# Purification and Characterization of the Corticosteroid-Binding Globulin of Pregnant Guinea Pig Serum†

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ABSTRACT: The corticosteroid-binding globulin from guinea pig pregnancy serum was purified by the sequential use of affinity chromatography, hydroxylapatite chromatography, and gel filtration chromatography at a cumulative yield of 80%. The protein was found to be homogeneous by analytical gel electrophoresis, equilibrium sedimentation ultracentrifugation, immunoelectrophoresis, and stoichiometry (1:1) of steroid binding. Guinea pig corticosteroid-binding globulin has a molecular weight of 43 300 and contains 29% carbohydrate. The intrinsic fluorescence of the corticosteroid-binding globulin is quenched by about 73% when 1 mol of cortisol is bound. The association constants (pH 7.4) at 4 and 37 °C are $2.5 \times 10^7$ and $1.5 \times 10^6$ $M^{-1}$ for cortisol and $1.4 \times 10^6$ and $0.2 \times 10^6$ $M^{-1}$ for progesterone, respectively.

The existence of a protein that binds cortisol with a higher affinity than albumin has been demonstrated in the serum of 131 vertebrate species examined, representing all of the 60 vertebrate classes (Seal & Doe, 1963, 1965, 1966). This protein has been called corticosteroid-binding globulin (CBG)[1] or transcortin. CBG has been extensively purified and characterized from the sera of human, rabbit, and rat [for a review, see Westphal (1971)]. It is noteworthy that cortisol, a hydrophilic steroid, and progesterone, a hydrophobic steroid, are bound by human CBG with similar affinity (Stroupe et al., 1978a).

A progesterone-binding globulin (PBG) from the serum of the pregnant guinea pig, possessing high affinity for progesterone but not for cortisol, has been extensively studied for its steroid-binding properties (Blanford et al., 1978). Guinea pig CBG was purified and found to bind cortisol more than 1 order of magnitude more weakly than does human CBG (Schneider & Slaunwhite, 1971). Preliminary results have indicated that progesterone binds to guinea pig CBG with lower affinity than cortisol (Perot & Allouch, 1974). Similar results were obtained by Volchek et al. (1975). A comparison of the chemical and steroid-binding properties of pure human CBG and cavian CBG and PBG is considered to be of great interest since it can provide a basis for an understanding of the molecular characteristics which are relevant to interactions of corticosteroids and progestins with proteins. This paper describes a rapid, efficient method for the purification of CBG from pregnant guinea pig serum and a partial characterization of its properties.

## Materials and Methods

Pooled serum from guinea pigs in the last 7–14 days of pregnancy was obtained from Dutchland Laboratory Animals, Inc., Denver, PA, and stored at −80 °C. Amberlite XAD-2 was from Rohm and Haas; it was washed with boiling methanol, boiling distilled water, and finally with distilled water at room temperature. Sepharose 4B and Sephacryl S-200 were purchased from Pharmacia Fine Chemicals, Inc. Hydroxylapatite (Bio-Gel HT) was from Bio-Rad Laboratories, and gelatin (Type I, from swine skin), *N*-acetylneuraminic acid, 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide, and *p*-(chloromercuri)benzoic acid were from Sigma Chemical Co. 1,4-Butanediol diglycidyl ether was obtained from Aldrich Chemical Co., Inc. 5,5′-Dithiobis(2-nitrobenzoic acid) was a product of Calbiochem. Constant-boiling 6 N hydrochloric acid (Sequanal grade) and 4 N methanesulfonic acid containing 0.2% 3-(2-aminoethyl)indole were from Pierce Chemical Co., and guanidine hydrochloride (ultrapure) was from Schwarz/Mann. Rabbit anti guinea pig serum was obtained from Miles Laboratories, Inc. Cortisol and progesterone were recrystallized to a constant melting point from ethanol or acetone by the addition of water.

[1,2-$^3$H]Cortisol and [1,2-$^3$H]progesterone were from New England Nuclear; their radiopurity was checked by thin-layer chromatography (Randerath, 1966) and found to be at least 98% by using a Packard radiochromatogram scanner. All other chemicals were reagent grade. Distilled deionized water was used throughout.

*Synthesis of the Affinity Resin.* Aminoethylamine–1,4-butanediol diglycidyl ether–agarose was synthesized by the method previously described (Mickelson et al., 1978). 11β-Hydroxy-3-oxo-4-androstene-17β-carboxylic acid was synthesized according to a method previously described (Mason et al., 1937). This steroid (1 g) was added to 100 mL of the amino-substituted agarose previously equilibrated with 70% dioxane and resuspended in 100 mL of 70% dioxane. The pH was adjusted to approximately 5, and 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide (5 g) was added to the mixture in two equal portions 4 h apart. The mixture was gently agitated on a mechanical shaker for 36 h at 23 °C.

*Procedure for Affinity Chromatography.* Pregnant guinea pig serum (100 mL) was treated with Amberlite XAD-2 resin for 6 h at 23 °C to remove endogenous steroid. The serum was then filtered through a fritted disk funnel (coarse) and dialyzed for 16 h against 50 mM Tris–0.5 M NaCl (pH 7.4, 4 °C). The serum was passed through a column (2.2 × 25 cm) containing the affinity resin at a flow rate of approximately 100 mL/h at 4 °C. The column was washed with buffer (50 mM Tris–0.5 M NaCl, pH 7.4, 4 °C) until the UV absorbance was negligible (<0.05). The column was then placed at room temperature and after 1 h washed with a solution of 200 μg of cortisol per mL of 50 mM Tris–0.1 M

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[1] Abbreviations used: CBG, corticosteroid-binding globulin or transcortin; $K_a$, equilibrium association constant; PBG, progesterone-binding globulin; $NaDodSO_4$, sodium dodecyl sulfate.

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NaCl buffer, pH 7.4. The protein fractions were pooled and concentrated to 20 mL by ultrafiltration using Amicon PM-30 membranes.

*Hydroxylapatite Chromatography.* The 20 mL of protein solution obtained from the affinity chromatography procedure was dialyzed for 24 h against 1 L of 5 mM phosphate buffer (pH 6.8, 4 °C) containing 3 mg of cortisol. The dialyzed solution was passed through a column (2.2 × 7 cm) containing hydroxylapatite equilibrated with 5 mM phosphate buffer (pH 6.8, 4 °C) at a flow rate of 10 mL/h.

*Gel Filtration Chromatography.* The CBG fraction obtained from the hydroxylapatite column was concentrated to 5–7 mL and applied to a column (2.7 × 90 cm) containing Sephacryl S-200 previously equilibrated with 50 mM phosphate–1 mM dithiothreitol (pH 7.4, 4 °C). The protein was eluted at 15 mL/h. The CBG fraction was stored at −80 °C.

*Effect of p-(Chloromercuri)benzoic Acid.* Pure guinea pig CBG ($1.2 \times 10^{-5}$ M in phosphate buffer, pH 7.4) was incubated for 2 h at 23 °C or for 8–72 h at 4 °C with $2.4 \times 10^{-4}$ M *p*-(chloromercuri)benzoic acid. The incubation was carried out in the presence and absence of excess cortisol. An aliquot of 5–20 μg of CBG from the above reaction mixture was subjected to polyacrylamide gel electrophoresis at 4 °C in the discontinuous buffer system of Davis (1964).

*Determination of Amino Acid Composition.* Pure protein (0.5 mg) was hydrolyzed in either 1 mL of 6 N HCl at 110 °C or 1 mL of 4 N methanesulfonic acid containing 0.2% 3-(2-aminoethyl)indole at 115 °C for 24, 48, 72, and 96 h in evacuated, sealed tubes. Serine and threonine values were obtained by extrapolation to zero-time hydrolysis. Cystine and cysteine were determined as cysteic acid after performic acid oxidation by the method of Moore (1963).

Free sulfhydryl groups were also determined for CBG in 6 M guanidine hydrochloride by the method described by Ellman (1959). Amino acid analyses were performed in the laboratory of Dr. J. A. Yankeelov, Jr., using an automatic single column amino acid analyzer (Benson, 1973). Tryptophan and tyrosine content were determined by the method of Edelhoch (1967).

*Carbohydrate Analysis.* Standard colorimetric methods were employed for the measurement of hexose (Roe, 1955), hexosamine (Winzler, 1955), fucose (Dische & Shettles, 1948), and sialic acid (Warren, 1959). Reagent volumes were scaled down by a factor of 10.

*Molecular Weight Determination.* The long-column (12 mm) meniscus depletion sedimentation equilibrium technique described by Chervenka (1970) was used to determine the weight-average molecular weight. CBG (1 mg/mL) was dialyzed for 48 h against 10 mM Tris–0.10 M NaCl (pH 7.4, 4 °C). Centrifugation was performed at 13 000 rpm at 5 °C for 26 h in a Beckman-Spinco Model E analytical ultracentrifuge equipped with a sapphire cell window and a 49-mm polarizing filter for Raleigh interference optics. Photographic plates were analyzed with a microscope comparator and the weight-average molecular weight was calculated (Yphantis, 1964). Points which represented a fringe displacement of less than 50 μm were unreliable and were not used. The apparent specific volume was calculated from the amino acid composition and corrected for carbohydrate content as described by Cohn & Edsall (1943) and Longsworth (1953), respectively.

*Production and Analysis of Anti-CBG Antibodies.* Water (2 mL) containing 1.0 mg of CBG was filtered through a Millipore filter (0.45-μm pore) and thoroughly emulsified with an equal volume of complete Freund's adjuvant. This emulsion (1 mL) was injected subcutaneously at 8–10 sites on the dorsum of each of two female New Zealand white rabbits (4.5 kg). The rabbits were given boosters 2 weeks apart for the second and third time and four weeks apart for the fourth and fifth booster by use of incomplete Freund's adjuvant. About 40–50 mL of blood was drawn from the central artery of the ear 2 weeks after the fourth and fifth booster. The serum was frozen at −80 °C.

Immunoelectrophoresis was performed at pH 8.6 in 0.075 M barbital buffer using Pol-E-Film (Pfizer). After electrophoresis of the CBG preparations, the antiserum was added to the troughs. Following incubation for 48 h at 4 °C in a moist chamber, the film was soaked with 0.3 M sodium chloride for 16 h at 23 °C and then stained with Coomassie Brilliant Blue (0.25%) for 30 min.

*Removal of Cortisol from CBG.* Purified CBG was diluted to a concentration of 0.5 mg/mL with 0.5 M potassium phosphate buffer (pH 6.15). The pH was adjusted to 6.15 with 2 M sodium acetate buffer (pH 4.5), and 5 mL was placed on a Sephadex G-25 column (2.6 × 40 cm) at 23 °C and eluted with distilled water. The protein peak was isolated and either lyophilized or adjusted to pH 7.4 with 0.5 M sodium phosphate. CBG was also stripped of steroid by a modification of the method described by Chan & Slaunwhite (1977). A CBG solution (25 mL) (10.0 μg/mL of 50 mM phosphate buffer, pH 7.4, containing 0.1% gelatin) was shaken with 2.5 mL of settled Amberlite XAD-2 for 2 h at 23 °C. The protein solution was filtered through a fritted disk funnel (coarse), followed by passage through a Millipore filter (0.45 μm).

*Fluorescence Quenching Titration of CBG.* Fluorometric experiments were performed with an Aminco-Bowman ratio spectrophotofluorometer connected to a Hewlett-Packard 7035B X-Y recorder. Excitation was at 280 nm with a spectral band width of 5.5 nm; emission was either set at 340 nm or varied from 200–450 nm for production of emission spectra with a spectral band width of 11 nm. All measurements were made with a CBG solution that was stripped of steroid by the gel filtration method and diluted to a concentration of $4.1 \times 10^{-7}$ M with 50 mM phosphate buffer (pH 7.4, 23 °C). Aliquots of a methanolic cortisol solution ($8.2 \times 10^{-5}$ M) were added to a thermostat-regulated cell containing 1.0 mL of protein solution. The final methanol concentration was 1.5% which had a negligible effect on the CBG spectrum.

*Equilibrium dialysis* was essentially performed as previously described (Westphal, 1969) to determine the affinity constants for the binding to CBG of [1,2-$^3$H]cortisol and [1,2-$^3$H]-progesterone. A CBG solution (2 mL) was the inside volume and 20 mL of 50 mM phosphate buffer (pH 7.4) containing 0.02% sodium azide was the outside volume. The dialysis flasks were shaken for 48 h at either 4, 25, or 37 °C. Triplicate samples of the inside and outside solutions were taken for scintillation counting, and the triplicate values were averaged. Scatchard plots consisting of at least eight points were analyzed by a computer program which gave a least-squares fit of the data.

*Determination of Optical Absorptivity.* Guinea pig CBG (0.5 mg/mL) was either extensively dialyzed against water at 4 °C or passed through the Sephadex G-25 column (2.6 × 40 cm) using water for elution at 23 °C. The protein was lyophilized and further dried in a vacuum desiccator over phosphorus pentoxide. The extinction coefficient was determined at 278 nm in a Zeiss PMQ II spectrophotometer with triplicate protein solutions (1.0 mg of CBG per mL of 50 mM phosphate buffer, pH 7.4). Correction was made for a water content of 9.9%, determined by drying triplicate samples to a constant weight at 110 °C.

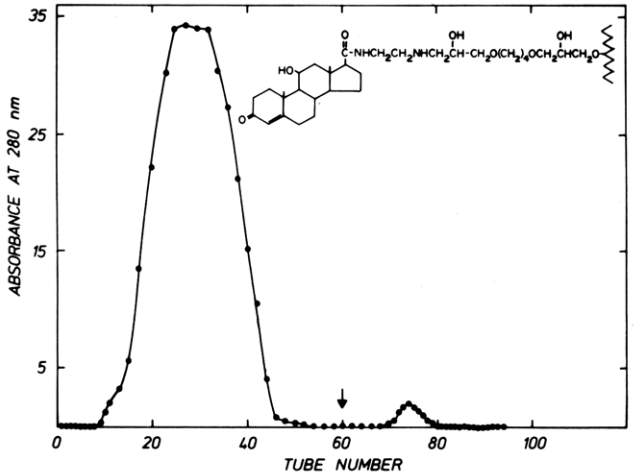


FIGURE 1: Affinity chromatography of pregnant guinea pig serum. Amberlite XAD-2 treated serum (100 mL) was applied at 4 °C to a 100-mL column of the affinity gel: 11β-hydroxy-3-oxo-4-androstene-17β-carboxyaminoethylamine–1, 4-butanediol diglycidyl ether–agarose. At the arrow the column was eluted with cortisol (200 μg/mL) at room temperature.

## Results

*Purification of CBG.* Adsorption of CBG to the affinity resin was performed at 4 °C while elution was at 23 °C in accordance with the inverse relationship between the temperature and the association constant of the CBG–cortisol complex (see below, Table II). The affinity column retained essentially all of the CBG steroid-binding activity of the Amberlite XAD-2 treated pregnant guinea pig serum based upon an equilibrium dialysis analysis of the nonadsorbing protein fraction. CBG was eluted from the affinity column by addition of cortisol to the buffer as illustrated in Figure 1. CBG was further purified by passage of the affinity-purified protein through a hydroxylapatite column which does not retain CBG at 5 mM sodium phosphate (pH 6.8, 4 °C). Final purification was achieved by gel filtration chromatography on Sephacryl S-200 with 50 mM phosphate–1 mM dithiothreitol (pH 7.4, 4 °C) as the eluting buffer. This procedure was included as a precaution since the progesterone-binding globulin (PBG) may have been partially bound to the affinity resin. PBG also does not adsorb to hydroxylapatite at 5 mM phosphate (Burton et al., 1971). The presence of small quantities of PBG is difficult to detect due to its low antigenicity and dye-binding properties. Therefore, gel filtration was used to remove any PBG present, since it has a much larger Stokes' radius than CBG (Burton et al., 1971). CBG purified by only affinity chromatography and gel filtration appeared to be homogeneous by the criteria of $NaDodSO_4$–polyacrylamide gel electrophoresis; however, hydroxylapatite chromatography was found to remove a minor γ-globulin contaminant (<2%) which was detectable only by immunochemical methods.

On the basis of the quantitation of CBG by equilibrium dialysis the cumulative yield of pure guinea pig CBG was 93 mg from 100 mL of serum which represents a yield of 80%. CBG was assayed after the serum or pure protein preparation was stripped of steroid by Amberlite XAD-2 treatment as described under Materials and Methods.

*Criteria of Purity.* CBG appeared to be homogeneous when analyzed by polyacrylamide gel electrophoresis in the presence and absence of 0.1% $NaDodSO_4$ (Figure 2A). CBG prepared by the above procedure was also found to be immunochemically pure. Anti-CBG serum, produced in the rabbit by using pure CBG as antigen, yielded one precipitin arc in immunoelectrophoresis when reacted with either pure CBG or pregnant guinea pig serum (Figure 2B).

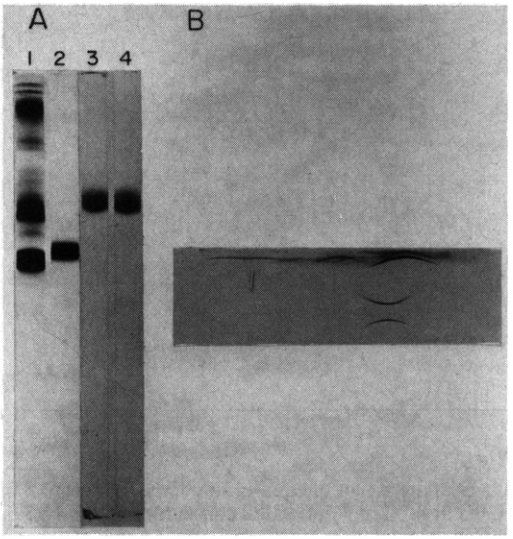


FIGURE 2: (A) Polyacrylamide disc gel electrophoresis of guinea pig CBG. Electrophoresis of gels 1 and 2 was done at 4 °C with the discontinuous buffer system of Davis (1964). Gels 3 and 4 are 7.5% $NaDodSO_4$ gels by using the method described by Weber et al. (1972). (1) Pregnant guinea pig serum (2.5 μL); (2) CBG (19 μg); (3) CBG (10 μg) + $NaDodSO_4$ + mercaptoethanol; (4) CBG (10 μg) + $NaDodSO_4$ without mercaptoethanol. Gels were stained overnight with Coomassie Brilliant Blue R-250 (0.25%). (B) Immunoelectrophoresis: (upper well) pregnant guinea pig serum (2 μL); (lower well) CBG (1.5 μg); (upper trough) rabbit anti guinea pig serum (20 μL); (lower trough) rabbit anti guinea pig CBG (20 μL).

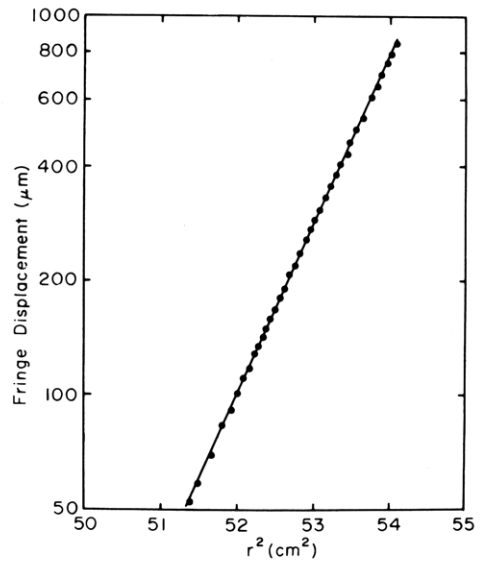


FIGURE 3: Yphantis plot of sedimentation equilibrium ultracentrifugation of CBG by the long-column meniscus depletion method (Chervenka, 1970). CBG (1.0 mg/mL) was centrifuged at 13 000 rpm at 5 °C for 26 h. The weight-average molecular weight of CBG was calculated from the slope to be 43 300.

Equilibrium sedimentation ultracentrifugation indicated a homogeneous species with a molecular weight of 43 300 (Figure 3). Purity was also indicated by the stoichiometry of binding: 1.0 molecule of CBG bound 1.0 molecule of steroid (Figure 4). An *n* value of 1.0 ± 0.1 was found as an average of 10 determinations.

*Chemical Composition of CBG.* The amino acid and carbohydrate composition of CBG is presented in Table I. Guinea pig CBG was found to contain about 29% carbohydrate based on colorimetric assays which is in agreement with

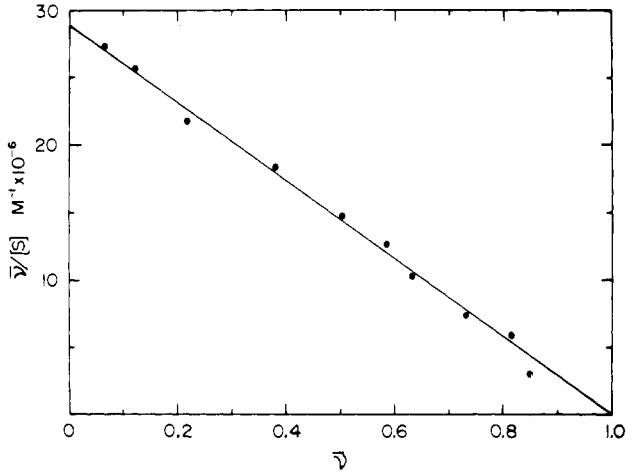


FIGURE 4: Scatchard plot of equilibrium dialysis of cortisol binding to pure CBG at 4 °C, pH 7.4. CBG concentration was 12.5 μg/mL. The line was calculated by a least-squares computer program.

Table I: Amino Acid Composition[a] of Guinea Pig CBG Reported from Various Laboratories

| | Rozen & Volchek (1970) | Schneider & Slaunwhite (1971) | present study[b] |
|---|---|---|---|
| Asp | 28.0 | 33.5 | 31.6 ± 0.8 |
| Thr | 21.7 | 18.5 | 20.1 ± 0.5 |
| Ser | 21.7 | 28.2 | 23.7 ± 0.8 |
| Glu | 28.8 | 26.2 | 25.1 ± 0.1 |
| Pro | 19.8 | 13.2 | 14.0 ± 0.5 |
| Gly | 19.2 | 13.9 | 13.9 ± 0.3 |
| Ala | 16.3 | 22.4 | 21.4 ± 0.2 |
| Val | 11.8 | 11.4 | 10.8 ± 0.4 |
| $^1/_2$-Cys | 2.6 | 0.9 | 2.9 ± 0.2[c] |
| Met | 2.2 | 1.8 | 7.5 ± 0.1 |
| Ile | 11.5 | 14.6 | 14.6 ± 0.4 |
| Leu | 21.0 | 39.6 | 39.3 ± 0.8 |
| Tyr | 1.7 | 4.5 | 5.5 ± 0.5[d] |
| Phe | 12.8 | 17.1 | 16.8 ± 0.4 |
| His | 8.5 | 8.5 | 9.2 ± 0.1 |
| Lys | 20.4 | 10.9 | 10.4 ± 0.2 |
| Arg | 8.0 | 11.6 | 11.6 ± 0.1 |
| Trp | N.D. | 1.5 | 1.9 ± 0.2[e] |
| | | % CHO | |
| hexose | | 8.3 | 13.1 |
| hexos-amines | | 5.5 | 7.1 |
| sialic acid | | 10.7 | 8.3 |
| fucose | | 0.1 | 0.1 |
| total | | 24.6 | 28.6 |

[a] The data are presented as the number of residues per mole of CBG. All values are standardized to a molecular weight of 43 300 of which 28.6% is carbohydrate. [b] The average of four determinations (±standard deviation) except Thr and Ser which are the average of three determinations extrapolated to zero time of hydrolysis. [c] Determined as cysteic acid. One sulfhydryl group was detected by the method of Ellman (1959). [d] Determined by amino acid analysis as well as by the method of Edelhoch (1967). [e] Determined by the method of Edelhoch (1967).

a 70% yield of polypeptide from amino acid analysis. This amount of carbohydrate is comparable with the 25% found by Schneider & Slaunwhite (1971). In general, the amino acid composition for guinea pig CBG prepared by affinity chromatography (Table I) is in agreement ($r^2 = 0.98$) with that prepared by the conventional methods used by Schneider & Slaunwhite (1971). The lower values of Schneider and Slaunwhite for $^1/_2$-Cys, Met, and Tyr are probably due to destruction during acid hydrolysis. The amino acid composition determined by Rozen & Volchek (1970) differs significantly from the other two analyses ($r^2 = 0.6$), which cannot be explained conclusively.

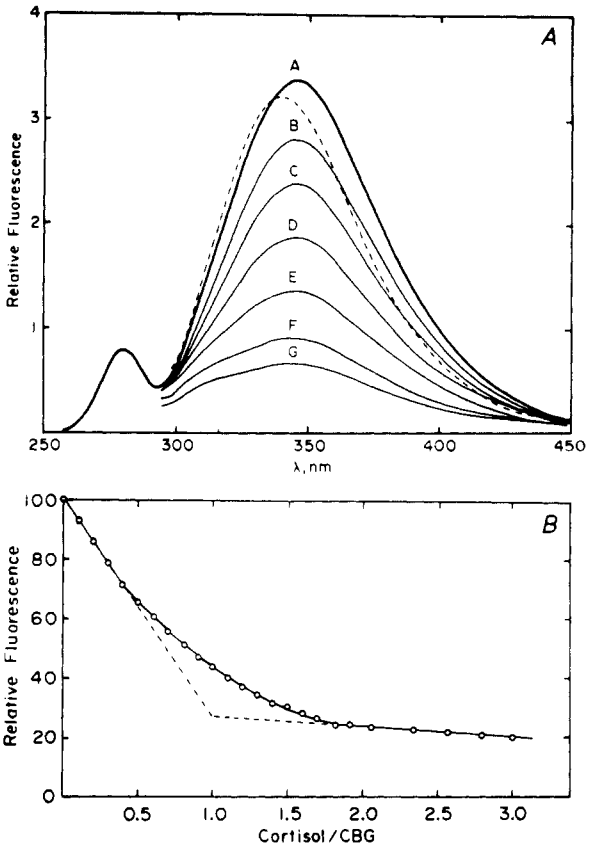


FIGURE 5: (A) Fluorescence emission spectra of guinea pig CBG at 23 °C, pH 7.4, in the presence of increasing concentrations of cortisol. [Cortisol]/[CBG]: (A) 0; (B) 0.025; (C) 0.05; (D) 0.20; (E) 0.25; (F) 1.0; (G) 3.0. CBG concentration was $4.1 \times 10^{-7}$ M. Excitation was at 280 nM. The dashed line represents the emission spectrum of CBG in the presence of $1.1 \times 10^{-6}$ M 5α-pregnane-3,20-dione. (B) Fluorescence quenching titration of CBG with cortisol at 23 °C, pH 7.4. Excitation was at 280 nm; emission was at 340 nm. CBG concentration was $4.1 \times 10^{-7}$ M. A $K_a$ of $2.5 \times 10^7$ $M^{-1}$ was calculated from the solid curve.

In addition, free sulfhydryl groups were measured by the method of Ellman (1959) by using 5,5′-dithiobis(2-nitrobenzoic acid). In 100 mM phosphate (pH 8.0) CBG was found to contain 0.9 mol of sulfhydryl per mol of protein. The addition of either 1% $NaDodSO_4$ or 6 M guanidine hydrochloride to the above buffer gave a value of 1.0 mol of sulfhydryl groups per mol of CBG. Performic acid oxidation of CBG resulted in the formation of three cysteic acid residues (Table I). The two additional cysteine residues, therefore, exist as either cystine or are still unreactive in the presence of 1% $NaDodSO_4$ or 6 M guanidine hydrochloride.

*Steroid Binding Properties of CBG.* The intrinsic fluorescence spectrum of CBG shows an emission maximum around 343 nm when excited at its excitation maximum of 280 nm as shown in Figure 5A. The excitation (not shown) and emission properties of CBG are characteristic of polypeptides containing tryptophan; an emission maximum at about 343 nm suggests that the tryptophan residues are located in a hydrophilic environment, possibly partially exposed to water (Williams & Bridges, 1964; Burstein et al., 1973). The fluorescence of CBG is quenched by the addition of cortisol (Figure 5A). This quenching is believed to result mainly from radiationless energy transfer from an excited tryptophan of CBG to the near-ultraviolet ($n \rightarrow \pi^*$) absorption band of steroids containing the 3-oxo-4-ene chromophore, as proposed

Table II: Temperature Dependence of Steroid Binding to CBG

| | $K_a$ ($M^{-1}$) × $10^{-7}$ [a] | | | | | |
|---|---|---|---|---|---|---|
| species: | guinea pig[b] | | | human[c] | | |
| temperature (°C): | 4 | 25 | 37 | 4 | 20 | 37 |
| cortisol | 2.5 | 0.76 | 0.15 | 79 | 39 | 4.0 |
| progesterone | 0.14 | 0.05 | 0.02 | 70 | 28 | 2.6 |

[a] Determined by equilibrium dialysis at pH 7.4. [b] Each value is the average of at least two determinations. [c] Data from Stroupe et al. (1978a).

Table III: Physicochemical Properties of Guinea Pig CBG

| | |
|---|---|
| molecular weight | 43 300 |
| carbohydrate content (%) | 28.6 |
| partial specific volume | 0.699 |
| $E_{1\,cm}^{1\%}$, 278 nm | 4.9 ± 0.2 |
| $A_{280}/A_{260}$ | 1.5 ± 0.1 |
| binding sites for cortisol, $n$ | 1.0 ± 0.1 |
| $K_a$ ($M^{-1}$) × $10^{-7}$, cortisol, 4 °C | 2.5 ± 0.6 |
| $K_a$ ($M^{-1}$) × $10^{-6}$, cortisol, 37 °C | 1.5 ± 0.4 |

for PBG (Stroupe et al., 1975). Occupation of the binding site by cortisol results in a blue shift of the emission wavelength maximum by about 3–5 nm. This is indicative of a decrease in the hydrophilicity of the environment of the fluorescent tryptophan which may be brought about by the binding of steroid. The addition of 5α-pregnane-3,20-dione, which lacks the 3-oxo-4-ene chromophore, does not quench the fluorescence of CBG, but the emission maximum is again shifted to the blue, as expected (Figure 5A). The blue shift observed (6–8 nm) for the 5α-saturated steroid is larger than that induced by cortisol; this may be due to 5α-pregnane-3,20-dione being more hydrophobic than cortisol. The addition of 5α-pregnane-3,20-dione to cortisol-containing CBG resulted in unquenching of the fluorescence spectrum which approached that obtained in the absence of cortisol, thus indicating a common binding site (data not shown).[2]

The fluorescence of CBG at 343 nm is quenched by about 73% when 1 mol of cortisol associates with 1 mol of CBG (Figure 5B). This value is generally in the range of 65–80%, depending on the specific activity of the CBG preparation which varies with the efficiency of the steroid removal procedure; the highest quenching value obtained was 85%. CBG is stripped of steroid by gel filtration at pH 6.15 and 23 °C, conditions necessary to remove 100% of the steroid. This procedure causes a variable (20–50%) loss of activity, thus altering the specific activity. Removal of steroid by Amberlite XAD-2 results in an $n$ value of 1.0 by equilibrium dialysis (Figure 4), but this method cannot be used in the fluorescence studies because of the presence of gelatin.

An association constant of $2.5 \times 10^7$ $M^{-1}$ was calculated from the data shown in Figure 5B by the method described by Velick et al. (1960), Attallah & Lata (1968), and Stroupe et al. (1975). This value is 2–3 times higher than that obtained by equilibrium dialysis at 23 °C.[3] The fluorescence quenching method also gave higher values than those obtained by equilibrium dialysis for PBG (Stroupe et al., 1975) and human CBG.[4]

In comparison with human CBG, progesterone is bound to guinea pig CBG with a much lower affinity than cortisol. The association constants for cortisol and progesterone of both proteins, however, decrease with increasing temperature (Table II). The physicochemical properties of guinea pig CBG are summarized in Table III.

## Discussion

The physiological significance of the steroid-binding serum proteins, especially those with high affinity, has been generally recognized (Westphal et al., 1978). As an overall effect, the binding to serum proteins results in the conservation of the steroid hormones. In man, cortisol and progesterone are bound with high affinity to CBG (Stroupe et al., 1978a). In 1969 a unique progesterone-binding protein (PBG) was detected in pregnant guinea pig serum (Diamond et al., 1969; Heap, 1969; Milgrom et al., 1970). The existence of PBG, or of related plasma proteins with a high affinity for progesterone, has been observed in five other hystricomorph species (Heap & Illingworth, 1974). Guinea pig CBG is elevated during pregnancy, similar to human CBG (Seal & Doe, 1966). In fact, the serum level of CBG is about 20 times higher in the pregnant guinea pig than in the pregnant woman. This higher concentration of guinea pig CBG may compensate for its lower affinity for cortisol in comparison with human CBG.

Equilibrium dialysis experiments with pregnant guinea pig serum at a dilution of 1:200 (Burton et al., 1974) indicated that essentially all of the progesterone binding activity was attributable to PBG (12 μM) although CBG was present at a high level (25–30 μM).[4] We determined that guinea pig CBG from nonpregnant guinea pig serum bound progesterone 20 times more weakly than cortisol. This lower progesterone binding affinity of guinea pig CBG may the the basis for the existence of PBG which is capable of binding progesterone, but not cortisol, with high affinity.

CBG has been previously purified from nonpregnant guinea pigs by using conventional methods (Rozen & Volchek, 1970; Schneider & Slaunwhite, 1971) which resulted in a low yield of an unstable protein. We have introduced the highly specific method of affinity chromatography in order to purify CBG efficiently and in high yield. From 100 mL of pregnant guinea pig serum, 93 mg of pure CBG is obtained (80% yield). This CBG preparation (in the presence of excess cortisol) was found to be relatively stable when stored at a concentration of 0.5–3.0 mg/mL of phosphate buffer (pH 7.4) at −80 to 4 °C. This is in contrast to CBG prepared by the conventional method used by Schneider & Slaunwhite (1971). Such increased stability has also been observed with affinity-purified human CBG in comparison with preparations obtained by conventional methods.[4] The amino acid and carbohydrate compositions of CBG from pregnant and nonpregnant guinea pig agree within experimental error, indicating that they are identical. Thus, the instability of CBG purified by conventional methods remains unexplained.

CBG prepared by the conventional methods used by Schneider & Slaunwhite (1971) formed aggregates when stored at 5 or 25 °C. In the present studies, no aggregation of affinity-purified CBG was detected after 6 months of storage at 4 °C in the presence of stoichiometric amounts of cortisol as judged by a single band on analytical polyacrylamide gels. Steroid-free CBG also did not aggregate after 3 months of storage at 4 °C although about 30% of the binding activity was lost in the absence of steroid. No aggregation of the affinity-purified CBG was observed by sedimentation equilibrium ultracentrifugation (Figure 3). Moreover, $p$-(chloromercuri)benzoate did not promote aggegation of affinity-purified CBG, in contrast to the aggregation observed for conventionally purified CBG (Schneider & Slaunwhite, 1971).

[2] Preliminary equilibrium dialysis experiments indicate that 5α-pregnane-3,20-dione competes for cortisol binding to guinea pig CBG with about half the affinity of progesterone.

[3] Unpublished experiments.

[4] Unreported observation.

The value of the equilibrium association constant ($2.5 \times 10^7$ $M^{-1}$) determined in this investigation is higher than that reported by Schneider & Slaunwhite (1971). The reported lower value may be due to the presence of endogenous steroid and/or aggregation of CBG in the experiments of Schneider & Slaunwhite (1971). A molecular weight of 43 300 for CBG determined in the present study is lower than the values previously determined, i.e., 48 600 (Rozen & Volchek, 1970) and 60 000 (Schneider & Slaunwhite, 1971). This difference may also be the result of aggregation in the case of conventionally purified CBG.

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References

Attallah, N. A., & Lata, G. F. (1968) *Biochim. Biophys. Acta 168*, 321–333.

Benson, J. R. (1973) in *Applications of the New Techniques of Analysis* (Simmons, I. L., & Ewing, G. W., Eds.) pp 223–236, Plenum Press, New York and London.

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

Burstein, E. A., Vedenkina, N. S., & Ivkova, M. N. (1973) *Photochem. Photobiol. 18*, 263–279.

Burton, R. M., Harding, G. B., Rust, N., & Westphal, U. (1971) *Steroids 17*, 1–16.

Burton, R. M., Harding, G. B., Aboul-Hosn, W. R., MacLaughlin, D. T., & Westphal, U. (1974) *Biochemistry 13*, 3554–3561.

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

Chervenka, C. H. (1970) *Anal. Biochem. 34*, 24–29.

Cohn, E. J., & Edsall, J. T. (1943) *Proteins, Amino Acids and Peptides*, p 370, Reinhold, New York.

Davis, B. J. (1964) *Ann. N.Y. Acad. Sci. 121*, 404–427.

Diamond, M., Rust, N., & Westphal, U. (1969) *Endocrinology 84*, 1143–1151.

Dische, Z., & Shettles, L. B. (1948) *J. Biol. Chem. 175*, 595–603.

Edelhoch, H. (1967) *Biochemistry 6*, 1948–1954.

Ellman, G. L. (1959) *Arch. Biochem. Biophys. 82*, 70–77.

Heap, R. B. (1969) *J. Reprod. Fertil. 18*, 546–548.

Heap, R. B., & Illingworth, D. V. (1974) *Symp. Zool. Soc. London 34*, 385–415.

Longsworth, L. G. (1953) *J. Am. Chem. Soc. 75*, 5705–5709.

Mason, H. L., Hoehn, W. M., McKenzie, B. F., & Kendall, E. C. (1937) *J. Biol. Chem. 120*, 719–741.

Mickelson, K. E., & Westphal, U. (1978) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 37*, 1449.

Mickelson, K. E., Teller, D. C., & Pêtra, P. H. (1978) *Biochemistry 17*, 1409–1415.

Milgrom, E., Atger, M., & Baulieu, E.-E. (1970) *Nature (London) 228*, 1205–1206.

Moore, S. (1963) *J. Biol. Chem. 238*, 235–237.

Perrot, M., & Allouch, P. (1974) *J. Steroid Biochem. 5*, 332.

Randerath, K. (1966) *Thin Layer Chromatography*, Academic Press, New York.

Roe, J. H. (1955) *J. Biol. Chem. 212*, 335–343.

Rozen, V. B., & Volchek, A. G. (1970) *Probl. Endokrinol. 16*, 81–85.

Schneider, S. L., & Slaunwhite, W. R., Jr. (1971) *Biochemistry 10*, 2086–2093.

Seal, U. S., & Doe, R. P. (1963) *Endocrinology 73*, 371–376.

Seal, U. S., & Doe, R. P. (1965) *Steroids 5*, 827–841.

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

Stroupe, S. D., Cheng, S.-L., & Westphal, U. (1975) *Arch. Biochem. Biophys. 168*, 473–482.

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

Stroupe, S. D., Gray, R. D., & Westphal, U. (1978b) *FEBS Lett. 86*, 61–64.

Velick, S. F., Parker, C. W., & Eisen, H. N. (1960) *Proc. Natl. Acad. Sci. U.S.A. 46*, 1470–1482.

Volchek, A. G., Polyntsev, I. U. V., & Rozen, V. B. (1975) *Probl. Endokrinol. 21*, 82–88.

Warren, L. (1959) *J. Biol. Chem. 234*, 1971–1975.

Weber, K., Pringle, J. R., & Osborn, M. (1972) *Methods Enzymol. 26*, 3–27.

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

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

Westphal, U., Stroupe, S. D., Cheng, S.-L., & Harding, G. B. (1978) *J. Toxicol. Environ. Health 4*, 229–247.

Williams, R. T., & Bridges, J. W. (1964) *J. Clin. Pathol. 17*, 371–394.

Winzler, R. J. (1955) *Methods Biochem. Anal. 2*, 279–311.

Yphantis, D. A. (1964) *Biochemistry 3*, 297–317.