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Estrogen Photoaffinity Labels. 2. Reversible Binding and Covalent Attachment of Photosensitive Hexestrol Derivatives to the Uterine Estrogen Receptor†

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ABSTRACT: The ability of two radiolabeled, photoreactive estrogen analogues, [^{3}H]hexestrol diazoketopropyl ether ([^{3}H]Hex-DKP) and [^{3}H]hexestrol azide ([^{3}H]Hex-N_3), to covalently label the uterine estrogen receptor is studied. Lamb uterine estrogen receptor preparations that have been partially purified (ammonium sulfate precipitation, Sephadex G-200 chromatography) and disaggregated by limited trypsinization can be electrophoresed on polyacrylamide gels under conditions where binding activity is preserved. This electrophoretic procedure was used to fractionate the proteins labeled by the two photoreactive estrogen analogues. Prior to photolysis, peaks of radioactivity indicating estrogen specific binding of [^{3}H]-Hex-N_3 and [^{3}H]Hex-DKP are evident on the gels, although dissociation of the latter compound is extensive. When preparations of uterine estrogen receptor that contain the photoreactive derivatives are irradiated and then electrophoresed, reversibly labeled proteins can be distinguished from irreversibly labeled ones (covalently bonded), by extraction of the individual gel slices with organic solvents. While no irreversible binding to receptor appears to result from irradiation with [^{3}H]Hex-DKP, irradiation with [^{3}H]Hex-N_3 does covalently label the estrogen receptor. The receptor covalently labeled with [^{3}H]Hex-N_3 has the same electrophoretic mobility as the unlabeled receptor; the covalent labeling process is estrogen-site specific, and the efficiency of labeling (15–20%) is consistent with the inactivation efficiency of Hex-N_3, previously measured by an indirect assay. This is the first example of the labeling of a steroid hormone receptor by photoaffinity labeling.

We have been investigating the technique of photoaffinity labeling as a means for studying the details of estrogen interaction with the uterine estrogen receptor (for reviews, see Katzenellenbogen, 1977; Katzenellenbogen et al., 1976, 1977a). Ideally, using this approach one should be able to covalently label the estrogen receptor in a manner that would be both selective and efficient; the covalently labeled receptor would then be available for rigorous and intensive studies that would no longer be limited to conditions consistent with normal, reversible hormone binding activity.

In principle, such a chemical approach is simple, but a number of problems are encountered in its reduction to practice: While considerable information about binding specificity of the receptor is available, little is known about the nature, distribution, and the chemical reactivity of the binding site residues; similarly, the chemical reactivity of affinity labeling reagents cannot be predicted completely. In the face of these circumstances, we considered it judicious to take an indirect but systematic approach to the development of reagents for affinity labeling the estrogen receptor. Our basic protocol has been first (1) to synthesize in nonradioactive form candidate compounds designed on the basis of the best knowledge available as to the binding specificity and chemical reactivity of the hormone binding site (Katzenellenbogen et al., 1973a), and then (2) to determine indirectly, by competition assays with radiolabeled estradiol (E_2), the binding affinity of these

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reagents (Katzenellenbogen et al., 1973b) and (3) the efficiency with which they react with the estrogen binding site (site-specific inactivation; Katzenellenbogen et al., 1974). On the basis of these indirect assays, certain individual compounds are then (4) selected for preparation in radioactive form (Katzenellenbogen et al., 1977b). These are (5) subjected to a complete characterization of their reversible binding with receptor. Finally, (6) their irreversible, covalent attachment to the receptor and to the various other protein components that may be present in the receptor preparation is determined.

On the basis of our previous studies concerning points 1-3 above, we have selected two photosensitive derivatives of hexestrol as being worthy of further investigation: hexestrol diazoketopropyl ether (Hex-DKP) and hexestrol azide (Hex-N_3).[1] In the forestanding report (Katzenellenbogen et al., 1977b), we described the radiochemical syntheses of these derivatives and their photochemical behavior in solution. This report describes an investigation of the reversible binding and the covalent attachment of these radiolabeled derivatives with the proteins in unpurified and partially purified preparations of estrogen receptor from rat and lamb uterus. In it we document the first example of the covalent labeling of a steroid hormone receptor using the technique of photoaffinity labeling.

Experimental Section

Materials. The following compounds were obtained from the sources indicated: 17β-estradiol (Searle, Steraloids); [6,7-^{3}H]-17β-estradiol (40-48 Ci/mmol, New England Nuclear); hydroxylapatite HT (Bio-Rad) or Type I (Sigma); charcoal, Norit A (Sigma); dextran, grade C (Schwarz/Mann); Triton X-114 (Central Solvents and Chemical Co.); 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-200 and Blue Dextran (Pharmacia); acrylamide (Matheson Coleman and Bell); *N,N'*-diallyltartardiamide (Eastman); periodic acid (G. F. Smith); monothioglycerol, trypsin, and soybean trypsin inhibitor (Sigma); γ-globulin (Schwarz/Mann).

Methods. The buffer used in these studies was 0.01 M Tris-HCl-0.0015 M EDTA-0.02% sodium azide, pH 7.4 at 25 °C (TEA buffer). The charcoal-dextran slurry consisted of 5% acid-washed Norit A and 0.5% dextran C in TEA buffer.

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. Rat uterine cytosol was prepared from immature Holtzman rats (21-25 day females) as previously described (Katzenellenbogen et al., 1973b). Lamb uterine cytosol was prepared from uteri that were collected at the abattoir and chilled rapidly in iced TEA buffer, usually within 10-20 min after the death of the animal. The uteri were transported to the laboratory in ice, where they were dissected free from nonuterine tissue. They were routinely stored whole in liquid nitrogen. Cytosol was prepared from fresh or frozen uteri by first mincing the uteri and then homogenizing at 0 °C with a Polytron PT 20 in 2 mL of TEA buffer per g of tissue. The homogenate was centrifuged at 12 350*g* for 15 min and the supernatant recentrifuged at 226 000*g* for 1 h. The resulting cytosol usually contained ca. 10 mg of protein per mL (Lowry et al., 1951) and varied between 4 and 12 nM in estradiol receptor. Cytosol was used immediately or refrozen in liquid nitrogen. Little loss of receptor activity was noted after storage of either whole uteri or cytosol for several months in liquid nitrogen.

Binding Assays. Binding of tritiated ligands to the receptor was determined by charcoal-dextran adsorption (Katzenellenbogen et al., 1973b).

Sedimentation Analysis. Sucrose gradient sedimentation analysis was performed as previously described (Katzenellenbogen et al., 1973c). The samples of cytosol were saturated with ligand at 30/RAC nM (0 °C, 2 h), treated with charcoal-dextran, mixed with ^{14}C-labeled standard proteins (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 45 000 rpm (200 000*g*) in a Beckman SW 56 Ti rotor for 16 h. Three-drop fractions were collected using the ISCO gradient fractionator.

Photolysis at 254 was conducted at 0-3 °C in an apparatus previously described (Katzenellenbogen et al., 1974).

Assay of Covalent Attachment. Attachment was monitored by spotting duplicate 50-μL aliquots onto 2.5-cm Whatman 3MM filter paper discs. The discs were collected at the end of the experiment, washed two times for 15 min in boiling 95% EtOH, rinsed at room temperature two times each in 1:1 ether-95% EtOH and in pure ether, and dried. Radioactivity was determined by liquid scintillation counting.

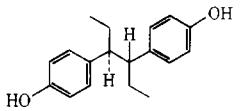
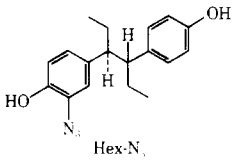
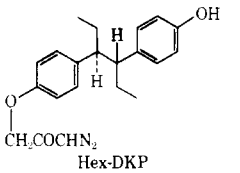
Purification. All procedures were carried out at 0-4 °C. The cytosol was brought to 30% $(NH_4)_2SO_4$ by adding the appropriate amount of a saturated solution of $(NH_4)_2SO_4$ in H_2O which had been adjusted to pH 7.2. After 15 min, the resulting precipitate was pelleted at 15 000*g* for 20 min, and the pellet was redissolved in TEA to the desired concentration. Trypsin (dissolved just before use at 10 mg/mL in 0.001 M HCl) was added to a final concentration of 20 μg/mg protein. After 1 h the trypsinization was halted by addition of 2.5 μg of soybean trypsin inhibitor per μg of trypsin. For greater details, see Carlson et al. (1977).

Further purification was achieved by passing the protein through a 1.5 × 50 cm column of Sephadex G-200, equilibrated with TEA buffer and eluted by inverted flow. For some experiments the column was equilibrated and eluted with TEA buffer containing 1 mM thioglycerol.

Polyacrylamide Gel Electrophoresis. Polyacrylamide gels were prepared following a modification of the method of Ornstein (1964) and Davis (1964). The gels contained 6% acrylamide and 0.48% *N,N'*-diallyltartardiamide (Anker, 1970). They were prerun at room temperature, with a cathode prerunning buffer that was a 1:10 dilution of the stacking gel buffer, until the bromphenol blue dye reached the stacking gel-running gel interface. Thereafter, the upper buffer was replaced with the running buffer; the gels were completely

[1] Common names (and abbreviations) used in this paper are: estradiol, 1,3,5(10)-estratriene-3,17β-diol; hexestrol, *meso*-3,4-bis(4'-hydroxyphenyl)hexane; THF, tetrahydrofuran; TEA buffer, 0.01 M Tris-0.0015 M EDTA-0.02% sodium azide (pH 7.4 at 25 °C) Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid; TLC, thin-layer chromatography; Hex-DKP, hexestrol diazoketopropyl ether, hexestrol 4-*O*-(3'-diazo-2'-oxopropyl) ether; Hex-N_3, hexestrol azide, 3-azidohexestrol; RAC, ratio of association constants (K_a(derivative)/K_a(estradiol)).

TABLE I: Binding Affinity and Inactivation Efficiency of Hexestrol Diazoketopropyl Ether (Hex-DKP) and Hexestrol Azide (Hex-N_3), as Determined in Competition Assays.

Compound	Rat			Lamb		
	RAC × 100[a] (Cytosol)	(AS)[c]	Inact. Eff (%)[b]	RAC × 100 (Cytosol)	(AS)[c]	Inact. Eff (%)
 hexestrol	300	1850	0	430	1430	0
 Hex-N_3	70	410	15	48	235	20
 Hex-DKP	2.9	4.1	15	2.8	10	10

[a] RAC = ratio of association constants K_a(derivative)/K_a(estradiol), determined by competitive binding. The data from compounds reported previously by us (Katzenellenbogen et al., 1973b) have been combined with the results from more recent experiments. Conversion of the assay data from the ratio of concentrations giving equivalent inhibitions to the ratio of association constants has been done using the equation given by Rodbard (1973) and Korenman (1970, 1975). (It should be noted that the term R in the expression given by Korenman should be the ratio of free to bound tracer ligand at the degree of competition used to determine the concentration ratio (Rodbard, 1973), rather than in the absence of competitor, as stated by Korenman (1975) and by ourselves previously (Katzenellenbogen et al., 1973b)). [b] Inactivation efficiency represents the percent of the estrogen-specific binding sites that are inactivated when the receptor bound derivative is photolyzed. Irradiation was done at 254 nm (cf. Katzenellenbogen et al., 1974). The rate of inactivation with hexestrol is the same as that with estradiol alone or in the absence of estradiol (cf. Katzenellenbogen et al., 1974, 1975b). [c] AS indicates that cytosol purified by precipitation with 30% ammonium sulfate was used in these experiments; see Experimental Section.

immersed in the lower buffer, and the lower buffer was magnetically stirred and chilled to 2 °C by circulating cold methanol through the lower reservoir jacket. All prerunning and running were done at 2 mA/gel. For a more complete description of the electrophoretic procedure, see Carlson et al. (1977).

After electrophoresis, the gels were frozen on dry ice and sliced into 2.3-mm slices. For extraction, each slice was soaked for 2 h in 0.5 mL of toluene or ethyl acetate at room temperature. The gel slice was then removed and dissolved using 0.5 mL of 2% periodic acid. The extract and gel slice were measured separately using liquid scintillation counting.

Results

The results of our investigations on hexestrol diazoketopropyl ether (HexDKP) and hexestrol azide (Hex-N_3) in nonradiolabeled form are given in Table I. The binding affinities for the uterine estrogen receptors were determined relative to that for estradiol by competitive binding assay as described previously (Katzenellenbogen et al., 1973b). The ortho azide function in Hex-N_3 is tolerated by the estrogen binding site with little reduction in binding affinity; however, etherification of the hydroxyl with the diazoacetonyl group as in Hex-DKP considerably reduces the binding affinity of this derivative. Ammonium sulfate precipitation removes the majority of the nonreceptor binding proteins (vide infra), causing substantial increases in the relative binding affinity of both derivatives. Such increases in the relative affinity of a lipophilic hormone analogue, which result from a lowering of nonspecific binding, were expected on theoretical grounds (Levitzski et al., 1975) and have been demonstrated with estradiol and estriol in an in vitro system (Anderson et al., 1974).

The estrogen site-specific photoinactivation efficiency was also determined with the nonradiolabeled derivatives, utilizing an exchange assay: the loss of exchange capacity of a receptor preparation, saturated with a photosensitive derivative, was monitored as a function of irradiation time (Katzenellenbogen et al., 1974). The inactivation due to the photosensitive reagent (as opposed to nonspecific photoinactivation) is determined by measuring the protective effect of an excess of unlabeled estradiol. Receptor preparations from both rat and lamb uterus show similar inactivation efficiencies (Table I).

Studies Utilizing Unfractionated Uterine Cytosol Preparations. Reversible Binding Studies. We endeavored to determine the estrogen specific binding affinity of the two radiolabeled derivatives using cytosol preparations from immature rat uterus. It was immediately apparent, however, that the level of nonspecific (i.e., nonreceptor) binding with these derivatives greatly exceeded that encountered with [^{3}H] estradiol. This caused serious interference with the charcoal–dextran adsorption binding assay.

Sucrose density gradient sedimentation is a convenient way to distinguish between estrogen receptor binding and nonspecific binding (Toft et al., 1967). Under conditions of low ionic strength (0.01 M Tris buffers), the rat uterine estrogen receptor sediments as a high-molecular-weight aggregate (ca. 8 S), while most of the nonspecific binding proteins sediment as 4S species. Figure 1 shows a sucrose gradient profile with [^{3}H]Hex-N_3.

While estradiol binding is found only in the 8S region of the gradient (see dashed line insert), most of the Hex-N_3 is bound

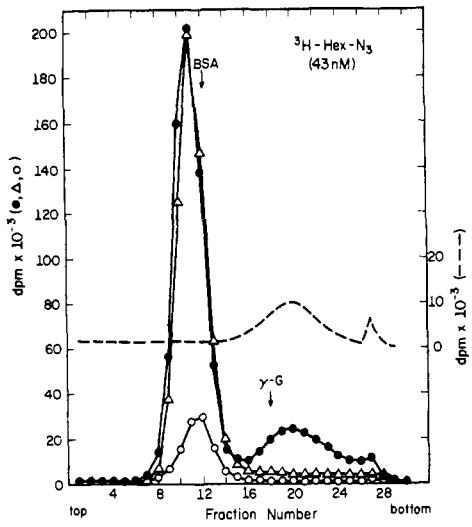


FIGURE 1: Sucrose density gradient profiles of [^{3}H]Hex-N$_3$-labeled cytosol. Rat cytosol was incubated with 43 nM [^{3}H]Hex-N$_3$ alone (●) or after preincubation with 75-fold excess unlabeled estradiol (E_2, Δ) or 100-fold excess unlabeled Hex-N$_3$ (○). The cytosol complex was charcoal treated and centrifuged for 13 h at 246 000 *g* through gradients of 5–20% sucrose. Bovine serum albumin (4.5 S) and γ-globulin (7.0 S) were added as internal markers. The dashed line, with scale on right vertical axis, is binding profile for 30 nM [^{3}H]estradiol alone.

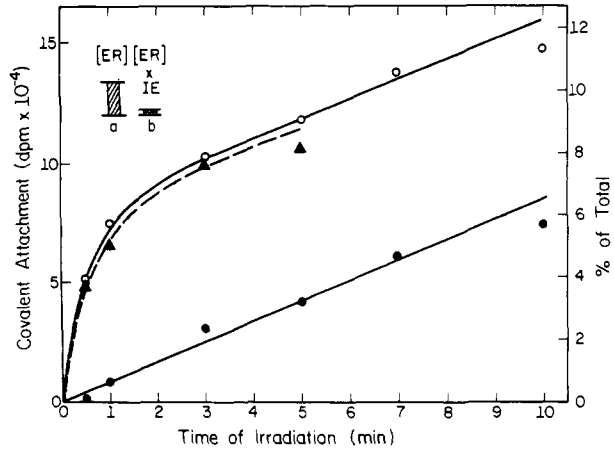


FIGURE 2: Photoattachment of [^{3}H]Hex-DKP to rat uterine cytosol proteins. Rat cytosol was incubated with 167 nM (3/RAC nM) [^{3}H]-Hex-DKP (○) or prephotolyzed [^{3}H]Hex-DKP (●) (prephotolyzed for 3 min at 254 nm). The protected sample (▲) ([^{3}H]Hex-DKP + E_2) was first incubated with unlabeled estradiol (3000 nM) to fill the receptor sites and then with [^{3}H]Hex-DKP. Following photolysis at 254 nm for the times indicated, the covalent attachment was assayed as described in Methods. The incorporation into dark controls (0.7–1 × 10^4 dpm, direct; 1–3 × 10^4 dpm, prephotolyzed) has been subtracted from each point. The amount of estrogen receptor in the cytosol ([ER], indicated as the striped bar a) was determined by charcoal–dextran adsorption (see Methods). Before photolysis, the amount of estradiol protectable binding seen with [^{3}H]-Hex-DKP or prephotolyzed [^{3}H]Hex-DKP was equivalent to the amount of estradiol receptor present ([ER]). As the inactivation efficiency (IE) of Hex-DKP is 15% (Table I), a maximum of only 15% of the receptor-bound reagent can be expected to become covalently attached ([ER] × IE, indicated as hatched bar b).

by nonreceptor proteins sedimenting in the 4S region. However, Hex-N$_3$ binding in the 8S region is evident and appears to be estrogen specific: it is quantitatively equivalent to the binding observed with [^{3}H]estradiol and is subject to competition by excess unlabeled estradiol. The 4S binding is not estrogen specific (no competition by unlabeled estradiol). It does show binding saturation in the presence of a 100-fold excess of unlabeled Hex-N$_3$, and from the data, a K_d of 0.6 μM can be estimated for the 4S Hex-N$_3$ binding. Similar sucrose gradient profiles showing predominately nonspecific binding were reported by us previously with the iodinated hexestrols (Katzenellenbogen et al., 1975a).

We have been unable to observe 8S binding of Hex-DKP on the sucrose gradients. This compound has a relative binding affinity much lower than that of Hex-N$_3$ (Table I), and it dissociates from the receptor with a $t_{1/2}$ of 3.5 h at 0 °C (determined by the method of Katzenellenbogen et al., 1973c). However, by using partially purified receptor preparations (vide infra), we have been successful in observing estrogen specific binding of Hex-DKP, using charcoal adsorption.

Photocovalent Attachment Studies. Despite the high level of nonspecific binding observed with these compounds in unpurified rat uterine cytosol, we proceeded to investigate their capacity for photocovalent attachment.

Figure 2 shows the time course of photocovalent attachment of [^{3}H]Hex-DKP to the rat uterine cytosol preparations. (Similar results were obtained with Hex-N$_3$.) In this experiment, the extent of covalent attachment was determined by boiling ethanol extraction of aliquots applied to filter paper discs (see Methods). In the dark, covalent attachment of these reagents to proteins proceeds only very slowly (see Figure 2, legend), but, upon irradiation, rapid attachment ensues (top curve). It is evident that the labeling is not highly specific for the receptor, however, since the amount of attachment that is obtained is in excess of the amount of labeling expected (curve A vs. inset values a and b; see Figure 2, legend).

In order to establish whether some of the incorporation in the curve was estrogen site specific, an experiment was run simultaneously with a sample in which the receptor sites had been blocked by the addition of unlabeled estradiol prior to the addition of [^{3}H]Hex-DKP (Figure 2, dotted curve). Protection by estradiol did cause a depression in the incorporation of [^{3}H]Hex-DKP that was of the order predicted from the inactivation efficiency of this compound (Table I and Katzenellenbogen et al., 1974; see inset value b).

The data in Figure 2 also indicate that not all the covalent attachment is dependent upon the photochemically reactive chromophore, as continued irradiation for times beyond which the chromophore has been completely consumed (ca. 1.5 min) still leads to additional covalent attachment, though at a slower rate. Furthermore, slow incorporation was also observed in the case where the compound was photolyzed prior to the addition of cytosol (Figure 2, bottom curve). Brunswick and Cooperman (1973) have also noted such secondary covalent attachment in their studies with diazomalonyl derivatives of cAMP, and we have described a slow, nonspecific photocovalent attachment of estrogens and other phenolic compounds to proteins (Katzenellenbogen et al., 1975b).

It was apparent from these studies that it would be essential to fractionate the radiolabeled protein components in order to determine whether the estrogen receptor had become labeled. However, despite repeated attempts using sucrose density gradient sedimentation and polyacrylamide gel electrophoresis (both with and without urea or sodium dodecyl sulfate), we were unable to provide a definitive delineation of the photochemical labeling of the estrogen receptor in unpurified rat uterine cytosol preparation with either [^{3}H]Hex-N$_3$ and [^{3}H]Hex-DKP. This work has been reported elsewhere (Katzenellenbogen et al., 1976).

Studies Utilizing Partially Purified Estrogen Receptor Preparations. Partial Purification of Lamb Cytosol. We have recently developed a procedure for the partial purification and

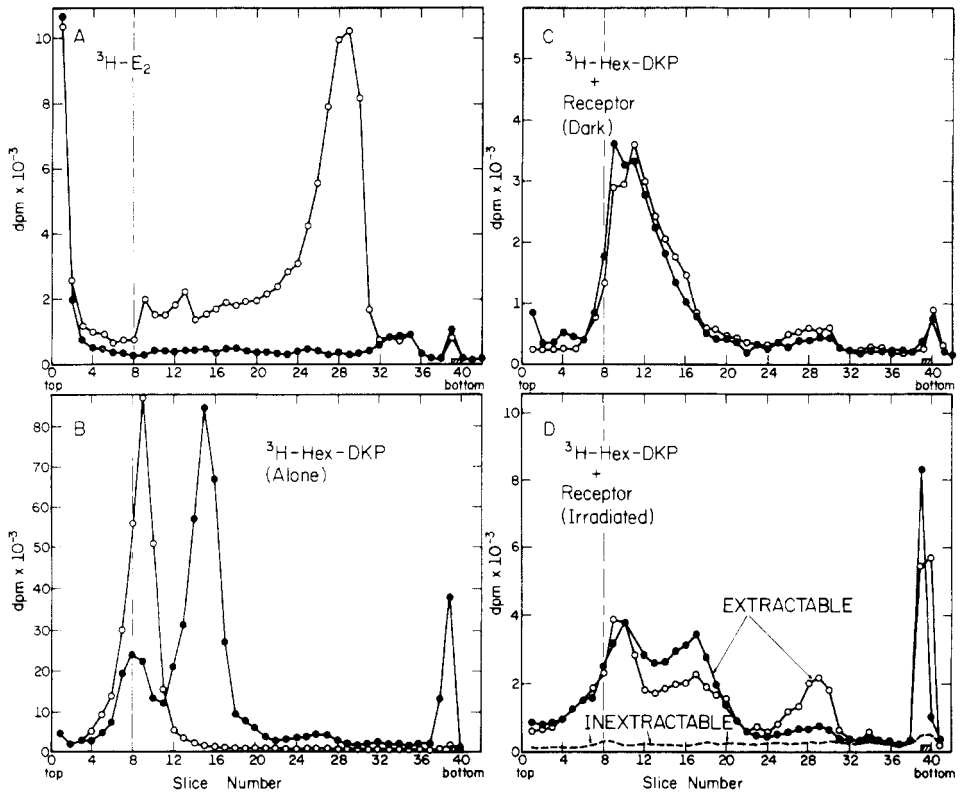


FIGURE 3: Electrophoresis of [^{3}H]estradiol and [^{3}H]Hex-DKP with Sephadex G-200 purified protein. (Panel A) Lamb uterine cytosol was incubated with [^{3}H]estradiol and then purified through the Sephadex G-200 column chromatography step before electrophoresis (○). The same procedure was followed on a sample exchanged against a 100-fold excess of unlabeled estradiol (●). (Panel C) An aliquot of the [^{3}H]Hex-DKP peak from the Sephadex G-200 column chromatography was electrophoresed directly (○) or after exchange for 3 h at 0 °C against a 100-fold excess of unlabeled estradiol (●). (Panel D) Portions of both [^{3}H]Hex-DKP samples from above (with (●) and without (○) unlabeled estradiol) were electrophoresed after irradiation for 1 min at 254 nm. The slices were extracted with 0.5 mL of ethyl acetate to remove noncovalently bound counts before being transferred to a new vial and dissolved in periodate. The inextractable activity is indicated by the dashed line (- - - -). Panel B shows the profile of [^{3}H]Hex-DKP that was (●) or was not (○) irradiated and then electrophoresed, all in the absence of proteins. The vertical dashed line at slice 8 in all panels indicates the interface between the stacking and separation gels, and the small shaded rectangle at slice 38 indicates the position of the tracking dye band (bromphenol blue).

disaggregation of the uterine estrogen receptor (Carlson et al., 1977), based on the limited trypsinization procedure first reported by Rat et al. (1974). Lamb uteri have proved to be a convenient and plentiful source of receptor for these purification studies, and, since Hex-N_3 and Hex-DKP showed similar binding affinity and inactivation efficiency with receptor from either rat or lamb uterus (Table I), these partially purified lamb receptor preparations seemed to be most suitable to continue our affinity labeling studies.

The two stage purification procedure (ammonium sulfate precipitation, Sephadex G-200 column chromatography) effects a 10–30-fold purification of receptor and removes a major portion of the proteins that contribute to the nonspecific binding. The trypsinization converts the lamb uterine receptor to a form that has an apparent molecular weight of ca. 40 000 and tolerates some further manipulation without reaggregation (Carlson et al., 1977).

Electrophoretic Fractionation of Estrogen Receptor Preparations: Analysis of Covalent Binding. We (Carlson et al., 1977) and others (Vallet-Strouve et al., 1976; Secco-Millet et al., 1977) have shown that the trypsinized forms of the estrogen receptor can be successfully subjected to polyacrylamide gel electrophoresis in the Ornstein–Davis buffer system while retaining their binding activity. Using this electrophoretic system (together with a solvent extraction assay, see below), we have been able to analyze the reversible and covalent binding of the photoreactive estrogen analogues to proteins. While we have found that Hex-DKP does not bind to proteins in the receptor preparations irreversibly, at least a portion of Hex-N_3 does become irreversibly associated with receptor through irradiation.

Figure 3 shows the electrophoretic profiles of [^{3}H]estradiol and [^{3}H]Hex-DKP bound to the partially purified lamb uterine cytosol protein preparation. A peak of estradiol binding activity migrates with high mobility (Figure 3A); the specificity of this binding is evident from the low level of nonspecific binding (Figure 3A, lower curve). Figure 3B shows the electrophoretic profile for [^{3}H]Hex-DKP before and after irradiation in the absence of proteins; none of the peaks due to this free material interferes with the analysis of the estrogen receptor.

After ammonium sulfate precipitation and Sephadex G-200 chromatography, the binding of [^{3}H]Hex-DKP to the estrogen receptor can be observed on gels (Figure 3C). The quantity of estrogen specific binding that is found with this compound, however, is considerably less than that seen with estradiol, since most of the receptor complex with this lower affinity derivative has undergone dissociation during the purification and electrophoretic procedures.

Figure 3D shows the profile of radioactivity obtained by electrophoresis of the same sample of semipurified receptor complex after photolysis. The intriguing feature of this profile is that there is more radioactivity in the estrogen receptor region than prior to photolysis.

In order to determine whether the radioactivity found in the gels after irradiation is covalently bound to protein, the indi-

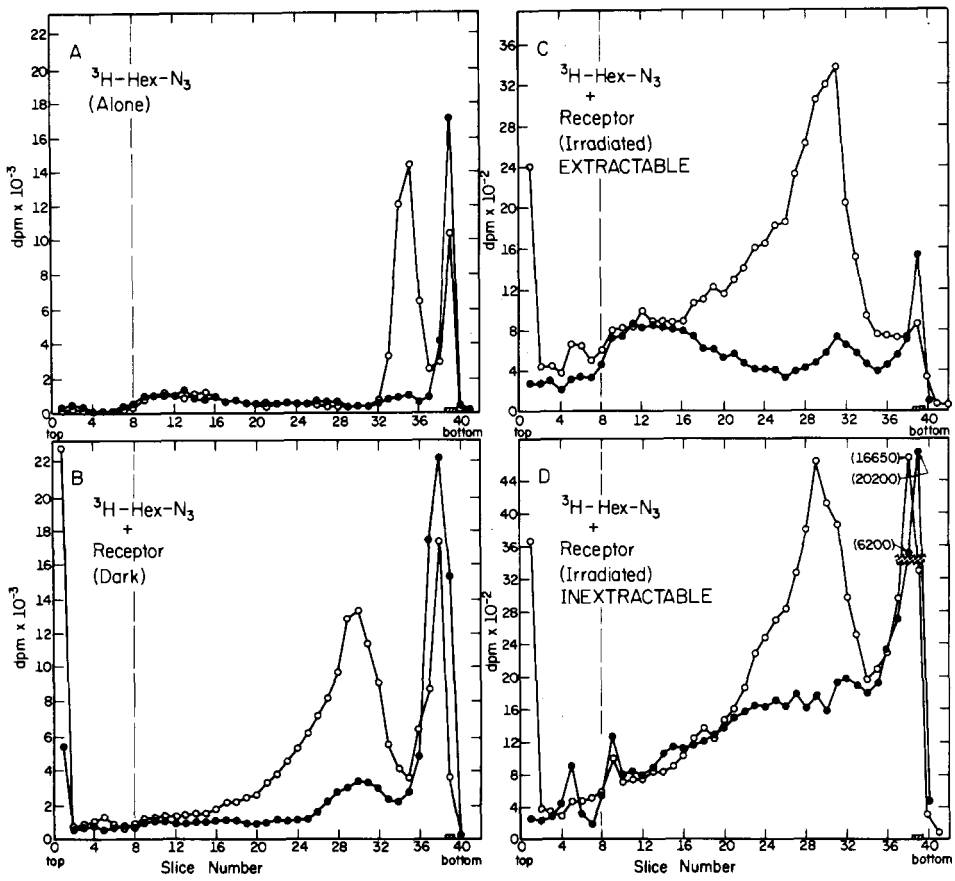


FIGURE 4: Acrylamide gel electrophoresis of [^{3}H]Hex-N$_3$. Panel A shows the electrophoretic profile of [^{3}H]Hex-N$_3$, before (○) and after (●) irradiation in the absence of protein. Panels B, C, and D represent partially purified lamb uterine cytosol incubated with 4.3 nM [^{3}H]Hex-N$_3$ in the absence (○) and presence (●) of 1000-fold excess of unlabeled estradiol. Samples for panels C and D were irradiated for 5 min at 254 nm before electrophoresis. Radioactivity extractable with toluene (panel C) represents [^{3}H]Hex-N$_3$ which is not covalently attached to the protein, while the inextractable radioactivity (panel D) may indicate covalent binding. The vertical dashed line at slice 8 in all panels indicates the interface between the stacking and separation gels, and the small shaded rectangle at slice 38 indicates the position of the tracking dye band (bromphenol blue).

vidual gel slices were extracted in an organic solvent, and the extract (noncovalent binding) and gel slice (covalent binding) were counted separately (see Methods). By using this technique, it was apparent that all of the radioactivity in the gel shown in Figure 3D was extractable and, therefore, that [^{3}H]Hex-DKP had not bound to the receptor covalently. Furthermore, the material that was reversibly bound to the receptor after photolysis was shown to be hexestrol by thin-layer chromatographic comparison with an authentic sample. In the preceding paper, we showed that hexestrol is one of the major photoproducts of Hex-DKP in water (Katzenellenbogen et al., 1977b). Thus, the higher (reversible) binding to receptor seen after photolysis of Hex-DKP (Figure 3D) can be explained by exchange in favor of the higher affinity ligand, hexestrol, which has been generated photolytically.

Hex-N$_3$ has a much higher binding affinity for the estrogen receptor than the diazo compound; therefore, fewer problems with complex dissociation and nonspecific binding were encountered upon electrophoresis. A large amount of estrogen-specific binding of [^{3}H]Hex-N$_3$ is evident in cytosol preparations that were only ammonium sulfate precipitated and trypsinized (Figure 4B). The profiles of free derivative, both before and after photolysis, are shown in Figure 4A.

Prior to photolysis, all the radioactivity associated with the receptor region is solvent extractable (data not shown); after photolysis, however, nearly half of the radioactivity that migrates in the receptor region is no longer solvent extractable (Figure 4C and D) and thus appears to be covalently bound. As an additional control, [^{3}H]Hex-N$_3$ was irradiated alone and then added to the receptor preparation and electrophoresed: no radioactivity at all was found in the receptor region of the gel (data not shown).

Simple calculations show that the quantity of radioactivity in the receptor region of the gel that is nonextractable after photolysis corresponds to approximately 15–20% of the material bound at the time of photolysis. This is the maximum incorporation that would be expected on the basis of the 20% inactivation efficiency of Hex-N$_3$ in lamb uterine cytosol as determined by the photolysis exchange assay (Table I).

Discussion

Estrogen receptor from lamb uterus that has been partially purified and disaggregated by limited trypsinization can be subjected to polyacrylamide gel electrophoresis without loss of binding activity. By combining this electrophoretic procedure with solvent extraction of the individual gel slices, one can perform a simultaneous determination for radioactivity that is both reversibly and irreversibly (covalently) bound to the receptor. Using this approach, we have been able to show that Hex-N$_3$, a photosensitive estrogen analogue, appears to covalently label the lamb estrogen receptor with an efficiency of about 15–20%. in contrast, Hex-DKP, a related derivative, does not, in fact, label the receptor covalently, even though there is reversible binding to receptor before and after irradiation.

The following points can be made to substantiate that the

species that has been labeled by Hex-N_3 is the estrogen receptor: (1) Both the reversible and the irreversible binding of this derivative is subject to competition with unlabeled estradiol; (2) the electrophoretic mobility of the species labeled with Hex-N_3, both reversibly and irreversibly, is the same as that of the receptor–estradiol complex; and (3) the extent of irreversible labeling is consistent with that determined to be an upper limit by our inactivation studies.

The following points can be made to substantiate that the linkage connecting a portion of the hexestrol derivative to the receptor after photolysis is, in fact, covalent in nature: (1) The unphotolyzed derivative or the derivative photolyzed alone, prior to the addition of protein, can be quantitatively extracted from the receptor, while a portion of the derivative is not extractable when it is bound to the receptor at the time of irradiation. In further studies to be described elsewhere, we have found that (2) the material that is not extractable with organic solvents can be extracted with buffer and that (3) the radioactivity in this buffer extract can be precipitated with trichloroacetic acid or hot ethanol (K. E. Carlson and J. A. Katzenellenbogen, unpublished data).

We have previously used the terms "binding affinity" and "attachment efficiency" to characterize the behavior of photoaffinity labeling reagents (Katzenellenbogen, 1977; Katzenellenbogen et al., 1976), and, in preliminary studies using nonradiolabeled reagents, we have determined the "photoinactivation efficiency" of a compound to assess the upper limit of its attachment efficiency (Katzenellenbogen et al., 1974). Through the present studies, we have seen one derivative (Hex-N_3) whose attachment efficiency appears to match closely its inactivation efficiency, while another derivative (Hex-DKP) with a similar inactivation efficiency failed to attach at all.

It is also clear that, for an affinity labeling reagent to act selectively, one must be concerned with more than just its binding affinity for receptor. The photosensitive derivatives we studied were in both cases more lipophilic than estradiol; as a consequence, their binding to nonreceptor proteins in unfractionated cytosol preparations was greatly elevated. This complicated the analysis of their reversible and irreversible binding to receptor and necessitated receptor purification prior to further attachment studies.

In our future development of affinity labels for steroid receptors, we will endeavor to reduce the level of nonreceptor binding by controlling the lipophilicity of the labeling reagents, so as to produce reagents that are both more efficient and more selective in their receptor labeling properties.

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