Kasper, C. B. (1975) in *Protein Sequence Determination* (Needleman, S. B., Ed.) 8th ed., pp 114–161, Springer-Verlag, New York.

Laskey, R. A., & Mills, A. D. (1975) *Eur. J. Biochem. 56*, 335–341.

Loh, Y. P., Sarne, Y., & Gainer, H. (1975) *J. Comp. Physiol. 100*, 283–296.

Mains, R. E., & Eipper, B. A. (1980) *Ann. N.Y. Acad. Sci. 343*, 94–110.

Nakanishi, S., Inoue, A., Kita, T., Nakamura, M., Chang, A.

C. Y., Cohen, S. N., & Numa, S. (1979) *Nature (London) 278*, 423–427.

Randerath, K. (1970) *Anal. Biochem. 34*, 188–205.

Roberts, J. L., Phillips, M., Rosa, P. A., & Herbert, E. (1978) *Biochemistry 17*, 3609–3168.

Steiner, D. F., Quinn, P. S., Chan, S. J., Marsh, J., & Tager, H. S. (1980) *Ann. N.Y. Acad. Sci. 343*, 1–16.

Stephens, R. E. (1978) *Anal. Biochem. 84*, 116–126.

Yates, M. E., & Berry, R. W. (1981) *Gen. Comp. Endocrinol. 43*, 308–314.

Steroid–Protein Interactions. Human Corticosteroid Binding Globulin: Some Physicochemical Properties and Binding Specificity[†]

Kenneth E. Mickelson,[*,‡] June Forsthoefel, and Ulrich Westphal

ABSTRACT: Reducing agents (dithiothreitol and β-mercaptoethanol) significantly decrease the affinity constants of the human corticosteroid-binding globulin (CBG)–cortisol complex in proportion to their concentration; the resulting K_a values are more consistent than those obtained in the absence of the reductants. The effect is reversible. The equilibrium association constants of the CBG complexes with cortisol and progesterone show a relatively broad pH maximum between pH 8 and 11. In this pH range, cortisol was found to be bound more strongly than progesterone; this relationship is reversed around pH 6. The van't Hoff plot of the temperature effect on K_a of the CBG–cortisol complex (4–41 °C) exhibits a nonlinear, possibly biphasic temperature dependency. The shape of the van't Hoff plot was similar in the presence of mercaptoethanol. The association of cortisol and progesterone to human CBG at 4 and 37 °C is enthalpy driven, compensating for the unfavorable change in entropy. Studies with 47 steroids served to elucidate the influence on binding affinity of polar and nonpolar groups and other structural alterations. The contribution of specific structural changes in the steroid molecule to the free energy of binding can be calculated from the results. Important structures for optimal binding are the 20-oxo group, a 10β-methyl group, and a double bond at the 4 position. A complementary image of the binding site with respect to the nature of binding at various locations is proposed.

The corticosteroid-binding globulin (CBG)[1] of human blood serum was the first high-affinity steroid-binding protein to be reported (Daughaday, 1956; Bush, 1957; Sandberg & Slaunwhite, 1958). CBG has been extensively purified and characterized from the sera of human, rabbit, and rat [for review, see Westphal (1971); Rosner, 1976; Ballard, 1979; Westphal, 1980] and, more recently, from guinea pig serum (Mickelson & Westphal, 1979). Guinea pig CBG has an affinity constant for cortisol that is 18 times larger than that for progesterone (Mickelson & Westphal, 1980). Human CBG, in contrast, binds both cortisol and progesterone with similar affinity (Stroupe et al., 1978). In the present study, the influence of reducing agents, temperature, and pH on the binding affinity has been investigated. Furthermore, the affinity constants of human CBG complexes with a number of steroids have been determined in order to define the structural features producing strong or weak interaction at various locations in the steroid molecule. A complementary image of

the binding site may be deduced from the results. This information is expected to contribute to a systematic investigation of the nature of the binding site, an effort that has entered a new phase recently by the crystallization of the cortisol complex of guinea pig CBG and α_1-acid glycoprotein (McPherson et al., 1980), opening the way for direct visualization of a high-affinity steroid binding site by X-ray diffraction analysis. These crystallizations follow the preparation of a crystalline lead salt of α_1-acid glycoprotein (Schmid, 1953) and the crystallographic studies on the progesterone-binding uteroglobin (Buehner & Beato, 1978; Mornon et al., 1980).

Materials and Methods

Term human pregnancy serum was obtained from Norton-Children's Hospital (Louisville, KY). Amberlite XAD-2 was from Rohm and Haas; it was washed 10 times with an equal volume of methanol and then in a similar way with distilled water at room temperature. Endogenous steroids were removed by gently shaking 1 L of pregnancy serum with 200 mL (settled volume) of Amberlite XAD-2 resin for 8 h at room temperature. The serum was then filtered through a fritted disk funnel (coarse), dialyzed for 40 h against 50 mM sodium phosphate–0.5 M KCl (pH 9.0, 4 °C, 0.02% sodium azide),

---

---

[1] Abbreviations used: BME, β-mercaptoethanol; CBG, corticosteroid-binding globulin; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); DTT, dithiothreitol; PBG, progesterone-binding globulin; EDTA, ethylenediaminetetraacetic acid; NaDodSO$_4$, sodium dodecyl sulfate.

centrifuged for 30 min at 12000g, 4 °C, and filtered through a Whatman No. 2 paper.

Purification of human CBG was achieved by affinity chromatography with 11β-hydroxy-3-oxo-4-androstene-17β-carboxyaminoethylamine–1,4-butanediol diglycidyl ether–agarose as the resin (Mickelson & Westphal, 1979). The steroid-free pregnancy serum (1 L) was passed through a column (2.2 × 25 cm, containing the affinity resin) at a flow rate of 150 mL/h at 4 °C. The column was washed with buffer (50 mM sodium phosphate–0.5 M KCl, pH 9.0, 4 °C) until the UV absorbance of the washings was negligible (<-0.05). The column was then placed at room temperature and, after 1 h, eluted with a solution of 280 μg of cortisol per mL of 50 mM sodium phosphate–0.1 M NaCl buffer, pH 7.4. The protein fractions were pooled and concentrated to 30 mL by ultrafiltration by using an Amicon PM-30 membrane. The 30 mL of protein solution was dialyzed for 48 h against 2 L of 5 mM phosphate buffer (pH 6.8, 4 °C) containing 10 mg of cortisol. The dialysis buffer was changed once. The dialyzed protein solution was passed at a flow rate of about 20 mL/h through a column (2.2 × 7 cm) containing hydroxylapatite equilibrated with 5 mM phosphate buffer (pH 6.8, 4 °C). The protein fractions were pooled, dialyzed at 4 °C against 50 mM phosphate buffer of pH 7.4 containing 1 mM EDTA and 0.02% sodium azide, and stored at –80 °C. The yield of pure protein from pregnancy serum was generally 55 mg/L as determined by the use of $E_{1cm}^{1\%} = 6.45$ (Muldoon & Westphal, 1967) and by the quantitative evaluation of the Scatchard plots.

Removal of Cortisol from CBG. Pure CBG was stripped of steroid by a modification of the method described by Chan & Slaunwhite (1977). A CBG solution (30 mL; 5.0 μg/mL of 50 mM sodium phosphate buffer, pH 7.4, containing 0.1% gelatin) was mixed by rotation with 6 mL of settled Amberlite XAD-4 for 4 h at 23 °C. The protein solution was then filtered through a Millipore filter (0.2 μm).

Equilibrium dialysis was performed essentially as previously described (Westphal, 1969; Mickelson & Westphal, 1980) in order to determine the affinity constants for the binding of radioactive steroids to CBG. Two milliliters of a CBG solution (9.2 × 10⁻⁹ M) was the inside volume and 20 mL of 50 mM sodium phosphate buffer, pH 7.4, at 4 °C, containing 1 mM EDTA and 0.02% sodium azide was the outside volume. A buffer consisting of citrate, phosphate, and borate (50 mM each), adjusted to the desired pH with NaOH, was used for the pH study at 4 °C. The dialysis flasks were gently shaken for 48 h at 4 °C or at the indicated 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. Individual determinations were performed on different days in order to maximize experimental error and were averaged.

The apparent binding affinity of radioinert steroids was determined by a competitive displacement method previously described (Mickelson & Westphal, 1980). The apparent dissociation constant of each competing steroid was determined by use of at least three different concentrations on 3 different days, giving a 20–80% increase in the apparent dissociation constant of radiolabeled cortisol. All steroids examined obeyed the criterion for competitive inhibition of an unaltered number of binding sites in the presence of the competing steroid.

Results

Effect of Reducing Agents on CBG. The data in Table I indicate that the association of cortisol with human CBG

Table I: Influence of Dithiothreitol (DTT) and β-Mercaptoethanol (BME) on the Human CBG–Cortisol Complex

DTT concn (mM)	$10^{-9}K_a \pm$ SD (M^{-1})	BME concn (mM)	$10^{-9}K_a \pm$ SD (M^{-1})
0	1.8 ± 0.3	0	1.6 ± 0.1
0.5	1.5 ± 0.6	10	1.0 ± 0.2
1	1.5 ± 0.4	20	0.7 ± 0.1
5	1.1 ± 0.5	50	0.5 ± 0.1
10	0.9 ± 0.3	100	0.3 ± 0.0
25	0.6 ± 0.2	200	0.2 ± 0.0

Table II: Influence of Various Oxidants and Reductants on the Human CBG–Cortisol Complex

agent	$K_a \pm$ SD [a]
none	1.4 ± 0.4
0.2 M sodium ascorbate	1.6
0.2 M sodium arsenite	1.5
10 mM sodium tetrathionate	1.6
10 mM potassium ferricyanide	1.4
10 mM hydrogen peroxide	1.9
O_2 gas	1.4 ± 0.3

[a] Determined by equilibrium dialysis at 4 °C, pH 7.4. Values are averages of two determinations, except for lines 1 and 7 which give standard deviations for three determinations.
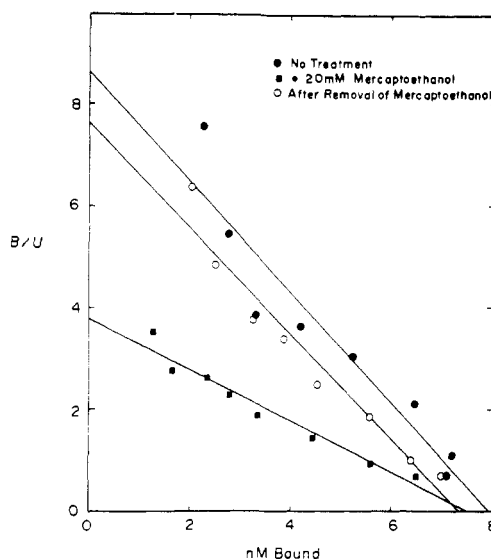

FIGURE 1: Reversal of the effect of reduction on the human CBG–progesterone complex. Scatchard plots of equilibrium dialysis data of progesterone binding to pure human CBG in the absence (filled circles), in the presence (squares), or after removal by dialysis (open circles) of 20 mM mercaptoethanol, pH 7.4, at 4 °C. Lines were calculated by a least-squares computer program.

decreases with increasing concentration of reducing agents. In the presence of 10 mM dithiothreitol, the association constant was decreased by 50%. Mercaptoethanol from Aldrich Chemical Co. reduced the association constant by 50% at a concentration of 20 mM (Table I, Figure 1). The concentration of 2-mercaptoethanol (Fisher, Sigma, Eastman, or Matheson, Coleman & Bell) required to reduce the association constant by 50% was 100 mM. The number of binding sites was not affected by the presence of the reductants. Full binding activity could be recovered by removal of mercaptoethanol by dialysis (Figure 1). Similar results were obtained with dithiothreitol or with cortisol instead of progesterone. No significant effect on binding activity of CBG was detected when the mild reductants, sodium ascorbate or arsenite, were added at a concentration of 200 mM (Table II). Oxidants such as hydrogen peroxide, tetrathionate, O_2 gas (bubbled

Table III: Sulfhydryl Content of Human CBG Determined by DTNB Titration

treatment	SH groups/mol of CBG
none	0.0
+cortisol	0.0
+2% NaDodSO$_4$	0.9
+10 mM DTT	0.9
+20 mM BME	0.8
+2% NaDodSO$_4$, 10 mM DTT	2.0
0.2 M ascorbate	1.0
0.2 M arsenite	0.9

Table IV: Thermodynamic Parameters of Steroid–Protein Complexes

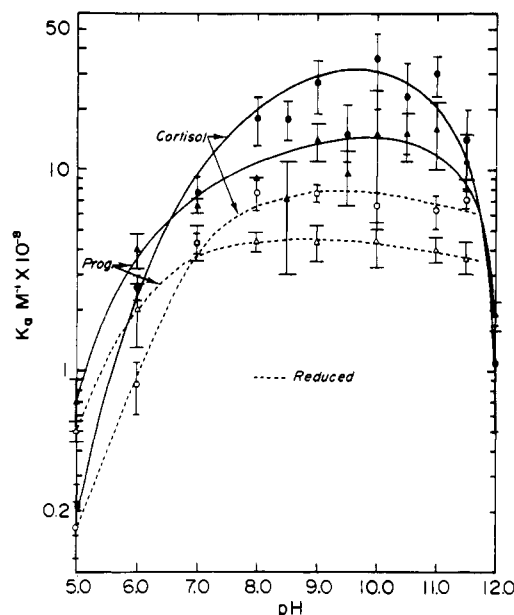| complex	$\Delta G°$ (kcal/mol) at		$\Delta H°$ (kcal/mol) at		$T\Delta S°$ (kcal/mol) at	
	4 °C	37 °C	4 °C	37 °C	4 °C	37 °C
human CBG–cortisol	−11.6	−10.8	−13.3	−32.9	−1.7	−22.1
human CBG–progesterone	−11.6	−10.6	−14.9	−26.4	−3.3	−15.8
guinea pig CBG–cortisol	−9.4	−8.8	−7.6	−28.4	+1.8	−19.6

FIGURE 2: Influence of pH on the association constants of the pure human CBG–cortisol (circles) and progesterone (triangles) complexes determined by equilibrium dialysis at 4 °C. No decrease of binding capacity was observed except that at pH 12.0, 20–40% of the binding capacity was lost. Solid lines and filled symbols are in the absence of mercaptoethanol whereas dashed lines and open symbols are in the presence of 20 mM mercaptoethanol.

through a CBG solution for 1 h), or potassium ferricyanide had no effect (Table II).

Sulfhydryl Group Determination. Amino acid analysis of human CBG indicates the presence of two half-cystines (Muldoon & Westphal, 1967; Le Gaillard et al., 1975). No sulfhydryl group was titratable by 5,5'-dithiobis(2-nitrobenzoic acid) in native CBG, free or complexed with steroid (Table III). One sulfhydryl group was detected after reduction of CBG by dithiothreitol, mercaptoethanol, sodium ascorbate, or sodium arsenite. One sulfhydryl group was also titratable in denatured CBG (2% NaDodSO$_4$, 37 °C, pH 7.4, 1 h). Two sulfhydryl groups, however, were detected in reduced, denatured CBG (Table III). These data confirm the results previously reported by Le Gaillard & Dautrevaux (1978), who assume the presence of one sulfenic acid group which is reducible by mild reductants. The data support the conclusion that CBG contains one sterically hindered cysteine that is exposed only after denaturation and one cysteine that is titratable only after reduction. This latter cysteine may be oxidized or complexed with some unknown molecule.

Influence of pH. The pH dependence of the equilibrium association constants for the CBG–cortisol and CBG–progesterone complexes is given in Figure 2. Relatively broad pH profiles are obtained for both steroids with maximal binding between pH 8.0 and 11.5. Significant binding still occurred at the pH extremes of 5.0 and 12.0. The association constants

determined at pH 12.0, however, are not reliable since the binding capacity was 20–40% lower than that obtained at other pH values.

It should be noted that the ionic strength of the mixed buffer used changes over the pH range applied (about 0.3 at pH 6.0 and 0.5 at pH 10.0). However, this variation is not assumed to affect the K_a values. Unpublished experiments by G. B. Harding in our laboratory have shown that the cortisol binding affinity values of human CBG, either in diluted pregnancy serum or in a purified CBG preparation, are the same in pH 7.4 phosphate buffers ranging in ionic strength from 0.3 to 1.2. Furthermore, we found that addition of 1 M NaCl to a 50 mM phosphate buffer did not significantly change the K_a value of the pure CBG–cortisol complex in the same phosphate buffer without added NaCl. These results agree with the observation of Chan & Slaunwhite (1977), who reported no significant differences of K_a for human CBG complexes with cortisol and progesterone in NaCl solutions ranging from 0.15 to 3.0 M.

An interesting observation is that CBG binds progesterone better than cortisol at pH 5.0 and 6.0 while cortisol is bound more tightly between pH 8 and 11. This may be due to the titration of an amino acid side chain capable of interacting with the hydroxyl group(s) of cortisol. The addition of 20 mM mercaptoethanol does not significantly alter the pH dependence. The association constants, however, are lower at all pH values in the presence of mercaptoethanol.

Influence of Temperature. Temperature has a dramatic effect on the binding of cortisol and progesterone to human CBG. Scatchard analyses of equilibrium dialysis experiments showed that the association constant decreased about 40-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 3. The complex exhibits a nonlinear, possibly biphasic temperature dependence over the range examined, with the break occurring around 27 °C for both steroids. Similar results were observed for guinea pig CBG interacting with cortisol (Mickelson & Westphal, 1980). The increasing slope with increasing temperature indicates that the enthalpy ($\Delta H°$) of the complex decreases as the temperature exceeds 27 °C. Table IV lists certain thermodynamic parameters for human CBG and guinea pig CBG. Since $\Delta G°$ is negative, the complex is at a thermodynamically lower energy state. The binding process, therefore, occurs spontaneously. Complex formation is enthalpy driven at both 4 and 37 °C since the change in entropy is unfavorable as evidenced by the negative value. Similar results were observed for guinea pig CBG, except at 4 °C where the change in entropy is favorable for complex formation. The shape of the van't Hoff plots was similar in the presence of mercaptoethanol.

Steroid-Binding Specificity. The apparent association constants of 47 steroids were determined as inhibition constants by competitive equilibrium dialysis (Table V) in the presence

Table V: Affinity Constants[a] of Human CBG Complexes with Steroids

no.	steroid	$10^{-7}K_a \pm SD$[b] (M^{-1})	$-\Delta G°$ (kcal/mol)
1	11β,17,21-trihydroxy-4-pregnene-3,20-dione (cortisol)	71 ± 7	11.24
2	14α,17,21-trihydroxy-4-pregnene-3,20-dione	0.7 ± 0.1	8.69
3	11β,17,21-trihydroxy-1,4-pregnadiene-3,20-dione (prednisolone)	37 ± 4	10.88
4	11β,17,21-trihydroxy-2α-methyl-4-pregnene-3,20-dione	60 ± 15	11.15
5	11β,17,21-trihydroxy-2α-methyl-9α-fluoro-4-pregnene-3,20-dione	0.17 ± 0.01	7.91
6	21-acetoxy-11β,17-dihydroxy-4-pregnene-3,20-dione (cortisol 21-acetate)	42 ± 6	10.95
7	17,21-dihydroxy-4-pregnene-3,11,20-trione (cortisone)	5 ± 1	9.78
8	11β,17,20α,21-tetrahydroxy-4-pregnen-3-one	1.7 ± 0.2	9.18
9	11β,17,20β,21-tetrahydroxy-4-pregnen-3-one	0.64 ± 0.03	8.64
10	11α,21-dihydroxy-4-pregnene-3,20-dione (epicorticosterone)	14 ± 5	10.34
11	11β,21-dihydroxy-4-pregnene-3,20-dione (corticosterone)	96 ± 23 (107 ± 2)	11.41
12	16α,17-dihydroxy-4-pregnene-3,20-dione	0.7 ± 0.1	8.69
13	17,21-dihydroxy-4-pregnene-3,20-dione (11-deoxycortisol)	64 ± 20	11.18
14	11β,21-dihydroxy-5β-pregnane-3,20-dione (5β-dihydrocorticosterone)	5 ± 1	9.78
15	2α-hydroxy-4-pregnene-3,20-dione	27 ± 1	10.71
16	6α-hydroxy-4-pregnene-3,20-dione	1.4 ± 0.2	9.07
17	6β-hydroxy-4-pregnene-3,20-dione	0.31 ± 0.03	8.24
18	11α-hydroxy-4-pregnene-3,20-dione	10 ± 1	10.16
19	16α-hydroxy-4-pregnene-3,20-dione	1.0 ± 0.4	8.89
20	17-hydroxy-4-pregnene-3,20-dione (17-hydroxyprogesterone)	63 ± 21	11.17
21	12α-hydroxy-5β-pregnane-3,20-dione	0.10 ± 0.02	7.62
22	17-acetoxy-4-pregnene-3,20-dione	0.08 ± 0.01	7.50
23	17-caproxy-4-pregnene-3,20-dione	0.0043 ± 0.0003	5.88
24	21-hydroxy-4-pregnene-3,20-dione (deoxycorticosterone)	68 ± 16 (69 ± 8)	11.22
25	17-hydroxy-6α-methyl-4-pregnene-3,20-dione	2.6 ± 0.3	9.42
26	17-hydroxy-16α-methyl-4-pregnene-3,20-dione	4.9 ± 1.4	9.77
27	4-pregnene-3,11,20-trione	3.7 ± 0.6	9.61
28	4-pregnene-3,20-dione (progesterone)	59 ± 9 (59 ± 6)	11.14
29	5-pregnene-3,20-dione	13 ± 2	10.30
30	5α-pregnane-3,20-dione	2.3 ± 0.5	9.35
31	5β-pregnane-3,20-dione	4.2 ± 1.9	9.68
32	3β-hydroxy-5-pregnen-20-one (pregnenolone)	0.05 ± 0.02	7.24
33	3α-hydroxy-5β-pregnan-20-one	0.23 ± 0.02	8.08
34	2α-methyl-4-pregnene-3,20-dione	34 ± 6	10.83
35	6α-methyl-4-pregnene-3,20-dione	7.1 ± 0.4	9.97
36	16α-methyl-4-pregnene-3,20-dione	11 ± 2	10.21
37	19-nor-4-pregnene-3,20-dione (19-norprogesterone)	5 ± 1	9.78
38	17-hydroxy-4-pregnen-3-one	0.6 ± 0.1	8.61
39	5α-pregnan-3-one	0.0025 ± 0.0007	5.58
40	18,11-hemiacetal of 11β,21-dihydroxy-3,20-dioxo-4-pregnen-18-al (aldosterone)	0.8 ± 0.1	8.77
41	9α-fluoro-16α-methyl-11β,17,21-trihydroxy-1,4-pregnadiene-3,20-dione (dexamethasone)	0.039 ± 0.006	7.10
42	17,21-dimethyl-19-norpregna-4,9-diene-3,20-dione (R5020)	0.5 ± 0.1	8.51
43	17β,19-dihydroxy-4-androsten-3-one	0.5 ± 0.1	8.51
44	17β-hydroxy-4-androsten-3-one (testosterone)	5 ± 1	9.78
45	17β-acetoxy-4-androsten-3-one	1.5 ± 0.3	9.11
46	17β-hydroxy-4-estren-3-one (19-nortestosterone)	0.5 ± 0.1	8.51
47	3,17β-dihydroxy-1,3,5(10)-estratriene (estradiol)	0.008 ± 0.001	6.23

[a] Affinity constants were determined in 20 mM mercaptoethanol (Aldrich) by competitive equilibrium dialysis at pH 7.4, 4 °C, with radiolabeled cortisol. The values in parentheses for steroids 11, 24, 28 in addition to the value for cortisol were obtained by direct equilibrium dialysis with the respective radiolabeled steroids. [b] SD, standard deviation of at least three determinations using a minimum of three different concentrations of competing steroid.

of 20 mM mercaptoethanol. Attempts to obtain the binding data in the absence of reducing agent were hampered by considerable variation (about ±50%) observed over several weeks. Addition of mercaptoethanol resulted in more consistent values (±10%). The K_a values were lower, but the relative binding affinities were not altered. Values obtained by the competition method are in agreement with the values obtained by direct equilibrium dialysis (Table V, 11, 24, and 28). All of the steroids examined yielded the same number of binding sites; therefore, all were competitive with radioactive cortisol for the binding to CBG.

Table VI indicates that a 3-oxo group is important for optimal binding. Reduction of the 3-oxo group to either a 3α-hydroxyl (33) or a 3β-hydroxyl (32) resulted in a loss of 1.6 and 3.1 kcal/mol, respectively. Table VI also indicates that a 20-oxo group is essential for optimal binding. Either removal (38 and 39) or reduction (8 and 9) resulted in a loss of 2-4 kcal/mol of free energy of binding. In contrast, introduction of an 11-oxo group decreases the binding affinity (7 and 27).

The 11β, 17α-, and 21-hydroxy groups are relatively unimportant for binding as shown in Table VII for a number of steroids. This results in the insignificant difference in affinity constants observed for cortisol and progesterone complexed with human CBG.

The presence of an 11α-hydroxy group, however, decreases the binding energy by about 1 kcal/mol (Table VIII, 18 and 10). Hydroxy groups also impair binding at the following positions: 6α, 6β, 12α, 14α, 16α, and 19. Introduction of a methyl group in the 6α position or 16α position also decreases binding, although not as much as a hydroxy group (Table IX). This may indicate that binding is reduced by steric hindrance. On the other hand, the 10β-methyl group is essential for optimal binding, as seen in comparison with 19-norsteroids (37 and 46). This methyl group contributes -1.4 kcal/mol of binding energy, probably the result of hydrophobic forces.
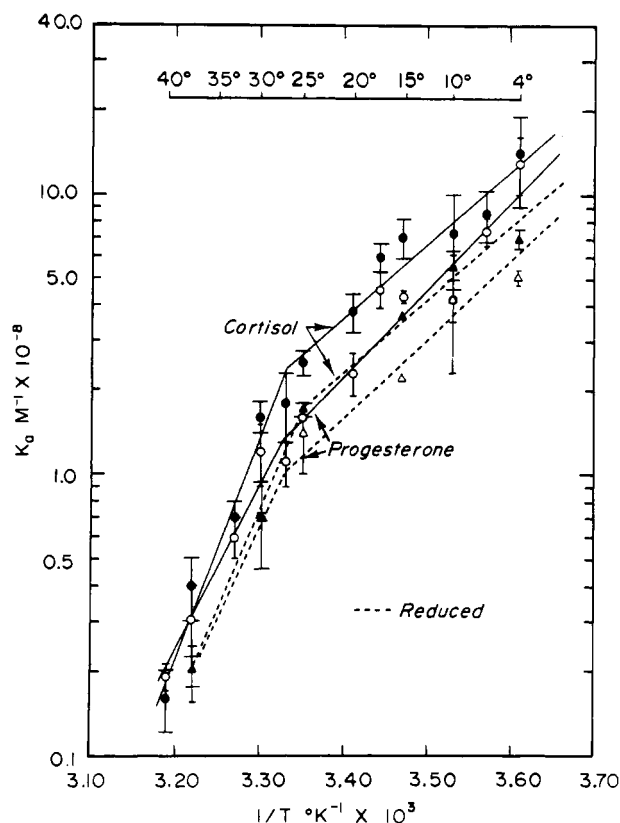
FIGURE 3: van't Hoff plots for pure human CBG at pH 7.4 in the absence and presence of mercaptoethanol. Solid lines and circles (filled for cortisol, open for progesterone) in the absence of mercaptoethanol; broken lines and triangles (filled for cortisol, open for progesterone) in the presence of 20 mM mercaptoethanol. The binding capacity was essentially the same at all temperatures examined, indicating no loss of binding sites during equilibrium dialysis at the various temperatures.

Table VI: Importance of Oxo Groups

no.[a]	steroid	$10^{-7}K_a \pm$ SD (M^{-1})	$\delta\Delta G°$ (kcal/ mol)[b]
	3-Oxo Group		
31	5β-pregnane-3,20-dione	4.2 ± 1.9	
33	3α-hydroxy-5β-pregnan-20-one	0.23 ± 0.02	+1.6
29	5-pregnene-3,20-dione	13 ± 2	
32	3β-hydroxy-5-pregnen-20-one	0.05 ± 0.02	+3.1
	20-Oxo Group		
1	cortisol	71 ± 7	
8	20α-hydroxycortisol[c]	1.7 ± 0.2	+2.1
9	20β-hydroxycortisol	0.64 ± 0.03	+2.6
20	17-hydroxyprogesterone	63 ± 21	
38	17-hydroxy-4-pregnen-3-one	0.6 ± 0.1	+2.6
30	5α-pregnane-3,20-dione	2.3 ± 0.5	
39	5α-pregnan-3-one	0.0025 ± 0.0007	+3.8
	11-Oxo Group		
13	11-deoxycortisol	64 ± 20	
7	cortisone	5 ± 1	+1.4
28	progesterone	59 ± 9	
27	11-oxoprogesterone	3.7 ± 0.6	+1.5

[a] The numbers in this and the following tables are taken from Table V. [b] Contribution of structural change to free energy of binding: $\delta\Delta G° = \Delta G°$ of structurally altered steroid minus $\Delta G°$ of reference steroid. A positive $\delta\Delta G°$ value indicates weakening of binding. [c] 11β,17,20α,21-tetrahydroxy-4-pregnen-3-one.

A tight fit around the 17α position is also seen by introduction of either an acetoxy group (22) or the larger caproxy

Table VII: Influence of Hydroxy Groups

no.	steroid	$10^{-7}K_a \pm$ SD (M^{-1})	$\delta\Delta G°$ (kcal/ mol)
	11β-Hydroxy Group		
1	cortisol	71 ± 7	
13	11-deoxycortisol	64 ± 20	+0.1
11	corticosterone	96 ± 23	
24	deoxycorticosterone	68 ± 16	+0.2
	17α-Hydroxy Group		
1	cortisol	71 ± 7	
11	corticosterone	96 ± 23	−0.2
20	17-hydroxyprogesterone	63 ± 21	
28	progesterone	59 ± 9	0.0
25	17-hydroxy-6α-methylprogesterone	2.6 ± 0.3	
35	6α-methylprogesterone	7.1 ± 0.4	−0.6
26	17-hydroxy-16α-methylprogesterone	4.9 ± 1.4	
36	16α-methylprogesterone	11 ± 2	−0.4
	21-Hydroxy Group		
24	deoxycorticosterone	68 ± 16	
28	progesterone	59 ± 9	+0.1
13	11-deoxycortisol	64 ± 20	
20	17-hydroxyprogesterone	63 ± 21	0.0

Table VIII: Addition of Other Hydroxy Groups

no.	steroid	$10^{-7}K_a \pm$ SD (M^{-1})	$\delta\Delta G°$ (kcal/ mol)
28	progesterone	59 ± 9	
15	2α-hydroxyprogesterone	27 ± 1	+0.4
16	6α-hydroxyprogesterone	1.4 ± 0.2	+2.1
17	6β-hydroxyprogesterone	0.31 ± 0.03	+2.9
19	16α-hydroxyprogesterone	1.0 ± 0.4	+2.3
28	progesterone	59 ± 9	
18	11α-hydroxyprogesterone	10 ± 1	+1.0
24	deoxycorticosterone	68 ± 16	
10	epicorticosterone	14 ± 5	+0.9
31	5β-pregnanedione	4.2 ± 1.9	
21	12α-hydroxy-5β-pregnanedione	0.10 ± 0.02	+2.1
13	11-deoxycortisol	64 ± 20	
2	14α-hydroxy-11-deoxycortisol	0.7 ± 0.1	+2.5
20	17-hydroxyprogesterone	63 ± 21	
12	16α,17-dihydroxyprogesterone	0.7 ± 0.1	+2.5
44	testosterone	5 ± 1	
43	19-hydroxytestosterone	0.5 ± 0.1	+1.3

group (23) which results in a greatly diminished interaction with CBG (Table X). Introduction of an acetoxy group at the 21 position (6), however, decreases the affinity constant by only 40%, indicating that a bulky group can be accommodated around C-21.

Reduction of the double bond at the 4 position to either the 5α or 5β derivative results in about the same reduction of binding (14, 30 and 31, Table X). Similar results have also been observed in the case of guinea pig CBG (Mickelson & Westphal, 1980). Guinea pig PBG, however, binds the 5α-dihydro derivative much better (6-fold) than the 5β-dihydro derivative (Blanford et al., 1978).

Discussion

The binding of cortisol and progesterone to human CBG was found to be sensitive to reducing agents. The association constant was significantly lower in the presence of dithiothreitol

Table IX: Influence of Methyl Groups

no.	steroid	$10^{-7}K_a \pm$ SD (M^{-1})	$\delta\Delta G°$ (kcal/mol)
	10β-Methyl Group		
37	19-norprogesterone	5 ± 1	
28	progesterone	59 ± 9	-1.4
46	19-nortestosterone	0.5 ± 0.1	
44	testosterone	5 ± 1	-1.3
	Other Methyl Groups		
1	cortisol	71 ± 7	
4	2α-methylcortisol	60 ± 15	$+0.1$
28	progesterone	59 ± 9	
34	2α-methylprogesterone	34 ± 6	$+0.3$
28	progesterone	59 ± 9	
35	6α-methylprogesterone	7.1 ± 0.4	$+1.2$
20	17-hydroxyprogesterone	63 ± 21	
25	6α-methyl-17-hydroxyprogesterone	2.6 ± 0.3	$+1.8$
28	progesterone	59 ± 9	
36	16α-methylprogesterone	11 ± 2	$+0.9$
20	17-hydroxyprogesterone	63 ± 21	
26	16α-methyl-17-hydroxyprogesterone	4.9 ± 1.4	$+1.4$

Table X: Influence of Additional Alterations

no.	steroid	$10^{-7}K_a \pm$ SD (M^{-1})	$\delta\Delta G°$ (kcal/mol)
	Double Bonds		
1	cortisol	71 ± 7	
3	prednisolone	37 ± 4	$+0.4$
11	corticosterone	96 ± 23	
14	5β-dihydrocorticosterone	5 ± 1	$+1.6$
28	progesterone	59 ± 9	
30	5α-pregnanedione	2.3 ± 0.5	$+1.8$
31	5β-pregnanedione	4.2 ± 1.9	$+1.5$
	Acylation		
20	17-hydroxyprogesterone	63 ± 21	
22	17-acetoxyprogesterone	0.08 ± 0.01	$+3.7$
23	17-caproxyprogesterone	0.0043 ± 0.0003	$+5.3$
1	cortisol	71 ± 7	
6	cortisol 21-acetate	42 ± 6	$+0.3$
	9α-F Group		
4	2α-methylcortisol	60 ± 15	
5	2α-methyl-9α-fluorocortisol	0.17 ± 0.01	$+3.2$

Table XI: Relative Binding Affinities of Human CBG–Steroid Complexes in the Presence and Absence of Mercaptoethanol

steroid	mercaptoethanol absent	mercaptoethanol present
cortisol	100	100
cortisone	7	7
corticosterone	143	135
epicorticosterone	19	20
11-deoxycortisol	107	90
deoxycorticosterone	107	96
17-hydroxyprogesterone	93	89
11α-hydroxyprogesterone	7	14
17-acetoxyprogesterone	2	1
progesterone	86	83
19-norprogesterone	0.5	7
cortisol acetate	31	59
prednisolone	28	52
5α-pregnane-3,20-dione	4	3
5β-pregnane-3,20-dione	8	6
testosterone	6	7
17-hydroxy-4-pregnen-3-one	1	1

presence of a disulfide bond as proposed by Seal & Doe (1966) appears unlikely; denaturation of CBG by NaDodSO$_4$ or exposure to ascorbate or arsenite resulted in the appearance of one titratable sulfhydryl group which could not result if a disulfide bond existed. It appears that CBG when isolated contains two distinguishable "cysteine" residues, one oxidized and one exposed only by denaturation (Le Gaillard & Dautrevaux, 1978).

The involvement of a cysteinyl residue in the steroid binding process of human CBG is not clear. The sulfhydryl reagents, p-mercuribenzoate and N-ethylmaleimide, do not inactivate CBG (Khan & Rosner, 1977; Le Gaillard & Dautrevaux, 1977; Mickelson et al., 1980), although in some preparations one thiol function could be detected by p-mercuribenzoate (Muldoon & Westphal, 1967) or DTNB (Khan & Rosner, 1977) without reduction. The state of oxidation–reduction of CBG does affect its steroid binding affinity. The association constant of the native CBG in vivo is, therefore, unknown. Khan & Rosner (1977) have reported a cysteinyl residue near the 6β position of progesterone by affinity labeling of CBG with 6β-bromoprogesterone. This only indicates the presence of a cysteinyl residue in the binding site. In fact, it is unlikely that a cysteinyl residue would interact with a steroid molecule around the 6β position. No sulfhydryl group in CBG was titratable by 5,5'-dithiobis(2-nitrobenzoic acid) in the presence or absence of cortisol, indicating that the cysteinyl residue in the binding site near the 6 position is not accessible to this reagent (Le Gaillard & Dautrevaux, 1977). Moreover, the lack of effect by p-mercuribenzoate or N-ethylmaleimide indicates that this cysteinyl residue is buried in the binding site and not accessible to chemical modification reagents.

Since the binding affinities of the various steroids for human CBG (Table V) were determined in the presence of 20 mM mercaptoethanol, it was necessary to determine whether or not the specificity of binding, i.e., the relative binding affinities of the steroids to CBG, was the same in the presence and absence of mercaptoethanol. Therefore, the K_a values of 17 selected steroids were determined with pure human CBG in parallel equilibrium dialysis experiments with and without mercaptoethanol added. The K_a values for cortisol were set to equal 100 and the relative binding affinities calculated. It is evident from Table XI that with only a few exceptions, the relative binding affinities in the presence and absence of mercaptoethanol are indistinguishable. The binding specificities as given in Table V, therefore, are considered valid for

and β-mercaptoethanol in proportion to their concentration. Removal of the reducing agent by dialysis reversed the effect. The number of binding sites was not altered in the presence of reducing agent in agreement with published results (Le Gaillard & Dautrevaux, 1978). A possible explanation of these findings is that a cysteine residue(s) becomes oxidized during the isolation of CBG, which results in a conformation capable of binding steroids more avidly. The putative conformation is probably an artifactual one since a more reducing environment would exist in vivo. Such oxidation–reduction effect with possible conformational change of CBG may explain the observed discrepancies in association constants reported for human CBG (pH 7.4 at 4 °C) from 1.5×10^8 (Basset et al., 1977) to 3.5×10^9 M^{-1} (Schiller & Petra, 1976).

One thiol group was titratable with DTNB after denaturation with NaDodSO$_4$ and a second one in the presence of reducing agents, in agreement with published results (Le Gaillard & Dautrevaux, 1978; Defaye et al., 1980). The
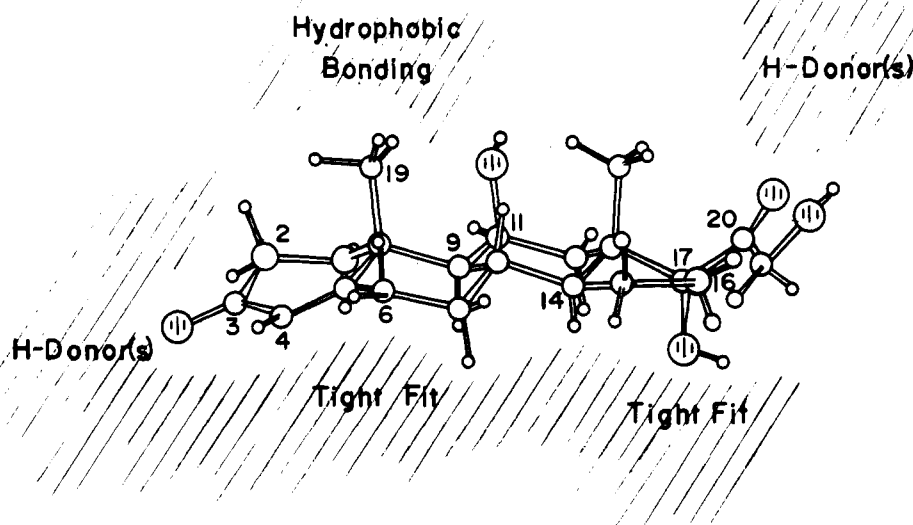
FIGURE 4: Human CBG steroid binding site, as deduced from specificity studies. Cortisol is shown as ligand.

nonreducing conditions as well as in the presence of mercaptoethanol.

The binding of cortisol and progesterone to CBG is sensitive to pH. A relatively broad pH profile was observed between pH 8 and 11.5, with cortisol having a higher affinity to CBG than progesterone. This finding is in disagreement with the results of Chan & Slaunwhite (1977), who found a relatively narrow pH profile, with progesterone binding to CBG more strongly than cortisol at all pH values examined. Le Gaillard et al. (1976) observed by circular dichroism measurements a large structural change in CBG occurring between pH 6.5 and 5.9. This conformational change may result in the binding specificity reversal observed in this study. Possibly, the CBG preparation isolated by Chan & Slaunwhite is locked into a specific conformation, resulting in a higher progesterone than cortisol binding affinity.

Similar to the results reported for guinea pig CBG (Mickelson & Westphal, 1980), the van't Hoff plot was not linear. The dependence of the enthalpy of binding upon temperature 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.

Since $\Delta H°$ is negative for all temperatures examined, the interaction of both human and guinea pig CBG with steroids is an exothermic reaction and, therefore, not mainly hydrophobic in nature. At 4 and 37 °C, $\Delta S°$ is also negative, further supporting the idea that complex formation is not entropy driven as expected if binding were hydrophobic.

By comparison of the K_a values for the individual steroids that differ in only one structural change, the effect of this change on the binding 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 free-energy contributions of steroid substituents in steroid interaction with human CBG, guinea pig CBG, and PBG are listed in Table XII.

In considering these energetic values, it should be realized that their assignment to individual substituents or single structural changes at a specific location of the steroid molecule is only a first approximation to the structure-binding problem. It is known that steroid association with a binding protein may result in a conformational change of the protein. It should be assumed, although it will be more difficult to prove, that

Table XII: Free-Energy Contributions of Steroid Substituents in Steroid Interaction with Various Proteins

	$\delta\Delta G°$ (kcal/mol)		
substituent	human CBG[a]	cavian CBG[b]	cavian PBG[c]
2α-OH	+0.4	+0.5	+4.6
3α-OH[d]	+1.6	+1.7	+1.1
3β-OH	+3.1	+1.6	+1.5
3-oxo			−2.7
6α-OH	+2.1	+2.0	+1.4
6β-OH	+2.9	+2.6	+1.0
11α-OH	+0.9	+1.0	+1.1
11β-OH	−0.1	−1.1	+1.4
11-oxo	+1.5	+0.5	+3.3
14α-OH	+2.4	+2.0	+0.2
17α-OH	+0.3	−0.2	+1.5
20α-OH[d]	+2.1	+3.0	
20β-OH[d]	+2.6	+3.2	+0.9
20-oxo	−3.2	−1.8	−0.1
21-OH	−0.1	−0.3	+0.4
2α-CH₃	+0.2	+0.6	+1.2
6α-CH₃	+1.5	+1.6	−0.8
10β-CH₃	−1.4	−1.7	+0.3
16α-CH₃	+1.2	+0.6	+0.8
17α-OAc	+3.6	+2.6	+1.3
9α-F	+3.2	+3.1	
Δ¹	+0.4	+1.2	+0.5
Δ⁴[e]	−1.6	−1.1	−1.3
Δ⁶		+0.2	−0.1

[a] This study. [b] Data from Mickelson & Westphal (1980).
[c] Data from Blanford et al. (1978). [d] Reduction of oxo group.
[e] Calculated as difference to both 5α- and 5β-dihydro compounds (averaged).

the complex formation may also lead to a conformational change of the steroid molecule. These alterations may affect the energy of binding. To what extent the steroid conformation as determined by X-ray structural analysis of crystallized steroids (Duax & Norton, 1975) can be considered indicative of the conformation in solution is not known at the present time. These various uncertainties call for caution in the assumption that the contributions to the free energy of binding seen in Table XII are solely attributable to the localized effect of the individual structural changes.

It is interesting to note that introduction of an 11β-hydroxy group has a different result for these three proteins. The free energy of binding is essentially not affected for human CBG,

Table XIII: Relative Binding of Steroids to CBG of Human and Guinea Pig

steroid	relative binding (%)		ratio human/ guinea pig
	human	guinea pig	
cortisol	100	100	1.0
progesterone	83	6	13.8
corticosterone	135	64	2.1
cortisone	7	6	1.2
11-deoxycortisol	90	14	6.4
deoxycorticosterone	96	11	8.7
11α-corticosterone [a]	20	1.8	11.1
5α-pregnanedione	3.2	2.1	1.5
5β-pregnanedione	5.9	1.4	4.2
19-norprogesterone	7	0.3	23.3
testosterone	7	0.8	8.8
aldosterone	1.1	0.1	11

[a] 11α,21-Dihydroxy-4-pregnene-3,20-dione.

decreased for PBG by 1.4 kcal/mol, and increased for guinea pig CBG by 1.1 kcal/mol. Introduction of an 11α-hydroxyl, however, results in a loss of about 1 kcal/mol of binding energy for all three proteins. A profound difference is also seen for the 20-oxo group. Binding is enhanced by 3.2 kcal/mol for human CBG, 1.8 kcal/mol for guinea pig CBG, and is not significantly affected for PBG.

The 10β-methyl group is essential for optimal binding for both guinea pig and human CBG but slightly hinders binding to PBG.

The steroid binding site of guinea pig PBG is predominantly hydrophobic in nature (Blanford et al., 1978) while the binding site in guinea pig CBG contains several hydrophilic residues capable of interacting with the hydroxy and oxo groups of cortisol (Mickelson & Westphal, 1980). Since human CBG binds cortisol and progesterone with essentially the same affinity, hydrophilic groups capable of forming hydrogen bonds with the three hydroxy groups of cortisol may be missing in human CBG. The nature of the binding site in human CBG, therefore, is somewhere between the predominantly hydrophobic site in PBG and the hydrophilic site of guinea pig CBG. Basset et al. (1978) concluded from their electron spin resonance studies that human CBG was hydrophobic in character and that the steroid binding site of PBG was more hydrophilic. These conclusions are in constrast with our binding specificity studies. This may result from the nitroxide label being removed from the steroid nucleus and therefore may not indicate the true nature of the steroid binding site.

Table XIII indicates that binding of steroids to guinea pig CBG has a higher specificity than the steroid complexes of human CBG. Although high-affinity binding of steroids to proteins generally means high specificity, the example of human vs. guinea pig CBG shows that the higher affinity can be connected with the lower specificity.

Figure 4 summarizes the nature of binding to the site in human CBG as derived from our specificity studies for the various locations in the steroid molecule. These binding characteristics complement the image of the human transcortin binding site as recently suggested by Defaye et al. (1980).

References

Ballard, P. L. (1979) in Glucocorticoid Hormone Action (Baxter, J. D., & Rousseau, G. G., Eds.) pp 25–48, Springer-Verlag, New York.

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

Basset, M., Chambaz, E. M., Defaye, G., & Metz, B. (1978) Biochimie 60, 715–724.

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

Buehner, M., & Beato, M. (1978) J. Mol. Biol. 120, 337–341.

Bush, I. E. (1957) Ciba Found. Colloq. Endocrinol. (Proc.) 11, 263–285.

Chan, D. W., & Slaunwhite, W. R., Jr. (1977) Arch. Biochem. Biophys. 182, 437–442.

Daughaday, W. H. (1956) J. Lab. Clin. Med. 48, 799–800.

Daux, W. L., & Norton, D. A. (1975) Atlas of Steroid Structure, Vol. 1, Plenum Press, New York.

Defaye, G., Basset, M., Monnier, N., & Chambaz, E. M. (1980) Biochim. Biophys. Acta 623, 280–294.

Khan, M. S., & Rosner, W. (1977) J. Biol. Chem. 252, 1895–1900.

Le Gaillard, F., & Dautrevaux, M. (1977) Biochim. Biophys. Acta 495, 312–323.

Le Gaillard, F., & Dautrevaux, M. (1978) FEBS Lett. 94, 63–67.

Le Gaillard, F., Han, K. K., & Dautrevaux, M. (1975) Biochimie 57, 559–568.

Le Gaillard, F., Aubert, J. P., Dautrevaux, M., & Loucheux-Lefebvre, M. H. (1976) FEBS Lett. 64, 278–284.

McPherson, A., Mickelson, K. E., & Westphal, U. (1980) J. Steroid Biochem. 13, 991–992.

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

Mickelson, K. E., & Westphal, U. (1980) Biochemistry 19, 585–590.

Mickelson, K. E., Forsthoefel, J. A., & Westphal, U. (1980) Fed. Proc., Fed. Am. Soc. Exp. Biol. 39, 1906.

Mornon, J. P., Fridlansky, F., Bally, R., & Milgrom, E. (1980) J. Mol. Biol. 137, 415–429.

Muldoon, T. G., & Westphal, U. (1967) J. Biol. Chem. 242, 5636–5643.

Rosner, W. (1976) Prog. Clin. Biol. Res. 5, 377–395.

Sandberg, A. A., & Slaunwhite, W. R., Jr. (1958) J. Clin. Invest. 37, 928.

Schiller, H. A., & Petra, P. H. (1976) J. Steroid Biochem. 7, 55–59.

Schmid, K. (1953) J. Am. Chem. Soc. 75, 60–68.

Seal, U. S., & Doe, R. P. (1966) in Steroid Dynamics (Pincus, G., Nakao, T., & Tait, J. F., Eds.) pp 63–90, Academic Press, New York.

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

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

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

Westphal, U. (1980) in Steroid Receptors and Hormone-Dependent Neoplasia (Wittliff, J. L., & Dapunt, O., Eds.) pp 1–17, Masson Publishing USA, Inc., New York.