Ultraviolet Photosensitivity of the Estrogen Binding Protein from Rat Uterus. Wavelength and Ligand Dependence. Photocovalent Attachment of Estrogens to Protein†

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ABSTRACT: Ultraviolet irradiation of the estrogen binding protein in rat uterine cytosol results in a progressive photoinactivation which is rapid at 254 nm and slower at >315 nm. Both unfilled and estradiol-filled sites are inactivated at approximately the same rates at 254 nm ($t_{1/2}$ = 8 min and 11 min, respectively), but at 315 nm, empty sites are consumed much more rapidly ($t_{1/2}$ = 3.4 hr) than filled ones ($t_{1/2}$ = 24 hr). The protective effect of the estrogen ligand at this wavelength appears to depend on its binding to the estrogen-specific binding site, as inactivation rate studies at different concentrations of estrone, estradiol, and estriol show a good correlation between the extent of protection and the fractional saturation of the high affinity estrogen binding sites. Scatchard analysis indicates that inactivation is the result of a loss of binding sites and not a decrease in their affinity, and sedimentation analysis shows that increased heterogeneity and aggregation of the estrogen binding species accompanies the photoinactivation process. Photoinactivation appears to be the result of direct irradiative damage of the amino acid residues, as the inactivation rate is the same under air and nitrogen atmospheres, and is unaffected by nucleophiles, reductants, and radical scavengers. When photoinactivation is measured by irradiation of cytosol containing [^{3}H]estradiol, a concurrent photocovalent attachment process is noted; the steroid becomes linked to protein in a solvent-inextractable manner (boiling ethanol inextractable). This attachment, however, does not appear to be related to the steroid binding at the estrogen binding site. Its rate is affected by reductants and scavengers. A similar photocovalent attachment reaction occurs when bovine serum albumin or ovalbumin is irradiated in the presence of [^{3}H]estradiol or [^{3}H]diethylstilbestrol. The detailed reactions involved in this photocovalent attachment process have not been defined at present.

One approach to elucidating the details of the molecular interaction between estrogens and the receptor components of target tissue cells involves the use of chemically reactive analogs of estradiol (Katzenellenbogen, 1974). Those derivatives having a suitable combination of binding affinity and chemical reactivity should act as affinity labeling reagents and be capable of covalently labeling the binding sites of the estrogen binding macromolecules. We have been interested in assessing the potential of photosensitive estrogen derivatives as photoaffinity labeling reagents for the cytoplasmic estrogen binding protein from rat uterus. The greater flexibility inherent in the control of the chemical reactivity of these light-sensitive species (light vs. dark) appeared to offer certain distinct advantages in studying this binding protein in unpurified uterine cytosol preparations and in elucidating the dynamical features displayed during its interaction with estradiol in the target cell (Katzenellenbogen, 1974). Earlier publications by us (Katzenellenbogen et al., 1974, 1973a–c) have described the preparation of several photosensitive diazo and azide derivatives of estradiol, estrone, and hexestrol, the determination of the binding affinity of these derivatives for the estrogen binding protein from rat uterine cytosol, and the measurement of the efficiency with which these derivatives react with the binding protein under irradiation, as determined by inactivation of binding capacity.

For a photoaffinity labeling reagent to be efficient in its reaction with a binding protein, it is a minimum requirement that the rate of photoactivation of the reagent must be fast relative to the rate of photoinactivation of the binding protein. In our earlier work, we had established that all the azide and diazo estrogen derivatives which we had prepared were highly photosensitive and were degraded by irradiation at 254 nm or >315 nm at a rate far greater than the photodegradation of estrogen binding activity (Katzenellenbogen et al., 1974). However, the extension of our investigations on estrogen photoaffinity labeling reagents to other less photoreactive derivatives (e.g., aryl iodides, ketones, and sulfonyl azides) has necessitated a quantitative study of the photosensitivity of the estrogen binding protein.

In this report, we describe an investigation of the rate of photoinactivation of the estrogen binding protein from rat uterus, and the dependence of this rate on wavelength of irradiation, the nature and concentration of ligands, radical, and electrophile scavengers, and incubation atmosphere. A peculiar nonspecific photoinduced covalent attachment reaction of estrogens to proteins is also described.

Experimental Section

Materials. The following compounds[1] were obtained from

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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; estriol (E_3), 1,3,5(10)-estratriene-3,16α,17β-triol; diethylstilbestrol (DES), 3,4-bis(4′-hydroxyphenyl)-*trans*-3-hexene; BHT, 2,6-di-*tert*-butyl-*p*-cresol.

the sources indicated: 17β-estradiol, estrone, estriol (Searle, Steraloids); [6,7-^{3}H]-17β-estradiol, estrone, and estriol (40–48 Ci/mmol, New England Nuclear); [^{3}H]-diethylstilbestrol (6.2 Ci/mmol, Amersham-Searle); phenol (Mallinckrodt); 2,6-di-*tert*-butyl-*p*-cresol (Eastman); Methylene Blue and Rose Bengal (Fisher); *p*-aminobenzoic acid, mercaptoethanol, and Acridine Orange (Matheson Coleman and Bell); hydroxylapatite HT (Bio-Rad) or Type I (Sigma); charcoal, Norit A (Sigma); dextran, grade C (Schwarz/Mann); Triton X-114 (Rohm and Haas); dimethylformamide (Baker); ethylenediaminetetraacetic acid, EDTA (Eastman); tris(hydroxymethyl)aminomethane, Tris (Nutritional Biochemicals); microtiter plates (Scientific Products); bovine serum albumin, recrystallized and lyophilized (Sigma); Sephadex G-25, fine and Blue Dextran (Pharmacia); acrylamide (Matheson Coleman and Bell); *N,N'*-diallyltartardiamide (Eastman); periodic acid (G. F. Smith); urea (Matheson Coleman and Bell; recrystallized from 95% ethanol).

Methods. The buffers used in these studies were 0.01 *M* Tris-HCl, 0.0015 *M* EDTA, and 0.02% sodium azide (pH 7.4) at 25° (TEA buffer) and 0.04 *M* Tris-HCl (pH 7.14) at 0° (Tris buffer). 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.

Unless noted otherwise, 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 glass (Demuth Glass) or plastic (Research Products) minivials containing 5 ml of scintillation fluid in a Nuclear Chicago Isocap 300 instrument (43–48% tritium efficiency).

Preparation and Storage of Cytosol. Cytosol was prepared from immature Holtzman rats (21–25 day females). Rats were decapitated and their uteri were excised and cleaned of adhering fat. The rat uteri were homogenized in TEA buffer at 0° at 5–10 uteri/ml using Duall all-glass grinders (Kontes). The homogenate was diluted to a concentration of 1–3 uterine equiv/ml with TEA buffer and centrifuged at 226,000*g* for 1 hr in a Beckman Model L ultracentrifuge to obtain the cytosol fraction. In most cases, the cytosol was used immediately; however, cytosol stored by freezing in 25% glycerol (Katzenellenbogen et al., 1974) was used occasionally, with identical results.

Photolyses at 254 and >315 nm were conducted at 0–3° in apparatuses previously described (Katzenellenbogen et al., 1974). Studies at 254 nm utilized 12 8-W GE germicidal lamps in a Rayonet photochemical reactor (Model RPR 100), with quartz annular photolysis tubes. At >315 nm, a 450-W mercury vapor lamp (Hanovia L 679A) jacketed with a copper sulfate solution filter and Pyrex sample tubes were used. In control irradiations, thermocouple measurements established that the sample temperature remains at 0–3° during the course of the photolysis.

Although the short-term stability of the 450-W lamp is good, its output does decrease with use, and the difference in rates of photoinactivation noted between Figures 2 and 4 is the result of using different lamps of different age.

Binding Assays. Charcoal–Dextran Adsorption. Duplicate incubations (50–150 μl) with 30 n*M* [^{3}H]E_2 (total binding) and 30 n*M* [^{3}H]E_2 plus 3000 n*M* E_2 (nonspecific binding) were prepared on microtiter plates; after an equilibration period (1–3 hr, 0°) free [^{3}H]E_2 was removed using 10–30% (v/v) of charcoal–dextran slurry, a 15-min adsorption time, and an 800*g* for 7 min centrifugation period (Katzenellenbogen et al., 1973a). The method of correcting for low affinity (nonspecific) binding by running parallel binding assays in the presence of 100-fold excess unlabeled ligand has been explained (Katzenellenbogen et al., 1973a).

Hydroxylapatite Adsorption. Aliquots (0.4 ml) of the cytosol were assayed in triplicate using the hydroxylapatite method (Erdos et al., 1970) as modified by Ruh and Ruh (1974). The hydroxylapatite was washed by centrifugation five times with Tris buffer, and then diluted to a slurry (1 volume of packed hydroxylapatite to 3 volumes of Tris buffer). The hydroxylapatite slurry (0.5 ml) was added to each tube and mixed intermittently for 30 min at 0° to adsorb the [^{3}H]estrogen–receptor complex. The pellet was then washed four times by adding 3 ml of Tris buffer, centrifuging at 500*g* for 5 min, and decanting the supernatant fraction. The [^{3}H]estrogen complex was extracted from the hydroxylapatite pellet with 3 ml of ethanol. The extract was added to 10 ml of scintillation fluid (0.5% 2,5-diphenyloxazole and 0.03% 1,4-bis[2-(5-phenyloxazolyl)]benzene in toluene). The radioactivity was determined in a Packard scintillation spectrometer with 24% efficiency.

Sephadex G-25 Columns. Binding was measured by chromatography on Sephadex G-25 fine micro columns (Ruh et al., 1973), patterned after Puca et al. (1971). Columns were prepared in Pasteur pipets and samples (150-μl incubation, 0.1% Blue Dextran) were eluted with TEA buffer; the Blue Dextran region was collected and counted.

Assay by Exchange. In those cases where the estrogen binding sites were filled with unlabeled estradiol prior to assay, estrogen-specific binding was assayed by the cytosol exchange procedure described by Katzenellenbogen et al. (1973a), using charcoal–dextran adsorption.

Scatchard and Sedimentation Analysis of Partially Photoinactivated Cytosol. Empty cytosol was irradiated at 254 nm for 0, 5, and 15 min. The estrogen specific binding capacity (picomole/uterine equivalent), determined by charcoal–dextran adsorption at 30 n*M* [^{3}H]E_2 and 30 n*M* [^{3}H]E_2 plus 3000 n*M* E_2, was: 0 min, 1.52 (100%); 5 min, 1.15 (76%); and 15 min, 0.66 (44%).

The estrogen specific binding affinity was determined by making a series of [^{3}H]E_2 binding measurements over a concentration range of 0.1–300 n*M*, by charcoal–dextran adsorption, after incubation at 0° for 18 hr. Scatchard plots (Scatchard, 1949) based on total binding showed evidence of a low affinity binding component (bound to free ratio reaches a minimum value (b/f) at high values of bound). The total binding levels were corrected for the contribution of the low affinity system, using the expression below, and the plot was reconstructed in terms of high affinity binding. (The b/f values were in the range 0.004–0.007.)

$$\text{bound}_{\text{high affinity}} = \text{bound}_{\text{total}} - (\text{b/f})\text{free}$$

By this method the following values were determined for the equilibrium dissociation constants (K_d in n*M*) and binding site concentration (n*M*), respectively: 0 min, 0.23, 0.40 (100%); 5 min, 0.23, 0.30 (75%); and 15 min, 0.28, 0.17 (43%).

Sucrose gradient sedimentation analysis was performed as previously described (Katzenellenbogen et al. 1973a). The same samples of irradiated cytosol were saturated with 30 n*M* [^{3}H]E_2 (0°, 2 hr), treated with charcoal–dextran, mixed with [^{14}C]bovine serum albumin (prepared according to the procedure of Rice and Means (1971)), layered onto linear 5–20% sucrose density gradients (4 ml, prepared in polyallomer tubes in TEA buffer), and centrifuged at

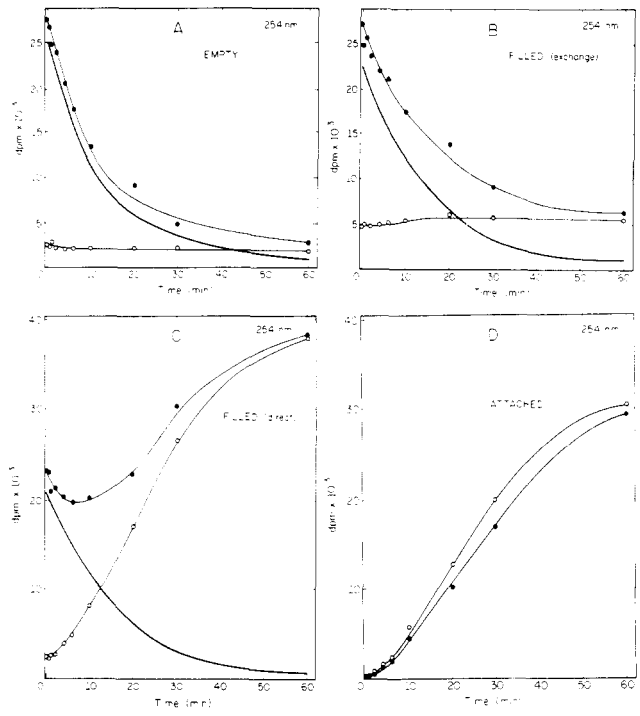

FIGURE 1: Photoinactivation (A–C) and photocovalent attachment (D) of rat uterine cytosol at 254 nm. Tubes containing empty cytosol (A), cytosol containing 30 n*M* E_2 (B), and 30 n*M* [^{3}H]E_2 and 30 n*M* [^{3}H]E_2 plus 3000 n*M* E_2 (C and D) were irradiated at 254 nm. Aliquots removed at the indicated times were assayed for estrogen binding by the charcoal-dextran technique (A and C; B by exchange) and for photocovalent attachment (D) by precipitation with chilled ethanol. Total binding or attachment (●) and low affinity binding or attachment (○) are noted with light curves; the heavy curve (specific binding) is the difference between the two light curves and represents estrogen-specific binding.

45,000 rpm (200,000*g*) in a Beckman SW 56 Ti rotor for 16 hr. Six-drop fractions were collected using the ISCO gradient fractionator.

Assay of Covalent Attachment. The photocovalent attachment of [^{3}H]E_2 or [^{3}H]DES was monitored by two assays. Aliquots (75 μl) were spotted onto numbered 2.4-cm disks of Whatman 3 filter paper, suspended on pins. After 1–3 min, the disks were placed in a beaker of boiling 95% ethanol (10 ml/disk) for 15 min. Following additional extractions (1 × boiling ethanol, 15 min; 2 × 1:1 ethanol–ether, 25°, 2 × ether, 25°), the disks were dried and counted in 5 ml of scintillation fluid.

Alternatively, aliquots were added to 200 μl of a 15 mg/ml solution of bovine serum albumin in water and precipitated by the addition of 3 ml of chilled (−20°) absolute ethanol. After 30 min at −20°, the precipitate was collected by centrifugation (800*g* for 10 min), and the supernatant was carefully removed by aspiration. The ethanol washing was repeated three times, and the final pellet was dissolved in 400 μl of distilled water and counted.

Polyacrylamide Gel Electrophoresis. The electrophoresis procedure was modified from Ornstein (1964) and Davis (1964) using the equivalent molar concentrations of *N,N'*-diallyltartardiamide in place of methylenebisacrylamide to cross-link the separation gel. Polyacrylamide gels (7.5%) containing 8 *M* urea were used, and gel slices were dissolved in 0.5 ml of 2% periodic acid (0.5 hr, 25°) prior to counting.

Results

Photoinactivation at 254 and >315 nm: Relative Rates and Ligand Dependence. Ultraviolet irradiation of cytosol prepared from immature rat uterus results in the inactivation of the estrogen binding capacity. The inactivation rate at 254 nm is quite rapid, as is illustrated in Figure 1. Figure 1A shows how the estrogen binding capacity of a cytosol preparation, irradiated at 254 nm in the absence of ligand (empty), undergoes degradation: the low affinity (nonspecific) binding sites are unaffected by the light, but the specific sites are degraded in a first-order process with a $t_{1/2}$ = ~8 min (Figure 3A). A very similar inactivation rate ($t_{1/2}$ = ~11 min; cf. Figure 3A) is observed when estrogen binding protein, filled with unlabeled estradiol, is irradiated, and binding capacity of aliquots determined by subsequent exchange with [^{3}H]estradiol (Figure 1B). Again the low affinity sites are unaffected.

In a parallel experiment (Figure 1C) in which the estrogen binding sites are filled with [^{3}H]estradiol (at 30 n*M* for measurement of total binding and 30 n*M* plus 3000 n*M* E_2 for low affinity binding), the rate of loss of estrogen-specific binding is nearly the same. However, there is a striking increase in the binding measured at the high estrogen concentration (considered nonspecific binding). It is apparent that the major portion of this increase is due to a covalent attachment process: assay of these same aliquots by ethanol precipitation revealed (Figure 1D) that irradiation produced an increase in radioactivity that was not solvent extractable, in an amount which nearly equalled the increase seen in the low affinity binding in Figure 1C. Of particular importance was the observation that in both the total and low affinity binding incubations, the time course and quantity of covalent attachment of radioactive material were nearly the same, indicating that this reaction is not restricted to the estrogen-specific binding sites (see also Discussion).

While photoinactivation at 254 nm is relatively fast, irradiation damage at >315 nm is relatively slow (see Figure 2). In addition, at this wavelength a pronounced effect of ligand binding on the inactivation rate is observed. Empty sites are degraded in a first-order process with a $t_{1/2}$ = 3.4 hr (Figure 3B). (Thermal lability of empty binding sites in long term incubations at 0° (Katzenellenbogen et al., 1973a) requires that binding levels be related to a dark control.) Filled sites, however, measured either by exchange (Figure 2B) or directly (Figure 2C), are 6–8 times more stable (cf. Figure 3B). Again, a nonspecific, covalent attachment is noted in the experiment in which binding protein is filled with labeled estradiol and irradiated (Figure 2D).

Quantitative Aspects of the Dependence of the >315 nm Inactivation Rate on Ligand. It is apparent that the presence of an estrogenic ligand in the irradiation incubations at >315 nm has an influence on the inactivation rate, and it is tempting to conclude that this is due to a "stabilization" of the binding protein which results from binding of a ligand at the estrogen binding site. (Ligand-induced stabilization against thermal degradation is well known (Katzenellenbogen et al., 1973a).) However, it is also possible that free estradiol, as a phenol, might slow the inactivation processes by scavenging radical species generated in the proteins by photolytic events. In order to distinguish between these two possibilities, the rates of inactivation at >315 nm were measured in the presence of varying concentrations of three es-

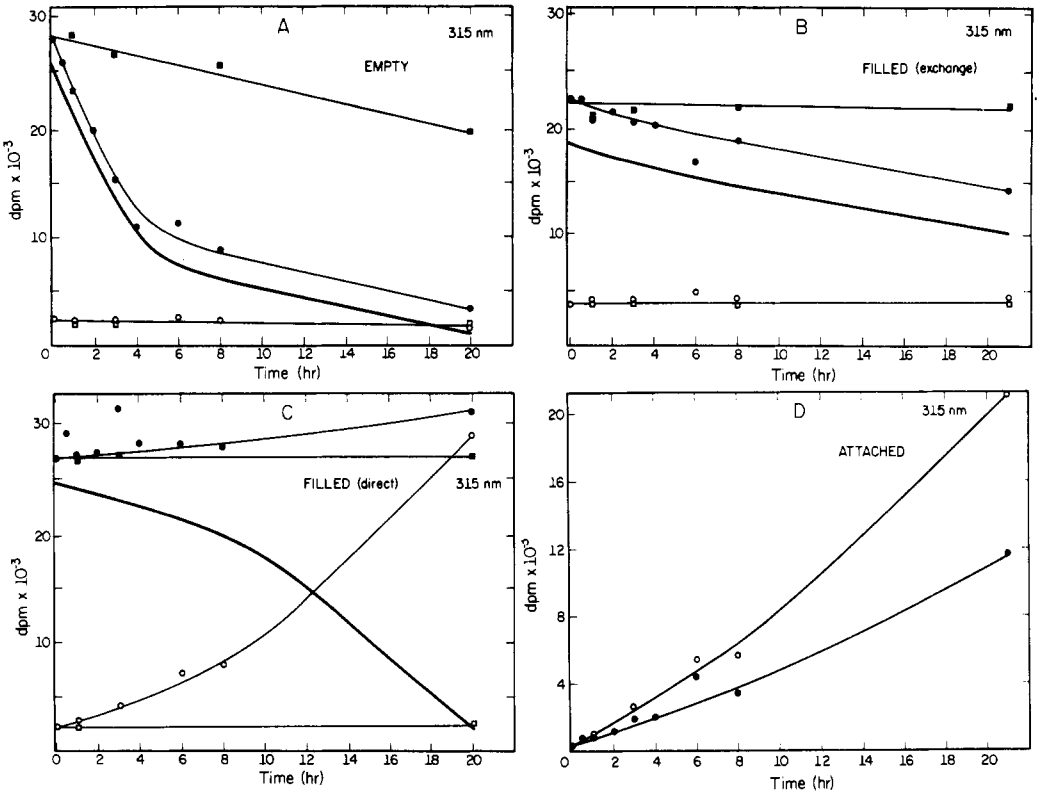

FIGURE 2: Photoinactivation (A-C) and photocovalent attachment (D) of rat uterine cytosol at >315 nm. Details are the same as in Figure 1. (●) Total binding or attachment, and (○) low affinity binding in irradiated samples; in dark controls, (■) total binding and (□) low affinity binding.

trogenic ligands, [^{3}H]estradiol, [^{3}H]estrone, and [^{3}H]estriol. These results are shown in Figure 4.

It is clear that all three ligands exert a profound protective effect against photoinactivation and that the effect is concentration dependent. Of greatest significance, though, is the fact that the effectiveness of these three ligands is different and that their relative effectiveness parallels their affinity for the estrogen binding protein. This can be seen best in Figure 5A, where the percent inactivation protection of each compound is plotted as a function of their concentration in the incubations. The order of effectiveness is estradiol > estriol > estrone, the same as the order of their relative binding affinity (Figure 5B). Furthermore, the correlation between the fractional saturation of the estrogen binding site and the fractional protection against photoinactivation is quite good and follows the same curve, regardless of the nature of the ligand (Figure 5A, insert). These data, together with the fact that high concentrations (up to 10^{-4} *M*) of small phenolic compounds (phenol and 2,6-di-*tert*-butyl-*p*-cresol) do not affect the inactivation rate (data not shown), strongly suggest that the protective effect of estrogens is related to their binding at the estrogen-specific binding site.

Nature of the Photoinactivation Process. It is important in any inactivation process to determine that a loss of activity is not the result of an artifact of the assay procedure. For example, it is possible that the loss of binding activity as monitored by hydroxylapatite adsorption might actually represent not a loss of the ability of the binding protein to bind estrogen, but of the protein-estradiol complex to bind to hydroxylapatite. We have, however, determined the inactivation rates of empty estrogen binding protein at both 254 and >315 nm, measuring binding by charcoal-dextran adsorption of free estradiol, by hydroxylapatite adsorption of

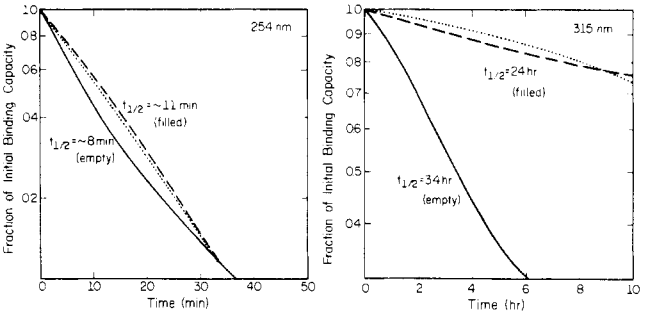

FIGURE 3: Semilogarithmic plot of the time course of photoinactivation at 254 nm and >315 nm. The data plotted are the heavy curves (representing estrogen specific binding) from Figures 1A-C and 2A-C. (—) Empty cytosol; (· · · ·) cytosol filled with unlabeled E_2; and (- - - -) cytosol filled with [^{3}H]E_2.

the protein-estrogen complex, and by separation of bound and free estradiol by chromatography on Sephadex G-25 columms. The inactivation rates determined by these three assays are within 25% of each other.

It is also important to establish that the inactivation represents an actual loss of the number of binding sites and not just a change in their affinity, so that they are no longer saturated by the concentration of ligand in the binding assay (30 n*M*). We have found that samples of empty estrogen binding protein inactivated to 75 and 43% of their initial binding capacity by irradiation at 254 nm maintain the same binding affinity but show a corresponding loss in binding capacity, as determined by Scatchard analysis (see Experimental Section). Sucrose density gradient sedimentation analysis of these three samples under low salt conditions (see Experimental Section) show that the typical 8S peak seen in the unirradiated control both decreased in area

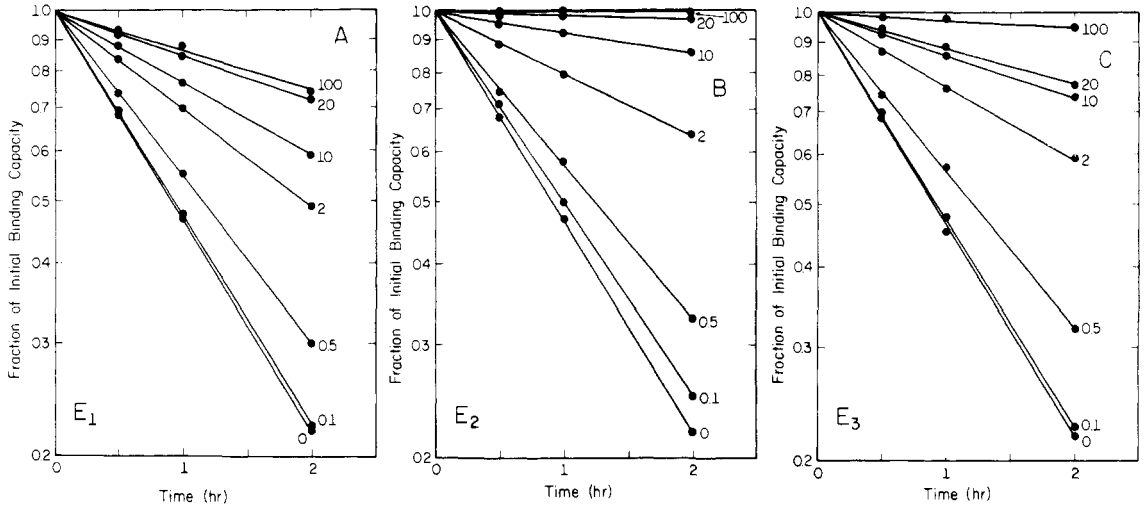

FIGURE 4: Effect of various concentrations of [^{3}H]estrogens on the photoinactivation rate at >315 nm of estrogen binding in uterine cytosol. Cytosol (1 uterine equiv/ml) was incubated for 15 hr at 0° with the indicated nanomolar concentrations of [^{3}H]E_1 (A), [^{3}H]E_2 (B), or [^{3}H]E_3 (C) (all at 40 Ci/mmol), and then irradiated for the time periods shown at >315 nm. Aliquots of the irradiated cytosol were transferred to test tubes, and all remaining sites were saturated for 2 hr at 0° with 20 n*M* [^{3}H]E_2 (40 Ci/mmol) and assayed by hydroxylapatite adsorption. Low affinity binding was determined in the presence of a 100-fold excess of unlabeled E_2. Each point is the mean of 2–4 determinations in triplicate.

with irradiation and became broader and rapidly sedimenting, which is indicative of greater heterogeneity and aggregation.

Nature of the Photocovalent Attachment Reaction. The studies described in Figures 1D and 2D showed that irradiation of cytosol protein in the presence of [^{3}H]estradiol resulted in the covalent attachment of the steroid to protein. We investigated the effect of some reductants (sodium thiosulfate and mercaptoethanol) and some radical scavengers (phenol, 2,6-di-*tert*-butyl-*p*-cresol (BHT), and *p*-aminobenzoic acid) on the time course of both photoinactivation and photocovalent attachment. None of these compounds (at concentrations of up to 10^{-3} *M*) had a significant effect on the rate of photoinactivation of estrogen binding at 254 nm or >315 nm; some, however, did reduce the extent of estrogen covalent attachment after a 30-min irradiation of [^{3}H]E_2 filled cytosol at 254 nm (percent decrease relative to control: BHT, 20%, sodium thiosulfate, 46%, and mercaptoethanol, 58%). In contrast, at >315 nm, covalent attachment actually took place more rapidly in the presence of these reagents (percent increase above control, amount of attachment of [^{3}H]E_2 to cytosol proteins after 20 hr at >315 nm: BHT, 5%, mercaptoethanol and sodium thiosulfate, 30%, phenol, 45%, and *p*-aminobenzoic acid, 100%).

It appears from the lack of saturability of the photocovalent attachment process (Figures 1D and 2D) that it is nonspecific, i.e., it is not related to the binding of steroid at the estrogen binding site. In order to characterize more completely the nature of the photocovalently labeled cytosol proteins, polyacrylamide gel electrophoresis in 8 *M* urea was run on irradiated samples, and the pattern of stained protein bands and radiolabel was compared (Figure 6). Panel A of Figure 6 shows a scan of a gel of unirradiated uterine cytosol; the prominent peak corresponding in mobility to slices 6–8 is presumed to be serum albumin on the basis of the reported prevalence of this protein in uterine tissue (Peck et al., 1973). Irradiation at 254 nm for 30 min causes a marked change in the pattern of stained protein (Figure 6C): many peaks in the albumin region, formerly well resolved, now appear as a broad, featureless band, indicative of extensive alteration of protein electrophoretic mobility. The radioactive E_2, covalently attached under these conditions, is seen throughout the gel in a quantity that corresponds closely to the intensity of stained protein. Irradiation at >315 nm for 20 hr causes only minor alterations in the stained pattern (Figure 6B); here as well, the various protein components appear to have been labeled in proportion to their relative concentrations. The lack of specificity in the photocovalent attachment reaction is further affirmed by the fact that the count profiles on the gels were essentially identical whether irradiation at 254 nm or >315 nm was conducted in the presence of 30 n*M* [^{3}H]E_2 or 30 n*M* [^{3}H]E_2 plus 3000 n*M* E_2. If a portion of the attachment had been dependent upon the binding of ligand to the estrogen-specific binding site, competition at the higher steroid concentration should have been evident as a depressed level of label incorporation in a specific region of the gel.

Discussion

Two basic mechanisms of photodegradation of proteins are recognized: (a) direct, irradiative destruction of amino acid residues, which involves complex photohomolytic cleavages of principally cystine, tryptophan, phenylalanine, and tyrosine residues, and (b) photooxidation, which requires oxygen and generally utilizes a dye capable of sensitizing the production of singlet oxygen; histidine, tryptophan, tyrosine, methionine, and cysteine residues are most often affected (Neckers, 1973; Westhead, 1972; Smith and Hanawalt, 1969; Spikes, 1968).

Although no dyes were used in these experiments, concentrated cytosol preparations have a noticeable red-yellow coloration, and weak visible absorbance maxima at 475 and 538 nm (plus intense Soret band at 418 nm) are indicative of the presence of low concentrations of hemoglobin (Mahler and Cordes, 1971). As hemes are active sensitizers of photooxidation (Westhead, 1972) it is conceivable that all or a portion of the photoinactivation might be due to a dye-mediated photooxidation process. This appears unlikely, however, because when the irradiation at 254 nm or >315 nm is conducted under an atmosphere of nitrogen, the same

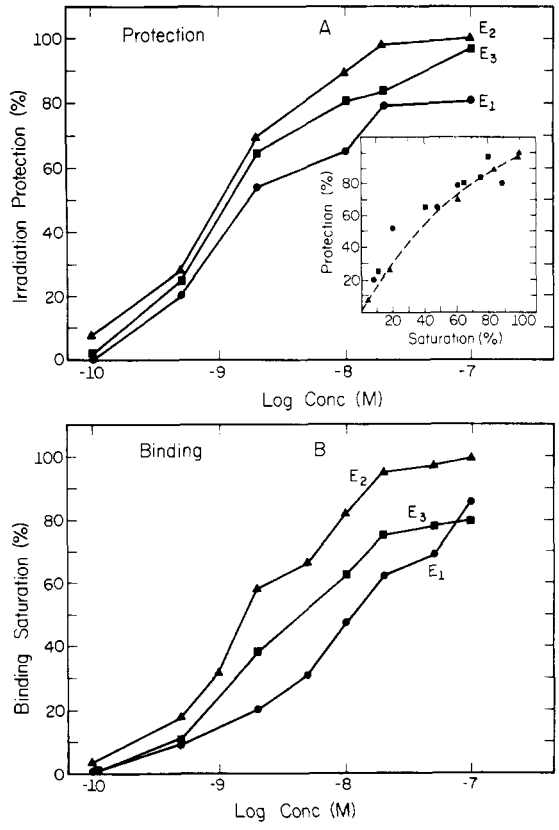

FIGURE 5: The effect of estrogen concentration on the relative degree of protection against photoinactivation at >315 nm (A) and on the degree of saturation of the estrogen binding sites (B). Percents of irradiation protection were calculated from Figure 4, using the rate of photoinactivation of empty cytosol as 0% protection and 100 n*M* [^{3}H]E_2 as 100% protection. The binding curves of [^{3}H]estrogens to rat uterine cytosol (B) are taken from data of Ruh et al. (1973) and more recent data, using Sephadex G-25 column chromatography. The insert in Figure 5A is the correlation between the fractional saturation of estrogen binding sites in rat uterine cytosol by E_1 (●), E_2 (▲), or E_3 (■) and the degree of protection afforded against photoinactivation at >315 nm.

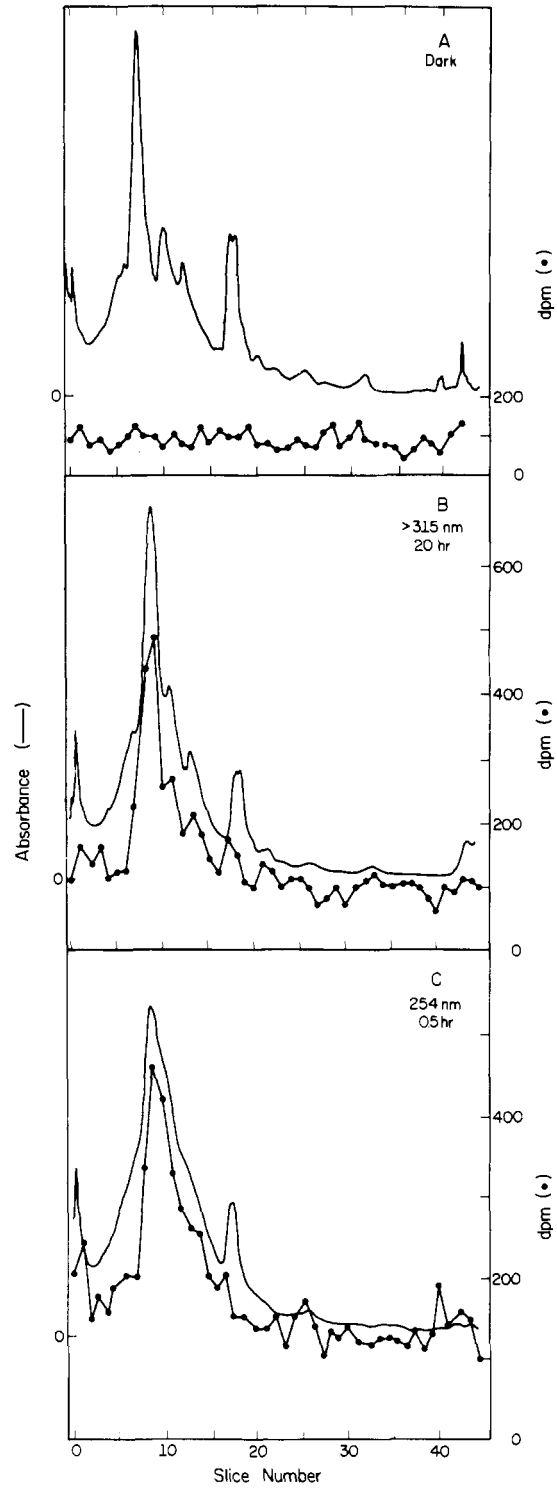

FIGURE 6: Acrylamide gel electrophoresis of rat uterine cytosol irradiated in the presence of [^{3}H]estradiol. Rat uterine cytosol, either 30 n*M* in [^{3}H]E_2 or 30 n*M* in [^{3}H]E_2 plus 3000 n*M* E_2, was left in the dark (control, A), irradiated at >315 nm for 20 hr (B) or at 254 nm for 0.5 hr (C), and then precipitated in chilled ethanol without carrier protein. The pellet was resuspended in electrophoresis sample buffer (containing 8 *M* urea) and applied to 7.5% polyacrylamide-8 *M* urea gels. For optical tracing, gels were stained with 1% Fast Green in acetic acid, electrophoretically destained in 7.5% acetic acid, and scanned at 650 nm on a Beckman DU spectrophotometer equipped with a Gilford linear transport system. After scanning, gels were frozen on Dry Ice, sliced into 2.3-mm sections, dissolved in 0.5 ml of 2% aqueous periodic acid, and counted. In duplicate runs, no significant differences were noted between the patterns of counts photocovalently attached at 30 n*M* [^{3}H]E_2 or at 30 n*M* [^{3}H]E_2 plus 3000 n*M* E_2. (A was run with 30 n*M* [^{3}H]E_2, B and C with 30 n*M* [^{3}H]E_2 plus 3000 n*M* E_2).

inactivation rate is seen as when an air atmosphere is used (data not shown). Furthermore, the estrogen binding protein does not seem to be particularly sensitive to photooxidation sensitized by the commonly used dyes, Methylene Blue, Rose Bengal, or Acridine Orange. Irradiation of empty cytosol at >315 nm in the presence of 0.05 mg/ml of these dyes resulted in photoinactivation at a rate only ca. 1.5 times greater than the control rate at this wavelength (data not shown).

Postulated mechanisms of the direct photodegradation of amino acid residues involve the generation of radical and electron deficient species (Vladimirov et al., 1970). It was on this basis that we investigated the effect of the addition of high concentrations of nucleophiles, reductants, and radical scavengers on the rate of binding inactivation and [^{3}H]estradiol covalent attachment. Little effect was noted on the rate of inactivation, but the rate of photocovalent attachment was significantly affected, indicating that this process may represent the capture by the phenol of a reactive species generated within the protein.

In other experiments (J. A. Katzenellenbogen and H. S. Iwamoto, unpublished), we have noted that [^{3}H]estradiol, [^{3}H]DES, and [^{14}C]phenol undergo photocovalent attachment when irradiated in the presence of bovine serum albumin. The extent of reaction is dependent on the concentration of the ligand and appears to saturate at high ligand

concentrations. This suggests that the covalent attachment process is dependent upon a protein–ligand interaction. Further experiments which are currently underway, investigating model photoreactions between phenols and amino acids in solution and isolating labeled fragments from photolabeled proteins, may serve to elucidate this interesting reaction.

There have been some interesting reports of metabolic processes which result in the covalent attachment of estrogens to protein: Jellinck and Lyttle (1972) have partially characterized an estrogen-induced uterine peroxidase which affects such an attachment; certain liver enzymes cause similar covalent attachment (Kappus et al., 1973); more recently, Cowan et al. (1975) have reported a curious stimulatory effect of manganous ion on estrogen covalent attachment in vitro in certain fractions of rabbit uterine cytosol.

Regardless of the precise mechanism of the photoinactivation process, it is clear that its rate and sensitivity to ligand protection are wavelength dependent. Further, the protective effect of an estrogen ligand against photoinactivation at >315 nm appears to be mediated by binding at the estrogen specific site. This is supported by the correlation between the extent of protection and the degree of binding site saturation, which holds for the three estrogens, estrone, estradiol, and estriol. The precise manner in which the protection is afforded is not clear, however.

A number of studies (see Jensen and DeSombre (1973) for a review) have suggested that the binding of an estrogenic ligand to the binding site in the rat uterine estrogen binding protein results in a conformational change: Thus, the estrogen binding protein complex can stimulate nuclear biosynthetic events in vitro, while the binding protein alone cannot (Mohla et al., 1972); further, the complex is considerably more thermally stable than the free protein (Katzenellenbogen et al., 1973a; Rochefort and Baulieu, 1971). The protective effect of the ligand may be related to this structural reorganization, which may have a more pronounced effect on the residues important in the photosensitivity at >315 nm than those active in the photolytic processes at 254 nm. Alteration of photoinactivation rates by ligand binding is well documented in other systems, e.g., mutarotase (Fishman et al., 1973) and thymidine kinase (Cysyk and Prusoff, 1972).

Ultimately, when homogeneous, purified rat uterine estrogen binding protein is available, more detailed photoinactivation studies can be conducted, with more precise delineation of action spectra, quantum yields, and specific amino acids affected by the degradation. Nevertheless, the results of the present study are indicative of several interesting phenomena: wavelength dependence and ligand dependence of the photoinactivation process and a curious, but general photostimulated covalent attachment of phenolic compounds (estradiol, phenol, and DES) to protein. Furthermore, now that the rate of direct photoinactivation of the rat uterine estrogen binding protein, at different wavelengths and degrees of binding saturation, has been determined, we can hope to make a reasonable assessment of the potential of the new, photosensitive steroid derivatives which we are preparing as photoaffinity labeling reagents.

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References

Anderson, L. E., and McClure, W. O. (1973), *Anal. Biochem. 51,* 173.

Cowan, R. A., Kim, U. H., and Mueller, G. C. (1975), *Steroids 25,* 135.

Cysyk, R., and Prusoff, W. H. (1972), *J. Biol. Chem. 247,* 2522.

Davis, B. (1964), *Ann. N.Y. Acad. Sci. 121,* 404.

Erdos, T., Best-Belpomme, M., and Bessada, R. (1970), *Anal. Biochem. 37,* 244.

Fishman, P. H., Kusiak, J. W., and Bailey, J. M. (1973), *Biochemistry 12,* 2540.

Jellinck, P. H., and Lyttle, C. R. (1972), *Adv. Enzyme Regul. 11,* 17.

Jensen, E. V., and DeSombre, E. R. (1973), *Science 182,* 126.

Kappus, H., Bolt, H. M., and Remmer, H. (1973), *Steroids 22,* 203.

Katzenellenbogen, J. A. (1974), *Annu. Rep. Med. Chem. 9,* 222.

Katzenellenbogen, J. A., Johnson, H. J., Jr., Carlson, K. E., and Myers, H. N. (1974), *Biochemistry 13,* 2986.

Katzenellenbogen, J. A., Johnson, H. J., Jr., and Carlson, K. E. (1973a), *Biochemistry 12,* 4092.

Katzenellenbogen, J. A., Johnson, H. J., Jr., and Myers, H. N. (1973b), *Biochemistry 12,* 4085.

Katzenellenbogen, J. A., Myers, H. N., and Johnson, H. J., Jr. (1973c), *J. Org. Chem. 38,* 3525.

Mahler, H. R., and Cordes, E. H. (1971), Biological Chemistry, 2nd ed, New York, N.Y., Harper and Row, pp 666–669.

Mohla, S., DeSombre, E. R., and Jensen, E. V. (1972), *Biochem. Biophys. Res. Commun. 46,* 661.

Neckers, D. C. (1973), *J. Chem. Educ. 50,* 164.

Ornstein, L. (1964), *Ann. N.Y. Acad. Sci. 121,* 321.

Peck, E. J., Jr., DeLibero, J., Richards, R., and Clark, J. H. (1973), *Biochemistry 12,* 4603.

Puca, G. A., Nola, E., Sica, V., and Bresciani, F. (1971), *Biochemistry 10,* 3769.

Rice, R. H., and Means, G. E. (1971), *J. Biol. Chem. 246,* 831.

Rochefort, H., and Baulieu, E. E. (1971), *Biochemie 8,* 893.

Ruh, T. S., Katzenellenbogen, B. S., Katzenellenbogen, J. A., and Gorski, J. (1973), *Endocrinology 92,* 125.

Ruh, T. S., and Ruh, M. F. (1974), *Steroids 24,* 209.

Scatchard, G. (1949), *Ann. N.Y. Acad. Sci. 51,* 660.

Smith, K. C., and Hanawalt, P. C. (1969), Molecular Photobiology, New York, N.Y., Academic Press, Chapters 5 and 9.

Spikes, J. D. (1968), in Photophysiology, Vol. III, Geise, A. C., Ed., New York, N.Y., Academic Press, Chapter 2.

Vladimirov, Yu. A., Roshchupkin, D. I., and Fesenko, E. E. (1970), *Photochem. Photobiol. 11,* 227.

Westhead, E. D. (1972), *Methods Enzymol. 25B,* 401.