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THE SPECIFICITY OF THE ESTROGEN RECEPTOR OF HUMAN UTERUS

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SUMMARY

The specificity of the estrogen receptor in human uterus was determined by incubating the cytosol fraction with tritiated estradiol-17β alone or in the presence of other steroids. If the steroid competed with the estradiol-17β for binding sites on the receptor the binding of the estradiol-17β was diminished.

Highest affinity to the receptor is found if the steroid has a phenolic hydroxyl group on C-3 and an alcoholic hydroxyl group on C-17 in the β-configuration. The presence of the free phenolic hydroxyl on ring A is essential and its position is of critical importance. The presence of a second oxygen function on ring D is important and its state of oxidation and its position influence the binding activity. Additional oxygen functions on ring D, additional substituents on ring A, and unsaturation of ring B decrease the affinity for the receptor while presence or absence of the angular methyl group on C-13 has no influence.

INTRODUCTION

ALTHOUGH some detailed information on the *in vitro* specificity of the estrogen receptors of the uterus of various animals is available (including rat[1–5], rabbit [6–9], calf[10, 11], sow[12], ewe[13], mouse[14]) relatively little is known about this property of the estrogen receptor in the human uterus[15–17]. In view of the possible implications for carcinogenesis and the mechanism of hormone action it was decided to study the specificity of the estrogen receptor in human uterus in greater detail.

To determine the specificity of the estrogen receptor, uterine cytosol was incubated with tritiated estradiol-17β alone and in the presence of the steroid to be tested. If the steroid competed with the estradiol-17β for binding sites on the receptor the binding of the tritiated estradiol-17β was diminished. This decrease could serve as a measure of the affinity to the estrogen receptor of the steroid under investigation.

MATERIALS AND METHODS

Tissues

Human uterine tissue was obtained at hysterectomy. The uteri were put on ice immediately and, after taking a sample for histopathological investigation, were homogenized as described below. Some of the uteri contained benign tumors but only the macroscopically normal parts were included in the homogenization.

Steroids

The following steroids were used in this study (the source is given in brackets, see key at end of list).

1. *Estrogens.* Estradiol-17β (a, b, q), estradiol-17α (b), estriol (b, q), 17-epiestriol (c), 16-epiestriol (a), 16, 17-epiestriol (c), estrone (b, q), equilin (i), equilenin (c), 16(α or β) hydroxyestrone (o), 16-oxo-estradiol-17β (a), 3-deoxy-

estradiol-17β (m), 3-deoxyestrone (m, p), 17-deoxyestrone (m, p), estradiol-3,17β-diacetate (n), 17α-ethynyl-estradiol-17β (f, r), 18-norestradiol-17β (m), 16α-estradiol [(a), 1,3,5(10)-estratrien-3-16α-diol], estratriene [(h), 1,3,5(10)-estratriene], 2-hydroxy-3-deoxyestradiol-17β[(f), 1,3,5(10)-estratriene-2,17β-diol), 1-methyl-estradiol-17β (i), 2-methylestradiol-17β (i), 1,2-dimethylestradiol-17β (i), 4-methyl-2,17β-estradiol [(s), 4-methyl-1,3,5(10)-estratriene-2,17β-diol], 17β-methoxy-estradiol (d), 19-nortestosterone (a), 16β-estradiol-3-methylether [(a), 3-methylether of 1,3,5(10)-estratriene-3,16β-diol], 17β-estradiol-3-methyl-ether (q), estriol-3-methylether (t), estrone-3-methylether (q), 2-methoxy-4-methyl-estradiol-1,17β[(s), 2-methoxy-4-methyl-1,3,5(10)-estratriene-1,17β-diol], 2-methoxy-4-methylestrone [(s), 2-methoxy-4-methyl-1,3,5(10)-estratriene-17-one], 6-dehydro-1,2-dimethyl-estradiol-17β [(i, p), 1,2-dimethyl-1,3,5(10), 6-estratetraene-3,17β-diol], estratetraenol [(c), 1,3,5(10),16-estratetraene-3-ol], estratetraenediol diacetate [(c), 1,3,5(10),16-estratetraene-3,17-dioldiacetate], 17β-hydroxy-5(10)-estren-3-one (i, m), 2-methoxy-4-methyl-1,3,5(10)-estratrien-17β-ol (s).

2. *Estrogen conjugates.* 17β-estradiol-3-glucosiduronic acid (b), 17β-estradiol-17-glucosiduronic acid (e), estriol-3-glucosiduronic acid (b), estriol-16-glucosiduronic acid (c), 17β-estradiol-3-sulfate (g), 17β-estradiol-17-sulfate (g), estrone-3-sulfate (q), estriol-3-sulfate (g).

3. *Androgens.* Testosterone (a), dehydroepiandrosterone (a), 5α-androstane-3α,17β-diol (a), 5α-androstane-3β,17β-diol (a), 5β-androstane-3α,17β-diol (a), 4-androstene-3β,17β-diol (a), 2α-methyldihydrotestosterone propionate [(k), dromostanolone propionate].

4. *Pregnane derivatives.* Pregnenolone (b), progesterone (b), cortisol (f), 17α-hydroxyprogesterone (b).

5. *Non-steroids.* Hexestrol [(a), 3,4-bis [4-hydroxyphenyl]-hexane m.p. 187–189], diethylstilbestrol [(a), 3,4-bis[4-hydroxyphenyl]-3-hexene, m.p. 170–173].

The steroids were obtained from the following sources: (a) Steraloids Ltd., Croydon, Surrey, England, (b) Sigma Chemical Co., St. Louis, Missouri, U.S.A., (c) Ikapharm Ltd., Ramat-Gan, Israel, (d) Synthesized from estradiol-3-benzoate (Steraloids Ltd.) according to the method of Sa'at and Slaunwhite[20], (e) Calbiochem Inc., Los Angeles, California, U.S.A., (f) British Drug Houses, Poole, Dorset, England, (g) Leo, Hälsingborg, Sweden, (h) Dr. E. Caspi, Worcester Foundation for Experimental Biology, Shrewsbury, Massachusetts, U.S.A., (i) Dr. J. A. Edwards, Syntex Ltd., Palo Alto, California, U.S.A., (k) Eli Lilly Co., Indianapolis, U.S.A., (l) Dr. J. Fishman, Institute for Steroid Research, Montefiore Hospital, Bronx, N.Y., U.S.A., (m) Dr. W. F. Johns, G. D. Searle and Co., Chicago, Illinois, U.S.A., (o) Dr. W. McCrae, Syntex Ltd., Palo Alto, California, U.S.A., (p) Dr. W. H. Rooks, Syntex Ltd., Palo Alto, California, U.S.A., (q) Schering Corporation, Berlin, Germany, (r) G. D. Searle and Co., Chicago, Illinois, U.S.A., (s) Sterling-Winthrop Laboratories, Rensselaer, N.Y., U.S.A., (t) Organon Laboratories, Crown House, Morden, Surrey, England, (n) Mann Research Laboratories, New York.

The purity and identity of most steroids used was confirmed by i.r. spectroscopy in KBr pellets.

Miscellaneous. [6,7-^{3}H]-estradiol-17β, (S.A. 40 Ci/mmole) was purchased from Radiochemical Centre, Amersham, England. Bray scintillator, dextran-coated charcoal buffer and Tris buffer pH 8·0 were prepared as described[18].

Preparation of tissue fraction

The fresh tissue was immersed in twice its weight of ice-cold Tris buffer pH 8·0 and cut into pieces approximately 2 × 2 × 2 mm with scissors. The suspension was then homogenized with a Sorvall Omnimixer in a 50 ml stainless steel chamber with a 1 in. rotor knife blade at high speed (200 V setting). Each of the runs lasted for 15 sec with intervals of 60 sec between the runs. During the entire process the homogenizer chamber was cooled in ice-water.

The homogenate was centrifuged for 30 min at 40,000 r.p.m. (105,000 *g*) at 4°C in a M.S.E. 75 preparative ultracentrifuge. The soluble fraction (cytosol) was poured off, frozen and stored at − 20°C in aliquots of 5–10 ml.

Incubation of tissue extracts and charcoal adsorption

The cytosol was diluted 1 to 10 with Tris buffer pH 8·0 and 1 ml aliquots were incubated with 2×10^{-13} moles tritiated estradiol-17β. After 30 min incubation at 25° the reaction mixture was cooled in ice water. 1 ml of the suspension of dextran-coated charcoal in Tris buffer was added. After briefly mixing the contents (vortex) the mixture was kept in ice-water for 20 min after which time the charcoal was spun down at 3500 r.p.m. for 10 min. The supernatant was poured directly into counting vials, 10 ml Bray scintillator was added and the radioactivity measured. Counting efficiency was 20 per cent on average.

For competitive inhibition experiments various amounts, 50, 100, 200, 1000, 5000, 10000 pg of non-radioactive estradiol-17β or other steroid was added to the reaction mixture together with the tritiated estradiol-17β and incubated as described.

The incubation mixture containing tritiated estradiol-17β only was used as control (= 100 per cent). This control value was obtained in 5 replicates with each run. In addition, incubations in the presence of non-radioactive estradiol-17β were done in triplicate for five concentrations (50–5000 pg) with each run in which other steroids were tested. Similarly, the influence of the steroid under test on the binding of tritiated estradiol-17β was determined in triplicate for each concentration.

Method of plotting results

The results were expressed as bound radioactivity divided by the radioactivity in the incubation medium (bound d.p.m./medium d.p.m.). These values were plotted over the logarithm of the total steroid concentration (pg) present in the incubation, i.e. tritiated + non-radioactive steroid.

Estimation of protein concentration

The protein concentration was measured by the method of Lowry[19] based on the Folin-reaction. In general, aliquots of 50 μl were used for the estimation.

Measurement of radioactivity

Radioactivity was measured in a Tri-carb model 3375 liquid scintillation spectrometer with automatic external standardization. The counting error was always below 2·5% and most often about 1·0%.

RESULTS

1. *Competitive inhibition of the binding of tritiated estradiol-17β to the estrogen receptor by other steroids*

The influence of increasing concentrations of non-radioactive estradiol-17β on the binding of tritiated estradiol-17β by a human uterus cytosol is shown on Fig. 1. The graph is based on 33 replicate estimations for each of the points (with the exception of the 10,000 pg point for which only 3 tests were done and the control [no cold estradiol-17β] for which 60 tests were done). The assays were done in 11 different runs (of 3 replicates for each point) spread over 6 days. The standard deviation of the means at the different concentrations was also calculated. The reproducibility of this graph during the various runs attests to the stability of the uterine cytosol receptor and to the reliability of the method employed.

It can be seen on the graph that the addition of 50 pg of non-radioactive estradiol-17β decreased the binding of tritiated estradiol-17β to 77% of the control value. The steep decrease continued when 100 pg (to 58% of control) or 200 pg (to 41% of control) were added. The binding continued to decrease on a gentler slope reaching 27% of the control value in the presence of 1000 pg non-radioactive estradiol-17β and 18% with 5000 pg.

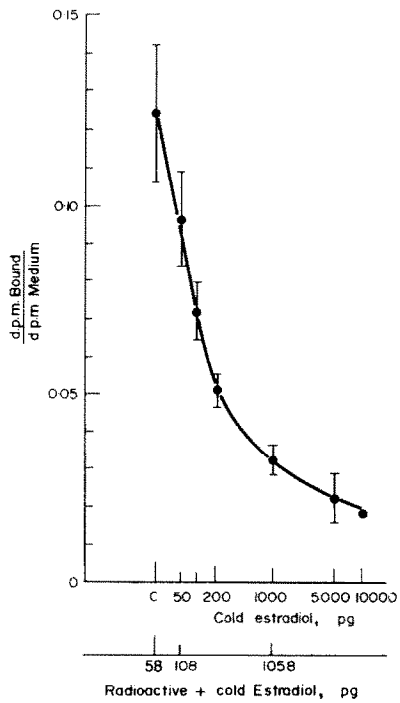

Fig. 1. Influence of increasing concentrations of non-radioactive estradiol-17β on the binding of tritiated estradiol-17β. On the abscissa the logarithm of the sum of tritiated and non-radioactive estradiol-17β is plotted. Point C (= control) shows the binding of 58 pg tritiated estradiol-17β in the absence of non-radioactive estradiol-17β. On the ordinate binding is expressed as (bound d.p.m./medium d.p.m.), where bound d.p.m. is the radioactivity in the charcoal supernatant (receptor-bound radioactivity) and medium d.p.m. is the radioactivity in the incubation medium at the beginning of the experiment. The means are based on 33 replicate estimations (50, 100, 200, 1000, 5000 pg) spread over 6 days. The mean for the control was calculated from 60 replicates. The vertical bars show the standard deviations.

The affinity of other steroids to the uterine cytosol receptor was tested by measuring their influence on the binding of tritiated estradiol-17β in the same way.

None of the C_{21}-steroids tested (up to 10,000 pg of cortisol, progesterone, 17α-hydroxyprogesterone or pregnenolone) had any influence on the binding of tritiated estradiol-17β by uterine cytosol.

Most of the C_{19}-steroids tested (up to 10,000 pg of dehydroepiandrosterone, 2-methyldihydrotestosterone, testosterone, 5α-androstane-3α,17β-diol, 5β-androstane-3α,17β-diol, 4-androstene-3β,17β-diol) had no effect either. 5α-Androstane-3β,17β-diol had some affinity to the estrogen receptor.

Of the C_{18}-steroids tested the following did not influence the binding of tritiated estradiol-17β: 17β-estradiol-3-methylether, estrone-3-methylether, estriol-3-methylether, 19-nortestosterone, estratriene, 16β-estradiol-3-methylether, 3-deoxyestrone, 1-methylestradiol-17β, 1,2-dimethylestradiol-17β, 6-dehydro-1,2-dimethylestradiol-17β, 2-methoxy-4-methylestratrien-17β-ol, 2-methoxy-4-methylestradiol-1,17β, 17β-hydroxy-5-estren-3-one, estriol-3-glucosiduronic acid, estriol-16-glucosiduronic acid, 4-methyl-2,17β-estradiol, 2-methoxy-4-methyl-estrone, estradiol-3,17β-diacetate, 17β-estradiol-3-sulfate, estriol-3-sulfate, estrone-3-sulfate, estradiol-17β-sulfate, estriol-17-sulfate, 17β-estradiol-3-glucosiduronic acid (Group 1, Table 1). The results of a representative example of this group (pregnenolone) as well as the mean results of all steroids in this group, together with standard deviations, lowest and highest results at the various steroid concentrations are given in Table 1.

Table 1. Steroids that do not compete with tritiated estradiol-17β for receptor sites

	Inhibitions in per cent of control					
	50 pg	100 pg	200 pg	1000 pg	5000 pg	10,000 pg
Example pregnenolone	109	111	108	106	111	101
Mean of group 1 steroids	109·1	110·2	111·7	108·2	99·1	97·0
Standard deviation	±8·2	±8·2	±8·3	±9·7	±10·9	±15·5
Minimum	90	98	100	90	80	72
Maximum	127	127	131	130	119	128

Binding of tritiated estradiol-17β in the presence of increasing amounts of steroids is given in percent of control (binding in the absence of other steroids). The individual steroids of this group (1) are listed in the text. As an example the results for pregnenolone are given as well as the mean results (with standard deviations and ranges) of all steroids in this group.

The following C_{18}-steroids had the same or nearly the same effect as non-radioactive estradiol-17β on the binding of tritiated estradiol-17β: 17α-ethynyl-estradiol-17β, 18-norestradiol-17β and 2-methylestradiol-17β. In addition, hexestrol and diethylstilbestrol competed with the receptor sites with the same efficiency as estradiol-17β (Group 2, Table 2).

Two more groups were discernible amongst the remaining steroids that were tested: One (group 3) includes estrone, estriol, 16-epiestriol, 17-epiestriol, 16α-estradiol and 17α-estradiol. These steroids showed considerable affinity towards the receptor (Table 3), but not as much as estradiol-17β and the compounds in group 2.

Group 4 comprises 16-hydroxyestrone, 16,17-epiestriol, 3-deoxyestradiol-

Table 2. Estrogens that have the same affinity to the receptor as estradiol-17β (group 2)

Compound	50 pg	100 pg	200 pg	1000 pg	5000 pg	10,000 pg
Estradiol-17β	77 ± 13·1	58 ± 10·4	41 ± 9·1	27 ± 11·7	18 ± 27·4	15
2-methylestradiol-17β	72	59	42	30	17	18·5
17α-ethynylestradiol-17β	77	62·5	53	—	13	11·5
18-norestradiol-17β	85	70	53	34·5	21	19
Diethyelstilbestrol	82	74	63	26	13	14
Hexestrol	80·5	72	53	32	19·5	16
Mean	79·3	67·5	52·8	30·6	16·7	15·8
Standard deviation	±5·8	±6·4	±7·4	±4·5	±3·8	±3·1

Binding of tritiated estradiol-17β in the presence of increasing amounts of competing estrogens is given in percent of control. Estradiol-17β results are given for comparison but are not included in the means.

Table 3. Steroids that compete strongly with estradiol-17β for binding sites on the receptor (group 3)

Compound	Inhibition of percent of control					
	50 pg	100 pg	200 pg	1000 pg	5000 pg	10,000 pg
Estrone	89·5	84	60	49	30·5	25·5
Estriol	—	71	65	40	24	23
16-epi-estriol	96	85	71	39·5	24	19
17-epi-estriol	93	74	69·5	40·5	25	23
16α-estradiol	91·5	72·5	70·5	43	21	19
17α-estradiol	101	84	75·5	47	23·5	17
Mean	94·2	78·4	68·6	43·2	24·7	21·1
Standard deviation	±4·3	±6·5	±5·6	±3·8	±3·3	±3·4

Binding of tritiated estradiol-17β in the presence of increasing amounts of competing steroids is given in percent of control.

17β, 17-deoxyestrone, 16-oxo-estradiol-17β, 17β-methoxyestradiol, 2-hydroxy-3-deoxyestradiol-17β, 1,3,5(10,16-estratetraen-3-ol, 1,3,5(10),16-estratetraene-3, 17-diol diacetate, estradiol-17β-glucosiduronic acid, 17β-estradiol-3-glucosiduronic acid, equilin, equilenin and 5α-androstan-3β, 17β-diol. All these compounds showed some affinity towards the estrogen receptor, but they competed only at considerably higher concentrations (1000 pg or more) with the (58 pg) tritiated estradiol-17β for the binding sites (Table 4).

The mean results of Tables 1–4 are compared on Fig. 2. This graph demonstrates that the distinction between some groups is not clearcut at every concentration. For instance, steroids of groups 1 and 4 are indistinguishable at low concentrations and have different effects only if present in larger amounts. On the other hand, the difference between groups 2 and 3 appears to decrease at high concentrations.

With compounds of groups 1 and 4 an increase rather than a decrease of

Table 4. Steroids that compete only at high concentrations with estradiol-17β for binding sites on the receptor (group 4)

Compound	Inhibition in percent of control					
	50 pg	100 pg	200 pg	1000 pg	5000 pg	10,000 pg
16-hydroxyestrone	103	99	98	83·5	37·5	29·5
16,17-epiestriol	113·5	115	107	103·5	75·5	63
3-deoxyestradiol-17β	106	98	93	89	48·5	42·5
17-deoxyestrone	113	124	105	86·5	51·5	47·5
16-oxo-estradiol-17β	117	122	114	96	68	62
1,3,5(10),16-estratetraen-3-ol	104	108	104	86	67·5	48
Estratetraenediol diacetate	113	106	107·5	92	63·5	64
2-hydroxy-3-deoxyestradiol-17β	99	106	98	88	52	37·5
Estradiol-17β-glucosiduronate	123	114	119	97·5	74	59
17β-methoxyestradiol	99	106	102·5	72	38·5	29·5
Equilin	115·5	106	96	70	32	28
Equilenin	104	111·5	97·5	80	68	53
5α-androstane-3β,17β-diol	106	106·5	111	101·5	70·5	57
Mean	108·9	109·4	104·0	88·1	57·5	47·7
Standard deviation	±7·5	±7·6	±7·6	±10·4	±13·8	±13·2

Binding of tritiated estradiol-17β in the presence of increasing amounts of competing steroids is given in percent of control.

tritiated estradiol-17β binding was observed in the presence of small amounts of steroid. Although statistically this increase was not significant it nevertheless may have some meaning as it occurred regularly.

2. *Structural requirements of the ligand for binding by the estrogen receptor of human uterus*

Highest affinity to the human uterine receptor was found if the steroid structure had all three of the following features:

1. An unsaturated ring A (1,3,5(10)-estratriene), 2. A phenolic hydroxyl group on carbon-3, and 3. An alcoholic hydroxyl group on carbon-17 in the β-configuration. The most important of these functions was the phenolic hydroxyl group on C-3. If it was missing (3-deoxysteroids) or substituted (3-methylethers, 3-glucosiduronates, 3-sulfates, 3-acetates) the affinity towards the receptor was largely or entirely abolished. The position of the hydroxyl group on the ring was also of importance: 2-hydroxy-3-deoxyestradiol-17β showed only a very limited affinity to the receptor, comparable to that of 3-deoxyestradiol-17β. An estradiol-17β with a free hydroxyl group at C-1 did not compete at all with estradiol-17β for binding sites on the receptor. In that case the result could have been influenced, however, by an additional methyl group in ring A (2-methoxy-4-methylestradiol-1,17β) as it was shown that methyl groups on ring A influence the affinity of the steroid to the receptor: Introduction of a methyl group on C-4 abolished the small but significant affinity towards the receptor of 2-hydroxy-3-deoxyestradiol-17β; presence of a methyl group at C-1 (1-methylestradiol-17β) or of methyl groups at C-1 and C-2 (1,2-dimethylestradiol-17β) led to the loss of affinity towards the receptor; 2-methoxy-4-methylestrone competed considerably less with estradiol-17β for receptor sites than did estrone. Surprisingly if a methyl group was substituted on C-2 (2-methylestradiol-17β) no change in affinity was observed.

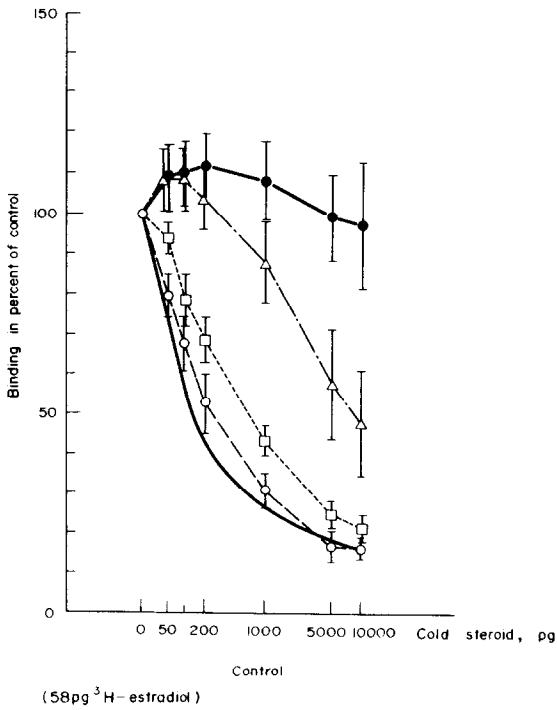

Fig. 2. Inhibition of the binding of tritiated estradiol-17β by other steroids. The abscissa is the same as in Fig. 1. On the ordinate is given the binding in percent of control (58 pg tritiated estradiol-17β only). The vertical bars show the standard deviations: ○—○ estradiol-17β (taken from Fig. 1); ●—● group 1 (Table 1): (virtually) no affinity; ○---○ group 2 (Table 2): Affinity like estradiol-17β; □----□ group 3 (Table 3): strong affinity; △-·-·-△ group 4 (Table 4): weak affinity.

Further it was essential that ring A is a benzene ring. Steroids that are similar to estradiol-17β as far as the number and positions of the hydroxyl groups are concerned did not show any affinity towards the receptor if the hydroxyl group at C-3 was alcoholic rather than phenolic. Neither 5α-androstan-3α,17β-diol, nor 5β-androstan-3α,17β-diol, nor 4-androstan-3β,17β-diol showed any binding activity, and 5α-androstan-3β,17β-diol exhibited a very limited receptor affinity. There is, however, the possibility that the presence of the angular methyl group on C-10 is responsible for the loss of affinity instead of the saturation of ring A. A 19-nor-androstanediol could be used to decide which alternative is correct.

An oxygen function on ring D was also necessary but not as essential as the hydroxyl group in ring A. This was demonstrated by the fact that 17-deoxyestrone, 17β-methoxyestradiol, 1,3,5(10),16-estratetraen-3-ol, and estradiol-17β-glucosiduronic acid still had some limited affinity to the receptor while 17β-estradiol-3-glucosiduronic acid had none. The oxygen function on C-17 may also be an oxo group (estrone) even though this reduced the receptor affinity. Nor is the position as critical as in ring A: 16α-estradiol and 17α-estradiol both exhibited considerable receptor affinity. Additional oxygen functions on ring D diminished the receptor affinity to some extent (estriol, 16-epiestriol, 17-epiestriol) or considerably (16,17-epiestriol, 16-oxo-estradiol-17β, 16-hydroxyestrone).

Presence or absence of the angular methyl group on C-13 (estradiol-17β vs. 18-norestradiol-17β) had no influence on the affinity.

Unsaturation of ring B decreased the affinity towards the estrogen receptor (estrone > equilenin > equilin).

DISCUSSION

The correlation of the shape and nature of the binding site of a receptor with the structure-affinity relationships of different ligands is a highly speculative undertaking fraught with many uncertainties and assumptions. Substitution of a single atom for another in the substrate molecule may affect its electron distribution and conformation and can alter chemical reactivity and the binding capacity of other groups.

The experimental results described in this article indicate the necessity for the ligand to have a two point attachment to the receptor site. The optimum requirement for interaction exists in estradiol-17β in which the phenolic hydroxyl group at C-3 and C-17β hydroxyl function are the active binding centres. As already mentioned the former functional grouping seems to be of primary importance, while the latter although necessary is not as essential.

It is generally assumed that attachment of small molecules to receptors can induce conformational changes in the receptor[21–24]. A receptor possessing a flexible active site could consist of two parts: (1) an attractive center determining the specificity and orientation of the substrate, (2) a second attractive center which is less specific than the first. Presumably, attachment of the substrate at the first center could then trigger a configuration change in the receptor bringing the substrate into closer proximity of the second center.

One can readily visualize a similar type of binding site for the uterine estrogen receptor. Binding is initiated by attachment of the C-3 phenolic hydroxyl group (almost certainly via the agency of a hydrogen bond) to a highly specific center. The strength of this attachment is influenced by varying functionalization of the A-ring as well as steric factors. Thus replacement of the hydrogen atom of the phenolic hydroxyl group by another grouping (methoxy, sulfoxy, etc.) eliminates binding. This is possibly because of the inability to form a sufficiently strong hydrogen bond.

Saturation of the A-ring has a similar effect. This may be due to the formation of a weaker hydrogen bond through the C-3 alcoholic group and may also be influenced by steric hindrance caused by the chair or boat shaped A-ring as compared to the planar aromatic ring in the unsaturated case.

Substitution of a methyl group at C-1 or C-4 decreases affinity drastically indicating that the initial binding site cannot tolerate bulky groups at these positions. Similar substitution at C-2 however does not decrease affinity to a marked extent.

Once the initial attachment between the C-3 phenolic hydroxyl group and the primary binding center has been established a stereospecific change in configuration of the receptor may occur in order to facilitate the second attachment of the C-17β hydroxyl group to the less specific binding center.

The two non-steroid estrogens tested, hexestrol and diethylstilbestrol, had binding affinities to the receptor similar to estradiol-17β. It may be assumed that binding occurs via the two phenolic hydroxyl groups in these two compounds. In order to test this possibility the distances between these functional groups and the

hydroxyl groups in estradiol-17β were measured on Dreiding models of these compounds. The hexestrol molecule is very flexible and there was no difficulty in obtaining a configuration in which the two phenolic hydroxyl groups were equidistant to those in estradiol ($\sim$ 10·6 Å). Diethylstilbestrol is a more rigid molecule due to the presence of the carbon–carbon double bond and the closest distance obtainable was approximately 12·0 Å. In this instance attachment to the second binding center may be enhanced by the presence of the phenolic hydroxyl group which is capable of forming a strong hydrogen bond. This could also be an indication that the interfunctional distance is not very critical. The considerable receptor affinity shown by 16α-estradiol, in which the distance between the C-16α hydroxyl group and the C-3 phenolic hydroxyl group is approximately 11·2 Å, is consistent with this line of thought.

It is conceivable that the compounds of Group 3 whose affinity for the receptor is considerable, but not as high as that of estradiol-17β, can induce only a partial configurational change in the receptor. This leads to an overall weaker attachment.

Although it has been possible to rationalize to some extent the results described in this paper through the flexible binding site, it will be necessary for the uterine receptor to be isolated and purified to a degree which will permit the application of physical measurements (such as circular dichroism, optical rotatory dispersion measurement or X-ray diffraction) in order to provide accurate details of the basis of structure-affinity relationships of molecules acting at the binding site and their mechanism of action.

It is interesting to compare the affinity of the steroids to the estrogen receptor with their ability to induce uterine growth in the rat *in vivo*[25]. *In vivo* the most efficient promoter of growth was estradiol-17β. Absence of one hydroxyl group reduced its activity, but the removal of the 3-hydroxyl group (3-deoxyestradiol-17β) caused a greater decrease in the activity than the removal of the 17-hydroxyl group (17-deoxyestradiol-17β). Oxidation of the 17-hydroxyl group reduced the growth promoting activity, so that estrone was 10-times less efficient than estradiol-17β, and 3-deoxyestrone less efficient than 3-deoxyestradiol-17β. Introduction of an oxo-group at C-16(16-oxo-estradiol-17β) reduced its activity considerably. Similarly an adjacent hydroxyl group (estriols) reduced the growth-promoting activity.

Identical effects were observed in our *in vitro* studies with regard to the affinity of the above estrogens for the receptor in human uterus.

Comparison of our results obtained with human uterus cytosol with published data on the specificity of uterine receptors in animals reveals a general similarity. The most extensive study is that of Korenman[7] who used the uterus cytosol of pregnant rabbits. Significant differences between his results and ours were observed in the following instances: The affinity of diethylstilbestrol and 17α-ethynyl-estradiol-17β for rabbit uterine cytosol was considerably stronger than that of estradiol-17β, while in the human the affinities were about the same. 2-methyl-estradiol-17β had the same affinity for human uterine cytosol receptors as unsubstituted estradiol-17β while Korenman found that substitution at C-2 markedly inhibited the binding activity (2-methoxyestradiol-17β and 2-methoxyestrone) to rabbit uterine cytosol.

Another steroid deserves mention, 17-methoxyestradiol-17β, which can be produced by intestinal bacteria from bile acids. Hill *et al.*[26] suggested that this

production of estrogens, which is dependent on the dietary fat intake, may enhance the risk of breast cancer. 17-methoxyestradiol-17β showed some affinity towards the estrogen receptor but only at high concentrations, and it was much less active than, for instance, estriol which is commonly regarded as an impeded estrogen.

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