Iodohexestrols. II. Characterization of the Binding and Estrogenic Activity of Iodinated Hexestrol Derivatives, in Vitro and in Vivo†

John A. Katzenellenbogen,* Hansen M. Hsiung, Kathryn E. Carlson, William L. McGuire, Russell J. Kraay, and Benita S. Katzenellenbogen

ABSTRACT: The affinity of ortho-iodinated hexestrols for the estrogen binding protein from rat uterus, determined by competitive binding assay, decreases with progressive iodine substitution; 3-iodohexestrol (I-Hex) has a binding affinity 42% that of estradiol. Analysis of [^{3}H]-I-Hex binding in rat uterine cytosol by sucrose density gradient centrifugation shows both an estrogen-specific binding component (8 S) and a more abundant component (4 S) that is not estrogen specific. Scatchard analysis indicates that this latter binding is of high affinity (K_d = 3.7–8.3 × 10^{-9} M) but is not uterine specific. Polyacrylamide gel electrophoresis shows that most of the [^{3}H]-I-Hex binding activity in serum and uterine cytosol is distinct from and anodic to the principal protein component (albumin), and that it comigrates with [^{14}C]thyroxine binding activity. In in vitro incubations of rat uteri, I-Hex can block the specific uptake of [^{3}H]estradiol into the nuclear fraction; it itself causes a translocation of estrogen-specific binding capacity (as measured by exchange) from cytoplasm to nuclei, and can induce the synthesis of an estrogen-specific uterine protein, all under conditions where it is not metabolically deiodinated to hexestrol. The uterotrophic activities of the iodohexestrols are in most cases comparable to that expected on the basis of their competitive binding affinities. However, selective, estrogen-specific uptake of [^{3}H]-I-Hex into rat uterus, either in vitro or in vivo, cannot be demonstrated.

In our preceding report (Katzenellenbogen and Hsiung, 1975) we described the preparation and characterization of five iodinated derivatives of hexestrol. In this report we present the results of an investigation of the binding activity and estrogenic character of these derivatives, which was undertaken to establish the potential of the iodohexestrols as estrogenic ligands for characterizing estrogen-specific binding in uterine tissue.

Experimental Section

Materials. The iodinated hexestrols were prepared as described previously (Katzenellenbogen and Hsiung, 1975). The following compounds were obtained from the sources indicated: 17β-estradiol (Searle, Steraloids); *meso*-hexestrol (Mann); [6,7-^{3}H]-17β-estradiol (46.5–48 Ci/mmol, New England Nuclear); iodine-125, carrier free (New England Nuclear); [2-^{14}C]-DL-thyroxine (30.4 Ci/mol, New England Nuclear); [^{14}C]formaldehyde (10 Ci/mol, New England Nuclear); L-[4.5-^{3}H]leucine (2.0 Ci/mmol) and L-[^{14}C]leucine (316 Ci/mol, Schwartz Bioresearch); acrylamide (Matheson Coleman and Bell); *N,N'*-diallyltartardiamide (Eastman); periodic acid (G. F. Smith); chloramine T (Sigma); sodium metabisulfite (Baker); sodium borohydride and sodium cyanoborohydride (Ventron); bovine serum albumin, crystallized and lyophilized (Sigma); bovine γ-globulin, crystallized and lyophilized, fraction II (Nutritional Biochemicals); Sephadex G-25, fine (Pharmacia); charcoal and Norit A (Sigma); dextran, grade C (Schwartz/Mann); Triton X-114 (Rohm and Haas); Biosolv BBS 3 (Beckman); Protosol (New England Nuclear); dimethylforamide (Baker); ethylenediaminetetraacetic acid (EDTA) (Eastman); tris(hydroxymethyl)aminomethane (Tris) (Nutritional Biochemicals); Eagle's HeLa medium (Difco); microtiter plates (Scientific Products); silica gel plastic-backed chromatographic sheets, Eastman Chromatosheets 6060 (Eastman).

Methods. The buffers used in these studies were: 0.01 M Tris–0.0015 M EDTA (TE) (pH 7.4) at 25°, 0.01 M Tris–0.0015 M EDTA–0.02% sodium azide (TEA) (pH 7.4) at 25°, and 0.01 M Tris (pH 8.9) at 5°. 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; modified to contain 0.55% 2,5-diphenyloxazole, 0.01% *p*-bis[2-(5-phenyloxazolyl]benzene, and 25% Triton X-114) was used, and counting was done in minivials (Research Products) containing 5 ml of scintillation fluid in a Nuclear Chicago Isocap 300 instrument (43–48% tritium, 90% carbon-14). γ counting was done in a Nuclear-Chicago instrument.

Carbon-14 labeled marker proteins (bovine serum albumin and γ-globulin, for sucrose density gradient centrifugation, and rat uterine cytosol and serum proteins for gel electrophoresis) were prepared by the method of Rice and Means (1971). A modification of that method, using sodium cyanoborohydride in place of sodium borohydride and run in 0.2 M phosphate buffer (pH 7.0) at protein concentrations ca. 0.1 mg/ml, was used to obtain labeled proteins with higher specific activity (J. A. Katzenellenbogen and B. S. Schwartz, unpublished). Homogenizations were done in

† From the Department of Chemistry (J.A.K., H.M.H., and K.E.C.) and the Department of Physiology and Biophysics and the School of Basic Medical Sciences (B.S.K.), University of Illinois, Urbana, Illinois 61801, the Department of Medicine, University of Texas Health Sciences Center, San Antonio, Texas 78284 (W.L.McG.), and The Eli Lilly Company, Indianapolis, Indiana 46206 (R.J.K.). *Received July 23, 1974.* Support of this research by the National Institutes of Health (Grants NIAMDD AM-15556 and NICHD HD-06726 and Contract NCI N01-CB-23862) and the Ford Foundation (Training Grant 700-0333) is gratefully acknowledged.

a motor-driven conical all-glass tissue grinder (Kontes Duall).

Preparation and Storage of Rat Uterine Cytosol and Serum. Cytosol was prepared in TEA or Tris buffer from uteri of immature (21–25 day) female Holtzman rats, according to procedures previously described (Katzenellenbogen et al., 1973a). Serum was prepared from the blood of the same rats, collected after decapitation: After clotting for 1–2 hr at 25°, the serum was separated by centrifugation (800*g* for 10 min). Both cytosol and serum could be stored at −20° in 25% glycerol; no differences were noted between fresh and frozen serum and cytosol. In most cases cytosol was diluted to 0.5–3.0 uterine equiv (0.5–3 mg of protein)/ml and serum to 1 mg of protein/ml just prior to use.

Competitive Binding Assay. The binding affinity of the iodinated hexestrols 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 ($K_a^{\text{estradiol}}/K_a^{\text{derivative}}$), was determined (Korenman, 1970), and the binding affinities were expressed as RAC × 100 values, which can be considered as percents of estradiol binding affinity.

Binding Assays. Sephadex G-25 Columns. The binding of [^{3}H]E_2[1] and [^{3}H]-I-Hex was measured by chromatography on Sephadex G-25 micro columns, prepared in Pasteur pipettes, patterned after Puca et al. (1971).

Charcoal–Dextran Adsorption. Rapid assay of [^{3}H]E_2 and [^{3}H]-I-Hex binding was accomplished using charcoal–dextran adsorption (Katzenellenbogen et al., 1973b). Incubations were generally conducted on microtiter plates, using a 10–30% (v/v) charcoal–dextran slurry, a 15-min adsorption time, and a 800*g* × 7 min centrifugation period. 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., 1973b).

Scatchard Analysis. The binding of [^{3}H]E_2 and [^{3}H]-I-Hex was determined in uterine cytosol over the range 1–10,000 n*M* by charcoal–dextran adsorption, after incubation at 0° for 18 hr. Scatchard plots (Scatchard, 1949) of the data 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

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

The *b/f* values were generally in the range 0.015–0.040.

Sucrose Density Gradient Centrifugation. Linear 5–20% sucrose density gradients were prepared in polyallomer tubes in TEA buffer. Sample preparation, centrifugation, and gradient fractionation are described in Figure 2 and in Katzenellenbogen et al. (1973a). No 8S (estrogen specific) peak was found when the gradients were run in Tris buffer (pH 8.9).

Polyacrylamide Gel Electrophoresis. The electrophoresis procedure was modified from Ornstein (1964) and Davis (1964): 7.5% polyacrylamide gels (70 × 6 mm), containing 0.22% *N,N'*-diallyltartardiamide (in place of methylenebisacrylamide), were polymerized with ammonium persulfate and run at room temperature (3 hr; 2 mA/gel). The analysis of electrophoretically separated components is described in the legend to Figure 3.

Incubation of Uteri, in vitro. Incubations of immature rat uteri, in vitro, were performed according to a previously described predure (Ruh et al., 1973), for the times indicated in Table II, footnote a, and Table III. For uptake measurements (Table II), the tissue was homogenized and separated into a washed nuclear pellet and a cytosol fraction (Ruh et al., 1973); nuclear-bound radioactivity was determined by ethanol extraction (Ruh et al., 1973) and cytosol binding by charcoal–dextran adsorption. For receptor translocation studies (Table III), the estrogen-specific binding capacity of the washed nuclear pellet was determined by a nuclear exchange assay (Anderson et al., 1972) and of the cytosol fraction by cytosol exchange (Katzenellenbogen et al., 1973a).

Induction and Measurement of the Relative Rate of Synthesis of a Specific, Uterine, Estrogen-Induced Protein (IP). Uteri from day 21–24 rats were incubated (37°, 1 hr) in Eagle's HeLa medium containing designated concentrations of 17β-estradiol, hexestrol, iodohexestrol, or vehicle control. Uteri were then allowed to incorporate amino acid (either [^{3}H]leucine for estrogen-stimulated uteri or [^{14}C]leucine for control uteri) for 2 hr at 37° in the presence of actinomycin D. Cytosol fractions prepared from combined (experimental plus control) uterine homogenates were analyzed by polyacrylamide gel electrophoresis. These methods have been described in detail previously by Katzenellenbogen and Gorski (1972).

Tissue Uptake of [^{3}H]-I-Hex, in Vivo. Mature (ca. 20-week old) female Sprague Dawley rats were ovariectomized, and 19 hr later injected with 36 μCi of [^{131}I]-I-Hex in 1 ml of 10% ethanol–0.9% saline. At the indicated times, the rats were exsanguinated; the tissues were excised and debrided, and the total tissue was either γ counted directly or extracted as follows. Tissues were homogenized in ether–methanol (2:1; 10 ml/0.5 g) in a Polytron P-10 homogenizer (Brinkman), left to extract overnight, and then centrifuged at 800*g* for 5 min. Aliquots of the clear or slightly yellow supernatant were taken for scintillation counting. Controls, in which the pellets were solubilized in Protosol, showed that extraction removed 70–95% of the total tissue radioactivity.

Metabolism Studies of [^{3}H]-I-Hex. Tissues excised from injected rats or uteri from in vitro incubations were either extracted as described above or were homogenized in TEA buffer, and the homogenate was precipitated with ethanol. After centrifutagion (800*g* for 10 min) the supernatant was evaporated under a gentle stream of nitrogen, redissolved in a minimum volume of anhydrous ether, and applied to a 15-cm silica gel chromatosheet together with ca. 10 μg of Hex and I-Hex. After development in methylene chloride (two developments), the markers were visualized with iodine vapor. The chromatogram was cut into 1.5-cm strips and placed in minivials with 0.5 ml of tetrahydrofuran for a few minutes, before the addition of 5 ml of scintillation fluid.

For a more rapid and convenient estimation of polar conjugates, 2 ml of the ether–methanol (2:1) extract was applied onto a 2-g silica gel (Brinkman) column (prepared in a 15-ml centrifuge tube with a hole blown out of the bot-

[1] Abbreviations and common names used in this paper are: hexestrol (Hex), *meso*-3,4-bis(4′-hydroxyphenyl)hexane; I-Hex, *erythro*-3-(3′-iodo-4′-hydroxyphenyl)-4-(4′-hydroxyphenyl)hexane; I_2s-Hex, *meso*-3,4-bis(3′-iodo-4′-hydroxyphenyl)hexane; I_2u-Hex, *erythro*-3-(3′,5′-diiodo-4′-hydroxyphenyl)-4-(4′-hydroxyphenyl)hexane; I_3-Hex, *erythro*-3-(3′,5′-diiodo-4′-hydroxyphenyl)-4-(3′-iodo-4′-hydroxyphenyl)hexane; I_4-Hex, *meso*-3,4-bis(3′,5′-diiodo-4′-hydroxyphenyl)hexane; estradiol (E_2), 1,3,5(10)-estratriene-3,17β-diol.

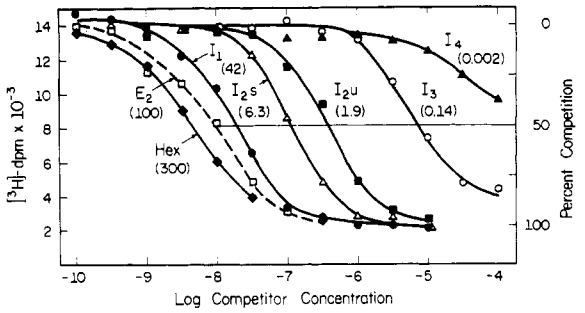

FIGURE 1: Competitive binding assay of iodohexestrols with rat uterine cytosol. This assay was conducted according to a previously described procedure (Katzenellenbogen et al., 1973b). Numbers in parentheses are RAC × 100, or percent of E_2 binding values.

Table I: Scatchard Analysis of [^{3}H]-3-Iodohexestrol Binding in Rat Tissue Cytosol and Serum.[a]

Tissue	$K_d \times 10^9$ (M^{-1})	Total Binding Sites (pmol/mg of Protein)[b]
Uterus	7.2	48
Kidney	5.9	27
Stomach	4.2	13
Liver	3.7	8
Serum	8.3	170

[a] Cytosols at 0.1–0.4 mg of protein/ml were incubated with varying concentrations of [^{3}H]-I-Hex for 16 hr at 0° in Tris buffer. Free ligand was removed by charcoal–dextran absorption. Scatchard analysis of the binding of [^{3}H]E_2 under these conditions indicated: $K_d = 0.14 \times 10^{-9}\ M^{-1}$; $P_0 = 1.7$ pmol/mg of protein. [b] Protein determinations by the method of Lowry (Lowry et al., 1951).

tom). Free, unmetabolized derivatives were eluted with 10 ml of the same solvent and polar conjugates with 5 ml of ethanol–2% ammonium hydroxide (1:1) (Schubert and Hobe, 1962). Column recoveries were quantitative, and TLC analysis showed that the free *vs.* conjugate separation was >95%.

Determination of the Uterotrophic Activities of the Iodinated Hexestrols. Immature standard Cox female mice, weighing 11–13 g, were injected subcutaneously daily for 3 days. On the day following the last injection, the mice were sacrificed and the uteri were quickly removed, carefully blotted, and weighed. All materials were dissolved or suspended (for high concentrations) in corn oil and a third of the total dose was given each day in 0.1 ml/mouse. The control group and the three standard estrone groups consisted of 10 mice each, whereas, each experimental group had 5 mice. The results are expressed as mean uterine weight ± the standard error. Statistical analysis was determined using the Student's *t* test.

Results

Characterization of the Binding Affinity of the Iodinated Hexestrols

Determination of the Binding Affinity to the Estrogen-Specific Binding Protein from Rat Uterus by a Competitive Binding Assay. A competitive protein binding assay, based on charcoal–dextran adsorption, is a convenient method for determining the binding affinity of various estrogen derivatives for the estrogen binding protein from rat uterus (Katzenellenbogen et al., 1973b). The competition curves determined by such an assay for estradiol, hexestrol, and the five iodinated hexestrols are shown in Figure 1. From the progressive displacement of these curves to the right, it is clear that iodine substitution decreases the binding affinity of the hexestrol ligand for the estrogen binding site. Introduction of each iodine atom lowers the binding affinity by approximately a factor of 10–20, and the symmetrical diiode derivative I_2s-Hex is bound with a substantially higher affinity than its unsymmetrical isomer I_2u-Hex. In terms of binding affinity, the most attractive derivative is I-Hex, with an equilibrium association constant 42% that of 17β-estradiol.

Direct Binding Studies. Scatchard Analysis of [^{3}H]-I-Hex Binding in Serum and Tissue Cytosols. Preliminary studies indicated that the solubility of [^{3}H]-I-Hex in pH 7.4 TEA buffer was exceedingly low, several orders of magnitude below that of estradiol (ca. 10^{-5} *M;* Hahnel, 1971). Solutions of [^{3}H]-I-Hex prepared at 10^{-9} *M* assayed correctly immediately after preparation, but after standing for 18 hr at 4° only a few percent of the counts remained in solution; vigorous remixing, however, did restore the original titer. Although solubility was improved by the addition of organic solvents (dimethylformamide, 10%; propylene glycol, 20%), proteins (bovine serum albumin, 0.75 mg/ml), or nonionic detergents (Tween 80—0.05%), all of which have only minimal effect on the estrogen specific binding activity found in rat uterine cytosol, we found it preferable to do quantitative binding measurements in Tris buffer at pH 8.9. The estrogen binding activity is reasonably stable at this pH (Talwar et al., 1968; Notides, 1970) and no solubility problems with [^{3}H]-I-Hex were encountered in the range 10^{-10}–10^{-7} *M*. The higher solubility at this pH is due most likely to a partial ionization of the relatively acidic phenolic hydroxyl on the iodinated ring (the pK_a of 2-iodo-*p*-cresol is 8.88 (Mayberry, 1967)).

Binding studies of [^{3}H]-I-Hex with rat uterine cytosol were first done using Sephadex G-25 micro columns. On these colums, estrogen binding activity elutes in the void volume, and its specificity and high affinity can be ascertained by competition with excess unlabeled E_2 or I-Hex. Column profiles of [^{3}H]-I-Hex were substantially different from those of [^{3}H]E_2; however, the binding capacity for [^{3}H]-I-Hex was several times greater than that for [^{3}H]E_2, and the major portion of the binding did not appear to be estrogen specific, as competition with E_2 had only minimal effect; competition by I-Hex was effective, however, indicating that the binding of [^{3}H]-I-Hex is of high affinity.

For convenience, further studies were done using the charcoal–dextran adsorption technique. Control studies showed that free [^{3}H]-I-Hex was removed from solution by charcoal within 3 min, while minimal dissociation of bound material occurred within 30 min.

Scatchard analysis (Scatchard, 1949) of the binding of [^{3}H]-I-Hex and [^{3}H]E_2 to uterine cytosol preparations was done using this technique. The equilibrium dissociation constant and binding capacity obtained for estradiol are in agreement with literature values: $K_d = 1.4 \times 10^{-10}\ M^{-1}$; $P_0 = 1.7$ pmol/mg of cytosol protein (Jensen and DeSombre, 1973). The amount of [^{3}H]-I-Hex bound with high affinity is much greater ($P_0 = 48$ pmol/mg of cytosol protein), as had been suggested by the preceding Sephadex G-25 experiments; its binding affinity, however, is about 50-fold lower than that of estradiol ($K_d = 7.2 \times 10^{-9}\ M^{-1}$).

Because the amount of [^{3}H]-I-Hex bound in the uterine cytosol is so much greater than the capacity of the specific

estrogen binding protein, we felt that most of the binding might be due to a serum protein or a cytoplasmic protein common to different tissues. Table I summarizes the results of binding measurements (by Scatchard analysis) on serum and on several tissue cytosols. In each case the equilibrium dissociation constants are essentially the same, although the binding capacities do vary considerably from tissue to tissue.

Analysis of Binding by Sucrose Density Gradient Centrifugation. Because the binding of [^{3}H]-I-Hex to other cytosol components is so extensive, direct binding measurements on whole uterine cytosol using Sephadex G-25 chromatography or charcoal-dextran adsorption failed to indicate whether [^{3}H]-I-Hex is actually binding to the specific estrogen binding sites. Sucrose density gradient centrifugation (Chamness and McGuire, 1972; Stancel et al., 1973) is a technique that has reliably been used to distinguish estradiol-specific binding from lower affinity binding to other cytosol components (generally termed nonspecific binding; Toft et al., 1967). The application of this technique to the binding of [^{3}H]-I-Hex is shown in Figure 2.

On low salt, 5–20% sucrose density gradients, estradiol binding activity sediments as a broadened peak at ca. 8 S; very little binding is evident elsewhere in the gradients (Figure 2A). [^{3}H]-I-Hex too shows a binding peak coincident with the 8S estradiol-specific peak, but the major portion of bound ligand sediments at 4 S. Gradient studies by others (Toft et al., 1967) have shown that low affinity, nonspecific binding is generally found in the 4–5 S region of 5–20% sucrose gradients.

It is evident from Figure 2B why [^{3}H]-I-Hex binding in whole uterine cytosol was not subject to competition by estradiol: addition of excess estradiol does abolish the binding of [^{3}H]-I-Hex to the 8S peak (evidence for the estrogen-specific nature of this binding), but fails to diminish the larger 4S binding. High concentrations of I-Hex, however, abolish both 4S and 8S [^{3}H]-I-Hex binding, which is consistent with the high binding affinity noted for I-Hex in both the competition (Figure 1) and the direct binding studies.

In order to examine the saturability (i.e., relative affinity) of I-Hex binding to the 4S and 8S species in uterine cytosol, a series of gradients were run using progressively increasing concentrations of [^{3}H]-I-Hex (from 10 to 1000 n*M;* data not shown). Saturation of the 8S binding at 1.1 pmol/uterine equiv was reached at 30 n*M,* while the 4S binding was much greater (ca. 120 pmol/uterine equiv), and saturation did not become apparent until concentrations exceeded 300 n*M*.

In gradients run with serum (not shown), no 8S binding is found for either [^{3}H]E_2 or [^{3}H]-I-Hex; both compounds, however, do show 4S binding. Others have noted (King and Mainwaring, 1974) that the 4S [^{3}H]E_2 binding in serum is of quite high affinity, and is thus competible with excess estradiol. The [^{3}H]-I-Hex binding in serum is competible by I-Hex, but not by E_2, as was true for its binding to the 4S component in uterine cytosol.

Analysis of [^{3}H]-I-Hex Binding by Polyacrylamide Gel Electrophoresis. Although the sucrose density gradient studies gave clear indication that [^{3}H]-I-Hex binds to the 8S, estrogen-specific binding protein from rat uterus, they also revealed that most of the binding occurred to proteins in the 4S region of the gradient. As many proteins in serum and cytosol have sedimentation coefficients in the range 3–5 S (Sober, 1970), we sought to investigate further the nature of this binding by electrophoresis.

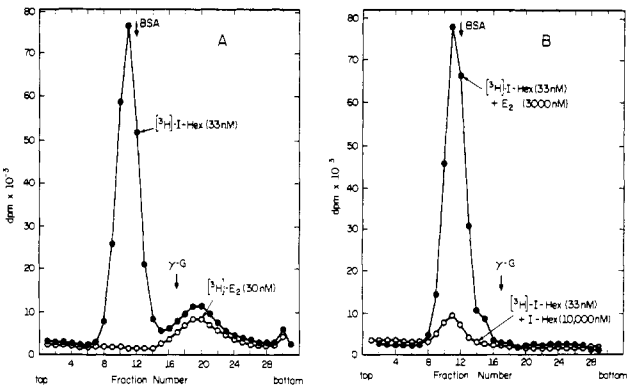

FIGURE 2: Sucrose density gradient centrifugation analysis of [^{3}H]E_2 and [^{3}H]-I-Hex binding in rat uterine cytosol. Samples of rat uterine cytosol (200 μl; 0.45 uterine equiv) were incubated with the indicated concentration of [^{3}H]- or unlabeled E_2 or I-Hex for 5 hr at 0°, charcoal-dextran treated, and then layered onto 3.6-ml 5–20% linear sucrose density gradients and centrifuged at 246,000*g* for 13 hr. Gradients were fractionated from the top by displacement with 50% sucrose, and 3-drop fractions were collected for scintillation counting. Carbon-14 labeled bovine serum albumin (4.7 S) and γ-globulin (7.0 S; Sober, 1970) were included as markers in some runs.

Figure 3 shows polyacrylamide gel electrophoresis profiles of rat uterine cytosol and serum proteins. Each panel shows a profile for bound I-Hex (tritium pattern) and two profiles for total protein (optical trace after fast green staining and carbon-14 pattern).

Within the resolution limits of our gel system, the major protein components in both serum and uterine cytosol (by both optical scanning and carbon-14) have identical electrophoretic mobilities and are presumed to be albumin (Peck et al., 1973). [^{3}H]-I-Hex binding in uterine cytosol appears as two closely migrating peaks which run slightly ahead of albumin (Figure 3A). In serum, two somewhat more widely separated [^{3}H]-I-Hex binding peaks are seen; the slower migrating one appears coincident with albumin (Figure 3B). Both peaks of [^{3}H]-I-Hex binding activity in serum and uterine cytosol are effectively competed by I-Hex, but competition by E_2 has little effect. Electrophoretic profiles of [^{3}H]-I-Hex binding in kidney cytosol (not shown) are nearly identical with those seen in uterine cytosol.

The electrophoretic mobility of the [^{3}H]-I-Hex binding activity is inconsistent with that of two serum hormone-binding globulins, sex steroid binding globulin (Westphal, 1970; Corvol et al., 1971) and thyroxine binding globulin (Sterling et al., 1971), and the cytosol thyroxine-binding protein described recently in the dog (Davis et al., 1974) all of which migrate behind albumin. Its sedimentation velocity and electrophoretic mobility are, however, suggestive of that of prealbumin (Tanabe et al., 1969; Raz and Goodman, 1969), which is known to bind thyroxine and a variety of other iodinated phenols with high affinity (Pages et al., 1973). Indeed, incubation of both uterine cytosol and serum with 10^{-4}–10^{-5} *M* thyroxine or triiodothyronine, prior to the addition of [^{3}H]-I-Hex, reduces the binding of [^{3}H]-I-Hex by 50–70%, as measured by charcoal-dextran adsorption. (Under these conditions [^{3}H]E_2 binding is unaffected.) Furthermore, double-labeled gel electrophoresis shows that the peaks of [^{3}H]-I-Hex binding activity in serum correspond in mobility with those for ^{14}C binding (Figure 3C); the major thyroxine binding under these conditions is to albumin. A similar pattern (not shown) is seen on electrophoretic analysis of uterine cytosol.

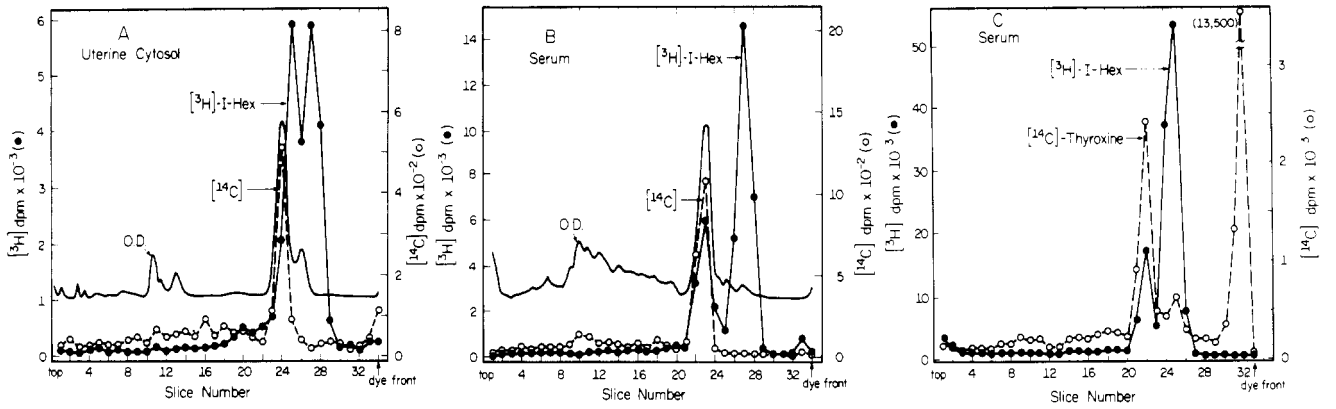

FIGURE 3: Gel electrophoretic analysis of [^{3}H]-I-Hex binding to rat uterine cytosol and serum. A sample of rat uterine cytosol (A; 150 μl; 0.5 uterine equiv) or rat serum (B; 150 μl, 1 mg/ml) was incubated with 10 nM [^{3}H]-I-Hex for 1 hr at 0°, or a sample of rat serum (450 μl; 0.5 mg/ml) was incubated with 10 nM [^{3}H]-I-Hex and 1.5×10^{-5} M [^{14}C]thyroxine for 1 hr at 0° (C). The samples were charcoal-dextran treated; carbon-14 labeled cytosol proteins were added, and the mixtures were layered onto a 7.5% polyacrylamide gel (see Experimental Section). For optical tracing, gels were stained with 1% Fast Green in 7.5% acetic acid, destained in 7.5% acetic acid, and scanned at 650 nm on a Beckman DU equipped with a Gilford linear transport system. For counting, gels were frozen on Dry Ice, sliced into 2.3-mm sections, and dissolved in 0.5 ml of 2% aqueous periodic acid. Optical trace and [^{3}H]-I-Hex counts are from different gels, since [^{3}H]-I-Hex binding does not survive the staining procedure. Both gels have the same carbon-14 peak, which is coincident with the major stained band. The large carbon-14 peak near the dye front in panel C is due to free [^{14}C]thyroxine.

Table II: Uptake of [^{3}H]Estradiol and [^{3}H]-I-Hex by Uterine Cytosol and Nuclei in Vitro.

Concn (nM)				Binding (pmol/uterine equiv ± SEM)[a]	
[^{3}H]E_2	[^{3}H]-I-Hex	E_2	I-Hex	Washed Nuclear Pellet	Cytosol (C-D Treated)
1. 30				1.63 ± 0.12 [2.30 ± 0.56][b]	0.17 ± 0.03 [0.23 ± 0.04][b]
2. 30		3000		0.52 ± 0.14 [0.40 ± 0.11]	0.09 ± 0.03 [0.05 ± 0.01]
3. 30			10,000	1.13 ± 0.10 [0.49 ± 0.16]	0.14 ± 0.03 [0.15 ± 0.08]
4.	100			2.16 ± 0.73	0.70 ± 0.02
5.	100	3000		2.86 ± 0.54	1.02 ± 0.26
6.	100		10,000	9.95 ± 0.07	0.40 ± 0.02

[a] Uteri are incubated at 37° in Eagle's HeLa medium either for 1 hr (with both radiolabeled and nonlabeled derivative) or for 2 hr (first hour with nonlabeled derivative, second hour with radiolabeled derivative, square brackets). Values are the mean of 2–4 incubations ± standard error of the mean. See Experimental Section for the details of incubation and fractionation procedures. [b] For data shown in square bracket (entry 1, only), [^{3}H]E_2 (30 nM) was present throughout the 2-hr incubation.

Characterization of the Estrogenic Activity of the Iodinated Hexestrols

Uptake and Metabolism of [^{3}H]Iodohexestrol by the Uterus in Vitro. Many studies have shown that incubation of immature rat uteri with [^{3}H]E_2 at 37°, under in vitro conditions, results in a rapid uptake of labeled steroid by the tissue (Jensen and DeSombre, 1973). This uptake is specific for estrogens and is limited in capacity; so it can be blocked by the addition of a large excess of unlabeled E_2. Within 1 hr, the bulk of the labeled E_2 is found in the nuclear pellet fraction, and the extent of metabolism is negligible. The ability of I-Hex to compete for estrogen binding sites in uterine cytosol and of [^{3}H]-I-Hex to bind to the estrogen-specific binding protein (8S peak on sucrose gradients) suggested that it too might be taken up by uterine nuclei. To pursue this point, we investigated in an in vitro uterine organ culture system, the nuclear uptake of I-Hex by direct binding, uptake competition, and receptor translocation. The results are given in Tables II and III.

Incubation of uteri at 37° for 1 hr with 30 nM [^{3}H]E_2 results in nuclear uptake of *ca.* 1.6 pmol of steroid/uterus (Table II, entry 1); about 1 pmol of this is E_2 specific, as indicated by the competitive effect observed when incubation is conducted with a 100-fold excess of unlabled E_2 (entry 2). Competition by I-Hex (entry 3), though less effective than E_2, does significantly reduce the uptake of [^{3}H]E_2.

The lower competitive effectiveness of I-Hex vs. E_2 may be due in part to differences in the kinetics of their uptake. In fact, Milgrom et al. (1973) have shown that uterine uptake of estrogens appears to be assisted by a specific transport system that facilitates the cellular entry of steroidal, but not nonsteroidal estrogens. When the uteri are incubated with the competitors for 1 hr, prior to the addition of [^{3}H]E_2 (Table II, square brackets, entries 1–3), conditions under which kinetic differences between the two compounds are minimized, E_2 and I-Hex are equally effective as competitors. The low levels of cytoplasmic binding found in these incubations are consistent with the near complete translocation of estrogen binding sites to the nucleus.

The results of [^{3}H]-I-Hex uptake by uteri (Table II, entries 4–6) are less clear. Although reasonable quantities of [^{3}H]-I-Hex are found to be nuclear bound after 1 hr at 37°, the uptake values were generally less reproducible than with [^{3}H]E_2. Addition of excess unlabeled E_2 or I-Hex, however, failed to reduce the nuclear uptake, which suggests that low affinity, high capacity binding processes are predominating. Furthermore, no macromolecularly bound radioactivity is evident when the washed nuclear pellet from entry 4 was extracted with 0.4 M KCl and analyzed by sucrose density gradient centrifugation, conditions under which specifically bound [^{3}H]E_2 sediments as a 5S

Table III: Translocation of Estrogen-Specific Binding Capacity, in Vitro.

Compd	Concn (n*M*)	Incubation Conditions	Specific Exchange Capacity (pmol/uterine equiv)[a] Nuclear	Cytoplasmic
1.		(unincubated)	0.30 ± 0.03	1.57 ± 0.04
2.		1 hr, 37°	0.26 ± 0.05	1.10 ± 0.04
3. E_2	30	1 hr, 37°	1.10 ± 0.15	0.06 ± 0.02
4. E_2	30	1 hr, 0°	0.36 ± 0.02	1.24 ± 0.03
5. I-Hex	100	1 hr, 37°	0.83 ± 0.03	0.24 ± 0.09
6. I-Hex	100	1 hr, 0°	0.37 ± 0.07	1.19 ± 0.07
7. [^{3}H]E_2/E_2	30/3000	1 hr, 37°	1.52[b]	0.24[b]

[a] Uteri are incubated in Eagles Hela medium. Tissue fractionation and determination of exchange capacities are detailed in the Experimental Section. The values are the mean of duplicate or quadruplicate determinations, ± the range or standard error of the mean. [b] Measured by direct uptake of [^{3}H]E_2, as in Table II, entry 1–entry 2.

complex (Jensen and DeSombre, 1973).

Two other features of the I-Hex uptake data are of note in light of the results of the preceding solubility and binding studies. (a) Competition with excess I-Hex (entry 6) consistently resulted in grossly elevated amounts of nuclear binding; this may represent a precipitation phenomenon resulting from the low water solubilities of the iodinated derivatives at neutral pH. Attempts to minimize this apparent precipitation effect, by competing with lower concentrations of I-Hex, also failed to demonstrate the saturability of nuclear uptake (data not shown). (b) Although E_2 does not compete with [^{3}H]-I-Hex bound in cytosol (entry 5 vs. 4), significant competition with I-Hex is observed (entry 6 vs. 4), consistent with the high affinity binding of [^{3}H]-I-Hex to cytoplasmic components other than the estrogen-specific binding protein.

Another approach to measuring estrogen-specific steroid uptake by uterine nuclei is to use exchange assays to monitor the movement or translocation of receptor sites from the cytoplasmic (Katzenellenbogen, 1973a) into the nuclear (Anderson et al., 1972) compartment. The results from such studies are shown in Table III. The bulk of the receptor is cytoplasmic in uteri which have not been incubated or have been incubated for 1 hr at 37° in the absence of steroid (entries 1 and 2). Estradiol-induced receptor translocation to the nucleus can be noted after 1 hr at 37° (entry 3), but not after 1 hr at 0° (entry 4) (Williams and Gorski, 1971); the nuclear exchange assay does not appear to measure the full nuclear binding capacity (entry 3 vs. 7), but the translocation is clearly evidenced by a depletion of cytoplasmic receptor. Exposure to I-Hex results in a similar temperature dependent nuclear translocation (entries 5 and 6).

Chromatographic analysis of extracts from the three subcellular fractions, cytosol, high-speed (mitochondrial/microsomal) pellet, and nuclear pellet, showed that the extent of metabolism of [^{3}H]-I-Hex during the 1-hr uptake period (37°) was negligible; in particular, no [^{3}H]Hex, from deiodination of [^{3}H]-I-Hex, is evident. Therefore, none of the above results can be ascribed to Hex, and in terms of [^{3}H]E_2 uptake competition and estrogen receptor translocation, I-Hex is behaving as an estrogen.

Stimulation of the Synthesis of an Estrogen-Induced Protein (IP), in Vitro. The synthesis of a specific protein in rat uterus is stimulated by the administration of 17β-estradiol (Notides and Gorski, 1966). This induced protein (IP) is detectable within 1 hr after hormone administration, and the response can be obtained in an in vitro incubation (Katzenellenbogen and Gorski, 1972). Earlier studies (Katzenellenbogen and Gorski, 1972; Ruh et al., 1973)

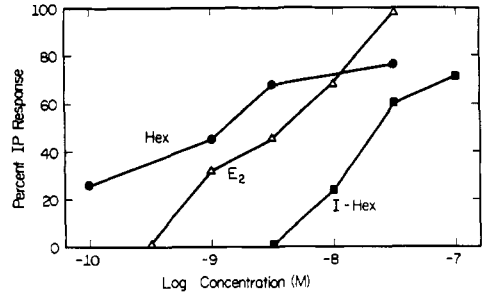

FIGURE 4: Dose–response curves for the induction of the synthesis of induced protein (IP) by Hex, E_2, and I-Hex, in vitro. Rat uteri were incubated for 1 hr at 37° in Eagle's HeLa medium under 95% O_2–5% CO_2, with the concentrations of derivatives shown, and were then transferred to flasks containing either [^{3}H]-L-leucine or [^{14}C]-L-leucine, both with actinomycin D, and incubated an additional 2 hr at 37°. Quantitation of the relative rate of IP synthesis are found in Katzenellenbogen and Gorski (1972). Values are the mean of two closely corresponding determinations; with the response obtained with 3×10^{-8} *M* E_2 considered 100%.

have demonstrated the parallel relationship between (1) the binding affinity of different estrogenic ligands and their ability to stimulate IP synthesis and (2) the quantity of estrogen-filled nuclear receptor sites and the magnitude of the IP response.

We have used this in vitro IP induction system as another means of characterizing the estrogenic nature of 3-iodohexestrol. Figure 4 shows the concentration dependence of the IP response with E_2, Hex, and I-Hex. It is apparent that the concentration of Hex, E_2, and I-Hex required to attain half-maximal induction roughly parallels the binding affinities of these ligands (cf. Figure 1). In vitro metabolism studies (see above) have shown that no conversion of I-Hex to Hex occurs during this 1 hr in vitro incubation.

In Vivo Uptake and Uterotrophic Activity of Iodinated Hexestrols. The time course of radioactivity uptake by various tissues following an intravenous injection of [^{131}I]-I-Hex into mature, ovariectomized rats is shown in Table IV. It is apparent that the pattern of uptake and retention by the uterus, pituitary, and mammary tissue differs little from that of control tissues, such as liver, stomach, kidney, and spleen. The major portion of radioactivity is found in the gut (and gut contents, not shown) where it appears initially in the small intestine and later in the colon and cecum; pronounced enterohepatic circulation is typical for estrogen distribution and elimination (Slaunwhite et al., 1973) and was previously noted by Albert et al. (1949) in his study of iodinated estradiols. From the progressively increasing uptake by the thyroid, it is apparent that some free iodide is being produced through metabolism; the thyroid uptake,

Table IV: Tissue Uptake [^{131}I]Iodohexestrol, in Vivo.[a]

Tissue	Tissue Content (cpm/g) 2 hr	6 hr	26 hr
Uterus	47,056	31,449	7,926
Pituitary	45,819	25,727	3,134
Mammary	27,091	12,421	3,402
Liver	58,837	33,167	14,547
Stomach	58,497	48,161	11,535
Kidney	24,207	14,759	4,420
Spleen	16,706	8,029	4,322
Small intestine[b]	350,046	135,819	56,128
Colon/cecum[b]	29,828	193,871	44,333
Thyroid	119,595	225,679	603,868

[a] Female rats (20-weeks old) were ovariectomized 19 hr prior to i.v. injection of 37 μCi of [^{131}I]-I-Hex in 1 ml of ethanol–saline. At the indicated times, the rats were exsanguinated and the tissues removed for γ counting. Three rats were used at each time point; data are the average value. [b] Washed free of contents with 0.9% saline prior to counting.

Table V: The Uterotrophic Activity of Hexestrol and Iodinated Hexestrols.[a]

Group	Total Dose (μg in 3 days)	N	Mean Uterine Weight ± SE
Control		10	8.5 ± 0.8
Estrone	0.3	10	48.9 ± 2.6[b]
	0.1	10	19.3 ± 1.3[b]
	0.03	10	9.3 ± 0.4
Hex	0.15	5	35.1 ± 4.8[b]
	0.015	5	8.9 ± 0.5
I_1-Hex	1.5	5	34.6 ± 5.6[b]
	0.15	5	20.3 ± 1.8[b]
I_2s-Hex	15.0	5	49.1 ± 3.9[b]
	1.5	5	15.2 ± 1.9[c]
I_2u-Hex	45.0	5	15.6 ± 3.5
	4.5	5	8.8 ± 1.4
I_3-Hex	750	5	20.5 ± 3.2[b]
	75	5	8.9 ± 1.5
I_4-Hex	3000	5	50.0 ± 3.9[b]
	300	5	32.5 ± 5.1[b]

[a] Immature female mice were injected daily for 3 days with the dose indicated. The mean uterine weight is given in mg ± SE. [b] $P < 0.01$. [c] $P < 0.05$.

however, represents less than 0.5% of the injected dose.

A similar pattern of 2 hr uptake is found in the immature, intact female rat, using either [^{3}H]-, [^{125}I]- or [^{131}I]-I-Hex (W. L. McGuire and J. A. Katzenellenbogen, unpublished observations). Under conditions where the uptake of [^{3}H]E_2 by the uterus is highly selective (30–60 times control tissue uptake) and estrogen-specific (competable by unlabeled E_2), the uptake of [^{3}H]-I-Hex by the uterus is neither selective, nor estrogen competable.

In order to assess the extent to which the tissue-bound radioactivity is comprised of conjugates and metabolites of the original compound, aliquots of the tissue extracts were chromatographed (silica gel, thin-layer, or column chromatography). At 2 hr after injection, free iodohexestrol constituted 60–75% of radioactivity in the ether–methanol (2:1) extract of uterus and control tissues, but only 25–30% of that extractable from the intestine or intestinal contents; no free hexestrol was found, and the remaining material was very polar (presumed to be sulfates and glucuronides) and was not investigated further.

In one instance, the fate of a double-labeled preparation ([^{3}H]- and [^{131}I]-I-Hex) was examined. The isotopic content of the tissue extracts at 2 hr, determined by differential liquid scintillation counting, indicated that metabolic deiodination is a very minor process.

The results from an investigation of the uterotrophic activities (3 day uterine weight gain assay) of Hex and the five iodinated derivatives is shown in Table V. A dose of 0.15 μg was selected for Hex which, based on previous experience, would fall in the upper part of the dose response curve. One-tenth this dose was arbitrarily selected to establish a minimum or ineffective dose. The dosage level for each derivative with the exception of I_4-Hex was adjusted according to its RAC value and molecular weight, to be equivalent to Hex. Practical considerations made it impossible to administer I_4-Hex at an estimated dose of 60 mg/mouse. The effectiveness of most derivatives in promoting uterine growth parallels their binding affinity, although on this basis, I_2u-Hex and I_3-Hex appear somewhat weaker than predicted and I_4 somewhat stronger. The possible effects of metabolism in this long term weight gain assay were not investigated.

Discussion

Two potential uses for an iodinated estrogen can be considered (Katzenellenbogen and Hsiung, 1975): (1) to study the details of the subcellular movement of estrogen binding proteins (Williams and Gorski, 1973) using two radioisotope tracers (tritium and iodine-131), and (2) to localize estrogen responsive tissues using γ radioimaging techniques. If an iodinated estrogen derivative is to be utilized for these purposes, it must fulfill three criteria : (1) the linkage of the iodine atom to the ligand must be chemically and metabolically stable; (2) the site of iodine substitution must be such that the derivative is still capable of binding with high affinity to the specific-estrogen binding proteins found in estrogen target tissues, and (3) the physicochemical properties of the derivative (polarity, etc.) must be such that it does not bind excessively to the low affinity, high capacity binding proteins found in serum and tissues. (A similar analysis of the factors required for high biological potency of estrogens has been presented recently by Raynaud et al., 1973.)

Aliphatic iodine compounds have a high susceptibility toward substitution and elimination reactions (Gould, 1959); therefore, on the grounds of chemical stability, we considered it preferable to have the iodine bonded to an aryl group. The preparation of both 2- and 4-monoiodo-17β-estradiol and 2,4-diiodo-17β-estradiol (Albert et al., 1949; Hillmann-Elies et al., 1953; Nambara et al., 1971; Matkovics et al., 1971) had been reported. Although the iodine in these derivatives appeared to be metabolically stable, these compounds showed either low or negligible uterotrophic activity (Albert et al., 1949; Hillmann-Elies, 1953; Peck et al., 1961), and they were not taken up selectively by the uterus (Albert et al., 1949). It is not certain whether this was the result of the failure to satisfy criteria 2 or 3, but as the estrogen receptor is known to be relatively intolerant of bulk at A-ring positions 2 and 4 (Katzenellenbogen et al., 1973b), it appeared that functionalization of these positions in a steroidal ligand was not consistent with high receptor binding (criterion 2).

Keeping in mind the desirable stability of an aryl iodine linkage and high binding affinity, we considered accommodating the iodine at the 3 position in hexestrol (ortho to hydroxyl). This nonsteroidal estrogen has a higher binding affinity for the estrogen binding protein from rat uterus than

17β-estradiol itself. In addition, its symmetry and conformational flexibility enable a substituent to adopt a number of alternative orientations in a binding site (Katzenellenbogen et al., 1973b). Earlier studies by us (Katzenellenbogen et al., 1973b) had established that bulky groups (NO_2, NH_2, N_3) at hexestrol position 3 were tolerated quite well by the rat uterine binding protein. Furthermore, quantitative comparison of these hexestrol derivatives with their analogs in the estradiol series (2- and 4-substituted) suggested that the hexestrol derivative is actually bound in an orientation in which its substituent projects into a region of the binding protein which is normally complementary to the D-ring of estradiol (Katzenellenbogen et al., 1973b). Of the five ortho-iodinated hexestrol derivatives we have prepared, 3-iodohexestrol (I-Hex) was shown to have, by competitive binding studies, the highest affinity for the estrogen specific binding protein from rat uterus. As it appeared that I-Hex satisfied criteria 1 and 2, subsequent studies were focused on this derivative.

The ability of I-Hex to bind to the estrogen receptor could be demonstrated indirectly in a variety of ways: Competition for $[^3H]E_2$ binding in uterine cytosol, blockage of $[^3H]E_2$ uptake in in vitro uterine organ culture, movement of estrogen receptors from cytoplasmic to nuclear compartment and temperature dependence of this movement, and the stimulation of uterine growth and IP synthesis.

Although introduction of an iodine at position 3 in hexestrol caused only a modest decrease in its binding affinity toward the specific estrogen receptor, it had a more profound effect on other physical properties. The iodine substitution caused a precipitous drop in the water solubility of the ligand at neutrality, and even though reasonable solubilities could be attained at higher pH, the binding of the derivative to other components found in serum and tissue cytosol preparations was greatly enhanced (criterion 3 not satisfied). This is demonstrated most clearly by the sucrose density sedimentation profiles.

As a result of the failure of I-Hex to satisfy criterion 3 it is not surprising that selective, estrogen-specific uptake by the uterus could not be demonstrated either in vitro or in vivo. We will be continuing our investigation by attempting to prepare iodinated estrogen analogs and derivatives which will adequately satisfy all three criteria needed to achieve selective uptake by uterine tissue.

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Steroidal 21-Diazo Ketones: Photogenerated Corticosteroid Receptor Labels†

Manfred E. Wolff, David Feldman,‡ Panayotis Catsoulacos,§ John W. Funder,# Curtis Hancock,‡‡ Young Amano,§§ and Isidore S. Edelman*

ABSTRACT: The 21-diazo derivatives of 9α-fluoro- and 9α-bromo-21-deoxycorticosterone, 21-deoxycorticosterone, and progesterone were synthesized for use as photoaffinity labels for corticosteroid receptors. In the isolated toad bladder system, 9α-bromo-21-diazo-21-deoxycorticosterone was as active as *d*-aldosterone and more active than 9α-fluorocortisol in augmenting active Na^+ transport. The activities of 21-diazoprogesterone and progesterone were equal; both were much less potent than *d*-aldosterone, however. These results indicate that the 21-diazo derivatives had significant functional activity in the toad bladder system. The rat kidney slice system was used to estimate the relative affinities of the diazo steroids for aldosterone receptor sites by competition experiments. At 100-fold excess of competitor to [^{3}H]aldosterone, the order of affinities was 9α-fluoro-21-diazo-21-deoxycorticosterone > 9α-bromo-21-diazo-21-deoxycorticosterone > 21-diazoprogesterone. Moreover, 9α-bromo-21-diazo-21-deoxycorticosterone reduced binding of [^{3}H]aldosterone to cytoplasmic and nuclear forms of the receptor proportionately. On the basis of competition for [^{3}H]corticosterone binding, presumably to corticosteroid-binding globulin (CBG), the order of affinities was 21-diazo-21-deoxycorticosterone > 21-diazoprogesterone > 9α-bromo-21-diazo-21-deoxycorticosterone. These findings indicate that 21-diazo steroids may be suitable as photogenerated affinity labels for mineralocorticoid receptors. The tritiated derivative, [1,2-^{3}H]-9α-bromo-21-diazo-21-deoxycorticosterone (specific activity 25 Ci/mol) was synthesized and used in model experiments on photogenerated covalent binding to rat plasma proteins. Irradiation with uv light resulted in binding of [1,2-^{3}H]-9α-bromo-21-diazo-21-deoxycorticosterone to plasma proteins, that was resistant to extraction with methylene dichloride and did not exchange with unlabeled corticosterone. The diazocorticosteroids, therefore, may have the requisite functional and selectivity properties for photoaffinity labeling of corticosteroid-binding proteins. Further studies are needed, however, to assure that photogenerated labeling with these steroids was site specific.

A growing body of evidence indicates that induction of protein synthesis mediates the action of steroid hormones on growth, differentiation, and metabolism in target tissues (Feldman et al., 1972). The initial events involve binding to a steroid-specific receptor protein and attachment of the resulting complex to the genome.

Cytoplasmic receptors, characterized by specificity in binding steroid hormones with high affinity, have been demonstrated for all of the physiological steroids. Partial success has been achieved in the isolation of stereospecific aldosterone binding proteins from rat kidney (Herman et al., 1968). Use of affinity chromatography to purify these receptors, however, has been frustrated by release of the steroid ligand from the agarose matrix on addition of the cytosol fractions (Ludens et al., 1972). Recently, significant progress has been reported in the purification of uterine estrogen receptors by affinity chromatography (Sica et al., 1973). The lability of the corticosteroid receptor complexes and in particular the susceptibility of the aldosterone receptors to irreversible loss of binding activity, however, may compromise the application of this method to purification of corticosteroid receptors (Herman and Edelman, 1968).

Purification of corticosteroid receptors should be facilitated by covalent linkage of a labeled analog to the receptor. Affinity-labeled receptors could be purified by rigorous

† From the Departments of Pharmaceutical Chemistry, of Medicine, and of Biochemistry and Biophysics, and the Cardiovascular Research Institute of the University of California, San Francisco, California 94143. *Received August 15, 1974.* This work was supported by National Institute of Arthritis, Metabolism and Digestive Diseases, Grant No. AM-14824, and National Heart and Lung Institute, Grant No HL-06285.

‡ During the tenure of a Postdoctoral Traineeship of the National Heart and Lung Institute, Grant No. HL-05725. Present address: Department of Medicine, Stanford University School of Medicine, Palo Alto, California 94305.

§ Present address: Laboratory of Pharmaceutical Chemistry, University of Athens, Athens, Greece.

During the tenure of a National Heart Foundation of Australia Overseas Research Fellowship. Present address: Medical Research Centre, Prince Henry's Hospital, St. Kilda Road, Melbourne, Vic. 3004, Australia.

‡‡ Present address: School of Medicine, University of Wisconsin, Madison, Wisconsin 53706.

§§ During the tenure of a Postdoctoral Traineeship of the National Heart and Lung Institute, Grant No. HL-05725.