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Structural Requirements for Maximal Inhibitory Allosteric Effect of Estrogens and Estrogen Analogues on Glutamate Dehydrogenase

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The inhibition of glutamate dehydrogenase by estrogens, estrogen analogues or polyphenylethylene derivatives (about one hundred molecules, most of them having estrogenic or antiestrogenic activities) was measured. The efficiency of these compounds in inducing allosteric inhibition of the enzyme was compared and correlated to their chemical structure: an aromatic ring A, a free phenolic group in the region of carbon 3 of the steroid nucleus and a lipophilic substitution in the region of C-12, C-13 or C-17 were found to be the main structural features required for maximal efficiency on glutamate dehydrogenase.

A tentative model for the relative orientation of the main inhibitor families is proposed. It accounts for most of the kinetic results and can be used as a tool for the selection of affinity labels directed towards the estrogen binding site of glutamate dehydrogenase.

Previous studies on the steroid binding site of bovine liver glutamate dehydrogenase were limited to a few steroids. Yielding *et al.* [1] and Douville and Warren [2] tested one or two steroids representative of the estrogen, androgen, progestagen and corticosteroid series. Estrogens were the most effective in lowering the glutamate dehydrogenase activity. Further studies performed by Yeates [3,4] using diethylstilbestrol demonstrated that a conformational change induced by this estrogen analogue was related to the enzyme inactivation.

Recently Titova and Klyueva [5], measuring the binding of different steroids to glutamate dehydrogenase by Sephadex filtration, demonstrated that the different degrees of inhibition of enzyme activity were correlated to the ability of steroids to bind the enzyme.

With the aim of affinity labelling of the 'estrogen binding site' of glutamate dehydrogenase [6,7], we carried out the present study as an attempt to define the structural requirements of this site. Most estrogens, estrogen analogues or polyphenylethylene derivatives tested on glutamate dehydrogenase have estrogenic or antiestrogenic activity. Their chemical structure was correlated to their effectiveness in lowering glutamate dehydrogenase activity. A tentative model is proposed.

Enzyme. Glutamate dehydrogenase or L-glutamate: $NAD(P)^+$ oxidoreductase (deaminating) (EC 1.4.1.3).

MATERIALS AND METHODS

Materials

NADH and 2-oxoglutarate were obtained from Boehringer (Mannheim). Buffer substances, all analytical grade, were products of Merck. Bovine liver glutamate dehydrogenase was purchased from Boehringer (Mannheim) as a crystalline suspension in ammonium sulphate. Before the experiments, the crystals were collected by centrifugation (30 min at $30000 \times g$), dissolved in 50 mM phosphate buffer, pH 7.6, 0.1 mM EDTA. The residual ammonium sulphate was removed by Sephadex G-25 filtration in the same buffer.

Enzyme Assay

Glutamate dehydrogenase activity was measured at 25 °C in 50 mM phosphate buffer, pH 7.6, by spectrophotometric measurement at 340 nm of the oxidation of NADH. The enzyme (1 μg/ml) was incubated for 1 min with 0.1 mM NADH, 8 mM 2-oxoglutarate and various amounts of inhibitor, then the kinetic was started by adding 0.16 M NH_4Cl.

Steroid or Analogues

These are described in the Appendix.

RESULTS

The binding of estrogens to glutamate dehydrogenase induces a conformational change resulting in a decrease of enzymatic activity [3,4]. We evaluated estrogen or analogue binding to the allosteric site by measuring the attendant allosteric inhibition, since this process is likely to be independent of possible binding to other sites on the enzyme. The concentration of effectors inducing a 50% inhibition (I_{50}) was determined and used to compare their effectiveness.

The upper limit of solubility of the different molecules tested being in the range of 50 – 100 μM, 50 μM was selected as the upper concentration limit for experimentation. When 50% inhibition could not be obtained at or below this effector concentration, the result was expressed in tables giving the percentage of inhibition measured at 50 μM.

The experimental results are indicated in Tables 1 – 9, viz: estratriene derivatives (Tables 1 – 5), comparison of different nuclei (Table 6), diethylstilbestrol derivatives (Table 7), polyphenylethylene derivatives (Table 8), azasteroids (Table 9).

In all these results the natural estrogens, estradiol and estrone, were taken as references, though some derivatives would be better effectors of glutamate dehydrogenase.

Influence of Substitutions on Ring A of the Steroid Nucleus: Table 1

The I_{50} of estradiol 3-methyl ether (IV) was not very different from that of estradiol (I), but larger substitutions in position 3, cf. acetyl (V) bromoacetyl (VI) or iodoacetamido (VII), severely decreased the efficiency.

Thus, the phenolic hydroxyl group is not absolutely necessary to obtain an allosteric effect. However, it should be noticed that, as confirmed and discussed below, when the phenolic group was substituted, the I_{50} never decreased below 10 μM.

The allosteric effect was slightly increased by small lipophilic substitutions: II, III, XVI were better effectors than estradiol (I).

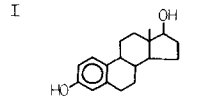
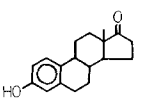
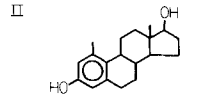
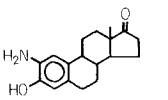
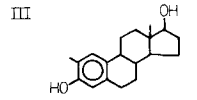
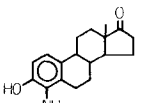
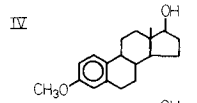
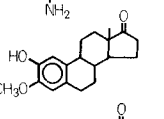
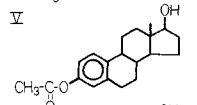
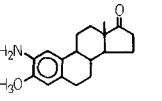
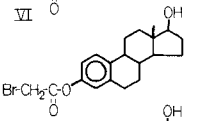
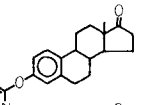
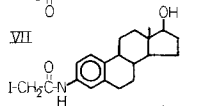
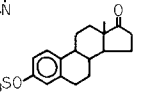
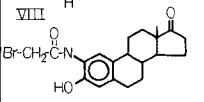
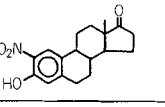
Small polar substitutions (X, XI, XII) or bulky substitutions (VII, VIII, XIV) decreased the efficiency: when these two factors were present no inhibition could be observed (XV).

Influence of the Aromaticity of Ring A: Table 2

The absence of aromaticity of ring A resulted in a dramatic decrease of the allosteric effect in the series of estradiol (left column) or 17β-estradiol 17-acetate (middle column).

Neither the nature of the function at carbon 3 of the steroid nucleus (oxo or hydroxyl) nor the planarity

Table 1. *Inhibition of glutamate dehydrogenase by estratriene derivatives: influence of substitutions on ring A*
I_{50} = the concentration of effector inducing 50% inhibition. The percentage inhibition measured at inhibitor concentration of 50 μM is given when 50% inhibition could not be obtained at or below this concentration

Derivative	I_{50} μM	50 μM %	Derivative	I_{50} μM	50 μM %
I 	30	70	IX 	50	50
II 	20	>95	X 		25
III 	15	>95	XI 		17
IV 	37	70	XII 		35
V 		25	XIII 		18
VI 	50	50	XIV 		38
VII 		18	XV 		0
VIII 		10	XVI 	28	

induced by the ethylenic bonding Δ4 or Δ5(10), compensated for the absence of aromatic structure in ring A. Furthermore, the 17β-acetyl group, which highly increased the efficiency of the estrogen structure (compare I and XX), was not sufficient to allow a measurable I_{50}.

It is surprising that conjugated double bonds in ring B and C (XIX) or naphthalenic structure of the equilenine (XXV) are unfavourable modifications. Thus the aromaticity, which is a necessary condition for good affinity, should not be extended towards ring B or C.

No allosteric effect could be detected with molecules lacking ring A (XXVI, XXVII, XXVIII).

Influence of Substitutions on Ring B: Table 3

Among the estrogens substituted in position 6 or 7, the 7α-hydroxy-estradiol (XXXIII) is less efficient than 6-oxo (XXXI), 7-oxo (XXXII) or 7α-methyl (XXX) derivatives. This result is confirmed in the

Table 2. *Inhibition of glutamate dehydrogenase by estrene derivatives: influence of the aromaticity of ring A.* Details as in Table 1

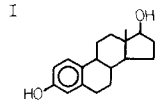
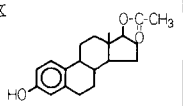
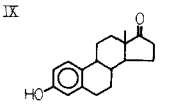
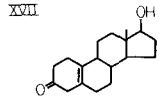
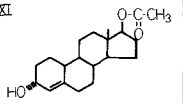
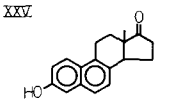
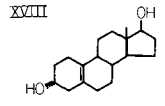
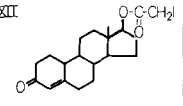
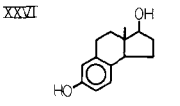
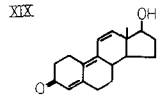
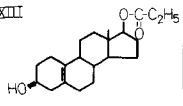
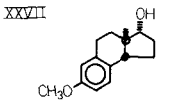
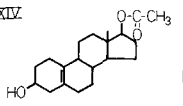
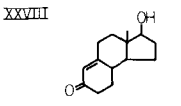
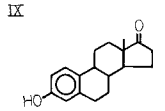
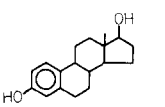
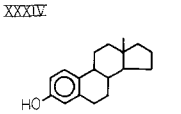
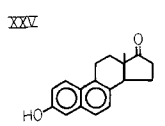
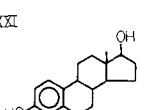
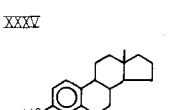
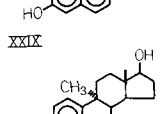
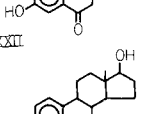
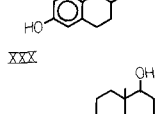
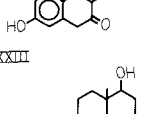
Derivative	I_{50} μM	50μM %	Derivative	I_{50} μM	50μM %	Derivative	I_{50} μM	50μM %
I 	30	70	XX 	2.6	100	IX 	50	50
XVII 		0	XXI 		0	XXV 		10
XVIII 		0	XXII 		10	XXVI 		0
XIX 		10	XXIII 		17	XXVII 		0
			XXIV 		0	XXVIII 		0

Table 3. *Inhibition of glutamate dehydrogenase by estratriene derivatives: influence of substitutions on ring B* Details as in Table 1

Derivative	I_{50} μM	50μM %	Derivative	I_{50} μM	50μM %	Derivative	I_{50} μM	50μM %
IX 	50	50	I 	30	70	XXXIV 	3	100
XXV 		10	XXXI 	50	50	XXXV 	40	60
XXIX 	29	80	XXXII 		38			
XXX 		40	XXXIII 		12			

series of 17-deoxy-estradiol: the 6α-hydroxyl group of (XXXV) increases more than 10 times the I_{50} of compound XXXIV.

The compound XXIX is equivalent to estradiol, showing that a 9α axial methyl group is well accepted by the estrogen binding site of glutamate dehydrogenase. This result and the better efficiency of 7α-methyl compared to 7α-hydroxy group suggest that the allosteric binding site has some ability to bind small lipophilic substitutions at the junction of rings B, C.

Influence of Substitutions on Ring C: Table 4

The hydroxyl or methoxy substitutions in position 11α or 11β of the steroid nucleus lowered the efficiency of the effector: whatever the series was, at 50 μM only 14 – 32% inhibition was obtained either in the estradiol series (I, XXXVI, XXXVII, XXXVIII), the estradiol 3-methyl ether series (IV, XL), the estradiol 17-methyl ether series (XLI, XLII) or the estradiol 17α-ethynyl series (XLIII – XLIV, XLV). This effect was lowered by the ethoxy group in compound XXXIX.

Table 4. *Inhibition of glutamate dehydrogenase by estratriene derivatives: influence of substitutions on ring C*
Details as in Table 1

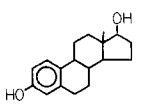
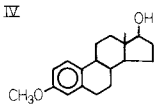
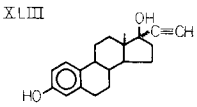
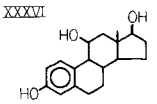
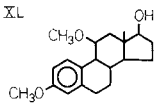
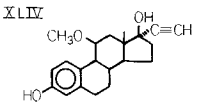
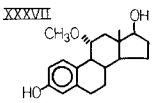
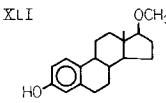
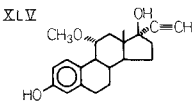
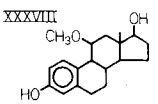
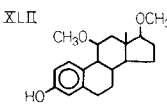
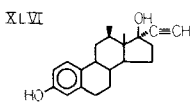
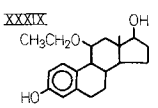
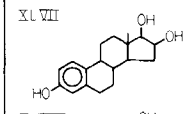
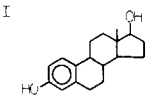
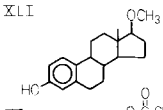
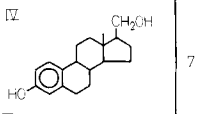
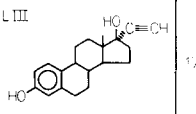
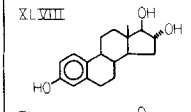
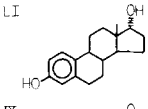
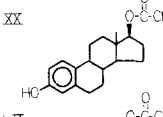
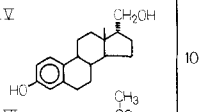
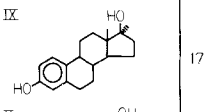
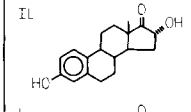
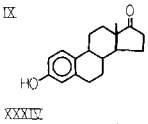
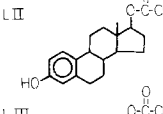
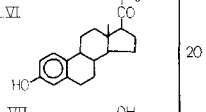
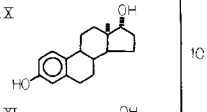
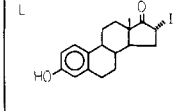
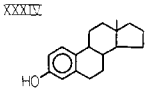
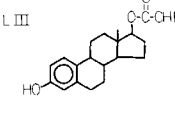
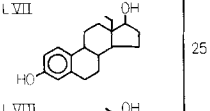
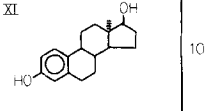
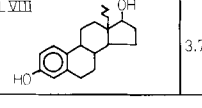
Derivative	I_{50} μM	50μM %	Derivative	I_{50} μM	50μM %	Derivative	I_{50} μM	50μM %
I 	30	70	IV 	37	70	XLIII 	17	85
XXXVI 		15	XL 		15	XLIV 		32
XXXVII 		20	XLI 	22	95	XLV 		24
XXXVIII 		14	XLII 		20	XLVI 	1.3	100
XXXIX 	46	55						

Table 5. *Inhibition of glutamate dehydrogenase by estratriene derivatives: influence of substitutions on ring D*
Details as in Table 1

Derivative	I_{50} μM	50μM %	Derivative	I_{50} μM	50μM %	Derivative	I_{50} μM	50μM %	Derivative	I_{50} μM	50μM %	Derivative	I_{50} μM	50μM %
XLVII 		0	I 	30	70	XLI 	22	95	LIV 	7	>95	XLIII 	17	85
XLVIII 		0	LI 	50	50	XX 	2.6	100	LV 	10	95	LIX 	17	85
IL 		0	IX 	47	53	LII 	0.4	100	LVI 	20	>95	LX 	10	
L 	27	95	XXXIV 	3	100	LIII 	1	100	LVII 	25	80	LXI 	10	
									LVIII 	3.7	>95			

The presence of a 12β-methyl group is specially favourable, since the I_{50} of compound XLVI is more than 10 times lower than the I_{50} of compound XLIII. As demonstrated below, it should be observed that hydrophobic substitutions at carbon 17, not very far from 12β substitution, also exerts a favourable effect (compare XLI and XLIII to I).

Influence of Substitution on Ring D: Table 5

The presence of 16α or 16β-hydroxyl groups (left column) completely suppresses the allosteric efficiency. This should be related to the polarity rather than to the hindrance of the hydroxyl group, since its substitution by the bulky atom of iodine (L) was favourable.

Either suppression of the polar function at carbon 17 (XXXIV) or lowering its polarity by etherification (XLI), esterification (XX – LII, LIII) or introduction of an alkyl chain at carbon 17 (LIV, LV, LVI, LIX, XLIII) highly increased the allosteric potency of the molecule. A 10 – 100-fold lowering of the I_{50} was observed after esterification of the 17β-hydroxyl group: compare I to XX, LII and LIII. A 10-fold increase in efficiency could also be obtained by sub-

stitution of a bulky hydrophobic group (ethyl, butyl) to the methyl 18 (LVII and LVIII).

Neither the orientation 17α nor 17β seems to be determinant, since in each pair of compounds (I, LI or LIV, LV and LX, LXI) the 17-epimers are equivalent. But it should be observed that the orientation 13α of methyl 18 is more favourable than 13β, since the 'lumiestradiols 17α and 17β' (LX, LXI) are better effectors than the corresponding estradiols 17α or 17β.

Influence of the Nature of the Nucleus of the Effector: Table 6

LXV, LXVI and LXVII are better effectors than estradiol itself. It should be noticed that LXV and LXVI are equivalent, an observation which agrees well with their almost superimposable structure. The better affinity of compound LXVII can be related to the absence of a hydroxyl group in the region of the molecule that can be considered as homologous to ring D (see Discussion). The *ent*-estradiol (LXII) is probably accepted via the ability of the site to bind bulky substitutions in the region of junctions B/C and C/D. The orientation of rings C and D relative to rings A and B is not determinant: compound LXIII (downward orientation of rings C and D) or compound LXIV (upward orientation of rings C and D) is of significant efficiency.

Influence of Substitutions on Diethylstilbestrol: Table 7

Monoesterification of diethylstilbestrol slightly increased the efficiency of the molecule (LXVI, LXIX, LXX), the allosteric effect becoming comparable with that of 17β esters of estradiol (XX, LII). In both series I_{50} was in the range of 1 μM. Therefore diethyl stilbestrol monoesters should be compared to 17β esters of estradiol keeping a free phenolic group in position 3.

The negative effect of esterification in position 3 on the estradiol molecule (V, VI, LXVIII) was also found with diesters of diethylstilbestrol (LXXI, LXXII). Thus, it appears that with 3 esters of estradiol, neither the 17β-hydroxyl group could replace the phenolic function nor could the 3-ester chain replace a 17β-ester chain by turning round the steroid nucleus in the binding responsible for the allosteric effect.

This situation is illustrated by compounds LXXIII and VIII, the first being a good allosteric effector (6 μM) in spite of the bulky substitution in *ortho* position of the phenolic group, whereas the latter cannot be accepted by turning round in the site.

Polyphenylethylene Derivatives and Analogues: Table 8

The I_{50} of most of the molecules listed in Table 8 are in the range of 3 μM, the I_{50} of compound XXXIV (Table 3).

Table 6. *Inhibition of glutamate dehydrogenase: influence of the nature of the nucleus of the effector in the estratriene, diethylstilbestrol and polyphenylethylene series*
Details as in Table 1

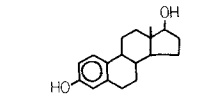
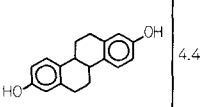
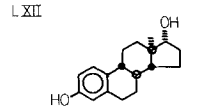
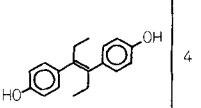
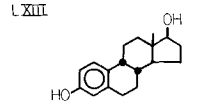
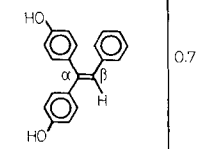
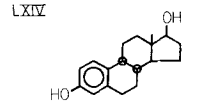
Derivative	I_{50} μM	50μM %	Derivative	I_{50} μM	50μM %
I 	30	70	LXV 	4.4	>95
LXII 		41	LXVI 	4	>95
LXIII 	50	50	LXVII 	0.7	100
LXIV 	33				

Table 7. *Inhibition of glutamate dehydrogenase by diethylstilbestrol derivatives: influence of substitutions on diethylstilbestrol and comparison with the corresponding estratriene derivatives*
Details as in Table 1

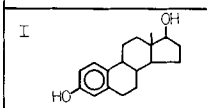
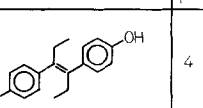
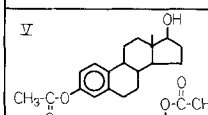
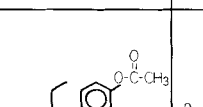
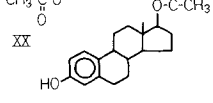
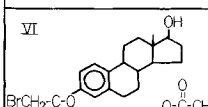
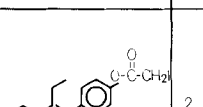
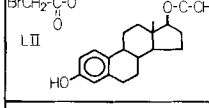
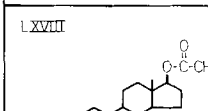
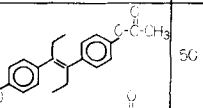
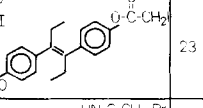
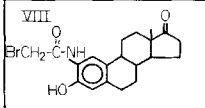
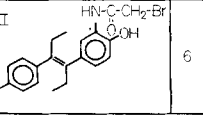
Derivative	I_{50} μM	50μM %	Derivative	I_{50} μM	50μM %
I 	30	70	LXVI 	4	>95
V 		25	LXIX 	2	100
XX 	2	100			
VI 	50	50	LXX 	2	100
LII 	0.4	100			
LXVIII 	35	80	LXXI 	50	50
			LXXII 	23	
VIII 		10	LXXIII 	6	>95

Table 8. *Inhibition of glutamate dehydrogenase by polyphenylethylene derivatives and analogues*
Details as in Table 1

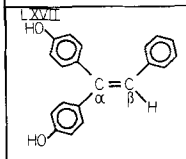
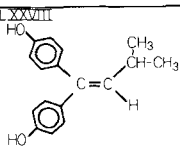
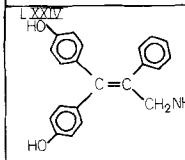
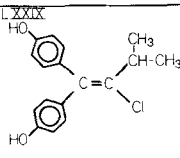
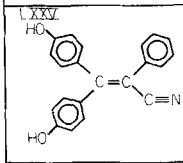
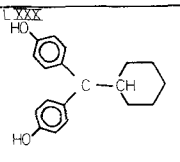
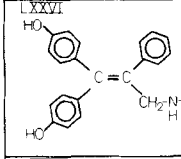
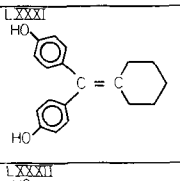
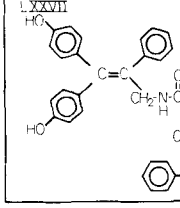
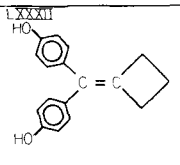
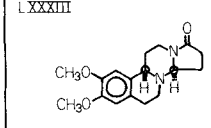
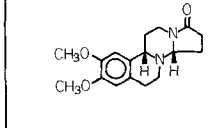
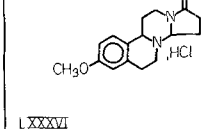
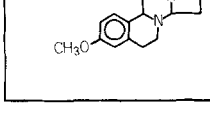
Derivative	I_{50} µM	50µM %	Derivative	I_{50} µM	50µM %
LXXVII 	0.7	100	LXXVIII 	3.5	>95
LXXIV 	1.2	100	LXXIX 	5	>95
LXXV 	3.2	>95	LXXX 	3	100
LXXVI 	3	100	LXXXI 	6	>95
LXXVII 	2	100	LXXXII 	7	>95

Table 9. *Inhibition of glutamate dehydrogenase by azasteroids*

Azasteroid	Inhibition
LXXXIII 	no inhibition for [I] < 0.4 mM
LXXXIV 	no inhibition for [I] < 0.4 mM
LXXXV 	no inhibition for [I] = 80 µM
LXXXVI 	20% inhibition for [I] = 80 µM

As discussed below the superimposition of these structures was possible according to the orientation given to the formulae in the Table 8. Bulky substitutions at carbon β (LXXIV, LXXV, LXXVI, LXXVII) were well accepted as were the other structures LXXX, LXXXI, LXXXII.

The hydrophobic isopropyl group (LXXVIII, LXXIX) could replace the bigger aromatic ring but the I_{50} was slightly increased.

Azasteroids: Table 9

No effect could be detected on glutamate dehydrogenase activity with compounds LXXXIII, LXXXIV or LXXXV in spite of the high concentration allowed by the solubility of these polar basic compounds. However, the abolition of the oxo group on ring D (LXXXVI) had a favourable effect, as in the estradiol and estrone series, since a 20% inhibition was observed but only at the high concentration of 80 µM.

DISCUSSION

The aim of this work was to define the structural features required for estrogens or analogues to induce the allosteric transition responsible for inactivation of glutamate dehydrogenase. Thus, we deliberately chose to compare the I_{50} of the molecules tested, since this parameter does not take into account eventual inefficient binding at other(s) region(s) of the enzyme.

Theoretically the enzyme inhibition could be the result of both binding and intrinsic ability of the effector to induce the conformational change. We observed that estradiol (at a concentration equal to its I_{50}) or poor effectors, like XXVI (Table 2), or LXXXV (Table 9), at 50 µM do not lower the effect of better inhibitors such as 17β-acetyl-estradiol, diethylstilbestrol or monoacetyldiethylstilbestrol and polyphenylethylene (LXVII, Table 8). If a high affinity binding of estradiol had occurred on the allosteric site and if this molecule had a poor ability to induce the conformational change, the estradiol molecule and analogues would have lowered the binding and the effect of other inhibitors by competition. Such is not the case, and the I_{50} should be considered as a good parameter for studying both binding and effectiveness. This agrees with the results of Titova and Klyueva [5], who compared the efficiency to the binding of several steroids by Sephadex filtration. They have shown that the regulatory effect of the steroid tested was correlated to their binding to the enzyme protein. The most important structural features for estrogen analogues to induce allosteric inhibition of glutamate dehydrogenase are as follows.

a) The presence of a free phenolic group at C-3 of the steroid nucleus.

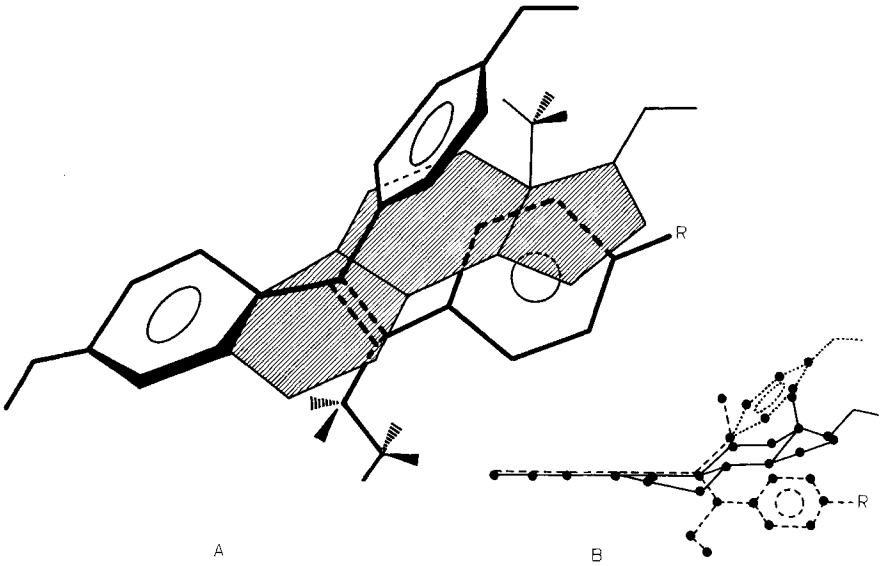


Fig. 1. *Postulated orientation of estratriene, diethylstilbestrol and triphenylethylene derivatives accounting for the results of glutamate dehydrogenase inhibition.* The phenolic ring of diethylstilbestrol and/or triphenylethylene is superposed on ring A of estradiol. Diethylstilbestrol is represented in the configuration 'diethylstilbestrol-2' according to the crystallographic results of Hospital *et al.* [8]. (A) Perspective graph of the inhibitors: fine lines and hatched area: rings B, C and D of 17β-estradiol. Heavy lines: diethylstilbestrol (R = OH) or triphenylethylene compound LXVII (R = H). (B) Lateral view of the model according to the conformation of 'diethylstilbestrol-2' described in [8]. (●——●) Estradiol; (● – –●) diethylstilbestrol (R = OH) or triphenylethylene compound LXVII (R = H); (● · · ·●) α'-phenolic ring of triphenylethylene compound LXVII. It should be noticed that the orientation of triphenylethylene dervatives relative to diethylstilbestrol or estradiol molecules is different from that proposed by Hospital *et al.* [8] for the interaction of these molecules with the uterine receptor of estrogens

b) A lipophilic substitution (ester group or alkyl chain) at C-12, 13 or 17.

The better effector was obtained when the same molecule had the above characteristics.

The aromaticity of ring A is necessary to obtain an inhibition. The supression of methyl 19 is not sufficient *per se*, since we did not observe inhibition with 19-nortestosterone even at 50 μM. Moreover, after esterification of this compound at C-17 (a modification known to considerably increase the efficiency of inhibitors in all the series tested; cf. Results Table 7), only 10% inhibition was obtained at 50 μM. The aromaticity of ring A is not the only factor influencing the efficiency: a free phenolic group is necessary to obtain effects at concentrations lower than 10 μM and it is possible in some cases to obtain effectors acting at less than 1 μM by esterification or alkylation in the region of carbons 12, 13, 17. An increase in efficiency by a factor 30 – 100 was observed when estradiol was diazoacetylated or bromoacetylated in position 17.

The polar substitutions tested around the steroid nucleus at carbons 2, 4, 6, 7, 11, 16, 17 were all unfavourable except the phenolic hydroxyl group at C-3.

7-Methyl or 11-methoxy derivatives of estradiol or estrone were less effective than estradiol but their effect remained relatively important at the concentration of 50 μM.

A tentative assay of integration of our kinetic results in a topological model of the orientation of the main inhibitor families (estratriene, diethylstilbestrol and triphenylethylene LXVII; Table 6) is given in Fig. 1. In this model the phenolic group (main orientation factor) of compounds of the three families are superimposed, the remaining part of the molecule being oriented in direction of ring D of estradiol. However, in the diethylstilbestrol series the second phenolic group is found below the α face of ring D of estradiol, such as the β benzene ring in triphenylethylenic compound LXVII (Table 6), and the α' phenolic group of this compound is directed in the direction of the well accepted hydrophobic substitutions on C-12, C-13 (cf. XLVI, LVII, LVIII in Tables 4 and 5). This representation is in complete agreement with the crystallographic data given by Hospital [8] for diethylstilbestrol and tamoxifene, a compound closely related to compound LXVII.

This speculative model gave useful information for the selection of affinity labels for the estrogen binding site of glutamate dehydrogenase as well as for the interpretation of the labelling experiments [6, 7].

APPENDIX

STEROIDS OR ANALOGUES ORIGIN

From Commercial Origin

Estriol (XLVIII), diethylstilbestrol (LXVI) from Merck; estrone sulphate (XV) from Sigma; 3-oxo-17β-hydroxy-5(10)-estrene; 16-epiestriol (XLVII); 16α-hydroxy-estrone (IL) from Steraloids.

Graciously Donated

J.P. Raynaud (Roussel Uclaf, Romainville, France): estradiol-17β (I), 1-methyl-estradiol-17β (II), 2-methyl-estradiol-17β (III), estradiol-17β 3-methyl ether (IV), estrone (IX), 3-oxo-4,9,11-estratriene-17β-ol (XIX); equilenine (XXV), *A*-desestradiol-17β (XXVI), *ent-A*-desestradiol-17β 5-methyl ether (XXVII), 5-oxo-17β-hydroxy-9-*A*-desestrene (XXVIII), 9α-methyl-estradiol-17β (XXIX), 7α-methyl-estradiol-17β (XXX), 7-oxo-estradiol-17β (XXXII), 7α-hydroxy-estradiol-17β (XXXIII), 17-deoxy-estradiol (XXXIV), 11β-hydroxy-estradiol-17β (XXXVI), 11α-methoxy-estradiol-17β (XXXVII), 11β-methoxy-estradiol-17β (XXXVIII), 11β-ethoxy-estradiol-17β (XXXIX), 11β-methoxy-estradiol-17β 3-methyl ether (XL), estradiol-17β 17-methyl-ether (XLI), 11β-methoxy-estradiol-17β 17-methyl ether (XLII); 17α-ethynyl-estradiol-17β (XLIII), 11β-methoxy-17α-ethynyl-estradiol-17β (XLIV); 11α-methoxy-17α-ethynyl-estradiol-17β (XLV); 12β-methyl-17α-ethynyl-estradiol-17β (XLVI); estradiol-17α (LI); 3-hydroxy-17β-hydroxymethyl-1,3,5(10)-estratriene (LIV); 3-hydroxy-17α-hydroxymethyl-1,3,5(10)-estratriene (LV); 17β-acetyl-1,3,5(10)-estratriene-3-ol (LVI); 18-methyl-estradiol-17β (LVII), 18-propyl-estradiol-17β (LVIII); 17α-methyl-estradiol-17β (LIX); lumiestradiol-17α (LX); lumiestradiol-17β (LXI); *ent*-estradiol-17β (LXII); 9-isoestradiol-17β (LXIII); 8-isoestradiol-17β (LXIV); 3,17-dihydroxy-*D*-homo-1,3,5(10), 13,15,17-gonahexaene (LXV).

M. Canet-Mousseron (CNRS, Ecole de Chimie, Montpellier, France): 5(10)-estrene-3α,17β-diol (XVIII); 4-estrene-3α,17β-diol 17-acetate (XXI); 5(10)-estrene-3α,17β diol 17-propionate (XXIII); 5(10)-estrene-3β,17β diol 17-acetate (XXIV).

J. F. Miquel (CERCOA Thiais, France): $\alpha\alpha'$-di(*p*-hydroxyphenyl)-β-phenylethylene (LXVII); $\alpha\alpha'$-di(*p*-hydroxyphenyl)-β-phenyl-β'-aminomethylethylene (LXXIV); $\alpha\alpha'$-di(*p*-hydroxyphenyl)-β-phenyl-β'-cyanoethylene (LXXV); $\alpha\alpha'$-di(*p*-hydroxyphenyl)-β-phenyl-β'-aminomethyl-acetamide (LXXVI); $\alpha\alpha'$-di(*p*-hydroxyphenyl)-β-phenyl-β'-aminomethyl-*N*-(*N'*-carboxybenzoxy-ε-aminocapronamide) (LXXVII); $\alpha\alpha'$-di(*p*-hydroxyphenyl)-β-isopropylethylene (LXXVIII); $\alpha\alpha'$-di(*p*-hydroxyphenyl)-β-isopropyl-β'-chloroethylene (LXXIX); di(*p*-hydroxyphenyl)-cyclohexylmethane (LXXX); cyclohexane-di(*p*-hydroxyphenyl)-methylene (LXXXI); cyclobutane-di(*p*-hydroxyphenyl)-methylene (LXXXII).

C. Viel (CERCOA, Thiais, France): 8,13-diaza-2,3-dimethoxy-17-oxo-1,3,5(10)-gonatriene (LXXXIII); 8,13-diaza-2,3-dimethoxy-17-oxo-1,3,5(10)-*ent*-gonatriene (LXXXIV); 8,13-diaza-3-methoxy-17-oxo-1,3,5(10)-gonatriene chlorhydrate (LXXXV); 8,13-diaza-3-methoxy-1,3,5(10)-gonatriene (LXXXVI).

Synthesized in Our Laboratory

Estradiol-17β 3-monoacetate (V), estradiol-17β 3-monobromoacetate (VI), 17-hydroxy-1,3,5(10)-estratriene-3-yl iodoacetamide (VII), 3-hydroxy-17-oxo-1,3,5(10)-estratriene-2-yl bromoacetamide (VIII), 2-aminoestrone (X), 4-aminoestrone (XI), 17-oxo-1,3,5(10)-estratriene-2,3-diol 3-methyl ether (XII), 2-aminoestrone 3-methyl ether (XIII), 2-nitroestrone (XVI), estradiol-17β 17-monoacetate (XX), 19-nortestosterone-17β-yl iodoacetate (XXII); 6-oxo-estradiol-17β (XXXI); 1,3,5(10)-estratriene-3,6-ξ-diol (XXXV), 16α-iodoestrone (L), estradiol-17β 17-monobromoacetate (LII), estradiol-17β 17-monodiazoacetate (LIII), estradiol-17β 3,17-diacetate (LXVIII), diethylstilbestrol monoacetate (LXIX); diethylstilbestrol monoiodoacetate (LXX), diethylstilbestrol diacetate (LXXI), diethylstilbestrol diiodoacetate (LXXII), 3-bromoacetamidodiethylstilbestrol (LXXIII).

Products V, XX, LXVIII

These products were obtained by acylation of estradiol. Infrared spectrum, and melting point are in agreement with values in the literature [9, 10].

Products VIII, X, XI, XIII, XVI

2-Nitroestrone (XVI) and 4-nitroestrone were obtained according to Werbin and Holoway [11]. The methylation of XVI according to Kuhn and Trischmann [12] gave 2-nitroestrone 3-methyl ether. These products reduced according to Kraychy [13] gave 2-aminoestrone (X), 4-aminoestrone (XI), and 2-aminoestrone 3-methyl ether (XIII). Infrared spectrum and melting point are in agreement with the literature [11, 13].

VIII was obtained by amidification of X by bromoacetic acid in the presence of dicyclohexylcarbodiimide according to Buzas *et al.* [14] with a yield of 45%. The main infrared absorption bands (KBr) were (OH phenolic, NH amide) 3325 cm^{-1}, (CO ketone) 1730 cm^{-1}, (CO amide) 1655, 1540 cm^{-1}. Melting point 196 – 198 °C.

Product XXII

This product was obtained by action of iodoacetic acid on nortestosterone in the presence of dicyclohexylcarbodiimide in the conditions described by Buzas [15]. Melting point 128 – 128.5 °C. The main infrared absorption bands (KBr) were (CO ester) 1725 cm^{-1}, (CO ketone) 1660 cm^{-1}.

Product XXXI

6-Oxo-estradiol was obtained by oxidation of estradiol diacetate (LXVIII) by tertiobutyl chromate

(yield 40% of the step) [16]. The saponification by potassium hydroxide gave 6-oxo-estradiol (80%). The physical properties are in agreement with the literature [9].

Product XXXV

This product was obtained from 6-oxo-3-hydroxy-1,3,5(10)-estratriene (Roussel Uclaf) after reduction by sodium borohydride, according to [17–18]. The nature of the epimer obtained is unknown. In the same conditions estradiol and estriol gave a 6α-hydroxy group exclusively [17,18]. Melting point 157–158 °C (dec). The main infrared absorption bands (KBr) were 3480 cm^{-1}, shoulder 3500 cm^{-1} (OH groups), 1610, 1590, 1495 cm^{-1} (aromatic nucleus).

Product LII

17β-Bromoacetoxy-estradiol was obtained with a 13% yield as by-product of the synthesis of VI described in [16]. Melting point: 176–180 °C. The main infrared absorption bands (KBr) were 3470 cm^{-1} (phenolic OH), 1730 cm^{-1} (CO ester) 1615, 1580, 1495 cm^{-1} (aromatic ring).

Product LXXI

Diethylstilbestrol (268 mg) in 3 ml of acetic anhydride/pyridine (1/2, v/v) mixture gave after 10 min at room temperature compound LXXI, which after precipitation by 1 M hydrochloric acid, was purified by column chromatography on kieselgel (Merck) eluted by chloroform with a yield of 85%. Melting point 120–122 °C. Main infrared absorption bands (KBr) were: 1760 cm^{-1} (CO ester), 1600, 1500 cm^{-1} (aromatic nucleus).

Product LXXII

Diethylstilbestrol (0.5 mmol) treated by iodoacetic acid in excess (2 mM) in the presence of dicyclohexylcarbodiimide (2 mM) in tetrahydrofurane (3 ml) at 4 °C gave compound LXXII with a 75% yield. Melting point 147–147.5 °C. Main infrared absorption bands (KBr) were 1740 cm^{-1} (CO ester), 1600, 1500 cm^{-1} (aromatic nucleus).

Product LXXIII

Aminodiethylstilbestrol was obtained by the method described by Werbin and Holoway [11] for amination of estrone.

Diethylstilbestrol (6 g) was nitrated in acetic acid 250 ml by 1.48 ml nitric acid overnight at room temperature. After purification on a kieselgel column, 2 g 3-nitrodiethylstilbestrol was obtained (mass spectrometry: $M_r = 313$), the melting point 92–95 °C. The main infrared absorption bands (KBr) were: 3420, 3500 cm^{-1} (phenolic OH) 1530, 1320 cm^{-1} (aromatic nitro).

3-Nitrodiethylstilbestrol (626 mg) was reduced by sodium dithionite (2 g) according to the conditions described by Kraychy [13] for estrone: 520 mg of 3 aminodiethylstilbestrol was obtained: melting point 150–155 °C. The main infrared absorption bands (KBr) were: 3200–3400 cm^{-1} (NH and OH aromatic) 1610, 1590, 1510 cm^{-1} (aromatic nucleus) 1250 cm^{-1} (C-N).

Bromoacetamidodiethylstilbestrol (LXXIII) was obtained by reaction of bromoacetic acid (138 mg) on 3-aminodiethylstilbestrol (286 mg) in 5 ml tetrahydrofuran in the presence of dicyclohexylcarbodiimide (250 mg). After 3 h at 4 °C, 320 mg diethylstilbestrol bromoacetamide was obtained, and purified on a kieselgel column eluted by a chloroforme/acetone mixture. Melting point: 155–156 °C. Mass spectrometry: M_r 403 and 405. The main infrared absorption bands (KBr) were: 3100–3400 cm^{-1} (NH and OH aromatic). 1650, 1550 cm^{-1} (amide) 1610, 1590, 1505 cm^{-1} (aromatic nucleus).

Product L

This product was obtained from 16α-iodoestrone 3-acetate (10 mg) synthesized as described in [19]. The 3-acetyl group was hydrolysed in 1 ml methanol/hydrochloric acid (95/5, v/v) for 15 h at room temperature. 16α-Iodoestrone (L) crystallized by adding few drops of water; melting point: 209–210 °C, main infrared absorption bands (KBr): 3350 cm^{-1} (phenolic OH), 1680 cm^{-1} (CO ketone), 1600, 1500 cm^{-1} (aromatic nucleus).

Product LIII

This product was obtained according to Katzenellenbogen *et al.* [20].

Products VI, VII, XII

The syntheses of these products are described by Pons *et al.* [16].

Products LXIX, LXX

The syntheses of these products are respectively described in [6,7].

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