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PREPARATION AND CHARACTERISTICS OF CORTICOSTEROID-BINDING GLOBULIN (CBG, TRANSCORTIN)

By

Ulrich Westphal

ABSTRACT

The corticosteroid-binding globulin (CBG, transcortin) rises in the blood of the developing mammal from small concentrations in the young to adult levels which are similar in man and woman, but twice as high in the female rat as in the male. The affinity of the CBG complexes with corticosteroids is maximal at approximately pH 8; it decreases with increasing temperature. Study of the relative binding strength for different steroids clearly indicated a species specificity of the CBG molecules. This was confirmed by the isolation of CBG from serum of man, rat and rabbit, accomplished by a variety of chromatographic procedures. The pure glycoproteins, homogeneous by physicochemical criteria, proved to be distinct molecules on the basis of amino acid and carbohydrate composition and other properties. They possess one principal steroid binding site. Thermodynamic data indicate a very tight fit of the interacting components in the complexes. Removal of about 90% of corticosterone from its complex with rat CBG by gel filtration at 23\° results in polymerization of the glycoprotein molecule to dimeric, tetrameric and octameric forms. Recombination of the polymeric mixture with 1 mole corticosterone per 53 000 g CBG re-forms the monomer. Possible biological significance of this reversible polymerization of a carrier protein under the regulatory control of the binding steroid hormone has to be explored.
The presence in the blood serum of a macromolecular component that binds corticosteroid hormones with high affinity is common to all vertebrate species examined to date. This has been found in extensive studies by Seal & Doe (1966), who concluded that the fundamental mechanism of serum protein binding of corticosteroids appeared early in the history of the vertebrates, mediated by specific proteins of unique conformational structure (Seal & Doe 1963). A few examples from the more than 130 different species tested are shown in Table 1 which gives the approximate binding capacity for cortisol in \( \mu g/100 \) ml, including extremes of high and low values. Most of the sera examined also bind corticosterone but no rational quantitative relation of corticosterone-binding capacity to cortisol-binding capacity or to the relative concentrations of the two corticosteroids in the serum has been observed. This large-scale survey was made possible by the development of the Sephadex gel filtration procedure (DeMoor et al. 1962; Seal & Doe 1963) which is capable of accurate determination of CBG capacity (Hoffmann & Westphal 1969).

The cortisol-binding activity of fetal serum is less than half that of the adult human (De Moor et al. 1962; Seal & Doe 1967); low values have been reported for newborn infants (Sandberg et al. 1966). The gradual increase of CBG activity in the growing rat has been studied by Gala & Westphal (1965); Fig. 1 shows the combining affinity, \( C \), for corticosterone in the serum of male and female rats up to about 8 weeks of age. The \( C \)-value (Daughaday 1958a) is defined as

\[
C = \frac{[S_{bd}]}{[S] \times [P]}
\]

where \([S_{bd}]\), \([S]\) and \([P]\) are the concentrations of bound steroid, of unbound steroid, and of total protein, respectively; it is used as a convenient term to assess the steroid binding activity which is a resultant of binding affinity and

<table>
<thead>
<tr>
<th></th>
<th>Lampey</th>
<th>Painted Turtle</th>
<th>Bass</th>
<th>Alligator</th>
<th>Iguana (green)</th>
<th>Bullfrog</th>
<th>Marine Toad</th>
<th>Whale</th>
<th>Horse</th>
<th>Man</th>
<th>Squirrel</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.5</td>
<td>2.7</td>
<td>6.8</td>
<td>43.0</td>
<td>110.0</td>
<td>1.0</td>
<td>18.0</td>
<td>2.9</td>
<td>10.0</td>
<td>22.0</td>
<td>92.0</td>
</tr>
</tbody>
</table>

Selected from Seal & Doe (1966).
concentration of binding sites (Westphal 1969). The upper part of Fig. 1 indicates that the CBG activity in the rat serum is low at 12 days of age; during the following days, it increases at a similar rate in the male and the female animal. At about day 30, the binding activity in the male rat appears to have reached its highest value, whereas that of the female continues to rise to a maximum of approximately twice the male value at 7 weeks of age. The corticosterone concentration (lower part of Fig. 1) seems to follow a similar pattern although the determinations were made only with ether-stressed animals in this experiment. The difference in the corticosteroid-binding activity in the male and female rat is clearly in contrast to observations in humans which show about the same CBG level in normal adult men and women (DeMoor et al. 1962).

Many properties of the corticosteroid-binding proteins and their steroid complexes in the blood serum of various species were studied before chemical isolation of the specific macromolecules. The results were confirmed later with the pure homogeneous proteins whenever such determinations were made. Early electrophoretic analyses on the hanging paper curtain (Daughaday 1958b; Slaunwhite & Sandberg 1959) or in starch gel (Doe et al. 1960) identified the cortisol-binding protein of human blood as an α-globulin. Subsequent studies by equilibrium paper-strip electrophoresis demonstrated the α-globulin nature of the corresponding corticosteroid-binding proteins in the serum of other

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**Fig. 1.**

Corticosterone-binding activity (C-values) and corticosterone levels in the serum of the developing rat. From Gala & Westphal (1965).
mammalian species (Westphal & DeVenuto 1966). Fig. 2 illustrates this for the corticosteroid-CBG complex in normal rat serum; similar results have been obtained for rabbit, bovine and horse serum. At low pH values, CBG of rat serum shows electrophoretic migration of a pre-albumin.

The binding affinity between the corticosteroid hormones and CBG is dependent on the pH of the solution. A maximal value has been observed at approximately pH 8 (DeMoor et al. 1963). Fig. 3 shows high-affinity binding for corticosterone in human and rat serum at different hydrogen ion concentrations. A maximal C-value is reached at pH 7.9 for both species; the binding activity is generally higher in the rat serum. The apparent affinity is somewhat lower in the ammediol than in the citrate-phosphate buffer; this is in accordance with observations in other steroid binding systems. Daughaday & Mariz (1961) and Seal & Doe (1962) reported irreversible denaturation with loss of binding activity for human CBG at pH 5 and below. To a great extent, the poor affinity values seen at the low pH in Fig. 3 may be ascribed to denaturation. However, even at pH 2.8 the very low binding activity is not entirely the result of irreversible denaturation. For example, neutralization to pH 7.35 of a rat serum solution, which had been kept at pH 2.8 for 48 h and

![Equilibrium paper electrophoresis of rat serum equilibrated with corticosterone-4-¹⁴C, in Veronal buffer, pH 8.6, at 25°. From Westphal (1964).](image)

**Fig. 2.**

Corticosterone-binding activity (C-values) of CBG in human and rat serum at different pH values, in 0.05 M buffers, 4°. From data reported by Westphal (1969).

Fig. 3.

gave a C-value of 0.09 (Fig. 3), yielded a C-value of 1.63 on subsequent binding analysis (Westphal 1969). The CBG molecule seems to be somewhat protected while in the serum. The decrease of binding affinity from the maximum at pH about 8 to the value observed at pH 9.8 was found to be completely reversible.

The first clear evidence of greater dissociation at 37° than at 4° was given by Slaunwhite & Sandberg (1959) for the cortisol-CBG complex in human serum. Fig. 4 shows that a similar relationship between temperature and binding affinity exists also for the association between corticosterone and human CBG, as well as for the corticosterone interaction with CBG in rat and rabbit serum. The substantial decrease of the combining affinity at the higher temperatures seen in Fig. 4 is not completely reversible. Daughaday et al. (1962) have reported that CBG in human serum is inactivated by heating to 60° for about 20 min. This inactivation is dependent on the concentration of cortisol present; a protective effect of the corticosteroids on CBG has been observed (Doe et al. 1964). Table 2 shows that exposure of steroid stripped human and rat serum to 50° for 48 h, the usual duration of the binding analysis by equilibrium dialysis, destroys the CBG activity almost completely. Similar treatment of rabbit serum seems to inactivate about 80% of the binding affinity; the apparent lower percentage destruction presumably results from a higher proportion of corticosterone binding in rabbit serum by the relatively thermostable albumin. In comparison to the heat lability of CBG at 50° and 60°, Slaunwhite & Sandberg (1959) observed stability of CBG in human serum.
Corticosterone-binding activity of human, rat and rabbit CBG in serum at different temperatures, in 0.05 M phosphate buffer at pH 7.4. From Westphal (1967).

**Fig. 4.**

![Graph showing combining affinity C for human, rat, and rabbit CBG at different temperatures.](image-url)

**Table 2.**
Effect of temperature on corticosterone binding to CBG in human, rat and rabbit serum.\(^a\)

<table>
<thead>
<tr>
<th>Species</th>
<th>4(^\circ)</th>
<th>50(^\circ)</th>
<th>4(^b)</th>
<th>Per cent of original activity lost by exposure to 50(^\circ)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human</td>
<td>2.62</td>
<td>0.03</td>
<td>0.19</td>
<td>93</td>
</tr>
<tr>
<td>Rat</td>
<td>7.92</td>
<td>0.04</td>
<td>0.15</td>
<td>98</td>
</tr>
<tr>
<td>Rabbit</td>
<td>0.60</td>
<td>0.04</td>
<td>0.12</td>
<td>80</td>
</tr>
</tbody>
</table>

\(^a\) Average C-values from duplicate multiple equilibrium dialysis experiments in 0.05 M phosphate, pH 7.4. The sera were cleared of endogenous steroids by gel filtration at 45\(^\circ\). From Westphal (1969).

\(^b\) Equilibrium dialysis at 50\(^\circ\) for 48 hours, then for an additional period of 48 hours at 4\(^\circ\).
exposed to 37° for at lest 72 h; the same result was obtained for the CBG activity in rat serum (Westphal 1961).

The relative affinities for steroids of varying polarity are different for the CBGs of different mammalian species. Table 3 shows that the association constant of the progesterone complex with human CBG at 37° is about three times as great as that of the cortisol complex, indicating validity of the polarity rule. In contrast, progesterone interacts with CBG in rabbit serum at 4° with an affinity approximately 2½ times smaller than that shown by cortisol. The polarity-rule relationship seems to be reversed again for guinea-pig CBG which binds progesterone much more strongly than cortisol – however, it must be remembered that the identity of CBG with the progesterone-binding protein has not been proven for this species (Diamond et al. 1969). These variations in the relative strength of interaction with the three steroids of increasing polarity (Table 3) suggest a high structural specificity in the different CBG molecules at or near the binding site. Elucidation of the chemical structures and their influence on binding affinity for different steroids requires the isolation of the various CBGs in pure, homogeneous form.

Knowledge of the general properties of CBG and its corticosteroid complexes discussed in the preceding paragraphs has been useful in the purification and final isolation of the binding protein from the blood serum of several species. The first to succeed in the preparation of a highly purified CBG from human serum were Seal & Doe (1962), followed several years later by

Table 3.
Concentration of binding sites and association constants, k, of steroid-CBG complexes in mammalian sera.a

<table>
<thead>
<tr>
<th>Species</th>
<th>[CBG]b in 10⁻⁷ M</th>
<th>k in 10⁸ M⁻¹ for</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Cortisol 4° 37°</td>
</tr>
<tr>
<td>Human</td>
<td>7.2</td>
<td>6 0.3</td>
</tr>
<tr>
<td>Monkey</td>
<td>9.3</td>
<td>3 0.3</td>
</tr>
<tr>
<td>Rat</td>
<td>11.3</td>
<td>3 0.1</td>
</tr>
<tr>
<td>Rabbit</td>
<td>3.4</td>
<td>10 0.4</td>
</tr>
<tr>
<td>Guinea Pig</td>
<td>5.7</td>
<td>0.5 0.04</td>
</tr>
</tbody>
</table>

a Determined by equilibrium dialysis in 0.05 M phosphate, pH 7.4. The sera had been stripped of endogenous steroids by gel filtration at 45°. Data from Westphal (1967).

b The concentrations of binding sites for the corticosteroids were approximately the same as those for progesterone.
Slaunwhite et al. (1966) and by Muldoon & Westphal (1967). Pure homogeneous CBG preparations were also obtained from rabbit (Chader & Westphal 1968a) and rat serum (Chader & Westphal 1968b). In all isolation procedures, the serum or plasma used as starting material was equilibrated with radiolabeled corticosterone to slight over-saturation of the high-affinity binding sites, and was fractionated by chromatographic procedures. The first step in the various purifications of human CBG was DEAE-cellulose chromatography initially reported by Westphal (1961). Fig. 5 shows this separation and indicates the removal of a large portion of inactive protein from the CBG fraction which is recognized by its association with bound cortisol-4-14C. In the procedures applied for the isolation of rat and rabbit CBG, DEAE Sephadex was used for the initial chromatographic fractionation.

Further purification of human CBG was obtained by chromatography on hydroxylapatite. This technique, introduced by Seal & Doe (1962), is signally efficient since CBG is not adsorbed by this reagent, in contrast to most other proteins. The high effectiveness of the hydroxylapatite chromatography is shown for an intermediate stage of rat CBG purification in Fig. 6. Nearly all of the radiosteroid appears in the early fractions in association with a very small portion of the protein, ahead of the bulk of inactive protein. The hydroxylapatite chromatography is repeated until symmetrical congruent peaks of radioactivity and protein are obtained as illustrated in Fig. 7 for human CBG.

In the isolation of rat and rabbit CBG, the hydroxylapatite adsorption was preceded by gel filtration over Sephadex G-200. This step is shown in Fig. 8 for rabbit CBG; it is evident that a substantial portion of inactive protein, free

![Fig. 5.](image)

Partial purification of human CBG by chromatography at 4°C of 10 ml serum, equilibrated with 2 µg cortisol-4-14C, on DEAE-cellulose. Highest CBG concentration in Fraction X. Trisphosphate gradient prepared from 0.005 M, pH 8.0, to 0.5 M, pH 4.5. Dotted line: unbound cortisol-4-14C; shaded area: protein-bound cortisol-4-14C. Modified from Westphal (1961).
Hydroxylapatite chromatography at 4° of rat CBG-corticosterone-4-14C complex after Sephadex G-200 filtration. Arrows indicate changes from 0.005 to 0.05 M and to 0.2 M phosphate buffer, pH 6.8. From Chader & Westphal (1968b).

of radioactivity, is removed. In all these separation steps, the fractions of high specific activity were combined, dialyzed, lyophilized and applied to the next column. It was found that gel filtration after hydroxylapatite chromatography further purified CBG from human serum and resulted in a homogeneous protein (Fig. 9). A similar final filtration over Sephadex yielded a pure rat CBG complex with corticosterone (Fig. 10). These gel filtrations were always repeated until symmetrical peaks were obtained, coinciding for protein concentration and radioactivity. The quantities of final CBG preparations obtained as corticosteroid complexes per liter of human, rabbit and rat serum were 14, 6.8 and 29 mg, respectively, representing yields of approximately 50 % in each case.

The purified CBG-corticosteroid complexes isolated from the three species in our laboratory were found to be homogeneous by several criteria. Immuno-electrophoretic studies gave single precipitation bands upon reaction of the pure CBG preparations with the antisera against whole serum of the given species; this is shown in Fig. 11 for rabbit CBG. No cross reactions between species were observed. The three proteins migrated as α1-globulins. Ultracentrifugation performed over a range of concentrations produced single symmetrical peaks as exemplified in Fig. 12 for rat CBG. As another indication
Hydroxylapatite chromatography at 4° of human CBG-cortisol-4-14C complex after separation on DEAE cellulose. (A) First, (B) final chromatography. In 0.001 M phosphate, pH 6.8. From Muldoon & Westphal (1967).

Sephadex G-200 filtration at 4° of rabbit CBG-corticosteroid complex after purification by DEAE-Sephadex chromatography. In 0.05 M phosphate, pH 7.4. From Chader & Westphal (1968a).
Sephadex G-200 filtration at 4° of human CBG-cortisol-4-14C complex after final chromatography on hydroxylapatite (see Fig. 7B). (A) First, (B), final gel filtration. In 0.05 M phosphate, pH 7.4. From Muldoon & Westphal (1967).

Final Sephadex G-200 filtration at 4° of rat CBG-corticosterone-4-14C complex in 0.05 M phosphate buffer, pH 7.4. From Chader & Westphal (1968b).
Fig. 11.
Immunoelectrophoresis of two preparations of pure rabbit CBG-corticosteroid complex. 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 through. Electrophoresis was performed in high resolution tris buffer, pH 8.9, at 23°. From Chader & Westphal (1968a).

Fig. 12.
Ultracentrifugation pattern of 0.9% and 0.2% solutions of rat CBG-corticosterone complex in 0.1 M NaCl, 20°. From Chader & Westphal (1968b).
Reciprocal $S_{30,w}$ values vs. concentration for rabbit CBG-corticosteroid complex in 0.1 M NaCl, $20^\circ$. The point circled twice was obtained with a second preparation.

From Chader & Westphal (1968a).

of homogeneity, linear relationships were found between the reciprocal sedimentation coefficients, $S_{30,w}$, and concentrations of CBG for the three species. This is illustrated in Fig. 13 for the rabbit protein. Table 4 shows some physicochemical properties of human, rabbit and rat CBG. The similarity of various parameters determined for the three proteins is evident; a relatively high carbohydrate content of 26–29 % is characteristic for these steroid-binding glycoproteins.

The chemical correspondence among the CBGs from the three species is also apparent in their amino acid and carbohydrate composition (Table 5). In reference to the large class of natural proteins in their numerous manifestations, a general similarity of the three glycoproteins is evident. Nevertheless, distinct differences in individual amino acids, such as the sulfur-containing amino acids, lysine, and others, attest to unique molecular species as might be expected from the binding properties seen in Table 3. Considering the carbohydrate constituents, the «backbone» residues of glycoproteins, i.e., hexose and hexosamine, are closer in their frequency than sialic acid and fucose, in analogy to other types of glycoproteins.

Table 6 shows the apparent association constants and other thermodynamic parameters of the isolated corticosteroid-CBG complexes. A comparison with Table 3 confirms the validity of the $k$ values obtained for the interactions in
Table 4.
Physicochemical properties of the corticosteroid complexes of human, rabbit and rat CBG.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Human CBG (Muldoon &amp; Westphal 1967)</th>
<th>Rabbit CBG (Chader &amp; Westphal 1968a)</th>
<th>Rat CBG (Chader &amp; Westphal 1968b)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sediment coefficient, ( s^0_{20,w} ) (S)</td>
<td>3.79</td>
<td>3.55</td>
<td>3.56</td>
</tr>
<tr>
<td>Diffusion coefficient, ( D_{20,w} ) (cm² sec⁻¹) × 10⁹</td>
<td>6.15</td>
<td>7.02</td>
<td>-</td>
</tr>
<tr>
<td>Partial specific volume, ( \bar{v} ) (ml/gm)</td>
<td>0.708</td>
<td>0.695</td>
<td>0.711</td>
</tr>
<tr>
<td>Molecular weight</td>
<td>51 700ᵃ</td>
<td>40 700</td>
<td>52 600 ± 300ᵇ</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>61 000 ± 1100ᶜ</td>
</tr>
<tr>
<td>Fractional ratio, ( f/fo )</td>
<td>1.42</td>
<td>1.37</td>
<td>-</td>
</tr>
<tr>
<td>( E_{1 cm}^1 ) at 279 nm</td>
<td>6.45</td>
<td>8.4</td>
<td>6.2</td>
</tr>
<tr>
<td>( A_{280}/A_{290} ), corticosteroid complex</td>
<td>1.13</td>
<td>1.38</td>
<td>1.58</td>
</tr>
<tr>
<td>( A_{280}/A_{290} ), complex stripped</td>
<td>1.57</td>
<td>-</td>
<td>1.71</td>
</tr>
<tr>
<td>Electrophoretic mobility at pH 8.6 ( (cm² vol⁻¹ sec⁻¹) \times 10^⁵ )</td>
<td>-4.9</td>
<td>-5.1</td>
<td>-</td>
</tr>
<tr>
<td>Carbohydrate (°/o)</td>
<td>26.1</td>
<td>29.2</td>
<td>27.8</td>
</tr>
<tr>
<td>Nitrogen (°/o)</td>
<td>12.7</td>
<td>12.1</td>
<td>-</td>
</tr>
</tbody>
</table>

ᵃ Molecular weights reported from other laboratories: 52 000 (Seal & Doe 1966); 58 500 (Slaunwhite et al. 1966).
ᵇ From corticosterone content of complex.
ᶜ From approach to sedimentation equilibrium.

d the steroid-stripped sera; evidently, other serum components do not interfere with the binding affinity. The number of binding sites was found to be \( n = 1 \) in each case (Table 6); the question of the presence of secondary binding sites of lower affinity has not been explored yet. The data show that the association constants at 4° are approximately 20 times greater than at 37°. The relatively high affinity of rabbit CBG for cortisol is also verified. Additional equilibrium dialysis studies with pure rabbit CBG and radiolabeled steroid at 4° and 37° have confirmed that the relative binding affinities do not follow the polarity rule; they decreased from cortisol \( \succ \) corticosterone \( \succ \) progesterone \( \succ \) aldosterone (Chader & Westphal 1968a). The free energy of binding has the high value of 10–11 kcal per mole. It 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 com-
ponents so that the enthalpy is drastically reduced; the total order of the system is much increased resulting in a negative entropy change.

The chemical characterization of CBG is in its beginning, and little is therefore known about the relationship of chemical structure to steroid-binding quality. Complete removal of sialic acid from human CBG by mild enzymatic hydrolysis did not affect the affinity for cortisol. This is in accordance with analogous observations on the sex steroid-binding β-globulin, the thyroxin-binding globulin and α1-acid glycoprotein which after elimination of sialic acid also retain full binding activity for estradiol, thyroxin, and progesterone, respectively. It would seem to follow from these results that sialic acid is not of general significance for the hormone-binding properties of these different types of glycoproteins.

Table 5.
Amino acid and carbohydrate composition of human, rabbit and rat CBG.a

<table>
<thead>
<tr>
<th>Residue</th>
<th>CBG-Hu</th>
<th>CBG-Rb</th>
<th>CBG-Rt</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lysine</td>
<td>15</td>
<td>10</td>
<td>20</td>
</tr>
<tr>
<td>Histidine</td>
<td>9</td>
<td>6</td>
<td>7</td>
</tr>
<tr>
<td>Arginine</td>
<td>10</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>Aspartic acid</td>
<td>32</td>
<td>25</td>
<td>39</td>
</tr>
<tr>
<td>Threonine</td>
<td>22</td>
<td>19</td>
<td>23</td>
</tr>
<tr>
<td>Serine</td>
<td>22</td>
<td>21</td>
<td>27</td>
</tr>
<tr>
<td>Glutamic acid</td>
<td>38</td>
<td>30</td>
<td>42</td>
</tr>
<tr>
<td>Proline</td>
<td>22</td>
<td>16</td>
<td>17</td>
</tr>
<tr>
<td>Glycine</td>
<td>19</td>
<td>19</td>
<td>21</td>
</tr>
<tr>
<td>Alanine</td>
<td>23</td>
<td>21</td>
<td>23</td>
</tr>
<tr>
<td>Half-cystine</td>
<td>2</td>
<td>6</td>
<td>9</td>
</tr>
<tr>
<td>Valine</td>
<td>26</td>
<td>18</td>
<td>20</td>
</tr>
<tr>
<td>Methionine</td>
<td>10</td>
<td>3</td>
<td>8</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>16</td>
<td>10</td>
<td>13</td>
</tr>
<tr>
<td>Leucine</td>
<td>40</td>
<td>26</td>
<td>34</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>10</td>
<td>8</td>
<td>10</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>19</td>
<td>11</td>
<td>16</td>
</tr>
<tr>
<td>Tryptophan</td>
<td>3</td>
<td>5</td>
<td>4</td>
</tr>
<tr>
<td>Hexose</td>
<td>37</td>
<td>26</td>
<td>32</td>
</tr>
<tr>
<td>Hexosamine</td>
<td>29</td>
<td>24</td>
<td>31</td>
</tr>
<tr>
<td>Sialic acid</td>
<td>7</td>
<td>12</td>
<td>12</td>
</tr>
<tr>
<td>Fucose</td>
<td>5</td>
<td>2</td>
<td>8</td>
</tr>
</tbody>
</table>

a Calculated as residues per mole glycoprotein from data in Muldoon & Westphal (1967); Chader & Westphal (1968a,b).
b Molecular weight used for calculation.
Table 6.
Binding parameters of corticosteroid-CBG complexes.
References (in parentheses) below table.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Human CBGa (1)</th>
<th>Rabbit CBGa (2)</th>
<th>Rat CBGb (3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of binding sites, n</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Association constant, k, 4° (M⁻¹)</td>
<td>5.2 × 10⁸</td>
<td>9.0 × 10⁸</td>
<td>5.1 × 10⁸</td>
</tr>
<tr>
<td>Association constant, k, 37° (M⁻¹)</td>
<td>2.4 × 10⁷</td>
<td>4.7 × 10⁷</td>
<td>2.8 × 10⁷</td>
</tr>
<tr>
<td>Free energy, ΔF°, 4° (kcal/mole)</td>
<td>-11.0</td>
<td>-11.3</td>
<td>-11.0</td>
</tr>
<tr>
<td>Free energy, ΔF°, 37° (kcal/mole)</td>
<td>-10.5</td>
<td>-10.9</td>
<td>-10.6</td>
</tr>
<tr>
<td>Enthalpy, ΔH° (kcal/mole)</td>
<td>-15.7</td>
<td>-15.0</td>
<td>-14.8</td>
</tr>
<tr>
<td>Entropy, ΔS° (cal mole⁻¹ deg⁻¹)</td>
<td>-17</td>
<td>-13</td>
<td>-14</td>
</tr>
</tbody>
</table>

a Cortisol complex.
b Corticosterone complex.
(1) (Muldoon & Westphal 1967).
(2) (Chader & Westphal 1968a).
(3) (Chader & Westphal 1968b).

Concerning the question of possible involvement of sulfhydryl in the steroid interaction with CBG, one free thiol group has been determined spectrophotometrically with p-hydroxymercuribenzoate in human CBG (Seal & Doe 1966; Muldoon & Westphal 1967). In contrast, no sulfhydryl was detected under identical conditions in rabbit CBG; however, addition of p-hydroxymercuribenzoate to an equilibrium dialysis system containing rabbit CBG and radiolabeled cortisol resulted in a 27-fold decrease of the association constant at 37° (Chader & Westphal 1968a).

Gel filtration at 45° of the corticosteroid complexes of pure human and rat CBG leads to dissociation and complete loss of steroid-binding activity. In case of rabbit CBG, about half of the bound steroid, and of the binding affinity, was lost under the same conditions. This is in contrast to similar gel filtration at 45° of whole serum which results in the complete removal of the steroid, but does not affect the binding ability of CBG; presumably, other serum proteins exert a protective influence.

When the pure rat CBG-corticosterone complex was subjected to gel filtration at 23°, 87% of the steroid was removed and the protein was found to undergo conformational alterations. Ultracentrifugal analysis showed that the homogeneous single peak characteristic of the CBG-corticosterone complex (Fig. 12) was changed to at least four molecular species with sedimentation coefficients of 3.4 S (= original), 5.4 S, 6.8 S and 8.1 S (Fig. 14). When this
Fig. 14.
Ultracentrifugation pattern of rat CBG after removal of 87% of the bound corticosterone at various times (min) after speed of 59,780 rpm was reached.
In 0.1 M NaCl, 20°. From Chader & Westphal (1968b).

Fig. 15.
Ultracentrifugation pattern of rat CBG used for polymerization study shown in Fig. 14, after recombination with 1 mole of corticosterone per mole CBG. Other conditions as in Fig. 14. From Chader & Westphal (1968b).
polymeric material was recovered from the ultracentrifuge cell and recombined with 1 mole corticosterone per 53 000 g of glycoprotein, the sedimentation pattern reverted to that of the original CBG-corticosterone complex as shown in Fig. 15. One homogeneous peak reappeared with a sedimentation of 3.45 S, a value indistinguishable from that of the original rat CBG complex under similar conditions. The polymerization phenomenon was confirmed by disc electrophoresis in polyacrylamide. After removal of corticosterone from the rat CBG complex by gel filtration under mild conditions, polymeric bands appeared whose migration rates indicated dimeric, tetrameric and octameric species in addition to the monomeric form (Chader & Westphal 1968b)*.

The reversible polymerization of the CBG molecule appears to be the first example of the control of the conformational structure of a carrier protein by the steroid hormone with which it forms a specific complex of high affinity. The biological significance of such hormone-controlled aggregation and deaggregation of a steroid-binding protein is unknown. An obvious speculation would be the regulation of the size of a carrier protein for transport, or blockage of transport, through cellular or subcellular membranes, including nuclear structures. Future research will show whether or not such assumption is justified.

REFERENCES

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* It should be mentioned that Freeman & Idler (1969) have observed polymerization with loss of steroid-binding activity on gel filtration of the sex steroid-binding globulin from the thorny skate. The question of analogy to the polymerism of rat CBG awaits clarification.
Abraham: whereas speculated Slaunwhite: about to groups,

Westphal: does observed rat Westphal: this Seal steroids.


DISCUSSION

Slaunwhite: I was very much interested in your observations on polymerization of the rat CBG. Dr. Schneider has recently isolated and purified guinea pig transcortin and observed the same sort of phenomenon. You did not mention human transcortin. This does not polymerize, at least it didn't in our hands.

Westphal: We have never observed polymerization with the human protein. Seal and Doe have not either. It seems that human CBG is different in this respect. We have speculated that perhaps the difference in the sulphur content might have something to do with it. The rat CBG has a half-cystine content of about 9 residues per molecule, whereas human CBG has only 2. Since such polymerizations may involve sulphhydryl groups, this difference may be of significance for the polymerism.

We have also done purification work on guinea pig CBG, which is so particularly interesting because of its very high concentration in the pregnant guinea pig that you observed some time ago, and which we have confirmed. We have similarly observed this polymerization.

Abraham: The interaction between steroids and their natural binding proteins seems to be enthalpy driven with high negative enthalpy and low negative entropy. A practical aspect of this is that if one wishes to use these proteins as reagents in assay procedures, lower temperature of incubation would be more efficient. We have studied the interaction between an anti-oestradiol antibody and 17β-oestradiol and found that this reaction is entropy driven. I would like to ask Dr. Westphal if he has any idea about the uniformity of reaction between naturally occurring proteins and their specific steroids.
Westphal: We have been puzzled about this for some time and have tried to find an interpretation. We have found that the typical steroid complexes with albumin look quite different from the CBG complexes in their thermodynamic properties; the free energies and enthalpies are always much lower. The entropies are positive for the albumin complexes with progesterone and all other steroids examined. This was mostly determined with human albumin, but I am sure that for bovine albumin it would be the same. Our explanation was that the displacement and dispersal of the ordered water molecules would lead to randomization according to the concept of Klotz and result in positive entropies. In the steroid interaction with the highly specific, high-affinity binding protein CBG we would have, in addition, a higher form of order in the complex, presumably resulting from a particularly good fit of a conformational structure which is optimal for that particular steroid and in this way would give us a negative entropy effect.

Rodbard: In connection with the use of Sephadex gel filtration and other forms of chromatography as a criterion for homogeneity and identity, I would make a plea that one calculates the number of theoretical plates. This is readily calculated by use of an approximation:

$$N = \left( \frac{V_e}{\sigma} \right)^2$$

where \( N \) = number of theoretical plates, \( V_e \) is the elution volume and \( \sigma \) \( V_e \) is the standard deviation of the elution volume. If the volume of the band between the two points at one half maximum peak height is designated \( V_B \), then the value \( \frac{\sigma^2}{V_e} \) is given by

$$\frac{\sigma^2}{V_e} = \frac{V_B^2}{5.55}$$

when dealing with a symmetrical peak of Gaussian distribution. For example, in your Fig. 9, \( N \approx 300 \); in Fig. 10, \( N \approx 20 \). Thus in one case we have excellent resolution, in the other a very poor method for establishment of homogeneity or identity.

Also, separation on gel filtration is optimized when \( K_{av} \approx 0.36 \); resolution is optimal when \( K_{av} \approx 0.13 \), where \( K_{av} \) is the partition coefficient,

$$K_{av} = \frac{V_e - V_0}{V_T - V_0}$$

where \( V_e \) is elution volume, \( V_0 \) is the void volume, and \( V_T \) is the total volume of the column.

The markedly improved resolution of gel electrophoresis when compared with gel filtration is due to the fact that gel electrophoresis is not a partition process, with its inherent binomial variance; band spreading in gel electrophoresis is governed by an apparent diffusion constant which is smaller than the free diffusion constant, and is usually orders of magnitude smaller than the band spreading seen in gel filtration (Rodbard & Chrambach 1970).

Westphal: Thank you for these valuable comments. I agree with you concerning the usefulness of considering the number of theoretical plates. However, we would not like to rely on this parameter alone as a criterion for homogeneity. We would rather apply as many protein criteria as possible, not only electrophoresis and various chromato-
graphies, but ultracentrifugation, reciprocal sedimentation coefficients vs. concentration, immuno-electrophoresis, and others. Of course, if one does not have anything else, I agree that it would be important to apply the criteria that you have outlined.

Baulieu: How do you explain this difference of molecular weight for the rabbit CBG, and how do you measure this molecular weight?

Westphal: We determined it from sedimentation velocity and diffusion data. I cannot say why the molecular weight of rabbit CBG is lower than that of human and rat CBG, but I would not consider it necessary for them to be the same, since they are different proteins. We can expect differences in such glycoproteins.

Baulieu: Because your extinction coefficient value was very different; this is a little difficult to understand.

Westphal: These are different proteins, and the molecular weights are different. However, I would like to mention that there have been some problems in the determination of the molecular weights of the CBG's. The first molecular weight of human CBG (Seal & Doe 1962) was lower than that of 52,000 reported later (Seal & Doe 1966), which agrees with the value of 51,700 determined in our laboratory from sedimentation velocity and diffusion data (Muldoon & Westphal 1967). Slaunwhite et al. (1966) published a value of 58,500 on the basis of sedimentation equilibrium. This technique was applied by us (Chader & Westphal 1967) for rat CBG, resulting in a molecular weight higher than the 53,000 calculated from the corticosterone content of the pure complex. We consider it possible that the polymerization of rat CBG is partly responsible for the high molecular weight found in the ultracentrifugation procedure which was done under conditions which do not exclude some dissociation of the CBG complex and subsequent polymerization. This is why we rely more on the value that we obtained by analysis of the corticosterone complex than on that derived from the sedimentation equilibrium technique.

Abraham: Tritium seems to behave as if it were more polar than hydrogen and forms stronger hydrogen bond than hydrogen itself. If you used tritiated steroids in your thermodynamic studies, I would suspect that the K values might have been lower with non-radioactive steroids. We found that for an entropy driven reaction between oestradiol and its antibody, the tritiated oestradiols studied had a lower affinity constant than non-radioactive oestradiol, and the effect of tritium was different depending on its position on the steroid molecule.

Westphal: We have not studied this problem in detail. Most of our affinity constants have been determined with ¹⁴C-labelled steroids. However, we assume in all these complexes a combination of hydrophobic and hydrophilic bonding. It is perhaps typical, when we compare the rabbit CBG complex with the human CBG complex that in the rabbit CBG complex in which cortisol is bound more firmly, hydrophilic bonding may be prevalent in comparison with human CBG. In most of the steroid-protein interactions hydrophobic bonding predominates. This is certainly the case for albumin, and also for orosomucoid ($\alpha_1$-acid glycoprotein). We believe that this is generally true also for CBG. I am afraid we have no results to answer your specific question concerning tritium versus other forms of label.

Baulieu: Our data with pig uterine oestradiol receptor and oestradiol did not show any difference between the non-radioactive and the 6,7-tritiated steroid. We deliberately investigated that point as far as affinity is concerned.
Pearlman: Does the dimeric form of CBG have any binding activity? And conversely, have you studied the possible dissociation of CBG into subunits? An interesting comparison might be made with the thyroxine-prealbumin complex which has recently been observed to undergo reversible dissociation into tetramers.

Westphal: We have not been able to study it in detail because of lack of pure CBG. However, we have some indications of material of lower molecular weight than the unity of molecular weights given.

Mester: To be completely happy about the purity of your final preparation it would be necessary to compare the specific activity with a number of binding sites per litre of the original plasma. Did you try to do this?

Westphal: I don’t know whether any protein chemist is ever happy with the final homogeneity of a protein. Anybody who has ever worked with albumin is certainly not. We have applied all possible criteria to establish homogeneity, and this is as far as we have gone. If we had more material, we would perhaps repeat all these determinations and possibly establish additional criteria, but speaking in practical terms, we believe that on the basis of today’s methods of protein chemistry we have reason to be sure that we have a homogeneous protein.

Mester: Have you compared the number of binding sites in your pure material with that in the original serum?

Westphal: Yes, we have determined the number of binding sites, always by analysis of the CBG complexes as we obtained them, which are the steroid-saturated complexes. The number of binding sites in the pure protein agrees with those present in the original plasma, calculated on the basis of about 50% yield which we obtained in our purification. We have never been able to study the question whether there are, in addition to the primary binding site of high affinity, secondary binding sites of lower affinity. We know that this is the case in many other proteins; we have found it for the α1-acid glycoprotein, but we have not studied this question with CBG.

References: