INHIBITION OF 17β-HYDROXYSTEROID DEHYDROGENASE (17β-HSD) ACTIVITIES OF HUMAN PLACENTA BY STEROIDS AND NON-STEROIDAL HORMONE AGONISTS AND ANTAGONISTS

Charles H. Blomquist (1), Nancy J. Lindemann and Erick Y. Hakanson

Department of Obstetrics and Gynecology
St. Paul-Ramsey Medical Center
St. Paul, Minnesota 55101
and
The University of Minnesota Medical School
Minneapolis, Minnesota 55455

Received 9-4-84.

ABSTRACT

Various naturally occurring steroids, synthetic steroid derivatives and non-steroidal hormone agonists and antagonists were assayed as inhibitors of human placental 17β-HSD activities. Microsomal 17β-HSD was inhibited by C_{18}-, C_{19}- and C_{21}-steroids. Soluble 17β-HSD was highly specific for C_{18}-steroids. In contrast to the soluble activity, the microsomal enzyme also had a strong affinity for ethinylestradiol (K_I=0.3 μM) and danazol (K_I=0.6 μM); anabolic steroids and norethisterone were weaker inhibitors. Of the non-steroids tested only diethylstilbestrol and o-demethyl CI-680 were inhibitors and they showed a greater affinity for soluble 17β-HSD.

K_I-values for estradiol-17β, (0.8 μM), progesterone (27.0 μM) and 20α-dihydroprogesterone (1.5 μM) were comparable to reported tissue levels of these compounds, consistent with a possible competition in vivo among naturally occurring C_{18}-, C_{19}-, and C_{21}-steroids for the active site of microsomal 17β-HSD.

INTRODUCTION

Membrane-bound and soluble 17β-hydroxysteroid and 20α-hydroxysteroid (20α-HSD) dehydrogenase activities are widespread in mammalian tissues. The relationship of these activities to steroid hormone action, however, is unclear. Current data suggest that steroid hormone receptors and 17β-HSD or 20α-HSD activities may function in concert, allowing for steroid-receptor complex formation and translocation while at the same time

regulating intracellular levels of hormone by the action of these steroid-metabolizing enzymes (2-5). As a result, steroid hormone agonists and antagonists could exert their effects not only by binding to or competing for steroid receptor but by inhibiting enzymes such as 17β-HSD and 20α-HSD, as well.

The human placenta is a rich source of a microsomal 17β,20α-HSD activity which recognizes C_{18}-, C_{19}- and C_{21}-steroids as substrates (6,7) with comparable V_{max} and K_M-values and a cytoplasmic activity, generally considered to be a soluble protein, which is maximally active with C_{18}-steroids, shows a slight activity with C_{21}-steroids, and has little affinity for C_{19}-steroids either as substrates or inhibitors (8,9).

In the experiments reported here we have investigated the ability of naturally occurring steroids, various synthetic derivatives of naturally occurring steroids, and some non-steroidal agonists and antagonists to inhibit the activity of microsomal 17β-HSD with testosterone and the soluble enzyme with estadiol-17β.

MATERIALS AND METHODS

Abbreviations and trivial names used: Bicine, N,N-bis(2-hydroxyethyl)glycine; 2-hydroxyestradiol, 1,3,5(10)-estratriene-2,3,17β-triol; 2-hydroxyestrone,2,3-dihydroxy 1,3,5(10)-estratrien-17-one; 19-nortestosterone, 17β-hydroxy-4-estren-3-one; 5α-dihydrotestosterone, 17β-hydroxy-5α-androstan-3-one; 5β-dihydrotestosterone, 17β-hydroxy-5β-androstan-3-one; androstenedione, 4-androstene-3,17-dione; 20α-dihydroprogesterone, 20α-hydroxy-4-pregnen-3-one; 17-hydroxyprogesterone, 17-

hydroxy-4-pregnene-3,20-dione; ethinylestradiol, 17α-ethinyl-1,3,5(10)-estratriene-3,17β-diol; ethinylestradiol 3-methyl ether, 17α-ethinyl-1,3,5(10)-estratriene-3,17β-diol 3-methyl ether; methyltestosterone, 17α-methyl-17β-hydroxy-4-androsten-3-one; methylandrostanolone, 17α-methyl-17β-hydroxy-5-androstan-3-one; norethisterone, 17β-ethinyl-17β-hydroxy-4-estren-3-one; danazol, 17α-pregna-4-en-20-yno[2,3-d] isoxazol-17-ol; medroxyprogesterone acetate, 6α-methyl-17-hydroxy-4-pregnene-3,20-dione 17-acetate.

Materials: Coumestrol was purchased from Eastman Kodak Co., Rochester, N.Y. Medroxyprogesterone acetate, U-11,100A(nafoxidine) and U-23,469 were gifts of the Upjohn Comp., Kalamazoo, MI. Tamoxifen citrate was a gift from Stuart Pharmaceuticals, Division of ICI Americas, Inc., Wilmington, DE. Enclomiphene citrate and zuclomiphene citrate were supplied by Merrell Dow Pharmaceuticals, Inc., Cincinnati, OH. A sample of danazol was supplied by Sterling-Winthrop Research Institute, Rensselaer, NY. CI-628, CI-680 and o-demethyl CI-680 were gifts from the Warner-Lambert Company, Ann Arbor, MI. Diethylstilbestrol and other steroids were purchased from Steraloids, Inc., Wilton, NH. NAD was from P-L Biochemicals, Div. of Pharmacia, Inc., Milwaukee, WI. Bicine was purchased from Calbiochem-Behring Corp., La Jolla, CA. [7-^{3}H]testosterone (25 Ci/mmol) was obtained from New England Nuclear, Boston, MA.

Tissue sources: Placentas were obtained as products of normal term, vaginal deliveries. Patient informed consent was obtained in accordance with the policy of the Institutional Review Board of St. Paul-Ramsey Medical Center. Tissue was kept on ice and used within 6h of delivery.

Preparation of microsomes: Microsomes were prepared from homogenates of placental tissue freed of umbilical cord and membranes, as previously described (10). They were stored in buffer (0.25 M sucrose, 10 mM 2-mercaptoethanol, 0.1 M bicine, pH 9.0) at 4°C at a protein concentration of 6-12 mg per mL as measured by the method of Bradford (11). Bovine serum albumin was used as the protein standard.

Preparation of soluble 17β-HSD: Soluble 17β-HSD was purified from placental homogenates by a procedure based on that developed by Jarabak (12), but modified by us to include an ammonium sulfate gradient solubilization step (8).

17β-HSD activity: The activity of soluble 17β-HSD was assayed fluorometrically (8) in an Aminco-Bowman spectrophotofluorometer fitted with a constant

temperature cuvette chamber connected to a circulating water bath. Reaction mixtures (1.0 mL final volume) contained 1.0 mM NAD, 1.0 or 10.0 μM estradiol-17β (added in 10 μL of methanol) and 15 μg of 17β-HSD (added in 10 μL of storage buffer) in 0.17 M bicine (pH 9.0). That steroid concentrations were within the solubility range for each compound assayed was confirmed by light-scattering (13).

Microsomal 17β-HSD activity was measured by a procedure simlar to that used by Pollow and coworkers (6). Reaction mixtures (1.0 mL or 0.5 mL final volume) contained 1.0 mM NAD, 1.0 or 10.0 μM [7-^{3}H]testosterone added in 20 μL of methanol, various concentrations of unlabeled compound to be tested as an inhibitor, also added in 20 μL of methanol, and 0.23 mg of microsomal protein in 0.17 M bicine (pH 9.0). Samples were incubated at 25°C for periods of time sufficient for approximately 20% conversion of substrate to product (15-20 min). Reaction was stopped by the addition of 3 mL of dichloromethane containing unlabeled testosterone and androstenedione, 0.1 mg per mL, as carriers. After vigorous vortexing, the samples were filtered through Whatman phase-separator filter paper and the filtrates taken to dryness in a stream of nitrogen. The residues were suspended in 100 μL of dichloromethane and 50 μL-aliquots spotted on silica gel G plates containing a fluorescent indicator (Eastman Chromagram sheets). After development at room temperature in benzene:acetone (4:1), the plates were air dried and the steroids located with uv-light. The spots were cut out and transferred to vials containing 10 mL of Liquifluor (New England Nuclear Boston MA) for scintillation counting in a Beckman LS-100 liquid scintillation spectrometer. In preliminary experiments it was established that recoveries were 75% or greater. Testosterone and androstenedione were the only compounds recovered on the plates. No aromatization of either testosterone or androstenedione was detectable in the reaction mixtures, in agreement with the results of Thomas and Veerkamp (7). Reaction velocities were calculated on the basis of cpm in product as percent of total cpm recovered.

Data analysis: The type of inhibition and values for inhibition constants were estimated from plots of 1/v versus [I] and [S]/v versus [I] as previously reported (8). Each potential inhibitor was initially tested at four concentrations, with two levels of testosterone, to establish the general inhibition pattern. It was then reassayed in triplicate or quadruplicate at a high concentration against two testosterone concentrations as described by Porter and Trager (14), for more accurate quantitation of inhibition constants.

RESULTS

Inhibition of microsomal 17β-HSD: Steroid concentrations and reaction conditions were such that linear inhibition patterns were observed. In such cases reaction velocity can be described by the general equation:

$$v = \frac{V_{max} \cdot [S]}{K_m (1 + [I]/K_I) + [S] (1 + [I]/K'_I)}$$

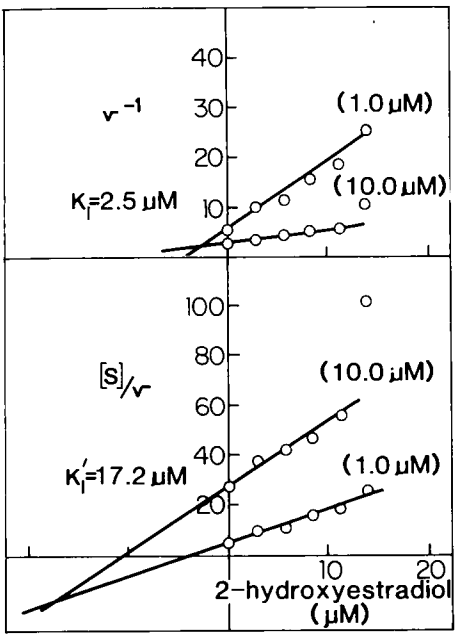
where K_I is generally taken as the dissociation constant of a competitive inhibitor from the enzyme-inhibitor complex and K'_I the dissociation constant of a second complex, possibly enzyme-inibitor-substrate (15). [S] and [I] are substrate and inhibitor concentrations. From plots of v against [I] and [S]/v against [I] it is possible to differentiate various types of inhibition. In competitive inhibition K_I has a finite value and K'_I approaches infinity. In uncompetitive inhibition K'_I is finite and K_I approaches infinity. K_I equals K'_I in noncompetitive inhibition and for mixed inhibition both K_I and K'_I have finite values (15). For competitive inhibitors and compounds which give mixed patterns, the extent of inhibition expected would be defined by substrate concentrations, inhibitor concentration and the quantitative relationship between K_I and inhibitor concentration, I/K_I.

Microsomal 17β-HSD activity with testosterone was inhibited by various C_{18}-, C_{19}-, and C_{21}- steroids

Table 1: Inhibition of microsomal 17β-HSD activity with testosterone by naturally-occurring steroids.

Steroid	Inhibition Type	K_I (μM)	K_I' (μM)
C_{18}			
2-hydroxyestradiol	mixed	2.5	17.2
2-hydroxyestrone	mixed	4.8	37.5
19-nortestosterone	mixed	2.0	19.5
estradiol-17β	compet.	0.8	∞
estriol	compet.	38.0	∞
estrone	compet.	11.8	∞
C_{19}			
5-androstene-3,17β-diol	mixed	1.4	39.0
androstenedione	compet.	37.0	∞
5α-dihydrotestosterone	mixed	1.7	44.0
5β-dihydrotestosterone	mixed	1.9	46.0
5α-androstane-3,17-dione	mixed	11.0	198
C_{21}			
20α-dihydroprogesterone	mixed	1.5	10.6
17-hydroxyprogesterone	mixed	29.0	110
progesterone	mixed	27.0	12.0

(Table 1). Mixed inhibition patterns were observed in the majority of cases consistent with an interaction of the inhibitor with more than one form of the enzyme, possibly free enzyme and an enzyme-substrate complex (15). A typical pattern is illustrated in Fig. 1 with data for inhibition by 2-hydroxyestradiol. Of the naturally occurring steroids tested estradiol-17β, estriol, estrone and androstenedione gave competitive inhibition patterns.



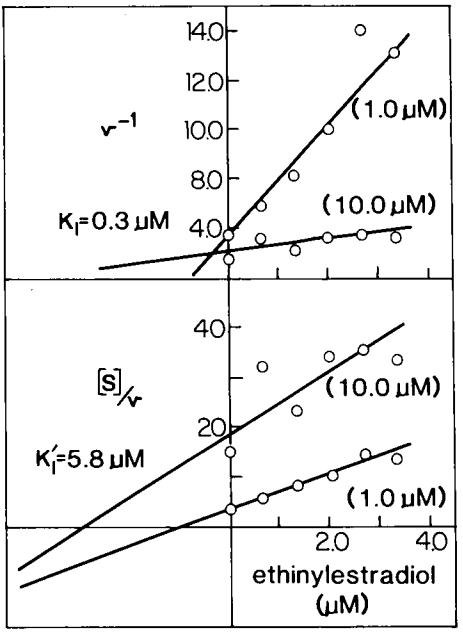


Fig. 1 (Left): Inhibition of microsomal 17β-HSD by 2-hydroxyestradiol. Substrate concentrations [S] are given in parentheses.
Fig. 2 (Right): Ethinylestradiol inhibition of microsomal 17 -HSD. Units are: v^{-1}=(μM/30 min)$^{-1}$; [S]/v=(30 min).

Certain synthetic steroids were also effective inhibitors of microsomal 17β-HSD. Ethinylestradiol gave a mixed inhibition pattern (Fig. 2) with inhibition constants, K_I and K_I', of 0.3 μM and 5.8 μM, respectively. Formation of the 3-methyl ether of ethinylestradiol abolished its inhibitory activity. Danazol was a competitive inhibitor with a K_I-value of 0.6 μM (Fig. 3). The two anabolic steroids, methyltestosterone and methylandrostanolone, both gave mixed inhibition patterns. Norethisterone was a competitive inhibitor, while an

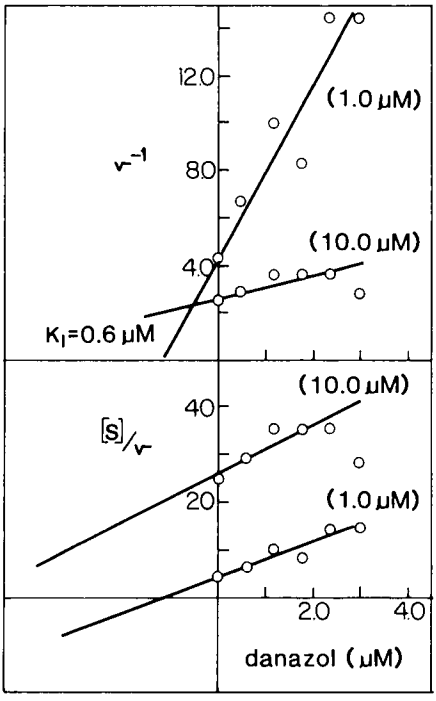


Fig. 3. Inhibition of microsomal 17β-HSD by danazol. Substrate concentrations are given in parentheses. Units are given in the legend to Figs. 1 and 2.

Table 2: Synthetic steroid derivatives as inhibitors of microsomal 17β-HSD activity with testosterone.

Synthetic Derivative	Inhibition Type	K_I (μM)	K'_I (μM)
ethinylestradiol	mixed	0.3	5.8
ethinylestradiol 3-methyl ether	no mixed inhibition detected		
danazol	competitive	0.6	∞
methyltestosterone	mixed	23.6	45.8
methylandrostanolone	mixed	9.3	25.2
norethisterone	competitive	21.0	∞
medroxyprogesterone acetate	uncompetitive		8.6

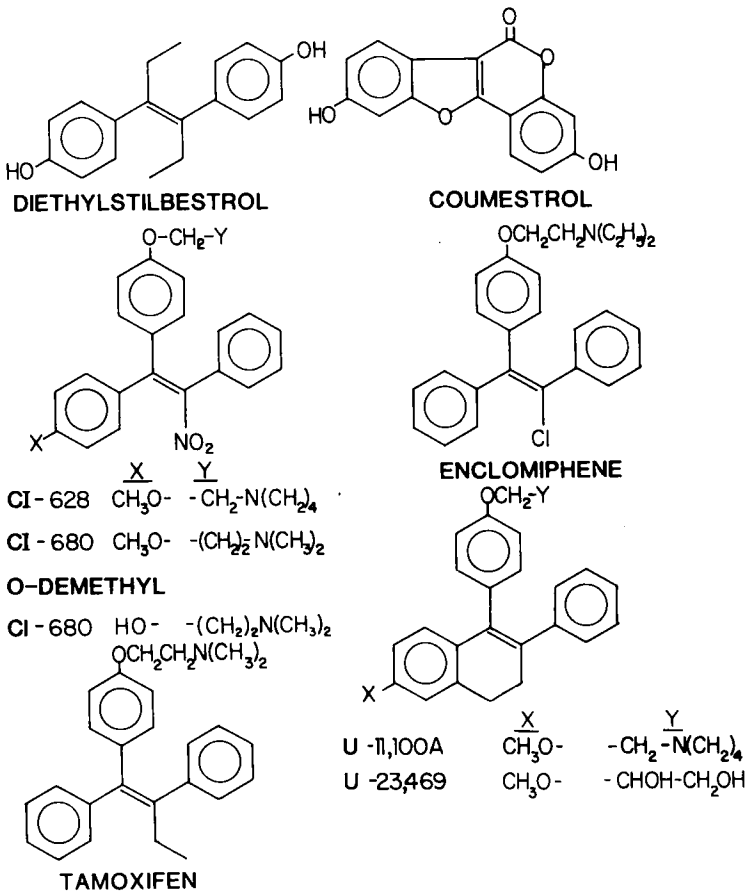


Fig. 4. Non-steroidal compounds tested as inhibitors

uncompetitive pattern was observed with medroxyprogesterone acetate (Table 2).

Structures of the various non-steroidal compounds tested are illustrated in Fig. 4. Diethylstilbestrol was an effective inhibitor (Table 3). No inhibition was observed with the phytoestrogen, coumestrol, and with

the exception of o-demethyl CI-680, none of the other non-steroidal compounds gave any inhibition.

Table 3: Effects of non-steroidal hormone agonists and antagonists on microsomal 17β-HSD activity with testosterone.

Compound	Type	Inhibition K_I (μM)	K_I (μM)
diethylstilbestrol	mixed	15.3	47.0
coumestrol	no inhibition detected		(4.6 μM)[a]
CI-628	no inhibition detected		(2.0 μM)
CI-680	no inhibition detected		(5.7 μM)
O-demethyl CI-680	compet.	19.0	
tamoxifen citrate	no inhibition detected		(2.0 μM)
enclomiphene citrate	no inhibition detected		(2.1 μM)
zuclomiphene citrate	no inhibition detected		(2.1 μM)
U-11,100A (nafoxidine)	no inhibition detected		(1.3 μM)
U-23,469	no inhibition detected		(12.3 μM)

[a]Maximum concentration tested

Inhibition of soluble 17β-HSD: In agreement with previous reports (8,9), the soluble form of 17β-HSD showed a high degree of specificity for naturally occurring C_{18}-steroids (Table 4). Of the synthetic steroid derivatives tested only ethinylestradiol was an inhibitor. The K_I was 50 μM, significantly greater than that observed with the microsomal activity.

In contrast to the microsomal enzyme, soluble 17β-HSD showed a greater affinity for diethylstilbestrol (K_I = 0.8 μM

versus 15.3 μM). As with microsomal 17β-HSD, however, o-demethyl CI-680 was the only other non-steroidal compound to exert a significant inhibition.

Table 4: Inhibitors of soluble 17β-HSD activity with estradiol-17β.

Compound	Inhibition Type	K_I (μM)	K_I' (μM)
Naturally-occurring steroids			
2-hydroxyestradiol	substrate	(K_M =1.7 μM)	-
2-hydroxyestrone	compet.	6.0	∞
testosterone	compet.	225[a]	∞
androstenedione	compet.	160[a]	∞
progesterone	compet.	200[b]	∞
20α-dihydro-progesterone	compet.	2500[b]	∞
Synthetic steroid derivatives			
ethinylestradiol	compet.	50	∞
danazol	no inhibition detected (14.8 μM)[c]		
Non-steroidal compounds			
diethylstilbestrol	compet.	0.8	∞
CI-680	no inhibition detected (5.7 μM)		
O-demethyl-CI-680	mixed	2.5	11.8

[a]Values from ref. 8
[b]Values from ref. 9
[c]Highest assayable concentration

DISCUSSION

We have extended previous studies of the specificity of soluble, cytoplasmic 17β-HSD (8,16,17) and for the first time used inhibition kinetics to characterize microsomal 17β-HSD of human placenta. The results allow for a discussion of the potential not only of synthetic steroids and non-steroidal compounds but naturally occurring steroids, as well, to compete for the active sites of the two activities.

As shown in Table 1, microsomal 17β-HSD is susceptible to inhibition by a variety of naturally occurring steroids. In contrast, soluble 17β-HSD is highly specific for C_{18}-steroids (Table 4); C_{19}- and C_{21}-steroids are bound as inhibitors and/or substrates, but with affinities orders of magnitude less than that of estradiol-17β (8,9).

Placental tissue levels of 8.0 μM and 1.4 μM have been reported for progesterone and 20α-dihydroprogesterone, respectively (18), and 0.2 μM and 1.5 μM for estradiol-17β and estrone (19), levels at which a significant competition for microsomal 17β-HSD would be expected if the reported values represent steroid available for binding to the enzyme.

Microsomal 17β-HSD is also more susceptible than the cytoplasmic enzyme to inhibition by synthetic steroid derivatives, particularly ethinylestradiol and danazol

(Table 2). Gurpide and colleagues (20) reported that in an *in vitro* perfusion system ethinylestradiol at 10 μM significantly increased the release of estradiol-17β and decreased the release of estrone into the fetal perfusate from placental cotyledons perfused *via* the fetal vessels with ^{3}H-labeled androstenedione. Since only microsomal 17β-HSD has a strong affinity for ethinylestradiol, this suggests not only that this form of the enzyme is a significant component in placental estrogen metabolism but also that it may influence the direction of release of products of the aromatase reaction. Such an inhibition by ethinylestradiol *in vivo* would not be expected during pregnancy but, as suggested above, the presence of naturally occurring competitors for the active site of 17β-HSD, such as testosterone, progesterone or 20α-dihydroprogesterone, could exert a similar effect.

17β-HSD of human corpus luteum is inhibited by danazol (21). Our data suggest that a similar form of 17β-HSD is associated with placental microsomes. Plasma levels of danazol as high as 2 to 3 μM have been reported for some dosage regimens (22), concentrations which could significantly inhibit microsomal 17β-HSD. Data consistent with the presence of this form of 17β-HSD in human brain and pituitary (23), liver (24), adipose tissue (25), endometrium (26) and breast (27) have also been reported. Thus it may be reasonably speculated that the ability of danazol to inhibit microsomal 17β-HSD, which

as a result lengthens the intracellular lifetime of testosterone within various tissues, could contribute to its androgenic potency.

Two anabolic steroids, methyltestosterone and methylandrostanolone, were tested. Both inhibit microsomal 17β-HSD, as does the progestational derivative, norethisterone. Their K_I-values were significantly greater than those of ethinylestradiol or danazol, however. The uncompetitive inhibition observed with medroxyprogesterone acetate indicates that it is not competing with substrate for the active site.

Only two non-steroidal compounds were inhibitors. Diethylstilbestrol inhibited both 17β-HSD activities (Tables 3 and 4) as did o-demethyl CI-680. Of all the steroidal and non-steroidal compounds tested, these were the only ones which showed a greater affinity for soluble 17β-HSD. None of the other non-steroidal compounds assayed, including coumestrol, an estrogenic isoflavone of plant origin (28), was inhibitory. This lack of effect in vitro must be interpreted with caution, however, since the range of concentrations that were used was limited in most cases by solubility.

In summary, various naturally occurring steroids, synthetic steroid derivatives in common use and non-steroidal hormone agonists and antagonists were assayed as inhibitors of microsomal and soluble forms of 17β-HSD. The microsomal enzyme was more susceptible to inhibition.

The observed K_I-values, when compared with data on *in vivo* levels of estrogens, progesterone, 20α-dihydro-progesterone, danazol and ethinylestradiol (5, 18, 19, 29), suggest that competition for the active site of microsomal 17β-HSD could be a normally occurring as well as a pharmacologic means for influencing cellular hormonal status. Because of the known ability of tissues to concentrate steroidal compounds (5), more data on the inhibition of 17β-HSD in intact cellular systems are of particular interest.

ACKNOWLEDGEMENTS

This work was supported by grants from the St. Paul-Ramsey Medical Education and Research Foundation. A preliminary report was presented at The Society for Gynecologic Investigation, 30th Annual Meeting, Washington, D.C., March, 1983. The authors are grateful to Ms. Carol Collins for typing the mannuscript and to Mr. Davey Smith for the figures.

REFERENCES

1. Address correspondence to this author at the Department of Obstetrics and Gynecology, St. Paul-Ramsey Medical Center, 640 Jackson St., St. Paul, MN 55101.
2. Weinberger, M.J., Aten, R.F. and Eisenfeld, A.J., BIOCHEM. PHARMACOL. 27:2469 (1978).
3. Gurpide, E. and Marks, C., J. CLIN. ENDOCRINOL. METAB. 52:252 (1981).
4. King, R.J.B., Townsend, P.T. and Whitehead, M.I., J. STEROID BIOCHEM. 14:235 (1981).
5. Gurpide, E., J. TOXICOL. ENVIRON. HEALTH 4:249 (1978).
6. Pollow, K., Runge, W. and Pollow, B., Z. NATURFORSCH. 30c:17 (1975).
7. Thomas, C.M.G. and Veerkamp, J.H., ACTA ENDOCRINOL. 82:150 (1976).
8. Blomquist, C.H., Kotts, C.E. and Hakanson, E.Y., ARCH. BIOCHEM. BIOPHYS. 186:35 (1978).
9. Strickler, R.C., Tobias, B. and Covey, D.F., J. BIOL. CHEM. 256:316 (1981).
10. Blomquist, C.H., Kotts, C.E. and Hakanson, E.Y., J. STEROID BIOCHEM. 9:685 (1978).

11. Bradford, M.M., ANAL. BIOCHEM. 72:248 (1976).
12. Jarabak, J., METH. ENZYMOL. 15:746 (1969).
13. Blomquist, C.H., Kotts, C.E. and Hakanson, E.Y., ANAL. BIOCHEM. 87:631 (1978).
14. Porter, W.R. and Trager, W.F., BIOCHEM. J. 161:293 (1977).
15. Cornish-Bowden, A., BIOCHEM. J. 137:143 (1974).
16. Jarabak, J. and Sack, G.H., Jr., BIOCHEMISTRY 8:2203 (1969).
17. Karavolas, H.J. and Engel, L.L., ENDOCRINOLOGY 8:1165 (1971).
18. Runnebaum, B., Stober, I. and Zander, J., ACTA ENDOCRINOL. 80:560 (1975).
19. Ferre, F., Breuiller, M., Tanguy, G., Janssens, Y. and Cedard, L., AM. J. OBSTET. GYNECOL. 138:500 (1980).
20. Gurpide, E., Marks, C., de Ziegler, D., Berk, P.D. and Brandes, J.M., AM. J. OBSTET. GYNECOL. 144:551 (1982).
21. Barbieri, R.L., Osathanondh, R. and Ryan, K.J., OBSTET. GYNECOL. 57:722 (1981).
22. Cook, D.B. and Gibb, I., J. STEROID BIOCHEM. 13:1325 (1980).
23. Jenkins, J.S. and Hall, C.J., J. ENDOCRINOL. 74:425 (1977).
24. Littmann, K.-P., Gerdes, H. and Winter, G., ACTA ENDOCRINOL. 67:473 (1971).
25. Perel, E. and Killinger, D.W., J. STEROID BIOCHEM. 10:623 (1979).
26. Tseng, L., Mazella, J. and Tseng, L., J. STEROID BIOCHEM. 14:437 (1981).
27. MacIndoe, J.H. and Woods, G.R., ENDOCRINOLOGY 108:1407 (1981).
28. Martin, P.M., Horwitz, K.B., Ryan, D.S. and McGuire, W.L., ENDOCRINOLOGY 103:1860 (1978)
29. Fotherby, K., J. STEROID BIOCHEM. 19:817 (1983).