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pH Dependence of Progesterone Interaction with Progesterone-Binding Globulin. Kinetic and Equilibrium Studies†

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ABSTRACT: The kinetics of binding and dissociation for the progesterone-binding globulin (PBG)·progesterone complex have been measured as a function of pH. The association rate constant appears to be independent of pH from pH 5 to 10 with an average value of $k_{on} = 8.5 \times 10^7\ M^{-1}\ s^{-1}$. The dissociation rate constant is strongly pH dependent with the dependency defined by: $k_{off} = k_0(1 + [H^+]/K_1 + K_2/[H^+])(1 + K_3^*/[H^+])/(1 + K_3/[H^+])$. The best values for the various parameters were $k_0 = 0.0785\ s^{-1}$, $pK_1 = 5.30$, $pK_2 = 10.54$, $pK_3^* = 7.41$, and $pK_3 = 7.21$. Simpler expressions were inadequate to fit the data, and it was concluded that at least three ionizing residues are responsible for the stability of the PBG·progesterone complex. The affinity constant was determined by equilibrium dialysis over the range of pH 3 to 12. The ratio of the association and dissociation rate constants is in agreement with the affinity constant from pH 6.5 to 10.5. The influence of pH on the conformation and binding activity of PBG was also investigated. Denaturation by acid, base, or guanidine hydrochloride leads to a reversible loss of binding activity. Regain of binding activity in all cases is slow with half-times of 0.5 to 2.7 h, depending on conditions. The rate of acid denaturation was determined as a function of pH. The protein was found to be incompletely protonated at pH 1.4, suggesting a buried carboxylic acid residue. The slow renaturation of PBG might be due to the difficulty of burying a charged residue in the protein's interior coupled with steric hindrance by the large carbohydrate moiety of PBG.

A detailed investigation of the physicochemical and steroid binding properties of the progesterone-binding globulin (PBG)[1] of the pregnant guinea pig has been performed in this laboratory (Burton et al., 1974; Stroupe and Westphal, 1975a). This unique high-affinity steroid binder can be obtained in pure form and in appreciable amounts (Cheng et al., 1976). It is a polydisperse glycoprotein with the unusually high carbohydrate content of about 70%, including 17% sialic acid; the polypeptide core has a molecular weight of 27 000. The marked alteration in the intrinsic fluorescence of PBG upon binding steroids (Stroupe et al., 1975) has made possible the determination of the association and dissociation rate constants for complex formation (Stroupe and Westphal, 1975b). Chemical-modification studies have been initiated to identify the amino acids involved in binding (Westphal et al., 1976). To gain additional insight into the amino acid residues responsible for the integrity of the steroid-binding site, we have undertaken a study on the influence of pH on the association and dissociation rate constants. The kinetic results were compared with the affinity constants determined under equilibrium conditions. To aid in the interpretation of these findings, the influence of pH on the conformation of PBG was investigated.

Materials and Methods

PBG was prepared from pooled pregnant guinea pig serum (Grand Island Biologicals Co.) by SP-Sephadex and affinity chromatographies as described by Cheng et al. (1976). Steroids were commercial products; their melting points were verified. [^{3}H]Progesterone was from New England Nuclear; its purity was checked by thin-layer chromatography. Guanidine hydrochloride was Mann "ultrapure". All other chemicals were reagent grade, and water was glass redistilled.

Stopped-Flow Fluorometry. A Gibson-Durrum stopped-flow apparatus equipped with a fluorescence cuvette and connected to a Nova 1200 computer for automatic data collection was used as described previously (Stroupe and Westphal, 1975b). The association and dissociation rates of PBG and progesterone were measured as before (Stroupe and Westphal, 1975b). To avoid errors due to possible differences in concentration, a single unbuffered stock solution of PBG (1.1 μM in 0.1 M NaCl) and a single progesterone solution of the same concentration in 0.1 M NaCl were used in measuring the pH dependence of the association rate. The pH was adjusted prior to loading the samples into the instrument by adding 50

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[1] Abbreviations used are: PBG, progesterone-binding globulin; Tris, 2-amino-2-hydroxymethyl-1,3-propanediol.

μL of 1 M buffer of the proper pH to 5 mL of the PBG and progesterone stock solutions. Similarly, for the dissociation rate studies an unbuffered PBG·progesterone complex stock solution (0.50 μM in 0.1 M NaCl) and an unbuffered dihydrotestosterone stock solution (50 μM in 0.1 M NaCl containing 0.25% ethanol) were utilized. This concentration of ethanol does not measurably alter the dissociation rates (Stroupe and Westphal, 1975b).

Measurement of pH. After all experiments, the mixed solutions were collected and the pH was measured to obtain the reported values. A Radiometer pH meter, Model 26, equipped with a GK 2322C combined glass electrode and standardized with pH 4.01 and 7.00 buffers was used for all determinations.

The rate of acid denaturation was measured by observing the decrease in intrinsic fluorescence of PBG when unbuffered PBG was mixed with an acidic buffer of the appropriate pH in the stopped-flow instrument.

The rate of renaturation was obtained by determining the time course of the recovery of steroid-induced quenching of the intrinsic fluorescence of PBG. Fluorescence was measured with an Aminco Ratio Spectrofluorometer equipped with an ellipsoidal condensing mirror. Excitation was at 280 nm and emission at 340 nm. A relatively concentrated (approximately 5 μM in 0.1 M NaCl) PBG sample was mixed with an equal volume of acidic or basic buffer and the pH was recorded (in the case of guanidine hydrochloride solid reagent was added to obtain a 6 M concentration). When steroid was absent from the denaturing medium, the time of exposure to low pH (0.5–30 min) did not affect the rate of renaturation. Renaturation was initiated by diluting the PBG solution tenfold with 0.1 M Tris buffer, giving a final pH of 7.40 ± 0.05 (dilution was 20-fold in the case of guanidine hydrochloride denatured PBG). Aliquots of the renaturing sample were placed in a cuvette and the fluorescence was measured; a 40-fold excess of progesterone was then added and the fluorescence was measured again giving the progesterone-induced quenching termed $\%Q$, where

$$\%Q = \frac{F \text{ of PBG} - F \text{ of PBG·P complex}}{F \text{ of PBG}} \times 100$$

(F is fluorescence and P is progesterone). Denatured PBG does not bind steroids and, hence, its fluorescence is not quenched by progesterone. Therefore, an increase in quenching is due to recovery of binding activity. The rate of renaturation was determined by plotting ln $(\%Q_\infty - \%Q_t)/\%Q_\infty$ vs. time. $\%Q_\infty$ was determined after 24 h and was found to be 82 to 85%, which is the value previously determined for affinity-purified PBG (Cheng et al., 1976).

Equilibrium dialysis was used to determine the pH dependence of the affinity constant. Scatchard plots were performed at pH 5.50, 6.50, 7.40, 8.50, and 10.50. The affinity constant was obtained over a wider pH range by measuring the nK_a value. It has been demonstrated (Harding et al., 1974) that PBG is stable within the pH range applied in the present studies. Therefore, changes in the nK_a value reflect alterations in K_a and not in n.

Computer fitting of the dependence of the dissociation rate constant to the expression

$$k_{off} = k_0(1 + [H^+]/K_1 + K_2/[H^+]) \times \frac{1 + K_3^*/[H^+]}{1 + K_3/[H^+]} \quad (1)$$

(Mattis and Laskowski, 1973) was performed by the Nova 1200. K_1 and K_2 are the dissociation constants for two amphoteric groups. K_3 and K_3* are the dissociation constants for a third group with K_3 corresponding to the PBG·progesterone complex and K_3* to free PBG. K_1 and K_2 were fixed, and k_0, K_3, and K_3* were allowed to vary to find the minimum deviation of calculated from observed values for that pair of K_1 and K_2. K_1 and K_2 were then systematically varied at 0.02 pH intervals until the overall minimum deviation of calculated from observed dissociation rate constants was obtained.

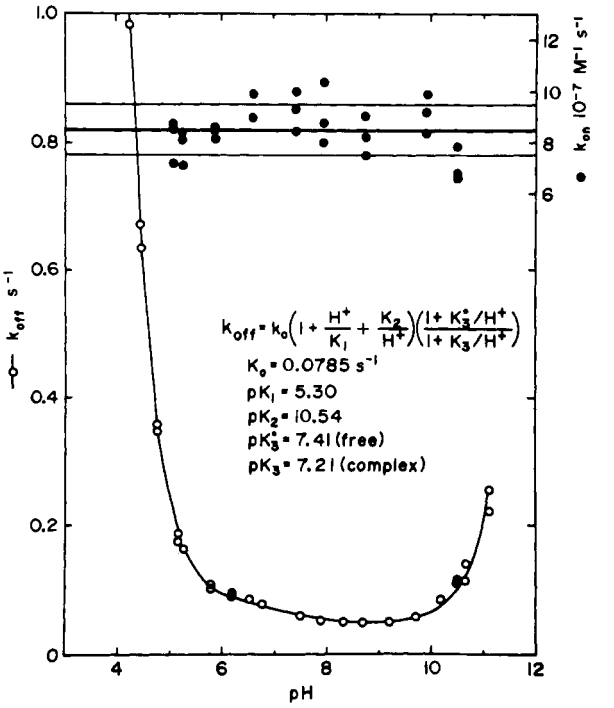

FIGURE 1: Dependence of the dissociation and association rate constants on pH. (○) (Left ordinate) Experimental values of k_{off} with the solid curve drawn from the equation and parameters given in the figure. (●) (Right ordinate) Experimental values of k_{on}; the heavy line is the mean of all values and the two lighter lines give ± one standard deviation. Buffers used were: pH 4.24–5.27, sodium acetate; pH 5.79–6.79, sodium cacodylate; pH 7.42–8.74, Tris-HCl; pH 9.22–11.13, sodium glycinate. Each data point is the mean of four independent determinations.

Results

The pH dependence of the association and dissociation rate constants for the PBG·progesterone complex is given in Figure 1. The association rate constant is pH independent from approximately pH 5 to 10, giving a value of 8.5 ± 1.1 × 10^7 M^{-1} s^{-1} in good agreement with the previously reported value of 8.7 × 10^7 M^{-1} s^{-1} (Stroupe and Westphal, 1975b). On the other hand, the dissociation rate constant describes a broad catenary with a minimum at pH 9. The solid curve is that calculated using k_0 = 0.0785 s^{-1}, pK_1 = 5.30, pK_2 = 10.54, pK_3 = 7.21, and pK_3* = 7.41. Visual inspection indicates the fit to be satisfactory; Figure 2A confirms the good fit. The percentage deviation of calculated from observed values of k_{off} is less than 11% over the entire pH range.

If a simpler expression is used:

$$k_{off} = k_0(1 + [H^+]/K_1 + K_2/[H^+]) \quad (2)$$

a reasonable fit to the data can be obtained with the values of k_0 = 0.048 s^{-1}, pK_1 = 5.55, and pK_2 = 10.50. These were the best values computed for eq 2 using a similar minimizing scheme as described above. However, the percent deviation of calculated from observed values presented in Figure 2B reveals

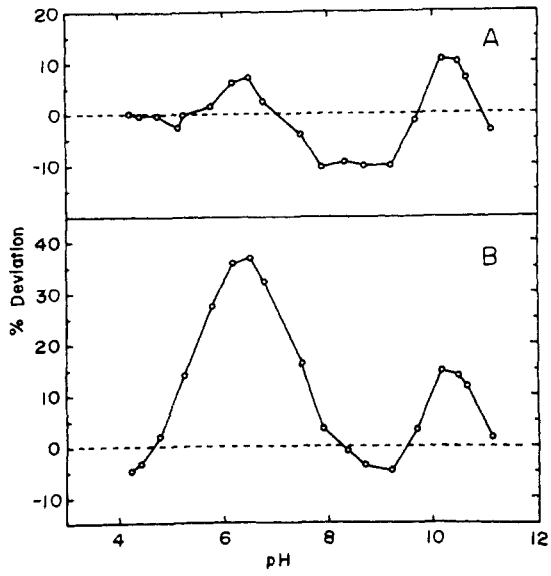

FIGURE 2: Deviation of calculated from observed values of the dissociation rate constant. (A) Calculated according to eq 1; (B) according to eq 2.

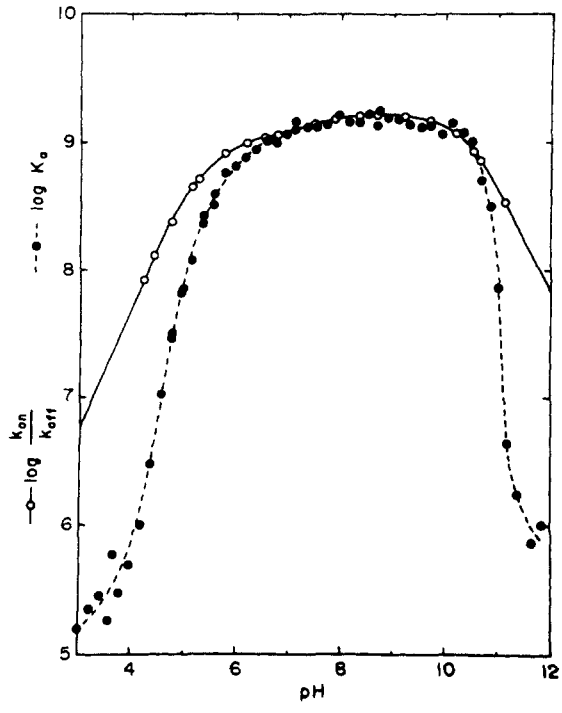

FIGURE 3: Dependence of K_a and k_{on}/k_{off} on pH. The logarithms of the affinity constant, K_a (●), and of the ratio of the association and dissociation rate constants, k_{on}/k_{off} (○), are presented as a function of pH. The values for K_a were obtained by equilibrium dialysis (see text) using the following buffers: pH 3.0–3.6, glycine hydrochloride; pH 3.6–5.6, sodium acetate; pH 5.4–7.4, sodium cacodylate; pH 7.2–8.8, Tris-HCl; pH 8.6–12.0, sodium glycinate. The values for k_{on}/k_{off} and the solid curve were obtained using the average k_{on} of 8.5×10^7 M^{-1} s^{-1} and the k_{off} was calculated from the parameters in Figure 1.

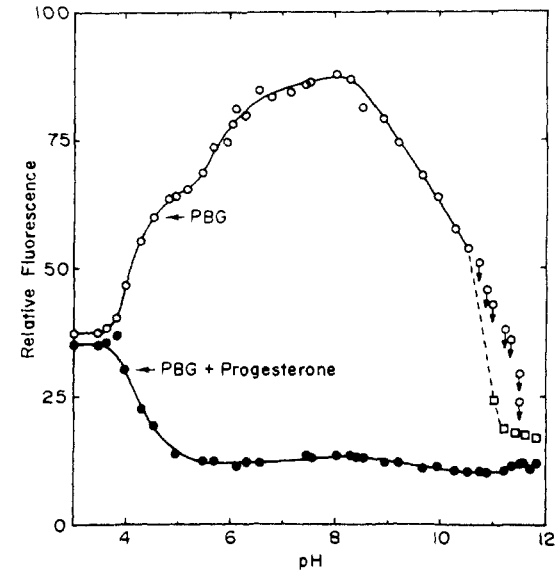

FIGURE 4: The pH dependence of the intrinsic fluorescence of PBG and of the PBG-progesterone complex. Unbuffered PBG (0.14 μM in 0.1 M NaCl) was titrated by adding small volumes of concentrated acid or base to the neutral solution. After pH adjustment, the fluorescence of a 1-mL aliquot was measured (○); a 40-fold excess of progesterone was then added, and the fluorescence measured again (●). Excitation was at 280 nm and emission at 340 nm. Above pH 10.5 there was a slow, time-dependent decrease (indicated by arrows) in the fluorescence of PBG to the values indicated by the broken line (□). Since the titration curve of PBG is not rapidly reversible (see text and Table I), separate samples were used for the acidic and basic limbs.

a large systematic error centered around pH 6.5. It is concluded that at least three ionizing residues are involved in control of the dissociation reaction.

Figure 3 gives the values of the equilibrium affinity constant K_a as well as the ratios k_{on}/k_{off} over the pH range studied. The average value of 8.5×10^7 M^{-1} s^{-1} for k_{on} and the values of k_{off} were calculated from the data in Figure 1 using eq 1. Agreement is satisfactory from pH 6.5 to 10.5; however, at lower and higher pH values the equilibrium constant deviates systematically from the calculated ratio. This deviation is best explained by a pH dependency of the association rate constant at the extreme pH values.

Further information concerning the pH dependence of PBG's physical properties is available in Figure 4, which gives the pH profile of the intrinsic fluorescence of PBG and its progesterone complex. Fluorescence is maximal at pH 8 with an almost linear decrease as the pH is increased. Above pH 10.5, the fluorescence exhibits a slow time-dependent decrease to the values indicated by the broken line. Acidic to pH 8 the fluorescence gradually declines and the curve forms a shoulder at pH 5; between pH 5 and 3.5, the fluorescence decreases sharply with a midpoint at about pH 4. The fluorescence changes indicate that above pH 10.5 and below pH 5 structural alterations occur.

The fluorescence of the PBG-progesterone complex is relatively pH independent from pH 12 to 5. Below pH 5 the fluorescence increases, mirroring the decrease seen for free PBG over this pH range. The increase in fluorescence is not due to the inability of PBG to bind progesterone; using the equilibrium value of the affinity constant (Figure 3) PBG is 97% saturated with progesterone at pH 4.5 and about 75% saturated at pH 4.0.

The acidic side of the fluorometric titration curve is only slowly reversible. Neutralization of a sample of PBG at a pH below 4 does not result in an immediate restoration of the fluorescence; rather, there is a slow increase in fluorescence to that of native PBG. Accompanying the regain of intrinsic fluorescence, PBG recovers its native conformation as measured by the increase in steroid-induced fluorescence quenching. The slow renaturation of PBG appears to follow first-order kinetics, as determined by full recovery of intrinsic fluorescence and binding activity. Table I summarizes several

TABLE I: Half-Times for Renaturation of PBG.

Denaturant	Half-Time (h)
pH 4.0	1.2
pH 3.5	1.3
pH 3.0	2.1
pH 3.0[a]	2.2
pH 2.6	2.7
pH 2.6[b]	1.3
pH 12.4	0.5
6 M Gdn-HCl	0.6

[a] A 100 molar excess of dihydrotestosterone was included in the renaturing buffer. [b] Two moles of dihydrotestosterone per mol PBG was included in the denaturing buffer.

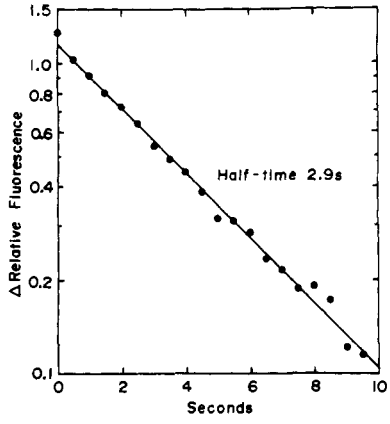

FIGURE 5: Denaturation of PBG via pH jump from pH 7.4 to 2.6. Unbuffered PBG (2.3 μM in 0.1 M NaCl, pH 7.4) was mixed with 0.1 M sodium citrate buffer (final pH 2.58). The change in intrinsic fluorescence of PBG occurred at a rate of 0.24 s^{-1}.

renaturation experiments in which the original %Q was recovered.

Several comments on the data are appropriate. First, PBG recovers binding activity slowly. Second, the presence of steroid during the renaturation process does not hasten renaturation. The apparent acceleration of renaturation when steroid is included in the denaturating medium is due to incomplete unfolding, which in turn results from stabilization of PBG by bound steroid. Third, recovery from acid denaturation is slower than from the effects of base or guanidine hydrochloride. Fourth, the acid denaturation is a multistep transition because the rate of renaturation depends on the hydrogen ion concentration with which the denatured PBG was equilibrated (Tanford, 1968, 1970).

The rate of acid denaturation can also be measured. When unbuffered, neutral PBG was mixed with an acidic buffer, there was a 40% reduction of the intrinsic fluorescence within the dead time of the instrument (3 ms). Following the instantaneous quench, there was a slower, first-order decrease in the remaining PBG fluorescence. A typical example is shown in Figure 5 where PBG was rapidly titrated from pH 7.4 to 2.58 in the stopped-flow fluorometer. The slower fluorescence change is seen to describe a first-order process with a rate of 0.24 s^{-1}. Figure 6 gives the pH dependence of the rate of denaturation. This rate appears to be pH independent above pH 3.3; however, on the acidic side, the rate increases and continues to increase down to pH 1.40, the lowest value investigated.

The acid-denatured protein was studied by difference

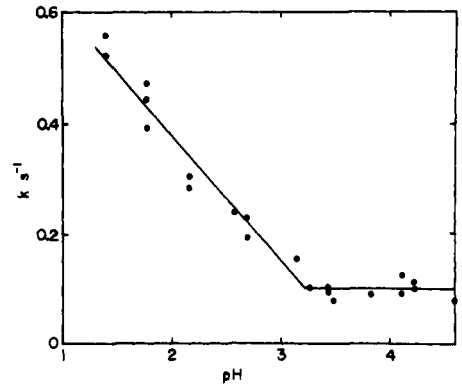

FIGURE 6: The pH dependence of the rate of acid denaturation of PBG. Unbuffered PBG samples (2.3 μM in 0.1 M NaCl, pH 7.4) were mixed with 0.1 M sodium citrate buffer and the time course of the change in the fluorescence of PBG was recorded. To obtain pH 1.78 and 1.40, PBG was mixed with 0.05 and 0.1 M HCl, respectively. Each data point is the mean of four individual measurements on the stopped-flow fluorometer.

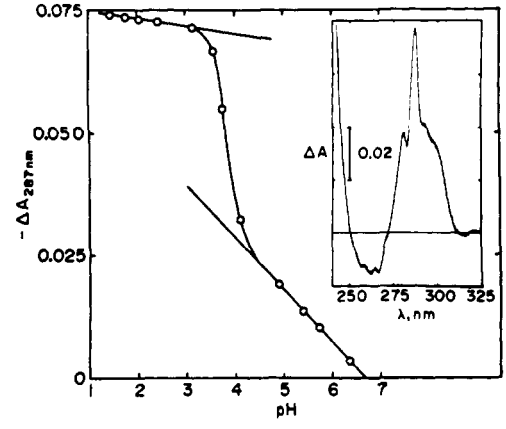

FIGURE 7: Acid denaturation of PBG. Using a Cary 15 recording spectrophotometer and the 0.1 slide-wire, a baseline was obtained for PBG vs. PBG (16.4 μM in 0.1 M NaCl, pH 7.15). Samples of the same stock solution were acidified by blowing HCl gas over the surface of the solution; the pH values were measured immediately after the difference spectra were recorded. The curve connecting the two linear portions is a theoretical curve for a transition having a $\Delta pH_{90}{}^{10}$ of 0.61 (Weber, 1975) and a midpoint at pH 3.80. Inset: Typical acid difference spectrum of PBG, determined separately. The sample solution and the reference solution (15.5 μM in 0.1 M NaCl) were at pH 7.40 and 2.30, respectively; the ΔA value at 287 nm, therefore, indicates decreased absorbance upon denaturation. The baseline (pH 7.40 vs. 7.40) was flat within ±0.002 A.

spectroscopy; Figure 7, inset, gives the near-UV difference spectrum of PBG (pH 7.40 vs. pH 2.30). The spectrum is a typical acid-denaturation spectrum with the denatured protein exhibiting a blue shift and decrease in extinction (Donovan, 1969). Not shown is the short-wavelength peak at 233 nm which has $\Delta\epsilon_{233}$ of 26 300 M^{-1} cm^{-1}. Utilizing the difference extinction coefficients proposed by Donovan (1973), the value of $\Delta\epsilon_{287}$ = 4970 M^{-1} cm^{-1} corresponds to the transfer of seven tyrosine residues from the interior of the protein to solvent. The $\Delta\epsilon_{293}$ of 2650 M^{-1} cm^{-1} is consistent with the exposure of 1 to 2 tryptophan residues to solvent upon denaturation (Donovan, 1973). The pH dependence of the difference signal at 287 nm is given in Figure 7. The curve describes a cooperative transition, since 0.61 pH unit corresponds to 10–90% completion (Weber, 1975); the midpoint is at pH 3.80.

Discussion

The pH dependence of steroid binding to human albumin, human α_1-acid glycoprotein, and corticosteroid-binding

globulin from human and rat serum has been reported (Westphal, 1971). In every case, as in the present report, maximal binding was found above physiological pH with decreasing affinity at both lower and higher pH. However, no previous study on serum high-affinity steroid binders has dealt with the kinetic basis for the pH dependence of the affinity constant.

The relative pH invariance of the association rate constant and the strong pH dependence of the dissociation rate constant are in agreement with the previous finding that the dissociation rate constant controls the specificity of steroid binding to PBG (Stroupe and Westphal, 1975b). Furthermore, Weber (1975) has argued that, in general, it is the dissociation rate constant which controls ligand affinity. Therefore, it is in the pH dependence of the dissociation rate constant that one would expect to find information concerning the amino acid residues which maintain the integrity of the steroid-binding site.

The dissociation rate data in Figure 1 fitted best an expression which invoked three ionizing residues (eq 1). The most acidic residue gave a pK of 5.30; this would suggest a carboxyl or histidyl. However, it is impossible to decide between these two groups without further information; the pK is higher than that expected for a carboxyl and lower than usually observed for a histidyl. Values of 3.9–4.35 and 6.4, respectively, are expected as the intrinsic pK's of carboxyl and histidyl residues in proteins (Nozaki and Tanford, 1967). There are examples of either residue titrating with a pK of approximately 5.3. For instance, Tanford and Roxby (1972) have assigned pK's of 5.6 and 6.3, respectively, to His-15 and Glu-35 of lysozyme.

The basic residue deduced from eq 1 has a pK of 10.54 and is provisionally assigned to a lysine. Both lysine and tyrosine titrate with pK's in this range and both have been implicated as being in or near the binding site by chemical-modification studies (Kute, 1975; Westphal et al., 1976). However, a pK of 10.5 is closer to that expected for a lysyl residue (Nozaki and Tanford, 1967) and the acid-denaturation spectrum of Figure 7 suggests that most tyrosyl residues are buried and would not be expected to exhibit normal titration behavior. Furthermore, a positively charged residue has been assumed in the difference spectrum between PBG and its progesterone complex (Stroupe and Westphal, 1975a).

The third residue whose pK shifts from 7.41 in free PBG to 7.21 in the progesterone complex is suggested to be a histidyl residue. There are no free sulfhydryl groups in PBG (Westphal et al., 1976) and the α-amino group appears to be blocked (unpublished results with S. L. Cheng). The shift in pK may appear to be minor; however, as demonstrated in Figure 2, its inclusion is necessary to avoid a large systematic error in the calculated curve near pH 6.5. The shift in pK from 7.41 to 7.21 upon binding a progesterone molecule results in the calculated release of 0.11 proton from PBG at pH 7.4. The equilibrium data in Figure 3 give a value of d log K_a/d pH of 0.1 at pH 7.4; therefore, both the kinetic and equilibrium pH dependency of progesterone binding at physiological pH is due to this small alteration in pK of one residue.

The term k_0 is the intrinsic dissociation rate constant of progesterone from PBG when group 1 is completely ionized and group 2 is completely protonated. The value for k_0 of 0.048 s^{-1} derived using eq 2 is closer to the observed value between pH 8 and 9 than the 0.0785 s^{-1} obtained with eq 1. The discrepancy is resolved by noting that with eq 1, k_0 is the intrinsic dissociation rate constant when group 1 is ionized and both groups 2 and 3 are completely protonated. In other words, when group 3 is protonated, the intrinsic dissociation rate of progesterone from PBG is 0.0785 s^{-1}, and it is 0.048 s^{-1} when group 3 is deprotonated. In the complex at pH 7.4, group 3 is about 60% deprotonated. If proton equilibrium with group 3 were slow, one would observe a biphasic dissociation rate with 40% occurring at 0.0785 s^{-1} and 60% at 0.048 s^{-1}. No such biphasic reaction is observed and the determined rate at pH 7.4 of 0.060 s^{-1} is exactly that predicted for a 40–60 weighted average of 0.0785 and 0.048 s^{-1}, respectively. One may thus conclude that proton equilibration with group 3 is, as expected, more rapid than that of the steroid with its binding site.

As indicated above, agreement exists between K_a, determined by equilibrium dialysis, and k_{on}/k_{off} between pH 6.5 and 10.5 (Figure 3), with K_a being systematically lower at more acidic and more basic pH. Calculated values of k_{on}/k_{off} at the extreme pH values are based on the assumption that the association rate constant is pH independent; this assumption may be incorrect.

Acid denaturation and renaturation of PBG exhibit certain distinct features. Renaturation from acid denaturation is very slow with the rate dependent on the denaturing pH. This very slow renaturation of PBG from acid, base, or guanidine hydrochloride denaturation is in contrast to the rapid renaturation observed from guanidine hydrochloride denaturation of carbonic anhydrase (Wong and Tanford, 1973), cytochrome *c* (Ikai et al., 1973), lysozyme (Tanford et al., 1973), and ribonuclease A (Garel et al., 1976). The slow renaturation of PBG from any form of denaturation appears to be a unique property of this protein.

The pH dependence of the denaturation reaction is also unique. The rate of denaturation appears to be pH independent above pH 3.3; in more acidic media, the rate increases down to pH 1.40, the most acidic medium utilized. The pH-independent region corresponds to the absorbance change (Figure 7); however, the increase in denaturation rate at low pH indicates that PBG is not completely unfolded at pH 3. Indeed, the increase in denaturation rate down to pH 1.4 suggests that at least one residue in PBG is not completely protonated at that pH and that the protein is still unfolding. The acidic group is not a sialic acid residue; these residues are located at the ends of carbohydrate branches and, hence, are remote from the peptide core. The presence of very acidic carboxylic acids in proteins has been reported; for example, the α-carboxyl of lysozyme has a pK of 1.2 in a medium of 0.1 ionic strength (Tanford and Roxby, 1972).

The slow refolding of PBG might be due to two factors. The incomplete protonation of PBG at pH 1.4 suggests that at least one carboxylate is buried in the interior of the molecule as an anion. For energetic reasons, this anion is probably combined with a positively charged residue in an ion pair. Formation of this ion pair might be slow considering that PBG is about 70% carbohydrate, and this large mass might offer steric hindrance to refolding as well as stabilize the unfolded structure by its hydrophilic nature.

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Isolation and Identification of Cytokinins from *Euglena gracilis* Transfer Ribonucleic Acid†

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ABSTRACT: Three ribonucleosides responsible for cytokinin activity in *Euglena gracilis* var Bacillaris tRNA have been isolated and identified as 6-(3-methyl-2-butenylamino)-9-β-D-ribofuranosylpurine, 6-(4-hydroxy-3-methyl-*cis*-2-butenylamino)-9-β-D-ribofuranosylpurine, and 6-(4-hydroxy-3-methyl-2-butenylamino)-2-methylthio-9-β-D-ribofuranosylpurine. The structures of these compounds were assigned on the basis of their chromatographic properties and ultraviolet and mass spectra which were identical with those of the corresponding synthetic compounds. The elution profiles of cytokinin bioassay activity and of ^{35}S radioactivity suggest the presence of a trace amount of 6-(3-methyl-2-butenylamino)-2-methylthio-9-β-D-ribofuranosylpurine.

Cytokinin activity has been reported in tRNA preparations from a wide variety of organisms (Skoog and Armstrong, 1970; Hall, 1973). The cytokinin-active ribonucleosides which have been identified as constituents of tRNA are *c*-io^{6}A,[1] *t*-io^{6}A, i^{6}A, ms^{2}i^{6}A, and ms^{2}io^{6}A. Studies on the distribution of cytokinins in tRNAs from various organisms suggest that i^{6}A and ms^{2}i^{6}A are the two major cytokinins generally found in bacterial tRNAs. However, *t*-io^{6}A has been reported recently in the tRNA of plant pathogen *Agrobacterium tumefaciens* (Chapman et al., 1976). Also ms^{2}io^{6}A has been reported in *Pseudomonas aeruginosa* tRNA preparation (Thimmappaya and Cherayil, 1974). Unlike bacteria, animal tRNAs are reported to contain only i^{6}A (Robins et al., 1967). The predominant cytokinin-active ribonucleoside found in plant tRNA is *c*-io^{6}A. Minor amounts of i^{6}A, ms^{2}io^{6}A, and ms^{2}i^{6}A are also present. Hall et al. (1967) have reported the occurrence of *c*-io^{6}A and i^{6}A in the tRNAs from spinach and peas. The cytokinins responsible for activity in wheat germ tRNA were identified as i^{6}A, io^{6}A, and their thiomethyl derivatives (Burrows et al., 1970). Pea shoot tRNA hydrolysates have been shown to contain i^{6}A, ms^{2}i^{6}A, *c*-io^{6}A, *c*-ms^{2}io^{6}A, and *t*-ms^{2}io^{6}A (Vreman et al., 1972, 1974). Cytokinin-dependent tobacco callus tissue, grown in the presence of synthetic benzylaminopurine (bzl^{6}Ade) to test possible incorporation of this base into tRNA, contained relatively high amounts of *c*-io^{6}A and i^{6}A and a small amount of ms^{2}io^{6}A as well as a trace of bzl^{6}A derived from the exogenous base bzl^{6}Ade (Burrows et al., 1971). Thus the cytokinins in plant tRNAs show greater diversity than those generally found in animal and bacterial tRNAs. The significance of the occurrence of several cytokinins in the tRNAs of a single organism is not known. Since eukaryotes contain organelles such as chloroplasts and mitochondria which have their own transcriptional and transla-

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[1] Abbreviations used: i^{6}A, 6-(3-methyl-2-butenylamino)-9-β-D-ribofuranosylpurine; *c*-io^{6}A, 6-(4-hydroxy-3-methyl-*cis*-2-butenylamino)-9-β-D-ribofuranosylpurine; *t*-io^{6}A, 6-(4-hydroxy-3-methyl-*trans*-2-butenylamino)-9-β-D-ribofuranosylpurine; ms^{2}i^{6}A, 6-(3-methyl-2-butenylamino)-2-methylthio-9-β-D-ribofuranosylpurine; ms^{2}io^{6}A, 6-(4-hydroxy-3-methyl-2-butenylamino)-2-methylthio-9-β-D-ribofuranosylpurine; bzl^{6}A, 6-(benzylamino)-9-β-D-ribofuranosylpurine; bzl^{6}Ade, 6-(benzylamino)purine; CTAB, cetyltrimethylammonium bromide; EDTA, ethylenediaminetetraacetate; KE, kinetin equivalent; Me_3Si, trimethylsilyl; BSTFA, *N,O*-bis(trimethylsilyl)trifluoroacetamide; Tris, tris(hydroxymethyl)aminomethane; DEAE, diethylaminoethyl; UV, ultraviolet; GLC, gas-liquid chromatography.