On the Specificity of Steroid Interaction with Mammary Glucose 6-Phosphate Dehydrogenase*

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ABSTRACT: This report describes an investigation of the interaction between rat mammary glucose 6-phosphate dehydrogenase and steroids. Certain steroids were known to act as potent inhibitors of glucose 6-phosphate dehydrogenase. The effects of altering the steroid structure on this inhibition were employed in delineating features of the steroid binding site on the enzyme. Fifty-two steroids and 8 steroid analogs were tested as inhibitors; for 14 of these, K_i values were determined and the free energy of binding calculated. All 14 compounds showed uncompetitive inhibition with respect to glucose 6-phosphate. For 3β-hydroxy-5-pregnen-20-one (pregnenolone) and 3β-hydroxy-5-androsten-17-one (dehydroepiandrosterone, in an earlier study), inhibition was also tested with respect to oxidized nicotinamide–adenine dinucleotide phosphate and found to be uncompetitive. Inhibition required the presence of a keto group at C-17 (for androstanes and estranes) or at C-20 (for pregnanes). The best inhibitor tested, 5α-androstan-17-one (K_i = 0.296 μM), contains no other substituents and is characterized by a large, planar surface (on the α side) which appears to play the dominant role in binding the steroid to the enzyme.

Presumably this binding occurs through hydrophobic interactions. Essentially all structural alterations which disrupt the planarity of the ring system diminish inhibition. Certain noninhibitory steroids, such as 5α-androstan-3β-ol, interfered with inhibition by 5α-androstan-17-one. It could be inferred, therefore, that although the keto group at C-17 or C-20 is essential for inhibition, it plays no crucial role in binding. Studies with steroid analogs possessing various portions of the ring system suggest that the entire steroid structure, with the possible exception of portions of ring D, is required for inhibition. The possible presence of two types of steroid binding sites was suggested by studies with high concentrations of certain steroids.

Many enzymes interact reversibly with activators or inhibitors known to bind at a site distinct from the catalytic site. This is true, for example, with most "allosteric" enzymes. The specificity at such modifier sites is as stringent as at the catalytic site, but because no catalytic transformations occur, binding specificity is more simply determined.

Steroids are particularly attractive molecules for binding studies because they are relatively rigid, with many possibilities for attaching various groups in different steric orientations. These facts were pointed out by Ringold *et al.* (1966) and utilized by them in an elegant investigation of the "topochemical pattern" of the steroid binding site of 3α-hydroxysteroid dehydrogenase from *Pseudomonas testosteroni*. Their studies with various steroid analogs, possessing limited portions of the steroid ring system, enabled them to identify the role of individual carbon atoms in allowing the enzyme to discriminate between stereoisomers as well as in providing rate enhancement during catalysis. The limitations imposed on these conclusions, inherent in the fact that the steroid is a substrate for their enzyme, were recognized by Ringold *et al.* (1966, 1967).

The inhibition of glucose 6-phosphate dehydrogenase by steroids was first reported by Marks and Banks (1960) and by McKerns and coworkers [McKerns and Bell (1960); McKerns and Kaleita (1960)]. These studies showed that there was a considerable degree of specificity with regard to the steroid structure, as well as a high affinity by glucose 6-phosphate dehydrogenase for the inhibitory steroids. Marks and Banks (1960) also demonstrated that whereas glucose 6-phosphate dehydrogenases from various mammalian sources were sensitive to steroids, glucose 6-phosphate dehydrogenases from spinach or yeast were not. Rat mammary glucose 6-phosphate dehydrogenase is inhibited by steroids (Levy, 1961, 1963). We have investigated the specificity of this inhibition in order to gain some insight into the requirements for steroid–enzyme interaction.

Experimental Procedures

Enzyme Assays. Glucose 6-phosphate dehydrogenase (GlcPD)[1] was prepared from the mammary glands of lactating rats by the procedure of Levy (1963). It was further purified[2] by using Sephadex G-200 and isoelectric focussing. Unless noted otherwise, all kinetic and competition experiments were performed with this highly purified enzyme obtained from a single band on isoelectric focussing and shown to have a single band on acrylamide gel electrophoresis.[2] The qualitative comparisons of inhibitors (Tables I–IV) were performed using enzyme which was only partially purified. Unless otherwise noted, enzyme assays were performed with

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[1] Abbreviation used is: GlcPD, glucose 6-phosphate dehydrogenase.

[2] B. H. Nevaldine, unpublished.

a Zeiss PMQ11 spectrophotometer at 25° by observing the absorbancy increase at 340 nm accompanying reduced coenzyme formation. Care was taken to obtain initial rates in all assays. All activity figures are the average of at least two assays. The concentrations of assay components, unless otherwise specified, were as follows: 66.7 mM Tris-acetate, pH 8.6; 83.3 μM $NADP^+$ or 5.00 mM NAD^+ (neutralized); and 3.33 mM glucose 6-phosphate. For the NADP-linked reaction these conditions ensured that both substrate and coenzyme were saturating; for the NAD-linked reaction this was only true for the substrate. Reactions were initiated with enzyme. Steroids and steroid analogs were dissolved in dioxane and added last before the enzyme. In the competition experiments (Tables V and VI) inhibitory steroid was added before the competitor compound. In all experiments control assays without steroids received identical increments of dioxane. The volume of dioxane never exceeded 0.83% of that of the final assay solution; usually it was 0.67%. Under these conditions dioxane had no effect on enzyme activity. Cuvets with 1-cm light paths were used for some experiments, but for many of the kinetic assays it was advantageous to use cuvets with 5-cm light paths.

Determination of Steroid Solubility. The maximum solubility of various steroids at 25° was determined by light scattering. A Farrand spectrofluorometer was employed, using excitation and emission wavelengths of 320 nm. Steroids dissolved in dioxane were added in small increments (usually 5 μl) to 3.0 ml of Tris-acetate buffer, pH 8.6. The maximum solubility was taken as that steroid concentration beyond which a sharp increase in light scattering was observed. The validity of such measurements was confirmed by noting that Dixon plots (reciprocal velocity *vs.* steroid concentration; Dixon, 1953) gave a sharp break at the same steroid concentration, the lines becoming horizontal at higher concentrations.

Materials

Glucose 6-phosphate, NAD^+, and $NADP^+$ were obtained from Sigma Chemical Co. Reagent grade dioxane was distilled twice and stored in a dark bottle. Courtauld space-filling atomic models were purchased from the Ealing Corp. The following steroids were obtained from Steraloids, Inc.: 5α-androstane; 5α-androstane-3β,17β-diol; 5α-androstane-3,17-dione; 5α-androstan-3α-ol; 5α-androstan-3β-ol; 5α-androstan-3-one; 5α-androstan-17-one; 5α-androst-1-ene-3,-17-dione; 4-androstene-3,17-dione; 3β,17β-dihydroxy-5α-androstan-17-one; 3β,17α-dihydroxy-5α-pregnan-20-one; 3β,21-dihydroxy-5-pregnen-20-one; 4-estrene-3,17-dione; 4-estren-17-one; 3α-hydroxy-5α-androstan-17-one; 3β-hydroxy-5α-androstan-16-one; 3β-hydroxy-5α-androstan-17-one; 3β-hydroxy-5β-androstan-17-one; 17β-hydroxy-5α-androstan-3-one; 3β-hydroxy-5-androsten-17β-carboxylic acid; 3β-hydroxy-5-androsten-17-one (dehydroepiandrosterone); 3β-hydroxy-16α-bromo-5-pregnen-20-one; 3β-hydroxy-16α,17α-epoxy-5-pregnen-20-one; 3β-hydroxy-6-methyl-5-pregnen-20-one; 3β-hydroxy-5α-pregnane-11,20-dione; 3β-hydroxy-5α-pregnan-20-one; 3β-hydroxy-5β-pregnan-20-one; 3β-hydroxy-5-pregnen-20-one (pregnenolone); 17-oxo-5α-androstan-3β-yl-acetate; 20-oxo-5α-pregnan-3β-yl acetate; 5α-pregnane-3β,20-diol; 5α-pregnane-3,20-dione; and 5α-pregnan-3β-ol. The following steroids were obtained from Mann Research Laboratories: 2α-bromo-5α-androstane-3,17-dione; 3β-chloro-5α-androstan-17-one; and 4-pregnene-3.20-dione (progesterone). Sigma Chemical Co. was the source for 1,3,5(10)-estratriene-3,17α-diol (17α-estradiol) and 1,3,5(10)-estratriene-3,17β-diol (17β-estradiol), and Calbiochem supplied 3-hydroxy-1,3,5(10)-estratrien-17-one (estrone). The following steroids were kindly supplied by Dr. John C. Babcock, of the Upjohn Co.: 4,9-androstadiene-3,17-dione; 5α-androstane-11,17-dione; 5α-androst-9-en-17-one; 1,3,5(10)-estratrien-17-one; 11β-hydroxy-5α-androstan-17-one; 11α-hydroxy-4-androstene-3,17-dione; 11β-hydroxy-4-androstene-3,17-dione; 3α-hydroxy-7α-methyl-5α-androstan-17-one; 3α-hydroxy-4α-methyl-5α-androstan-17-one; 3β-hydroxy-7α-methyl-5-androsten-17-one; 3β-hydroxy-7α-methyl-5-estren-17-one; 3β-hydroxy-7β-methyl-5-estren-17-one; 7α-methyl-4-estren-17-one; and 7α-methyl-1,3,5(10)-estratrien-17-one. Dr. Howard Ringold, from Syntex Research, kindly made the following two steroid analogs available to us: *dl-trans*-2-decalone, and *dl-trans*-tetradecahydro-2-acetylphenanthren-7-one. Dr. Charles J. Sih, of the School of Pharmacy, University of Wisconsin, kindly provided samples of 7,7α-dihydro-7α-methyl-1,5-(6*H*)-indan dione and (+)-*anti-trans*-1-keto-8β-methyl-4,5-(4-keto-1,2,3,4-tetrahydrobenzo)hydrindan. Cyclohexanol and cyclopentanone were obtained from Eastman; *cis,trans*-decahydro-2-naphthol and 2-nonanone from Aldrich Chemical Co. All steroids and their analogs were used without further purification. Spot checks were made on melting points of commercially supplied compounds and these agreed in every instance with the reported value. The procedure of Glazier (1962) was used to synthesize 3β-hydroxy-16-α-bromo-5α-androstan-17-one from 3β-hydroxy-5α-androstan-17-one. Its melting point (161–162°) agreed with the value (161.5–163°) reported by Glazier (1962).

Results

Inhibition of the NADP-Linked Reaction by Steroids. In the first phase of these studies a large number of steroids and steroid analogs were surveyed for their capacity to inhibit the NADP-linked reaction. A few of these compounds were also treated with NAD^+ as coenzyme. In these experiments all compounds were used at a final concentration of 10 μM, or at lower concentrations when this exceeded the solubility.

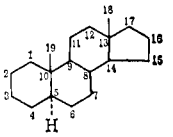
In Table I are collected data on the effects of steroids related to androstane. The results can be summarized as follows.

(a) A 17-keto group is required for inhibition. Thus 5α-androstan-17-one (2) is the minimum structure leading to inhibition. Steroids possessing no functional groups (1), a keto group (3) or α- or β-hydroxyl groups at C-3 (10, 4) are inactive. Neither a β-OH group (5) nor a β-carboxyl group (*cf.* 16 *vs.* 15) can replace the keto group at C-17, nor can a 16-keto group serve this function (*cf.* 7 *vs.* 6).

(b) Changing the configuration at the A–B ring juncture to A/B *cis* (8) markedly reduces inhibitory activity (*cf.* 6).

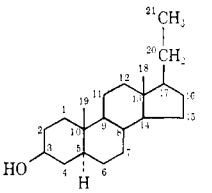
(c) Polar substituents at C-3 diminish the inhibitory activity of the steroid. This effect is least with a β-OH group (*cf.* 6 *vs.* 2), greater with a keto group (9), and most marked with an α-OH group (11). However, in the β configuration, a larger group has a more deleterious effect on inhibition (*cf.* 12 and 28)

TABLE I: Inhibitory Activity of Compounds Related to Androstane.

Compound[a]	NADP-Linked Activity[b]	NAD-Linked Activity[c]
1. 5α-Androstane 	102[d]	
2. 5α-Androstan-17-one	11,[e] 26[f]	80[g]
3. 5α-Androstan-3-one	97[h]	
4. 5α-Androstan-3β-ol	101[g]	102
5. 5α-Androstane-3β,17β-diol	100	
6. 3β-Hydroxy-5α-androstan-17-one	20	93, 66[i]
7. 3β-Hydroxy-5α-androstan-16-one	103	95
8. 3β-Hydroxy-5β-androstan-17-one	88	
9. 5α-Androstane-3,17-dione	60	
10. 5α-Androstan-3α-ol	102[e]	
11. 3α-Hydroxy-5α-androstan-17-one	90	100
12. 17-Oxo-5α-androstan-3β-acetate	73	
13. 5α-Androst-1-ene-3,17-dione	77	
14. 4-Androstene-3,17-dione	82	
15. 3β-Hydroxy-5-androsten-17-one	52	
16. 3β-Hydroxy-5-androsten-17β-carboxylic acid	97	
17. 5α-Androst-9-en-17-one	37[f]	
18. 4,9-Androstadiene-3,17-dione	92	
19. 5α-Androstane-11,17-dione	23	
20. 11β-Hydroxy-5α-androstan-17-one	58	
21. 11β-Hydroxy-4-androsten-3,17-dione	95	
22. 11α-Hydroxy-4-androstene-3,17-dione	101	
23. 3α-Hydroxy-4α-methyl-5α-androstan-17-one	70	
24. 3α-Hydroxy-7α-methyl-5α-androstan-17-one	91	
25. 3β-Hydroxy-7α-methyl-5-androsten-17-one	77	
26. 2α-Bromo-5α-androstane-3,17-dione	77	105
27. 3β-Hydroxy-16α-bromo-5α-androstan-17-one	10	76
28. 3β-Chloro-5α-androstan-17-one	35	66[i]

[a] Concentrations are 10 μM unless otherwise indicated. [b] Velocity of NADP-linked reaction in the presence of steroid, expressed as per cent of velocity observed in the presence of dioxane only. [c] Velocity of NAD-linked reaction in the presence of steroid, expressed as per cent of velocity observed in the presence of dioxane only. [d] <1 μM (see text). [e] 3 μM. [f] 1 μM. [g] 4 μM. [h] 5 μM. [i] 50 μM.

TABLE II: Inhibitory Activity of Compounds Related to Pregnane.

Compound[a]	Activity[b]
1. 5α-Pregnan-3β-ol 	104[c]
2. 3β-Hydroxy-5α-pregnan-20-one	11[d]
3. 5α-Pregnane-3β,20-diol	99
4. 3β-Hydroxy-5β-pregnan-20-one	74
5. 5α-Pregnane-3,20-dione	97
6. 20-Oxo-5α-pregnan-3β-ylacetate	78
7. 4-Pregnene-3,20-dione	101
8. 3β-Hydroxy-5-pregnen-20-one	36
9. 3β-Hydroxy-5α-pregnane-11,20-dione	69
10. 3β,17α-Dihydroxy-5α-pregnan-20-one	23
11. 3β,21-Dihydroxy-5-pregnen-20-one	63
12. 3β-Hydroxy-16α,17α-epoxy-5-pregnen-20-one	99
13. 3β-Hydroxy-6-methyl-5-pregnen-20-one	34
14. 3β-Hydroxy-16α-bromo-5-pregnen-20-one	100

[a] Concentrations are 10 μM unless otherwise indicated. [b] Velocity of NADP-linked reaction observed in the presence of the steroid, expressed as per cent of velocity observed in the presence of dioxane only. [c] 4 μM. [d] 5 μM.

(d) The introduction of double bonds diminishes inhibition. This effect is small at C-1 (13 *vs.* 9), C-4 (14 *vs.* 9), or C-9 (17 *vs.* 2) and somewhat more marked at C-5 (15 *vs.* 6). The introduction of 2 double bonds results in further loss of inhibitory activity (18 *vs.* 14).

(e) Polar substituents at C-11, like those at C-3, diminish the inhibitory activity of the steroids. Again, an α-OH is most deleterious (22), but the effect of a keto group (19) is less marked than that of a β-OH group (20 and 21).

(f) The effect of methyl substituents at C-4 or C-7 is not great. A 4α-CH_3 group appears to enhance inhibition slightly (23 *vs.* 11) and a 7α-CH_3 group has little effect (24 *vs.* 11) or is somewhat deleterious (25 *vs.* 15).

(g) The introduction of a bromine group at C-2 appears to have little effect (26 *vs.* 9) and a 16α-Br group actually enhances inhibition (27 *vs.* 6).

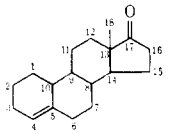
Table II contains the results obtained with steroids related to pregnane. These data can be summarized as follows:

(a) The 20-keto group appears to serve the same essential function in these steroids as the 17-keto group does in the androstane series (2 and 1). An OH-group cannot replace the function of the keto group (3 *vs.* 2).

(b) As in the androstane series, the A/B *cis* configuration (4) is much less inhibitory than the A/B *trans* configuration (2).

(c) Less limited data on the substitution of functional groups at C-3 shows that, as in the androstane series, either

TABLE III: Inhibitory Activity of Compounds Related to Estrane.

Compound[a]	Activity[b]
1. 4-Estren-17-one 	22
2. 4-Estrene-3,17-dione	88
3. 7α-Methyl-4-estren-17-one	30
4. 3β-Hydroxy-7α-methyl-5-estren-17-one	101
5. 3-β-Hydroxy-7βmethyl-5-estren-17-one	70
6. 1,3,5(10)-Estratrien-17-one	44
7. 3-Hydroxy-1,3,5(10)-estratrien-17-one	84
8. 1,3,5(10)-Estratriene-3,17β-diol	99; 89[c]
9. 1,3,5(10)-Estratriene-3,17α-diol	101; 100[c]
10. 7α-Methyl-1,3,5(10)-estratrien-17-one	84

[a] Concentrations are 10 μM unless otherwise indicated. [b] Velocity of NADP-linked reaction observed in the presence of the steroid, expressed as per cent of velocity observed in the presence of dioxane only. [c] 50 μM.

a 3β-acetate (6) or a 3-keto group (5) allows less inhibition than a 3β-OH group (2).

(d) Double bonds diminish inhibition in the pregnane series, as in the androstane series. Thus 7 is not inhibitory, and 8 is less inhibitory than 2.

(e) An 11-keto group diminishes inhibition (9 *vs.* 2), as it does in the androstane series.

(f) The introduction of a methyl group at C-6 has no effect (*cf.* 13 and 8).

(g) Inhibition is diminished by introducing OH groups at C-17α (*cf.* 10 *vs.* 2) or C-21 (11 *vs.* 8). A 16α,17α-epoxy substituent totally abolishes inhibition (12 *vs.* 8).

(h) In contrast to the result in the androstane series, a 16α-bromo substituent completely abolishes inhibition (14 *vs.* 8).

Data on compounds in the estrane series, collected in Table III, are more limited. They are summarized as follows:

(a) A 17-keto group is virtually essential for inhibition. At the relatively high concentration of 50 μM, 1,3,5(10)-estratrien-3,17β-diol (estradiol, 8) causes slight inhibition. The 17α-analog (9) is inactive at this concentration, whereas the 17-keto analog (estrone 7) gives some inhibition at 10μM.

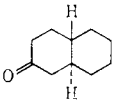
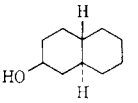
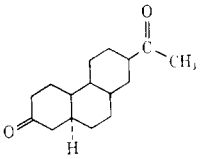
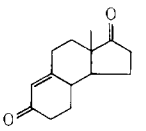
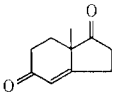
(b) Polar substituents at C-3 diminish inhibition (*cf.* 2 *vs.* 1, and 7 *vs.* 6) as they do in the androstane and pregnane series.

(c) No compounds containing a saturated A ring were tested, but comparison of 6 with 1 shows that the aromatic A ring, diminishes inhibition as double bonds do in the other two series of steroids tested.

(d) The effect of methyl groups in these steroids appears to be more deleterious than it is in the androstane compounds (*cf.* 3 *vs.* 1, 10 *vs.* and 4 and 5).

The side chain at C-17 in the pregnane series appears to enhance inhibition (*cf.* 2, 4, and 8, Table II *vs.* 6, 8, and 15, Table I, respectively) or to have little effect (6 Table II *vs.* 12 Table I). This is not seen when other substituents in the pregnene series (*e.g.*, C-3 keto or 16α-bromo) render the compounds inactive (*cf.* 9, Table I, with 5, Table II; 14, Table I, and 2, Table III, with 7, Table II; 27, Table I, with 14, Table II).

TABLE IV: Inhibitory Activity of Steroid Analogs.

Compound	Concentration (mM)	Activity[a]
1. Cyclohexanol 	10	100
2. *dl-trans*-2-Decalone 	0.50	97
	1.0	95
3. *cis,trans*-Decahydro-2-naphthol[b] 	1.0	100
4. *dl-trans*-Tetradecahydro-2-acetylphenanthren-7-one[c] 	0.10	91
	0.50	68
	1.0	52
5. (+)-*anti-trans*-1-Keto-8β-methyl-4,5-(4-keto-1,2,3,4-tetrahydrobenzo)hydrindan 	0.10	95
	0.50	89[d]
6. 7,7α-Dihydro-7α-methyl-1,5-(6*H*)-indan dione 	0.10	99
	0.50	99
7. Cyclopentanone 	18.8	97
	75.2	97
	113	87
8. 2-Nonanone	0.010	103

[a] Velocity of NADP-linked reaction observed in the presence of the analog expressed as per cent of velocity observed in the presence of dioxane only. [b] Only *trans* compound is analogous to steroid inhibitor. [c] Only *d* compound is analogous to steroid inhibitor. [d] Only single assay performed because of limited supply of compound.

Inhibition of the NADP-Linked Reaction by Steroid Analogs. Several compounds which contain various portions of the steroid ring system were tested for inhibitory activity. The results of these studies are shown in Table IV. Of the compounds listed, 1 is analogous to ring A of steroids; 2 and 3 to rings AB; 4 to rings ABC; 5 to rings BCD; 6 to rings CD; and 7 to ring D. Compound 8, 2-nonanone, has the same overall length as 5α-androstan-17-one. Slight, but significant inhibition was observed with compounds 2 and 5, and with a very high concentration of cyclopentanone (7). Compound 4 exhibited considerable inhibition.

TABLE V: Effect of Noninhibitory Steroids on Inhibition by 5α-Androstan-17-one.

Expt	Steroids Added	Per Cent Activity of Control	Activity Increase[a]
1	5 μM 5α-Androstan-17-one	7.15	
	+ 5 μM 5α-Androstan-3β-ol	36.0	5.03
	+ 5 μM 5α-Androstan-3-one	13.7	1.92
	+ 5 μM 5α-Androstan-3α-ol	10.0	1.40
2	1.0 μM 5α-Androstan-17-one	27.4	
	+ 5α-Androstane[b]	34.2	1.25
	+ 1.5 μM 5α-Androstan-3β-ol	32.2	1.18
	+ 1.5 μM 5α-Androstan-3α-ol	31.3	1.14
	+ 1.5 μM 5α-Androstan-3-one	29.3	1.07

[a] Ratio of the velocity observed in the presence of 5α-androstan-17-one plus the other steroid divided by velocity observed with 5α-androstan-17-one only. [b] Concentration < 1 μM; see text.

TABLE VI: Effect of 5α-Androstan-3β-ol on Inhibition by 5α-Androstan-17-one.

Concentration of 5α-Androstan-17-one (μM)	Concentration of 5α-Androstan-3β-ol (μM)	Velocity[a]
0	0	0.184
0.1	0	0.147
	1	0.144
	5	1.154
1.0	0	0.059
	1	0.066
	5	0.091
2.5	0	0.026
	1	0.044
4.0	0	0.0205
5.0	0	0.0185

[a] Velocity is expressed as change in absorbance at 340 nm per min.

Various combinations of pairs of compounds listed in Table IV (1 + 7, 2 + 7, 3 + 7, 4 + 7, 1 + 6) failed to produce greater inhibition than that produced by either one alone.

Inhibition of the NAD-Linked Reaction by Steroids. Table I contains data on several steroids which were tested for their inhibitory effect on the NAD-linked reaction. The inhibition is very much less than for the NADP-linked reaction, and only the most potent inhibitors show any effect.

Interference with Steroid Inhibition of the NADP-Linked Reaction by Noninhibitory Steroids. From the foregoing section it is clear that the most inhibitory steroids possess a single polar group, *i.e.*, the 17-keto group, on a large, hydrophobic molecule (Table I, compounds 2 and 17; Table III, compounds 1, 3, and 6). The observation that virtually any substituent except, in some instances, a methyl group, diminishes inhibition, suggests that nonpolar interactions are involved in binding the steroid to the enzyme. To test this possibility, noninhibitory steroids containing no 17-keto group were examined for their effect on the inhibition of mammary GlcPD by 5α-androstan-17-one. The first experiment (Table V) provides evidence that 5α-androstan-3β-ol, 5α-androstan-3-one, and 5α-androstan-3α-ol, in that order of effectiveness, compete with 5α-androstan-17-one for the enzyme. It is noteworthy that this is the same order as the order of effectiveness of the 17-keto analogs as inhibitors (see Table I, compounds 6, 9, and 11). Other steroids found not to compete with 5α-androstan-17-one were: 5α-androstane-3α,17α-diol; 17β-hydroxy-5α-androstan-3-one; 3β-hydroxy-5α-androstan-16-one; and 3β-hydroxy-5β-androstan-17-one. 5α-pregnan-3β-ol, however, was found to be about as effective as 5α-androstan-3β-ol, which is consistent with the close structural similarity between these two steroids. All steroids were tested at a concentration of 5 μM.

The maximum solubility of 5α-androstan-17-one was found to be 4 μM by light scattering, and 4–5 μM from Dixon plots. The possibility, therefore, had to be considered that apparent competition resulted from precipitation from saturated solution of 5α-androstan-17-one by the addition of 5α-androstan-3β-ol. Although this seemed unlikely in view of the failure of several other steroids, including 5α-androstan-3α-ol, to cause a similar effect, a second experiment was performed at which the combined concentration of the two steroids was maintained below 4 μM. Under these conditions (expt 2, Table V) competitive effects were less marked, but clearly evident. Especially significant is the finding that 5α-androstane was apparently the most effective competitor. The sensitivity of our techniques was not sufficient to determine the solubility of this steroid accurately, but it did indicate that it is below 1 μM. Thus although this compound was present at an unknown concentration below 1 μM, it could compete more effectively than 1.5 μM 5α-androstan-3β-ol. This, too, is in keeping with the relative inhibitory effectiveness of the analogous 17-keto analogs (Table I, compounds 2 and 6).[8] No significant competition was observed in this experiment using the following steroids at 1.5 μM concentration: 3β,11β-dihydroxy-5α-androstan-17-one; 3β-hydroxy-5α-androstan-16-one; 5α-androstane-3,17-dione; 5α-pregnan-3β-ol; and 4-pregnene-3,20-dione.

Although 5α-androstane appeared to be the best competitor, it was more convenient to work with 5α-androstan-3β-ol since its solubility was known. Further experiments were therefore performed with this compound, one of which is illustrated in Table VI. The rate of the GlcPD-catalyzed reaction decreased with increasing concentration of 5α-androstan-17-one, until it leveled off at approximately 4 to 5 μM. At total steroid concentrations of below 4 μM, 5α-androstan-3β-ol could partially overcome inhibition by 5α-androstan-17-one.

[8] The fact that, in expt 2, the -3α-ol steroid competed better than the -3-keto steroid is probably only apparent. At this concentration the rate differences are probably not significant.

TABLE VII: Apparent Michaelis Constants and Inhibitor Constants Calculated from Lineweaver–Burk Plots

	pH 7.2		pH 8.6	
	Varying GlcP	Varying $NADP^+$	Varying GlcP	Varying $NADP^+$
K_m (GlcP)	12.1 μM[a]		22.3 ± 1.3 μM[b]	
K_m ($NADP^+$)		1.54 μM[a]		1.59 ± 0.27 μM[b]
K_i (pregnenolone)	4.35 μM[a]	5.72 μM[a]	4.77 ± 0.60 μM[b]	4.68 ± 2.6 μM[c]

[a] Single expt. [b] Average of 2 expt. [c] Average of 3 expt.

Certain of the steroid analogs were also tested to determine whether they could compete with 5α-androstan-17-one. Using 0.5 μM 5α-androstan-17-one, concentrations of cyclohexanol up to 10 mM; of *dl-trans*-2-decalone up to 2 mM; and of *cis,trans*-decahydro-2-naphthol up to 0.5 mM, produced no significant diminution of inhibition. At 2.5 μM 5α-androstan-17-one, 2 mM *cis,trans*-decahydro-2-naphthol also gave no loss of inhibition.

Determination of Inhibitor Constants. In order to obtain quantitative comparisons of the affinities of various steroids to mammary GlcPD inhibitor constants (K_i) were determined. For these measurements we routinely employed the procedure of Dixon (1953) in which reaction velocities are determined at various concentrations of an inhibitor and at two different concentrations of substrate. Because the K_m for $NADP^+$ is so low, it was convenient to perform all experiments at a saturating concentration of $NADP^+$ and two different concentrations of glucose 6-phosphate. For one steroid, 3β-hydroxy-5-pregnen-20-one (pregnenolone) we also determined K_i values by varying either glucose 6-phosphate at saturating $NADP^+$, or $NADP^+$ at saturating glucose 6-phosphate, both with and without the steroid, and at both pH 7.2 and pH 8.6. The inhibition was strictly uncompetitive with respect to both glucose 6-phosphate and $NADP^+$ at both pH values. All the kinetic constants from this experiment, derived from Lineweaver–Burk plots, are presented in Table VII.[4]

K_i values and standard free energies of binding were determined for 13 steroids and for *dl-trans*-tetradecahydro-2-acetyl-phenanthren-7-one. These data are assembled in Table VIII. For purposes of comparison, data are also included on the more qualitative experiments with these compounds described in an earlier section. For all the inhibitors shown in Table VIII the mechanism of inhibition was strictly uncompetitive with respect to glucose 6-phosphate.

Effect of High Steroid Concentrations. In the experiments for determining K_i values, the concentrations of inhibitor generally did not exceed 2 K_i. It was of interest to examine inhibition at higher concentrations, but only three steroids possessed the appropriate combination of a sufficiently low K_i and high solubility to permit such experiments. An example of the results seen under these conditions is illustrated in Figure 1 for 5α-androstan-17-one. A biphasic Dixon plot results, with a marked break separating two linear portions. This change in slope occurred at 2.6, 2.4, and 2.4 μM in three separate experiments. Similar results were seen with 3β-hydroxy-5α-androstan-17-one and 3β-hydroxy-16α-bromo-5α-androstan-17-one, although the change in slope was less marked with these steroids. From the ratio of slopes of the two linear portions of the Dixon plots and the determined K_i value, a second, apparent K_i could be calculated for the three steroids which was 2.00, 1.43, and 1.22 times, respectively, the first K_i for 5α-androstan-17-one, 3β-hydroxy-5α-androstan-17-one, and 3β-hydroxy-16α-bromo-5α-androstan-17-one, respectively. Possible implications of these results will be discussed below.

Discussion

The interaction between GlcPD and steroids has been studied by noting the effect of structural changes in the steroid on its inhibitory effectiveness. These studies, therefore, provide information only on those interactions between steroid and enzyme which affect catalytic activity. They permit deductions about topological features of a specific steroid binding site on the enzyme, and about the nature of the binding forces at that site, but do not pertain to any nonspecific interactions between steroid and enzyme which might occur without affecting catalytic activity.

Mammary GlcPD catalyzes an NAD-linked oxidation of glucose 6-phosphate which differs from the NADP-linked reaction in its response to various reagents and conditions (Levy, 1963; Levy *et al.*, 1966). This NAD-linked reaction appears to be much less sensitive to steroid inhibition (Table I). However, we have not investigated whether this differential sensitivity may be related to the fact that the NAD^+ concentration used in these assays is below its K_m, whereas the NADP-linked reaction is assayed at saturating $NADP^+$ concentration. The following discussion will concern the NADP-linked reaction only.

The inhibitor constants determined for 13 steroids and 1 steroid analog correlate well with the extent of inhibition observed at a single steroid concentration (Table VIII). This agreement justifies the use of data from Tables I to IV to reach conclusions about binding specificity.

Kinetics of Inhibition. Marks and Banks (1960) showed that dehydroepiandrosterone inhibits human erythrocyte GlcPD uncompetitively with respect to both glucose 6-phosphate and $NADP^+$. Our studies confirmed this for mammary GlcPD (Levy, 1963). In the present work we have shown that

[4] The K_m values previously reported at pH 8.6 were: glucose 6-phosphate, 30 μM, and $NADP^+$, 8.9 μM (Levy, 1963). These differences may result from the fact that dioxane was not present in the earlier experiments, and that they were performed with less highly purified enzyme.

TABLE VIII: Inhibitor Constants.

Compound	Per Cent Activity of Control[a]	K_i (μM)[b]	$-\Delta G°$ (kcal/mole)[c]
5α-Androstan-17-one	11[d]	0.296	8.95
5α-Androst-9-en-17-one	37[e]	0.525	8.60
3β-Hydroxy-5α-pregnan-20-one	11[f]	0.568	8.55
3β-Hydroxy-16α-bromo-5α-androstan-17-one	10	0.571	8.55
5α-Androstane-11,17-dione	23	2.28	7.72
3β-Hydroxy-5α-androstan-17-one	20	3.56	7.46
4-Estren-17-one	22	4.29	7.35
3β-Hydroxy-5-pregnen-20-one	36	5.41[g]	7.22
3β-Hydroxy-5-androsten-17-one	52	18.5	6.47
5α-Androstane-3,17-dione	60	20.0	6.44
3β-Hydroxy-5β-pregnan-20-one	74	21.0	6.41
11β-Hydroxy-5α-androstan-17-one	58	53.2	5.85
4-Androstene-3,17-dione	82	60.7	5.78
dl-trans-Tetradecahydro-2-acetylphenanthren-7-one	91[h]	667 (334)[i]	4.35 (4.75)[i]

[a] Data from Tables I–IV. Concentration for all compounds, 10 μM unless noted. [b] Average of two determinations except where noted. [c] Calculated from: $\Delta G° = -RT \ln (1/K_i)$, where $T = 298°K$. [d] 4 μM. [e] 1 μM. [f] 5 μM. [g] Average of 7 determinations, including the 5 at pH 8.6. noted in Table VII. [h] 100 μM. [i] Data in parentheses are calculated on the assumption that only the *d* isomer is active and that the *l* isomer is not bound.

pregnenolone also shows uncompetitive behavior with respect to both glucose 6-phosphate and $NADP^+$. All the other steroids and the steroid analog for which K_i values were determined (Table VIII) showed uncompetitive behavior with respect to glucose 6-phosphate; their behavior with respect to $NADP^+$ was not tested. Several steroids showed uncompetitive inhibition with respect to $NADP^+$ for GlcPD from bovine corpora lutea (Nielson and Warren, 1965). However Criss and McKerns (1969), working with bovine adrenal cortex GlcPD, reported competitive behavior with some steroids and uncompetitive behavior for others.

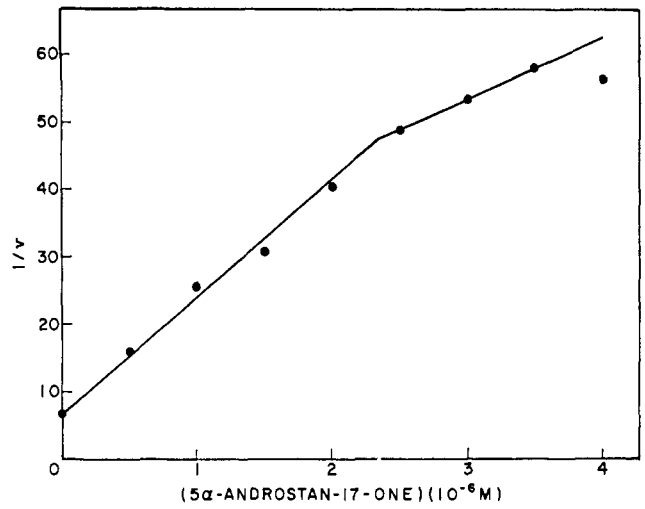


FIGURE 1: Dixon plot for 5α-androstan-17-one including high steroid concentrations. Each point represents the average of two determinations. Assay conditions as described under Experimental Procedure; Volume, 3.0 ml. Steroid added in 25 μl of dioxane.

The kinetic mechanism of mammary GlcPD has not been reported yet. Thus, the enzyme complex with which the steroid interacts is unknown. We shall refer to the interaction of steroid with enzyme in the following discussion, but it should be understood that the interaction is actually with the appropriate binary or ternary complex.

Structural Requirements for Inhibition by Steroids. Steroids which can inhibit mammary GlcPD possess a keto group at C-17 (in the estrane and androstane series) or at C-20 (in the pregnane series). The only exception we have found is 17β-estradiol, which is slightly inhibitory at high concentrations. Our results are in general agreement with those found with human erythrocyte GlcPD (Marks and Banks 1960) and with GlcPD from bovine corpora lutea (Nielson and Warren, 1965). However, McKerns has reported data on GlcPD from the bovine adrenal cortex (McKerns, 1962; Criss and McKerns, 1969) which show that 17β-estradiol, 5α-androstane-3β,17β-diol (completely inert in our system, see Table I), and several other steroids with a 17β-ol substituent inhibit that enzyme.

Construction of models reveals that the relative positions of the 17-keto and 20-keto groups are quite similar (Figure 2). In contrast, the position of the 16-keto group in 3β-hydroxy-5α-androstan-16-one, which does not inhibit (Table I), is quite different from that of the 17-keto group in the inhibitory 3β-hydroxy-5α-androstan-17-one (Figure 3). Also, whereas 5-pregnen-3β-ol-20-one is an excellent inhibitor (K_i = 5.41 μM, Table VIII), 3β,21-dihydroxy-5-pregnen-20-one is less inhibitory (Table II) and 3β-hydroxy-5-androsten-17β-carboxylic acid is essentially without inhibitory activity (Table I). Thus, the C-21-methyl group may play an important role in binding steroids of the pregnane series.

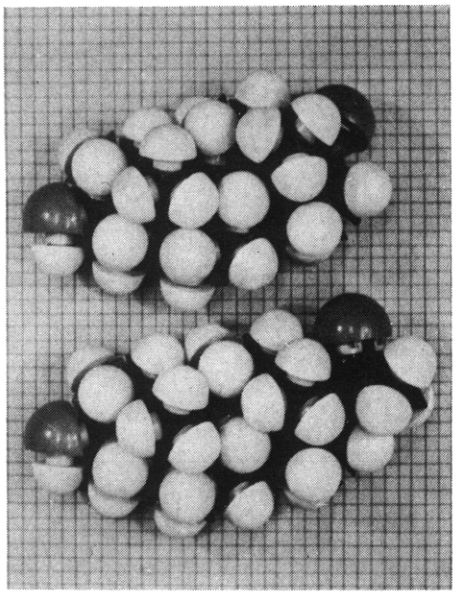


FIGURE 2: Models of 3β-hydroxy-5α-androstan-17-one (top) and 3β-hydroxy-5α-pregnan-20-one (bottom), as seen from front (nonplanar side) with A ring at left, D ring at right. Grid in background for this and other models (Figures 3–6) is 0.5 Å.

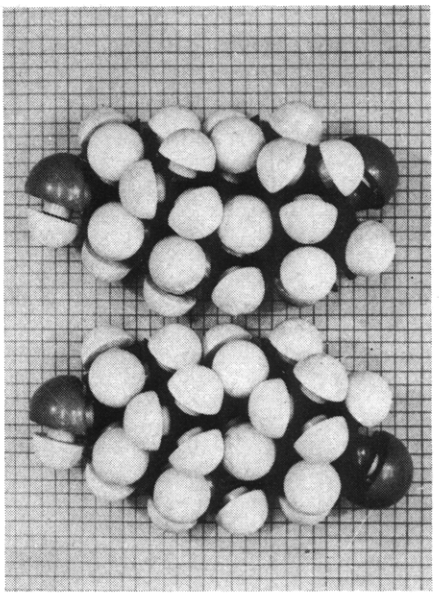


FIGURE 3: Models of 3β-hydroxy-5α-androstan-17-one (top) and 3β-hydroxy-5α-androstan-16-one (bottom), as seen from front (nonplanar side) with A ring at left, D ring at right.

The most effective inhibitor we tested, 5α-androstan-17-one (K_i = 0.296 μM, Table VIII), contains no other functional groups besides the ketone at C-17. A striking feature of this molecule is its flat surface on the α side (Figure 4). Our data suggest that this planar surface plays a critical role in binding, presumably through hydrophobic bonding. In steroids possessing the A/B-*cis* configuration the A ring projects approximately 60° from the planar surface (Figure 4). Such steroids are much less inhibitory, as illustrated by comparing the K_i for 3β-hydroxy-5β-pregnan-20-one (21.0 μM) and 3β-hydroxy-5α-pregnan-20-one (0.568 μM) (Table VIII), and by comparing the inhibition with 10 μM 3β-hydroxy-5β-androstan-17-one (12%) and 10 μM 3β-hydroxy-5α-androstan-17-one (80%) (Table I). The importance of the stereochemistry of A–B ring fusion for interaction between a steroid and an enzyme was first clearly demonstrated by Marcus and Talalay (1955) with β-hydroxysteroid dehydrogenase from *P. testosteroni*. Similarly, substituents which project from the planar surface (*i.e.*, which are axial and α) interfere much more seriously with inhibition than equatorial substituents on the same carbon atoms. This is illustrated in Figure 5 for the 3α- and 3β-OH groups. From Table VIII it can be seen that substitution of a 3β-OH group for an H in 5α-androstan-17-one produces a 12-fold increase in K_i. Table I shows that whereas 10 μM 3β-hydroxy-5α-androstan-17-one inhibits 80%, an equal concentration of 3α-hydroxy-5α-androstan-17-one inhibits only 10%. A 3-keto group lies between the 3α- and 3β-OH groups, projecting less than a 3α-OH; 10 μM 5α-androstan-3,17-dione shows 40% inhibition, intermediate between the 3α-ol and 3β-ol compounds (Table I). Similarly, in the pregnane series (Table II) 3β-hydroxy-5α-pregnan-20-one is a much more potent inhibitor than the corresponding 3-keto compound; the corresponding 3α-ol was not tested. At C-7, also, an axial α-CH_3 group interferes more with inhibition than an equatorial β-CH_3 group (Table III, compounds 4 and 5). Finally, the presence of an α-epoxide ring at C-16,17 in the pregnane series totally abolishes inhibition, but a C-17α-OH group has a much less drastic effect upon inhibition (Table II).

Interference with inhibition is not confined to axial groups on the α side. Introduction of an 11α-ol group completely prevents inhibition, although this group is equatorial. Thus, 10 μM 4-androstene-3,17-dione gives 18% inhibition whereas 10 μM 11α-hydroxy-4-androstene-3,17-dione does not inhibit at all; the same concentration of the 11β-ol gives 5% inhibition (Table I). Interestingly, at C-11 a β-OH group interferes more with inhibition than a keto group, as seen by comparing the K_i values for 5α-androstan-17-one (0.296 μM), its 11-keto analog (2.28 μM) and the corresponding 11β-ol (53.2 μM) (Table VIII). An 11-keto group also interferes with inhibition in the pregnane series (*cf.* compounds 2 and 9, Table II). The equatorial bromine in the 2α-bromo-5α-androstane-3,17-dione has virtually no effect on inhibition (Table I). Models show that this bulky atom is completely without effect on steroid planarity.

The introduction of double bonds causes a partial loss of the chair configuration of the affected ring, thus diminishing planarity of the steroid. Some illustrations taken from Table VIII, show that this effect is quite serious when it occurs in the B ring (3β-hydroxy-5α-androstan-17-one, K_i = 3.56 μM and 3β-hydroxy-5-androsten-17-one, K_i = 18.5 μM; 3β-hydroxy-5α-pregnan-20-one, K_i = 0.568 μM and 3β-hydroxy-5-pregnen-20-one, K_i = 5.41 μM); it is less serious in the A ring (5α-androstane-3,17-dione, K_i = 20.0 μM and 4-androstene-3,17-dione, K_i = 60.7 μM); and it is quite small in ring C (5α-androstan-17-one, K_i = 0.296 μM and 5α-androst-9-en-17-one, K_i = 0.525 μM).

One of the few substituents which causes an increase in inhibitory activity is a 4α-CH_3 group (Table I, compounds 11 and 23). Inspection of models reveals that the equatorial 4α-CH_3 group causes a planar extension of the A ring, possibly allowing the formation of an additional hydrophobic bond.

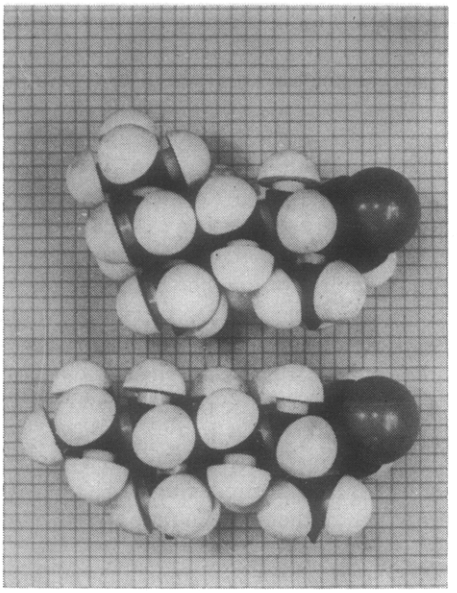


FIGURE 4: Models of 5β-androstan-17-one (top) and 5α-androstan-17-one (bottom), as seen edge-on from above, with A ring at left, D ring at right.

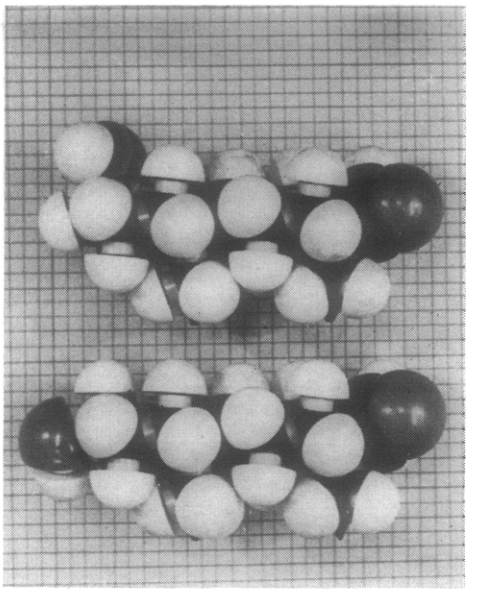


FIGURE 5: Models of 3α-hydroxy-5α-androstan-17-one (top) and 3β-hydroxy-5α-androstan-17-one (bottom) as seen edge-on from above with A ring at left, D ring at right.

These data emphasize the importance of the planar (α) side of the steroid in binding to the enzyme and suggest that hydrophobic bonding occurs between this planar surface and a corresponding, apolar region of the enzyme. Models of 5α-androstan-17-one reveal that 7 hydrophobic bonds might participate in binding. In addition a hydrogen bond could form between the C-17-keto group and a donor on the enzyme. Thus the calculated ΔG° of binding of -8.95 kcal/mole (Table VIII) can be reconciled with the principal forces postulated to operate in steroid–enzyme interaction. Of course, this merely suggests that the ΔG° is consistent with the type of bonding which could occur.

The 7α-CH_3 group interferes less with inhibition of saturated steroids (Table I, compounds 11 and 24; Table III, compounds 1 and 3) than Δ^5-steroids (Table I, compounds 15 and 25; Table III, compounds 4 and 5) or steroids with aromatic A rings (Table III, compounds 6 and 10). These differences can be reconciled, with the aid of models, by noting that the 7α-CH_3 group projects from the plane of the steroid less in saturated steroids than in Δ^5-steroids and steroids with an aromatic A ring.

Axial substituents on the nonplanar (β) side of the steroid also effect inhibition, although less than axial, α substituents. The C-19-CH_3 group actually appears to aid somewhat in binding, as seen by comparing the inhibition observed with compounds 2 and 4, Table III, and compounds 14 and 25, Table I, respectively. The 11β-OH group, however, causes substantial diminution of inhibition. The K_i of 5α-androstan-17-one is increased 180-fold by the presence of an 11β-OH group (Table VIII). Although the equatorial 3β-OH diminishes inhibition only moderately, the larger 3β-Cl group (Table I) interferes more with binding. In both the androstane and pregnane series, a compound containing a 3β-acetate group is much less inhibitory than one with the corresponding 3β-ol [80% inhibition reduced to 27% in the former (Table I), and 89% inhibition reduced to 22% in the latter (Table II)].

The presence of a keto group at C-3 or C-11 produces a much more marked loss of inhibition in androstanes than pregnanes. Especially noteworthy is the fact that the introduction of a 16α-bromo group completely abolishes inhibition in 3β-hydroxy-5-pregnen-20-one whereas it enhances inhibition in 3β-hydroxy-5α-androstan-17-one. These differences may arise from the fact that the freely rotating carbonyl group in the former permits more efficient hydrogen bonding, and that thereby the rest of the molecule is oriented slightly differently in the two classes of compounds. Since the carbonyl is free to rotate in the pregnane compound, a 16α-bromo group may also result in repulsion between the strongly electronegative Br atom and the electronegative carbonyl oxygen, displacing it from the orientation favored for hydrogen bonding. The important role which the C-21-CH_3 group may play in binding of the pregnane steroids was noted earlier. The enhanced inhibition of 3β-hydroxy-16α-bromo-5α-androstan-17-one may arise from an electronic interaction between the Br atom and a group on the protein. Although this compound is an α-halo ketone, an exhaustive study failed to yield any evidence for its irreversible inhibition of GlcPD.

Structural Requirements for Inhibition by Steroid Analogs. Support for the nature of steroid binding was sought by testing the effects of various steroid analogs containing an incomplete ring system. Cyclopentanone, analogous to the D ring with its 17-keto group, was found to be slightly inhibitory at a concentration 400,000 times greater than the K_i of 5α-androstan-17-one. The indandione derivative, which bears a ring system analogous to rings C plus D, was not inhibitory. This could be either because the concentrations used were too low to allow significant binding, or because the keto group on the six-membered ring cannot be accommodated in what is presumably a hydrophobic region of the enzyme. The hydrindane derivative, possessing a ring system analogous to rings B, C, and D, showed a slight inhibition

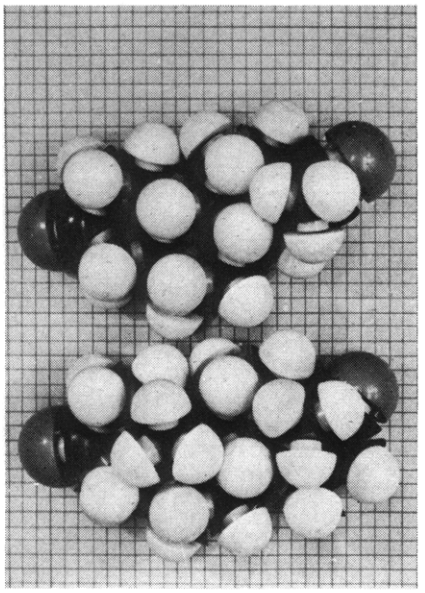


FIGURE 6: Models of ABC analog (top) and 5α-androstane-3,17-dione (bottom) as seen from front (nonplanar side) with A ring at left. Note almost identical positioning of oxygens of acetyl group (top) and C-17 carbonyl (bottom).

when tested at a concentration 1700 times greater than the K_i of 5α-androstan-17-one.

Since a 17- or 20-keto group is required for inhibition, it was not anticipated that compounds lacking the D ring would be inhibitory. Cyclohexanol and the two AB ring analogs were not inhibitory at the concentrations tested. However, the ABC ring analog gave significant inhibition at 0.5 and 1.0 mM (of the racemic mixture). Inspection of the model revealed that this compound bears a striking resemblance to 5α-androstane-3,17-dione (Figure 6). The acetyl group on ring C is readily positioned so that the oxygen is virtually superimposable over the C-17 oxygen of the steroid, and the methyl group is oriented similarly to either the C-18-CH_3 or the C-16-methylene carbon of the steroid. Thus, although it appears from the formula that this analog lacks the two angular CH_3 groups (C-18 and C-19) and carbon 15 of ring D, it may also be considered as functionally lacking the C-19-CH_3 group and carbon atoms 15 and 16 of ring D. On the assumption that only the *d* isomer is inhibitory and that the *l* isomer is not bound, a K_i value of 334 μM could be determined for this analog, and $\Delta G°$ of binding calculated to be −4.75 kcal/mole (Table VIII). This latter value is approximately 1.7 kcal less negative than the $\Delta G°$ of binding calculated for 5α-androstane-3,17-dione. The C-19-CH_3 group, as noted above, does not appear to make a major contribution to binding. Thus, the C-15-methylene carbon plus either the C-18-CH_3 group or the C-16-methylene carbon account for about one fourth of the binding energy of 5α-androstane-3,17-dione. It can be concluded, therefore, that in the overall binding, except for the carbonyl group, ring D does not play an overriding role. This is in contrast to the binding of substrate by 3α-hydroxysteroid dehydrogenase from *P. testosteroni*, in which carbon atoms 15 and 16 play a critical role in rate enhancement and are essentially entirely responsible for the stereospecificity which enables the enzyme to select the *d* steroid over the *l* steroid (Ringold *et al.*, 1966).

Pairs of steroid analogs were tested to determine whether any combination could produce greater than additive inhibition. All these attempts gave negative results. These data are in marked contrast to those of Inagami and Murachi (1964) who found that the rate of hydrolysis of acetylglycine ethyl ester was enhanced in the presence of small alkylammonium ions. Their data are in accord with the activation of the catalytic site, upon binding of one portion of the substrate, α-*N*-acetyl-L-lysine ethyl ester, to the specificity-determining site of the enzyme, *i.e.*, they are in accord with Koshland's induced-fit theory (Koshland, 1958). No such interaction between the hydophobic portion of the steroid and the functional carbonyl group was detected in our studies. These results are thus consistent with the requirement of a rigid steroid ring system in order to place the functional C-17- or C-20-carbonyl group into its appropriate configuration with respect to the enzyme.

Competition by Noninhibitory Steroids. The data reported in Tables V and VI strongly suggest that steroids lacking the C-17-carbonyl, but satisfying the other structural requirements for binding, are bound to the site occupied by the steroid inhibitor, displacing it from the enzyme.

A puzzling aspect of these competition experiments is the observation that competition appears to be more effective at higher inhibitor concentration. We considered the possibility that two steroid binding sites might exist on the enzyme, possessing different affinities for the inhibitor, but not for the competitor. This situation could arise, for example, if only one of the two sites required a 17- or 20-keto group on the steroid for binding. Some support for a second steroid binding site was obtained when it was found that three steroids, most notably 5α-androstan-17-one, yielded biphasic Dixon plots when the steroid concentration was increased beyond 2 K_i. These findings are only suggestive, however, and other explanations for the biphasic Dixon plots, as well as for the paradoxical competition behavior, must be sought in future experiments. The possibility that the biphasic Dixon plots result from steroid micelle formation is considered unlikely for two reasons: first, such micelle formation was not detected during the light-scattering tests for solubility; and second, the change in slope occurred at 2.35 μM for 5α-androstan-17-one, yet for 3β-hydroxy-16α-bromo-5α-androstan-17-one which is 66 times more soluble than the former steroid, the slope changed at a lower concentration, 1.80 μM.

Two bicyclic compounds, analogous to steroid rings A plus B, and cyclohexanol, equivalent to ring A, were also tested for their capacity to interfere with 5α-androstan-17-one inhibition. They had no effect at high concentrations. These experiments give only an indirect measure of binding, of course, but they do suggest that these compounds are only weakly bound, if at all. Yet *dl-trans*-tetradecahydro-2-acetylphenanthren-7-one, which contains additional carbon atoms equivalent to carbons 11, 12, 13, and 14 plus part of ring D, is reasonably well bound with a K_i of 0.334 mM (assuming binding of the *d* isomer only). These data suggest the importance of ring C (and part of ring D) for binding. But the extremely low activity of cyclopentanone, the CD analog, and the BCD analog as inhibitors (Table IV) points to the fact that the A ring plays the predominant role in

binding. The low activities of the CD and BCD analogs may be due to the presence of the keto groups on the analogous rings C and B, respectively, as mentioned above. However, a more likely explanation is that the entire steroid ring system allows a cooperative effect of the several hydrophobic bonds which cannot be achieved if even one ring is missing. Marcus and Talalay (1955) invoked multiple hydrophobic interactions between the surface of the planar steroid and β-hydroxysteroid dehydrogenase to explain the high affinity and specificity exhibited by that enzyme.

The general picture which emerges from these studies is that inhibitory steroids are bound to a hydrophobic pocket in the enzyme which has the approximate dimensions of $15 \times 8 \times 6$ Å. One side of this pocket appears to be virtually planar under optimum conditions of binding, and to permit very little flexibility. Insufficient information is available on the shape of the opposite side of the pocket since steroids were not tested with bulky groups on their nonplanar (β) side. The fact that various structural alterations on this side of the steroid plane influence inhibition, however, does support the idea that the steroid is bound in a pocket and not just *via* one plane. At one end of this pocket, corresponding to the position of the C-17- or C-20-keto group of the bound steroid, there may be located an amino acid with a polar group which can donate a hydrogen for hydrogen bonding with this keto group. Alternately, the hydrogen may be donated by a group on one of the bound substrates. The tight binding of the most inhibitory steroids, such as 5α-androstan-17-one, may best be attributed to the cooperative hydrophobic bonding afforded by the entire condensed rings system.

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