MOLECULAR PHARMACOLOGY, 8, 438-445

X-ray Crystallography of Estrogens and Their Binding to Receptor Sites

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(Received February 22, 1972)

SUMMARY

Estradiol, a natural estrogen and a relatively rigid molecule, and diethylstilbestrol, a synthetic, nonsteroidal estrogen and more flexible molecule, compete for the same sites of the uterus estrogen receptors in cytosol and non-histone chromatin protein preparations. When crystallized under certain conditions, these compounds have very different over-all structures. The stilbene derivative possesses a much thicker, more symmetrical structure, with a phenol group at each extremity of the molecule, and it is difficult to reconcile the difference in shape of these two estrogens with binding to the same receptor sites. However, when diethylstilbestrol is crystallized from methanol–water and from ethanol, each molecule is linked to 1 alcohol and 1 water molecule in the former case, and with a molecule of ethanol by two different hydrogen bonds in the latter. The molecule thus becomes asymmetrical and assumes a conformation more closely simulating estradiol, with two distinguishable oxygen-hydrogen bonds.

It is proposed that the solvated asymmetrical form of diethylstilbestrol resembles more closely its "active" conformation when present in receptor binding sites. This concept is compatible with the estrogenic activity and binding affinity of (a) the estradiol derivatives (11β-methoxy-7α-methyl), which closely resemble asymmetrical diethylstilbestrol structurally, and (b) the stilbene derivatives (4,4′-dihydroxy-7-ethyl-6′,7′-dimethylstilbene and 4,4′-dihydroxy-7′-ethyl-6′,7′-dimethylstilbene), which are more like estradiol in structure.

INTRODUCTION

The estrogenic activity of diethylstilbestrol, (4,4′-dihydroxy-*trans*-7,7′-diethylstilbene) a synthetic, nonsteroidal compound (1) that was the first effective tool employed in cancer chemotherapy (2), has been well established. In all species diethylstilbestrol gives rise to the same effects as the natural

These experiments were partially supported by the Délégation Générale à la Recherche Scientifique et Technique, la Fondation pour la Recherche Médicale Française, and the Ford Foundation.

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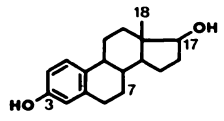
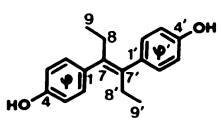


Estradiol



Diethylstilbestrol

estrogen, estradiol, thereby suggesting a "qualitative" similarity between the two compounds. It is therefore logical to assume that they are bound to the same hormone receptor sites (3), and it has been shown that both compounds have the same equilibrium association constant for binding to the cytosol receptor protein from pig uterus (4). Any "quantitative" differences in activity, as given by the ratio of the estradiol and diethylstilbestrol doses required to induce the same uterine weight increase, may be ascribed to different metabolic fates in the living animal and/or to different affinities for she same receptor sites.

Estradiol, unlike diethylstilbestrol, is a relatively rigid molecule. Their structures reveal several similarities,[5] which are generally thought to account for their identical binding to receptor sites. We report here that detailed structural studies on both compounds crystallized from different solvent systems have yielded more precise information. Whereas estradiol has only one structure, diethylstilbestrol has two: a symmetrical structure in solvent-free crystals, and an asymmetrical structure in alcohol-containing crystals. Several arguments point to a similarity between one of the two enantiomorphs of the asymmetrical form and the conformation that diethylstilbestrol might adopt when bound to estrogen receptors. The asymmetrical form could thus be considered to resemble more closely the "active" form of this nonsteroidal estrogen than the symmetrical one.

MATERIALS AND METHODS

The identity of estradiol and diethylstilbestrol was checked by melting point determination and X-ray powder diagrams, and their purity was ascertained by thin-layer chromatography (at least 99% pure).

[5] Such an analogy has been contested on purely chemical grounds (5).

The crystal structures of estradiol and diethylstilbestrol were analyzed by X-ray diffraction, using an automatic diffractometer and CuK_α radiation. Initial estimates, obtained either by direct-phase determination, by the study of the Patterson function, or by direct minimization, were refined by computer analysis using a program adapted for an IBM 360-44 system (6).[6]

RESULTS

Table 1 gives the values for parameters describing the crystal structures of estradiol and diethylstilbestrol containing various solvent molecules.

Estradiol. When estradiol was crystallized from methanol or ethanol, the crystals contained 1 water molecule for every 2 estradiol molecules. The crystals were orthorhombic and belonged to the $P2_12_12$ space group (7). Moreover, the lengths of the intermolecular O_3— and O_{17}—hydrogen bonds were different, as well as those of the intramolecular C_3— and C_{17}—oxygen bonds (Table 2).

Crystallization of anhydrous estradiol from solutions of varying propanol–H_2O ratios gave a crystal of the $P2_12_12_1$ space group containing 1 molecule of propanol per molecule of estradiol. However, the over-all conformation of the estradiol molecule was the same as in the previous case (8) and furthermore was identical with that of 4-bromoestradiol (12), a convenient derivative crystallized from methanol and yielding crystals containing methanol. The maximum thickness of estradiol, calculated as the projection of the distance between carbons 7 and 18 on the z-axis (Fig. 1), was estimated to be approximately 3.5 A.

Diethylstibestrol. Diethylstilbestrol crystallized in anhydrous form from hexane was also orthorhombic, but belonged to the Pbca space group. The molecule was found to be centrosymmetrical. The two phenol rings were parallel and oriented at 63 degrees from the central plane containing the double bond (carbons 8, 7, 7′, and 8′) (Table 3); carbons 9 and 9′ were on either side of the plane of the double bond; and the C_4—

[6] Final positional and thermal parameters and structural factors are available on request.

TABLE 1
Crystallographic data

	Estradiol–water (7)	Estradiol–propanol (8)	Diethylstilbestrol (9)	Diethylstilbestrol–water–methanol (10)	Diethylstilbestrol–ethanol (11)
Spatial group	$P2_12_12$	$P2_12_12_1$	Pbca	$P\bar{1}$	$P\bar{1}$
Unit cell					
a(α)[a]	12.054	12.215	18.941	9.281 (73.45)	9.294 (71.95)
b(β)	19.182	24.251	14.933	13.657 (70.82)	13.841 (80.26)
c(γ)	6.634	6.671	5.298	7.626 (100.92)	7.713 (98.60)
Residual factor	0.065	0.061	0.068	0.069	0.060
σ[b](C—C) (Angstroms)	0.008	0.005	0.005	0.003	0.005
σ(C—C—C) (degrees)	0.8	0.5	0.5	0.3	0.5

Detailed structures are reported in the above references.
[a] a, b, and c are the unit cell dimensions (Angstroms); α, β, and γ are the unit cell angles (degrees).
[b] σ is the standard deviation.

TABLE 2
Intramolecular and hydrogen bond lengths

Interval	Estradiol–water	Estradiol–propanol	Symmetrical diethylstilbestrol	Diethylstilbestrol–methanol–water	Diethylstilbestrol–ethanol
Intramolecular bond length					
$O_{(3)}$—$O_{(17)}$	10.920	10.988			
$O_{(4)}$—$O_{(4')}$			12.130	12.138	12.139
$C_{(3)}$—$O_{(3)}$	1.387	1.371			
$C_{(4)}$—$O_{(4)}$			1.389	1.375	1.399
$C_{(17)}$—$O_{(17)}$	1.424	1.429			
$C_{(4')}$—$O_{(4')}$			1.389	1.379	1.394
Hydrogen bond length					
$O_{I(3)}$[a]—$O_{II(17)}$	2.774	2.759			
$O_{I(4)}$—$O_{II(4)}$			3.003		
$O_{I(4')}$—$O_{II(4')}$				2.759	2.844
$O_{(3)}$—O_W	2.792				
$O_{(17)}$—O_W	2.854				
$O_{(3)}$—O_P		2.636			
$O_{(17)}$—O_P		2.724			
$O_{I(4')}$—$O_{W \text{ or } E}$				3.006	2.991
$O_{II(4)}$—$O_{M \text{ or } E}$				2.606	2.718
O_M—O_W				2.630	

[a] W, water; E, ethanol; M, methanol; P, propanol; I and II, two different molecules of estradiol or diethylstilbestrol.

and $C_{4'}$—oxygen bond lengths were equal as well as the O_4— and $O_{4'}$—hydrogen bond lengths. The thickness of the molecule along the *z*-axis was 5.90 ± 0.05 A (Fig. 1A). These data corroborate results recently published independently (9, 14, 15) and confirm that the over-all molecular shape of symmetrical diethylstilbestrol is very different from that of estradiol. Sublimation or crystallization from no more than 10% (v/v) methanol or ethanol in water gave diethylstilbestrol of the same crystal structure and conformation.

Diethylstilbestrol was also crystallized from more concentrated alcoholic solutions: methanol–water (1:1, v/v), ethanol, and ethanol–water (1:1, v/v). In the first case (10), triclinic racemic crystals of the P1

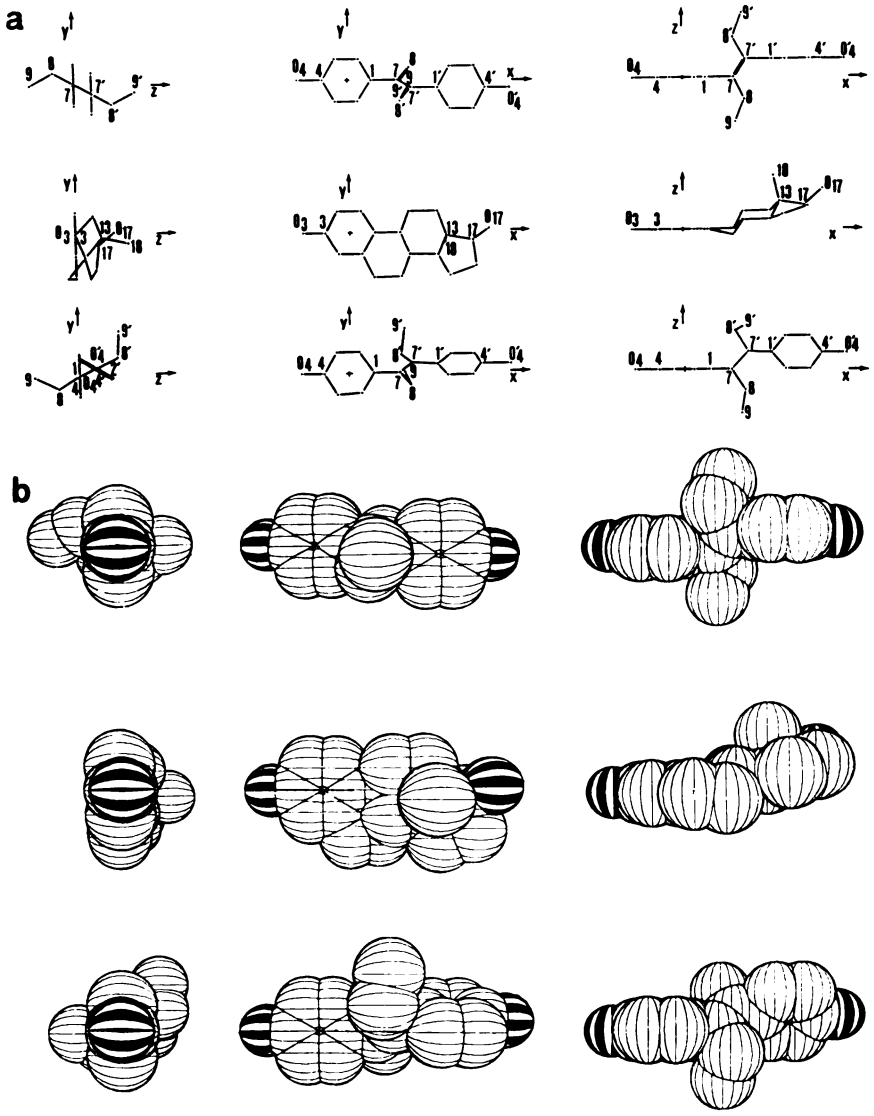


FIG. 1. *Projections of estradiol and inactive (top row) and active (bottom row) diethylstilbestrol in the same axis systems*

A. x is in the benzene ring plane and passes through the carbon-oxygen bond. y is in the benzene ring plane, perpendicular to x. z is perpendicular to the benzene ring in its center. For diethylstilbestrol, the reference benzene ring is +.

B. Perspective views using for each carbon and oxygen atom a sphere of the van der Waals radius (as in space-filling models). The computer program for these drawings was developed by Cohen (13).

space group were formed (Table 1) in which each diethylstilbestrol molecule was linked to 1 water and 1 methanol molecule. Centrosymmetry was thus destroyed. The angle between the two phenol rings, φ and φ', was 59 degrees. The dihedral angles formed by the plane of the double bond with the φ ring (methanol-bound) and φ' ring (water-bound) were 63 and 58 degrees, respectively, but were oriented in opposite directions (Table 3). The C_9 and $C_{9'}$ groups were on the same side of the double bond. The 4- and 4'-

TABLE 3

Plane angles in diethylstilbestrol molecule

The central plane contains carbons 1, 7, 8, 8′, 7′, and 1′. φ and φ' are the two phenolic rings. See the text for the directions of the angles.

Angle	Diethyl-stilbestrol	Diethyl-stilbestrol–water–ethanol	Diethyl-stilbestrol–ethanol
φ/φ'	0°	59°	61°
φ/central plane	63°	63°	62°
φ'/central plane	63°	58°	56°

oxygens were in different electronic states, as indicated by the strong hydrogen bond of O_4 with methanol (2.606 A) and the weaker hydrogen bond of $O_{4'}$ with water (3.006 A).

This crystal and asymmetrical molecular arrangement was observed only when the methanol–water medium from which crystals were obtained contained at least 15% alcohol. In order to maintain the medium composition, crystallizations were performed by slow cooling (1°/hr) in sealed tubes. Solvated crystals were very unstable when separated from the solvent medium, and there was a rapid transformation into the symmetrical form, as checked by X-ray powder diagrams.

The structure and over-all conformation of diethylstilbestrol crystallized from ethanol (11, 16) were very similar. The crystals with 1 molecule of ethanol per molecule of diethylstilbestrol, belonged to the same space group, P1. The angle between the two phenol rings was 61 degrees, and the dihedral angles between the φ and φ' rings and the plane of the double bond were 62 and 56 degrees, respectively (Table 3). Hydrogen bonding was also asymmetrical: the φ ring has a stronger hydrogen bond (2.718 A) than the φ' ring (2.991 A), suggesting that the ethanol oxygen in this water-free crystal is linked differently with 2 molecules of diethylstilbestrol.

Figure 1A shows projections, in the same system of axes (*xyz*), of estradiol (middle row), symmetrical diethylstilbestrol (top row), and one enantiomorphous form of asymmetrical diethylstilbestrol (bottom row). A set of perspective views of the carbon and oxygen skeleton of these molecules, drawn by a mechanical plotter controlled by a computer program, is shown in Fig. 1B (13).

DISCUSSION

Structures of estradiol, the two forms of diethylstilbestrol, and some chemical derivatives. In the absence of X-ray studies, a sound comparison between the molecular characteristics of estradiol and diethylstilbestrol was not available (see a thorough review in ref. 17). The results of the present studies shed new light on this comparison. No steric or chemical similarity between estradiol and symmetrical diethylstilbestrol has been revealed, despite the suggestion of Weeks *et al.* (15). Symmetrical diethylstilbestrol (see Fig. 1) has two identical phenol groups, is much "thicker" in the center of the molecule than estradiol (5.9 A for the C_9—$C_{9'}$ distance compared to 3.4 A for the C_{18}—C_7 ordinate on the *z*-axis for estradiol), and is too "flat" in the ring φ' region if compared to ring D and C_{18} of estradiol.

When diethylstilbestrol is crystallized from methanol–water or ethanol, "intrinsic" asymmetry is imparted to the molecule as a result of "extrinsic" over-all asymmetry. The two hydroxyl groups are distinguished by a difference in the strength of their hydrogen bonding and may be compared to the two different hydroxyls of estradiol, 3-phenolic and 17β-hydroxyl. The thickness at the center of the molecule decreases to 4.8 A as a result of the *cis* conformation of C_9 and $C_{9'}$. Ring φ', which has rotated with respect to ring φ, imparting greater thickness to this region of the molecule, may be considered a counterpart of ring D of estradiol.

This last point may be very important, and is clearly apparent when comparing the perspective views in the last column of Fig. 1B. In the diethylstilbestrol molecule the phenol ring on the left (φ) adopts exactly the position of the phenolic A ring of estradiol. However, the protuberance caused by the methyl group at carbon 18 of estradiol, on the upper face of the molecule in the immediate vicinity of the hydroxyl at C_{17}, has no counterpart in the centrosymmetrical form of diethylstilbestrol, whereas in the asymmetrical form the rotation of the phenol

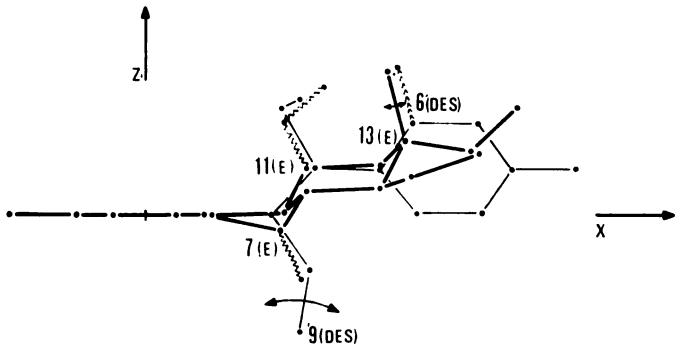
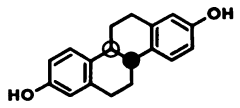


FIG. 2. *Superimposition of estradiol, estradiol derivatives, and asymmetrical diethylstilbestrol*
Axes are the same as in Fig. 1. On the estradiol structure, two groups have been added: 11β-methoxy and 7α-methyl. On the asymmetrical diethylstilbestrol structuie, a 6′-methyl group has been added. See DISCUSSION.

ring φ' imparts a corresponding steric bulkiness. This steric hindrance may in fact be essential for estrogenic activity, which has been shown to be almost totally supressed in the absence of the methyl group at C_{18} in estradiol [18-norestradiol is 2×10^{-5} times less active than estradiol (18)]. Taken together, these features show the asymmetrical, rather than the symmetrical, form of diethylstilbestrol to be more similar to estradiol (see superimposition of estradiol and of the asymmetrical form of diethylstilbestrol in Fig. 2).

Several differences nervertheless remain between asymmetrical diethylstilbestrol and estradiol; in particular, a difference in the $O_{(4)}$—$O_{(4')}$ and $O_{(3)}$—$O_{(17)}$ distances (Table 2), for which even hydrogen bonding does not compensate. Indeed, the over-all length poses an unsolved problem. For instance, although the oxygen-oxygen distance in the rigid diphenolic hexahydrochrysene derivative (12 A as measured on a molecular model) is very close to that of diethylstilbestrol, its estrogenic activity and binding to the receptor are very weak (19). As in symmetrical diethylstilbestrol, however, the two phenol rings are symmetrical, parallel, and identical. ICI compound 47, 499 (20), on the other hand, has its φ and φ' rings arranged as in asymmetrical diethylstilbestrol (φ/φ' 60 degrees, φ/central plane 53 degrees, φ'/central plane 60 degrees; see also Table 3) and is a rather active estrogen. Finally, dienestrol (20), with both phenol rings situated in parallel planes because of



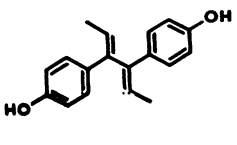
Diphenolic hexahydrochrysene

the rigidity of the two central double bonds, exhibits very weak estrogenic activity, and it has been impossible to obtain crystals containing an alcohol molecule.

Since the structure of the natural hormone estradiol is rigid, it may be possible to infer the conformation of diethylstilbestrol at the receptor sites, on the assumption that it is as much like estradiol as possible, by studying the structure of certain estrogenic derivatives of estradiol. Consequently, the following estrogens, all exhibiting high affinity for the uterine receptor, were studied: (a) two estradiol derivatives which resemble asymmetrical diethylstilbestrol more closely than estradiol itself, i.e., 11β-methoxyestradiol (21) and 7α-methylestradiol (22), in which the 11β- methoxy substituent corresponds to the $C_{8'}$—$C_{9'}$ group of diethylstilbestrol and the 7α-methyl substituent corresponds to the C_8—C_9 group; (b) two intrinsically asymmetrical stilbene derivatives, 4,4′-dihydroxy-7-ethyl-7′,6′-dimethylstilbene and 4,4′ - dihydroxy - 7′ - ethyl - 6′,7 - dimethylstilbene (23),[7] in which the methyl group on the φ' ring may play the part of the C_{18} methyl of estradiol.

Binding of estradiol and diethylstilbestrol to

[7] Unpublished observations.



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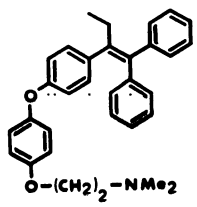

Dienestrol

various proteins. It is now widely accepted that a specific interaction of the hormone with intracellular binding proteins is a prerequisite for estrogen action (3). In all species studied, diethylstilbestrol is bound by the same uterine "cytosol receptor" sites as estradiol, with equal, if not higher, affinity ($\cong 10^{10}$ M^{-1} at 4° at equilibrium). It was also bound with high affinity ($\geqq 10^{14}$ M^{-1} at 4° at equilibrium) by the recently described non-histone chromatin protein of calf endometrium (24).

Pseudomonas testosteroni $\Delta^{5\to4}$-3-oxosteroid isomerase (EC 5.5.5.1) is an enzyme catalyzing the intramolecular transfer of a proton of $\Delta^{5,3}$-oxosteroid from the $C_{4\beta}$ position to the $C_{6\beta}$ position, thus producing the corresponding $\Delta^{4,3}$-oxo compound. It has been observed recently in these laboratories that diethylstilbestrol, like estradiol (25), is a competitive inhibitor of this reaction and that it has exactly the same inhibition constants, (10 and 18 μM at 25° in buffer containing 5 and 10% methanol, respectively). In other studies on several parameters of the enzymatic reaction in various organic solvents (26, 27), a striking similarity with estrogen binding has been observed, although the catalytic site of isomerase does not closely resemble a receptor site. This led to an investigation of the interactions of various alcohols with the hormone.

Some estradiol-binding proteins, such as those of human (28) and rat plasma (29), do not bind diethylstilbestrol, and there is no evidence that these plasma proteins play a role in hormone action at the cellular level. Certain antibodies to estradiol 17-hemisuccinate–albumin complexes also do not bind diethylstilbestrol (30). These dissimilarities may be explained by steric differences in the binding sites of these proteins. Moreover, an interaction may occur at the binding sites of certain estradiol-binding proteins, especially those directly implicated in hormone response, between diethylstilbestrol and amino acid residue(s), similar to those observed in solvated crystals between phenolic hydroxyl groups and alcohol and water molecules. The questions which remain to be answered are how specific is the conformational change in the artificial "flexible" molecule, and whether changes occur in the receptor protein conformation upon binding to these two hormone ligands. Moreover, the conformation of diethylstilbestrol in solution, especially in biological media, is unknown. Should it be different from that of diethylstilbestrol occupying the binding sites, the calculated K values may not express the true affinity for the receptor sites. Indeed, even the estradiol molecule might be more flexible than generally implied from the appearance of the molecule in the solid state.

Combined approaches of ligand binding and X-ray crystallography studies (31) may contribute to the elucidation of the structural requirements of receptor sites. Of course, precise information will only be obtained when the structure of the proteins and of their binding sites, alone or in the presence of ligands, is known (32). It is hoped, however, that because of the relatively simple and rigid structure of the natural ligand, estradiol, the predictions made here will be of some value.

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