.*, 1973a), regardless of whether they are occupied by a ligand or not. In this report, we describe the application of the cytosol exchange assay to the problem of affinity labeling. Binding sites, filled with a photosensitive labeling reagent, are irradiated with ultraviolet light; the decrease in estrogen binding capacity, followed as a function of time, is measured by exchange. Such experiments, coupled with suitable controls, allow the determination of the efficiency with which the labeling reagent undergoes reaction at the binding site.

Using this assay, we have examined a number of azide and diazo derivatives of estradiol and hexestrol that we have prepared, as well as a variety of other chromophoric estrogens and antiestrogens. We have been able to measure the reactivity of the different photosensitive functions, to uncover certain orientational preferences, and to distinguish two different kinetic modes of photoreactive behavior. These data permit the selection of the most promising derivatives for preparation in radiolabeled form as possible photoaffinity labeling reagents for the estrogen binding protein of rat uterus.

Experimental Section

Materials. The following compounds were obtained from the sources indicated: estradiol[1] and estrone (Searle; Steraloids); Δ^6-estradiol (Steraloids); [6,7-^{3}H]estradiol (46.5–48 Ci/mmol; New England Nuclear); *meso*-hexestrol and dienestrol (Mann); diethylstilbestrol (Pfaltz-Bauer); antiestrogens CI-628, CI-680, and 9411X27 (all as monocitrates; Parke-Davis) and nafoxidine U 11,100A (as the hydrochloride, Upjohn); anisoin (Aldrich); charcoal, Norit A (Sigma); dextran, grade C (Schwarz-Mann); Triton X-114 (Rohm and Haas); dimethylformamide (Baker); ethylenediaminetetraacetic acid (East-

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[1] Abbreviations and common names used are: estradiol (E_2), 1,3,5(10)-estratriene-3,17β-diol; estrone (E_1), 3-hydroxy-1,3,5(10)-estratrien-17-one; hexestrol (Hex), *meso*-3,4-bis(4′-hydroxyphenyl)hexane; diethylstilbestrol, 3,4-bis(4′-hydroxyphenyl)-*trans*-3-hexene; dienestrol, 3,4-bis(4′-hydroxyphenyl)-2,4-hexadiene; U 11,100A, 1-(2-(*p*-3,4-dihydro-6-methoxy-2-phenyl-1-naphthyl)phenoxy)ethyl)pyrrolidine hydrochloride; CI-628, 4-methoxy-α-(*p*-2′-(*N*-pyrrolidino)ethoxy)-phenyl-α′-nitrostyrene; CI-680, 4-methoxy-α-(*p*-3′-(dimethylamino)-propoxy)phenyl-α′-nitrostyrene; 9411X27, 4-hydroxy-α-(*p*-3′-(dimethylamino)propoxy)phenyl-α′-nitrostyrene; DKP ether, 3-diazo-2-ketopropyl ether; cAMP, cyclic adenosine 3′,5′-monophosphate.

man); trishydroxymethylaminomethane (Nutritional Biochemicals); microtiter plates (Scientific Products).

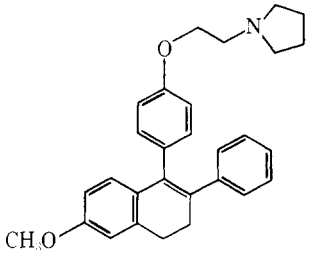
Methods. The buffer used in these studies was Tris-EDTA (TEA) (0.01 M Tris, 0.0015 M EDTA, and 0.02% sodium azide (pH 7.4) at 25°). The charcoal–dextran slurry consisted of 5% acid-washed Norit A and 0.5% dextran C in 0.01 M Tris (pH 7.4) at 25°, containing 0.02% sodium azide. A xylene-based scintillation fluid (Anderson and McClure, 1973) was modified to contain 0.55% 2,5-diphenyloxazole, 0.01% *p*-bis[2-(5-phenyloxazolyl)]benzene, and 25% Triton X-114. Counting was done in minivials (Research Products) containing 5 ml of scintillation fluid in either a Nuclear Chicago Isocap 300 (43–48% tritium efficiency) or a Packard 314 EX (18–22% tritium efficiency) instrument.

The exchange procedure was carried out in blood microtiter plates. All volumetric additions and withdrawal of samples were made with either Eppendorf pipets or Pipetman adjustable pipets.

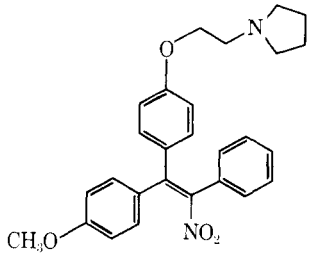
Nmr spectra were obtained on a Varian A60A spectrometer and are expressed as parts per million downfield from tetramethylsilane as internal standard. Other spectroscopic data were obtained as follows: infrared, Perkin Elmer 237; mass spectra, Varian MAT CH5; and uv, Cary 15. Melting points are uncorrected.

Estrogen Derivatives. The structure and preparation of most of the estrogen derivatives used in this study and the determination of their binding affinities have been described previously (Katzenellenbogen *et al.*, 1973b,c). The preparation of derivatives not previously described is given at the end of this section. The structures of certain antiestrogens and chromophoric estrogens are given in Scheme I.

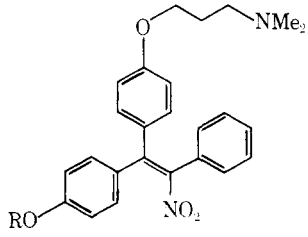
SCHEME I: Photosensitive Estrogen Derivatives.



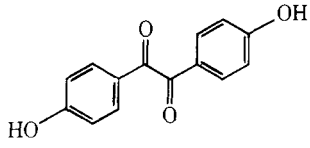
U 11,100A



CI-628



CI-680, R = CH_3
9411X27, R = H



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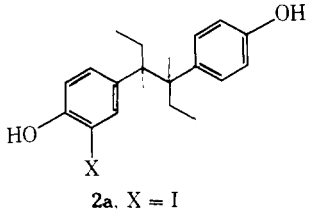
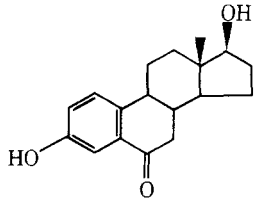


2a, X = I
b, X = NO_2



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Preparation and Storage of Cytosol. Cytosol was prepared from immature Holtzman rats (21–25-day females) in TEA buffer at 5 uterine equiv/ml according to Katzenellenbogen *et al.* (1973a).

Cytosol could be stored by freezing. Freshly prepared cytosol was brought to 25% in glycerol (3.75 uterine equiv/ml) and then frozen quickly in a Dry Ice–acetone slurry and stored at −20°. A 30% decrease in binding capacity results from the initial freezing; thereafter, prolonged storage for several months causes no further decrease (K. E. Carlson, unpublished results). Frozen samples were thawed slowly in an ice bucket.

All cytosols were diluted to 3 uterine equiv/ml just prior to use in the assays. No differences in exchange rate or reaction efficiencies were noted between fresh and frozen samples; generally, frozen cytosol was used.

Incubations. The minimum concentration of estradiol required to achieve effective saturation of the estrogen binding protein of rat uterus is 30 nM. For derivatives having binding affinities different from estradiol, the equivalent minimum saturating concentration is 30/RAC nM, where RAC is the ratio of association constants (see Determination of Binding Affinity). The stock solutions (10^{-3}–10^{-4} M in dimethylformamide) were diluted with TEA buffer to 90/RAC nM in each case, and photolysis exchange assay incubations were prepared as follows (see Scheme III).

(1) UNPROTECTED INCUBATIONS (E–X ALONE). Cytosol (3 uterine equiv/ml) was mixed 2:1 with the derivative solution (90/RAC nM) to give a final concentration of 30/RAC nM in derivative and 2 uterine equiv/ml and was kept at 4° for 1–2 hr.

(2) PROTECTED INCUBATION (E_2 PRIOR TO E–X). Cytosol was brought to 30 nM in estradiol by the addition of the appropriate small volume of a 12×10^{-7} M estradiol solution in TEA buffer, to presaturate the estrogen binding sites. A compensatory volume of TEA buffer is added to the first incubation to make all final volumes equal. After 1–2 hr at 4°, the protected incubation is diluted 2:3 with 90/RAC nM solution of derivative to give the same final concentration (30/RAC nM) as in incubation 1. Photolysis is started 5 min after the last addition.

Photolysis Conditions. The apparatus for photolysis at 254 nm is shown in Figure 1a. The lucite tube holder is housed in a Rayonet photochemical reactor (Model RPR 100) and is attached to a motor through a drive shaft which turns at 50 rpm. Four symmetrically placed 8-W GE germicidal lamps (G8T5) are used for irradiation of samples; 95% of the output of these lamps is at 254 nm. Cooling is effected by means of a quartz ice bath (see Figure 1a). Photolysis is conducted for a total time of 10 min, with periodic mixing.

The absorbance by protein (in these samples *ca.* 2 mg/ml) is large; however, by using quartz photolysis tubes of annular design in which the 0.8-ml sample is dispersed in a film of 0.4 mm thickness, self-absorbance effects can be minimized. (Control experiments (H. N. Myers, unpublished) have established this: the rate of photolysis at 254 nm of solutions of 16-diazoestrone and 3-azidohexestrol, placed inside the center tube, is not retarded when the annular space is filled with a 3 mg/ml solution of bovine serum albumin.) The center tube can be removed to facilitate the mixing of samples and the removal of aliquots from the annular space.

The apparatus shown in Figure 2 was used for photolysis at 315 nm. A 450-W mercury vapor lamp (Hanovia L679A) is cooled by circulating water, and is surrounded by a solution filter of saturated aqueous copper(II) sulfate. This filter effectively screens all irradiation shorter than 315 nm. Sample tubes are placed in a lucite holder immersed in a 1:1 mixture of ethylene glycol–water. The temperature of this bath is maintained

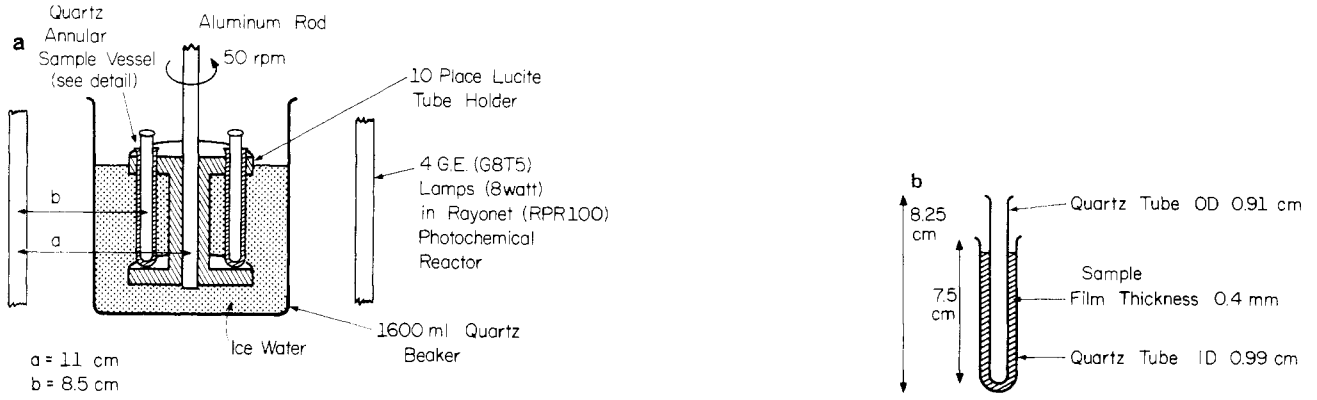


FIGURE 1: (a) Apparatus for photolysis at 254 nm. Samples (0.8 ml) are placed in the annular space between two quartz tubes (Figure 1b). The tubes are placed in a lucite holder which is rotated at 50 rpm within a 1600-ml quartz beaker filled with an ice–water slurry. This apparatus is positioned within a Rayonet photochemical reactor (RPR 100). Irradiation is with four 8-W G. E. Germicidal lamps (G8T5).

at 2° by circulating chilled methanol from a Landa Ultra Kryomat K70DW. Annular photolysis tubes are not needed at this wavelength because of the low absorbancy of protein at 315 nm. Samples are irradiated at 315 nm with periodic mixing for a total time of 12 min.

Samples irradiated under nitrogen were degassed by bubbling a stream of nitrogen through the sample for 2 min and stoppering immediately. Tubes are reflushed with nitrogen after removal of aliquots.

Assay of Photolytic Consumption of Binding Sites by Exchange. The basic procedure described by Katzenellenbogen *et al.* (1973a) was followed in these experiments. Duplicate aliquots (75 μl), removed from a photolysis experiment, were placed on a microtiter plate cooled on ice. When all the samples from the irradiation time course were collected, 25 μl of charcoal–dextran slurry was added to each well to adsorb the excess free reagent. The samples were vortexed several times over a 15-min period, and the plates were then covered with adhesive film and centrifuged for 7 min at 1500*g*. Aliquots (75 μl) of the supernatant were transferred to new wells and 37.5 μl of 90 nM [^{3}H]estradiol in TEA buffer ("hot" exchange) or 37.5 μl of 90 nM [^{3}H]estradiol and 9000 nM estradiol in TEA ("hot plus cold" exchange) were added. After incubating for 18–22 hr at 25° to allow complete exchange of labeled and unlabeled ligand, the samples were treated with 25 μl of charcoal–dextran slurry and centrifuged for 7 min at 1500*g*. Aliquots (100 μl) of the supernatant were counted in 5 ml of scintillation fluid. The duplicate values generally differ by less than 10%.

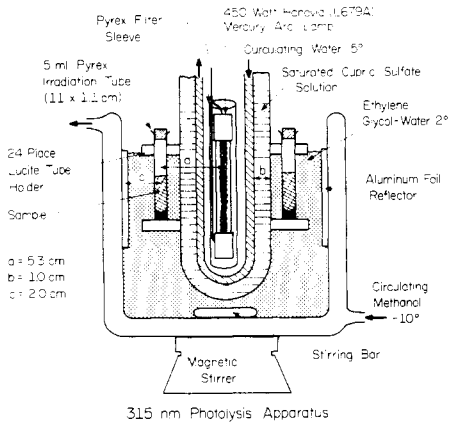


FIGURE 2: Apparatus for photolysis at 315 nm. Samples (up to 5 ml) are placed in Pyrex tubes which are positioned around a 450-W Hanovia (L679A) medium-pressure mercury arc. Cooling (2°) is achieved by circulation of water (inner jacket), methanol (outer jacket), and 1:1 ethylene glycol–water (main bath). A copper(II) sulfate solution filters out irradiation shorter than 315 nm, and an aluminum foil reflector increases the irradiation intensity.

The difference in binding determinations in the "hot" exchange (total binding—high affinity plus nonspecific) and the "hot plus cold" exchange (only nonspecific binding) is taken as the specific high-affinity binding (Katzenellenbogen *et al.*, 1973b). The nonspecific binding generally constitutes less than 20% of the total binding and increases by a few per cent during a 10-min irradiation at 254 nm. Results are generally expressed as specific binding capacity (difference between the averaged duplicate values in the "hot" and "hot plus cold" exchanges). In order to facilitate comparison of data between different experiments, the binding capacities determined after irradiation are expressed as per cent of the original binding capacity.

Exchange at 0°. The rates of exchange of 16-diazoestrone, 3-azidohexestrol, CI-628, and 6-oxoestradiol, at 0°, were determined by the cytosol exchange assay (Katzenellenbogen *et al.*, 1973a).

Determination of Binding Affinity. The binding affinity of the estrogen derivatives not previously reported was determined by a competitive binding assay using charcoal–dextran adsorption, according to the procedure of Katzenellenbogen *et al.* (1973b). The ratio of association constants, RAC ($K_a^{\text{estradiol}}/K_a^{\text{derivative}}$) are determined, and the binding affinities are expressed as RAC × 100 values (see Table I) which can be considered as per cents of estradiol binding affinity.

*4,4′-Dihydroxybenzil (**1**).* 4,4′-Dihydroxybenzil was prepared by Jones oxidation of anisoin, followed by ether cleavage with 49% hydrobromic acid. Purification by preparative thin-layer chromatography and recrystallization from nitroethane gave white crystals: mp 251–252° (lit. 244–246°; Leonard *et al.*, 1949).

*3-Iodohexestrol (**2a**).* Hexestrol (1.08 g, 4 mmol) was dissolved in 15 ml of methanol and 5 ml of concentrated ammonia. A solution of 1.016 g of iodine (4 mmol) in 5 ml of tetrahydrofuran (THF) was added with stirring. The brown iodine color was discharged within a few minutes. Stirring was continued for 30 min, after which time the pH was adjusted to 5 by addition of glacial acetic acid, and the reaction mixture was diluted with 50 ml of water and extracted with three 50-ml portions of ethyl acetate. The extracts were dried ($MgSO_4$) and concentrated to dryness. Analytical thin-layer chromatography indicated five components in addition to starting material.

Separation of the iodination products was carried out by col-

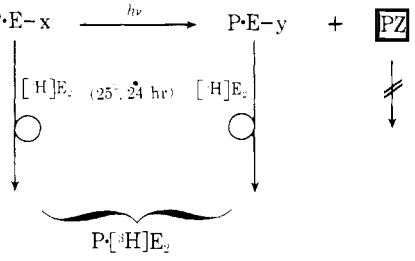
umn chromatography on silica gel, eluting with benzene. Elution order of iodohexestrols was: 3,3′,5,5′-tetraiodohexestrol, 3,3′,5-triiodohexestrol, a mixture of 3,3′- and 3,5-diiodohexestrol, and 3-iodohexestrol. Hexestrol was strongly adsorbed on the column and not easily eluted with benzene. Fractions containing the desired monoiodohexestrol were combined and recrystallized from benzene–hexane and then from methanol–water to give white crystals of 3-iodohexestrol: mp 125–126°; nmr (THF) δ 6.80 (m, 6 H, aromatic), 7.48 (d, 1 H, J = 1 Hz), 7.96 (s, 1 H, phenolic OH), 8.63 (s, 1 H, phenolic OH); mass spectrum (10 eV) m/e (rel intensity) 396 (M^+, 10), 135 (15), 134 (23), 72 (100), and 71 (59). *Anal.* Calcd for $C_{18}H_{21}O_2I$: C, 54.60; H, 5.32; I, 32.05. Found: C, 54.65; H, 5.43; I, 31.87.

6-Oxoestradiol-17β (3). 6-Oxoestradiol-17β was prepared according to the procedure of Longwell and Wintersteiner (1940) by chromium trioxide–acetic acid oxidation of estradiol-17β 3,17-diacetate, followed by hydrolysis; recrystallization from ethanol gave **3** as white plates, mp 281–283° (lit. 281–283°, Longwell and Wintersteiner, 1940; Dean *et al.*, 1971).

Results

Use of the Exchange Assay to Monitor the Reaction of Photosensitive Estrogen Derivatives with the Estrogen Binding Protein. If a sample of estrogen binding protein (P) is treated with a saturating concentration of a photosensitive (nonradiolabeled) estrogen derivative (E-x) and the complex (P·E-x) is irradiated (Scheme II), the ensuing reaction of the intermediate generated by photolysis of the reagent molecules can proceed either (1) by a process that consumes the binding protein in some fashion (generation of PZ), or (2) by a process (presumably reaction with water) that discharges the reagent, leaving the binding protein in an unaltered state (as the new complex P·E-y).

SCHEME II: Photolysis-Exchange Assay.



Although the actual nature of the "consumed" binding protein (PZ) cannot be established without data from experiments utilizing radiolabeled reagents, it is presumably the product of either one of two different reaction processes: the reagent molecule and the binding protein may have become covalently linked together, so that the binding site is permanently blocked, or the binding site may no longer be capable of binding ligand because it has been damaged by some photolytic process, mediated by the reagent molecule, but not resulting in its covalent attachment (see Discussion). In either case, this consumed species can be detected by the fact that it can no longer undergo ligand exchange reactions, and thus the time course of the photoreaction can be conveniently monitored using an exchange assay. As only the complexes with unreacted reagent (P·E-x) or discharged reagent (P·E-y) undergo exchange, the progressive accumulation of the consumed species (PZ) is measured as a diminution of the total exchange capacity. As the binding protein itself is somewhat sensitive to ultraviolet irradiation (particularly at 254 nm), the curve representing the loss of binding capacity during irradiation in the presence of a photoreactive compound is, in practice, compared to the curve obtained when the binding sites are filled with a nonphotoreactive ligand such as estradiol.

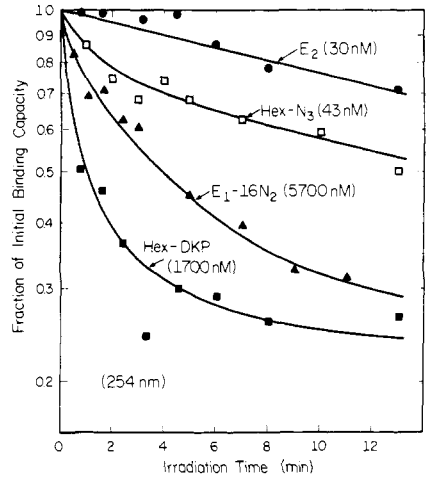


FIGURE 3: Time course of irradiation of some photosensitive estrogen derivatives at 254 nm. The derivatives (E_2-estradiol; Hex-N_3; E_1-16N_2; Hex-DKP ether) were incubated with rat uterine cytosol (2 uterine equiv/ml) at the concentrations shown in parentheses (30/RAC nM; see Methods) and irradiated. The binding capacity of aliquots removed at various times was determined by exchange with [^{3}H]estradiol at 25° for 18–22 hr (see Methods); correction has been made for nonspecific binding. Aliquots contained 0.082 uterine equiv, and initial specific binding capacity constituted 19,000 dpm or 2.15 pmol/uterine equiv.

Photoreactivity of Photosensitive Estrogen Derivatives. Plots of the fraction of initial estradiol binding capacity as a function of irradiation time at 254 nm for three different photosensitive estrogen derivatives are shown in Figure 3. In each case, the concentration of the derivative used is that required to effectively saturate the estrogen binding sites (30/RAC nM, *ca.* 99% saturation; see Methods). Binding sites filled with estradiol are degraded slowly ($t_{1/2}$ = 26 min); this degradation is most likely a direct effect of uv light on the protein, as degradation proceeds at the same rate in the absence of estradiol. However, in each case where binding sites are filled with a photosensitive ligand, the binding capacity is consumed more rapidly than in the presence of estradiol. This is an indication that these derivatives are undergoing some reaction with the estrogen binding protein.

Further evidence for the role played by these particular derivatives comes from the kinetics of the reaction: it is clear from the curvature of the lines on this semilog plot that the reaction is not first order, but rather is biphasic. The initial rapid component of the biphasic reaction corresponds to the rate of photolysis of the photoreactive derivative in aqueous solution (H. N. Myers and H. J. Johnson, Jr., unpublished data); the slower, second reaction roughly parallels the curve for estradiol-filled sites, and presumably represents the rate of photodegradation of the P·E-y complex.

Establishing Photoreaction at the Estrogen Binding Site: Reaction Efficiency. Although data such as shown in Figure 3 indicate that certain estrogen derivatives are photoreactive, they do not rigorously establish that the photolytic reaction is taking place at the estrogen binding site. Particularly in the cases where high concentrations of reagent are needed to achieve saturation, the possibility exists that a portion of the loss in binding capacity is due to an external reaction, *i.e.*, the reaction of a reagent molecule, photoactivated in solution, with the estrogen binding protein, but at a location other than the binding site. Such a reaction, through some allosteric or con-

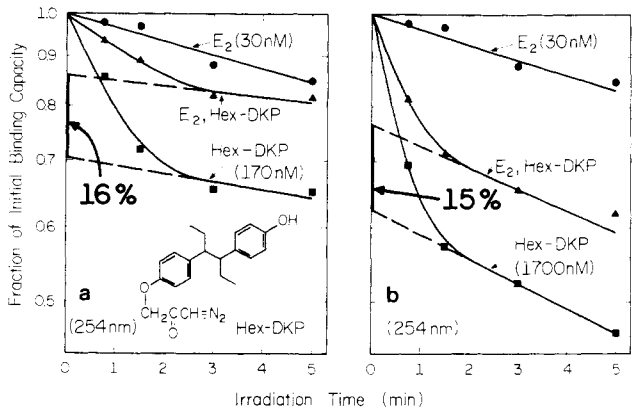


FIGURE 4: Effect of concentration on photoreactivity of hexestrol-diazoketopropyl ether at 254 nm. This compound (■) at two concentrations, (a) 170 nM (0.1 × 30/RAC nM) and (b) 1700 nM (30/RAC nM, see Methods), was irradiated in the presence of rat uterine cytosol at 2 uterine equiv/ml. The irradiation time courses of binding sites filled only with estradiol (30 nM, ●), or protected with estradiol prior to the addition of Hex-DKP ether (▲) are also shown. The reaction efficiency is determined by extrapolation of the linear portion of the lower two curves back to zero time. Binding capacity was determined by exchange with [^{3}H]estradiol and is corrected for nonspecific binding (see Methods). Aliquots contained 0.082 uterine equiv and initial binding capacity was 22,000 dpm or 2.2 pmol/uterine equiv.

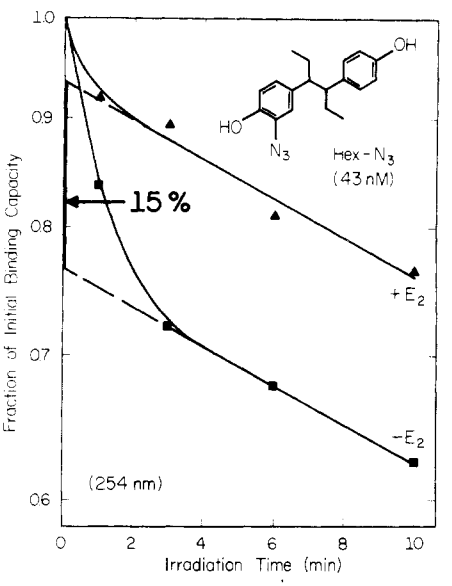
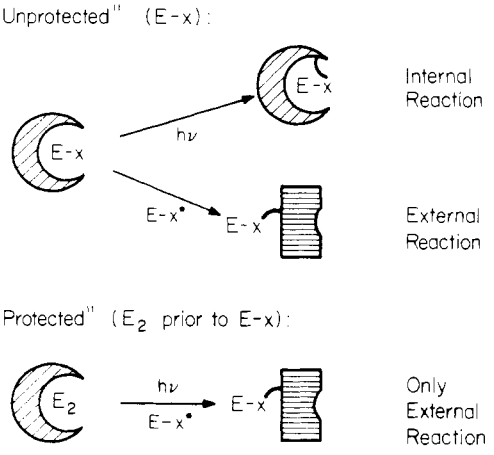


FIGURE 5: Time course of irradiation of 3-azidohexestrol at 254 nm. This compound at 43 nM was irradiated in the presence of rat uterine cytosol (at 2 uterine equiv/ml) with estradiol (30 nM) preincubation (▲) and without estradiol preincubation (■). Determination of binding capacity and reaction efficiency are as in Figure 4.

formational effect, could render the binding protein unable to bind estrogenic ligands (Scheme III).

SCHEME III: Two Types of Covalent Attachment Reactions Producing "Consumed" Estrogen Binding Protein.



The question of apportioning an observed consumption of binding sites between (1) a binding-preceded, binding-site localized reaction (internal reaction) and (2) a random, bimolecular reaction at the surface of the protein (external reaction) can be answered by a competition experiment. This experiment, run in parallel to the reaction with E-x alone, involves photolysis under conditions in which all the estrogen binding sites have first been filled with estradiol (30 nM), prior to the addition of the photosensitive derivative E-x. In the first photolysis (unprotected, E-x alone), both internal and external reactions can take place, while only the external reaction can occur in the second (protected; E_2, prior to E-x). Any difference that is observed in the rate or extent of binding site consumption in these two experiments can thus be classified quite reliably as reactions occurring within the estradiol binding site, as only this internal reaction is subject to protection by prior addition of estradiol. The slowness of the ligand exchange reaction at 0° is used to advantage in such an experiment, as less than 2% of the estradiol-protected binding sites undergo exchange during the time course of the reaction.

Figure 4 shows the irradiation time course at 254 nm of hexestrol-diazoketopropyl (DKP) ether at two different concentrations. In Figure 4a, binding protein exposed to the hexestrol derivative (170 nM or 3/RAC nM; *ca.* 90% saturation) is degraded rapidly (bottom curve); however, when the binding sites are protected by estradiol (middle curve), degradation proceeds at a rate only somewhat faster than when only estradiol is present (top curve). As the diazo ketone is consumed, the rapid initial rate in both lower curves slows until it approximates the rate with estradiol. Extrapolates of the final portion of these curves back to zero time encompass a certain percentage of the initial binding capacity. This is a measure of the estrogen binding sites that have been affected by the reaction of photosensitive reagent molecules within the binding site; expressed as a percentage of the initial binding capacity, it is referred to as the "reaction efficiency" of the particular reagent, which in this case is 16%.

Comparison of the results from an experiment conducted at tenfold higher concentration of hexestrol-DKP ether (Figure 4b; 30/RAC nM, *ca.* 99% saturation) with Figure 4a shows both a similarity and a difference. In each case the reaction efficiency is nearly the same; that is, estradiol is able to protect 15-16% of the estrogen binding sites, regardless of whether a small or a large excess of the photosensitive reagent is provided. This "saturation" effect is the parallel of the "rate saturation" effect predicted for reaction by an affinity labeling mechanism (Baker, 1967; Davidoff *et al.*, 1973). In contrast, the external reaction, which does not depend on prior binding, does not show this saturation effect and is thus much more pronounced at the tenfold higher concentration. (Note downward displacement of both of the hexestrol DKP ether curves in Figure 4b).

Figures 5 and 6 present similar data for 3-azidohexestrol and 16-diazoestrone. Reaction efficiencies on the order of 15-20% are found for these compounds as well, despite great differences in their binding affinities (hence, irradiation concentrations).

Reaction Efficiency Dependence on the Orientation of the Photoreactive Function. A comparison of hexestrol and estradiol azides suggests significant dependence of reaction efficien-

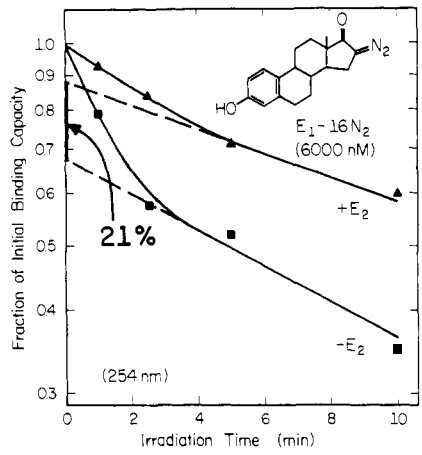


FIGURE 6: Time course of irradiation of 16-diazoestrone at 254 nm. This compound at 6000 nM was irradiated in the presence of rat uterine cytosol, with estradiol (30 nM) preincubation (▲) and without estradiol preincubation (■). Determination of binding capacity and reaction efficiency are as in Figure 4.

cy on the orientation of the reactive function (Table I). Both 3-azidohexestrol and 3,3′-diazidohexestrol have significant reaction efficiencies; however, neither 2- nor 4-azidoestradiol shows any photoreactivity whatsoever.

In our previous analysis of the binding of these derivatives (Katzenellenbogen *et al.*, 1973b), it was suggested that the high affinity of azidohexestrol was a result of the symmetry of the hexestrol ligand, which permitted accommodation of the bulky azide function of one of the azidohexestrol enantiomers in a region of the estrogen binding site complementary to the steroid D-ring. The diazidohexestrol, of necessity, would also present an azide in this region. With both steroidal derivatives, however, the azide functions are positioned without ambiguity, in binding regions complementary to their positions at C-2 and C-4. As only the hexestrol azides are reactive, it appears that the region of the protein that binds the D-ring is considerably more susceptible toward reaction with the species generated upon photolysis of an *o*-azidophenol than the region which binds the A-ring. As the hexestrol diazide has no greater reactivity than the monoazide, it can be presumed that the second azide function, accommodated in the A-ring binding region, makes no contribution to the photoreaction. So, it is not only the nature of the photoreactive function, but its orientation relative to vulnerable regions within the estrogen binding site, that is crucial to the success of photoaffinity labeling.

A similar, though less pronounced difference is noted in the reaction efficiency of the DKP ethers of hexestrol and estradiol (Table I).

Photoreactivity of Chromophoric Derivatives Other than Azides and Diazo Compounds. The photolysis exchange assay for determining the reaction efficiency of photosensitive estrogen derivatives is by no means limited to the simple azides and diazo compounds whose preparation we have described previously (Katzenellenbogen *et al.*, 1973c); actually, the photoreactivity of any compound with estrogen binding activity can be determined. The results of a survey of the azides and diazo compounds together with a variety of different estrogenic ligands are summarized in Table I. A number of features are notable.

Reaction efficiencies of the photoactive azides and diazo compounds determined at 315 nm are comparable to, though somewhat lower than, the 254-nm values. This dependence of the reaction efficiency of diazo compounds on wavelength has previously been noted (Vaughan, 1970; Brunswick and Cooperman, 1971).

TABLE I: Reaction Efficiencies of Photosensitive Estrogen Derivatives.

Compound	Reaction Efficiency (%)[a] 254 nm	315 nm	Binding Affinity (RAC × 100)
Diazo compounds			
Hexestrol diazoketopropyl ether	16, 15	15	1.8
Estradiol 3-diazoketopropyl ether	5	0	1.4
Estradiol 17-diazoacetate	10		1.6
16-Diazoestrone	21	12	0.5
Azides			
3-Azidohexestrol	15	9	69
3,3′-Diazidohexestrol	16, 16	12	12
2-Azidoestradiol	0		3
4-Azidoestradiol	0, 0		0.9
Triarylethylenes			
U 11,100A	11	28	5.9
CI-628	28	51, 50, 47 (37[b])	3.8
CI-680	16, 22	26, 25	33.9
9411X27	10, 17 (16[c])	3, 0 (15[c])	222
Miscellaneous			
4,4′-Dihydroxybenzil (**1**)	0		0.6
3-Iodohexestrol (**2a**)	0	0	37
3-Nitrohexestrol (**2b**)		0	15
Dienestrol	0	5	36
16-Oximoestrone		0	4.9
Estrone	0		12
Δ^6-Estradiol	0	0	21
Diethylstilbestrol	7		300

[a] Duplicated values are determinations from separate experiments. [b] The last value (37), determined under nitrogen atmosphere. [c] The last value (15), determined at 7 × 30/RAC nM, that is at the same concentration as compound CI-680.

Most members of the triarylethylene class have high reaction efficiency at 315 nm. The nitro-substituted chromophore in the Parke-Davis antiestrogen CI-628 is most effective, and its time course of photoreaction at 315 nm is shown in Figure 7. This figure also illustrates the great stability of estradiol-filled and empty sites toward irradiation at this wavelength (see Figure 7 legend).

The divergent photoreactivities of the antiestrogens CI-680 and 9411X27, most evident at 315 nm, is curious. They differ only in the nature of the terminal substituent, and the more reactive one (CI-680; methoxy) is actually bound only one-seventh as well as the other (9411X27; hydroxy). An interesting parallel to these differences in photoreactivity is found in the photoreduction of para,para′-disubstituted benzophenones in 2-propanol: benzophenone and *p,p′*-dimethoxybenzophenone are reduced with a quantum efficiency of 1.0; *p,p′*-dihydroxybenzophenone, at an efficiency of only 0.02 (Porter and Suppan, 1964). The explanation advanced in the case of the benzophenone derivatives involved deactivation of the excited electronic state of the hydroxy-substituted member through ionization (Porter and Suppan, 1964). A similar mechanism may be responsible for the low reactivity of 9411X27 compared to CI-680.

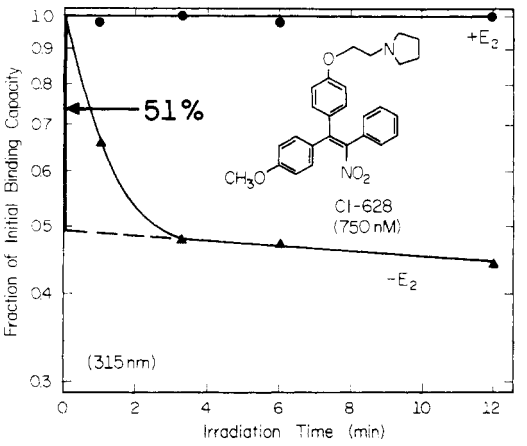


FIGURE 7: Time course of irradiation of Parke-Davis antiestrogen CI-628 at 315 nm. This compound at 750 nM was irradiated in the presence of rat uterine cytosol, both with estradiol (30 nM) preincubation (●) and without estradiol preincubation (▲). Determination of binding capacity and reaction efficiency are as in Figure 4. Sites irradiated in the absence of either derivative (empty sites) gave data superimposable with the protected line (●).

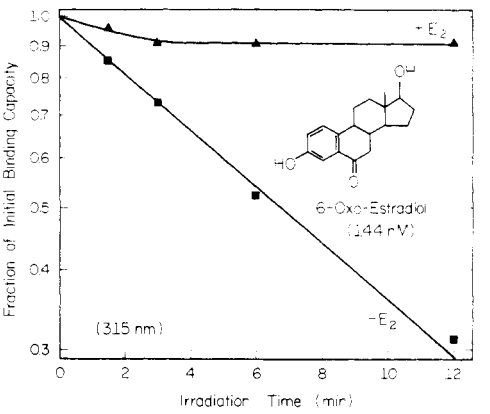


FIGURE 8: Time course of irradiation of 6-oxoestradiol (**3**) at 315 nm. This compound at 144 nM was irradiated in the presence of rat uterine cytosol, both with estradiol (30 nM) preincubation (▲) and without estradiol preincubation (■). Determination of binding capacity is as in Figure 4.

Several ligands bearing chromophores with fairly well-established reactivity in solution photochemistry are completely unreactive toward the estrogen binding protein: 4,4′-dihydroxybenzil (**1**) (α-dione chromophore, Bunbury and Wang, 1968; Rubin, 1969; Munroe, 1971), diethylstilbestrol (stilbene chromophore), estrone (cyclopentanone chromophore, Butenandt and Poschmann, 1944), iodohexestrol (**2a**) (*o*-iodophenol, Wolf and Kharasch, 1961), nitrohexestrol (**2b**) (nitrobenzene, Letsinger and McCain, 1969), dienestrol and Δ^6-estradiol (styrene, Fujita *et al.*, 1971; Kropp, 1969; Cookson *et al.*, 1969), and 16-oximoestrone (α-oximo ketone, Stojiljkovic and Tasovac, 1970).

Photoreactivity of 6-Oxoestradiol; Two Modes of Photoreactive Behavior: Photoexcitable and Photolabile. The irradiation time course of 6-oxoestradiol (**3**) is shown in Figure 8. The apparent first-order rate of reaction displayed by this compound stands in marked contrast to that of all the other compounds we have studied. If one considers the probable nature of the photoreactive species in these cases, however, this difference is not surprising (Scheme IV).

SCHEME IV: Kinetic Models for the Two Modes of Photoreactivity.

photolabile reagent (biphasic)

$$P\cdot E{-}x \xrightarrow{h\nu} [P\cdot E{-}x^*] \longrightarrow PZ + P\cdot E{-}y$$

photoexcitable reagent (first order)

$$P\cdot E{-}x \rightleftharpoons [P\cdot E{-}x^*] \longrightarrow PZ$$

Photoactivation of the azide and diazo derivatives is irreversible; nitrogen is lost, and the reactive species thus generated has only a single opportunity to react with amino acid residues in the binding site. If reaction fails to occur, the reagent is discharged and is degraded to a form that is no longer photoreactive (E-y). This type of photoreactivity can be termed "photolabile," and reagents in this class should exhibit biphasic kinetics.

Photoactivation of the oxoestradiol involves, most likely, conversion to an electronically excited state. This species has an opportunity to react, but if it is discharged without reacting, it can return to the ground state and thus has the capacity to become photoactivated once again. This mode of photoreactivity can be called "photoexcitable," and the reaction rate by this mode should follow first-order kinetics.

Discussion

In affinity labeling experiments with enzymes, preliminary experiments, usually done with nonradioactive labeling reagents, utilize the maximum velocity (v_{max}) of the enzymatic activity as a measure of the extent of active site modification; the fraction of the initial activity, determined after a period of exposure to a candidate labeling compound, is taken as a measure of the active sites that have survived without impairment of their catalytic activity. This determination, when made at different reagent concentrations and coupled with suitable competition experiments, can establish that reaction is occurring at the catalytic site by an affinity labeling mechanism (Baker, 1967; Davidoff *et al.*, 1973).

Although a binding protein such as the rat uterine estrogen binding protein has no catalytic activity, the parameter analogous to v_{max} is binding capacity; so measurement of the effect of a labeling reagent on the binding capacity should give information analogous to that from rate studies on enzymes. A problem peculiar to the estrogen binding protein arises because of its high binding affinity: the rate of ligand dissociation is so slow, that the exchange of a reactive (unlabeled) ligand by a radiolabeled ligand (for binding capacity determinations) takes several hours at 25° (Katzenellenbogen *et al.*, 1973a). In enzymatic systems exchange times are generally on the order of a few milliseconds. We have previously reported a method of assaying estrogen binding sites in rat uterine cytosol by exchange (Katzenellenbogen *et al.*, 1973a). In this report we show how this assay can be applied to determine the reactivity of photosensitive estrogen derivatives so as to assess their promise as photoaffinity labeling reagents for the estrogen binding protein of rat uterus.

Advantages and Problems Associated with the Use of the Exchange Assay to Measure Photoattachment. In addition to the obvious advantage that a wide variety of nonradiolabeled compounds can be surveyed conveniently, the exchange assay permits a separation of the questions of labeling selectivity and labeling efficiency: only nonradiolabeled compounds are used during the phase when covalent attachment is occurring; so any attachment that may be taking place to the many other proteins present in the rat uterine cytosol preparation is not detected; only those reactions that have affected the integrity of the estrogen binding site are determined in the exchange-binding measurements.

In spite of this significant advantage, a drawback of the exchange assay is that it does not distinguish a decrease in bind-

ing capacity due to a covalent attachment of a reagent molecule in the binding site, from the destruction of the binding activity through some photolytic process, mediated by the reagent molecule in the binding site, but not resulting in its covalent attachment. Therefore, the "reaction efficiency" determined for each derivative by this assay procedure does not necessarily correspond to an "attachment efficiency," although it does represent a valid upper limit to the attachment efficiency. However, indications of particularly reactive functions as well as unreactive ones, and orientational differences in reactivity can be obtained rapidly and conveniently by this indirect approach; so the photolysis-exchange assay of photoreactivity is an important means of screening compounds for further study.

One conceivable process that could result in a binding site reaction without covalent attachment is a photosensitized oxidation. Binding-site localized oxidations of this type are known to occur with certain dyes (Rippa and Pontremoli, 1969; Gennari *et al.*, 1970; Sawada, 1969), and it is possible that some of the reagents could be acting as sensitizers. However, we have found that when irradiation of the two most reactive derivatives, CI-628 and 6-oxoestradiol, at 315 nm is conducted under nitrogen, only small changes in photoreactivity are observed: the reaction efficiency of CI-628 decreases from 51 to 37% and the reaction rate of 6-oxoestradiol actually increases slightly.

Recently Ruoho *et al.* (1973) have encountered a number of pitfalls, in the course of photoaffinity labeling the acetylcholine esterase in intact human erythrocyte membranes. Two of these pitfalls must be considered in our system as well.

The first potential misinterpretation can arise when one is attempting to protect a binding site from labeling by filling it with an unreactive, protector ligand. The protecting ligand itself may be acting as a scavenger, so that what appears to be a decrease in the extent of labeling due to protection of the binding site may actually be suppression of a nonselective, external labeling process by scavenging.

It is very unlikely that significant scavenging by the protector is taking place in our system, because in all experiments the concentration of protector (estradiol, 30 nM) is lower, and generally much lower, than the concentration of labeling reagent (43–6000 nM). Effective protection can be attained with low estradiol concentrations because of the high affinity and slow exchange rate of estradiol. Furthermore, in two instances (CI-628 and 6-oxoestradiol) we have compared the rate of binding site consumption in the experiments: E-x alone and E_2 prior to E-x, with that of E-x prior to E_2. In the last case, the binding site is occupied with E-x and estradiol is present only externally, so that if estradiol does scavenge the reactive intermediate involved in the external reaction, it should do so in this case as well. However, no difference in rate or quantity of binding site consumption was noted between this experiment (E-x, prior to E_2) and that with E-x alone.

The second potential problem was termed "pseudo photoaffinity labeling." In this case labeling of the binding site which can be protected and appears to result from the photolysis of a reagent molecule in the binding site is actually just a conventional labeling process in which a reactive intermediate, generated in solution, diffuses into the binding site and reacts. This pseudo photoaffinity labeling can be scavenged, while true photoaffinity labeling cannot.

Again, our system does not appear to suffer from this artifact. The high binding affinity of all the photosensitive derivatives ($K_d = 7 \times 10^{-9}$–5×10^{-7} M) has the result that the exchange at 0° is extremely slow. This has been verified experimentally with several of the derivatives (6-oxoestradiol, CI-628, 16-diazoestrone, and 3-azidohexestrol show less than 5% exchange within 1 hr at 0°). Since the concentration of reagent in each photolysis experiment is adjusted to achieve saturation of the binding sites, it is highly unlikely that significant reaction could be occurring through the interaction of an externally activated reagent molecule with these filled sites.

Reactivity of Photosensitive Estrogen Derivatives. The reaction efficiencies of the photolabile estrogen derivatives we have prepared span the range 0–51%, with most of the reactive ones falling between 15 and 30%. These values are quite comparable to attachment efficiencies which have been reported for photoaffinity labeling studies in other systems. For example, studies on the NAD binding site of yeast alcohol dehydrogenase using diazo (Browne *et al.*, 1971) and azide derivatives ($K_d \simeq 10^{-4}$ M, Hixson and Hixson, 1973) gave attachment efficiencies of 5–10%. Higher efficiency (35%) was attained in labeling the cAMP binding site in phosphofructokinase using a diazomalonyl-cAMP derivative ($K_d \simeq 10^{-5}$ M; Brunswick and Cooperman, 1971). Still higher efficiencies (65–90%) have been noted in antibody labeling sites, both with diazo derivatives ($K_d \simeq 10^{-5}$–10^{-4} M; Converse and Richards, 1969; Rosenstein and Richards, 1972) and azides ($K_d \simeq 10^{-7}$; Fleet *et al.*, 1972).

Although in most of the literature examples, there seems to be a correspondence between attachment efficiency and binding affinity, there is no clear correlation between reaction efficiency and binding affinity among the reagents we have prepared. The most deviant example is the lowest affinity derivative 16-diazoestrone (RAC × 100 = 0.5) which actually reacts with higher efficiency (21%) than the most strongly bound azidohexestrol (RAC × 100 = 69; efficiency = 16%). Further, it is evident that reaction efficiency is not just a function of the nature of the photoreactive group or the binding affinity of the reagent; the dependence of reaction efficiency of both the azides and the diazoketopropyl ethers on the ligand structure (hexestrol *vs.* estradiol) establishes that orientational differences can be crucial as well. Thus, the tenet, that the high, indiscriminate reactivity of the intermediate generated upon irradiation of a photosensitive ligand should assure efficient covalent attachment, must be qualified (Singer, 1967; Shaw, 1970); the reaction of photoaffinity labeling reagents may well be bound by the same sort of geometrical requirements which constrain conventional affinity labeling reagents, whose reaction is based on alkylation or acylation.

The only example of a labeling reagent of the photoexcitable type that has been fully described in the literature is 3-oxo-4-estran-17β-yl acetate (Martyr and Benisek, 1973). Essentially complete inactivation with first-order kinetics of the steroid isomerase enzyme (*Pseudomonas testosteroni;* EC 5.3.3.1) could be achieved with this reagent, although the attachment efficiency determined directly by incorporation of radioactivity was only 50%.

Comparison of the Different Modes of Photoreaction: Photolabile and Photoexcitable. A major criteria for evaluating the success of an affinity labeling process, particularly in an impure preparation such as rat uterine cytosol, is its selectivity; that is, the extent to which label is introduced at the desired binding locus without labeling other proteins. In the most simple terms, selectivity will depend on two factors, the binding affinity that the labeling reagent displays for the site to be labeled and the rate or efficiency with which it is able to react at that site (Katzenellenbogen *et al.*, 1973b).

Laying aside for the moment considerations of relative binding affinities, one can raise the question of which reagent type, photolabile or photoexcitable, should be capable of a more selective labeling. A number of factors come into play in a com-

parison of these two reactivity modes. First, reagents of the photoexcitable type, because of their opportunity for repetitive reaction, should in principle be capable of completely labeling all of the estrogen binding sites, compared to a photolabile reagent where labeling is limited by the reaction efficiency. On the other hand, if only a small fraction of the photoexcited intermediate undergoes reaction during each photoactivation cycle, then a larger and potentially more damaging dose of irradiation would be required to achieve a reasonable degree of labeling. In such a situation, a very photosensitive reagent of the photolabile type with only a modest attachment efficiency might prove superior.

In certain systems it has been possible to enhance the efficiency of a photolabile reagent by periodic or continuous reloading of unlabeled sites with fresh labeling reagent (Cooperman and Brunswick, 1973; Rosenstein and Richards, 1972). However, this approach to increasing labeling efficiency is applicable only to those systems in which the rate of ligand exchange is rapid.

Conclusion

The photolysis-exchange assay provides a rapid and convenient means of establishing to what degree a photosensitive estrogen ligand is capable of photoreaction with the estrogen binding site; thus, it can be used to survey a wide variety of potentially interesting compounds. The kinetics of the photoinactivation process gives some idea of how the reaction is proceeding and permits classification of the reagents as either photolabile or photoexcitable. Reaction efficiencies give at least an upper limit estimate of the extent of labeling that is taking place, and, coupled with orientational effects which indicate the vulnerable regions of the binding site, provide valuable information for the selection and design of effective photoaffinity labeling reagents.

Acknowledgment

We are grateful to Dr. H. DeWald, Parke-Davis (CI-628, CI-680, 9411X27), and Dr. A. Nathan, Upjohn (U 11,100A) for providing us with samples of the antiestrogens and to Mr. H. M. Hsiung and Mr. B. S. Schwartz for the preparation of compounds **2a** and **1**, respectively.

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