Steroid-Protein Interactions

XV. ISOLATION AND CHARACTERIZATION OF CORTICOSTEROID-BINDING GLOBULIN FROM HUMAN PLASMA*

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THOMAS G. MULDOON† AND ULRICH WESTPHAL

From the Biochemistry Department, University of Louisville, School of Medicine, Louisville, Kentucky 40208

SUMMARY

Corticosteroid-binding globulin was isolated from normal human plasma by successive anion exchange chromatography and gel filtration. Ammonium sulfate fractionation was employed as a preliminary step when large volumes were processed. The purification was about 6000-fold. Homogeneity was shown by free boundary, agar and paper electrophoresis, sedimentation velocity, and diffusion. Immunoelectrophoretic behavior against antisera to human serum and serum fractions indicated purity. Other immunoelectrophoretic analyses showed lack of reaction with antisera produced against sera from several species other than human.

The molecular weight of the protein was calculated as 51,700 from sedimentation and diffusion data. Chemical composition was ascertained by amino acid and carbohydrate analyses, nitrogen determination, and biuret. The physicochemical properties described include sedimentation coefficient (s20,w = 3.79 S), diffusion coefficient (D20,w = 6.15 × 10⁻⁹ cm² sec⁻¹), electrophoretic mobility (u = 4.9 × 10⁻⁵ cm² volt⁻¹ sec⁻¹), extinction coefficient (ε₂₄₅ = 6.45), partial specific volume (V = 0.708 ml g⁻¹), and frictional ratio (f/f₀ = 1.42).

One binding site for cortisol was found by equilibrium dialysis at 4°C and at 37°C; the association constants at these temperatures were 5.2 × 10⁴ M⁻¹ and 2.4 × 10⁴ M⁻¹, respectively. Complete removal of sialic acid did not significantly affect the cortisol-binding affinity. Pure corticosteroid-binding globulin was inactivated by gel filtration at 45°C or by dialysis against glass-redistilled water.

One of the objectives of the studies in this series on steroid-protein interactions is the elucidation of the relationship between the chemical structure of the interacting components and their binding affinity and specificity (2, 9). Results have been obtained on the significance of certain structural features of Δ⁴-3-ketosteroids for their interaction with human serum albumin (3-5), a protein which can readily be obtained in a high state of purity. Another serum protein, the corticosteroid-binding globulin, binds several steroid hormones with much higher affinity than albumin does (for references, see References 2, 3, 6-8) but occurs in low concentration and is therefore more difficult to isolate. Steroid-binding affinities and concentrations of this specific globulin have been determined in sera of several species after removal of the endogenous steroids; considerable species specificity was reflected in the association constants for cortisol, corticosterone, and progesterone (2). For an interpretation of these species differences in the binding of the three steroid hormones in terms of protein structure, the corticosteroid-binding globulins must be available in pure form. The present paper describes the isolation and physicochemical characterization of CBG from human plasma and an evaluation of its binding affinity for cortisol.

Early purification studies have shown that human CBG can be separated from the major part of the serum proteins by chromatography on a DEAE-cellulose column (Fig. 2 and 3 in Reference 3) when a combined pH-ionic strength gradient of Tris phosphate buffer is used as eluent. While the present study was in progress, Seal and Doe reported the preparation of a highly purified CBG from human plasma (9, 10). An important step in the purification (9) was the use of hydroxylapatite as adsorbent; this technique was adopted with some modification. The purification factor achieved by the fractionation procedures applied in our laboratory indicates a higher degree of purity of the CBG obtained; the properties and composition differ significantly from reported values (9, 10, 7). Recently, Slaunwhite et al. (11) published a procedure for the preparation of a highly purified transcortin from human plasma. Although the compo-

The abbreviations used are: CBG, corticosteroid-binding globulin; ACD, human acid citrate-dextrose.
tion of the polypeptide portion of this glycoprotein (11) is in fairly close agreement with our CBG preparation, no valid comparison can be made since carbohydrate values were not determined for the transcortin (11). The present account of our experimental results includes physicochemical parameters not previously reported.

**EXPERIMENTAL PROCEDURE**

**Materials and Methods**

Solutions were prepared with glass-redistilled water of specific resistance, 2 × 10^6 ohm cm. Purification procedures were performed at 4°C, unless otherwise specified.

**Protein Source**—Either plasma or serum from outduted human acid citrate-dextrose blood was suitable as a source of CBG. The plasma was removed from the cells by gentle aspiration, centrifuged for 20 min at 8000 × g, and dialyzed against two changes of 4 volumes of water for 24 hours. The cuglobulin precipitate was separated by centrifugation and discarded. Serum was prepared by addition of a 1.0 M solution of CaCl₂ to dialyzed plasma at a final concentration of 11.5 mg of Ca²⁺ per 100 ml. Fibrin precipitation was allowed to proceed for 1 hour at 25°C, and then 8 hours at 4°C. The clot was removed by centrifugation. Plasma and serum were stored at -85°C.

**Labeled Steroids and Chemical Reagents**—Cortisol-4-¹⁴C was obtained in benzene-methanol (9:1) solution from New England Nuclear. The average specific activity of different lots was 40 mCi per mmole. Radiopurity was assayed by descending paper chromatography in either a Zaffaroni (benzene-methanol-formamide) (12) or a Bush B₅ (13) system. Preparations of a radioactive chemical purity lower than 95%, were subjected to preparative paper chromatography. To determine cortisol concentration, the benzene solvent was exhaustively evaporated and the remaining steroid was dissolved in methanol. Absorbance was measured at 241 nm and cortisol concentration was calculated on the basis of a molecular extinction coefficient of 15,900 (14). Stock solutions containing 0.2 μg per ml were prepared in methanol or 10% methanol in benzene and stored at -85°C. All organic solvents were redistilled.

This was reagent grade, recrystallized from 95% ethanol, or Fisher primary standard grade. Ammonium sulfate was reagent grade, recrystallized from 1.5 × 10⁻³ M disodium EDTA, or special enzyme grade (Mann). Synthetic N-acetylneuraminic acid was obtained from Koch-Light Laboratories, Ltd. All other chemicals were reagent grade, used without further purification.

**Chromatography**—DEAE-cellulose was obtained from Eastman Kodak Company and prepared for use as described by Peterson and Sober (15). Hydroxylapatite was either purchased from Bio-Rad or prepared by the method of Tiselius, Hjerten, and Levin (16). Sephadex was obtained from Pharmacia. The adsortbents were equilibrated with buffer and packed under gravity in glass columns equipped with coarse sintered plates. Gradient elution was performed with a nine-chambered Buchler Varigrad.

**Determination of Specific Activity**—The purification of CBG from cortisol-4-¹⁴C-equilibrated plasma was assessed from successive increases in specific activity expressed as counts per min per mg of protein. Protein content of eluate fractions in early stages of purification was estimated by measurement of the absorbance at 279 mμ, on the basis of an average absorbance of 0.8 for a solution containing 1 mg of protein per ml in 1-cm cells; with CBG preparations of 5% purity or greater, an absorbance of 0.65 was assumed. The protein concentrations obtained were in good agreement with biuret values and with dry weight of protein. Radioactivity was measured either in a Nuclear-Chicago automatic gas flow counter (17) or in a Packard Tri-Carb liquid scintillation spectrometer. The aqueous scintillation system of DeMoor and Steen0 (18) was employed, it measures ¹⁴C with an efficiency of 55%. Counting was performed at a level which permitted an error of ±2.4% at a 95% confidence level. Corrections were made for background, self-absorption (where applicable), and variations in response to a ¹⁴C standard.

**Tests for Homogeneity**—Boundary electrophoresis and free diffusion were performed in a Spinco model H apparatus. Sedimentation velocity data were obtained with a Spinco model E analytical ultracentrifuge. An LKB Produkter apparatus was utilized for paper strip electrophoresis. Strips were stained for protein with Amido schwarz 10B and for carbohydrate with periodic acid-Schiff reagent (19). The same apparatus was adapted for agar microelectrophoresis and immunoelectrophoresis, with the slide technique of Schniedegger (20). Antisera were obtained from Mann and from Behringwerke AG.

**Compositional Analysis**—Protein-bound hexose (21), hexosamine (22), and fucose (23) were determined as described by Winzler (24). Sialic acid was assayed according to Warren (25). For all carbohydrate analyses, standard curves were prepared and the content of the sample was read directly from these curves. An α₂-acid glycoprotein (orosomucoid) preparation (24) was analyzed along with the CBG samples as an additional check for validity of the assays. Polyptide content of pure CBG was analyzed by a biuret technique (26), calibrated with Armour standard bovine serum albumin. Amino acid analyses were performed on a Technicon model NC-1 and on a Spinco model 120 analyzer. Samples were prepared and digested with 6 N HCl under reduced pressure for 24 hours at 110°C according to the method recommended by Moore and Stein (27), or with Streptomycyes griseus Pronase (Calbiochem B grade) for 5 days at 37°C (28). Kjeldahl nitrogen was determined by the microtechnique of Lang (29). Analysis of reactive sulfhydryl groups was performed by the method of Boyer (30). Tryptophan was determined colorimetrically (31).

**Binding Studies**—Strips of seamless cellulose dialysis tubing (Visking Company) were washed with water and then with a solution of 0.002 M disodium EDTA and 0.002 M ascorbate in 0.05 M phosphate, pH 7.4, for 24 hours with gentle shaking. They were rinsed over a period of 24 hours with at least six changes of the solution to be used as dialysate.

A series of equilibrium dialyses was performed in 0.05 M phosphate buffer, pH 7.4, at 4°C and at 37°C with a constant CBG concentration in the presence of different amounts of cortisol-4-¹⁴C.

1. J. Kerkay, unpublished results.
2. The authors are indebted to Drs. B. V. Van Osdol for his expert assistance in the studies involving ultracentrifugation, diffusion, and free boundary electrophoresis.
3. The authors are indebted to Dr. R. J. Winzler, Dr. M. Ganguly, and Mr. J. Kerkay for samples of pure orosomucoid, including results of their carbohydrate analyses.
4. The authors wish to thank Dr. C. A. Lang for advice regarding the nitrogen determination.
Experimental conditions have been described previously (32). Exchange equilibrium (33) was used as a test for equilibration. Sialic acid was removed by treatment of 5 g of CBG with 100 units of neuraminidase (Behringwerke AG) over a 5-day period at 4°C with gentle shaking. One unit of the enzyme preparation was defined as the amount required to liberate 1 µg of protein-bound sialic acid in 15 min at 37°C. The resulting preparation did not contain any detectable sialic acid when examined by the method of Warren (25). Endogenous steroid was removed from pure CBG to an extent of 97% by two successive gel filtrations on Sephadex G-25, fine grade, at 45°C, as described by Westphal (2, 8). Cortisol levels at various stages of CBG purification were determined according to Peterson, Karrer, and Guerra (34).

**Procedure**

**Isolation of CBG.**—Plasma or serum volumes greater than 1 liter were subjected to ammonium sulfate fractionation. The sample (2 to 4 liters) was brought to 40% saturation with a saturated solution of ammonium sulfate, shaken gently for 8 hours at 4°C, and centrifuged at 10,000 g for 20 min; the precipitate was discarded. The supernatant was adjusted to pH 6.4 with 0.1 N H₂SO₄ and brought to 60% saturation with ammonium sulfate solution. The precipitate was dissolved in a minimum of water, dialyzed against water to remove ammonium sulfate, centrifuged, and adjusted to a concentration of 7 g/100 ml in water. This fraction contained 85% of the total CBG activity and 17% of the total protein.

Plasma or serum samples of 1 liter or less, or ammonium sulfate fractions, were equilibrated for 1 hour at 25°C and 23 hours at 4°C with a small amount of cortisol-4-¹⁴C, which was added to the plasma samples at a final concentration not greater than 2.0 µg/100 ml. This level was chosen on the basis of preliminary experiments which were conducted to determine optimal amounts of radioactive cortisol to be added to the samples. The total cortisol level after equilibration with 2 µg of cortisol-4-¹⁴C per 100 ml exceeded the binding capacity of the CBG present, as seen from the appearance of unbound cortisol in the DEAE-cellulose eluate immediately following the void volume. This appearance of unbound cortisol was not observed in all samples. Since the endogenous cortisol level of the normal plasma pools was found to be approximately 20 µg/100 ml and did not fluctuate excessively, the total cortisol concentration in our samples was high enough to saturate the CBG-binding sites. This assumption was confirmed by the content of radiolabeled and cold cortisol in the final pure CBG. Calculation of the endogenous plasma cortisol level according to the procedure of isotope dilution gave a value of 23.5 µg/100 ml.

The sample was then applied to a water-equilibrated column (25 × 460 mm) of DEAE-cellulose, which, under the conditions used, was suitable for fractionation of protein quantities of 75 g or less. Elution with 800 ml of water removed 85 to 90% of the used, was suitable for fractionation of protein quantities of 75 g

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**Fig. 1.** DEAE-cellulose chromatography of 400 ml of plasma. The sample was equilibrated with 2.0 µg of cortisol-4-¹⁴C. Protein was eluted at a flow rate of 1 ml per min from a column (48 × 460 mm) containing 70 g of adsorbent. Fractions of 20-ml volume were collected. The arrow indicates the start of the Tris phosphate gradient; at this point, elution with water (900 ml) had removed approximately 85% of the total protein, free of radioactivity.

**Fig. 2.** Hydroxylapatite chromatography of active fractions after separation on DEAE-cellulose. Protein was applied at a concentration of 50 µg per ml and eluted with 0.001 M phosphate buffer, pH 6.8, at a flow rate of 0.2 ml per min. Fractions of 3-ml volume were collected. A, first chromatography, 140 mg of protein on a column (25 × 120 mm); B, final chromatography, 10.1 mg of protein on a column (10 × 120 mm).
dialyzed, lyophilized, and stored at -86°. Prolonged exposure to the low temperature did not affect the activity of CBG at any stage of purity.

Active protein fractions, at a concentration of 20 mg per ml, were subjected to a single hydroxylapatite gel batch separation as described by Seal and Doe (9). The gel was then washed twice with volumes of buffer each equivalent to half of the original sample volume. Supernatant solutions were combined, dialyzed, and lyophilized. Recovery of radioactivity was 87% or higher. The dry protein was dissolved in 0.001 M phosphate buffer, pH 6.8, at a concentration of 50 to 60 mg per ml, and not more than 5 ml of this solution was applied to a column of hydroxylapatite equilibrated with the same buffer. Columns were poured and allowed to settle under gravity for 2 hours at maximal flow rate, and the sample was applied. Elution was performed with 0.001 M phosphate buffer, pH 6.8, at a constant flow rate of 0.2 ml per min. Sample concentration and column preparation were critical for achievement of reproducible separation by hydroxylapatite chromatography. Of the total radioactivity applied to the column, 85 to 90% was recovered in the frontal portion of the eluted protein peak, as shown in Fig. 2A. Active fractions were pooled, desalted by gel filtration on Sephadex G-25, and lyophilized. Hydroxylapatite column chromatography was repeated under identical conditions until a product of constant specific activity was obtained; this required up to five chromatographies, depending on the nature of the material.

Protein which was homogeneous on hydroxyapatite (Fig. 2B) was dissolved at a concentration of 10 mg per ml in 0.05 M phosphate buffer, pH 7.4, containing 5 x 10^{-4} M ascorbate. Ascorbic acid stabilizes the sensitive CBG (37). Not more than 3 ml of this solution was applied to a column of Sephadex G-200 and the protein was eluted with the same buffer. As may be seen in Fig. 3A two protein peaks appeared, the second of which contained all the radioactivity applied to the column. The filtration was repeated until protein and radioactivity were eluted in a single symmetrical peak of constant specific activity (Fig. 3B). The results obtained by this purification procedure are summarized in Table I. The over-all yield was about 50% from plasma samples of 1 liter or less, and about 30% from larger volumes, in which preliminary ammonium sulfate fractionation was employed. The final purification factor achieved was about 4000. Our results indicated a plasma CBG concentration of about 28 mg per liter. Despite its lower efficiency, salt fractionation was an advantageous large scale procedure since greater quantities of CBG could be isolated in shorter periods of time.

RESULTS AND CONCLUSIONS

Physicochemical Properties of CBG—The purified protein appeared homogeneous with respect to protein and carbohydrate, according to paper strip electrophoresis in Veronal buffer (μ = 0.1) at pH 8.6. Agar electrophoresis and immunoelectrophoresis showed the presence of a single homogeneous α-globulin (Fig. 4) in either Tris buffer at pH 8.9 or cacodylate buffer at pH 6.2. The electrophoretic mobility of a 0.12% solution in Veronal buffer at pH 8.6 (μ = 0.1) was determined by moving boundary electrophoresis at 1.0° at -4.87 x 10^{-5} cm² per volt per sec. The protein was immunochemically unreactive in the presence of antiserum to crystallized human serum albumin, human orosomucoid, and human serum Fractions III-1 and VI. This lack of antigenicity indicates that the CBG preparation is free of these protein components. It also shows that the various preparations used for the formation of the antiserum were free of immunologically reactive CBG. It was found that the α-globulin-rich Fractions IV-4 and IV-5,6 bound cortisol with an appreciably lower affinity than whole human serum did. The results show that these serum fractions, which are not obtained under mild conditions, do not contain appreciable amounts of CBG activity. The pure human CBG did not react immunochemically with antiserum prepared against sera of rabbit, rat, or guinea pig; pure rabbit CBGα does not react with antiserum to whole human serum. Since human CBG reacts with antiserum to whole human serum, and pure rabbit CBGα reacts with antiserum to whole rabbit serum, the negative result of the cross reactions with the heterologous antiserum suggests that human and rabbit CBG are immunologically different. Details will be given in a later publication.

Sedimentation velocity ultracentrifugation indicated homogeneity at a concentration of 0.9%, as shown in Fig. 5. Sedimentation coefficients were determined at concentrations of 0.94%, 0.63%, 0.235%, and 0.094%. Solutions were prepared in 0.1 M NaCl and centrifugation was performed at 20°. A linear proportion was found between the reciprocal of the observed s₂₀,₃₆ values and concentration (Fig. 6). Extrapolation to infinite dilution gave an s₂₀,₃₆ of 3.79 S.

* For preparation see Chader (38).
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Summary of CBG isolation procedure from 600 ml of ACD plasma

<table>
<thead>
<tr>
<th>Purification step</th>
<th>Protein mg</th>
<th>Specific activity cpm/mg protein</th>
<th>Purification*</th>
<th>Recovery % Total</th>
<th>Recovery % Utilized</th>
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<tr>
<td>DEAE-cellulose column eluate</td>
<td>3600</td>
<td>24</td>
<td>11.3</td>
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<tr>
<td>Hydroxylapatite batch fractions</td>
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<td>141</td>
<td>6.0</td>
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<tr>
<td>Hydroxylapatite column eluate</td>
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<td>5460</td>
<td>42.2</td>
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<td>85</td>
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<tr>
<td>Sephadex G-200 column eluate</td>
<td>7.2</td>
<td>7530</td>
<td>1.38</td>
<td>98</td>
<td>91</td>
</tr>
<tr>
<td>Final CBG preparation</td>
<td>7.2</td>
<td>7530</td>
<td>3950</td>
<td>68</td>
<td>49</td>
</tr>
</tbody>
</table>

* Purification is calculated on the basis of increase in specific activity at each step in the isolation procedure, after correction for losses of radioactivity upon dialysis.

The percentage actually introduced into the next fractionation step. For instance, 100,000 cpm in starting plasma would give 92,000 cpm total recovery in DEAE-cellulose eluate; 73,000 cpm, constituting the fractions of highest specific activity, were subjected to hydroxylapatite batch fractionation. The remaining active fractions (corresponding to 19,000 cpm) were combined from several experiments and rechromatographed. This improves the over-all yield to a value greater than 49% and not higher than 68%. However, this additional yield is not included in the 7.2-mg final preparation listed in this table.

The diffusion pattern of a 0.25% solution of CBG is shown in Fig. 7. The sample was homogeneous with respect to size of the diffusing species. The true diffusion coefficient for these conditions was determined as the intercept of the linear plot of individually calculated apparent diffusion coefficients plotted against the reciprocal of time. Reduction to water at 20°C gave a $D_{20,w}$ value of $6.15 \times 10^{-7}$ cm$^2$ per sec.

Carbohydrate determinations indicated that CBG contained 9.0% hexosamine, 11.5% hexose, 1.5% fucose, and 4.1% sialic acid. Biuret analysis showed that 75% of CBG was polypeptide in nature. The ultraviolet absorption of a 1% solution in phosphate buffer of pH 7.4 at 279 m$\mu$ was 6.45, in 1-cm cells. The ratio $A_{230}:A_{280}$ was 1.13 for the isolated protein. This ratio was increased to 1.57 when the protein was cleared of steroid by gel filtration at 45°C. The water content of lyophilized CBG was determined as 6.2% by drying to constant weight at 110°C, and all physicochemical parameters were corrected to dry weight.

Amino acid analyses performed on HCl and Pronase hydrolysates of purified CBG gave results which were generally in good agreement. Values for methionine, valine, leucine, and half-cystine were calculated from analysis of a Pronase digest; all other values are from an HCl digest. It has been reported (39) that valine, leucine, and isoleucine are released slowly from proteins, especially when they appear in sequence, and that acid hydrolysis for 22 hours is sometimes not sufficient to liberate these residues completely. After Pronase digestion, the percentages of valine and leucine obtained were, respectively, 4.91 and 8.54; the corresponding values after acid hydrolysis were 4.20 and 7.73. The higher values are considered more representative of the true content and are used in the calculation of partial specific volume. Values obtained for isoleucine by the two procedures were in good agreement.

Observed percentages of acidic amino acids and ammonia were appreciably higher after HCl hydrolysis than after Pronase hydrolysis. There is good evidence that carbohydrate is covalently bound in glycoproteins through N-glycosidic linkages with either asparagine or glutamine (40). The lower acidic amino acid values observed after Pronase digestion were considered the result of incomplete hydrolysis of the protein-carbohydrate bonds (41); the percentages reported are those obtained from acid hydrolysis. Losses of serine and threonine upon hydrolysis were compensated by the use of correction factors, as recommended by Hirs, Stein, and Moore (42). After these corrections, the values from the two types of hydrolysis were in agreement.

The composition of CBG is given in Table II. Values obtained by Seal and Doe (9, 10) are presented for comparison (Section A). Since both Slaunwhite et al. (11) and Seal and Doe (9) utilized the figure of 14.1% as total carbohydrate content, the amino acid composition reported is not directly comparable to that found in the present study for a protein containing 26.1% carbohydrate. The amino acid content of the three preparations has therefore been recalculated on the basis of carbohydrate-free protein, and these values are given in Section B of Table II. A single titratable sulfhydryl group was observed by spectrophotometric analysis (30). Two moles of half-cystine were found per mole of protein by amino acid analysis, suggesting that CBG contains 2 cysteine residues, 1 of which was not readily accessible to mercaptoform formation (39). From data presented in Table II, the partial specific volume was calculated as 0.708 ml per g. The Kjeldahl nitrogen content was measured as 12.7%. Calculation of nitrogen on the basis of amino acid and carbohydrate composition gave a value of 13.0%.

Molecular weight was calculated as 51,700 from sedimentation and diffusion data. The frictional ratio, $f/f_s$ (43), was 1.42.
This figure probably reflects a high degree of hydration owing to the high carbohydrate content of the protein. The physicochemical properties of CBG are summarized in Table III.

Cortisol-binding Properties of CBG—Removal of steroid from pure CBG by gel filtration at 45° resulted in irreversible inactiva-

<table>
<thead>
<tr>
<th>Table II</th>
<th>Amino acid and carbohydrate composition of CBG</th>
</tr>
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<tbody>
<tr>
<td>Residue</td>
<td>Section A: glycoprotein</td>
</tr>
<tr>
<td></td>
<td>Present study</td>
</tr>
<tr>
<td></td>
<td>%</td>
</tr>
<tr>
<td>Lys</td>
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</tr>
<tr>
<td>His</td>
<td>2.43</td>
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<td>Asp</td>
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<td>4.23</td>
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<td>Ser</td>
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<td>Cys</td>
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<tr>
<td>Val</td>
<td>4.91</td>
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<td>Met</td>
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<td>Fucose</td>
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</tr>
<tr>
<td>Amide NH₂</td>
<td>1.93</td>
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</table>

* Calculated on the basis of carbohydrate-free protein.
* Determined by colorimetric method.
* Determined by spectrophotometric method.

FIG. 7. Diffusion pattern of a 0.25% solution of CBG in 0.1 M NaCl; 1°. The exposure shown was made 412 min after initial boundary formation.

FIG. 5. Sedimentation pattern of CBG, at 4°; 59,780 rpm. Concentration was 9.0 mg per ml in 0.05 M phosphate buffer, pH 7.4. The exposure shown was made 121 min after attainment of maximum speed. Bar angle was 60°. Sedimentation is from left to right.

FIG. 6. Relationship between reciprocal sedimentation coefficient and CBG concentration. The straight line was calculated by the method of least squares.
were analyzed according to the methods of Klotz (44) and Scatchard (45). Average values of the calculated association constants were $5.2 \times 10^3$ M$^{-1}$ at 4° and $2.4 \times 10^5$ M$^{-1}$ at 37°. One binding site for cortisol was observed per CBG molecule at either temperature. Reciprocal plot analysis of the data obtained at 4° is shown in Fig. 8.

The enzymatic removal of sialic acid from CBG did not significantly alter the cortisol-binding affinity. Association constants of $4.9 \times 10^3$ M$^{-1}$ and $3.0 \times 10^5$ M$^{-1}$ were calculated from equilibrium dialysis data obtained at 4° and 37°, respectively.

### DISCUSSION

The CBG preparation isolated in the present study differs in various respects from those described previously (7, 9-11). In the course of our purification, somewhat different techniques were applied which in our hands improved the efficiency and reproducibility. A consistently higher purification factor was achieved by use of gradient (3) rather than stepwise (9) elution in DEAE-cellulose chromatography. It was found advantageous to elute with distilled water prior to application of the buffer gradient since this technique resulted in the removal of 85 to 90% of the total protein applied. This protein material was free of CBG since it did not contain radioactive cortisol and did not show cortisol-binding affinity by gel filtration; equilibrium dialysis with the eluted fractions did not reveal any protein which bound cortisol with an affinity greater than that of albumin.

CBG preparations of constant specific activity and homogeneity with respect to hydroxylapatite chromatography (Fig. 2B) were obtained only by close adherence to the techniques described. The purification at this stage was about 2900-fold, which is similar to that reported by Seal and Doe (10) for their final product. Immunelectrophoresis against antihuman serum showed the presence of a second component at this stage of fractionation, when the protein was analyzed at a concentration of 8 g/100 ml. Slawnhite et al. (11) have recently observed a second band in disc electrophoresis after attainment of transcorin homogeneity on hydroxylapatite. The impurity in our preparation migrated as an $\alpha_2$-globulin and stained poorly with Amido schwarz. Gel filtration on Sephadex G-200 separated the two proteins; the inactive material constituted about 30% of the total protein applied to the column (Fig. 3A). The final CBG preparation (Fig. 3B) was approximately 4000-fold purified and differed significantly in composition (Table II) and physicochemical properties (Table III) from that described by Seal and Doe (9, 10).

From the over-all yield of 49% for the pure CBG preparation described in Table I, it can be calculated that the original ACD plasma contained 24.5 mg of CBG per liter. This corresponds to 27.8 mg of CBG per liter of undiluted plasma since the ACD plasma contains about 12% diluent. This value of approximately 28 mg of CBG per liter seems somewhat low considering the level of about $7 \times 10^3$ M ($36$ mg per liter) reported for normal human plasma (6, 7). However, analyses for values of a large number of individual samples show a wide range of 23 to 45 mg per liter (33) or 27 to 43 mg per liter (7). In the earlier work of Seal and Doe (9, 10), a level of 20 mg of CBG per liter of normal human plasma has been stated (approximately one-third of 00 mg per liter present in diethylstilbestrol-treated patients). The plasma used as starting material by Seal and Doe (10), i.e. blood bank plasma, is similar to that used in the present work, whereas the reported values (6, 7) in general refer to fresh plasma.

On the basis of Seal and Doe's and our own experience, it is conceivable that plasma samples obtained from outdated blood bank blood may have a somewhat lower CBG concentration than fresh material. Murphy, Engelberg, and Pattow (46) described inactivation of CBG by citrate when stored in the frozen state. Since we have never frozen CBG-containing solutions while fresh material. Murphy, Engelberg, and Pattow (46) described inactivation of CBG by citrate when stored in the frozen state. Since we have never frozen CBG-containing solutions while fresh material. Murphy, Engelberg, and Pattow (46) described inactivation of CBG by citrate when stored in the frozen state. Since we have never frozen CBG-containing solutions while fresh material. Murphy, Engelberg, and Pattow (46) described inactivation of CBG by citrate when stored in the frozen state. Since we have never frozen CBG-containing solutions while fresh material. Murphy, Engelberg, and Pattow (46) described inactivation of CBG by citrate when stored in the frozen state. Since we have never frozen CBG-containing solutions while fresh material. Murphy, Engelberg, and Pattow (46) described inactivation of CBG by citrate when stored in the frozen state. Since we have never frozen CBG-containing solutions while fresh material. Murphy, Engelberg, and Pattow (46) described inactivation of CBG by citrate when stored in the frozen state.
The amino acid composition agreed reasonably well with that determined by Slaunwhite et al. (11), when calculated on a polypeptide basis. The extinction coefficient and the partial specific volume found in the present study were lower than those reported (9), in accordance with the observed higher carbohydrate content.

The sedimentation coefficient of CBG was obtained by extrapolation to infinite dilution of the s_{20, w} values determined at four concentrations (Fig. 6). In previous studies, the molecular weight of CBG was estimated on the basis of binding parameters (45,000 (9)), compositional analysis (43,000 (10)), and microsedimentation equilibrium (58,500 (11)). The molecular weight reported in the present paper (51,700) was calculated from the sedimentation coefficient, diffusion coefficient, and partial specific volume determined for the homogeneous CBG prepared in our laboratory; the value obtained by this more rigorous procedure is considered more definitive than those of earlier publications. It is in agreement with the molecular weight of 52,000 recently given by Seal and Doe (7).

Dialysis against water of active fractions at different stages of purity showed a tendency toward irreversible dissociation of the cortisol-CBG complex, which increased sharply as the preparations approached homogeneity. Inactivation of pure CBG occurred upon subjection to gel filtration at 45°. Since it had been shown (2) that removal of steroids from whole serum at 45° does not significantly alter the binding affinity of CBG, it would appear that other plasma components exert a protective influence. Equilibrium dialysis of CBG in the presence of added cortisol at 37° for 48 hours does not result in loss of activity, suggesting that the steroid substrate protects the purified protein in a similar way as observed for CBG in serum (33).

In the present study, a single cortisol-binding site has been found per molecule of protein, confirming earlier reports (10, 7). The association constants at 4° and at 37° agree well with those determined by Seal and Doe (9, 7) and with those observed in steroid-free human serum (2). It appears that, at low steroid concentrations, the cortisol-binding affinity of CBG is not influenced by the presence of other plasma components.

It was observed that, under the mild conditions used, complete enzymatic removal of sialic acid from CBG does not affect the cortisol-binding affinity of the protein. This finding suggests that CBG lability at pH levels below 5 is probably not the result of cleavage of the ketosidic carbohydrate linkage, as has been suggested (10). Thyroxine-binding globulin (47) and human α1-acid glycoprotein (48) also retain complete binding activity for thyroxin and progesterone, respectively, after removal of sialic acid, indicating that this moiety may not be of general significance for hormone-binding of proteins.

REFERENCES
