References
Atger, M., and Milgrom, E. (1976), *Biochemistry 15,* 4298.
Buller, R. E., Schrader, W. T., and O'Malley, B. W. (1975a), *J. Biol. Chem. 250,* 809.
Buller, R. E., Toft, D. O., Schrader, W. T., and O'Malley, B. W. (1975b), *J. Biol. Chem. 250,* 801.
Burton, K. (1968), *Methods Enzymol. 12B,* 163.
Giannopoulos, G. (1975), *J. Biol. Chem. 250,* 2896.
Higgins, S. J., Rousseau, G. G., Baxter, J. D., and Tomkins, G. M. (1973), *J. Biol. Chem. 248,* 5866.
Jensen, E. V., and DeSombre, E. R. (1972), *Annu. Rev. Biochem. 41,* 203.
Kalimi, M., Colmen, P., and Feigelson, P. (1975), *J. Biol. Chem. 250,* 1080.
King, E. G. (1932), *Biochem. J. 26,* 292.
Lohmar, P. H., and Toft, D. O. (1975a), 57th Annual Meeting of the Endocrine Society, New York, N.Y., Abstract No. 25.
Lohmar, P. H., and Toft, D. O. (1975b), *Biochem. Biophys. Res. Commun. 67,* 8.
Lowry, O. H., Rosebrough, N. J., Farr, H. L., and Randall, R. J. (1951), *J. Biol. Chem. 193,* 265.
Milgrom, E., Atger, M., and Baulieu, E.-E. (1973), *Biochemistry 12,* 5198.
Milgrom, E., Atger, M., and Bailly, A. (1976), *Eur. J. Biochem. 70,* 1.
Moudgil, V. K., and Toft, D. O. (1975), *Proc. Natl. Acad. Sci. U.S.A. 72,* 901.
Moudgil, V. K., and Toft, D. O. (1976), *Proc. Natl. Acad. Sci. U.S.A. 73,* 8443.
Moudgil, V. K., and Toft, D. O. (1977), *Biochim. Biophys. Acta 490,* 477.
Notides, A. C., and Nielsen, S. (1975), *J. Steroid Biochem. 6,* 483.
Schrader, W. T., Toft, D. O., and O'Malley, B. W. (1972), *J. Biol. Chem. 247,* 2401.
Schrader, W. T., Heuer, S. S., and O'Malley, B. W. (1975), *Biol. Reprod. 12,* 134.
Spelsberg, T. C. (1974), in Acidic Proteins of the Nucleus, Cameron, I. L., and Jetter, J. R., Ed., New York, N.Y., Academic Press, p 247.
Spelsberg, T. C., Knowler, J. T., and Moses, H. L. (1974), *Methods Enzymol. 31,* 263.
Toft, D. O. (1973), in Receptors for Reproductive Hormones, O'Malley, B. W., and Means, A. R., Ed., New York, N.Y., Plenum Press, p 247.
Toft, D. O., Lohmar, P., Miller, J., and Moudgil, V. K. (1977a), *J. Steroid Biochem. 7,* 1053.
Toft, D. O., Moudgil, V., Lohmar, P., and Miller, J. (1977b), *Ann. N.Y. Acad. Sci. 286,* 29.
Yamamoto, K. R., and Alberts, B. M. (1972), *Proc. Natl. Acad. Sci. U.S.A. 69,* 2105.

Kinetic and Equilibrium Studies on Steroid Interaction with Human Corticosteroid-Binding Globulin†

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ABSTRACT: Kinetic and equilibrium studies on the interaction of steroids with human corticosteroid-binding globulin (CBG, transcortin) were performed with pH, temperature, and steroid structure as variables. Dissociation rate constants were determined fluorometrically; the values for cortisol, corticosterone, deoxycorticosterone, and progesterone are 0.031, 0.047, 0.10, and 0.16 s^{-1}, respectively, at 20 °C, pH 7.4. The pH dependence of the dissociation rate constant for the corticosterone complex below pH 10.5 at 20 °C is given by $k_{off} = 0.043\ (1 + [H^+]/10^{-6.50})\ s^{-1}$; above pH 11, $k_{off} = 0.030\ (1 + 10^{-12.15}/[H^+])\ s^{-1}$. A temperature-dependence study of k_{off} for the cortisol and progesterone complexes gave values of 0.0028 s^{-1} and 0.012 s^{-1} at 4 °C, respectively, and 0.88 s^{-1} and 4.5 s^{-1} at 37 °C, with progesterone dissociating about four to five times faster over the entire temperature range. The affinity constants, determined by equilibrium dialysis, for the binding of cortisol, corticosterone, and progesterone at 4 °C were 7.9, 7.2, and 7.0 × 10^8 M^{-1}; values of 0.40 and 0.26 × 10^8 M^{-1} were determined at 37 °C for cortisol and progesterone. The close similarity of the affinity constants of the three steroids combined with differing dissociation rates implies that the association rate changes with steroid structure, in contrast to our earlier findings with progesterone-binding globulin.

Human corticosteroid-binding globulin (CBG)[1] was the first high-affinity steroid binding protein to be reported (Daughaday, 1956; Bush, 1957; Sandberg and Slaunwhite, 1958). It has been purified in several laboratories and some of its physicochemical properties have been described in detail (Westphal, 1971; Le Gaillard et al., 1975). Advances in affinity chromatography have simplified the purification of CBG (Rosner and Bradlow, 1971; Le Gaillard et al., 1974). However, only a few studies have been published on the mechanism of steroid interaction with the high-affinity binding protein. In the present report, we attempt, by means of kinetic and equiilibrium studies, to establish an understanding of the binding of steroids to CBG.

The kinetic studies on CBG–steroid interaction utilized the method of fluorescence quenching (Stroupe et al., 1975). The intrinsic fluorescence of the binding protein is quenched upon

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[1] Abbreviations used: CBG, corticosteroid-binding globulin or transcortin; E_a, energy of activation; PBG, progesterone-binding globulin; Tris, 2-amino-2-hydroxymethyl-1,3-propanediol.

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association with a 3-oxo-4-ene steroid by radiationless energy transfer from protein fluorophores (tryptophan and tyrosine) to the steroid chromophore. Removal of the steroid or its replacement by a nonquenching steroid results in restoration of the protein fluorescence, thereby allowing measurement of the rate of dissociation of steroid ligand from the protein (Gibson and Roughton, 1955). These principles have been applied to the quantitative analysis of steroid binding to and dissociation from progesterone-binding globulin (Stroupe and Westphal, 1975). Marver et al. (1976) have used the fluorescence quenching method to study the interaction of photoreactive steroids with CBG.

Materials and Methods

Steroids were commercial products. Their melting points were verified; if necessary, they were recrystallized from ethanol or acetone and water. Steroid solutions of given concentrations were prepared in ethanol gravimetrically (microbalance); the concentrations of 4-ene-3-one steroids were checked spectrophotometrically. The 5β-pregnane-3,20-dione showed negligible absorption at 241 nm, indicating the absence of contaminating 4-ene-3-one steroids. [1,2-^{3}H]Cortisol was obtained from Amersham/Searle; both [1,2-^{3}H]corticosterone and [1,2-^{3}H]progesterone were from New England Nuclear; their radiopurity was checked by thin-layer chromatography. Gelatin was purchased from Sigma; addition of 0.1% to the equilibrium dialysis systems (see below) did not change the pH.

Purification of CBG from outdated human blood or pregnancy serum was achieved by affinity chromatography essentially according to the method of Le Gaillard et al. (1974). CBG was eluted from the affinity column at room temperature with 50 mM phosphate buffer of pH 7.4, containing 0.2 M NaCl and 36.5 μM corticosterone. After pressure concentration and equilibration to 5 mM phosphate, pH 6.8, the protein was chromatographed on hydroxylapatite (Bio-Rad) using the same buffer. The purified CBG·corticosterone complex was concentrated, equilibrated with water in an Amicon pressure device with a PM 30 membrane, and lyophilized. The concentration of the CBG·steroid complex was measured using an extinction coefficient of $\epsilon_{280}^{1\%} = 6.45$ (Muldoon and Westphal, 1967). Complexes of CBG with steroids other than corticosterone were prepared by first incubating the CBG·corticosterone complex with a 20-fold excess of the desired steroid for 1 h at room temperature; the unbound steroid was then removed by rapid gel filtration over Sephadex G-25 at 4 °C, and the exchange step was repeated. Unbound steroid was again removed by gel filtration at 4 °C and the CBG·steroid complex was lyophilized. Tests with radioactive steroids indicated the exchange to be greater than 98%.

Steroid Removal from CBG. Corticosterone was "stripped" from CBG prior to the dialysis experiments by three procedures. In a modification of the method of Chan and Slaunwhite (1977), 25 mL of CBG solution (5 μg/mL) in phosphosaline buffer (75 mM NaCl, 25 mM sodium phosphate, 0.02% sodium azide, pH 7.4) containing 0.1% gelatin was shaken with 4 mL of settled XAD-4 resin (Rohm and Haas) for 4 h at room temperature. This technique removed 95–100% of the steroid with less than 10% loss in binding sites. In a second method, equal volumes of CBG solution in 50 mM phosphate of pH 7.4 (~1 mg/mL) and dextran-coated charcoal suspension in 50 mM phosphate, pH 7.4 (0.01% dextran and 0.1% Norite A), were shaken at room temperature for about 4 h. The charcoal was removed by centrifugation and Millipore filtration. The protein concentration in the clear solution was estimated spectrophotometrically. In a gel filtration procedure, 100 μL of 1 mg of CBG/mL in 50 mM phosphate buffer, pH 7.4, was applied to a 2.5 × 45 cm column of Sephadex G-25 at 25 °C and eluted with the same buffer at the relatively slow flow rate of 1 mL/min (Dixon, 1976). The same affinity constants were obtained for CBG stripped by any one of the three methods. However, most experiments were done with the XAD procedure, because gel filtration often gave incomplete removal of steroid, and the charcoal method resulted in loss of binding sites.

Equilibrium dialysis was used as previously described (Westphal, 1969) to determine the affinity constants for the binding to CBG of [1,2-^{3}H]cortisol, [1,2-^{3}H]corticosterone and [1,2-^{3}H]progesterone. Two milliliters of inside solution (5 μg of CBG/mL of phosphosaline buffer containing 0.1% gelatin) and 20 mL of outside phosphate buffer (50 mM, pH 7.4) were used for the dialysis experiments. Control experiments showed that gelatin does not bind corticosterone; progesterone, deoxycorticosterone, and cortisol previously were found not to interact with gelatin (Westphal, 1971, p 157). The equilibrium dialysis flasks were shaken at the various temperatures for 44 to 48 h, a time found to be adequate for complete equilibration. Triplicate samples of the inside and outside solutions were taken for scintillation counting. Scatchard plots were determined by a computer program which gave a least-squares fit of the data.

Fluorometric determination of dissociation rate constants was performed with an Aminco Bowman ratio fluorometer connected to a Perkin-Elmer Coleman 165 strip chart recorder using 20 mV full scale. Excitation was at 280 nm with a spectral band width of 0.55 nm; emission was at 340 nm with a spectral band width of 11 nm. The narrow excitation slit width was used to avoid photochemical damage to the protein. All measurements were made with 0.2 μM CBG·steroid complex having an absorbance at 280 nm of about 0.007. A 4.9 mM stock solution of 5β-pregnane-3,20-dione was prepared in ethanol, and the pregnanedione was added in 100-fold molar excess to replace the steroid in the CBG complex; this resulted in a reversal of the fluorescence quenching. The final ethanol concentration was 0.5%. The amount of pregnanedione added was found to be more than sufficient to completely replace the bound steroid thereby allowing an accurate determination of the dissociation rate (Gibson and Roughton, 1955). As in the analogous case of interaction of PBG with steroids lacking the conjugated ene-one chromophore (Stroupe et al., 1975), 5β-pregnanedione did not quench the fluorescence of CBG. The cell holder was thermostated; the temperature of the cell contents was measured with a calibrated thermistor at the completion of each run. Measurements were made in 50 mM phosphate, pH 7.4, except for the pH dependency study, where 0.1 M solutions of sodium acetate (pH 5.53), sodium cacodylate (pH 5.98, 6.54), sodium phosphate (pH 7.03, 7.49), Tris-Cl (pH 8.00, 8.98), and sodium glycinate (pH 9.99–12.15) were used. The reaction mixtures were collected after each determination and the pH was measured as reported (Stroupe et al., 1977). Computer fitting of the pH dependence to the expressions $k_{off} = k_0(1 + [H^+]/K_1)$ and $k_{off} = k_0(1 + K_2/[H^+])$ (Dixon and Webb, 1964) was performed as previously described for PBG (Stroupe et al., 1977).

Results

Kinetics of Steroid Dissociation. Figure 1 demonstrates the increase in intrinsic fluorescence of CBG when bound cortisol is replaced by 5β-pregnane-3,20-dione. The overall signal change corresponded to about 17 mV and could be observed easily with the available apparatus. Figure 2 gives the same data in the form of a semilog plot yielding a first-order disso-

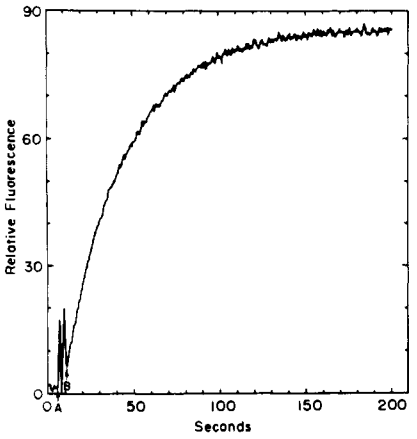

FIGURE 1: Dissociation of CBG·cortisol complex. At time 0, the fluorescence of 1.0 mL of 0.2 μM CBG·cortisol complex (pH 7.4, 20 °C) was offset to ~0 using the blank subtract control of the fluorometer. At A, 5 μL of 4.9 mM 5β-pregnane-3,20-dione in ethanol was added with mixing; at B, reliable recording of the change in fluorescence begins.

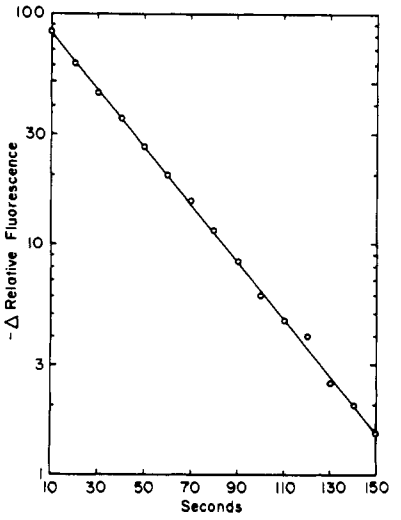

FIGURE 2: Dissociation rate constant of the CBG·cortisol complex obtained by plotting the data from Figure 1. A first-order dissociation rate constant of 0.029 s^{-1} was found. The time scale is that of Figure 1.

ciation rate constant of 0.029 s^{-1} for the CBG·cortisol complex. In Table I, we compare the dissociation rate constants of four CBG·steroid complexes at 20 °C, pH 7.4. Cortisol dissociates with the lowest rate constant while progesterone dissociates most rapidly. The progression of rate constants follows exactly the degree of hydrophobicity; as hydroxyl groups are removed from the cortisol molecule, the dissociation rate constant increases.

As seen in Figure 3, the dissociation rate constant (k_{off}) of the CBG·corticosterone complex is strongly pH dependent below pH 7, but is essentially invariant between pH 7 and 10.5. For comparison, the pH dependence of k_{off} for PBG·progesterone is given (Stroupe et al., 1977). Below pH 10.5, the pH dependence of k_{off} for CBG·corticosterone may be described quantitatively by the expression:

$$k_{off} = 0.043(1 + [H^+]/10^{-6.50})\ s^{-1} \quad (1)$$

indicating that only one ionizing residue affects the dissociation rate over a very wide pH range. Above pH 10.5 the pH dependence is more complex with the observed value of k_{off} decreasing abruptly from 0.0431 ± 0.0035 s^{-1} at pH 10.53 to 0.0324 ± 0.0012 s^{-1} at pH 11.01. The increase in k_{off} near pH 12 is then described by the expression

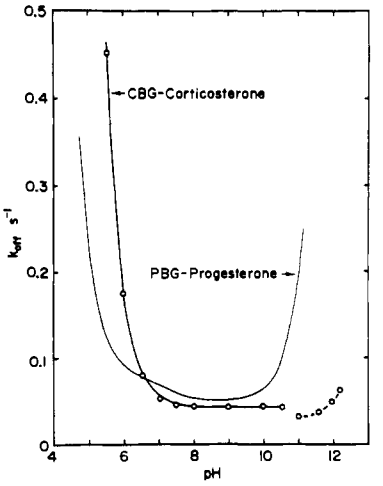

FIGURE 3: Influence of pH on the dissociation rate constants of the CBG·corticosterone and PBG·progesterone complexes at 20 °C. The curve for the CBG·corticosterone complex below pH 10.5 (—O—) was drawn according to eq 1. From pH 11 to 12.2 (- - O - -) the data are described by eq 2. The discontinuity between pH 10.5 and 11 is discussed in the text. Each data point is the average of four separate experiments. The pH dependence of k_{off} for PBG·progesterone is given for comparison (Stroupe et al., 1977).

TABLE I: Dissociation Rate Constants of CBG·Steroid Complexes Determined Fluorometrically at 20 °C, pH 7.4.

Steroid	k_{off} (s^{-1})	n
Cortisol	0.031 ± 0.002[a]	11
Corticosterone	0.047 ± 0.002	9
Deoxycorticosterone	0.10 ± 0.01	4
Progesterone	0.16 ± 0.01	4

[a] Standard deviation of n determinations. A value of 0.04 s^{-1} was obtained with an affinity-purified CBG preparation from Dr. W. Rosner.

$$k_{off} = 0.030(1 + 10^{-12.15}/[H^+])\ s^{-1} \quad (2)$$

The temperature dependencies of the dissociation rate constants of the CBG·cortisol and CBG·progesterone complexes are given as Arrhenius plots in Figure 4. Both complexes exhibit a similar biphasic temperature dependence with the progesterone complex dissociating about five times faster than the cortisol complex over the entire temperature range studied. The CBG·cortisol complex dissociates with an E_a of 23 kcal/mol below 20 °C; at higher temperatures, the E_a rises to 36 kcal/mol. The CBG·progesterone complex exhibits activation energies of 26 kcal/mol and 35 kcal/mol over the corresponding temperature ranges. For comparison, the PBG·progesterone complex has an E_a of 17 kcal/mol, rising to 24 kcal/mol above 30 °C (Stroupe and Westphal, 1975).

The affinity constants for the binding of several steroids to CBG at 4, 20, and 37 °C are given in Table II. By utilizing the simple kinetic description of the affinity constant $K_a = k_{on}/k_{off}$, the association rate constants for the binding of three steroids to CBG were calculated. With the purified CBG, the K_a values do not show great differences, although cortisol appears to be bound most firmly. As shown above (Figure 4), the CBG·progesterone complex dissociates about five times faster than the CBG·cortisol complex at all temperatures measured. Therefore, if steroids bind to CBG in a simple one-step mechanism, progesterone would associate with CBG faster than cortisol. This can be seen in the higher calculated

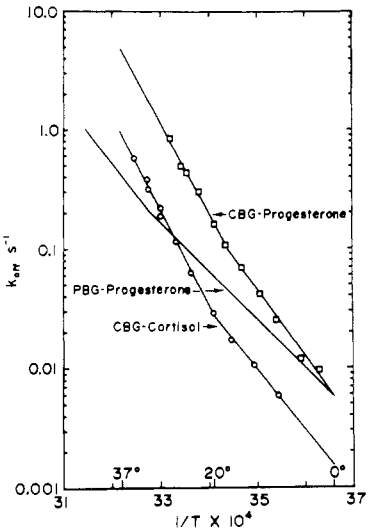

FIGURE 4: Temperature dependence of k_{off} for CBG·cortisol (— O —) and CBG·progesterone (— □ —) in 50 mM phosphate, pH 7.4. Each data point is the average of four separate experiments. For comparison, the dissociation rate constant of progesterone from PBG (—) is also given (Stroupe and Westphal, 1975).

values of k_{on} for progesterone over the temperature range applied (Table II).

TABLE II: Temperature Dependence of Steroid Binding to CBG.

Steroid	$K_a \times 10^{-8}$ [a] (M^{-1})	k_{off} [b] (s^{-1})	$k_{on} \times 10^{-6}$ [c] (M^{-1} s^{-1})
		At 4 °C	
Cortisol	7.9	0.0028	2.2
Corticosterone	7.2 [d]		
Progesterone	7.0	0.012	8.4
		At 20 °C	
Cortisol	3.9	0.031	12
Corticosterone	2.8	0.047	13
Progesterone	2.8	0.16	45
		At 37 °C	
Cortisol	0.40	0.88	35
Progesterone	0.26	4.5	117

[a] Determined by equilibrium dialysis. [b] Determined fluorometrically. [c] Calculated from $K_a = k_{on}/k_{off}$. [d] 7.2 ± 2.3 × 10^8 M^{-1} (from 11 determinations, including CBG preparations stripped by the three methods described under Materials and Methods). All other K_a values are averages from duplicate experiments using XAD-treated CBG.

Discussion

Equilibrium Studies. The K_a values reported in this communication (Table II) are in reasonable agreement with previously published values for classically purified CBG (Westphal, 1971). The lower K_a values reported for affinity purified CBG (Rosner and Bradlow, 1971; Le Gaillard et al., 1975; Chan and Slaunwhite, 1977) cannot be reconciled with our observations.

Kinetics of CBG·Steroid Complexes. In our previous studies on the kinetics of steroid interaction with PBG a computerized stopped-flow fluorometer was used (Stroupe and Westphal, 1975). The present investigation was made with conventional equipment, i.e., a fluorometer and a recorder. This apparatus is less expensive than the stopped-flow fluorometer but can accurately measure rate constants up to about 1 s^{-1}. Indeed, the manual fluorometer is preferable for measuring relatively slow dissociation reactions because of its greater signal stability, sensitivity, and spectral resolution. Furthermore, the manual mixing method allows greater flexibility in the choice of competing steroid ligands since solubility problems with aqueous buffer systems required for the stopped-flow device can be circumvented.

The kinetics of steroid dissociation from CBG have been reported from several laboratories. Only Rosner et al. (1973) used purified CBG; Dixon (1968) and Paterson (1973) worked with whole plasma. All three studies entailed use of radiolabeled steroids and a method of physically separating bound from free steroid. The advantages of utilizing an intrinsic parameter of the system to follow steroid–protein interactions have been discussed previously (Stroupe and Westphal, 1975).

By extrapolation of the data in Figure 4, cortisol was found to dissociate from CBG with a rate constant of 0.0028 s^{-1} at 4 °C. This value is in good agreement with the constant of 0.0023 s^{-1} determined by Rosner et al. (1973), but is about five times faster than the values reported by Dixon (1968) and Paterson (1973) for CBG·cortisol in whole plasma at 2–4 °C. In view of unknown factors involving studies on protein mixtures, we will compare our results only with the other study on pure CBG. The activation energy of 21 kcal/mol obtained by Rosner et al. (1973) for the CBG·cortisol complex is in agreement with our value of 23 kcal/mol below 20 °C. Extrapolation of our data to 37 °C yields a k_{off} of 0.88 s^{-1}, while the data of Rosner et al. give 0.13 s^{-1}. The rather large discrepancy probably is due to the fact that the earlier report did not incorporate measurements above 18 °C, a temperature range where the activation energy increases from 23 to 36 kcal/mol.

Influence of Temperature. Both the CBG·cortisol and CBG·progesterone complexes have essentially the same temperature dependence of k_{off} thereby indicating that it is the protein portion of the complex, not the steroid, which controls the effect of temperature on the dissociation reaction. The previously determined kinetic data on the PBG·progesterone complex (Stroupe and Westphal, 1975) agree with a dominant role of the protein. For the CBG·progesterone complex, E_a = 26 kcal/mol below 20 °C, rising to 35 kcal/mol near physiological temperature. With PBG·progesterone, E_a at lower temperatures is 17 kcal/mol, increasing to only 24 kcal/mol at 37 °C. In both complexes, the same steroid, progesterone, leaves the binding site, where presumably it was partly removed from water, and becomes surrounded completely by water. If the rate at which the steroid is hydrated were limiting, one would expect similar k_{off} and E_a values for the progesterone complexes of both proteins. Clearly, it is the bonding forces between the protein and the steroid which are dominant since the same steroid has different k_{off} and E_a values for CBG and PBG.

In addition to temperature, k_{off} is also a function of pH; therefore, it might be argued that the increase in E_a is due to a change in the pK of the acidic group (Figure 3). Extrapolation of the low-temperature k_{off} data for CBG·cortisol to 37 °C gives a value of about 0.25 s^{-1}, compared with 0.88 s^{-1} from the experimentally determined high temperature data. The pK of the acidic group would have to change from 6.50 to 7.87 to cause an increase of the rate from 0.25 s^{-1} to 0.88 s^{-1}. This increase in pK requires a ΔH of +33.5 kcal/mol, an unreasonable value. If the pK of 6.50 corresponds to a histidyl residue, a ΔH of 7 kcal/mol is expected (Tanford, 1961). Assuming a ΔH of 7 kcal/mol, the change in pK of the acidic group would increase the extrapolated dissociation rate constant from 0.25 s^{-1} to 0.28 s^{-1}. Evidently, the properly obtained value of 0.88 s^{-1} must be due to changes in binding

TABLE III: Thermodynamic Parameters of CBG-Steroid Complexes.

	$\Delta G°$ (kcal/mol), 4 °C	$\Delta H°$ (kcal/mol), 4–20 °C	$\Delta S°_u$[a] (eu), 4 °C
Cortisol	−11.3	−7.1	+23
Corticosterone	−11.2	−9.5	+14
Progesterone	−11.2	−9.2	+15
	20 °C		
Cortisol	−11.5		
Corticosterone	−11.3		
Progesterone	−11.3		
	37 °C	20–37 °C	37 °C
Cortisol	−10.8	−24.2	−36
Progesterone	−10.5	−25.2	−39

[a] The unitary change of $\Delta S°$ is used because it more accurately reflects the true energy relationship (Kauzman, 1959).

forces between protein and steroid which are largely independent of the degree of ionization of the crucial residue.

Table III shows certain thermodynamic parameters for the CBG-steroid complexes. The values of $\Delta G°$ and $\Delta H°$ over the temperature ranges 4–20 and 20–37 °C were obtained from the equilibrium association constants in Table II. The van't Hoff equation was used to calculate $\Delta H°$ as discussed by Tanford (1968). At 4 °C, association of cortisol, corticosterone, and progesterone with CBG is predominantly entropy driven, the unitary change (Kauzman, 1959), ΔS_u, being positive in all cases. The situation is reversed at 37 °C with enthalpy being the driving force. At 37 °C, ΔS_u assumes a large negative value while ΔH becomes even more negative. The potential error of extrapolating low temperature observations to physiological temperature is obvious.

The pH dependence of k_{off} for the CBG-corticosterone complex is similar to that previously reported for the PBG-progesterone complex (Figure 3). Both complexes have a rather broad region at neutral and slightly basic pH where k_{off} is invariant. At lower and higher pH values, k_{off} increases; the acid limb of the CBG-corticosterone complex is controlled by a pK of 6.50 according to eq 1. The basic limb above pH 11 is described by eq 2. The pH profile has a discontinuity between pH 10.5 and 11 which is not due to buffer effects; 0.1 M sodium glycinate was used for all measurements above pH 10. The simplest interpretation is that the discontinuity results from an abrupt conformational change between pH 10.5 and 11 in which the intrinsic dissociation rate constant, k_0, decreases from 0.043 s^{-1} to 0.030 s^{-1}. Thus the pH dependence of CBG-steroid dissociation is governed by two normally ionizing residues of pK = 6.50 and 12.15 plus an unidentified number of residues which are deprotonated between pH 10.5 and 11.

This dependency is in contrast to the pH influence on the PBG complex where three residues are involved (Stroupe et al., 1977); the most acidic residue has a pK of 5.30 compared with 6.50 for CBG. The basic residue of PBG gave a pK of 10.54 compared with 12.15 for CBG. The third residue in PBG alters its pK from 7.41 to 7.21 when a steroid is bound. For PBG the most acidic residue is probably either a carboxyl or a histidyl residue; the neutral residue has been suggested to be a histidyl, and the most basic residue a lysyl group. In the case of CBG the amino acid residue with a pK of 6.50 may be assumed to be histidyl and that with the pK of 12.15 to be an arginyl. Van Baelen et al. (1972) observed a midpoint at approximately pH 6.5 in studies on the pH dependence of the cortisol binding activity of CBG, which further implicates a histidyl residue. Khan and Rosner (1977) have demonstrated the presence of a cysteinyl residue in the binding site of CBG by means of affinity labeling. Unless the SH group ionizes with an abnormally low or high pK, the degree of ionization of this residue is unlikely to affect the dissociation rate constant of CBG-steroid complexes.

Since only the protein is amphoteric while the steroid is neutral, the pH dependence of k_{off} should be the same for different steroids with only the intrinsic dissociation rate constant assuming different values. In confirmation, the pH dependence of the CBG-cortisol complex was found to be $k_{off} = 0.03(1 + [H^+]/10^{-6.5})$ s^{-1} for a less extensive series of experiments than undertaken for the CBG-corticosterone complex. As expected, the same pK found with the corticosterone complex controls the low-pH increase in k_{off} for the cortisol complex; only the intrinsic dissociation rate constant is changed.

Synopsis

The result that both k_{on} and k_{off} vary for the interaction of different steroids with CBG differs from our observations with PBG (Stroupe and Westphal, 1975). The affinity constant for steroid binding to PBG is quantitatively defined by $K_a = k_{on}/k_{off}$. For all steroids with $K_a > 10^8$ M^{-1}, k_{on} varied by less than ±10% while k_{off} increased in direct proportion to the decrease in K_a. However, for the CBG complexes with the three steroids measured at 4 and 20 °C, and the two measured at 37 °C, K_a was approximately the same while k_{off} varied by a factor of about five from cortisol to progesterone (Table II). If $K_a = k_{on}/k_{off}$, the variation in k_{off} requires a corresponding change in k_{on} as calculated in Table II. Direct experimental verification of the change in k_{on} with steroid structure is presently not available for the CBG complexes.

The data for CBG in Table II allow a recalculation of the half-times of association and dissociation. Using previously published values obtained with CBG in serum (Paterson, 1973), Stroupe and Westphal (1975) calculated for the CBG-cortisol complex at 37 °C a half-time of dissociation of 1.1 s and a half-time of association of 44 ms under first-order conditions with CBG in excess. Using the data of Table II, we obtain $t_{1/2}$ = 0.79 s for dissociation and $t_{1/2}$ = 56 ms for association for the CBG-cortisol complex. For the dissociation and association of progesterone at 37 °C, half times of 0.15 s and 17 ms, respectively, are calculated. The equilibration of progesterone with CBG is faster than that of cortisol, although both steroids are bound with approximately the same affinity.

CBG and PBG are the two best characterized serum proteins that bind steroids with high affinity. Both are glycoproteins which interact with progesterone; however, only CBG binds both cortisol and progesterone with comparable affinity. Apparently, the CBG binding site is as well or better suited for binding polar steroids whereas the binding of PBG follows the polarity rule in that the highly polar cortisol is bound one thousand times less firmly than progesterone. This ability to bind both hydrophilic and hydrophobic steroids places human CBG in a unique position since other steroid-binding serum proteins show predominantly hydrophobic bonding. Although CBG and PBG have different binding specificities, they share a common trait in that, unlike cellular steroid receptors, they equilibrate rapidly with steroids, displaying both fast association and dissociation under physiological conditions.

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References

Bush, I. E. (1957), *Ciba Found. Colloq. Endocrinol. [Proc.] 11*, 263–285.
Chan, D., and Slaunwhite, W. R., Jr. (1977), *J. Clin. Endocrinol. Metab. 44*, 983–985.
Daughaday, W. H. (1956), *J. Lab. Clin. Med. 48*, 799–800.
Dixon, H. B. F. (1976), *Biochem. J. 159*, 161–162.
Dixon, M., and Webb, E. C. (1964), Enzymes, New York, N.Y., Academic Press, pp 116–145.
Dixon, P. F. (1968), *J. Endocrinol. 40*, 457–465.
Gibson, Q. H., and Roughton, F. J. W. (1955), *Proc. R. Soc. London, Ser. B 143*, 310–334.
Kauzman, W. (1959), *Adv. Protein Chem. 14*, 1–63.
Khan, M. S., and Rosner, W. (1977), *J. Biol. Chem. 252*, 1895–1900.
Le Gaillard, F., Aubert, J. P., Dautrevaux, M., and Loucheux-Lefebvre, M. H. (1976), *FEBS Lett. 64*, 278–284.
Le Gaillard, F., Han, K. K., and Dautrevaux, M. (1975), *Biochimie 57*, 559–568.
Le Gaillard, F., Racadot, A., Racadot-Leroy, N., and Dautrevaux, M. (1974), *Biochimie 56*, 99–108.
Marver, D., Chiu, W. H., Wolff, M. E., and Edelman, I. S. (1976), *Proc. Natl. Acad. Sci. U.S.A. 73*, 4462–4466.
Muldoon, T. G., and Westphal, U. (1967), *J. Biol. Chem. 242*, 5636–5643.
Paterson, J. Y. F. (1973), *J. Endocrinol. 56*, 551–570.
Rosner, W., and Bradlow, H. (1971), *J. Clin. Endocrinol. 32*, 193–198.
Rosner, W., Darmstadt, R., and Tauster, S. J. (1973), *J. Steroid Biochem. 4*, 249–255.
Sandberg, A. A., and Slaunwhite, W. R., Jr. (1958), *J. Clin. Invest. 37*, 928.
Schneider, S. L., and Slaunwhite, W. R., Jr. (1971), *Biochemistry 10*, 2086–2093.
Stroupe, S. D., Acree, K., and Westphal, U. (1977), *Biochemistry 16*, 1350–1355.
Stroupe, S. D., Cheng, S. L., and Westphal, U. (1975), *Arch. Biochem. Biophys. 168*, 473–482.
Stroupe, S. D., and Westphal, U. (1975), *J. Biol. Chem. 250*, 8735–8739.
Tanford, C. (1961), Physical Chemistry of Macromolecules, New York, N.Y., Wiley, p 548.
Tanford, C. (1968), *Adv. Protein Chem. 23*, 121–282.
Van Baelen, H., Beck, M., and DeMoor, P. (1972), *J. Biol. Chem. 247*, 2699–2703.
Westphal, U. (1969), *Methods Enzymol. 15*, 761–796.
Westphal, U. (1971), Steroid-Protein Interactions, New York, N.Y., Springer-Verlag.

Effect of Stacking Interactions with Poly(riboadenylic acid) on the Triplet State Properties of Tryptophan†

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ABSTRACT: Optically detected magnetic resonance (ODMR) signals of tryptophan (Trp) have been measured in L-lysyl-L-tryptophyl-L-lysine (Lys-Trp-Lys) and in its complex with poly(riboadenylic acid) [poly(rA)]. Measurements were made with optical narrow band detection through the Trp O–O band. Plots of $|D|$ and $|E|$ vs. λ are distinctly different for Lys-Trp-Lys and its complex with poly(rA). A reduction of $|D|$, in particular, is consistent with stacking of Trp with adenine in the complex, since this effect is expected from charge-transfer contributions in the excited triplet state. Triplet energy transfer from poly(rA) to Lys-Trp-Lys is nearly complete at 77 K, with a Trp:adenine ratio of 0.1. The energy transfer efficiency is considerably reduced at 4.2 K and below, probably resulting from reduction of the triplet mobility in the polymer. Analysis of the phosphorescence decays shows that the triplet states of poly(rA), Lys-Trp-Lys, and their complex decay nonexponentially. Binding of polylysine to poly(rA) has no effect on the phosphorescence spectrum, but the decay kinetics are changed.

Interactions between proteins and nucleic acids play a vital role in the functioning of biological systems. These interactions may involve electrostatic forces between charged groups, hydrogen bonding, and stacking forces (Yarus, 1969; Hwang, 1971). Exposed aromatic side chains—tryptophan (Trp),[1] tyrosine, and phenylalanine—could be important in selective recognition of base sequences through stacking interactions involving π overlap. Quenching of Trp fluorescence as a result of such interactions has been observed in Trp-nucleoside aggregates formed in frozen aqueous solutions (Montenay-Garestier and Hélène, 1968, 1971; Hui Bon Hoa and Douzou, 1970) and from complexation of indole derivatives and small Trp-containing peptides such as Lys-Trp-Lys with DNA and with various RNAs (Wagner, 1969; Smythies and Antun, 1969; Hélène, 1971a; Hélène et al., 1971; Raszka and Mandel, 1971; Brun et al., 1975). Spectroscopic methods also have been used to study stacking interactions in covalently linked indole-base dimer models (Mutai et al., 1975), tRNA-synthe-

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[1] Abbreviations used are: poly(rA), poly(riboadenylic acid); Trp, tryptophan; Lys-Trp-Lys, L-lysyl-L-tryptophyl-L-lysine; poly(Lys), polylysine; CD, circular dichroism; ODMR, optical detection of magnetic resonance; ZFS, zero field splitting; λ, wavelength; ^{1}H NMR, proton magnetic resonance; EPR, electron paramagnetic resonance.

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