Steroid-Protein Interactions

XVI. ISOLATION AND CHARACTERIZATION OF THE CORTICOSTEROID-BINDING GLOBULIN OF THE RABBIT

(Received for publication, September 29, 1967)

GERALD J. CHADER† AND ULRICH WESTPHAL
From the Biochemistry Department, University of Louisville School of Medicine, Louisville, Kentucky 40208

SUMMARY

Comparative determination of high affinity binding activity for cortisol and corticosterone in the sera of various species by equilibrium dialysis indicated considerable differences, which were independent of the ratio of cortisol to corticosterone present in the blood plasma. Corticosteroid-binding globulin (CBG) was isolated from pooled rabbit serum by chromatography on diethylaminoethyl Sephadex, gel filtration over Sephadex G-200, and hydroxylapatite chromatography. Ammonium sulfate fractionation was found useful as a preliminary purification step. A quantity of 13.5 mg of pure CBG was obtained from 2 liters of serum, with an overall 6200-fold purification and 50 to 60% yield. The protein was homogeneous by free boundary and paper electrophoresis, sedimentation velocity at different concentrations, and diffusion. Immunoelectrophoretic analysis with antisera to rabbit serum indicated a pure α-globulin; no precipitation was obtained with antisera to human serum, to rat serum, or to guinea pig serum.

Amino acid and carbohydrate composition showed a general similarity to human CBG, in spite of distinct differences in certain residues. The extrapolated sedimentation coefficient was found to be $s_{20,w} = 3.55$ S; diffusion coefficient, $D_{20,w} = 7.02 \times 10^{-7}$ cm$^2$ sec$^{-1}$; electrophoretic mobility at pH 8.6, $\Gamma/2 = 5.1 \times 10^{-3}$ cm$^2$ volt$^{-1}$ sec$^{-1}$; extinction coefficient at 279 mμ, $E_{\text{1cm}}^{\text{1%}} = 8.4$; partial specific volume, $\beta = 0.695$ ml per g; and frictional ratio, $f/f_0 = 1.37$. A molecular weight of 40,700 was calculated from sedimentation velocity and diffusion data.

The pure CBG, isolated under conditions of saturation with endogenous corticosterone and added cortisol-4-14C, contained 1 mole of corticosteroid per 40,700 g of CBG; the number of high affinity binding sites was determined to be $n = 1$. The association constants at 4° and 37° were $k = 9.0 \times 10^4 \text{ M}^{-1}$ and $4.7 \times 10^4 \text{ M}^{-1}$, respectively. Thermodynamic calculations gave negative enthalpy change and negative entropy change for the interaction. Removal of corticosteroid from the isolated pure CBG by gel filtration at 45° resulted in loss of binding activity.

The presence in human serum of a protein with high binding affinity for the corticosteroid hormones (2) is well established (3, 4). The isolation and characterization of this corticosteroid-binding globulin, or transcortin, has been reported by Scal and Doe (3, 6), by Slaunwhite et al. (7), and by Muldoon and Westphal (1). Much less is known about the corticosteroid-binding proteins of other species, although the techniques of equilibrium dialysis (8-10), electrophoresis (9, 11), and gel filtration (12, 13) have revealed the presence of such proteins in the serum of all vertebrate species tested. The strength of steroid binding varies considerably in different species, from very low values in dog (14) and sheep (15) plasma to those considerably higher, as in the rat (16-18). From recent equilibrium dialysis studies with the serum of the rabbit, rat, guinea pig, and monkey, in addition to human serum, association constants have been calculated for CBG binding with several steroids (19). Estimates were also made of the concentration of CBG in the serum of these species.

Gel filtration (12, 13, 20) and equilibrium dialysis (8, 9, 10) studies have clearly established the high affinity of a rabbit serum protein component for the glucocorticoid hormones and progesterone. Electrophoretic studies show this component to

† Supported in part by United States Public Health Service Training Grant 65901. Present address, Department of Biological Chemistry, Harvard University Medical School, Boston, Massachusetts 02115.

The abbreviations used are: CBG, corticosteroid-binding globulin or transcortin; HMB, p-hydroxymercurobenzoate.
be an α1-globulin (11). Seal and Doe (21) have described the use of hydroxyapatite in obtaining highly purified CBG preparations from seven species, including the rabbit, and have given preliminary data on the carbohydrate content of these proteins. Besides this, little information is available on either the physio-

chemical or the steroid-binding properties of corticosteroid-

binding globulins of any species other than the human.

The isolation and characterization of corticosteroid-binding globulin from the rabbit are of interest for several reasons. First, a comparison is made possible with the human protein as to the chemical specificity of steroid binding. It is known that cortisol is the major plasma corticosteroid in the human and that corticosterone is predominant in the rabbit. It has also been found that rabbit CBG in unfractionated serum has a higher binding affinity for cortisol than for corticosterone, whereas the opposite is true at 4°C for human CBG (19). An analysis of differences in binding of these and other steroids to CBG may lead to an identification of functional groups in the steroid and in the protein which are involved in the interactions. Binding differences might thus be related to specific differences in the chemical structure of the proteins. A second point of interest would be a comparison between the physical and chemical properties of CBG molecules in different species. Since Seal and Doe have demonstrated the ubiquitous occurrence of corticosteroid binding macromolecules in widely divergent vertebrate species (12), this may indicate the existence in blood of a protein type generally responsible for functional regulation of corticoids and possibly other steroid hormones (21). The characterization of functional groups of the protein involved in steroid binding may also provide a valuable chemical analogy for highly specific steroid-protein interactions at receptor tissue sites.

MATERIALS AND METHODS

The experimental procedures were previously described (1) except for the following additions and changes. All experiments were conducted at 4°C unless otherwise noted.

**Protein Sources**—Sera from monkey (Macaca mulatta), rat (Sprague-Dawley and Wistar), guinea pig, wild starling, mongrel dog, and cat were prepared by allowing freshly drawn blood to clot and carefully drawing off the serum after 1 hour. Cow, horse, sheep, and pig sera were prepared similarly from blood obtained at the slaughterhouse. Other serum samples used for species comparison were purchased from Colorado Serum Company, Denver; rabbit serum, Pool I and Pool II (see Table 11), was obtained from normal male albino from local sources and from Pel-Freeze Biologicals, respectively. Female rabbit serum from seven species, including the rabbit, and have given preliminary data on the carbohydrate content of these proteins. Besides this, little information is available on either the physio-

chemical or the steroid-binding properties of corticosteroid-

binding globulins of any species other than the human.

The isolation and characterization of corticosteroid-binding globulin from the rabbit are of interest for several reasons. First, a comparison is made possible with the human protein as to the chemical specificity of steroid binding. It is known that cortisol is the major plasma corticosteroid in the human and that corticosterone is predominant in the rabbit. It has also been found that rabbit CBG in unfractionated serum has a higher binding affinity for cortisol than for corticosterone, whereas the opposite is true at 4°C for human CBG (19). An analysis of differences in binding of these and other steroids to CBG may lead to an identification of functional groups in the steroid and in the protein which are involved in the interactions. Binding differences might thus be related to specific differences in the chemical structure of the proteins. A second point of interest would be a comparison between the physical and chemical properties of CBG molecules in different species. Since Seal and Doe have demonstrated the ubiquitous occurrence of corticosteroid binding macromolecules in widely divergent vertebrate species (12), this may indicate the existence in blood of a protein type generally responsible for functional regulation of corticoids and possibly other steroid hormones (21). The characterization of functional groups of the protein involved in steroid binding may also provide a valuable chemical analogy for highly specific steroid-protein interactions at receptor tissue sites.

**Protein Sources**—Sera from monkey (Macaca mulatta), rat (Sprague-Dawley and Wistar), guinea pig, wild starling, mongrel dog, and cat were prepared by allowing freshly drawn blood to clot and carefully drawing off the serum after 1 hour. Cow, horse, sheep, and pig sera were prepared similarly from blood obtained at the slaughterhouse. Other serum samples used for species comparison were purchased from Colorado Serum Company, Denver; rabbit serum, Pool I and Pool II (see Table 11), was obtained from normal male albino from local sources and from Pel-Freeze Biologicals, respectively. Female rabbit serum from seven species, including the rabbit, and have given preliminary data on the carbohydrate content of these proteins. Besides this, little information is available on either the physio-

chemical or the steroid-binding properties of corticosteroid-

binding globulins of any species other than the human.

The isolation and characterization of corticosteroid-binding globulin from the rabbit are of interest for several reasons. First, a comparison is made possible with the human protein as to the chemical specificity of steroid binding. It is known that cortisol is the major plasma corticosteroid in the human and that corticosterone is predominant in the rabbit. It has also been found that rabbit CBG in unfractionated serum has a higher binding affinity for cortisol than for corticosterone, whereas the opposite is true at 4°C for human CBG (19). An analysis of differences in binding of these and other steroids to CBG may lead to an identification of functional groups in the steroid and in the protein which are involved in the interactions. Binding differences might thus be related to specific differences in the chemical structure of the proteins. A second point of interest would be a comparison between the physical and chemical properties of CBG molecules in different species. Since Seal and Doe have demonstrated the ubiquitous occurrence of corticosteroid binding macromolecules in widely divergent vertebrate species (12), this may indicate the existence in blood of a protein type generally responsible for functional regulation of corticoids and possibly other steroid hormones (21). The characterization of functional groups of the protein involved in steroid binding may also provide a valuable chemical analogy for highly specific steroid-protein interactions at receptor tissue sites.

**Protein Sources**—Sera from monkey (Macaca mulatta), rat (Sprague-Dawley and Wistar), guinea pig, wild starling, mongrel dog, and cat were prepared by allowing freshly drawn blood to clot and carefully drawing off the serum after 1 hour. Cow, horse, sheep, and pig sera were prepared similarly from blood obtained at the slaughterhouse. Other serum samples used for species comparison were purchased from Colorado Serum Company, Denver; rabbit serum, Pool I and Pool II (see Table 11), was obtained from normal male albino from local sources and from Pel-Freeze Biologicals, respectively. Female rabbit serum from seven species, including the rabbit, and have given preliminary data on the carbohydrate content of these proteins. Besides this, little information is available on either the physio-

chemical or the steroid-binding properties of corticosteroid-

binding globulins of any species other than the human.

The isolation and characterization of corticosteroid-binding globulin from the rabbit are of interest for several reasons. First, a comparison is made possible with the human protein as to the chemical specificity of steroid binding. It is known that cortisol is the major plasma corticosteroid in the human and that corticosterone is predominant in the rabbit. It has also been found that rabbit CBG in unfractionated serum has a higher binding affinity for cortisol than for corticosterone, whereas the opposite is true at 4°C for human CBG (19). An analysis of differences in binding of these and other steroids to CBG may lead to an identification of functional groups in the steroid and in the protein which are involved in the interactions. Binding differences might thus be related to specific differences in the chemical structure of the proteins. A second point of interest would be a comparison between the physical and chemical properties of CBG molecules in different species. Since Seal and Doe have demonstrated the ubiquitous occurrence of corticosteroid binding macromolecules in widely divergent vertebrate species (12), this may indicate the existence in blood of a protein type generally responsible for functional regulation of corticoids and possibly other steroid hormones (21). The characterization of functional groups of the protein involved in steroid binding may also provide a valuable chemical analogy for highly specific steroid-protein interactions at receptor tissue sites.
equilibration was complete by 48 hours at both 4° and 37°. The tests for sulfhydryl involvement were performed at 37° with addition of 2 x 10^{-4} M HMB (29). Association constants were determined as previously reported (1) from quadruplicate experiments at each temperature (4° and 37°); the values were calculated from those dialyses to which no further radiolabeled cortisol was added. Changes of the Gibbs free energy, enthalpy, and entropy were calculated by standard equations (30).

**Sephadex Gel Filtration**—Binding capacity in serum was measured according to the method of De Moor et al. (20) and Seal and Doe (21) on Sephadex G-50. One milliliter of the serum sample was allowed to equilibrate with 2.0 μg of corticosterone-4-¹⁴C for 15 min at 37° with gentle shaking, and for 1 hour at 4°. It was then applied to a column (2.5 x 27 cm) of Sephadex G-50 equilibrated with phosphate buffer and eluted with the same buffer. Samples were assayed for protein content at 279 μm; radioactivity was determined as previously described (1).

For characterization of the ammonium sulfate fractions (see below) and for analysis of the binding proteins in unfractonated serum, a column (2.5 x 28 cm) of Sephadex G-200 equilibrated with 0.05 M phosphate buffer, pH 7.4, was used. The protein sample (90 mg dissolved in 1.0 ml of buffer or 1 ml of a serum sample diluted to contain 50 mg of total protein) was allowed to equilibrate with 0.2 μg of cortisol-4-¹⁴C for 15 min at room temperature and 1 hour at 4° with gentle shaking. Equilibration of added radioactive steroid with endogenous corticosteroid has been found to be complete under similar conditions (31). The protein was then subjected with phosphate buffer; the fractions were analyzed for protein and radioactivity as previously described (1).

**Isolation of CBG**—Ammonium sulfate fractionation at 4° was found useful for rapid preliminary purification of CBG. A convenient volume of serum (up to 1 liter) was brought to 40% saturation with a saturated ammonium sulfate solution (previously prepared at room temperature) and stirred for 4 hour, and the precipitate (Fraction 1) was separated by centrifugation at 10,000 × g for 20 min. The supernatant was adjusted to pH 6.4 with dilute H₂SO₄, brought to 60% saturation with saturated ammonium sulfate, and stirred, and the precipitate (Fraction II) was centrifuged as before. The supernatant was brought to 75% saturation with saturated ammonium sulfate, stirred, and centrifuged; the precipitate was termed Fraction III. The three fractions and the final supernatant were dialyzed, lyophilized, and subjected to Sephadex G-200 gel filtration as described above. Fraction II was used for further purification.

**DEAE-Sephadex chromatography** as a preliminary step afforded a more rapid purification of liter quantities of serum, primarily because of the high anion exchange capacity of the adsorbent. One liter (or less) of female rabbit serum was dialyzed for 24 hours against two to three changes of water; it was found unnecessary to remove the small quantities of euglobulins precipitated. The serum was then equilibrated with 2 to 3 μg of cortisol-4-¹⁴C at room temperature with stirring for at least 1 hour, and for an additional 2 to 3 hours at 4°, and applied to a DEAE-Sephadex column (4.5 x 5 cm) which had been equilibrated with 0.004 M Tris-phosphate buffer, pH 8.0. Cortisol-4-¹⁴C was chosen in this case rather than corticosterone-4-¹⁴C because of its slightly higher binding affinity to rabbit CBG (19); it was added in a quantity sufficient to oversaturate the CBG binding sites slightly. For elution of the major protein peaks, 450 ml of 0.0025 M Tris-phosphate buffer, pH 7.5, and 1.5 liters of 0.06 M Tris-phosphate buffer, pH 7.0, were used. A gradient was then applied as outlined in Table 1 by use of a Buchler Varigred apparatus with a total of 440 ml in each of seven chambers; 0.0075 M Tris-phosphate, pH 8.0, was used as starting buffer, and 0.4 M Tris-phosphate, pH 5.5, as the limit buffer.

Fractions were assayed for radioactivity in a Packard Tri-Carb liquid scintillation spectrometer as previously described (1). Since considerable variations in color and protein content were observed in these fractions, samples were mixed with a standard amount of cortisol-4-¹⁴C and reanalyzed to check for quenching or other influences on the counting rate. In all cases, the total sample count agreed within 4% with the theoretical amount calculated to be present.

Fractions of high specific activity were dialyzed, lyophilized, and applied to a column (4.5 x 5 cm) of Sephadex G-200 equilibrated with 0.05 M phosphate buffer, pH 7.4. The same buffer was used for elution. The fractions of higher specific activity were combined, partially dialyzed, lyophilized, and subjected in a similar manner to a second filtration on Sephadex G-200 for further purification. This second Sephadex filtration was not always found essential for purification and therefore was often omitted. The protein was then subjected to hydroxylapatite chromatography essentially as outlined by Seal and Doe (5). Lyophilized protein was dissolved in 0.005 M phosphate buffer, pH 6.8 (50 to 80 mg per ml), and applied to a hydroxylapatite column equilibrated with the same buffer. Stepwise elution was performed with 0.005 M, 0.02 M, 0.05 M, and 0.2 M phosphate buffers of pH 6.8; the next higher buffer strength was applied when the protein concentration in the eluate fell markedly. Fractions of high specific activity were collected, equilibrated with 0.005 M phosphate buffer, pH 6.8, by passage over Sephadex G-25, and recycled through the hydroxylapatite chromatographic procedure until a single, homogeneous product was obtained. The final product was desalted on Sephadex G-25.

In some cases, CBG partially purified by hydroxylapatite was finally subjected to another Sephadex G-200 filtration under conditions similar to those described for the testing of the ammonium sulfate fractions, except that no new cortisol-4-¹⁴C was added.

**Methods of Characterization**—Techniques for paper strip, free boundary and immunoelectrophoresis, ultracentrifugation, diffusion, and carbohydrate analysis have been previously described (1). Some immunoelectrophoretic studies were per-
formed simultaneously with both pure rabbit and human (1) CBG preparations. In all carbohydrate determinations, two human orosomucoid preparations from different sources were analyzed as controls along with the rabbit CBG. For all quantitative measurements on pure rabbit CBG, concentrations were determined by spectrophotometric measurement at 279 m\(\mu\). A water content of 4.4% for the lyophilized protein was determined by drying quadruplicate samples to constant weight at 110°; the extinction coefficient after correction to dry weight was found to be \(E_{410}^\text{em} = 8.4\).

Amino acid analyses were performed on a Technicon model NC-1 and a Spinco model 120 amino acid analyzer for the HCl-digested and Pronase-digested material, respectively. Experimental details have been given (1). A human orosomucoid sample analyzed on the Technicon instrument within 2 days of the CBG analysis gave excellent agreement with literature values. Tryptophan was determined by the method of Benez and Schmid (32) as well as that of Opieńska-Blaith, Charzinski, and Berbee (33). The results obtained by the two techniques were in good agreement and were therefore averaged.

**RESULTS**

**CBG Activity in Various Species**—Table II gives the relative binding affinities (C values) for various serum samples at 4° and 37° with cortisol (F) and corticosterone (B). In all cases, the binding activity was higher at 4° than at 37°; no correlation was found between affinity to the two corticosteroids and F:B ratio. These values are in general agreement with binding capacities determined by the gel filtration technique (12, 13, 21).

Serum samples used in this study were mainly pools obtained from several animals. Binding values of such pooled serum samples correctly represent the mathematical averages of the individual sample values (34). In the present study, the calculated average of the binding values of 20 individual samples of rabbit serum (C = 0.14) agreed closely with the value obtained with the pooled serum supply (C = 0.15, Pool II, interaction with cortisol at 37°), even though there was considerable variation in the individual binding values and in the endogenous corticosteroid levels.

Rabbit serum bound cortisol better than corticosterone, although the ratio of the two corticoids in the circulating plasma was 0.05 (Table II). A large variation was found among binding values of different serum pools. Rabbit Pool I was a mixture of normal albino males and showed high steroid-binding affinity. Pool II from mixed male and female rabbits (generally 60%; New Zealand whites (albinos), 35%; Californians (albinos), and 5%; various colored breeds) according to the supplier, Pel-Freez Biologicals had considerably lower binding affinity but showed the same over-all binding pattern as other pools of rabbit serum tested. Serum from a single mature male New Zealand rabbit showed high binding affinity for cortisol at 4°C at 37° (C = 0.65), comparable with that of Pool I. Samples of Pel-Freez serum from female rabbits tended to have somewhat higher binding affinities for the glucocorticoids, and therefore only serum from female rabbits was used for the purification studies.

Since pools of rabbit serum from various sources exhibited marked differences in their corticosteroid-binding affinities as determined by equilibrium dialysis, the binding behavior was further investigated by an independent method. For this purpose, the Sephadex G-50 gel filtration technique was used, since it gives estimates proportional to CBG concentration rather than to a resultant of binding affinity and capacity as obtained from equilibrium dialysis (26). The results were found to be in good agreement with the affinity or C values given in Table II; serum from Pool I had approximately 3.0 times the corticosterone-binding capacity as the serum from Pool II, whereas it showed a 2.9-fold greater C value. The gel filtration technique also corroborated the higher CBG activity found in the serum of female rabbits by equilibrium dialysis (for details, see Reference 35); serum pools from female rabbits used for the purification studies had about 20% higher corticosterone-binding capacity than serum pools mixed from male and female animals.

Table III shows the results of equilibrium dialysis experiments in which the binding activity of stripped rabbit serum was measured at 4° and 37°. A number of steroid hormones of different structures and polarities were applied. The C values at 4° indicate the highest binding affinity for cortisol, in accordance with the observations on serum containing the endogenous corticosteroids; the value for corticosterone, however, is lower than might be expected from results obtained with unstripped (Table II) and stripped (19) rabbit serum. In comparing binding of cortisol with that of the other steroids, especially progesterone and estradiol, it should be recalled that the C value is a resultant of interaction with albumin as well as with CBG. The relatively high binding affinity for testosterone may
Relative steroid-binding activities (C values) of stripped rabbit serum

<table>
<thead>
<tr>
<th>Steroid</th>
<th>4°</th>
<th>37°</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cortisol</td>
<td>4.3</td>
<td>0.68</td>
</tr>
<tr>
<td>Corticosterone</td>
<td>1.9</td>
<td>0.79</td>
</tr>
<tr>
<td>Progesterone</td>
<td>2.7</td>
<td>1.3</td>
</tr>
<tr>
<td>Testosterone</td>
<td>2.3</td>
<td>0.73</td>
</tr>
<tr>
<td>Estradiol</td>
<td>1.4</td>
<td>1.2</td>
</tr>
<tr>
<td>Aldosterone</td>
<td>0.17</td>
<td>0.20</td>
</tr>
</tbody>
</table>

Fig. 1. Sephadex G-200 filtration of rabbit serum containing 14C-labeled steroids. One milliliter of diluted serum containing 50 mg of total protein was equilibrated with 0.2 µg of cortisol-4-14C (A) or progesterone-4-14C (B).

be attributed to a specific protein, as demonstrated for human serum by Pearlman and Crépy (36). Binding of aldosterone is low in comparison with that to human serum proteins (3), as was also observed with the pure rabbit CBG (see below). Since steroid-CBG interaction is markedly decreased at elevated temperature (3, 4), greater involvement of albumin binding in the C values would be expected at 37°. The data of Table III verify this.

Gel Filtration—Steroid binding to serum proteins of the rabbit can be demonstrated by Sephadex G-200 filtration, as shown in Fig. 1. Binding of cortisol (Fig. 1A) is slightly higher than with corticosterone (see Fig. II of Chader (35)), in agreement with equilibrium dialysis data; progesterone binding (Fig. 1B) is considerably greater as a result of binding by albumin as well as by CBG. Since albumin also binds corticosteroids, it was necessary to show that albumin-steroid interactions did not contribute to the values obtained when cortisol or corticosterone was applied. If a mixture of rabbit serum albumin and radio-labeled cortisol was subjected to gel filtration under the usual conditions, no corticosteroid remained bound to the protein fraction (Fig. 2A). Progesterone, on the other hand, was strongly bound to rabbit albumin (Fig. 2B), and particularly well to the apparently heavier component preceding the main albumin peak. This technique therefore affords not only a relative estimate of binding affinity, but an assessment of the distribution of binding proteins as well. Fig. 3 shows that cortisol binding to human albumin follows the same pattern as that to rabbit albumin, this behavior forms the basis for the purification of human CBG by Sephadex G-200 gel filtration (1) and may well indicate the general applicability of this technique for fractionation of CBG from various species.

Gel Filtration Analysis of Ammonium Sulfate Fractions—As Fig. 4 shows, ammonium sulfate Fractions I and III bound only small amounts of cortisol. Fraction II had a much higher specific activity (counts per min per mg of protein), containing over 90% of the CBG activity and about 30% of the total protein. Similar results were obtained when the fractions were analyzed by the equilibrium dialysis technique. The small amounts of supernatant protein did not show CBG activity. Specific activity could be greatly increased by precipitating the protein in a narrower concentration range of ammonium sulfate. The highest specific activity was obtained in the 50 to 62% saturated ammonium sulfate fraction, but the total yield was

Fig. 2. Sephadex G-200 filtration of rabbit serum albumin with 14C-labeled steroids. Fifty milligrams of albumin were dissolved in 1 ml of 0.05 m phosphate, pH 7.4, and equilibrated with 0.2 µg of cortisol-4-14C (A) or progesterone-4-14C (B).
low. The method provides a convenient 3 to 1 fold concentration of serum CBG. Purification on a large scale is cumbersome, however, because of the large volumes of liquid and long dialysis times involved. The Sephadex G-200 test system gives a rough estimate of the different molecular sizes of proteins in the samples and is considered a useful extension of the filtration procedure of DeMoor et al. (20).

Isolation of Rabbit CBG—Fig. 5 shows the pattern obtained when 1 liter of rabbit serum is subjected to DEAE-Sephadex chromatography with stepwise and gradient elution. Results of paper strip electrophoretic analyses of selected fractions are given below the elution pattern; for comparison, the figure shows the electrophoretic separation of unfractionated rabbit serum and the pattern obtained with the final pure CBG, indicating a single band with an $\alpha_1$ mobility. Small amounts of radioactivity eluted with the breakthrough volume and with the major protein peak were not strongly bound to protein, as determined by gel filtration and equilibrium dialysis studies; presumably they represented cortisol-4-14C that exceeded the CBG binding capacity. Steroid was bound with high affinity to the protein contained in tubes 250 to 300, as revealed by Sephadex G-200 gel filtration experiments (35). A 15- to 20-fold concentration was usually achieved in this way. Further purification was obtained by filtration over Sephadex G-200 (Fig. 6). This technique not only gave a 2- to 4-fold increase in specific activity, but further revealed the high binding affinity of the sample, since all radio-active steroid remained protein bound during the filtration.

The final purification of CBG was usually achieved by repeated chromatography on hydroxylapatite columns. Fig. 7 shows a typical fractionation by stepwise elution of partially purifed material from Sephadex G-200 chromatography. It was necessary to perform such hydroxylapatite chromatography three or four times before a homogeneous product was obtained. In accord with the studies on human CBG by Seal and Doe (5, 6) and Muldoon and Westphal (1), rabbit CBG was found in greatest concentration in the leading edge of the first protein peak eluted. When a smaller amount of partially purified protein was applied to the column, the CBG activity was often seen to be concentrated in a discrete, small shoulder on the leading edge of this first peak. Subsequent Sephadex G-200 filtration was also found to be useful for final purification, in a way similar to that reported for human CBG (1). Fig. 8 shows such gel filtration of material after partial purification with DEAE-Sephadex, Sephadex G-200, and a single hydroxylapatite chromatography. The protein peak containing bound cortisol-4-14C was found to be homogeneous by several techniques (see next section), and the CBG was similar in all respects to that obtained after DEAE-Sephadex, Sephadex G-200, and successive hydroxylapatite chromatography.

Table IV shows the results of a purification procedure starting with 2 liters of rabbit serum. A yield of 13.5 mg of CBG was obtained, with an over-all 6200-fold purification and a 53% recovery of cortisol-4-14C. When larger amounts of serum were used initially, the recovery was 60 to 70%.

Physicochemical Characterization—All data on physicochemical properties and chemical composition were obtained with the pure CBG-corticoiosteroid complex as isolated. The paper strip electrophoresis technique showed a single band at pH 8.6 in Veronal buffer (Fig. 5). Two preparations of rabbit CBG from different purifications showed identical immunoelectrophoretic patterns (Fig. 9), i.e. a single precipitation crescent in the $\alpha_1$ globulin region. In studies performed with Dr. T. Muldoon, rabbit CBG was found not to give a precipitation line when tested against human antiserum; likewise, pure human CBG (1) did not give a precipitation line when run against rabbit antiserum. Neither pure protein reacted with rat or guinea pig antisera. These immunoelectrophoretic experiments did not utilize the full potential of the technique, mainly because the available quantity of pure CBG was insufficient to permit its application as an antigen for preparation of CBG antiserum. Nevertheless, the results indicate distinct antigenic differences between human and rabbit CBG, as well as a probable lack of antigenic similarity of the two CBG proteins to any component of rat and guinea pig serum.
A single homogeneous component with a mobility of $-5.1 \times 10^{-4}$ cm$^2$ volt$^{-1}$ sec$^{-1}$ was observed when rabbit CBG was subjected to moving boundary electrophoresis in Veronal buffer ($1/2 \times 0.1$) of pH 8.6. Diffusion studies of a 0.15% solution of CBG in 0.1 M NaCl indicated homogeneity of the sample with respect to size of the diffusing species (see Fig. XI of Chader (35)); the diffusion coefficient was calculated to be $7.02 \times 10^{-7}$ cm$^2$ sec$^{-1}$. The sedimentation pattern of a 1% solution of rabbit CBG is shown in Fig. 10; a single, homogeneous boundary was observed.
Pronase digestion of rabbit CBG also yielded amino acid percentages different from those obtained after Pronase digestion of human CBG; serine, threonine and half-cystine

![Graph: Absorbance of 270 nm vs Fraction Number](image)

**Fig. 8.** Sephadex G-200 filtration of CBG partially purified by DEAE-Sephadex, Sephadex G-200, and a single hydroxylapatite chromatography. The buffer was 0.05 M phosphate, pH 7.4.

at this and all other concentrations tested. The question of whether a small shoulder develops on the leading edge of the peak in the late stages of the centrifugation (see Fig. 10C) will not be discussed here. Evidence of polymer formation has been obtained for CBG preparations of some species and will be presented in a later publication. A linear proportion was found between the reciprocal values of the observed $s_{20, w}$ and the protein concentration (Fig. 11). Extrapolation of this line to infinite dilution gave $s_{20, w} = 3.55$ S. Sedimentation of a 0.7% sample of a second CBG preparation gave an $s_{20, w}$ value of 3.1 S, which is in line with the others in the reciprocal plot (see Fig. 11).

The amino acid composition of rabbit CBG after HCl digestion is given in Table V, along with that for human CBG as isolated in our laboratory (1). A considerable difference was found between the results after digestion with HCl and with Pronase. A low recovery of acidic amino acids after Pronase incubation was probably caused by incomplete digestion with the Pronase as compared with the more complete hydrolysis by HCl. The Pronase digestion of rabbit CBG also yielded amino acid percentages different from those obtained after Pronase digestion of human CBG; serine, threonine and half-cystine

![Image: Immunoelectrophoresis of two preparations of pure rabbit CBG](image)

**Fig. 9.** Immunoelectrophoresis of two preparations of pure rabbit CBG. Top wells contained 8% CBG (A) and 5% CBG (B); bottom wells contained normal rabbit serum. Goat antiserum to whole rabbit serum was added in the center trough. Electrophoresis was performed in high resolution Tris buffer, pH 8.9, at 20°C.

![Image: Ultracentrifugal pattern of a 1% solution of rabbit CBG](image)

**Fig. 10.** Ultracentrifugal pattern of a 1% solution of rabbit CBG after 29, 88, and 104 min. The buffer was 0.05 M phosphate, pH 7.4; 4°C; 59,780 rpm. Bar angle for A, 75°; B, 50°; C, 75°.

### Table IV

**Purification procedure for CBG from 2 liters of rabbit serum**

<table>
<thead>
<tr>
<th>Purification step</th>
<th>Protein applied</th>
<th>Activity applied</th>
<th>Purified fraction</th>
<th>Activity recovered</th>
<th>Cumulative recovery</th>
<th>Specific activity*</th>
</tr>
</thead>
<tbody>
<tr>
<td>DEAE-Sephadex</td>
<td>156</td>
<td>993,556</td>
<td>8.73</td>
<td>544,975</td>
<td>92</td>
<td>68</td>
</tr>
<tr>
<td>Sephadex G-200</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>First</td>
<td>8.73</td>
<td>544,975</td>
<td>3.18</td>
<td>524,370</td>
<td>88</td>
<td>160</td>
</tr>
<tr>
<td>Second</td>
<td>3.18</td>
<td>524,370</td>
<td>2.17</td>
<td>473,200</td>
<td>80</td>
<td>218</td>
</tr>
<tr>
<td>Hydroxylapatite</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>First</td>
<td>2.17</td>
<td>472,200</td>
<td>0.800</td>
<td>367,455</td>
<td>62</td>
<td>412</td>
</tr>
<tr>
<td>Second</td>
<td>0.89</td>
<td>367,055</td>
<td>0.471</td>
<td>356,880</td>
<td>60</td>
<td>758</td>
</tr>
<tr>
<td>Third</td>
<td>0.471</td>
<td>357,280</td>
<td>0.102</td>
<td>340,095</td>
<td>57</td>
<td>3,330</td>
</tr>
<tr>
<td>Fourth</td>
<td>0.102</td>
<td>340,095</td>
<td>0.0135</td>
<td>317,720</td>
<td>53</td>
<td>23,500</td>
</tr>
</tbody>
</table>

* Specific activity of the starting material was 3.8 cpm per mg of protein.
Fig. 11. Reciprocal $s_{20\text{w}}$ values plotted with respect to concentration for a rabbit CBG-corticosteroid complex. Conditions for 1% concentration are given in Fig. 10; all other solutions were in 0.1 M NaCl, 20°C. O, Preparation I; Q, Preparation II.

**Table V**

Amino acid composition of rabbit and human CBG

Values for human CBG are according to Muldoon and Westphal (1).

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>Relative to polypeptide moiety</th>
<th>Relative to glycoprotein</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lysine</td>
<td>4.60  5.03</td>
<td>25.4  29.0</td>
</tr>
<tr>
<td>Histidine</td>
<td>2.80  3.29</td>
<td>14.9  17.7</td>
</tr>
<tr>
<td>Arginine</td>
<td>1.22  1.50</td>
<td>20.7  18.0</td>
</tr>
<tr>
<td>Aspartic acid</td>
<td>9.84  9.72</td>
<td>60.6  62.4</td>
</tr>
<tr>
<td>Threonine</td>
<td>6.70  7.33</td>
<td>46.9  41.9</td>
</tr>
<tr>
<td>Serine</td>
<td>0.61  1.05</td>
<td>15.0  16.9</td>
</tr>
<tr>
<td>Glutamic acid</td>
<td>13.33 12.77</td>
<td>73.1  73.1</td>
</tr>
<tr>
<td>Proline</td>
<td>5.31  5.48</td>
<td>38.7  41.7</td>
</tr>
<tr>
<td>Glycine</td>
<td>3.67  2.86</td>
<td>45.5  37.0</td>
</tr>
<tr>
<td>Alanine</td>
<td>5.12  4.34</td>
<td>50.9  43.8</td>
</tr>
<tr>
<td>Half-cystine</td>
<td>2.10  0.64</td>
<td>14.6  3.9</td>
</tr>
<tr>
<td>Value</td>
<td>6.10  6.66</td>
<td>43.6  49.5</td>
</tr>
<tr>
<td>Methionine</td>
<td>1.51  3.52</td>
<td>8.2  19.8</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>3.74  4.76</td>
<td>23.4  31.1</td>
</tr>
<tr>
<td>Leucine</td>
<td>10.05 11.98</td>
<td>62.9  78.2</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>4.41  4.22</td>
<td>19.1  19.1</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>5.80  7.29</td>
<td>27.9  30.6</td>
</tr>
<tr>
<td>Tryprophan</td>
<td>3.39  1.49</td>
<td>12.9  5.9</td>
</tr>
</tbody>
</table>

Values, especially, were markedly higher in the rabbit protein. Micro-Kjeldahl determination of nitrogen (37) gave a value of 12.1% as compared with 12.0% for the calculated amount of nitrogen in the protein. No free sulfhydryl groups were found by the spectrophotometric titration technique (29) with HMB: at the same time and under identical conditions, one free sulfhydryl was determined for human CBG, in agreement with published results (4). This may indicate that such free groups are not present in the protein or that the groups are "buried" or otherwise inaccessible, and thus blocked from forming the mercaptide.

Rabbit CBG was found to have a total carbohydrate content (Table VI) of 29.2%, including 10.4% hexose, 9.5% hexosamine, 0.8% fucose, and 8.5% sialic acid. Hexose and sialic acid determinations on the second CBG preparation gave values of 10.3% and 8.8%, respectively, in close agreement with those of the first preparation. All values are corrected to water-free protein.

Calculation of the partial specific volume from the amino acid and carbohydrate composition yielded a value of 0.695 ml per g (Table VII). By using this figure with the diffusion and sedimentation coefficients, the molecular weight of rabbit CBG was calculated to be 40,700. The frictional ratio, $f/f_o$, was found to be 1.37. The $1200:1260$ ratio for isolated corticosteroid-containing rabbit CBG was 1.38, as compared with reported values for human CBG of 1.36 (5) and 1.13 (1). No accurate value could be determined for the steroid-free protein, since all attempts to remove the steroid led to slightly turbid solutions. A compilation of the physicochemical parameters of rabbit CBG is given in Table VII.

**Table VI**

Carbohydrate composition of rabbit and human CBG

Values for human CBG are according to Muldoon and Westphal (1).

<table>
<thead>
<tr>
<th>Carbohydrate</th>
<th>Rabbit CBG</th>
<th>Human CBG</th>
</tr>
</thead>
<tbody>
<tr>
<td>g/100 g glycoprotein</td>
<td>residues/100,000 g glycoprotein</td>
<td></td>
</tr>
<tr>
<td>Hexose</td>
<td>10.4</td>
<td>11.5</td>
</tr>
<tr>
<td>Hexosamine</td>
<td>9.5</td>
<td>9.0</td>
</tr>
<tr>
<td>Sialic acid</td>
<td>8.5</td>
<td>4.1</td>
</tr>
<tr>
<td>Fucose</td>
<td>0.8</td>
<td>1.5</td>
</tr>
</tbody>
</table>

**Table VII**

Physicochemical properties of rabbit and human CBG

Values for human CBG are according to Muldoon and Westphal (1).

<table>
<thead>
<tr>
<th>Property</th>
<th>Rabbit CBG</th>
<th>Human CBG</th>
</tr>
</thead>
<tbody>
<tr>
<td>Carbohydrate (%)</td>
<td>29.2</td>
<td>26.1</td>
</tr>
<tr>
<td>Nitrogen (%)</td>
<td>12.1</td>
<td>12.7</td>
</tr>
<tr>
<td>Electrophoretic mobility at pH 8.6, $x 10^5$</td>
<td>-5.1</td>
<td>-4.0</td>
</tr>
<tr>
<td>$s_{20\text{w}}$</td>
<td>3.55</td>
<td>3.9</td>
</tr>
<tr>
<td>$D_{20\text{w}}$ $x 10^7$</td>
<td>7.02</td>
<td>6.15</td>
</tr>
<tr>
<td>$P$ (mol/g)</td>
<td>0.093</td>
<td>0.238</td>
</tr>
<tr>
<td>Mol wt</td>
<td>40,700</td>
<td>51,700</td>
</tr>
<tr>
<td>$f/f_o$</td>
<td>1.37</td>
<td>1.42</td>
</tr>
<tr>
<td>$A_{20\text{w}}$ $-1250$</td>
<td>1.38</td>
<td>1.13</td>
</tr>
<tr>
<td>$F^{15}$ $cm$, 270 nm</td>
<td>8.4</td>
<td>7.4</td>
</tr>
</tbody>
</table>

The authors wish to thank Dr. C. A. Lang for advice regarding the nitrogen determinations.
and methods applied. Nevertheless, the total is close to the theoretical amount of 8.5 μg of corticosterone per mg of CBG, calculated on the basis of a single binding site per protein molecule and 100% saturation. Equilibrium dialysis of this fully saturated CBG without addition of further steroid permitted the calculation of an average value of $9.0 \times 10^8$ M$^{-1}$ for $k$, the apparent association constant at 4°, and of $4.7 \times 10^8$ M$^{-1}$ at 37°. Further addition of radiolabeled steroid in successive increments led to a marked increase of the concentration of unbound steroid accompanied by a drop in the calculated value of $k$; this seeming decrease of the association constant is explained by oversaturation of the binding sites. This result shows that the principal number of binding sites is $n = 1$, and that there is no second set of binding groups of comparably high affinity. Addition of HMB (at a molar ratio of HMB to protein of 3:1) lowered the $k$ value to $1.8 \times 10^4$ M$^{-1}$ at 37°.

Dialysis of the CBG with small amounts of various radiolabeled steroids at 4° and 37° indicated generally that the more polar steroids were bound with the highest affinity, decreasing from cortisol $>$ corticosterone $>$ progesterone $>$ aldosterone. This is in contrast to the "polarity rule" (38) which governs steroid interaction of most serum proteins (16), including human CBG (4, 19). Aldosterone may not be directly comparable with the other $C_28$ steroids because of its predominant hexanoic acid form.

Attempts at removal of steroid from the protein on Sephadex G-50 at 45° resulted in removal of 48% of the steroid, as compared to 97% for the human protein under similar conditions (1). Equilibrium dialysis studies of CBG treated in this way indicated that about half of it was inactivated by the stripping procedure. Further studies on the steroid-binding properties of rabbit CBG and an analysis of these observations will be given in a subsequent publication.

**DISCUSSION**

Recent reports (1, 7) have extended the pioneering work of Seal and Doe (5) on the isolation of the corticosteroid-binding globulin of human serum and have confirmed the general physical and chemical characteristics of the CBG as an $\alpha$-glycoprotein of relatively low molecular weight. The introduction of hydroxylapatite chromatography in the isolation procedure (5) was of particular significance, since it affords a highly specific purification for this trace protein, not only from human serum but also from the sera of other species (21). Throughout the fractionation procedure in the present study, rabbit CBG behaved in a way similar to that reported for the human protein (1, 5, 7), except that higher buffer concentrations were necessary for elution from hydroxylapatite columns. This may have been due to differences in the composition of the proteins applied to the column or, more probably, to variations in the adsorptive properties of the hydroxylapatite preparations used. Several repetitions of apatite chromatography were always found necessary before a homogeneous product was obtained. The adsorptive properties of hydroxylapatite in the batch method also were different from those described for the human CBG (1, 5), and, as it was difficult to obtain quantitative recovery of the CBG-steroid complex, the batch technique was not incorporated into the present procedure.

The use of DEAE-Sephadex was advantageous as a first purification step, owing to its high adsorptive capacity; the combination of stepwise and gradient elution was also helpful in improving purification and yield. Essentially, this chromatographic procedure was a modification of DEAE-cellulose techniques published before the start of the present studies (5, 16).

Sephadex G-200 filtration afforded a method for assaying CBG activity as well as for further purification before or after hydroxylapatite chromatography. The use of Sephadex G-25 and G-75 in the purification of human CBG has been previously reported (7).

From the results of the purification procedure, it can be estimated that the original female rabbit serum contains about 13.5 mg of CBG per liter, or a concentration of $3.3 \times 10^{-7}$ M. In good agreement with this value, the concentration of CBG binding sites in the same sample of rabbit serum has been found (19) to be $3.4 \times 10^{-7}$ M by an equilibrium dialysis technique with whole serum after removal of endogenous steroids. The concentration thus is approximately half that found in normal human serum and also is lower than binding capacities previously estimated for rabbit serum of different origins (12, 21). A value of $3.5 \times 10^{-7}$ M was found for the total endogenous corticosteroids in the starting serum sample. Assuming one corticosteroid-binding site per CBG molecule, this would indicate that the serum CBG binding sites were saturated before and during the isolation procedure.

The purified CBG contained about 8.5 μg of corticosterone per mg of protein. Assuming 13.5 mg of CBG per liter of serum, this would correspond to about 115 μg of steroid per liter, a value similar to that found in the original starting serum (120 μg). The concentration of CBG thus appeared to be virtually equimolar with the corticosteroid in serum. Equimolarity is also present in the isolated corticosteroid-saturated protein, indicating the presence of one steroid-binding site per protein molecule. During the isolation procedure, a negligible amount of radiolabeled steroid was lost through dialysis and in desalting by gel filtration. Preliminary studies on the inactivation of rabbit CBG indicated that prolonged dialysis of the purified preparations, or low pH (below pH 5), reduced the steroid-binding activity of the protein.

The association constants for rabbit CBG were obtained with a preparation containing essentially endogenous corticosterone (97%), plus a small quantity of endogenous and added cortisol (3%). The association constant at 4° ($9.0 \times 10^8$ M$^{-1}$) is in close agreement with those previously found (19) for interaction of rabbit CBG with cortisol (8 X 10$^8$ M$^{-1}$) and with cortisol (10 X 10$^8$ M$^{-1}$). The value for $k$ at 37° ($4.7 \times 10^8$ M$^{-1}$) is closer to that previously obtained (19) for cortisol (1 X 10$^8$ M$^{-1}$) than for corticosterone (2 X 10$^8$ M$^{-1}$), which might be expected, since the calculation of the association constant was based on measurement of cortisol-4-14C.

In Table VIII, the association constants, number of binding sites, and thermodynamic data are given for the interactions of rabbit and human CBG (1) with their corticosteroids. For comparison, corresponding data are presented for the progesterone complex of $\alpha$-acid glycoprotein and of human serum albumin as examples of steroid-serum protein complexes of relatively high affinity. The number of binding sites is $n = 1$ for the three glycoproteins, whereas $n = 2$ has been found for human serum albumin-progesterone. It is evident that the very high values of about 11 kcal per mole for the Gibbs free energy of the corticosteroid interaction with rabbit and human CBG is composed of a very high negative enthalpy change, in association with a negative change of entropy. These thermodynamic
data are interpreted as indicative of a very tight fit of the interacting components, so that the enthalpy is drastically reduced; the total order is much increased, so that a negative entropy change results. As seen in Table VIII, this is in contrast to the positive entropy changes observed with the progesterone complexes of \( \alpha_1 \)-acid glycoprotein and of human serum albumin. The positive \( \Delta S^\circ \) values may be interpreted by the assumption, first advanced by Klotz for anion-albumin interaction (39), that the highly structured hydration water is displaced and randomized as solvent water in the complex formation. Details of this interpretation will be published separately.\(^7\)

Competitive equilibrium dialysis with the isolated CBG and results from studies on unfractionated serum (19) indicate that cortisol is bound more strongly than corticosterone, although the major plasma glucocorticoid in the rabbit is corticosterone. This relative predominance of corticosterone in the rabbit can be changed, since Kass et al. found a shift to cortisol secretion by rabbit adrenals after prolonged treatment with adrenocorticotropic (40). Progesterone was bound with less affinity to rabbit CBG than either cortisol or corticosterone, although human CBG interacts more strongly with the progesterational hormone than with either of the glucocorticoids at 37°. The serum used in the present study came from mature, normal, female rabbits only; it is not known whether there are any differences in the steroid-binding properties between the CBG of female and male rabbits. The possibility of analogous differences in humans has also not been explored.

The question of sulfhydryl involvement in steroid binding of rabbit CBG is unresolved. Whereas spectrophotometric evidence of free thiol groups by HMB titration could not be obtained, addition of HMB to an equilibrium dialysis system containing CBG and cortisol-L\(^{14}\)C resulted in a 27-fold decrease of the association constant at 37°. An analysis of these observations will be reported later.

It has been pointed out that rabbit CBG differs from human CBG in that its binding affinity increases with the number of polar groups in the steroid molecule. This is contrary to the polarity rule, as previously discussed, and can be assumed to indicate significant variations in those parts of the two glycoprotein molecules which are involved in the steroid-binding process. A major objective of this study, therefore, was the investigation of the differences between the human and rabbit proteins with reference to their steroid-binding abilities in relation to chemical structure, and also the exploration of possible species similarities or variations. In this regard, a striking overall similarity in physical characteristics and chemical composition was found (Tables V to VII) between rabbit CBG and published results for the human protein (1, 5-7). Both glycoproteins have the mobility of \( \alpha_1 \)-globulins in free boundary electrophoresis. The sedimentation coefficients are also quite similar, but the diffusion coefficients are substantially different. The molecular weight of the rabbit CBG was found to be about 41,000, i.e. approximately 23% less than that of human CBG. It should be noted that the present determination is based on sedimentation velocity and diffusion data, as were calculations for human CBG (1, 4). A higher value for human CBG (38,500) was obtained by the equilibrium sedimentation technique (7).

The total carbohydrate percentage (Table VI) was found to be very close to that recently reported for the human CBG (1). The carbohydrate pattern also appears similar to that published by Seal and Doe (21) for purified rabbit CBG on the basis of absorbance. The hexose and hexosamine contents were almost the same, while sialic acid and fucose varied inversely with the percentages found for the human glycoprotein. This relationship may indicate a species specificity in the two more variable carbohydrate groups, while hexose and hexosamine may be more invariant, forming the “backbone constituents” (41) of the carbohydrate units. The enzymatic removal of sialic acid from human CBG has been found to have no effect on cortisol-binding affinity (1); other carbohydrate components are present in virtually the same quantities in the rabbit as in the human protein. The assumption may be justified, then, that the carbohydrate portion of the glycoprotein does not play a significant role in the differences in binding affinities observed, but may rather be a characteristic of the species. This interpretation of the significance of the carbohydrate moiety of CBG is analogous to the conclusions of Eylar (42) concerning the relationship of the carbohydrate portion of other glycoproteins to their biological activity.

The amino acid composition of the rabbit CBG determined after acid hydrolysis is quite close to that of the human protein (Table V). Since binding of nonpolar ligands to proteins presumably involves hydrophobic areas of the protein molecules, and since interaction of polar groups of the steroid molecule will take place preferentially in a hydrophilic environment, it was of interest to compare the amino acid compositions of the two proteins with respect to polar and nonpolar groups as well as other types of amino acid residues (43) that might vary significantly.

It is evident from Table V that the rabbit CBG contains slightly fewer nonpolar and more polar residues than the human protein; no significant variations were observed in the amino acid composition of the two proteins. The question of sulfhydryl involvement in steroid binding of rabbit CBG is unresolved. Whereas spectrophotometric evidence of free thiol groups by HMB titration could not be obtained, addition of HMB to an equilibrium dialysis system containing CBG and cortisol-L\(^{14}\)C resulted in a 27-fold decrease of the association constant at 37°. An analysis of these observations will be reported later.

The question of sulfhydryl involvement in steroid binding of rabbit CBG is unresolved. Whereas spectrophotometric evidence of free thiol groups by HMB titration could not be obtained, addition of HMB to an equilibrium dialysis system containing CBG and cortisol-L\(^{14}\)C resulted in a 27-fold decrease of the association constant at 37°. An analysis of these observations will be reported later.

It has been pointed out that rabbit CBG differs from human CBG in that its binding affinity increases with the number of polar groups in the steroid molecule. This is contrary to the polarity rule, as previously discussed, and can be assumed to indicate significant variations in those parts of the two glycoprotein molecules which are involved in the steroid-binding process. A major objective of this study, therefore, was the investigation of the differences between the human and rabbit proteins with reference to their steroid-binding abilities in relation to chemical structure, and also the exploration of possible species similarities or variations. In this regard, a striking overall similarity in physical characteristics and chemical composition was found (Tables V to VII) between rabbit CBG and published results for the human protein (1, 5-7). Both glycoproteins have the mobility of \( \alpha_1 \)-globulins in free boundary electrophoresis. The sedimentation coefficients are also quite similar, but the diffusion coefficients are substantially different. The molecular weight of the rabbit CBG was found to be about 41,000, i.e. approximately 23% less than that of human CBG. It should be noted that the present determination is based on sedimentation velocity and diffusion data, as were calculations for human CBG (1, 4). A higher value for human CBG (38,500) was obtained by the equilibrium sedimentation technique (7).

The total carbohydrate percentage (Table VI) was found to be very close to that recently reported for the human CBG (1). The carbohydrate pattern also appears similar to that published by Seal and Doe (21) for purified rabbit CBG on the basis of absorbance. The hexose and hexosamine contents were almost the same, while sialic acid and fucose varied inversely with the percentages found for the human glycoprotein. This relationship may indicate a species specificity in the two more variable carbohydrate groups, while hexose and hexosamine may be more invariant, forming the “backbone constituents” (41) of the carbohydrate units. The enzymatic removal of sialic acid from human CBG has been found to have no effect on cortisol-binding affinity (1); other carbohydrate components are present in virtually the same quantities in the rabbit as in the human protein. The assumption may be justified, then, that the carbohydrate portion of the glycoprotein does not play a significant role in the differences in binding affinities observed, but may rather be a characteristic of the species. This interpretation of the significance of the carbohydrate moiety of CBG is analogous to the conclusions of Eylar (42) concerning the relationship of the carbohydrate portion of other glycoproteins to their biological activity.

The amino acid composition of the rabbit CBG determined after acid hydrolysis is quite close to that of the human protein (Table V). Since binding of nonpolar ligands to proteins presumably involves hydrophobic areas of the protein molecules, and since interaction of polar groups of the steroid molecule will take place preferentially in a hydrophilic environment, it was of interest to compare the amino acid compositions of the two proteins with respect to polar and nonpolar groups as well as other types of amino acid residues (43) that might vary significantly.

It is evident from Table V that the rabbit CBG contains slightly fewer nonpolar and more polar residues than the human protein; no significant variations were observed in the amino acid composition of the two proteins.

### Table VIII

**Binding parameters of steroid protein complexes**

<table>
<thead>
<tr>
<th>Complex</th>
<th>( n )</th>
<th>( k )</th>
<th>( \Delta F^\circ )</th>
<th>( \Delta H^\circ )</th>
<th>( \Delta S^\circ )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rabbit CBG-cortisol</td>
<td>1</td>
<td>9.0 ( \times 10^9 )</td>
<td>4.7 ( \times 10^9 )</td>
<td>-11.3</td>
<td>-10.9</td>
</tr>
<tr>
<td>Human CBG-cortisol</td>
<td>1</td>
<td>5.2 ( \times 10^9 )</td>
<td>2.4 ( \times 10^9 )</td>
<td>-11.0</td>
<td>-10.5</td>
</tr>
<tr>
<td>Human ( \alpha_1 )-acid glycoprotein-progesterone</td>
<td>1</td>
<td>11.0 ( \times 10^9 )</td>
<td>4.9 ( \times 10^9 )</td>
<td>-7.7</td>
<td>-8.1</td>
</tr>
<tr>
<td>Human serum albumin-progesterone</td>
<td>2</td>
<td>1.0 ( \times 10^9 )</td>
<td>4.6 ( \times 10^9 )</td>
<td>-6.3</td>
<td>-6.6</td>
</tr>
</tbody>
</table>

\( \Delta F^\circ \) in kcal/mole, \( \Delta H^\circ \) in kcal/mole, \( \Delta S^\circ \) in cal/mole deg.

---

\(^7\) J. Kerkay and U. Westphal, to be published.

\(^8\) Unpublished observations.
protein. No difference in content was seen in the total of acidic amino acids or of basic amino acids, but the rabbit protein contained about 30% more arginine than the human CBG. The content of hydroxyl-containing amino acids was also slightly higher in the rabbit protein. A major divergence between the two proteins was found in the biosynthetically related, sulfur-containing amino acids methionine and half-cystine. Rabbit CBG contained about 4 times as much half-cystine, but only half the amount of methionine as human CBG. It should be noted, however, that the sum of the two sulfur-containing amino acids on a molar basis is almost identical in the two glycoproteins. In general, there appears to be remarkable similarity in the total content of the various types of amino acids in rabbit and human CBG, in spite of differences in amounts of individual residues.

It would seem to be an attractive hypothesis to attribute the differences between rabbit and human CBG, with respect to binding of steroids of varying polarities, to the lower percentages of nonpolar amino acids and to the higher content of polar amino acids, especially hydroxyl-containing residues, in rabbit CBG. However, this interpretation of protein composition in relation to steroid binding would be simplistic. Factors such as location of the amino acids in the protein molecule relative to the binding site, conformational structure in relation to steroid configuration, and specific binding affinities must also be considered. For example, the difference in half-cystine may be significant, in that HMB was found to inhibit markedly the binding of cortisol to rabbit CBG.

The close similarity of the two corticosteroid-binding glycoproteins in their polypeptide moiety is considered significant from a phylogenetic viewpoint. This likeness may have its basis in a similar steroid-binding function of this type of protein, even though synthesised in widely differing species. Also, the fundamental structure of the steroid hormones known to interact strongly with CBG, i.e., essentially progesterone and the corticoids, is the same in all vertebrate species studied.

Seal and Doe (21) have suggested, on the basis of the wide species distribution of CBG molecules, and in view of their unusual property of not being adsorbed on hydroxylapatite, that this type of protein is characterized by a unique structure which appeared early in the history of the vertebrates. The similarities in physicochemical properties and composition of the CBG molecules for man and rabbit (Tables V to VII) corroborates this suggestion, at least for these two species.

REFERENCES