Seo, H., Vassart, G., Brocas, H., & Refetoff, S. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 2054–2058.
Silpananta, P., & Goodridge, A. G. (1971) *J. Biol. Chem. 246*, 5754–5761.
Sonenshein, G. E., & Brawerman, G. (1976) *Biochemistry 15*, 5501–5506.
Sonenshein, G. E., & Brawerman, G. (1977) *Biochemistry 16*, 5445–5448.
Sun, J. D., & Holton, D. (1978) *J. Biol. Chem. 253*, 6832–6836.
Tarentino, A. L., Richert, D. A., & Westerfield, W. W. (1966) *Biochim. Biophys. Acta 124*, 295–309.
Tepperman, H. M., & Tepperman, J. (1964) *Am. J. Physiol. 206*, 357–361.
Towle, H. C., Dillmann, W. H., & Oppenheimer, J. H. (1979) *J. Biol. Chem. 254*, 2250–2257.
Tsai, S. Y., Tsai, M. J., Schwartz, R., Kalimi, M., Clark, J. H., & O'Malley, B. W. (1975) *Proc. Natl. Acad. Sci. U.S.A. 72*, 4228–4232.
Wise, E. M., & Ball, E. G. (1964) *Proc. Natl. Acad. Sci. U.S.A. 52*, 1255–1263.
Yeung, K. K., & Carrico, R. J. (1976) *Anal. Biochem. 74*, 369–375.

Steroid–Protein Interactions. Influence of Steroid Structure and Temperature on the Binding of Steroids to Guinea Pig Corticosteroid-Binding Globulin†

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ABSTRACT: To better understand the nature of the interactions between steroids and proteins, we have analyzed the thermodynamic and structural requirements of steroid binding by the corticosteroid-binding globulin purified from pregnant guinea pig serum. The affinity constant of the protein–cortisol complex is inversely related to temperature. At 4 °C the association of cortisol is enthalpy and entropy driven, whereas at 37 °C enthalpy is the driving force, $\Delta S°$ being negative. By comparison of the K_a values determined by equilibrium dialysis for individual steroids that differ in only one structural change, the effect of this change on the binding affinity can be assessed. The steroid-binding site appears to be best adapted to bind cortisol; alteration of this structure invariably decreases the affinity. The free energy contributions by individual substituents are approximately additive in determining the total free energy of binding. For example, the low affinity of progesterone relative to cortisol for guinea pig corticosteroid-binding globulin results from the absence of the three hydroxyls at C-11, C-17, and C-21. The total free energy contribution of the three hydroxyls, calculated as the sum of their individual contributions, is −1.6 kcal/mol, a value which is also found experimentally as the difference in the binding energy between the cortisol and progesterone complexes. Important structures for optimal binding are the 3- and 20-oxo groups, a double bond at the 4 position, a 19-methyl group, and an 11β-hydroxy group. In contrast to the steroid-binding site of the guinea pig progesterone-binding globulin, which is predominantly hydrophobic in nature, the binding site of guinea pig corticosteroid-binding globulin apparently contains several hydrophilic groups capable of forming hydrogen bonds. Half of the free energy of binding at 4 °C can be attributed to the three hydroxy and the two oxo groups of cortisol. Based on the evaluation of the binding constants of 45 steroids, a model for the steroid binding site of guinea pig CBG is proposed.

The corticosteroid-binding globulin (CBG)[1] of the guinea pig exists at a relatively high concentration during late pregnancy and can be isolated from serum in pure form with a high yield (Mickelson & Westphal, 1979a). Guinea pig CBG has an affinity constant for cortisol that is ~20 times larger than that for progesterone. Human CBG, in contrast, binds both cortisol and progesterone with similar affinity (Stroupe et al., 1978). In the present study, the affinity constants of guinea pig CBG complexes with a number of steroids have been determined in order to define the conditions producing strong or weak interaction at various locations in the steroid molecule. As a result, a complementary image of the binding site may be deduced. The influence of temperature on the binding activity was also investigated. The information derived from this study will serve as a prelude to a systematic investigation of the nature of the steroid binding site using chemical modification techniques. Recently, the guinea pig CBG–cortisol complex prepared in our laboratory has been crystallized and X-ray crystallographic analysis has been initiated.[2]

Materials and Methods

Pooled serum from guinea pigs in the last 7–14 days of pregnancy was obtained from Dutchland Laboratory Animals,

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[1] Abbreviations used: CBG, corticosteroid-binding globulin or transcortin; K_a, equilibrium association constant; PBG, progesterone-binding globulin.

[2] Personal communication from Dr. Alex McPherson, Jr., University of California, Riverside.

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Inc., Denver, PA, and from Grand Island Biologicals, Grand Island, NY, and stored at −80 °C. CBG was purified by affinity chromatography, hydroxylapatite, and gel filtration as previously described (Mickelson & Westphal, 1979a). The steroids used were from our collection obtained over the years from commercial sources or research laboratories as previously acknowledged (Blanford et al., 1978). Steroid solutions were prepared and concentrations were verified by using the molar absorptivities as previously published (Blanford et al., 1978). [1,2-^{3}H]Cortisol and [1,2-^{3}H] progesterone were from New England Nuclear; their radiopurity was checked by thin-layer chromatography (Randerath, 1966) and found to be at least 95–98% by using a Packard radiochromatogram scanner. All chemicals were reagent grade. Distilled deionized water was used throughout.

Equilibrium dialysis was performed essentially as previously described (Westphal, 1969) in order to determine the affinity constants for the binding of radioactive steroids to CBG. Two milliliters of a CBG solution was the inside volume, and 20 mL of 50 mM phosphate buffer (pH 7.4) containing 0.02% sodium azide was the outside volume. The dialysis flasks were shaken for 48 h at 4 °C or at the specified temperature. Triplicate samples of the inside and outside solutions were taken for scintillation counting, and the triplicate values were averaged. Scatchard plots consisting of eight points were analyzed by a computer program which gave a least-squares fit of the data.

The apparent binding affinity of radioinert steroids was determined by a competitive displacement method previously described (Mickelson & Pêtra, 1975, 1978). An apparent dissociation constant of radioactive cortisol to CBG was determined in the presence of a constant amount of radioinert steroid. The inhibition constant (K_i), which in this case is equal to the equilibrium dissociation constant, can be determined from the relationship (Dixon & Webb, 1964)

$$K_p = K_d\left(1 + \frac{[I]}{K_i}\right)$$

where K_p = the apparent equilibrium constant of dissociation in the presence of competitive inhibitor, K_d = the equilibrium constant of dissociation in the absence of competitor, [I] = concentration of competitive inhibitor, and K_i = the equilibrium dissociation constant of the competitive inhibitor. At least three different concentrations of competitive steroid giving a 20–80% increase in the apparent dissociation constant were used for calculating an average K_i. All steroids examined obeyed the criterion for competitive inhibition of an unaltered number of binding sites in the presence of competitive steroid, within experimental error (±5%).

Results

Influence of Temperature. Temperature has a dramatic effect on the binding of cortisol to guinea pig CBG. Scatchard analyses of equilibrium dialysis experiments showed that the association constant decreased about 17-fold from 4 to 37 °C while the number of binding sites remained the same. The temperature dependence of the CBG–cortisol complex from 4 to 41 °C is given as a van't Hoff plot in Figure 1. The complex exhibits a nonlinear, biphasic temperature dependence over the range examined with the break occurring around 24 °C. The increasing slope with increasing temperature indicates that the enthalpy ($\Delta H°$) of the complex decreases as the temperature exceeds 24 °C. The entropy ($\Delta S°$) of the complex was calculated from the Gibbs–Helmholtz equation: $\Delta S° = (\Delta H° - \Delta G°)/T$. The free energy of binding, $\Delta G°$, of the

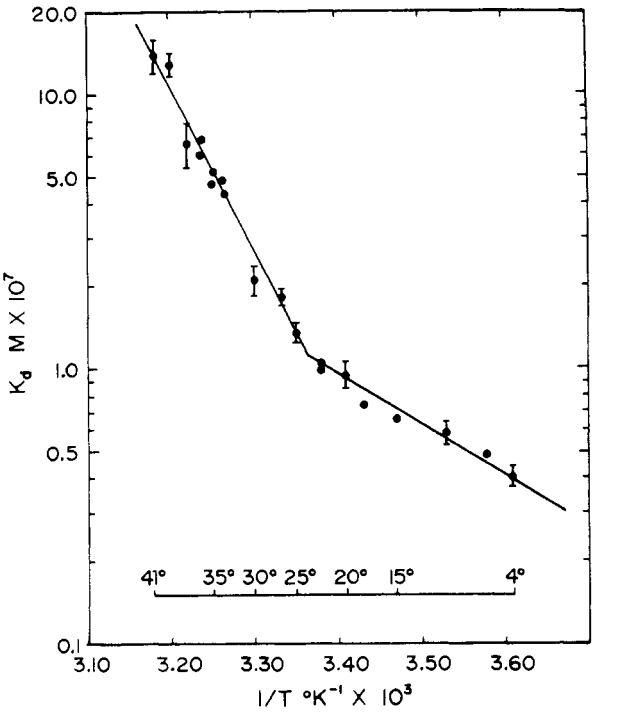

FIGURE 1: van't Hoff plot for thhe guinea pig CBG–cortisol complex. Each point is the average of two to five determinations of the dissociation constant by equilibrium dialysis at pH 7.4. The enthalpy ($\Delta H°$) of the complex is equal to the slope of the line times the molar gas constant.

Table I: Thermodynamic Parameters of the Guinea Pig CBG–Cortisol Complex

parameter	value	parameter	value
$\Delta G°$ (4 °C)	−9.4 kcal/mol	$\Delta G°$ (37 °C)	−8.8 kcal/mol
$\Delta H°$ (4–24 °C)	−7.6 kcal/mol	$\Delta H°$ (24–41 °C)	−28.4 kcal/mol
$\Delta S°$ (4 °C)	+6 eu	$\Delta S°$ (37 °C)	−63 eu
$T\Delta S°$ (4 °C)	+1.8 kcal/mol	$T\Delta S°$ (37 °C)	−19.6 kcal/mol

complex was calculated according to the relationship $\Delta G° = -RT \ln K_a$. Table I shows certain thermodynamic parameters for guinea pig CBG. Since $\Delta G°$ is negative at all temperatures examined, the binding process occurs spontaneously; the complex is at a thermodynamically lower energy state. At 4 °C the binding of steroid to protein is driven by both the entropy and enthalpy (Table I). Complex formation at 37 °C, however, is enthalpy driven. In fact, the enthalpy of binding compensates for the large, unfavorable, entropy value.

Steroid-Binding Specificity. The apparent association constants of 45 steroids were determined as inhibition constants by competitive equilibrium dialysis (Table II). This method, in contrast to other inhibition experiments, yields quantitative results. In essence two equilibrium dialysis experiments of cortisol binding to CBG are performed, one in the presence of the competing steroid and the other in its absence. The effect on the binding constant of cortisol to CBG in the presence of competing steroids depends upon the binding constant of the competing steroid relative to cortisol. Figure 2 represents a competitive equilibrium dialysis experiment for the determination of the binding constant of progesterone to CBG. As required for competitive inhibition, both lines intersect at the same point on the abscissa, indicating the same number of binding sites. An affinity constant value of 1.2×10^6 M^{-1} for the progesterone–CBG complex determined from Figure 2 agrees with the value obtained by direct equilibrium dialysis using radioactive progesterone (Table II, no. 29).[3]

Table II: Affinity Constants[a] of Guinea Pig CBG Complexes with Steroids Determined by Equilibrium Dialysis

no.	steroid	$K_a \pm SD^b$ (M^{-1}) × 10^{-7}	$-\Delta G^\circ$ (kcal/mol)
1	11β,17,21-trihydroxy-4-pregnene-3,20-dione (cortisol)	2.8 ± 0.3[c]	9.46
2	11β,17,21-trihydroxy-5β-pregnane-3,20-dione	0.05 ± 0.02	7.24
3	14α,17,21-trihydroxy-4-pregnene-3,20-dione	0.010 ± 0.003	6.35
4	11β,17-21-trihydroxy-1,4-pregnadiene-3,20-dione (prednisolone)	0.60 ± 0.06	8.61
5	11β,17,21-trihydroxy-2α-methyl-4-pregnene-3,20-dione (2α-methylcortisol)	0.9 ± 0.1	8.83
6	11β,17,21-trihydroxy-2α-methyl-9α-fluoro-4-pregnene-3,20-dione	0.0034 ± 0.0002	5.75
7	11β,17,21-trihydroxy-6α-methyl-1,4-pregnadiene-3,20-dione	0.036 ± 0.002	7.06
8	11β,17,21-trihydroxy-16α-methyl-4-pregnene-3,20-dione (16α-methylcortisol)	0.90 ± 0.09	8.83
9	21-acetoxy-11β,17-dihydroxy-4-pregnene-3,20-dione (cortisol 21-acetate)	0.93 ± 0.18	8.85
10	21-acetoxy-11β,17-dihydroxy-9α-chloro-4-pregnene-3,20-dione	0.005 ± 0.001	5.97
11	21-acetoxy-11β,17-dihydroxy-9α-fluoro-4-pregnene-3,20-dione	0.004[d]	5.84
12	17,21-dihydroxy-4-pregnene-3,11-20-trione (cortisone)	0.16 ± 0.04	7.88
13	17,21-dihydroxy-1,4-pregnadiene-3,11,20-trione (prednisone)	0.011 ± 0.002	6.40
14	11β,17-dihydroxy-4-pregnene-3,20-21-trione	1.0 ± 0.2	8.89
15	11β,17,20α,21-tetrahydroxy-4-pregnen-3-one	0.012 ± 0.007	6.45
16	11β,17,20β,21-tetrahydroxy-4-pregnen-3-one	0.008 ± 0.001	6.23
17	11β,17-dihydroxy-4-pregnene-3,20-dione	1.6 ± 0.3	9.15
18	11α,21-dihydroxy-4-pregnene-3,20-dione (epicorticosterone)	0.05 ± 0.01	7.24
19	11β,21-dihydroxy-4-pregnene-3,20-dione (corticosterone)	1.8 ± 0.2 (1.5)	9.21
20	17,21-dihydroxy-4-pregnene-3,20-dione (11-deoxycortisol)	0.38 ± 0.04 (0.42)	8.36
21	2α-hydroxy-4-pregnene-3,20-dione (2α-hydroxyprogesterone)	0.066 ± 0.002	7.39
22	6α-hydroxy-4-pregnene-3,20-dione (6α-hydroxyprogesterone)	0.0041 ± 0.0004	5.86
23	6β-hydroxy-4-pregnene-3,20-dione (6β-hydroxyprogesterone)	0.0015 ± 0.0005	5.30
24	11α-hydroxy-4-pregnene-3,20-dione (11α-hydroxyprogesterone)	0.03 ± 0.01	6.96
25	11β-hydroxy-4-pregnene-3,20-dione (11β-hydroxyprogesterone)	1.3 ± 0.4	9.03
26	17-hydroxy-4-pregnene-3,20-dione (17-hydroxyprogesterone)	0.16 ± 0.02	7.88
27	17-acetoxy-4-pregnene-3,20-dione	0.0014 ± 0.0005	5.27
28	21-hydroxy-4-pregnene-3,20-dione (deoxycorticosterone)	0.31 ± 0.06 (0.37)	8.24
29	4-pregnene-3,20-dione (progesterone)	0.16 ± 0.04 (0.11, 0.12)	7.88
30	5-pregnene-3,20-dione	0.016 ± 0.005	6.61
31	5α-pregnane-3,20-dione	0.06 ± 0.01	7.34
32	5β-pregnane-3,20-dione	0.04 ± 0.01	7.11
33	3β-hydroxy-5-pregnen-20-one (pregnenolone)	0.0009 ± 0.0004	5.02
34	3α-hydroxy-5β-pregnan-20-one	0.0019 ± 0.0005	5.43
35	4,6-pregnadiene-3,20-dione	0.11 ± 0.02	7.67
36	19-nor-4-pregnene-3,20-dione (19-norprogesterone)	0.007 ± 0.001	6.15
37	17-hydroxy-4-pregnen-3-one	0.006 ± 0.002	6.07
38	18,11-hemiacetal of 11β,21-dihydroxy-3,20-dioxo-4-pregnen-18-al (aldosterone)	0.003 ± 0.002	5.69
39	9α-fluoro-16α-methyl-11β,17,21-trihydroxy-1,4-pregnanediene-3,20-dione (dexamethasone)	0.0012 ± 0.0002	5.18
40	17,21-dimethyl-19-norpregna-4,9-diene-3,20-dione (R5020)	0.028 ± 0.003	6.92
41	17β-hydroxy-4-andosten-3-one (testosterone)	0.022 ± 0.001	6.78
42	17β-hydroxy-5α-androstan-3-one (dihydrotestosterone)	0.005 ± 0.001	5.97
43	11β,17β-dihydroxy-17α-methyl-4-androsten-3-one	0.009 ± 0.002	6.29
44	estradiol	<0.001[e]	
45	4-cholesten-3-one	0.0002	4.19

[a] The values given for steroids 1, 19, and 29 were determined by direct equilibrium dialysis using the radiolabeled steroids. The values in parentheses for steroids 19 and 29 were determined by competitive equilibrium dialysis. The values in parentheses for steroids 20 and 28 were obtained by direct equilibrium dialysis using the radiolabeled steroids. All other values were determined by competitive equilibrium dialysis using radiolabeled cortisol. [b] SD, standard deviation of at least three determinations using three different concentrations of competing steroid. [c] Average of 27 determinations; correlation coefficient for 27 determinations was −0.99 ± 0.01 (SD). [d] Average of two values (0.003 and 0.004). [e] No competition was obtained at a ratio of steroid/protein of 50:1.

Values obtained by direct equilibrium dialysis were also comparable to those obtained by competitive equilibrium dialysis (see Table II) for corticosterone (no. 19), 11-deoxycortisol (no. 20), and deoxycorticosterone (no. 28). All of the steroids examined yielded the same number of binding sites; therefore, all were competitive with cortisol for binding to CBG.

Table III shows that a 20-oxo group is essential for optimal affinity. Reduction of this group to either 20α (no. 15) or 20β (no. 16) lowers the affinity 230-fold and 350-fold, respectively. The importance of the 20-oxo group is also evident by comparing 17-hydroxyprogesterone (no. 26) with the 17-hydroxy-20-deoxopregnane derivative (no. 37). The K_a is reduced 27-fold by removal of the 20-oxo group which corresponds to a loss of 1.8 kcal/mol of free energy of binding.

[3] The numbers given in Table II will be used in this paper to identify individual steroids.

Table III also indicates that a 3-oxo group is important for optimal binding. Reduction of the 3-oxo group to either a 3α-hydroxyl (no. 34) or a 3β-hydroxyl (no. 33) results in a 20-fold reduction in K_a.

The importance of an 11β-hydroxy group is shown in Table IV. Either removal or oxidation of the 11β-hydroxy group significantly lowers the binding affinity. Introduction of an 11β-hydroxyl into progesterone results in an increase in K_a while an 11α-hydroxyl lowers the K_a significantly. Similarly, corticosterone binds to CBG with about a 6-fold higher K_a than deoxycorticosterone while epicorticosterone has a 36-fold lower K_a than corticosterone.

Table IV shows the lesser importance of the 17-hydroxy group. Removal of the 17-hydroxyl in cortisol or 11-deoxycortisol lowers the K_a only marginally. Moreover, introduction of a 17-hydroxy group in the progestins has little if any effect. However, introduction of the large acetoxy group at the 17

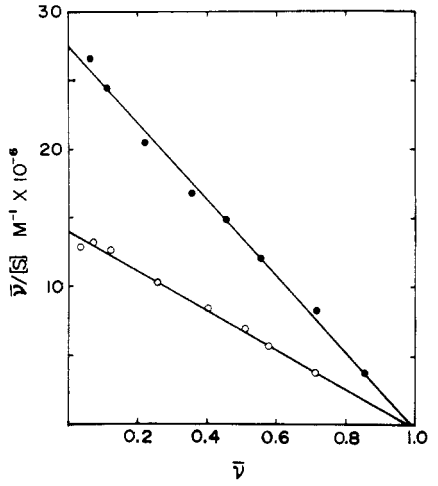

FIGURE 2: Determination of the equilibrium association constant of progesterone by the "competitive" equilibrium dialysis method. A Scatchard plot of data for equilibrium dialysis of guinea pig CBG (1.1×10^{-7} M) is shown in the presence of the radioactive cortisol (closed circles) and in the presence of radioactive cortisol plus 7.2×10^{-7} M radioinert progesterone (open circle).

Table III: Importance of the Oxo Group

no.[a]	steroid	K_a (M^{-1}) × 10^{-7}	$\delta \Delta G^\circ$ (kcal/mol)[b]
	3-Oxo Group		
32	5β-pregnanedione	0.04 ± 0.01	
34	3α-hydroxy-5β-pregnan-20-one	0.0019 ± 0.0005	+1.7
30	Δ^5-progesterone	0.016 ± 0.005	
33	pregnenolone	0.0009 ± 0.0004	+1.6
	20-Oxo Group		
1	cortisol	2.8 ± 0.3	
15	20α-hydroxycortisol	0.012 ± 0.007	+3.0
16	20β-hydroxycortisol	0.008 ± 0.001	+3.2
26	17-hydroxyprogesterone	0.16 ± 0.02	
37	17-hydroxy-4-pregnen-3-one	0.006 ± 0.002	+1.8

[a] The numbers in this and the following tables are taken from Table II. [b] Contribution of structural change to free energy of binding: $\delta \Delta G^\circ = \Delta G^\circ$ of reference steroid minus ΔG° of structurally altered steroid.

position decreases the binding more than 2 orders of magnitude (Table VI, no. 27).

The 21-hydroxy group is also of minor importance as shown in Table IV. Removal of the 21-hydroxyl of cortisol significantly lowers the binding affinity, but only by about 40%.

Introduction of either a 2α-hydroxy group (no. 21, Table IV) or a 2α-methyl group (no. 5, Table V) lowers the K_a about threefold. Introduction of either a 6α-hydroxyl (no. 22, Table IV) or a 6α-methyl group (no. 7, Table V) decreases the affinity 20- to 40-fold. The 6β-hydroxy group (no. 23, Table IV) lowers the binding affinity by 2 orders of magnitude. Introduction of a hydroxy group at the 14 position (no. 3, Table IV) lowers the K_a almost 40-fold while a methyl group at the 16 position (no. 8, Table V) lessens the affinity only 3-fold. Removal of the 19-methyl group results in a 20-fold reduction in K_a (no. 36, Table V).

Table VI shows that introduction of chlorine or fluorine at the 9 position greatly hinders binding. The K_a is reduced by more than 2 orders of magnitude. About 3 kcal/mol of free energy of binding is lost by this addition. Oxidation of the 21-hydroxyl of cortisol significantly lowers the binding, but only threefold (no. 14, Table VI). Similarly, replacing the 21-hydroxyl by the bulky acetoxy group reduces the binding affinity threefold (no. 9).

Table IV: Influence of Hydroxy Groups

no.	steroid	K_a (M^{-1}) × 10^{-7}	$\delta \Delta G^\circ$ (kcal/mol)
	11β-Hydroxy Group		
1	cortisol	2.8 ± 0.3	
20	11-deoxycortisol	0.38 ± 0.04	+1.1
12	cortisone	0.16 ± 0.04	+1.6
4	prednisolone	0.60 ± 0.06	
13	prednisone	0.011 ± 0.002	+2.2
28	deoxycorticosterone	0.31 ± 0.06	
19	corticosterone	1.8 ± 0.2	−1.0
18	epicorticosterone	0.05 ± 0.01	+1.0
29	progesterone	0.16 ± 0.04	
25	11β-hydroxyprogesterone	1.3 ± 0.4	−1.2
24	11α-hydroxyprogesterone	0.03 ± 0.01	+0.9
	17-Hydroxy Group		
1	cortisol	2.8 ± 0.3	
19	corticosterone	1.8 ± 0.2	+0.3
20	11-deoxycortisol	0.38 ± 0.04	
28	deoxycorticosterone	0.31 ± 0.06	+0.1
17	11β,17-dihydroxyprogesterone	1.6 ± 0.3	
25	11β-hydroxyprogesterone	1.3 ± 0.4	+0.1
29	progesterone	0.16 ± 0.04	
26	17-hydroxyprogesterone	0.16 ± 0.02	0.0
	21-Hydroxy Group		
1	cortisol	2.8 ± 0.3	
17	21-deoxycortisol	1.6 ± 0.3	+0.3
19	corticosterone	1.8 ± 0.2	
25	11β-hydroxyprogesterone	1.3 ± 0.4	+0.2
28	deoxycorticosterone	0.31 ± 0.06	
29	progesterone	0.16 ± 0.04	+0.4
	Other Hydroxy Groups		
20	11-deoxycortisol	0.38 ± 0.04	
3	14α-hydroxy-11-deoxycortisol	0.010 ± 0.003	+2.0
29	progesterone	0.16 ± 0.04	
21	2α-hydroxyprogesterone	0.066 ± 0.002	+0.5
22	6α-hydroxyprogesterone	0.0041 ± 0.0004	+2.0
23	6β-hydroxyprogesterone	0.0015 ± 0.0005	+2.6

Table V: Influence of Methyl Groups

no.	steroid	K_a (M^{-1}) × 10^{-7}	$\delta \Delta G^\circ$ (kcal/mol)
1	cortisol	2.8 ± 0.3	
5	2α-methylcortisol	0.9 ± 0.1	+0.6
8	16α-methylcortisol	0.90 ± 0.09	+0.6
4	prednisolone	0.60 ± 0.06	
7	6α-methylprednisolone	0.036 ± 0.002	+1.6
29	progesterone	0.16 ± 0.04	
36	19-norprogesterone	0.007 ± 0.001	+1.7

Reduction of the double bond at the 4 position to either the 5α or 5β derivative results in a reduction of the K_a (no. 31 and 32, Table VI). A double bond at the 5 position instead of the 4 position in progesterone results in a 10-fold decrease in binding (no. 30). Introduction of a double bond at the 1 position causes a significant decrease in K_a (no. 4 and 13) while introduction at the 6 position (no. 35) lowers the binding affinity only slightly.

Discussion

The binding of cortisol to guinea pig CBG was found to be dependent on temperature. The van't Hoff plot of the dissociation constant (Figure 1) was biphasic with a transition occurring at about 24 °C. A biphasic plot has also been observed for human CBG, with the transition at about 20 °C (Basset et al., 1977; Stroupe et al., 1978). Plotting the limited

Table VI: Influence of Additional Alterations

no.	steroid	K_a (M^{-1}) × 10^{-7}	$\delta\Delta G°$ (kcal/mol)
	9α-Halogens		
9	cortisol acetate	0.93 ± 0.18	
11	9α-fluorocortisol acetate	0.004	+3.0
10	9α-chlorocortisol acetate	0.005 ± 0.001	+2.9
5	2α-methylcortisol	0.9 ± 0.1	
6	9α-fluoro-2α-methylcortisol	0.0034 ± 0.0002	+3.1
	Other Substitutions		
26	17-hydroxyprogesterone	0.16 ± 0.02	
27	17-acetoxyprogesterone	0.0014 ± 0.0005	+2.6
1	cortisol	2.8 ± 0.3	
9	cortisol acetate	0.93 ± 0.18	+0.6
14	21-dehydrocortisol	1.0 ± 0.2	+0.6
	Double Bonds		
1	cortisol	2.8 ± 0.3	
2	5β-dihydrocortisol	0.05 ± 0.02	+2.2
4	prednisolone	0.60 ± 0.06	+0.9
29	progesterone	0.16 ± 0.04	
31	5α-pregnanedione	0.06 ± 0.01	+0.5
32	5β-pregnanedione	0.04 ± 0.01	+0.8
30	Δ^5-progesterone	0.016 ± 0.005	+1.3
35	4,6-pregnadiene-3,20-dione	0.11 ± 0.02	+0.2
41	testosterone	0.022 ± 0.001	
42	dihydrotestosterone	0.005 ± 0.001	+0.8
12	cortisone	0.16 ± 0.04	
13	prednisone	0.011 ± 0.002	+1.5

temperature–K_a data reported for guinea pig PBG by Stroupe & Westphal (1975) also appears to result in a biphasic plot with a transition temperature at about 23 °C. The dependence of the enthalpy of binding upon temperature (Table I) indicates a different binding mechanism for the higher and lower temperatures. This may be the result of a structural change occurring in the CBG molecule and/or a change in the pK_a of an essential amino acid residue.

At 4 °C the association of cortisol with CBG is enthalpy and entropy driven, with $\Delta H°$ being negative and $\Delta S°$ being positive. However, at 37 °C the change in entropy is negative. Binding still occurs spontaneously because of the larger, negative $\Delta H°$ which overcomes the unfavorable $\Delta S°$. A decrease in entropy indicates an increase in order which is unfavorable. A possible explanation of the negative $\Delta S°$ may be that the binding of steroid decreases the intramolecular vibrations (Sturtevant, 1977) of CBG. One would predict a higher entropic state for CBG at 37 °C than at 4 °C which could explain a negative $\Delta S°$ at higher temperatures and a positive $\Delta S°$ at lower temperatures.

By comparison of the K_a values for individual steroids that differ in only one structural change, the effect of this change on the binding affinity can be assessed. Since the free energy of binding ($\Delta G°$) is directly related to the binding affinity, the free energy contribution for the individual substituents can be determined. The contributions to free energy of binding to guinea pig CBG for a 20-oxo group, 11β-hydroxyl, and 19-methyl are −1.8, −1.1, and −1.7 kcal/mol, respectively. These large free energy values indicate the relative importance of these groups for optimal binding. The calculated free energy contributions of substitutent groups (Table VII) may be added together to predict the affinity of different steroids for CBG. For example, the experimentally measured low affinity of dexamethasone (no. 39) to guinea pig CBG ($\Delta G°$ = −5.18 kcal/mol) could have been predicted. Dexamethasone resembles cortisol except for the following alterations: 1-ene (+1.2 kcal/mol, $\delta\Delta G°$), 9α-fluorine (+3.1 kcal/mol), and

Table VII: Free Energy Contributions of Steroid Substituents in Steroid Interaction with Guinea Pig CBG

substituent	$\delta\Delta G°$ (kcal/mol)
2α-OH	+0.5
3α-OH from 3-oxo	+1.7
3β-OH from 3-oxo	+1.6
6α-OH	+2.0
6β-OH	+2.6
11α-OH	+0.9; +1.0
11β-OH	−1.1; −1.0; −1.2
11-oxo	+0.5
14α-OH	+2.0
17α-OH	−0.3; −0.1; −0.1; 0.0
20α-OH from 20-oxo	+3.0
20β-OH from 20-oxo	+3.2
20-oxo	−1.8
21-OH	−0.3; −0.2; −0.4
2α-CH_3	+0.6
6α-CH_3	+1.6
16α-CH_3	+0.6
10β-CH_3	−1.7
17α-OAc	+2.6
9α-Cl	+2.9
9α-F	+3.0; +3.1
Δ^1	+0.9; +1.5
Δ^4	−0.5; −0.8; −2.2; −0.8
Δ^6	+0.2

16α-CH_3 (+0.6 kcal/mol). One would, therefore, predict that the free energy of binding of dexamethasone to CBG would be 4.9 kcal/mol higher than that of cortisol binding. The experimentally determined difference of 4.3 kcal/mol is in agreement. Neglecting the influence of the C-20 and C-21 carbon atoms, we would predict testosterone (no. 41) to have a 3.4 kcal/mol higher energy of binding than cortisol. The experimentally observed value is 2.7 kcal/mol. The presence of the three hydroxy groups in cortisol that are absent in progesterone would be predicted to result in a 1.6 kcal/mol lower energy of binding which is the value observed experimentally. The influence of given substituents at specific locations is, therefore, additive within a reasonable error. A similar additivity of the substituent contributions to free energy of binding has been observed for the glucocorticoid receptors from rat hepatoma cells (Wolff et al., 1978).

The importance of certain hydrophilic amino acid residues in the binding site is evident from the binding specificity studies. The presence of a 20-oxo group results in a gain of free energy contribution of −1.8 kcal/mol, and reduction of the 20-oxo to a 20α- or 20β-hydroxy group gives a loss of about 3 kcal/mol. Similarly, reduction of the 3-oxo group to 3α- or 3β-hydroxyl diminishes the free energy of binding by about 1.7 kcal/mol. This relationship of binding affinity may result from hydrogen-bond formation with a hydrogen donor located near the 20- and 3-carbon atoms of the steroid in the binding site. On the other hand, a hydrogen acceptor near the 11β-hydroxy group may be the reason for a gain in energy of binding of −1.1 kcal/mol.

The total energy of binding gained by the 20-oxo and three hydroxy groups of cortisol is calculated to be −3.4 kcal/mol at 4 °C. Assuming a similar contribution by the 3-oxo group as the 20-oxo group (−1.7 kcal/mol), a total energy gain by the hydrophilic groups of approximately −5 kcal/mol is estimated. Since the total free energy of binding of cortisol to CBG was experimentally determined to be −9.4 kcal/mol, more than 50% of the binding energy is accounted for by hydrophilic groups. The remaining energy may result from van der Waals and hydrophobic bonds.

The binding affinity would be directly proportional to temperature if hydrophobic interactions were the principal forces

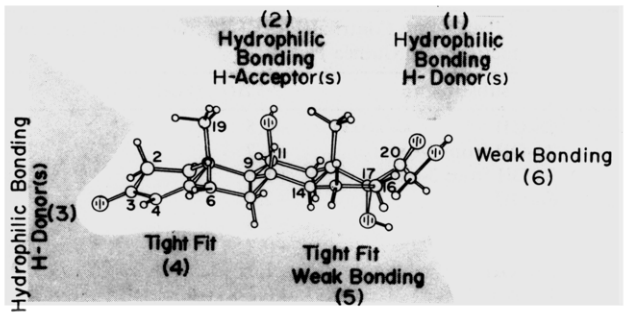

FIGURE 3: Guinea pig CBG steroid-binding site, as deduced from specificity studies.

for binding. However, an inverse relationship of K_a and temperature is observed experimentally for guinea pig CBG as well as for other high-affinity steroid-binding proteins (Westphal, 1971) and the glucocorticoid receptors (Koblinsky et al., 1972; Schaumberg & Bojensen, 1968; Wolff et al., 1978), progesterone receptor (Schrader & O'Malley, 1978), and estrogen receptor (Notides et al., 1975). For hydrophobic bonds to be the principal driving force for the binding process, $\Delta H°$ would be positive or nearly athermal and $\Delta S°$ would also be positive (Kauzman, 1959). For guinea pig CBG and human CBG (Stroupe et al., 1978), $\Delta H°$ is negative for all temperatures examined. At temperatures approaching physiological, $\Delta H°$ as well as $\Delta S°$ is negative for the glucocorticoid receptor (Wolff et al., 1978). Therefore, the principal driving force for the interaction of steroids with high-affinity binding proteins or receptors is not hydrophobic in nature, contrary to the proposal of Wolff et al. (1978).

Synopsis of Binding Specificity. An interpretation of the effects of the various structural changes on the binding affinity leads to a conceptual image of the cortisol-binding site of guinea pig CBG. The present results suggest the following characteristics for the binding site (Figure 3). (1) A hydrogen donor forms a hydrogen bond with the 20-oxo group. Reduction to a hydroxyl or removal of the carbonyl lowers the K_a 27–350-fold. (2) A hydrogen acceptor forms a hydrogen bond with the 11β-hydroxy group. Removal of the hydroxyl lowers the K_a by about 1 order of magnitude. Oxidation to a carbonyl group decreases the K_a 18–55-fold. This may be the result of an undesirable dipole–dipole interaction of the 11-keto group with the putative hydrogen-bond acceptor of the protein (Wolff et al., 1978) or may be caused by the reported effect of the 11 position substituent on the bowing of the A ring relative to the remainder of the steroid: the A/B–C–D angle is −32.3° for cortisone and −19.8° for cortisol (Duax & Norton, 1975). (3) A hydrogen donor forms a hydrogen bond with the 3-oxo group. Reduction to a hydroxyl decreases the K_a about 20-fold. (4) Reduction of the 4 double bond in the A ring results in a decreased K_a (3–56-fold). Introduction of a Δ^1 double bond diminishes K_a about 5–10-fold. Shift of the double bond from C-4 to C-5 also decreases the affinity 10-fold. Introduction of either a hydroxyl or a methyl at C-2 or C-6 results in a lower K_a (about 2–10-fold). Introduction of halogens at C-9 lowers the K_a more than 200-fold. These data indicate a tight fit around the A and B rings. Possibly, these alterations may lower the K_a by interfering with the hydrogen-bond formation of the 3-oxo group with the putative hydrogen donor of the protein. (5) A tight fit is proposed for the C-17 because introduction of an acetoxy group lowers the K_a more than 2 orders of magnitude. A strong hydrogen bond is not proposed at this position because removal of the hydroxyl lowers the K_a only slightly (0–40%). (6) Oxidation of the 21-hydroxyl to an oxo group or conversion to a hydrogen lowers the K_a only slightly (2–3-fold).

The steroid binding site of guinea pig CBG appears to be best adapted to bind cortisol, the predominant corticosteroid in the guinea pig; alteration of this structure invariably decreases the affinity. In contrast to the steroid-binding site of the guinea pig PBG, which is predominantly hydrophobic in nature (Blanford et al., 1978), the binding site of CBG apparently has more hydrophilic groups capable of interacting with the hydroxy and oxo groups of cortisol. Based on studies of steroid binding to human CBG (Murphy, 1969; unpublished results from this laboratory) and on the present results, it is concluded that the steroid-binding site of guinea pig CBG is more specific in comparison with human CBG which binds cortisol, progesterone, and certain other steroids with a similar affinity.

References

Basset, M., Defaye, G., & Chambaz, E. M. (1977) *Biochem. Biophys. Res. Commun. 79*, 380–387.

Blanford, A. T., Wittman, W., Stroupe, S. D., & Westphal, U. (1978) *J. Steroid Biochem. 9*, 187–201.

Dixon, M., & Webb, E. C. (1964) in *Enzymes*, 2nd ed., pp 82–83, Academic Press, New York.

Duax, W. L., & Norton, D. A. (1975) in *Atlas of Steroid Structure*, Vol. 1, IFI-Plenum, New York.

Kauzmann, W. (1959) *Adv. Protein Chem. 14*, 1–63.

Koblinsky, M., Beato, M., Kalimi, M., & Feigelson, P. (1972) *J. Biol. Chem. 247*, 7897–7904.

Mickelson, K. E., & Pētra, P. H. (1975) *Biochemistry 14*, 957–963.

Mickelson, K. E., & Pētra, P. H. (1978) *J. Biol. Chem. 253*, 5293–5298.

Mickelson, K. E., & Westphal, U. (1979a) *Biochemistry 18*, 2685–2690.

Mickelson, K. E., & Westphal, U. (1979b) *International Congress of Biochemistry, 11th*, Toronto, Canada, Abstract No. 03-1-S46.

Murphy, B. E. P. (1969) *Recent Prog. Horm. Res. 25*, 563–610.

Notides, A. C., Hamilton, D. E., & Auer, H. E. (1975) *J. Biol. Chem. 250*, 3945–3950.

Randerath, K. (1966) *Thin Layer Chromatography*, Academic Press, New York.

Schaumberg, B. P., & Bojensen, E. (1968) *Biochim. Biophys. Acta 170*, 172–188.

Schrader, W. T., & O'Malley, B. W. (1978) in *Receptors and Hormone Action* (O'Malley, B. W., & Birnbaumer, L., Eds.) Vol. II, pp 189–224, Academic Press, New York.

Stroupe, S. D., & Westphal, U. (1975) *J. Biol. Chem. 250*, 8735–8739.

Stroupe, S. D., Harding G. B., Forsthoefel, M. W., & Westphal, U. (1978) *Biochemistry 17*, 177–182.

Sturtevant, J. M. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 2236–2240.

Westphal, U. (1969) *Methods Enzymol. 15*, 761–796.

Westphal, U. (1971) *Steroid-Protein Interactions*, Springer-Verlag, New York.

Wolff, M. E., Baxter, J. D., Kollman, P. A., Lee, D. L., Kuntz, I. D., Bloom, E., Matulich, D. T., & Morris, J. (1978) *Biochemistry 17*, 3201–3208.