Interaction of Steroids with *Pseudomonas testosteroni* 3-Oxosteroid Δ^4-Δ^5-Isomerase†

Hadassa Weintraub, Francoise Vincent,‡ Etienne-Emile Baulieu,* and Annette Alfsen

ABSTRACT: The structural features of a number of steroids and synthetic derivatives were related to their potency as competitive or noncompetitive inhibitors of the isomerization of 5-androstene-3,17-dione by *Pseudomonas testosteroni* 3-oxosteroid Δ^4-Δ^5-isomerase. Any substituent introduced at the C-11 (α or β) position of C_{18}, C_{19}, and C_{21} steroids hinders the interaction with the isomerase. With phenolic C_{18} derivatives, the C-3 hydroxyl is essential for firm interaction; removal or replacement of this group by a methyl or methoxy group weakens binding. The absence of a substituent at the C-17β position or the lengthening of the C-17β side chain increases the affinity of both C_{18} and C_{19} steroids. With C_{19} and C_{21} steroids, the absence of the angular C-19 methyl group as well as the presence of a conjugated double bond system at C-9 or C-9 and C-11 favors binding. Substituents introduced at the C-13 and C-17α positions have different effects on phenolic steroids and 3-oxo-Δ^4 derivatives. Lengthening the C-18 hydrocarbon side chain increases markedly the affinity of 3-oxo-Δ^4-monounsaturated steroids, but does not affect binding of phenolic steroids. This affinity increase is less pronounced with polyunsaturated $C_{18}\Delta^{4,9,11}$ derivatives (with 17α-substituents). The presence of a methyl, hydroxyl, ethynyl, or acetoxy group at C-17α markedly decreases the affinity of 3-oxo-Δ^4-C_{19} and C_{21} derivatives, but not of phenolic steroids. It is suggested that the fit of rings C and D in the binding site of isomerase differs for 3-oxo-Δ^4 and phenolic derivatives. Some ligands, which are structurally similar to competitive inhibitors, exhibit pure noncompetitive or mixed noncompetitive behavior. Estradiol is a competitive inhibitor, whereas estrone and its derivatives are noncompetitive. Diethylstilbestrol and the 4,4′-dihydroxy-2′,7-dimethyl-7′-ethyl-*trans*-stilbene are competitive, whereas 4,4′-dihydroxy-2′,7′-dimethyl-7-ethyl-*trans*-stilbene is a noncompetitive inhibitor. 3-Deoxyestradiol and coumestrol are mixed noncompetitive inhibitors. The affinities of estradiol and estrone for the isomerase show the same pH dependence, and equilibrium dialysis studies suggest that estrone and estradiol compete for the same binding site of isomerase. These findings complement the previously reported half-of-the-sites reactivity of the isomerase dimeric protein, and suggest that a "flip-flop" mechanism may be involved.

Pseudomonas testosteroni 3-oxosteroid Δ^4-Δ^5-isomerase (isomerase, EC 5.3.3.1) is induced in this soil microorganism when sources of carbon other than steroids are limited in the culture medium (Kawahara, 1962). The enzyme promotes the isomerization of a wide variety of Δ^5- and $\Delta^{5(10)}$-oxo substrates giving the corresponding Δ^4-3-oxo reaction products; no coenzyme is required. Various physicochemical studies have been performed on the homogeneous enzyme: the primary sequence (Benson et al., 1971) and the molecular weight and the absorbancy coefficient (Weintraub et al., 1973) have all been determined. In a previous report (Vincent et al., 1976), it was shown that isomerase exhibits a "half-of-the-sites" reactivity for some substrate analogues tested. This paper reports studies with a large variety of steroids in an attempt to define the topochemical pattern of the isomerase binding site for steroids. The structural features of the steroids which are required for a "high" affinity binding to isomerase are described in kinetic parameters.

Experimental Section

Materials

Steroids and Measurement of Radioactivity. Unlabelled steroids (a gift of Roussel-UCLAF) were more than 95% pure as determined by thin-layer chromatography and were used without further purification.

Labeled Steroids. 6,7-[^{3}H]Estradiol (sp act. 43 Ci/mmol), 6,7-[^{3}H]estrone (sp act. 55 mCi/mmol) were purchased from New England Nuclear Corporation (NEN); 4-[^{14}C]estradiol (sp act. 54.5 mCi/mmol) was purchased from C.E.A. (Saclay, France).

Radioactivity was measured with a Packard liquid scintillation counter in Bray's solution (efficiency 16–20%) or with an Omnifluor (NEN)–toluene system (efficiency 40%).

Chemicals. The buffers used (potassium phosphate, sodium acetate, Tris[1]-glycine, and Tris-HCl) were prepared from Merck products.

Methanol and ethanol were purchased from Prolabo, and dioxane (Uvasol for spectroscopy) was from Merck.

Methods

The isomerase was purified by a modification of a previously described method (Jarabak et al., 1969). The initial steps of their procedure were retained; thus the isomerase was adsorbed on calcium phosphate gel in ethanol (66% v/v) with 5 mM $MgCl_2$ as previously described. We have introduced dialysis against poly(ethylene glycol) (40% v/v) (Prolabo) to concentrate the large volumes of isomerase solution. The enzyme was further purified by DEAE-cellulose (DE 52 Whatman, high capacity) column chromatography using a continuous phosphate buffer gradient (0.01–0.1 M). The active eluates

† From Cnrs, ER 125 (H.W.), Unité de Recherches sur le Métabolisme Moléculaire et la Physiopathologie des Stéroïdes de l'Institut National de la Santé et de la Recherche Médicale, Université Paris-Sud, Département de Chimie Biologique, 94270 Bicêtre, France (H.W., F.V., E.-E.B.), and Laboratoire des Etats Liés Moléculaires, Cnrs, Faculté de Médecine, 75006 Paris, France (A.A.). *Received March 3, 1977; revised manuscript received July 15, 1977.*

‡ This paper is a part of Thèse de Doctorat ès Sciences Physiques.

[1] Abbreviations used are: Tris, tris(hydroxymethyl)aminomethane; DEAE, diethylaminoethyl; EDTA, ethylenediaminetetraacetic acid.

were passed through hydroxylapatite columns (Bio-Gel HTP, Bio-Rad), preequilibrated with 1 mM potassium phosphate buffer. Isomerase is excluded from this column but the denatured enzyme is retained. The active enzyme in the void volume is precipitated by $(NH_4)_2SO_4$, and after dialysis is rechromatographed on an HTP column.

The homogeneity of the isomerase preparation was checked by polyacrylamide gel electrophoresis (where a single narrow band is observed) and by the very characteristic ultraviolet (UV) spectrum of isomerase. Homogeneous isomerase has a specific activity of about 55 000 units/mg using the new $E_{1\,cm}^{0.1\%}$ value of 0.328 ± 0.004 at 280 nm (Weintraub et al., 1973). One unit of enzyme isomerizes 1 μmol of 5-androstene-3,17-dione per min under standard conditions (Kawahara and Talalay, 1960) and corresponds to an enzyme concentration of 0.67 nM.

Kinetic Determinations of Apparent Dissociation Constants K_I. Initial isomerization rates (v_i) at 25 °C were recorded within the first 2 min by following changes in absorbance at 248 nm using a Cary 15 recording spectrophotometer fitted with a thermostated cell compartment; the temperature was checked during the kinetic measurements with a digital thermometer (Limited Systems Corp., Dayton, Ohio) and varied ±0.05 °C. The standard reaction cell (unless otherwise stated) contained 3 mL of 0.03 M potassium phosphate buffer (pH 7.0, ionic strength (μ) = 0.066), methanol (10% v/v), and 5-androstene-3,17-dione (50–750 μM) as substrate; the reaction was initiated by the addition of isomerase diluted with bovine serum albumin (0.5% w/v). The control "blank" cell contains all components except the enzyme; under the conditions used spontaneous isomerization of the substrate is negligible. K_I determinations were made according to Dixon (1953); see Figure 1, for estrone and estradiol.

When the inhibition observed with certain steroids was neither competitive nor noncompetitive by the Dixon or Lineweaver–Burk (1934) plots, an estimate of the affinity of the steroid inhibitor was obtained using the method of Friedenwald and Maengwyn-Davies (1954). A plot of the slopes and ordinate intercept of Lineweaver–Burk plots vs. inhibitor concentration gives two straight lines, corresponding to two different K_I values [K_{Is} (from the slope) and K_{Ii} (from intercept)].

Since steroids are rather rigid molecules with only a few polar groups, with limited solubility in aqueous media, organic solvents are required for steroid solubilization over the extended steroid concentration range necessary to determine kinetic parameters quantitatively. The influence of different organic solvents on kinetic parameters has previously been described (Falcoz-Kelly et al., 1968; Weintraub et al., 1972; Jones and Gordon, 1973). The main effect observed with straight aliphatic alcohols is that the apparent affinity of substrates and steroid inhibitors as well as V_{max} are progressively reduced, as the concentration of organic solvent is increased. Most previous kinetic studies on isomerase have been performed at low alcohol concentrations (≤5%) (Jarabak et al., 1969; Kawahara, 1962; Wang et al., 1963; Martyr and Benisek, 1973; Jones and Wigfield, 1968; Weintraub et al., 1970). According to Wigfield and Jones, the isomerization of substrates more hydrophobic than 5-androstene-3,17-dione (as with C-17β side chains) requires high concentrations of organic solvents. This suggests that isomerization occurs only when steroids are in a monomeric state. Nevertheless, even with such substrates, only a small proportion of the steroid need be in a monomeric state to achieve rapid and complete isomerization, assuming that there is a rapid equilibrium between the solvated monomer and the micellar form of the substrate. To date there are no precise data concerning the equilibrium between the monomer–polymer (micelles) of steroid molecules under these solvent conditions.

In the present work, a concentration of 10% (v/v) of methanol was chosen. It provides the best balance of properties, allowing solubilization of a wide variety of steroids with different polarities, as checked by turbidity measurements, while producing minimal enzymatic denaturation during the 2–3 min interval necessary for kinetic measurements. The apparent K_m of 5-androstene-3,17-dione is doubled in 10% (v/v) methanol (K_m = 625 μM) compared to the K_m in 3.3% (v/v) methanol (320 μM). Other examples of the solvent effects with different substrates and inhibitors have been previously published (Weintraub et al., 1972).

The study of K_I dependence on pH with estrone (E_I) and estradiol (E_{II}) was performed at a single ionic strength (0.066) using different buffers: (i) sodium acetate in the 3.8–5.7 pH range, (ii) potassium phosphate in the 5.7–8.0 pH range. Determination of pH was made with a Radiometer pH meter Model 25 (precision ±0.05 pH unit). In the acidic pH range (from 4 to 5.5), isomerase is not irreversibly inactivated during the time necessary for v_i measurements, and the enzyme is stable from pH 6 to 9. The effects of pH on K_I values with isomerase have been interpreted in terms of the theory of Dixon and Webb (1958) which assumes a bifunctional catalysis.

Equilibrium Dialysis. Dialysis tubings (Visking tubing $^{8}/_{100}$ ft, Union Carbide Corp.) were extensively washed with boiling sodium bicarbonate solutions containing EDTA and then with distilled water. Dialyses were routinely performed at 4 °C for 20 h (the time necessary to achieve equilibrium was found to be about 16 h). One-milliliter portions of an isomerase solution (1 μM) in 0.03 M potassium phosphate buffer (pH 7.0) were dialyzed with magnetic stirring against 15 mL of the same medium. To minimize enzyme denaturation which becomes important after about 1 h of dialysis in 10% (v/v) methanol these experiments were performed using 3.0% (v/v) methanol. Radioactive steroids were introduced outside the tubing in a concentration range between 0.2 and 7.7 μM, in a final volume of 15 mL. After the equilibrium was reached, the radioactivity inside and outside the tubing was measured and steroid concentrations were calculated. The unbound steroid concentration ([U]) is equal to the steroid concentration outside the tubing; the bound steroid concentration ([B]) is obtained from the difference between the total steroid concentration ([T]) inside the tubing and [U]. When estrone and estradiol were simultaneously present, to demonstrate their mutual exclusion by the enzyme, equilibrium dialysis was performed with a mixture of [^{3}H]estrone and [^{14}C]estradiol at various concentrations.

The data were plotted according to Best-Belpomme and Dessen (1973) derived from the law of mass action. Considering a system containing a protein and two ligands (eq 1 to 5) which bind to the same binding site, then:

$$S + U_1 \underset{K_{D1}}{\rightleftharpoons} B_1 \qquad K_{D1} = \frac{[S][U_1]}{[B_1]} \tag{1}$$

$$S + U_2 \underset{K_{D2}}{\rightleftharpoons} B_2 \qquad K_{D2} = \frac{[S][U_2]}{[B_2]} \tag{2}$$

The concentration of free protein sites, [S], appears in the two equilibria, where $[U_1]$ and $[U_2]$ are the concentrations of unbound ligands 1 and 2, whereas $[B_1]$ and $[B_2]$ are the concentrations of bound ligands 1 and 2, respectively. K_{D1} and K_{D2} are the dissociation constants of the two ligands. Thus, at equilibrium:

$$[S] = \frac{K_{D1}[B_1]}{[U_1]} = \frac{K_{D2}[B_2]}{[U_2]} \quad (3)$$

The total concentration of ligand 2, $[T_2]$, is equal to $[U_2] + [B_2]$; then:

$$\frac{K_{D1}[B_1]}{[U_1]} = \frac{K_{D2}[B_2]}{[T_2] - [B_2]} \quad (4)$$

and

$$([T_2]/[B_2]) - 1 = [U_1]K_{D2}/[B_1]K_{D1} \quad (5)$$

In our work estradiol is considered as ligand 1 and estrone as ligand 2.

Results

Kinetic Study with Steroid Ligands

A set of 94 steroids (designated **1–94**) have been tested for their activity in inhibiting the enzymatic isomerization of 5-androstene-3,17-dione, and are shown in Table I together with their inhibition constants (K_I).

The effects of chemical modifications resulting from introduction of various substituents at different positions of estrogens, androgens, and progestagens have been tested with the following results.

Substitutions on Ring A. Replacement of the 3-hydroxyl in estradiol by a 3-methyl or a methoxy group markedly decreases the affinity of the steroid for isomerase (compounds **1** to **5**).

Addition of two methyl groups at C-2 in 17α-methyl-3-oxo-4,9,11-trien-17β-ol decreases more than twofold the affinity (steroids **37** and **38**). Substitution of the C-2 hydrogen in estrone decreases the affinity about 2.5-fold; 2-fluoroestrone (**9**) like estrone (**8**) is a pure noncompetitive inhibitor.

A similar affinity decrease upon introduction of bromine at C-2α in testosterone is reported by Jones and Ships (1972). On the contrary, these authors observed an affinity increase of steroids having a saturated ring A; the affinity of the 2α-bromodihydrotestosterone was threefold increased relative to dihydrotestosterone.

Substitutions on Ring B. Introduction of a C-7α methyl group in 19-nortestosterone or estradiol was without significant effect on affinity (**39** and **40**, **1** and **10**); however, with estrone an increase in affinity occurs (**8** and **11**).

The differential effect upon addition of a C-7α methyl group in estradiol and estrone suggests that the binding sites for these two molecules are different when substrate is present. Estrone interacts with a site to produce noncompetitive inhibition, whereas estradiol blocks the substrate binding site. Therefore, it is difficult to draw any conclusion on the importance of the effect with the C-7α CH_3-substituted estrone.

If a four-carbon chain is introduced at the 7α position in estradiol (**1**) the affinity is markedly decreased. The potency of estradiol to inhibit isomerase is reduced 20- and 4-fold, respectively, by introduction of a 7-α-ylbutyric acid or 7-α-ylbutyric alcohol residue (compare **1**, **12**, and **13**). This suggests that the drastic decrease in affinity is due not only to steric hindrance but also to a charge effect.

Junction between Rings A and B. Removal of the C-19 methyl group, to give the corresponding nor derivatives, markedly increases affinity of inhibitors (**39** and **41**, **64** and **65**). A similar increase in apparent affinity of 19-nor substrates has been previously reported (Falcoz-Kelly et al., 1968; Weintraub et al., 1972); thus, the K_m of 5-androstene-3,17-dione is 300 μM whereas the K_m of 5-estrene-3,17-dione is 130 μM. The fact that removal of the C-19 methyl has identical effects on K_I and K_m suggests that the presence of the C-19 angular methyl group does not favor the formation of the enzyme–substrate complex. Likewise, hydroxylation of the angular C-19 methyl group at C-10 (giving CH_2OH) produces a further decrease in binding (compare steroids **39**, **41**, and **63**).

Substitution on Ring C. Introduction of a hydroxyl or a methoxy group at the C-11 position (whether α or β) or of an 11-oxo group completely abolishes binding as illustrated by the steroid pairs (**1** vs. **14**; **15** vs. **16** and **17**; **8** vs. **18**; **41** vs. **42**; **66** vs. **67**; **68** vs. **70**). This effect, in part at least, appears to be due to a steric hindrance, and would be compatible with the increase of affinity previously described, with 19-nor derivatives compared to the corresponding 19-methyl compounds.

Junction between Rings C and D. Introduction of an ethyl or propyl group at the C-13 position in estradiol does not significantly affect binding (compare steroids **1**, **19**, and **20**). With 3-oxo-Δ^4 derivatives, on the other hand, lengthening of the hydrocarbon side chain at C-13 enhances affinity for isomerase (**39** vs. **43**; **44** vs. **45** and **46**; **48** vs. **49**; and **54** vs. **55**). With polyunsaturated Δ^4-3-oxo steroids, introduction of an additional methyl or ethyl group at C-18 has a less pronounced effect on binding (**48** vs. **49** and **54** vs. **55**). A slight affinity increase with the 13β-ethyl-4-estrene-3,17-dione compared to 4-estrene-3,17-dione has previously been observed (Falcoz-Kelly et al., 1968).

Substitutions on Ring D. 17α Derivatives. Addition of a methyl or ethynyl group at C-17α in estradiol does not significantly affect binding; if the side chain at 17α is lengthened to give the 17α-ethynyl derivative, affinity is increased (steroids **1**, **15**, **21–25**). Contrariwise, with 3-oxo-Δ^4, -$\Delta^{4,9}$, or -$\Delta^{4,9,11}$ derivatives, addition of a 17α-hydroxyl, 17α-methyl, 17α-ethynyl, or acetoxy group markedly increases binding (steroids **41** vs. **50**; **39** vs. **51**; **52** vs. **37**; **39** vs. **44**; **43** vs. **45**; **47** vs. **41**; **52** vs. **54**; **53** vs. **48**; **65** vs. **71**; **73** vs. **74**).

17β Derivatives. The enhanced inhibition efficiency associated with lengthening the C-17β side chain is best illustrated with three series of inhibitors: 19-nortestosterone compared to 19-norprogesterone, testosterone to progesterone or 21-hydroxyprogesterone, and estradiol to 1,3,5(10)-pregnatriene-3,20-dione (**39** vs. **65**, **41** vs. **64** and **66**, **1** vs. **26**). The effect of this structural modification on an increase in affinity has been previously reported, using other alcohol concentrations (Falcoz-Kelly et al., 1968; Weintraub et al., 1972).

Esterification of the 17β-hydroxyl group of estradiol likewise increases affinity. The K_I values of the 17β-hemisuccinate and of the 17β-acetate are 10- and 20-fold increased relative to estradiol (**1**, **27–28**). The same enhancement is observed with 4-estrene and 4-androstene derivatives (compare **41** to **57**; **39** to **56**).

C-17 Deoxy Derivatives. Independent of the nature of ring A, 17-deoxy steroid derivatives have higher affinities than the corresponding oxygenated compounds. Thus, 17-deoxyestradiol is more effective than estradiol and estrone (**1**, **6**, and **8**); similarly, 4-estren-3-one is a more effective inhibitor than 4-estrene-3,17-dione and 19-nortestosterone (steroids **58** and **61** and **39**); 17-deoxytestosterone is bound more tightly than testosterone and 4-androstene-3,17-dione (steroids **41** and **59** and **60**).

Comparative Efficiency of 17-Oxo and 17β-Hydroxy Groups. The respective binding affinities of 17-oxo and 17β-hydroxyl compounds are markedly influenced by the nature of ring A. With inhibitors containing a phenolic ring, the 17-oxo derivative is more efficient than the corresponding 17β-hydroxyl compound; conversely, in the 4-estrene and 4-androstene series, the 17β-hydroxy derivatives have higher

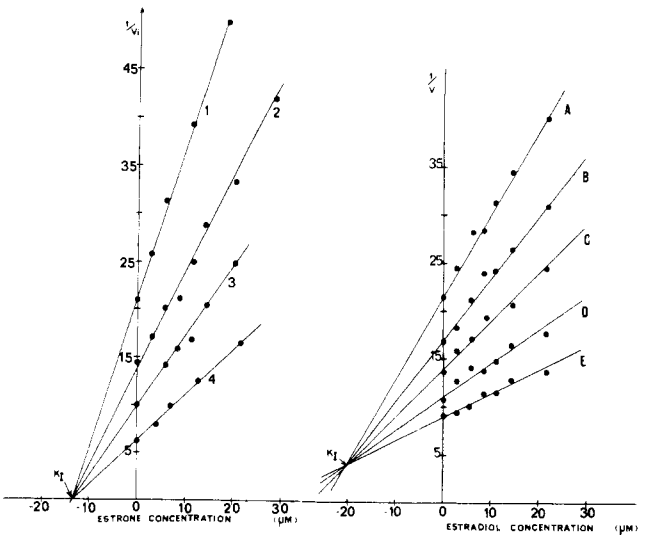


FIGURE 1: Dixon plots (1953) of the inhibition of the enzymatic isomerization of 5-androstene-3,17-dione by estradiol and estrone. K_I values are expressed in micromolar ligand concentrations. Experiments were performed in potassium phosphate buffer (0.03 M, pH 7.2) in 10% (v/v) methanol at 25 °C. With estradiol, four substrate concentrations were used: (1) 93 μM; (2) 135.5 μM; (3) 232.5 μM; and (4) 345 μM. With estrone five substrate concentrations were used: (A) 135 μM; (B) 196 μM; (C) 232.5 μM; (D) 325 μM; and (E) 465 μM. Isomerase was 7.4×10^{-6} μM

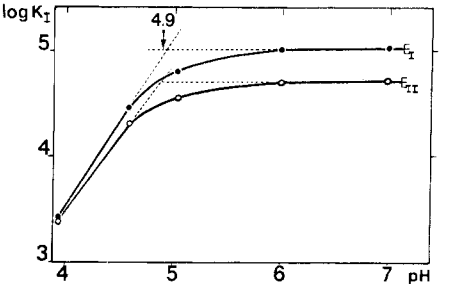


FIGURE 2: Affinity of estrone (E_I) and estradiol (E_{II}) as functions of pH. K_I values are expressed in micromolar concentrations and were determined according to Dixon (1953), in 0.03 M potassium phosphate buffer (pH 7.0) in 10% (v/v) methanol at 26 °C with 5-androstene-3,17-dione as substrate. More details are outlined in the Experimental Section.

affinity than the 17-oxo compounds (compare steroids **39** and **61; 41** and **60**).

Comparative Efficiency of the C-17α and -β Hydroxyl Groups. The 17α-estradiol exhibits higher affinity for isomerase than 17β-estradiol (**31** and **1**); in contrast, the 17α-testosterone does not bind isomerase (**41** and **62**), whereas testosterone (with a 17β-hydroxy) does bind.

16α-Hydroxylation of Estradiol. A supplementary hydroxylation of estradiol at C-16α to give estriol reduces affinity fourfold (**32** vs. **1**).

Double Bond(s) in the B and C Rings. Introduction of one or two double bonds in ring B in 1,3,5(10)-estratriene derivatives slightly increases affinity (**1** vs. **30, 8** vs. **29** and **33**). With the 4-estrene series, introduction of double bonds in ring B, or B and C, results in a greater increase in affinity (**39** vs. **53** and **52; 44** vs. **48** and **54; 51** vs. **37**).

For 17-α-methyl derivatives of norprogesterone, the introduction of a double bond between C-9 and C-10 enhances affinity (**72** and **74**), whereas for the 19-norprogesterone $\Delta^{4,5}$ derivative, no significant effect on binding was observed (**65** vs. **73**).

Stereoisomers of Natural Steroids. The stereoisomer of estradiol (antipodal) has higher affinity for isomerase than estradiol (**1** vs. **34**). 8-Isoestradiol (**35**) on the other hand has only slightly higher affinity than estradiol (**1**), whereas 9-isoestradiol shows drastic reduction in binding (**1** vs. **36**).

Stilbene Derivatives (78–82). Diethylstilbestrol (**78**) is a competitive inhibitor and has almost the same K_I value as estradiol.

Dimethylstilbestrol (**79**), which has a slightly lower affinity than diethylstilbestrol, shows that removal of the two CH_3 groups in diethylstilbestrol reduces binding.

If one considers that the two phenol rings of diethylstilbestrol represent rings A and D of estradiol and that the 4,4′-hydroxyl groups are equivalent to the 3- and 17β-hydroxyl groups in estradiol then the results obtained with modified stilbene and estradiol derivatives show a striking parallelism. Thus, when two methoxy groups are added in estradiol (**5**) or in compound **80** to give **81,** binding was no more detectable.

Two asymmetrical stilbene derivatives (**80** and **82**) are also effective inhibitors of isomerase. Compound **80,** like diethylstilbestrol (**78**), is competitive (K_I = 14 μM), whereas derivative **82** is surprisingly noncompetitive but binds to isomerase with the same affinity as diethylstilbestrol (K_I = 19 μM).

Miscellaneous Compounds. The *D*-homo derivative of estradiol, the bis-(*p*-hydroxyphenyl)hexahydrochrysene (derivative **83**), which is a rigid molecule and resembles the diethylstilbestrol, is a competitive inhibitor with a slightly higher affinity (K_I = 15 μM) than diethylstilbestrol (K_I = 18 μM) and estradiol (K_I = 20 μM).

Two *B*-nor derivatives, *B*-norestrone and *B*-nor-17α-methyltestosterone, are, respectively, noncompetitive and competitive inhibitors with significantly lower affinity than their reference compounds (compare **8** and **84; 50** and **85**).

The polycyclic cyclofenil and nafoxidine and some phytoestrogens bind to isomerase (derivatives **86–90**). However, with miroestrol, binding is not detectable and this could be related to the presence of the two methyl groups at C-11.

Finally, four derivatives which are bicyclic derivatives and resemble a part of the steroidal nucleus (A and B or A and D) do not bind isomerase (derivatives **91–94**).

Comparative Study of Estradiol and Estrone Interaction with Isomerase

To investigate the differential behavior of estradiol and estrone (Figure 1), we attempted to identify the pH-dependent amino acid residues which could be involved in the complex formation of isomerase and these two steroids.

Figure 2 shows the plot of log $1/K_I$ for estrone and estradiol as a function of pH, in 10% (v/v) methanol. The shape of the two curves and the pK_E value (pH 4.9) are identical.

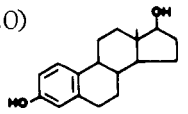
According to the Dixon rules and from the convexity of these curves, the pK value of 4.9 may be assigned to a group of the free enzyme (pK_E), since the other components (estrone, estradiol, and 5-androstene-3,17-dione) do not ionize in this pH range. An almost identical pK_E value for the isomerization of 5-androstene-3,17-dione in 3.3% methanol has previously been obtained (Weintraub et al., 1970).

The fact that estrone and estradiol show identical pK_E values for binding does not support the idea that two distinct sites in isomerase are present which might be expected from the purely noncompetitive behavior of estrone in contrast to the pure competitive inhibition of estradiol.

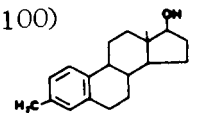
In a previous work it has been shown by equilibrium dialysis, using either radioactive nortestosterone or estradiol, that there is only one steroid binding site per isomerase dimer in the enzyme concentration range studied (Vincent et al., 1976). In the present work, equilibrium dialysis studies were carried out with a mixture of [^{14}C]estradiol and [^{3}H]estrone. Isomerase

TABLE I[a]

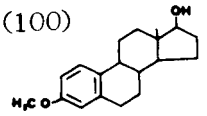
1. C(20)



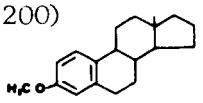
2. C(100)



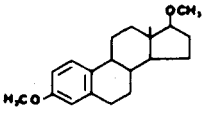
3. C(100)



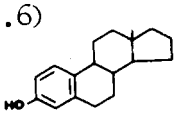
4. C(200)



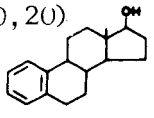
5. U



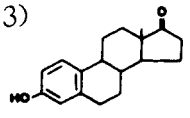
6. C(2.6)



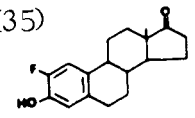
7. M(400, 20)



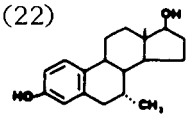
8. N(13)



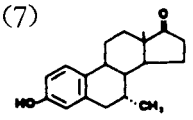
9. N(35)



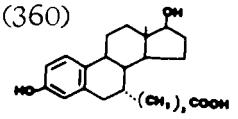
10. C(22)



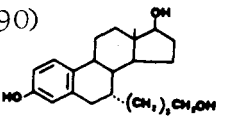
11. N(7)



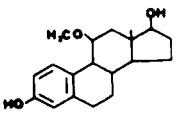
12. C(360)



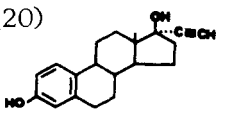
13. C(90)



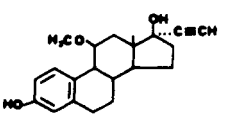
14. U



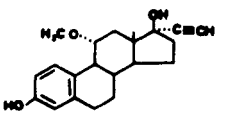
15. C(20)



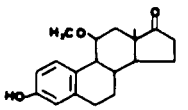
16. U



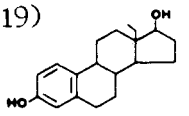
17. U



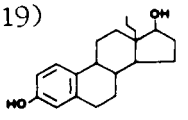
18. U



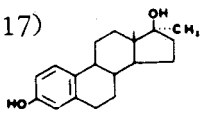
19. C(19)



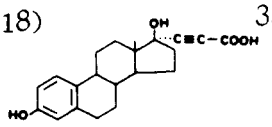
20. C(19)



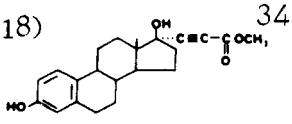
21. C(17)



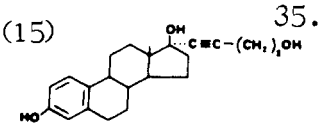
22. C(18)



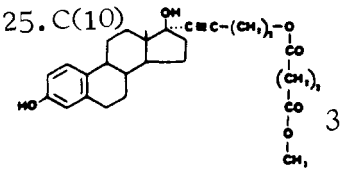
23. C(18)



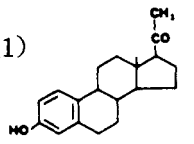
24. C(15)



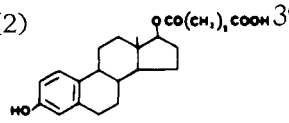
25. C(10)



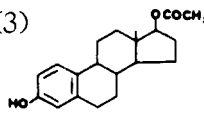
26. C(1)



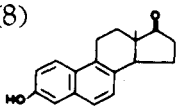
27. C(2)



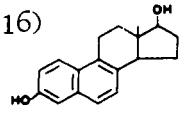
28. C(3)



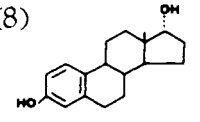
29. C(8)



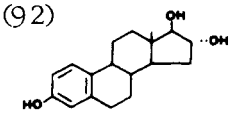
30. C(16)



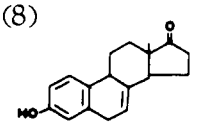
31. C(8)



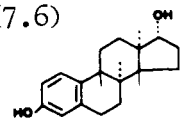
32. C(92)



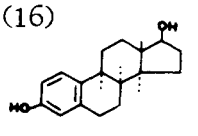
33. C(8)



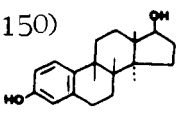
34. C(7.6)



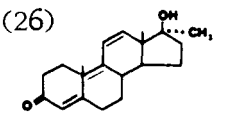
35. C(16)



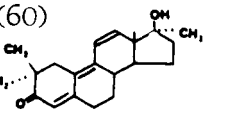
36. C(150)



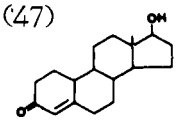
37. C(26)



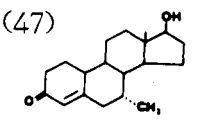
38. C(60)



39. C(47)



40. C(47)



41. C(150)

42. U

43. C(6.5)

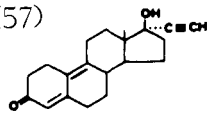
44. C(110)

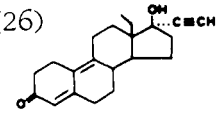
45. C(32)

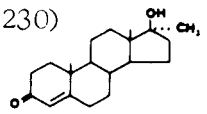
46. C(26)

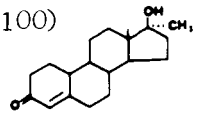
47. C(427)

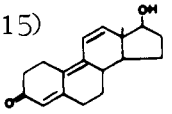
TABLE I (continued)

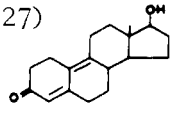
48. C(57) 

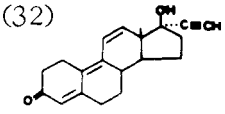
49. C(26) 

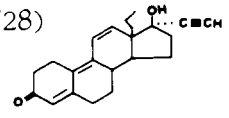
50. C(230) 

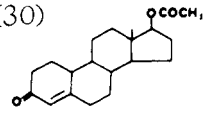
51. C(100) 

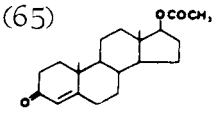
52. C(15) 

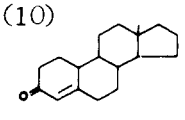
53. C(27) 

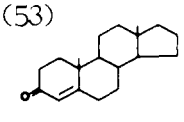
54. C(32) 

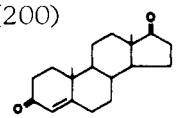
55. C(28) 

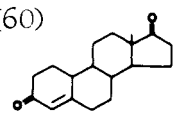
56. C(30) 

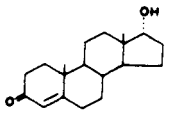
57. C(65) 

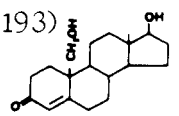
58. C(10) 

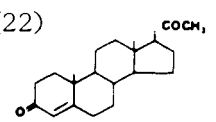
59. C(53) 

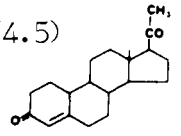
60. C(200) 

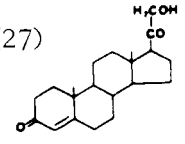
61. C(60) 

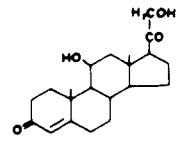
62. U 

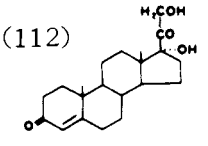
63. C(193) 

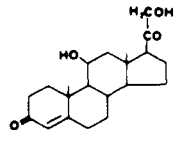
64. C(22) 

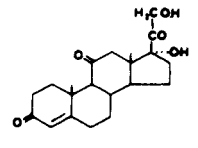
65. C(4.5) 

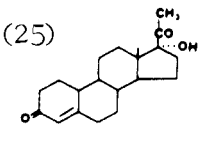
66. C(27) 

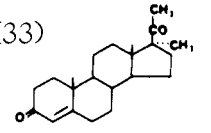
67. U 

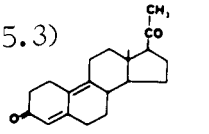
68. C(112) 

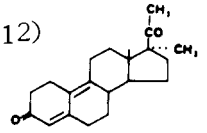
69. U 

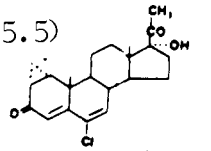
70. U 

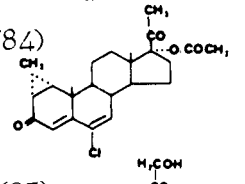
71. C(25) 

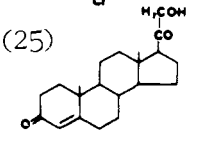
72. C(33) 

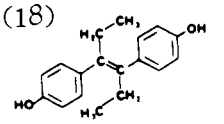
73. C(5.3) 

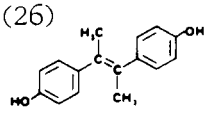
74. C(12) 

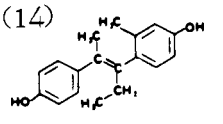
75. C(5.5) 

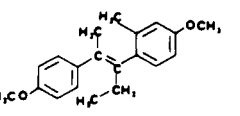
76. C(84) 

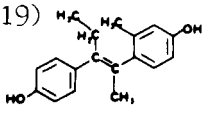
77. C(25) 

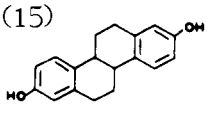
78. C(18) 

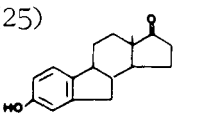
79. C(26) 

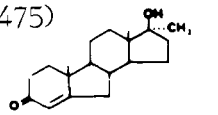
80. C(14) 

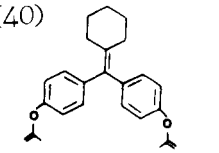
81. U 

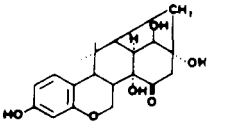
82. N(19) 

83. C(15) 

84. C(25) 

85. C(475) 

86. C(40) 

87. U 

88. M(17,64)

89. C(6)

90. C(90)

91. U

92. U

93. U

94. U

TABLE 1 *(continued, footnotes)*

[a] Experiments were performed in potassium phosphate buffer (0.03 M, pH 7.0) containing 10% (v/v) methanol at 25 °C. 5-Androstene-3,17-dione was used as substrate in the concentration range 50–750 μM and inhibitor concentrations varied from 1 to 60 μM. K_I (inhibition constants) were determined at least with four substrate concentrations according to Dixon (1953) and expressed in micromolar concentration. The code letters are: C, competitive inhibitors; NC, noncompetitive inhibitors; M, mixed noncompetitive inhibitors; U, unbound steroids. K_I values expressed in micromolar concentration are presented in parentheses. For mixed noncompetitive inhibitors the two numbers indicate, respectively, K_{Is} and K_{Ii}. Names of compounds (1 to 94) tested were: **1**, 1,3,5(10)-estratriene-3,17β-diol (estradiol-17β), C; **2**, 3-methyl-1,3,5(10)-estratrien-17β-ol, C; **3**, 3-methoxy-1,3,5(10)-estratrien-17β-ol, C; **4**, 3-methoxy-1,3,5(10)-estratriene, C; **5**, 3,17β-dimethoxy-1,3,5(10)-estratriene, U; **6**, 1,3,5(10)-estratrien-3β-ol, C; **7**, 1,3,5(10)-estratrien-17β-ol, M; **8**, 3-hydroxy-1,3,5(10)-estratrien-17-one (estrone), NC; **9**, 3-hydroxy-2-fluoro-1,3,5(10)-estratrien-17-one, NC; **10**, 7α-methyl-1,3,5(10)-estratriene-3,17β-diol, C; **11**, 3-hydroxy-7-methyl-1,3,5(10)-estratrien-17-one, NC; **12**, 3,17β-dihydroxy-1,3,5(10)-estratrien-7α-ylbutyric acid, C; **13**, 3,17β-dihydroxy-1,3,5(10)-estratrien-7α-ylbutyric alcohol, C; **14**, 11β-hydroxyl-1,3,5(10)-estratriene-3,17β-diol, U; **15**, 17α-ethynyl-1,3,5(10)-estratriene-3,17β-diol, C; **16**, 11β-methyl ether of 17α-ethynyl-1,3,5(10)-estratriene-3,17β-diol, U; **17**, 11α-methyl ether of 17α-ethynyl-1,3,5(10)-estratriene-3,17β-diol, U; **18**, 3-hydroxy-11β-methoxy-1,3,5(10)-estratrien-17-one, U; **19**, 13β-ethyl-1,3,5(10)-estratriene-3,17β-diol, C; **20**, 13β-propyl-1,3,5(10)-estratriene-3,17β-diol, C; **21**, 17α-methyl-1,3,5(10)-estratriene-3,17β-diol, C; **22**, 3,17β-dihydroxy-1,3,5(10)-estratrien-17α-ylpropiolic acid, C; **23**, methyl ester of 3,17β-dihydroxy-1,3,5(10)-estratrien-17α-ylpropiolic acid, C; **24**, 17α-hydroxybutylidyne-1,3,5(10)-estratriene-3,17β-diol, C; **25**, methyl succinate of 17α-hydroxybutylidyne-1,3,5(10)-estratriene-3,17β-diol, C; **26**, 3-hydroxy-1,3,5(10)-pregnatrien-20-one, C; **27**, 3-hydroxy-1,3,5(10)-estratriene 17β-hemisuccinate, C; **28**, 3-hydroxy-1,3,5(10)-estratriene 17β-acetate, C; **29**, 3-hydroxy-1,3,5(10),6,8-estrapentaen-17-one (equilenin), C; **30**, 1,3,5(10),6,8-estrapentaene-3,17β-diol (dihydroequilenin), C; **31**, 3-hydroxy-1,3,5(10)-estratrien-17α-ol (17α-estradiol), C; **32**, 1,3,5(10)-estratriene-3,16α-17β-triol (estriol), C; **33**, 3-hydroxy-1,3,5(10),7-estratetraen-17-one (equilin), C; **34**, estradiol antipodal C; **35**, 8-isoestradiol, C; **36**, 9-isoestradiol, C; **37**, 17α-methyl-3-oxo-4,9,11-estratrien-17β-ol, C; **38**, 17β-hydroxy-2,2,17α-trimethyl-4,9,11-estratrien-3-one, C; **39**, 17β-hydroxy-4-estren-3-one (19-nortestosterone), C; **40**, 7α-methyl-3-oxo-4-estren-17β-ol, C; **41**, 17β-hydroxy-4-androsten-3-one (testosterone), C; **42**, 11β-hydroxy-3-oxo-4-androsten-17β-ol, U; **43**, 13β-ethyl-3-oxo-4-estren-17β-ol, C; **44**, 17α-ethynyl-3-oxo-4-estren-17β-ol, C; **45**, 17α-ethynyl-13β-ethyl-3-oxo-4-estren-17β-ol, C; **46**, 17α-ethynyl-13β-propyl-3-oxo-4-estren-17β-ol, C; **47**, 17α-ethynyl-3-oxo-4-androsten-17β-ol (ethisterone), C; **48**, 17α-ethynyl-3-oxo-4,9-estradien-17β-ol, C; **49**, 17α-ethynyl-13-ethyl-3-oxo-4,9-estradien-17β-ol, C; **50**, 17α-methyl-3-oxo-4-androsten-17β-ol, C; **51**, 17α-methyl-3-oxo-4-estren-17β-ol, C; **52**, 3-oxo-4,9,11-estradien-17β-ol (trienolone), C; **53**, 3-oxo-4,9-estradien-17β-ol, C; **54**, 17α-ethynyl-3-oxo-4,9,11-estratrien-17β-ol, C; **55**, 17α-ethynyl-13β-propyl-3-oxo-4,9,11-estratrien-17β-ol, C; **56**, 3-oxo-4-estrene 17β-acetate (19-nortestosterone acetate), C; **57**, 3-oxo-4-androstene 17β-acetate (testosterone acetate), C; **58**, 4-estren-3-one (17-deoxy-19-nortestosterone), C; **59**, 4-androsten-3-one (17-deoxytestosterone), C; **60**, 4-androstene-3,17-dione, C; **61**, 4-estrene-3,17-dione, C; **62**, 3-oxo-4-androsten-17α-ol (17α-testosterone), U; **63**, 3-oxo-4-androstene-19,17β-diol (19-hydroxytestosterone), C; **64**, 4-pregnene-3,20-dione (progesterone), C; **65**, 19-nor-4-pregnene-3,20-dione (19-norprogesterone), C; **66**, 21-hydroxy-4-pregnene-3,20-dione, C; **67**, 11β,21-dihydroxy-4-pregnene-3,20-dione (corticosterone), U; **68**, 17α,21-hydroxy-4-pregnene-3,20-dione (11-deoxy-17α-hydroxycorticosterone), C; **69**, 11β,17α,21-trihydroxy-4-pregnene-3,20-dione (cortisol), U; **70**, 17α,21-dihydroxy-4-pregnene-3,11,20-trione (cortisone), U; **71**, 17α-hydroxy-19-norpregnene-3,20-dione, C; **72**, 17α-methyl-4-pregnene-3,20-dione (methylprogesterone), C; **73**, 4,9-pregnadiene-3,20-dione, C; **74**, 17α-methyl-4,9-pregnadiene-3,20-dione, C; **75**, 1α,2α-dimethylene-6-chloro-17α-hydroxy-4,6-pregnadiene-3,20-dione (cyproterone), C; **76**, 1α,2α-dimethylene-6-chloro-4,6-pregnadiene-3,20-dione 17α-acetate (cyproterone acetate), C; **77**, 21-hydroxy-4-pregnene-3,20-dione, C; **78**, 4,4′-dihydroxy-7,7′-diethyl-*trans*-stilbene (diethylstilbestrol), C; **79**, 4,4′-dihydroxy-7,7′-dimethyl-*trans*-stilbene (dimethylstilbestrol), C; **80**, 4,4′-dihydroxy-2′, 7-dimethyl-7′-ethyl-*trans*-stilbene, C; **81**, 4,4′-dimethoxy-2′,7-dimethyl-7′-ethyl-*trans*-stilbene, U; **82**, 4,4′-dihydroxy-2′,7′-dimethyl-7-ethyl-*trans*-stilbene, NC; **83**, bis(*p*-hydrophenyl)hexahydrochrysene, C; **84**, *B*-norestrone, NC; **85**, 17α-methyl-*B*-nortestosterone, C; **86**, cyclofenil, C; **87**, miroestrol, U; **88**, coumestrol, M; **89**, genistein, C; **90**, nafoxidine, C; **91**, 1-methylbicyclo[4.4.0]-5-nonene-4,9-dione, U; **92**, 1-methylbicyclo[4.4.0]-5-decene-4,10-dione, U; **93**, α,α-dimethyl-β-ethylallenolic acid, U; **94**, methyl ester of α,α-dimethyl-β-ethylallenolic acid, U.

was not denatured during the 16-h period necessary to achieve equilibrium as established from the enzymatic activities determined before and after dialyses. We plotted $[T_2]/[B_2]$ vs. $[U_1]/[B_1]$ (Best-Belpomme and Dessen, 1973). The linearity of the plot and the intercept on the ordinate at a value of 1 indicate that estrone competes with estradiol for the same binding site. The slope corresponding to the ratio of the dissociation constants of estrone ($K_{D,2}$) and estradiol ($K_{D,1}$) is equal to 0.41. The reciprocal slope, when considering estrone as ligand 1 and estradiol as ligand 2, has a value of 2.4.

Discussion

The Structural Basis of Ligand–Isomerase Interaction. The present findings demonstrate that a wide variety of C_{18}, C_{19}, and C_{21} steroids of the androgen, estrogen, and progestagen series, as well as synthetic nonsteroidal compounds, bind isomerase. It is possible to describe in part structural features of the isomerase binding site, based upon affinity of these different ligands. It appears from this investigation that the isomerase steroid binding site exhibits flexibility, since ligands of different size (e.g., 17-deoxy-19-nortestosterone and nafoxidine) bind equally well (the K_I values are 47 and 40 μM, respectively). A more detailed examination of ligand structure shows the following requirements for binding. The presence of a polar group at C-3 is an essential requirement for a firm interaction. Hydroxyl or oxo groups at C-3 are probably hydrogen bonded with some residues of the isomerase binding site.

Substituents at C-1 and C-2 are tolerated with only small reduction in affinity, suggesting that the isomerase region of the active site where the steroidal ring A binds is relatively open. The absolute rejection of C-11 substituted steroids suggests that there is a rather precise stringent fit of the isomerase binding site with the C-11 area of the steroid. The inhibitory effect of C-10 substituents on binding introduced whether on 3-oxo-Δ^4 C_{19}-nor or C_{21}-nor steroids is consistent with this view.

Introduction of bulky substituents in estradiol at C-17α or β or at C-17β in 19-nortestosterone does not significantly affect affinity, suggesting that the active-site region of isomerase facing the C_{17} part of ring D is relatively open. This region may involve hydrophobic bonding of steroid to enzyme. The existence of such hydrophobic interaction was previously shown by partition coefficient measurements (Weintraub et al., 1973); it was demonstrated that about 30% of the ΔG of steroid–isomerase interaction arises from hydrophobic contribution.

In contrast, substituents introduced at C-13 and C-17α carbon positions of steroids produce different effects on affinity which depend upon the specific ring A structure (phenolic or 3-oxo-Δ^4). A study of Dreiding molecular models of estradiol and testosterone suggests a possible structural explanation of the differential effects of substituents on affinity observed with these two classes of steroids. Figure 3 shows a schematic model where ring A of estradiol and testosterone "fits" an identical region in the isomerase binding "pocket", but where ring D of these two steroids binds to a different surface of the isomerase "binding pocket". This assumption is based upon the fact that

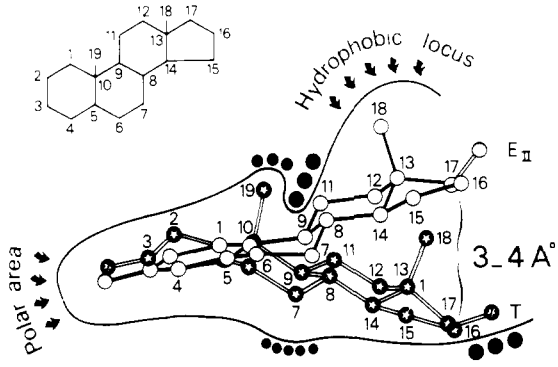


FIGURE 3: A schematic model of a possible adaptation of 3-oxo-Δ^4 and phenolic steroids in the isomerase steroid binding "pocket". Models of testosterone (✪) and estradiol (○) were taken from Duax (1975) and Precigoux et al. (1975) (pp 74 and 262): (●●●) binding site region of the isomerase with a stringent precise fit of the steroid nucleus or a region which rejects any C-11 substituted steroids; (●●●) binding site area of the isomerase which recognized substituted steroids but with a decrease in affinity.

it is possible to superimpose either ring A or D of estradiol and testosterone. Since the enzymatic proton transfer reaction involves C-4, -5, -6, and -10 of rings A and B of steroids, it seems likely that the steroidal ring A has a precise position in the isomerase binding "pocket". If one assumes the same precise position of ring A for estradiol and testosterone in the active site, Dreiding molecular models show that rings D diverge beyond a C-5–C-11 axis by a distance of 3–4 Å between the two C-17 positions. With such a schematic Dreiding molecular model it is now possible to explain in part the different substitution effects observed with these two classes of steroids. Thus, the decreased binding observed with 3-oxo-Δ^4 steroids when 17α substituents are introduced may result from steric hindrance in the vicinity of the α face of ring D with isomerase.

The fact that 17α substituents introduced on phenolic derivatives do not influence binding would be explicable in terms of the different position of estradiol derivatives in the isomerase binding "pocket" as compared to 3-oxo-Δ^4 steroids, as illustrated in Figure 3.

In general, 3-oxo-4-estrene steroids exhibit lower affinities than the corresponding phenolic derivatives. Their affinities can be markedly enhanced by replacing at C-13 the methyl by an ethyl or propyl group, while the identical substitutions on phenolic steroids are without effect on binding. The variation of affinity associated with lengthening the side chain at C-13 suggests that the aliphatic hydrocarbon moiety makes an effective contribution to binding via hydrophobic interaction with the added methyl or ethyl group at C-18 of 3-oxo-4-androstene or 4-estrene steroids and a region of the isomerase binding "pocket" which is exposed to the β face of the steroid C/D rings junction.

Introduction of conjugated double bonds in rings B and C of 3-oxo-4-estrene derivatives strongly enhances their binding to isomerase. This result is consistent with the proposed molecular model which shows an upward displacement for the 4,9-estradiene and 4,9,11-estratriene molecules beyond the C-5–C-10 axis relative to the monounsaturated 4-estrene derivative. Another possibility is that π electrons of the conjugated double bond system participate in specific binding. The slight strengthening of interaction observed with equilin and equilenin compared to estrone, as well as dehydroquilenin compared to estradiol, suggests that the enhanced affinity observed with 3-oxo polyunsaturated steroids may be due to participation of the π-electron system and the upward displacement of the steroidal rings C and D.

The enhancement of affinity previously observed upon introduction of a C-13 ethyl group in 3-oxo-Δ^4 monounsaturated steroids is markedly reduced when this group is introduced in $\Delta^{4,9}$ or $\Delta^{4,9,11}$ 17α-substituted derivatives. This latter effect is likewise consistent with the upward displacement of these molecules in the isomerase binding site; thus, a single C-13 methyl group is now sufficient to form the previously suggested hydrophobic interaction.

Comparison of molecular models of different estradiol isomers (8-isoestradiol and 9-isoestradiol) shows that the situation of C-11 relative to a plane formed by ring A is a critical parameter for the recognition of the steroid by the enzymatic binding site.

It appears, in terms of the model proposed in Figure 3, that the best position of C-11 in estradiol isomers for an effective strong binding is a forward and upward position of C-11 with respect to the plane formed by ring A of 17β-estradiol.

The high affinity of the antipodal estradiol similar to that of 17α-estradiol is difficult to explain on a structural basis. The study of stilbene derivatives is consistent with the structural resemblance of diethylstilbestrol to estradiol. Moreover, its conformation in the active site could be relatively planar since bis(*p*-hydrophenyl)hexahydrochrysene binds with higher affinity than diethylstilbestrol.

Although the geometrical approach to define the topology of the binding site of isomerase appears to account for many of the observations reported, it must be emphasized that this approach by itself cannot account for the complex behavior of interaction of ligands for the isomerase.

Thus, the existence of noncompetitive or mixed inhibitors is difficult to explain on the basis solely of their molecular structure since they are very similar to competitive inhibitors. The attempt in this study to demonstrate the existence of a second steroid binding site on isomerase failed. No ternary complexes were detectable by equilibrium dialysis; furthermore, estrone and estradiol were shown to compete for the same binding locus. The existence of an interprotomer binding locus in the dimeric isomerase molecule, which is yet another possibility, has not been substantiated. A catalytic mechanism wherein there is strong interdependence and coupling between the two protomers of isomerase in the binding and catalytic step, as described by Lazdunski (1972, 1974) and Gache (1974) in the "flip-flop" mechanism, could explain these apparently paradoxical results with isomerase: (a) half-of-the-sites reactivity (Vincent et al., 1976), (b) pure Michaelian behavior, (c) existence of binary complexes in equilibrium dialysis studies (isomerase concentrations in the micromolar range), and (d) ternary complexes under enzymatic assay conditions where the substrate is present and isomerase concentrations are very low (1–100 pM).

Acknowledgments

This work has been partially supported by the Centre National de la Recherche Scientifique, the Délégation Générale à la Recherche Scientifique et Technique, and Drs. D. Bertin, R. Bucourt, O. Martel, and L. Penasse of Roussel-Uclaf.

The authors thank J. Leger for technical help, Professor O. Hechter for editorial assistance, and Paule Soper for secretarial aid.

References

Benson, A. M., Jarabak, R., and Talalay, P. (1971), *J. Biol. Chem. 246,* 7514.

Best-Belpomme, M., and Dessen, P. (1973), *Biochimie 55,* 11.

Dixon, M. (1953), *Biochem. J. 55,* 170.

Dixon, M., and Webb, E. C. (1958), Enzymes, London, Longmans, Green and Co., p 120.
Duax, W. L. (1975), in Atlas of Steroid Structure, Duax, W. L., and Norton, D. A., Ed., New York, London, Washington, IFI Plenum Press, p 74.
Falcoz-Kelly, F., Baulieu, E.-E., and Alfsen, A. (1968), *Biochemistry 7,* 4119.
Friedenwald, J. S., and Maengwyn-Davies, G. D. (1954), *Johns Hopkins Univ., McCollum-Pratt Inst., Contrib. No. 70,* 154.
Gache, C. (1974), *FEBS Lett. 49,* 5.
Jarabak, R., Colvin, M., Moolgavkar, S. H., and Talalay, P. (1969), *Methods Enzymol. 15,* 642.
Jones, B. J., and Gordon, K. D. (1973), *Biochemistry 12,* 71.
Jones, B. J., and Ships, S. (1972), *Biochim. Biophys. Acta 258,* 800.
Jones, B. J., and Wigfield, D. C. (1968), *Can. J. Chem. 235,* 1.
Kawahara, F. S. (1962), *Methods Enzymol. 5,* 527.
Kawahara, F. S., and Talalay, P. (1960), *J. Biol. Chem. 235,* 1.
Lazdunski, M. (1972), *Curr. Top. Cell. Regul. 6,* 267.
Lazdunski, M. (1974), *Prog. Bioorg. Chem. 3,* 81.
Lineweaver, H., and Burk, D. (1934), *J. Am. Chem. Soc. 56,* 658.
Martyr, R. J., and Benisek, W. F. (1973), *Biochemistry 12,* 2172.
Precigoux, T. G., Hospital, M., and Van den Bosche, G. (1975), in Atlas of Steroid Structure, Duax, W. L., and Norton, D. A., Ed., New York, Washington, London, IFI Plenum, p 262.
Talalay, P., and Wang, S. F. (1955) *Biochim. Biophys. Acta 18,* 300.
Vincent, F., Weintraub, H., Alfsen, A., and Baulieu, E.-E. (1976), *FEBS Lett. 62,* 126.
Wang, S. F., Kawahara, F. S., and Talalay, P. (1963), *J. Biol. Chem. 238,* 576.
Weintraub, H., Alfsen, A., and Baulieu, E.-E. (1970), *Eur. J. Biochem. 12,* 217.
Weintraub, H., Baulieu, E.-E., and Alfsen, A. (1972), *Biochim. Biophys. Acta 258,* 655.
Weintraub, H., Vincent, F., Baulieu, E.-E., and Alfsen, A. (1973), *FEBS Lett. 37,* 82.

Purification and Characterization of an Acetylcholine Receptor from Mammalian Skeletal Muscle†

J. Oliver Dolly* and Eric A. Barnard

ABSTRACT: The acetylcholine receptor from denervated mammalian skeletal muscles has been purified 2400-fold. Receptor activity was followed by an assay based upon the binding of a [^{3}H]triacetyl derivative of α-bungarotoxin. Efficient extraction from the muscle of the native receptor was obtained in 1.5% Triton X-100, 15 h at 4 °C, in the presence of inhibitors of proteolysis. Gel filtration of the extract separated much other protein, including acetylcholinesterase, from the receptor. The next stage employed an affinity column containing an immobilized cholinergic (quaternary ammonium) ligand, with biospecific elution by 2 mM gallamine triethiodide. The final stages involved ion-exchange chromatography with a salt gradient elution and salt removal by continuous-flow dialysis, in 0.1% Triton X-100. The muscle receptor is a protein with 6000 nmol of toxin-binding sites per g of protein. It is homogeneous in gel electrophoresis. The receptor–toxin complex has an isoelectric point of pH 5.3. The association rate constant for the reaction with toxin, 2.9×10^5 M^{-1} s^{-1} at 25 °C (pH 8), is consistent with the rates for vertebrate muscle receptors in less pure forms reported elsewhere with related toxins. Protection from the toxin binding, exerted specifically by cholinergic ligands, leads to values of affinities for those ligands in agreement with their known pharmacological degrees of effectiveness.

Recent advances in the purification of the acetylcholine receptor (ACh.R)[1] have mainly been possible due to the discovery of snake polypeptide α-neurotoxins which bind specifically and virtually irreversibly to the nicotinic cholinergic receptor of fish electric organs and of vertebrate skeletal muscle (Chang and Lee, 1963; Changeux et al., 1970; Barnard et al., 1971; Miledi and Potter, 1971). A number of investigators have obtained preparations of ACh.R from electric organs, and shown it to be a glycoprotein that binds cholinergic ligands and inorganic cations. (For reviews, see Karlin et al., 1975; Changeux, 1975). In contrast, much less information is available on the biochemistry of the ACh.R from skeletal muscle, although its electrophysiology and pharmacology have been studied in great detail [reviewed, e.g., by Hubbard (1974) and Rang (1974)]. Therefore, it is important to isolate and characterize the ACh.R from mammalian muscle so that its biochemical properties can be correlated with, and used to fully interpret, the physiological data. Furthermore, it would enable

† From the Department of Biochemistry, Imperial College, London SW7, England. *Received February 14, 1977.* Supported in part by National Institutes of Health Grant GM-111754, and in part by an M.R.C. Programme Grant. The earlier parts of this work were performed at the Department of Biochemistry, State University of New York, Buffalo, N.Y., and at the Division of Molecular Pharmacology, National Institute for Medical Research, Mill Hill, London NW7, England, where E.A.B. held a Faculty Scholar Award of the Macy Foundation, New York, and J.O.D. held a Fellowship of the Muscular Dystrophy Associations of America.

[1] Abbreviations: ACh, acetylcholine; AChE, acetylcholinesterase; ACh.R, acetylcholine receptor; BuTX, α-bungarotoxin; [^{3}H]BuTX, [^{3}H]triacetyl-α-bungarotoxin; *d*-TC, *d*-tubocurarine; Flaxedil, gallamine triethiodide; $PhCH_2SO_2F$, phenylmethylsulfonyl fluoride; EDTA, ethylenediaminetetraacetic acid; DEAE, diethylaminoethyl.