Nature of Steroid–Glucocorticoid Receptor Interactions: Thermodynamic Analysis of the Binding Reaction†

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ABSTRACT: To understand the nature of the interactions of hormones with receptors and other proteins, we analyzed the thermodynamics and structural requirements of steroid binding by glucocorticoid receptors from rat hepatoma tissue culture (HTC) cells. The entropy (ΔS) for association is positive at 0 °C and the enthalpy (ΔH) and entropy of association decrease as temperature increases, effects typical of reactions dominated by hydrophobic interactions. The association rate constants at various temperatures indicate that the entropy of activation ($\Delta S^{\ddagger}$) is positive, also characteristic of hydrophobic interactions. A mathematical reexamination of the thermodynamics of the reported association of progesterone and corticosterone with plasma proteins was also consistent with a binding process involving principally hydrophobic effects, implying that steroid protein interactions may generally be driven by this process. To test whether such hydrophobic interactions could account for the observed high affinity steroid receptor binding, the binding free energy was calculated using the surface areas of the steroids and literature estimates from other protein–protein interactions. When the association of both faces of the steroid with the receptor was assumed, the calculated estimates of binding free energy accounted for about half of the observed values, whereas the interaction of only one face of the steroid would result in a positive free energy of association. On this basis, the steroid must be enveloped by the receptor for binding to occur. We also examined the effects of individual substitutions on the binding of the steroid molecule. The free energy contributions by the individual substituents are approximately additive in determining the total free energy of the binding and can be used roughly to predict the affinity of unknown steroids for the receptor. Four structural features of the steroids were found to account for receptor affinity: surface area, A-ring conformation, size of the 9α-substituent, and polarity. A general mathematical expression was developed relating affinity to the four variables; the resulting equation accurately predicts the affinity of steroids for the glucocorticoid receptor. This approach should be useful generally in understanding the major determinants of hormone receptor interactions and in predicting the activity of hormonal drugs prior to synthesis.

In several studies of steroid–protein interactions, efforts were made to examine the topography of the active site through comparative binding or biological studies (Ashley & Westphal, 1958, 1959, 1962; Rousseau et al., 1972; Smith et al., 1974; Kontula et al., 1975) or through affinity labeling (Wolff et al., 1975; Marver et al., 1976; Chin & Warren, 1968, 1970; Katzenellenbogen et al., 1973, 1977; Liarakos & May, 1969; Solo & Gardner, 1968, 1971). In a few cases, in addition to the affinity and kinetic constants, the entropy and enthalpy for the receptor–steroid interaction were calculated (Koblinsky et al., 1972; Schaumberg & Bojensen, 1968). However, little information is available about the chemical nature of the steroid–receptor interaction, the corresponding transition state, or the quantitative contributions of the individual substituents on the steroid molecule to the binding reaction. It would be expected that the receptor–steroid interaction is due to hydrophobic bonding reinforced by hydrogen bonding by the hydroxyl and keto groups of the steroid, but no analyses have been made to determine whether the observed thermodynamic functions for association are consistent with this hypothesis, or whether hydrophobic bonding can explain quantitatively the high affinities that are common in steroid–protein associations.

In this paper we describe some thermodynamic aspects of the interactions of a number of steroids with the glucocorticoid receptors from cultured rat hepatoma cells. The relative potencies of steroid analogues in producing enzyme induction in these cells are well correlated with their ability to compete with [^{3}H]dexamethasone binding to isolated cytosol (Baxter & Ivarie, 1977; Baxter & Tomkins, 1971; Rousseau et al., 1972; Rousseau & Schmidt, 1973). The measured thermodynamic parameters have been considered in an examination of the determinants of binding, the topography of the receptor site and the nature of the binding forces. These data have also been incorporated into a quantitative expression relating structure and binding that may have general applicability.

Materials and Methods

We are grateful for gifts of the following steroids:[1] triamcinolone and triamcinolone acetonide (Lederle Laboratories); fluorandrenolide, paramethasone, and 6α-fluoro-16α-hydroxycortisol (Eli Lilly and Co.); dexamethasone, 21-deoxydexamethasone, and 9α-fluorocortisol (Merck Sharp and Dohme); dichlorisone (Schering Corp.). Other steroids were purchased from Steraloids, Inc., or Sigma and were the best grade available. [^{3}H]Dexamethasone (22.6 Ci/mmol) and [^{3}H]corticosterone (92 Ci/mmol) were from New England Nuclear.

Cultured rat hepatoma (HTC) cells were grown in the absence of added steroid, harvested, and stored at −20 °C; cytosol was prepared as previously described (Ballard et al., 1975). Solutions of steroid were prepared as previously described (Ballard et al., 1975). Cytosol was incubated with [^{3}H]dexamethasone (3 nM final concentration) with or without competing steroids (14–18 h at 0 °C) and specific binding was

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0006-2960/78/0417-3201$01.00/0

TABLE I: Calculated Values of the Enthalpic and Entropic Terms for Corticosterone Binding to the Glucocorticoid Receptor and for Steroid Binding to Plasma-Binding Proteins as Analyzed by a Second Degree Polynomial Fit to the Data Points.[a]

system		temperature (°C) 0	5	10	15	20
1. glucocorticoid receptor (corticosterone) (Figure 4)	ΔH[b]	1200	0	−1000	−2 200	−3 200
	ΔS[c]	29	24	20	15	12
2. human CBG corticosterone (Figure 1)	ΔH	2200	~320	~2700	−5 000	−7 200
	ΔS	48	38	30	21	13
3. human serum albumin (HSA) (progesterone) (Figure 4)	ΔH	2400	520	−1250	−3 000	−4 700
	ΔS	37	30	23	16	10
4. serum α_1-acid-protein (AAG)[a] (progresterone) (Figure 4)	ΔH	1354	−2888	−6980	−11 100	−14 740
	ΔS	42	29	12	0.6	−12.5

[a] Data with the glucocorticoid receptors are taken from the present study. The data for CBG are from Westphal (1967), those for AAG are from Ganguly et al. (1967), and those for HSA are from Westphal (1964, 1971). The polynomial functions employed were: corticosterone-glucocorticoid receptor, $1.62 \times 10^7(1/T^2) + 118\,000(1/T) - 195 = \ln K_a$; progesterone HSA, $-4.58 \times 10^6(1/T)^2 + 32900(1/T) - 47.5 = \ln K_a$; progesterone-AAG, $-7.07 \times 10^6(1/T)^2 + 50\,600(1/T) - 47.5 = -9.43 \times 10^6(1/T) - 77 = \ln K_a$; and corticosterone-CBG, $\ln C = -9.43 \times 10^6(1/T)^2 + 6.8 \times 10^4(1/T) - 122.36$. [b] cal/mol. [c] eu.

measured by the charcoal assay as previously described (Ballard et al., 1975). Similar conditions were used for incubations with [^{3}H]corticosterone except that, for temperature dependency studies, the incubations at higher temperatures (to 14 °C) involved progressively shorter incubation times (down to 2.5 h) that allowed for maximal steroid binding (based on kinetic studies) but minimized denaturation. For kinetic studies with [^{3}H]corticosterone, the time of incubation was varied; otherwise the conditions were identical.

Steroid analogue affinities were calculated from competition studies. The ratio of the concentration of a particular steroid required to inhibit by 50% the binding of 1.0 nM [^{3}H]dexamethasone (C_{50}) to the C_{50} for dexamethasone was obtained. This ratio, when multiplied by the K_D for dexamethasone, gives the receptor affinity of the steroid. The K_D for dexamethasone, determined by Scatchard analyses of the binding of tritiated material (Rousseau & Schmidt, 1973), ranged from 2.1 to 5.3 nM in 20 experiments. For this approach to be valid the C_{50} must reflect the concentration of free (rather than total) analogue; under our conditions about 80% of the total steroid was free as shown by independent determination using ^{3}H-labeled steroid. The results were adjusted by a small correction to account for this.

All calculations and curve fitting and regression analyses were carried out on the PROPHET system. A detailed description of this specialized computer resource developed by the Chemical/Biological Information Handling Program of the National Institutes of Health is published elsewhere (Raub, 1974).

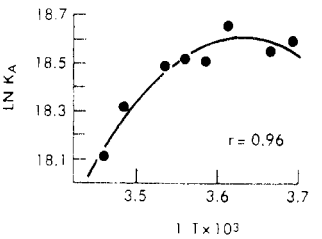


FIGURE 1: Plot of ln K_A vs. $1/T$ for corticosterone binding to the glucocorticoid receptor at −2 to 16 °C (T in K).

Results

Free Energy, Enthalpy, and Entropy of Steroid-Glucocorticoid Receptor Binding. Whereas the free energy for the steroid-receptor interactions can be calculated from the equilibrium association constants (K_A), determinations of the enthalpy and entropy require knowledge of the temperature influence on the binding constant. We undertook such a temperature dependency study of the binding of corticosterone to the glucocorticoid receptor and analyzed ln K_A as a function of $1/T$. Unlike the results for many simple association reactions (Moore, 1962), ΔH has marked temperature dependence over the range examined and the plot of ln K_A as a function of $1/T$, therefore, is not a straight line. Since $d \ln K_A/d(1/T) = -\Delta H/R$, the enthalpy ($\Delta H$) could be determined from the slope of the second degree polynomial least-squares fitted to the data points. The curve shown in Figure 1 provides a good fit to the data points ($r = 0.96$) and the decreasing slope with increasing $1/T$ indicates that the enthalpy decreases as the

[1] Abbreviated names for compounds employed in this study are: 6α-methylprednisolone, 6α-methyl-11β,17,21-trihydroxypregna-1,4-diene-3,20-dione; dexamethasone, 9-fluoro-11β,17,21-trihydroxy-16α-methylpregna-1,4-diene-3,20-dione; betamethasone, 9-fluoro-11β,17,21-trihydroxy-16β-methylpregna-1,4-diene-3,20-dione; 9α-fluorocortisol, 9-fluoro-11β,17,21-trihydroxypregn-4-ene-3,20-dione; dichlorisone, 9,11β-dichloro-17,21-dihydroxypregn-4-ene-3,20-dione; corticosterone, 11β,21-dihydroxypregn-4-ene-3,20-dione; 9α-chlorocortisol, 9-chloro-11β,17,21-trihydroxypregn-4-ene-3,20-dione; 6α-fluorocortisol, 6α-fluoro-11β,17,21-trihydroxypregn-4-ene-3,20-dione; prednisolone, 11β,17,21-trihydroxypregna-1,4-diene-3,20-dione; cortisol, 11β,17,21-trihydroxypregn-4-ene-3,20-dione; 11α-hydroxyprogesterone, 11α-hydroxypregn-4-ene-3,20-dione; aldosterone, 11β,21-dihydroxy-3,20-dioxopregn-4-en-18-al; deoxycorticosterone, 21-hydroxypregn-4-ene-3,20-dione; cortexolone, 17,21-dihydroxypregn-4-ene-3,20-dione; progesterone, pregn-4-ene-3,20-dione; 17α-hydroxyprogesterone, 17-hydroxypregn-4-ene-3,20-dione; prednisone, 17,21-dihydroxypregna-1,4-diene-3,11,20-trione; 9α-bromocortisol, 9-bromo-11β,17,21-trihydroxypregn-4-ene-3,20-dione; cortisone, 17,21-dihydroxypregn-4-ene-3,11,20-trione; 9α-methoxycortisol, 9-methoxy-11β,17,21-trihydroxypregn-4-ene-3,20-dione; 11-oxoprogesterone, pregn-4-ene-3,11,20-trione; 21-deoxydexamethasone, 9-fluoro-11β,17-dihydroxy-16α-methylpregna-1,4-diene-3,20-dione; paramethasone, 6α-fluoro-11β,17,21-trihydroxy-16α-methylpregna-1,4-diene-3,20-dione; fluocinolone, 6α,9-difluoro-11β,16α,17,21-tetrahydroxypregna-1,4-diene-3,20-dione; fluocinolone acetonide, 6α,9-difluoro-11β,21-dihydroxy-16α,17[(1-methylethylidene)bis(oxy)]pregna-1,4-diene-3,20-dione; 6α-fluoro-16α-hydroxycortisol, 6α-fluoro-11β,16α,17,21-tetrahydroxypregn-4-ene-3,20-dione; flurandrenolide, 6α-fluoro-11β,21-dihydroxy-16α,17-[(1-methylethylidene)bis(oxy)]pregn-4-ene-3,20-dione; triamcinolone acetonide, 9-fluoro-11β,21-dihydroxy-16α,17-[(1-methylethylidene) bis(oxy)]pregna-1,4-diene-3,20-dione; 9α-fluoroprednisolone, 9-fluoro-11β,17,21-trihydroxypregna-1,4-diene-3,20-dione.

TABLE II: Estimation of the Extent of Hydrophobic Binding of Steroid to the Glucocorticoid Receptor.

free energies (kcal/mol)	one face of steroid involved			two faces of steroid involved		
	progesterone	corticosterone	cortisol	progesterone	corticosterone	cortisol
$\Delta G_{(obsd)}$[a]	−8.6	−10.2	−9.6	−8.6	−10.2	−9.6
ΔG_t[b]	+12.3	+12.3	+12.3	+12.3	+12.3	+12.3
$\Delta G_{s(steroid)}$[c]	−4.0	−4.3	−4.5	−8.1	−8.6	−9.0
$\Delta G_{s(receptor)}$[c]	−4.0	−4.3	−4.5	−8.1	−8.6	−9.0
$\Delta G_{(calcd)}$[d]	+4.3	+3.7	+3.3	−3.9	−4.9	−5.7
$(\Delta G_{s(steroid)} + \Delta G_{s(receptor)})/(\Delta G_{(obsd)} - \Delta G_t)$	38%	38%	41%	78%	76%	82%

[a] $\Delta G_{(obsd)} = -RT \ln K_{assoc}$ at 0 °C. [b] Determined by Page & Jencks (1971) to be the average translational and rotational energy loss for a bimolecular reaction at 0 °C. Calculation of the translational and rotational entropy loss through statistical thermodynamics of the steroid molecule in a bimolecular reaction finds the entropy loss to be approximately 76 eu (20 kcal). The translational and rotational energy loss of the receptor in a bimolecular reaction would be expected to be minimal as association of the acceptor with the relatively small steroid molecule would have very little effect on the translational and rotational entropy of the receptor as a whole. Moreover, Page & Jencks have noted that, in equilibrium complexes, some of the translational and rotational entropy loss in forming the complex is converted into a low frequency stretching vibration, into an internal rotation, and into four low-frequency bending modes. These may contribute up to 30 eu (9 kcal) of residue entropy to the loose complex. Thus, the estimate of 45 eu for the translational and rotational entropy loss for steroid complex formation appears reasonable. [c] Determined by multiplying one-half or one times the Bondi surface area of the steroids times 22.5 cal/Å^2 (see text). [d] $\Delta G_{(calcd)} = \Delta G_t + \Delta G_{s(steroid)} + \Delta G_{s(receptor)}$.

temperature increases. The entropy (ΔS) was obtained from the free energy of the binding and the enthalpy; the entropy also decreases as the temperature increases. These determinations are shown in Table I. The observed temperature influences on the thermodynamic parameters would be expected if hydrophobic interactions were the main driving forces for the association; in such interactions, there is a negative change in heat capacity (ΔC_p) upon association, and consequently both ΔH and ΔS decrease as the temperature increases (Brandts, 1969). We observed a change of −240 cal deg^{-1} mol^{-1} in ΔC_p for the corticosterone-receptor association, in reasonable agreement with the figure of about −20 cal deg^{-1} mol^{-1} given by Edelhock & Osborne (1976) for each $-CH_2-$ group transferred from water to a nonpolar medium. Some of this change in ΔC_p may result from conformational changes in the protein which occur on binding. For example, typical ΔC_p values for protein denaturation are as large as 3000 cal deg^{-1} mol^{-1} (in this case the sign of ΔC_p is opposite (positive) since denaturation involves the exposure of nonpolar groups to water).

Energy of Activation. To elucidate the nature of the transition state for steroid-glucocorticoid receptor complex formation, the activation energy for dexamethasone binding to the receptors was determined from the kinetics of the process at several temperatures. The second-order association rate constants were calculated (Moore, 1962) from the slopes of plots of $1/(A-B)/\ln[B(A-X)/A(B-X]$ vs. time at each temperature, where A = initial concentration of bound steroid at time t (Figure 2). As in the work of Hansen et al. (1976), the rate constant was more affected by temperature changes than was the equilibrium constant. From the temperature dependence of the rate constant (Moore, 1962), we found the enthalpy of activation to be 12.8 kcal/mol and the entropy of activation ($\Delta S^{\pm}$) to be 17.2 eu. The fact that $\Delta S^{\pm}$ is positive is further evidence that the driving forces for the formation of the transition state are hydrophobic since, for example, a negative value would be expected if there were more hydrogen bonds in the transition state than the reactants.

In using the above equation to determine the association rate constant, we neglected the dissociation reaction (k_r), but this does not vitiate our conclusions about the nature of the transition state. The incorporation of k_r into the analysis *increases* k_f and the increases will be greater at higher temperatures. Hence, consideration of k_r will *increase* $\Delta H^{\pm}$ and $\Delta S^{\pm}$ giving even more positive values for these parameters. For example,

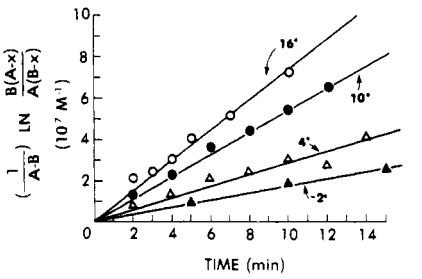


FIGURE 2: Kinetics of dexamethasone binding by the glucocorticoid receptor.

by using the polynomial expression in Figure 1 as an estimate of the temperature dependence of the reciprocal of the dissociation constant of dexamethasone, k_f and k_r values consistent with these data have been calculated. The incorporation of k_r into the model increases k_f by ~30% at the highest temperature and by ~10% at the lowest temperature. This increases the $\Delta H^{\pm}$ by about 1 kcal/mol and increases the $\Delta S^{\pm}$ by about 8 eu, changes which are in harmony with our model.

Analysis of the Role of Hydrophobic Binding. It is likely that hydrogen bonding contributes very little to the overall driving force for protein-protein interactions because the net difference in free energy between protein-protein hydrogen bonding in the bound state and hydrogen bonding between protein and water in the unbound state has been shown to be small (Klotz & Franzen, 1962). Steroid-protein binding systems would not be significantly different and the main function of hydrogen bonds would be in the structural recognition of different steroids. Therefore, it is pertinent to inquire (1) if hydrophobic interactions could account for high affinity receptor binding of steroids and (2) the extent of the surface (i.e., one side or both sides of the steroid) that would have to be involved in the binding to achieve a high-affinity interaction. Chothia has noted a relationship between the surface area of proteins and the strength of protein-protein interactions (Chothia, 1974, 1975; Janin & Chothia, 1976):

$$\Delta G = \Delta G_t + \Delta G_S$$

where ΔG is the observed free energy of association, ΔG_s is the free energy of association which is proportional to the surface area involved in hydrophobic bonding and is equal to ΔG_s(steroid) + ΔG_s(receptor). ΔG_t is the free energy associ-

TABLE III: Steroids Employed in Binding Study along with Parameters Employed in the Linear Regression Analysis.[a]

compound[b]	surface area (Å)^2	polar interaction	C-3–C-17 (Å)[c]	9α size	obsd log K_D	calcd log K_D
6α-methylprednisolone	50.8	1	−0.26	0	−8.94	−8.65
dexamethasone	60.9	1	−0.26	0	−8.47	−8.88
betamethasone	60.9	1	−0.26	0	−8.55	−8.88
9α-fluorocortisol	47.7	1	−0.18	0	−8.27	−8.46
dichlorisone	55.3	0	−0.26	0	−8.64	−8.16
corticosterone	22.7	2	−0.05	0	−8.18	−8.29
9α-chlorocortisol	58.3	1	0.13	0	−8.24	−8.24
6α-fluorocortisol	44.2	1	0.05	0	−8.14	−8.04
prednisolone	28.6	1	−0.26	0	−8.07[d]	−8.15
cortisol	37.5	1	0.06	0	−7.67	−7.87
11β-hydroxyprogesterone	11.3	1	0.00	0	−7.51	−7.37
aldosterone	22.7	1	0.10	0	−7.46[d]	−7.48
deoxycorticosterone	11.5	1	−0.01	0	−7.36[d]	−7.38
cortexolone	26.2	0	0.08	0	−7.06[d]	−6.99
progesterone	0.0	0	0.00	0	−6.85	−6.52
17α-hydroxyprogesterone	14.8	−1	−0.04	0	−6.48[d]	−6.32
prednisone	22.4	−2	−0.26	0	−6.18[d]	−6.21
9α-bromocortisol	62.8	1	0.05	0.3	−6.16	−6.63
cortisone	31.4	−2	−0.20	0	−6.00[d]	−6.32
9α-methoxycortisol	73.2	1	−0.02	0.5	−5.90	−5.63
11-oxoprogesterone	5.2	−2	−0.20	0	−5.51	−5.72
21-deoxydexamethasone	49.5	0	−0.26	0	−8.21	−8.03
paramethasone	57.5	1	−0.26	0	−8.88	−8.80
fluocinolone	56.6	0	−0.34	0	−8.50	−8.31
fluocinolone acetonide	89.0	0	−0.34	0	−8.84	−9.04
6α-fluoro-16α-hydroxycortisol	55.5	0	0.05	0	−7.45	−7.70
flurandrenolide	87.8	0	0.05	0	−8.74	−8.43
triamcinolone acetonide	82.4	0	−0.26	0	−8.95	−8.77
6α-fluoroprednisolone	35.2	1	−0.26	0	−8.38	−8.30

[a] The surface area, polar interaction term, C-3–C-17 distance, 9α-size function, and the calculated (calcd) log K_D were obtained as described in the text. The observed (obsd) log K_ds were determined as described in Materials and Methods. The values reported reflect the mean of 3–13 experiments (except that the data for 11β-OH-progesterone were taken from a single binding curve) in which at least six different concentrations of competitor were employed. The experimental variations were similar to those previously reported (Ballard et al., 1975). [b] See footnote 1 regarding nomenclature. [c] C-3–C-17 distance taken from x-ray structures where available; where no x-ray structure is available, we used a steroid which had a similar A and B ring substitution pattern to determine this distance. The corresponding distance in progesterone (8.56 Å) has been subtracted from all listed values. [d] Data taken from Ballard et al. (1975).

ated with translational and rotational entropy loss when two molecules are brought together. By analyzing model peptide systems it was found that the relation between "hydrophobic energy" and surface area was approximately 24 cal/Å^2.

We applied this concept to the steroid–receptor complex. For ΔG_t we calculated the value of 45 eu (12.3 kcal/mol at 0 °C, legend to Table II) from considerations (Page & Jencks, 1971) of the average translational and rotational energy loss for a bimolecular reaction. Bondi surface areas (Bondi, 1965) were utilized in the determination of the surface area. Janin & Chothia's (1976) value of 24 cal/Å^2 at 25 °C was corrected[2] to 22.5 cal/Å^2 for processes occurring at 0 °C and used in the determination of the contribution of the receptor (ΔG_s(receptor)) to the hydrophobic free energy of binding. The surface areas of the receptor and steroid required for the calculation of ΔG_s(receptor) and ΔG_s(steroid), respectively, were taken as one-half (for a one-face binding analysis) or equal to (for a two-face analysis) the Bondi surface area of the steroid. The results (Table II) indicate that the "one-face" model is incorrect since a *positive* value for ΔG is obtained. By contrast, the "two-face" model predicts a negative ΔG of about half the magnitude observed.

Most of the shortfall in the calculated free energy of the two-face model could be due to neglecting van der Waals forces. Assuming the protein density at the receptor site to be ~1.4 g/cm^3 (Low & Richards, 1954) and the free energy contribution for each van der Waals contact as −0.2 kcal/mol (Ramachandran & Sasisekharan, 1968), a free energy of binding of ca. −4 kcal/mol could result from van der Waals forces in the two-face model. Any remaining deficit may be caused by overestimating ΔG_t through too low a value for entropy associated with low frequency vibrations of the steroid–receptor complex. Even though there is uncertainty in these estimates, the results are consistent only with the view that these cases of high affinity steroid–protein can be generated through hydrophobic interactions and that most of the steroid is enveloped by the receptor.

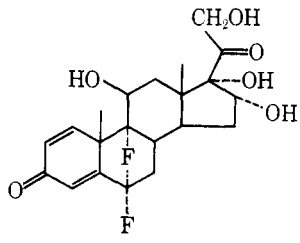
An alternative attempt was made to use Tanford's procedure (Nozaki & Tanford, 1971) to estimate directly the hydrophobicity (ΔG_s(steroid)) of progesterone and cortisol from their solubilities in ethanol and water (Bischoff & Pilhorn, 1948; Merck Index, 1976). The values obtained had only 33% and 57% the magnitude of the corresponding figures derived from the surface area of cortisol and progesterone, respectively. Such underestimates are not unexpected since polar groups on

[2] Janin & Chothia's value of 24 cal/Å for processes occurring at 25 °C was corrected to the appropriate value at 0 °C by calculating the difference in hydrophobicity per CH_2 group at 298 and 273 K. From the data of Tanford (1973), the free energy of transfer from the neat liquid to water is 821 cal/CH_2 group at 298 K and 768 cal/CH_2 group at 273 K. Thus, we used a value of 24 cal/Å^2 × 768/821 = 22.5 cal/Å in our surface area calculations.

the steroid decrease solubility in ethanol but may form specific hydrogen bonds within the receptor.

Contributions of Individual Substituents to the Steroid-Glucocorticoid Receptor Interactions. We next analyzed the contributions of specific structural features of the steroid to receptor binding. The apparent equilibrium dissociation constants of a series of steroids for binding to the hepatoma cell receptor were measured (Table III). The difference or average difference in the free energy of binding of one to three pairs of steroids which differed in only one substituent was calculated to afford the empirical free energy contribution to binding for that substituent (Table IV).

The free energy group increments can be added to predict the binding constants of steroids of unknown affinity. For example, we calculated the free energy of binding of fluocinolone



(Table III) from the individual contributions listed in Table IV (fluocinolone was not used to determine the individual group increments). This calculation predicts that fluocinolone would bind to the receptor with a free energy −1.32 kcal/mol more negative than progesterone; the actual value is −1.65 kcal/mol. Although the agreement is reasonable, it appears that the increments are not fully independent of one another. Thus, the free energy increment due to the 21-hydroxy group was found to be −0.64 kcal in deoxycorticosterone and −0.72 kcal in cortexolone, but only −0.32 kcal in dexamethasone. the data in Table IV indicate that the free energy contribution of a given group becomes smaller in a molecule incorporating several of such groups. Therefore, the specific contributions on particular structural features were examined in detail.

The receptor present in the cytosol of HTC cells appears to have specific sites for hydrogen bonding. The C-3 and C-20 ketone groups are of major importance; reduction to hydroxyl greatly diminishes binding (Baxter & Tomkins, 1971; Rousseau et al., 1972; Ballard et al., 1975), suggesting hydrogen bond donors in the receptor site at corresponding positions. Likewise, C-11 and C-21 hydroxyl groups are important for binding, indicating hydrogen bond acceptors in their proximity. Unlike the C-11 and C-21 hydroxyl groups and unlike the case with glucocorticoid receptors from human sources (Ballard et al., 1975), the C-17 hydroxyl group decreases binding. The receptor either cannot accommodate the size of the group in that position, or cannot tolerate the introduction of a polar group into a nonpolar environment.

The addition of certain other relatively nonpolar groups to the steroid molecule (e.g., the 6α-Me) generally increases the binding; there is a good correspondence between the surface area of the substituent added and the free energy of binding gained.

The conformation of the A ring (Weeks et al., 1973) has a pronounced effect on the binding of the steroid to the receptor. We employed the difference in the C-3 to C-17 distance in the steroids, relative to progesterone, as a measure of the A-ring conformation, since we found this distance to be strongly influenced by such conformational changes; the shorter the C-3 to C-17 distance, the greater was the binding. The inclusion of a fluoro group at C-9 or a double bond at C-1 and C-2 had the greatest effect on the A-ring conformation; other substituents had varying effects (Table III).

TABLE IV: Steroid Substituent Free Energy Contributions to Binding to the Glucocorticoid Receptor (kcal/mol).

substituent	ΔG (kcal/mol)[a]
Δ1 [b]	−0.29
6α-F [c]	−0.36
6α-methyl [d]	−1.09
9α-F [e]	−0.57
9α-Cl [f]	−0.71
9α-Br [g]	+1.89
9α-methoxy [h]	+2.21
11β-OH [i]	−0.89
11α-OH [j]	−0.82
11β-OH → 11-keto [k]	+2.23
11-keto [l]	+1.67
16α-methyl [m]	−0.11
16β-methyl [n]	−0.21
16α-OH [o]	+0.86
17α-OH [p]	+0.49
21-OH [q]	−0.56

[a] Calculated by the formula $RT \ln (K_D$ substituted steroid$/K_D$ unsubstituted steroid) using the observed K_D values from Table IV for the steroid pairs indicated, and averaging the results where more than a single pair was available. [b] 9α-Fluoroprednisolone vs. 9α-fluorocortisol, prednisolone vs. cortisol, prednisone vs. cortisone. [c] Triamcinolone acetonide vs. fluocinolone acetonide, 6α-fluoro-16α-hydroxycortisol vs. 6α-fluorocortisol. [d] 6α-Methylprednisolone vs. prednisolone. [e] 9α-Fluorocortisol vs. cortisol, 9α-fluoroprednisolone vs. prednisolone. [f] 9α-Chlorocortisol vs. cortisol. [g] 9α-Bromocortisol vs. cortisol. [h] 9α-Methoxycortisol vs. cortisol. [i] Corticosterone vs. deoxycorticosterone, cortisol vs. cortexolone. [j] 11α-Hydroxyprogesterone vs. progesterone. [k] Cortisone vs. cortisol, prednisone vs. prednisolone. [l] 11-Oxoprogesterone vs. progesterone. [m] Dexamethasone vs. 9α-fluoroprednisolone. [n] Betamethasone vs. 9α-fluoroprednisolone. [o] 6α-Fluoro-16α-hydroxycortisol vs. 6α-fluorocortisol. [p] Cortisol vs. corticosterone, cortexolone vs. deoxycorticosterone, 17α-hydroxyprogesterone vs. progesterone. [q] Deoxycorticosterone vs. progesterone, cortexolone vs. 17α-hydroxyprogesterone, dexamethasone vs. 21-deoxydexamethasone.

It is noteworthy that C-9 methoxy and bromo substituents result in derivatives with low binding affinities, notwithstanding the surface area increases they produce. Evidently, the size of these substituents prevents the proper engagement of the steroid within the receptor site, or induces a conformational change in the receptor such that binding is significantly altered.

General Expression of Receptor Binding Affinity. The data presented above allow the possibility of formulating a mathematical expression to predict the binding affinity of the steroid and receptor on a structural basis. Such quantitative structure–activity relationships have been derived for the pharmacological action of many drugs (Hansch, 1971). Normally such an analysis is carried out stochastically through an empirical, probabilistic approach. By contrast, our analyses of the qualitative structure–activity relationships and the thermodynamics of steroid–receptor binding suggest the use of a more deterministic approach in formulating a model. In fact, the binding of the steroids to receptor appears to be determined principally by four factors: (1) the hydrophobicities (surface areas) of substituents on the steroid; (2) the presence or absence of polar groups in specific areas of the steroid, i.e., C-11, C-17, and C-21; (3) the conformation of the A ring as measured by the C-3 to C-17 distance; and (4) the size of any given substituent. Clearly, 1 and 2 are directly related to hydrophobic bonding as previously discussed. Factors 3 and 4 are related

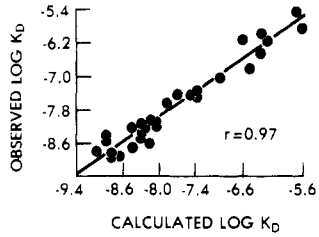


FIGURE 3: Plot of calculated eq 1 vs. observed logarithm of the equilibrium dissociation constant (K_D).

to the ability of the steroid to fit into its site in the receptor.

To test this hypothesis, a computer-assisted linear multiple regression analysis was made relating the four parameters to the logarithm of the equilibrium dissociation constant. The first parameter, the surface area (SA) employed for each derivative, was obtained from the summation of the Bondi surface area of each substituent in excess to that of the basic progesterone skeleton (Table III). To represent the interaction of polar groups with the receptor, recourse was had to a de novo variable. For each hydroxyl group at C-11 and/or C-21, an arbitrary value of +1 was assigned to account for the specific attraction to an H-bond donor in the receptor. If no group resided in those positions, a value of 0 was assigned. A value of −1 was made for the presence of each C-17 or C-16 polar group, since here it was necessary to account for the consequence of placing a polar group in a nonpolar region of the receptor. A value of −2 was established for the presence of an 11-keto functionality to express, first, the conformational change associated with a change in hybridization from sp^3 to sp^2 at C-11 and, second, the undesirable dipole–dipole interaction of the 11-keto group with the putative hydrogen bond acceptor of the receptor in that position. The total of these de novo values was used for the second parameter as the polar interaction term.

The third parameter (tilt) is related to the overall shape of the steroid; it is known that the shape of the steroid can range from a quite planar structure to a bowed structure (Weeks et al., 1973) and this can be parameterized through the C-3 to C-17 distance in angstroms. A computer analysis of the change in intercarbon distances indicated that the C-3, C-17 distance is the most sensitive intercarbon distance in the steroid molecule to the bowing of the molecule as a whole. The value employed was the difference in this distance for a given steroid from the corresponding value in progesterone.

The fourth parameter (X) is a means for expressing the size limitation of the presumed hydrophobic pocket into which the 9-substituent projects. The value employed was $R_X - R_{Cl}$ where R_X is the radial extension in angstroms which is projected by the 9-substituent from the pregnane ring system and R_{Cl} is the corresponding distance for the case of Cl. Any value of $R_X - R_{Cl}$ less than 0 is set equal to 0. Thus, this parameter has values of 0 for H, F, and Cl but expresses a size limitation for groups larger than Cl, such as methoxy and bromo.

The steroids, along with the values for each parameter, are shown in Table IV. An excellent correlation was found relating these four parameters to the logarithm of the equilibrium dissociation constant:

$$\log K_D = -0.022(\pm 0.002)SA - 0.59(\pm 0.05)P + 1.50(\pm 0.35)tilt + 6.10(\pm 0.49)X - 6.52 \quad (1)$$

For this equation: n, the number of data points, is 29; r, the multiple correlation coefficient, is 0.97; s, the standard deviation of the regression, is 0.26; and F, a measure of the significance of the regression, is 106. This value of F indicates that the equation is statistically significant at better than the 0.999 level (Croxton & Cowdon, 1955). The F statistic for each variable in the equation is SA, 10.4; X, 28.7; tilt, 17.8; and P, 67.2; each parameter is significant at better than the 0.999 level. A squared correlation matrix of the parameters employed showed no coefficient that exceeded 0.08, indicating that all of the variables are independent. The percentage of variance (r^2) accounted for by each parameter is X, 35%; P, 29%; SA, 22%; and tilt, 4%. Shown in Figure 3 is a plot of the calculated vs. the observed logarithm of the equilibrium dissociation constant. The straight line in the graph represents a 1:1 correspondence between observed and calculated K_Ds. As can be seen in Figure 3, the above equation describes the steroid-receptor interactions in a quantitative manner. An examination of the physical significance of this equation is of interest, since it should reflect the thermodynamic contributions of the substituents. The equation represents the effects of substituents on the K_D of progesterone, given by the intercept (−6.52). By multiplying by $2.303RT$, the equation is transformed[3] to

$$\Delta G_{(assoc)}\ (cal) = -27\ (\pm 2)SA\ (cal/Å^2) - 734(\pm 62)P\ (cal/P) + 1865(\pm 436)\ tilt\ (cal/Å) + 7585(\pm 609)X\ (cal/Å) - 8143\ (cal)$$

giving the thermodynamic equivalent of each parameter. The surface area term shows a contribution of 27 cal/Å^2, in good agreement with the temperature corrected value of 22.5 cal/Å^2 based on the work of Chothia (Chothia, 1974, 1975). The absolute value of 0.76 K_c per P unit agrees well with the values ranging from −0.89 K_c (attractive) to +0.86 K_c (repulsive) for hydroxyl groups (Table IV) and with the figure of −600 calories per hydrogen bond in the binding of trisaccharides to lysozome (Rupley et al., 1967). The huge size of the X term is indicative of the disruptive effect on binding due to the introduction of a group larger than the corresponding "pocket" in the receptor.

Discussion

We have examined the thermodynamic and steroid structural requirements of the glucocorticoid-receptor interaction in order to understand further the nature of steroid–protein interactions and the determinants for binding specificity. These observations have been used as the basis for a generalized approach for predicting steroid drug receptor affinity. We determined the required thermodynamic parameters by measuring the effect of temperature on the equilibrium dissociation constant (K_D) and the association rate constant. A positive ΔS (at 0 °C) and a negative change in heat capacity (ΔC_p) upon association was found as well as a progressive decrease in the entropy as the temperature increases. For the transition state for association, the entropy of activation ($\Delta S^{\pm}$) was found to be +17.2 eu, also characteristic of a hydrophobic effect. Finally, a series of calculations using the known binding constants and a direct estimate of the hydrophobicity of a steroid from the analysis of other binding systems indicates that hydrophobic interactions can account for a tight hormone–receptor interaction. A model in which both sides of the steroid are enveloped by the receptor, increasing the areas of hydrophobic contact, readily accounts for the high affinity of association; a model assuming only one side of the steroid in contact with receptor is not in harmony with such a binding process.

To ascertain if hydrophobic interactions could account generally for other steroid protein interactions, we recalculated some of the data of the more comprehensive studies in this area. Shown in Figure 4 is the relationship of the equilibrium association constant to $1/T$ for three steroid–protein binding sys-

[3] We thank Dr. Stephen Dietrich for suggesting this approach.

tems: progesterone with human serum albumin, corticosterone with corticosterone-binding globulin (CBG), and progesterone with α-acid glycoprotein (AAG). In all cases, a relationship similar to that of corticosterone and the specific glucocorticoid receptors was observed. The calculated entropy and enthalpy terms are shown in Table I for comparison. Of note are the same trends of decreasing ΔH and ΔS with increasing temperature. Thus, in all cases the primary driving forces clearly are hydrophobic, which is not in agreement with the recent suggestion that hydrogen bonds and van der Waals forces are principally responsible for steroid–protein binding (Mornon et al., 1977).

We also examined the data obtained by Schaumberg & Bojensen (1968) and Koblinsky et al. (1972) who studied glucocorticoid–receptor binding. Schaumberg & Bojensen failed to find a temperature dependence of ΔH or ΔS, as there was a linear relationship between log K_A and $1/T$. Similarly, Koblinsky et al. (1972) interpreted their data as being linear. Unfortunately, only three temperature points were examined, and the data suggest that there could have been a curvilinear relationship. It is not clear why these discrepancies exist, but recently Rousseau & Schmidt (1977) have obtained findings similar to ours. The most likely source for error in our studies is that receptors in the uncomplexed state are less stable than those bound by the steroid. Any equilibrium studies performed under conditions of receptor instability would therefore result in a lower binding at the lower steroid concentrations and an overestimate of the true affinity. Similarly, in kinetic experiments, this effect would result in a low value for the association rate constants as the total receptor concentration at time zero would be understated. Care was taken to minimize this, and, under the experimental conditions employed, there was less than 10% degradation of the receptor. The receptor is more stable at low temperature, and in the temperature range from -2 °C to 6 °C we still noted a curved ln K vs. $1/T$ relationship. The results of Schaumberg & Bojensen may reflect other fundamental differences in the reaction since the experiments were performed in whole cells. In these, a number of different factors, including the influence of temperature on steroid uptake by the cells and on nuclear binding, could produce results that differ from our own. However, the general hydrophobic phenomena are supported not only by our measurements of the influence of temperature on equilibrium constant as well as the association rate studies, but also by the studies by Westphal & colleagues. Any conceivable receptor degradation would not have influenced our data sufficiently to yield artifactually a positive $\Delta S^{\ddagger}$.

Although the hydrophobic effect accounts for most of the binding energy, it is clear that the shape of the steroid and other types of interactions, such as hydrogen bonds, are also of importance. An analysis of the free energy contributions associated with individual substituents on the steroid was made by a number of comparisons of the binding of two steroids differing only by the substituents in question (Table IV). In those cases where the presence of a hydroxyl group resulted in increased binding (i.e., at the 11α, 11β, and 21 positions), the free energy contribution to binding was -0.56 to -0.89 kcal/mol for each potential hydrogen bond. This result is in good agreement with the value of -0.6 kcal/mol[4] estimated for each of the six hydrogen bonds in the lysozyme–trisaccharide complex (Rupley et al., 1967). The effect of a hydroxyl group in a region where a hydrogen bond with the receptor is apparently not possible is seen in the case of substituents at C-16.

[4] This value is incorrectly given as -800 cal/mol in the reference, apparently because of an arithmetical error or misprint.

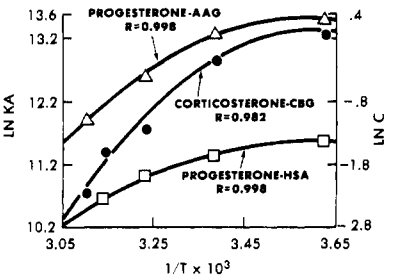


FIGURE 4: Corticosterone–CBG plot of ln C vs. $1/T$ (T in K); progesterone–AAG plot of ln K_A vs. $1/T$ (T in K); progesterone–HSA plot of ln K_A vs. $1/T$ (T in K).

Here the 16α-hydroxyl group has a free energy contribution of +0.86 kcal/mol, suggesting a repulsive interaction with a hydrophobic pocket in the receptor. In confirmation, the 16α-methyl group exhibits an attractive interaction of -0.11 kcal/mol, presumably due to a hydrophobic effect.

The calculated free energy contributions of substituent groups (Table IV) could be added together to predict quantitatively the affinity of different steroids for the receptor. However, the influence of substitutions was not strictly additive, and we sought a more accurate method to analyze the binding reaction and to understand the actual determinants for high affinity associations.

Utilizing knowledge of the free energy contributions of the substituents and the hydrophobicity and A-ring conformation of the steroids, we found that receptor affinity for a large number of compounds could be described in terms of four parameters. Thus a general relationship was derived relating the equilibrium dissociation constant to a surface area term, a polar interaction term, an A-ring tilt term and a size limitation function for the 9α substituent. The excellent correlation obtained suggests that these four factors are the major determinants of glucocorticoid receptor interactions.

The inclusion of other spatial terms would allow the expression to be comprehensive for more radical substitutions of the steroid molecule. It would have been of interest to compare the structures of nonsteroidal glucocorticoid antagonists or agonists. Unfortunately, after examining the binding of a large number of drugs (Ballard et al., 1975), we found only a few nonsteroidal substances whose inhibition constant ($K_{I\pm}$) is $<10^{-2}$ for the glucocorticoid receptor. This may be due to the large surface contact necessary for such hydrophobic interactions. It would be interesting to extend this type of analysis to other steroid–protein systems to know how generally true this is, especially in the case of nonsteroidal analogues of estradiol which interact with the estrogen receptor with high affinity (Katzenellenbogen et al., 1976).

It is clear from our studies that use of a mathematical relationship that defines the strength of steroid–receptor interaction is a valuable tool for investigating structure–activity relationships. This would be especially true in the design of steroid drugs. The current results show that the use of a linear free-energy model (eq 1) is superior to the assumption of substituent additivity in predicting binding affinities. This type of relationship will be useful in the preparation of steroids for use in affinity labeling studies and should be adaptable to other binding systems in which it is desirable to obtain synthetic analogs for more potent activity or specificity.

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