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STEROID INTERACTIONS WITH PROGESTERONE-BINDING GLOBULIN

ULRICH WESTPHAL,* STEPHEN D. STROUPE, TIMOTHY KUTE†
and SU-LI CHENG
Department of Biochemistry, University of Louisville School of Medicine, Health Sciences Center, Louisville KY 40201, U.S.A.

SUMMARY

The progesterone-binding globulin (PBG) of the pregnant guinea pig has been purified on sulfopropyl Sephadex and subsequent affinity chromatography on 19-nortestosterone-Sepharose. A pure glycoprotein of molecular weight 88,000 was obtained that contained about 70% carbohydrate. PBG is polydisperse and has a polypeptide core of about 27,000 daltons. It has one binding site for progesterone ($K_a^{37°} = 4 \times 10^8\,M^{-1}$). Studies on chemical modification of PBG show the involvement of tryptophan, lysine, and tyrosine in steroid binding. In typical reactions, the binding sites were inactivated by the chemical substitutions; complex formation with progesterone protected the binding site from the modification. Association of progesterone or other steroids with PBG results in conformational changes as seen from circular dichroism and ultraviolet difference spectra. The intrinsic tryptophan fluorescence of PBG is quenched by more than 80% when one mol progesterone is bound. This is the basis for a fluorescence quenching titration method to determine binding site concentrations and association constants. Association and dissociation rate constants were measured by stopped-flow fluorescence techniques; it was found that the affinity of the steroid–PBG complexes is controlled by the rate of dissociation. Steroid complexes of high-affinity binders from serum have generally higher dissociation rates than those of cellular receptor proteins. This is in harmony with the physiological functions of the two types of steroid-binding proteins.

INTRODUCTION

In the blood of the pregnant guinea pig a glycoprotein has been detected [1,2] that binds progesterone with relatively high affinity and specificity. This progesterone-binding globulin (PBG) appears around the 15th day of pregnancy and increases during gestation to high levels so that the concentration of binding sites far exceeds the amounts of progesterone present [3]. The high content of approximately 1 mg/ml of late pregnancy serum, a high stability to denaturing conditions, and other properties make PBG uniquely suited for an investigation of some basic problems of steroid–protein interactions.

A. General properties of PBG

Early observations on PBG as a serum binder distinct from the corticosteroid-binding globulin (CBG, transcortin) have been reviewed previously [4]. Purification by chromatographic and other methods has been reported from several laboratories [5,6,7]. A useful simplification of the purification procedure was achieved [8] by taking advantage of the acid stability of PBG and its extremely low isoelectric pH of 2.8 [9]: chromatography at pH 4.5 of the serum over a column of sulfopropyl (SP) Sephadex, a strong cation exchanger, results in adsorption of most serum proteins except PBG which is eluted in the void vol. of the column. A single run gives a PBG preparation free of albumin and of CBG which presumably is inactivated under the acidic conditions.

Final purification of PBG was achieved by affinity chromatography on columns of immobilized steroids [10]. Sepharose 4B was coupled with diaminodipropylamine to which the hemisuccinate of 19-nortestosterone or of deoxycorticosterone was then attached through an amide bond. When PBG is applied to the affinity column, a portion of the protein is not adsorbed and is readily eluted by the starting buffer; this material does not bind progesterone. The active PBG fraction is eluted with 10 μM progesterone or 5α-pregnane-3,20-dione.

Some properties of the affinity-purified PBG are shown in Table 1. Sedimentation equilibrium ultracentrifugation [11] gave an apparent average molecular weight of 88,000. The preparation consisted of approximately 30% polypeptide and had the unusually high carbohydrate content of about 70%. Almost one

Table 1. Properties of PBG

Molecular weight:	88,000; polydisperse
Isoelectric pH:	2.8
Peptide content:	~ 30%
Carbohydrate content:	~ 70%
K_a, 4° (progesterone):	$2.2 \times 10^9\,M^{-1}$
K_a, 37° (progesterone):	$3.5 \times 10^8\,M^{-1}$
Number of steroid binding sites:	$n = 1$

* Research Career Awardee of the United States Public Health Service.

† Present address: Department of Biochemistry, University of Rochester, Rochester, New York.

fourth of the carbohydrate was sialic acid. It has been previously reported [7] that PBG is a polydisperse molecule resulting from the attachment of varying amounts of carbohydrate to a polypeptide core of about 27,000 molecular weight. Such polypeptide-carbohydrate relationships are known for other polydisperse glycoproteins. The polydisperse nature was confirmed [10] for the affinity-purified PBG; on the basis of the compositional data given in Table 1, a polypeptide core of approximately 27,000 daltons can again be calculated. The amino acid composition and carbohydrate content of PBG are given in Tables 2 and 3, respectively.

The unusually high carbohydrate values for pure PBG have been obtained consistently in our laboratory. It should be noted, however, that lower amounts have been found by other investigators; Milgrom *et al.* [5] reported 48.7% for their preparation, and Lea [6] determined only 42%. No definite explanation can be given for these dissimilarities, except for the possibility that different strains of guinea pigs produce variant glycoproteins. We have eliminated differences in the purification methods as the cause of the variations of the carbohydrate content [7].

Equilibrium dialysis and Scatchard evaluation of the affinity-purified PBG gave a value of 1.03 for n, the number of binding sites per molecule, as an average of 6 analyses at different temperatures. The association constant of the progesterone complex, K_a, was $2.2 \times 10^9 M^{-1}$ at 4°, $1.1 \times 10^9 M^{-1}$ at 22°, and decreased further with increasing temperature (Table 1 and Table 6 below).

In an attempt to elucidate the chemical nature of the progesterone binding site, individual amino acid residues in PBG were modified by chemical reactions. Amino acid side chain groupings were substituted by specific reagents, and the effect on the binding activity was analyzed. In other experiments, the binding site was first "protected" by complex formation with pro-

Table 2. Amino acid composition of affinity-purified PBG

Residue	g per 100 g polypeptide	mols/mol PBG
Asp	10.6	22
Thr	8.1	19
Ser	5.1	14
Glu	10.8	20
Pro	4.5	11
Gly	2.2	9
Ala	3.6	12
Val	4.2	10
1/2 Cystine	0.85	2
Met	2.2	4
Ile	2.4	5
Leu	13.3	28
Tyr	6.9	10
Phe	6.2	10
Lys	5.4	10
His	4.1	7
Arg	5.9	9
Trp	3.9	5

Table 3. Carbohydrate content of affinity-purified PBG

Carbohydrate	%
Hexose	29.0
Hexosamine	23.5
Fucose	1.5
Sialic Acid	17.0
Total	71.0

gesterone, and the modifying reaction was done subsequently. The chemical reactions were performed [12] with PBG purified by chromatographic methods exclusive of affinity chromatography. Although the use of such PBG preparations having n values < 1 may influence some results in a quantitative manner, the basic effects of the chemical modifications on progesterone binding are not affected by it.

Reaction of *tryptophan* in PBG with hydroxynitrobenzylbromide (HNBB) resulted in a decrease of binding sites while the association constant of the remaining sites was not changed. Table 4 shows the parallelism between HNBB applied per PBG molecule, number of hydroxynitrobenzyl (HNB) residues introduced, and decrease of n measured by fluorescence quenching titration (see below) and by equilibrium dialysis. The tryptophan residues in PBG could be protected from substitution by complex-formation with progesterone (Table 5). Whereas 90% of the binding sites are inactivated by reaction of PBG with HNBB alone, a loss of only 20% of the binding sites occurs in the presence of an excess of progesterone. The K_a values of the remaining binding sites are not affected.

Modification of *lysine* in PBG by trinitrobenzenesulfonic acid (TNBS) also results in decreased steroid binding activity (nK_a). Complex formation with progesterone protects the lysine residues from substitution. Figure 1 shows that this protection starts immediately, with the first residues reacting. This would indicate that the lysine residues most reactive to TNBS are the ones with which progesterone interacts, presumably, in the binding site.

In experiments using different conditions, we analyzed the effect of chemical modification of lysine on the binding activity of PBG with and without protection by progesterone. In the absence of progesterone, there was a rapid decrease of the binding activity (Fig. 2) as the number of trinitrophenyl groups incorporated into PBG increased. For example, at 2.5 min after addition of TNBS, 3.8 amino groups were modified and the binding activity was reduced to 84% and 83% of the original value as measured by multiple equilibrium dialysis and fluorescence quenching titration, respectively; only 63% of the original quenching was now observed. After 15 min of TNBS reaction, the binding activity was down to 1/10 of the original value while the quenching was only 8% of that of the unmodified control. In contrast, the PBG

Table 4. Chemical modification of PBG by HNBB at pH 7.2

$\frac{HNBB}{PBG}$	$\frac{HNB}{PBG}$	Fluorescence titration % Quench	n*	Scatchard analysis n*	$K_a(M^{-1} \times 10^{-9})$
0	0	56	1.00	1.00	1.1
15	0.5	47	0.61	0.70	1.7
30	1.0	39	0.54	0.46	2.5
45	1.4	33	0.39	0.41	1.9

* Values adjusted relative to control $n = 1.00$.

Table 5. Inactivation of PBG by HNBB and protection of the binding site by progesterone

Reagent per mol PBG	Trp modified per mol PBG	Equilibrium dialysis, 4° n*	$K_a(M^{-1} \times 10^{-9})$
None	0	1.0	1.1
20 HNBB	1.2	0.1	1.1
10 Progesterone, 20 HNBB	0.7	0.8	0.8

* Values adjusted relative to control $n = 1.0$.

that was protected by complex formation with progesterone showed a much slower loss of binding activity as determined by multiple equilibrium dialysis (Fig. 2). After 15 min of reaction, 83% of the binding sites were still active, and equilibrium dialysis indicated 60% of the original binding activity. Even after 60 min, there was still 42% of the original binding activity present.

Reaction of PBG with tetranitromethane (TNM) reduced the number of progesterone binding sites to 3–10% of its original value, concomitantly with introduction of nitro groups into *tyrosine* residues [12]. The formation of nitrotyrosine was suppressed, and under the conditions applied about two thirds of the binding sites remained active when PBG was shielded from TNM by association with progesterone.

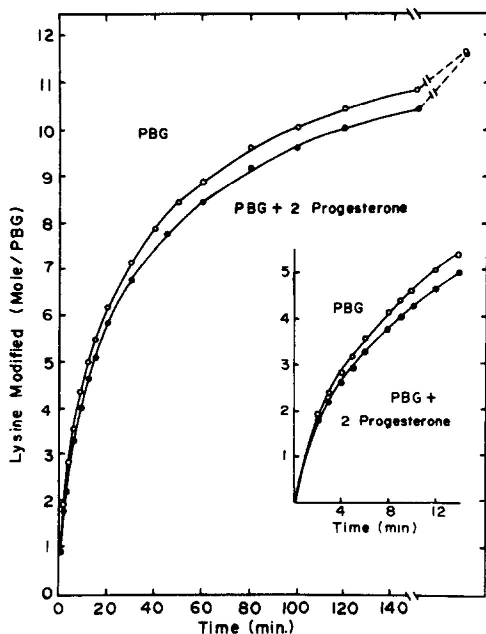

Fig. 1. Modification of PBG by trinitrobenzenesulfonic acid at 22° in the presence and absence of 2 mol progesterone per mol PBG. At 150 min the temperature was raised to 70° for 15 min to complete the reaction. The inset shows the lysine modification (mol/PBG) on an expanded time scale. From [12].

The results of the chemical modification studies with PBG indicate that tryptophan, lysine, and tyrosine residues are involved in the binding of progesterone. Covalent substitution suppresses the binding activity. The side chains of these amino acids are in different environments in free and progesterone-complexed PBG. The altered reactivity of the residues could result from direct interaction with progesterone at the binding site, or from a conformational change accompanying complex formation as will be discussed subsequently.

B. *Conformational changes of PBG upon interaction with steroids*

With the exception of a few results on CBG [13, 14], no observations have been reported on the induction of conformational changes by binding of steroids to proteins that form high-affinity complexes with steroids. The readily available and comparatively

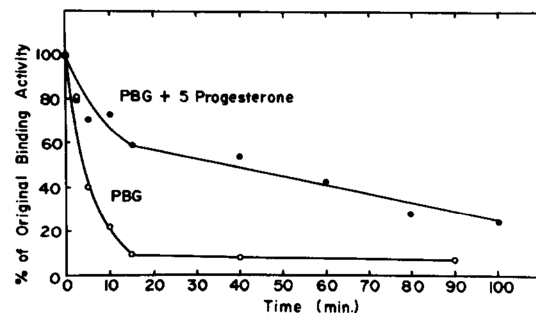

Fig. 2. Rate of inactivation of PBG and PBG–progesterone complex by trinitrobenzenesulfonic acid. The binding activity was determined by multiple equilibrium dialysis. For details see [12].

stable PBG provided a suitable steroid–protein system to study this conformational influence [8]. The circular dichroism spectra of PBG and of the PBG-progesterone complex (see Fig. 3 in [8]) show a more negative ellipticity between 240 and 227 nm for the steroid complex compared to free PBG. These and other differences in the CD spectra demonstrate a conformational change of PBG upon binding the steroid. A simple titration experiment indicated that this change in conformation is indeed due to complex formation. Attempts to apply available methods of interpreting the CD spectra in terms of α-helix, β-pleated sheet, and random coil segments were unsuccessful [8]. The reason may be the contribution to the observed ellipticity of the amide chromophores present in the carbohydrate moiety of PBG.

Conformational differences between PBG and steroid-liganded PBG were also revealed by ultraviolet difference spectra [8]. Such spectra of PBG against its complexes with progesterone and 5-pregnen-3β-ol-20-one (see Fig. 4 in [8]) show the induction of a large positive signal at 294–295 nm due to the perturbation of a tryptophan residue. In the case of the progesterone complex, a broad and intensive negative signal appears at 268–270 nm, resulting from solvent perturbation of the 4-ene-3-keto chromophore; this is the basis of a previously published spectral method for measuring protein interactions with 4-ene-3-ketosteroids [15]. In accordance with this interpretation the PBG complex with pregnenolone, a steroid lacking the 4-ene-3-keto chromophore, does not give the 268–270 nm minimum in its difference spectrum. A large positive peak at 233–234 nm in both difference spectra, resulting primarily from perturbation of the short wavelength transitions of aromatic amino acids, shows that the conformational change in PBG upon binding progesterone or pregnenolone is the same [8].

C. Fluorescence quenching of PBG upon interaction with 4-ene-3-ketosteroids

The involvement of tryptophan in the steroid binding to PBG, revealed by the ultraviolet difference spectra and the chemical modification studies, can also be seen in fluorometric measurements [16]. The intrinsic fluorescence spectrum of PBG shows an excitation maximum at 280 nm and an emission maximum at 340 nm characteristic of polypeptides containing tryptophan (see Fig. 1 in [16]). Occupation of the binding site by progesterone results in strong quenching without shift of the wavelength maxima; for affinity-purified PBG the reduction of fluorescence is over 80% [10]. This quenching phenomenon can be utilized in a simple titration method to determine the concentration of binding sites and the association constant. Addition of small aliquots of a progesterone solution to a given PBG solution results in a gradual decrease of the relative fluorescence until the binding site is saturated (Fig. 3). Further addition of progesterone does not reduce the fluorescence more, except for a small quenching contribution caused by ethanol in the steroid solution and by inner filter effects. The intersect of the extended initial linear portion and the extrapolated baseline gives the equivalence point. This determines the concentration of binding sites since the concentration of the added progesterone solution is known.

The association constant can be determined by the expression

$$K_a = \alpha/(1 - \alpha)^2 P$$

where α is the degree of association as given by the ratio of quenching at the equivalence point over total quenching, and P is the binding site concentration at the equivalence point. Drawing a smooth curve through all data points between the two linear portions (Fig. 3) makes it possible to base the value of α not on a single point but rather on an average. The data representing the quenching curve between the linear portions can be presented as a Scatchard plot (Fig. 3, inset). As to be expected, the affinity constant obtained is the same as that calculated by the simple expression given above.

The main advantage of the fluorescence quenching titration for determination of association constant and binding site concentration are the small amount of binding protein required (typically 0.1 nmol or less than 10 μg of PBG per experiment), the greater rapidity compared to equilibrium dialysis, and the possibility of using unlabeled steroids. The method has been applied in our laboratory for the determination of association constants of PBG complexes with a large number of steroids with 4-ene-3-keto and other absorbing groupings.* The association constants

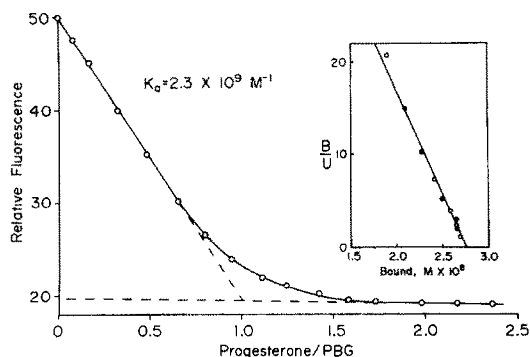

Fig. 3. Fluorescence quenching titration of PBG with progesterone. The equivalence point gives a binding site concentration of 2.70×10^{-8} M; the solid curve yields a K_a of $2.3 \times 10^9\ M^{-1}$. Inset: Scatchard plot of the data from the nonlinear region, giving a binding site concentration of 2.77×10^{-8} M and a K_a of $2.1 \times 10^9\ M^{-1}$. The filled circles correspond to the actual data points whereas the open circles are taken from the solid curve. From [16].

* Unpublished results with A. T. Blanford and W. L. Wittman.

obtained are in general agreement with those determined by equilibrium dialysis techniques.

If a steroid lacking the 4-ene-3-keto grouping binds to the same site in PBG as progesterone, competitive displacement of bound progesterone by the nonabsorbing steroid should occur and the fluorescence quenching should be reversed. Figure 4 shows that this is indeed the case: addition of 5α- or 5β-pregnane-3, 20-dione to a PBG solution that is maximally quenched by a stoichiometric amount of progesterone, results in restoration of the fluorescence [16]. The PBG complex formed with the saturated pregnane derivative approaches the intrinsic fluorescence of the unbound PBG as the concentration of pregnanedione is increased. The inset of Fig. 4 indicates that the fluorescence of PBG does not change even when several mol of 5-β-pregnanedione are added per mol of protein. It is also evident from Fig. 4 that greater amounts of the 5β-steroid than of the 5α-isomer are needed to displace progesterone from PBG. This is explained by a lower binding affinity of the 5β-pregnanedione, in agreement with competitive equilibrium dialysis studies showing that the association constant of the PBG complex with 5α-pregnane-3,20-dione is almost 10 times higher than that of the 5β-stereoisomer [17]. We will see below that the change of fluorescence by competitive displacement can be utilized to measure dissociation rates of PBG–steroid complexes.

D. Kinetics of steroid–protein interactions

Our present knowledge on interactions between steroids and proteins [4] has been attained essentially under the viewpoint of systems in equilibrium. Serum proteins with high affinity for steroid hormones have been isolated and characterized, and the strength of their association with steroids has been determined under various conditions. The emphasis has been on the end products of the reversible binding reaction, i.e., the steroid–protein complex and the unbound components formed by dissociation. For a meaningful interpretation of the interactions as they occur *in vivo* between hormones and proteins, and for an understanding of their biological significance, the association complex has to be viewed as a dynamic system; detailed information is required on the rates of association and dissociation, as well as on thermodynamic parameters. In the area of steroid-binding serum proteins, only a few quantitative kinetic studies on the interactions have been reported, as will be seen below.

The unusual stability [9], ready availability in pure form [10], binding affinity for a wide variety of steroids [5, 17–19], and suitable fluorescence properties [16] make PBG a very useful protein to study the rates of association and dissociation for progesterone and other steroids [20]. Quenching of the strong fluorescence signal of PBG upon binding a 4-ene-3-ketosteroid provides a sensitive indicator for association; the high affinity of PBG for progesterone, androgens, and other steroids permits the use of dilute reactant solutions to slow down the rapid bimolecular association reaction while still attaining complete association. Displacement of the quenching steroid from the complex by an ultraviolet-transparent steroid such as 5α-pregnanedione or dihydrotestosterone results in restoration of the intrinsic PBG fluorescence so that the dissociation rate of the PBG-4-ene-3-ketosteroid complex can be measured. These unique features allow the use of stopped-flow fluorometry for the determination of association and dissociation rates of PBG–steroid complexes.

The rate constant of association, k_{on}, of PBG and progesterone was measured over the temperature range from 5.9° to 41.2°; the corresponding dissociation rate constant, k_{off}, was determined from 20.0° to 41.4°. Utilizing these data, the general relationship $K_a = k_{on}/k_{off}$ was obtained and compared with the association constants measured by equilibrium dialysis [20]. Table 6 gives the rate constants of the progesterone–PBG complex and the ratios k_{on}/k_{off} at various temperatures. The K_a values determined by the two independent procedures agree at all temperatures. The close coincidence between the kinetic and the equilibrium association constants gives assurance that the observed fluorescence changes provide a true measure for complex formation and dissociation.

Introduction of hydroxy groups into the progesterone molecule results in a decrease of binding affinity to PBG [17], in proportion to the number of hydroxy groups involved. This binding behavior is in accordance with the polarity rule [4] indicating that the PBG–steroid interaction is predominantly of a hydrophobic nature. The association and dissociation rate constants, as well as their ratios which are equal to K_a, are given in Table 7 for progesterone, deoxycorticosterone, corticosterone, and cortisol, in addition to testosterone, its acetate, and medrogestone (6,16α-dimethyl-4,6-pregnadiene-3,20-dione). For compari-

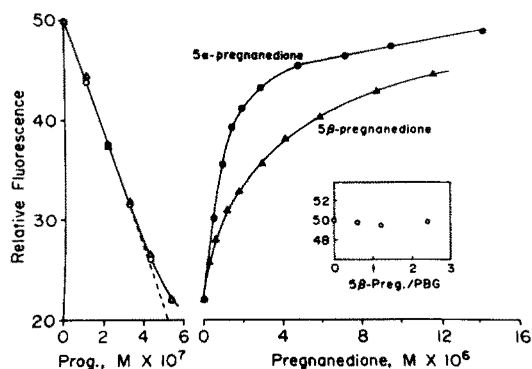

Fig. 4. Fluorescence quenching titration of PBG with progesterone (open circles and triangles) followed by addition of an excess of 5α-pregnane-3,20-dione or 5β-pregnane-3,20-dione. Note the "unquenching" by displacement of progesterone. The inset shows that the fluorescence of PBG does not change by addition of more than one mol of 5β-pregnanedione per mol PBG. From [16].

Table 6. Association constants of PBG–progesterone complex from kinetic and equilibrium data [20]

Temperature °C	k_{on} $M^{-1}s^{-1} \times 10^{-7}$	k_{off} s^{-1}	k_{on}/k_{off} $M^{-1} \times 10^{-9}$	K_a† $M^{-1} \times 10^{-9}$
4	2.2	0.0095‡	2.4	2.2
14	4.3	0.031	1.4	1.4
23	7.4	0.083	0.89	1.0
37	16.2	0.39	0.41	0.35
45	24.6	1.05	0.23	0.22

† Equilibrium dialysis; unpublished results with G. B. Harding.
‡ A value of 0.011 s^{-1} was determined independently by radiosteroid displacement using gel filtration.

son, the association constants determined independently by fluorescence quenching titration are listed. The latter values for most steroids in Table 7 are somewhat higher than the association constants obtained from kinetic data, in accordance with our previous observation that the fluorescence method slightly overestimates the affinity constants when compared with equilibrium dialysis results [16].

The association of PBG with the various steroids occurs at a very rapid rate which is essentially the same for all steroids that bind with affinity constants of $> 10^8\ M^{-1}$. Complexes of lower affinity appear to associate at a somewhat slower rate, although the values for the weakly bound corticosteroids are less certain. The rate constant for association of progesterone to PBG, $k_{on} = 8.7 \times 10^7\ M^{-1}\ s^{-1}$ at 20°, is similar to those of many protein–ligand complexes reported to be in the range of 10^6 to $10^8\ M^{-1}\ s^{-1}$ [21]. The association rates of the PBG–steroid complexes are not limited by diffusion [20].

In contrast to the relatively invariant rates of association, the dissociation rate constants show considerable differences among the steroids studied, covering a relative range of 1–3750 (Table 7). The dissociation rate thus becomes the controlling factor in steroid binding to PBG, conferring specificity to the interaction. This may be seen in several examples. Medrogestone binds to PBG with an affinity 2–3 times that of progesterone, yet both steroids associate at the same rate. It is the 3-fold slower dissociation of medrogestone which gives the higher affinity. Similarly testosterone acetate binds 3 times more tightly than testosterone and dissociates about 3 times more slowly. And the ratio between the affinity constants of progesterone and deoxycorticosterone is about 2 to 1 with a ratio of dissociation of 1 to 2. All these steroids associate with PBG at the same rate. The phenomenon of the dissociation rates controlling complex stability is similar to observations with antibody–hapten systems [22] and has been argued to be generally valid for protein–ligand interaction [22a].

What is the physiological meaning of the association and dissociation rates of various steroid–protein complexes? Obviously, the dissociation rate of the serum protein complex controls the availability of the steroid hormone to the target cell. Binding of the steroid to an intracellular receptor protein serves a different function: the hormone remains attached to the receptor while conformational changes and nuclear transfer reactions take place. It is worth examining, therefore, in which proportions association and dissociation rates contribute to the stability of the different types of steroid protein complexes. Table 8 shows these rate constants for steroid complexes with several high-affinity binders from serum, including PBG, CBG, and the testosterone-estradiol binding globulin (TeBG), and with some cytosol receptor proteins from chick and rat. A striking difference is evident: all serum protein complexes have dissociation rate constants that are one or two orders of magnitude higher than those of the group of receptor proteins.

These differences (Table 8) appear to be in accordance with physiological function. At late term preg-

Table 7. Association and dissociation rate constants of PBG and various steroids at 20° [20]

Steroid	k_{on} $M^{-1}s^{-1} \times 10^{-7}$	k_{off} s^{-1}	k_{on}/k_{off} $M^{-1} \times 10^{-8}$	K_a $M^{-1} \times 10^{-8}$
Progesterone	8.8	0.060	14.5	20*
Deoxycorticosterone	8.3	0.12	6.7	10
Corticosterone	~5	1.4	0.4	0.2†
Cortisol	~5	90	—	0.02
Testosterone	7.8	0.43	1.8	2.9†
Testosterone acetate	9.3	0.15	6.1	9.2†
Medrogestone‡	8.5	0.024	35.3	45.5†

* Extrapolation of equilibrium dialysis data in Table 6 gives $K_a = 11 \times 10^8\ M^{-1}$ at 20°.
† Determined by fluorescence quenching at 23°. Unpublished results with A. T. Blanford and W. L. Wittman.
‡ 6,16α-Dimethyl-4,6-pregnadiene-3,20-dione.

Table 8. Association and dissociation rate constants for various steroid binders at 0–4°

Protein	Steroid*	k_{on} $M^{-1}s^{-1} \times 10^{-4}$	k_{off} $s^{-1} \times 10^{5}$	k_{on}/k_{of} f $M^{-1} \times 10^{-8}$	Reference
PBG, guinea pig	P	2200	950	24	Table 6
CBG, human	C	20	41	4.9	[23]
CBG, human	C	—	45	—	[24]
CBG, human	C	—	230	—	[25]
CBG, rat	C	—	50	—	[26]
TeBG, human	T	—	90	—	[27]
TeBG, human	DHT	—	15	—	[27]
TeBG, rabbit	DHT	—	220	—	[28]
Chick oviduct receptor A	P	28	1.9	150	[29]
Chick oviduct receptor B	P	63	2.4	260	[29]
Chick oviduct receptor, purified	P	—	1.6	—	[30]
Rat liver receptor	D	1.4	2.0	7.3	[26]
Rat uterus receptor	E	—	3.9†	—	[31]

* P, progesterone; C, cortisol; T, testosterone; DHT, dihydrotestosterone; D, dexamethasone; E, estradiol.
† 25°.

nancy concentrations of progesterone and PBG, the half-time of association at 37° is 0.36 ms, that of dissociation of the complex is 1.8 s [20]. The corresponding half-time values for the CBG–cortisol complex at 37° are 44 ms and 1.1 s. By comparison, the association rate constant of progesterone with the chick oviduct receptors, and that of dexamethasone with the rat liver binder resemble that found for CBG. However, the half-time of dissociation for receptor complexes in general is of the order of 10 h at 0–4°; at 25°, the rat uterus receptor–estradiol complex dissociates with a half-time of 5 h. The half-time of dissociation for the dexamethasone-binding receptor protein at 37° is approximately 13 min.

The slow dissociation rates of the cytosol receptor complexes is in accordance with their postulated role in mediating cellular response to steroid hormones. It assures that the steroid remains bound to the cytosol receptor protein for a sufficiently long time so that the subsequent reactions leading to nuclear hormonal effects can take place. In contrast, the requirements of a hormone-transporting protein in the blood serum are different: rapid dissociation of the complex is necessary to provide immediate availability of the steroid at the target site. At the same time, the association must be fast, resulting in a high affinity so that the major portion of the circulating hormone is present in bound, biologically inactive form, for the protection of the organism as well as for the preservation of the steroid hormone.

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DISCUSSION

De Moor. May I ask you a stupid question: is there a progesterone binding globulin in milk?

Westphal. We don't know, we have never investigated it.

De Moor. I asked you this question because we found (H. Van Baelen, R. Bouillon and P. De Moor, *J. biol. Chem.* (in press) that transcalciferin, the serum protein binding 25-hydroxycholecalciferol, has a higher molecular weight in human milk than in human serum. We could show that this is due to aggregation of the binding protein of serum with a cytosolic factor that does not bind 25-hydroxycholecalciferol itself and that is secreted into the milk. We could not prove, however, that the reverse situation, i.e. the serum binding protein penetrating into the cell and forming an intracellular complex with the cytosolic factor, does also occur.

Westphal. Thank you for these interesting comments. We have been interested in the relationship of the PBG in serum to the progesterone receptor that is present in the pregnant uterus of the guinea pig, and although there are many properties that seem to be identical we have clearly separated the two now by chemical methods. This shows that they are definitely not identical, that they are two different species. However, whether there is a chemical relationship, which of course is of considerable interest, has not yet been established.

De Moor. The cytosolic factor involved in the aggregation does not bind 25-hydroxycholecalciferol itself.

Posner. The observation of the more marked effect of temperature on the dissociation rate constant as opposed to its minimum effect on the association rate constant has been made in other systems as well. Why do you think temperature elevation augments predominantly the dissociation rate constant?

Westphal. I don't think I can give a good answer to this question. Obviously the association rate which is so much faster is subject to a much smaller temperature effect. It must be a molecular reason which we do not understand because we know so little about the steric relationships of the different steroids to the amino acid residues located in the binding site. It may also involve a different temperature effect on the bimolecular vs the monomolecular reactions.

Pasqualini. I remember that you demonstrated many years ago that progesterone circulates in the maternal compartment of guinea pig mainly bound to the plasma proteins. On the other hand in our laboratory we found that in the fetal plasma of the same animal species progesterone circulates mainly in unbound form, and very little is bound (5–10%). Do you have some explanation for this difference in the binding?

Westphal. Not really, we have never investigated the binding in the fetus.

Challis. I wondered if you had any explanation for the source of PBG and what you really think the biological significance of it is, because the guinea pig is one of the few species in which it is very difficult to demonstrate any inhibitory effect of progesterone on myometrial activity.

Westphal. The best reason I can give is preservation of progesterone. I am sure you are familiar with the work of Heap and his coworkers in England who have investigated this system many years ago. As a matter of fact when we found the PBG they had just published an abstract in which they concluded from metabolic clearance rates that there must be a high-affinity binding protein in that serum. I believe the physiological function is essentially the preservation of progesterone. This is the biological significance that Heap gives who investigated the biology of this system much more than we have.

Challis. And the source?

Westphal. The source is unknown. We had assumed at one time that the placenta is the source. We have done some studies on this but this work is not completed.