Journal of Steroid Biochemistry, 1977, Vol. 8, pp. 1011–1015. Pergamon Press. Printed in Great Britain

INHIBITION OF HUMAN PLACENTAL PROGESTERONE SYNTHESIS BY ESTRANES: A NOVEL RELATIONSHIP OF STRUCTURE TO ACTIVITY

DEAN P. EDWARDS*, EDWIN D. BRANSOME, JR., JAMES L. O'CONNER and W. EMMETT BRASELTON, JR.
Departments of Medicine and Endocrinology Medical College of Georgia, Augusta, GA 30901, U.S.A.

(*Received* 2 *March* 1977)

SUMMARY

The inhibitory effect of a series of estranes on the human placental enzyme which catalyzes conversion of pregnenolone to progesterone was investigated *in vitro*. Inhibition tended to increase with structural changes such as unsaturation of ring B, increased planarity, and oxidation of the 17-hydroxyl to a ketone, while biological "estrogenic" activity decreased. The finding that weak estrogens inhibit progesterone synthesis suggests a natural mechanism for control of hormonogenesis in the human placenta and a new approach to pharmacologic control of fertility.

INTRODUCTION

Since the advent of the hypothesis that the actions of estrogens and other steroid hormones on target tissues involve binding to cytosol "receptor" proteins [1] little attention has been paid to the direct interaction of steroids and enzyme proteins. Estrogens for example, have significant effects on a number of electron transport enzymes [2–4], and on a variety of dehydrogenases [5]. Evidence that other steroids may regulate the synthesis of hormonally active steroids has been provided by several groups of investigators who have studied enzymes involved in the synthesis of estrogens by the human placenta [6–9].

Our laboratory has been interested in the inhibition of the synthesis of progesterone (4-pregnene-3,20-dione), a hormone necessary for establishing and maintaining mammalian pregnancy [10–12]. Previous studies have shown that the conversion of pregnenolone (3β-hydroxy-5-pregnen-20-one) to progesterone is mediated by two coupled enzymes: 3β-hydroxysteroid: NAD^+ oxidoreductase (EC 1.1.1.51 (3β-HSD)) and 3-ketosteroid 4-ene, 5-ene-isomerase (EC 5.3.3.1. (5-ene-isomerase)). These studies have also shown that dehydrogenation is the initial and rate limiting reaction [13–18]. We have now measured the effect of other steroids on the rate of conversion of radiolabeled pregnenolone to progesterone using an *in vitro* test system consisting of a partially purified human placental enzyme.

EXPERIMENTAL

Preparation of the enzyme and measurement of enzyme reaction velocities were according to procedures which we have described in previous publications [10–12]. Briefly, the enzyme reaction mixture consisted of 50 μM Na_2HPO_4 (pH 9.5) in 30% glycerol. Pregnenolone was the varied substrate, ranging in concentration from 1.0–10.0 μM; NAD^+ was the invariant substrate (200 μM). The soluble enzyme preparation was diluted to a protein concentration between 0.2 and 0.8 mg/ml. The final vol. of the reaction medium was 1.0 ml. Steroidal inhibitors were added in 25 μl ethanol; equivalent vols of ethanol were added to control assays. All reaction components were allowed to incubate for two min prior to initiation of the enzymatic reaction by addition of [^{3}H]-pregnenolone in 50 μl ethanol. Incubations were carried out in a Dubnoff metabolic incubator at 30°. Separation of pregnenolone from its conversion product, progesterone, was achieved by selective precipitation with digitonin. The formation of enzyme products was then determined by liquid scintillation counting. Incubations were carried out for 5 min so that reaction rates could be regarded as initial velocities: the relationship of the reaction rate and the concentration of the enzyme protein was always linear.

To quantitate inhibition of progesterone synthesis, we used K_I values (inhibition constants) which approximate the dissociation constants for enzyme–inhibitor complexes. The K_I is thus an inverse measurement of the strength of the binding of the inhibitor as long as the interaction between inhibitor and enzyme is competitive with formation of an enzyme–substrate complex. The smaller the value of the K_I, the greater the potency for inhibition. K_I Values were determined graphically using Lineweaver–Burk ($1/v$ *vs* $1/s$) plots [19] or Dixon ($1/v$ *vs* i) plots [20]. The data were statistically fitted to linear regression curves by the method of least squares.

* Now at University of Texas Health Science Center, San Antonio, Texas.

Table 1. Inhibition of Placental 3βHSD:5-ene Isomerase by Estranes

Compound	Inhibition K_I(M)	Substituents on positions Ring B	C3	C-16	C17	Uterotropic activity*	Binding potency†
Diethylstilbestrol	0	—		(Synthetic, not a steroid)		452	246
Estrenol (4-estrene-3,17β-diol)	0		OH	—	OH	0	
Estrenone (4-estrene-3β-ol-17-one)	2.0×10^{-5}	—	OH	—	=O	2	—
		Estratrienes (1,3,5(10))					
Mestranol	0		H_3CO	—	βOH,αC≡CH	14	8
Estradiol-17-sodium sulfate	0	—	OH		OSO_3Na		
Estradiol-17α-3-sodium sulfate	0		NaO_3SO	—	αOH		
Estrone methyl ether	0	—	H_3CO		=O		
Estradiol-17-hemisuccinate	0		OH	—	OHemiSO		
Estradiol-17-glucuronide	4.0×10^{-5}	—	OH		O Gluc.		
Estriol	3.0×10^{-5}		OH	αOH	βOH	<1	16
Estradiol-17α	1.0×10^{-5}	—	OH	—	αOH	<2	49
Epiestriol	8.0×10^{-6}	—	OH	βOH	βOH	1	44
Estradiol-17β	2.8×10^{-6}	—	OH		βOH	100	100
Ethinyl estradiol	2.0×10^{-6}		OH	—	βOH,αC≡CH	276	191
Estrone	3.5×10^{-7}	—	OH	—	=O	29	66
Estradiol-17-acetate	3.0×10^{-7}	—	OH		OAc		29
Estradiol-3,17-diacetate	2.0×10^{-7}	—	OAc	—	OAc	—	11
		Estratetraenes (1,3,5(10),6 or 7)					
7-Dehydroestrone-3-acetate	6.5×10^{-6}	Δ7	OAc	—	=O		
7-Dehydroestradiol-17α	4.0×10^{-6}	Δ7	OH	—	αOH		
6-Dehydroestradiol-17β	2.7×10^{-6}	Δ6	OH	—	βOH	<1	10
7-Dehydroestradiol-17β	2.5×10^{-6}	Δ7	OH	—	βOH	26	—
6-Dehydroestrone	7.0×10^{-7}	Δ6	OH	—	=O	2	10
Equilin (7-dehydroestrone)	2.0×10^{-7}	Δ7	OH	—	=O	20	24
17-dihydroequilin-3,17-diacetate	1.0×10^{-7}	Δ7	OAc	—	OAc		
		Estrapentaenes (1,3,5(10),6,8)					
Equilenin-3-methyl ether	0	Δ6,Δ8	H_3CO	—	=O		
Equilenin-3-benzyl ether	0	Δ6,Δ8	⬭-CH_2—O—		=O		
17-dihydroequilenin-17-sodium sulfate	0	Δ6,Δ8	OH	—	OSO_3Na		
6,8-dehydroestradiol-17α	7.0×10^{-7}	Δ6,Δ8	OH		αOH	0	
Equilenin-3-sodium sulfate	2.0×10^{-7}	Δ6,Δ8	NaO_3SO	—	=O		
17-dihydroequilenin-3-triethyl-ammonium sulfate-17-acetate	2.0×10^{-7}	Δ6,Δ8	$(CH_2CH_3)_3NSO_4$	—	αOH,OAc		
6,8-dehydroestradiol 17β	7.0×10^{-8}	Δ6,Δ8	OH		βOH		
Equilenin	4.0×10^{-8}	Δ6,Δ8	OH	—	=O	<2	8
17-dihydroequilenin-3,17-diacetate	1.0×10^{-8}	Δ6,Δ8	OAc		OAc		

* The values taken from Endocrine Bioassay Data (A. G. Hilgar and L. C. Trench, eds., National Cancer Institute, 1968), are expressed as percent relative potency in uterine response assigning a value of 100 to the reference steroid, estradiol 17β. †Estrogenic potency determined by comparative binding affinity to rabbit uterine cytosol receptors assigning a value of 100 to the reference steroid, estradiol 17β. S. G. Korenman: *Steroids* **13** 163 (1969).

RESULTS

A large number of estranes were found to inhibit progesterone synthesis by the placental enzyme. In Table 1 the compounds are arranged in three main structural groups: estratrienes, estratetraenes, and estrapentaenes. Within each group the steroids are listed in descending order of the K_I values, the more potent inhibitors having the lower K_I values. Figure 1(a) provides an example of K_I estimation for equilenin, a very potent inhibitor of the enzyme reaction: the point of intersection on the horizontal axis indicates that the K_I for equilenin was approximately 4×10^{-8} M. The parallel regression lines of the same data on the Cornish-Bowden (s/v *vs* i) plot (Fig. 1(b)) show that the inhibition by equilenin was competitive [21]. The values given for biological potency listed in Table 1 are based on data selected as representative of the literature. Compounds with little or no estrogenic activity were the most effective inhibitors while diethylstilbestrol, the most potent (non-steroidal) estrogenic compound tested as far as uterotropic effects are concerned, did not inhibit 3β-HSD:5-ene isomerase activity.

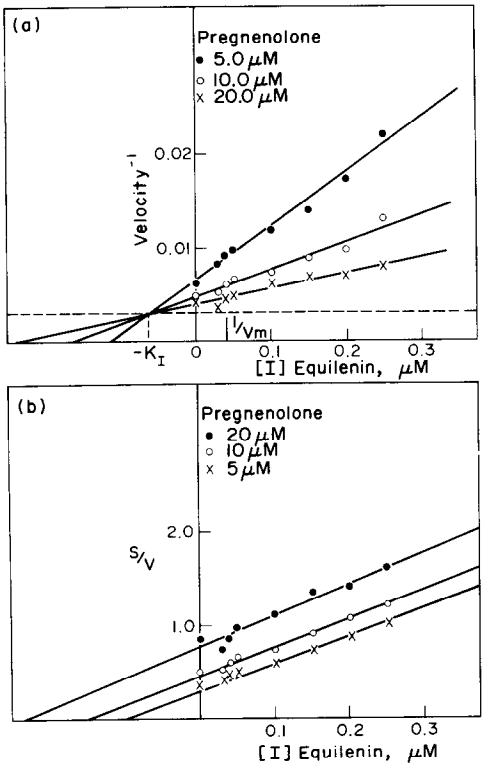

Fig. 1. Effect of equilenin on the human placental 3β-HSD:5-ene isomerase as a function of inhibitor concentration. Dixon plots (A) and Cornish-Bowden plots (B). (s: concentration of substrate; v: velocity of substrate conversion to product; i: concentration of inhibitor). Incubation conditions: 0.05 M sodium phosphase in 30% glycerol (pH 9.5), NAD^+ as the invariant substrate (200 μM) and soluble enzyme preparation (0.75 mg protein/ml). Measurement of the reaction velocity (nmol of product formed per min per mg protein) was over a 5 min period at 30°, with increasing concentrations of equilenin and with the three different fixed concentrations of pregnenolone indicated in the figure. Each value is the mean determination from duplicate incubations. Regression lines were calculated by the method of least squares.

Certain generalizations on the relationship between inhibitory potency and specific structural features may be deduced. Inhibitory potency is related to the degree of unsaturation of carbon–carbon bonds in rings A and B of the estrane nucleus. Estranes which did not have an aromatized A ring were poor inhibitors. Estrenol did not inhibit and estrenone was a very weak inhibitor. Ring A aromatized estranes (1,3,5(10) estratrienes) were significant inhibitors. Unsaturation in ring B (estratetraene) at either C-6 or C-7 increased inhibition further; the position of the double bond in ring B seemed to be of little consequence. Equilenin and its derivative dihydroequilenin-diacetate which are ring A + B aromatized estrogens (1,3,5(10,6,8-estrapentaenes) were the most potent inhibitors tested.

Large substituent end groups on the estrane nucleus prevented inhibition: estradiol-hemisuccinate, estradiol-glucuronide, estradiol 17α-sodium sulfate, estradiol-17α-3-sodium sulfate, equilenin-benzyl ether and dihydroequilenin-17-sodium sulfate proved to be ineffective as inhibitors. The inability of dihydroequilenin-17-sodium sulfate and estradiol-17-sodium sulfate to inhibit even at very high concentrations suggests that esterification *in vivo* at C-17 with a sulfate ester may inactivate steroids as inhibitors.

17α-Ethinyl estradiol, commonly used as the estrogen component in oral contraceptives, proved to be a moderately potent inhibitor. Its 3-methyl ether derivative (mestranol) showed no inhibitory action *in vitro*. The presence of an ether at C-3 appears to prevent inhibition, whereas esterification at C-3 seems to have less dramatic effects. Although addition of the 17α-ethinyl group to estradiol enhances estrogenic (eg. uterotropic) activity, it did not significantly affect inhibition of 3β-HSD:5-ene-isomerase. The effect of inhibition of the substituents at C-17 is predictable for each estrane series: 17-acetoxy > 17-keto > 17-ethinyl = 17β-hydroxy > 17α-hydroxy. A comparison of the K_I values for estradiol, estriol and epiestriol shows that hydroxyl substitution at C-16 decreases inhibition. Epiestriol which has the 16-hydroxy group in the β-orientation as opposed to α-orientation in estriol, is the more effective inhibitor of the two.

DISCUSSION

Our findings thus show a definite relationship between structural characteristics of estranes and the ability to inhibit the human placental 3β-HSD:5-ene-isomerase. The following generalizations seem to apply: (1) Increased planarity of the estrane nucleus enhances inhibition, suggesting that the effectiveness of compounds in the estrane series may be due in part to the conformation of the steroid carbon skeleton. Three dimensional Dreiding models show that

aromatization in ring A and ring B results in rings A and B being more nearly planar to rings C and D. Figure 2 shows that the phenolic ring B aromatized estrane equilenin approximates the planarity of the 21 carbon substrate pregnenolone to a much greater extent than the less potent inhibitor estradiol 17β. (2) Substituents *cis* to ring D or in the β-orientation tend to increase inhibition, whereas substituents *trans* to ring D or in the α-orientation tend to decrease inhibition. (3) Small polar substituents have predictable effects on inhibition whereas large end group substitutents (C-3 and C-17) appear to abolish inhibition, presumably because they impede interactions of the steroid and the active site of the enzyme.

Although the structure–activity relationships observed in the present study bear some resemblances to the results obtained with the effects of steroids on other enzymes [2, 3; 6–8], these relationships are novel and may be unique to inhibition of the 3β-HSD:5-ene isomerase activity. Our data from experiments with 3β-HSD:5-ene isomerase enzymes from ovary and adrenal cortex are consistent with the data on inhibition reported in this paper [10–12].

Progesterone production by the human placenta has been presumed to be relatively autonomous because the placenta is a temporary steroidogenic organ of pregnancy, and because a mechanism for regulating synthesis of this steroid has not been demonstrated. We have found however that the magnitude of the K_I values for inhibition of the placental enzyme *in vitro* by estrone, estradiol and estriol is consistent with the endogenous concentrations of these steroids normally found in the human placenta [9, 22]. Therefore progesterone synthesis by the human placenta *in vivo* could be, as has been suggested by Wiener [23–24], influenced by a feedback mechanism involving enzyme inhibition.

The synthetic steroidal analogue cyanoketone (2α-cyano-17β-hydroxy-4, 4,17α-trimethyl-androst-5-en-3-one) has been used extensively as an inhibitor of the 3β-HSD and 5-ene-isomerase reactions [25–30]. Cyanoketone has the advantage of being a potent inhibitor at low concentrations ($K_I = 1.8 \times 10^{-9}$ M) as well as having little other hormonal activity. It has the disadvantage however of undesirable side-effects *in vivo* including interference with adrenocortical hormonogenesis. We have found that ring B unsaturated estrapentaenes (1,3,5(10),6,8) are also effective inhibitors at very low concentrations and that equilenin, which has very weak estrogenic activity, is nearly as potent an inhibitor of the human placental enzyme system as is cyanoketone. Low levels of both steroids (10^{-8} M) also inhibit the conversion of pregnenolone to progesterone in cultured human trophoblast cells [31].

A substance which would lower levels of circulating progesterone before or after conception might be of

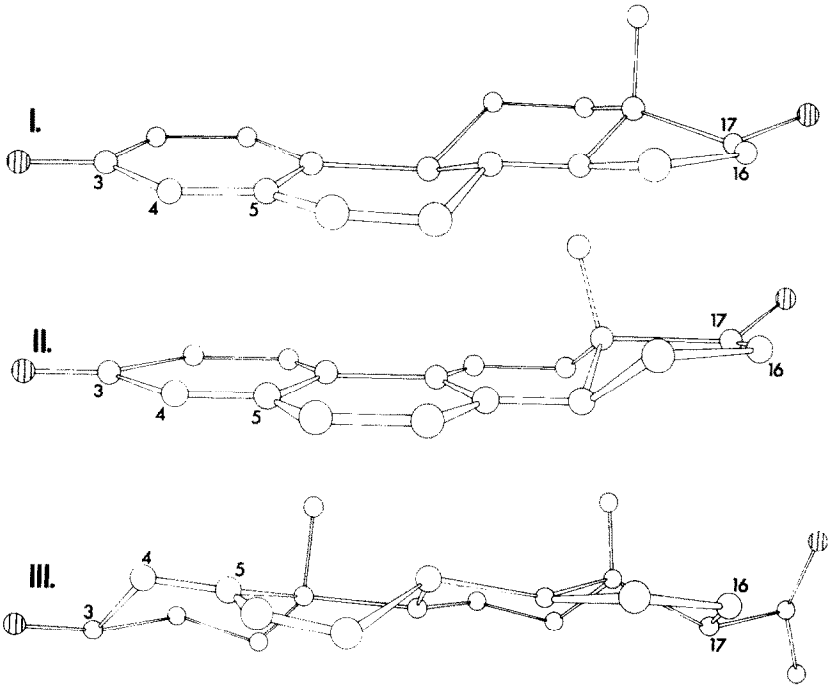

Fig. 2. Three-dimensional Dreiding models of the carbon skeletons of estradiol-17β (I), equilenin (II) and pregnenolone (III). The molecules are drawn as though in space as viewed from an angle of approximately 5 degrees above the plane of the junction of the A/B rings. The larger the circles denoting carbon atoms are, the closer they are to the viewer. Carbons 3, 4 and 5 are loci involved in the transformation of pregnenolone to progesterone by 3β-HSD:5-ene isomerase. See the text for further discussion.

considerable value as an antifertility agent. If the uterine endometrium is deprived of sufficient progesterone, implantation will not occur; if the feto-placental unit is deprived of progesterone, pregnancy will not proceed [32]. Both *in vivo* and *in vitro* experiments with rats and rhesus monkeys have shown that equilenin can inhibit progesterone synthesis, thus suggesting that very planar estranes are potential antifertility agents. The partial definition in this paper of structure–activity relationships for inhibition of the enzymatic conversion of pregnenolone to progesterone may also provide a basis for identification of other steroidal inhibitors which would also have potential as antifertility agents.

Acknowledgements—From the Center for Population Studies at the Medical College of Georgia. This work was supported in part by US AID contract csd/2491 to the Population Council and NIH Training Grant No. HD 00112.

REFERENCES

1. Jensen E. V., DeSombre E. R.: *Ann. Rev. Biochem.* **41** (1972) 203–230.
2. Stoppani A. O., Brignone M. C. and Brignone A. J.: *Archs Biochim. Biophys.* **127** (1968) 463–475.
3. Villee C. A.: *J. biol. Chem.* **215** (1955) 171–182.
4. Chyen J. L., Bitensky L, Butcher R. G. and Altman F. P.: In *Advances in Steroid Biochemistry and Pharmacology* (Edited by M. H. Briggs and G. H. Christie). Academic Press, New York, Vol. 4 (1974) pp. 1–60.
5. Douville A. W. and Warren J. C.: *Biochemistry* **7** (1968) 4052–4059.
6. Schwarzel W. C., Kruggel W. G. and Brodie H. J.: *Endocrinology* **92** (1973) 866–880.
7. Siiteri P. K. and Thompson E. A.: *J. steroid Biochem.* **6** (1975) 317–322.
8. Townsley J. D.: *Endocrinology* **93** (1973) 172–181.
9. Townsley J. D.: *Acta endocr., Copenh.* **79** (1975) 740–748.
10. Bransome E. D. Jr., Edwards D. P. and O'Conner J. L.: *J. clin. Invest.* **52** (1973) 12a.
11. Bransome E. D. Jr., Edwards D. P., O'Conner, J. L. and Braselton W. E. Jr.: *56th Meeting of the Endocrine Society* (1974), abstract 325.
12. Edwards D. P., O'Conner J. L., Bransome E. D. Jr. and Braselton W. E. Jr.: *J. biol. Chem.* **251** (1976) 1632–1638.
13. Kawahara F. S.: *Meth. Enzymol.* **5** (1962) 527–532.
14. Ewald W., Werbin H. and Chaikoff I. L.: *Biochim. Biophys. Acta* **81** (1964) 199–201.
15. Ewald W., Werbin H. and Chaikoff I. L.: *Steroids* **3** (1964) 505–522.
16. Kruskemper H. L., Forchielli E. and Ringold H. J.: *Steroids* **3** (1964) 295–309.
17. Neville A. M., Orr J. C. and Engel L. L.: *J. Endocr.* **43** (1969) 599–608.
18. McCune R. W., Roberts S. and Young P. O.: *J. biol. Chem.* **245** (1970) 3859–3867.
19. Lineweaver H. and Burk D.: *J. Am. chem. Soc.* **56** (1934) 658–666.
20. Dixon M.: *Biochem. J.* **55** (1953) 170–171.
21. Cornish-Bowden A.: *Biochem. J.* **137** (1974) 143–144.
22. Diczfalusy E. and Mancuso S.: In *Foetus and Placenta* (Edited by A. Klopper and E. Diczfalusy). Blackwell, Oxford (1969) pp. 191–248.
23. Weiner M. and Allen S. H. G.: *Steroids* **9** (1967) 567–582.
24. Weiner M.: *Biol. Reprod.* **14** (1976) 306–313.
25. Goldman A. S.: *J. clin. Endocr. Metab.* **27** (1967) 325–332.
26. Goldman A. S.: *J. clin. Endocr. Metab.* **28** (1968) 1539–1546.
27. Neville A. M. and Engel L. L.: *Endocrinology* **83** (1968) 873–876.
28. Neville A. M. and Engel L. L.: *J. clin. Endocr. Metab.* **28** (1968) 49–60.
29. Depp R., Cox D. W., Plon R. J., Conrad S. H. and Henrichs W. L.: *Gynec. Invest.* **4** (1973) 106–120.
30. Handler R. P. and Bransome E. D. Jr.: *J. clin. Endocr. Metab.* **29** (1969) 1117–1119.
31. Pattillo R. A., Rinke M. L. and Hussa R. O.: *In Vitro* **10** (1974) 77–82.
32. Warren J. C.: *Biol. Reprod.* **8** (1973) 259–274.