Corticosteroid-binding Globulin

I. ISOLATION FROM PLASMA OF DIETHYLSTILBESTROL-TREATED MEN*

ULYSES S. SEAL AND R. P. DOE

From the Metabolic Research Laboratory, Veterans Administration Hospital, and the Departments of Physiological Chemistry and Medicine, University of Minnesota, Minneapolis, Minnesota

(Received for publication, May 17, 1962)

The presence in human plasma of a specific corticosteroid-binding protein different from albumin was observed by Daughaday (2) and Bush (3); using equilibrium dialysis methods. Sandberg, Slaunwhite, and Antoniades (4) provided additional evidence for the presence of a specific binding protein and named it transcortin (5). Electrophoretic studies by Daughaday (6) have established that the binding protein has the mobility of an $\alpha_2$-glycoprotein and that it probably is different from the acidic $\alpha_2$-glycoprotein and thyroxine-binding protein. Daughaday (7) has suggested that a second corticosteroid-binding protein with different properties was the basis of the increased serum binding of cortisol previously noted during pregnancy or treatment with estrogens (8-11). The possible role of corticostereoid-binding globulin in the transport, distribution, and metabolism of cortisol has been the subject of continuing investigation and review (12-16) with the accumulation of much data on the properties of this protein as present in whole serum or electrophoretically separated fractions.

To assess quantitatively the contribution of this specific binding protein to cortisol binding by whole serum and to investigate the possible presence of several binding proteins, it is essential that the properties of highly purified preparations be determined. This report describes a procedure for the 1000-fold purification of a corticosteroid-binding globulin from the plasma of estrogen-treated patients with cancer of the prostate. Some observations on the purity of the preparation, on its composition, and on its properties are presented.

MATERIALS AND METHODS

Source Material—Serum or heparinized plasma were equally satisfactory. Blood was collected from patients with cancer of the prostate who had been receiving 5 mg of diethylstilbestrol daily for at least 1 month. The cells were removed by centrifugation, and the serum (or plasma) was stored at $-20^\circ\text{C}$ until used. This source was chosen since, in accordance with other studies (9-11), it was found that the amount of cortisol binding was elevated several fold in these patients. Also chromatographic experiments at $2^\circ\text{C}$ indicated no difference between the normal and estrogen-induced proteins when tracer amounts of cortisol-4-$\text{C}^{14}$ were used for equilibration with endogenous cortisol.

* Supported in part by grants from the National Cancer Institute, National Institutes of Health, United States Public Health Service (C-5371 and C-5993) and by the Veterans Administration Cooperative Urological Research Project. A preliminary note has appeared (1).

Radioactive Compounds—Cortisol-4-$\text{C}^{14}$ (dissolved in benzene) was obtained from the Endocrinology Study Section of the National Heart Institute and used without further purification. It had a specific radioactivity of 24.7 $\mu$C per mg. The identity was verified and the radio purity established by descending paper chromatography in a Zaffaroni (benzene-methanol-formamide) (17) and a Bush B$_2$ system (18). The cortisol-4-$\text{C}^{14}$ and its acetate were chromatographed in these systems and found to be at least 96% pure. For use, a stock solution containing 2.5 $\mu$C per ml was made up in 0.9% NaCl and stored in the refrigerator. The $\text{T}^{3}$-thyroxine was obtained from Abbott Laboratories, and the Co$^{57}$-vitamin B$_{12}$ was from Merck and Company. Both preparations were used without further purification. The water used in all of the procedures was distilled, deionized, and passed through a cellulose column. The last step removed ultraviolet-absorbing impurities present in the deionized water.

Preparation of Columns—DEAE-cellulose was obtained from Eastman Kodak Company. The ion exchange agent was prepared for use as described by Peterson and Sober (19, 20). The remaining operations were carried out at $2^\circ\text{C}$. The columns were poured and allowed to settle under gravity, and then packed under a hydrostatic head of 15 inches. Finally, before use, 2 to 4 liters of starting buffer were passed through the column, and equilibration was checked by comparison of the pH values of the effluent and eluent. Hydroxylapatite was prepared according to the method of Tiselius, Hjerten, and Levin (21). Preparations 1 week and 1 year old gave identical results except for somewhat slower flow rates in columns packed with the older material.

Preparation of Sample for Chromatography—The whole serum was thawed, centrifuged, and equilibrated with a quantity of cortisol-4-$\text{C}^{14}$. The equilibration was accomplished by the addition of labeled cortisol to the serum at room temperature (26$^\circ\text{C}$), and the mixture was allowed to stand for 1 hour with occasional mixing, followed by standing for 18 hours at $2^\circ\text{C}$. It was then dialyzed at $2^\circ\text{C}$ against several changes of 0.0175 M phosphate buffer, pH 6.3, centrifuged, and applied to the column. Elution was usually performed stepwise as described by Sober and Peterson (22) for whole serum. Elutions from the hydroxylapatite columns were also stepwise with pH 6.8 phosphate buffers and increasing ionic strength (21).

Assays of Effluents—$\text{C}^{14}$ was estimated by direct plating of aliquots of the original samples and the effluent fractions from the columns. Self-absorption was either corrected for from empirical curves or minimized by appropriate dilution of the sample so that less than 1 mg of protein was plated. Radioactiv-
ity was measured in an automatic windowless gas flow counter. Counting times were chosen so that the counting error was 3% or less. Duplicate samples agreed within 5%. Co\(^{60}\) and \(^{14}\)C were counted in a manual scintillation counter with a 4-ml well. The effluents from several columns were also assayed for 17-hydroxycorticosteroids by the Peterson modification (23) of the method of Silber and Porter (24). Elution of protein was followed by measurement of the ultraviolet absorbancy at 280 m\( \mu \) in a Beckman model DU spectrophotometer. Volumes of the eluted fractions were measured, and recoveries of the radioactivity and ultraviolet-absorbing material calculated by summation of the values for the individual fractions. Recoveries routinely ranged from 93 to 100%.

Other Analytical Procedures—Sialic acid was assayed by the method of Warren (25). The purified preparation was assayed for fucose (26), hexose (27), and hexosamine (28) essentially as described by Winder (29) except that the alcohol precipitation step was omitted. Paper electrophoresis was performed in Spinco Durrum-type cells with 0.075 ionic strength barbital buffer, pH 8.6. The strips were stained with bromphenol blue and scanned, without oiling, in a recording densitometer. Immunoelectrophoresis on agar was done by the slide technique of Scheidegger (30). Sedimentation measurements were made in a Spinco model E ultracentrifuge.

**EXPERIMENTAL PROCEDURE**

Purification—The distribution of protein-bound cortisol among the fractions obtained when whole serum was chromatographed on DEAE-cellulose is shown in Fig. 1. The indicated elution procedure of Sober and Peterson for human serum was used (22). The radioactivity of the cortisol \(^{14}\)C appeared as a peak on the trailing edge of the third protein or albumin peak. A smaller peak of radioactivity appeared in the fourth or \(\alpha\)-globulin peak. The recoveries of radioactivity and material absorbing at 280 m\( \mu \) were quantitative. The radioactivity associated with the major peak was protein-bound, as indicated by equilibrium dialysis. It was readily extractable with organic solvents and thus not bound by covalent linkages. Proof that the added 4 \(\mu\)g of radioactive cortisol had equilibrated with the 100 \(\mu\)g of cortisol already present was provided by chemical analysis (23). The distribution of the chemically determined material and the radioactivity were identical within the limits of error of the measurements. On this basis, the purification steps were followed by assay of radioactivity, with only occasional checks by chemical analysis. The fact that the major portion of the cortisol was present in this peak and that it was not symmetrically distributed with the eluted protein suggested that the binding was not due to albumin but to the \(\alpha\)-globulin-binding protein appearing as a "contaminant" in the albumin peak. Support for this possibility was provided by paper electrophoresis of samples of this fraction, which showed the presence of \(\alpha\)-globulin as well as \(\beta\)-and \(\gamma\)-globulins. The presence of these globulins was verified by immunoelectrophoresis. In addition, equilibrium dialysis experiments with crystalline human serum albumin and the material eluted from the column indicated a much tighter binding of radioactive cortisol by the fractions from the albumin peak from the column. On the basis of these observations, further efforts at purification of the binding protein were directed at those fractions from the columns which showed maximal radioactivity. For further purification, the fractions from the selected peak were combined, dialyzed, dried from the frozen state, and stored at \(-20^\circ\) until used. Repeated dialysis and freeze-drying had no effect on the binding capacity at any stage of purification, provided the dialyzing solution was buffered at pH 7.0. If the pH fell to 5 or lower, or if distilled water was used, irreversible inactivation occurred with preparations of 5% or greater purity.

When normal serum was chromatographed under conditions similar to those described for estrogen-treated plasma (Fig. 2), but with an excess of added cortisol, all of the eluted protein-bound radioactive cortisol was found associated with the same albumin fraction. The additional peak of radioactivity asso-
Fraction

FIG. 3. Hydroxylapatite gel chromatography of 800-fold purified preparation of corticosteroid-binding globulin. The column was equilibrated with the elution buffer, 0.001 M phosphate, pH 6.8. Fractions of 5 ml were collected. ---, absorbancy; ---, radioactivity.

TABLE I

Summary of purification procedure for corticosteroid-binding globulin

<table>
<thead>
<tr>
<th>Step</th>
<th>Purification</th>
<th>Recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. DEAE-cellulose</td>
<td>2</td>
<td>83</td>
</tr>
<tr>
<td>2. Hydroxylapatite gel batch</td>
<td>25</td>
<td>90</td>
</tr>
<tr>
<td>3. First hydroxylapatite column</td>
<td>4</td>
<td>100</td>
</tr>
<tr>
<td>4. Second hydroxylapatite column</td>
<td>2</td>
<td>100</td>
</tr>
<tr>
<td>5. Third hydroxylapatite column</td>
<td>2</td>
<td>100</td>
</tr>
<tr>
<td>6. Fourth hydroxylapatite column</td>
<td>1.25</td>
<td>100</td>
</tr>
<tr>
<td>7. Final preparation</td>
<td>1000</td>
<td>75</td>
</tr>
</tbody>
</table>

Physicochemical Properties—The sedimentation coefficient, corrected to 20°, of a 1.2% solution of the purified material was 3.0 S. Only a single peak was observed in this run as shown in Fig. 4. Insufficient material was available for determination of the partial specific volume and diffusion constant, and so an accurate molecular weight could not be calculated from the sedimentation data. The purified protein migrated as an α₁-globulin on paper electrophoresis and immunoelectrophoresis. The

FIG. 4. Unretouched photograph of ultracentrifuge pattern taken at 88 minutes after start of run. The bar angle was 50°. The rotor attained full speed of 59,780 r.p.m. at 6 minutes and 20 seconds. The protein (1.2 mg per ml) was dissolved in pH 7.2 phosphate buffer of 0.100 ionic strength.
absoorbancy at 280 μm of a 0.1% solution, in 1-cm cells, of the protein in 0.01 M phosphate buffer, pH 7.4, or in distilled water was 0.74. The ratio of the 280 μm to 260 μm ultraviolet absorbancies was 1.74 for the cortisol-free protein and 1.36 for the cortisol-saturated protein at 25°C.

Evidence for Homogeneity—No protein was lost on further recycling of the purified material on hydroxylapatite gel. Also, there was no detectable change in the binding capacity per mg of protein. When examined in the ultracentrifuge, only a single peak was evident. The purified preparation and material from each step of the purification procedure were examined by immunoelectrophoresis. Rabbit and horse antisera to whole human serum and to electrophoretically separated fractions were utilized. Fig. 5 shows the results obtained with the purified preparation in comparison with the “albumin” fraction from the DEAE-cellulose column. Antiserum to human γ-globulin, β-globulins, albumin, and α₁-globulins were negative. A rabbit antiserum to a mixture of human albumin and α₁-globulins likewise showed a single line in the same position as the one shown here. Equilibrium dialysis of the purified protein with 1³¹P-thyroxine and Co²⁺-vitamin B₁₂ indicated that the preparation had no affinity for these compounds and hence was probably not contaminated with the respective binding proteins. The conditions used would have detected a 0.01% contamination with the proteins. Both of these trace proteins had followed the cortisol-binding globulin closely on the DEAE-cellulose columns.

Carbohydrate Composition—Analysis of the protein for carbohydrate yielded values, as percentage of dry weight, of 5.4 for hexose, 4.7 for hexosamine, 3.2 for sialic acid, and 0.8 for methylpentose (fucose). Identification of the individual carbohydrates will require larger amounts of protein than were available.

Cortisol-binding Properties—The actual content of cortisol (17,21-dihydroxy-20-ketosteroid) in the isolated protein was determined chemically by the Silver-Porter method (23, 25). Also, the cortisol was extracted from the protein with methylene chloride and the ultraviolet spectrum was compared with that of an authentic specimen of cortisol. The spectra were identical. Values for the quantity of cortisol present obtained by the chemical and spectrophotometric methods agreed within 3%. The amount actually found in any given preparation depended on the amount of cortisol originally present, the amount of cortisol added, the dialysis conditions, and the number of dialyses during the course of preparation.

The number of binding sites and the association constant were estimated by an equilibrium dialysis technique. The number of binding sites was estimated by successive addition of increments of radioactive cortisol. The radioactivity of the dialysate and bag contents were measured after equilibration at 2°C with each quantity of cortisol-4-C¹⁴; 1 mg of the preparation bound 8 μg of cortisol. If one binding site is present per molecule of glycoprotein, the molecular weight is approximately 45,000. This value is consistent with the sedimentation coefficient of 3.0 S. Estimation of the association constant at 2°C from these data yielded a value of 6 × 10⁶ liters per mole, comparable with that reported by Slaunwhite and Sandberg (5) and Daughaday (15).

The effect of temperature on the binding reaction was examined, with the results shown in Fig. 6. The dialyses were performed under uniform conditions except for the variation of temperature. Controls were run at each temperature. Evidence for reversibility was provided by re-equilibrating each flask at a different temperature. In each case the value obtained for the original equilibration at that temperature was obtained. The data obtained indicated a decline in binding affinity with increasing temperature.

Discussion

The cortisol-binding α₁-glycoprotein described here constitutes about 0.1% of the total serum proteins and 3 to 5% of the α₁-proteins of these diethylstilbestrol-treated patients. The proportion in normal persons would be approximately one-third of these values, assuming the proteins are the same (9–11). Purification of the protein from normal plasma would then have to be about 3000-fold as compared to the 1000-fold described here for the elevated levels found in these patients.

The relationship of the binding protein described here to the novel binding protein, appearing in the plasma of estrogen-treated patients, described by Daughaday (7) is not clear. The over-all yield of binding protein in the purification procedure (75%) would suggest that this preparation contained both components, if they are different. The properties that Daughaday

![Fig. 5](image-url)
(7) found to differentiate the novel binding protein were a lower affinity for cortisol at 4°C and a lesser effect of temperature on the binding reaction. The behavior of the purified protein described here was similar to that of normal serum with regards to the association constant and the decreasing affinity with rising temperature. The relationship of the binding protein of normal plasma to the one described here will require isolation of the normal protein.

Several aspects of the nature of the binding reaction and binding protein would appear to be settled by the present data. The participation of enzymes in the binding reaction either from liver or in blood was not necessary, since the reaction was rapidly reversible and the binding was stronger at lower than at high temperatures and there was no evidence of modification of the cortisol. Evidence for the lack of alteration of the cortisol was provided by recovery of Porter-Silber chromogens, the ultraviolet spectrum of the material extracted by methylene chloride, the partition of the radioactivity between the aqueous and methylene chloride phases, and by paper chromatography, which yielded a single zone corresponding to cortisol-4-C14. The decreased affinity of the binding protein for cortisol at 37°C raises the question of its precise contribution to cortisol binding in circulating blood. A detailed answer to this question will require precise estimates of the dissociation constants of purified corticosteroid-binding globulin and human serum albumin separately and in combination as well as the effect of whole normal plasma added to the purified binding protein. However, on the basis of the present and published data it would seem that unbound plasma cortisol is in equilibrium with both the binding protein and albumin under the temperature and concentration conditions of normal circulating blood.

**SUMMARY**

1. A procedure for the 1000-fold purification in 75% yield of a corticosteroid-binding globulin from the plasma of estrogen-treated male patients with cancer of the prostate has been described.

2. The purified protein appeared homogeneous in the ultracentrifuge and by immunoelctrophoresis. It contained hexose, hexosamine, methyl pentose, and sialic acid.

3. The sedimentation coefficient was 3.0 S. The extinction coefficient of a 1% solution in 1-cm cells was 7.4, and the cortisol-free protein had a 280 mg-to-280 mg ratio of 1.74.

4. The number of binding sites per molecule was one for a molecule of 45,000 molecular weight. The binding reaction was temperature-dependent, and the affinity decreased with rising temperature from 4°C to 42°C.

**Acknowledgments**—We wish to express our appreciation to Dr. R. A. Bridges for the immunoelctrophoretic analyses and to Dr. D. R. Briggs for the ultracentrifugal analysis.

**REFERENCES**

Corticosteroid-binding Globulin: I. ISOLATION FROM PLASMA OF DIETHYLSTILBESTROL-TREATED MEN
Ulysses S. Seal and R. P. Doe


Access the most updated version of this article at http://www.jbc.org/content/237/10/3136.citation

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 0 references, 0 of which can be accessed free at http://www.jbc.org/content/237/10/3136.citation.full.html#ref-list-1