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A SERIES OF OPTICAL, STRUCTURAL AND ISOMERIC ANALOGS OF ESTRADIOL: A COMPARATIVE STUDY OF THE BIOLOGICAL ACTIVITY AND AFFINITY TO CYTOSOL RECEPTOR OF RABBIT UTERUS

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SUMMARY

Competitive binding between optical, structural and spatial isomers of estradiol with estradiol receptors of cytosol from rabbit uterus has been studied.

The concept is put forward that the 3–17 distance and the conformation of substituents in the molecule of estrogen, is of major importance for the specific binding with the rabbit cytosol receptor to take place and for the biological activity to be displayed.

It is established that, for the binding with the receptor to be effective, the steric agreement is indispensable between the steroid molecule and the binding site of the receptor. L-estradiol-17β which has the same 3–17 distance as natural estradiol, but an opposite configuration of the asymmetric centres, was found to have a low affinity for the receptor. The affinity of D,L-estradiol-17β (racemic mixture of D- and L-estradiol-17β) is only 50%. The ability of the optical isomers to bind with the receptor correlates with their biological activity.

Alteration in the configuration of asymmetric centre in the molecule of estradiol decreases the binding and the biological activity.

The binding and biological activity also decrease if the D-ring becomes six-membered. It is shown that removal of any of the hydroxyl groups decreases the affinity for the cytoreceptor and the estrogenic activity. The binding disappears if both hydroxyl groups are removed, indicating that 3–17 hydroxyl groups are indispensable for the specific binding with the receptor and manifestation of the biological activity.

A seemingly illogical fact that D,L-8-iso-D-homoestradiol-17$\alpha\beta$ has a higher estrogenic activity and a higher affinity to the receptor than D,L-D-homoestradiol-17$\alpha\beta$ of natural configuration, may be explained in terms of 3–17 hydroxyl groups distances.

INTRODUCTION

The problem of uptake of hormones by target cells is one of the most interesting in present day endocrinology. Research in recent years [1–6] has demonstrated that in target organs estrogens bind with specific complex forming proteins (receptors). Formation of an estrogen–protein complex in the cytoplasm of sensitive cells is the first and indispensable step for the hormonal effect to be realized. A dependence between the character and the degree of biological activity of synthetic and natural analogs of estrogens, phytoestrogens and other compounds of steroid and non-steroid hormones has been established [7–18].

To throw further light on the mechanism of action of estrogens, the present work describes the influence of structural and steric changes in the molecule of estradiol on the binding with cytosol receptor from rabbit uterus and compares this effect with estrogenic activity of the compounds involved.

MATERIALS AND METHODS

The following labelled compounds have been used: [6,7-^{3}H]-estradiol-17β; S.A. 45 Ci/mmol and [2,4,6,7-^{3}H]-estradiol-17β; S.A. 100 Ci/mmol (Amersham, Great Britain). The radiochemical purity of the compounds was 98–99%.

The non-labelled steroids used were: D-estradiol-17β (I) (Jena-Pharm, DDR); D,L-estradiol-17β (II) this was obtained by reduction of D,L-estrone [19] by $NaBH_4$ [20]; L-estradiol-17β (III) (Jena-Pharm DDR); D-3-deoxyestradiol-17β (IV) obtained as described in [21]; D-17-deoxyestradiol (V), obtained according to [22]; D-3,17-bisdeoxyestradiol (VI), obtained from D-17-deoxyestradiol as described in [23]; D,1-8-isoestradiol-17β (IX); D,L-D-homoestradiol-17$\alpha\beta$ (VII) and D,L-8-iso-D-homoestradiol-17$\alpha\beta$ (X) obtained by total synthesis [24]; D,L-3-deoxy-D-homoestradiol-17$\alpha\beta$ (VIII) m.p. 178–180° (MeOH) prepared from D,L-D-homoestradiol-17$\alpha\beta$ obtained

similarly to (IV); D-estrone (XI) manifactured by "Reanal" (Hungary). All the compounds were identified by thin-layer and gas-liquid chromatography.

Estrogenic activity was determined using 20–22 g ovariectomized albino mice. The Allen–Doisy assay was employed to determine the threshold dose, i.e. the dose which induces estrus in 50% of experimental animals. In these experiments D-estradiol-17β was used as a standard. The compounds were dissolved in peach kernel oil and were administered at 10 a.m. for two days, the doses being from 0·05 to 200 μg per animal. Vaginal smears were examined 24, 32, 48, 72 and 96 h after the first injection. The data obtained were treated by the method of Student–Fisher.

Experiments in which the binding of different structural analogs and optical isomers of D-estradiol-17β with rabbit uterus receptors were studied were carried out with 2·5 kg female rabbit which had been given, 6 days before the experiments, 100 units of chorionic gonadotropin HCG ("Gedeon Richter", Hungary). Administration of HCG caused an increase in the weight of the uterus from 4·0 to 10·0 g. The animals were then decapitated. All the subsequent procedures were performed at 0–4°C. The uterine tissue was washed with 0·02 M Tris–HCl-0·0015 M EDTA buffer pH 7·34 and then homogenized in the same buffer for 5 min in a P-32 homogenizer at 14,000 rev/min. The homogenate was centrifuged for 90 min in a B rotor of a Spinco $\mathscr{L}$ centrifuge at 105,000 g to obtain the soluble part of the cell (cytosol). The protein content determined by the method of Lowry[25], varied from 1·5 to 10 mg/ml, depending on the weight of the uterus and degree of dilution: the average protein content was 6 mg/ml.

Radioactivity was measured in Intertechnique, ABAC, SL-40, 4K and Marck II liquid scintillation counters, the tritium counting efficiency in Bray scintillator [26] was 20–25%.

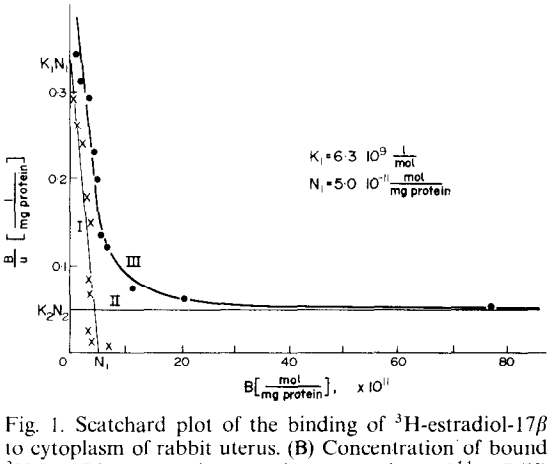


Fig. 1. Scatchard plot of the binding of ^{3}H-estradiol-17β to cytoplasm of rabbit uterus. (B) Concentration of bound ^{3}H-E_2-17β espressed as mol/mg protein $\times 10^{11}$; (B/U) Ratio of bound to unbound ^{3}H-E_2-17β; (K) Association constant of binding protein; (N) Number of binding sites per molecule of protein; (I) A plot of the binding of ^{3}H-E_2-17β to first specific component of the binding system; (II) A plot of the binding of ^{3}H-E_2-17β to second non-specific component of the binding system; (III) A common plot of the binding of ^{3}H-E_2-17β to I and II components of the binding system.

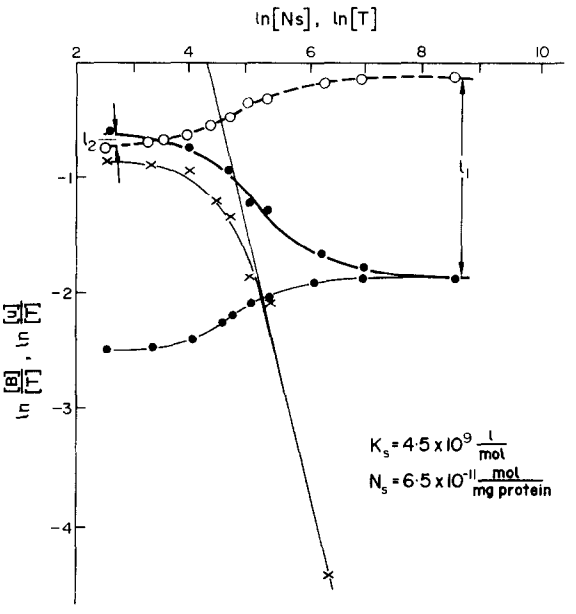


Fig. 2. "Proportion graph" of the binding of ^{3}H-estradiol-17β to cytoplasm of rabbit uterus. Fig. 2 represents in the "proportion graphs" the ln([B]/[T]) and ln([U]/[T]) as a function of ln [T]: (Bs and Bns) ^{3}H-E_2-17β Bound to specific and non-specific components of the binding system; (Ks and Kns) Association constant of specific and non-specific components of the binding system; (Ns and Nns) Number of binding sites of specific and non-specific components of the binding system; (U) Unbound ^{3}H-E_2-17β; (T) Total ^{3}H-E_2-17β in the sample of the cytoplasm. All concentrations [ ], are in pg/ml and K is in M^{-1}. ×—×—× is a plot of ln([Bs]/[T]); ·—·—· is a plot of ln([Bns]/[T]); ●—●—● is a plot of ln([Bs + Bns]/[T]); O---O is a plot of ln([U]/[T]) e_1 is ln Kns [Nns]; e_2 is ln(Ks[Ns] + Kns[Nns]).

The cytosol was freed from endogenous steroids by Dextran-coated charcoal (10% charcoal "Norit-A", Holland or "OU-B", RJAP, U.S.S.R.; and 0·5% Dextran, mol. wt. 80,000); treatment for 2 min at 37°. The percentage of endogenous steroids removed amounted to 88–90.

Determination of the binding of ^{3}H-estradiol-17β with rabbit uterus cytosol was carried out by Dextran-coated charcoal adsorption at 0–4°C (of 2·6% charcoal and 0·5% Dextran) after the protein-bound and free forms of the steroid had been equilibrated (18–20 h). Additions of ^{3}H-estradiol-17β varied from 15 to 10,000 pg/ml.

To determine the concentration of the binding sites (N) and the association constants (K_{ass}), the analysis of Scatchard plot [27] according to Sandberg[28] was used, Fig. 1; the recently suggested method by Baulieu and Raynaud[29] based on "proportion graph" technique was also used, Fig. 2. The two methods yielded similar results.

Analysis of estradiol binding by uterine cytosol of rabbit indicates two-component protein binding system. One of the binding proteins (first component) had a high K_{ass} ($5·0 \times 10^9 M^{-1}$, the average of four

determinations, ranging from 3·8 to 6·3 × $10^9 M^{-1}$), a limited binding capacity (N = 2·3–8·2 × 10^{-11} mol/mg protein, the average being 5·6 × 10^{-11} mol/mg protein), and a high specificity (no, or almost, no binding was observed with testosterone, progesterone, D-estriol-16α, 17β and a low affinity to D-estrone); this evidence allows the conclusion to be made that the first component is a specific receptor for estradiol. The second binding component is non-specific.

The affinity of the above steroids studied for uterine receptors was judged by the ability of different doses of these compounds (from 50 pg to 100 ng) to compete with 25 pg of ^{3}H-estradiol-17β for binding complex-forming proteins in 1·0 ml of cytosol. The competitive ability (the relative affinity to cytosol receptor) was estimated as described in Korenman[8,9], by determining the ratio between the dose of unlabelled D-estradiol-17β reducing the initial percentage of the labelled steroid by half and the respective dose of the competitor, i.e. the binding inhibitor (competitive ability = (dose of estradiol/dose of inhibitor) × 100%). The calculation was made with the assumption that the value of the dose is inversely proportional to the relative affinity to cytoreceptor. The relative affinity of D-estradiol-17β was assumed as being 100%. The respective doses of the competitor were determined from the curves showing the decrease of the initial percentage of the binding of the labelled estradiol in relation of the logarithm of the concentration of the inhibiting steroid in 1·0 ml of cytosol (Fig. 3).

RESULTS

The data presented in Table 1 and Fig. 3 show that the structural and steric changes in the molecule of estradiol result both in decrease in the degree of affinity to the cytosol receptor of rabbit uterus and decrease in the estrogenic activity.

Change in the estrogenic activity of the optical isomers of estradiol proceeds in parallel with change in the degree of the affinity to cytosol receptor. For example, for D,L-estradiol-17β (II), the threshold dose of which, as judged by the Allen–Doisy test is 1·58 μg, the degree of affinity, to cytosol receptor amounts to 50% of that of D-estradiol-17β (I), the threshold dose of which is 0·05 μg.

l-Estradiol-17β (III), which possesses almost no estrogenic effect proves to give a poor binding with

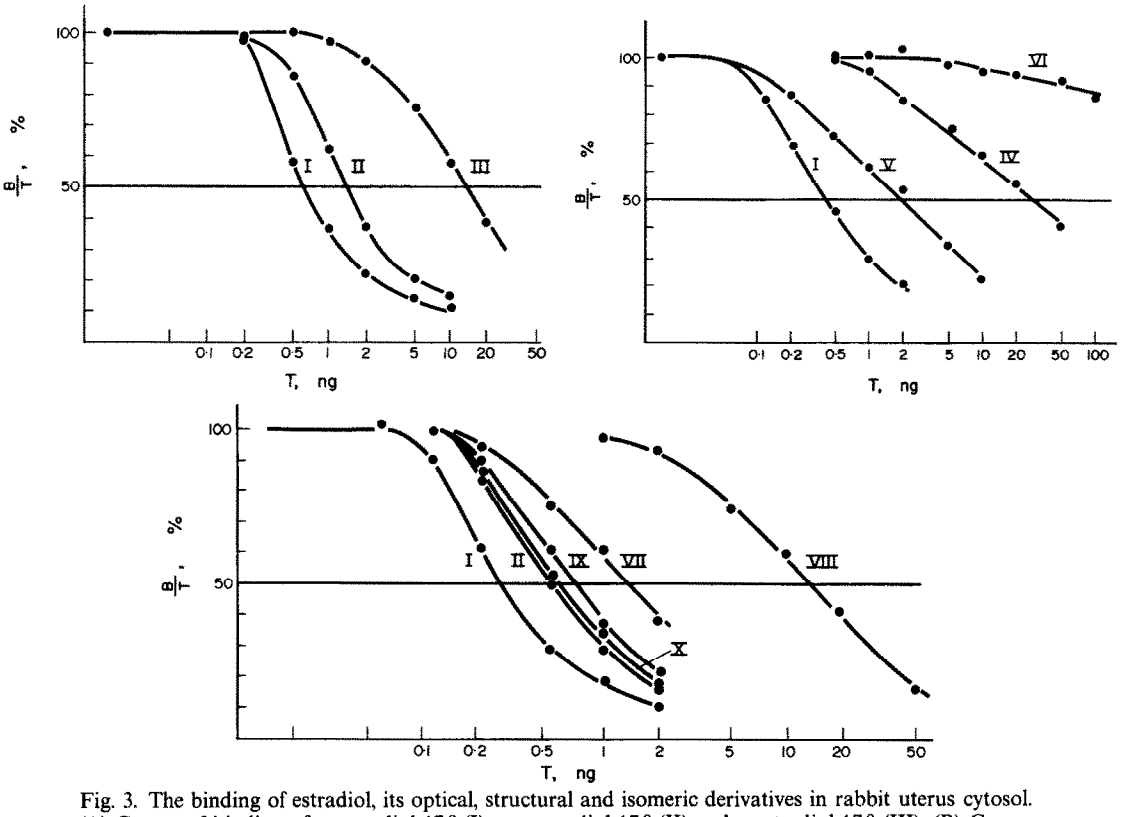
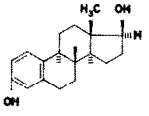
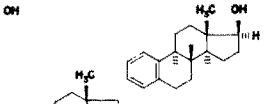
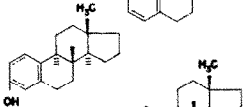
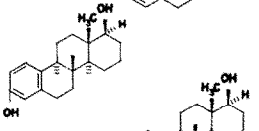
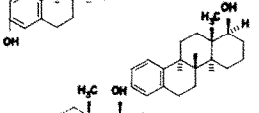
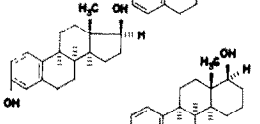
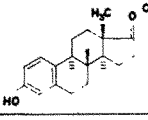


Fig. 3. The binding of estradiol, its optical, structural and isomeric derivatives in rabbit uterus cytosol. (A) Curves of binding of D-estradiol-17β (I), D,L-estradiol-17β (II) and L-estradiol-17β (III). (B) Curves of binding of D-estradiol-17β (I), D-3-deoxyestradiol-17β (IV), D-17-deoxyestradiol (V) and D-3,17-bis-deoxyestradiol (VI). (C) Curves of binding of D-estradiol-17β (I), D,L-estradiol-17β (II), D,L-D-homoestradiol-17αβ (VII), D,L-3-deoxy-D-homoestradiol-17αβ (VIII), D,L-8-isoestradiol-17β (IX), D,L-8-iso-D-homoestradiol-17αβ (X). Abscissa—quantity of the competitive steroid (in pg) added to 25 pg of ^{3}H-estradiol-17β in logarithmic scale. Ordinate—binding of labelled estradiol in per cent; binding of labelled estradiol in the absence of the competitor is assumed as being 100%. The dose of steroid reducing by half the initial percentage of labelled estradiol may be determined from the curves. A perpendicular drawn from the intersection of the curve with the line showing reduction of the initial percentage of binding by half, will indicate this dose on the abscissa.

Table 1. Relative affinity to cytoreceptor of rabbit uterus cytosol and estrogenic activity in a series of optical, structural and isomeric analogs of estradiol

Compound	Chemical development	Relative affinity to cytoreceptor in per cent		Estrogenic activity (according to Allen–Doisy) in μg	
		Mean ± SE	n	μg	n_1
I. d-estradiol-17β		100		0·05	(45)
II. d,l-estradiol-17β		49·2 ± 2·5	(20)	1·5	(35)
III. l-estradiol-17β		6·0 ± 0·6	(17)	500	(35)
IV. d-3-deoxyestradiol-17β		1·7 ± 0·4	(7)	5	(40)
V. d-17-deoxyestradiol		14·4 ± 2·3	(6)	25	(40)
VI. d-3,17-bisdeoxyestradiol		0	(2)	35	(30)
VII d,l-D-homoestradiol-17αβ		22·0 ± 3·0	(9)	200	(40)
VIII. d,l-3-deoxy-D-homoestradiol-17αβ		1·4 ± 0·4	(5)	500	(25)
IX. d,l-8-isoestradiol-17β		38·0 ± 3·5	(7)	5	(40)
X. d,l-8-iso-D-homoestradiol-17αβ		46·0 ± 5·6	(7)	50	(30)
XI. d-estrone		24·0 ± 5·8	(3)	0·1	(30)

n = number of experiments
n_1 = number of animals

cytosol receptor (6%), which may be explained by its weak antiestrogenic action [13, 30].

Removal of the hydroxyl group in the molecule of estradiol in positions 3 or 17 [3-deoxy-(IV) and D-17-deoxyestradiol (V)] brings about a decrease in the degree of their affinity to cytosol receptor. It should be noted that the decrease in the competitive ability is more pronounced in D-3-deoxyestradiol-17β (IV) than in D-17-deoxyestradiol (V) as in the former it is 1·7% and in the latter −14·4%, at the same time, according to our data, the estrogenic activity in (IV) is higher than in (V), *i.e.* 5 μg and 25 μg respectively.

Removal of the two hydroxyl groups in positions 3 and 17 of the estradiol molecule [D-3,17-bisdeoxyestradiol (VI)] results in a still greater reduction of the estrogenic activity (35 μg) and the compound almost completely loses its ability to bind with the receptor.

If the 17-hydroxyl group is oxidized into a keto group [D-estrone (XI)], a certain decrease in the estrogenic activity is observed (0·1 μg), and a marked decrease in the degree of affinity to cytosol receptor.

If the D ring in the molecule of estradiol becomes six-membered [D,L-D-homoestradiol-17αβ (VII)], the degree of the affinity to the receptor decreases to 22%, and the estrogenic activity decreases as well (200 μg). Removal of 3-hydroxyl group in D,L-D-homoestradiol-17αβ (VII) [D,L-3-deoxy-D-homoestradiol-17αβ (VIII)] causes an almost complete disappearance of both the estrogenic activity (500 μg) and in the receptor binding ability (1·4%).

Alteration in the configuration of the asymmetric centre at carbon 8 in estradiol [D,L-8-iso-estradiol-17β (IX)] results in both the estrogenic activity (5 μg) and the degree of binding with cytosol receptor (38%) being decreased.

It turned out, unexpectedly that D,L-8-iso-D-homoestradiol-17αβ (X), as compared with D,L-D-homoestradiol-17αβ (VII) of natural configuration, possesses a higher estrogenic activity [24] and a higher affinity to the receptor.

DISCUSSION

It is an accepted point of view that the 3 and 17 hydroxyl groups play an important role in the complex formation with the receptor protein [5, 9, 18, 31, 32]. The results obtained in this work with 3 and 17 deoxycompounds lend more support to the conclusion of Hähnel *et al.*[18] that hydroxyl group in estradiol are indispensable for binding with uterus cytosol receptor.

Comparison of the competitive ability of D-3-deoxyestradiol-17β (IV) and D-17-deoxyestradiol (V) allows to conclude that the phenol hydroxyl group at C_3 is of major importance for the binding to occur, as D-3-deoxyestradiol-17β (IV) has a much less affinity to cytosol receptor than D-17-deoxyestradiol. This conclusion is substantiated by the results about the binding of D-3,17-bisdeoxyestradiol (VI) with cytosol receptor. Elimination of both hydroxyl groups from the molecule of estradiol completely deprives the steroid of the ability to bind with the receptor. A similar phenomenon is observed in a series of D,L-D-homoestradiol-17$\alpha\beta$ (VII), where removal of 3-hydroxyl group (D,L-3-deoxy-D-homoestradiol-17$\alpha\beta$ (VIII)] results in a sharp decrease in the competitive ability (relative affinity of VIII is 1·4%).

In contrast to our *in vitro* experiments, D-3-deoxyestradiol-17β (IV) proved to be a more active estrogen than D-17-deoxyestradiol (V) as judged by threshold dose determination according to the Allen–Doisy test in mice subjected to ovariectomy. It should be mentioned that these data do not agree with those of Huggins and Jensen[33]; these authors, using a different test and different quantitative estimations, reported a higher estrogenic activity for D-17-deoxyestradiol (V) than for D-3-deoxyestradiol-17β (IV).

That we have observed no correlation between the estrogenic activity *in vivo* and the affinity to cytosol receptor *in vitro* in a series of D-3, 17-bisdeoxyestradiols (VI) may be explained by that in the organism D-3-deoxyestradiol-17β (IV) is 3-hydroxylated to form estradiol.

No unequivocal evidence is as yet available for the presence in organisms of a 3-hydroxylating system for aromatic ring of the estrogens. However, it has shown [34–35] that in male rabbit liver there is a system hydroxylating steroids of androstan and 19-norandrostan series at C_3.

The fact that D-3,17-bisdeoxyestradiol (VI), which does not bind with cytosol receptor, possesses an estrogenic activity similar to that of D-17-deoxyestradiol (V), also testifies to the possibility of estrane compound being hydroxylated at C_3.

Our results have shown that the affinity of D-estrone to the receptor is significantly lower than that of D-estradiol-17β, which agrees with the data of Jensen *et al.*[36], Hähnel *et al.*[18] etc. The high estrogenic activity of D-estrone is accounted for by its being reduced to D-estradiol-17β in the organism.

The relatively high degree of binding in D-L-D-homoestradiol-17$\alpha\beta$ (VII) with the estrogenic effect being rather low, is apparently due to another kind of activity, antiestrogenic. According to our unpublished results, this compound displays a higher antiestrogenic effect than L-estradiol-17β.

Taking into account that hydroxyl groups in the molecule of estrogen are necessarily involved in the receptor binding, we offer the following explanation for the receptor-binding ability in different structural and spatial analogs of estradiol.

It is possible that cytosol receptor possesses a certain estrogen-binding site which is complementary to the molecule of natural estradiol. Structural and steric alterations in the molecule of estradiol should change the parameters of the molecule and especially the distance between hydroxyl groups 3 and 17. New parameters alter the conformation of the binding site [5, 31], the change which involves some energy and reduces the effectivity of the binding.

We have used the molecular models of Dreiding to determine approximately the distances between hydroxyl groups 3 and 17. Table 2 shows these distances in relation to the affinity to rabbit uterus receptor. One sees that the distances and the affinities correlate. Estradiol of natural configuration, in which the 3–17 distance is 10·5 Å, binds with the receptor with no conformational rearrangements in the binding site being involved, hence the highest effectivity of the binding. A decrease in the distance of 0·2 Å in D,L-8-iso-D-homoestradiol-17$\alpha\beta$ (X) results in a somewhat lower affinity to the receptor (94%) because of a small amount of energy required to alter the conformation of the binding site. The binding of D,L-8-iso-estradiol-17β (IX) in which the 3–17 distance is by 0·4 Å lower than in natural estradiol, apparently involves more energy of the binding site rearrangement and its affinity to the receptor decreases by 22%.

Greater distances between the hydroxyl groups also affects the receptor binding, the effect being expressed even higher. For example, in D,L-D-homoestradiol-17$\alpha\beta$ (VII), the 3–17 distance of which is by 0·2 Å larger than in the natural compound, the affinity to the receptor is lower by 55%. Presumably, in this case, more energy is required for conformational rearrangements in the binding site.

The somewhat unexpected result that D,L-8-iso-D-homoestradiol-17$\alpha\beta$ (X) displays a higher affinity to the receptor and a higher estrogenic activity than D,L-D-homoestradiol-17$\alpha\beta$ (VII) of natural configuration, may also be explained in terms of 3–17 distances.

At the same time, experiments with L-estradiol-17β show that for the steroid to bind with the receptor, the molecule of the former should possess steric congruence with the binding site.

L-estradiol-17β has the same 3–17 distance as natural D-estradiol-17β, but the asymmetric centres have opposite configurations; as a result, the receptor-binding ability is low (Table 1).

Table 2. 3–17 hydroxyl groups distances and affinity of D,L-estradiol-17β analogues to rabbit uterus cytoreceptor

Nos.	Compounds	3-17 distances (Å)	Relation affinity to receptor in per cent
II.	d,l-estradiol-17β	10·5	100*
X.	d,l-8-iso-D-homoestradiol-17aβ	10·3	94
IX.	d,l-8-isoestradiol-17β	10·1	78
VII.	d,l-D-homoestradiol-17aβ	10·7	45

* Affinity of d,l-estradiol-17β to cytoreceptor is assumed as being 100 per cent.

Table 3. 3-17 hydroxyl groups distances and affinity to cytoreceptor of some derivatives of estradiol and androstandiol

Nos	Compounds	3-17 distances (Å)	Relative affinity to receptor in per cent
I	d-estradiol-17β	10·5	100
XII	d-estradiol-17α	10·4	17*
X.	d,l-8-iso-D-homoestradiol-17$\alpha\beta$	10·3	46
XIII.	d-estradiol-16α	10·8	22*
VII	d,l-D-homoestradiol-17$\alpha\beta$	10·7	22
XIV	5α-androstandiol-3β. 17β	10·7	1·4*
XV	5β-androstandiol-3α. 17β	9·4	0*

* Calculated from results of Hähnel *et al.*[18].

The data of Hähnel *et al.*[18] may also be analysed in terms of the concept of the 3-17-distance being the most important factor for the binding.

As seen in Table 3, in D-estradiol-17α (XII) the 3-17-hydroxyl groups distance (10·4 Å) is the mean between those in D-estradiol-17β (10·5 Å) and D,L-8-iso-D-homoestradiol-17$\alpha\beta$ (10·3 Å); but its affinity to the receptor is weak (17%). This may be accounted for by the change in the spatial localization of the 17-hydroxyl group (it is pseudoxial); this means that the energy required for the conformation of the binding site to be altered is greater. D-estradiol-16α (XIII), in which the 3-17 hydroxyl distance is 10·8 Å, i.e. close to that of D,L-D-homoestradiol-17$\alpha\beta$ (VII) $-$10·7 Å; the conformation of the 17$\alpha\beta$ –OH and 16α –OH group (equatorial and pseudoequatorial) are also similar; it is only logical therefore that the two compounds display a similar affinity to the receptor.

Among the compounds of the androstane series, which have, as was reported by Hähnel *et al.*[18], an insignificant affinity to the receptor, there is 5α-androstane-3β, 17β-diol (XIV) in which the 3-17 distance is similar to that in D,L-D-homoestradiol-17$\alpha\beta$ (VII). The fact that the affinity in XIV is lower than in VII may be explained by that in the former the binding is realized at the expense of two alicyclic hydroxyl groups. As was shown by Hähnel *et al.*[18] and by us with D-3-deoxyestradiol-17β (IV) the phenolic OH-group, due to its chemical nature, is more responsible for the binding than the respective alicyclic 17β-OH-group.

The change in the 3-17 hydroxyl groups distance by 1·1 Å leads in the case of 5β-androstane-3α, 17β-diol (XV) to a complete loss of the receptor binding ability (Table 3).

The concept about major role of 3-17-OH distances in steroid estrogens is not consistent with the data on the high estrogenic activity and the high binding ability of synthetic estrogen, diethylstilbestrol, in which the distance between OH-groups is, according to X-ray data, 12·14 Å [31]. However, due to flexibility of the structure of this compound, its true conformation in solution, and hence the true distances between the hydroxyl groups are unknown.

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