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STEROID–PROTEIN INTERACTIONS—XXXVIII. INFLUENCE OF STEROID STRUCTURE ON AFFINITY TO THE PROGESTERONE-BINDING GLOBULIN*

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SUMMARY

The association constants, K_A, of the complexes of more than a hundred steroids with the progesterone-binding globulin (PBG) were determined by fluorescence quenching titration and competitive binding equilibrium dialysis. By comparison of the K_A values for individual steroids that differed only by one structural change, the effect of this change on the binding affinity and on the free energy of binding was assessed. The binding to PBG is provided predominantly by hydrophobic forces; hydrogen bonding also occurs, for example, at the 3-oxo group. The progesterone molecule appears to be in almost perfect contact with the binding site of PBG; introduction of most substituents decreases the binding affinity. Testosterone is more loosely associated with PBG, but the affinity constant approaches that of the progesterone complex when the 17β-hydroxy group is acetylated. Changing the steroid conformation to a more planar structure results in stronger binding; this is seen with 5α-pregnane-3,20-dione, with the 4,6-dienes, and with the 19-norsteroids. A synoptic interpretation of the effects of the various structural changes on binding affinity leads to a conceptual image of the binding site.

INTRODUCTION

The progesterone-binding globulin (PBG)¶ of the pregnant guinea pig was first discovered [2] when distinct differences from the corticosteroid-binding globulin were observed. It has proved to be well suited for an investigation of the physicochemical nature of a site to which a hydrophobic steroid is bound with high affinity. PBG has one binding site either for progesterone or for certain other steroids [3, 4, 5]. Fluorescence quenching studies [6] have shown that tryptophan is present in the binding site; chemical modification experiments [7, 8] have suggested that tryptophan, lysine, and tyrosine are involved in the interaction with progesterone. Affinity labeling studies are expected to provide further information on the amino acid residues at the binding site [9].

Another approach to discern the nature of the binding site of PBG is to determine the affinity constants, K_A, of a large number of steroids that differ from each other either by limited changes in structure or in steric arrangement. In this way, the positive or negative influence on binding by a given substituent can be assessed, and the effects of polarity, size, and of steric or conformational characteristics can be investigated. Since the relatively high binding affinities of many steroids to PBG imply a close association of ligand and protein [6], a complementary image of the most likely shape of the binding site can be deduced, and hydrophobic or hydrophilic bonding areas may be defined. Information may be derived on availability or limitation of space, on predominance of hydrogen-donor or acceptor groups in hydrogen bonding, and on other affinity-sensitive characteristics.

In the present study, the affinity constants of the PBG complexes with more than a hundred steroids of the pregnane and androstane series have been determined. An attempt is made to recognize the conditions for strong or weak interaction at the different locations in the steroid molecule. These findings and the deduced concept of the steroid binding site in PBG may eventually be correlated with chemical results on the nature and sequence of amino acid residues involved in the binding.

MATERIALS AND METHODS

PBG was prepared by published procedures [10] from pregnant guinea pig serum obtained from Grand Island Biologicals, Grand Island, NY. The steroids

* For the preceding paper in this series see reference [1].

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¶ Abbreviation used: PBG, progesterone-binding globulin.

used were taken from our collection obtained over the years from commercial sources or research laboratories*. After determination of the melting point, the steroids were either used without further purification, or they were recrystallized one or more times from ethanol, methanol, or acetone, with addition of water. Glass-redistilled deionized water was used throughout this study. Comparison of the concentration of steroid solutions derived from weighing on a microbalance with that determined by the ultraviolet absorbance measured with a Zeiss PMQ II spectrophotometer provided an additional check of purity; the molar absorptivities used are given in Table 1.

The methyl ester of β-3-oxobisnor-4-cholenic acid [11] was prepared essentially by the esterification procedure of Riegel *et al.*[12]. To 40 mg of the acid (from Steraloids, Inc.) dissolved in 5 ml methanol, 0.25 ml of acetylchloride was added and the reaction mixture was stirred overnight. Precipitation with water gave a crystalline product that was recrystallized three times from methanol and water, and once from acetone and water. The fine needles melted at 167–170° [11].

The fluorescence quenching titrations were performed according to the method of Stroupe *et al.*[6]. A stock solution of purified PBG was diluted with 10 mM Tris buffer of pH 7.5, containing 0.1 M KCl, to a binding site concentration of approximately 0.03 μM. For weakly binding steroids, PBG concentrations up to μM were used. The steroids were dissolved to a concentration of approximately 10 μM in 10% or 50% ethanol in water, depending on their solubility. For each titration, increments of 0.5, 1.0, or 5.0 μl of the steroid solution were added to 2.0 ml of the PBG solution; thorough mixing after each addition was essential. Fluorescence was measured at 23°C in an Aminco–Bowman spectrophotofluorometer equipped with a constant-temperature water circulating system. The wavelengths used for excitation and emission were 280 and 340 nm, respectively. The titration was extended sufficiently (to several times binding site concentration) to obtain a clearly linear baseline for extrapolation in the graphic determination of K_A [6]. The affinity constant was calculated according to $K_A = \alpha/(1-\alpha)^2 P$, where α is the ratio of quenching at the equivlalence point to total quenching, and P is the binding site concentration at the equivalence point.

For the determination of K_A of steroids that could not be measured by fluorescence quenching because of lack of a suitable chromophore or because of an extremely low binding affinity, the method of competitive binding equilibrium dialysis [13] was used, with the following modifications. The dialysis bag contained 2.0 ml of a PBG solution (approximately 5 μg/ml) in 50 mM phosphate buffer, pH 7.4; the buffer contained 1 mM EDTA, 20 μg streptomycin/ml, and 50,000 units penicillin/ml. Ten ml of this buffer plus [^{3}H]-progesterone (2 ng/ml) and the unlabeled test steroid were put into the dialysis flask. Triplicate dialysis runs were performed at 4° ± 0.2°C and three 0.5 ml aliquots from the inside solutions and three 1.0 ml aliquots from the outside solutions were counted with a Packard Tricarb liquid scintillation spectrometer (Model 3380). Whenever necessary, several different concentrations of the unlabeled steroids were used so that the decrease of bound over unbound steroid, Δ(B/U), was between 5 and 90% of the B/U value for radioactive progesterone alone [13].

RESULTS

Table 1 shows the association constants, K_A, of steroids derived from pregnane and androstane, as well as data on a few other steroids. The 19-norandrogens and 19-norprogesterone are listed with the C_{19} and C_{21} steroids, respectively. Average values of at least 4 determinations by fluorescence quenching titration, and of triplicate dialysis runs are given. The figures in parentheses indicate the range of values obtained. Stroupe *et al.*[6] have calculated a fiducial limit (for 99% confidence level) of about ±25% from 15 independent fluorescence quenching titrations of the PBG–progesterone complex. The range seen in some of the present determinations may exceed these limits in accordance with the smaller number of observations. A fiducial limit of ±25% for the K_A values results in a range of error of ±0.15 kcal/mol for the free energy of binding, $\Delta G°$.

Earlier we reported [6] that the fluorescence quenching titration method gives higher association constants than equilibrium dialysis. In a strict sense, therefore, only values obtained by the same method are comparable with respect to the influence of variations of steroid structure on binding affinity; this limitation is important when relatively small differences are compared. However, in the cases utilized for critical evaluation, the difference in K_A values of two complexes is one, two or more orders of magnitude, and leaves no doubt about its significance.

Affinity constants of some PBG–steroid complexes shown in Table 1 have been determined previously by competitive binding equilibrium dialysis [13]. Furthermore, relative binding affinities to PBG in pregnant guinea pig serum or plasma have been assayed for numerous steroids by use of Florisil [26] or charcoal [27, 28] for the separation of bound and unbound steroid.

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Table 1. Affinity constants, K_A, of PBG complexes wiith steroids, determined by fluorescence quenching titration or competitive binding equilibrium dialysis

No.	steroid	ϵ*	K_A† ($M^{-1} \times 10^{-8}$)
	I. Pregnanes		
	A. 1 Oxygen		
1	5α-Pregnan-3-one	NA	16.9 (10.7–22.4)‡
2	3,5-Pregnadien-20-one	20,400 [14]	0.23 (0.19–0.29)‡
3	β-3-Oxobisnor-4-cholenaldehyde	16,500 [11]	12.3 (10.4–14.0)
4	β-3-Oxobisnor-4-cholenic acid	NA	1.2 (1.1–1.3)‡
5	β-3-Oxobisnor-4-cholenic acid methyl ester	NA	5.8 (4.0–7.9)
	B. 2 Oxygens		
6	5α-Pregnane-3,20-dione	NA	21.0 (17·5–23.3)‡
7	5β-Pregnane-3,20-dione	NA	3.3 (3.1–3.5)‡
8	4-Pregnene-3,20-dione (progesterone)	16,700 [15]	18.5 (10.2–24.0)¶
8a	19-Norpregn-4-ene-3,20-dione (19-norprogesterone)	16,500	29 (23–35)
9	5-Pregnene-3,20-dione	NA	12.1 (11.5–12.6)‡
10	1,4-Pregnadiene-3,20-dione	16,150 [14]	7.8 (7.3–8.7)
11	4,6-Pregnadiene-3,20-dione	25,100 [16]	22.0 (16.3–25.4)
12	4,9(11)-Pregnadiene-3,20-dione	17,700 [15]	3.2 (2.7–3.9)
13	4,16-Pregnadiene-3,20-dione	24,100 [15]	5.8 (4.4–8.7)
14	2α-Methyl-4-pregnene-3,20-dione	NA	2.3 (1.7–3.2)
15	16α-Methyl-4-pregnene-3,20-dione	20,400 [14]	4.9 (4.5–5.4)
16	16β-Methyl-4-pregnene-3,20-dione	21,900 [14]	5.6 (4.2–6.2)
17	16α-Cyano-4-pregnene-3,20-dione	17,000 [14]	0.08 (0.07–0.09)‡
18	6,17-Dimethyl-4,6-pregnadiene-3,20-dione (medrogestone)	25,000 [17]	45.5 (37.5–54.2)
18a	17,21-Dimethyl-19-norpregna-4,9-diene-3,20-dione (R-5020)	NA	0.85 (0.7–1.0)
19	3α-Hydroxy-5β-pregnan-20-one	NA	0.44 (0.41–0.47)‡
20	3β-Hydroxy-5-pregnen-20-one	NA	0.83 (0.81–0.84)‡
21	3-Hydroxy-3,5-pregnadien-20-one-3-D-triacetylglucosiduronic acid methyl ester	NA	1.4 (1.3–1.7)‡
22	20β-Hydroxy-4-pregnen-3-one	17,000 [14]	4.2 (3.1–4.8)
23	17-Acetoxy-6α-methyl-4-pregnen-20-one (anagestone acetate)	NA	0.04 (0.02–0.06)‡
24	5β-Pregnane-3α,20α-diol	NA	0.03 (0.02–0.04)‡
	C. 3 Oxygens		
25	5β-Pregnane-3,12,20-trione	NA	0.03 (0.027–0.035)‡
26	4-Pregnene-3,11,20-trione	15,500 [14]	0.072 (0.060–0.080)
27	3,20-Dioxo-4-pregnen-21-al	NA	3.8 (3.2–5.1)
28	12α-Hydroxy-5β-pregnane-3,20-dione	NA	0.12 (0.11–0.13)‡
29	2α-Hydroxy-4-pregnene-3,20-dione	16,600 [14]	0.02 (0.015–0.027)‡
30	6α-Hydroxy-4-pregnene-3,20-dione	15,570 [14]	1.6 (1.2–2.2)
31	6β-Hydroxy-4-pregnene-3,20-dione	12,390 [14]	3.2 (2.1–4.4)
32	11α-Hydroxy-4-pregnene-3,20-dione	15,600 [15]	2.8 (2.4–3.1)
33	11β-Hydroxy-4-pregnene-3,20-dione	16,000 [15]	1.8 (1.6–2.2)
34	17-Hydroxy-4-pregnene-3,20-dione	16,500 [16]	1.5 (1.1–2.0)
35	17-Acetoxy-4-pregnene-3,20-dione	16,900 [18]	2.1 (1.2–2.5)
36	17-Caproxy-4-pregnene-3,20-dione	17,000 [14]	7.0 (5.6–8.1)
37	17-Hydroxy-6α-methyl-4-pregnene-3,20-dione (medroxyprogesterone)	15,500 [15]	5.9 (3.2–8.9)
38	17-Acetoxy-6α-methyl-4-pregnene-3,20-dione	16,000 [14]	6.6 (5.7–8.0)
39	17-Hydroxy-16α-methyl-4-pregnene-3,20-dione	16,600 [14]	0.032 (0.028–0.037)
40	17-Hydroxy-6-methyl-4,6-pregnadiene-3,20-dione (megestrol)	22,300 [15]	10.2 (9.0–11.2)
41	17-Acetoxy-6-methyl-4,6-pregnadiene-3,20-dione	21,800 [15]	2.0 (1.7–2.3)
42	6-Chloro-17-acetoxy-4,6-pregnadiene-3,20-dione (chlormadinone acetate)	22,700 [18]	2.1 (2.0–2.1)
43	6-Chloro-17-hydroxy-1α,2α-methylene-4,6-pregnadiene-3,20-dione (cyproterone)	NA	7.9 (6.3–8.8)
44	6-Chloro-17-acetoxy-1α,2α-methylene-4,6-pregnadiene-3,20-dione	17,280 [19]	5.4 (4.4–6.1)
45	6,11β-Dichloro-17-acetoxy-19-nor-4,6-pregnadiene-3,20-dione (GR 2/1159)	NA	3.2 (2.7–3.8)
46	21-Hydroxy-4-pregnene-3,20-dione (deoxycorticosterone)	16,300 [15]	8.6 (6.5–10.1)
47	Deoxycorticosterone acetate	16,400 [15]	5.5 (4.3–6.2)
48	Deoxycorticosterone hemisuccinate	NA	2.3 (2.0–2.6)
49	21-Hydroxy-2α-methyl-4-pregnene-3,20-dione	15,200 [15]	2.2 (1.9–3.1)
50	3-[3-Oxo-17β-hydroxy-4-androsten-17α-yl]-propionic acid γ-lactone (SC-5233)	17,000 [20]	4.1 (3.4–4.4)
51	3-[3-Oxo-17β-hydroxy-19-norandrost-4-en-17α-yl]-propionic acid γ-lactone (SC-8109)	NA	2.1 (2.0–2.1)‡
52	3-[3Oxo-7α-acetylthio-17β-hydroxy-4-androsten-17α-yl]-propionic acid γ-lactone (SC-9420, aldactone)	20,200 [18]	0.21 (0.18–0.23)‡
53	3-[3-Oxo-9α-fluoro-11β,17β-dihydroxy-4-androsten-17α-]-propionic acid γ-lactone (SC-9837)	16,100 [21]	0.21 (0.16–0.26)‡

Table 1 (*continued*)

No.	steroid	ϵ*	K_A† ($M^{-1} \times 10^{-8}$)
	D. 4 Oxygens		
54	11β-17-Dihydroxy-4-pregnene-3,20-dione	14,200 [14]	0.066 (0.050–0.080)
55	11α,21-Dihydroxy-4-pregnene-3,20-dione (epicorticosterone)	15,400 [15]	3.5 (2.8–4.2)
56	11β,21-Dihydroxy-4-pregnene-3,20-dione (corticosterone)	15,800 [15]	1.4 (0.7–2.2)
57	Corticosterone 21-acetate	16,000 [22]	0.078 (0.072–0.090)
58	16α,17-Dihydroxy-4-pregnene-3,20-dione	15,200 [14]	3.6 (3.1–4.3)
59	16α,17-Epoxy-4-pregnene-3,20-dione	NA	4.7 (4.0–5.3)
60	17,21-Dihydroxy-4-pregnene-3,20-dione	16,600 [14]	3.3 (2.6–4.1)
61	17-Hydroxy-21-acetoxy-4-pregnene-3,20-dione	17,400 [16]	4.5 (4.2–4.8)
62	17,21-Dihydroxy-1,4-pregnadiene-3,20-dione	15,900 [14]	0.11 (0.08–0.14)‡
	E. 5 Oxygens		
63	11β,17,21-Trihydroxy-4-pregnene-3,20-dione (cortisol)	15,900 [15]	0.022 (0.021–0.023)
64	2α-Methyl-11β,17,21-trihydroxy-4-pregnene-3,20-dione	15,300 [15]	0.002 (0.0015–0.0033)‡
65	6α-Methyl-11β,17,21-trihydroxy-1,4-pregnadiene-3,20-dione (medrol)	14,900 [17]	0.005 (0.004–0.006)‡
66	18,11-Hemiacetal of 11β,21-dihydroxy-3,20-dioxo-4-pregnen-18-al (aldosterone)	15,000 [23]	0.03 (0.021–0.031)‡
67	14α,17,21-Trihydroxy-4-pregnene-3,20-dione	NA	2.5 (2.3–2.8)
	II. Androstanes		
	A. 2 Oxygens		
68	4-Androstene-3,17-dione	16,300 [15]	2.3 (1.6–2.9)
69	(5α)-Androst-1-ene-3,17-dione	10,200 [16]	2.1 (1.8–2.4)
70	6α-Methyl-4-androstene-3,17-dione	15,800 [15]	9.9 (8.5–11.0)
71	6β-Methyl-4-androstene-3,17-dione	16,200 [15]	3.2 (2.6–3.6)
72	17β-Hydroxy-5α-androstan-3-one (dihydrotestosterone)	NA	2.1 (1.9–2.3)‡
73	17β-Acetoxy-5α-androstan-3-one	NA	10.4 (9.3–12.3)‡
74	17β-Hydroxy-(5α)-androst-1-ene-3-one	10,000 [16]	5.2 (5.0–5.6)
74a	17α-Hydroxy-4-androsten-3-one (epitestosterone)	15,900 [15]	0.11 (0.093–0.124)
75	17β-Hydroxy-4-androsten-3-one (testosterone)	15,900 [15]	2.9 (2.4–3.3)
76	Testosterone acetate	NA	9.2 (6.3–13.2)
77	Testosterone propionate	16,900 [16]	9.9 (8.2–11.6)
78	Testosterone hemisuccinate	NA	4.4 (3.5–5.4)
79	Testosterone benzoate	NA	12.0 (10.2–13.9)
80	17β-Hydroxy-19-norandrost-4-ene-3-one	16,200 [15]	8.5 (5.6–9.8)
81	17β-Hydroxy-2α-methyl-4-androsten-3-one	15,000 [15]	2.3 (2.0–2.7)
82	17β-Hydroxy-4-methyl-4-androsten-3-one	15,300 [15]	8.4 (7.5–10.6)
83	17β-Hydroxy-6α-methyl-4-androsten-3-one	14,900 [15]	8.5 (5.6–9.7)
84	17β-Hydroxy-6β-methyl-4-androsten-3-one	15,500 [15]	3.6 (2.3–4.8)
85	17β-Hydroxy-17α-methyl-4-androsten-3-one	16,000 [15]	3.7 (3.5–3.9)
86	17β-Hydroxy-17α-ethinyl-4-androsten-3-one	16,400 [14]	1.4 (1.1–1.7)
87	17β-Hydroxy-17α-ethinyl-19-norandrost-4-ene-3-one (norethindrone)	16,000 [18]	2.9 (1.5–4.0)
88	17β-Acetoxy-17α-ethinyl-19-norandrost-4-ene-3-one	17,200 [18]	2.6 (2.0–3.1)
89	17β-Hydroxy-17α-ethinyl-19-norandrost-5(10)-en-3-one (norethynodrel)	NA	2.9 (1.5–4.0)
90	17β-Hydroxy-4-butyl-4-androsten-3-one	15,000 [24]	5.3 (4.0–7.3)
91	17β-Hydroxy-6α,17α-dimethyl-4-androsten-3-one	15,400 [15]	13.0 (9.0–16.0)
92	17β-Hydroxy-6β,17α-dimethyl-4-androsten-3-one	16,400 [15]	4.1 (2.5–6.0)
	B. 3 Oxygens		
93	4-Androstene-3,11,17-trione (adrenosterone)	14,800 [15]	0.03 (0.01–0.04)‡
94	1,4-Androstadiene-3,11,17-trione	15,100 [25]	0.01 (0.002–0.027)‡
95	11α-Hydroxy-4-androstene-3,17-dione	14,800 [14]	1.3 (0.8–2.0)
96	11β-Hydroxy-4-androstene-3,17-dione	15,300 [14]	1.1 (0.4–1.6)
97	11β-Hydroxy-1,4-androstadiene-3,17-dione	14,500 [25]	0.02 (0.01–0.03)‡
98	11α,17β-Dihydroxy-17α-methyl-4-androsten-3-one	15,000 [15]	0.7 (0.5–0.8)
99	11β,17β-Dihydroxy-17α-methyl-4-androsten-3-one	15,600 [15]	1.5 (0.7–2.0)
	III. Estranes; other steroids		
100	Estrone	2,300 [14]	0.004 (0.003–0.004)‡
101	Estradiol	2,000 [14]	0.001 (0.0011–0.0016)‡
102	Estradiol-17-acetate	2,640 [16]	0.002 (0.0017–0.0021)‡
103	4-Cholesten-3-one	18,000 [16]	0.10 (0.073–0.15)

* Molar absorptivity; NA, not available or not applicable; literature references in brackets. † Determined by fluorescence quenching titration or when marked by ‡, by competitive binding equilibrium dialysis; range in parentheses. ¶ Average of 26 titrations.

DISCUSSION

The strength of interaction between a steroid molecule and a binding protein is controlled by a number of factors some of which have been reviewed previously [15]. For the protein, a specific sequence of amino acid residues with a given conformational arrangement constitutes the binding site. This component of the steroid–protein complex will not be discussed in the present report, except for certain features evolving from the recognition of the binding site as an image complementary to the steroid structure.

The results compiled in Table 1 are interpreted in terms of the following aspects of steroid structure: (1) Polarity: the influence of hydrophilic and of hydrophobic groups. (2) Spatial relations: steric hindrance and best fit. (3) Steroid conformation: relation to crystal structure.

* The steroid numbers used in this paper are those given in Table 1.

Influence of hydroxy groups on binding affinity

Progesterone binds to PBG with the high affinity of $K_A = 18.5 \times 10^8\ M^{-1}$. Introduction of almost any substituent results in a decrease of the association constant. This shows that the particular structure and conformation of this relatively hydrophobic steroid hormone, progesterone, provide an optimal fit to the binding site in PBG.

Table 2 shows the effect of hydroxylation of progesterone (steroid No. 8)* or other steroids on the apparent association constant, K_A, and the free energy of binding, $\Delta G°$, of the complexes with PBG. The last column indicates the contribution of the substituting group(s) to $\Delta G°$, calculated as $\delta\Delta G°$, i.e., the difference between the $\Delta G°$ values of the PBG complexes with a given steroid and with the corresponding hydroxysteroid. A positive sign for this $\delta\Delta G°$ means a decrease of K_A and a corresponding increase of the free energy of the complex.

Introduction of hydroxy groups into progesterone results, without exception, in a lowering of the bind-

Table 2. Introduction of hydrophilic groups

Section	No.	Steroid*	K_A ($M^{-1} \times 10^{-8}$)	$\Delta G°$† (kcal/mol)	$\delta\Delta G°$‡ (kcal/mol)
A	8	Progesterone	18.5	−12.55	—
	46	21-Hydroxy-	8.6	−12.11	+0.4
	34	17-Hydroxy	1.5	−11.08	+1.5
	32	11α-Hydroxy-	2.8	−11.45	+1.1
	33	11β-Hydroxy-	1.8	−11.19	+1.4
	30	6α-Hydroxy-	1.6	−11.12	+1.4
	31	6β-Hydroxy-	3.2	−11.52	+1.0
	29	2α-Hydroxy-	0.02	− 7.99	+4.6
	17	16α-Cyano-	0.08	− 8.75	+3.8
	60	17,21-Dihydroxy-	3.3	−11.54	+1.0
	55	11α,21-Dihydroxy-	3.5	−11.58	+1.0
	56	11β,21-Dihydroxy-	1.4	−11.04	+1.5
	54	11β,17-Dihydroxy-	0.066	− 9.24	+3.3
	58	16α,17-Dihydroxy-	3.6	−11.59	+1.0
	63	11β,17,21-Trihydroxy-	0.02	− 8.54	+4.0
	67	14α,17,21-Trihydroxy-	2.5	−11.38	+1.2
B	14	2α-Methylprogesterone	2.3	−11.33	—
	49	21-Hydroxy-	2.2	−11.30	0.0
	64	11β,17,21-Trihydroxy-	0.002	− 6.72	+4.6
C	15	16α-Methylprogesterone	4.9	−11.78	—
	39	17-Hydroxy-	0.032	− 8.81	+3.0
D	7	5β-Pregnane-3,20-dione	3.3	−10.80	—
	28	12α-Hydroxy-	0.12	− 8.98	+1.8
E	10	1,4-Pregnadiene-3,20-dione	6.1¶	−11.14	—
	62	17,21-Dihydroxy-	0.11	− 8.93	+2.2
F	68	Androstenedione	2.3	−11.33	—
	95	11α-Hydroxy-	1.3	−10.99	+0.3
	96	11β-Hydroxy-	1.1	−10.90	+0.4
G	85	17-Methyltestosterone	3.7	−11.61	—
	98	11α-Hydroxy-	0.7	−10.63	+1.0
	99	11β-Hydroxy-	1.5	−11.08	+0.5

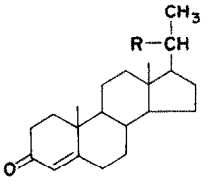
* Pertinent substitution only; for complete names see Table 1. † Although the second decimals are not significant (see experimental part), they are left for best values of $\delta\Delta G°$. ‡ Contribution of entering group(s) to free energy of binding. ¶ Determined by competitive binding equilibrium dialysis.

ing affinity to PBG, as evident from the positive $\delta\Delta G^\circ$ of the group contributions. However, the magnitude of this lowering depends on the location of the entering hydroxy group and on the presence of other groups in the steroid. For example, a hydroxyl at C-21 (Table 2A, steroid No. 46) increases ΔG° by 0.4 kcal/mol, whereas the increase of ΔG° by a 2α-hydroxy group (steroid No. 29) is more than 4 kcal/mol. Conversely, the $\delta\Delta G^\circ$ of an 11β-hydroxy group varies between 0.4 kcal/mol (Table 2F, No. 96) and 3.0 kcal/mol (steroid No. 63 vs 60). The effect of a 12α-hydroxy group is within this range (Table 2D). Accumulation of three hydroxy groups (11β, 17α, 21) in cortisol (Table 2A, No. 63) and in steroid No. 64 (Table 2B) decreases the binding affinity about a thousand fold.

The data in Table 2 demonstrate that the interaction between steroids and PBG follows the polarity rule indicating that the binding is predominantly of hydrophobic nature [15]. The examples also show that there is no simple "additive" effect of a hydroxy group on the free energy of binding; structure and conformation of the whole steroid molecule determine the free energy that a particular group contributes to the association complex.

The acetylation of the 17- and 21-hydroxy groups in the C_{21} series in most cases lowers the binding affinity to PBG or is without effect (Table 3). The decrease of K_A is substantial when either an 11β-hydroxy group (Table 3B, No. 57 vs 56) or the affinity-increasing 4,6-diene structure (steroid No. 41 vs 40) is also present. Hydrophobic and hydrophilic effects of the acyl groups are evident in steroids No. 36 and 48 (Table 3A). Spatial consequences of the acylation of the hydroxy group in testosterone will be discussed below.

The relatively low K_A value of the hemisuccinate of deoxycorticosterone (Table 3A, No. 48) is partly caused by the strong polarity of the carboxylate anion. A similar decrease of the affinity is seen when the 20β-hydroxy group in steroid No. 22 (structural formula **Ia**) is replaced by a 20β-carboxyl (**Ib**). Reducing this polarity (**Ib**) by formation of the 20β-carboxy methylester (**Ic**) or 20β-carboxaldehyde (**Id**) strengthens the complex with PBG. The distribution of the two oxo groups in steroid No. 3 (**Id**) and in progesterone differs only by the attachment at C-20.



I

	Steroid No.	R	K_A ($M^{-1} \times 10^{-8}$)	ΔG° (kcal/mol)
Ia	22	OH	4.2	−11.7
Ib	4	COOH	1.2	−11.0
Ic	5	$COOCH_3$	5.8	−11.9
Id	3	CHO	12.3	−12.3

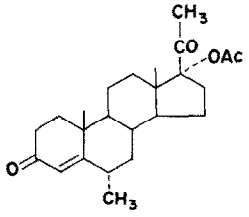
Table 3. Acylation of hydroxy groups

Section	No.	Hydroxyl or Acyl	Steroid*	K_A ($M^{-1} \times 10^{-8}$)	ΔG° (kcal/mol)	$\delta\Delta G^\circ$† (kcal/mol)
A	46	21-Hydroxy	progesterone	8.6	−12.11	—
	47	21-Acetoxy	progesterone	5.5	−11.84	+0.3
	48	21-Succinoxy	progesterone	2.3	−11.33	+0.8
	34	17-Hydroxy	progesterone	1.5	−11.08	—
	35	17-Acetoxy	progesterone	2.1	−11.28	−0.2
	36	17-Caproxy	progesterone	7.0	−11.99	−0.9
	37	17-Hydroxy	6α-methylprogesterone	5.9	−11.88	—
	38	17-Acetoxy	6α-methylprogesterone	6.6	−11.95	−0.1
B	60	21-Hydroxy	17-hydroxyprogesterone	3.3	−11.54	—
	61	21-Acetoxy	17-hydroxyprogesterone	4.5	−11.73	−0.2
	56	21-Hydroxy	11β-hydroxyprogesterone	1.4	−11.04	—
	57	21-Acetoxy	11β-hydroxyprogesterone	0.08	− 9.34	+1.7
	40	17-Hydroxy	6-methyl-4,6-pregnadiene-3,20-dione	10.2	−12.21	—
	41	17-Acetoxy	6-methyl-4,6-pregnadiene-3,20-dione	2.0	−11.25	+1.0
	43	17-Hydroxy	1α,2α-methylene-6-chloro-4,6-pregnadiene-3,20-dione	7.9	−12.06	—
	44	17-Acetoxy	1α,2α-methylene-6-chloro-4,6-pregnadiene-3,20-dione	5.4	−11.83	+0.2
	87	17β-Hydroxy	17α-ethinyl-19-norandrost-4-ene-3-one	2.9	−11.47	—
	88	17β-Acetoxy	17α-ethinyl-19-norandrost-4-ene-3-one	2.6	−11.40	+0.1

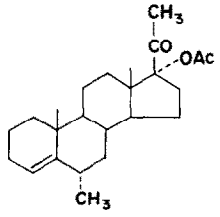
* For systematic names see Table 1. † Contribution of acylation to free energy of binding.

Influence of oxo groups on binding affinity

The 3-keto group in the progesterone structure is essential for the interaction with PBG. This follows from a comparison of the K_A and $\Delta G°$ values for 6α-methyl-17-acetoxyprogesterone (steroid No. 38, formula **II**) with those of the corresponding 3-deoxo compound (steroid No. 23, formula **III**). The contribution of the 3-oxo group to the free energy of binding in this example is 2.7 kcal/mol.



II Steroid No. 38
$K_a = 6{\cdot}6 \times 10^8\ M^{-1}$
$\Delta G° = -11{\cdot}1$ kcal/mol



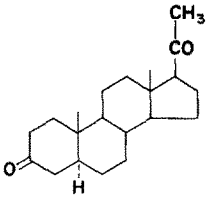
III Steroid No. 23
$K_a = 0{\cdot}04 \times 10^8\ M^{-1}$
$\Delta G° = -8{\cdot}4$ kcal/mol

A similar, if less rigid, indication for the importance of the 3-keto group is given by a comparison of the K_A and $\Delta G°$ values for 5-pregnene-3,20-dione (steroid No. 9, $K_A = 12.1 \times 10^8\ M^{-1}$, $\Delta G° = -11.5$ kcal/mol) and 3,5-pregnadien-20-one (steroid No. 2, $K_A = 0.23 \times 10^8\ M^{-1}$, $\Delta G° = -9.3$ kcal/mol).

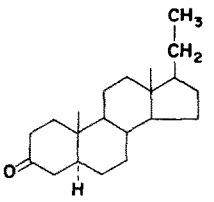
These results can be interpreted by the assumption of a hydrogen-donor group in the binding site which is located close to the 3-oxo group of the ligand.

In contrast to the oxo group at C-3, the 20-oxo group has little, if any, influence on the binding affinity. The K_A value of the PBG complex of the 20-deoxopregnane derivative V (steroid No. 1) is not significantly lower than that of the complex with the 3,20-dione IV (steroid No. 6); the $\Delta G°$ values of the two complexes are identical within the range of error.

Introduction of the oxo groups in other locations in the pregnane or androstane series results in a marked decrease of K_A, usually by 1–2 orders of magnitude (Table 4). The decrease is less marked in the 21-aldehyde (No. 27) than in the derivatives that have the oxo groups at the "hydrophilic edge" [29] of the steroid, i.e., at C-11 and C-12 (No. 25, 26, and 93);



IV Steroid No. 6
$K_a = 21{\cdot}0 \times 10^8\ M^{-1}$
$\Delta G° = -11{\cdot}8$ kcal/mol



V Steroid No. 1
$K_a = 16{\cdot}9 \times 10^8\ M^{-1}$
$\Delta G° = -11{\cdot}7$ kcal/mol

in these cases, the $\delta\Delta G°$ values are from +2.6 to 3.3 kcal/mol.

The importance of the 3-keto group for the binding affinity (see above, formulas **II** and **III**) follows also from the strong decrease of K_A upon reduction to a hydroxy group (Table 5A). Although the contribution of the 20-oxo group to $\Delta G°$ is about the same as that of $C(20)H_2$ (see above, formulas **IV** and **V**), reduction to a 20β-hydroxy derivative (Table 5A, No. 22) lowers the binding affinity significantly. If we assume hydrogen bonding in these interactions, the oxo group at C-3 or C-20 as hydrogen acceptor provides stronger association than the hydrogen donor hydroxyl.

In contrast to these effects at C-3 and C-20 in the pregnane series, reduction of the 17-oxo group of the androstene derivatives to 17β-hydroxyl does not result in a decrease of the binding affinity; the K_A values are unchanged or may even increase (Table 5B). Evidently, the 17β-hydroxy group maintains or strengthens the interaction given by the 17-oxo group. In contrast, reduction to the axial 17α-hydroxyl decreases the binding affinity drastically (Table 5B, No. 74a).

Whereas the oxo groups at C-3 or C-20 provide better affinity for PBG than the corresponding hydroxy groups, this situation is reversed at C-11, and to a lesser extent at C-21 (Table 5C). Reduction of the 11-oxo group to 11α- or 11β-hydroxyl in progesterone (Table 5C, No. 26, 32, 33) and in androstenedione (No. 93, 95, 96) decreases the $\Delta G°$ values markedly. As an overall interpretation of the interaction at the important "hydrophilic edge" at C-11, a

Table 4. Introduction of oxo groups

No.	Oxo-	Steroid*	K_A ($M^{-1} \times 10^{-8}$)	$\Delta G°$ (kcal/mol)	$\delta\Delta G°$† (kcal/mol)
8		Progesterone	18.5	−12.55	—
26	11-Oxo-	progesterone	0.072	− 9.29	+3.3
27	21-Oxo-	progesterone	3.8	−11.63	+0.9
7		5β-Pregnanedione	3.3	−10.80	—
25	12-Oxo-	5β-pregnanedione	0.03	− 8.21	+2.6
68		Androstenedione	2.3	−11.33	—
93	11-Oxo-	androstenedione	0.03	− 8.21	+3.1

* For systematic names see Table 1.
† Contribution of entering group to free energy of binding.

hydrophobic methylene structure provides a higher binding affinity than a hydroxy group (Table 2); however, if substitution by oxygen occurs, a hydroxy group as hydrogen donor interacts more firmly with PBG than an oxo group as hydrogen acceptor (Table 5C).

Influence of hydrophobic groups on binding affinity

Contrary to expectation for hydrophobic bonding, introduction of methyl groups into 3-oxo-4-pregnene derivatives generally decreases the binding affinity to PBG, as seen in the positive $\delta\Delta G^\circ$ values in Table 6A. This decrease of K_A by alkyl groups in a predominantly hydrophobic complex is interpreted as the result of steric hindrance: in the tightly fitting progesterone–PBG complex there is not enough space for the attachment of methyl groups. Testosterone forms a more loosely fitting complex with PBG, with a lower K_A value. Introduction of alkyl groups into the androgen structure mostly strengthens the association (Table 6B); enough space seems to be available to accommodate alkyl substituents even as large as *n*-butyl (steroid No. 90). A 17-ethinyl group in testosterone or in 19-nortestosterone decreases the binding affinity to PBG (steroid Nos. 86 and 87); the accumulation of π-electrons or steric reasons may be responsible for this effect.

In the androstane series, 6α-methylation without exception causes a greater increase of K_A than is observed for substitution in the 6β position (Table 6B, Nos. 70, 83, and 91 in comparison with the adjacent 6β-compounds). The 6α-methyl group, located in an unhindered equatorial position at the "hydrophobic edge" of the steroid molecule [29, p. 8], can thus provide a stronger hydrophobic interaction with PBG than the axial 6β-methyl group which is sterically hindered by the C-19 methyl group. The 6α-methyl group is the only one that strengthens the affinity to PBG in a progesterone derivative (Table 6A, No. 38). In contrast to these effects of 6α vs 6β-methyl, no difference in the decreasing effect on K_A is seen between the 16α and 16β methyl groups in progesterone (Table 6A, No. 15, 16), presumably because the hydrogens attached to C-16 of the five-membered D-ring are eclipsed, like those of cyclopentane, and the conformational distinction of axial and equatorial does not apply [30].

Table 6C shows that entrance of methyl groups at both C-6 (non-axial) and C-17 (axial) of 4,6-pregnadiene-3,20-dione increases K_A to the highest value observed for any steroid–PBG complex (steroid No. 18 vs 11). This 4,6-diene structure has a conformation which is highly favorable for binding to PBG (see below, p. 198). The high binding affinity of medrogestone (No. 18) for PBG has been observed previously [26, 31]. It is noteworthy that the highest affinity in the androgen series is also obtained by introducing both equatorial 6α-methyl and axial 17α-methyl groups (Table 6B, No. 91).

Table 5. Reduction of oxo to hydroxy groups

Section	No.	Steroid*	K_A ($M^{-1} \times 10^{-8}$)	ΔG° (kcal/mol)	$\delta\Delta G^\circ$† (kcal/mol)
A	7	5β-Pregnane-3,20-dione	3.3	−10.80	—
	19	3α-Hydroxy-5β-pregnan-20-one	0.44	− 9.69	+1.1
	24	3α,20α-Dihydroxy-5β-pregnane	0.03	− 8.21	+2.6
	9	5-Pregnene-3,20-dione	12.1	−11.52	—
	20	3β-Hydroxy-5-pregnen-20-one	0.83	−10.04	+1.5
	8	4-Pregnene-3,20-dione	18.5	−12.55	—
	22	20β-Hydroxy-4-pregnen-3-one	4.2	−11.68	+0.9
B	68	4-Androstene-3,17-dione	2.3	−11.33	—
	75	17β-Hydroxy-4-androsten-3-one	2.9	−11.47	−0.1
	74a	17α-Hydroxy-4-androsten-3-one	0.11	− 9.54	+1.8
	69	5α-Androst-1-ene-3,17-dione	2.1	−11.28	—
	74	17β-Hydroxy-5α-androst-1-en-3-one	5.2	−11.81	−0.5
	70	6α-Methyl-4-androstene-3,17-dione	9.9	−12.19	—
	83	17β-Hydroxy-6α-methyl-4-androsten-3-one	8.5	−12.10	−0.1
	71	6β-Methyl-4-androstene-3,17-dione	3.2	−11.52	—
	84	17β-Hydroxy-6β-methyl-4-androsten-3-one	3.6	−11.59	−0.1
C	26	11-Oxoprogesterone	0.072	− 9.29	—
	32	11α-Hydroxyprogesterone	2.8	−11.45	−2.2
	33	11β-Hydroxyprogesterone	1.8	−11.19	−1.9
	27	21-Oxoprogesterone	3.8	−11.63	—
	46	21-Hydroxyprogesterone	8.6	−12.11	−0.5
	93	11-Oxoandrostenedione	0.03	− 8.21	—
	95	11α-Hydroxyandrostenedione	1.3	−10.99	−2.8
	96	11β-Hydroxyandrostenedione	1.1	−10.90	−2.7
	94	11-Oxo-1,4-androstadiene-3,17-dione	0.01	− 7.61	—
	97	11β-Hydroxy-1,4-androstadiene-3,17-dione	0.02	− 7.99	−0.4

* For systematic names see Table 1. † Contribution of structural change to free energy of binding.

Spatial relations

Polarity is only one of the parameters that control the affinity of a steroid to a binding protein. Another factor is the spatial relationship of the liganded steroid to the three-dimensional structure of the binding site. Steric hindrance of a substituent may decrease the association constant, optimal contact and interaction of a group that narrowly fits into the "groove" in the binding site may increase it. In many cases, polar and steric effects may overlap.

An example for steric hindrance is the introduction of the equatorial 2α-methyl group in the pregnane series (Table 6A, No. 14, 49). The affinity constant of 2α-methylprogesterone is still 100 times greater than that of 2α-hydroxyprogesterone (Table 2A, No. 29), indicating that a hydrophobic alkyl group favors binding in comparison with the hydrophilic substituent. Steric hindrance is therefore the most likely explanation for the K_A-lowering influence of the 2α-methyl group.

In contrast, the effect of methyl substitution at C-6 of the androstanes (see above) indicates availability of space and hydrophobic contact; this is more marked in the equatorial 6α-position than in the axial 6β-position. The stronger influence of the 6α-position on K_A can also be concluded from a comparison of 6α- and 6β-hydroxyprogesterone (Table 2A, No. 30 and 31) with the greater weakening of the complex by the polar group in α-position.

The affinity-increasing effect of a space filling group is seen when the 17β-hydroxy group of androgenic hormones is acylated (Fig. 1). This increase is reflected in $\delta\Delta G°$ values of -0.7 to -0.9 kcal/mol in the acetoxy derivatives (steroids No. 73 and 76) versus the parent steroids No. 72 and 75; it is also seen in testosterone propionate (No. 77) and benzoate (No. 79). In the testosterone hemisuccinate (No. 78), the affinity-enhancing influence of the carbon chain is counteracted by the strong polarity of the carboxyl anion.

The structure of testosterone acetate differs from progesterone only by the insertion of an ester oxygen between the acetyl group and C-17; the attachment to C-17 is in both cases in the equatorial β-position. The molecular models show the close similarity in the spatial requirement of these two compounds, in

Table 6. Introduction of alkyl groups

Section	No.		Steroid*	K_A ($M^{-1} \times 10^{-8}$)	$\Delta G°$ (kcal/mol)	$\delta\Delta G°$† (kcal/mol)
A	8		Progesterone	18.5	−12.55	—
	14	2α-Methyl-	progesterone	2.3	−11.33	+1.2
	15	16α-Methyl-	progesterone	4.9	−11.78	+0.8
	16	16β-Methyl-	progesterone	5.6	−11.85	+0.7
	46		Deoxycorticosterone	8.6	−12.11	—
	49	2α-Methyl-	deoxycorticosterone	2.2	−11.30	+0.8
	34		17-Hydroxyprogesterone	1.5	−11.08	—
	37	6α-Methyl-	17-hydroxyprogesterone	5.9	−11.88	−0.8
	39	16α-Methyl-	17-hydroxyprogesterone	0.032	− 8.81	+2.3
	35		17-Acetoxyprogesterone	2.1	−11.28	—
	38	6α-Methyl-	17-acetoxyprogesterone	6.5	−11.95	−0.7
B	68		Androstenedione	2.3	−11.33	—
	70	6α-Methyl-	androstenedione	9.9	−12.19	−0.9
	71	6β-Methyl-	androstenedione	3.2	−11.52	−0.2
	75		Testosterone	2.9	−11.47	—
	81	2α-Methyl-	testosterone	2.3	−11.33	+0.1
	82	4-Methyl-	testosterone	8.4	−12.09	−0.6
	90	4-n-Butyl-	testosterone	5.3	−11.82	−0.4
	83	6α-Methyl-	testosterone	8.5	−12.10	−0.6
	84	6β-Methyl-	testosterone	3.6	−11.59	−0.1
	85	17-Methyl-	testosterone	3.7	−11.61	−0.1
	86	17-Ethinyl-	testosterone	1.4	−11.04	+0.3
	85		17-Methyltestosterone	3.7	−11.61	—
	91	6α-Methyl-	17-methyltestosterone	13.0	−12.35	−0.7
	92	6β-Methyl-	17-methyltestosterone	4.1	−11.67	−0.1
	80		19-Nortestosterone	8.5	−12.10	—
	87	17-Ethinyl-	19-nortestosterone	2.9	−11.47	+0.6
C	11		4,6-Pregnadiene-3,20-dione	22.0	−12.66	—
	18	6,17-Dimethyl-	4,6-pregnadiene-3,20-dione	45.5	−13.09	−0.4
	42		6-Chloro-17-acetoxy-4,6-pregnadiene-3,20-dione	2.1	−11.28	—
	44	1α,2α-Methylene-	6-chloro-17-acetoxy-4,6-pregnadiene-3,20-dione	5.4	−11.83	−0.6

* For systematic names see Table 1. † Contribution of entering group(s) to free energy of binding.

contrast to the three-dimensional structure of testosterone itself where the bulky C-17 side chain is missing (Fig. 1). If a C_2 side chain is already present at C-17β or C-17α, as in the pregnanes and the 17α-ethinyl-androstanes, acetylation of a C-17 hydroxyl group has no effect or decreases K_A (see Table 3, No. 35, 41, 44, 88), presumably by steric hindrance. Steric hindrance is also seen as the reason why the long paraffinic side chain at C-17β of cholestenone (steroid No. 103) decreases the binding affinity to PBG more than hundredfold compared to progesterone; lack of the 20-oxo group is not responsible for such decrease as discussed above (Formulas **IV** and **V**).

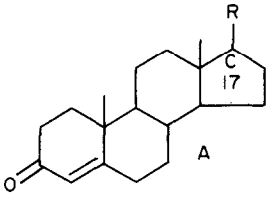
Steroid conformation: relationship to crystal structure

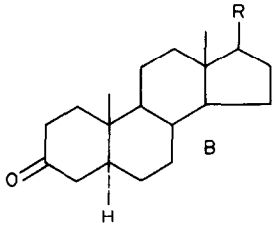
The discussion given so far illustrates the complexity of the factors controlling steroid affinity to PBG. Hydrophobicity or hydrophilicity, spatial requirements, optimal contact, and steric hindrance influence the K_A values. We have attempted to define distinct effects of certain groups in the steroid molecule by comparing pairs differing in only one parameter—however, in many cases we cannot distinguish between overlapping effects such as polarity and spatial requirements. Such distinction would be easier if we knew the exact conformation of the steroid molecule under study.

Filling a need expressed more than 10 years ago [32], Duax and Norton have published an Atlas of Steroid Structure [29] in which the crystallographic data of over 100 steroids are transformed to conformational structures and dimensional data which can be directly applied by a biochemist not expert in crystallography. Knowing the exact conformation of a pair of steroids that we compare in their binding to PBG would enable us to pinpoint more clearly the influence of structural changes on binding.

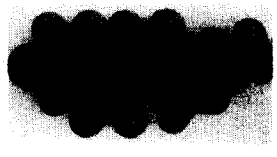
The validity of the application of crystallographic steroid conformation to the problem of structure and biological function, or more specifically of structure and protein binding, depends upon whether the conformational structure determined from X-ray crystallographic data is the same as that of the steroid in aqueous solution, and in contact with a binding protein. It has been stated that the influence of crystal packing on conformation is minimal, and that the conformations observed in the solid state closely approximate the most probable conformations regardless of environment. Steroid interaction with the immediate environment is considered highly controlled by intramolecular forces [ref. 29, p. 45].

One molecule of progesterone in the packed crystal occupies a space of about 5.2 × 6.3 × 13.8 Å or 448 Å^3 [ref. 29, p. 414] as can be approximated directly with Stuart-type molecular models [33]. In a

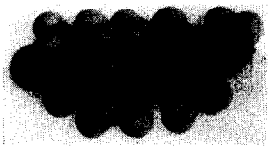




Testosterone



Testosterone Acetate



Progesterone

	No.	Structure	R	K_a $M^{-1} \times 10^{-8}$	$\Delta G°$ kcal/mol
Testosterone	75	A	-OH	2.9	-11.5
DHT°	72	B	-OH	2.1	-11.3
Testosterone					
-acetate	76	A	$-OCOCH_3$	9.2	-12.2
-propionate	77	A	$-OCOC_2H_5$	9.9	-12.2
-benzoate	79	A	$-OCOC_6H_5$	12.0	-12.3
-hemisuccinate	78	A	$OCOC_3H_5O_2$	4.4	-11.7
DHT acetate	73	B	$-OCOCH_3$	10.4	-12.2
Progesterone	8	A	$-COCH_3$	18.5	-12.6

°DHT = "dihydrotestosterone" or 17β-hydroxy-5α-androstan-3-one.

Fig. 1. Space-filling effect of acylation at C-17 of androgenic hormones.

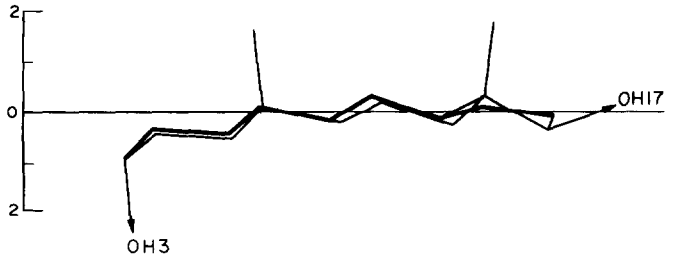


Fig. 2. Conformation of 5α-androstane-3α,17β-diol [ref. 29, p. 202].

pregnancy serum, containing about 12 μg total progesterone per 100 ml, each unbound progesterone molecule is surrounded, on the statistical average, by a solvent volume more than 500 million times its own. Any influence between progesterone molecules, therefore, can be virtually excluded. In contrast, there are indications that the crystal packing forces distort the steroid conformation, especially in head to tail packing*.

Realizing our lack of knowledge of the true steroid conformation in solution and of its possible difference from the conformation in the packed crystal, we are utilizing in this study the crystallographic data only for such major conformational aspects that appear to be caused by close-neighbor influences and are considered independent of the crystal packing forces.

In addition to the assumption of a given intrinsic steroid conformation controlled only by intramolecular forces and solvent interaction, we have to consider an influence of the steroid-protein association on the conformational structure of the steroid. This is a problem awaiting investigation.

Reduction of the double bond in progesterone leads to 5α- and 5β-pregnane-3,20-dione. The 5α-isomer (**IV**, steroid No. 6) has the same or a somewhat higher affinity for PBG [6, 34] than progesterone (Table 2A, No. 8). A similar relationship exists between testosterone and its 5α-dihydro derivative (Fig. 1, steroid Nos. 75 and 72), and their acetates (Fig. 1, steroid Nos. 76 and 73). The crystallographic analysis shows a close conformational similarity between the Δ^4-3-ketosteroids and their 5α-dihydro products [29]; the bowing of the A-ring appears slightly less in the 5α-dihydro series resulting in a somewhat more planar structure.

It has long been known that a large conformational difference exists between 5α-and 5β-steranes [30]. Competitive binding equilibrium dialysis shows that 5α-pregnane-3,20-dione (**IV**) has a 6 times greater affinity for PBG than the 5β-isomer (Table 5A, No. 7). In the absence of a crystallographic analysis for these two steroids, the conformational structure of the 5α- and 5β-series can be seen from the data for 5α-androstane-3α,17β-diol (Fig. 2) and 5β-androstane-3α,17β-diol (Fig. 3).

* The following randomly chosen examples are given for conformational variations in the crystalline state: (a) In crystallographically independent analyses of different crystal forms of testosterone, the distance between the angular methyl groups varied from 4.48 to 4.82 Å, and the orientation of oxygen O(3) was from 1.44 to 1.86 Å below the C(5)–C(17) plane [ref. 29, p. 257]. (b) In cortisone, the "bowing" of the A-ring relative to the remainder of the steroid (plane B–C–D) is −32.3°, making the distance of O(3) to the C(5)–C(17) plane about 2.5 Å; in cortisone-21-acetate, the corresponding values are −21.5° and 1.5 Å [ref. 29, pp. 464, 468]. It is not likely that intramolecular forces would produce such conformational changes at one end of the molecule if acetylation occurs at the other end. (c) In deoxycorticosterone, the bowing of the A-ring is −26.1°, the distance of O(3) to the C(5)–C(17) plane 2.1 Å. Entrance of a hydroxyl group at the opposite end of the molecule, at C-17, changes thes values to −18.9° and about 1.2 Å [ref. 29, pp. 436, 440]. It is more likely that this conformational difference results from intermolecular forces in crystal packing than from intramolecular effects. (d) The torsional angle C(13)–C(17)–C(20)–O(20) has a range from 75.2° to 115.8° in 31 independent observations on different steroids; it is 75.2° in 16β-bromo-3β, 17α-dihydroxy-5α-pregnane-11,20-dione. Acetylation of OH(3) at the opposite end of this molecule changes the angle from 75.2 to −4.9° [ref. 29, pp. 380, 383]. It is difficult to see how this conformational distortion can result from intramolecular forces.

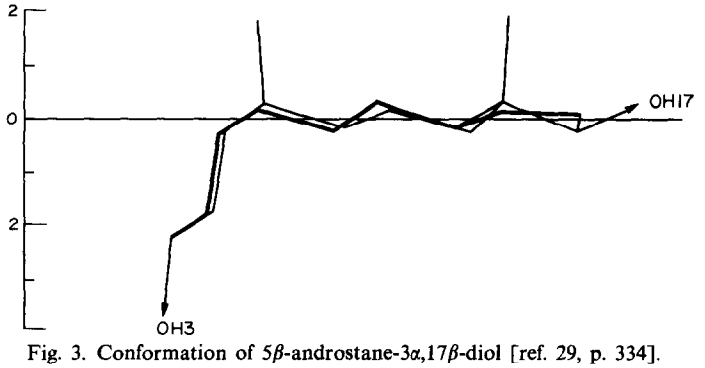


Fig. 3. Conformation of 5β-androstane-3α,17β-diol [ref. 29, p. 334].

Table 7. Introduction of double bonds

No.	Steroid	K_A ($M^{-1} \times 10^{-8}$)	ΔG° (kcal/mol)	$\delta\Delta G^\circ$* (kcal/mol)
8	4-Pregnene-3,20-dione	18.5	−12.55	—
10	1,4-Pregnadiene-3,20-dione	7.8	−12.05	+0.5
11	4,6-Pregnadiene-3,20-dione	22.0	−12.66	−0.1
37	17-Hydroxy-6α-methyl-4-pregnene-3,20-dione	5.9	−11.88	—
40	17-Hydroxy-6-methyl-4,6-pregnadiene-3,20-dione	10.2	−12.21	−0.3
60	17,21-Dihydroxy-4-pregnene-3,20-dione	3.3	−11.54	—
62	17,21-Dihydroxy-1,4-pregnadiene-3,20-dione	0.11	− 8.93	+2.6
96	11β-Hydroxy-4-androstene-3,17-dione	1.1	−10.90	—
97	11β-Hydroxy-1,4-androstadiene-3,17-dione	0.02	− 7.99	+2.9

* Contribution of structural change to free energy of binding.

The bowing of the A-ring relative to the B–C–D plane is −13.1° in Fig. 2 and −65.1° in Fig. 3. We conclude that the nearly planar 5α-pregnane structure provides better contact with the binding site in PBG than the bent 5β-structure, for example, between the essential C(3)=O group and a hydrogen donor in the protein. Greater binding affinity to PBG of 5α- than 5β-pregnane-3,20-dione has been reported previously [6, 13, 26, 27].

Crystallographic analysis shows that the introduction of a Δ^1-double bond into 17β-hydroxy-4-androsten-3-one causes bending of the A-ring [ref. 29, p. 326] similar to that seen in 5β-steranes; the bowing angle is −49.1°. This conformational change again results in a marked decrease of K_A as seen in Table 7 in three examples of $\Delta^{1,4}$-dienes.

In contrast, the 4,6-pregnadiene derivatives show the same or somewhat stronger binding to PBG than the Δ^4-analogs (Table 7, No. 11, 40). The conformational analysis of 4,6-pregnadienes [ref. 29, p. 551, structures PR43, 54, and 56] shows less bowing of the A-ring, and therefore a more planar structure than that of 4-pregnenes.

Removal of the axial 19-methyl group in progesterone and androst-4-en-3-ones results in greater binding affinity to PBG (Table 8). The flat conformation of the 19-nor structure [ref. 29, p. 58] again appears to provide a better fit to the binding site than the natural hormones. A higher binding affinity for PBG has been reported for 19-nortestosterone compared to testosterone [26, 27] as well as for 19-norprogesterone compared to progesterone [27].

Comparison of PBG with other progesterone-binding proteins

Table 9 shows the relative binding affinities of various steroids to PBG, to several progesterone receptors, and to rabbit uteroglobin. Each of these progesterone binding sites has a unique pattern of affinities for the steroids tested. One has to assume, therefore, that chemical make-up and binding site conformation in each of these proteins from five vertebrates are distinct. This is in contrast to a very low species specificity of vertebrate peptide hormones. Even in the same species, the guinea pig uterus receptor for progesterone when compared to PBG has clearly a lower binding affinity for hydroxyprogesterones, 5α-pregnane-3,20-dione, testosterone and other steroids.

One binding characteristic is common to all proteins listed in Table 9: they associate more strongly with a planar steroid molecule. This is seen in the tighter binding of 5α-pregnane-3,20-dione than of the 5β-isomer. The advantage of a planar steroid structure is also evident in the enhanced affinity of 19-norsteroids for the mammalian uterine receptors in Table 9. Similarly, binding of 1,4-pregnadiene-3,20-dione is weaker than that of progesterone. Introduction of hydroxy groups into progesterone reduces the binding affinity in all cases, except for binding of deoxycorticosterone to the chick oviduct receptor. Marked differences are seen in the binding of 17-acetoxyprogesterone. All these uterine receptors have a lower affinity for testosterone than exhibited by PBG. Other

Table 8. Removal of the 19-methyl group

No.	Steroid*	K_A ($M^{-1} \times 10^{-8}$)	ΔG° (kcal/mol)	$\delta\Delta G^\circ$† (kcal/mol)
8	Progesterone	18.5	−12.55	—
8a	19-Norprogesterone	29	−12.81	−0.3
75	Testosterone	2.9	−11.47	—
80	19-Nortestosterone	8.5	−12.10	−0.6
86	17β-Hydroxy-17α-ethinyl-4-androsten-3-one	1.4	−11.04	—
87	17β-Hydroxy-17α-ethinyl-19-norandrost-4-en-3-one	2.9	−11.47	−0.4
89	17β-Hydroxy-17α-ethinyl-19-norandrost-5(10)en-3-one	2.9	−11.47	−0.4

* For systematic names see Table 1. † Contribution of structural change to free energy of binding.

Table 9. Relative affinities of steroids to progesterone-binding proteins

No.	Steroid*	PBG	G.P. uterine recept.† [28]	Human uterine recept. [35]	Rabbit uterine recept. [28]	Sheep uterine recept. [28]	Chick oviduct recept. [35]	Rabbit utero-globin [36]
8	Progesterone	100	100	100	100	100	100	100
46	21-Hydroxyprogesterone	46	14	28	25	25	94	32
34	17-Hydroxyprogesterone	8	0.3	3	0.8	0.5	3	9
35	17-Acetoxyprogesterone	11	0.2	40	52	40	1	48
32	11α-Hydroxyprogesterone	15	0.8	1	2	0.7	—	6
33	11β-Hydroxyprogesterone	10	—	36	—	—	—	5
6	5α-Pregnane-3,20-dione	114	25	12	55	30	86	222
7	5β-Pregnane-3,20-dione	18	5	6	12	4	15	24
10	1,4-Pregnadiene-3,20-dione	42	44	52	20	16	30	—
11	4,6-Pregnadiene-3,20-dione	119	—	51	—	—	82	—
38	Medroxyprogesterone acetate	36	18‡	90	100	73	<1	—
41	Megestrol acetate	11	0.5	88¶	100	33	—	—
42	Chlormadinone acetate	11	0.8	50	146	60	<1	—
8a	19-Norprogesterone	156	115	168	258	135	89	40
75	Testosterone	16	1	2	0.6	1	5	7
80	19-Nortestosterone	46	40	22	10	10	2	3
87	Norethindrone	16	9	150	169	75	110	16

* For systematic names see Table 1. † Uterine receptor from non-pregnant guinea pig. ‡ Ref. [37]. ¶ Ref. [28].

characteristic differences in binding affinity and specificity may be seen in Table 9.

CONCLUSIONS

If we visualize the progesterone binding site in PBG as a folded polypeptide chain surrounding in some fashion the steroid molecule, the present results suggest the following characteristics for the binding site (Fig. 4).

(1) A hydrogen donor forms a hydrogen bond with the 3-oxo group which is essential for strong interaction; reducing this group to hydrogen lowers K_A more than 100-fold. Reduction of the carbonyl to hydroxyl decreases K_A by only about one order of magnitude.

(2) The binding of $C(2)H_2$ of pregnane compounds to a hydrophobic region of PBG is tight, so that introduction of a methyl group lowers K_A 4–8 times, presumably by steric hindrance. The contact region is hydrophobic because a hydroxyl at C-2 reduces K_A by three orders of magnitude. The smaller molecule of testosterone does not show the steric hindrance effect of a 2-methyl group; presumably, it fits more loosely into the binding groove.

(3) Removal of the C-10 methyl group increases K_A indicating tight hydrophobic bonding which is favored by a planar steroid structure.

(4) The hydrophobic bonding to $C(11)H_2$ and $C(12)H_2$ is greatly disturbed by an oxo group at these positions, decreasing K_A about 100-fold. However, the higher binding affinity of the 11α- and 11β-hydroxy

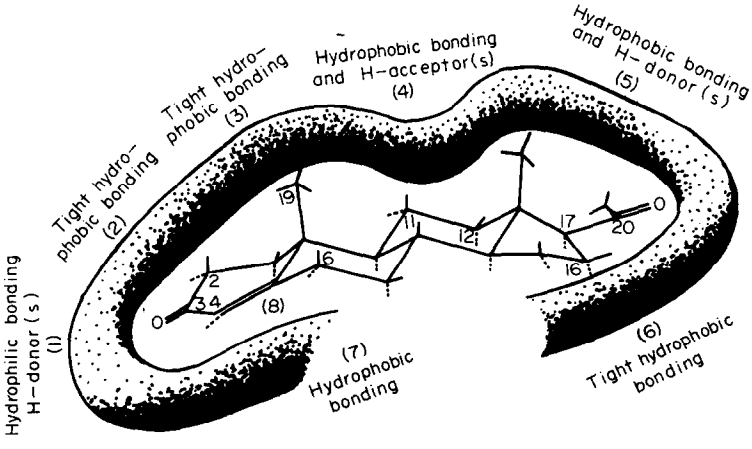


Fig. 4. Suggested characteristics of the steroid binding site in PBG. Schematic; the binding groove may be formed by more than one segment of the polypeptide chain.

derivatives suggests H-bonding to hydrogen acceptors at this region of PBG.

(5) Excellent fit into the binding groove is given by the C(13)–C(17)–C(16) edge and the C-17 side chain of progesterone. The binding is essentially hydrophobic; introduction of hydroxyl at C-17α results always in a decrease of K_A. Hydrophobic and hydrophilic bonding seems to be involved at the side chain: the 20-deoxo derivative has the same K_A as the 20-oxo compound. Hydrogen bonding may be provided by H-donors in the protein since reduction of the 20-oxo group to hydroxyl increases $\delta\Delta G^\circ$ significantly.

Since the testosterone molecule is smaller than progesterone (Fig. 1), the androgen structure does not have optimal contact with the binding site in PBG; this results in a lower K_A. However, if the 17β-substituent of testosterone is enlarged by addition of acetyl or other acyls, the binding affinity increases and approaches that of progesterone.

(6) The binding to C-16 is comparable to interaction at C-2; a 16-methyl group decreases K_A, but an electrophilic cyano group decreases K_A much more.

(7) Introduction of a methyl group at C-6α increases K_A in the pregnane series; this effect is more marked with the androgens. The hydrophobic bonding is stronger to the equatorial 6α-CH_3 than to the axial 6β-CH_3. The greater involvement of the equatorial 6α-positions follows also from the interaction of PBG with the 6-hydroxy compounds: the 6α-hydroxyl weakens K_A more than the 6β-hydroxy group does. Increase of K_A by hydrophobic bonding of alkyl is also apparent at C-4 of testosterone.

(8) The bending of the pregnane structure seen in the 5β-pregnane derivatives (Fig. 3) results in diminished contact, possibly of the 3-oxo group with the H-donor(s) of PBG, and is accompanied by a decrease of K_A. Flattening the steroid structure by lifting the A-ring relative to the B–C–D plane, as seen in 5α-pregnane-3,20-dione, the 4,6-pregnadiene compounds, and the 19-norsteroids, provides better contact and increases the affinity constant.

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